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# External gills and adaptive embryo behavior facilitate synchronous development and hatching plasticity under respiratory constraint

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## SUMMARY

**Plasticity in hatching timing allows embryos to balance egg- and larval-stage risks, and depends on the ability of hatching-competent embryos to continue developing in the egg. Hypoxia can slow development, kill embryos and induce premature hatching. For terrestrial eggs of red-eyed treefrogs, the embryonic period can extend ~50% longer than development to hatching competence, and development is synchronous across perivitelline oxygen levels ($P_{O_2}$) ranging from 0.5–16.5 kPa. Embryos maintain large external gills until hatching, then gills regress rapidly. We assessed the respiratory value of external gills using gill manipulations and closed-system respirometry. Embryos without external gills were oxygen limited in air and hatched at an external $P_{O_2}$ of 17 kPa, whereas embryos with gills regulated their metabolism and remained in the egg at substantially lower $P_{O_2}$. By contrast, tadpoles gained no respiratory benefit from external gills. We videotaped behavior and manipulated embryos to test if they position gills near the air-exposed portion of the egg surface, where $P_{O_2}$ is highest. Active embryos remained stationary for minutes in gills-at-surface positions. After manipulations and spontaneous movements that positioned gills in the $O_2$-poor region of the egg, however, they returned their gills to the air-exposed surface within seconds. Even neural tube stage embryos, capable only of ciliary rotation, positioned their developing head in the region of highest $P_{O_2}$. Such behavior may be critical both to delay hatching after hatching competence and to obtain sufficient oxygen for normal, synchronous development at earlier stages.**

Key words: embryo behavior, gills, hatching, hypoxia, phenotypic plasticity, respiration.

## INTRODUCTION

Egg envelopes, such as shells, capsules and egg cases, both protect developing embryos and impose constraints on their microenvironment, including diffusion barriers to oxygen. Development is an aerobic process, and embryonic oxygen demand increases as yolk is converted to metabolically active tissue. Thus hypoxia can slow development, kill eggs, and select for traits that improve the oxygen supply to embryos (Bradford and Seymour, 1988; Booth, 1995; Cohen and Strathmann, 1996; Seymour et al., 2000). Both parental traits and egg mass features that improve egg oxygenation are well documented [e.g. egg ventilation (Fernández et al., 2000), clutch size and shape (Strathmann and Strathmann, 1995), convection channels (Pinder and Friet, 1994)]. Embryo traits could also improve oxygen uptake ability. Here, we assess the contribution to respiration of embryo behavior and the external gills of embryos and hatchlings of red-eyed treefrogs.

Hatching frees animals from the diffusion barrier of the egg and can be induced prematurely by hypoxia in many taxa (Petranka et al., 1982; Warkentin, 2007). In some species, hatching is both advanced by hypoxia and delayed by hyperoxia, suggesting that it occurs at a respiratory threshold (Latham and Just, 1989). While solving a physiological problem, hatching earlier may, however, create an ecological one. More mature hatchlings typically have greater sensory development and/or locomotor abilities (Fuiman, 2002), both of which can reduce mortality from predation (Sih and Moore, 1993; Warkentin, 1999a; Gomez-Mestre et al., 2008). If predators or other factors in the post-hatching environment impose selection for later hatching, then mechanisms to extend embryonic development will be favored (Warkentin, 2007). For embryos under respiratory constraint these include traits that improve oxygen supply or oxygen uptake capacity.

Anurans have four respiratory surfaces: skin, lungs, external gills and internal gills. Their external gills are transient embryonic structures that are, in many species, little more than short externalized capillary loops of equivocal functional value (Burggren and Just, 1992). Moreover, some amphibian and fish embryos develop normally without convective oxygen transport, suggesting that diffusion is sufficient to supply their oxygen needs (Flores and Frieden, 1969; Pelster and Burggren, 1996; Territo and Burggren, 1998). In other anurans, both morphological elaboration of the external gills and environmentally regulated gill regression suggest that these structures serve an important function (del Pino and Escobar, 1981; Channing, 1993).

The behavior of embryos and fetuses has been viewed primarily from a developmental perspective (Smotherman and Robinson, 1996). For instance, embryo movements play a role in neuromuscular and skeletal development (Robinson et al., 2000; Pitsillides, 2006). Embryo movements have seldom been examined for immediate utility, and functional roles for embryo behavior are documented in few contexts, mostly late in embryonic development [e.g. environmentally cued hatching (Warkentin and Caldwell, in press); care solicitation vocalizations (Brua, 2002)]. Behavior may, however, have immediate utility at earlier stages (Goldberg et al., 2008).

### Study organism and hypotheses

Red-eyed treefrogs, *Agalychnis callidryas* (Cope), attach their eggs to vegetation over ponds and swamps, and tadpoles fall into the water upon hatching. The embryos hatch rapidly, up to 30% before

their modal spontaneous hatching age, in response to egg-stage threats, including egg-eating snakes and wasps, pathogenic fungus and submergence underwater, which drowns eggs too young to hatch (Warkentin, 2007). Early hatched tadpoles are developmentally premature (Warkentin, 1999b), and more vulnerable to aquatic predators than are full-term hatchlings (Warkentin, 1995; Warkentin, 1999a). Embryos hatch by performing movements that rupture the egg capsule and propel them from it. This behavior is cued by vibrations in snake attacks (Warkentin, 2005) and by hypoxia in flooding (Warkentin, 2002).

Red-eyed treefrog eggs are large (~5 mm diameter when hatching-competent) and closely packed in gelatinous clutches, so each egg is only partly exposed to air. The perivitelline oxygen level (partial pressure; $P_{O_2}$) at the center of eggs varies substantially, and embryos develop synchronously at $P_{O_2}$ from 0.5–16.5 kPa. Moreover, hatching-competent embryos tolerate $P_{O_2}$ as low as 0.5 kPa without hatching; such hypoxia can slow development, induce hatching and kill embryos of other anurans with terrestrial eggs (Warkentin et al., 2005).

