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When and where to hatch? Red-eyed treefrog embryos use light cues in two contexts

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Hatching timing is under strong selection and environmentally cued in many species. Embryos use multiple sensory modalities to inform hatching timing and many have spontaneous hatching patterns adaptively synchronized to natural cycles. Embryos can also adaptively shift their hatching timing in response to environmental cues indicating immediate threats or opportunities. Such cued shifts in hatching are widespread among amphibians; however, we know little about what, if anything, regulates their spontaneous hatching. Moreover, in addition to selection on hatching timing, embryos may experience benefits or suffer costs due to the spatial orientation of hatching. Amphibian eggs generally lack internal constraints on hatching direction but embryos might, nonetheless, use external cues to inform hatching orientation. The terrestrial embryos of red-eyed treefrogs, *Agalychnis callidryas*, hatch rapidly and prematurely in response to vibrational cues in egg-predator attacks and hypoxia if flooded. Here we examined *A. callidryas’* use of light cues in hatching timing and orientation. To assess patterns of spontaneous hatching and the role of light cues in their diel timing, we recorded hatching times for siblings distributed across three light environments: continuous light, continuous dark, and a 12L:12D photoperiod. Under a natural photoperiod, embryos showed a clear diel pattern of synchronous hatching shortly after nightfall. Hatching was desynchronized in both continuous light and continuous darkness. It was also delayed by continuous light, but not accelerated by continuous dark, suggesting the onset of dark serves as a hatching cue. We examined hatching orientation and light as a potential directional cue for flooded embryos. Embryos flooded in their clutches almost always hatched toward open water, whereas individual eggs flooded in glass cups often failed to do so, suggesting the natural context provides a directional cue. To test if flooded embryos orient hatching toward light, we placed individual eggs in tubes with one end illuminated and the other dark, then flooded them and recorded hatching direction. Most embryos hatched toward the light, suggesting...
they use light as a directional cue. Our results support that *A. callidryas* embryos use light cues to inform both when and where to hatch. Both the spatial orientation of hatching and the timing of spontaneous hatching may affect fitness and be informed by cues in a broader range of species than is currently appreciated.
Title

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Short title

Red-eyed treefrog embryos use light cues

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Abstract

Hatching timing is under strong selection and environmentally cued in many species. Embryos use multiple sensory modalities to inform hatching timing and many have spontaneous hatching patterns adaptively synchronized to natural cycles. Embryos can also adaptively shift their hatching timing in response to environmental cues indicating immediate threats or opportunities. Such cued shifts in hatching are widespread among amphibians; however, we know little about what, if anything, regulates their spontaneous hatching. Moreover, in addition to selection on hatching timing, embryos may experience benefits or suffer costs due to the spatial orientation of hatching. Amphibian eggs generally lack internal constraints on hatching direction but embryos might, nonetheless, use external cues to inform hatching orientation. The terrestrial embryos of red-eyed treefrogs, *Agalychnis callidryas*, hatch rapidly and prematurely in response to vibrational cues in egg-predator attacks and hypoxia if flooded. Here we examined *A. callidryas’* use of light cues in hatching timing and orientation. To assess patterns of spontaneous hatching and the role of light cues in their diel timing, we recorded hatching times for siblings distributed across three light environments: continuous light, continuous dark, and a 12L:12D photoperiod. Under a natural photoperiod, embryos showed a clear diel pattern of synchronous hatching shortly after nightfall. Hatching was desynchronized in both continuous light and continuous darkness. It was also delayed by continuous light, but not accelerated by continuous dark, suggesting the onset of dark serves as a hatching cue. We examined hatching orientation and light as a potential directional cue for flooded embryos. Embryos flooded in their clutches almost always hatched toward open water, whereas individual eggs flooded in glass cups often failed to do so, suggesting the natural context provides a directional cue. To test if flooded embryos orient...
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**Introduction**

Maternally provided structures such as egg capsules and shells provide embryos with protection, yet limit their interaction with the surrounding environment. Thus, at hatching animals enter new physical environments, with new dangers and resources. Many biotic and abiotic factors differentially affect eggs and hatchlings, creating selective tradeoffs that determine the optimal time or developmental stage at which to hatch, often in a context-dependent manner (Christy 2011; Doody 2011; Korwin-Kossakowski 2012; Warkentin 2011a). A substantial body of research has documented adaptive shifts in hatching timing in response to cues indicating immediate, direct threats to embryo survival [e.g., hypoxia (Petranka et al. 1982; Warkentin 2007), predation, dehydration, and pathogens (reviewed in Warkentin 2011a)]. However, embryos may also use environmental cues to inform their hatching timing or process in the absence of immediate threats. For instance, embryos may time hatching to occur at favorable points during natural photoperiod, tidal, and lunar cycles (Christy 2011). Embryos may also orient hatching in relation to the structure of their egg capsule or its immediate surroundings in
ways that improve their hatching process or post-hatching fate (Korwin-Kossakowski 2012; Oppenheim 1972).

