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Hatching plasticity and the adaptive benefits of extended embryonic development in glassfrogs

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Abstract

Phenotypic plasticity in hatching age has been documented in many animals. A growing body of research indicates that embryos can rapidly hatch to escape egg-stage risk. However, fewer studies have tested whether selective tradeoffs in post-hatching stages favor trait plasticity. We assessed hatching plasticity and its benefits to the larval stage in five species of Neotropical glassfrogs (Centrolenidae). Glassfrog embryos develop on terrestrial vegetation and larvae in benthic stream sediments; thus hatching involves a dramatic habitat shift, when hatchlings must dive past stream fish to reach larval refuges. We found that all five species have extensive plasticity in hatching age and can delay hatching to more than double their minimum embryonic period. Along a stream in Panama, we found evidence that early hatching is induced by the risk of embryo predation, dehydration, and fungal infections. Differences in hatching timing were coupled with changes in hatchling phenotypes, such that younger hatchlings were smaller and less developed than older individuals. To assess locomotor function we measured diving speed, a key performance trait for newly hatched larvae. Older hatchlings dove 1.4–3.8 times faster than younger ones, which would reduce their exposure to predators in the water column. To assess the potential for exotrophic growth, we measured digestive system morphology and feeding onset across hatching ages. Younger hatchlings had intact yolk sacs and spent 4.5–6 days as larvae before feeding, while older hatchlings entered the water with well-developed guts and fed immediately. Therefore, while early hatching enables embryos to escape egg-stage risk, it is associated with initial performance costs and a lag before feeding in the larval stage. We recovered consistent results across multiple genera of glassfrogs, supporting that hatching plasticity is widespread, ancient, and has been maintained by shared selective trade-offs in this family.

Keywords Centrolenidae · Embryo behavior · Frog · Development · Environmentally cued hatching · Phenotypic plasticity

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Introduction

Phenotypic plasticity in the age and/or developmental stage of hatching has been documented across animals, from echinoderms to mammals (Martin 1999; Warkentin 2011a; Armstrong et al. 2012). It results from multiple processes, ranging from inevitable environmental effects that alter the duration of the egg stage (e.g., thermal effects on embryogenesis), to sophisticated embryo adaptations that improve survival in dynamic environments (Warkentin 2011a). The evolution of adaptive hatching plasticity depends, in part, on selective trade-offs across life stages, such that the optimal time to hatch is contingent on environmental conditions (Via and Lande 1985; Moran 1992). This form of plasticity can be evaluated using theory on life-history transitions (e.g., metamorphosis, Wilbur and Collins 1973), which predicts that natural section should optimize the ratio of growth to mortality in each life stage and thereby reduce time spent in more dangerous or less productive stages (Williams 1966; Shine 1978; Werner and Gilliam 1984). When stage-specific costs or benefits vary, selection can favor plasticity in the timing of life-stage transitions (Wilbur and Collins 1973; Werner and Gilliam 1984; Werner 1986; Day and Rowe 2002). A growing number of studies document that embryos hatch earlier in response to egg-stage risk, such as predators, pathogens, and abiotic hazards (reviewed by Warkentin 2011a, b). However, fewer studies have assessed if or how selection in post-hatching environments favors plastic extensions in embryonic development.

Hatching plasticity is widespread in amphibians, with environmentally cued shifts documented in at least 15 families (Warkentin 2011b; Van Buskirk 2016; Poo and Bickford 2014). In many cases, these shifts match directional predictions based on stage-specific risk and confer immediate survival benefits, such as hatching early to escape egg-stage predators (e.g., Warkentin 2011b). However, a recent meta-analysis examining the generality of predator-cued hatching across 20 species of amphibians found equivocal results (Van Buskirk 2016). This result could be due, in part, to how selective environments vary with reproductive modes. This analysis focused predominately on species with aquatic eggs and larvae (17 species), where hatching might not allow successful escape from aquatic predators. In contrast, in species with semi-terrestrial reproduction the egg and larval habitats are separated, so hatching mediates exposure to stage-specific risks—embryos can avoid dangers in the water by remaining in their terrestrial egg, and escape terrestrial threats by fleeing to the water. Understanding variation in risk-induced hatching requires understanding how selection acts on both pre- and post-hatching stages. However, research evaluating selective tradeoffs across life-stages is limited to relatively few amphibian families (e.g., Eleutherodactylidae: Buckley et al. 2005; Hylidae: Touchon and Warkentin 2010; Touchon et al. 2011; Hyperoliidae: Vonesh 2005; Vonesh and Bolker 2005; Phyllomedusidae: Warkentin 1995; Gomez-Mestre et al. 2008; Ranidae and Bufonidae: Gomez-Mestre et al. 2006).

Multiple lineages of amphibians have independently evolved terrestrial eggs, while retaining aquatic larvae (Gomez-Mestre et al. 2012). This separation of egg and larval habitats could produce strong selection on hatching plasticity. Moreover, it facilitates the identification of stage-specific selective factors and experimental tests of how hatching timing affects fitness correlates. In *Agalychnis callidryas*, for example, early hatching allows embryos to escape multiple egg-stage risks (Warkentin 1995, 2000; Warkentin et al. 2001; Salica et al. 2017). However, younger hatchlings experience higher rates of aquatic predation relative to older hatchlings and, in some contexts, relatively lower viability even without predation (Warkentin 1995; Touchon et al. 2013; Willink et al. 2014). Such tradeoffs

are likely relevant and possibly widespread for species with semi-terrestrial reproductive modes (Vonesh and Bolker 2005; Touchon and Warkentin 2010; Poo and Bickford 2014).

In this study we document the existence of hatching plasticity and examine its benefits to the larval stage in five species of Neotropical glassfrogs (Centrolenidae). These frogs have a semi-terrestrial reproductive mode, where eggs develop on vegetation and rocks above streams, and larvae develop in the water until metamorphosis. There is evidence in some species that embryos accelerate hatching in response to egg-stage risk, as early hatching has been documented in association with clutch mortality (Hawley 2006), physical disturbance (Lehtinen and Georgiadis 2012), and parental abandonment (Delia et al. 2014). Less is known about whether larval environments favor plastic extensions in embryonic development. In at least two species of *Hyalinobatrachium*, differences in hatching age are coupled with the size and developmental stage of hatchlings (Delia et al. 2014; Nokhbatolfoghahai et al. 2015)—it is possible that early hatching carries performance costs in the larval stage. Many glassfrog larvae face immediate risk of predation when they first enter the water. Tadpoles appear to inhabit benthic sediments and leaf packs along slower sections of streams (e.g., Villa and Valerio 1982; Hoffman 2010). At our study site in Panama, we have observed hatchlings immediately dive to the stream bottom, then travel along or take cover in gravel and leaf litter. We have also observed the abundant fish *Poecilia gillii* readily capturing hatchlings as they dove through the water column (Fig. 1, Delia and Bravo-Valencia unpublished observations). In the absence of egg-stage risk, delaying hatching could improve the development of swimming-related traits used to escape aquatic predators. In addition to changing risks, hatching offers access to external food resources. In at least Mexican *H. fleischmanni*, substantial gut development occurs during the facultative embryonic period, during which an intact yolk sac is converted into a presumably functional digestive system (Delia et al. 2014). If the egg-stage is safe, delaying hatching could allow embryos to maximize growth and development on maternal yolk *in ovo*, and hatch once they are capable of feeding and yolk is depleted.

