

# Genetic variation in pathogen-induced early hatching of toad embryos

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## Abstract

Accelerated hatching is one of few defences available to embryos, and is effective against many egg-stage risks. We present the first analysis of genetic variation in hatching plasticity, examining premature hatching of American toad embryos in response to pathogenic water moulds. We reared eggs from half- and full-sib families in the presence and absence of water mould. Hatching age and hatchling size showed low cross-environment genetic correlations, suggesting that early-induced hatching can evolve largely independently of spontaneous hatching. We found less phenotypic and additive genetic variation for early-induced hatching than spontaneous hatching, and a stronger correlation between egg and induced hatchling sizes. Directional selection by the pathogen may have eroded variation in early-induced hatching, pushing it against the constraint of hatching gland development. Later hatching has a second, muscular component. This pattern of variation may characterize defences based on developmental transitions, although other inducible defences show more variation in induced phenotypes.

## Introduction

Inducible defences are adaptive plastic changes in phenotype that enable organisms to cope with heterogeneous biotic risks (Karban & Myers, 1989; Tollrian & Harvell, 1998). Theory predicts that inducible, rather than constitutive, defences will evolve in response to spatially and/or temporally heterogeneous selective pressures if there are reliable cues, a fitness trade-off between induced and uninduced phenotypes, and appropriate genetic variation (Dodson, 1989; Harvell, 1990; Tollrian & Harvell, 1998).

Inducible defences have been described for a wide array of organisms, including plants, vertebrates and invertebrates (Tollrian & Harvell, 1998). Studies of inducible defences have revealed the fitness trade-offs and costs shaping plasticity (Van Buskirk, 2000; Kopp & Tollrian, 2003), the nature of cues mediating the response (Schoeppner & Relyea, 2005; Warkentin,

2005; Warkentin et al., 2006), and the demographic and ecological consequences of plasticity (Vos et al., 2002). There is also ample evidence for the existence of genetic variation for inducible defences from a wide array of taxa (Parejko & Dodson, 1991; De Meester, 1996; Harvell, 1998; Agrawal et al., 2002; Relyea, 2005; Kraft et al., 2006). There remain, however, many unanswered questions about the genetics of inducible defences, including the relative contribution of additive and non-additive components, the amount of variance for induced and uninduced phenotypes, and potential limits on inducible phenotypes set by developmental constraints. It is unclear if we should expect a consistent trend in the amount and type of genetic variation for induced vs. uninduced phenotypes, or if such trends would be specific to different types of inducible traits. Many studies have analysed genetic variation for inducible defences in zooplankton species comparing clonal lines (reviewed in Lass & Spaak, 2003). Clonal analysis, however, provides only broad-sense heritability estimates and does not allow separation of maternal and other non-additive effects from additive variation (Lynch & Walsh, 1998). From the few studies to date from which it could be quantified, induced defensive phenotypes appear to have

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higher coefficients of additive genetic variation than uninduced phenotypes (Agrawal et al., 2002; Relyea, 2005; Kraft et al., 2006), although it may be premature to consider this a general trend.

We present an analysis of the genetic variation in inducible premature hatching of American toad (*Bufo americanus*) embryos in response to water mould infection. To the best of our knowledge, this is the first study on the genetic variation of an egg-stage inducible defence. Water moulds are important pathogens of fish and amphibian eggs worldwide (Blaustein et al., 1994; Kiesecker & Blaustein, 1995; Robinson et al., 2003), and are a contributing factor to the global amphibian decline (Kiesecker et al., 2001; Collins & Storfer, 2003). Embryos have only a small defensive repertoire (Hamdoun & Epel, 2007; Warkentin, 2007), and most egg-stage defences are of parental, often maternal, origin (Gomez-Mestre et al., 2006). Pathogens, however, may induce adaptive shifts in hatching timing, enabling embryos to escape infection (Warkentin et al., 2001; Moreira & Barata, 2005). *Bufo americanus* clutches are often infected by water moulds, although there is substantial variation in infection rates among ponds (Gomez-Mestre et al., 2006). Toad embryos reduce mortality from mould infections by hatching precociously in response to physical contact with the hyphae (Gomez-Mestre et al., 2006; Touchon et al., 2006). We used a full-sib/half-sib nested breeding design to obtain a series of *B. americanus* clutches and reared them in a common garden experiment where we manipulated the presence of pathogenic water moulds. Our aims were (1) to assess the level of genetic variation in hatching-related traits within pathogen-rich and pathogen-free environments; and (2) to assess the amount of genetic variation for adaptive hatching plasticity in *B. americanus* and its evolutionary potential.

