Review

Oxygen, gills, and embryo behavior: mechanisms of adaptive plasticity in hatching

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Abstract

Many species alter the timing of hatching in response to egg or larval predators, pathogens, or physical risks. This plasticity depends on separation between the onset of hatching competence and physiological limits to embryonic development. I present a framework based on heterokairy to categorize developmental mechanisms and identify traits contributing to and limiting hatching plasticity, then apply it to a case of predator-induced hatching. Red-eyed treefrogs have arboreal eggs, and tadpoles fall into ponds upon hatching. Egg and tadpole predators select for earlier and later hatching, respectively. Embryos hatch up to 30% early in predator attacks, and later if undisturbed. They maintain large external gills throughout the plastic hatching period, delaying gill regression while development otherwise continues. Rapid gill regression occurs upon hatching. Prolonged embryonic development depends on external gills; inducing gill regression causes hatching. External hypoxia retards development, kills eggs, and induces hatching. Nonetheless, embryos develop synchronously and without hatching prematurely across a broad range of perivitelline PO2, from 0.5–12.5 kPa. Embryos exploit spatial variation of PO2 within eggs by positioning gills against patches of air-exposed surface. Respiratory plasticity and oxygen-sensitive behavior appear critical for the hatching plasticity that balances a predation risk trade-off across life stages.

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Keywords: Agalychnis callidryas; Anura; Developmental physiology; Hatching; Heterokairy; Phenotypic plasticity; Predation; Respiration

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1. Introduction

Hatching is a critical event in the lives of most animals. It is both a developmental event and a life history switch point, changing the physical environment, mobility, and interactions with other organisms including resources and natural enemies. The developmental timing of hatching can evolve in response to selection pressures on eggs and young (Shine, 1978), contributing to diversity such as between precocial and altricial birds, or direct-developing amphibians and those with larvae. Hatching timing can also be plasticly adjusted in response to environmental conditions. Physical conditions, such as flooding of terrestrially-incubated eggs of fishes and amphibians, have long been known to affect hatching timing (reviewed in Martin, 1999). Hatching plasticity in response to the biotic environment, specifically predators and pathogens, is a more recent discovery (Sih and Moore, 1993; Warkentin, 1995). Shifts in hatching timing in response to predators and pathogens have been demonstrated in several amphibians, two fishes, a lizard and a spider (Table 1). These organisms respond to cues in different sensory modalities, including chemical information (Chivers et al., 2001; Wedekind, 2002) and movement (Griem and Martin, 2000; Warkentin, 2005). They also use different mechanisms to escape from the egg (e.g. behavior, Warkentin, 2005; enzymatic digestion of the egg capsule, Touchon et al., 2006). Thus hatching plasticity has evolved multiple times, in different ways.

Hatching plasticity is adaptive if the optimal hatching stage or timing varies with environmental conditions and either embryos or, in species with parental care, a parent can detect the relevant conditions and modify hatching accordingly. Because the structure of eggs both protects and constrains animals developing within them, trade-offs that make optimal hatching the structure of eggs both protects and constrains animals developing within them, trade-offs that make optimal hatching possible are probably widespread. I present a general conceptual framework for mechanisms of adaptive plasticity in hatching. I address commonalities and differences between previous work on embryo responses to the physical environment and more recent work focusing on the biotic environment. I then use the best-studied case of adaptive hatching plasticity in response to predators and pathogens, the embryos of red-eyed treefrogs, to examine the interplay between physical and biotic factors. Specifically I ask: Does plasticity in respiratory physiology play a role in predation-sensitive hatching?

1.1. Hatching plasticity in red-eyed treefrogs

Red-eyed treefrogs, Agalychnis callidryas, inhabit lowland wet tropical forests from the Yucatan through Panama. They have semi-terrestrial development; eggs are laid on vegetation overhanging ponds and swamps and tadpoles fall into the water upon hatching. This creates an experimentally tractable separation between embryonic and larval environments, with non-overlapping sets of natural enemies in each. Undisturbed eggs typically hatch after 6 days of development in Panama, or 7 d in Costa Rica, but embryos are capable of hatching up to 30% prematurely. They hatch as early as 4 (Panama) or 5 d (Costa Rica) in attacks by egg-eating snakes or wasps, pathogenic fungus infections, or if submerged underwater (Warkentin, 1995, 2000b, 2002; Warkentin et al., 2001). In all cases, hatching allows tadpoles to escape from egg-stage risks. Hatchlings then face a new suite of aquatic predators. Premature hatchlings are more vulnerable than full-term hatchlings to five of six aquatic predators we have tested (Warkentin, 1995, 1999a, J. Vonesh and Warkentin unpublished). Thus egg predators and pathogens, and larval predators, exert opposing selection pressures on hatching timing.