In contrast to other well-studied frogs with hatching plasticity, glassfrogs also exhibit parental care of eggs, and embryos alter hatching age in response to parental abandonment. Prolonged care has evolved repeatedly in this family, and within such species the duration of care varies widely (Delia et al. 2017). Elsewhere, we found that *H. fleischmanni* embryos hatch early when abandoned, but delay hatching under continued parental care (Delia et al. 2014). Variable parental care is thought to alter selection on offspring traits that increase the efficiency with which care is converted into offspring benefits (e.g., begging behavior, Kölliker et al. 2012). Considering the variable nature of parental care in glassfrogs, hatching plasticity could provide offspring with a mechanism to convert facultative extensions in care into direct benefits. Testing this hypothesis requires information on selection in the post-hatching stage.

We studied five species of glassfrogs from three genera; *Hyalinobatrachium colymbiphyllum*, *H. fleischmanni*, *Cochranella granulosa*, *Teratohyla pulverata*, and *T. spinosa*. First, we monitored natural patterns of hatching in the field to assess the presence and extent of hatching plasticity. We evaluated whether early hatching is associated with the risk of embryo mortality, using direct observations of attacks on clutches and comparisons of hatching age between undisturbed clutches and those that suffered mortality. To examine potential benefits of delayed hatching for the larval stage, we tested how hatching age affects diving speed—a key performance trait that determines how long hatchlings are exposed to fish in the water column before reaching refuge in the stream bottom. In addition, we tested how hatching age affects the onset of feeding, which determines when larvae begin to benefit from access to external food resources. We predict that delaying hatching
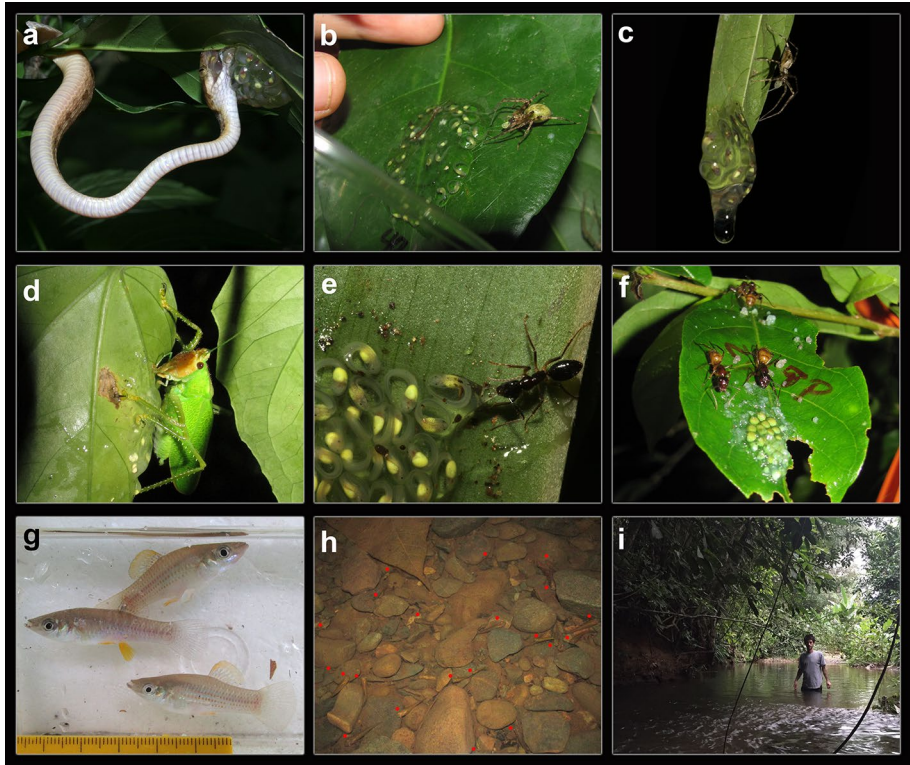
Fig. 1 Predators of glassfrog embryos and larva in Rio Frijoles, Panama. **a** A *Leptodeira septentrionalis* attacking a *H. fleischmanni* clutch, many embryos of which rapidly hatched out during the attack. Anyphaenid spiders capturing a *H. colymbiphyllum* embryo (**b**) and a *T. spinosa* embryo (**c**)—nearby siblings successfully hatched during the attack in both species. **d** The katydid *Copiphora brevirostris* consuming a *H. colymbiphyllum* clutch. Ants (likely *Camponotus*) extracting a *H. colymbiphyllum* embryo (**e**) and consuming a young *T. pulverata* clutch (**f**); nearby siblings in e successfully hatched during the attack. **g** and **h** Stream fish *Poecilia gillii* (Poeciliidae); red dots in **h** indicate individual fishes. **i** The study stream, Rio Frijoles. All photos were taken on Rio Frijoles, except (**a**) from Oaxaca, Mexico; we observed multiple attacks by this snake on clutches of both *Hyalinobatrachium* on Rio Frijoles. (Color figure online)

provides a performance benefit relevant to hatchling survival, and that undisturbed hatchlings will remain in the egg until they can gain a nutritional benefit from hatching.

Methods

Field monitoring

We monitored and collected egg clutches of all five species along Rio Frijoles in Parque Nacional Soberanía near the Smithsonian Tropical Research Institute (STRI) in Gamboa, Panamá. Field monitoring occurred from June to October 2011–2013, and field collections for lab experiments in Gamboa from June to November of 2016. We monitored nightly breeding activity of adults along stream transects, locating pairs in amplexus and recording

the date and locations of their clutches. When we did not observe oviposition, clutch age was determined based on when embryos reached Gosner (1960) stage 17, which occurs 2.5–3 days past oviposition at this site (unpublished data).

Natural timing of hatching and escape hatching observations

To determine natural variation in hatching age, we followed a set of clutches in the field for each species, checking them nightly until all embryos either hatched or died (*C. granulosa* $n=19$, *H. colymbiphyllum* $n=30$, *H. fleischmanni* $n=20$, *T. pulverata* $n=25$, *T. spinosa* $n=20$). We determined hatching timing using small plastic cups attached beneath clutches with small-gauge wire to catch hatchlings (sensu Hayes 1983). Here, we present data on hatching age; more detailed information on embryo mortality, including additional clutches with complete mortality, has been analyzed elsewhere (Delia et al. 2017; *in revision*). During the course of fieldwork, we also made opportunistic observations of predator-induced hatching.

