Hatching Timing, Oxygen Availability, and External Gill Regression in the Tree Frog, *Agalychnis callidryas*

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ABSTRACT

The physiological role of the embryonic external gills in anurans is equivocal. In some species, diffusion alone is clearly sufficient to supply oxygen throughout the embryonic period. In others, morphological elaboration and environmental regulation of the external gills suggest functional importance. Since oxygen stress is a common trigger of hatching, I examined the relationships among hatching timing, oxygen stress, and external gill loss. I worked with the red-eyed tree frog, *Agalychnis callidryas*, a species with arboreal eggs and aquatic tadpoles in which gill regression is associated with hatching, and hatching timing affects posthatching survival with aquatic predators. Both exposure to a hypoxic gas mixture and submergence in water, a natural context in which hypoxic stress can occur, induced early hatching. Exposure to hyperoxic gas mixtures induced regression of external gills, and subsequent exposure to air induced early hatching. Prostaglandin-induced external gill regression also induced hatching, and this effect was partially ameliorated by exposure to hyperoxic gas. Together, these results suggest that external gills enhance the oxygen uptake of embryos and are necessary to extend embryonic development past the onset of hatching competence.

Introduction

Gills are a defining feature of amphibian larvae. The long-lasting external gills of salamanders and internal gills of anurans have been relatively well studied and are physiologically important (reviewed in Burggren and Just 1992). The external gills of anurans have received less attention. These are ostensibly embryonic structures that are lost soon after hatching, and their physiological role is equivocal (Burggren and Just 1992).

Some amphibian and fish embryos can develop to hatching, and beyond, with simple diffusion as their only gas exchange mechanism. For instance, cardiac lethal mutants of the salamander *Ambystoma mexicanum* survive until several days after hatching without blood circulation (Humphrey 1972). Functional ablation of hemoglobin by CO in the fish *Danio rerio* and the frog *Xenopus laevis* does not impair development or metabolic rate in embryos or early larvae (Pelster and Burggren 1996; Territo and Burggren 1998), suggesting that gills, as well, are unnecessary for these animals. However, these experiments were conducted in well-oxygenated water. Under even moderate hypoxia, most premetamorphic stages of *X. laevis* are oxygen conformers, reducing metabolic rate. The exception is a brief period of oxygen regulation shortly after hatching, when external gills are present and P_{crit}, the point where oxygen level begins to limit metabolism, drops to less than half that of earlier and later stages (Hastings and Burggren 1995). Many amphibian embryos and larvae can experience hypoxic conditions in nature (e.g., Seymour and Roberts 1991; Pinder and Friet 1994; Seymour et al. 1995), and anuran external gills may be functionally important in this context. Furthermore, embryos of *Ambystoma* and *Xenopus* have relatively small, simple gills. Some anurans have much larger, more elaborate external gills, and in at least a few species, their regression is environmentally regulated (Pyburn 1963; del Pino and Escobar 1981; Kluge 1981; Channing 1993; Seymour and Loveridge 1994; Warkentin 2000*a*). Both morphological elaboration and environmental regulation suggest physiological importance.

Here, I test whether external gills facilitate delayed hatching in anuran embryos (i.e., extend the period of embryonic development beyond the point when hatching could first occur). Hatching stage is ecologically important; later hatching can improve larval survival because larger, more developed hatchlings are better able to avoid and evade predators (Petranka et al. 1987; Sih and Moore 1993; Parichy and Kaplan 1995; Warkentin 1995, 1999*a*). Oxygen stress can accelerate hatching in amphibians, fish, and invertebrates and may be a common natural trigger of hatching (DiMichele and Taylor 1980; Petranka et al. 1982; Bradford and Seymour 1988; Miller 1992; Mills and Barnhart 1999; Seymour et al. 2000; but see Griem and Martin 2000 for a different trigger). If external gills enhance oxygen uptake, they may extend the period of embryonic development before oxygen stress stimulates hatching.

I worked with embryos of the red-eyed tree frog, *Agalychnis*

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callidryas, chosen for their large external gills and variable hatching stage (Pyburn 1963; Warkentin 1995, 1999*b*). This species attaches clumps of eggs to vegetation overhanging ponds and swamps. The eggs are thus incubated in air, and tadpoles fall into the water upon hatching. Aerial incubation is obligate because eggs too young to hatch will die if submerged, probably due to respiratory stress (Pyburn 1970; K. M. Warkentin, personal observation). Hatching releases embryos from the diffusion barrier of the egg capsule and surrounding clutch and allows direct exposure of skin and gills to water and lungs to air; hatchlings normally fill their lungs within a few minutes of hatching.

The structure of *A. callidryas* clutches changes with development. Typical clutch dimensions are 2–3 × 3–4 cm. Newly laid clutches are roughly hemi-circular in cross section, hemioval in long section, with on average 40 2.25-mm-diameter eggs embedded in a gelatinous matrix attached to a leaf (Pyburn 1963; Warkentin 2000*a*). As the eggs develop, the perivitelline space swells, and the gelatinous egg capsule shrinks. Well-hydrated eggs at age 5 d average 5.2 mm in diameter (± 0.1 mm SE; $N = 10$ clutches; range of individual eggs 4.1–6.1 mm; K. M. Warkentin, unpublished data) and are essentially not separated by jelly. Most hatchable eggs are partly exposed to air; eggs in the interior of a clutch have about 25% of their surface exposed, while peripheral eggs can be up to 50% exposed (Warkentin 2000*a*).

Within populations, embryonic development rate is very consistent. In Panama, where this study was conducted, embryos become capable of hatching at age 4 d, but in undisturbed egg clutches, most embryos delay hatching until age 6 d (Warkentin 2000*b*). Under natural conditions, *A. callidryas* embryos maintain large, fully perfused external gills until hatching, after which the gills regress rapidly. In embryos hatched into normoxic water at a normal stage of development (6 d after oviposition in Panama; 7 d after oviposition in Costa Rica; Warkentin 1995, 2000*b*), blood flow through the external gills ceases and the gills regress completely within a few hours. Over 60% of the loss of length and 90% of the reduction in perfusion occurs in the first 10 min posthatching (Warkentin 2000*a*). In embryos induced to hatch 2 d earlier, which have smaller lungs and less developed internal gills than normal stage hatchlings, posthatching gill regression can take 1 d. Hatchlings also maintain their gills if hatched into hypoxic water and denied access to air. In contrast, separating eggs to maximize the exposed surface area available for gas exchange induces gill regression in embryos (Warkentin 2000*a*).

