Hatching plasticity in two temperate anurans: responses to a pathogen and predation cues

J.C. Touchon, I. Gomez-Mestre, and K.M. Warkentin

Abstract: Water molds are widespread in aquatic environments and are important causes of mortality in amphibian and fish eggs. We tested the ability of two species of North American anurans with different breeding phenologies (*Rana sylvatica* LeConte, 1825 and *Bufo americanus* Holbrook, 1836) to alter their hatching timing in response to three indicators of environmental risk: infection with a water mold, exposure to simulated egg predation cues, or exposure to simulated larval predation cues. When infected with water mold (Saprolegniaceae), *B. americanus* eggs hatched, on average, 44% earlier than the controls and *R. sylvatica* eggs hatched 19% earlier than the controls. In addition, *B. americanus* but not *R. sylvatica* eggs hatched significantly earlier than the controls when exposed to simulated egg and larval predation cues. *Bufo americanus* embryos hatched before developing muscular response, suggesting that hatching occurs through enzymatic egg capsule degradation combined with ciliary movement, not through behavior. *Bufo americanus* breeds later than *R. sylvatica* and responded to infection and simulated predation cues more strongly. This may reflect a history of stronger selection by pathogens and predators that accumulate in ponds as the breeding season progresses. To our knowledge, these are the first examples of induced hatching of amphibians in response to aquatic pathogens.

Résumé : Les moisissures sont répandues dans les milieux aquatiques et sont d'importantes causes de mortalité des oeufs d'amphibiens et de poissons. Nous vérifions si deux espèces nord-américaines d'anoures possédant des phénologies de reproduction différentes (*Rana sylvatica* LeConte, 1825 et *Bufo americanus* Holbrook, 1836) peuvent modifier le moment de l'éclosion de leurs oeufs en réaction à trois indicateurs de risque environnemental, soit une infection avec des moisissures aquatiques, une exposition à des signaux simulés de prédation des oeufs et une exposition à des signaux simulés de prédation des larves. Les oeufs de *B. americanus* infectés par une moisissure aquatique (Saprolegniaceae) éclosent en moyenne 44 % plus tôt que les oeufs témoins et ceux de *R. sylvatica* 19 % plus tôt. De plus, lors d'expositions à des signaux simulés de prédation embryonnaire ou larvaire, les oeufs de *B. americanus*, mais non ceux de *R. sylvatica*, éclosent significativement plus tôt que les témoins. Les embryons de *B. americanus* éclosent avant d'avoir développé leur réaction musculaire, ce qui laisse croire que l'éclosion se fait grâce à une dégradation enzymatique de la capsule de l'oeuf combinée à un mouvement ciliaire plutôt que par une réaction comportementale. *Bufo americanus* se reproduit plus tard que *R. sylvatica* et réagit plus fortement à l'infection et aux signaux simulés de prédation. Cela peut être le reflet d'une sélection plus importante au cours de l'évolution par les pathogènes et les prédateurs qui s'accumulent dans les étangs à mesure que la saison de reproduction progresse. À notre connaissance, ces exemples constituent les premières mentions chez les amphibiens d'éclosions provoquées en réaction à des pathogènes aquatiques.

[Traduit par la Rédaction]

Introduction

Adaptive developmental plasticity allows organisms to maintain fitness across a wider range of environments than fixed phenotypes (e.g., Karban and Baldwin 1997; Schlichting and Pigliucci 1998; Tollrian and Harvell 1999; West-Eberhard 2003). While natual selection in heterogeneous environments shapes plastic phenotypic responses, the historical association between environmental cues and particular conditions is also critical (West-Eberhard 1989, 2003; Lima and Dill 1990; DeWitt et al. 1998; Schlichting and Pigliucci 1998; Pigliucci 2001). Two organisms with different evolutionary histories of either selection or association between cues

and context can show markedly different plastic responses to the same cue (West-Eberhard 2003). Variation in phenotypic plasticity may therefore reflect different histories of selection, information, or both.

Early life stages, such as eggs and larvae, often suffer high mortality from stage-specific risks, including predators and pathogens (Werner and Gilliam 1984; Kiesecker and Blaustein 1997). Variation in these risks may select for plasticity in hatching timing (Sih and Moore 1993). Several anamniote vertebrates are known to alter hatching time in an apparently adaptive manner in response to predators, hatching early in response to embryo predators (Warkentin 1995, 2000; Chivers et al. 2001; Johnson et al. 2003; Saenz et al. 2003; Kusch and Chivers 2004; Vonesh 2005) or delaying hatching in response to larval predators (Sih and Moore 1993; Moore et al. 1996). In other cases, embryos do not alter hatching timing in response to egg-stage or larval-stage risk (Van Buskirk 2002; Anderson and Petranka 2003; Johnson et al. 2003). Furthermore, hatching stage may be plastic without conferring a fitness benefit. For instance, *Triturus* Rafinesque, 1815 newts hatch less developed, but

J.C. Touchon,[1] I. Gomez-Mestre, and K.M. Warkentin. Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215, USA.

