Development, Surface Exposure, and Embryo Behavior Affect Oxygen Levels in Eggs of the Red-Eyed Treefrog, *Agalychnis callidryas*

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ABSTRACT

Oxygen stress can slow development, induce hatching, and kill eggs. Terrestrial anamniote embryos face a potential conflict between oxygen uptake and water loss. We measured oxygen levels within eggs to characterize the respiratory environment for embryos of the red-eyed treefrog, *Agalychnis callidryas*, a Neotropical frog with arboreal egg masses and plastic hatching timing. Perivitelline oxygen partial pressure (Po_2) was extremely variable both within and among eggs. Po_2 increased with air-exposed surface of the egg and declined over the developmental period before hatching competence. Through the plastic hatching period, however, average Po_2 was stable despite continued rapid development. Development was synchronous across a wide range of perivitelline Po_2 (0.5–16.5 kPa), and hatching-competent embryos tolerated Po_2 as low as 0.5 kPa without hatching. The variation in Po_2 measured over short periods of time within individual eggs was as great as that measured across development or surface exposure, including sharp transients associated with embryo movements. There was also a strong gradient of Po_2 across the egg from superficial to deep positions. Ciliary circulation of fluid within the egg is clearly insufficient to keep it mixed. Embryos may maintain development under hypoxic conditions by strategic positioning of respiratory surfaces, particularly external gills, to exploit the patchy distribution of oxygen within their eggs.

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Introduction

Development is essentially an aerobic process. Development rates are sensitive to oxygen availability, and embryos may also be killed by hypoxia (Bradford and Seymour 1988; Lutz et al. 1992; Booth 1995; Strathmann and Strathmann 1995; Mills and Barnhart 1999; Woods and Hill 2004), although some embryos tolerate periods of hypoxia or anoxia (Pinder and Friet 1994; Padilla and Roth 2001). Many features of reproduction—such as parental egg ventilation, oviposition site choice, and the structure of egg clutches—function to improve or secure the oxygen supply for embryos, sometimes at a substantial cost (Seymour and Bradford 1995; Strathmann and Strathmann 1995; Seymour 1999; Fernández et al. 2000; Brante et al. 2003). As well, both embryos and the maternally constructed structures surrounding them show adaptations to enhance oxygen uptake (Tuft 1950; del Pino and Escobar 1981; Ragsdale and Ingermann 1993).

A major feature of oviposition site that affects oxygen availability is the medium surrounding eggs; terrestrial oviposition has evolved several times from aquatic oviposition in both fishes and amphibians (Martin and Strathmann 1999). Because boundary layers are a much larger impediment to oxygen diffusion in water than in air, terrestrial oviposition could substantially improve the respiratory environment of embryos. However, both gravity and surface tension tend to reduce the space between eggs in terrestrial clutches, compared with aquatic clutches, potentially impeding gas exchange (Seymour 1999; Strathmann and Hess 1999). Furthermore, thinning of jelly capsules due to evaporative water loss both reduces diffusion distance to the air and may reduce space between eggs, depending on clutch structure. Indeed, many aspects of clutch structure that improve oxygen uptake also increase water loss, thus the morphology of terrestrial egg clutches may reflect a balance between the water and oxygen needs of embryos (Woods and Hill 2004). In this context, it is not clear whether or to what extent terrestrial egg deposition improves gas exchange for anamniote embryos.

The respiratory environment changes substantially at hatching, often improving oxygen availability by reducing boundary layers, allowing microhabitat selection, providing direct access to air, and allowing the use of new respiratory surfaces (Seymour and Bradford 1995; Warkentin 2002). Indeed acute, externally generated oxygen stress commonly triggers hatching of well-developed embryos in invertebrates, fishes, and amphibians, for instance, when terrestrial eggs are flooded (DiMichele

and Taylor 1980; Petranka et al. 1982; Bradford and Seymour 1988; Miller 1992; Czerkies et al. 2001). The role of slower-onset, internally generated oxygen stress is less clear. Embryonic oxygen demand increases developmentally, since relatively inert yolk is converted to metabolically active tissue (Seymour and Bradford 1987; Burggren et al. 1990; Hastings and Burggren 1995). In amphibians, the oxygen conductance of the egg capsule also increases developmentally as water moves into the perivitelline space and the egg capsule thins (Seymour and Bradford 1987; Seymour 1999). However, depending on the developmental rate and environment outside the egg, this may not keep pace with embryonic oxygen demand (Seymour et al. 1991; Seymour and Roberts 1995). Thus, it is possible that oxygen stress is also an internal trigger of "spontaneous" hatching, as embryonic oxygen consumption exceeds and becomes limited by capsular conductance. If so, we would expect a decline in oxygen levels within the egg before hatching (Warkentin 2002).