To assess the role of external gills in extending embryonic development (i.e., delaying hatching), I conducted a series of experiments. The first experiments examine the relationship between oxygen stress and hatching in *A. callidryas* and test critical methods for subsequent experiments. A natural context in which oxygen stress induces hatching is the flooding of terrestrial eggs (DiMichele and Taylor 1980; Petranka et al. 1982; Bradford and Seymour 1988). I therefore determined whether submergence in pond water stimulates hatching in *A. callidryas*. I also used gas manipulations to test whether hypoxia would induce hatching and to establish whether hyperoxia could be used to induce gill regression in embryos. I then tested whether external gill regression induces hatching using two independent methods to induce gill loss: temporary hyperoxia and prostaglandin treatment. Finally, if the effect of prostaglandin on hatching is due to oxygen stress caused by gill regression, the effect should be weaker in a hyperoxic environment. Therefore, I tested whether hyperoxia reduced the effect of prostaglandin on hatching.

Material and Methods

Collection, Care, and Developmental Staging of Embryos

I collected young egg clutches (age ≤ 3 d) and the leaves on which they were attached from an *Agalychnis callidryas* breeding site 2 km south of Gamboa, Panama, and brought them to an open-air laboratory at the Smithsonian Tropical Research Institute in Gamboa. Any dead or damaged eggs were removed, excess leaf area around the clutch was removed, and each clutch was hung in an individual container over aged tap water approximately 1 cm deep. Containers were covered with fine screening to exclude insects, placed in the shade, and misted with rainwater daily before the start of experiments. They were exposed to ambient temperatures (daily highs: $29.3° \pm 0.2°$C; lows: $25.9° \pm 0.1°$C; $N = 40$ d) and ambient high humidity (unmeasured).

Because standard staging tables (e.g., Gosner 1960) use gill regression as a marker of development, they are not useful for hatching-competent *A. callidryas* embryos or recently hatched tadpoles. Instead, to indicate stage of development, I refer to the age of embryos in days and the time of day. Most clutches are laid between 10:00 P.M. and 2:00 A.M. Within a population, under ambient conditions, development is quite consistent within and among *A. callidryas* clutches. Briefly, in my Panamanian study population, embryos pass through the cleavage stages and begin gastrulation in their first day. By the morning of their second day (age 1 d, 8:00 A.M.), they are in the yolk plug stage. That afternoon, they form a neural tube, and they reach the tail bud stage in the evening. Muscular response develops in the afternoon at age 2 d. By that evening, gill circulation is evident (stage 20, Gosner 1960), but gill branches are still very short. At age 3 d, gill branches are much longer, and melanophores have begun to appear. By 8:00 A.M. at age 4 d, tail circulation is present, an opercular fold has formed, and large external gills are still present on both sides (stage 23, Gosner 1960). Many embryos are competent to hatch, if sufficiently disturbed; the rest become competent later in the day. At age 5 d, iridophores are evident in the iris and over the heart. The first furrow in the yolk sac develops in the evening at age 5 d. At age 6 d, 1–1.5 gut coils are externally visible.

The bulk of spontaneous hatching occurs in the evening at age 6 d, some in the evening at age 7 d, and a few embryos remain unhatched until age 8 d, still in Gosner stage 23 (Warkentin 2000*b*). For more detailed descriptions of morphological change through the plastic hatching period, see Warkentin (1999*b*).

Oxygen Manipulations

Embryos were exposed to experimental gas mixtures in 400-mL glass jars with metal lids sealed with vacuum grease. Each jar lid was provided with two glass tubes (inlet and outlet) sealed with silicone into holes in the lid. Experimental jars were connected in a single series with Nalgene tubing; control jars were similarly connected in a separate series. Connecting jars in parallel was not feasible due to gas supply limitations. Cylinders of experimental gas mixtures (7%, 35%, and 42% oxygen, balanced with nitrogen) were purchased from Acetioxigeno in Panama City. Air (21% oxygen, 78% nitrogen, 1% other gases) was pumped with a Whisper 1000 aquarium pump (Second Nature, Blacksburg, Va.). All gas mixtures, including air, were first bubbled through an air stone in a jar of rainwater to humidify them, then led into a series of jars with egg clutches. The outlet tube from the last egg jar ended underwater in a final eggless jar, allowing me to monitor gas flow by bubbling and hence to maintain similar flow rates in both jar series. Oxygen levels in each series of jars were measured with the oxygen meter of a Horiba U-10 Water Quality Checker (Horiba Instruments, Irvine, Calif.) enclosed in a plastic bag with the end of the appropriate outflow tube. Immediately before the experiments, oxygen levels in the outflow from the humidifying bubble jars were measured. Egg jars were then connected to the appropriate gas source, and the oxygen level was continuously monitored in the outflow from the final experimental jar. Gas flow was maintained at a high level until jars were completely flushed, as indicated by outflow oxygen levels equal to inflow levels. The flow rate was then reduced to conserve gas supply. Periodic checks of outflow oxygen levels from experimental and control jar series confirmed that flow rates remained sufficient to ensure a consistent oxygen level throughout each series of jars.

Effect of Submergence in Water on Hatching

A natural context in which arboreal embryos may experience hypoxia is by submergence in pond water, if their clutch falls into the pond or if pond levels rise. Thus, I tested whether *A. callidryas* embryos would hatch when submerged. Fourteen clutches were paired by size (32 ± 2 eggs/clutch [mean ± SE]) and then randomly assigned to treatments within pairs. Experimental clutches were hung in 266-mL plastic cups. Control clutches were hung in similar cups, with drain holes cut about 5 mm from the bottom of the cup. At 10:00 A.M., age 5 d, I collected water from the surface of an *A. callidryas* breeding pond and, within 5 min, poured 250 mL into each cup to fully submerge eggs in the flooding treatment. The water was unstirred, mimicking conditions in the pond. The number of embryos hatched from each clutch was counted at 5-min intervals for 45 min.