Hatching age manipulation

We conducted laboratory trials to assay swimming performance and onset of feeding for larvae hatched at two different ages. For the early treatment, hatching age was 7.5 days past oviposition, which is about 0.5 days after the onset of hatching competence (in all species). For the late treatment, hatching age was 14.5 days old (in four species) or 15.5 days (in *H. colymbiphyllum*), near the mean spontaneous hatching age under good conditions (i.e., adequate rain/no predation; see results)—the hatching age of *H. colymbiphyllum* was based on that of embryos with continued paternal care (Delia unpublished data). Clutches were left to develop in the field until 2–4 days prior to treatment, and then transported to an open-air laboratory at STRI in Gamboa. Clutches were misted at set intervals using an automated misting system to maintain hydration. We induced embryos to hatch by jiggling them with a plastic pipette or forceps (between 11:00 and 14:00 h); glassfrog embryos hatch in response to physical disturbance and predators (Lehtinen and Georgiadis 2012, Delia unpublished observations). For some early-treatment embryos that were less responsive to this stimulus, we manually decapsulated them to speed the process; these were confirmed to be hatching-competent based on their developmental stage (Gosner 1960 stages 24–25).

Hatchling phenotypes

To assess differences in hatchling morphology, we preserved a subset of individuals from each hatching-age treatment in 9% formalin immediately after hatching (14–26 individuals per species, see results). We photographed specimens in ventral and lateral view with a scale, using a Canon DSLR with a MPE-65 mm macro lens, then measured morphological features from images using ImageJ 1.48 v (Schneider et al. 2012). We measured 10 features of external morphometry and internal organ development for each individual; total length (TL), tail length (TAL), tail height (TH), tail musculature height and width (TMH, TMW), head length and width (HL, HW), yolk length and width (i.e., undivided sac or yolk-filled gut coils; YL, YW), and the number of gut-coil rotations (GC; to the nearest quarter rotation). We were unable to accurately count gut-coil number in *C. granulosa*

(without dissection) due to the semi-opaque venter of preserved hatchlings in this species, and therefore only compare the first 9 measures.

Diving performance

For an ecologically relevant measure of locomotor performance, we compared diving (swimming) speed between hatching-age treatments. We conducted diving trials in plastic buckets with a clear observation panel in one side, filled to a depth of 32 or 36 cm with aged tap water. For each trial, we induced an individual to hatch, then immediately dropped it from near the surface into the water. We recorded the amount of time it took to swim to the bottom of the bucket, to the nearest 0.01 s. Upon entering the water, all hatchlings immediately dove, swimming continuously or swimming and coasting until reaching the bottom. We tested 5–7 individual hatchlings of the same age from each clutch (*C. granulosa* $n_{clutches} = 18$, $n_{hatchlings} = 50$ early and 55 late; *H. colymbiphyllum* $n_{clutches} = 24$, $n_{hatchlings} = 60$ early and 60 late; *H. fleischmanni* $n_{clutches} = 18$, $n_{hatchlings} = 50$ early and 50 late; *T. pulverata* $n_{clutches} = 13$, $n_{hatchlings} = 65$ early and 45 late; *T. spinosa* $n_{clutches} = 14$, $n_{hatchlings} = 72$ early and 72 late).

Onset of feeding

We measured the onset of feeding to determine when individuals begin to benefit from access to external resources. We placed each hatchling into an individual cup with aged tap water and a small amount of detritus (soil) and checked them twice daily for signs of feeding, at 11:00–14:00 h and 20:00–23:00 h. The ventral tissues and gut coils of glassfrog tadpoles are transparent to semi-transparent in life, which allowed us to assess if they had begun feeding with minimal disturbance. We gently captured tadpoles with a large, clear plastic pipette, and examined their gut coils for detritus from below with a hand lens. In the early treatment, we observed that no tadpoles began feeding prior to 12 days post-oviposition. Therefore, we reduced checks to once daily prior to age 11.5 d to further minimize disturbance. We assayed 3–5 hatchlings of the same age from each clutch (*C. granulosa* $n_{clutches} = 21$, $n_{hatchlings} = 30$ early and 33 late; *H. colymbiphyllum* $n_{clutches} = 21$, $n_{hatchlings} = 31$ early and 30 late; *H. fleischmanni* $n_{clutches} = 20$, $n_{hatchlings} = 29$ early and 30 late; *T. pulverata* $n_{clutches} = 15$, $n_{hatchlings} = 36$ early and 26 late; *T. spinosa* $n_{clutches} = 20$, $n_{hatchlings} = 43$ early and 41 late).

Statistical analyses

All statistical analyses were conducted in R version 3.3.3 (R Core Team 2017). For field-monitored clutches, we calculated the modal hatching age of individual clutches, estimated means ($\pm$ se) of modal hatching age across clutches, and calculated the range of hatching ages within clutches as first–last day of hatching. We used Mann–Whitney *U* tests to compare the modal hatching age (count data) for disturbed clutches that experienced mortality from predation, dehydration, and/or fungal infection to undisturbed clutches with no evidence of mortality from these sources (note our sample size was too small for GLMs). We did not consider developmental abnormalities or eggs falling off clutches during rain as sources of disturbance, since these do not indicate a threat to remaining embryos. To compare hatchling phenotypes between age treatments, we used

principle component analyses (PCA) to summarize the measured features of hatchling phenotypes for each species with the prcomp function, and compared principle components (PC) between groups using *t* tests. For diving and feeding assays, we used linear and generalized linear mixed models to compare between hatching-age treatments for each species, while accounting for clutch-of-origin random effects (multiple hatchlings from the same clutch) in the package lme4 (Bates et al. 2015). Diving speed was calculated as cm per second, and modeled using a Gaussian error distribution with the lmer function. For the onset of feeding parameters, we modeled the number of observation-intervals (2 per day) until feeding using a Poisson error distribution and a log link function with glmer. We compared the number of observation intervals until feeding from both hatching and oviposition, estimated effect sizes from these models, and converted to number of days for presentation. We computed *P* values using likelihood ratio tests (LRT) comparing nested models with and without the age-treatment predictor ($df = 1$).

Results

Natural timing of hatching

In all five species the earliest detected hatching in the field occurred at age 7 days, at Gosner (1960) stages 23–25. The latest detected hatching varied from age 19–21 d among species (Table 1). The average modal age of hatching for all clutches ranged from 12.1 to 12.56 d across species. However, undisturbed clutches hatched significantly later than disturbed clutches in four species (Table 1, Fig. 2). We did not detect any difference for *C. granulosa*, but the sample size of undisturbed clutches was very small for statistical comparisons ($n = 4$). Using the earliest hatching detected to estimate the minimum (obligate) embryonic period, average relative delays in hatching for undisturbed clutches range from 75 to 112.6% beyond the obligate embryonic period, with maximum delays (latest hatching) up to 171–200% across species.

