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Corresponding Author: Professor Karen Michelle Warkentin, PhD

Corresponding Author's Institution: Boston University

First Author: Karen Michelle Warkentin, PhD

Order of Authors: Karen Michelle Warkentin, PhD; Juliana Cuccaro Diaz, BSc; Brandon A Güell, BS; Julie Jung, BA; Su Jin Kim; Kristina L Cohen, MA

Abstract: Developmental onset of escape-hatching responses in red-eyed treefrogs depend on cue type

Hatching is an essential and often behaviorally mediated process. Many animals can hatch at different developmental stages, and embryos time hatching based on cues indicating threats to eggs or opportunities outside them. However, the specific mechanisms enabling such responses, and how their ontogenies combine to determine when environmentally cued hatching is possible, are largely unknown. Many embryos use stimuli in multiple sensory modalities as cues to hatch. Thus, comparing the onset of responses across cue types can distinguish shared ontogenetic constraints, such as hatching ability, from constraints specific to a single modality, such as sensor development. The arboreal embryos of red-eyed treefrogs, *Agalychnis callidryas*, hatch rapidly in response to physical disturbance in predator attacks and hypoxia if submerged in water. Prior experiments and field monitoring documented both responses beginning at age 4 days at our study site in Panama. We tested embryos, starting at 3 d, for hypoxia-cued hatching (HCH) and mechanosensory-cued hatching (MCH), submerging eggs in degassed water to provide a strong hypoxia cue and manually jiggling eggs as a stimulated attack cue. We tested developmental series for HCH, MCH, and both responses, and identified morphological markers to distinguish developmental stages across the onset of hatching. Hatching competence begins substantially earlier than previously reported. Across sibships, HCH began at a smaller size, less developed stage, and on average 8 h earlier than MCH. Both responses increased from 0-100% over just a few hours. Latency to hatch, after stimulation, was much longer in HCH and uncorrelated with stage, whereas latency in MCH decreased with development. HCH appears constrained by the development of hatching ability, while MCH appears constrained by mechanosensor development. Hatching ability is not the sole constraint on the developmental onset of escape-hatching responses to attacks.

Suggested Reviewers: Karen Martin PhD
Professor, Pepperdine University
Karen.Martin@Pepperdine.edu

She has published extensively on environmentally cued hatching in California grunion, which hatch in response to a combination of flooding and mechanosensory stimulation. She knows the environmentally cued hatching literature well.

Hiromi Mukai PhD

Forest Entomology, Forestry & Forest Prod Res Inst, Ibaraki, Japan
mhisa8088@ffpri.affrc.go.jp

She has published on vibration-cued hatching and mother-embryo interactions in insects.

J. Sean Doody PhD

Asst. Prof., SE Louisiana University
jseandoody@gmail.com

He has published on environmentally cued hatching in reptiles, including flooding-cued hatching of turtles and mechanosensory-cued hatching of skinks.

Doug Chivers PhD

Professor, Biology, University of Saskatchewan
doug.chivers@usask.ca

Doug has published extensively on predator-prey interactions, including work with fish and amphibian embryos, environmentally cued hatching, and embryo learning. This includes assessments of the role of different sensory modalities in such interactions. [Maud Ferrari, also at U.Sask and a collaborator of Doug's in much of this work, would be another possible reviewer.]

Opposed Reviewers:



Karen M. Warkentin, Professor, Boston University
Research Associate, Smithsonian Tropical Research Institute

15 December 2016

Dr. Susan Foster,
Editor, *Animal Behaviour*

Dear Dr. Foster,

Please consider our manuscript “Developmental onset of the escape-hatching response in red-eyed treefrogs depends on cue type” for publication in *Animal Behaviour*.

This research examines the development of adaptive embryo behavior in the frog *Agalychnis callidryas*. We found that hypoxia-cued hatching begins earlier in ontogeny than does mechanosensory-cued hatching; thus their onset is limited by different developmental events. Behavioral responses to hypoxia begin long before hatching competence, and hatching responses start once a hatching mechanism develops. However, escape-hatching in response to predator attacks, or simulated attack cues, does not begin until later, apparently limited by mechanosensor development. As an essential and often environmentally cued embryo behavior, hatching offers excellent opportunities to study mechanisms underlying developmental changes in behavior.

The research was conducted under research permits from the Panamanian Environmental Ministry (SC/A-15-14, SE/A-46-15, SE/A-59-16) and an institutional animal care and use protocol from the Smithsonian Tropical Research Institute (protocol 2014-0601-2017), secondarily approved by Boston University.

The manuscript has not been published, nor is it under consideration for publication elsewhere, and all authors have approved the manuscript. Some of the research in the manuscript has been presented at meetings of the *Society for Integrative and Comparative Biology*, in combination with other related work, and thus the associated abstracts are available, as listed below.

Cohen, KL and KM Warkentin. 2014. Do distinct hatching glands mediate hatching at different ontogenetic stages in red-eyed treefrogs? **Integr Comp Biol** 54:E255-E255.

Jung, J, SJ Kim, BA Güell, KL Cohen, and KM Warkentin. 2016. Ontogeny of escape hatching in red-eyed treefrogs: onset of response to flooding and attack cues. **Integr Comp Biol** 56:E310-E310.

Kim, SJ, J Jung, SMP Arias, JG McDaniel, and KM Warkentin. 2016. Shake and roll: testing the ontogenetic correlation of vibration-cued hatching and otic mechanoreception in red-eyed treefrogs. **Integr Comp Biol** 56:E315-E315.

Warkentin, KM, KL Cohen, JC Diaz, BA Guell, and J Jung. 2016. Development of embryo behavior: hatching mechanisms, performance, and decisions in red-eyed treefrogs. **Integr Comp Biol** 56:E234-E234.

We have provided these abstracts as “Related Material” and cite them all in the manuscript.

Thank you for considering our manuscript.

Sincerely,

Karen Warkentin

TITLE

Developmental onset of escape-hatching responses in red-eyed treefrogs depend on cue type

RUNNING TITLE

Onset of escape-hatching

AUTHOR INFORMATION

Karen M. Warkentin^{1,2,*}, Juliana Cuccaro Diaz³, Brandon A. Güell⁴, Julie Jung¹, Su Jin Kim¹, Kristina L. Cohen¹

¹Department of Biology, Boston University, MA, USA

²Smithsonian Tropical Research Institute, Panamá

³Universidad de los Andes, Bogota, Colombia

⁴University of California, San Diego, USA

Word count: 8388 (including everything)

*Author for correspondence: kwarken@bu.edu; Department of Biology, Boston University, 5 Cummington Mall, Boston, MA, 02215, USA; Phone: 617-358-2385; Fax: 617-353-6340

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9 Karen M. Warkentin^{1,2,*}, Juliana Cuccaro Diaz³, Brandon A. Güell⁴, Julie Jung¹, Su Jin Kim¹,

