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Ontogeny of escape-hatching decisions: vibrational cue use balances sampling and false alarm costs

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Lay Summary: Embryos change how they assess risk cues as their development changes the costs of mistakes. Premature red-eyed treefrog embryos use many properties to distinguish snake and rain vibrations, hatching to escape attacks and avoiding false alarms in benign disturbances. Near-term embryos accept more false alarms to reduce the risk of sampling vibrations that might indicate danger, but they continue to use rapidly evident cue properties to inform their hatching response to vibrations.

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Abbreviated title: Adaptive ontogeny of escape-hatching decisions

ABSTRACT: As animals develop, their abilities and needs change, altering possible and optimal behavior in multiple contexts. Some embryos hatch prematurely in response to cues indicating threats to eggs. We asked if cued-hatching decisions change ontogenetically as predicted from cost/benefit trade-offs. Arboreal embryos of red-eyed treefrogs hatch rapidly and prematurely to escape egg predators, cued by physical disturbance in attacks. These embryos use multiple vibration properties to assess risk, avoiding false alarms in benign disturbances such as rain. Hatching earlier increases tadpole mortality, but this cost decreases developmentally while egg predation remains lethal. Thus, older embryos should accept more false alarms to reduce the predation-risk cost of vibration-sampling in attacks. To compare responses of premature and near-term embryos, we designed a playback system to present vibrational cues with minimal

disturbance during set-up. We designed three stimuli based on responses of premature embryos, combining frequencies and temporal properties that elicit hatching (low-fast), and altering either one to elicit little hatching (high or slow). Information accrues more rapidly from frequency than temporal properties, so embryos might use frequency to avoid false alarms until later stages. Latency to hatch in playbacks decreased with development, indicating reduced cue-sampling. Hatching increased with development in a stimulus-dependent manner, with the largest increase in low-slow playbacks. At both stages, hatching response differed between frequencies, but only younger embryos discriminated between temporal patterns. Thus even nearly full-term embryos show selective hatching responses to cues. Developmental changes in discrimination appear well-matched to changing trade-offs, suggesting ontogenetic adaptation of embryo behavior.

KEY WORDS: *Agalychnis callidryas*, biotremology, egg predation, embryo behavior, ontogenetic adaptation, vibration playback

INTRODUCTION

Animal behavior changes ontogenetically for many reasons (Wiedenmayer 2009). Development of sensory systems allows individuals to perceive and respond to cues (Romagny et al. 2012), maturation of effectors enables and improves performance of actions (Bate 1999), and learning can alter the likelihood or type of response to a stimulus (Guibe et al. 2012; Harshaw and Lickliter 2011). In addition, because it changes animals'

abilities and needs, development can alter the costs and benefits of expressing a behavior in particular context (Wiedenmayer 2009). Thus behavioral ontogeny may be adaptively matched to changing effects on fitness.

Behavioral optimization requires balancing trade-offs at multiple levels. First, there are costs and benefits of performing a behavior, which often depend on environmental context. Second, animals depend on cues to assess environmental context, and they can make mistakes. Given imperfect information, optimal decisions must balance the trade-off between missed cues and false alarms (Bradbury and Vehrencamp 1998). Third, while information has value in improving decisions, its acquisition entails costs, such as sampling time and associated risks. Animals must balance this value and cost in determining how much information to use for a behavioral decision (Dall et al. 2005). Fourth, both the value and cost of information can vary with the form it takes and manner in which it is acquired, for instance differing among sensory modalities (e.g., visual, olfactory, acoustic, vibrational) or cue properties within a modality (e.g., dominant frequency, call repetition rate)(Bradbury and Vehrencamp 1998). Animals may use single or multiple forms and sources of information, and alter their use of different information sources contextually.

Hatching is both a critical embryo behavior and an irreversible transition between distinct life stages, with different risks and opportunities. Fitness trade-offs at hatching are common, so that environmental variation in either the egg or post-hatching environment generates variation in the optimal timing of hatching (Warkentin 2011a, b). Environmentally cued hatching is widespread in animals and, in some species, hatching

can be expressed as a very rapid response to a transient stimulus (Cohen et al. 2016; Martin et al. 2011; Whittington and Kearn 1988). Like other behavioral decisions, the appropriate expression of cued hatching requires balancing the fitness costs of missed cues and false alarms but, for developing embryos, one or both of those costs may change developmentally.

Physical disturbance of eggs is a widespread cue for hatching. For instance, vibrations from siblings cue hatching of turtles (Doody et al. 2012) and locusts (Nishide and Tanaka 2016), maternal vibrations cue hatching in burrower and shield bugs (Mukai et al. 2012, 2014), tumbling in waves cues hatching of grunion (Griem and Martin 2000), and physical disturbance by hosts cues hatching in parasitic flatworms (Whittington and Kearn 1988, 2011). Vibration may be particularly useful as an indicator of predation risk, since predators cannot eat eggs without moving them and, at least for active foragers, vibrational crypsis may be more difficult to achieve than acoustic or visual crypsis. Physical disturbance of eggs cues escape-hatching responses to predators in delicate skinks, various frogs, and nudibranchs (Doody and Paull 2013; Gomez-Mestre et al. 2008; Oyarzun and Strathmann 2011; Warkentin 2005).

Nonetheless prey, including embryos, may also experience benign disturbances and need to distinguish them from predator cues to avoid false alarms (Caldwell et al. 2010). In general, the accuracy of cue discrimination will increase with greater cue sampling, but extended sampling of predator cues can increase risk, so optimal sampling must balance the benefits of more accurate discrimination against the risks incurred in gathering more information (Dall et al. 2005; Warkentin and Caldwell 2009). Moreover,

the rate at which information accumulates over time varies among cue properties (Bradbury and Vehrencamp 1998). Thus, if the costs of false alarms or missed cues, and the importance of accurate decisions, change as embryos develop, then the set of cue properties they use to assess risk may also change.

The hatching responses of embryos to environmental cues may vary ontogenetically and with stimulus properties in different ways. Depending on the relative importance of sensory system development, hatching effector development, and changing cost/benefit trade-offs at multiple levels, we predict different ontogenetic patterns (Fig. 1). First, we know that across some developmental period embryos should remain in the egg in some contexts and hatch in others, and they use cues to assess context and respond appropriately; i.e., embryos show strong hatching responses to some stimuli and little or no response to other stimuli (Warkentin and Caldwell 2009). As a null hypothesis, particularly across short developmental periods, these responses may not change (Fig. 1A). We also know that, in general, hatchling likelihood increases developmentally. This might reflect the development of hatching effectors, improved ability to survive outside the egg capsule, decreased ability to continue developing inside it, or improved ability to sense cues. As a second null hypothesis, across some developmental period, differential responses based on cue properties might be independent of this developmentally changing likelihood of hatching (Fig. 1B). Either null hypothesis might pertain if development does not change how embryos discriminate among stimuli.

A developmental increase in hatching response with a decrease in discrimination among stimuli (Fig. 1C) might occur for two reasons. First, as embryos develop toward

the point of spontaneous hatching, the cost of false alarms decreases. Thus we expect a developmental increase in the hatching response to ambiguous cues, with a greater range of ambiguous cues passing the threshold to elicit a hatching response. Second, the development of sensory systems increases the range of perceptible stimuli. If this occurs during the period of hatching competence, it could generate a similar response pattern (Fig. 1C). Alternatively, it is also possible that sensory and cognitive development improve embryos' ability to perceive differences in some stimulus properties, so that more developed embryos may show increasingly distinct responses to different cues (Fig. 1D), either across some period during which the cost of false alarms is not declining, or if embryos simply lack responses matched to a decreasing false-alarm cost.