Table 1 Hatching age (days) for egg clutches of five glassfrog species monitored along Rio Frijoles, Panama

Species	All clutches		Undisturbed		Disturbed		MWU	
	Age range	Mean	*n*	Mean	*n*	Mean	*W*	*p*
C. granulosa	7–20	12.1 (±0.56)	4	12.25 (±1.97)	15	12.06 (±0.53)	29.5	1
H. colymbiphyllum	7–20	12.46 (±0.45)	9	14.88 (±0.84)	21	11.42 (±0.36)	166	0.001
H. fleischmanni	7–19	12.4 (±0.53)	13	13.38 (±0.57)	7	10.57 (±0.72)	77	0.013
T. pulverata	7–19	12.56 (±0.36)	14	13.29 (±0.41)	11	11.63 (±0.52)	121	0.042
T. spinosa	7–21	12.36 (±0.68)	9	14.77 (±0.82)	11	11.9 (±0.9)	72	0.027

Means (±se) are calculated using the modal hatching age of each clutch, and separated into groups that did or did not experience embryo mortality from external sources (disturbed); Mann–Whitney *U* (MWU) were used to test for risk-induced shifts in hatching age between groups
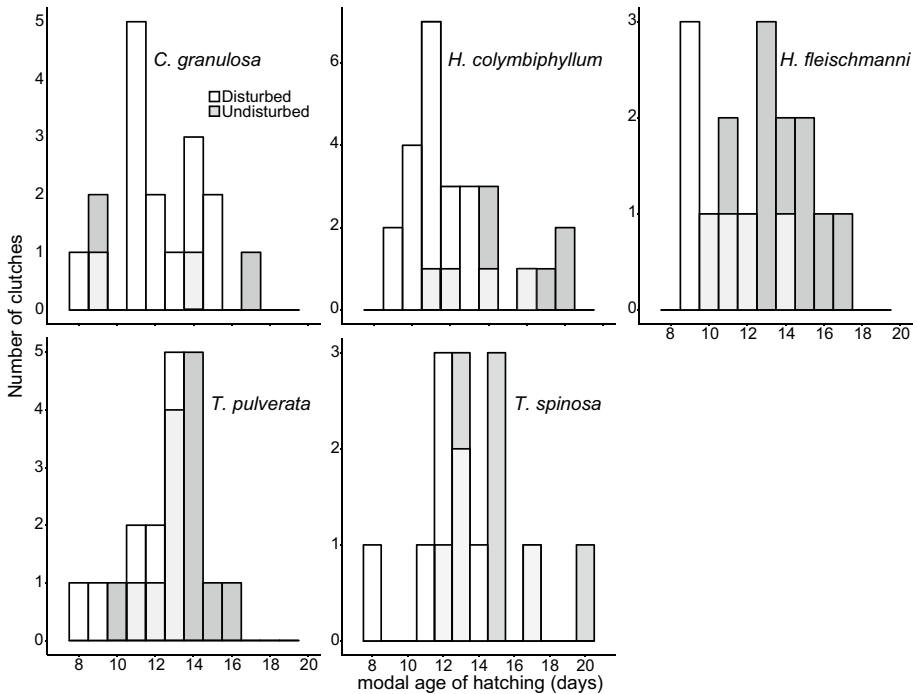
Fig. 2 Modal age of hatching for egg clutches of five glassfrog species monitored along Rio Frijoles, divided into clutches that were undisturbed (grey) or disturbed (white) by external sources of mortality (predators, dehydration, fungus). Lighter grey indicates overlap between the two categories of clutches

Escape hatching observations

Over the course of fieldwork we observed that embryos hatch rapidly and escape during attacks by invertebrate and vertebrate predators (Fig. 1). We directly observed attacks by anyphaenid spiders, katydids, and/or ants (*Azteca*, *Camponotus*, and a species of formicine) on both species of *Hyalinobatrachium*, *C. granulosa*, and *T. spinosa*; many embryos escaped in most cases, except when they were not yet hatching competent. We did not directly observe attacks in *T. pulverata*, but found spiders on clutches and early hatchings in cups. We also observed embryos rapidly hatching and escaping during attacks by cateyed snakes, *Leptodeira septentrionalis,* in both species of *Hyalinobatrachium*. Predators including ants, snakes, and katydids can consume entire clutches and multiple clutches within hours. Others such as spiders eat fewer embryos in an evening, but continue to feed on the same clutch over multiple nights until all embryos hatch or are captured.

Hatchling phenotypes

Principle components analyses indicate that PC1 accounts for 54–73% of the total variance in hatchling phenotypes, with moderately positive component loadings for 3–7 measures of body size (Sup. Table 1). In addition to size measures, gut-coil number loaded positively on PC1 (32–40%) for all species except *C. granulosa*, for which we were unable to count

gut-coil number. Total length (5 species) and gut-coil number (4 species) loaded most heavily on PC1. Therefore, this component can be interpreted as a measure of overall size and development, with higher values indicating larger individuals with more gut coils. In *T. pulverata*, yolk length loaded moderately and negative on PC1, such that higher values also indicate shorter yolk lengths. The remaining 3–7 measures loaded on PC2 for all species, which accounts for 13.7–37% of total variance (Sup Table 1); the measures and directions of component loading varied among species. However, yolk length and width loaded most heavily on PC2 for all but *T. pulverata*. All other PCs each accounted for less than 5.0% of total variance (Sup Table 1).

Early hatchlings had significantly lower PC1 scores than late hatchlings in all species (i.e., were smaller and less developed), whereas differences between PC2 scores were non-significant (Fig. 3); *C. granulosa*: PC1 $t_{17.99} = -11.79$, $p = 6.812e{-}10$, and PC2 $t_{17.92} = -0.01$, $p = 0.98$, $n = 20$; *H. colymbiphyllum*: PC1 $t_{18.35} = -14.86$, $p = 1.109e{-}11$, and PC2 $t_{14.23} = 0.39$, $p = 0.69$, $n = 26$; *H. fleischmanni*: PC1 $t_{12.32} = -7.56$, $p = 5.614e{-}06$, and PC2 $t_{17.29} = -1.55$, $p = 0.13$, $n = 20$; *T. pulverata*: PC1 $t_{8.6} = -7.68$, $p = 3.923e{-}05$, and PC2 $t_{9.09} = 0.96$, $p = 0.36$, $n = 14$; *T. spinosa*: PC1 $t_{17.11} = -13.29$, $p = 1.895e{-}10$, and PC2 $t_{16.6} = 1.11$, $p = 0.27$, $n = 20$.

Diving performance

Older hatchlings (14.5 or 15.5 d) swam significantly faster than the younger hatchlings (7.5 d) in all five species (Fig. 4, Table 2). The developmental increase in average diving speed ranged from 3.9 to 11.94 cm/s across species, with older hatchlings diving 1.4–3.8 times as fast as younger hatchlings.

Onset of feeding

Post-hatching delays until feeding were significantly longer for early hatchlings in all five species (Fig. 4, Table 3). Food was evident in most late-hatchling's guts within 12–24 h after hatching, indicating that these animals fed immediately or shortly after hatching. In contrast, early hatchlings spent on average 4.5–5.9 days developing in the larval stage before feeding, with average post-hatching delays 5.0 to 7.9 times longer than those of late hatchlings.

While post-hatching delays until feeding were longer for early hatchlings, overall they began feeding at a younger absolute age than late hatchlings (Fig. 4, Table 4). Early hatchlings began feeding at 12–13.45 days past oviposition, before late-hatching animals entered the water (at 14.5 or 15.5 d); on average 1.8–4.04 days sooner depending on the species.