Hatching during low-risk periods of natural cycles has been widely documented among crustaceans and fishes. For example, many tropical brachyuran crabs and reef fishes release their larvae during high-amplitude, nocturnal high tides, facilitating larval transport away from high-predation locations (Christy 2003; Johannes 1978; Morgan & Christy 1995; Robertson 1991).

This regulation of hatching timing in response to abiotic cycles in fish and crustacean embryos is hypothesized to be an adaptive strategy which serves as an antipredator defense mechanism (Christy 2003; McAlary & McFarland 1993; Morgan & Christy 1995). It can also serve, for parasites, to increase their chances of finding a host (e.g. monogenean flatworms, Whittington & Kearn 2011). However, such hatching responses are complex and may be endogenously or exogenously regulated, or both (see DeCoursey 1983). For instance, in different species of brachyuran crabs, hatching timing may be controlled by either the mother or embryos’ endogenous rhythms, which are set and may be altered by environmental cues and cycles (reviewed in Christy 2011). One common and widespread environmental factor which entrains endogenous hatching rhythms are light-dark cycles (Asoh & Yoshikawa 2002; Brännäs 1987; Brüning et al. 2011; DeCoursey 1983; McAlary & McFarland 1993; Salmon et al. 1986).

Environmentally cued shifts in hatching timing are widespread in amphibians (Warkentin 2011b). However, hatching rhythms that may concentrate hatching at favorable times in natural cycles have received little attention, despite the likelihood of diel variation in risks to eggs and hatchlings. Such cycles of risk may be particularly relevant for species that lay terrestrial eggs...
above water, into which tadpoles fall upon hatching. Embryos presumably have no direct
information about current conditions in their larval habitat but, for instance, a history of tadpole
predation by visual predators, such as fishes or odonates, could select for hatching under the
cover of darkness. Here, we use one such species, the red-eyed treefrog *Agalychnis callidryas*
(Cope 1862), to assess the effect of light-dark cycles on the timing of hatching.

In addition to better and worse times to hatch, there may be better and worse spatial orientations
for hatching. The impact of fetal orientation on the emergence process is well-known for
mammalian birth, where breech positions are associated with increased complications (Hannah et
al. 2000). However, embryo position during hatching is also relevant for oviparous species. For
instance, most bird embryos pip internally, gaining access to the air-space within their egg some
time before cracking the shell; this allows them to begin breathing air and increase their oxygen
uptake to support the exertion of external pipping and exit from the shell (Oppenheim 1972;
Pettit & Whittow 1982; Visschedijk 1968a; Visschedijk 1968b; Visschedijk 1968c). Some fish
eggs are asymmetrically shaped and attached to substrates; at the time of hatching, embryos
orient their heads to the free end of their egg to exit from it (Korwin-Kossakowski 2012; Olivotto
et al. 2003). Correct orientation appears essential for hatching to occur in several of these
species. In amphibians, egg capsules lack opercula or rupture planes. In some species the entire
vitelline membrane is enzymatically degraded (Altig & McDiarmid 1999), while in others
embryos digest only a small escape hole (Cohen et al. 2016). In both cases, embryos seem able to
exit in any direction. Nonetheless, for eggs closely packed into gelatinous masses, hatching
outward to directly enter the outside medium may be more efficient or successful than hatching
toward the center of the mass and then moving through the matrix of jelly and other eggs to
escape. We examine hatching orientation and test the role of light as a possible orientation cue for embryos of *A. callidryas*.