10 Kristina L. Cohen¹

11 ¹Department of Biology, Boston University, MA, USA

12 ²Smithsonian Tropical Research Institute, Panamá

13 ³Universidad de los Andes, Bogota, Colombia

14 ⁴University of California, San Diego, USA

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20 *Author for correspondence: kwarken@bu.edu; Department of Biology, Boston University, 5

21 Cummington Mall, Boston, MA, 02215, USA; Phone: 617-358-2385; Fax: 617-353-6340

Hatching is an essential and often behaviorally mediated process. Many animals can hatch at different developmental stages, and embryos time hatching based on cues indicating threats to eggs or opportunities outside them. However, the specific mechanisms enabling such responses, and how their ontogenies combine to determine when environmentally cued hatching is possible, are largely unknown. Many embryos use stimuli in multiple sensory modalities as cues to hatch. Thus, comparing the onset of responses across cue types can distinguish shared ontogenetic constraints, such as hatching ability, from constraints specific to a single modality, such as sensor development. The arboreal embryos of red-eyed treefrogs, *Agalychnis callidryas*, hatch rapidly in response to physical disturbance in predator attacks and hypoxia if submerged in water. Prior experiments and field monitoring documented both responses beginning at age 4 days at our study site in Panama. We tested embryos, starting at 3 d, for hypoxia-cued hatching (HCH) and mechanosensory-cued hatching (MCH), submerging eggs in degassed water to provide a strong hypoxia cue and manually jiggling eggs as a stimulated attack cue. We tested developmental series for HCH, MCH, and both responses, and identified morphological markers to distinguish developmental stages across the onset of hatching. Hatching competence begins substantially earlier than previously reported. Across sibships, HCH began at a smaller size, less developed stage, and on average 8 h earlier than MCH. Both responses increased from 0–100% over just a few hours. Latency to hatch, after stimulation, was much longer in HCH and uncorrelated with stage, whereas latency in MCH decreased with development. HCH appears constrained by the development of hatching ability, while MCH appears constrained by mechanosensor development. Hatching ability is not the sole constraint on the developmental onset of escape-hatching responses to attacks.

KEYWORDS: antipredator defense, anuran, embryo behavior, environmentally cued hatching, developmental constraint, ontogenetic adaptation, oxygen, phenotypic plasticity, sensory development, vibration

Developmental changes in behavior reflect a combination of maturational processes and ontogenetic adaptations (Wiedenmayer 2009). For instance, the sequential development of different sensory systems increases the range of stimuli to which animals can respond (Romagny et al. 2012) and the appropriate response to a potential threat changes with ontogenetic changes in vulnerability (Wiedenmayer 2009). Most research on the development of behavior in embryos focuses on behaviors that will function later in life, including motor skills (Bate 1999; Grillner 2000), communication (Colombelli-Negrel et al. 2012; Lickliter 2005), anti-predator defense (Ferrari and Chivers 2009; Mathis et al. 2008) and foraging (Darmaillacq et al. 2008; Guibe et al. 2012). Nonetheless, embryo behavior is of widespread and immediate functional consequence in one context: hatching. Both embryos and hatchlings often suffer high mortality, but causes of death (e.g., predators, pathogens, harsh abiotic conditions, starvation) are often stage-specific (Warkentin 2011a; Warkentin 2011b). Thus embryos can avoid or escape from threats, and exploit opportunities, by timing their hatching appropriately. Environmentally cued hatching (ECH) is widespread among animals (Warkentin 2011a); embryos respond to diverse biotic and abiotic factors, including predators, pathogens, conspecifics, hosts, food resources, dehydration, and flooding, using cues in multiple sensory modalities (Warkentin 2011a; Warkentin 2011b). Responses range from substantially premature hatching, to escape threats to eggs, to long delays in hatching under conditions unfavorable to hatched young. Nonetheless, there must be constraints on the developmental period when hatching is sensitive to environmental cues.

68

69 The factors that limit the period of cued, plastic hatching depend on the type of ECH. For
70 instance, for embryos that delay hatching until conditions are favorable, energy reserves can
71 limit the maximum delay (Bradford and Seymour 1985; Darken et al. 1998). For embryos that
72 hatch early in response to cues, two factors may constrain the onset of hatching responses. First,
73 embryos must have reached hatching competence in order to show a hatching response to any
74 cue. The development of any component of the hatching mechanism could, therefore, limit the
75 onset of cued hatching, and this constraint would apply equally across all cue types. Second,
76 embryos must detect an environmental cue in order to respond to it. Cue detection depends on
77 the development of the relevant sensory system, and cue assessment may also require further
78 neural processing (Romagny et al. 2012; Wiedenmayer 2009). Thus, the developmental stage
79 when embryos begin responding to threats may differ for cues in different sensory modalities.
80 We studied the ontogenetic onset of an early “escape-hatching” response to two different threats,
81 cued by two sensory modalities, to test if they shared or differed in their period of sensitivity,
82 and thus were limited by development of the same or different aspects of the underlying
83 mechanisms.

84

85 We worked with red-eyed treefrogs, *Agalychnis callidryas*, one of the best-studied examples of
86 environmentally cued hatching. This species lays eggs on vegetation over ponds, where
87 undisturbed embryos develop for 6 or 7 days before they hatch and drop into the water below.
88 Hatching is a rapid switch between two environments, allowing embryos to escape egg-stage
89 threats by hatching early. Egg predation is the most common threat; snakes have attacked from
90 24% to over 60% of monitored clutches, across years and ponds, while wasps attacked up to 50%

of clutches (Gomez-Mestre and Warkentin 2007; Warkentin 1995; Warkentin 2000b). Embryos can hatch in seconds up to 30% prematurely to escape from attacking snakes and wasps (Warkentin 1995; Warkentin 2000b). They also hatch early, but less synchronously, in response to the slower-acting threats of pathogenic fungus and dehydration (Salica et al. 2012; Warkentin et al. 2001). Furthermore, submergence underwater can be deadly for eggs not yet capable of hatching (Pyburn 1970) and elicits hatching within tens of minutes (Warkentin 2002). Hatching responses to these different threats are mediated by different cue types, in different sensory modalities. Predator-induced hatching depends on mechanosensory cues, including vibrations, during the physical disturbance of clutches, and embryos can distinguish snake vibrations from those caused by benign sources (Warkentin 2005; Warkentin and Caldwell 2009). In contrast, flooding-induced hatching is cued by hypoxia (Warkentin 2002; Warkentin 2007). As early as the neural tube stage, long before hatching has been observed, embryos sense and orient in oxygen gradients within the egg (Rogge and Warkentin 2008). Thus we hypothesize that the onset of flooding-induced hatching is limited by the development of hatching ability rather than oxygen sensing ability. The sensory system mediating predator-induced hatching is unknown; however, mechanosensory structures in anurans include the inner ear and lateral line (Hill 2008), both of which develop later (Bever et al. 2003; Nieuwkoop and Faber 1956). Thus the onset of predator-induced hatching might be limited by the development of either hatching ability or mechanosensory ability.