[1]Corresponding author (e-mail: jtouchon@bu.edu).

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not earlier, when exposed to egg predator cues (Orizaola and Braña 2004).

Although a moderate amount of research has explored the responses of vertebrate embryos to predation risk, there has been less research on embryo responses to pathogens. Pathogens infect and kill amphibian, fish, and reptile eggs in North America (Blaustein et al. 1994; Kiesecker and Blaustein 1997; St. Mary et al. 2004), Europe (Czeczuga et al. 1998; Green 1999; Paxton and Willoughby 2000; Czeczuga et al. 2002; Robinson et al. 2003; Moreira and Barata 2005), and the Neotropics (Ramnarine 2001; Warkentin et al. 2001). Water molds ("oomycetes", Stramenopila: Peronosporomycotina: Dick 2001) such as species of *Saprolegnia* Thuret spread rapidly via contact with neighboring eggs and can be especially harmful to communal breeders (Kiesecker and Blaustein 1997; Green 1999). Water molds can also be saprotrophic, first infecting dead eggs and then sometimes spreading to live ones (J.C. Touchon and I. Gomez-Mestre, personal observation). Anuran clutch mortality as high as 80% has been attributed to water mold infection (Kiesecker and Blaustein 1997). Water molds from the family Saprolegniaceae also cause frequent adult and egg mortality of fishes in both hatcheries and nature (Taylor and Bailey 1979; Czeczuga et al. 2002; Wedekind 2002; St. Mary et al. 2004).

In at least one amphibian (*Agalychnis callidryas* (Cope, 1862)), embryos respond to fungal infection by hatching precociously (Warkentin et al. 2001). Two other vertebrates, a fish (genus *Coregonus* L., 1758) and a lizard (*Lacerta* (= *Iberolacerta*) *monticola* (Boulenger, 1905)), have also been shown to hatch in response to an egg parasite and pathogen, respectively (Wedekind 2002; Moreira and Barata 2005). The prevalence of egg pathogens and predators, combined with the growing evidence for embryo responses to risk, suggests that predator-induced and pathogen-induced hatching might be widespread and ecologically important.

Here we test embryo responses to both predation and pathogen cues in two North American anurans, *Rana sylvatica* LeConte, 1825 and *Bufo americanus* Holbrook, 1836. These frogs breed annually in vernal pools, small ponds that hold water in spring but are dry during much of the summer. Most vernal pools at our study site fill with fall rains, freeze during the winter, and thaw in early spring. *Rana sylvatica* is one of the earliest species to breed in such pools, followed several weeks later by *B. americanus* (Petranka et al. 1994; Paton and Crouch 2002). *Rana sylvatica* also breeds more synchronously than *B. americanus*. *Rana sylvatica* adults migrate to ponds and breed over a span of weeks, whereas *B. americanus* migrate and breed over a span of months (Paton and Crouch 2002). At our study site, the entire *R. sylvatica* population typically breeds within a few days. This breeding phenology affects embryo exposure to predators and pathogens.

When *R. sylvatica* breeds, pools contain few or no egg predators. It is possible for pools to contain larval predators that overwinter such as odonate nymphs and backswimmers (Notonectidae). During this period at our field site, water mold growth is slow and infection of *R. sylvatica* eggs is rare (Gomez-Mestre et al. 2006). Also, because of the synchronicity of *R. sylvatica* breeding and the length of embryo development, eggs and larvae are rarely if ever present at the same time. Therefore, *R. sylvatica* embryos may be exposed to some egg pathogens but are rarely exposed to conspecific cues indicating larval predation threats (e.g., alarm pheromones from larvae).

By the time *B. americanus* breeds 4–6 weeks later, potential egg predators such as *R. sylvatica* larvae are present (Petranka et al. 1994; Paton and Crouch 2002). In addition, water temperatures have risen, increasing primary productivity (Skelly et al. 2002) and facilitating rapid growth of water mold (Gomez-Mestre et al. 2006). *Bufo americanus* also breeds over a longer period of time than *R. sylvatica* (Paton and Crouch 2002), thereby increasing the chances that larvae and eggs will be present in the same pond. *Bufo americanus* embryos are more likely to be exposed to cues from larval conspecifics indicating post-hatching risk, in addition to facing higher infection rates by aquatic egg pathogens.