Discussion

We found evidence that hatching plasticity is widespread and adaptive in glassfrogs. All five species studied exhibit an extensive facultative embryonic period. Early hatching was associated with clutch mortality and directly observed during predation events, supporting that accelerated hatching helps embryos escape egg-stage risks. Morphological analyses revealed that differences in hatching timing are coupled with changes in hatchling phenotypes, such that younger hatchlings are smaller and less developed than older ones. Our results support that hatching early incurs a performance cost in the larval stage, as diving
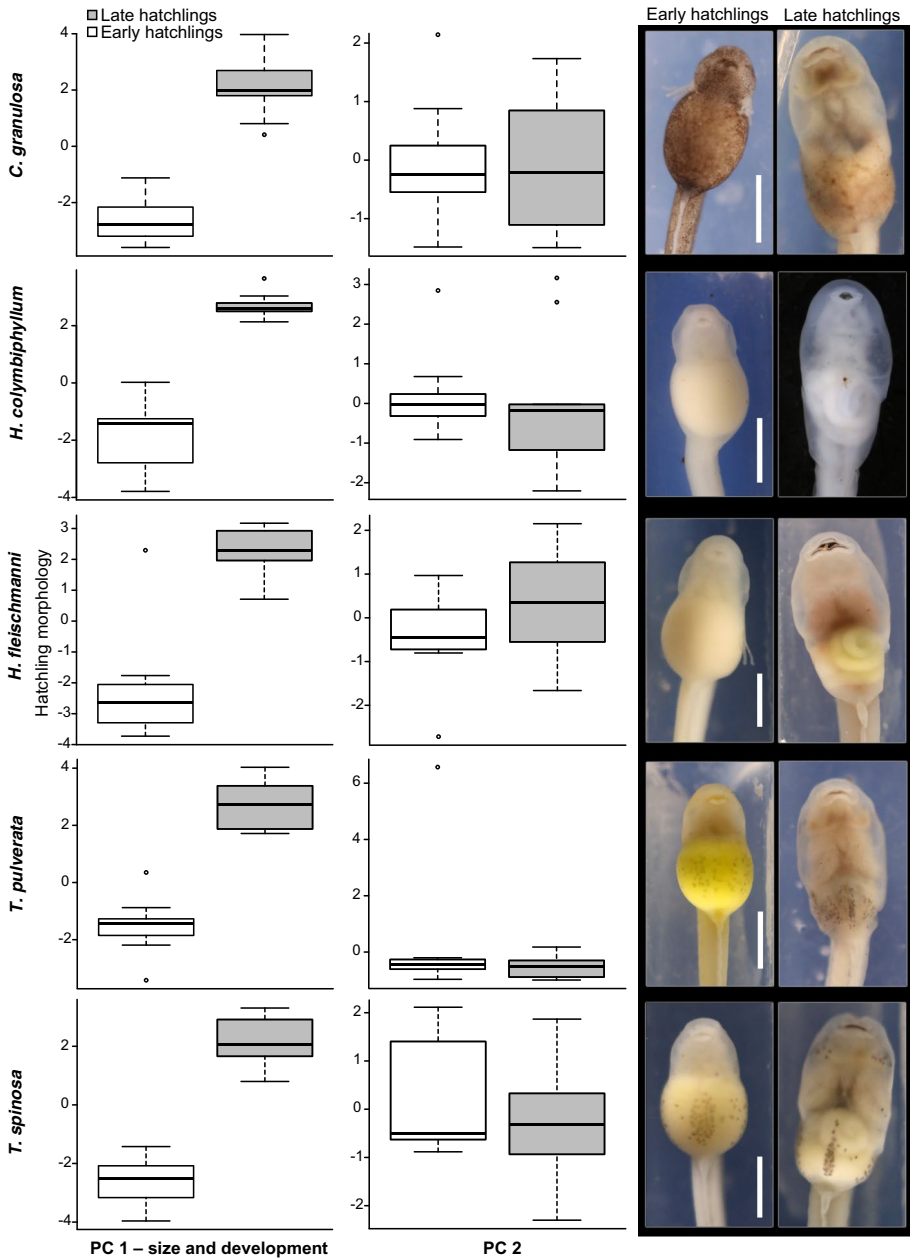
Fig. 3 Hatchling morphology in five species of glassfrogs hatched either early (7.5 d) or late (14.5–15.5 d). Box plots show principle component values summarizing multiple traits; PC1 represents overall size and development (left), in all species, while PC2 loadings vary among species (middle). Panel (right): hatchlings from both age-treatments (white bars = 1 mm)
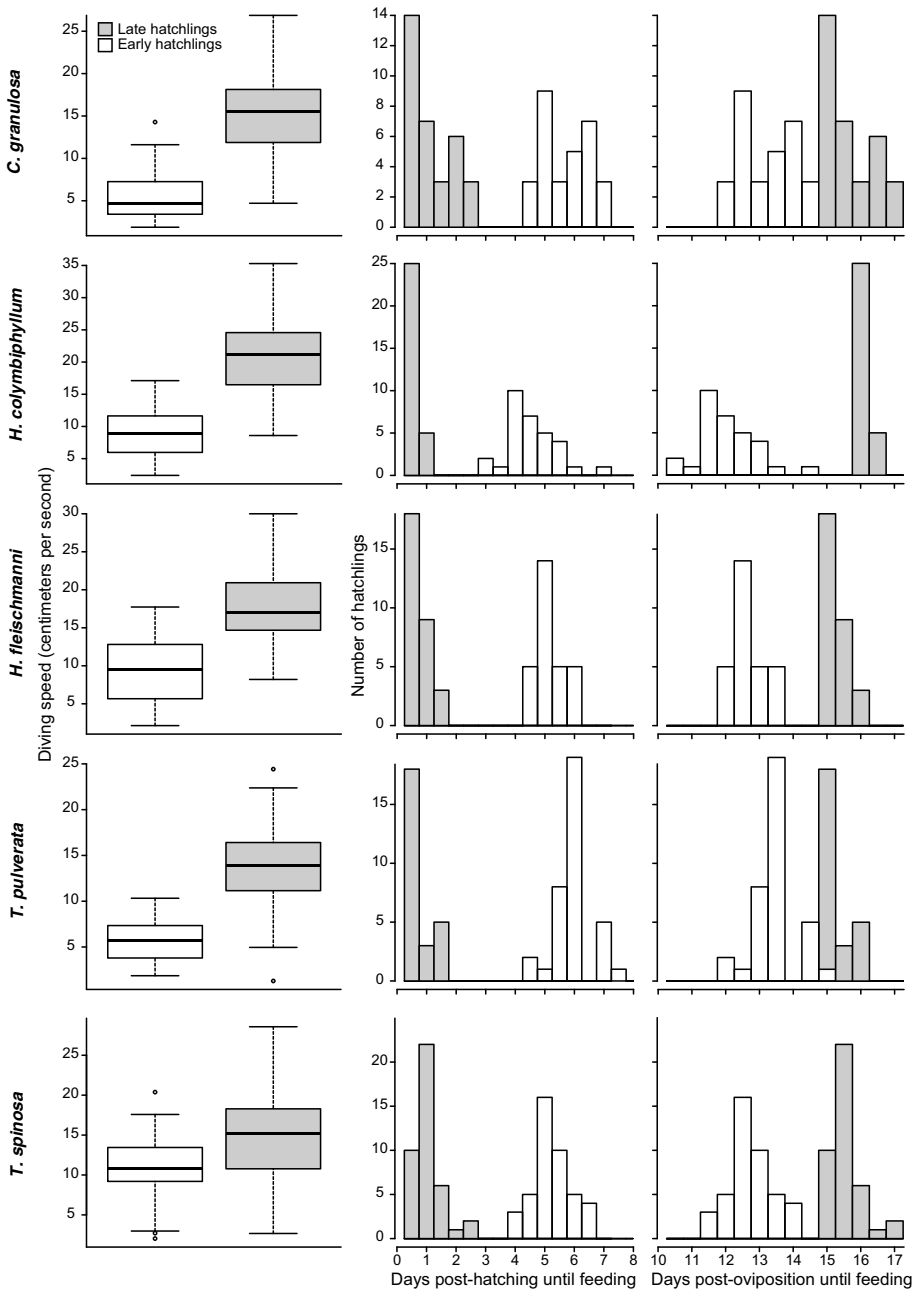
Fig. 4 Hatchling locomotor performance and feeding onset for five species of glassfrogs hatched early (7.5 d) or late (14.5–15.5 d). Box plots (left) show diving speed; histograms show first observation of food in larval guts, measured as days after hatching (middle) or oviposition (right)