Finally, hatching responses may be adaptively matched to developmentally changing risk trade-offs. If so, we hypothesize that responses to unambiguous stimuli might remain consistent across some developmental period; definite indicators of rapidly impending egg mortality should eliciting hatching, while stimuli that are clearly from benign sources should not (Fig. 1E, black and white symbols). In contrast, hatching responses to ambiguous stimuli (Fig. 1E, grey symbols), indicating possible threats to eggs, should increase as the cost of false alarms decreases. However, incidental environmental cues are unlikely to be perfectly unambiguous if cue sampling is limited, and embryos may require long samples for accurate discrimination between predators and benign disturbances (Warkentin and Caldwell 2009). Because sampling cues that might be caused by predators entails risk, the best embryo response should be a developmental increase in hatching wherein the magnitude of the change increases with both cue

ambiguity and cue sampling costs (Fig. 1F). We expect a greater developmental change (i.e., more sensitivity to the cost of false alarms) for very ambiguous cues (Fig. 1F, grey symbol) than for cues that are fairly good indicators of high or low risk (Fig. 1F, black and white symbols). Similarly, as the costs of false alarms decreases, embryos benefit less from accurate cue assessment, and should reduce risky cue sampling. We expect a greater developmental change in response to cues with high sampling costs (Fig. 1F, grey symbol) than cues with low sampling costs (Fig. 1F, black and white symbols). In both cases, this could generate an overall developmental increase in hatching response, but with stage-dependent discrimination among stimuli. While at earlier stages embryo responses to highly ambiguous cues or costly-to-assess indicators of low risk (Fig. 1F, grey symbol) might match responses to clearer, cheaper-to-assess indicators of low risk (white symbol), at some later stage we predict their responses will match those to clearer, cheaper-to-assess indicators of high risk (black symbol).

Here we use embryos of red-eyed treefrogs, *Agalychnis callidryas*, to assess the hypothesis of ontogenetic adaptation in hatching decisions. Specifically we tested for developmental changes in cue sampling and hatching responses, and for the predicted effect of sampling costs on the magnitude of the change in hatching (Fig. 1F). Red-eyed treefrog eggs are laid on vegetation over ponds. These arboreal embryos can hatch in seconds and up to 30% prematurely in attacks by egg-eating snakes (Cohen et al. 2016; Warkentin 1995). Hatchlings escape from snakes but face aquatic predators in ponds (Touchon and Vonesh 2016). Their risk of mortality in the water decreases with development, so the cost of false alarms declines as embryos approach the stage of

spontaneous hatching (Warkentin 1995, 1999; Willink et al. 2014). However, the cost of missing predator cues – i.e., being eaten if they fail to hatch when attacked – remains consistently high across development. Embryos hatch in response to physical disturbance, or vibrations, of their gelatinous egg clutches (Warkentin 2005; Warkentin et al. 2017). In their Neotropical rainforest habitat, *A. callidryas* embryos are subject to frequent benign disturbance in rainstorms, and they use multiple frequency and temporal properties of vibrations to assess risk and discriminate among cues (Caldwell et al. 2009, 2010; Warkentin et al. 2006). Previous vibration playbacks, to embryos midway through the plastic hatching period, have revealed that the hatching response to vibration is very specific and affected non-redundantly by multiple cue properties. Low frequencies are required; higher frequencies elicit little or no hatching and their presence reduces the hatching response to concurrently presented low frequencies (Caldwell et al. 2009, 2010). Vibrations must be intermittent and both the duration of vibrations and the spacing between them, two features of the temporal pattern, must be within limited ranges to elicit hatching (Warkentin and Caldwell 2009; Warkentin et al. 2006). Moreover, embryos alter their information sampling strategy based on the cost of information; they base hatching decisions on less information if the information accrues more slowly (Warkentin et al. 2007).

We hypothesized that: (1) *A. callidryas'* hatching response to vibrations increases ontogentically, due to the decreasing cost of false alarms, and (2) the magnitude of this increase varies among stimuli, based on the variation in information acquisition costs across vibration properties (Fig. 1F). Frequency spectra of vibrations are evident from

very short samples, thus low-risk to assess. Temporal pattern information accrues more slowly, at a rate dependent on the cycle length of the pattern; with long cycles it is particularly costly to assess. To assess hatching responses of embryos close to spontaneous hatching, when false alarm costs are low, we developed a new egg-tray vibration playback method to minimize the disturbance of embryos during set-up for playbacks. We used it to compare responses of younger and older embryos to three stimuli that varied in frequency spectra and temporal pattern. We predicted that, as in prior experiments using a tine-based vibration playback system, younger embryos would use frequency and temporal properties non-redundantly, hatching only if both were in particular ranges. We predicted that older embryos would spend less time sampling vibrations before deciding to hatch but, nevertheless, still use rapidly evident frequency properties to modulate their response. In contrast, we predicted that short samples of temporal patterns with long cycles would provide insufficient information to classify a disturbance as benign, so that a broader range of temporal patterns would elicit similarly high hatching.

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 them in an ambient temperature and humidity laboratory at the Smithsonian Tropical Research Institute. Clutches were mounted on plastic support cards, hung over aged tap water in plastic cups, and placed

inside humidors constructed from large plastic bins with screen lids connected to an automatic misting system (Mist King, Jungle Hobbies, www.mistking.com) set to provide 30 seconds of fine rainwater mist every 3 h. After experimental testing, all hatchlings were released into the Experimental Pond. Research was conducted under permits from the Panamanian Environmental Ministry (SE/A-46-15) and approved by the Institutional Animal Care and Use Committees of the Smithsonian Tropical Research Institute (2014-0601-2017-A3) and Boston University (14-008).

Egg-tray vibration playback system

At age 3 days, when embryos are insensitive to vibration (Warkentin et al. 2017), eggs were removed from their clutches and transferred to individual holes in custom-made egg trays. Egg trays (Fig. 2) were machined from 12.7 mm thick acrylonitrile butadiene styrene plastic (ABS). Each has 15 funnel-shaped holes, consisting of 4.5 mm diameter tunnels topped with 4 mm deep cones with walls angled at 69°. Thus eggs, at about 5 mm diameter, are held in the cones and hatched tadpoles slide down through the tunnels to exit the tray. The tunnels below eggs remain air-filled until hatching, ensuring sufficient air-exposed egg surface to support normal embryonic development. The cones allow eggs of slightly different sizes to sink to their own level, where they seal to the plastic by the surface tension of their moist membranes and maintain a stable position within their cone as the tray is vibrated (see Supplementary Movie 1 for high speed video of egg motion during playback).