As a step toward understanding both the oxygen availability for terrestrial anamniote embryos and the potential role of oxygen stress in spontaneous hatching, we quantified oxygen levels inside the eggs of red-eyed treefrogs across embryonic development. Red-eyed treefrogs, *Agalychnis callidryas*, deposit their eggs on vegetation overhanging water in wet forests from the Yucatán through Panama. The eggs are fairly large (3.7 mm diameter at oviposition, 5.2 mm when hatchable; Pyburn 1963; Warkentin 2002), are clumped together in gelatinous egg masses (average mass size is 40 eggs; Warkentin 1995), and develop rapidly in their warm tropical environment. Thus, among terrestrial eggs, they are likely candidates for oxygen limitation during development. The embryos have large, well-perfused external gills that they normally maintain as long as they are in the egg. At our Panama field site, embryos can hatch and lose their external gills as early as 4 d of age if disturbed by egg predators or remain in the egg and maintain the gills to 7 d (Warkentin 2000*b*, 2002). Manipulations of egg surface exposure and oxygen content of both the air around clutches and water around hatchlings indicate that external gill loss is associated with increased oxygen availability, not hatching per se, suggesting that hatching normally improves oxygen availability (Warkentin 2000*a*, 2002). Even late hatchlings still have substantial yolk reserves and do not feed immediately (Warkentin 1999*b*), suggesting that nutrient supply limitations are not involved in hatching timing. Several lines of evidence indicate that oxygen stress can induce hatching. Within the developmental period of hatching competence, embryos will hatch if exposed to hypoxic gas mixtures, if submerged underwater, or if induced to lose their external gills (Warkentin 2002). Nonetheless, development of eggs within and among clutches is highly synchronous across a wide range of egg surface exposures, excepting only eggs completely buried behind their siblings.

We hypothesized (1) that oxygen levels in *A. callidryas* eggs would depend on their surface exposure, with poorly exposed eggs being relatively hypoxic, and (2) that perivitelline Po_2 would decline over development, with the lowest levels just before hatching. We measured oxygen levels in the perivitelline fluid bathing the embryos from ages 2 d, just before the start of blood circulation, through 6 d, when most embryos hatch spontaneously. At each age, we included eggs with a wide range of surface exposures. Motivated by the results of these measurements, we also assessed the spatial distribution of oxygen inside eggs. We measured Po_2 at two positions, superficial and deep, within the perivitelline space of 4-d-old eggs, when embryos first become hatching competent.

Material and Methods

This research was conducted under permits from the Smithsonian Tropical Research Institute and the Panamanian National Environmental Authority and approved by the Animal Care and Use Committee of Boston University (IACUC protocol 02-013).

Effects of Development and Surface Exposure

We measured oxygen levels in the perivitelline space inside 210 *Agalychnis callidryas* eggs of five different ages (2–6 d) and varying proportions of air-exposed surface area (15%–80%). We used only clutches that were healthy, with no evidence of predator contact or pathogen infection, and only eggs that were developing in synchrony with their clutch mates and other eggs of the same age on the basis of external morphology. We used 10 clutches per age from 2 to 5 d. At 6 d, when some embryos hatched during measurements, we used 15 clutches to obtain sufficient measurements. Each egg was measured once.

Young egg clutches were collected, along with the leaves on which they were laid, from Ocelot Pond (2 km south of Gamboa, Panama), brought to an open-air laboratory in Gamboa, and maintained under ambient high humidity and natural temperatures (23.5°–29.5°C). Clutches on their leaves were attached to plastic support cards and hung in a natural, approximately vertical position over water in individual plastic cups. They were misted frequently with rainwater to maintain hydration. See Warkentin (2002) for details of clutch structure. Because standard staging tables (e.g., Gosner 1960) are of limited use for *A. callidryas* embryos as a result of their reliance on the highly plastic character of external gill regression as a marker, we use age as a proxy for developmental stage. Under ambient conditions, development is consistent within and among *A. callidryas* clutches. Briefly, in this population, 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 short. At age 3 d, gill branches are longer, and melanophores have begun to appear. In the morning, 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; 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, but a few embryos remain unhatched until age 8 d, still in stage 23 (Gosner 1960). See Warkentin (1999*b*, 2002) for additional discussion of development, age, and staging of *A. callidryas* embryos.

To measure oxygen, we used a fiber optic microprobe (Precision Sensing, Regensburg, Germany) mounted with the tip (<50 μm diameter) at a fixed position in the aperture of a 27-gauge needle. The extension of the angled needle tip beyond the probe tip protected the probe from direct contact with the embryo. The optode (probe) does not consume oxygen and even mounted in the needle tip has a rapid response (from air-saturated to anoxic water: 94% response in 3 s, 99% in 7 s; Fig. 2*I*). The probe was connected to an optical oxygen sensor (Microx TX2, Precision Sensing), and the partial pressure of oxygen (Po_2) was displayed and recorded on a laptop computer, sampling once per second. Before each day's measurements, the probe was calibrated in a fresh anoxic solution of water with $NaSO_3$ and in water-saturated air. The calibration was rechecked after each day's measurements. Optode drift was never >2%, thus no drift compensation was required. Measurements were temperature compensated using a thermal probe in contact with the egg clutch under measurement. Temperatures ranged from 25.5° to 29.4°C (27.4° ± 0.06°C; mean ± SE). Between succeeding eggs, the oxygen-probe response and high-point calibration were checked in air-saturated water. If the probe showed a slow or erratic response, presumably because of egg jelly caught in the needle, it was cleaned in water until the response was normal.