## Materials and methods

### Field collection and controlled breeding

We collected water moulds from two ponds in Lynn Woods Reservation (Lynn, Massachusetts, USA) in late March 2005, after the ponds thawed and weeks before *B. americanus* began to breed. We sank 20 tea bags filled with sterilized hemp seeds in each pond as water mould baits (Robinson et al., 2003). We retrieved the seeds a week later, plated them on a sterilized cornmeal agar medium in Petri dishes together with fresh sterilized seeds, and incubated them at 11 °C. This procedure resulted in a mixed culture of *Achlya* sp. and *Saprolegnia* sp. water moulds (Gomez-Mestre et al., 2006; Touchon et al., 2006).

On 20 April we collected 30 female *B. americanus* from a single pond, all of which had been amplexed already but had not begun to lay eggs, and 15 randomly selected calling males. Males and females were separately distributed in large plastic containers filled with shallow water

and leaf litter, and transported to an environmental chamber at Boston University set at 11 °C and a 10 : 14 h light : dark cycle, as observed in the field. We randomly assigned two females to each male and placed them in plastic containers holding 5 L of carbon-filtered tap water plus 1 L pond water. Each container was divided in two with a fine mesh, and females were placed on opposite sides. Overnight, each male was sequentially allowed to amplex each female, while the mesh divider prevented mixing of the clutches. Fourteen males successfully mated with two females each, for a nested series of crosses consisting of 28 full-sib families and 14 half-sib families. This breeding design (North Carolina I) does not require killing adults, which were returned to their pond within 24 h, but neither does it allow partitioning of dominance from maternal effects (Kearsey & Pooni, 1996; Lynch & Walsh, 1998). In this design, the difference between variance components due to sire, and to dam nested within sire, is a function of dominance and epistasis, plus variance caused by maternal environment (Lynch & Walsh, 1998). As there is no maternal care in this species, the latter is probably restricted to differences in egg provisioning.

### Experimental mould infection

*Bufo americanus* clutches consist of a thin gelatinous string, usually containing over 4000 eggs. We cut 16 segments of 10 eggs each from each clutch, placed them in plastic cups with 115 mL of carbon-filtered tap water, and randomly assigned them to either mould infection or control treatments, for a total of eight replicates per cross in each treatment. For the mould infection treatment, we added one heavily infected hemp seed (i.e. showing hyphal growth) per cup. We added one uninfected, sterilized seed to the control cups. Replicates were arrayed in random blocks across four shelves in the environmental chamber, so that two replicates per cross and treatment were allocated to each block. An extra 15-egg segment was taken from each clutch to measure egg diameter. We measured eggs to the nearest 0.01 mm using an ocular micrometer mounted on a dissecting microscope. All replicates were checked daily until all eggs had either hatched or died. We recorded the date of each event (i.e. death or hatching) and preserved the first hatchling from each replicate in 10% formalin. These first hatchlings were later staged, digitally photographed through a dissecting microscope, and measured for total length using IMAGE J (version 1.33; National Institutes of Health, Bethesda, MD, USA). Seven experimental units contained eggs that did not develop (probably unfertilized) and were discarded, resulting in 218 control and 223 water mould cups. An additional nine hatchlings (five control, four mould treatment) were poorly preserved, precluding accurate length measurements; for these animals we have only hatching age and stage.

## Statistical analysis

All statistical analyses were conducted in SAS version 9.12 (SAS Institute, 2003). Sire, dam nested within sire and experimental block were all considered random factors, whereas environment (mould infection or control) was considered a fixed effect. Experimental block never had a significant effect but was included in the models when it increased the goodness of fit [i.e. reduced the Akaike information criterion (AIC)]. We tested for differences in survival between water mould and control environments to assess the pathogenic effect of the water mould. We also tested for 'sire - environment' and 'dam-within-sire - environment' interactions to test for variation in susceptibility to water mould infection. Survival was analysed by fitting generalized linear mixed models with an underlying binomial distribution and a logit link function using the GLIMMIX macro.