To assess the responsiveness of *R. sylvatica* and *B. americanus* embryos to stage-specific risks, we experimentally tested the effects of simulated predation cues and infection by aquatic pathogens on the timing of hatching in both species. (*i*) We hypothesized that embryos from both species would hatch early if inoculated with pathogens. However, we predicted that *B. americanus* would show a stronger response than *R. sylvatica* because *B. americanus* breed when aquatic pathogens are more prevalent and when infection rates are higher. (*ii*) Because of its early breeding, *R. sylvatica* is less likely than *B. americanus* to be exposed to egg predators in the wild. As such, we predicted that *R. sylvatica* eggs would not respond to simulated egg predation cues, whereas *B. americanus* embryos would hatch early in response to the cues. (*iii*) In the wild, *B. americanus* embryos are present at the same time as both conspecific and *R. sylvatica* tadpoles. We therefore tested the response of *B. americanus* eggs to cues of simulated predation on conspecific and heterospecific (*R. sylvatica*) tadpoles. We hypothesized that both cues would delay hatching, but that the response would be stronger for conspecific cues. Because of the early and synchronous breeding of *R. sylvatica*, we were unable to test the effects of either conspecific larval predation cues or heterospecific egg or tadpole predation cues on *R. sylvatica* embryos.

Materials and methods

All experiments were conducted between April 2003 and May 2004. *Bufo americanus* and *R. sylvatica* clutches were collected from ponds in Lynn Woods Reservation, Massachusetts, USA. Each clutch contained several hundred to thousands of eggs, of which we used only a small subset for the experiments. Except where otherwise noted, clutches were collected before Gosner stage 13 (formation of the neural plate; Gosner 1960) and experiments commenced at Gosner stage 15 (neural folds coalesce). Based on the development of known age clutches of both species in the field (J.C. Touchon and I. Gomez-Mestre, unpublished data), we estimated the date of oviposition from the developmental stage at the time of collection. All experiments with *R. sylvatica* were maintained on a 10 h light : 14 h dark cycle at 12 °C (light) and 8 °C (dark). We maintained the same light schedule for the *B. americanus* experiments, but increased the light:dark temperatures to 15 and 11 °C, respectively, to reflect the warmer temperatures that we had measured at the

collection site. All experiments were checked twice daily for hatched, unhatched, and dead eggs (ca. 0800 and 2000). Eggs were considered hatched when the embryos had completely exited the egg capsule; embryos inside ruptured or deflated egg capsules were not considered hatched.

Simulated predation cue experiments

Predation-cue experiments were conducted in 21 cm diameter containers with 1.5 L of carbon-filtered dechlorinated water distributed in four randomized blocks on shelves in an environmental chamber. Simulated predation cues were crushed eggs or tadpoles (prepared using a manual glass homogenizer) and were added twice daily (ca. 0800 and 2000). To prevent depletion of water oxygen content resulting from the addition of organic matter (crushed eggs or tadpoles), we gently aerated each container. Water oxygen levels (as measured by a fibre-optic microprobe connected to an optical oxygen sensor; Microx TX2, Precision Sensing, Regensburg, Germany) did not differ between treatments. When the suspension containing crushed tadpoles or eggs was added to the water in the test containers, oxygen levels briefly dropped by up to 26%. Initial high levels of oxygen saturation were recovered within 30–40 s.

We collected nine *B. americanus* clutches on 3 May 2003 and removed four strings of 10 eggs each per clutch, assigning one to each of four cue treatments: 3 mL of filtered water added alone or with one crushed *B. americanus* egg, *B. americanus* tadpole, or *R. sylvatica* tadpole. For analyses of all experiments, our hatching timing data are means per container to avoid pseudoreplication (Hurlbert 1984). This resulted in nine replicates per cue treatment, with replicates randomly assigned within blocks. Crushed tadpoles were roughly size-matched to standardize the cue intensity between treatments. Three replicates (one in each experimental treatment) became contaminated during the experiment and were discarded.

We collected three *R. sylvatica* clutches on 15 April 2004 for use in this experiment. We removed eight groups of 10 eggs from each clutch, randomly assigning half to 3 mL of filtered water added alone (control) and half to four crushed *R. sylvatica* eggs. This resulted in a total of 12 replicates per cue treatment (4 replicates from each of three clutches per treatment), randomly assigned within blocks. Preliminary experiments had indicated that *R. sylvatica* embryos may be unresponsive to simulated predation cues, so cue intensity was increased with respect to the *B. americanus* experiments to test if a response would be elicited at an elevated level.