Effect of Hypoxia on Hatching

To test whether hypoxia induces hatching in *A. callidryas*, I exposed eggs to a gas mixture of 7% oxygen balanced with nitrogen and compared their hatching pattern with that of control eggs in air (21% oxygen). Clutches were paired by egg size and color, and one member of each pair was randomly assigned to control and experimental treatments ($N = 10$ pairs). Experimental and control jars were closed, and gas flow started at 9:00 A.M., age 5 d. Experimental jars were completely flushed (outflow oxygen level matched inflow level) within 16 min. The number of embryos hatched from each clutch was counted every 20 min for 4 h, then every hour to 15 h, then every few hours until all embryos had hatched.

Effect of Hyperoxia on External Gill Regression

I hypothesized that exposure to a hyperoxic gas mixture would stimulate regression of external gills before hatching in naturally clumped embryos. I tested this by dividing 12 clutches and rearing some of the eggs in air (21% oxygen) and some in a gas mixture of 35% oxygen balanced with nitrogen. Young (age 2 or 3 d) clutches were cut in half, dividing eggs, jelly, and the leaf to which they were attached. Each half-clutch contained at least 13 eggs, which is within the natural range of clutch sizes. The eggs were hung in glass jars, and at 8:00 A.M., age 4 d, the jars were closed and gas treatments started. Half the embryos were left in the treatments until 6:00 P.M., age 5 d, and half until 6:00 P.M., age 6 d. I then opened the jars and immediately preserved up to five unhatched embryos per half-clutch in 10% neutral-buffered formalin. Specimens were examined under a dissecting microscope at ×20 within 3 d, and external gill lengths were measured with an ocular micrometer. Although gill circulation could not be measured, per se, the area of the gills containing blood was ranked as an indicator of perfusion as follows: 0 = gills entirely white and bloodless or no external gills; 0.5 = blood present in the gills but some sections lacking blood; and 1 = blood throughout the entire external gill vasculature. I examined 97 embryos in 21 half-clutches (4.62 ± 0.2 embryos per half-clutch). In two control and one experimental age 6-d half-clutches, all embryos hatched before I could obtain specimens. All statistical comparisons use mean values for each half-clutch.

Effect of Hyperoxia-Induced External Gill Loss on Hatching

To assess whether the maintenance of embryonic external gills facilitates delayed hatching, I induced external gill regression with a hyperoxic gas mixture and then returned embryos to air. This experiment had three treatments. Experimental embryos were exposed to a gas mixture of 42% oxygen balanced with nitrogen starting at 9:00 A.M., age 4 d, and were later transferred to air (21% oxygen). Hyperoxia controls were exposed to the 42% oxygen gas mixture in series with experimental jars but were maintained in hyperoxia until they hatched. Air controls were maintained in air throughout their embryonic development. The experiment was conducted in two batches, each including eight clutches per treatment (total $N = 16$ clutches per treatment).

Experimental embryos were transferred from hyperoxia to normoxic air in the early morning, age 6 d. This time was initially selected based on the timing of gill regression in the previous experiment and the constraint that spontaneous hatching occurs in the evening at age 6 d. In addition, embryos in all three treatments were observed several times daily to monitor gill regression. Because embryos were inside jars, it was not possible to measure gill size or perfusion under a microscope. Nonetheless, to the naked eye, the gills of air-control embryos were always readily visible as long, red filaments. In the evening, at age 5 d, most hyperoxia-exposed embryos still had visible red gills. In the morning, age 6 d, most hyperoxia-exposed embryos had no visible external gills. I counted any hatched tadpoles and, at 6:40 A.M., disconnected experimental jars from the series with hyperoxia controls and reconnected them in series with air controls. Gas flow rate was temporarily increased in all treatments to flush jars. After 7 min, the outflow from experimental jars had dropped to ambient Po_2, and flow was reduced. I counted the number of embryos hatched from each clutch several times per hour after this switch to normoxia and then at gradually increasing intervals until all embryos had hatched.

Effect of Prostaglandin-Induced External Gill Loss on Hatching

As a second method to assess the effect of embryonic external gills on hatching timing, I induced gill regression with topical prostaglandin treatment, following Warkentin and Wassersug (2001). Clutches were hung in individual plastic cups and exposed to experimental or control treatments between 9:00 A.M. and 3:00 P.M., age 5 d ($N = 11$ pairs). Experimental clutches were sprayed with 2 mL of 2.61×10^{-4} mol/L of the prostaglandin E1 analog, misoprostol (Searle, Skokie, Ill.), to completely wet the clutch surface. Matched control clutches were treated concurrently with 2 mL of the carrier solution, 0.8% ethanol in rainwater. All run off solution was removed from the cup and replaced with aged tap water. Hatching was monitored continuously for at least 30 min in each pair of clutches, then several times per hour for several hours, and then at longer intervals until all embryos had hatched. Because the precise intervals between treatment and hatchling counts varied among pairs of clutches, for data presentation, I estimated the number hatched at standardized times by linear interpolation between adjacent data points where necessary.

Combined Effect of Hyperoxia and Prostaglandin on Hatching

If oxygen stress caused by gill regression is the reason that prostaglandin treatment induces hatching, then the effect should be reduced by concurrent exposure to hyperoxia. Therefore, I repeated the prostaglandin treatment and concurrently exposed half the egg clutches to a hyperoxic gas mixture, either 35% oxygen balanced with nitrogen ($N = 10$ pairs) or 42% oxygen ($N = 11$ pairs). Paired clutches were treated with misoprostol as above, between 9:00 and 11:30 A.M., age 5 d, but in glass jars. Within 2 min of prostaglandin treatment, each jar was capped and flushed with either hyperoxic gas (experimental) or air (control); oxygen levels in outflow gas stabilized at inflow levels within 4 min. Hatchlings were counted every 10 min for 1 h and then at increasing intervals until all embryos had hatched.