We hypothesize that the ability of *A. callidryas* to maintain rapid embryonic development and delay hatching despite very low $P_{O_2}$ depends on four things: (1) retention of external gills until hatching; (2) spatial variation in $P_{O_2}$ within eggs, with at least a small well-oxygenated region; (3) functional contribution of external gills to oxygen uptake; and (4) behavioral positioning of external gills in the well-oxygenated region of the egg. The first two have already been demonstrated; we briefly review the evidence. We then measure the value of *A. callidryas*' external gills for oxygen uptake and assess how embryos behaviorally position their gills.

The timing of gill regression in *A. callidryas* is plastic. It normally occurs immediately after hatching, regardless of hatching age; young hatchlings regress the gills rapidly but even the oldest embryos retain them (Warkentin, 2000b). Gill regression depends more on oxygen availability than on hatching *per se*. Embryos under hyperoxia or in eggs removed from the clutch to increase surface exposure regress the gills, whereas tadpoles in hypoxic water without access to air retain them (Warkentin, 2000b; Warkentin, 2002). Moreover, embryos induced to regress their external gills, under temporary hyperoxia or prostaglandin treatment, hatch rapidly from egg clutches in air (Warkentin, 2002).

Measurements of $P_{O_2}$ at two locations within 4-day-old *A. callidryas* eggs showed strong gradients, despite constant ciliary circulation of the perivitelline fluid. $P_{O_2}$ was 12.4±0.8 kPa just underneath the air-exposed surface, compared with 3.4±0.3 kPa deep inside the eggs (mean ± s.e.m. here and throughout, $N$=30) (Warkentin et al., 2005). Here we repeat those measurements with younger eggs (2 days old) to assess developmental change in the gradient.

The external gills of *A. callidryas* are long (~25% of total length), branched structures with great positional flexibility (Warkentin, 1999b). Hatching-competent embryos curl within the egg so most of their skin faces the hypoxic egg interior or is pressed against parts of the egg capsule adjacent to other eggs or their leaf substrate. By contrast, the gills offer a spatially flexible gas exchange surface that could be positioned adjacent to the air-exposed egg surface. We assess the contribution of gills to oxygen uptake by comparing metabolic rates of embryos and tadpoles with and without external gills across different oxygen levels. We quantify natural patterns of embryo position and movement, and embryo responses to experimental repositioning, to assess if embryos actively position their gills near the air-exposed egg surface.

## MATERIALS AND METHODS

### Animal collection, care and staging

We collected young *A. callidryas* egg clutches, with the leaves on which they were laid, from breeding ponds near Gamboa, Panama, and maintained them under natural temperatures and ambient high humidity in an open-air laboratory in Gamboa. Leaves were taped to plastic cards for support and clutches misted frequently with rainwater to maintain hydration. Tadpoles were returned to their collection sites after experiments. Because most aspects of development are synchronous within and among egg clutches at a site (Warkentin, 1995; Warkentin, 1999b), we use embryonic age as a proxy for developmental stage; plasticity in gill regression makes standard staging tables misleading for hatching-age animals. For detailed discussions of development, age and staging of *A. callidryas* see (Warkentin, 1999b; Warkentin, 2002; Warkentin, 2007).

This research was conducted between June and August 2006 and 2007 under permits from the Smithsonian Tropical Research Institute and the Panamanian National Authority for the Environment (Autoridad Nacional del Ambiente), and approved by the Animal Care and Use Committee of Boston University.

### Respirometry and manipulations of gill regression

We used closed system respirometry to measure metabolic rates of individual *A. callidryas* embryos and hatchlings with and without external gills across a range of oxygen levels. Measurements were conducted in airtight glass respirometry chambers (Unisense, Aartius, Denmark). We used a 0.41 ml chamber for measurements of embryos in air, and a larger chamber for measurements of tadpoles in aged tap water (2.09 ml water + tadpole volume). A glass-covered magnetic stirrer in the larger chamber, separated from the tadpole by stainless steel mesh supported on a glass ring, continuously mixed the water. The combination of stirring speed (240 r.p.m.) and mesh size (0.4 mm) was chosen to ensure complete mixing within the chamber based on dye visualization, and to not disturb tadpoles in pilot tests. For all measurements, we sealed the capillary tube probe port and the lid to the chamber with water. Seal effectiveness was verified by a pilot experiment in which the $P_{O_2}$ of degassed, hypoxic water in the chamber remained constant for 2 h.

We used an optical oxygen sensor (Microx TX2, Precision Sensing; Regensburg, Germany) with a fiberoptic microprobe (optode) to measure the partial pressure of oxygen ($P_{O_2}$) within the chamber. The optode does not consume oxygen. It was mounted in a needle with the tip slightly extruded. $P_{O_2}$ was sampled once per second and recorded on a laptop computer. The probe was calibrated daily before measurements in water-saturated air and anoxic water formulated with $NaSO_3$. The high point calibration was checked between animals and both calibration points were rechecked at the end of each day. Optode drift was never over 3%, thus we did not correct for it. Oxygen measurements were automatically temperature-compensated by the sensor using a thermal probe touching the respirometry chamber. Temperatures ranged from 25 to 28°C.

To assess bacterial oxygen consumption we conducted five blank trials of 2 h each, under each of the two measurement conditions (0.41 ml respirometry chamber filled with air, 2.09 ml respirometry chamber filled with aged tap water). Bacterial $O_2$ consumption was undetectable in air. We used the average bacterial $O_2$ consumption in aged tap water (0.044±0.019 μmol $O_2$ $h^{-1}$) to correct measurements of tadpole oxygen consumption.

We measured $P_{O_2}$ over time as animals consumed oxygen in four treatments: embryos with and without external gills and newly hatched tadpoles (hatchlings) with and without external gills ($N$=11

per treatment). All animals were 5 days old and of similar size. Their opercula had formed, yolk sacs were streamlined but large and still undivided into gut coils, and beaks had begun to keratinize. We only used clutches that were healthy, with no evidence of predator contact or pathogen infection, and only eggs that were developing in synchrony with their siblings and other eggs of the same age. No siblings were included in the same treatment, and each individual was measured only once.