**Study organism and hypotheses**

There are two contexts in which light cues might be important in the hatching behavior of *A. callidryas* embryos. First, embryos may use cues from light/dark cycles to hatch at particular times in diel cycles. *A. callidryas* embryos develop in gelatinous egg masses on leaves above ponds, in lowland wet forests from Mexico to Colombia. Most undisturbed embryos hatch during the night and enter the water after 6 or 7 days of arboreal development (Gomez-Mestre & Warkentin 2007; Warkentin 1995; Warkentin 2000b). Aquatic predator communities in *A. callidryas* breeding ponds vary substantially; however, hatchlings often face visually hunting insects or fishes (Touchon & Vonesh 2016). To evaluate the role of direct light cues and endogenous rhythms in the hatching timing of undisturbed *A. callidryas* embryos, we manipulated embryonic light environments. We raised embryos in one of three light treatments: continuous light, continuous darkness, and a 12L:12D photoperiod. We hypothesize that hatching is dark-cued or light-inhibited, thus we expect differences in hatching timing across photoperiod treatments. If embryos have an endogenous hatching rhythm, we expect hatching to be temporally patterned (e.g. synchronous or pulsed) even in the absence of photoperiod cues. In addition, because we know *A. callidryas* embryos hatch primarily at night (Gomez-Mestre & Warkentin 2007; Warkentin 1995), we hypothesized that embryos in continuous darkness would hatch earlier and those in continuous light would hatch later than those in other treatments.
The second context in which visual or light cues may be important to hatching of *A. callidryas* embryos is their orientation when exiting the clutch. The typical structure of hatching-competent *A. callidryas* clutches is a monolayer of eggs adhered to a core of jelly adhered to a leaf, with each egg having part of its surface exposed to air and part pressed against other eggs and the underlying jelly (Gomez-Mestre et al. 2008; Warkentin 2000a; Warkentin 2002). Thus, eggs have strong internal oxygen gradients with the highest concentration of oxygen at the air-exposed surface (Warkentin et al. 2005). As early as the neural tube stage, embryos orient their developing heads towards the air and reposition themselves after being displaced, and more developed embryos spend most of their time with their external gills toward the air (Rogge & Warkentin 2008). The fact that embryos orient in their egg’s oxygen gradient means they are usually positioned to hatch outward, into the air, without any active orientation at hatching. However, one common threat to eggs, flooding, disrupts this orientation. For *A. callidryas*, as for many species with terrestrial egg masses, flooding causes hypoxia, which stimulates embryos to hatch (Doody et al. 2001; Martin 1999; Petranka et al. 1982; Warkentin 2002). However, the initial response of *A. callidryas* embryos to flooding is to change position, many times, thus disrupting their outward-facing orientation (Rogge & Warkentin 2008). Therefore, to hatch into the water, instead of into jelly or other eggs, flooded *A. callidryas* embryos may need another orientation cue. To assess if the natural clutch environment might provide such a cue, we compared hatching orientations (toward open water or not) for embryos flooded within clutches on leaves, where the substrate and siblings filter or block light from most sides, and embryos flooded individually in glass cups, where light enters from all sides providing no indication which side is open to water. Because embryos flooded in glass cups often attempted to hatch into the glass, and hatching-competent embryos have well-developed eyes, we hypothesized that in
flooded clutches *A. callidryas* embryos use light cues, or positive phototaxis, to orient their hatching. To test this, we positioned individual eggs in tubes with one end illuminated and the other dark, then flooded them and recorded the direction of hatching.

**Methods**

**Egg collection and maintenance**

Red-eyed treefrogs lay eggs at night, with most eggs laid between 22:00 and 02:00 h; thus we report clutch ages starting from midnight of their oviposition night. We collected 0–3 day old *A. callidryas* egg masses on leaves from the Experimental Pond (9°7'14.77"N, 79°42'12.03"W) in Gamboa, Panama, and brought them to an open-air, ambient temperature and humidity laboratory at the Smithsonian Tropical Research Institute. We attached leaves with clutches to plastic cards and removed any dead or abnormally developed embryos to ensure all embryos used in experiments were healthy and normally developed. We kept clutches in cups over aged, dechlorinated tap water in large, mesh-lidded bins and misted them frequently with rainwater to maintain high humidity and clutch hydration. After experiments, we returned all hatched tadpoles to the Experimental Pond. Experiments were conducted under permit SC/A-15-14 and SE/A-46-15 from the Panamanian Ministerio de Ambiente, STRI IACUC protocol 2014-0601-2017, and BU IACUC protocol 14-008.

**Hatching timing experiment**
We used a split-clutch design to assess the role of light cues in diel hatching rhythms of *A. callidryas* embryos from August 4–7, 2015. We divided each clutch into three groups of 7–10 embryos, exposed them to three different light environments (continuous light, continuous darkness, and a 12L:12D photoperiod; 108 eggs per treatment), and recorded when embryos hatched. We initially set up 12 clutches, but a subset of one clutch dried, and hatched early, so we restrict our analyses to the 11 clutches whose hatching patterns were unaffected by any direct risk factor (*N* = 11). Including the dried clutch in our analyses does not change the statistical significance, or non-significance of any result.

We divided clutches and set up the treatments at 18:00 h when embryos were 3 days old, before physical disturbance induces hatching (Warkentin et al. 2017). To support each group of eggs, we glued two strips of plastic (30–45 mm long) into a 47 mm diameter plastic petri dish, creating a V-shaped compartment, attached the dish at a 75-degree angle to the inner wall of a square plastic cup, and partially lined the egg compartment with jelly from the clutch (Fig. 1). We placed eggs on jelly in a monolayer, keeping surface exposure to air within the natural range (Warkentin 2000b; Warkentin 2002). We constructed three lightproof walk-in chambers within the ambient laboratory using black plastic sheets. For the continuous light treatment and the light period of the photoperiod treatment (06:00–18:00 h), we illuminated chambers with a single LED light (320 lumens, 5000 K, 2CYSVB, Ottlight Technologies, Tampa, FL). For the dark treatment and during the dark period of the photoperiod treatment chambers were completely dark, except during observations which we made under dim red light. We placed one group of eggs per clutch into each compartment, inside a plastic bin with a mesh lid to maintain humidity.
For extra security, in the continuous dark and photoperiod treatments, we wrapped bins in black plastic, and we constructed screened portions of lids using multiple layers of black mesh to allow gas exchange but reduce light entry. For the continuous light treatment and the light period of the photoperiod treatment, we used lids with a single layer of light green mesh that allowed light penetration. We checked eggs every 2 hours from 06:00 h, 4 d, until all embryos had hatched. At each time point, we counted the unhatched eggs in each dish and/or the hatched tadpoles in each cup, and carefully misted the walls and lid of the container to maintain humidity without disturbing the embryos.

