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RESEARCH ARTICLE

How embryos escape from danger: the mechanism of rapid, plastic hatching in red-eyed treefrogs

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ABSTRACT

Environmentally cued hatching allows embryos to escape dangers and exploit new opportunities. Such adaptive responses require a flexibly regulated hatching mechanism sufficiently fast to meet relevant challenges. Anurans show widespread, diverse cued hatching responses, but their described hatching mechanisms are slow, and regulation of timing is unknown. Arboreal embryos of red-eyed treefrogs, *Agalychnis callidryas*, escape from snake attacks and other threats by very rapid premature hatching. We used videography, manipulation of hatching embryos and electron microscopy to investigate their hatching mechanism. High-speed video revealed three stages of the hatching process: pre-rupture shaking and gaping, vitelline membrane rupture near the snout, and muscular thrashing to exit through the hole. Hatching took 6.5–49 s. We hypothesized membrane rupture to be enzymatic, with hatching enzyme released from the snout during shaking. To test this, we displaced hatching embryos to move their snout from its location during shaking. The membrane ruptured at the original snout position and embryos became trapped in collapsed capsules; they either moved repeatedly to relocate the hole or shook again and made a second hole to exit. Electron microscopy revealed that hatching glands are densely concentrated on the snout and absent elsewhere. They are full of vesicles in embryos and release most of their contents rapidly at hatching. *Agalychnis callidryas*' hatching mechanism contrasts with the slow process described in anurans to date and exemplifies one way in which embryos can achieve rapid, flexibly timed hatching to escape from acute threats. Other amphibians with cued hatching may also have novel hatching mechanisms.

KEY WORDS: Hatching gland cells, Phenotypic plasticity, Frogs, Embryo behavior, Antipredator defense

INTRODUCTION

Hatching is an essential event in animal development and a critical transition between two distinct environments, within and outside the egg capsule. Its timing may be crucial to balance selective costs and benefits across life stages, and if either environment varies, so will the optimal time to hatch (Warkentin, 1995; Werner and Gilliam, 1984). Many animals, from all three major clades of Bilateria, negotiate this challenge by adaptively altering their timing of hatching in response to environmental factors, a phenomenon known as environmentally cued hatching that is particularly well documented in frogs (Warkentin, 2011a,b). Frogs exhibit remarkable diversity in reproductive modes; for example, eggs can be laid in the water, on land, in foam nests, or carried by a parent, and can hatch as larvae or froglets (reviewed in Haddad and Prado, 2005). Across this diversity, in at least 10 anuran families, embryos show adaptive accelerations, delays and in some cases remarkably precise and rapid hatching responses to diverse environmental factors, including hypoxia, dehydration, pathogens, predators and parental behavior (Delia et al., 2014; Warkentin, 2011b). Such responses require that embryos perceive and respond appropriately to environmental stimuli (Warkentin and Caldwell, 2009). They also require a mechanism to regulate the process of hatching.

The red-eyed treefrog, *Agalychnis callidryas* (Cope 1862) (Hylidae, Phyllomedusinae), provides an excellent example of environmentally cued hatching. It lays eggs on vegetation over ponds; undisturbed eggs hatch after 6 or 7 days, and the tadpoles drop into the water. These embryos can, however, hatch up to 30% early to escape from numerous threats, including pathogenic fungus (Warkentin et al., 2001), flooding (Warkentin, 2002), dehydration (Salica et al., 2012) and predators such as egg-eating snakes and wasps (Warkentin, 1995, 2000). Hatching early incurs a trade-off; early hatchlings are less developed and more vulnerable to aquatic predators compared with older hatchlings (Touchon et al., 2013; Warkentin, 1995, 1999a,b; Willink et al., 2014). During snake attacks, embryos must hatch rapidly to escape being eaten (Warkentin, 2005). They begin hatching, on average, 16 s after first contact with the snake (Warkentin et al., 2007), with about 80% escape success through most of the plastic hatching period (Gomez-Mestre et al., 2008b; Warkentin, 1995). Fine-tuned, flexibly timed hatching would not be possible without a proximate mechanism of regulation. However, a rapid mechanism of hatching has not been described for anurans. Thus, we sought to understand the hatching mechanism that enables these embryos to escape such acute threats.

Anuran hatching has not been previously described as a rapid event. In *Xenopus laevis*, hatching is divided into two phases that span over half of the embryonic period. In phase 1, the three jelly coats surrounding the egg are shed and/or dissolved. In phase 2, which takes about 3–6 h, an evagination in the weakening membrane, called a bleb, forms near the embryo's head, and embryos use movement to rupture the egg membrane at this locally weakened site (Carroll and Hedrick, 1974). In *Rana japonica*, examination of ultrastructural and biochemical changes in the egg membrane over development revealed that breakdown of the vitelline membrane begins about halfway (~55%) through the embryonic period, then continues gradually until hatching (Yoshizaki, 1978). Blebbing occurs in the final stages, and embryos take 25–45 min to exit the vitelline membrane and jelly layers. Timing is similar in *Alytes obstetricans*, for which exit from a localized rupture in the membrane takes 25–30 min (Noble, 1926). *Hyla avivoca* are in the same family as *A. callidryas*, but lay eggs in shallow water and hatch slowly. The inner jelly coat ruptures early

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Received 22 February 2016; Accepted 30 March 2016

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in development and, at the time of hatching, blebbing occurs near the head and embryos glide out gently (Volpe et al., 1961). Volpe et al. (1961) suggested this occurs by ciliary motion, with little or no pressure exerted by the vitelline fluid, but noted that embryo movements could rupture the membrane in some cases. This may indicate a general membrane weakening prior to the moment of hatching.

The mechanism of hatching has been investigated in detail for only a few anuran species, all of which lay aquatic eggs [*Xenopus* (Drysdale and Elinson, 1991), *Bufo* (Yamasaki et al., 1990), *Rana* (Yoshizaki and Katagiri, 1975)]. Unicellular hatching glands [hatching gland cells (HGCs)] on the surface of the head secrete a proteolytic hatching enzyme that digests components of the vitelline membrane (Altig and McDiarmid, 1999). Enzyme secretion and membrane degradation are usually gradual and begin several developmental stages prior to hatching (Yamasaki et al., 1990; Yoshizaki, 1978; Yoshizaki and Katagiri, 1975). A comparative study of 20 anuran species, spanning six families, identified HGCs in all but one, *Phyllomedusa trinitatis* (Nokhbatolfoghahai and Downie, 2007), which was the only phyllomedusine examined. *Phyllomedusa trinitatus* wrap their egg clutches in leaves, with jelly plugs above and below the eggs (Faivovich et al., 2010; Kenny, 1966). Downie et al. (2013) later found HGCs in this species, and suggested their primary role is to break down the jelly plugs, facilitating tadpole emergence from the nest, and that the hatching of individual embryos from their capsules occurs largely by wriggling behavior and osmotic stretching of the membrane.