Results

Effect of Submergence in Water on Hatching

Significantly more submerged eggs hatched during the observation period than did control eggs (Fig. 1; Mann-Whitney: $U = 49$, $N_{\text{flooded}} = N_{\text{control}} = 7$, $P = 0.0005$). Experimental eggs began hatching within 5 min of submergence, and all embryos

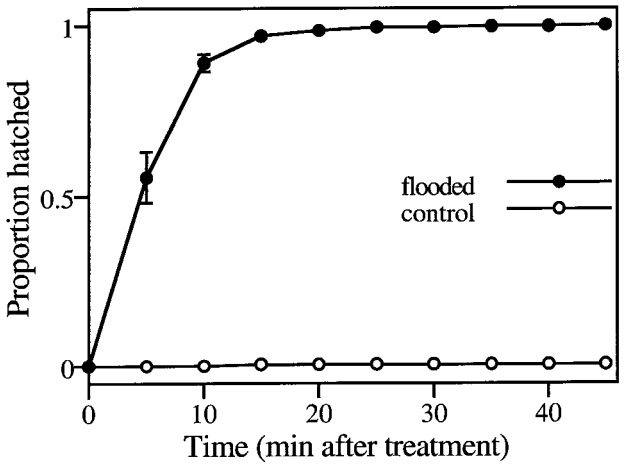

Figure 1. Hatching patterns of *Agalychnis callidryas* eggs submerged in pond water at 10:00 A.M., age 5 d, and control eggs in air. Data are means ± SE. In some cases, the SEs are smaller than the data points. Submergence induced hatching.

in all submerged clutches hatched within 41 min. Only a single control embryo hatched during this period.

Effect of Hypoxia on Hatching

Exposure to a gas mixture containing 7% oxygen induced accelerated hatching in *Agalychnis callidryas* (Fig. 2). Embryos began hatching within 20 min, and over 77% had hatched within 2 h. One hour after treatment, the proportion of embryos hatched was significantly higher in the hypoxic gas treatment than that in the air controls (Mann-Whitney: $U = 93$, $N_{exp} = N_{control} = 10$, $P = 0.0007$).

Effect of Hyperoxia on External Gill Regression

Embryos held in a hyperoxic gas mixture (35% oxygen) had smaller external gills and less blood in the gills compared to embryos raised in air (Fig. 3). Gills were significantly smaller under hyperoxia both in age 5-d embryos and in age 6-d embryos (Friedman tests; age 5 d: test statistic = 6, $N = 6$ pairs, $P = 0.01$; age 6 d: test statistic = 4, $N = 4$ pairs, $P = 0.046$). The extent of gill perfusion, however, was significantly less only at age 6 d (Friedman tests; age 5 d: test statistic = 1.5, $N = 6$ pairs, $P = 0.22$; age 6 d: test statistic = 4, $N = 4$ pairs, $P = 0.046$). At age 5 d, only five out of 30 experimental embryos had completely bloodless gills. Eleven showed blood throughout the gills, while 24 out of 29 control embryos had blood throughout the gills; the rest showed some unperfused area. At age 6 d, the control gills were relatively unchanged, with 14 out of 17 specimens showing blood throughout the gills, and three showing some reduction of perfused area. In contrast, only eight out of 21 experimental embryos still had external gills. Three of those had no blood in the gills, and in the remainder, the gills were only partially perfused.

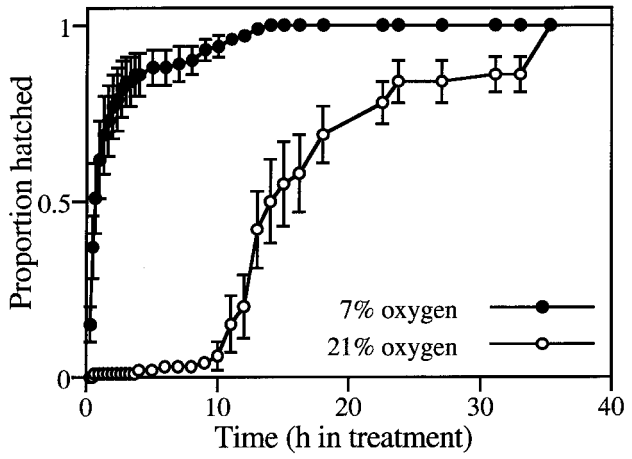

Figure 2. Hatching patterns of *Agalychnis callidryas* eggs exposed to a gas mixture containing 7% oxygen at age 5 d, and of control eggs in air (21% oxygen). Data are means ± SE. Hypoxia induced hatching.

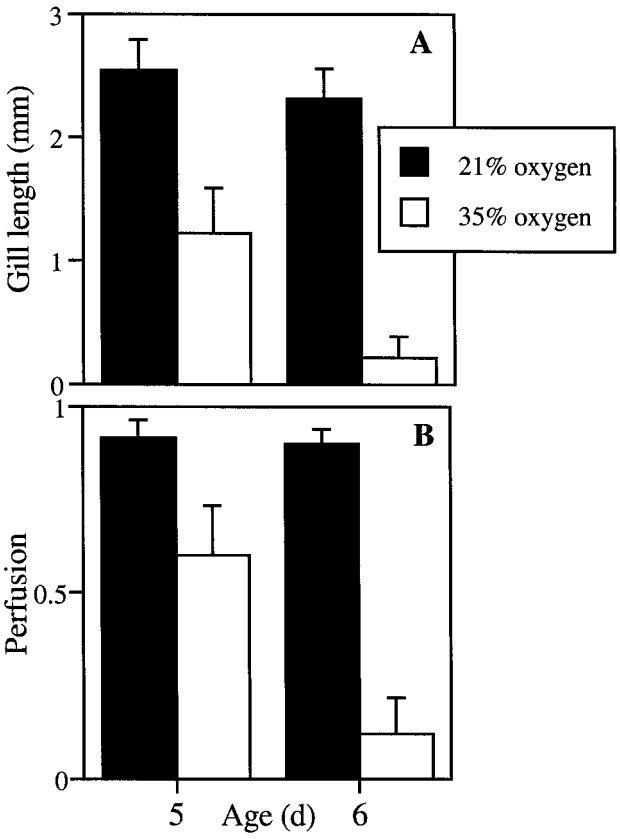

Figure 3. External gill length (*A*) and perfusion (*B*) for *Agalychnis callidryas* embryos in a hyperoxic gas mixture (35% oxygen) and in air (21% oxygen). Embryos were exposed to hyperoxia for 34 h (scored at 6:00 P.M., age 5 d) or 58 h (scored at 6:00 P.M., age 6 d). Perfused area: 0 = no blood in external gills; 0.5 = blood in part but not all of external gill vasculature; 1 = blood throughout the external gill vasculature. Data are means ± SE. Hyperoxia induced gill regression.