2. How does selection act on hatching?

Life history theory predicts that switch point timing should be adjusted to minimize the ratio of costs to benefits across stages (Werner and Gilliam, 1984; Werner, 1986). With some simplifying assumptions, this ratio is commonly formulated as $\mu/g$, measuring costs as mortality rate ($\mu$), benefits as growth rate ($g$), and both as functions of size. This theory is size-based because vital rates (growth, mortality, and fecundity) are often size-dependent. For instance, prey can achieve size refuges from gape-limited predators, and the capacity to exploit specific resources often changes with size. Furthermore, growth rate provides an assay of resource availability, an important aspect of habitat or stage-specific benefits. Minimizing $\mu/g$ maximizes the chance of reaching each size, including reproductive size (Werner and Gilliam, 1984).

Can we apply this theory, developed for later switch points, directly to hatching? Growth and development are partially coupled processes; however, in oviparous species embryos accrue no new resources. Rather they deplete energy reserves as they develop, and their growth consists simply of water uptake and the conversion of yolk into other tissues. This process will be affected by a different suite of environmental factors than those affecting growth rate after feeding has begun. For instance, temperature, water and oxygen availability may have strong affects on embryonic development, via physiological processes (Spicer and Burggren, 2003), whereas prey availability, competitor density, and the energetic cost of foraging are irrelevant. Moreover, the risk of egg and hatchling mortality from most physical and biotic sources as well as the capacity to
Table 1: Plasticity in hatching in response to biotic risks

<table>
<thead>
<tr>
<th>Species</th>
<th>Risk</th>
<th>Change in hatching</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Accelerated hatching in response to egg stage risks (earlier E, smaller S, less developed LD)</td>
<td></td>
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<tr>
<td>Amphibians</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambystoma maculatum</td>
<td>Water mold</td>
<td>E, S, LD</td>
<td>Gomez-Mestre et al. (2006)</td>
</tr>
<tr>
<td>Agalchnis annae</td>
<td>Snake</td>
<td>E, S, LD</td>
<td>Gomez-Mestre and Warkentin unpublished</td>
</tr>
<tr>
<td>Agalchnis moreletii</td>
<td>Snake</td>
<td>E, S, LD</td>
<td>Gomez-Mestre and Warkentin unpublished</td>
</tr>
<tr>
<td>Agalchnis saltator</td>
<td>Snake</td>
<td>E, S, LD</td>
<td>Gomez-Mestre and Warkentin unpublished</td>
</tr>
<tr>
<td>Agalchnis spurrelli</td>
<td>Snake</td>
<td>E, S, LD</td>
<td>Gomez-Mestre and Warkentin (in press)</td>
</tr>
<tr>
<td>Pachymedusa dacnicolor</td>
<td>Snake</td>
<td>E, S, LD</td>
<td>Gomez-Mestre and Warkentin unpublished</td>
</tr>
<tr>
<td>Hyla regilla</td>
<td>Leech</td>
<td>E, S, LD</td>
<td>Chivers et al. (2001)</td>
</tr>
<tr>
<td>Hyperolius spinigularis</td>
<td>Frog, fly larva</td>
<td>E, S, LD</td>
<td>Vonesh (2005)</td>
</tr>
<tr>
<td>Hyperolius cinnamomeoventris</td>
<td>Fly larva</td>
<td>S</td>
<td>Vonesh (2000)</td>
</tr>
<tr>
<td>Rana cascadae</td>
<td>Leech</td>
<td>E, S, LD</td>
<td>Chivers et al. (2001)</td>
</tr>
<tr>
<td>Rana arvalis</td>
<td>Leech</td>
<td>E, LD</td>
<td>Laurila et al. (2002)</td>
</tr>
<tr>
<td>Rana sphenochelata</td>
<td>Crayfish</td>
<td>E, S</td>
<td>Johnson et al. (2003), Saenz et al. (2003)</td>
</tr>
<tr>
<td>Rana sylvatica</td>
<td>Water mold</td>
<td>E, S, LD</td>
<td>Touchon et al. (2006), Gomez-Mestre et al. (2006)</td>
</tr>
<tr>
<td>Bufo americanus</td>
<td>Water mold</td>
<td>E, S, LD</td>
<td>Touchon et al. (2006), Gomez-Mestre et al. (2006)</td>
</tr>
<tr>
<td>Fishes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coregonus sp.</td>
<td>Bacterial pathogen</td>
<td>E</td>
<td>Wedekind (2002)</td>
</tr>
<tr>
<td>Reptiles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacerta monticola (lizard)</td>
<td>Fungus</td>
<td>E, S, LD</td>
<td>Moreira and Barata (2005)</td>
</tr>
<tr>
<td>Invertebrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syctodes pallida (spider)</td>
<td>Spider</td>
<td>E, S</td>
<td>Li (2002)</td>
</tr>
</tbody>
</table>