Trays with eggs were placed over aged tap water in the humidor boxes, on racks

that held them at about a 45° angle to allow excess moisture to run off. When each egg cohort reached the younger test age, we turned off the misting system to minimize any hatching induced by vibrations from the water pump, as embryos continued developing. For each vibration playback, a tray of eggs was gently removed from its humidor, rotated to horizontal, and attached via a custom-made interface to an electrodynamic minishaker (4810, Brüel & Kjær, Nærum, Denmark) mounted horizontally on a wooden stand on an adjacent table (Fig 2B). The minishaker-tray interface (MTI) was an 11.5 mm diameter, 105 mm long aluminum alloy 6061-t6 shaft, with one end threaded for attachment to the shaker. At the other end was affixed, perpendicularly, an aluminum cylinder with the right end terminating in a cone. The cylinder was enclosed in Thermomorph (thermomorph.co.uk) plastic hand-molded to interface with one edge of the trays, with the cone-end exposed. On the right, each tray had an extension drilled with a horizontal hole that was partially filled with Thermomorph to precisely seat the metal cone of the MTI; on the left, the MTI and trays both had extending flanges that we clamped together with a rubber-tipped spring clamp. This design gave excellent mechanical coupling of shaker-to-tray and enabled rapid set-up of trays for playback with little vibrational disturbance to eggs; we just gently slid the tray onto the MTI cone and clamped their flanges together. Part of the MTI shaft and the unattached edge of the egg tray were supported by pieces of open-cell memory foam to avoid torque on the shaker, suspending the central portion with egg-funnels over a container of aged tap water to catch hatchlings. Vibrational stimuli were played from Audacity 2.1.1 (www.audacityteam.org) on a MacBook Pro, output to the shaker via an external sound card (MSE-U33HB,

Onkyo, Japan) and a custom-designed amplifier (E. Hazen, Boston University Electronics Design Facility).

Experimental design and stimuli

We designed three simple synthetic stimuli that varied in two properties, frequency spectrum and temporal pattern, that are known to affect *A. callidryas* escape-hatching decisions and enabled us to vary information delivery rate (Fig. 3, Supplementary Movies 2–4). Both properties of the Low-Fast (LF) stimulus were set to values known to elicit a strong hatching response, in attempt to generate a relatively clear cue to danger. High-Fast (HF) and Low-Slow (LS) stimuli each had just one of the two properties set to a value that elicits high hatching, in attempt to generate ambiguity. The Slow temporal pattern was designed to deliver information more slowly, and thus entail high sampling costs per unit of information, particularly compared with frequency spectra.

Specifically, low-frequency stimuli were 1–80 Hz (Fig. 3A), similar to the frequency distribution of egg-clutch vibrations in predator attacks (Caldwell et al. 2009). The high-frequency stimulus was 50–300 Hz (Fig. 3B), resembling egg-clutch vibrations in rainstorms but with the lowest frequencies removed. This is within the frequency range sensed by *A. callidryas* embryos but, based on prior playbacks, we predicted that the combination of broad bandwidth, absence of very low frequencies, and presence of higher frequencies would elicit little hatching (Caldwell et al. 2009, 2010). Fast-cycle stimuli consisted of 0.5 s bursts of noise, separated by 1.5 s periods of silence, for a 2 s cycle length (Fig. 3C); we chose this temporal pattern because it elicited the strongest

hatching response in prior playbacks of 0–100 Hz noise (Warkentin et al. 2006). The slow-cycle stimulus consisted of 1.5 s bursts of noise, separated by 15.5 s periods of silence, for a 17 s cycle length (Fig. 3D); this is within the range of temporal patterns that elicited little hatching in prior playbacks (Warkentin et al. 2006), and the long cycle duration means that information about the pattern accrues relatively slowly (Warkentin et al. 2007). We re-recorded playback stimuli from an empty egg tray, using an AP19 accelerometer powered by an APC7 signal conditioner (AP Technology International, Oosterhout, The Netherlands), an Onkyo MSE-U33HB external sound card, and RavenPro 1.3 (Cornell Lab of Ornithology, Ithaca, NY) on a MacBook Pro. We adjusted frequency using playback.m in Matlab (R2015a 8.5.0.197613) following (Caldwell et al. 2009), to compensate for non-linearities in the playback system, and equalized the RMS acceleration amplitude of LF and HF stimuli. We played the LS stimulus at the same amplitude as the LF stimulus.

We filled trays with sibling eggs, splitting larger clutches across multiple trays, and in 3 cases included eggs from two clutches in the same tray. We used trays of siblings at different ages, or for different stimuli, so each age × stimulus combination was tested on at least as many clutches as trays (N = 13 trays per stimulus at 5.2 d, 14 at 5.7 d; 46 clutches). After coupling a tray to the MTI, we allowed 5 min for acclimation, then began playback. We only performed playbacks to trays with at least 8 test eggs remaining after set-up and acclimation (mean ± SD: 12.4 ± 2.0, total N = 1007 eggs). Each tray was watched continuously, recording the timing of first hatching as a measure of latency, number hatched after 5-min of playback, and number hatched after a 5-min undisturbed

post-playback period; for some trials we also recorded video (see Supplementary Movies 1–5). We then manually stimulated any remaining eggs with a blunt metal probe (Gomez-Mestre et al. 2008; Warkentin et al. 2017), to detect any developmentally abnormal embryos not competent to hatch; we did not consider these to be test subjects. Playbacks were performed between October 13–26, 2015, on 5 egg cohorts, with each cohort tested at two ages (henceforth younger and older, details below). Within each age block, we presented stimuli in random order to sets of 3 trays. To assess developmental differences across ages, during pre-playback acclimation we noted gut coil development for any suitably positioned embryos in each tray. Gut coil development was categorized in 4 stages (ranked 0–3): undivided yolk sac, straight furrow, curved furrow, full coil. After each playback we photographed a subset of three hatchlings in dorsal view with a ruler, and in frontal view, placing each hatchling in a horizontal, water-filled tube. To assess growth, we measured hatchling total lengths from dorsal images using NIH ImageJ (Schneider et al. 2012), and calculated the mean hatchling length for each tray. We used frontal photos to assess beak keratinization, as a developmental marker (Warkentin et al. 2017).

Developmental timing of testing

In prior vibration playbacks to whole *A. callidryas* egg clutches, using an interface of blunt metal tines inserted among the eggs, we limited our testing to the most tractable developmental period. This was bounded at the start by sufficient levels of hatching in response to a subset of test stimuli and at the end by our ability to set up clutches to test

without inducing excessive hatching. For most test series, this period fell between 5 d, 16 h and 6 d, 06 h (Caldwell et al. 2009, 2010; Warkentin et al. 2006; Warkentin et al. 2007); our first series of playbacks, conducted in 2000–2001 in a warmer laboratory, were run at 5 d, 07–13 h (Warkentin 2005). The present experiments were done during the 2015 El Niño, when warmer than usual temperatures in Gamboa accelerated *A. callidryas* development and the onset of hatching responses to mechanosensory stimuli (Warkentin et al. 2017) and many embryos in our ambient laboratory hatched spontaneously during the age 5–6 d night. We attempted to time our earlier test period to developmentally match previous playback series, based on embryo responsiveness in pilot experiments, and our later test period as late as was tractable, based on the timing of spontaneous hatching, and worked within narrow time windows of just 5 h. Younger embryos were tested at 5 d, 03–08 h (125.6 ± 0.2 h) and older ones at 5 d, 15–19.8 h (137 ± 1.2 h), estimating age from midnight of the night eggs were laid. The mean test ages of our older and younger embryos were, therefore, a few hours before most spontaneous hatching occurred and 11.4 h before that.

Statistics

We used t-tests of tray mean values to compare embryo sizes and Wilcoxon tests to compare ranked developmental stages across test ages. We used a generalized linear model (GLM) with a binomial distribution and logit link to test for effects of age, stimulus and age-by-stimulus interaction on the hatching response (number hatched out of test eggs). We followed this with separate binomial GLMs at each age and planned

contrasts to test hypothesized patterns. We used the period from the start of playback until the first embryo hatched in each tray as a measure of latency and used ANOVAs of log-transformed data to test for effects of age, stimulus and age-by-stimulus interaction on the latency to hatch. Because risk in predator attacks probably accrues as a function of time, but information from temporal properties accrues as a function of cycles of the pattern (Warkentin and Caldwell 2009), we conducted analyses of latency measured both in time (seconds) and in cycles (dividing time by the cycle length of the stimulus). We conducted analyses of latency both on the subset of trays from which at least one individual hatched and also on the full dataset, assigning a latency of 600 s (i.e., the full playback plus post-playback observation period) to trays in which no embryos hatched. All analyses were conducted in JMP Pro 12.

