# Risk-induced hatching timing shows low heritability and evolves independently of spontaneous hatching in red-eyed treefrogs

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*Keywords:*

*Agalychnis*;
heritability;
inducible defences;
life-history evolution;
limits of plasticity;
quantitative genetics.

## Abstract

Plasticity in the timing of transitions between stages of complex life cycles allows organisms to adjust their growth and development to local environmental conditions. Genetic variation in such plasticity is common, but the evolution of context-dependent transition timing may be constrained by information reliability, lag-time and developmental constraints. We studied the genetic architecture of hatching plasticity in embryos of the red-eyed treefrog (*Agalychnis callidryas*) in response to simulated predator attacks using a series of paternal and maternal half-sibs from a captive breeding colony of wild-collected animals. We compared the developmental timing of induced early hatching across sibships and estimated cross-environment genetic correlations between induced and spontaneous hatching traits. Additive genetic variance for induced early hatching was very low, indicating a constraint on the short-term evolution of earlier hatching timing. This constraint is likely related to the maturation of the hatching mechanism. The most plastic genotypes produced the most extreme spontaneous hatching phenotypes, indicating that developmental range, per se, is not constrained. Cross-environment genetic correlation in hatching timing was negligible, so the evolution of spontaneous hatching in this species has not depended on the evolution of risk-induced hatching and vice versa.

## Introduction

Most organisms have complex life cycles and timing of transitions between life stages is critical; it determines size and developmental state at transitions, which strongly affect how organisms interact with their sequential environments (Werner, 1988; Pechenik, 2006; Gomez-Mestre *et al.*, 2010). The timing of life-history switch points often varies plastically and variation in such plasticity has a genetic basis, whether it relates to hatching (Gebhardt & Stearns, 1988; Phillips & Furness, 1998), metamorphosis (Newman, 1988; Laurila *et al.*, 2002), diapause induction (Roff & Bradford, 2000) or time of first reproduction (Silverstein & Hershberger, 1992).

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For most animals, hatching is their first major life-history transition and substantially alters their abiotic and biotic environment. As with other such transitions, the timing of hatching in relation to both embryonic development and environmental conditions can be under strong selection (Sih & Moore, 1993; Gomez-Mestre & Warkentin, 2007). The evolution of hatching timing, which ranges across metazoa from the blastocyst stage to juveniles resembling small adults, has long been of interest (e.g. Shine, 1978). Recent syntheses have revealed that plastic, environmentally cued timing of hatching is widespread in bilateria (Christy, 2011; Doody, 2011; Warkentin, 2011a,b; Whittington & Kearn, 2011). Embryos respond to diverse cues associated with factors that affect survival within and outside the egg. Some taxa accelerate hatching in response to egg-stage risks, while others delay hatching to improve their chances of post-hatching survival. Some elements of the mechanisms that enable plasticity in hatching may be ancient, conserved

traits, while others have likely evolved convergently in multiple taxa (Warkentin, 2011a,b). However, we currently know very little about these mechanisms or their evolution.

As with any trait, the evolution of plasticity may be limited by reduced genetic variation, for instance in isolated, inbred populations (Auld & Relyea, 2010) or by strong genetic correlations with other traits (Falconer & MacKay, 1996). It may also be limited by cross-environment self-correlations (Via & Lande, 1985; Gomulkiewicz & Kirkpatrick, 1992; Scheiner, 1993). Rapid evolution of plasticity, however, suggests that wild populations must often harbour substantial genetic variation for trait plasticity (e.g. Pigliucci *et al.*, 1999; Van Buskirk & Arioli, 2005; Lind & Johansson, 2007). Hatching timing is often genetically variable (Phillips & Furness, 1998; Gomez-Mestre *et al.*, 2008a). However, consistent directional selection on hatching timing in particular inducing environments could deplete such variation, compared to that measured for uninduced or spontaneous hatching (Gomez-Mestre *et al.*, 2008a).

Of the various constraints on the evolution of plasticity that have been proposed (DeWitt *et al.*, 1998; Van Kleunen & Fischer, 2005; Auld *et al.*, 2009), three seem particularly relevant to hatching timing. These are information reliability, lag-time and developmental range limits. Information reliability limits phenotype-environment matching and may arise from imperfect correlation between cues and conditions or errors in cue assessment. For embryos, cue assessment will also be constrained by the development of sensory systems (Warkentin, 2011b). The lag-time from cue detection to phenotype expression limits the value of plastic responses if environments change rapidly. Lag-times for cued hatching responses depend on the mechanism and process of hatching, which can occur within seconds (e.g. grunion [Griem & Martin, 2000]; monogeneans [Whittington & Kearn, 2011]; red-eyed treefrogs [Warkentin, 2007]) or take place over days (birds, [Oppenheim, 1972]; *Rana japonica*, [Yoshizaki, 1978]). Hatching would not evolve as a plastic response to a particular cue if the duration of the hatching process exceeded the time between a cue to impending egg mortality and the occurrence of that mortality. Thus, if limited by lack of reliable cues or lag-time limits, adaptive plasticity will not evolve and we would expect bet-hedging to evolve instead (Simons, 2011).

In principle, the evolutionary success of plastic genotypes could be constrained by developmental range limits if extreme phenotypes are favoured, plastic genotypes produce a range of intermediate phenotypes, and environmental specialists produce the most extreme phenotypes (DeWitt *et al.*, 1998). To date there is little evidence for this, as plastic genotypes often produce the most extreme phenotypes (e.g. Auld *et al.*, 2009). Nonetheless, even if they do not limit the existence of plasticity, developmental constraints limit the range of possible phenotypes that an organism can produce. For instance, development affects the ability of animals to survive within and outside the egg as well as to exit from the egg, thus limiting possible hatching times (Warkentin, 2007, 2011a).