For measurements, each clutch on its card was set approximately vertically in a cutaway cup to allow access to the front air-exposed surface of the clutch, opposite the leaf and card. The probe, held stable in plasticine on a ceramic base, was positioned in front of an egg and then manually advanced so that the probe tip entered the perivitelline space. We recorded from a single probe position at the approximate center of the perivitelline space and initially placed the probe tip away from the body of the embryo. Embryos often moved during measurements, and thus their spatial relationship to the probe tip changed; however, all measurements are from the perivitelline space surrounding the embryo, not from within the embryo itself. We watched each embryo as we recorded from its egg and noted the time of gross movements (position changes and flexion without position change) in reference to the time trace on the oxygen recording. Embryos also flicked their external gills, but this was only visible in some body positions, so we did not record it. We recorded a total of 25 h of oxygen levels (7.2 ± 0.2 min/egg; mean ± SE). We recorded at least 5 min/egg, unless they hatched, and longer if levels were highly variable. If the egg leaked perivitelline fluid and lost turgor when the probe was inserted, we did not use it. We measured external egg diameters using calipers and estimated the proportion of the egg surface exposed to the air (i.e., not in contact with other eggs or attached to the leaf). *Agalychnis callidryas* eggs 2 d old and older usually fit together in roughly hexagonal arrays, facilitating exposure estimates. For more than half the eggs in this experiment, two observers (K. M. Warkentin and I. Gomez-Mestre) independently estimated exposure. We were usually in complete agreement and never differed by more than 10%. All oxygen measurements were conducted between 8 a.m. and 8 p.m. over a period of 9 d.

Analysis of Oxygen Data

For analysis of the Po_2 recordings, we developed algorithms to compute the overall minimum and maximum Po_2 from each egg, the mean Po_2, and the total duration of periods where Po_2 was relatively stable, and to identify points where the direction of oxygen transients reversed, that is, short-term highs and lows. A continuous set of observations that stayed within a range of ±0.2 kPa for at least 25 s was classified as a stable period. Short-term highs and lows were identified as those observations that were at least 0.2 kPa higher or lower than the previous and subsequent four observations. We did not include the initial drop in Po_2 readings on insertion of the probe into the egg or any transients associated with probe withdrawal or embryo hatching.

For graphical presentation of effects of surface exposure across development, we simply divided eggs into high- (≥50%) and low-exposure categories. Highly exposed eggs averaged 63% ± 1% exposed (range 50%–80%, $N = 70$). Eggs with low exposure averaged 34% ± 1% exposed (range 15%–45%, $N = 140$). For statistical analysis, we used the full range of exposure values as a continuous variable.

We tested the effects of age and surface exposure on mean Po_2, the proportion of time Po_2 was stable, and, as a measure of oxygen fluctuations, the frequency of short-term highs and lows using general linear models (PROC MIXED, SAS Institute 1999). Data met parametric assumptions; no transformations were necessary. We included clutch, nested within age, in all models because it improved goodness of fit. Clutch effects were significant for mean Po_2 and frequency of short-term highs and lows ($F_{50,154} = 1.73$, $P < 0.01$; $F_{50,154} = 1.76$, $P < 0.01$, respectively) but not for the proportion of time Po_2 was stable. External egg diameters averaged 4.8 ± 0.03 mm (range: 3.8–5.9 mm) and did not change significantly across the age range examined (Spearman $r = -0.035$, $P = 0.63$). We initially included egg diameter as a covariate, but it had no significant effect in any case and was thus removed from the final analyses.

Effect of Position within Egg

To assess the spatial variation in oxygen levels within eggs, we measured Po_2 at two locations within each of 30 eggs at age 4 d (three eggs each from 10 clutches). Eggs were 4.8 ± 0.03 mm with, on average, 47% ± 2% exposed surface area (range 25%–70%). The probe tip was placed initially just inside the perivitelline membrane and then later advanced to a deeper position or placed initially in a deep position and then withdrawn to just under the surface. For deep measurements, the probe tip was positioned between the center of the egg and the far wall, touching neither the embryo nor perivitelline membrane; exact depth varied with embryo position. Measurement order was haphazard unless embryo position made one initial placement easier. To calculate the Po_2 at a position, we used two methods. If the measurements did not stabilize, we averaged across the entire time that the probe tip was in that position, excluding only the initial transient associated with moving the probe. When there was a substantial period of stable oxygen values, we used the average Po_2 across the stable period, excluding both transients associated with moving the probe and any other transients, for example, associated with embryo movements. On average, we measured Po_2 for 163 ± 18 s at each position.

Results

Development, Surface Exposure, and Behavior

Average Po_2 levels within individual *Agalychnis callidryas* eggs ranged from 0.5 to 16.5 kPa and varied with both surface exposure and age (Figs. 1*A*, 2; $F_{1,154} = 109.39$, $P < 0.0001$; $F_{4,154} = 13.67$, $P < 0.0001$). Across all ages (developmental stages), more exposed eggs had higher oxygen levels (Fig. 2; exposure vs. Po_2 regression: $F_{1,208} = 77.8$, $P < 0.0001$, $R^2 = 0.27$). As embryos developed, perivitelline Po_2 initially declined, but once eggs became hatching competent (at 4 d), it remained stable or, for poorly exposed eggs, even increased slightly (Fig. 1*A*).

The variation in Po_2 over short periods of time within individual eggs was, on average, as great as the variation in mean Po_2 among eggs (Fig. 1*B*). Although some eggs had stable oxygen levels (e.g., Fig. 3*G*), most were characterized by persistent fluctuations in Po_2, including strikingly rapid transients (e.g., Fig. 3*A*–3*F*). The frequency of short-term highs and lows increased slightly with age ($F_{4,154} = 2.72$, $P = 0.032$), ranging from 0.68 ± 0.09 per minute at 2 d (range across eggs 0–1.99) to 1.11 ± 0.12 per minute at 5 d (0–2.63). Recordings were stable within ±0.2 kPa for only 26% ± 1.4% of the time (range 0%–81%). The proportion of time in such stable periods did not vary with age but was greater in less exposed eggs ($F_{1,154} = 5.66$, $P = 0.019$). In eggs with less than half their surface area exposed to air, oxygen levels were stable on average 32% ± 3% of the time (range 0%–81%, $N = 70$), while in eggs more than half exposed, levels were stable 24% ± 2% of the time (0%–78%, $N = 140$). Most of the striking oxygen transients and periods of instability were clearly associated with embryo movements (Fig. 3*D*–3*F*), although some oxygen instability also occurred without obvious movements, and not all visible embryo movements were associated with oxygen transients.