We ran pilot experiments to determine pre-test rearing conditions to induce or prevent gill regression. Gill condition was assessed at ×30 magnification under a dissecting microscope. To measure respiration of a gilled embryo, we separated a single egg from its clutch and placed it in the center of the floor of the respirometry chamber. Eggs thus had ~75% air-exposed surface, near the high end of exposures recorded in clutches (Warkentin et al., 2005). The chamber was sealed and $P_{O_2}$ of the air around the embryo recorded until the embryo hatched. Embryos were observed continuously throughout the experiment (9.17±0.66 h per embryo) and retained easily visible, well perfused external gills that extended past the end of their yolk sac (≥1.9 mm). When the embryo hatched, the $P_{O_2}$ at hatching was recorded, and the mass of the egg and embryo were determined.

To induce gill regression we reared embryos in an oxygen-enriched environment. Individual 3-day-old eggs were separated from their clutches and suspended on 3 mm diameter hexagonal nylon netting, fully exposing the egg surface except for a narrow strip occluded by the nylon filament (Warkentin, 2000b). The suspended embryos were placed in a container with a constant flow of a humidified 40% oxygen, 60% nitrogen mixture. Measurements of $P_{O_2}$ at the center of such eggs at 4 days ranged from 17.4 to 17.9 kPa (17.7±0.001 kPa, $N$=7), substantially higher than the 0.5–9.0 kPa (4.0±0.3 kPa, $N$=41) recorded from 4-day eggs in clutches (Warkentin et al., 2005). On the morning when embryos were five days old, individual eggs were placed in the respirometry chamber. The chamber was partially flushed with 40% $O_2$ gas (starting $P_{O_2}$: 32.6±1.1 kPa) and then sealed. $P_{O_2}$ was recorded until the embryo hatched, then hatching $P_{O_2}$ was recorded, the mass of the egg and embryo determined, and external gills examined. Final gill length was 0.08±0.05 mm; eight of 11 animals had no visible gills. Experiment durations were 9.64±0.75 h, similar to those with gilled embryos (Wilcoxon rank-sum test: $z$=0.296, $P$=0.77).

For measurements of gilled tadpoles we induced individual embryos to hatch directly into the larger chamber, which was filled with air-saturated aged tap water. We sealed the chamber immediately, excluding air before hatchlings could fill their lungs. For measurements of tadpoles without gills, we induced gill loss in embryos as above, then induced these animals to hatch into the water-filled chamber. Trials ran until the $P_{O_2}$ in the water surrounding the tadpole reached approximately 0.7 kPa (1.78±0.05 h), at which point the mass of the tadpole was determined and the external gills examined. All gilled tadpoles retained external gills (1.9±0.1 mm), and no animals induced to regress gills had visible gill remains.

### Analysis of respirometry data

We calculated the rate of oxygen consumption ($\dot{M}_{O_2}$; as a measure of metabolic rate) at different $P_{O_2}$ over 5 min and 30 min periods in the tadpole and embryo trials, respectively (~1 kPa decrease in both cases). To smooth noise in optode readings, we calculated $\dot{M}_{O_2}$ from the difference between $P_{O_2}$ averaged over 30 s at the start and end of each period. We used average $P_{O_2}$ over the entire period as a measure of the oxygen level corresponding with each $\dot{M}_{O_2}$ value. To determine the volume of medium (air or water) in the chamber from which $O_2$ was consumed, we subtracted individually estimated egg or tadpole volume from the chamber capacity. For eggs, we measured the diameter of five eggs near the test individual in its clutch, and used the average to estimate volume based on spherical geometry. For tadpoles, we used the wet mass of the experimental individual and the average density of tadpoles, based on the wet mass and volume of a sample of 5-day hatchlings with unfilled lungs ($N$=40 tadpoles from five clutches).

We calculated the critical oxygen level ($P_{crit}$), below which animals could no longer regulate their metabolic rate, for each individual following the method of Yeager and Ultsch (Yeager and Ultsch, 1989), as implemented in a MatLab script written by E. Dzialowski. $P_{crit}$ was determined as the break point between two linear regressions of $\dot{M}_{O_2}$ on $P_{O_2}$, minimizing $r^2$ for the regression above $P_{crit}$. We calculated unconstrained $\dot{M}_{O_2}$ for each animal by averaging $\dot{M}_{O_2}$ across all data points above $P_{crit}$. For animals with at least four data points below $P_{crit}$, we also calculated the slope of that regression. Data were analyzed in STATA v.9.0. We tested for effects of hatching, gill regression and their interaction on $P_{crit}$ and the slope of the regression below $P_{crit}$ with ANOVAs, and on unconstrained $\dot{M}_{O_2}$ with ANCOVA, with wet mass as a covariate. Hatching $P_{O_2}$ was heteroscedastic and therefore tested nonparametrically.

### Measurement of perivitelline $P_{O_2}$ gradient

We measured perivitelline $P_{O_2}$ at two locations within each of 23 2-day-old eggs, from five clutches (three to five eggs each): just under the air-exposed egg surface and deep within the egg, between the center of the egg and the far wall, touching neither embryo nor egg capsule. Embryos were in early Gosner stage 18 (muscular response) (Gosner, 1960). Measurements were made using the Microx TX2 and a needle-mounted probe following the method of Warkentin et al. (Warkentin et al., 2005).

### Manipulation of embryo position

To test the hypothesis that embryos actively maintain their gills near the air-exposed egg surface we manipulated embryo position and monitored subsequent behaviors. We selected 10 eggs of intermediate surface exposure in each of five clutches at three ages: 3 days (Gosner stage 22, tail fin circulation), 2 days (stage 18, muscular response) and 1 day (stage 16, neural tube). All eggs were fully surrounded by other eggs in the vertical plane of the clutch and adhered at the back to a leaf, thus exposed to air only at the front. All embryos used started with their external gills (3 days) or developing head (1 and 2 days) facing the air-exposed portion of the egg. We haphazardly assigned five eggs per clutch to each treatment. Using a blunt probe we carefully turned each embryo within its egg capsule. Test embryos ($N$=25/age) were positioned with their external gills or developing head in the oxygen-poor region at the back of the egg. Controls ($N$=25/age) were manipulated in a similar manner, but positioned with their external gills or developing head at the air-exposed surface. We recorded all behaviors of each manipulated embryo. We watched each 3-day embryo until it returned its gills to the surface, stopping after 60 s post-manipulation for those that did not move. We watched each 2-day embryo for 120 s. For 1-day embryos, whose only movement is slow ciliary rotation in the horizontal plane, we drew their position in dorsal view at 60 s intervals for 5 min then measured their angular rotation with reference to the exposed–deep axis of the egg and the covered and exposed egg surfaces. Temperatures during experiments were 28.6±2.1°C.