We hypothesize that the 'spontaneous' timing of hatching, and of life-history transitions more generally, that occurs without clear inducing factors is likely to be influenced by many genetic and environmental factors of small effect and hence show greater variation than when the transition is induced. Any bet-hedging evolved in response to selective factors that do not provide reliable cues (Simons, 2011) will also contribute to variation in 'spontaneous' hatching time. In contrast, when clear cues indicate a strong, immediate egg-specific risk (i.e. embryos must hatch or die) we expect embryos to hatch as soon as they are able to detect the cue and effect the response, substantially reducing the possible sources of variation. Indeed, hatching timing under acute risk may be limited by a single trait, the final requirement for hatching competence (Warkentin, 2011a), and variation in that trait may be eroded by consistent directional selection in the inducing context.

In this study, we assessed quantitative genetic variation of hatching timing and plasticity in the red-eyed treefrog, *Agalychnis callidryas*. Red-eyed treefrog embryos experience high predation rates in the field (Warkentin, 1995; Gomez-Mestre & Warkentin, 2007) and have evolved the ability to escape egg-stage risks by hatching early (Gomez-Mestre *et al.*, 2008b). Treefrog embryos obtained from a breeding colony in the laboratory were allowed to hatch spontaneously or induced to hatch early via physical disturbance simulating a predator attack, that is, an acute egg-stage risk. Environmental conditions for egg incubation in the laboratory were based on those experienced by the parental population in the wild, but standardized to minimize uncontrolled environmental effects on development rate and hatching timing. Heritability estimates obtained from laboratory-reared individuals are often strongly correlated with and reliable indicators of estimates obtained in the field (Roff, 1997). We determined the relative importance of additive vs. nonadditive genetic components and estimated the degree of cross-environment genetic correlation in hatching timing. We hypothesized that induced early hatching would show less genetic variation than spontaneous hatching.

## Materials and methods

### Study system

Red-eyed treefrogs are Central American phyllomedusines that lay eggs in gelatinous masses attached to plants over rainforest ponds, so hatched tadpoles fall into the water (Duellman, 2001). They suffer high egg mortality from predation by arboreal snakes (24% to

> 60% of clutches attacked, range across ponds and years; Warkentin, 1995; Warkentin, 2000; Gomez-Mestre & Warkentin, 2007), and are also exposed to other risks such as predation by social wasps, fungal infections, and hypoxia due to flooding (Warkentin, 2000, 2007; Warkentin *et al.*, 2001; Gomez-Mestre & Warkentin, 2007). Hatching-competent embryos hatch rapidly in response to physical disturbance by predators; in snake attacks, on average, the first embryo hatches in 16 s and all embryos have hatched, or been eaten, in 4.8 min (Warkentin *et al.*, 2007). This response to snakes is mediated at least in part by vibrations in egg clutches (Warkentin, 2005; Warkentin & Caldwell, 2009). Through most of the plastic hatching period about 80% of embryos in attacked clutches successfully escape from snakes, but escape rates are lower and more variable at the onset of hatching competence (Gomez-Mestre & Warkentin, 2007); the same pattern holds in wasp attacks (Warkentin *et al.*, 2006a). In flooding, young embryos drown but hatching-competent embryos all hatch; hatching in response to flooding is cued by hypoxia and slower than the response to physical disturbance (Warkentin, 2002, 2007).

A strong hatching response to flooding is evident in all phyllomedusines tested to date, and appears to be a conserved ancestral trait in the clade (Gomez-Mestre *et al.*, 2008b). Rapid snake-induced hatching occurs in several species of *Agalychnis* and the closely related *Pachymedusa*; however, two congeners syntopic to *A. callidryas* have low escape success in snake attacks, at developmental stages when they readily hatch if flooded (Gomez-Mestre *et al.*, 2008b). Thus, hatching responses to different risks and cues have changed independently. Nonetheless, the capacity for hatching acceleration, or proportional difference between the earliest induced and modal spontaneous hatching time, is highly conserved ranging from 28 to 36% across all species studied despite two-fold variation in modal hatching timing (Gomez-Mestre *et al.*, 2008b). All species achieve hatching competence early in Gosner developmental stage 23 (Gosner, 1960; Gomez-Mestre *et al.*, 2008b), suggesting a developmental constraint.

At field sites in Costa Rica and Panama, we have observed moderate variation among clutches in the onset of inducible hatching of *A. callidryas* embryos, with a similar range evident in clutches attacked by snakes or by wasps and clutches artificially stimulated by physical disturbance or by flooding in the laboratory. Clutches can first be induced to hatch from early morning to mid-day at age 4 days in Gamboa, Panama, or 5 days in Corcovado, Costa Rica. We observe greater variation among clutches in the peak of spontaneous hatching; this occurs during the evening at age 6 or 7 days in Panama, and 7 or 8 days in Costa Rica. In the field, and for clutches reared under semi-natural conditions at our field laboratory, there are many environmental variables that may contribute to this variation. Thermal differences could explain the geographical variation, and day-to-day fluctuations in temperature likely determine if spontaneous hatching peaks at age 6 or 7 days in Panama (Warkentin, personnel observation). Egg dehydration accelerates hatching (Salica *et al.*, 2012) so variation in humidity and rainfall could affect hatching patterns of clutches undisturbed by predators. Moreover, laying times vary, so diel peaks in temperature fall at slightly different developmental stages for different clutches. To assess genetic effects on hatching timing, and particularly to facilitate detecting such effects in the apparently small range of variation in the onset of inducible hatching, it was necessary to control these sources of environmental variation.