Effect of Hyperoxia-Induced External Gill Loss on Hatching

Regression of external gills occurred in embryos held in a gas mixture containing 42% oxygen but was relatively slow, as in 35% oxygen. In the evening at age 5 d, most embryos exposed to 42% oxygen still had perfused external gills (i.e., gills were bright red and easily visible to the naked eye). By age 6 d, 6:00 A.M., in most embryos under hyperoxia, no external gills were visible to the naked eye. Short, red gills were still visible in one to three embryos each in 13 of the 32 clutches. It is possible that a few more embryos were positioned so that their shortened gills were hidden, but long, perfused gills are not readily obscured.

When the hyperoxia-treated embryos were reexposed to air

(21% oxygen), they began hatching within 30 min and about half hatched within 2 h (Fig. 4). At 1 h after exposure to air, the proportion hatched from experimental clutches was higher than that hatched from controls (Mann-Whitney: $U = 50$, $N_{exp} = 16$, $N_{control} = 32$, $P < 0.0001$). There was no significant difference in the proportion hatched from air controls and hyperoxic controls (Mann-Whitney: $U = 119.5$, $N_{air} = N_{hyperoxic} = 16$, $P = 0.6862$). A substantial proportion (40%) of experimental embryos did not hatch until approximately 12–15 h after air exposure, when most of the control embryos hatched (Fig. 4).

Effect of Prostaglandin-Induced External Gill Loss on Hatching

Topical prostaglandin treatment, which induces rapid regression of external gills (Warkentin and Wassersug 2001), also stimulated accelerated hatching (Fig. 5*A*). The hatching pattern was very similar to that of clutches exposed to a hypoxic gas mixture (Fig. 2). Embryos began hatching within 20 min, and 68% had hatched within 2 h. At 1 h posttreatment, the proportion hatched from control and experimental clutches was dramatically different (Mann-Whitney: $U = 0$, $N_{exp} = N_{control} = 11$, $P = 0.0001$).

Combined Effect of Hyperoxia and Prostaglandin on Hatching

Embryos treated with prostaglandin in a hyperoxic gas mixture did not hatch as rapidly as those treated with prostaglandin in air, although the difference was small (Fig. 5*B*). Data from the tests using 42% and 35% oxygen were pooled, after no significant differences were found between the two air treatments and the two hyperoxia treatments (Mann-Whitney, air: $U = 68$, $N_1 = 10$, $N_2 = 11$, $P = 0.36$; hyperoxia: $U = 60$, $N_1 = 10$, $N_2 = 11$, $P = 0.69$). At 30 min posttreatment, significantly fewer misoprostol-treated embryos had hatched under hyperoxic conditions than in air (Mann-Whitney: $U = 318$, $N_{air} = N_{hyperoxic} = 21$, $P = 0.01$).

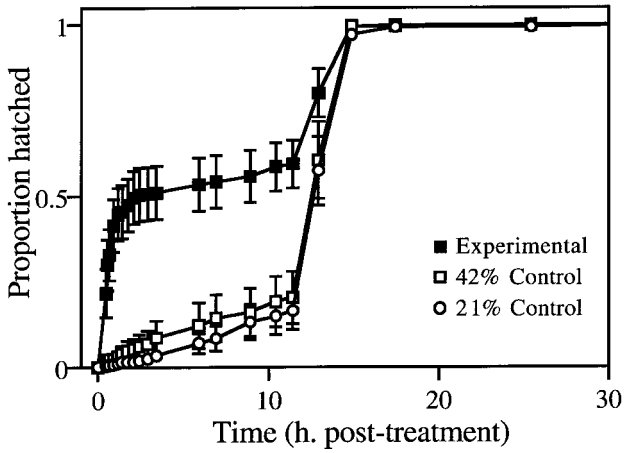

Figure 4. Hatching patterns of *Agalychnis callidryas* eggs in air, after a period of gill regression in a hyperoxic gas mixture, with air and continuous hyperoxia controls. Posttreatment time is measured from the transfer of experimental embryos to air (21% oxygen) at 6:40 A.M., age 6 d, after 45.7 h in 42% oxygen. Data are means ± SE. Transfer of hyperoxia-exposed embryos to air induced hatching.

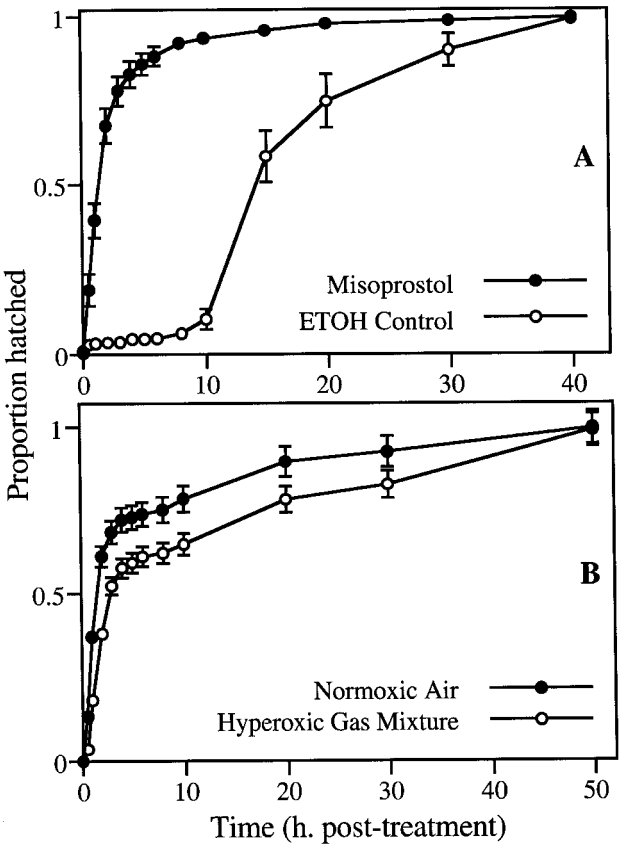

Figure 5. *A*, Hatching pattern of *Agalychnis callidryas* eggs treated with the prostaglandin E1 analog misoprostol, which induces external gill regression, or an ethanol carrier control. Pairs of egg clutches were treated asynchronously from 8:55 A.M. to 2:56 P.M., age 5 d; time is measured from the start of treatment. Data are means ± SE. Misoprostol treatment induced hatching. *B*, Hatching of *A. callidryas* eggs treated with misoprostol and then held in air (21% oxygen) or in a hyperoxic gas mixture (35% or 42% oxygen). Clutch pairs were treated asynchronously between 9:00 and 11:30 A.M., age 5 d. Hyperoxia reduced the hatching induced by misoprostol.