Analyses were conducted in STATA v.9.0. For 2- and 3-day eggs, we tested the effect of position on 'time-to-move' with ANOVA. We conservatively assigned time watched as time-to-move for control embryos that did not move in tests, and time to return gills to the surface as time-to-move for experimental embryos that took multiple movements to accomplish this. Neither clutch nor a clutch-by-position interaction were significant in either case, nor did they improve model fit as indicated by the Akaike Information Criterion (AIC). At 1 day, angular rotation of embryos was intractably heteroscedastic. Based on the lack of clutch effects for older eggs, we report Wilcoxon rank-sum test results, pooling eggs across clutches. Paired sign tests on clutch average values are also significant. For time-to-move we assigned 6 min for 1-day embryos that did not move in the 5 min observation period.

### Videotape analysis of embryo behavior

To quantify embryo behavior in relation to oxygen gradients within eggs we videotaped ten 3-day and ten 5-day *A. callidryas* clutches for 1 h each. After positioning each clutch vertically in front of the camera we left embryos undisturbed for 5 min before recording. Temperatures during recordings were 31.7±0.2°C. We estimated the proportional surface exposure of each egg in the field of view based on egg geometry (Warkentin et al., 2005). Rogge and Warkentin initially independently estimated exposure, and results were fully consistent. We analyzed the behavior of four embryos per clutch: the two with the least and the two with the most air-exposed surface in the field of view (27±2% and 67±2% exposed, respectively). We only used eggs for which we could see the entire air-exposed surface. We counted all gross muscular movements during the 1 h recording for each embryo, classifying each behavior as either a twitch (muscular contraction with no change in position) or a move (muscular contraction with position change). The final position after each move was scored on the basis of gill location: either 'surface' if at least some part of one gill was visible at the air-exposed surface or 'deep' if no part of either gill was at the exposed surface. To determine if the time embryos spend in a position depends on their gill location, we used the time trace on the videotape to record the time each embryo spent in up to six positions (following different moves): three surface and three deep positions. Individual positions sampled were at least 10 min apart except in a few cases where the embryo made three or fewer moves to a position type.

Data analyses were carried out in SAS v.9.1.2. We tested for effects of age, surface exposure, and their interaction on embryo activity (twitches + moves in 60 min) using ANOVA. Similarly, we tested for these effects on the number of moves to a deep position, relative to all position changes, assuming a binomial distribution. To assess effects of age, exposure, gill location and their interactions on the time spent in a position we used a repeated measures ANOVA, because individual embryos were sampled multiple times. For graphical presentation of these data we present means and s.e.m. across eggs, first averaging duration in each position type for individual eggs. The effect of clutch, nested within age, was

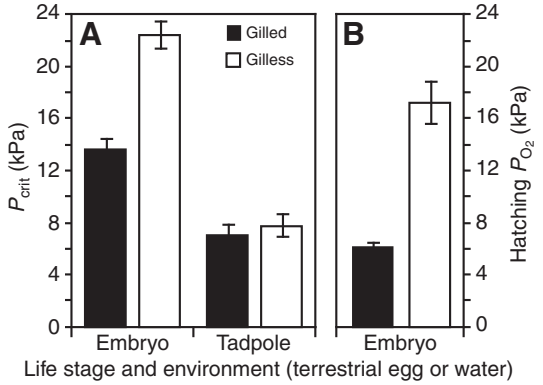


Fig. 2. Effect of external gills on oxygen levels that (A) limited metabolic rate in red-eyed treefrog embryos and tadpoles and (B) induced hatching, measured using closed-system respirometry. External gills improved oxygen uptake for embryos in terrestrial eggs, lowering both $P_{crit}$ and the $P_{O_2}$ that induced hatching. Embryos without gills were oxygen limited even in air (~20.5 kPa). External gills had no effect on the $P_{crit}$ of hatched tadpoles in water. Data are means ± s.e.m.; $N$=11 animals per treatment.

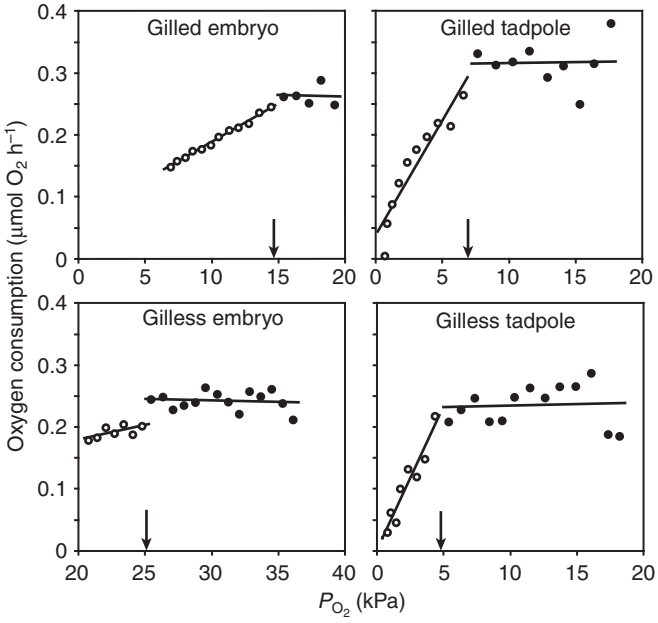


Fig. 1. Examples of oxygen consumption rates measured at different oxygen levels for individual 5-day-old embryos and newly hatched tadpoles of red-eyed treefrogs with and without external gills. Embryo measurements stopped at hatching. Lines show regression fits used to estimate $P_{crit}$ (arrows); data points above and below $P_{crit}$ are indicated by filled and open circles, respectively. Note that the *x*-axis location for the gilless embryo is shifted relative to that for the other animals.