Hatching orientation experiment

To test if the natural clutch environment provides a cue for hatching direction when submerged, we conducted whole-clutch and individual-egg flooding experiments with *A. callidryas* embryos and recorded hatching direction (into open water or not).

In 2014, we flooded individual embryos at 4 days of age (*N* = 21) and recorded their response using macro-videography. These video recordings are part of a larger dataset on the ontogeny of hatching performance in *A. callidryas* that will be reported elsewhere (Warkentin et al. unpublished data). At this stage embryos hatch readily in response to both mechanosensory and hypoxia cues and have large, well developed eyes (Warkentin 1995; Warkentin 2002; Warkentin et al. 2017). At age 3 days, eggs were placed into custom-made small glass cups (interior diameter 5 mm, depth 3–4 mm; Fig. 2) which fit them closely and provided natural surface area exposure (Rogge & Warkentin 2008; Warkentin 2002). Eggs in cups were placed in humidors.
and misted often with rain water to maintain hydration. For each test, we moved a single embryo, in its cup, into a small, glass-fronted, Plexiglas aquarium and then waited five minutes for acclimation before gently submerging it. To minimize the time for boundary layer formation and local oxygen depletion around eggs, we used hypoxic water. Water was degassed by boiling it for at least 10 minutes, allowed to cool in sealed glass jars, and used within 30 min of opening each jar (15 ± 1.3% air saturated at opening to 21 ± 3.0% air saturated at 30 min, \( N = 10 \) jars). Focal embryos were video-recorded using a Canon EOS 5D Mark III camera and MPE-65 mm macro lens until they hatched. Hatching direction was recorded from the videos.

We conducted whole-clutch flooding experiments from July 12–24, 2015, using 4 day old clutches \( (N = 14) \). We used Plasticene to anchor plastic cards, with attached clutches, to the bottom of small aquaria and waited five minutes after setting up each clutch, before testing it. We used aged, de-chlorinated tap water (initially fully air saturated) to flood clutches, then continuously observed them, recording hatching direction for one hour or until all embryos had hatched.

To test \( A. \) callidryas embryos for light-cued hatching orientation, we conducted hatching phototaxis experiments at 4 days of age from 12:00–17:00 h. We positioned each test egg in a close-fitting tube, with one end illuminated and the other dark, then flooded it with hypoxic water and recorded hatching direction.

To hold each egg, we placed a clear plastic tube, 5 mm inner diameter and 5 mm long, horizontally into a close-fitting hole in the wall dividing dark and light sides of a half-dark cup.
To construct the cups, we positioned a vertical plastic wall centrally in a clear plastic cup, sealed it to the cup floor and wall, then wrapped the divider and half of the cup wall in lightproof black duct tape and covered the top of the dark side with aluminum foil to prevent light from entering. For a subset of trials (51–92; Experiment 2), we also wrapped black tape around the half of the egg tube that protruded into the dark side of the cup to reduce the passage of light to the dark side. We inserted eggs into tubes the day before testing, prior to the onset of hatching responses to physical disturbance (Warkentin et al. 2017), alternating side of insertion (dark or light), and allowed embryos to acclimate overnight.

We tested 92 embryos for hatching orientation, from 39 clutches (up to 3 siblings per clutch), running 10–15 trials concurrently in 8 sets from July 4–20, 2015. Seven trials were eliminated from analysis because eggs slipped out of their tubes when flooded, leaving $N=85$. As a control for side-bias, we tested 20 additional embryos, from 5 clutches, in a dark room using only dim red light to observe hatching direction. For experimental half-dark trials, in addition to the natural light from windows and the overhead florescent lab lighting, cup sets were illuminated by two LED lights (320 lumens, 5000 K, 2CYSVB, Ottlight Technologies, Tampa, FL) positioned at a distance of about 10 cm (trials 1–50, Experiment 1) or 15–20 cm (trials 51–92, Experiment 2) above the cups. After switching on the LED lights, we gave embryos three minutes to acclimate to the light environment, then gently flooded their cups with hypoxic water. We observed all trials continuously until embryos had hatched, noting hatching direction and timing. Because eggs fit closely inside tubes, embryos were constrained to hatch either toward the light or dark, and exited their egg and tube simultaneously. Thereafter, hatched tadpoles were confined to one half of the cup, allowing us to confirm hatching direction after trials.
We performed all our statistical analysis using R v3.3.1 (R Core Development Team 2017). We used the package “lme4” (Bates et al. 2015) to analyze hatching over time and by treatment. We used a generalized linear mixed-effects model (GLMM) with an underlying binomial error structure and logit link function to test for differences in hatching timing of embryos across different light treatments. The response variable was the number of tadpoles hatched, of the initial number per clutch in each treatment (i.e., per dish). We included age, treatment, and their interaction as fixed predictors, and clutch as a random effect to account for non-independence of observations of embryos in the same clutch. We then compared increasingly parsimonious, nested models with likelihood ratio tests to estimate $p$ values of the predictors and their interactions. Tukey’s post-hoc pairwise comparisons among light treatments were made using the glht (general linear hypothesis test) function in the “multcomp” package (Hothorn et al. 2008). We used ANOVAs with Tukey’s post-hoc pairwise comparisons to test for a treatment effect on the proportion of embryos hatched just after the onset of darkness at age 5.75 d and on hatching synchrony within egg masses (i.e., maximum proportion of embryos hatched during any 2-h period). We used Levene’s tests for homogeneity of variance to determine the effects of light treatment on hatching synchrony among egg masses; we determined the 4-h period with the most hatching for each mass, then compared the distribution of these modal hatching times across treatments. Some clutches had equally high numbers of tadpoles that hatched during two different 2-h intervals, thus we based modal hatching times on 4-h intervals to ensure each clutch had a single mode of hatching. We used Kruskal-Wallis tests followed by Dunn's tests of
multiple comparisons to compare the proportion of embryos hatched by age 5.75 d and after age 6.0 d in each treatment to assess whether continuous darkness accelerated hatching and continuous light delayed hatching.