To avoid possible bias in analyses of hatchling traits (age, size, stage) caused by differential mortality between the treatments, in all cases we analysed only the first hatchling from each replicate, rather than averaging across surviving hatchlings. For hatching age and hatch-

ling size (total length), we tested for effects of environment (mould or control), sire, dam-within-sire and, to assess genetic variation in embryo response to infection, sire - environment and dam-within-sire - environment, via general linear mixed models using PROC GLM. Data on hatchling developmental stages violated parametric assumptions; we therefore used a Kruskal-Wallis analysis to test for environmental effects on hatching stage. To compare the overall variation in hatching age and hatchling size across sibships in each environment, we computed coefficients of variation and estimated their standard errors using 500 nonparametric bootstrapping replicates. Finally, we tested whether egg size was correlated with hatching age and hatchling size within each environment. As dams were nested within sires, we could not include all full-sibships in a single analysis because of potential sire effects on the hatching variables and hence non-independence of the data. We thus calculated Pearson's product-moment correlations from 14 full-sibships, randomly excluding one of every two dams nested within each sire. The results were robust to which dams were removed, and a similar pattern of correlations resulted if we used sire mean values or the full set of dams (ignoring non-independence of sires) instead.

Genotype - environment interactions may have a variety of biological causes. Thus, we calculated some within-environment variance components using restricted maximum likelihood (REML) in VARCOMP; these were in good agreement with estimates obtained through least squares. The variance component associated with differences among sires, assuming epistasis is negligible, estimates one-fourth of the additive genetic variance ( $V_A$ ) (Lynch & Walsh, 1998). All families were reared under

standardized laboratory conditions, so variance because of differences among dams, nested within sires, estimated one-fourth of  $V_A$  plus a non-additive component,  $V_{NA+M}$  (Via, 1984a). Assuming that epistatic sources of variation were negligible, we estimated a non-additive component of variance as  $V_{NA+M} = V_{DAM(SIRE)} + V_{SIRE}$  (Via, 1984a; Lynch & Walsh, 1998). Given our breeding design, this variance component encompasses both non-additive genetic ( $\frac{1}{4}$  dominance variance,  $V_D$ ) and maternal environmental effects. The residual variance includes the environmental variance ( $V_E$ ) plus  $\frac{1}{2} V_A$  and  $\frac{3}{4} V_D$  (Kearsey & Pooni, 1996; Laurila et al., 2002).

Heritability of hatching age and hatchling size within environment was calculated as:

$$h^2 = \frac{V_A}{V_P} = \frac{4r_{SIRE}^2}{r_{SIRE}^2 + \frac{1}{2}r_{DAM \& SIRE}^2 + \frac{1}{2}r_{BLOCK}^2 + r_{RESIDUAL}^2}$$

To facilitate comparisons of the estimates of genetic variability between infection and control treatments, and to those reported in other studies, we also calculated the coefficient of additive genetic variation (Houle, 1992):

$$CV_A = \frac{\sqrt{V_A}}{\bar{X}} \times 100$$

We tested for differences in  $CV_A$  between environments using the two-tailed test for differences between coefficients of variation described by Zar (1999).

We estimated the heritability of trait plasticities for hatching age and hatchling size across environments as (Groeters, 1988; Relyea, 2005):

$$h^2 = \frac{4V_{SIRE \times ENV}}{V_P} = \frac{4r_{SIRE \times ENV}^2}{r_{ENV}^2 + r_{SIRE \times ENV}^2 + r_{DAM \& SIRE \times ENV}^2 + r_{BLOCK}^2 + r_{RESIDUAL}^2}$$

Standard errors for variance components and heritability were calculated using bootstrap sampling with 500 replicates. We calculated cross-environment additive genetic correlation for hatchling size from the REML-estimated variance components as (Via, 1984b; Fox et al., 1999):

$$r_A = \frac{r_{SIRE}^2}{r_{SIRE-MOULD} \times r_{SIRE-CONTROL}}$$

We estimated its standard error using a jackknife procedure across sires (Laurila et al., 2002) as the estimate required variance components from different models (within and across environments). This method for calculating cross-environment genetic correlation was not applicable to hatching age because the additive variance component in the infection treatment equalled zero. Instead, we estimated its cross-environment correlation by calculating Pearson's product-moment correlation among sire means (Roff, 1997; Laurila et al., 2002; Relyea, 2005). We calculated standard errors for

Pearson's correlations using a bootstrap procedure across sires with 500 replicates.

## Results

### Embryo response to pathogenic water moulds

Water mould reduced embryo survival by 32% on average ( $v_{1,13}^2 = 25.40$ ,  $P < 0.0001$ ; Fig. 1). No sire effects were detected in susceptibility to pathogen infection ('sire - environment' interaction had no significant effect on survival), but there was a significant 'dam-within-sire - environment' interaction ( $Z = 2.15$ ,  $P < 0.016$ ), indicating that not all crosses suffered equal mortality. Mortality was 100% in only 17 of the 223 replicates in the water mould treatment (distributed across five crosses); all other replicates produced hatching data. Further comparisons among environments were thus based on 218 and 206 replicates in the control and water mould treatments respectively.