Agalychnis callidryas shows very little additive genetic variance for the onset of hatching in response to physical disturbance; this suggests a developmental constraint, hypothesized to be the maturation of the hatching mechanism (Gomez-Mestre and Warkentin 2013). A comparative

study of the onset of hatching competence across *Agalychnis* species and other phyllomedusine treefrogs used both flooding and mechanosensory cues concurrently, without testing for a possible difference in the response to them (Gomez-Mestre et al. 2008). In Gamboa, Panama, escape-hatching begins on the morning embryos are 4 days old, both in field observations of predation and flooding and in predation and pathogen experiments (Gomez-Mestre and Warkentin 2013; Gomez-Mestre et al. 2008; Warkentin 1995; Warkentin 2000b; Warkentin et al. 2006a; Warkentin et al. 2001). Work at other sites shows some geographic, possibly thermal, variation in the onset of hatching but has been, nonetheless, consistent with the hypothesis that hatching onset is limited by development of the hatching mechanism (Gomez-Mestre and Warkentin 2013; Gomez-Mestre et al. 2008; Warkentin 1995).

However, an embryo's experience of hypoxia and risk of mortality in flooding must depend substantially on both the aquatic oxygen level and the exposed surface area of the egg. Thus, to test for congruence or divergence in the onset of hatching across cue types, and the possibility that different traits limit the onset of responses to different cues, we developed a stronger, more consistent assay for hypoxia-cued hatching. First, we exposed a developmental series of embryos to this strong hypoxia cue and found the escape-hatching response begins substantially earlier than revealed in any prior research. Then, with a second developmental series, we assessed the ontogenetic onset of both hypoxia-cued hatching and mechanosensory-cued hatching in the same sibships. We also tested for developmental changes in latency to hatch in response to both cues. Finally, because El Niño weather during the second series may have altered the timing of development and hatching, we tested a third developmental series with mechanosensory cues the following year to assess the developmental consistency of response onset.

Standard staging tables (Gosner 1960; Pyburn 1963) offer no resolution of developmental variation across the hatching period in *A. callidryas* (Warkentin 2000a; Warkentin 2002). Embryos pass through 22 stages in 4 days then, over the next 3–4 days, from the earliest predator-induced hatching to the latest spontaneous hatching, remain in stage 23 (Gosner 1960; Warkentin 2002). Stage 23 ends when external gills regress, which depends on hatching timing (Warkentin 2000a; Warkentin 2002; Warkentin 2007). During the plastic hatching period there is, however, substantial growth and development of mouthparts, digestive system, lungs, and other structures (Warkentin 1999b). Our prior time-based methods for characterizing development have revealed ecologically important changes across the plastic hatching period, affecting viability, survival with aquatic predators, and onset of feeding (Touchon et al. 2013; Warkentin 1995; Warkentin 1999a; Warkentin 1999b; Warkentin et al. 2006a; Willink et al. 2014). Nonetheless, they offer insufficiently detailed developmental resolution for the study of mechanisms underlying changes in embryo behavior. Therefore, in the second developmental series, we photographed test animals to identify characters that could be used as markers of development across the onset of cued hatching responses. This is a step toward a detailed staging table across the entire period of hatching competence and, together with our cued hatching tests, should make the onset of cued hatching abilities morphologically identifiable.

METHODS

Egg collection and care

We collected young *A. callidryas* egg clutches from the Experimental Pond in Gamboa, Panama (9.120894 N, 79.704015 W) and maintained and tested them in an ambient temperature and

humidity laboratory at the Smithsonian Tropical Research Institute. Most clutches are laid between 10 pm and 2 am, so we age embryos starting from midnight of the oviposition night. Clutches were mounted on plastic support cards, hung over aged tap water in plastic cups, and misted with rainwater frequently to maintain hydration. After experiments, hatchlings were maintained in predator-free water in the lab for 2–3 days and released into the Experimental Pond at a size typical for spontaneous hatching. Research was conducted under permits from the Panamanian Environmental Ministry (SC/A-15-14, SE/A-46-15, SE/A-59-16) and approved by the Institutional Animal Care and Use Committees of the Smithsonian Tropical Research Institute and Boston University.

Ontogeny of hatching response to hypoxia

To assess when *A. callidryas* embryos begin hatching in response to hypoxia, in 2014 we tested developmental series of embryos from 10 clutches (i.e., 10 full sibships: four on 3 July and six on 7 July). Based on pilot experiments, we began testing at age 3 days, at noon, and tested embryos every two hours until midnight. We tested 3 eggs per clutch per time point except for the smallest clutch, from which we used 2 eggs per time point. To provide a strong hypoxia cue and minimize time required for boundary layer formation and local oxygen depletion, we used degassed water. We boiled tap water for at least 10 min then allowed it to cool to ambient temperature in sealed glass bottles; water was $15 \pm 1.3\%$ air saturated (mean \pm SD, from a sample of 10 bottles). We carefully removed each egg from its clutch with blunt forceps and placed it in a small glass jar, filled the jar with hypoxic water, and sealed it to prevent air contact. We observed test embryos continuously, recording if and when each individual hatched. Hatched tadpoles were immediately transferred to air-saturated aged tap water. After 30 min, eggs

remaining unhatched were returned to air, and embryos that had ruptured their capsule but failed to exit were manually decapsulated and placed in air-saturated water.

Ontogeny of hatching responses to hypoxia and physical disturbance cues

In 2015, we tested developmental series of embryos from 11 clutches to assess the onset of hatching responses to both hypoxia and physical disturbance cues (six clutches Aug. 11–12, five Aug. 12–13). We tested 2 eggs per clutch per stimulus at 3 h intervals. We attempted to begin testing each stimulus before embryos would respond to it, and continued testing each clutch with a stimulus until both siblings had hatched in response to it twice. Due to the 2015 El Niño, temperatures in Gamboa were warmer and *A. callidryas* eggs developed faster than usual (daily mean temperatures 27.6 ± 0.8 C during the testing period, mean \pm SD). We therefore began testing with hypoxia at age 3 d, 9 am on Aug. 11 and at 3 d, 6 am Aug. 12. For all but one clutch, we began testing the response to physical disturbance only after embryos had hatched in response to hypoxia, to ensure sufficient siblings were available for testing both cues. Our assay for hypoxia-cued hatching followed 2014 methods, except we ran trials for 40 min. To test the hatching response to physical disturbance, we manually stimulated eggs with a simulated attack cue. We placed each egg in a small petri dish with a drop of water and used a blunt metal probe to jiggle, prod, slide and roll it in the dish, providing motion and tactile cues (Gomez-Mestre and Warkentin 2013; Gomez-Mestre et al. 2008). We stimulated embryos intermittently, alternating 15-s periods of jiggling and of rest for 5 min, or until the egg hatched (Supplementary video, Egg Jiggling Assay). Within a few minutes after hatching trials, we manually decapsulated unhatched embryos and photographed all test animals in dorsal view with a ruler, for measurement, and in frontal view inside a close-fitting water-filled tube, to assess morphological markers of

development. For individuals tested with mechanosensory cues, we also measured their vestibulo-ocular reflex, an indicator of otic mechanosensory function (Jung et al. 2016); details will be reported elsewhere. In 2016, under more typical thermal conditions (daily mean temperatures 26.4 ± 0.3 C), we repeated the physical disturbance assay for a series of 11 clutches, starting at 4 d, 6 am on June 22. After trials, we photographed hatchlings and manually decapsulated embryos in frontal view for staging.