RESULTS

All hatchlings photographed showed beak keratinization, excepting one individual that appeared less developed than its siblings photographed at the same age; thus they were at least stage 7 following Warkentin et al. (2017). Embryos developed between test ages as indicated by division of the yolk sac into gut coils (Figs 4A, 5). Younger embryos showed a range of stages from intact yolks to curved furrows while older embryos had straight furrows to full coils (N = 35 and 38; modes: straight furrow and full coil; Wilcoxon test, S = 718.5, P < 0.0001). Embryos grew between test ages. Hatchling total length increased from 11.1 ± 0.07 to 11.6 ± 0.05 mm (mean ± SE, N = 39 and 42 trays respectively; t-test, $t_{72.5} = 6.416$, P < 0.0001; Fig. 4B). Embryos also hatched

spontaneously between test ages. From trays tested, more of the older embryos had hatched spontaneously before the test period (0.9 ± 0.1 vs. 0.5 ± 0.1 embryos per tray; Wilcoxon test S = 1139.5, P = 0.0074) and relatively fewer of the trays with older eggs had sufficient individuals to attempt setup (KMW unquantified personal observation). In addition, more of the older embryos hatched during setup and acclimation (2.5 ± 0.3 vs. 0.9 ± 0.2 per tray; Wilcoxon test S = 1189, P < 0.0001), resulting in smaller numbers of test eggs per tray (Wilcoxon test, S = 2062.5, P < 0.0001; older 11.5 ± 0.3, younger 13.5 ± 0.2, range 8–15 at both ages).

Age, stimulus, and their interaction affected the hatching response of embryos in playbacks (Fig. 6A). Older embryos hatched more than did younger ones (binomial GLM, age effect: $\chi^2 = 107.71$, df = 1, P < 0.0001), and the LF stimulus elicited the strongest hatching response (stimulus effect: $\chi^2 = 57.57$, df = 2, P < 0.0001). However, the significant age × stimulus interaction revealed that the developmental increase in hatching was not uniform across stimuli (interaction effect: $\chi^2 = 12.55$, df = 2, P = 0.0019). Younger embryos showed equally little response to both the HF and LS stimuli but a substantial hatching response to the LF stimulus (Fig. 6A, stimulus effect: $\chi^2 = 57.57$, df = 2, P < 0.0001; HF–LS contrast $\chi^2 = 1.48$, P = 0.22; (HF+LS)–LF contrast $\chi^2 = 56.75$, P < 0.0001). In contrast, older embryos showed similarly strong hatching responses to both LF and LS stimuli and a weaker response to the HF stimulus (stimulus effect: $\chi^2 = 73.84$, df = 2, P < 0.0001; LF–LS contrast $\chi^2 = 2.46$, P = 0.12; (LF+LS)–HF contrast $\chi^2 = 71.61$, P < 0.0001).

In trays where hatching occurred, the latency until the first embryo hatched

ranged from 9–420 s. Latency varied among stimuli and decreased with age, with a marginally non-significant interaction effect (Fig. 6B,C). This pattern held whether measuring latency in seconds or in cycles of vibration (seconds: age, $F_{1,58} = 44.07$, $P < 0.0001$; stimulus, $F_{2,58} = 13.88$, $P < 0.0001$; interaction, $F_{2,58} = 2.91$, $P = 0.0625$; cycles: age, $F_{1,58} = 44.05$, $P < 0.0001$; stimulus, $F_{2,58} = 31.78$, $P < 0.0001$; interaction, $F_{2,58} = 2.91$, $P = 0.0625$). The shortest latency times were for the LF stimulus (younger: 39.5 ± 10.1 s; older 18.6 ± 1.9 s), while latencies for the LS stimulus included the fewest cycles of the vibration pattern (younger: 6.5 ± 1.6; older: 1.9 ± 0.3 cycles). If we assign a latency of 600 s to trays in which no embryos hatched, age and stimulus effects remain strong and the interaction effect is much weaker (latency in seconds: age, $F_{1,75} = 27.98$, $P < 0.0001$; stimulus, $F_{2,75} = 7.77$, $P = 0.0009$; interaction $F_{2,75} = 0.75$, $P = 0.47$).

DISCUSSION

Development changes how red-eyed treefrog embryos use vibrational cues in their hatching decision. Prior research, with egg clutches mid-way through the plastic hatching period, has shown that these embryos use multiple temporal and frequency properties of vibrational cues to assess risk (Caldwell et al. 2009, 2010; Warkentin et al. 2006), and that they alter their sampling of vibrational cues with the cost of information (Warkentin et al. 2007). Here we found that, with development, the overall hatching response increases, cue sampling decreases, and discrimination among stimuli changes as predicted. For younger, less developed embryos, either frequency or temporal properties alone can substantially reduce the hatching response, even though the other property is

highly stimulatory of hatching; both must be stimulatory to elicit substantial hatching. Older embryos continue to use frequency properties, which are rapidly evident, to decide not to hatch. However, as their cost of false alarms decreases (Touchon et al. 2013; Warkentin 1995, 1999; Willink et al. 2014), they cease using a slow, costly to assess, temporal pattern as an indicator of safety. These changes are consistent with the adaptive ontogenetic pattern we predicted based on developmental changes in the missed cue/false alarm trade-off and the different assessment costs of the two cue properties (compare Fig. 1F, Fig. 6A).

Developmental change in behavior

We tested embryos just a few hours before spontaneous hatching and not quite 12 hours earlier, representing an 8.3% difference in developmental age since oviposition. During these experiments, mechanosensory-cued hatching of *A. callidryas* began at 4.125 days (4 d 03 h, Warkentin et al. 2017), and spontaneous hatching was well underway by 6.0 days. Thus, the difference between our younger and older test embryos represents about 25% of the period of mechanoresponsive hatching plasticity. During this time embryos developed measurably but there was, nonetheless, still overlap in stage and size between the two age classes.

Despite this relatively modest age and developmental difference between younger and older embryos, we found clear differences in their behavioral response to our vibration playback stimuli. The specific, stimulus-dependent magnitude of the increase in hatching with development indicates that this change reflects changing embryo decisions,

not simply improved hatching ability. Indeed, the 37% of younger embryos that hatched in response to the LF stimulus did so, on average, in under 40 s, and no embryos attempted to hatch but failed. Thus, the patterns of cue use and embryo decisions described previously, based on a single convenient developmental period, are not consistent across the entire plastic hatching period. We compared changes in response to only two levels of two stimulus properties. However, there are likely to be a suite of developmental changes in the environmentally cued hatching behavior of *A. callidryas*. These may include changes in embryo responses to additional cue properties across the period we tested here. Responses probably also change across other subsets of the plastic hatching period, potentially for different reasons. For instance, comparing embryo responses at the onset of cued hatching with those midway through the plastic hatching period might reveal different kinds of changes than the relatively late developmental period we assessed. Because the onset of escape-hatching responses to mechanosensory cues lags behind the first response to hypoxia cues, we hypothesize that it is limited by mechanosensor development, not by hatching ability (Warkentin et al. 2017). If so, early changes in embryo responses to physical disturbance, soon after they first appear, might reflect continuing changes in sensory capacities, while later changes might be more likely to reflect ontogenetic adaptations.