Discussion

Oxygen Stress as a Hatching Stimulus

As expected, hypoxia stimulates the hatching of *Agalychnis callidryas* eggs. Immediate hatching in response to acute oxygen stress and accelerated hatching in response to chronic oxygen

stress have been described in several amphibians, as well as in fish and invertebrates (e.g., DiMichele and Taylor 1980; Petranka et al. 1982; Bradford and Seymour 1988; Miller 1992; Mills and Barnhart 1999; Seymour et al. 2000). It appears to be a widespread and evolutionarily ancient response.

The arboreal eggs of red-eyed tree frogs are normally not submerged in water. However, eggs are occasionally submerged by rising water levels or if the leaf to which they are attached falls (Warkentin 2000*b*). If this occurs before the embryos are capable of hatching, they die (Pyburn 1970; K. M. Warkentin, personal observation). Submergence of hatchable eggs, however, induces immediate hatching. Several species of amphibians and fish have terrestrial eggs from which aquatic larvae hatch when the eggs are flooded, and oxygen stress is the proximate mechanism by which flooding induces hatching in many of these cases (reviewed in Martin 1999). Although I did not measure perivitelline oxygen levels in submerged red-eyed tree frog eggs, oxygen stress is likely the proximate cause of hatching in this case as well.

Oxygen Availability and Gill Regression

The timing and speed of external gill regression in *A. callidryas* varies both with development and with oxygen availability. Natural regression of the gills occurs immediately after hatching, at different developmental stages depending on hatching age. It is faster in hatchlings with larger lungs and more developed internal gills (Warkentin 1999*b*, 2000*a*). Gill regression can also be induced before hatching by separating eggs to increase the surface area for gas exchange or delayed after hatching by keeping tadpoles in hypoxic water and denying them access to air (Warkentin 2000*a*).

The fact that exposure of egg clutches to hyperoxic gas mixtures induces external gill regression provides additional support for the hypothesis that gill regression is a response to enhanced oxygen availability, not to hatching per se. The much greater speed of gill regression in hatchlings, compared to embryos in a hyperoxic gas mixture, suggests that even 42% oxygen may not improve oxygen availability to clumped embryos as much as does hatching. Hatching allows ventilation of internal gills with water, rather than perivitelline fluid, and air ventilation of lungs, a previously unused respiratory surface. The failure of some embryos, particularly those with little exposed surface area, to completely lose their external gills after over 2 d in hyperoxia (K. M. Warkentin, personal observation) also suggests that oxygen availability to embryos was only moderately enhanced by the gas manipulation. Gill regression in separated eggs is gradual, as under hyperoxia, but it is more consistent among embryos (Warkentin 2000*a*). This suggests that variation in surface exposure, due to position within the clutch, may affect gill regression under hyperoxia. There is, however, no absolute constraint on rapid gill loss in embryos. Prostaglandin treatment induces loss of gill perfusion and substantial gill shortening within minutes, similar to the natural posthatching process (Warkentin and Wassersug 2001).

Gill Regression and Hatching Timing

Two independent methods of inducing external gill regression in *A. callidryas* embryos induce hatching. First, after a period of gill regression in a hyperoxic gas mixture, many embryos transferred to air hatch rapidly, well before the normally gilled controls maintained in air. This is clearly an effect of the transfer to air, not of exposure to the hyperoxic gas per se, since the hatching pattern of clutches maintained in hyperoxia was indistinguishable from control clutches maintained in air. The early hatching of embryos without external gills in air was similar to that of normally gilled embryos in a hypoxic gas mixture, suggesting that both conditions created a similar oxygen stress (cf. Figs. 2, 4). However, not all the hyperoxia-exposed embryos hatched immediately upon exposure to air; some hatched up to 12 h later, along with the controls. Two factors may have affected the variation in hatching timing among experimental embryos. First, not all hyperoxia-treated embryos had completely lost their external gills, and a few embryos may have even increased the size or perfusion of their gills after the transfer to air (K. M. Warkentin, personal observation). Also, as embryos hatch, their egg capsules collapse, increasing the surface exposure and presumably improving gas exchange for neighboring eggs. In fully exposed eggs separated from their clutch, the loss of external gills is not associated with hatching (Warkentin 2000*a*). Thus, three factors may have combined to induce early hatching: lack of external gills, limited exposure of the egg surface within a clutch, and normoxic conditions in air.

The second method of inducing gill regression, topical prostaglandin treatment, also induces hatching. Misoprostol induces rapid regression of external gills in *A. callidryas*; in some embryos, gill perfusion is lost, and the gills shorten to less than half their pretreatment length within 10 min (Warkentin and Wassersug 2001). The hatching pattern of misoprostol-treated egg clutches was similar to that of clutches in a hypoxic gas mixture and to the initial hatching of clutches transferred from a hyperoxic gas mixture to air (cf. Figs. 2, 4, 5*A*), suggesting that the same oxygen stress mechanism could explain all three patterns. The fact that concurrent exposure to a hyperoxic gas mixture reduced the effect of misoprostol in inducing early hatching supports the hypothesis that at least part of the mechanism for misoprostol-induced early hatching is oxygen stress, presumably due to external gill regression. However, hyperoxia did not fully rescue embryos from misoprostol-induced early hatching. There may be another pathway by which misoprostol stimulates hatching, and/or the hyperoxia treatment may not have fully compensated for the loss of oxygen uptake via the external gills. The latter seems plausible, considering the much more gradual regression of gills under hyperoxia or the en-

hanced surface exposure, than with either hatching or prostaglandin treatment (Warkentin 2000*a*; Warkentin and Wassersug 2001). Measurements of oxygen levels in the perivitelline fluid under different egg surface exposures and external oxygen levels and comparisons of the respiratory rates of embryos with and without external gills at appropriate oxygen levels would resolve this question.