Because standard staging tables (e.g., Gosner 1960) offer little or no resolution of *A. callidryas* development through the period of hatching competence (Warkentin 2002), we created a staging system to assess development across the onset of hatching. Concurrent with the 2015 hatching trials, we took a time-lapse series of macro-photographs, every 2 min, of another egg clutch from the same cohort as our test sibships. We generated an initial list of candidate traits from these photographs. We then scored the traits in all the frontal photos of hatchlings from the test sibships. We eliminated several traits that did not change in a consistent sequence, relative to other traits, across all 11 clutches. This left seven morphologically identifiable developmental stages (see results). We used these to classify hatchling development.

Analysis

We generated cumulative hatching response curves based on the mean proportion of test eggs hatched at each time point in the 2014 hypoxia-cued developmental series. We did the same for each cue type separately in the 2015 developmental series. Some hypoxia-cued individuals ruptured their membrane but remained inside it for some time. If they freed themselves by the end of the experiment, we counted them as hatched. We counted the 4 that needed rescue as not

hatched. One mechanosensory-cued individual ruptured its egg after the observation period, then hatched; it was also counted as not hatched. Treating these 5 individuals as hatched in our analyses either strengthens or does not change the statistical support for all effects tested. We used paired t-tests to compare the age and size, and Wilcoxon signed-rank tests to compare the developmental stage, of siblings hatched in response to each cue type at two points in the ontogeny of hatching: the earliest hatching and when the response became consistent, defined as the second time both siblings hatched. For earliest hatching, we compared traits of the first individuals hatched from each clutch in response to each cue, averaging length and stage of siblings in cases where both test embryos hatched at the first response. For the point of consistent response, we averaged trait values of siblings for analysis across clutches. We hypothesized that hypoxia-cued hatching begins before mechanosensory-cued hatching, thus used one-tailed tests. We imposed a Bonferroni correction for the six comparisons of cue effects on hatching timing, adjusting the *P*-value for significance to 0.0083. We compared the latency to hatch after the start of stimulation between cue types, and within cue types from earliest to consistent hatching, using Wilcoxon signed-rank tests. We also tested for correlations of latency to hatch with developmental stage and with size, for each cue type. Finally, we compared the size and stage of siblings that differed in their response to each cue. We also compared age and developmental stage at the point of consistent response to mechanosensory cues across years (2015 and 2016). Analyses were performed in JMP Pro 12 and RStudio Version 0.98.1091.

RESULTS

We identified a series of six morphological markers that changed in a consistent sequence across all clutches examined, based on melanophore distribution on the body, changes in venation over

the surface of the yolk, eye development, and beak development. These demarcate seven developmental stages (Table 1) encompassing the onset of hatching responses to hypoxia and mechanosensory cues. We used these stages to characterize development. Neither tail fin circulation nor operculum formation, which demarcate Gosner stages 22 and 23 (Gosner 1960), were visible in our frontal images. However, our stage 1 embryos were at least Gosner stage 21 and embryos reached Gosner stage 23 by our stage 5.

Table 1. Stages in the development of *Agalychnis callidryas* embryos through the onset of hatching, based on traits visible in frontal view.

Stage	Morphological criteria
1	Melanophores extend less than halfway down sides over yolk.
2	Melanophores extend at least halfway down sides over yolk. Two dominant veins on yolk surface enter heart separately, fairly symmetrically under center of heart.
3	Two dominant veins on yolk surface join to enter heart as a single vessel. Cornea close to lens and granular or slightly cloudy, partially obscures view of lens.
4	Cornea clear and well separated from lens; lens readily visible. Yolk vein enters medially below heart, within edge of cement gland in direct frontal view
5	Yolk vein enters heart dextrally, at or lateral to cement gland in direct frontal view. Eyes angled in dorsally.
6	Eyes parallel in frontal view, not angled in dorsally. Beaks unkeratinized.
7	Edge of upper and/or lower beaks keratinized.

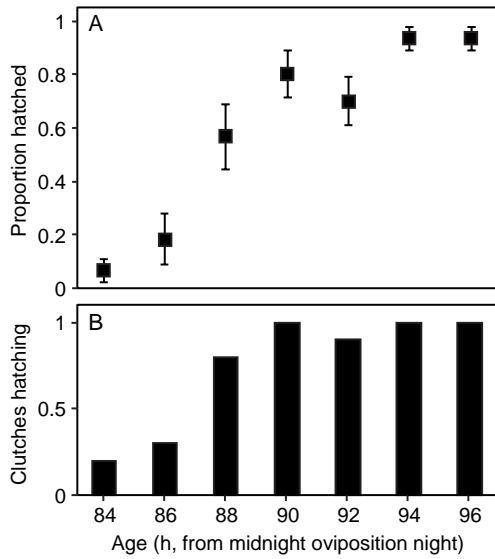


Fig. 1. Developmental onset of hatching of *Agalychnis callidryas* embryos in response to submergence in hypoxic water. (A) Mean proportion hatched, of 2–3 embryos per clutch (sibship) tested at each age, \pm SE across clutches. (B) Proportion of clutches with any eggs hatching at a time point. $N = 10$ clutches, tested in 2014 starting at noon, age 3 days.

In the 2014 developmental series, two embryos, from two different clutches, hatched at our first hypoxia test point (84 h; age 3 d, noon). The hatching response increased gradually over the afternoon, with individuals hatching from all clutches tested by 6 pm (90 h, Fig. 1). In 2015, the earliest hypoxia-cued hatching was at 3 d, 6 am (78 h), and again all clutches had begun hatching by 6 pm. Across clutches, hypoxia-cued hatching both began and became consistent earlier than did mechanosensory-cued hatching, whether measured by age (Fig. 2; first hatching: $t_{10} = 7.4198$, $P = 2.26e^{-05}$; consistent: $t_{10} = 6.8472$, $P = 4.47e^{-05}$), embryo size (Fig. 3A; first hatching: $t_{10} = 4.6818$, $P = 0.0009$; consistent: $t_{10} = 6.2058$, $P = 0.0001$), or developmental stage (Fig. 3B; both first and consistent hatching: Wilcoxon signed-rank test, $V = 0$, $P = 0.0036$). In the modal pattern, hypoxia-cued hatching began at 87 h in stage 2 (3 d, 3 pm; Fig. 4A), and was consistent at 90 h in stage 3 (3d, 6 pm; Fig. 4B); mechanosensory-cued hatching began at 93 h in stage 5 (3 d, 9 pm; Fig. 4C) and was consistent at 99 h in stage 6 (4 d, 3 am; mean \pm SD = 101.5 \pm 4.2 h; Fig. 4D). Stage at the onset of hatching was very consistent under hypoxia and more variable in response to the mechanosensory cue (First hatching: Fig. 3B; Levene's test, $F_{1,20} = 16.988$, $P = 0.0005$). However, the time from first until consistent hatching did not differ between stimulus