Our older embryos were very close to spontaneous hatching, many with siblings that had already hatched, thus their false alarm cost was presumably relatively low. Nevertheless, they did not show an indiscriminate hatching response to all vibrational stimuli. Rather, they continued to avoid hatching using some, but not all, cue properties

that younger embryos use. Specifically, they responded much less to higher, broad-band, more rain-like vibration frequencies than to lower, more snake-like frequencies, but ceased to differentiate between our tested temporal patterns. Younger embryos are behaviorally responsive to high frequency vibrations in the range presented; specifically, such vibrations reduce their hatching response to concurrently presented low frequency vibrations (Caldwell et al. 2010). Thus we consider the continued low response of older embryos to our HF stimulus to reflect behavioral decisions, rather than an inability to sense the stimulus. Others have asserted that *A. callidryas* specifically, and leaf-breeding frogs more generally, show a hatching response to vibration in order to hatch during rainstorms, which might aid hatchlings in reaching the water or make their arrival less detectable by aquatic predators (e.g. Savage 2002). Our data do not support this hypothesis. They are, however, consistent with our prediction of developmental changes in cue use based on sampling cost; assessing the slow temporal pattern would take longer than assessing a frequency distribution, particularly one that is consistent across vibration bursts.

The data presented here do not enable us to distinguish if older embryos cease attending to all temporal pattern information, while continuing to use frequency cues, or if they show a more nuanced response based on the specific assessment costs of particular patterns. We are testing this by comparing responses to stimuli that vary only in temporal pattern, with different sampling costs. Initial results suggest that, like younger embryos, older embryos do use very fast temporal patterns as cues of relative safety (Jung et al. 2017).

Information sampling and latency to hatch

The induced hatching process in *A. callidryas* can be very rapid, taking as little as 6.5 s, and typically involves an easily identifiable shaking behavior associated with hatching enzyme release (Cohen et al. 2016). For trays with very short latencies (minimum 9 s) until the first embryo hatched, the hatching process represents a substantial portion of that time. With longer latencies, much of the delay from stimulus onset to hatching represents the embryos' information sampling period, as they did not exhibit shaking behavior for most of that time (see Supplementary Movies 2, 3). Thus, we consider latency to be a useful indicator of cue sampling. Latencies were substantially shorter for older embryos, indicating that they based their decision to hatch on less information, consistent with predictions based on their lower cost of false alarms. Comparing stimuli, embryos responded most quickly to the least ambiguous stimulus (LF), which elicited the most hatching. However, they sampled the fewest cycles of the slow pattern (LS), for which each cycle took 8.5 times longer to assess (17 vs. 2 s). Although most of the younger embryos decided not to hatch in response to the LS stimulus, the first that did hatch sampled on average 6.5 cycles before hatching. Perhaps just a little more information would have been sufficient for them to decide it was safe. In contrast, the older embryos, which did not distinguish between LS and LF stimuli, sampled on average less than 2 cycles of the slow stimulus before the first ones hatched. The overall developmental reduction in sampling time, combined with slow rate of information accumulation for the LS stimulus, appears to bar older embryos from using the long cycle duration as a low-

risk cue.

Vibration playback device for motion-only cues

Our new egg-tray vibration playback system has several advantages over the previous tine-based playback systems (Caldwell et al. 2009, 2010; Warkentin 2005; Warkentin et al. 2006; Warkentin et al. 2007). First, it enables the presentation of clean, uniform, egg-motion cues to groups of embryos, avoiding the concurrent presentation of tactile or pressure cues to a subset of embryos as occurs in tine playbacks. Thus, our results clearly demonstrate that motion alone, without tactile cues, is sufficient to induce hatching of *A. callidryas* embryos. Moreover, the responses of younger embryos to the frequency and temporal properties of vibrational stimuli presented through the egg-tray playback system matched expectations based on responses of developmentally similar embryos tested using tine-based playbacks. Thus, these elements of embryo behavior appear robust to the details of stimulus presentation methodology.

Second, we can place eggs into trays before embryos begin hatching in response to mechanosensory cues (Warkentin et al. 2017) and thereafter avoid touching them directly. Setting up trays for playback inevitably requires moving eggs but, unlike the set-up for tine playbacks, it does not provide any new direct-contact stimulation. This reduces the amount of hatching that occurs in setup, and enables us to work with more developed embryos than is possible with tine playbacks, making questions about vibration-cued behavioral decisions of extremely mechanoresponsive embryos much more tractable. Thus, we now know that even very close to spontaneous hatching

embryos continue to respond selectively to physical disturbance cues, albeit with a different pattern of selectivity than they showed earlier.

Third, the physical separation of individual eggs reduces the potential influence of embryos' behavior on each other. This separation and their standardized positions also facilitate individual-level observations and video-recordings of behavior, as well as split clutch designs, and increase the amount and quality of data that can be collected per egg clutch. This adds to the value of vibration-cued hatching as a study system for research on a variety of behavioral questions including, but not only, questions focused on development. For instance, vibration-cued hatching offers an excellent system in which to examine how animals use incidental, non-stereotyped cues to inform critical behavioral decisions. The basic design of this tray-based playback system could potentially be modified for use with a variety of other terrestrial eggs, such as other frogs, lizards, turtles, or insects (Brown and Iskandar 2000; Buckley et al. 2005; Doody and Paull 2013; Doody et al. 2012; Nishide and Tanaka 2016).

Fourth, a second component to present controlled tactile cues could be added. Embryos could be stimulated through motion alone, touched without moving eggs, or touched and moved simultaneously. Real predator attacks include complex combinations of mechanosensory stimuli in different sensory modalities. Independent manipulation of different components of such cues could, for instance, assess if embryos use both motion and tactile cues, examine how they combine information from these different channels, and test if this changes developmentally.

Conclusions

Embryo behavior is less studied than the behavior of later life stages. However, embryos sense their environments, perform actions, and learn things that affect their immediate and future success (Colombelli-Negrel et al. 2012; Darmaillacq et al. 2008; Ferrari and Chivers 2009; Kempster et al. 2013; Mathis et al. 2008; Romagny et al. 2012). Moreover, they do all this during a period of phenotypic change that alters their perceptual and performance abilities and changes the trade-offs that shape their optimal decisions. Thus embryos are excellent subjects for integrative research on behavioral development and ontogenetic adaptations. The relative simplicity of their lives, compared to adults, is also useful for asking some general behavioral questions. Hatching is often behaviorally mediated, and variable selective trade-offs across egg and post-hatching environments shape embryo hatching responses to environmental cues in many animals (Warkentin 2011a). Vibration-cued hatching, in particular, enables the use of playback experiments to probe how embryos make this critical decision, and the new egg-tray playback system will facilitate such experiments across a broad range of developmental stages.

Our first egg-tray playback experiment revealed that highly sensitive embryos, just hours away from spontaneous hatching and facing only low costs of false alarms, continue to respond selectively to different types of physical disturbance. At no point during development does hatching appear to be an indiscriminate response to disturbance. Moreover, embryo responses changed developmentally as predicted based on property-specific sampling costs and ontogenetic adaptation to the changing trade-off between missed cues and false alarms. Older embryos made their hatching decision more rapidly,

and ceased using a slow-to-assess cue property to discriminate, but they continued to use a rapidly evident property. We varied only two cue properties in a simple proof-of-concept experiment. However, there are other plausible ways how, and reasons why, the way that embryos use environmental cues to make escape-hatching decisions may change developmentally. For the vibration-cued hatching of *A. callidryas*, such changes are now amenable to investigation using playback experiments.