### Animal housing and breeding

Adult red-eyed treefrogs were collected in summer 2003 from ponds in the humid premontane forest of Costa Rica, near Guayacán, Limón, under permits from the Ministerio de Ambiente y Energía. Frogs were brought to an animal facility at Boston University where they were housed in groups of up to three in 60 × 30 × 60 cm glass aquaria with screen lids. Each aquarium was provided with a potted plant (*Epipremnum* sp.) and a water bowl with carbon-filtered dechlorinated water. Temperature in the room ranged from 23 to 28 °C, and relative humidity from 45 to 90%. Frogs were maintained on a 12 : 12 light cycle using full spectrum bulbs and gradually clock-shifted so their nocturnal activity period occurred during our daytime to facilitate monitoring of breeding and other activity. Frogs were fed crickets every other day, and crickets were dusted with a vitamin complex powder once a week.

Breeding was stimulated using three rain chambers (Fenolio, 1996). Glass aquaria as above were filled with water to 10 cm depth and covered with a 3 cm deep plexiglass reservoir in which small holes (1.5 mm diameter) had been drilled every 3 cm. A small electric water pump (Mini-Jet 606, Aquarium Systems, Mentor, OH, USA) submerged in the tank pumped water through a PVC tube to the reservoir, which drained into the tank simulating rain. The water pump was controlled by a timer, allowing custom rain cycles. Each rain chamber was divided in half with a plexiglass wall to allow six simultaneous pairings. We placed one unrooted cutting of *Epipremnum* or *Philodendron* inside each subchamber to provide cover and perching sites for the frogs while keeping the water clear. Despite the plants, frogs sometimes laid eggs on the chamber's walls. We therefore lined the chambers with clear plastic so we could remove clutches intact by cutting the plastic. Frogs moved from their home tanks into the rain chambers were allowed to acclimate for 24 h with a short pulse of rain (30 min). Rain cycles were

gradually increased to 3 h duration (1 h before dark and 2 h afterwards) until eggs were laid. Pre-recorded *A. callidryas* mating calls were played to simulate a breeding chorus, but no hormonal manipulation was necessary. Frogs normally came into amplexus a few hours to a day after we placed them in rain chambers, and females laid eggs on average 2.7 days after being placed in the chambers (±0.46 SE, $n = 22$). As soon as any amplexus was observed, we monitored the chambers every two hours to record egg-laying time. Time of oviposition was taken as the time when the eggs were first found, hence introducing a maximum error of 2 h. After eggs were laid, frogs were returned to their housing tanks. We allowed at least three months in between breeding attempts.

### Experimental design

Our breeding programme followed a partial diallel with partial overlap design (Lynch & Walsh, 1998), where 11 males were crossed with 13 females to obtain a series of 22 sibships. This breeding design maximizes the ability to estimate genetic effects when only a limited number of families can be raised (Travis *et al.*, 1987; Roff, 1997). Each male was mated to two females, but only nine females could be mated to two males because two of the females initially crossed died before they could be mated again. All crosses took place between May 2004 and September 2006, under identical environmental conditions.

Egg colour variation suggests that environmental maternal effects were reduced under our standardized housing conditions. Most females collected were found laying eggs or did so shortly after collection, and egg colour varied among individuals. In *A. callidryas*, egg colour is uniform within and variable among clutches; most eggs are green or yellow, and a minority are turquoise blue (Garcia, McCoy, Hughey, Vonesh & Warkentin, in prep.). However, all clutches laid by those same females in our lab colony were turquoise, indicating that egg colour is plastic and that standardized conditions, presumably diet, had eliminated variation in that nongenetic maternal effect.

Clutches were removed from the rain chambers, attached to plastic support cards, and placed above 3 mm of water in 350-mL plastic cups. We kept all clutches in incubators (Percival Scientific Inc., Perry IA, USA) at 26 °C, 80–90% relative humidity, and a 12 : 12 photoperiod, and sprayed them with distilled water every few hours to maintain egg hydration. To obtain replicate measures of the timing of earliest induced hatching and spontaneous hatching for each sibship, we split each clutch into several groups of eggs. This was necessary because hatching times within a contiguous egg mass might be influenced nonindependently by shared environmental factors or sibling interactions. We allowed embryos to develop for 3 days before splitting the clutches at Gosner stage 22 (tail fin circulation and melanophores extending across the venter [Gosner, 1960; Pyburn, 1963]). This minimized risk of egg dehydration but ensured that clutch partitioning was done before embryos reached hatching competence (Gomez-Mestre & Warkentin, 2007; Gomez-Mestre *et al.*, 2008b). We used 8–10 subsets of three to five eggs per sibship, depending on clutch size, to assess early-induced hatching. Using minimum subsets of three eggs kept egg surface exposure and direct sibling contact within the range commonly found in entire clutches. Another 8–10 subsets of three to five eggs were monitored for spontaneous hatching timing. In both cases, replicates were distributed among three shelves inside the incubator, keeping an even number of replicates per treatment ('induced' or 'spontaneous') and randomizing their position within the shelf. We conducted both induction trials and surveys of undisturbed eggs every 2 h from 8:00 h to 24:00 h, including the entire dark period when most spontaneous hatching occurs (Gomez-Mestre *et al.*, 2008b), and every 4 h in between. Our previous experience of clutch monitoring in the field and in the laboratory with *A. callidryas* show consistency across these environments in the onset of hatching competence and patterns of undisturbed hatching.