Agalychnis callidryas has also been hypothesized to hatch by mechanically, rather than enzymatically, rupturing the vitelline membrane (Rogge and Warkentin, 2008). Unlike in other anurans, *A. callidryas* eggs remain turgid and do not visibly degrade prior to hatching (K.M.W. and K.L.C., unpublished observation). Moreover, the gradual enzyme secretion and membrane degradation described in other anurans is too slow to enable escape from snake and wasp attacks. However, rapid, predator-induced hatching is not unique to *A. callidryas*. Two other *Agalychnis* species and the closely related *Pachymedusa dacnicolor* also have high escape success in snake attacks, and a more distantly related phyllomedusine, *Cruziohyla calcarifer*, hatches rapidly in response to mechanically simulated attack, suggesting that this ability may be ancestral or common in the group (Gomez-Mestre et al., 2008b). The glassfrogs, Centrolenidae, evolved terrestrial eggs independently of phyllomedusines (Gomez-Mestre et al., 2012), and some species can also hatch rapidly, to escape from predators, with no prior degradation of the membrane (J. Delia, personal communication). Myobatrachid anurans from multiple genera lay terrestrial eggs that are 'ready and waiting' (Martin, 1999) to hatch when flooded (reviewed in Warkentin, 2011b). For example, the Australian frog *Pseudophryne bibronii* can hatch after 36 days of development, but can wait up to 120 days for inundation before hatching (Bradford and Seymour, 1985). Our current mechanistic understanding of anuran hatching does not include such species with terrestrial eggs that hatch rapidly, nor can the known mechanisms explain the diverse cued hatching patterns observed across amphibians (Warkentin, 2011b). *Agalychnis callidryas* offers a robust, tractable and well-documented example for mechanistic investigation of rapid, flexibly timed, environmentally responsive hatching.

In the first part of this study, we used high-speed video recordings of individual embryos hatching to characterize the hatching process in *A. callidryas*. We identified distinctive pre-rupture behavior and observed the context of egg membrane rupture and process of exiting the egg capsule. Based on these results, we hypothesized (i) that membrane rupture is enzymatic, not mechanical or muscular, and (ii) that embryos release hatching enzymes rapidly and locally from their snout. If hatching is mediated by local, acute enzyme release from the snout, we predicted that the membrane would rupture where the snout was located when the pre-rupture behavior was initiated, even if the embryo was subsequently displaced from this position. Moreover, if localized enzymatic digestion is required for embryos to exit the egg, displacing the embryo from the position where it initiated hatching should delay its exit. To test these predictions, in the second part of this study we experimentally spun embryos within their egg capsule after the apparent initiation of hatching and recorded the location and timing of membrane rupture and exit from the egg. Finally, to understand the role of hatching glands (HGs) in this process, we used scanning electron microscopy to locate and characterize HGCs in *A. callidryas* and scanning transmission electron microscopy to examine the contents of HGCs before and just after hatching.

MATERIALS AND METHODS

Egg collection and care

Agalychnis callidryas eggs are laid in masses of, on average, 40 eggs (Gomez-Mestre et al., 2008b) that are attached to a substrate by a layer of jelly. Egg masses on leaves were collected from Ocelot Pond (9°6′8.62″N, 79°40′56.96″W), Bridge Pond (9°6′50.26″N, 79°41′48.13″W) and Experimental Pond (9°7′14.77″N, 79°42′12.03″W) near and in Gamboa, Panamá, under permits from the Panamanian Autoridad Nacional del Ambiente (SE/A-41-08, SE/A-13-11, SC/A-19-11 and SC/A-16-12). Eggs were collected prior to hatching competence, based on embryo morphology (Warkentin, 2002), and brought to an open-air laboratory at the Smithsonian Tropical Research Institute in Gamboa. Clutches on leaves were attached to plastic support cards, suspended over aged tap water to catch hatchlings, and misted frequently to maintain hydration. Videos were recorded in an air-conditioned room adjacent to the ambient-conditions laboratory. Each clutch was individually carried to the camera location just prior to recording to minimize its time in cooler, drier air, taking care to limit physical disturbance so eggs did not hatch before recording. All embryos used were morphologically normal, developmentally in synchrony with their siblings in the clutch, and in intact, turgid, eggs. Other than individual embryos preserved for electron microscopy, all tadpoles were returned to their egg collection site after hatching. The Smithsonian Tropical Research Institute IACUC approved all work.

High-speed video analysis of hatching

We used a Redlake MotionPro X3 camera (DEL Imaging Systems, Cheshire, CT, USA) to record high-speed macro video of embryos hatching in October–November 2008. We mounted a Petri dish at a 30 deg angle from vertical, adhered a small Plasticine shelf in the camera field of view, and illuminated it with two high-intensity fiberoptic gooseneck lights at an angle from behind, for darkfield, and two small LED lamps at an angle from the front. Individual eggs were carefully removed from their clutch with moistened blunt forceps and placed on the shelf for recording. If they did not begin hatching shortly, they were gently rubbed or prodded with a blunt probe. We used 5 and 6 day old embryos that hatch readily in response to physical disturbance and tried to provide a minimal hatching stimulus in order to record the entire hatching process without obstructing the view. We recorded at 200 Hz to capture potential pre-rupture behavior plus exit from the egg. We analyzed only videos in which the egg membrane was intact at the start, the

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embryo's body was fully outside the egg by the end, and nothing suggested that probe contact after the initiation of hatching may have altered the process (N=62 embryos, 44 at 5 days and 18 at 6 days, from 19 clutches; 3.3±2.1 embryos per clutch; mean±s.d. here and throughout unless noted). Recording durations were 27.9±13.9 s (range 8.2–63.5 s) at f8 or f11 with a shutter speed of 1.9 ms. In 20 cases, part of the embryo's tail remained within the egg at the end of the recording.