Embryos in the water mould treatment hatched significantly earlier than in the control treatment (by  $4.9 \pm 0.48$  days, mean  $\pm$  SE; Table 1, Fig. 1). Such early hatching resulted in less developed (Kruskal-Wallis,  $H_{1,N=413} = 86.50$ ,  $P < 0.0001$ ) and smaller hatchlings ( $23 \pm 3\%$  smaller, Table 1, Fig. 1). The modal hatching developmental stages for infected and control treatments were Gosner stages 17 and 18, respectively, which correspond to early tail bud and muscular contraction stages (Gosner, 1960). Early induced hatchlings in the water mould treatment showed no sign of infection and were viable.

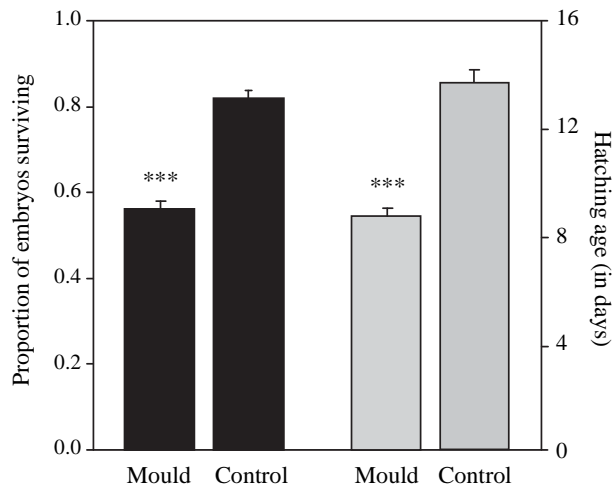


Fig. 1 Embryo survival (black bars) and average hatching age (grey bars) of *Bufo americanus* eggs in the presence or absence of pathogenic water moulds (*Saprolegniaceae*). Water mould exposure decreased embryo survival and embryonic period. Error bars denote SE. Asterisks indicate significant differences ( $P < 0.001$ ).

### Within-environment variation

Within-environment variance components, coefficients of additive variation and heritabilities are presented in Table 2. In the pathogen-free environment, additive genetic variances for hatching age and hatchling size were significantly higher than zero ( $F_{13,203} = 5.67$ ,  $P < 0.0001$  and  $F_{13,197} = 7.66$ ,  $P < 0.0001$ ), and resulted in heritability estimates of 0.20 and 0.55 respectively. In contrast, additive genetic variance for hatching age in the water mould treatment was not significantly different from zero ( $F_{13,178} = 0.65$ ,  $P = 0.774$ ). Hatchling size in the water mould treatment showed some additive genetic variation (significantly higher than zero,  $F_{13,175} = 5.17$ ,  $P < 0.001$ ), and heritability was higher than for hatching age, but  $V_A$  was lower than in the pathogen-free environment. Coefficients of additive genetic variation were significantly smaller in the infection treatment than in the control for both traits (hatching age:  $Z = 10.14$ ,  $P < 0.001$ ; hatchling size:  $Z = 3.21$ ,  $P < 0.002$ ). Non-additive genetic and environmental maternal effects ( $V_{NA+M}$ ) tended to be higher than additive effects for hatching age within each environment, whereas the reverse was true for hatchling size (Table 2). We tested the significance of these differences empirically by comparing the degree of overlap in the distribution of the estimates obtained by bootstrapping. We only detected significant differences between  $V_A$  and  $V_{NA+M}$  for hatching age, and only in the water mould environment ( $P = 0.008$ ). Egg size was not significantly correlated with hatching age in any case (control:  $r = 0.02$ ,  $P = 0.95$ ; mould:  $r = 0.07$ ,  $P = 0.82$ ), and was only significantly correlated with hatchling size in the presence of water mould (control:  $r = 0.25$ ,  $P = 0.40$ ; mould:  $r = 0.74$ ,  $P < 0.01$ ;  $N = 14$ ).