Water mold infection experiments

Water mold experiments were conducted in 8 cm diameter containers with 150 mL of carbon-filtered dechlorinated water, blocked by replicate, on shelves in an environmental chamber. We collected water mold by submerging tea bags containing sterilized hemp seeds in ponds at Lynn Woods Reservation (Robinson et al. 2003). All tea bags were collected once hyphal growth was evident. This collection method may have harvested several varieties of water mold (oomycete), as well as other microbes. To select only water mold hyphae for use in our experiments, infected seeds were plated on autoclaved cornmeal agar with uninfected, sterilized seeds to encourage hyphal growth. Only filamentous

water mold hyphae were used to inoculate egg clutches for the experiments. Our water mold cultures were identified by J.E. Longcore as a mixture of species from the genera *Achlya* Nees and *Saprolegnia* based on characteristic morphology and zoosporangia (J.E. Longcore, personal communication). Species-level identification of water molds is nearly impossible without molecular techniques, but genus-level identification was suitable for our purposes.

We collected six *R. sylvatica* clutches on 15 April 2004 for use in this experiment. We removed eight groups of 10 eggs from each clutch and randomly assigned them to two treatments (four replicates per clutch to each treatment): control (eggs alone) or inoculation with three seeds visibly infected with water mold. This resulted in 40 replicated strings of eggs for each treatment. The mean hatching time for each group of 10 eggs was considered a single data point to avoid pseudoreplication (Hurlbert 1984)

We collected three healthy *B. americanus* clutches on 29 April 2004 and a fourth on 4 May 2004. On 29 April, we also collected one *B. americanus* clutch heavily infected with water mold and with nearly complete mortality. Segments of 10 eggs each were removed from each healthy *B. americanus* clutch and randomly assigned to three treatments: control, inoculation with seed-collected water mold (three infected seeds), or inoculation with *B. americanus* egg-collected water mold (three infected eggs with hyphae). Eggs were inoculated the day of collection; two clutches were at Gosner stage 16 (formation of neural tube; Gosner 1960), a third clutch was at Gosner stage 14 (formation of neural folds), and the fourth clutch was at Gosner stage 13 (formation of the neural plate) when inoculated.
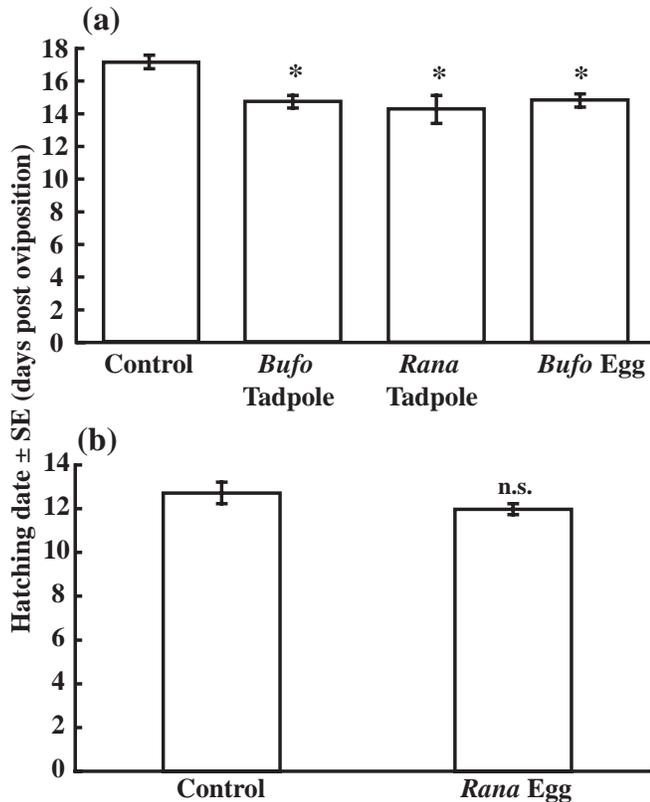
Statistical analyses

All analyses were performed in SAS® version 8.02 (SAS Institute Inc. 1999). Survival was analyzed using an underlying binomial error distribution and by fitting a generalized linear model (GLM) with a logit link function using PROC GENMOD. Hatching date was analyzed with GLMs using PROC MIXED. In one experiment, hatching date showed significant heteroscedasticity and was therefore ranked prior to the fitting of GLMs (*R. sylvatica* predation cue). Clutch and experimental block were included in the models as random factors. Block effect was never significant and was therefore excluded from the final analyses. GLMs were chosen following goodness-of-fit criteria. Both likelihood-ratio tests and Akaike's information criterion (AIC_c) suggested exclusion of clutch and clutch × treatment parameters from the model in the cue experiments and these were therefore excluded from the final analyses.