We used ImageJ v1.49 (Schneider et al., 2012) to analyze embryo behavior and the hatching process, quantifying timing from video frame numbers. Based on an initial review of recordings, we identified a set of events and behaviors during the hatching process (see Results); we then reviewed all recordings at multiple speeds, including frame-by-frame as needed, to quantify their incidence and timing. We distinguished events that occurred before, and thus may have contributed to, membrane rupture, whether the initial evidence of rupture was protrusion of the embryo's snout or leaking of fluid, and the post-rupture process by which the embryo exited from its membrane. We then calculated the durations of various periods during the hatching process. None differed significantly between 5 and 6 day old embryos (Wilcoxon tests, all P>0.11); thus, we present data pooled across ages.

Embryo displacement experiment

In July 2011 we manipulated embryo position within the egg capsule after hatching was initiated to determine the timing and location of enzyme release. We tested embryos in their clutches, with eggs naturally adhered to the jelly, which prevented the capsules from rotating during manipulation, and minimized disturbance that could induce hatching during setup. Embryos were tested between 15:00 h and 24:00 h at age 5 days. We laid each clutch horizontally on a platform within a shallow dish of aged tap water, to catch hatchlings, and viewed it through a dissecting microscope. We manipulated a series of embryos sequentially, haphazardly interspersing individuals assigned to displacement and rotation control treatments (see below) within each clutch. We recorded all embryo manipulation and subsequent events until hatching through the microscope using a Nikon D7000 camera at 24 Hz. For each focal embryo (N=62), we used a moistened blunt probe to gently prod and rub the embryo through its egg membrane until it began low amplitude shaking, a behavior associated with hatching (see Results; 4.79±2.37 s of stimulation, range 1.13–14.5 s). In a small number of pilot trials to determine the approximate timing of hatching events and how long to wait before rotating embryos, we allowed the embryos to hatch with no further manipulation after initial stimulation. During experimental trials, we waited for 7.22±0.465 s of shaking (range 0.46–20.5 s) before applying one of two treatments. For the displacement treatment, we swiftly rotated the embryo so that the snout faced a different part of the vitelline membrane, whereas in the rotation control treatment, we rotated the embryo away and then back to its original position (Movie 1).

We performed 245 trials but analyzed only those that met the following criteria: (i) experimental manipulation was performed without error: membrane rupture did not occur while the probe was in contact with the membrane, rotation controls were returned to the correct position, and embryo rotation was complete before membrane rupture occurred; (ii) the entire egg and embryo were visible throughout the video; (iii) spontaneous position changes did not prevent implementation of the rotation protocol; and (iv) if neighboring embryos hatched during the video, neither their bodies nor fluid from their egg capsules came into physical contact with the focal egg. Because of the greater time and precision required for success, the fraction of rotation control trials (N=26) that met the criteria was lower than that for displacement trials (N=36). Displacement trials included embryos from 12 clutches; rotation controls included embryos from 11 of those.

We analyzed recordings in ImageJ v1.43u (Schneider et al., 2012). Event timing, including the start and end of behaviors, was quantified from frame numbers. We defined shaking duration prior to displacement as the summed duration of all shaking bouts before the first frame when the embryo's snout left its original position. For membrane rupture time, we used the first frame in which fluid was visible outside the capsule (Movie 1). Displaced embryos sometimes became trapped in the collapsed membrane. Time spent trapped was measured from the last frame of membrane collapse to the first frame where the snout protruded through the membrane. To compare how long embryos took to begin their exit from the egg capsule, we measured the interval from the first frame of embryo shaking to the first frame where the snout protruded through the membrane. Because data were not normally distributed, we used non-parametric Wilcoxon rank sum tests to compare the distribution of mean ranks between treatment groups. Using the Bonferroni correction for multiple comparisons we adjusted the P-value for significance to 0.0125. All analyses were performed using R version 3.0.1 (R Development Core Team, 2013).

Electron microscopy

We examined *A. callidryas*' surface HG morphology at age 5 days, and compared HG cellular ultrastructures between embryos and hatchlings at age 6 days. To obtain specimens of unhatched embryos, we chilled eggs on ice until embryos were unresponsive, then manually decapsulated them. Others from the same clutches were manually stimulated to hatch, as above, and placed immediately in buffered MS-222 for anesthesia. We preserved embryos in 5% glutaraldehyde plus 2% paraformaldehyde in phosphate-buffered saline (PBS) for 16–24 h at 4°C. To observe HG morphology and distribution, specimens were washed in PBS, dehydrated in an ethanol series, critical point dried, sputter coated in gold/palladium, and imaged on a Zeiss EVO 40vp scanning electron microscope (N=5 embryos spanning four clutches). To examine HGC contents and changes associated with hatching, specimens preserved before and immediately after hatching were post-fixed in osmium tetroxide for 1 h, embedded in Spurr's medium, sectioned at ∼100 nm on a Leica ultra-microtome, and the sections mounted on Formvar/Carbon grids. We stained for contrast with uranyl acetate and lead citrate prior to imaging on the Zeiss EVO 40vp using a STEM detector.

RESULTS

High-speed video analysis of hatching

Our 200 Hz video recordings revealed three sequential stages in the hatching process of *A. callidryas*. First, most embryos performed distinctive behaviors within the egg that have not been observed in non-hatching contexts; second, a hole formed in the egg membrane; and third, the embryo exited the egg (Fig. 1). The hatching process was rapid, taking just 20.6 s, on average, from the first identifiable pre-rupture behavior to complete exit from the egg membrane (Table 1).

In most recordings (82%), embryos exhibited axial muscle contractions that generated low-amplitude lateral shaking (Fig. 1; Movies 2 and 3). Shaking was strongest at, and sometimes restricted to, the base of the tail but was often more extensive, sometimes including the entire body and tail. This did not displace embryos

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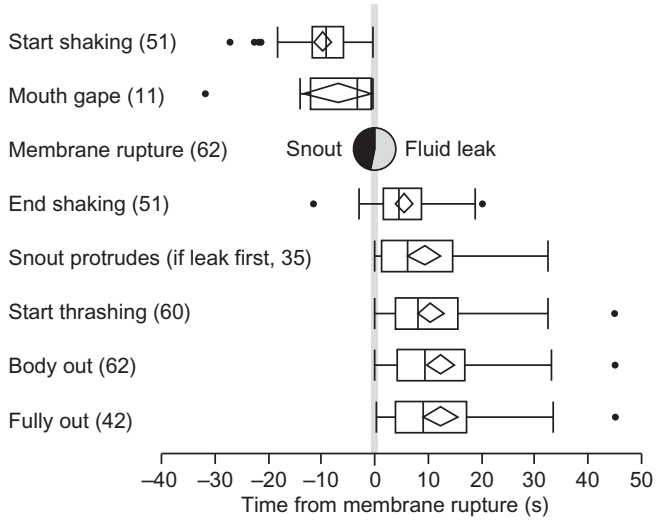