### Genotype-by-environment interaction

We observed differences among crosses in their hatching response to water mould, indicated by non-parallel reaction norms in Fig. 2, and significant 'dam-within-sire - environment' effects (Table 1). Variation in hatching age was lower in the presence of water moulds than in the pathogen-free environment, as indicated by a lower coefficient of variation ( $CV_A$ , Table 2) and a converging pattern of reaction norms (Fig. 2). This pattern was similar for hatchling size, and in both cases the overall phenotypic variance also differed significantly between environments (Levene's test of heterogeneity of variances, hatching age:  $F_{55,368} = 2.94$ ,  $P < 0.0001$ ; hatchling size:  $F_{55,359} = 4.49$ ,  $P < 0.0001$ ).

Additive genetic variances for plasticity in hatching age and hatchling size (reflected in  $V_{SIRE-ENV}$ , Table 3) were not significantly different from zero (Table 1), and hence neither was the heritability of plasticity for these traits. In contrast, the variance in plasticity because of variation among dams nested-within-sires ( $V_{DAM(SIRE)-ENV}$ ,

Table 1 Effects of pathogenic water moulds, sire and dam identity, and their interactions on hatching age and hatchling size of *Bufo americanus*.

Factor	Hatching age				Hatchling size			
	MS	df	F	P	MS	df	F	P
Environment	927.21	1	473.37	<0.0001	95.79	1	216.62	<0.0001
Sire	10.34	13	0.69	0.745	3.47	13	1.18	0.416
Dam-within-sire	11.73	14	2.07	0.094	1.66	14	1.03	0.481
Sire - environment	8.99	13	1.59	0.201	2.90	13	1.79	0.145
Dam-within-sire - environment	5.68	14	2.90	<0.001	1.62	14	3.66	<0.0001
Block	1.27	5	0.65	0.661	0.91	5	2.06	0.071
Error	1.96	362			0.44	353		

'Sire - environment' interactions indicate additive genetic effects for the plasticity of the trait. The 'dam-within-sire - environment' interaction includes non-additive genetic and environmental maternal components of plasticity in addition to any additive effects. Significant effects are shown in bold.

Table 2 Within-environment variance components and narrow-sense heritability ( $h^2$ ) of age and size at hatching for *Bufo americanus* exposed and not exposed to pathogenic water moulds as eggs.

Environment	$V_A$	$V_{NA+M}$	$V_{RES}$	$CV_A$	$h^2$
Hatching age					
Mould	0 (0.052)	0.616 (0.158)	1.014 (0.126)	0	0 (0.114)
Control	0.862* (0.266)	1.085 (0.421)	2.696 (0.296)	9.1	0.198 (0.245)
Hatchling size					
Mould	0.097* (0.017)	0.027 (0.023)	0.168 (0.019)	11.3	0.398 (0.274)
Control	0.661* (0.088)	0.141 (0.108)	0.699 (0.071)	21.8	0.554 (0.272)

All analyses are based on the first hatchling per replicate only. Variance components are additive genetic ( $V_A$ ), non-additive and maternal ( $V_{NA+M}$ ), and residual ( $V_{RES}$ ).  $CV_A$  are coefficients of additive variation. Asterisks indicate where additive genetic variance was significantly greater than zero, as revealed by likelihood ratio tests. Numbers in parenthesis indicate standard errors.

Table 3) was significantly different from zero in both traits (Table 1). Cross-environment genetic correlations for hatching age and hatchling size were low and not significant (Table 3).

## Discussion

The hatching of American toad embryos was substantially accelerated in the presence of pathogenic water moulds. The mean hatching time of mould-exposed embryos was 36% earlier, compared with hatching in the pathogen-free environment. This acceleration is within the range previously observed for *B. americanus* (Gomez-Mestre et al., 2006; Touchon et al., 2006) and close to the highest mean hatching acceleration reported for other taxa (39% in *Hyperolius spinigularis*, Vonesh 2005). These mean values, however, collapse substantial variation. Among crosses, hatching acceleration varied from undetectable (negative values) to 54% (Fig. 2). Unlike hatching timing, development rate was not affected by pathogen exposure. Thus variation in hatching timing generated concurrent variation in the size and developmental stage of animals entering the larval stage. We examine the sources of this variation in hatchling phenotypes, presenting the first study of genetic variation in hatching plasticity.