types ($t_{10} = 1.047$, $P = 0.32$). Mechanosensory-cued hatching became consistent later in 2016 than in 2015 (2016 mode, 4 d noon, 108 h; mean \pm SD = 108.8 ± 2.7 h; $t_{10} = -4.9796$, $P = 0.0006$), but at the same developmental stage (stage 6, Wilcoxon rank-sum test, $Z = 0.5570$, $N_{2015} = N_{2016} = 11$, $P = 0.58$). When we began testing in 2016 at 4 d, 6 am, all but one clutch had at least 1 individual hatch. Thus, it is possible a few might have hatched earlier. The clutch that began hatching at 9 am hatched consistently by 6 pm (9 h lag). All hatching data are included as supplementary material.

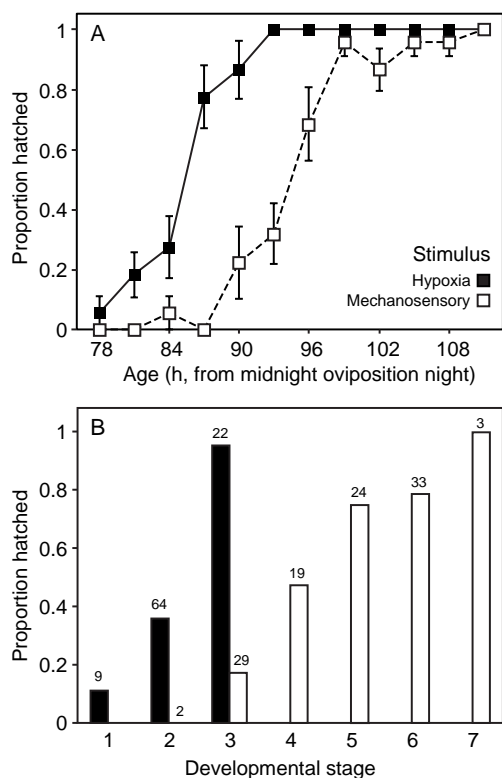


Fig. 2. Onset of hatching of *Agalychnis callidryas* embryos in response to two cue types. Embryos began responding to hypoxia, which occurs when arboreal eggs are submerged in water, earlier than they did to mechanosensory cues, which can indicate egg predator attack. (A) Mean proportion of embryos that hatched at each age, of 2 tested per clutch, \pm SE across clutches, and (B) overall proportion of embryos tested at each developmental stage that hatched in response to each cue. Embryos were from developmental series of 11 clutches tested in 2015 with both cues; N for each stage is indicated. See Table 1 for morphological criteria defining developmental stages.

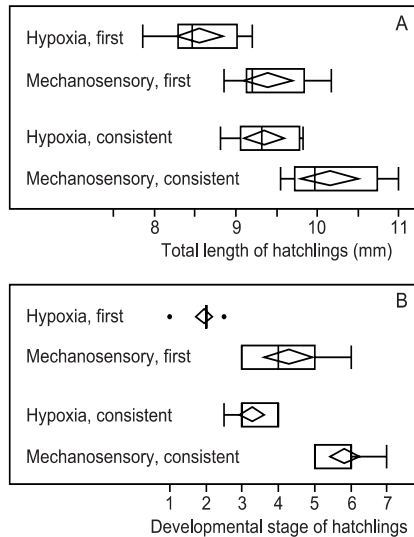


Fig. 3. The onset of environmentally cued hatching in *Agalychnis callidryas* occurred at (A) a smaller size and (B) an earlier developmental stage with hypoxia than with mechanosensory cues. Data are from developmental series of 11 clutches tested with both cues, with 2 siblings tested per time point. We considered the hatching response to a cue to be consistent in a clutch the second time that both siblings hatched, and averaged size and stage values across the siblings. Both siblings hatched at first response to hypoxia in 2 clutches, and at first response to mechanosensory cues in 5 clutches; other first values are from the individual that hatched. The graph shows means and 95% confidence intervals (diamonds), mode, interquartile range (IQR) and extent of data to $\pm 1.5 \times \text{IQR}$ (box and whiskers), and outliers.

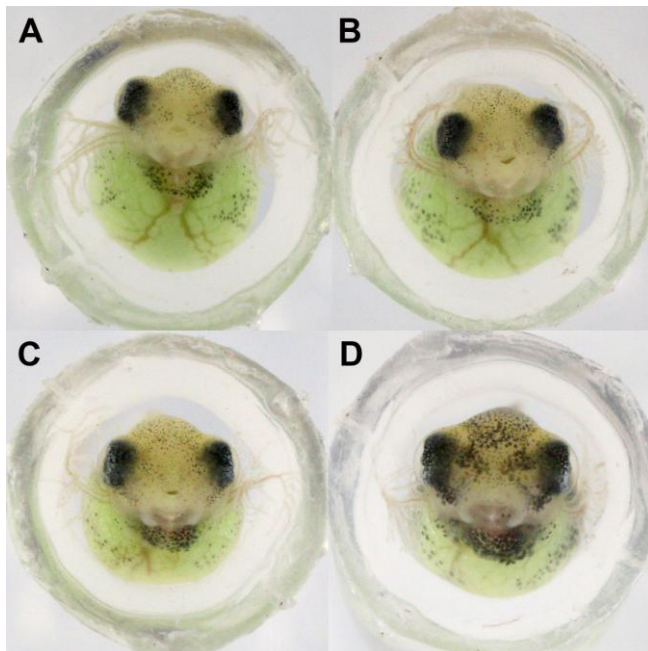


Fig. 4. Frontal views of *Agalychnis callidryas* hatchlings at the modal developmental stages of earliest and consistent hatching in response to strong hypoxia and mechanosensory cues: A) stage 2, earliest hypoxia-cued hatching; B) stage 3, consistent hypoxia-cued hatching; C) stage 5, earliest mechanosensory cued hatching; D) stage 6, consistent mechanosensory cued hatching.