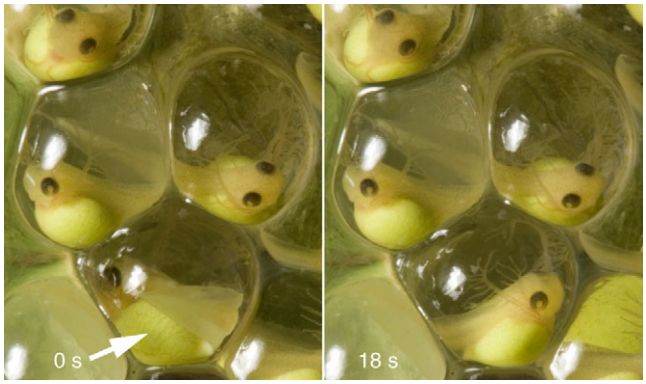


Fig. 3. Red-eyed treefrog embryo rapidly moves to reposition itself with external gills in the well-oxygenated region near the air-exposed egg surface, after experimental manipulation to position gills in the hypoxic rear of the egg. Embryos are 3 days old and egg diameter is ~4.9 mm; the arrow marks the experimental individual. Photos by K.M.W.

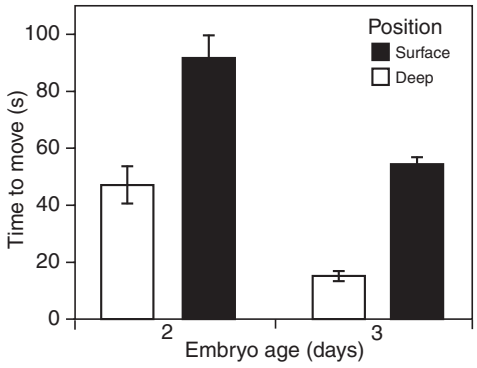


Fig. 4. Time required for red-eyed treefrog embryos to reposition gills or head near air-exposed egg surface after experimental manipulation turning animals to face hypoxic rear of egg, and time until the next movement for controls manipulated similarly but positioned facing air-exposed egg surface. Embryos were watched until they moved or for 120 s (2 days old) or 60 s (3 days old). Many control embryos did not move; time watched was used as time to move for these animals. Data are means ± s.e.m.; *N*=25 embryos per treatment per age.

significant only for the repeated measures analysis. It was not included in other final models, where its effect was nonsignificant, since based on AIC it did not improve model fit.

## RESULTS

### Respirometry

All embryo and hatchling measurements included a sufficient range of oxygen levels to estimate $P_{crit}$ (examples in Fig. 1). At the end of experiments, wet masses of embryos (13.7±0.6 mg) were slightly less than those of tadpoles (15.2±0.4 mg; $F_{1,40}$=4.25, $P$=0.046) but gilled and gilless animals did not differ in mass ($F_{1,40}$=1.84, $P$=0.18). At $P_{O_2}$ above individual $P_{crit}$ values, oxygen consumption increased with wet mass (ANCOVA: $F_{1,38}$=17.39, $P$=0.0002), but we found no evidence that hatching, gill regression or their interaction affected unconstrained metabolic rate (whole animal $\dot{M}_{O_2}$=0.261±0.006 µmol $O_2$ $h^{-1}$, $N$=44; mass-specific $\dot{M}_{O_2}$= 18.46±0.39 µmol $O_2$ $h^{-1}$ $g^{-1}$, $N$=43; all $F_{1,38}\leq$2.7, all $P\geq$0.11; mass data are lacking for one embryo).

There was a significant hatching-by-gill regression interaction effect on $P_{crit}$ ($F_{1,40}$=19.5, $P$=0.0001). For embryos, $P_{crit}$ was lower with external gills than without them, whereas for tadpoles gill regression did not change $P_{crit}$ (Fig. 2A; Tukey's *post-hoc* tests, embryo $P$=0.0002, tadpole $P$=0.89). Hatching also decreased $P_{crit}$ for animals both with and without external gills (Fig. 2A; Tukey's *post-hoc* tests, both $P$=0.0002). There was no effect of gill regression, or its interaction with hatching, on the slope of the regression of $\dot{M}_{O_2}$ on $P_{O_2}$ below $P_{crit}$ (both $F_{1,36}\leq$0.9, both $P\geq$0.35), but the slope was steeper for tadpoles than for embryos (tadpoles: 0.038±0.003 µmol $h^{-1}$ $kPa^{-1}$, $N$=22; embryos: 0.015±0.003, $N$=18; $F_{1,36}$=36.9, $P$<0.0001; Fig. 1). Embryos without external gills hatched at a higher $P_{O_2}$ than did embryos with gills (Fig. 2B; Wilcoxon rank-sum test, $z$=3.98, $P$=0.0001). Embryos without external gills also tolerated less of a drop in $P_{O_2}$ below $P_{crit}$, before hatching, than did embryos with gills (Fig. 2; 4.6±0.7 *vs* 7.5±0.7 kPa; Wilcoxon rank-sum test, $z$=–2.40, $P$=0.017).

### Oxygen gradient within eggs

As with 4-day-old eggs (Warkentin et al., 2005), there was an oxygen gradient in 2-day-old eggs (Sign test: $P$<0.0001). In all 2-day eggs measured, the surface $P_{O_2}$ was higher than the $P_{O_2}$ deeper within the egg. The difference was 7.0±0.7 kPa (range 1.1–16.2 kPa); interior $P_{O_2}$ averaged 6.9±0.6 kPa, whereas surface $P_{O_2}$ was 14.0±0.7 kPa.

### Embryo response to position manipulation

All embryos capable of muscular response returned their external gills or head to the well oxygenated part of the egg, near the air-exposed surface, soon after we positioned their gills deep inside the egg (Fig. 3). Experimental animals moved sooner than did controls with gills positioned at the air-exposed egg surface (3-day embryos, $F_{1,48}$=163.9, $P$<0.0001; 2-day embryos $F_{1,48}$=18.9, $P$=0.0001; Fig. 4). All 3-day experimental embryos rapidly returned their gills to the surface, on average in 15±2 s, while only 24% of controls moved within 60 s. All 2-day experimental embryos, newly capable of muscular response, returned their gills to the surface in 47±7 s, while only 44% of controls moved within 120 s.