Developmental changes in embryo behavior due to ontogenetic adaptations and changing constraints are likely to be widespread. Rapid hatching responses to changed conditions or transient stimuli are widespread across vertebrates and invertebrates (Doody and Paull 2013; Martin et al. 2011; Mukai et al. 2012; Strathmann et al. 2010; Warkentin 2011a, b; Whittington and Kearn 2011). Such cases of environmentally cued hatching offer an ecologically relevant and often experimentally tractable behavior during a period of rapid development with which to probe how and why development changes behavior.

Data availability

All data will be available from the Dryad Digital Repository (details to be added).

Supplementary information

Video recordings of egg-tray vibration playbacks are available online at (details to be added).

FIGURE CAPTIONS

Figure 1. Alternative hypotheses for the ontogeny of environmentally cued hatching. Panels represent possibilities for how the hatching response to different stimuli could change as embryos develop. Embryos might show: (A) different responses to different stimuli, but no developmental change in the response to each stimulus; (B) discrimination among stimuli and a general developmental increase in response, but no developmental change in discrimination; (C) a developmental increase in response and a decrease in discrimination among stimuli; (D) no overall change in response but a developmental increase in discrimination; (E) a developmental increase in the response to ambiguous or costly-to-assess stimuli (grey symbols) but no change in responses to clear indicators of danger or safety (black and white symbols), resulting in altered patterns of discrimination; (F) a stimulus-dependent increase in response magnitude, with a greater increase in response to more ambiguous stimuli and/or stimuli with higher assessment costs, also resulting in altered patterns of discrimination.

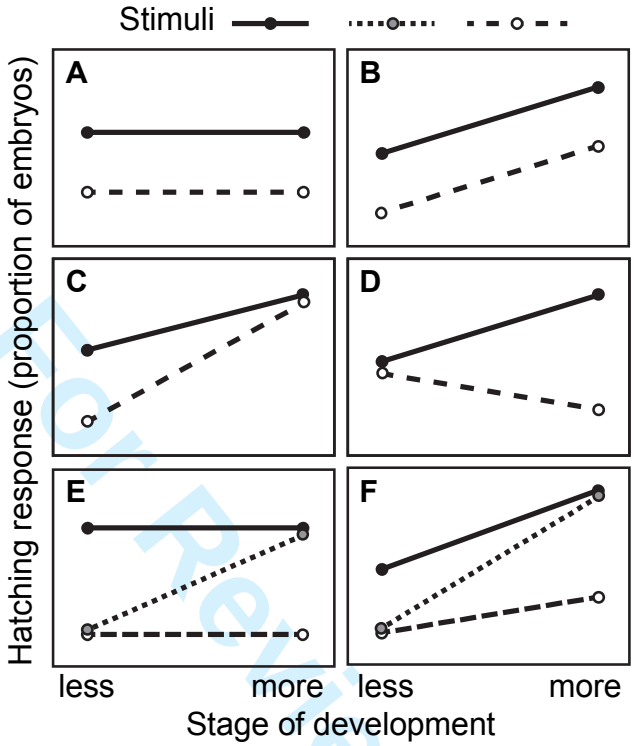
Figure 2. Egg-tray vibration playback system. Eggs are transferred to individual cones in egg trays (A) just before the embryos develop mechanosensory sensitivity. When eggs reach the desired age or developmental stage for testing, their tray is connected to the minishaker to present vibrational cues (B). Hatched tadpoles fall through tunnels below their eggs, into a container of water below the tray.

Figure 3. Vibration playback stimuli. Stimuli were bursts of noise with roughly rectangular amplitude envelopes separated by intervals of silence, and varied in two ways. The frequency spectrum included either (A) a relatively narrow band of low frequencies, characteristic of predator attacks, or (B) a broader distribution of higher frequencies, somewhat similar to rain but with the low frequencies attenuated. The temporal pattern had either a fast, 2-s cycle length (C), or a slow, 17-s cycle length (D). The low frequency range and fast temporal pattern elicited high hatching in prior playbacks, while the alternative values of each property did not. We combined the two property values that stimulate hatching (LF) to generate a relatively clear risk cue. We combined one stimulatory and one less stimulatory property value to generate cues with either low sampling costs (HF) or high sampling costs (LS) that did not clearly indicate risk.

Figure 4. Development and growth. Distributions of gut coil developmental stages (A) and hatchling total lengths (B) of *Agalychnis callidryas* subjected to vibration playbacks at two developmental ages. Data are tray means of individuals visible in ventral view or photographed for length. Box plots show median, interquartile range, extent of the data to 1.5 × IQR and outlier.

Figure 5. Gut coil developmental stages of *Agalychnis callidryas* embryos. Straight furrow (A) and full coil (B) gut coil stages, representing the modal stages of younger and older embryos tested, respectively.

Figure 6. Hatching responses of *Agalychnis callidryas* embryos to vibration playbacks at two ages. Overall, development increased the proportion that hatched (A) and decreased the latency to hatch (B, C). It also changed how embryos discriminated among stimuli that varied in frequency and temporal pattern. (A) Younger embryos showed similarly low hatching responses in response to both the slow cycle and high frequency stimuli; older embryos showed similarly high responses to both low frequency stimuli, but continued to respond weakly to the higher frequency stimulus. For embryos that hatched, they responded fastest to the low frequency–short cycle stimulus (B), but sampled fewest cycles of the long cycle stimulus before hatching (C). Latency data include only trays from which at least one embryo hatched; sample sizes are shown in (C).



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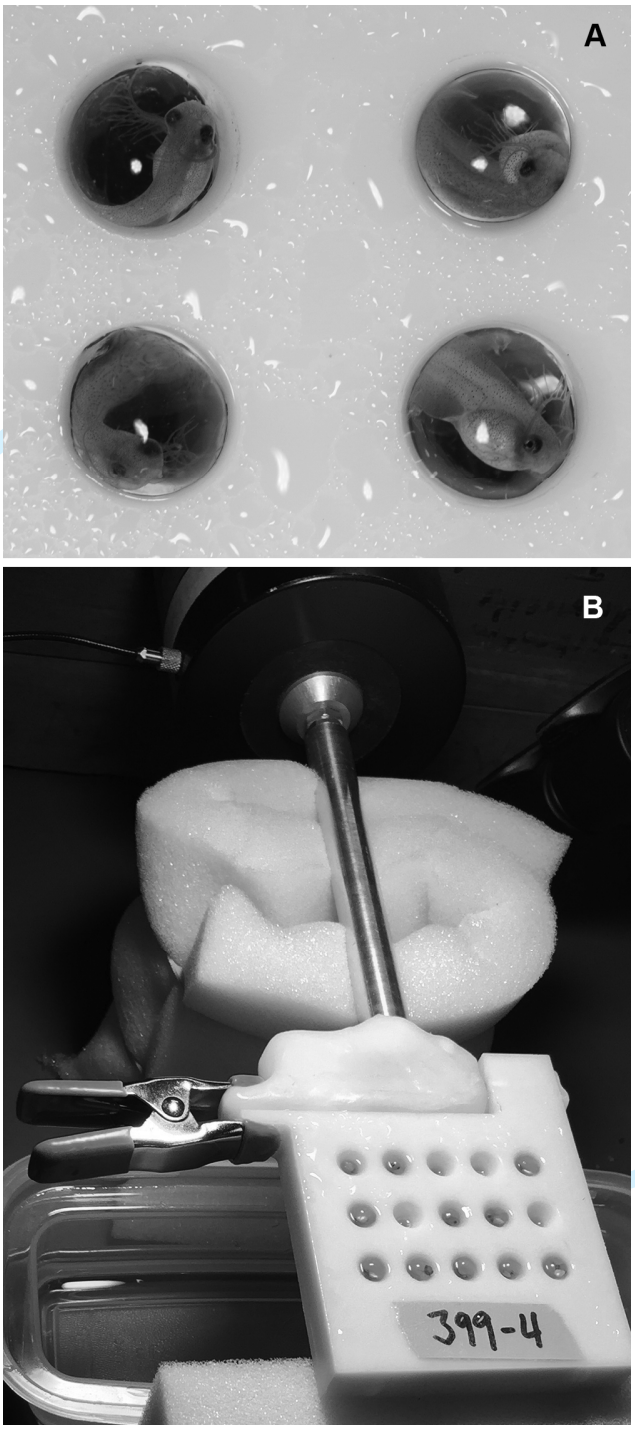
629 **Figure 1**