287 The latency to hatch after onset of the stimulus was an order of magnitude longer for hypoxia
 288 than for mechanosensory cues (Fig. 5; Wilcoxon signed-rank test on clutch mean values, $V = 66$,
 289 $N = 11$, $P = 0.00098$). Under hypoxia, embryos took 20.6 ± 5.2 min to hatch (mean \pm SD, $N =$
 290 108, range 12.0 – 34.2 min), while in response to the mechanosensory stimulus they hatched in
 291 2.4 ± 1.6 min ($N = 112$, range 0.1 – 5.8 min). Latency did not change developmentally from first
 292 to consistent hatching in response to hypoxia (Fig. 5; Wilcoxon signed-rank test, $V = 46$, $N = 11$,
 293 $P = 0.2662$) and, across the tested developmental range, latency to hatch in response to hypoxia
 294 was uncorrelated with developmental stage or size (Fig. 5; Pearson's correlations; stage: $t_{52} = -$
 295 1.4314, $P = 0.1583$; size: $t_{54} = 0.334$, $P = 0.7440$). In contrast, latency to hatch in response to the
 296 mechanosensory cue decreased from first to consistent hatching (Fig. 5; $V = 60$, $N = 11$, $P =$
 297 0.01367) and with developmental stage and size (Fig. 5; Pearson's correlations, stage:
 298 $t_{60} = -5.3341$, $P = 1.538e^{-06}$; size: $t_{60} = -3.5418$, $P = 0.0007759$).

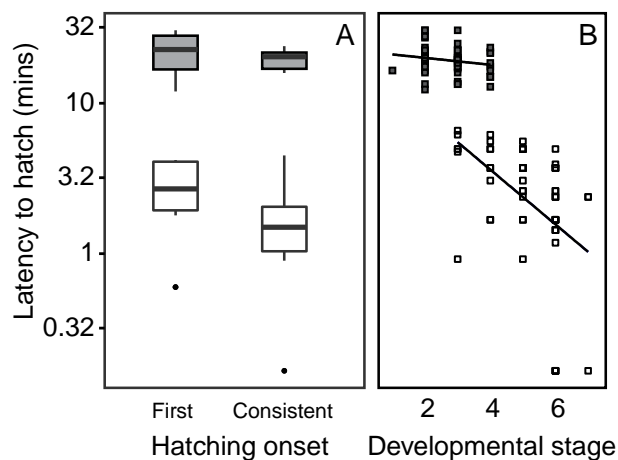


Fig. 5. Latency of *Agalychnis callidryas* embryos to hatch after exposure to a hypoxia cue (filled symbols) or a mechanosensory cue (open symbols). (A) Latency for the first hatching in response to each cue and when embryos hatched consistently, across clutches. Box plots show medians, interquartile range and extent of data to $\pm 1.5 \times \text{IQR}$ (box and whiskers), and outliers. (B) Latency for individual embryos hatching in response to each cue, across developmental stages. Lines are linear regressions. Latency is plotted on a log scale.

300

301 At the onset of hatching, the developmental stages of embryos that hatched in response to
302 hypoxia and their same-age siblings that did not hatch were identical, and their size did not differ
303 (paired t -test, $t_{12} = -0.196$, $P = 0.85$). Similarly, the earliest individuals that hatched in response
304 to mechanosensory cues were not developmentally ahead of their unresponsive siblings
305 (Wilcoxon signed-rank test with continuity correction, $V = 5$, $N = 17$, $P = 1$), nor were they
306 larger (paired t -test, $t_{15} = 1.533$, $P = 0.15$).

307

308 **DISCUSSION**

309 The developmental onset of environmentally cued hatching in red-eyed treefrogs depends on cue
310 type. At stages when embryos are clearly capable of hatching, as demonstrated by their
311 consistent hatching response to strong hypoxia, they respond neither to a strong artificial
312 mechanosensory threat cue nor to their natural predators (Warkentin 1995; Warkentin et al.
313 2006a). From field data on predation rates, up to 10% of eggs laid can be consumed during this
314 hatching-competent but unresponsive-to-predators period (Gomez-Mestre and Warkentin 2007;
315 Warkentin 1995; Warkentin 2000b). Why don't those embryos hatch to escape and, more
316 generally, what different factors limit the onset of cued hatching in different contexts?

317

318 Ontogenetic differences in behavior may reflect variation in abilities. Hatching is only possible
319 after embryos develop the ability to open a hole in their egg capsule and move through it to the
320 outside world. Responses to environmental cues are only possible after animals develop the
321 sensory systems required to perceive the cues. However, animals equally able to perform a
322 behavior may express it, or not, in a particular context. The optimal behavioral decision often

differs depending on the current state of an animal (Houston and McNamara 1999). Thus, ontogenetic changes in embryo responses may be adaptively matched to changing cost-benefit trade-offs, as their ability to survive in their post-hatching environment improves (Touchon et al. 2013; Warkentin 1995; Warkentin 1999a; Willink et al. 2014). In this context, variation in responses to different cues at the same developmental stage might reflect either real differences in the indicated risk level, or differences in cue quality that alter the optimal decision (Dall et al. 2005; Koops 2004; Trimmer et al. 2011). Finally, developmental changes in behavior may be due to learning, but we consider this the least likely alternative (Wiedenmayer 2009). Hatching is a behavior expressed once in a lifetime, which reduces the value of any potential learned responses. Moreover, embryos that fail to hatch in a direct and persistent predatory attack on their egg, or if their egg falls to a hypoxic depth in a pond, are unlikely to survive, which minimizes opportunities for learning in these contexts. We address hypotheses of changing abilities and changing decisions below.

Onset of hypoxia-cued hatching

Hatching responses to hypoxia are phylogenetically widespread (Warkentin 2011a) and some embryos express oxygen-cued behavior long before hatching; for instance, pond snails respond to oxygen as soon as their first pair of neurons develops (Goldberg et al. 2008; Kuang et al. 2002). *Agalychnis callidryas* embryos orient in oxygen gradients within their eggs as early as the neural tube stage (Rogge and Warkentin 2008), so development of oxygen-sensing ability cannot constrain the onset of hypoxia-cued hatching (HCH). The later onset of HCH previously reported (Gomez-Mestre et al. 2008), compared with the early onset documented here, must therefore reflect different behavioral decisions. While submergence in pond water, the stimulus used in our

prior work (Gomez-Mestre et al. 2008; Warkentin 2002), can kill *A. callidryas* embryos (Pyburn 1970), oxygen levels in ponds vary widely. Both aquatic oxygen level and egg surface-exposure to the medium presumably affect the severity of respiratory constraint, and thus the likelihood that flooding will be lethal and time before mortality occurs (Seymour 1999; Strathmann and Strathmann 1995; Warkentin 2007). The variation in HCH onset across flooding contexts therefore reveals a developmental period during which embryos tolerate sub-lethal hypoxia, at least temporarily, rather than accept the risks of hatching, and opens questions about risk assessment and decision-making in response to hypoxia cues. Hypoxia-cued hatching may be more nuanced and interesting than previously thought (Moskowitz et al. 2016; Vasquez et al. 2016).