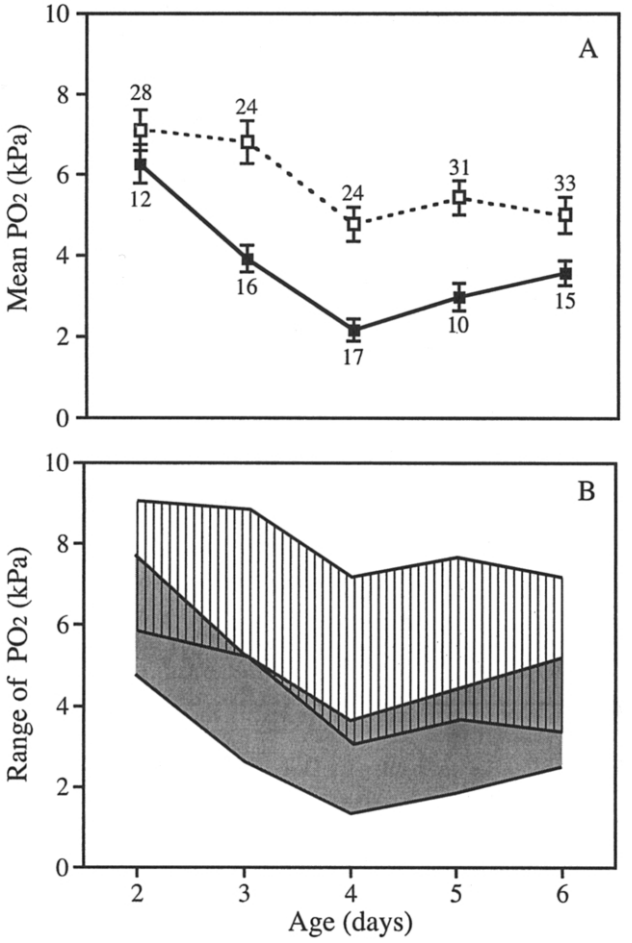

Figure 1. Oxygen levels inside *Agalychnis callidryas* eggs of different ages. *A*, Average of mean perivitelline Po_2 in the approximate center of eggs ±SE of the mean across eggs for eggs with high surface exposure (≥50% exposed; *open symbols and dashed line*) and low surface exposure (<50%; *filled symbols and solid line*). Sample sizes are indicated. *B*, Range of Po_2 within eggs of high (*hatched*) and low (*gray*) surface exposure. Data are the average maximum Po_2 and average minimum Po_2 recorded in eggs of each age.

Position within Egg

Oxygen levels were, on average, 9 ± 0.8 kPa higher near the surface of eggs than deep within them (Fig. 3; Wilcoxon matched pairs test: $Z = 4.78$, $P = 0.000002$, $N = 30$). However, there was considerable variation in surface Po_2 among

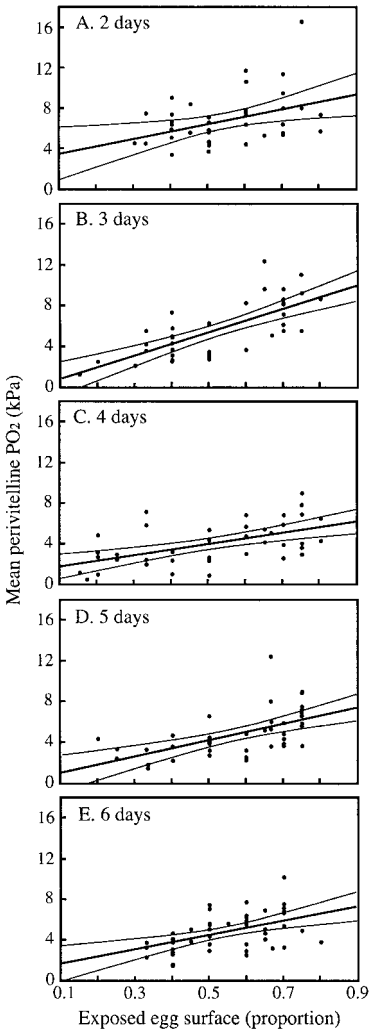

Figure 2. Effect of the amount of egg surface area exposed to air on average perivitelline Po_2 at age 2 d through 6 d (*A–E*). Each data point represents an individual egg. Lines are least squares regression and 95% confidence interval. Exposure increases perivitelline Po_2 at all ages in a similar manner.

eggs, ranging from near air saturated (20.5 kPa) down to 3.9 kPa. Oxygen levels deeper within the egg were somewhat less variable (Fig. 4).

Discussion

We recorded a broad range of oxygen levels from the perivitelline fluid within *Agalychnis callidryas* eggs from very hypoxic levels (0.5 kPa, in the center of an egg) to near air saturated (20.5 kPa, a surface measurement). We also recorded striking transients in Po_2 (e.g., Fig. 2*D*; 5.3 kPa change in 4 s). Among eggs, the average Po_2 in the center of the egg ranged from 0.5 to 16.5 kPa.