Fig. 1. Timing of events in the hatching process of *Agalychnis callidryas*. Hatching was analyzed from 200 Hz video recordings of 62 embryos, but not all individuals showed every behavior; *N* for each event type is indicated in parentheses. Event timing is shown relative to membrane rupture, evidenced by snout protrusion or leaking of perivitelline fluid, in the proportions shown. Data are means and 95% confidence intervals (diamonds), mode, interquartile range (IQR) and extent of data to ±1.5×IQR (box and whiskers), and outliers.

from their position in the egg, and in some cases pushed the embryo's snout against the membrane (Movie 3). Shaking often continued through rupture (Fig. 1; Movie 3), and in one case the embryo only shook after rupture. In some recordings, embryos gaped open their mouths shortly before hatching (30% of cases where embryo position allowed visibility). This behavior differed from buccal pumping in the larger amplitude and longer duration of the gape (Movies 2 and 3). Nine embryos performed similar behavior post-rupture; three of these visibly filled their lungs upon gaping. Seven embryos (six with visible mouths) neither shook nor mouth-gaped before rupture.

Membrane rupture was identified by protrusion of the embryo's snout or leaking of perivitelline fluid (26 and 33 cases, respectively, plus three in which the two occurred simultaneously; Fig. 1; Movies 2 and 3). Fluid always leaked from a single location on the egg surface, near the embryo's snout. In one case, where the embryo slid forward during shaking, the leak formed at the snout's position earlier in the process. In a few cases, fluid began leaking without visible contact between the embryo and the membrane at the rupture site (Fig. 1; Movie 2).

As an embryo's snout moved into the rupture site, it often prevented fluid from leaking. Instead, turgor pressure within the elastic egg membrane or small, shaking contractions of axial musculature slowly forced the embryo forward, sometimes with a ring of body constriction marking its progression through the membrane (Fig. 1; Movie 3). Almost all embryos also performed thrashing motions – high-amplitude body undulations traveling from snout to tail, as in swimming (Fig. 1; Movies 2 and 3). Embryos began thrashing soon after rupture, often with their snout already protruding; only two embryos began thrashing before rupture was evident (Table 1). In most cases, thrashing rapidly propelled embryos from the egg but in eight cases the exit took much longer (Table 1); in all slow exits, thrashing was discontinuous.

Table 1. Duration of the hatching process of *Agalychnis callidryas* and some components and periods within it, from analysis of video recorded at 200 Hz

	Duration (s)		
Component of the hatching process	Mean±s.d.	Range	*N*
Entire hatching process*	20.59±11.31	6.52–49.08	38
Pre-rupture behavior to rupture	9.99±6.45	0.50–31.77	55
Pre-rupture behavior to body out	21.11±10.90	6.48–49.04	55
Total duration of shaking	15.71±5.85	4.76–34.30	52
Rupture to snout protrusion	5.22±8.35	0–32.52	62
Snout protrusion to body out	6.97±7.92	0.02–32.99	62
Snout protrusion to fully out	7.96±8.40	0.13–33.04	42
Rupture to start of thrashing	10.52±9.35	–0.12–44.94	60
Snout protrusion to start of thrashing	5.31±6.87	–0.21–32.89	60
Start of thrashing to body out (fast)	0.22±0.14	0.05–0.66	52
Start of thrashing to body out (slow)	7.74±9.65	1.4–30.11	8

*Time from first pre-rupture behavior, shaking or gaping, to complete exit from the egg membrane. In two cases, the embryo changed position and the rupture formed at the second snout position; in those cases, time is measured from the behavior in the second position. One embryo changed position after gaping, then shook. The other began shaking, turned, and continued shaking.

Embryo displacement experiment

Timing and location of membrane rupture

The timing of membrane rupture, whether measured from the start of shaking or from the start of spinning, did not differ between treatments (from shaking: Wilcoxon rank sum test W=404, P=0.365, Fig. 2A; from spinning: Wilcoxon rank sum test W=487.5, P=0.786, Fig. 2B). Of 36 embryos displaced from their original position, all but two showed evidence of membrane degradation at the original snout location (Table 2). In 29 displaced embryos, the leaking of perivitelline fluid was clearly visible as evidence of membrane rupture (Movie 1). In four cases, a bleb

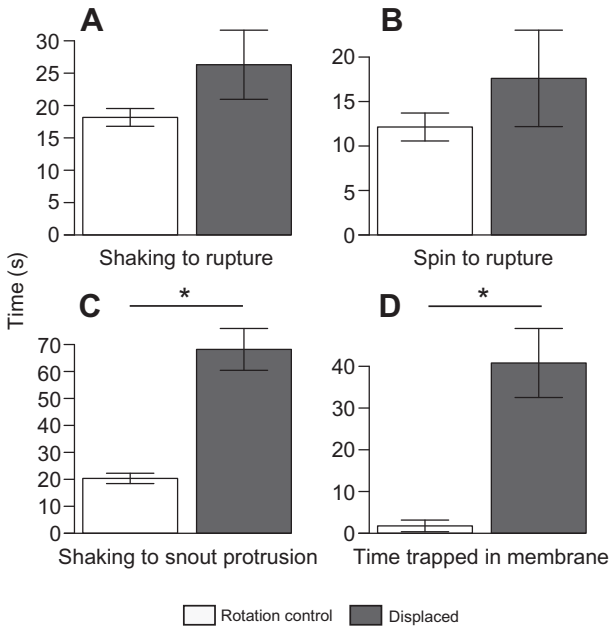

Fig. 2. Effect of experimental rotation of *A. callidryas* embryos on timing of events during hatching. The lag from (A) initiation of shaking to membrane rupture and (B) start of the spinning manipulation to rupture did not differ significantly between embryos in the rotation control and displaced treatments. Displaced embryos (C) took longer to begin their exit from the egg capsule and (D) spent more time trapped in the collapsed membrane. Wilcoxon rank sum tests with Bonferroni correction for multiple comparisons, *P<0.0125; N=26 rotation controls, N=36 displaced. Data are means±s.e.m.

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Table 2. Evidence and location of membrane rupture after experimental rotation of *A. callidryas* embryos during hatching

Treatment	Outcome	Number (%) of embryos
Displaced *N*=36	Fluid leak at original site	29 (80.6%)
	Bleb at original site	4 (11%)
	Tail emerges through original site	1 (2.8%)
	No membrane rupture at original site (rupture at new location of snout)	2 (5.6%)
Rotation control *N*=26	Fluid leak at original site	14 (54%)
	No fluid leak visible; exit at original site	12 (46%)

Embryos were moved on average 7 s after they began shaking, displacing their snout from its original site.