Table 2 Hatchling diving speed improvement between early and late hatching in five species of glassfrogs (LRT of Linear MMs)

Species	Diving speed (cm/s)			
	Differences	χ^2	p value	$n_{hatchlings}$
C. granulosa	4.3 versus 16.24	96.94	2.20E−16	105
H. colymbiphyllum	9.04 versus 20.78	41.84	9.90E−11	120
H. fleischmanni	8.18 versus 18.18	62.55	2.59E−15	100
T. pulverata	6.03 versus 13.88	53.54	2.53E−13	110
T. spinosa	10.9 versus 14.8	24.72	6.61E−07	144

Table 3 Onset of feeding after hatching in early versus late hatchlings of five species of glassfrogs (LRT of Poisson MM)

Species	Onset of feeding (days) past hatching			
	Differences	χ^2	p value	$n_{hatchlings}$
C. granulosa	5.71 versus 1.15	42.69	6.39E−11	63
H. colymbiphyllum	4.54 versus 0.58	60.6	6.98E−15	61
H. fleischmanni	5.17 versus 0.75	63.02	2.05E−15	59
T. pulverata	5.95 versus 0.75	51.68	6.51E−13	62
T. spinosa	5.24 versus 1.04	56.99	4.37E−14	84

Table 4 Onset of feeding measured as developmental time since oviposition for early versus late hatchlings of five species of glassfrogs (LRT of Poisson MM)

Species	Onset of feeding (days) past oviposition			
	Differences	χ^2	p value	$n_{hatchlings}$
C. granulosa	13.21 versus 15.65	12.9	0.0003	63
H. colymbiphyllum	12.04 versus 16.08	30.47	3.39E−08	61
H. fleischmanni	12.67 versus 15.24	14.04	0.0001	59
T. pulverata	13.45 versus 15.25	6.77	0.009	62
T. spinosa	12.74 versus 15.54	22.32	2.30E−06	84

speed of older hatchlings is much faster than that of younger individuals. This has direct relevance to larval survival in streams, where hatchlings entering the water must dive past predatory fishes to reach refuge in the benthos. Early hatchlings also spend on average 4.5–5.9 days as larvae before they can begin feeding—thus gain no immediate benefit from access to external food—whereas late-hatching individuals enter the water capable of feeding. Therefore, delaying hatching in safe eggs maximizes embryo growth and development on yolk reserves, which improves a key larval performance-trait and reduces time until larvae begin to benefit from food resources. We recovered consistent results across multiple genera of centrolenids, supporting that hatching plasticity is widespread, likely ancient, and potentially maintained by shared selective trade-offs in this family.

Glassfrogs exhibit substantial plasticity in hatching age. All five studied species are capable of hatching at 7 days old, but can delay hatching until 19–21 days—doubling to tripling their embryonic period. Based on the onset of hatching competence, undisturbed clutches exhibited average facultative delays of 75–112.7% and maximum delays of up to

171–200% across species. We likely underestimated average shifts, as field clutches were assumed to be "undisturbed" when we did not detect externally caused embryo mortality. This would not exclude clutches from which all embryos successfully escaped biotic or abiotic risk by hatching early. The magnitude of hatching plasticity in centrolenids is large compared to other semi-terrestrial breeding frogs that hatch in response to biotic and abiotic threats (reviewed by Warkentin 2011b). For example among sympatric species at our study site in Panama, *Agalychnis callidryas* exhibit maximum delays of 100% and *Dendropsophus ebraccatus* of ca.160% (Warkentin et al. 2017; Touchon et al. 2011). Moreover, due to the slower development of glassfrogs, their facultative embryonic periods are also absolutely long. Hatching-competent glassfrogs may spend weeks *in ovo* exposed to egg-stage threats, while sympatric *D. ebraccatus* embryos spend no more than 2 days and *A. callidryas* no more than 4 days past hatching competence (Touchon and Warkentin 2010; Touchon et al. 2011; K. Cohen unpublished, Warkentin et al. 2017).

While we did not experimentally test cued-hatching responses, our results support that embryos hatch in response to biotic and abiotic risk. Egg dehydration and predation are the two most common causes of embryo mortality for all five glassfrog species at this site, on average accounting for 56–89% of total mortality during the egg-stage (Delia et al. 2017; *in revision*). Field-monitored clutches that experienced these sources of mortality hatched earlier than did undisturbed clutches. We directly observed rapid early hatching during attacks by several kinds of invertebrate and vertebrate predators in all species except *T. pulverata*. Predators and egg-dehydration are known to induce escape hatching across clades of arboreal-breeding frogs with aquatic larvae, including the Hylidae (Touchon and Warkentin 2010; Touchon et al. 2011), Hyperoliidae (Vonesh 2005), Phyllomedusidae (Warkentin 1995; Gomez-Mestre et al. 2008; Salica et al. 2017), and Rhacophoridae (Poo and Bickford 2014). Similar risks to terrestrial frog eggs may have promoted convergent or parallel cued-hatching mechanisms across repeated origins of semi-terrestrial reproduction. More experimental research in glassfrogs is needed to assess hatching responses to particular cues and the associated mechanisms enabling cued hatching.