All of the eggs we recorded from had apparently healthy, normal embryos developing in synchrony with others of their age. We determined this on the basis of external morphology before measuring their Po_2. Moreover, *A. callidryas* embryos are well ciliated (Warkentin 1999*b*) and constantly circulate the perivitelline fluid around them by ciliary motion and later also by buccal pumping; small flecks visible in the perivitelline fluid make a complete circuit of the egg in ca. 11 s (K. M. Warkentin, personal observation). Thus, this amount of variation in Po_2 was surprising. Steep spatial gradients in Po_2 from egg (embryo) surface to interior are known (Woods and Hill 2004), but we are unaware of data showing comparable rapid temporal transients within eggs.

Is the Variation in Perivitelline Po_2 Real?

Because the perivitelline fluid is constantly circulated, we expected it to be well mixed (as in Burggren 1985) and thus to record relatively stable oxygen levels from eggs, such as the traces shown in Figure 2*G* and 2*H*. Previous reports of perivitelline Po_2 measured in amphibian eggs make no mention of measurement instability (Seymour and Bradford 1987; Seymour and Roberts 1995). Inside *A. callidryas* eggs, however, stable Po_2 values were rare. We are convinced that within-egg variation is real and not a measurement artifact for several reasons. (1) The fiber optic probes we used consume no oxygen. Thus, the changes in Po_2 that we recorded cannot be artifacts of sensor oxygen consumption. This also could not explain sudden increases in Po_2. (2) Contact between the open aperture of the needle (probe guard) and the embryo's body might block the aperture and cause a decrease in Po_2; then, if contact were broken, the Po_2 would rise. However, we positioned the probe to minimize such contact, and it was infrequent. Moreover, most of the oxygen fluctuations that we observed clearly involved no contact between embryo and needle. (3) We have used these probes in a variety of other media, including standard aqueous solutions, air, pond water, and frog egg jelly. In no other context have we seen the large erratic fluctuations in Po_2 that are typical of our recordings from the perivitelline fluid in live eggs. (4) We checked the probe response and cleaned it if necessary immediately before each individual egg recording. Any erratic behavior of the sensor and recording equipment should have been evident in these checks, but they were consistently stable. (5) Before collecting the data set presented here, we measured perivitelline Po_2 in 17 eggs using an exposed fiber optic probe unprotected by a needle. The probe was then damaged by an embryo, motivating the change in method, but the exposed-probe recordings were also variable within eggs, similar to those from the needle-protected probe. This suggests that jelly blocking the needle aperture was not a substantive problem.