(evagination of the membrane) was visible at the original site, indicating weakening of the membrane, and in one case the tail poked through at the original site, indicating rupture. In all 26 rotation controls, rupture occurred at the original site; a fluid leak was clearly visible in 14 of these, and in all cases embryos exited from the original site, indicating location of rupture.

Timing and location of exit

Displaced embryos took longer to begin exiting the egg capsule, from the onset of shaking to snout protrusion (Wilcoxon rank sum test W=43, P=3.5e−12, Fig. 2C). Of the 29 displaced eggs that had a visible fluid leak at the original snout location, 24 experienced membrane collapse due to leaking of perivitelline fluid from the rupture site. In the cases where collapse did not occur, either the tail or the egg jelly appeared to be blocking the rupture site. Displaced embryos spent 40.79±8.27 s (mean±s.e.m.) in the collapsed membrane before snout protrusion. Only 3 of the 26 rotation control embryos experienced membrane collapse and, overall, rotation controls spent less time trapped in a collapsed membrane than did displaced embryos (Wilcoxon rank sum test: W=159, P=1.793e−06, Fig. 2D).

Once embryos became trapped in the collapsed membrane, many used large undulations to change position, often multiple times, until their snout encountered the original hole in the membrane. Displaced embryos performed more position changes than did rotation controls (Wilcoxon rank sum test: W=304, P=0.0055); in one case, a displaced embryo changed position 14 times before exiting the membrane. Among rotation controls, all 26 embryos exited through the original rupture site. Among displaced embryos, 13 of 36 found and exited through their original hole, while 23 formed a second rupture site in the membrane and exited through it (Movie 1). All but two displaced embryos resumed shaking after they were spun. Of the two that did not shake, one found and exited through the original rupture and the other formed a second rupture and exited through that.

Electron microscopy

We found HGCs highly concentrated on the snout of 5 day old embryos (Fig. 3A,B). HGCs have short microvilli and appear recessed among common epithelial cells (Fig. 3C,D). HGCs were most abundant in the area between the nares, above the mouth, but

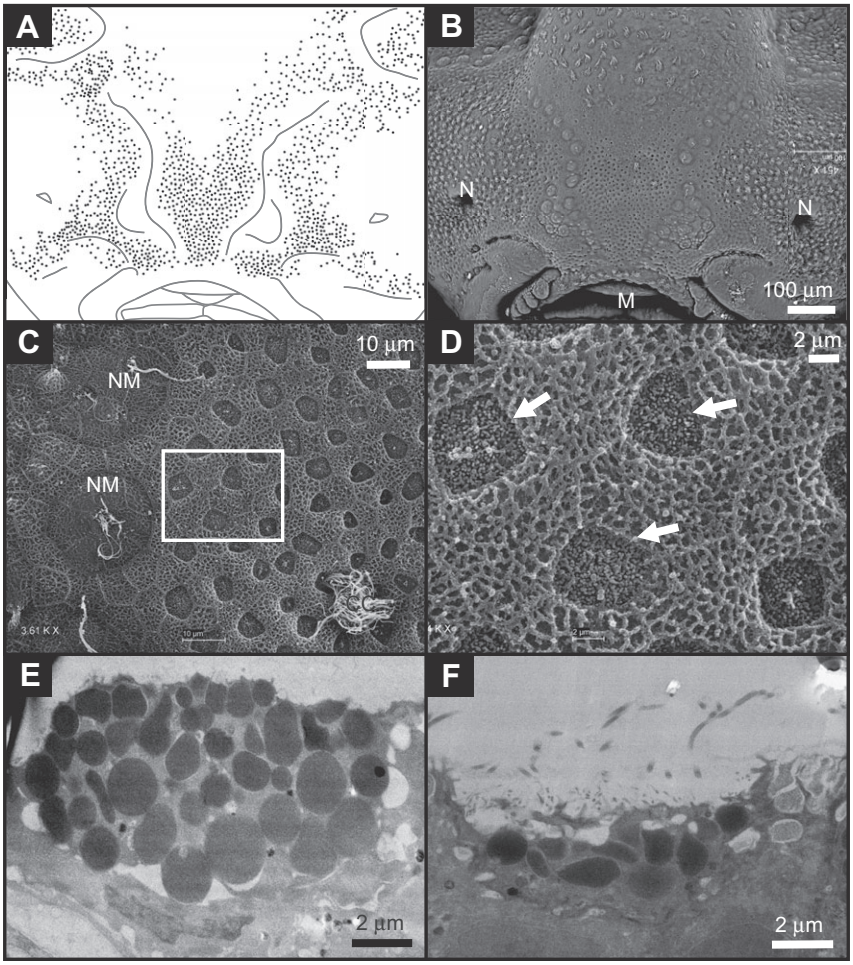

Fig. 3. Hatching gland cells in *A. callidryas*. Distribution of hatching gland cells (HGCs) on the snout of a 5 day old embryo in (A) schematic representation, as black dots and (B) the corresponding scanning electron micrograph (N, nares; M, mouth). (C) HGCs appear recessed between common epithelial cells (NM, neuromast; CC, ciliated cell). The boxed region is enlarged in D. (D) Close-up of HGCs (arrows) among common epithelial cells. Cross-sections of HGCs (E) full of secretory vesicles in an unhatched 6 day old embryo and (F) largely emptied of vesicles in a 6 day hatchling preserved seconds after hatching.

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also extended around the nares and above the eyes. We did not observe HGCs on the dorsal surface of the head, along the dorsal midline, or on the tail. Prior to hatching, the HGCs were full of secretory vesicles close to the surface of the cell (Fig. 3E). In embryos anesthetized seconds after hatching and preserved within minutes, the HGs had few secretory vesicles (Fig. 3F). We did not observe any unhatched samples with HGCs that appeared to have already secreted a portion of their vesicles, nor did we observe any full, intact HGCs in hatchlings. Ciliated and epithelial cells did not differ in morphology between individuals preserved before and after hatching (not shown).

DISCUSSION

Hatching in *A. callidryas* is a very rapid, flexibly regulated process. At any point during the last third of the typical undisturbed embryonic period, embryos can hatch within seconds in response to a physical disturbance, enabling them to escape from predator attacks. We found hatching to be a three-stage process including stereotyped pre-rupture behavior, rapid enzyme release and localized membrane rupture, and muscular exit through the rupture site. Rapid hatching appears to be enabled by a novel HGC distribution, highly concentrated on the snout, and by rapid, bulk release of hatching enzyme.