We tested for non-random hatching orientation in flooded whole clutches using a test of equal proportions (prop.test function), based on the null hypothesis that the probability of hatching into open water by chance was equal to the natural proportion of exposed surface area of embryos (25–50%; Warkentin 2000a; Warkentin 2002). We also used a test of equal proportions to test for a difference in the incidence of hatching into the jelly or glass between whole-clutch and individual egg flooding experiments, based on the null hypothesis that the proportion of hatching into the glass in individual trials was equal to the observed average incidence of embryos hatching into the jelly in whole-clutch flooding experiments (3.4%). We then tested for non-random hatching orientation in the presence of a light cue using a test of equal proportions, based on the null hypothesis that the probability of hatching in each direction was 50%. We used a GLMM to test whether the ‘side of insertion into the tube’ affected hatching direction, including clutch as a random factor (N = 57 trials for which side of insertion was recorded). For all statistical tests, α = 0.05.

Results

Effect of light-dark cycle on diel hatching pattern
Light environments affected hatching timing of *A. callidryas* embryos. Embryos hatched more gradually and later in continuous light, showed more variation in hatching timing among clutches in continuous darkness, and showed the strongest hatching synchrony in the 12L:12D photoperiod, with a sharp peak of hatching shortly after dark at 5.75 days. Hatching timing was influenced by age, treatment, and a time-by-treatment interaction (Fig. 3; GLMM, age $\chi^2 = 10317$, $p < 0.001$; treatment $\chi^2 = 272.56$, $p < 0.001$; interaction $\chi^2 = 92.442$, $p < 0.001$).

Unsurprisingly, the proportion hatched increased over time in all treatments; all embryos hatched by 04:00 h, 7 d. Post hoc tests revealed differences in the proportion of embryos hatched over time between the photoperiod and both light and dark treatments (Tukey’s post hoc tests, photoperiod vs. light; $p < 0.001$; photoperiod vs dark $p < 0.001$). Embryos in the photoperiod treatment had pulsed, synchronous hatching shortly after the onset of darkness at age 5.75 d; ca. 71% of all embryos in the photoperiod treatment hatched immediately after the onset of darkness (vs. 21% and 14% during the same time period in the light and dark treatments, respectively; Fig. 4; ANOVA, $F_{2,30} = 20.596$, $p < 0.001$; Tukey’s post hoc tests, photoperiod vs. light $p < 0.001$; photoperiod vs. dark $p < 0.001$). Synchrony of hatching within egg masses was highest in the photoperiod treatment (Fig. 5A; highest proportion hatched during any two-hour period, ANOVA, $F_{2,30} = 5.6252$, $p = 0.008$; Tukey’s post hoc tests, photoperiod vs. light $p = 0.006$; photoperiod vs. dark $p = 0.135$). Similarly, the photoperiod treatment also had the most synchronous hatching across egg masses. Clutches in the photoperiod treatment clearly had more similar modal hatching times compared to clutches in other treatments (Fig. 5B; Levene’s test, photoperiod vs. light $F_{1,20} = 14.76$, $p = 0.001$; photoperiod vs. dark $F_{1,20} = 7.0203$, $p = 0.0154$; light vs. dark $F_{1,20} = 0.93$, $p = 0.7635$). We found no evidence that continuous darkness accelerated hatching; at age 5.75 days all treatments had a similar proportion of embryos hatched.
(44% dark, 45% light, 44% photoperiod; Kruskal-Wallis, $\chi^2 = 0.096, p = 0.953$). However, a larger proportion of embryos in the light treatment hatched after age 6.0 days compared to those in the other two treatments (Fig. 6; Kruskal-Wallis, $\chi^2 = 11.476, p = 0.003$; Dunn's post hoc tests, light vs. dark $p = 0.027$, light vs. photoperiod $p = 0.003$).