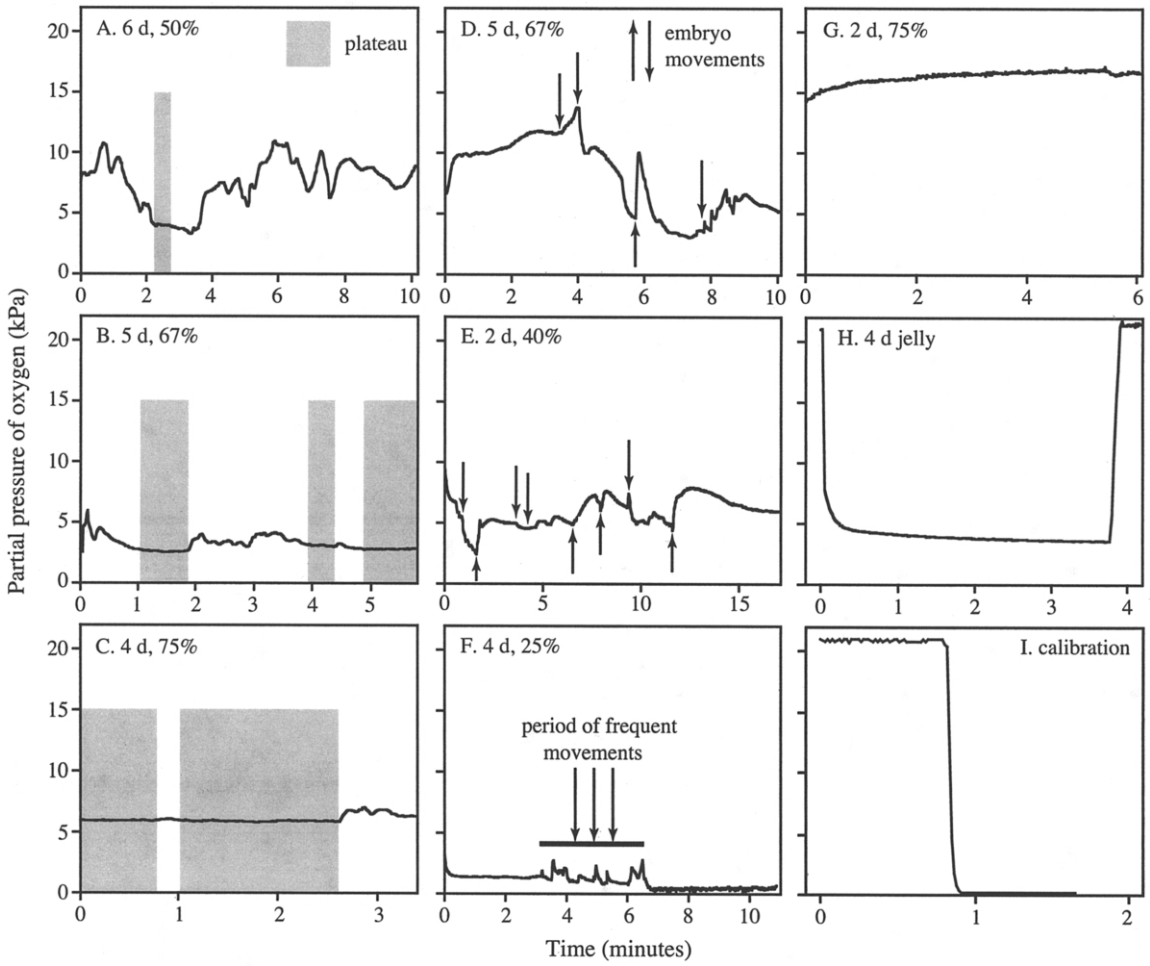

Figure 3. Representative oxygen recordings from *Agalychnis callidryas* egg clutches. Recordings (*A–G*) were each from a single location at the approximate center of the perivitelline space within an egg, with the probe tip initially positioned away from the embryo's body. Egg ages and surface exposures are noted. *A–C*, Three examples showing periods of stable and variable oxygen levels, with measured plateaus indicated. *D–F*, Three examples showing relationship of embryo behavior to oxygen levels. The times of individual embryo movements (position change or flexion) are indicated as well as periods of frequent movements. *G*, Unusually stable recording from an egg. *H*, Oxygen recording from a probe inserted into jelly between eggs and then later withdrawn into air. The probe response and stability is typical of nonegg recordings (water, jelly, air, etc.). *I*, Calibration curve from a probe in humid air moved to anoxic water. Po_2 is to the same scale for all graphs; time scale varies with recording length.

What Causes the Variation in Po_2 among and within Eggs?

Not surprisingly, eggs with more of their surface area exposed to air have higher average Po_2. Also, as expected, there is an effect of development on perivitelline Po_2; it decreases from age 2 to 4 d. This is consistent with an increase in oxygen demand as embryos convert yolk into metabolically active tissue (e.g., Bradford and Seymour 1985; Seymour and Roberts 1991, 1995). It also suggests that any increase in capsular conductance (e.g., Seymour and Bradford 1987) does not keep pace with increasing oxygen demand across this developmental period.

Once embryos become hatching competent, however, there is no further decline in perivitelline Po_2, although they continue to consume yolk and produce other tissue (Warkentin 1999*b*). Indeed, the lowest oxygen levels we recorded were from 4-d eggs just at the onset of hatching competence. In some other species, embryos enter a period of reduced or stable metabolic rates and slowed or static development once they achieve hatching competence, conserving energy until environmental conditions are suitable for posthatching survival (Bradford and Seymour 1985; Darken et al. 1998). Such a slowing of development and metabolism could explain why Po_2 does not continue to decline after hatching competence. We have no direct measurements of embryo metabolic rates, but the continued rapid development (Warkentin 1999*b*) and behavioral sensitiv-

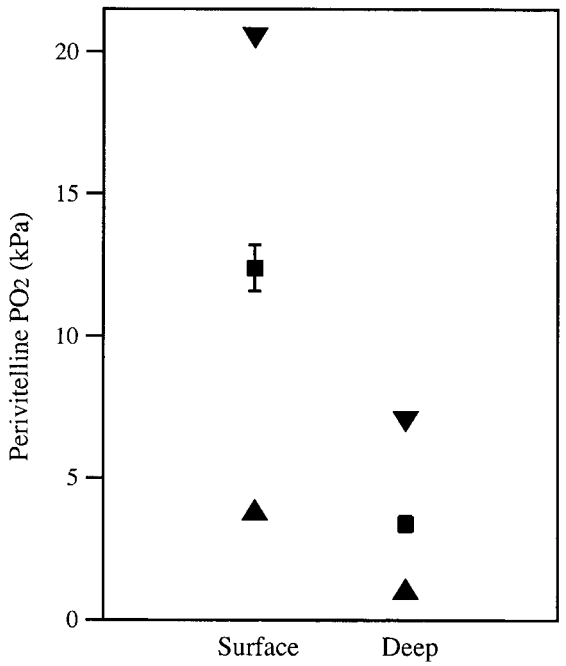

Figure 4. Oxygen levels at superficial and deep positions within 4-d-old *Agalychnis callidryas* eggs. Data are averages of mean Po_2 within eggs (*squares*) ± SE and the maxima and minima of mean Po_2 across eggs (*triangles*).

ity (Warkentin 1995, 2005) of hatching-competent embryos suggest that their metabolism may remain high or even continue to increase. Likewise, increased capsular conductance due to continued swelling of the perivitelline space and thinning of the jelly (Seymour and Bradford 1987) seems unlikely to explain the relatively high perivitelline Po_2 late in development. Jelly capsule thickness decreases during the period before hatching competence from an average of 0.8 mm on the day of oviposition to 0.23 mm at 2 d and 0.08 mm at 4 d (I. Gomez-Mestre, unpublished data), leaving hatching-competent eggs with little jelly barrier left to lose. Egg diameters also did not increase during the relevant developmental period (4–6 d).

As well as the expected variation in oxygen levels among eggs, we found surprisingly high variation in Po_2 within eggs. Despite the constant circulation of the perivitelline fluid, it is evidently not well mixed. First, there is a strong spatial gradient in oxygen: levels are high close to the air-exposed surface where oxygen enters and much lower deeper within the egg. This spatial gradient is over twice the magnitude of the greatest developmental difference (2–4-d eggs) and substantially larger than the effect of surface exposure on average Po_2 in the center of eggs (cf. Figs. 1, 3, 4). Perivitelline fluid moving past the air-exposed egg surface will pick up oxygen and then lose that oxygen to the embryo as it passes over the body and gills. The dramatic among-egg variation in superficial oxygen measurements presumably reflects this process, with little oxygen present in fluid that has just reached the surface and much more oxygen in fluid that has already passed across the exposed surface. As well, the strong spatial gradient within the egg suggests that the oxygen flux through the egg is high.