Gill Regression Heterochrony and Hatching Plasticity

The early hatching caused both by return to normoxia following temporary hyperoxia and by prostaglandin treatment is most parsimoniously attributed to the common factor of gill loss, since other potential effects differ between treatments. *Agalychnis callidryas* also exhibit substantial natural variation in the timing of external gill regression, associated with variation in hatching timing. Most undisturbed embryos hatch at age 6 d in Panama (age 7 d in Costa Rica), but some remain unhatched as long as 9 or 10 d, and embryos disturbed by predators hatch as early as age 4 d in Panama (age 5 d in Costa Rica). Embryos progress steadily through the series of developmental stages described for other anurans until stage 23 (Gosner 1960), defined by the presence of bilateral external gills and an opercular fold covering the base of the gills. They remain in stage 23 until they hatch. Meanwhile, the development of other structures continues. Animals 8 mm in length with a simple, undifferentiated yolk sac and no keratinized mouthparts can hatch, and rapidly lose their external gills, while others 12 mm in length with a 17-mm-long coiled gut and their jaw sheaths and all labial tooth rows keratinized can remain in the egg and maintain large, bilateral external gills (Warkentin 1999*b*, 2000*a*).

The natural association of gill regression with hatching and the hatching associated with experimentally induced gill regression suggest that, under normoxic conditions, external gills are necessary for hatching-competent embryos to remain in the egg. Experimentally induced oxygen stress clearly induces hatching. If spontaneous hatching occurs at the point in development when some critical level of oxygen stress is reached, the maintenance of external gills could delay this point by enhancing oxygen uptake. This may be particularly relevant for species such as *A. callidryas* that inhabit warm tropical environments and have large eggs with rapidly developing embryos packed closely together in clutches, limiting the surface area for gas exchange (Seymour and Bradford 1995).

Delaying hatching beyond the stage when embryos are first competent to hatch clearly improves the survival of hatchlings with aquatic predators (Warkentin 1995, 1999*a*), and at the point of undisturbed hatching, *A. callidryas* are substantially more developed than many frog hatchlings. An extended period of external gill maintenance appears to be a critical component of the physiological mechanism that allows delayed hatching and therefore enhances hatchling survival. Selection by aquatic predators for later hatching may have favored extended maintenance of the external gills. However, respiratory structures are often highly plastic and sensitive to oxygen availability. Thus, it is also plausible that preexisting plasticity in the timing of external gill regression facilitated the evolution of plasticity in hatching. These hypotheses could be tested by comparisons of the relationships among external gill regression, oxygen stress, and hatching in related species that differ in their hatching timing and hatching plasticity.

Oxygen and the Optimal Timing of Hatching

The timing of hatching, and other life history transitions, should reflect the costs and benefits accruing in the adjacent stages (Werner and Gilliam 1984; Werner 1988). Costs are commonly measured as mortality. Benefits are often measured as growth, but for nonfeeding, encapsulated embryos development seems a more appropriate parameter (Warkentin 1999*b*). Lack of sufficient oxygen can both retard development and kill embryos (Seymour et al. 2000 and citations therein). Hatching improves gas exchange by removing the diffusion barrier of the egg capsule and allowing direct access to water and air. This allows the use of lungs, a previously nonfunctional gas exchange surface, and probably improves the respiratory function of internal gills, although, at least in *A. callidryas*, these are ventilated with perivitelline fluid before hatching. Hatching also allows selection of favorable microhabitats and disruption of boundary layers around the skin by movement. Thus, early hatching may be an adaptive response of embryos to the risk of mortality posed by acute oxygen stress or to the slower rate of development imposed by moderate oxygen limitation. Based on this, we would expect hatching to occur when gas exchange begins to limit embryonic development because development rate can then be improved by hatching.

Oxygen is, however, not the only factor affecting development and mortality. Risks from egg predators or pathogens might favor hatching earlier than required to maximize development rate. Similarly, limited yolk reserves may require embryos to hatch and begin feeding before their development becomes oxygen limited in the egg. Larval stage risks such as predators might favor delayed hatching, with a cost to development rate, if more developed hatchlings are more competent to survive. Likewise, intermittent larval habitats may force delayed hatching, and slower development, until conditions are suitable for posthatching life. These other factors may shift the optimal hatching stage away from the point when embryonic development becomes oxygen limited. There may be no relation of hatching timing to oxygen stress in cases where a different stage-specific selective agent would cause certain death. For instance, most fish embryos do not hatch out of water, even under severe hypoxia (DiMichele and Taylor 1980; Yamagami 1988; Yamahira 1996; Griem and Martin 2000). Where other risks are less severe, or less certain, we expect a balancing of different factors. In such cases, even if hatching does not occur

precisely when development becomes oxygen limited, we would still expect embryos to alter hatching stage in response to sublethal changes in oxygen availability.

In *A. callidryas*, growth and development rates increase at hatching, suggesting that embryonic metabolism may be constrained by gas exchange (i.e., that hatching timing does not maximize development rate; Warkentin 1999*b*). However, as in several other amphibians, fish, and invertebrates (e.g., DiMichele and Taylor 1980; Petranka et al. 1982; Mills and Barnhart 1999; Seymour et al. 2000), *A. callidryas* eggs do hatch in response to increased oxygen stress. This response allows hatchable embryos submerged in pond water to survive, where younger embryos drown (Pyburn 1970). *Agalychnis callidryas* eggs also hatch in response to attack by egg-eating snakes and wasps and an egg-killing fungus (Warkentin 1995, 2000*b*; Warkentin et al. 2001). While physical disturbance mediates predator-induced hatching, the fungus-induced hatching might be triggered by oxygen stress, if fungal mycelium impedes gas exchange through the egg capsule or competes with the embryo for oxygen in the perivitelline fluid. If so, fungus-induced early hatching may be a fortuitous effect of a preexisting behavioral response to oxygen stress, rather than a specific adaptation to the fungal pathogen.

It would be worthwhile to investigate the extent to which "spontaneous" hatching is actually triggered by oxygen stress, as the increasing oxygen needs of developing embryos meet the limitations of oxygen diffusion into their egg capsules.

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Literature Cited

Bradford D.F. and R.S. Seymour. 1988. Influence of environmental Po_2 on embryonic oxygen consumption, rate of development, and hatching in the frog *Pseudophryne bibroni*. Physiol Zool 61:475–482.

Burggren W.W. and J.J. Just. 1992. Developmental changes in physiological systems. Pp. 467–530 in M.E. Feder and W.W. Burggren, eds. Environmental Physiology of the Amphibians. University of Chicago Press, Chicago.