## Inducible embryo defences and early life stage risks

Early life stages, including embryos and hatchlings, often suffer high mortality and hence may experience strong selection for defences (Houde, 2002). Field studies at our collection locality (Lynn Woods, MA, USA) documented 25% egg mortality in water mould-infected clutches (Gomez-Mestre et al., 2006), which is close to the 32% mortality of pathogen-exposed eggs in our experiment. Water mould infections of amphibian eggs often result in much higher mortalities, particularly when they occur in combination with additional stressors (Blaustein et al., 1994; Beebee, 1996). Early hatching, however, is an effective defence against egg pathogens (Warkentin et al., 2001; Wedekind, 2002; Moreira & Barata, 2005; Gomez-Mestre et al., 2006).

Hatching plasticity is the most broadly documented inducible self-defence of embryos. In a diversity of taxa, environmentally cued hatching functions as a defence against a variety of risks, including egg and larval predators, harsh physical conditions in egg and larval environments, and larval resource limitation (reviewed in Warkentin, 2007; Warkentin & Caldwell, in press). Moreover, the variety of cues to which, and hatching mechanisms by which, embryos respond suggests that such responses have evolved multiple times

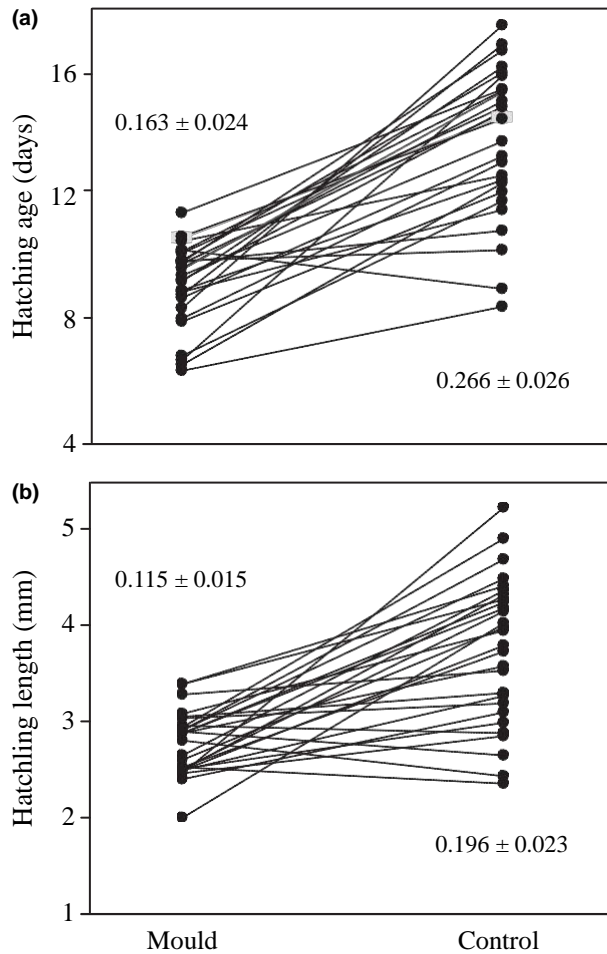


Fig. 2 Reaction norms for hatching age (embryonic period) and hatching size of *Bufo americanus* across environments differing in the presence of pathogenic water mould. Data are mean values for each of 28 full sibships, based on the first hatching in each replicate. Numbers indicate the coefficients of variation for each trait in each environment ( $\pm$ SE). Lack of parallelism among these reaction norms indicates the existence of genotype-by-environment interactions for both traits. Variation among crosses was substantially lower in the water mould environment, as indicated by the lower coefficients of variation.

	$V_{\text{SIRE-ENV}}$	$V_{\text{DAM(SIRE)-ENV}}$	$r_m$	$r_A$	Plasticity $h^2$
Age of first hatching	0.078 (0.121)	0.993 (0.211)	0.108 (0.254)	–	0.038 (0.064)
Size of first hatching	0.093 (0.046)	0.182 (0.052)	0.136 (0.162)	0.287 (0.227)	0.314 (0.144)

Variance components for the interactions of both sire and dam nested-within-sire with the environment ( $V_{\text{SIRE-ENV}}$  and  $V_{\text{DAM(SIRE)-ENV}}$  respectively) are shown. The 'sire - environment' component indicates additive genetic variance for plasticity and was used for calculating the narrow-sense heritability of plasticity ( $h^2$ ). The 'dam-within-sire - environment' component includes both additive and non-additive genetic effects plus maternal environmental effects on plasticity. Cross-environment correlations are also given (genetic,  $r_A$ , and Pearson's product-moment,  $r_m$ ). Numbers in parenthesis indicate standard errors.

independently. In this context, understanding the genetic variation underlying hatching plasticity is of value.