Furthermore, at a single position within the egg, oxygen levels vary substantially over time. The range of Po_2 in the center of individual eggs is, on average, lower than the spatial gradient across eggs, but it is comparable to the developmental changes and effects of surface exposure (Figs. 1, 2, 4). Moreover, oxygen transients at a single point can be extremely rapid (Fig. 3). In general, these transients occur when the embryo moves, necessarily altering the flow pattern of the perivitelline fluid, presumably mixing and moving patches of fluid of different oxygen levels, and often moving toward or away from the probe tip. It is possible that transients not associated with gross movements of the embryo are caused either by gill flicking, which we did not record, or by changes in buccal pumping of older embryos.

Our oxygen recordings showed a higher frequency of short-term high and low points in older eggs. This could reflect a developmental increase in the frequency or strength of embryo movements. Alternatively, as embryos develop and increase their oxygen demand, the oxygen gradient within the egg may increase, potentially magnifying the effect of embryo movements on Po_2 at any position within the egg. We also found that Po_2 was stable for a higher proportion of the time in eggs with less exposed surface area, suggesting a difference in embryo behavior between highly and poorly exposed eggs.

What Effects Does the Variation in Po_2 Have?

Embryos of *A. callidryas* tolerate a broad range of oxygen levels without showing effects on development, at least at the level of external morphology. Eggs that entirely lack air-exposed surface do not develop normally. Those submerged underwater early in development or fully covered by other eggs in the clutch are at least developmentally retarded and often die (Pyburn 1970; K. M. Warkentin, personal observation). However, even as little as 15% exposed surface is enough to support normal development. Moreover, although unexposed eggs are obviously developmentally abnormal, development is highly synchronous among embryos exposed to a wide range of oxygen levels. Thus, more exposed embryos appear to gain little from their more favorable respiratory environment, although small internal differences in development cannot be ruled out without further investigation. Embryos of *A. callidryas* must be proficient at oxygen uptake under fairly hypoxic conditions; development rate regulation implies similar regulation of metabolic rates across a broad range of perivitelline Po_2.

Comparable perivitelline Po_2 measurements are available for only a few species. Oxygen levels calculated from capsular conductance under ideal incubation conditions, in air or air-saturated water, are often quite high, ~15 kPa for eggs of seven

species of terrestrial and pond-breeding amphibians (Seymour 1994; Seymour and Bradford 1995). Field data indicate that oxygen levels around aquatic eggs can vary widely (e.g., 2.9–19.3 kPa for *Crinia georgiana*), causing egg mortality at the low extreme (Seymour et al. 2000). The oxygen levels in poorly exposed *A. callidryas* eggs are well into the range that is associated with reduced metabolism, slower development, and induced hatching in other amphibians, although there is substantial variation in embryonic tolerance of hypoxia (Table 1). The best comparative data from terrestrial eggs are for *Pseudophryne bibroni*. At 12°C, when development to hatching competence at stage 26 or 27 (Gosner 1960) takes approximately 39 d, perivitelline Po_2 below 10.3–10.6 kPa limits metabolism (Seymour and Bradford 1987; Bradford and Seymour 1988). This corresponds to an external Po_2 of 14.7 kPa, that is, a drop of 4.4 kPa across the egg capsule (Bradford and Seymour 1988). Embryos incubated at an external Po_2 of 6.9 kPa died without hatching (Bradford and Seymour 1988). Since capsular conductance does not change with metabolic demand or ambient Po_2 (Seymour et al. 1991), and metabolism is declining with Po_2 within this range, this would correspond to a perivitelline $Po_{2(in)}$ of no less than 2.5 kPa. The much faster developing terrestrial eggs of *A. callidryas* develop normally at substantially lower average oxygen levels. Comparable tolerance of hypoxia may be more common in aquatic eggs (e.g., *C. georgiana*; Seymour et al. 2000).

Hatching-competent *A. callidryas* embryos also tolerate very low oxygen levels without hatching. Normal, healthy 5- and 6-d-old embryos that hatch readily and rapidly in response to a variety of biotic risks (Warkentin 1995, 2000*a*; Warkentin et al. 2001) remain in eggs with center-average Po_2 as low as 1.5 kPa. Four-day embryos that are becoming hatching competent and may hatch in response to risk remain in eggs with center-average Po_2 as low as 0.5 kPa. Like several other amphibians (Petranka et al. 1982; Bradford and Seymour 1988), *A. callidryas* embryos hatch in response to oxygen stress imposed either by submerging egg clutches underwater or by exposing them to hypoxic gas mixtures (7 kPa; Warkentin 2002). Perivitelline Po_2 was not measured in those experiments, but our measurements presented here suggest that the perivitelline Po_2 levels required to maintain *A. callidryas* within the range of regulated development and behavioral tolerance are comparatively low (Table 1). However, the level of temporary hypoxia that embryos tolerate without hatching may in general be lower than that which

Table 1: Comparison of embryonic tolerance of hypoxia in aquatic and terrestrial amphibians