We placed eggs for scoring of early-induced hatching inside Petri dishes (40 mm in diameter) lined with water-soaked absorbent paper. We began hatching induction trials on the evening of their third day of age (3 day, ca. 90 h from oviposition). During each trial, we applied intermittent physical disturbance (Warkentin *et al.*, 2006b) to the eggs in the Petri dish, using forceps to roll them and apply gentle pressure to simulate a predation event, being careful not to damage the egg capsule (Gomez-Mestre *et al.*, 2008b). Eggs were physically disturbed for a total of 1 min each, spread over a period of several minutes. The time of the first hatching event within each Petri dish was recorded, and the hatchling preserved in 10% buffered formalin for morphological analysis. Regardless of the number of eggs in each dish (3–5), we only used data from the first hatchling to avoid pseudoreplication, resulting in 8–10 replicates per sibship.

The stimulus was designed to elicit as strong a hatching response as possible without damaging embryos or eggs, informed by observations of disturbance patterns in predator attacks (Warkentin, 2005), responses of embryos to vibration playback experiments (Warkentin, 2005; Warkentin *et al.*, 2006b, 2007) and our experience using similar physical disturbance of egg clutches to generate premature hatchlings for tadpole experiments (e.g. Warkentin, 1995, 1999; Gomez-Mestre *et al.*, 2008a,b; Rogge & Warkentin, 2008). We used manual physical disturbance rather than recorded vibrations in this experiment because the former provides a stronger and more multifaceted stimulus that more reliably induces hatching of newly hatching-competent eggs in our fieldwork

(Warkentin and Caldwell unpublished). Predator cues include pressure, displacement and tactile elements in addition to vibrations. Vibrations are an important factor in snake attacks, but may be unimportant in wasp attacks, or at least insufficient, based on playback experiments (Caldwell and Warkentin unpublished). We did not use flooding or hypoxia because embryos at the onset of hatching competence may take over an hour to hatch in response to this cue (Warkentin unpublished), meanwhile suffering reduced metabolic rates (Rogge & Warkentin, 2008). Since we needed to test embryos repeatedly, starting from before hatching competence, to determine when they would first respond, a hypoxia stimulus would have altered their development rate.

Egg subsets for determination of spontaneous hatching times were left attached to leaves and mounted on plastic cards over 3 mm of water in plastic cups (Gomez-Mestre *et al.*, 2008b). Except for periodic gentle misting, these clutch portions were left undisturbed. We recorded the time of the first hatching event per clutch subset and preserved the hatchling, also resulting in 8–10 replicates per sibship. To avoid pseudoreplication, we only kept one data point per replicate, and by using the data on the first hatchling per replicate instead of the average across all siblings in each replicate we avoided common-environment effects. We took digital images of all preserved hatchlings through a dissecting microscope and took four measurements using Image J (version 1.33, National Institutes of Health, Bethesda, MD): total length, yolk depth, body length, tail depth.

### Statistical analyses

As a first approach to quantify variation in hatching timing among sibships, we used a multivariate linear model to test for effects of sibship identity (entered as a random factor), experimental treatment (induced or spontaneous hatching) and their interaction on hatching time and all four morphological measurements. We included shelf as a random factor in the model but found it to have no effect and thus removed it from further analyses. We then used the software ASReml 3 (VSN International, UK; Gilmour *et al.*, 2006) to fit a general linear model for each variable within each environment including 'sire' and 'dam' as main and random factors, plus their interaction, and obtained restricted maximum likelihood estimates of the variance components associated with each term in the model and their standard errors. We used linear combinations of the variance components obtained to estimate additive, maternal and dominance variance components ($V_A$, $V_M$, $V_D$) such that $V_A = 4 \times \sigma^2_{sire}$; $V_M = \sigma^2_{dam} - \sigma^2_{sire}$; $V_D = 4 \times \sigma^2_{sire \times dam}$ (Falconer, 1981; Lynch & Walsh, 1998). We also calculated narrow-sense heritability as $h^2 = V_A/V_P = V_A/(V_A + V_M + V_D + V_E)$ where $V_E$ was the environmental variance ($V_E = \sigma^2_{residual} - (1/2\ V_A) - (3/4\ V_D)$) (Travis *et al.*, 1987). Moreover, we calculated the coefficient of additive genetic variation ($CV_A = (\sqrt{V_A}/x) \times 100$; Houle, 1992), and tested for differences in $CV_A$ among environments with the two-tailed test for differences between coefficients of variation described by (Zar, 1999). We calculated the heritability of trait plasticity as $h^2 = 4 \times \sigma^2_{SIRE \times ENV}/V_P = 4 \times \sigma^2_{SIRE \times ENV}/\sigma^2_{SIRE} + \sigma^2_{DAM} + \sigma^2_{SIRE \times DAM} + \sigma^2_{SIRE \times ENV} + \sigma^2_{DAM \times ENV} + \sigma^2_{RESIDUAL}$. To test for cross-environment genetic correlations, we fitted a series of bivariate linear models where we tested for covariance of the same trait across environments (Via, 1984), so that $r_A = \sigma^2_{SIRE}/\sigma_{SIRE\_induced} \times \sigma_{SIRE\_spontaneous}$.

## Results

Embryos hatched within seconds of simulated predation, and much earlier in development that they would have hatched if left undisturbed. The average onset of spontaneous hatching across sibship means was 154.7 h after oviposition (± 11.3 SD; range 118–178), but embryos could be induced to hatch 29.9% earlier on average (range 19.5–35.4%) by mechanical stimulation (108.9 ± 3.3 h after oviposition, mean ± SD; range 98–120). We observed important differences among sibships in their response to embryonic environment, reflected in a significant multivariate 'sibship × environment' interaction (Wilkinson's $\lambda = 0.017$, $F_{86, 782} = 60.21$, $P < 0.0001$). Moreover, there was consistent asymmetry in the extent of trait variation across environments, with the coefficients of variation always being greater for spontaneous hatching (Fig. 1b). This asymmetry was particularly marked for hatching time, where reaction norms were highly convergent for early-induced hatchlings and strongly divergent for spontaneous ones (Fig. 1b).