Pre-rupture behavior

We observed two common behaviors shortly before membrane rupture, shaking and mouth gaping. Of these, the most frequently expressed and distinctive was low-amplitude lateral shaking. Shaking was visible at all video speeds, and expressed by most hatching embryos, but has not been observed in videos of non-hatching embryos (Hughey et al., 2015; Rogge and Warkentin, 2008). Our data do not address the incidence of shaking behavior in spontaneous hatching. However, in the context of cued hatching, our displacement experiment showed that shaking was a reliable indicator of initiation of the hatching process and temporally associated with enzyme release. Thus, we can use shaking behavior as an indicator of the decision to hatch, or start of the hatching process. Mouth gaping behavior was also common, but not essential for hatching. Although shaking did not occur in all cases, and thus is not essential for hatching to occur, this behavior could function to press the snout against the membrane, thereby reducing dispersal of released hatching enzyme through the egg. As HGs are densely concentrated on the snout, mouth gaping might facilitate enzyme release by squeezing or stretching the epithelial layer of cells. Alternatively, because some hatchlings gape to fill their lungs with air as soon as their snout penetrates the egg capsule, mouth gaping may simply be an air-breathing behavior that is also expressed, without function, at earlier stages of the hatching process.

Hatching-related behaviors have also been observed in other frogs. Just prior to hatching, *Bufo vulgaris formosus* embryos stop moving and adhere to the already softened membrane, pressing against it. As they are not yet capable of muscular movement, their ciliary currents play an important role in hatching (Kobayashi, 1954). *Xenopus laevis* embryos sink to the bottom of the egg and rotate every 10–15 min, which Bles (1905) hypothesized to spread hatching enzyme on the membrane. Some teleost fish embryos have a period of rapid, intense movement as they approach hatching, but stop moving just prior to hatching, during release of hatching enzyme and localized digestion of the egg envelope (reviewed in Korwin-Kossakowski, 2012). In contrast, California grunion embryos increase their activity after receiving a hatching stimulus (Speer-Blank and Martin, 2004). This behavior may function to distribute hatching enzyme along the inside of the egg, as this species has HGs along the lateral surface of the body. In all of these cases, the behavior of the embryo has been hypothesized to either spread or concentrate hatching enzyme on the membrane.

Localized, enzymatic rupture of the membrane

Our prior hypothesis that *A. callidryas* use vigorous movement to rupture the egg membrane (Rogge and Warkentin, 2008) is incorrect, as may be the similar hypothesis for *P. trinitatus* (Downie et al., 2013; Nokhbatolfoghahai and Downie, 2007). Our high-speed video revealed that most *A. callidryas* form a small, localized rupture in the membrane before beginning the vigorous movements that propel them from the egg (Table 1, Fig. 1). Indeed, in a few videos, the rupture formed apparently without contact between embryo and membrane at the rupture site. In our high-speed videos, we observed some embryos thrashing when they lost the rupture site. While thrashing visibly stretched the membrane, it was not sufficient to cause additional rupture. We conclude that embryos do not rupture the membrane through physical force; they must do so chemically. Our embryo displacement experiment supports that enzyme release occurs locally at the snout during shaking behavior. When embryos were displaced from their original position seconds after shaking began, fluid subsequently leaked from where their snout was located during the initial shaking.

In fishes, contact between the HGs and egg envelope facilitates hatching; examination of eggs has shown rupture at locally digested sites, and digestion occurs faster with direct contact than when enzyme is diluted in perivitelline fluid (reviewed in Korwin-Kossakowski, 2012). The same comparison has not been made in a frog, and the few species investigated vary in their need for contact with the membrane. In two ranid species, culture media containing secretions from decapsulated embryos are capable of digesting vitelline membranes of other eggs (Cooper, 1936; Katagiri, 1973). However, in *X. laevis*, contact between the embryo and the vitelline membrane is important for hatching; Bles (1905) found that when eggs were rotated so embryos lost contact with the membrane, their hatching was delayed for hours until they were returned to their normal position. Although direct contact between *A. callidryas* embryos and the membrane does not appear essential for rupture, it is usual. Moreover, the orientation of the embryo, and its ability to maintain a consistent position through the hatching process, is clearly important for the speed of escape from the egg. In high-speed videos, some embryos turned within the capsule after initiating hatching and were therefore not positioned properly to exit through their rupture site. Experimental displacements also resulted in membrane collapse, with embryos becoming trapped for several minutes (Fig. 2). Indeed, even slight displacements of the snout, in failed displacement control trials, could result in membrane collapse and slower hatching. Nonetheless, embryos still had some remaining enzyme to secrete; most displaced embryos formed a second rupture in the membrane, through which they exited. Together, these observations support the hypothesis of rapid and highly localized membrane degradation, and suggest that it depends on acute release of enzyme from the snout.

HG distribution and function

Our electron microscopy revealed a dense concentration of HGCs in a very small area on the snout (Fig. 3A,B), and an absence of such cells elsewhere on the head and body. Such tight packing of HGCs enables highly localized enzyme release, concentrating membrane degradation in a very small area. We observed HGCs to be acutely, but only partially, emptied of their contents when embryos were

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stimulated to hatch (Fig. 3E,F). The rapid release of enzyme is likely critical for rapid membrane rupture and escape. However, the small reserve of enzyme that remained in cells could be available for the formation of a second rupture, should the initial hatching attempt fail. It is also possible that not all the HGCs empty at once. We did not observe full HGCs remaining in hatched individuals; however, the proportion of cells we could image with scanning transmission electron microscopy was small.