All experimental embryos at the neural tube stage, before developing gills and capable only of ciliary rotation, rotated their developing heads toward the air-exposed surface (Fig. 5). They were more likely to move, moved sooner, and moved farther than control embryos (Wilcoxon rank-sum tests: moved at all, $z$=–4.802, $P$<0.0001; time-to-move, $z$=5.386, $P$<0.0001; angle moved, $z$=–6.1, $P$<0.0001; Fig. 6). On average they moved 83±7 deg. in 5 min; eight embryos by then had their heads fully exposed at the egg-air interface, six were partially exposed, and 11 still next to the egg-covered surface. By contrast only 36% of controls moved; average rotation was 6±2 deg.

### Videotaped embryo behavior

Comparing overall activity level, 3-day embryos moved more frequently than 5-day embryos, but neither surface exposure nor its

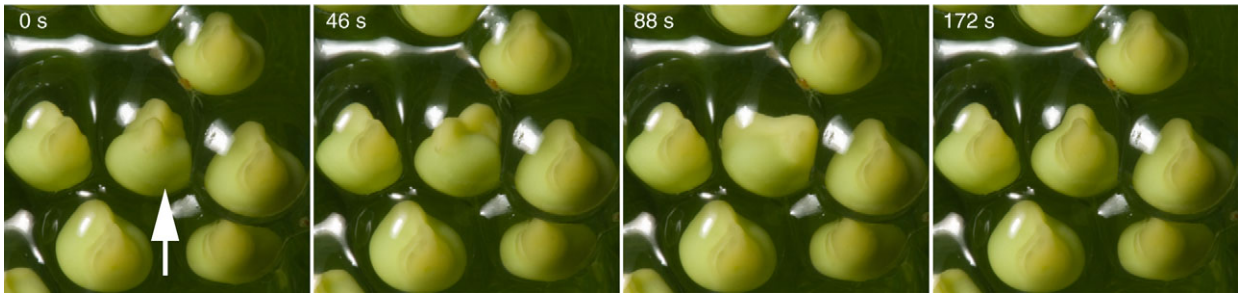


Fig. 5. Red-eyed treefrog embryo uses ciliary rotation to return its developing head to the well-oxygenated region under the air-exposed egg surface, after experimental manipulation to position it facing the hypoxic rear of egg. Embryos are 1 day old and ~3.2 mm long; the arrow marks the experimental individual. Photos by K.M.W.

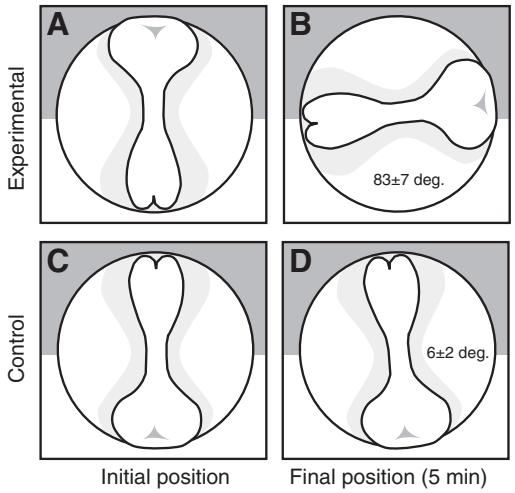


Fig. 6. Angular rotation of 1-day-old (neural tube stage) embryos of red-eyed treefrogs in the 5 min after moving them to different initial positions within the egg. Grey background indicates egg surface covered by other eggs and leaf substrate; white background represents air exposure and zone of higher perivitelline oxygen levels. Experimental embryos positioned with their developing head at the hypoxic rear of the egg moved it toward the air-exposed surface (A,B), whereas controls positioned with their developing head at the front of the egg remained there (C,D). Data are means ± s.e.m.; *N*=25 embryos per treatment.

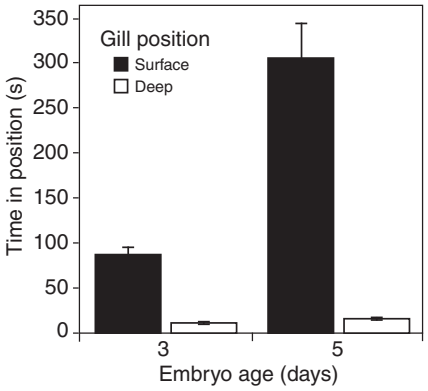


Fig. 7. Effects of embryo development (age) and position of external gills in the oxygen gradient within eggs on the time red-eyed treefrog embryos remained in a position, after spontaneous movements. Deep positions within the egg are hypoxic, and positions near the air-exposed surface are better oxygenated. No embryos remained long with their gills in hypoxic regions of the egg, and younger embryos were more active. Data were taken from videotapes (means ± s.e.m., *N*=40 embryos per age).

interaction with age affected activity (Table 1). Embryos with little air-exposed egg surface made relatively more movements that positioned their gills away from that surface, compared with embryos in highly exposed eggs (Table 1; 26±2% and 12±2% of movements, respectively). Neither age, nor its interaction with exposure affected the relative number of movements to deep and surface positions (Table 1). The amount of time embryos spent in a position was affected by age, gill location, and an age-by-gill location interaction, as well as by clutch, nested within age (Table 1). Younger embryos spent less time in surface positions than did older embryos, but neither stayed long in deep positions (Fig. 7).

## DISCUSSION

Red-eyed treefrog embryos reach hatching competence in 4–5 days, depending on locality. If undisturbed, they typically remain in the egg ~50% longer, developing substantially and, thereby, increasing their chances of surviving as tadpoles with aquatic predators (Warkentin, 1995; Warkentin, 1999b). Among amphibians, this species has relatively large eggs and develops at warm temperatures; consistent with this, *A. callidryas* embryos have high metabolic rates [for a review, see Seymour and Bradford (Seymour and Bradford, 1995)]. These embryos maintain rapid development and refrain from hatching at perivitelline $P_{O_2}$ as low as 0.5 kPa, and develop synchronously across a broad range of $P_{O_2}$ (Warkentin et al., 2005), even though hypoxia in natural flooding or experimentally manipulated gas mixtures causes hatching or death (Pyburn, 1970; Warkentin, 2000a; Warkentin, 2002). How can we reconcile these apparently conflicting facts? We propose that the concentrated,