Induced early hatchlings were less developed than spontaneously hatched ones, judging from overall morphology. Standard anuran developmental staging tables are not useful for hatching-competent or newly hatched *A. callidryas* (Warkentin, 2007). These embryos reach hatching competence at Gosner stage 23 (Gosner, 1960), the last stage with bilateral external gills. Successive stages are based on gill resorption, but *A. callidryas* retain gills until hatching, then reabsorb them rapidly after entering the water, regardless of age or the development of other traits (Warkentin, 2007). Substantial development occurs within the egg during the plastic hatching period, despite gill maintenance (Warkentin, 1999). Induced hatchlings were on average 22% shorter than spontaneous hatchlings (early hatched: 9.98 mm, spontaneously hatched 12.75; $F_{1,434} = 971.38$, $P < 0.0001$, Fig. 1a, c). Controlling for body size, induced hatchlings had more bulbous yolk sacs (Fig. 1a, c; 77% deeper relative to total length; ANCOVA: $F_{1,433} = 115.66$, $P < 0.0001$) and relatively deeper tails (9.3% deeper; ANCOVA: $F_{1,433} = 52.81$, $P < 0.0001$).

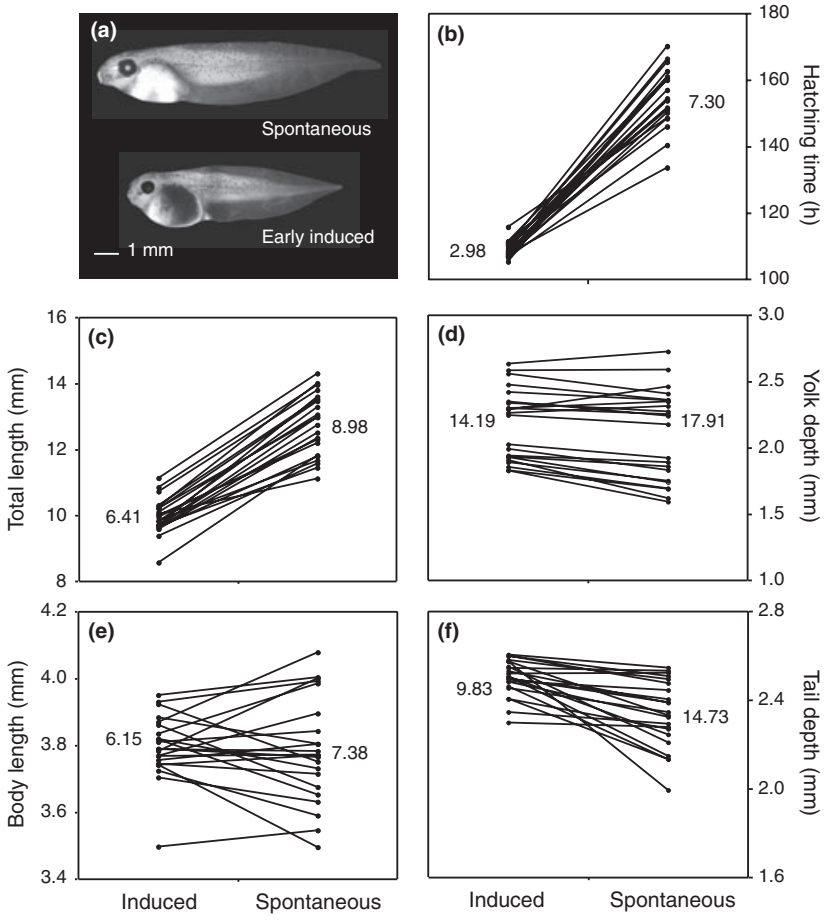


**Fig. 1** (a) Examples of spontaneous and induced early hatchlings of the red-eyed treefrog, *Agalychnis callidryas*. (b–f) Reaction norms for hatching time and four morphological traits of hatchlings (raw data, uncorrected for body size). Each line connects the average trait value of each sibship ($n = 22$) in each environment (induced vs. spontaneous hatching). Numbers on each end of reaction norms represent the coefficient of variation (CV) for the trait in that environment, across all sibships. Spontaneous hatchlings showed greater CV than induced hatchlings in all cases.

Induced hatching timing and the morphology of both induced and spontaneous hatchlings had small additive variance components, resulting in low trait heritabilities (Table 1). Spontaneous hatching time, however, had a significant additive genetic component and a heritability of 0.37. Both induced and spontaneous hatching time had a strong nonadditive maternal component, whereas morphological traits often showed high dominance variance components (Table 1). The coefficient of additive genetic variation was significantly lower for induced than spontaneous hatching time ($Z = 0.493$, $P < 0.0005$). Within-sibship coefficients of variation in hatching time were greater for spontaneous hatching than for induced hatching (Fig. 3; $F_{1,43} = 46.73$, $P < 0.0001$), and were also higher in spontaneous hatchlings for all morphological variables (all $P < 0.002$). Cross-environment genetic correlations were generally low (Table 2); they were significantly greater than zero only for yolk depth and body length.