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632 **Figure 2 in greyscale**

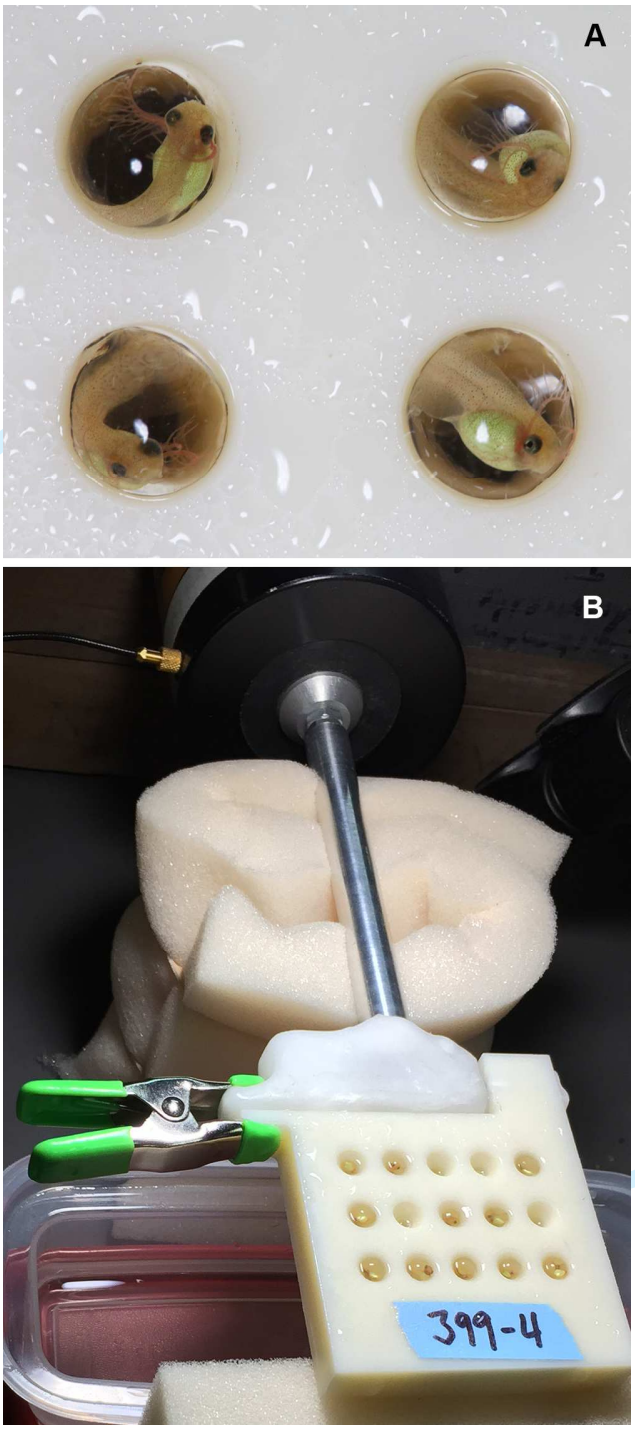


Figure 2 in color

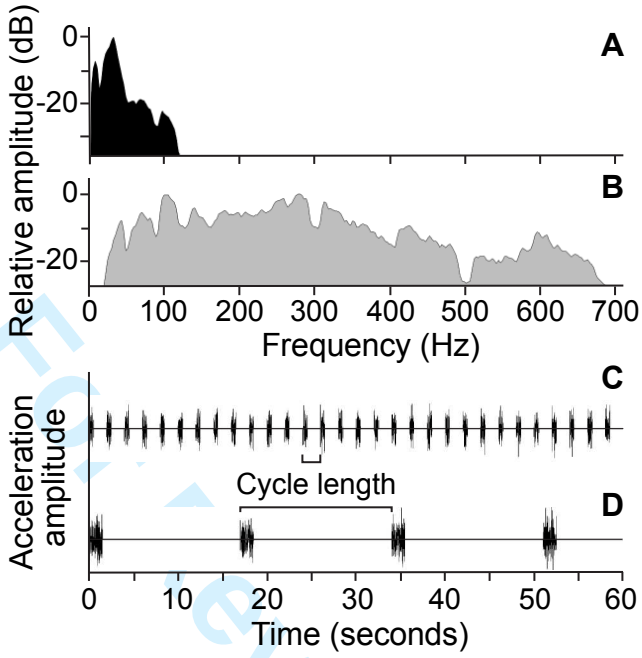


Figure 3

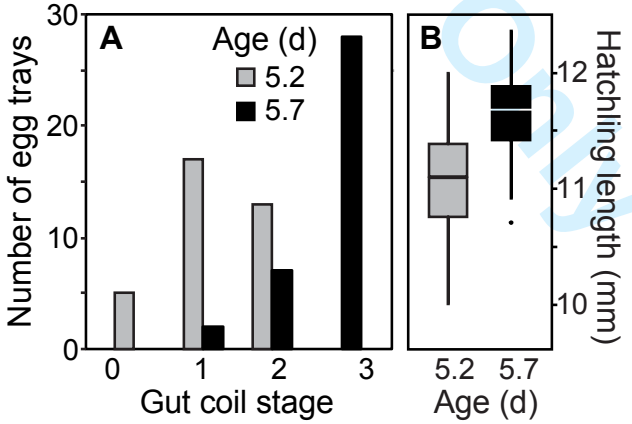


Figure 4

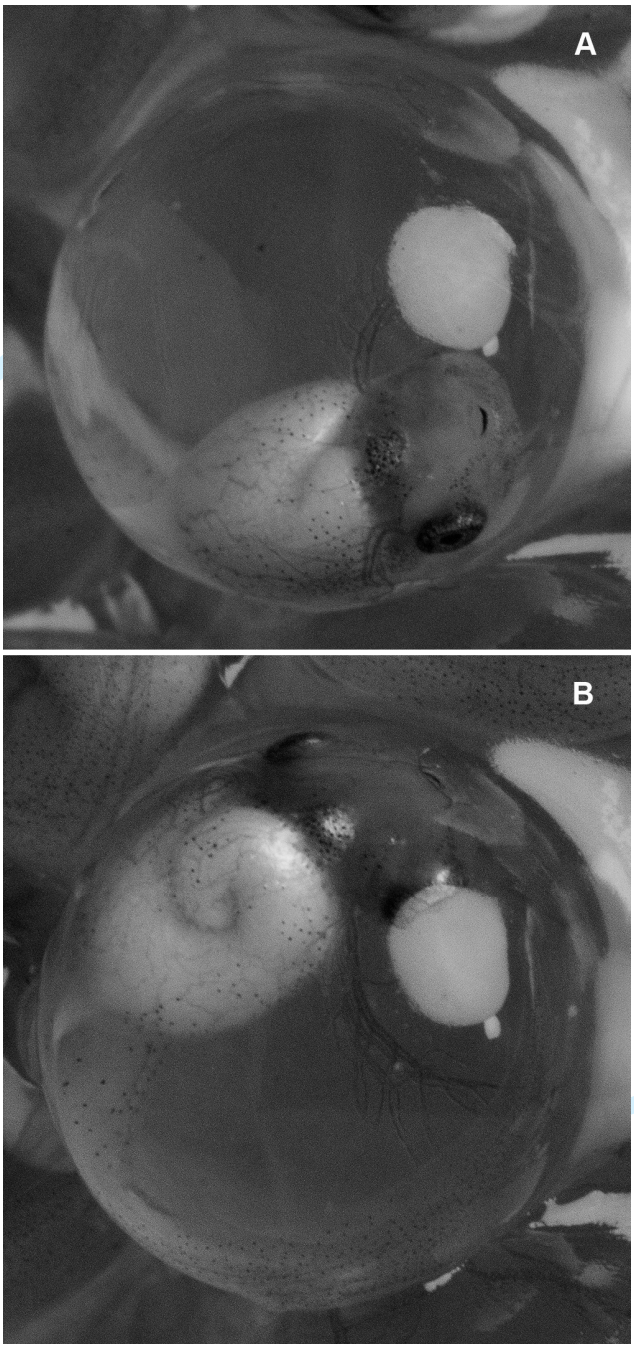
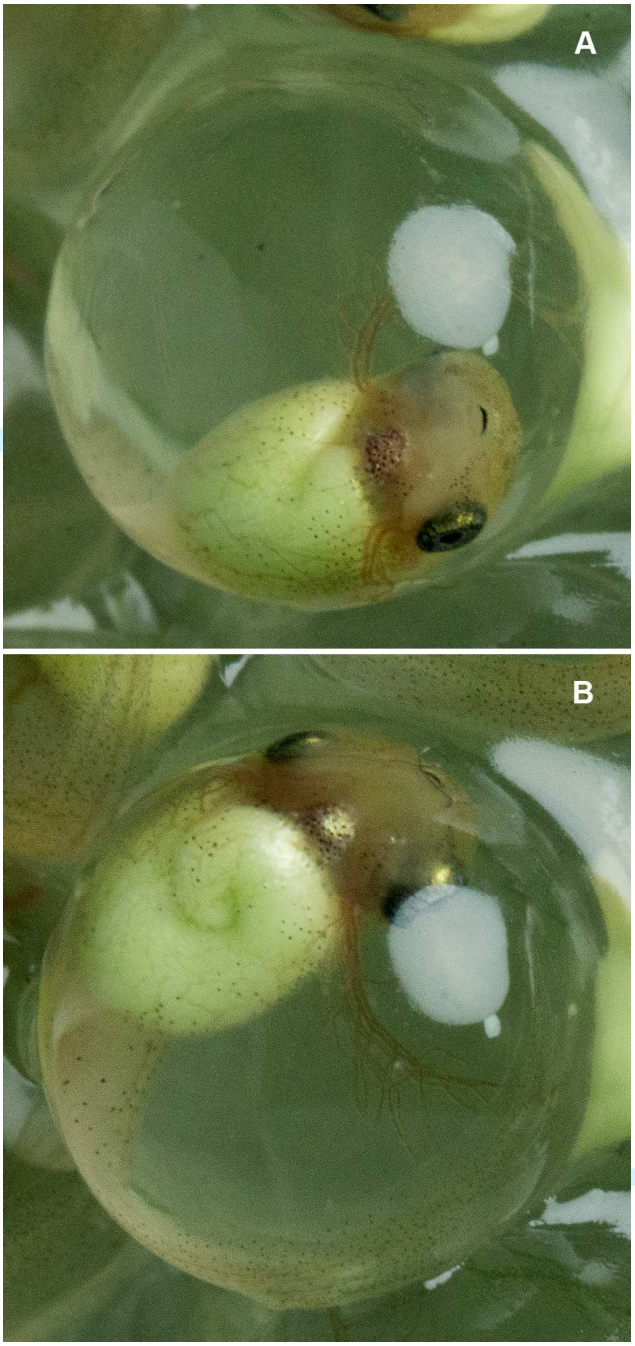