Results

Simulated predation cues experiments

Exposure to simulated predation cues induced early hatching in *B. americanus* eggs ($F_{[3,29]} = 5.54$, $P = 0.004$; Fig. 1a). All three treatments (crushed *Bufo* eggs, crushed *Bufo* tadpoles, and crushed *Rana* tadpoles) differed significantly from the control (post hoc Tukey tests, all $P < 0.05$), but did not differ from each other (Fig. 1a). Control eggs hatched (from estimated date of oviposition) after 17.2 ± 0.4 days (mean ± SE). *Bufo americanus* eggs exposed to si-

Fig. 1. The hatching date of *Bufo americanus* and *Rana sylvatica* embryos exposed to simulated predation cues. (*a*) *Bufo americanus* eggs exposed to crushed *Bufo* tadpoles, *Rana* tadpoles, or *Bufo* eggs hatched earlier than those in the controls. All experimental treatments differed from the controls but not from each other. (*b*) *Rana sylvatica* eggs exposed to crushed conspecific eggs did not hatch earlier than the controls.
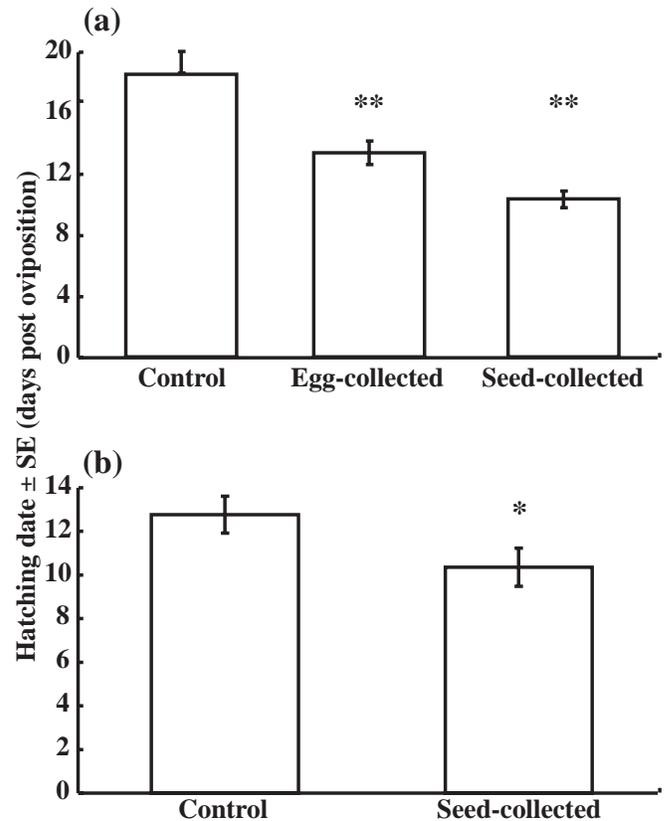

Fig. 2. The hatching date of *B. americanus* and *R. sylvatica* embryos inoculated with a mixed culture of water mold (Saprolegniaceae: genera *Saprolegnia* and *Achlya*). (*a*) *Bufo americanus* eggs exposed to water mold hatched earlier than the controls. Hatching time of eggs exposed to seed-collected water mold or to hyphae from a naturally infected clutch differed from that of the controls. (*b*) *Rana sylvatica* eggs exposed to seed-collected water mold hatched earlier than the controls. *, $P < 0.05$; **, $P < 0.001$.

mulated predation cues hatched 2.6 ± 0.6 days earlier than the controls. Overall survival across the experiment was high ($89.7\% \pm 2.2\%$, mean ± SE) and did not vary among treatments.

Addition of simulated conspecific egg predation cues had no significant effect on hatching timing in *R. sylvatica* ($F_{[1,22]} = 1.8$, $P = 0.193$; Fig. 1*b*). Survival was close to 100%. Only one egg in the entire experiment ($N = 240$ eggs) failed to hatch.

Water mold infection experiments

Bufo americanus eggs infected with water mold hatched significantly earlier than the controls ($F_{[2,45]} = 7.47$, $P < 0.001$). Control clutches hatched after 18.6 ± 0.7 days. Eggs inoculated with the seed-collected mold hatched, on average, 8.2 days earlier than the controls (post hoc Tukey test, $P < 0.001$; Fig. 2*a*). Mold collected from a naturally infected clutch had a smaller, but significant, effect on hatching timing, causing eggs to hatch, on average, 5.2 days earlier than the controls ($P = 0.008$; Fig. 2*a*). Clutch also had a significant effect on hatching timing ($F_{[3,42]} = 5.01$, $P = 0.005$). Specifically, eggs from *B. americanus* clutch 4, inoculated at Gosner stage 13 (Gosner 1960), hatched the earliest of the four clutches used (12.1 ± 0.5 days earlier than the control eggs). Although we did not stage all hatchlings from all clutches,

the most precocious hatchlings from this clutch were determined to be at Gosner stage 17 (Gosner 1960). Survival was high and did not differ across treatments ($95.8\% \pm 1\%$).