We found that extended development *in ovo* improves an ecologically relevant performance trait. Across species, 14.5–15.5 day-old hatchlings dove on average 1.4–3.8 times faster than 7.5 day-old hatchlings. Burst swimming speed correlates with escape success from predators in tadpoles of many species (e.g., Watkins 1996; Dayton et al. 2005; Teplitsky et al. 2005). In glassfrogs, diving speed affects the time needed for hatchlings to reach refuge in the stream bottom and, consequently, their exposure to predatory fishes in the water column. The study stream in Panama hosts a diversity of fishes (Rio Frijoles, Angermeier and Karr 1983), and egg clutches can be laid over water up to several meters deep. We observed poeciliid fishes (including *P. gillii*) catching and consuming hatchlings as they dove through the water in Rio Frijoles. In *H. colymbiphyllum*, older hatchlings have greater escape-success from *P. gillii* than do younger individuals (Delia unpublished data). The enhanced diving performance associated with prolonged embryonic development seems likely to confer a survival advantage for other species as well, although this needs to be tested. Our results are consistent with some studies evaluating the adaptive benefits of delayed hatching to the post-hatching stage. Research on phyllomedusid frogs found that older, more developed hatchlings are better at escaping multiple larval-stage predators (Warkentin 1995; Gomez-Mestre et al. 2008). Older hatchlings of the direct-developing Coqui frog (*Eleutherodactylus coqui*) have better jumping performance, which may be beneficial to escape predators on the forest floor (Buckley et al. 2005). In species with smaller magnitude shifts in hatching, studies have found mixed results as to whether there are immediate survival costs of early hatching

(e.g., Gomez-Mestre et al. 2006; Touchon and Warkentin 2010). There is also some evidence that costs of early hatching can appear later in development (Vonesh and Bolker 2005; Touchon et al. 2013).

We found that older hatchlings gain immediate foraging benefits when they enter the larval stage. Younger hatchlings receive no such benefits at hatching, as they are unable to feed until on average 4.5–5.9 days later. This difference in feeding onset is a direct consequence of digestive-system development that occurs during the facultative embryonic period; at hatching competence all species exhibit an intact yolk sac, which is converted into a functioning digestive system during the plastic embryonic period. Therefore, delaying hatching allows embryos to maximize growth and development on maternal yolk *in ovo* and hatch ready to forage. Like other amphibians, young glassfrog hatchlings do not lose access to their remaining yolk reserves. However, a post-hatching delay until feeding means they cannot yet accrue external resources for exotrophically based growth. The inability of younger hatchlings to feed is not a *cost* of early hatching, but it reveals that there is no resource-acquisition benefit to be gained by hatching at that stage. Early hatched *A. callidryas* develop faster than embryos of the same age—perhaps due to metabolic constraints in the egg—and compensatory growth may extend for some time into the larval period, so that early-hatched tadpoles become larger than later-hatched ones (Warkentin 1999; Touchon et al. 2013). Similarly, we found that early hatchlings reach feeding competence before older hatchlings do, despite the difference in post-hatching lag time. Embryos hatched at 7.5 days began feeding on average 1.8–4.04 days younger than did those hatched at 14.5–15.5 days, while the late-hatching treatment was still *in ovo*. It is possible that glassfrogs are capable of compensatory growth to offset some costs of early hatching.

Our results suggest that hatching plasticity could help embryos benefit from facultative extensions in parental care. Across independent origins of prolonged care in glassfrogs (*Centrolene*, *Hyalinobatrachium*, and *Ikakogi*), we have found that embryos hatch early when abandoned by their caregiving parent and delay hatching under continued care (Delia et al. 2014; Delia unpublished data). It is not known what cues early hatching in these species, but it might occur in response to deteriorating egg environments in the absence of care (e.g., predation, dehydration, and the accumulation of embryonic wastes; Delia et al. 2014; Méndez-Narváez and Delia unpublished data). Here, our results support that delaying hatching provides a mechanism that could convert facultative extensions in care into direct offspring benefits. The evolution of parental care is thought to alter selection on offspring traits that increase the efficiency with which care is converted into offspring fitness (Kölliker et al. 2012). For example, begging behavior can indicate offspring need and/or solicit care, and likely coevolves with parental traits in many taxa (e.g., Royle et al. 2004; Hinde et al. 2010; Yoshioka et al. 2016). There is no evidence suggesting that glassfrog embryos can solicit longer care durations. However, hatching plasticity may allow embryos to cope with and/or exploit behavioral changes in parenting, mitigating fitness costs of early abandonment and converting increased parental effort into direct benefits.

Hatching plasticity is widespread and likely ancient in centrolenids. It has been detected in 7 of 12 genera, including the genus sister to all other glassfrogs (this work, Delia unpublished data). Based on this distribution, it appears that some level of hatching plasticity is ancestral in this family and, if so, it has been conserved for some ~ 19–35 million years (according to divergence-time estimates by Hutter et al. 2013 and Castroviejo-Fisher et al. 2014). We found consistent results across three genera of glassfrogs, supporting that semi-terrestrial reproduction generates clear stage-specific tradeoffs that maintain hatching plasticity across species. Selection for this plasticity may have been generated or enhanced by an initial transition from aquatic to semi-terrestrial reproduction, which occurred in the

ancestor of the family or perhaps even earlier—the reproductive mode of the sister family, the Allophrynidae, remains unknown.

Summary

A recent meta-analyses testing the generality of cued hatching in amphibians found equivocal results across species (Van Buskirk 2016). However, this analysis focused predominately on species with aquatic oviposition, where hatching might not allow successful escape from aquatic predators. The greatest cued shifts in hatching occur among amphibians with terrestrial eggs and aquatic larvae (reviewed by Warkentin 2011b), supporting that this reproductive mode is associated with strong selective trade-offs. Our results establish glassfrogs as another lineage—with an independent origin of semi-terrestrial reproduction—in which clear trade-offs occur between egg- and larval-stage risks and embryos have evolved substantial plasticity in hatching age. This strengthens the generality of the association of reproductive ecology with the nature and magnitude of hatching plasticity. Stage-specific selection on eggs and larvae should be assessed for other lineages in which these life stages share, and do not share, habitats.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All research was conducted in accordance with approved IACUC protocols from the Smithsonian Tropical Research Institute (2011-0426-2014-04, 2014–0601-2017-2-A4), and permits provided from the Ministerio de Ambiente, Panama (SE/A-47-11, SC/A-24-12, SE/A-65-13, SE/A-70-13, SE/A-51-14, SEX/A-93-14, SE/A-63-15, SC/A-28-16).

References

Angermeier PL, Karr JR (1983) Fish communities along environmental gradients in a system of tropical streams. Environ Biol Fishes 9:117–135

Armstrong AF, Blackburn HN, Allen JD (2012) A novel report of hatching plasticity in the phylum echinodermata. Am Nat 181:264–272

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1–48

Buckley CR, Michael SF, Irschick DJ (2005) Early hatching decreases jumping performance in a direct-developing frog, *Eleutherodactylus coqui*. Funct Ecol 19:67–72

Castroviejo-Fisher S, Guayasamin JM, Gonzalez-Voyer A, Vilà C, Ebach M (2014) Neotropical diversification seen through glassfrogs. J Biogeogr 41:66–80

Day T, Rowe L (2002) Developmental thresholds and the evolution of reaction norms for age and size at life-history transitions. Am Nat 159:338–350

Dayton GH, Saenz D, Baum KA, Langerhans RB, DeWitt TJ (2005) Body shape, burst speed and escape behavior of larval anurans. Oikos 111:582–591

Delia JRJ, Ramirez-Bautista A, Summers K (2014) Glassfrog embryos hatch early after parental desertion. Proc R Soc B Biol Sci 281:2013–3237