## Discussion

Both the developmental timing of hatching and the extent and nature of plasticity in hatching have diversified greatly in animals (Warkentin, 2011a). Anurans can hatch at stages ranging from tailbud embryos not yet capable of muscular movement to fully metamorphosed froglets (Buckley *et al.*, 2005; Gomez-Mestre *et al.*, 2006). The reported magnitude of hatching acceleration in response to threats to eggs ranges across species from 2 to 67% of the embryonic period of undisturbed eggs; threats to hatchlings elicit hatching delays of 7–614% (Warkentin, 2011b). We know relatively little about the developmental or genetic mechanisms underlying this variation in plasticity or how it evolves.

The hatching accelerations we found in red-eyed treefrogs (20–35% across sibships) seem common in frogs, as 13 of 22 species reviewed in Warkentin (2011b) showed accelerations between 19 and 36%. However, the range across sibships is lower than that found for oomycete-induced early hatching in toads (0–54% acceleration across sibships; Gomez-Mestre *et al.*, 2008a). The spontaneous hatching times and developmental accelerations observed across sibships in this laboratory experiment closely matched the hatching times and developmental accelerations observed in the field for this species (Warkentin,

**Table 1** Within-environment variance components and narrow-sense heritability ($h^2$) of hatching time and hatchling morphology for *Agalychnis callidryas* embryos allowed to hatch spontaneously or induced to hatch early via simulated predator attack.

| | $V_A$ | $V_M$ | $V_D$ | $V_E$ | $CV_A$ | $h^2$ |
|---|---|---|---|---|---|---|
| Induced early hatching | | | | | | |
| Hatching | 1.599 | **4.221** | – | 7.036 | 1.161 | 0.123 |
| | (1.961) | (2.256) | – | (1.396) | | (0.140) |
| Total length | $0.47\times10^{-4}$ | 0.058 | **0.854** | 0.171 | 0.217 | 0.0001 |
| | (0.342) | (0.155) | (0.514) | (0.017) | | (0.317) |
| Yolk | 0.066 | 0.017 | **0.249** | 0.018 | 12.067 | 0.209 |
| | (0.101) | (0.025) | (0.108) | (0.002) | | (0.314) |
| Body | 0.764 | 4.221 | **1.213** | 7.036 | 24.902 | 0.180 |
| | (2.954) | (2.256) | (3.576) | (0.717) | | (0.295) |
| Tail | $0.85\times10^{-7}$ | **0.018** | 0.014 | 0.028 | 0 | 0 |
| | ($0.86\times10^{-8}$) | (0.009) | (0.012) | (0.003) | | |
| Spontaneous hatching | | | | | | |
| Hatching | **69.676** | **56.043** | – | 64.638 | 5.396 | 0.366 |
| | (47.374) | (34.699) | – | (6.411) | | (0.189) |
| Total length | $0.14\times10^{-5}$ | 0.450 | **1.547** | 0.518 | 0.009 | 0 |
| | ($0.15\times10^{-6}$) | (0.319) | (0.820) | (0.052) | | |
| Yolk | 0.127 | 0.032 | **0.405** | 0.021 | 16.514 | 0.243 |
| | (0.168) | (0.042) | (0.173) | (0.002) | | (0.311) |
| Body | $0.94\times10^{-7}$ | 0.010 | 0.152 | 0.045 | 0.008 | 0 |
| | ($0.95\times10^{-8}$) | (0.019) | (0.079) | (0.005) | | |
| Tail | $0.34\times10^{-6}$ | 0.040 | **0.224** | 0.063 | 0.022 | 0 |
| | ($0.35\times10^{-7}$) | (0.034) | (0.112) | (0.006) | | |

Bold values indicate variance components significantly different from zero according to likelihood-ratio tests between the saturated model and a model in which the component was constrained to zero. Standard errors of estimates are in parentheses.

1995; Gomez-Mestre & Warkentin, 2007), supporting the realism of the experimental data.

The low 'sire × environment' variance components found resulted in low heritabilities for plasticity in hatching timing and hatchling morphology (Table 2). Nonetheless, we observed substantial maternal variance for plasticity (dam × environment interaction) in hatching timing and hatchling size (Table 2). Such maternal variance can allow adaptive responses to selection even with little additive genetic variation (Mousseau & Fox, 1998). We also observed a marked asymmetry in genetic variation for hatching timing across environmental contexts. *Agalychnis callidryas* showed substantial additive genetic variation (plus a large nonadditive maternal component) for spontaneous hatching time, but almost none for induced early hatching (Fig. 1, Table 1). This asymmetry is similar to, but more extreme than, the pattern found in *Bufo americanus*, which shows substantial genetic variation in spontaneous hatching timing and much less variation in the timing of oomycete-induced early hatching (Gomez-Mestre *et al.*, 2008a). Unlike the toads, all sibships of *A. callidryas* hatched early in response to the inducing stimulus.

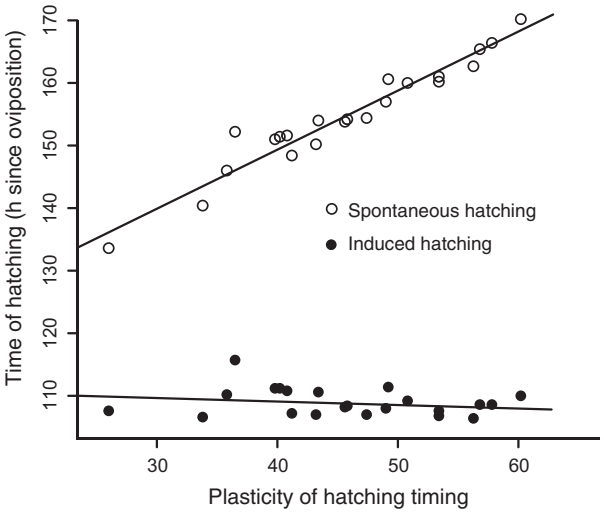


**Fig. 2** Relationship between hatching timing and hatching plasticity, estimating plasticity as the difference between the mean hatching times for each sibship in the two environments. Given the reduced variation for hatching time in the 'induced hatching' environment, plasticity showed a very strong association with spontaneous hatching time, so the most plastic genotypes were also the ones producing the most extreme (i.e. late hatching) phenotypes.