Exposure to infection by a seed-collected water mold induced early hatching in *R. sylvatica* eggs (Fig. 2*b*). Control eggs hatched, on average, after 12.7 ± 0.9 days. Eggs exposed to water mold hatched 2.5 days earlier than the controls ($F_{[1,41]} = 4.1$, $P = 0.049$). Survival was high in both treatments ($98.1\% \pm 0.6\%$).

Discussion

When infected with pathogenic water mold, both *B. americanus* and *R. sylvatica* embryos hatched precociously (Fig. 2). However, when exposed to cues indicating predation threats, only *B. americanus* responded by hatching early (Fig. 1). *Rana sylvatica* also does not alter hatching timing in response to cues from odonate tadpole predators (Anderson and Petranka 2003). Water molds are a widespread threat to fishes, urodeles, and anurans (e.g., Blaustein et al. 1994; Green 1999; St. Mary et al. 2004). To our knowledge, this is the first documented example of anamniote vertebrates accelerating hatching timing in response to infection from these ubiquitous pathogens.

Bufo americanus embryos responded to a greater variety of cues, and appeared to do so to a greater extent, than did *R. sylvatica*. *Bufo americanus* eggs hatched, on average, 44% earlier than the controls, and *R. sylvatica* hatched 19% earlier than the controls (Figs. 1, 2). Because we have observed high clutch mortality from water mold infection in both nature and in other laboratory experiments (Gomez-Mestre et al. 2006), but had low mortality in these experiments, these data probably provide a conservative estimate of how early embryos can hatch. *Bufo americanus* clutch 4, inoculated the earliest in development, had high survival (only 1 egg died out of 120 eggs) and showed the most dramatic change in hatching time: eggs inoculated with the seed-collected water mold hatched, on average, 63% (12.1 ± 0.5 days) earlier than the controls.

The difference in hatching time between *B. americanus* eggs infected with seed-collected and egg-collected water mold may reflect differences in the species of oomycetes included in the different inocula. Variation in either pathogenicity of or the embryonic hatching response to different pathogen strains would not be surprising. This does not, however, alter our fundamental conclusion that infection with pathogenic water mold can induce precocious hatching in both species examined. Of the eggs infected with water mold, *B. americanus* eggs inoculated with the seed-collected strain showed the most dramatic shift in hatching time. Many eggs infected with this strain of the water mold hatched at Gosner stage 17 (Gosner 1960), before achieving muscular response (at Gosner stage 18). Furthermore, embryos only hatched after physical contact with the growing water mold hyphae. We suggest two possible mechanisms for such early hatching. First, hyphae from the water mold grew directly into the egg capsules and may have actually initiated the hatching by perforating the capsules. Hyphal damage to the physical structure of the egg capsule may have enabled embryos to escape prematurely.

A second potential mechanism for such precocious hatching lies in the enzymatic nature of the hatching process, wherein proteolytic enzymes are released from hatching glands and are combined with physical movement of the embryo to escape the weakened egg capsule (Duellman and Trueb 1986). In anurans, this has been most extensively studied in *Xenopus laevis* (Daudin, 1802), *Hyla avivoca* Viosca, 1928, *R. japonica*, *B. bufo*, and *Bufo japonicus* Temminck and Schlegel, 1838 (Carroll & Hedrick 1974; Yoshizaki and Katagiri 1975; Yoshizaki 1978; Urch and Hedrick 1981; Yamasaki et al. 1990). In *X. laevis*, hatching glands are detectable at formation of the neural tube (Gosner stage 16; Gosner 1960) and secrete enzyme granules before formation of the tail bud occurs (Gosner stage 17; Yoshizaki and Yamasaki 1991; Yoshizaki 1991). We regularly observed *B. americanus* eggs (more often in infected eggs than in the controls) that appeared deflated, indicating a loss of integrity of the egg capsule (Fig. 3a compared with Fig. 3b). These eggs, however, were not considered hatched and did not factor into our measurements of hatching time. Enzymatic assays and histological examination of hatching glands would clarify if accelerated secretion of hatching enzymes is involved in oomycete-induced early hatching. At this point we cannot rule out a role for simple physical disruption of the egg capsule by water mold hyphae.