#### Variation in hatching traits within environments

We found substantial additive genetic variation for hatching age and size of *B. americanus* in the pathogen-free environment (heritability 0.20 and 0.55 respectively). These values are similar to those reported for other species in previous quantitative genetic studies of hatching time. For instance, heritability of hatching age was estimated to be 0.44 for a colony of lesser snow geese (*Anser caerulescens*; Findlay & Cooke, 1982), and  $0.22 \pm 0.72$  for a population of parasitic jaegers (*Stercorarius parasiticus*; Phillips & Furness, 1998). In a galling aphid (*Kaltenbachella japonica*), the within-tree, between-gall components of hatching time accounted for 0.18 and 0.37 of total phenotypic

secutive years (Komatsu & Akimoto, 1995).

We found less genetic variation for pathogen-induced

spontaneous hatchlings. This difference was indicated by a converging pattern of the reaction norms, and confirmed analytically through significant heterogeneity of variances and differences in the coefficients of additive genetic variation (Fig. 2, Table 2). Complete mortality in a few replicates reduced our sample size by 5.5% for the mould treatment, compared with the controls. This difference, however, is insufficient to account for the substantial difference in variance between treatments.

and long embryonic periods) to reduce variance in the mould-free environment and equalize sample size across treatments did not eliminate the variance heterogeneity. Non-additive and/or maternal effects on hatching age were, however, stronger than additive genetic effects in the mould environment.

#### Variation in hatching plasticity

The non-parallel reaction norms for hatching age and hatching size in Fig. 2 indicate variation in plasticity

Table 3 Quantitative genetic analysis of hatching plasticity of *Bufo americanus* embryos in response to pathogenic water moulds.



among crosses; some responded strongly to pathogen exposure and others not at all. Survival in the pathogen environment – not a trait in itself, but an indicator of the effectiveness of the anti-pathogen response – also varied among crosses. The ‘sire - environment’ interaction was, however, not significant for survival, hatching age or hatchling size. Thus we found no measurable additive genetic variance for the response to the pathogen, nor significant heritability of plasticity in hatching time and hatchling size. This may in part reflect our small sample size for sires. Nonetheless, it contrasts with the substantive additive genetic variation and clear heritability of hatching traits we found in the control environment.

It also contrasts with the significant ‘dam-within-sire - environment’ interactions for all three variables (survival, age and size; Tables 1 and 3). As all eggs were reared under standardized laboratory conditions, these indicate a non-additive genetic and/or maternal component to hatching plasticity and to its effectiveness as a defence. Our experimental design does not, however, allow us to separate these two sources of variation.

We observed low cross-environment genetic correlations for hatching age and hatchling size. This suggests that variation in early induced and spontaneous hatching may be under the control of different loci, with little linkage disequilibrium (Via, 1984b; Kraft et al., 2006). Relative independence of phenotypes between environments allows selection to shift the trait mean in one environment without incurring antagonistic pleiotropic effects in the other environment, and is a minimal requirement for the evolution of a new reaction norm (Via & Lande, 1985, 1987). In *B. americanus*, the ability of embryos to hatch prematurely in response to pathogens apparently does not entail correlated changes in hatching age under pathogen-free conditions. Such decoupling of genetic variation in early induced and spontaneous hatching could occur via differences in the mechanism of hatching at different ontogenetic stages.

### Non-additive effects and egg size variation

Non-additive genetic and maternal environmental effects often account for large amounts of phenotypic variance, particularly for life history traits and in early life stages (Falconer, 1981; Wade, 1998; Wolf et al., 1998), and may allow an adaptive response to selection even when additive genetic variation is low (Mousseau & Fox, 1998; Shaw & Byers, 1998). Maternal effects in amphibians are often associated with egg size and/or the amount and quality of protective jelly in the clutch (Parichy & Kaplan, 1995; Rasanen et al., 2003; Gomez-Mestre et al., 2006). The egg size variation among clutches in our study ( $1.06 \pm 0.03$  mm; CV = 8.6%) falls within the range observed in several other amphibians, which has been associated with maternal effects on hatchling size (reviewed in Kaplan, 1998).

Egg size was correlated with hatchling size in the presence of water mould, but not in the pathogen-free environment. Particularly given the greater variance in hatchling size in the absence of mould, its lower correlation with egg size in that context suggests that different mechanisms affect hatchling size in the two environments. There may be tighter developmental constraints on early hatching in the mould environment than on spontaneous hatching without the pathogen. Egg size was not correlated with hatching age in either environment, congruent with other amphibian studies (Parichy & Kaplan, 1995; Rasanen et al., 2003). This suggests that if the observed non-additive variation in hatching age, reflected in the ‘dam-within-sire’ main and interaction effects, has any maternal environmental component, it is not mediated by egg size.