Hatching orientation

In whole-clutch flooding experiments, we found that $87.8 \pm 9.1\%$ (mean ± SD across clutches) of embryos that attempted to hatch did so without delays or complications, simply making a rupture in their membrane and emerging directly through it into the water. This was significantly different from hatching into open water by chance when compared to either extreme of the natural range of exposed surface area for individual eggs (25%, $\chi^2 = 6225.5$, df = 1, $p < 0.001$; 50%, $\chi^2 = 3011.7$, df = 1, $p < 0.001$). Among embryos that did not hatch immediately into open water, many failed to exit through their initial rupture site and were trapped in their collapsed vitelline membranes or the egg mass for prolonged periods. These and other hatching complications will be addressed in more detail elsewhere. Only $3.4 \pm 4.2\%$ of embryos that attempted to hatch emerged into the jelly rather than toward the open water. In contrast, when individual embryos were flooded with similar surface exposure in glass cups, 28.6% of embryos attempted to hatch toward the glass, rather than through the exposed portion of their egg surface. Using a test of equal proportions, we found a significant difference in the proportion of poorly oriented hatching between these two contexts ($\chi^2 = 33.21$, df = 1, $p < 0.001$).
We found no evidence of a side bias in our half-dark cups. When the “light” side was not illuminated 9 embryos hatched towards the “light” and 11 towards the “dark” side of cups ($\chi^2 = 0.05$, df = 1, $p = 0.823$). However, when one side of the cups was illuminated, *A. callidryas* embryos were more likely to hatch towards the light. In both sets of trials, significantly more embryos hatched toward the light (trials 1–50: 67%, $\chi^2 = 4.356$, df = 1, $p = 0.037$; trials 51–92, 73%, $\chi^2 = 7.225$, df = 1, $p = 0.007$). We found no difference between the two sets of trials ($\chi^2 = 0.611$, df = 1, $p = 0.434$) and therefore pooled them. Overall, 59 of 85 tested embryos hatched toward the light (69.4%, Fig. 7; $\chi^2 = 12.047$, df= 1, $p < 0.001$). Embryos hatched 16.27 ± 13.24 minutes after flooding (mean ± SD; range 3.07–63.28 min), and the initial side of insertion into the tube had no effect on hatching direction (GLMM, $\chi^2 = 1.799$, $p = 0.407$).

**Discussion**

We found that embryos of *A. callidryas* use light cues to inform their hatching in two different ways. First, under natural photoperiods, embryos show a clear diel pattern of hatching, with a strong peak shortly after nightfall. Their hatching is desynchronized by continuous light and darkness and is delayed by continuous light but not accelerated by continuous darkness. Second, embryos use light cues to orient their hatching direction when escaping from flooded eggs, showing positive phototaxis. These results add another sensory modality to the information sources *A. callidryas* embryos use to inform their hatching process, and another aspect of hatching – orientation as well as timing – that is responsive to environmental cues.

**Diel pattern of hatching**
Embryos with plastic hatching timing can make crucial and often swift decisions to leave their egg to escape immediate threats (Warkentin 1995; Warkentin 2000b). Thus, diel hatching patterns are likely most relevant for undisturbed egg clutches, in which embryos apparently hatch spontaneously. In *A. callidryas*, most undisturbed embryos hatch at night (Gomez-Mestre & Warkentin 2007; Warkentin 1995). Nocturnal hatching may be favorable for embryos of many species, since hatchings are often susceptible to diurnal visual predators (Bradbury et al. 2004; Gustafson-Marjanen & Dowse 1983; Touchon et al. 2013; Warkentin 1995; Witherington et al. 1990). This is consistent with an inhibitory or delaying effect of light on hatching, as is evident in *A. callidryas* and some fish species (e.g. Brüning et al. 2011; Downing & Litvak 2002). In addition, diel hatching patterns may be particularly valuable for species which exhibit habitat switches at the time of hatching, with different diel patterns of risk in each habitat. For instance, a generally higher risk from terrestrial egg-predators at night, and from aquatic predators of tadpoles during the day, could favor hatching at dusk (e.g. nocturnal snakes vs. diurnal fishes; Gomez-Mestre et al. 2008).

Under natural photoperiods, *A. callidryas* embryos showed a clear peak of hatching (i.e., relative synchrony) shortly after the onset of darkness. Synchronous hatching may reflect a simple convergence of many embryos on a shared optimal hatching time. Synchrony could also, in itself, be beneficial. Synchronous hatching and synchronous emergence from nests have been suggested to reduce predation through predator-swamping or avoidance (Christy 2003; Ims 1990). If predators are alerted by initial hatchlings and converge on sites where hatchlings are emerging, higher predation on later-hatching individuals could also favor synchrony (Ims 1990;
Observations of fish predation on hatchlings suggest that hatching after dark may improve *A. callidryas* survival in some contexts (M. Hughey & K. Warkentin unpublished data); however, to our knowledge relatively little research has examined diel patterns of tadpole predation.