Location of measurement	Po_2 (kPa)	T (°C)	Effect	Source
Aquatic eggs:				
Crinia georgiana:				
External (water)	8–21[a]	15	Reduced $\dot{V}o_2$	Seymour and Roberts 1995
Perivitelline	13.3[b]	15	Reduced $\dot{V}o_2$	
External (air)	15	15	Early hatching	Seymour et al. 2000
External (air)	2	15	Abnormal/slow development	
External (jelly)	2.9	12–17	Death	
Ambystoma maculatum and *annulatum*:				
External (water)	15.9	15	Slowed development	Mills and Barnhart 1999
Terrestrial eggs:				
Pseudophryne bibroni:				
Perivitelline	10.6	12	Reduced $\dot{V}o_2$	Seymour and Bradford 1987
External (air)	14.7	12	Reduced $\dot{V}o_2$	Bradford and Seymour 1988
External (air)	6.9	12	Death	
External (air)	10	17	Slowed development	Seymour et al. 1991
Chiromantis xerampelina:				
External (foam)	10	25	Reduced $\dot{V}o_2$	Seymour and Loveridge 1994
Philoria (=*Kyarranus*) *loveridgei*:				
External (jelly)	7.6	20	Slowed development	Seymour et al. 1995
Bryobatrachus nimbus:				
Perivitelline	3.3–8.7[c]	5–20	Reduced $\dot{V}o_2$	Mitchell and Seymour 2003
Agalychnis callidryas:				
External (air)	7	26–29	Early hatching	Warkentin 2002
Perivitelline	.5–16.5	26–29	Normal development	This study

Note. We include reports of Po_2 that limits metabolism ($\dot{V}o_2$) or causes developmental retardation, developmental abnormalities, or embryo mortality and indicate whether values are for perivitelline Po_2 or for Po_2 external to the egg.

[a] Range across developmental stages.

[b] For near-hatching embryos, calculated from $Po_{2(out)}$ and change in Po_2 across capsule.

[c] Estimated from young larvae.

limits development. For instance, *P. bibroni* hatch in air at a $Po_{2(out)}$ of 5.2 kPa but do not hatch at an external Po_2 of 6.9 kPa that would, eventually, be lethal to younger eggs (Bradford and Seymour 1988). We also found no evidence that spontaneous hatching is associated with declining oxygen availability within *A. callidryas* eggs. To the contrary, the lowest oxygen levels we recorded were at the onset of hatching competence, not at 6 d when most spontaneous hatching occurs.

How Do Agalychnis callidryas *Embryos Tolerate Such Low* Po_2*?*

Agalychnis callidryas maintain rapid, synchronous development across a broad range of perivitelline Po_2, unlike many embryos that show developmental delays or stasis under hypoxia (Booth 1995; Mills and Barnhart 1999; Seymour et al. 2000; Padilla and Roth 2001). We suggest that three factors contribute to this ability: (1) the patchy oxygen environment within eggs, (2) prolonged maintenance of large external gills (Warkentin 2000*a*, 2002), and (3) embryo behavior.

In a patchy environment, the oxygen available to the embryo will depend not on the average Po_2 in the egg but on the amount of oxygen in the subset of fluid passing over its respiratory surfaces. Although this could be worse than average, it could also be substantially better. For instance, the surface Po_2 in poorly exposed 4-d-old eggs ranged up to 15.2 kPa and in all but one case was higher than the average center Po_2 in highly exposed eggs of the same age (9.5 ± 1.1 kPa, $N = 12$; 4.8 ± 0.4 kPa, $N = 24$, respectively). The ability of embryos to benefit from this patchiness of oxygen within their eggs will depend on spatial positioning of respiratory surfaces.

Anuran embryos exchange gases through external gills, skin, and potentially also internal gills. Of these surfaces, the most spatially flexible is the external gills. *Agalychnis callidryas* embryos can move their external gills independently of the body, with close to hemispherical freedom of position on each side, and the gills can extend to the far side of the egg, away from the embryo's body. Thus, in a variety of body positions, embryos can and often do position their gills against the egg membrane. Appropriate positioning of gills within higher oxygen patches might substantially improve oxygen uptake rates. Consistent with this, the maintenance of embryonic development past the point of hatching competence appears to depend on the external gills because inducing gill regression also induces hatching (Warkentin 2002). As much as reducing the total available respiratory surface, gill regression could compromise the ability of embryos to exploit the patchiness of their oxygen environment.

In addition to the positioning of respiratory surfaces, a second aspect of embryo behavior might affect oxygen uptake. Gross movements of the embryo mix the perivitelline fluid and so could alter the diffusion gradient across the egg surface, potentially increasing oxygen flux into the egg. These movements must also disrupt boundary layers around the embryo's body, which could improve cutaneous gas exchange, as flicking the gills may improve branchial gas exchange. The embryos of *A. callidryas* perform frequent movements within their egg unrelated to hatching. Such movements may play a role in the development of musculoskeletal and nervous systems (Müller 2003). However, they may also have a role in the oxygen uptake required for development (Kuang et al. 2002).

Work with predators and pathogens of eggs and hatchlings has demonstrated that *A. callidryas* embryos respond to conditions in their environment, altering the timing of hatching to balance shifting pre- and posthatching risks (Warkentin 1995, 2000*a*; Warkentin et al. 2001). In this species, when to hatch is clearly an important behavioral decision. However, it may not be the first important behavioral decision. In the sparse and patchy oxygen environment within *A. callidryas* eggs, the precise position of an embryo's external gills and body and the nature and frequency of its movements may be critical for adequate oxygen uptake. Thus, embryo behavior may not only allow early hatching to escape from egg predators. It may also facilitate the extended development and delayed hatching that improves survival with tadpole predators (Warkentin 1995, 1999*a*).

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