### Selection, constraints and genetic variation in risk-induced phenotypes

Given the pervasiveness of Saprolegniaceae in temperate water bodies (Blaustein et al., 1994; Robinson et al., 2003), the high levels of infection observed at our field site (Gomez-Mestre et al., 2006), and the effectiveness of hatching as a defence, water moulds are likely to have imposed strong and consistently directional selection for earlier hatching in *B. americanus*. This is congruent with the lower variation we found in induced vs. spontaneous hatching (Fig. 2, Table 2). In contrast, the optimal hatching age or stage in a pathogen-free environment probably varies with multiple factors that affect embryo and hatchling development and survival (e.g. yolk reserves, oxygen availability, temperature, aquatic predators, water chemistry; Warkentin, 2007).

In contrast to our results, other studies of additive genetic variation in plastic defences found greater variation in the induced phenotypes, rather than the uninduced phenotypes (Agrawal et al., 2002; Relyea, 2005; Kraft et al., 2006). Similarly, most studies of zooplankton clones found patterns of equal or greater genetic variation for predator-induced phenotypes than for uninduced phenotypes (Boersma et al., 1998; Stirling & Roff, 2000). The main exception is neonate size, which shows less variance in the presence of predator cues (Boersma et al., 1998). However, predators also induce smaller size of females at maturation, which may constrain variance in offspring size. As all these induced defences presumably evolved under selection, the argument that selective history by itself is enough to explain lower variation in induced phenotypes appears insufficient as a general explanation.

The range of phenotypes in inducible defences could be limited by costs of plasticity or of defence (Agrawal et al., 2002; Relyea, 2002), but such costs may rarely impose sharp boundaries. In contrast, accelerating the timing of ontogenetic switch points depends on the prior development of mechanisms for both detecting cues and

effecting the life stage transition (Warkentin, 2007). Responses such as accelerating metamorphosis to escape a desiccating pond or hatching prematurely to evade a pathogen are therefore subject to clear developmental constraints. Consistent with this, spadefoot toad tadpoles reared in short-lasting ponds show less additive genetic variance for age and size at metamorphosis than tadpoles raised in long-lasting ponds (Newman, 1988). Similarly, our studies of hatching plasticity in red-eyed treefrogs also reveal less genetic variation in induced premature hatching than in spontaneous hatching (I. Gomez-Mestre & K. M. Warkentin unpublished).

We have never observed *B. americanus* to hatch before the tail bud stage (Gosner, 1960; stage 17); less developed embryos overgrown by water mould die (Gomez-Mestre et al., 2006; Touchon et al., 2006). Hatching of full-term bufonid embryos occurs by a combination of enzymatic weakening and muscular rupture of the egg capsule (Duellman & Trueb, 1986). Premature *B. americanus* hatchlings are not capable of muscular contraction, thus must rely solely on enzymatic degradation (Touchon et al., 2006). In *Bufo japonicus* and *Xenopus laevis*, hatching glands become active just before the tail bud is formed, i.e. Gosner stage 17 (Yamasaki et al., 1990; Yoshizaki, 1991). If hatching gland development is similarly timed in *B. americanus*, this could constrain how early embryos can hatch. The low additive genetic variance we observed for hatching time under pathogen exposure may thus reflect a combination of past directional selection and this developmental constraint. This could be tested by an analysis of variation in hatching gland development in *B. americanus*.

## Conclusions

Early hatching is an effective defence against pathogenic water moulds in *B. americanus*, allowing relatively high survival even with high infection rates. Low cross-environment genetic correlations between spontaneous hatching and pathogen-induced early hatching suggest that premature hatching could evolve as an inducible defence in *B. americanus* without pleiotropic effects on spontaneous hatching time. Additive genetic variation for early hatching is substantially lower than that for spontaneous hatching, suggesting that genetic variation has been eroded by selection imposed by water moulds and/or other egg-stage risks. This reduction in additive genetic variation might have been accentuated by a constraint on the developmental onset of hatching competence, which takes place at the early stages of tail bud development and depends on prior formation of the hatching glands. We need further studies of genetic variation for inducible defences to assess the importance of non-additive effects, the limits of the induced phenotypic response, and whether the pattern of reduced genetic variation in induced phenotypes is characteristic of plasticity in ontogenetic switch points.

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