Channing A. 1993. Observations on the natural history of *Stephopaedes anotis* (Bufonidae). J Herpetol 27:213–214.

del Pino E. and B. Escobar. 1981. Embryonic stages of *Gastrotheca riobambae* (Fowler) during maternal incubation and comparison of development with that of other egg-brooding hylid frogs. J Morphol 167:277–295.

DiMichele L. and M.H. Taylor. 1980. The environmental control of hatching in *Fundulus heteroclitus*. J Exp Zool 214: 181–187.

Gosner K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Griem J.N. and K.L.M. Martin. 2000. Wave action: the environmental trigger for hatching in the California grunion *Leuresthes tenuis* (Teleostei: Atherinopsidae). Mar Biol 137: 177–181.

Hastings D. and W. Burggren. 1995. Developmental changes in oxygen consumption regulation in larvae of the South African clawed frog, *Xenopus laevis*. J Exp Biol 198: 2465–2475.

Humphrey R.R. 1972. Genetic and experimental studies on a mutant gene (*c*) determining absence of heart action in embryos of the Mexican axolotl (*Ambystoma mexicanum*). Dev Biol 27:365–375.

Kluge A.G. 1981. The life history, social organization, and parental behavior of *Hyla rosenbergi* Boulenger, a nest-building gladiator frog. Univ Mich Mus Zool Misc Publ 160:1–170.

Martin K.L.M. 1999. Ready and waiting: delayed hatching and extended incubation of anamniotic vertebrate terrestrial eggs. Am Zool 39:279–288.

Miller P.L. 1992. The effect of oxygen lack on egg hatching in an Indian dragonfly, *Potamarcha congener*. Physiol Entomol 17:68–72.

Mills N.E. and M.C. Barnhart. 1999. Effects of hypoxia on embryonic development in two *Ambystoma* and two *Rana* species. Physiol Biochem Zool 72:179–188.

Parichy D.M. and R.H. Kaplan. 1995. Maternal investment and developmental plasticity: functional consequences for locomotor performance of hatchling frog larvae. Funct Ecol 9: 606–617.

Pelster B. and W.W. Burggren. 1996. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (*Danio rerio*). Circ Res 79:358–362.

Petranka J.W., J.J. Just, and E.C. Crawford. 1982. Hatching of amphibian embryos: the physiological trigger. Science 217: 257–259.

Petranka J.W., A. Sih, L.B. Kats, and J.R. Holomuzki. 1987. Stream drift, size-specific predation and the evolution of ovum size in an amphibian. Oecologia 71:624–630.

Pinder A.W. and S.C. Friet. 1994. Oxygen transport in the egg masses of the amphibians *Rana sylvatica* and *Ambystoma maculatum*: convection, diffusion and oxygen production by algae. J Exp Biol 197:17–30.

Pyburn W.F. 1963. Observations on the life history of the treefrog, *Phyllomedusa callidryas* (Cope). Tex J Sci 15:155–170.

———. 1970. Breeding behavior of the leaf-frogs *Phyllomedusa callidryas* and *Phyllomedusa dacnicolor* in Mexico. Copeia 1970:209–218.

Seymour R.S. and D.F. Bradford. 1995. Respiration of amphibian eggs. Physiol Zool 68:1–25.

Seymour R.S. and J.P. Loveridge. 1994. Embryonic and larval respiration in the arboreal foam nests of the African frog *Chiromantis xerampelina*. J Exp Biol 97:31–46.

Seymour R.S., M.J. Mahony, and R. Knowles. 1995. Respiration of embryos and larvae of the terrestrially breeding frog, *Kyarranus loveridgei*. Herpetologica 51:369–376.

Seymour R.S. and J.D. Roberts. 1991. Embryonic respiration and oxygen distribution in foamy and nonfoamy egg masses of the frog *Limnodynastes tasmaniensis*. Physiol Zool 64: 1322–1340.

Seymour R.S., J.D. Roberts, N.J. Mitchell, and A.J. Blaylock. 2000. Influence of environmental oxygen on development and hatching of aquatic eggs of the Australian frog, *Crinia georgiana*. Physiol Biochem Zool 73:501–507.

Sih A. and R.D. Moore. 1993. Delayed hatching of salamander eggs in response to enhanced larval predation risk. Am Nat 142:947–960.

Territo P.R. and W.W. Burggren. 1998. Cardio-respiratory ontogeny during chronic carbon monoxide exposure in the clawed frog, *Xenopus laevis*. J Exp Biol 201:1461–1472.

Warkentin K.M. 1995. Adaptive plasticity in hatching age: a response to predation risk trade-offs. Proc Natl Acad Sci USA 92:3507–3510.

———. 1999*a*. The development of behavioral defenses: a mechanistic analysis of vulnerability in red-eyed tree frog hatchlings. Behav Ecol 10:51–262.

———. 1999*b*. Effects of hatching age on development and hatchling morphology in the red-eyed treefrog, *Agalychnis callidryas*. Biol J Linn Soc 68:443–470.

———. 2000*a*. Environmental and developmental effects on external gill loss in the red-eyed tree frog, *Agalychnis callidryas*. Physiol Biochem Zool 73:557–565.

———. 2000*b*. Wasp predation and wasp-induced hatching of red-eyed treefrog eggs. Anim Behav 60:503–510.

Warkentin K.M., C.C. Currie, and S.A. Rehner. 2001. Egg-killing fungus induces early hatching of red-eyed treefrog eggs. Ecology 82:2860–2869.

Warkentin K.M. and R.J. Wassersug. 2001. Do prostaglandins regulate external gill regression in anurans? J Exp Zool 289: 366–373.

Werner E.E. 1988. Size, scaling, and the evolution of complex life cycles. Pp. 60–81 in B. Ebenman and L. Persson, eds. Size-Structured Populations. Springer, Berlin.

Werner E.E. and J.F. Gilliam. 1984. The ontogenetic niche and species interactions in size structured populations. Annu Rev Ecol Syst 15:393–425.

Yamagami K. 1988. Mechanisms of hatching in fish. Pp. 447–499 in W.S. Hoar and D.J. Randall, eds. The Physiology of Developing Fish. Academic Press, San Diego, Calif.

Yamahira K. 1996. The role of intertidal egg deposition on the survival of the puffer, *Takifugu niphobles* (Jordan et Snyder), embryos. J Exp Mar Biol Ecol 198:291–306.