The asymmetric pattern of variation in hatching timing, combined with the low cross-environment genetic correlation, could be interpreted as an indication that different genes may contribute to variation in hatching timing in the two contexts (Falconer & MacKay, 1996). However, the magnitude of detectable cross-environment genetic correlation may be constrained by the lack of additive genetic variance detected for induced early hatching (Riska *et al.*, 1989; Simons & Roff, 1994).

For red-eyed treefrogs, and for many species, spontaneous hatching timing is more likely to be under stabilizing selection than directional selection, and many genetic and environmental factors may have small effects on its actual and optimal timing. In contrast, predators specific to the egg stage impose consistent directional selection on hatching timing. If an egg is clearly about to be eaten, the embryo should hatch immediately unless there is no possibility of survival outside the egg. Thus, given clear cues of impending egg death, all the variation in the initiation of hatching should come from variation in hatching competence. Since the hatching process in *A. callidryas* is rapid (seconds), this will not contribute to variation in hatching timing as we measured it. With more ambiguous cues, or an elevated but still uncertain chance of egg death, more factors should affect hatching timing.

Although multiple traits contribute to the ability of animals to exit from and survive outside the egg, the onset of hatching competence will depend only on the last of those to develop (Warkentin, 2007). In

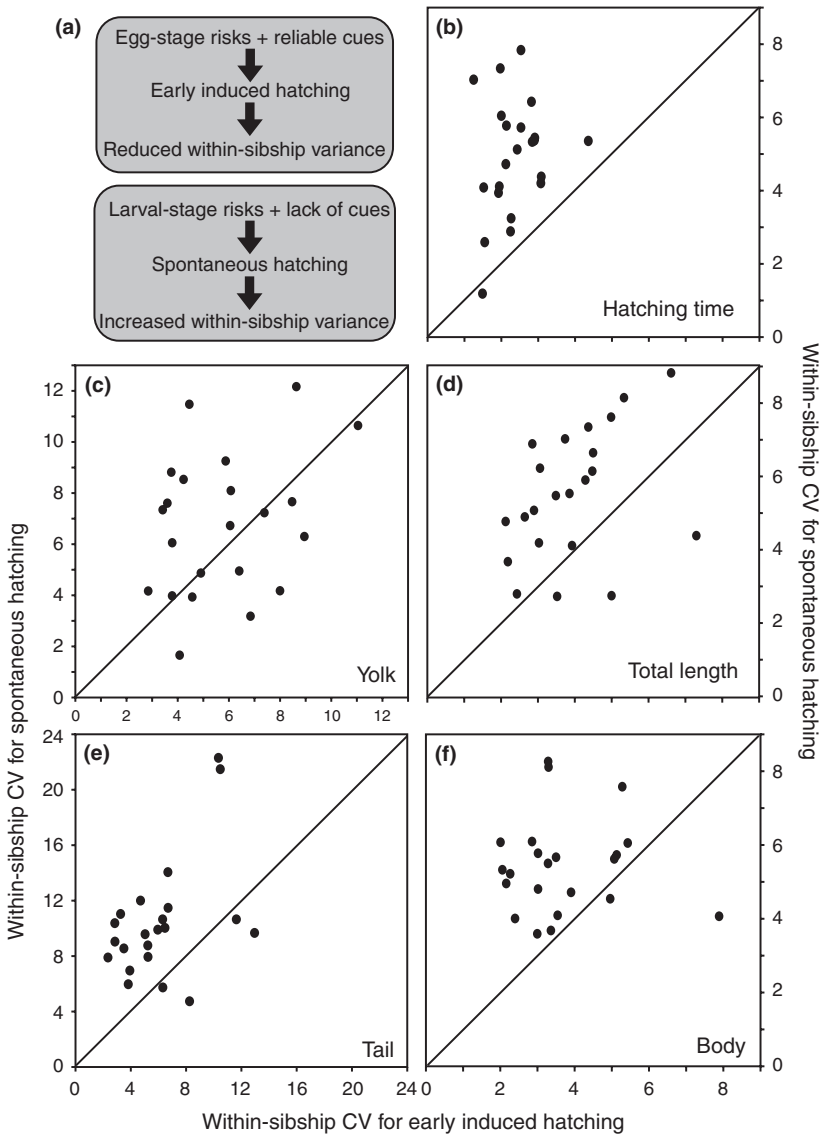


**Fig. 3** (a) Early-induced hatching timing evolves in response to reliably cued egg-stage risks (e.g. predator attacks, hypoxia), whereas spontaneous hatching responds to selective factors in the larval stage and no cues are available for embryos, resulting in increased within-sibship variance. (b–f) Observed relationship between within-sibship coefficients of variation for early-induced *vs.* spontaneous hatchlings in their hatching timing, total length and morphology. Dashed lines indicate $CV_{induced} = CV_{spontaneous}$ so that sibships mapping above that line showed greater CV for spontaneous than for induced hatching time.