Synchronous, nocturnal hatching is clearly a potential adaptive strategy for many species, and likely also relevant to *A. callidryas*. This strategy has been widely documented in fishes [e.g., tropical reef damselfish (Asoh & Yoshikawa 2002; Doherty 1983; McAlary & McFarland 1993); Baltic salmon, *Salmo salar* L. (Brännäs 1987; Gustafson-Marjanen & Dowse 1983); rainbow smelt, *Osmerus mordax* (Bradbury et al. 2004)]. Across different photoperiods, these species all show synchronous hatching shortly after the onset of darkness. This pattern is hypothesized to be a predator avoidance strategy that evolved due to high risk of predation when fry emerge.

However, very few studies have directly, empirically linked this hatching pattern to diel variation in predation pressure (Bradbury et al. 2004). In the case of reptiles, most species develop in nests with temperature gradients which influence incubation and hatching times; thus, apart from some well documented cases in sea turtles (Spencer & Janzen 2011) and pig-nosed turtles, *Carettochelys insculpta* (Doody et al. 2001), synchronous hatching is less common since hatching prematurely has immediate and long term costs on individuals (Colbert et al. 2010; Doody 2011). A review of the timing of larval release in brachyuran crabs found that of the 81 species examined, 78 release larvae at or after sunset, and mostly during high amplitude high tides, presumably as a predator avoidance strategy (Christy 2003; Christy 2011). Amphibians provide some of the best-studied examples of environmentally cued hatching, with documented
cases of hatching plasticity widely distributed across the clade (Warkentin 2011b); yet, to our
knowledge, our study is the first to test for a shift in hatching timing in response to light cues.

Our finding of reduced hatching synchrony in continuous darkness, but no general acceleration,
suggests that the change from light to darkness, rather than darkness *per se*, may serve as
hatching cue in *A. callidryas*. Similarly, Baltic salmon (*Salmo salar* L.) embryos kept in dark
conditions hatched continuously and unsynchronized; 50% of all eggs hatched within 6 days
after the onset of hatching competence, while embryos in a 16L:8D photoperiod environment
hatched more synchronously; 50% of eggs hatched within 2 days after the onset of hatching
competence, with peaks of hatching after the onset of darkness (Brännäs 1987). However,
embryo responses to photoperiod may differ among species. For instance, hatching of haddock
(*Melanogrammus aeglefinus*) embryos is delayed in darkness and advanced in light (Downing &
Litvak 2002). In freshwater fish, continuous light delayed hatching in roach (*Rutilus rutilus*) and
bleak (*Alburnus alburnus*), but accelerated hatching timing in chub (*Leuciscus cephalus*), and
only in one species, perch (*Perca fluviatilis*), was the onset of darkness found to elicit more
hatching (Brüning et al. 2011). Clearly, the specific effects of light, darkness, and light-dark
transitions on hatching timing vary among species. Further examination of both the adaptive
significance of diel hatching patterns and the underlying mechanisms eliciting these responses in
light- or dark-cued species is needed.

Undisturbed hatching may appear spontaneous in red-eyed treefrogs, but it is clearly not simply a
developmental process under full endogenous control. Rather, like induced early hatching, its
timing is environmentally informed in ways that may confer selective benefits. This may also be true for apparently spontaneous hatching in other species as well.

**Hatching orientation**

For some embryos, the physical structure of their egg dictates hatching direction. For instance, in non-spherical eggs, egg shape may limit the range of positions embryos can occupy, and thus their potential hatching orientation (e.g. some desersal fish eggs, Korwin-Kossakowski 2012; Olivotto et al. 2003). Other embryos, including *A. callidryas* and other amphibians, move freely within their vitelline chamber and could potentially exit through any part of their capsule. Their hatching orientation may, nonetheless, affect fitness. Specifically, for terrestrial eggs laid in masses on solid substrates, embryos that hatch toward the interior of their mass may become trapped between sibling eggs and the substrate. In our field and laboratory observations of *A. callidryas*, such cases are relatively rare but can be fatal (B. Güell & K. Warkentin pers. obs.), revealing the importance of hatching orientation. Thus, there must be cues that allow embryos to hatch correctly oriented. Prior work has demonstrated that *A. callidryas* embryos in clutches in air orient toward the exposed surface of their egg capsules, presumably in response to the oxygen distribution within eggs (Rogge & Warkentin 2008). Our results show that these embryos use light cues to orient their hatching when submerged. This adds another sensory modality to the suite of cues that affect *A. callidryas* hatching behavior, beyond oxygen and mechanosensory cues (Warkentin 2002; Warkentin 2005). It also raises the possibility that oxygen gradients may not be the only cue informing *A. callidryas* orientation and hatching direction in clutches in air.
Moreover, it suggests that other species might use light – or more complex visual cues – to inform hatching in some way beyond diel cycles.