Fig. 3. (*a*) A *B. americanus* embryo from a control treatment, at Gosner stage 17 (tail-bud formation; Gosner 1960), surrounded by a turgid egg capsule. (*b*) A *B. americanus* embryo from a water mold infection treatment, also at Gosner stage 17, shown within a deflated egg capsule. (*c*) A string of control *B. americanus* embryos at Gosner stage 19–20 (heart beat and gill circulation). All embryos are still in the egg capsules as evidenced by the curvature of the body axis. (*d*) *Bufo americanus* hatchlings, also at Gosner stages 19–20, with water mold hyphae invading the egg capsules (in the upper left corner). Embryos have hatched and are adhered to the outside of the jelly string.
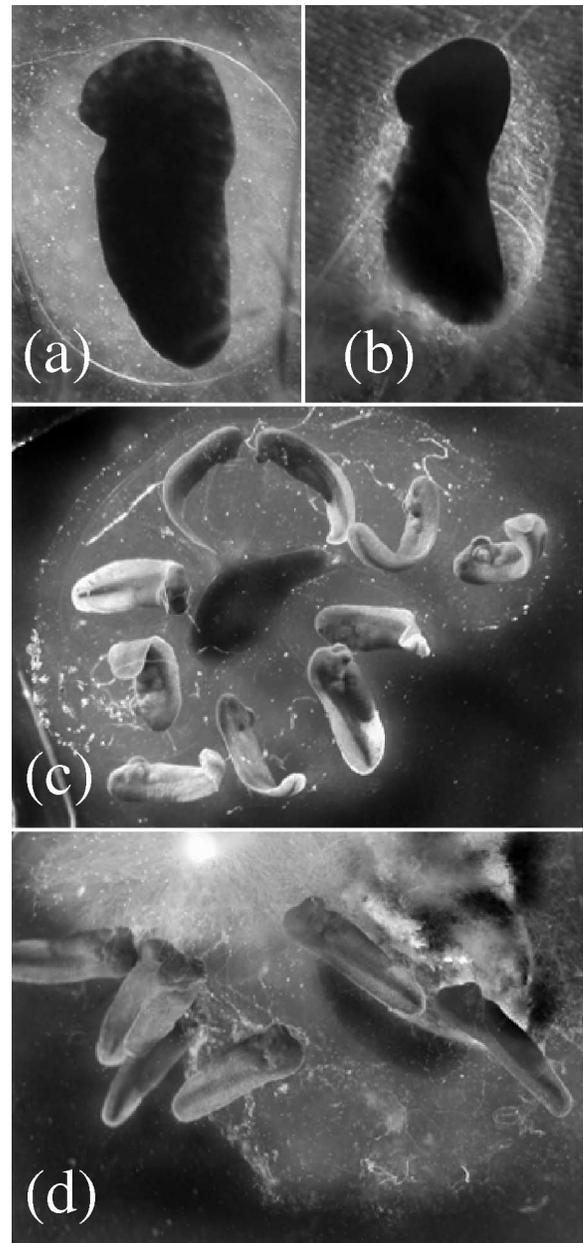
Puncturing of the egg capsule by water mold hyphae or enzymatic breakdown of the egg capsule may have occurred, but this does not explain the movement of embryos to outside the egg. An active embryonic role in exiting the deflated egg capsule appears necessary and appears to be a

response to the mold. Ciliary movement begins before muscular movement (Gosner stage 15; Gosner 1960) and aids hatching of *H. avivoca* and *Bufo vulgaris formosus* Okada, 1931 (= *B. japonicus*) (Volpe et al. 1961; Kobayashi 1954 cited in Duellman and Trueb 1986). Hatching enzymes combined with ciliary movement may also assist *B. americanus* in leaving the egg capsule before muscular response occurs.

As hypothesized, *B. americanus* embryos seemed to respond more strongly to water mold infection than did *R. sylvatica*. This may reflect different historical levels of water mold exposure faced by each species' eggs. *Rana sylvatica* breeds early in the year, when vernal pools are still cold (Paton and Crouch 2002; Skelly et al. 2002) and water mold growth is inhibited (Gomez-Mestre et al. 2006). *Bufo americanus*, however, breeds only after ponds have warmed (Paton and Crouch 2002; Skelly et al. 2002) and water mold growth rates have increased. Thus, *B. americanus* may have experienced greater exposure to active water mold than has *R. sylvatica*, resulting in the observed differences in hatching plasticity.