*A. callidryas* strong directional selection to escape from attacks by egg predators (Warkentin, 1995, 2000; Gomez-Mestre & Warkentin, 2007), and potentially other egg-stage threats, appears to have depleted whatever genetic variation may have existed in this limiting trait. One consequence of such asymmetry in variation of hatching time across environments is that most of the variation in plasticity across sibships is due to differences in their spontaneous hatching time (Fig. 1b). The genotypes with greatest plasticity show the most extreme (latest) hatching times, when allowed to hatch spontaneously (Fig. 2); there are no nonplastic late-hatching specialists. A correlation of extreme trait values with greater plasticity is congruent with other predator and herbivore-induced responses (Auld *et al.*, 2009). Inducible defences, however, often show greater genetic variation for induced (defended) vs. uninduced phenotypes (Agrawal *et al.*, 2002; Relyea, 2005). Such patterns may, however, depend on the type of trait in question, as well as on the nature of the cue triggering the phenotypic response and its relationship to the source of risk.

In addition to higher coefficients of additive genetic variation across sibships, we observed higher within-sibship coefficients of variation in spontaneously hatching eggs than in early-induced ones (Fig. 3). Such within-sibship variance might reflect adaptive plastic responses to microenvironmental variation, bet-hedging diversification (Simons, 2011), or a combination of both. Due to trade-offs between risks to eggs and to hatchlings (e.g. aquatic predation), embryos that have recently developed hatching competence are very unlikely to hatch unless they perceive a strong, unequivocal environmental cue indicating risk, such as the acute

**Table 2** Variance components for the interaction effects of sire- and dam-by-environment (induced vs. spontaneous hatching).

| Variable | $V_{sire\ x\ env}$ | $V_{dam\ x\ env}$ | $r_A$ | Plasticity $h^2$ |
|---|---|---|---|---|
| Hatching | **6.025** | **38.552** | 0.258 | 0.024 |
| | (4.707) | (12.436) | (0.456) | (0.026) |
| Total length | **0.072** | **0.181** | 0.557 | 0.047 |
| | (0.005) | (0.009) | (0.268) | (0.055) |
| Yolk | 0.003 | 0.001 | **0.981** | 0.007 |
| | (0.0002) | (0.0009) | (0.018) | (0.005) |
| Body | 0.005 | 0.004 | **0.807** | 0.054 |
| | (0.0003) | (0.0003) | (0.149) | (0.054) |
| Tail | 0.017 | 0.026 | 0.248 | 0.249 |
| | (0.010) | (0.013) | (0.368) | (0.234) |

The 'sire × environment' variance was used to compute the additive genetic variance for plasticity, whereas the 'dam × environment' component describes the additive and nonadditive genetic maternal component of plasticity. The 'sire × environment' component was also used to estimate the narrow-sense heritability of plasticity. A significant across-environment genetic correlation, $r_A$, indicates nonindependence of the trait across induced and spontaneous hatching contexts. Bold values indicate estimates significantly different from zero. Standard errors of estimates are in parentheses.

physical disturbance caused by a predator attack. In contrast, selective factors shaping spontaneous hatching times in undisturbed *A. callidryas* may have been more subtle and stochastic. In nature, hatching decisions of undisturbed eggs might differ within clutches due to microenvironmental variation across the clutch (e.g. oxygen availability, partial dehydration). This is unlikely in our study because the way we split clutches reduced variation in egg surface exposure, and thus oxygen supply, and temperature and hydration were carefully controlled. In addition, conditions in the post-hatching aquatic environment will affect the optimal hatching time for undisturbed *A. callidryas*. However, it seems unlikely that arboreal embryos can assess the abundance of aquatic predators or competitors, the algal food resources, or the water depth and quality below them. Hence, fluctuating selection in the post-hatching environment could favour increased within-sibship variance in hatching times as a long-term adaptive response to unpredictability (Simons, 2009, 2011).

The timing of ontogenetic transitions (e.g. hatching, birth, metamorphosis and reproductive maturation) is likely constrained by development, perhaps especially so early in development. The lower limit to hatching timing is highly conserved across *Agalychnis* and *Pachymedusa* (Gomez-Mestre *et al.*, 2008b), suggesting that they are bound by the same developmental constraint. We hypothesize that maturation of the hatching mechanism is this constraint, against which selection has eroded additive genetic variation for the earliest inducible hatching. In *A. callidryas* the first stage of hatching is rapid, highly localized rupture of the vitelline membrane, presumably via enzymes released from their highly localized hatching glands (Cohen *et al.*, 2012).

Phenotypes in different environments are partly the results of differential gene expression, that is, up- and down-regulation of the same genes and/or differences in which genes are transcribed (Aubin-Horth & Renn, 2009). Different patterns of environmentally induced gene expression may translate into low cross-environment genetic correlation and a high degree of evolutionary independence among trait values expressed in different environments. Here, we found low genetic correlation between risk-induced early hatching and spontaneous hatching time in a tropical treefrog, suggesting that these two phenomena are likely to be under different genetic regulation and evolving independently in response to different selective factors. The evolution of plasticity and the environment-specific values for the timing of life-stage transitions in early ontogeny are likely to be constrained by the timing of development of their regulatory mechanisms.

## Acknowledgments

We thank D. Fenolio for advice in setting up the frog colony, and J. Perry for assistance in building the rain chambers. We thank E. Collins, A. D'Amato, A. Gehrke, C. Hanlon, T. Iijima, S. Poo, J. Rogge and V. Saccoccio, for their help in frog care. We also thank two anonymous reviewers for their constructive comments. This study was funded by grants from National Science Foundation (IBN-0234439), National Geographic Society (7541-03) and Boston University. It was conducted under Boston University IACUC protocols #03-019 and 06-016.

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