While the precise distribution of HGCs varies among previously studied anurans, a comparative examination of 20 species described HGs to be primarily on the dorsal anterior of the head and in most cases extending some distance along the dorsal midline (Nokhbatolfoghahai and Downie, 2007). The extreme concentration of HGs on the snout in *A. callidryas* contrasts notably with this pattern and appears functionally important for their rapid hatching process. Additionally, the acute timing of enzyme release contrasts with other anurans for which the hatching mechanism has been investigated in detail. *Xenopus laevis* and *B. japonicus* both secrete hatching enzyme gradually, as it is synthesized (Yamasaki et al., 1990; Yoshizaki, 1991), but both species hatch slowly and lack documented adaptive plasticity in hatching timing. Nonetheless, slower hatching mechanisms can also be regulated in response to chronic threats, such as dehydration or pathogens, or cues to predation risk level (Warkentin, 2011b). For instance American toads, *Anaxyrus americanus*, accelerate hatching to escape water mold before they are capable of muscular movement (Gomez-Mestre et al., 2008a; Touchon et al., 2006); this species likely shares a hatching mechanism with better-studied bufonids. Other phyllomedusines can also hatch rapidly from their terrestrial eggs (e.g. *Agalychnis annae*, *A. moreletti*, *Pachymedusa dacnicolor*, *Cruziohyla calcarifer*; Gomez-Mestre et al., 2008b) and may share the rapid hatching mechanism of *A. callidryas*. Clades that have independently evolved terrestrial eggs and rapid escape-hatching [e.g. glassfrogs, Centrolenidae (J. Delia, personal communication), or *Limnocetes arathooni* (Brown and Iskandar, 2000)] may also have densely localized HGs and acute enzyme release, or they may have evolved alternative mechanisms with similar function. Indeed, the mechanism of hatching in most anurans remains to be investigated.

Exit from the capsule

From our videos, it was clear that when an embryo maintained its position in the egg, its snout moved into the membrane rupture as or soon after it formed, blocking the release of perivitelline fluid. Turgor pressure was thus maintained inside the egg as the embryo squeezed its body through the tiny rupture site. *Xenopus laevis* also appear to use turgor pressure to exit the egg (Bles, 1905), but *A. callidryas* contrasts with observations in some other hylids [*H. avivoca* (Volpe et al., 1961); *Dendropsophus ebraccatus* (K.L.C. and K.M.W., unpublished data)] whose embryos slide out through relatively large holes under little or no pressure. In *A. callidryas*, maintaining position to immediately plug the rupture with the snout seems important for the timing of exit; even a minor displacement of the embryo prevents this and leads to membrane collapse, which delays escape from the egg. A 40 s hatching delay (Fig. 2D), although trivial in many contexts, can be lethal while a snake is consuming an egg mass. Thus, the embryo's behavior and position in the egg are crucial for rapid hatching to escape from predators. As early as the neural tube stage, *A. callidryas* orient their head toward the air-exposed side of their egg capsule, rather than the side attached to the leaf (Rogge and Warkentin, 2008). This behavior aids oxygen uptake and, at later stages, facilitates rapid escape from egg capsules under attack. Unlike anurans, some fishes hatch tail-first instead of head-first; the orientation of exit usually depends on the location of HGs on the embryo, and hatching head-first generally enables faster exit from the egg capsule (reviewed in Korwin-Kossakowski, 2012). However, the importance of embryo positioning may vary, depending on the time course of hatching enzyme release, the distribution of HGs, embryo behavior, and egg structure or environment.

In the final stage of hatching, *A. callidryas* embryos used thrashing movements, similar to swimming, to propel themselves from the egg capsule. In most cases, this took only a fraction of a second; however, the delay between rupture formation and the start of thrashing or, in some cases, temporary cessations of thrashing contributed substantially to the total duration of hatching (Table 1, Fig. 1). Thus, overall hatching speed depends strongly on embryo behavior, which may vary across hatching stimuli. In addition, our analysis focused on 5 day old embryos, which are premature but quite well developed. However, the effectiveness of thrashing for rapid exit from a ruptured capsule might vary with embryo size and axial muscle development; younger embryos might take longer or rely more on turgor pressure.

Speed of hatching

Hatching of *A. callidryas* took from 6 to 50 s (Table 1), consistent with the speed of hatching during snake attacks (Warkentin et al., 2007). This is substantially faster than in previously studied amphibians, which take from 25 min (Noble, 1926), to 6 h (Carroll and Hedrick, 1974), to half their embryonic period (Yoshizaki, 1978; see Introduction). For video recordings, we tried to provide a minimal disturbance cue to induce hatching without obscuring our view, but under more intense stimulation in attacks, *A. callidryas* probably achieve even faster hatching. In particular, the delay between membrane rupture and the onset of thrashing motions, which propel embryos from their capsule, accounted for about half the measured duration and need not occur at all (Table 1). Rapid, cued hatching exists in other anurans (see Introduction and above), but fast hatching in other anuran species has not been measured or investigated in the detail presented in our study.

Remarkable examples of cued hatching have also been described in some fishes and invertebrates. For instance, the California grunion, *Leuresthes tenuis*, is a beach-spawning fish with terrestrially incubated embryos that hatch in response to inundation and wave action. In contrast to most other fishes, which take 30–60 min to hatch after releasing enzyme (reviewed in Martin et al., 2011), grunion embryos hatch in under a minute after receiving the environmental cue and, following rupture, 90% of embryos emerge from the egg in under 2 s (Griem and Martin, 2000; Speer-Blank and Martin, 2004). The hatching process in grunion is in some ways similar to what we have observed in *A. callidryas*; a fluid leak initially occurs at a small rupture site. However, in contrast to *A. callidryas*, the chorion then rips open more broadly as the grunion embryo pushes at it (Speer-Blank and Martin, 2004). Some parasitic marine flatworms also hatch extremely rapidly, in about 2 s, in response to cues from their highly mobile hosts (Whittington and Kearn, 2011). Thus, across taxa, rapid hatching mechanisms enable embryos to deploy effective cued-hatching responses both to escape from sudden-onset, severe threats and to take advantage of transient abiotic and biotic opportunities.

Conclusions

The general model of anuran hatching is a fundamentally developmental process, with gradual degradation of the membrane beginning long before membrane rupture and the embryo's exit from the egg (e.g. Altig and McDiarmid, 1999; Carroll and Hedrick, 1974; Nokhbatolfoghahai and Downie, 2007).

The hatching process in *A. callidryas* is different, and its distinctive features enable embryos to remain within or rapidly escape from the egg, and its predators, over a broad developmental period. This species illustrates that the effective performance and precise timing of anuran hatching can depend critically on embryo behavior, and behavior combines with HG distribution and function to affect the speed of hatching. Moreover, synchronized release of stored hatching enzyme must also require coordination of HGC physiology. Our new understanding of *A. callidryas*' hatching mechanism suggests that hatching performance and the risk of potential hatching complications may change developmentally, for instance as enzyme reserves accumulate or musculature develops. It could also vary contextually, for instance if physical disturbance of eggs impairs the place-holding ability of embryos. Moreover, both factors might alter optimal embryo hatching decisions if risk cues are ambiguous (Warkentin and Caldwell, 2009). Thus, this species offers an opportunity to examine how the ability to hatch and the cost of early hatching change and interact over development.