Water mold infection of egg clutches at our field site is substantially more frequent in *B. americanus* than in *R. sylvatica* (Gomez-Mestre et al. 2006). If levels of infection have been consistently high, we might expect a fixed early hatching response instead of a plastic one. However, field surveys indicate that water mold presence is also highly variable between ponds, even within close proximity of one another (Gomez-Mestre et al. 2006). Such spatial variation in infection rates could favor the evolution of a plastic response instead of a fixed one. There may also be temporal variation both within and between years. Given the behavioral plasticity in oviposition-site choice demonstrated by many anurans (e.g., Petranka et al. 1994), it would be interesting to know if *R. sylvatica* or *B. americanus* can discriminate between ponds with different levels of water mold.

The fact that both *R. sylvatica* and *B. americanus* hatched early in response to water mold infection, but that only *B. americanus* hatched early in response to egg or larval predation cues, may also reflect different historical risks from predators and pathogens. Like water molds, egg and larval predators are more likely to be present when *B. americanus* breeds, as they will have had more time to colonize the pond. The only predators that are potentially present when *R. sylvatica* breeds are overwintering odonates or notonectids. However, field surveys of our site have failed to find amphibian egg predators during *R. sylvatica* embryonic development (Gomez-Mestre et al. 2006). Since *R. sylvatica* altered hatching time in response to infection, but not to embryo predation cues (which were at an elevated concentration compared with those that *B. americanus* was exposed to), this pattern is consistent with the hypothesized differences in selection intensity by these different risks.

Bufo americanus embryos hatched early in response to crushed conspecific egg cues, as predicted. However, *B. americanus* eggs unexpectedly hatched early in response to both simulated conspecific and heterospecific (*R. sylvatica*) larval predation cues. We expected *B. americanus* to delay hatching when exposed to simulated larval predation cues, allowing them to increase size and developmental stage prior to hatching. Our results suggest three possible explanations. (1) *Bufo americanus* embryos may not have

been responding to alarm cues from crushed tadpoles, but instead to the presence of increased organic matter in the water, as an indicator of feeding opportunities. This, however, seems unlikely, as the most precocious hatchlings were at Gosner stage 17 (Gosner 1960) and they would not have been able to feed until at least Gosner stage 25 when the requisite mouthparts and gut have formed. (2) Predation cues released by embryos and larvae may be similar and evolutionarily conserved across taxa. If so, embryos would be unable to distinguish stage-specific or species-specific cues, and might thus interpret any predation cue as dangerous, regardless of the specific threat involved. (3) Even if cues are chemically distinct, *B. americanus* embryos may be unable to distinguish between conspecific and heterospecific, or embryo and larval predation cues. Therefore, *B. americanus* embryos would respond similarly to all of these cues no matter their origin. Hatching in response to larval predation cues would seem to be a maladaptive response for *B. americanus*, a pleiotropic cost of the potentially adaptive response to egg-stage risks (DeWitt et al. 1998).

Bufo americanus precocious hatchlings were not free swimming. Induced hatchlings remained adhered to the outside of the jelly surrounding the eggs, whereas control embryos remained in the egg capsule at the equivalent stage (Fig. 3c compared with Fig. 3d). Since precocious hatchlings were not free swimming, simply exiting the egg capsule would seem maladaptive, as it would likely make them more vulnerable to larval predators. We need to measure the relative importance of egg versus larval predators, and their effects on precocious hatchlings, to understand the potentially adaptive or maladaptive nature of induced hatching. Comparative analyses of embryonic responses in a phylogenetic framework would contribute to understanding the evolutionary context of hatching plasticity.

Organisms experience environmental variation during development, and appropriate responses to this variation can often improve survival and, ultimately, reproductive fitness (West-Eberhard 1989, 2003; Schlichting and Pigliucci 1998; Pigliucci 2001). Here, we supply evidence that the embryos of two anurans alter hatching timing in response to threats to current (both *B. americanus* and *R. sylvatica*) and subsequent life stages (only *B. americanus*). Furthermore, we document remarkable variability in hatching timing of *B. americanus* embryos, which responded to clutch infection by hatching an average of 44%, and as much as 63%, earlier than uninfected eggs. Induced hatching in response to infection may be adaptive, since water molds are known pathogens of these and other aquatic vertebrate eggs (e.g., Kiesecker and Blaustein 1997; Green 1999; Czeczuga et al. 2002; Wedekind 2002). Presumably, such a response to clutch infection is not fixed because of a cost or trade-off incurred in a different environmental context (e.g., Tollrian and Harvell 1999). We now need to measure the consequences of hatching early in different ecological contexts (e.g., Warkentin 1995) to assess the adaptive value of these responses.

Acknowledgements

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