Embryonic visual learning and its effect on post-hatching behavior has been demonstrated in several animal taxa (cuttlefish, Darmaillacq et al. 2008; bobwhite quail, Honeycutt & Lickliter 2002; e.g., leopard gecko, Sleigh & Birchard 2001). These studies suggest that embryos may specifically receive and retain visual information presented to them in ovo, but not necessarily that they use this information prior to hatching. Human fetuses have also been shown to respond behaviorally to face-like visual patterns presented in utero, more than to inverted patterns (Reid et al. 2017). Red-eyed treefrog embryos clearly respond behaviorally to patterns of illumination around their eggs in at least one context, flooding. However, our study does not address to what extent these embryos are capable of perceiving images, such as embryos in neighboring eggs, the leaf to which the clutch is attached, or predators nearby. Nor does it address if, beyond simple light and oxygen gradients, they might use such visual information to inform hatching. However, these are now open questions.

Clearly the hatching response of A. callidryas embryos to light is context-dependent, reversing in polarity between the two cases we studied. In flooded clutches, A. callidryas embryos hatch toward light, or away from the dark, thereby avoiding potentially fatal hatching complications. In contrast, for the more common context of embryos in air, light inhibits or delays hatching and the onset of darkness appears to stimulate hatching, which might reduce larval predation risk. We know A. callidryas embryos show context-dependent cue use in at least two ecologically relevant contexts using different sensory modalities (Warkentin 2002; Warkentin 2005; Warkentin et al.
Here we show that the presumably adaptive use of a novel cue type by *A. callidryas* embryos is also context-dependent.

**Conclusions**

Like threat-induced early hatching, the timing of undisturbed, apparently spontaneous hatching in *A. callidryas* is environmentally cued. In undisturbed egg clutches and in flooded clutches suffering hypoxia, embryos use light cues in two different ways, to inform when and where to hatch. These findings add to the range of sensory modalities these embryos use to guide their behavior and support the generality of context-dependent cue use across sensory modalities. We propose that further investigation of how embryos use light and visual cues in hatching is worthwhile.

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**Author contributions**
B.A.G. and K.M.W. designed the experiments. B.A.G. performed the experiments, analyzed the videos, and did the statistical analysis. B.A.G. and K.M.W. wrote the paper.

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Figure 1 (on next page)

Mass of sibling *Agalychnis callidryas* embryos in dish over dechlorinated tap water for experiment testing effect of light environment on diel patterns of hatching.
**Figure 2** (on next page)

*Agalychnis callidryas* embryos in their natural clutch environment (**A**) and in a glass cup (**B**).
Figure 3 (on next page)

Hatching timing of *Agalychnis callidryas* embryos in three light environments.

Data are mean proportion hatched ± SE (shaded ribbons) for embryos in each treatment, recorded every 2 h (*N* = 11 clutches, split across treatments).
Figure 4 (on next page)

Proportion of *Agalychnis callidryas* embryos that hatched between 16:00–20:00 h at age 5 d.

Hatching was more concentrated in this 4-h period when it included the onset of darkness than under constant dark or constant light. Data are means ± SE for proportion of embryos hatched per sibship in each treatment (*N* = 11 split clutches).
Proportion hatched between 5.67–5.83 days

Light treatment

- Dark
- Light
- Photoperiod

The graph shows the proportion hatched for each light treatment, with the light treatment having a significantly higher proportion compared to the dark treatment.
**Figure 5** (on next page)

Synchrony of hatching of *Agalychnis callidryas* embryos within and among egg masses in three light environments

(A) Synchrony within egg masses. Data are means ± SE for the highest proportion of embryos hatched in each mass in any 2-h period (*N* = 11 clutches split across treatments). Embryos within masses hatched more synchronously in the photoperiod treatment. (B) Synchrony of hatching of *Agalychnis callidryas* embryos among egg masses (sibships) in three light environments. Data are number of clutches whose peak of hatching occurred in each 4-h time period. Grey bars indicate dark periods. Hatching was more synchronous across sibships in the photoperiod treatment.
Max proportion hatched in any 2 hour period

Light treatment

[Graph A]

[Graph B]

Number of clutches

Age (days)

Treatment
- dark
- light
- photoperiod
Figure 6 (on next page)

Proportion of *Agalychnis callidryas* embryos that hatched after age 6 d in three light environments.

More embryos hatched late in development in the continuous light treatment. Data are means ± SE for proportion of embryos hatched per sibship in each treatment (*N* = 11 split clutches).
Proportion hatched after age 6.0 days

Light treatment:
- Dark
- Light
- Photoperiod

Legend:
- a
- b
Figure 7 (on next page)

Hatching direction of *Agalychnis callidryas* embryos submerged in hypoxic water with one side illuminated (phototaxis experiments) or neither side illuminated (control).

More embryos hatched towards the light in experiments and there was no evidence of side-bias. Data are number of embryos exiting their egg, and egg-holding tube, in each direction.
Experiment 1

Experiment 2

Side-bias Control

(Both sides dark)

Number of embryos

Number of embryos

Embryo Hatching Direction

dark side light side