Amphibian embryos, in general, can survive periods of hypoxia or even anoxia, simply arresting development and then continuing when oxygen is restored (Valls and Mills 2007). In nature, *A. callidryas* embryos experience hypoxia in four contexts. First, all embryos experience frequent, brief periods of hypoxia after random movements position their gills away from the air-exposed egg surface; they rapidly relieve this by changing position again (Rogge and Warkentin 2008). Second, some eggs within clutches have insufficient surface exposure to air. This slows embryo development or causes mortality, depending on the severity of oxygen limitation. Third, clutches attached to vegetation near the water surface may be temporarily submerged, then emerge into air again, as pond levels vary with rainfall (KW personal observation). Embryos in such clutches might experience a limited, survivable period of hypoxia. Fourth, individual eggs and entire clutches can fall into ponds. These eggs sink to rest on a substrate where they experience position- and exposure-dependent hypoxia that, if severe enough to be lethal, is unlikely to be

relieved. To probe the onset of earliest hatching competence, our hypoxia cue was designed to be lethal if experienced in nature and uniform across individuals.

Embryos initially responded to our hypoxia cue by changing position multiple times, as if they might just be poorly positioned. They then began the shaking behavior that is associated with hatching enzyme release (Cohen et al. 2016), made holes in their vitelline membranes, and squeezed out. Some embryos shook and appeared to be trying to exit the egg, without actually rupturing the membrane, and four ruptured the membrane but failed to squeeze through the hole. Eventually, embryos that remained within intact and ruptured eggs ceased all visible movement. They recovered after trials, when we returned them to air or removed their collapsed membranes and placed them in air-saturated water, but would presumably have died if left in the hypoxic environment. Hatching occurred from 12.0 – 34.2 min after flooding, and our 2015 trials lasted 40 min, thus we are confident we allowed sufficient time to assess embryo responses. Embryos that failed to rupture and exit their egg in these trials were presumably unable to do so.

The general mechanism of hatching, for anurans with a tadpole stage, is through secretion of proteolytic enzymes from hatching gland cells (HGCs) on the head and back of the embryo (Altig and McDiarmid 1999; Nokhbatolfoghahai and Downie 2007). In the few species previously examined in detail, hatching enzymes are secreted slowly, over an extended period, and gradually weaken the vitelline membrane (Yamasaki et al. 1990; Yoshizaki 1978; Yoshizaki and Katagiri 1975). In contrast, the rapid, regulated hatching of *A. callidryas* occurs by acute, highly localized release of hatching enzyme to form a small hole for immediate exit (Cohen et al. 2016). Working with 5-d embryos, we found large hatching gland cells full of secretory vesicles

highly concentrated on embryos' snouts, and absent elsewhere on the body (Cohen et al 2016). Our examination of 3-d embryos, however, revealed that these large HGC are not yet present (Cohen and Warkentin 2014); thus they cannot mediate the earliest hatching. We found another, smaller and more anatomically dispersed type of HGC in 3-d embryos (Cohen and Warkentin 2014; Warkentin et al. 2016). Presumably these smaller HGC mediate hypoxia-cued hatching at its onset, although they disappear before spontaneous hatching. We posit that their development constrains the onset of hypoxia-cued hatching, and that in our least developed test embryos these small HGCs were simply unable to release sufficient hatching enzyme to make an escape hole. Nonetheless, *A. callidryas* embryos clearly are capable of hatching substantially earlier than previously known.

Onset of mechanosensory-cued hatching

Physical disturbance of eggs is a common cue to hatch, across taxa, functioning in both antipredator responses and the host-cued hatching of parasites (Warkentin 2011a; Warkentin 2011b). In our prior experiments and clutch monitoring at ponds in Gamboa, we found predator-induced hatching to begin at age 4 d (Warkentin 2000b; Warkentin et al. 2006a). This is congruent with the onset of response to our mechanosensory stimulus. At our field sites in Costa Rica, predator-induced hatching begins at age 5 d (Gomez-Mestre and Warkentin 2007; Gomez-Mestre et al. 2008; Warkentin 1995). This difference probably reflects simply slower development at slightly cooler temperatures, as early hatchlings in Costa Rica are not more developed compared to those in Panama; their beaks are unkeratinized and yolk sacs undivided at the onset of predator-induced hatching (Warkentin 1999b). Variation in development rate, presumably due to temperature, also explains the variation in timing across our 2014–2016

experiments; age at the onset of responses varied, but developmental stage did not.

Clearly, there is a developmental period when embryos can hatch, and do in response to hypoxia, but do not in response to predators or artificial mechanosensory cues. Might this lack of response reflect adaptive embryo decisions, given the costs of early hatching? We think not. Embryos do experience frequent benign disturbances, such as rainstorms, and overlap in the properties of vibrations in predator attacks and benign disturbances generates ambiguity (Warkentin 2005). Nonetheless embryos are able to discriminate among these stimuli, using complex risk assessment mechanisms that combine information from multiple vibration properties (Caldwell et al. 2009; Caldwell et al. 2010; Warkentin and Caldwell 2009; Warkentin et al. 2006b; Warkentin et al. 2007). Based on our knowledge of mechanosensory risk assessment, we attempted to design a very clear threat cue, simulating intense attacks focused on individual eggs – an occurrence likely to be rapidly lethal. The complete hatching response of relatively young embryos supports that they perceive this cue to indicate high risk. Moreover, the rapid developmental change in response to this cue (average 8.3 h from 0–100% response) also contrasts with the relatively slow decrease in the cost of false alarms. For instance, hatchling survivorship with aquatic predators improves gradually across several days of embryonic development (Warkentin 1995; Willink et al. 2014) due to changes in multiple traits (Warkentin 1999a). We do not know how hatching at the earliest possible stage affects survivorship in nature. However, most hypoxia-cued hatchlings survived and appeared to develop normally in the laboratory. Thus, we think a dramatic decrease in the cost of false alarms over a 6–9 h period at age 4 d is unlikely to explain the complete unresponsiveness of younger hatching-competent embryos to strong mechanosensory threat cues. A developmental constraint on cue perception

seems more likely.

We hypothesize that embryos sense motion cues in predator attacks using inner ear mechanoreceptors. The complete amphibian inner ear contains eight mechanosensory surfaces with specialized sensitivity to angular acceleration, linear acceleration and gravity, substrate-borne vibration, and sound (Lewis et al. 1982; Lewis and Narins 1999). Ears are not fully developed until well after hatching (Bever et al. 2003; Quick and Serrano 2005), but show mechanosensory function much earlier. In *Xenopus laevis*, a clear behavioral indicator of otic mechanoreception is the vestibuloocular reflex (VOR) – eye rotation counter to head rotation that improves stability of the visual field (Horn and Sebastian 1996; Sebastian and Horn 1998). A weak gravitational VOR first appears at Nieuwkoop-Faber stage 42 (Horn et al. 1986), concurrent with the appearance of stereocilia in the first patch of hair cells in the otic vesicle (Quick and Serrano 2005). Red-eyed treefrog embryos show substantial ear development after the onset of hatching competence, suggesting that otic sensory function changes during this period (Warkentin et al. 2014). Their VOR first appears at age 4 d, concurrent with hatching responses to vibrational risk cues and predator attacks (Kim et al. 2016). Thus, the onset of mechanosensory-cued escape-hatching in *A. callidryas* may be limited by ear development.