The mechanisms regulating hatching timing in fishes have been relatively well studied, partly due to the importance of hatching synchronization in commercial hatcheries (Korwin-Kossakowski, 2012). However, prior mechanistic investigations of hatching in anurans have not examined what enables embryos to alter its timing in response to environmental cues. Environmentally cued hatching responses have been documented across 10 anuran families, in response to diverse environmental factors, such as hypoxia, drying, predators, pathogens and parents (Warkentin, 2011b). It seems likely that different anuran lineages have also evolved diverse mechanisms of hatching and its regulation, adapted to their reproductive mode and the ecological threats and opportunities facing embryos and larvae.

Acknowledgements
We thank Bill Wcislo for access to the high-speed camera and laboratory facilities, Jorge Ceballos and Gloria Vargas for assistance with electron microscopy, Camden Rouben, Jonathan Suh and Rebecca Wier for assistance with video analyses, and members of the Gamboa Frog Group at the Smithsonian Tropical Research Institute and the Egg Science Reading Group at Boston University for discussion of the research and comments on the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
K.M.W., M.A.S. and K.L.C. designed the study. K.M.W. and M.A.S. recorded the high-speed video and K.M.W. analyzed it. M.A.S. and K.L.C. developed techniques for imaging hatching gland cells. K.L.C. performed the embryo displacement experiment and video analysis and the electron microscopy. K.L.C. and K.M.W. did the statistical analysis and wrote the paper.

Funding
This research was funded by the National Science Foundation (DEB-0716923 and IOS-1354072), Boston University, the Smithsonian Tropical Research Institute (STRI), and an F. H. Levinson grant to STRI's Laboratory of Behavioral and Evolutionary Neurobiology (W. T. Wcislo PI). K.L.C. was supported by an Ernst Mayr short-term fellowship from STRI.

Data availability
All datasets are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.cj030

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.139519/-/DC1

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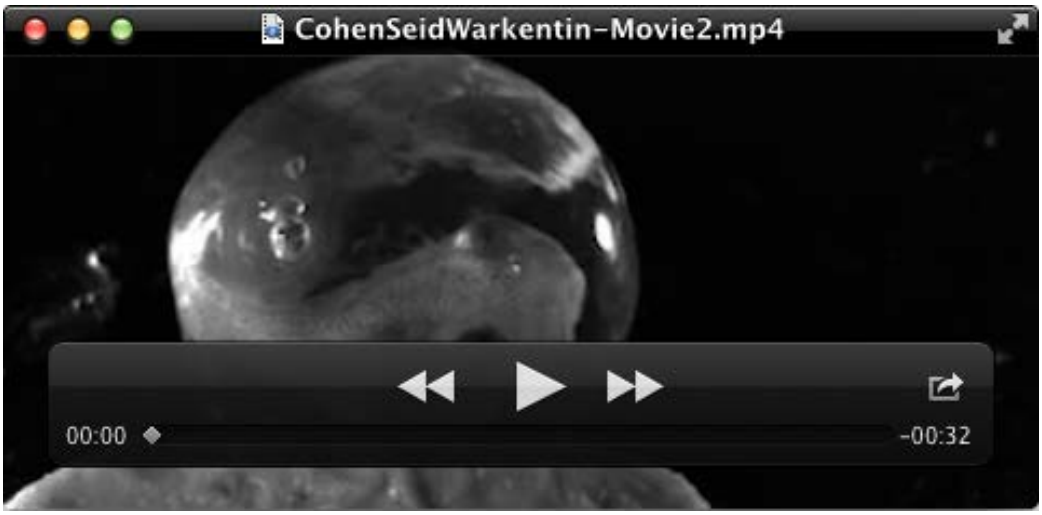
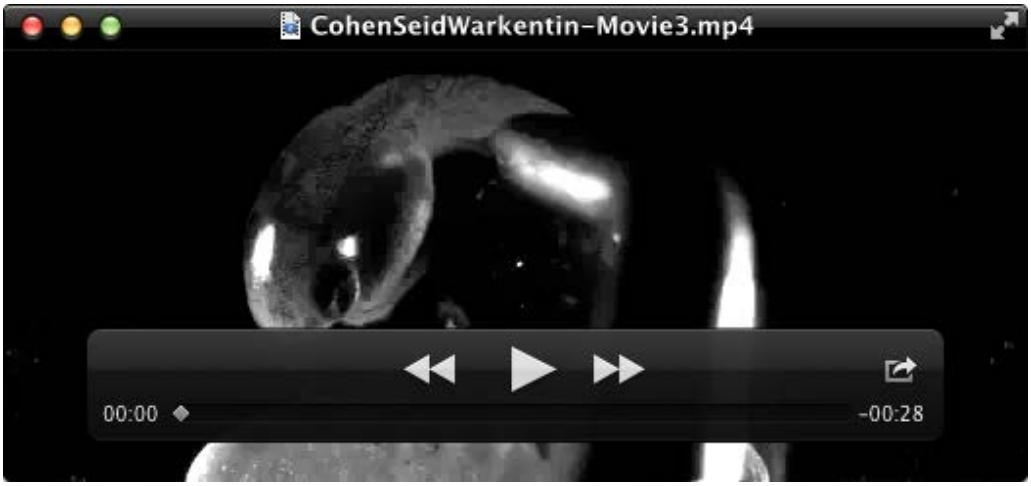
Movie 1. Experimental rotation of *Agalychnis callidryas* embryo during hatching. The embryo is stimulated to hatch using a moistened, blunt probe. After it shakes for several seconds, the embryo is rotated to a new position. A fluid leak forms at the original location of the embryo's snout, and the egg capsule shrinks slightly, indicating loss of turgor, but does not collapse completely; its tail may block further leakage. The embryo then digests a second hole in the membrane and exits through it. The embryo is 5 days old; video was recorded and plays in real time.



Movie 2. *Agalychnis callidryas* hatching. The embryo shakes briefly and gapes, without pressing its snout against the membrane, then a leak forms. The embryo passively slides forward so its snout protrudes, thrashes briefly so its head emerges, pauses, and thrashes again to complete its, exit. The embryo is 5 days old; video was recorded at 200 Hz and plays here in real time.



Movie 3. *Agalychnis callidryas* hatching. The embryo shakes continuously, pressing its snout against the membrane and gaping once. The snout protrudes slightly, a small amount of fluid leaks, and the snout protrudes further. The embryo continues shaking, pushing forward then, after one eye is free, amplifies its motions to exit in burst of continuous thrashing. The embryo is 5 days old; video was recorded at 200 Hz and plays here at 100 Hz (half-speed).