Delia J, Bravo-Valencia L, Warkentin KM (2017) Patterns of parental care in Neotropical glassfrogs: fieldwork alters hypotheses of sex-role evolution. J Evol Biol 30:898–914

Delia J, Bravo-Valencia L, Warkentin KM (in revision) The evolution of extended parental care in glass-frogs: egg-clutch phenotypes might mediate coevolution between the sexes. Ecol Monogr

Gomez-Mestre I, Touchon JC, Warkentin KM (2006) Amphibian embryo and parental defenses and a larval predator reduce egg mortality from water mold. Ecology 87:2570–2581

Gomez-Mestre I, Wiens JJ, Warkentin KM (2008) Evolution of adaptive plasticity: risk-sensitive hatching in neotropical leaf-breeding treefrogs. Ecol Monogr 78:205–224

Gomez-Mestre I, Pyron RA, Wiens JJ (2012) Phylogenetic analyses reveal unexpected patterns in the evolution of reproductive modes in frogs. Evolution 66:3687–3700

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190

Hawley TJ (2006) Embryonic development and mortality in *Hyalinobatrachium pulveratum* (Anura: Centrolenidae) of south-western Costa Rica. J Trop Ecol 22:731–734

Hayes MP (1983) A technique for partitioning hatching and morality estimates in leaf-breeding frogs. Herpetol Rev 14:115–116

Hinde CA, Johnstone RA, Kilner RM (2010) Parent-offspring conflict and coadaptation. Science 327:1373–1376

Hoffman H (2010) The glass frog tadpoles of Costa Rica (Anura: Centrolenidae): a study of morphology. Abh Senckenberg Ges Naturforschung 567:1–78

Hutter CR, Guayasamin JM, Wiens JJ (2013) Explaining Andean megadiversity: the evolutionary and ecological causes of glassfrog elevational richness patterns. Ecol Lett 16:1135–1144

Kölliker M, Royle NJ, Smiseth PT (2012) Parent-offspring co-adaptation. In: Royle NJ, Smiseth PT, Kölliker M (eds) The evolution of parental care. Oxford University Press, Oxford, pp 285–303

Lehtinen RM, Georgiadis AP (2012) Observations on parental care in the glass frog *Hyalinobatrachium orientale* (Anura: Centrolenidae) from Tobago, with comments on its natural history. Phyllomedusa 11:75–77

Martin KLM (1999) Ready and waiting: delayed hatching and extended incubation of anamniotic vertebrate terrestrial eggs. Am Zool 39:279–288

Moran NA (1992) The evolutionary maintenance of alternative phenotypes. Am Nat 139:971–989

Nokhbatolfoghahai M, Pollock CJ, Downie JR (2015) Oviposition and development in the glass frog *Hyalinobatrachium orientale* (Anura: Centrolenidae). Phyllomedusa 14:3

Poo S, Bickford DP (2014) Hatching plasticity in a southeast Asian tree frog. Behav Ecol Sociobiol 68:1733–1740

R Core Team (2017) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/. Accessed Mar 2017

Royle NJ, Hartley IR, Parker GA (2004) Parental investment and family dynamics: interactions between theory and empirical tests. Popul Ecol 46:231–241

Salica MJ, Vonesh JR, Warkentin KM (2017) Egg clutch dehydration induces early hatching in red-eyed treefrogs, *Agalychnis callidryas*. PeerJ 5:e3549

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675

Shine R (1978) Propagule size and parental care: the "safe harbor" hypothesis. J Theor Biol 75:417–424

Teplitsky C, Plenet S, Léna JP, Mermet N, Malet E, Joly P (2005) Escape behaviour and ultimate causes of specific induced defenses in an anuran tadpole. J Evol Biol 18:180–190

Touchon JC, Warkentin KM (2010) Short- and long-term effects of the abiotic egg environment on viability, development and vulnerability to predators of a neotropical anuran. Funct Ecol 24:566–575

Touchon JC, Urbina J, Warkentin KM (2011) Habitat-specific constraints on induced hatching in a treefrog with reproductive mode plasticity. Behav Ecol 22:169–175

Touchon JC, McCoy MW, Vonesh JR, Warkentin KM (2013) Effects of plastic hatching timing carry over through metamorphosis in red-eyed treefrogs. Ecology 94:850–860

Van Buskirk J (2016) A meta-analysis on facultative responses of embryonic amphibians to predation risk. Copeia 104:691–696

Via S, Lande R (1985) Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution 39:505–522

Villa J, Valerio CE (1982) Red, white and brown. Preliminary observations on the color of the centrolenid tadpole (Amphibia: Anura: Centrolenidae). Brenesia 19:1–16

Vonesh JR (2005) Egg predation and predator-induced hatching plasticity in the African reed frog, *Hyperolius spinigularis*. Oikos 2:241–252

Vonesh JR, Bolker BM (2005) Compensatory larval responses shift trade-offs associated with predator-induced hatching plasticity. Ecology 86:1580–1591

Warkentin KM (1995) Adaptive plasticity in hatching age: a response to predation risk trade-offs. Proc Natl Acad Sci 92:3507–3510

Warkentin KM (1999) Effects of hatching age on development and hatchling morphology in the red-eyed treefrog, *Agalychnis callidryas*. Biol J Linn Soc 68:443–470

Warkentin KM (2000) Wasp predation and wasp-induced hatching of red-eyed treefrog eggs. Anim Behav 60:503–510

Warkentin KM (2011a) Environmentally cued hatching across taxa: embryos respond to risk and opportunity. Integr Comp Biol 51:14–25

Warkentin KM (2011b) Plasticity of hatching in amphibians: evolution, trade-offs, cues and mechanisms. Integr Comp Biol 51:111–127

Warkentin KM, Currie CC, Rehner SA (2001) Egg-killing fungus induces early hatching of red-eyed treefrog eggs. Ecology 82:2860–2869

Warkentin KM, Cuccaro Diaz J, Güell BA, Jung J, Kim SJ, Cohen KL (2017) Developmental onset of escape-hatching responses in red-eyed treefrogs depends on cue type. Anim Behav 129:103–112

Watkins TB (1996) Predator-mediated selection on burst swimming performance in tadpoles of the pacific tree frog, *Pseudacris regilla*. Physiol Zool 69:154–167

Werner EE (1986) Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. Am Nat 128:319–341

Werner EE, Gilliam JF (1984) The ontogenetic niche and species interactions in size-structured populations. Annu Rev Ecol Syst 15:393–425

Wilbur HM, Collins JP (1973) Ecological aspects of amphibian metamorphosis. Science 182:1305–1314

Williams GC (1966) Adaptation and natural selection: a critique of some current evolutionary thought. Princeton University Press, Princeton

Willink B, Palmer MS, Landberg T, Vonesh JR, Warkentin KM (2014) Environmental context shapes immediate and cumulative costs of risk-induced early hatching. Evol Ecol 28:103–116

Yoshioka M, Meeks C, Summers K (2016) Evidence for begging as an honest signal of offspring need in the biparental mimic poison frog. Anim Behav 113:1–11

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