Table 1. Analysis of the effects of development (age), egg surface exposure to air, and position of external gills in relation to air-exposed surface on the behavior of embryos of red-eyed treefrogs, *Agalychnis callidryas*, videotaped in natural clutches

| Variable | Effect | d.f. | *F* | *P* |
|---|---|---|---|---|
| Activity* | Age† | 1, 18 | 67.46 | <0.0001 |
| | Air exposure‡ | 1, 58 | 1.76 | 0.19 |
| | Age × exposure | 1, 58 | 2.05 | 0.16 |
| Moves to deep position§ | Age | 1, 18 | 1.31 | 0.25 |
| | Air exposure | 1, 58 | 27.95 | <0.0001 |
| | Age × exposure | 1, 58 | 0.04 | 0.84 |
| Position duration¶ | Age | 1, 106 | 26.80 | <0.0001 |
| | Air exposure | 1, 106 | 0.59 | 0.44 |
| | Gill position | 1, 106 | 123.82 | <0.0001 |
| | Age × position | 1, 106 | 41.31 | <0.0001 |
| | Exposure × position | 1, 106 | 0.01 | 0.93 |
| | Clutch (age) | 18, 106 | 4.39 | <0.0001 |

*Total number of flexions plus position changes in 1 h; ANOVA results.
†Eggs were videotaped at ages 3 and 5 days; they become hatching competent at 4 days.
‡Low, 27±2%; high, 67±2% of egg surface exposed to air.
§Number of position changes after which neither gill was visible at the air-exposed egg surface, relative to all position changes; general linear model, assuming binomial distribution.
¶Total time embryo remained in the same location within egg, regardless of flexion within position; repeated-measures ANOVA.

spatially flexible gas exchange surface of the external gills, strong oxygen gradients within individual eggs, and oxygen-sensitive embryo behavior allow terrestrial *A. callidryas* embryos to exploit a spatial refuge from hypoxia, and that these traits may be critical for both normal development and hatching plasticity.

### External gills in embryonic oxygen uptake and delayed hatching

The external gills of *A. callidryas* embryos appear to confer a substantial oxygen uptake benefit. Embryos with external gills were able to regulate their metabolism at external $P_{O_2}$ well below the $P_{crit}$ of gilless embryos (Fig. 2), and indeed below the $P_{crit}$ of some smaller, cooler anuran eggs [e.g. *Pseudophryne bibroni*, 2.8 mm diameter eggs, hatching stage $\dot{V}_{O_2}$ 1.05 µl h$^{-1}$ (Seymour and Bradford, 1987; Seymour and Bradford, 1995) and $P_{crit}$ 19 kPa (Seymour et al., 1991) at 12°C, *vs A. callidryas* 4.9 mm, 5.85 µl h$^{-1}$ and 13.4 kPa at 25–28°C].

Age-matched externally gilled and gilless *A. callidryas* were indistinguishable in size, non-branchial aspects of gross morphology, and oxygen uptake under conditions of ample supply. Thus there is no evidence that rearing conditions affected their oxygen requirements. It is possible that the oxygen-enriched rearing conditions designed to induce gill regression also affected other respiratory traits, such as hematocrit or blood chemistry. We did not assess these variables, thus cannot be sure gill regression was the only factor affecting oxygen uptake ability. Nonetheless, the dramatic difference in $P_{crit}$ between gilled and gilless embryos is consistent with a major role of these structures in embryonic respiration. The rapid hatching of *A. callidryas* following rapid, prostaglandin-induced external gill regression (Warkentin, 2002) is also consistent with a respiratory role of the gills. Indeed, the fact that induced hatching begins under 20 min after prostaglandin treatment limits the possibility that other respiratory traits that change more slowly also contribute to the hatching induction.

The metabolic rate of embryos without external gills in eggs with ~75% of their surface exposed for gas exchange was oxygen limited even in air (Fig. 2). This surface exposure is near the highest we have observed in natural clutches (80%), and most eggs are substantially less exposed (Warkentin et al., 2005). Were such gilless embryos to occur in nature, they would be oxygen limited, and we would expect variation in surface exposure to generate variation in development rates. By contrast, externally gilled embryos in eggs with as little as 15% air-exposed surface develop in synchrony with their highly exposed clutchmates (Warkentin et al., 2005). Their rapid, synchronous development appears to depend on the external gills.

For both gilled and gilless embryos, the $P_{O_2}$ that induced hatching was well below $P_{crit}$ (Fig. 2), indicating that embryos tolerated some metabolic constraint within the egg. Consistent with this tolerance, in natural clutches, embryos with very low air exposure can develop – albeit more slowly than their clutchmates – and hatch late but not necessarily developmentally premature (K.M.W., personal observation). Tolerance of metabolic constraint by hatching-competent embryos suggests that, at least in the absence of other cues indicating egg-stage risk, a more advanced developmental stage at hatching is prioritized over maximizing development rate *per se*.

More extreme hypoxia can, however, kill embryos, for instance when clutches are submerged in pond water (Pyburn, 1970). Under such conditions, premature hatching is clearly a better option, and embryos hatch. The level of hypoxia that embryos tolerate without hatching differs more between gilled and gilless animals than does their $P_{crit}$ (Fig. 2). Gilless, hatching-competent embryos remain in only a small subset of eggs in natural clutches (Warkentin, 2002), suggesting that most eggs are sufficiently hypoxic to put gilless embryos beyond their tolerance for metabolic constraint. Although we do not know the safety margin that embryos allow between lethal hypoxia and hatching, eggs lacking any air-exposed surface typically fail to develop and die (K.M.W., personal observation). Poorly exposed eggs that support normal development of gilled embryos may also be sufficiently hypoxic to kill gilless embryos. Thus the presence and function of embryonic gills will affect the range of clutch structures that support normal development, as well as the balance between water loss and oxygen exchange in a terrestrial environment (Strathmann and Strathmann, 1989; Strathmann and Hess, 1999).