Evolutionary changes in ontogeny of mechanisms of cued hatching

The ability to hatch substantially before the point of spontaneous hatching is a shared, presumably ancestral, trait across *Agalychnis* and other phyllomedusines, as is a strong escape-hatching response to flooding (Gomez-Mestre et al. 2008). However, the response to snake attacks during this period of hatching competence varies; *A. callidryas* and several other species

have strong, effective escape-hatching responses, but *A. saltator* and especially *A. spurrelli* show much weaker responses (Gomez-Mestre et al. 2008). Even in clutches that have begun hatching spontaneously, most *A. spurrelli* embryos simply fail to respond to snakes (Gomez-Mestre and Warkentin 2007). The discovery of a developmental period in *A. callidryas* when hatching-competent embryos are unresponsive to physical disturbance cues suggests that the failure of hatching-competent *A. spurrelli* and *A. saltator* to flee from snakes may reflect simply a change in the extent of this developmental period, perhaps due to changes in the relative timing of ear development. Both species develop faster, and hatch at younger ages, than syntopic *A. callidryas* and four other phyllomedusines studied (Gomez-Mestre et al. 2008). If mechanosensor development is less impacted than other traits by the mechanism that speeds their embryonic development, their weak antipredator response might be simply a heterochronic byproduct of developmental acceleration.

Latency to hatch

The latency from cue exposure to hatching includes both the time required to perceive and assess the cue, and the time required to rupture the egg membrane and exit after a decision to hatch is made. Latency was an order of magnitude longer under hypoxia than in physical disturbance. Most of this difference probably reflects a difference in the risk assessment period. Embryos' initial response to flooding was to change position many times, repeating the same behavior they perform after they turn away from the air-exposed part of their egg (Rogge and Warkentin 2008). Such an oxygen-search or sampling period is presumably required to distinguish positional hypoxia from submergence. There was no change in latency through the onset of HCH; however, latency to hatching after flooding does decrease over longer developmental periods, as embryos

approach spontaneous hatching and the cost of false alarms declines (Moskowitz et al. 2016). The difference in latency might, in addition, include a difference in the time required to escape from the egg, since embryos use different types of hatching glands at different stages (Cohen and Warkentin 2014). However, separating these components will require more detailed analysis of changes in the hatching process across stages and contexts. Unlike HCH, latency to hatch decreased with development through the onset of mechanosensory-cued hatching. Costs of false alarms probably change little over this 6-h period. Thus, the reduced latency likely reflects improvements in mechanosensory or hatching ability, more than changing decision criteria.

Morphological markers of development

We identified a series of morphological markers that enable us to divide *A. callidryas* development through the onset of hatching into seven developmental stages (Table 1). Both hypoxia-cued hatching and mechanosensory-cued hatching began, and became consistent, within narrow morphologically defined developmental periods. Thus we can now use readily visible external traits to identify important functional changes in embryo behavior. Since ectotherm development rates vary with temperature, stage provides a more precise, consistent method to identify the developmental timing of events, improving on the age-based system previously used for research on *A. callidryas* hatching plasticity (Warkentin 1999b; Warkentin 2002). These developmental markers across the onset of hatching are a starting point toward a staging table through the entire plastic hatching period of *A. callidryas*, which will be essential for a better understanding of the ontogenetic changes that generate developmental changes in behavior. Such a staging table would also facilitate comparative research across phyllomedusines.

Conclusions

In red-eyed treefrogs, embryos begin hatching to avoid asphyxiation as soon as they are able to exit their egg capsules. Their failure to hatch even earlier appears due to inability, not lack of motivation. Escape-hatching responses to predators do not begin until later in development, and no evidence suggests that younger embryos try to hatch but are unable. Both the rapid increase in embryos' response to a mechanosensory cue, from 0–100% over a period of hours, and its congruence with an indicator of ear function suggest that the onset of antipredator responses is constrained by sensor development. It is unlikely that hatching-competent embryos perceive intense attack cues but decide not to hatch. Because embryos develop rapidly, their behavior changes substantially over relatively short periods. Such changes occur for many reasons, including development of effectors enabling performance of actions, development of sensors enabling receipt of signals and cues, and ontogenetic adaptations matching behavior to changing costs and benefits. As an essential embryo behavior mediating a critical life history switch point, for which optimal timing is context-dependent, hatching offers excellent opportunities to study how and why development changes behavior. In many animals, embryos use multiple cue types to respond to multiple environmental factors, including different kinds of threats and indicators of opportunity (Warkentin 2011a; Warkentin 2011b; Whittington and Kearn 2011), creating opportunities to distinguish shared vs. specific developmental constraints. The ontogeny of cued hatching responses may often reflect combinations of changing hatching and sensory abilities and changing decisions.

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AUTHOR CONTRIBUTIONS

KC did the pilot work that motivated these experiments; KW, KC, JC and BG designed the hypoxia experiments; KW and JJ designed the mechanosensory cue experiment; JC and KW did the 2014 hypoxia experiment; BG, JJ, SJK and KW did the 2015 experiment; KW and JJ did the 2016 mechanosensory experiment; KW and SJK developed the staging system; KW and JJ analyzed the data; KW and KC wrote the paper; all authors edited the paper.

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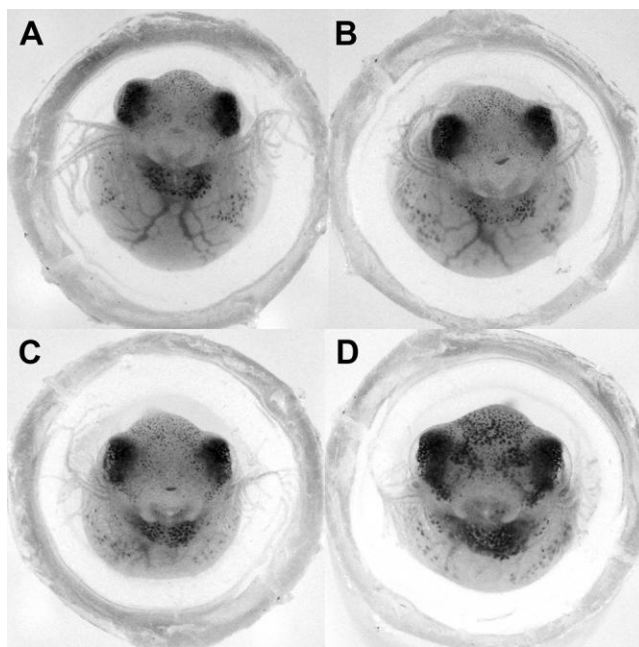


Fig. 4 in greyscale, for print version.

- Red-eyed treefrog embryos use multiple cue types to escape threats by hatching early.
- We compared escape-hatching onset in response to hypoxia and mechanosensory cues.
- Hypoxia-cued hatching begins earlier, when embryos fail to flee in snake attacks.
- Hypoxia-cued hatching begins when a hatching mechanism develops.
- Later-onset mechanosensory-cued hatching appears limited by mechnosensor development.

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