Figure 5 in greyscale



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649 **Figure 5 in color**

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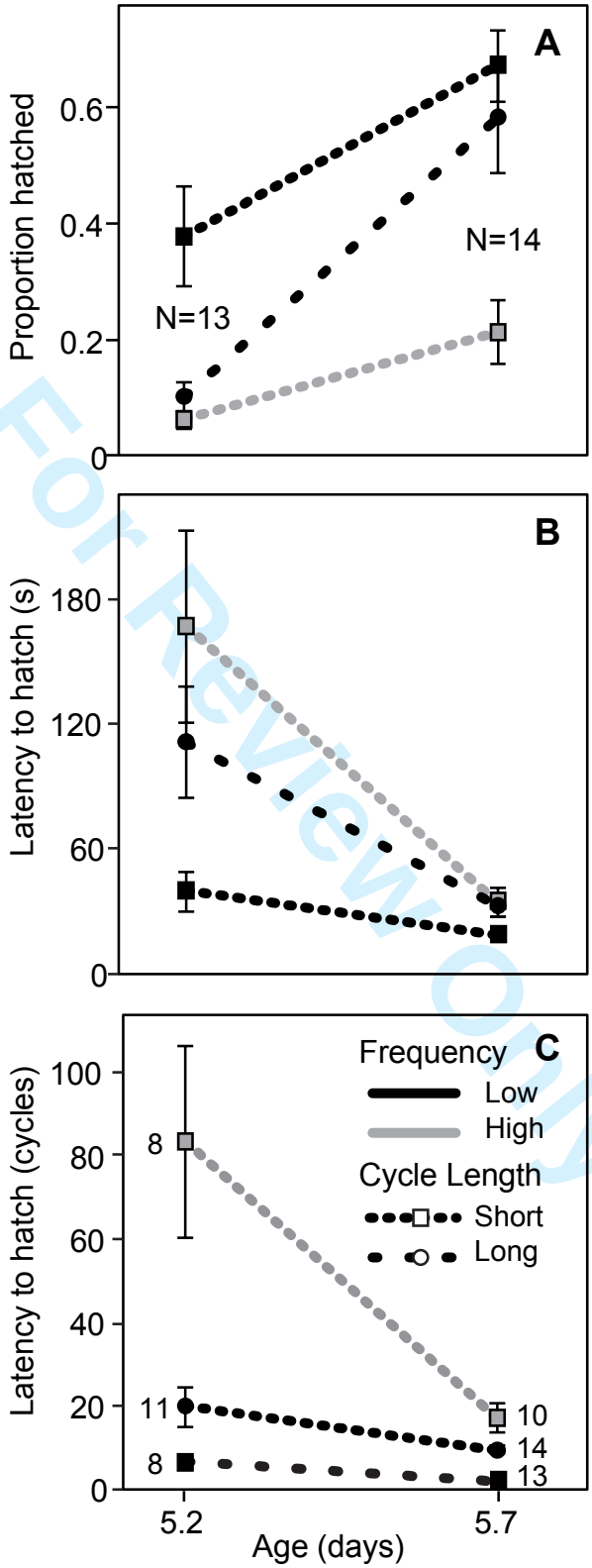

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652 **Figure 6**

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