### Tadpole respiration and rapid gill regression upon hatching

We detected no effect of external gills on the oxygen uptake of tadpoles in water without access to air. Moreover, the unconstrained metabolic rate of tadpoles did not differ from that of developmentally matched embryos. Consistent with results from other anamniotes (Barrionuevo and Burggren, 1999), $P_{crit}$ decreased upon hatching. The indistinguishable $P_{crit}$ of gilled and gilless tadpoles also suggests that non-branchial respiratory traits are unlikely to contribute substantially to the higher $O_2$ uptake capacity of gilled embryos, compared with gilless embryos. If such were the case, we would expect this superior capacity to also be evident in tadpoles.

Because neither embryos nor tadpoles in our experiment had access to air, the key difference in their respiratory environment was the spatial distribution of oxygen. Tadpoles were in well-mixed water, in which all respiratory surfaces – skin, internal gills and external gills – would be exposed to similar $P_{O_2}$. Embryos were exposed to oxygen gradients in the egg. Although the single eggs in our experiment were highly exposed, at most half the embryo's skin faced the air-exposed surface, with the rest facing the hypoxic egg interior or the portion of egg capsule against the impermeable glass bottom of the container. We tested *A. callidryas* early in the period of hatching competence, when their internal gills are poorly developed (Warkentin, 1999b). Thus most gas exchange presumably occurred *via* skin or external gills. Cutaneous respiration, although insufficient for *A. callidryas* within terrestrial eggs, therefore appears adequate to support unconstrained metabolism of hatchling tadpoles in even moderately hypoxic water (Fig. 2). This sufficiency of cutaneous gas exchange for tadpoles is consistent with the rapid regression of external gills that normally occurs upon hatching (Warkentin, 2000b).

Tadpoles hatched into very hypoxic water retain their external gills (Warkentin, 2000b). Since the tadpole gills alter neither $P_{crit}$ nor the slope of the $\dot{M}_{O_2}/P_{O_2}$ relationship below $P_{crit}$, under experimental conditions gill retention would confer no metabolic benefit. It might simply reflect mechanisms of gill retention more relevant within the egg. Alternatively, facultative retention of external gills might benefit hatchlings in hypoxic ponds, which often hang with gills near the air-water interface (K.M.W., personal observation). Similar behavior and facultative external gill retention has been reported for other anuran tadpoles [*Hypsiboas* (*Hyla*) *rosenbergi* (Kluge, 1981), *Stephopaedes anotis* (Channing, 1993)].

### Adaptive embryo behavior

The positions of *A. callidryas* embryos are highly non-random. Most of the time embryos have their external gills positioned near the air-exposed egg surface, where oxygen is highest. They may not specifically shape movements to position their gills near air; they

often end up with gills in other places, particularly in eggs with low air-exposure. However, embryos that come to rest with their gills in a high-oxygen region remain in that position for a relatively long period (Fig. 7). By contrast after experimental repositioning or spontaneous movements that place their gills in a low-oxygen region embryos move again within seconds. Indeed, the time that active embryos remained in such positions was remarkably consistent across ages and contexts (10±1 s for 3-day embryos after spontaneous movements, 15±2 s for 3-day embryos after experimental repositioning, and 15±1 s for 5-day embryos after spontaneous movements). This may reflect the time necessary for oxygen gradients, based on flow patterns of perivitelline fluid, to re-establish after the turbulence caused by embryo movements, and for embryos to either assess oxygen levels at their gills or begin to feel a constraint on $O_2$ uptake.

Considering the efficacy of cutaneous respiration even in moderately hypoxic water and its inadequacy in the egg, and the fact that external gills do not improve $O_2$ uptake in well-mixed water, it is unlikely that these gills would contribute much to embryonic respiration if they were not positioned in high-oxygen regions of the egg. Thus appropriate embryo behavior is necessary for the gills to confer a respiratory benefit, and appears critical both for rapid, synchronous development and for delayed hatching, after hatching competence. Indeed, the initial response of *A. callidryas* embryos to oxygen stress is position change; under hypoxia embryos change position within the egg many times at short intervals before hatching (J.R.R. and K.M.W., personal observations). These movements are like the gill repositioning movements and distinct from hatching movements (Warkentin et al., 2007).

Even before the development of gills that allow spatially focused oxygen uptake, and a circulatory system that distributes oxygen to tissues, the oxygen demand of different parts of the embryo varies. For instance, yolk is relatively inert and developing nervous tissue is more metabolically active. Embryos newly capable of muscular response (Gosner stage 18) took longer to turn 180 deg. than did more developed, active embryos, but nonetheless rapidly returned their developing head to the air-exposed region of their egg after displacement. The earliest we could rotate embryos within the egg was stage 16 (neural tube), shortly after the onset of ciliary rotation at stage 15. After displacement these early embryos, without heart, brain, blood or gills, rotated within the egg to bring their developing head back to the best-oxygenated region. We measured substantial oxygen gradients within eggs at early stage 18 (the onset of muscular response), and they probably exist before this. Local oxygen levels affect tissue metabolism, thus we hypothesize that developmental benefits accrue from positioning the developing head in a region of higher oxygen. If this is correct, the ciliary rotation of embryos to face the air-exposed surface represents an adaptive behavioral response to environmental variation. Pond snail embryos also respond behaviorally to hypoxia, increasing ciliary rotation and repositioning themselves within the oxygen gradient inside their eggs (Kuang et al., 2002; Goldberg et al., 2008). This behavior is mediated by the first neurons to develop in the embryo (Kuang et al., 2002).

The behavior of embryos is known to play a role in neuromuscular and skeletal development in preparation for functions that occur later in life, after hatching or birth (Colman and Lichtman, 1993; Pitsillides, 2006). Particularly at early developmental stages, this is the predominant functional framework for investigations of embryo behavior. Research on adaptive plasticity in hatching has demonstrated that embryo behavior, and appropriate responses to environmental cues, can be also of immediate importance for survival (Warkentin, 2007; Gomez-Mestre et al., 2008; Warkentin and Caldwell, in press). Similarly, behavioral interactions of bird embryos, shortly before hatching, with parents and siblings appear to have immediate functions in care solicitation and hatching synchronization (Brua, 2002). The behavioral responses of *A. callidryas* embryos to oxygen gradients within the egg, like the ciliary spinning of pond snail embryos under hypoxia (Goldberg et al., 2008), represent apparently adaptive behavioral responses to environmental variation *in ovo* at much earlier developmental stages.

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