B. Delayed hatching in response to post-hatching risks (later Lt, larger Lg, more developed MD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Risk</th>
<th>Change in hatching</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphibians</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambystoma barbouri</td>
<td>Flatworm, fish</td>
<td>Lt, Lg, MD</td>
<td>Sih and Moore, (1993), Moore et al. (1996)</td>
</tr>
<tr>
<td>Rana clamitans</td>
<td>Leech</td>
<td>Lt, Lg</td>
<td>Schalk et al. (2002)</td>
</tr>
<tr>
<td>Rana temporaria</td>
<td>Fish</td>
<td>Lt</td>
<td>Laurila et al. (2002)</td>
</tr>
</tbody>
</table>

* These species show predicted responses to the risks listed. Some of these and other species show no embryonic response to other risks, or potentially maladaptive shifts in hatching stage or timing (Laurila et al., 2002; Anderson and Petranka, 2003; Orizaola and Braña, 2004).

Exploit external resources are functions of development, because embryos are rapidly changing their morphology as well as physiological and behavioral capacities. Thus a development-based framework for understanding hatching timing may be more appropriate than a size-based framework (Petranka et al., 1982; Bradford and Seymour, 1993; Chivers et al., 2001; Wedekind, 2002), mechanical cues from predators and pathogens (Sih and Moore, 1993; Chivers et al., 2001; Wedekind, 2002), chemical cues from predators (Warkentin, 2005; Warkentin et al., 2006, 2007), oxygen stress cues indicating conditions in the physical environment (Petranka et al., 1982; Bradford and Seymou, 2002).
Hatching plasticity is essentially a form of heterokairy. Heterokairy is a word recently coined for plastic changes in developmental rate and sequence; it is the individual equivalent of evolutionary heterochrony (Spicer and Burggren, 2003). This provides a conceptual framework to organize different types of hatching plasticity as changes in rate, sequence, or both.

Considering rate, if hatching is an essentially developmental event embedded in an inflexible sequence, hatching timing can be shifted in two ways. First the overall rate of embryonic development could change, altering the timing but not stage of hatching (Fig. 1A, B). Such cases may often be simply direct effects of the environment on development. For instance, cooler eggs often develop more slowly and hatch later than warm eggs. In other cases they appear to be a specific adaptive response of embryos to poor conditions in the post-hatching environment, as indicated by cues from conspecifics (Voronezhskaya et al., 2004).

Second, hatching timing may be shifted by changing the rate of development only during a particular developmental period (Fig. 1A, C). This is likely to require specific regulatory mechanisms and reflect adaptive plasticity. For instance, under conditions unfavorable for larval survival some embryos develop to hatching competence then enter a period of slowed or static development, sometimes associated with reduced metabolism, delaying hatching until conditions for larval life improve (Bradford and Seymour, 1985; Darken et al., 1998; Martin, 1999). Others show one or more periods of diapause at earlier developmental stages, during which they are resistant to harsh conditions, then resume development and hatch after conditions improve (Wourms, 1972; Hamdoun and Epel, 2007).

If, instead, hatching is basically a life history switch point that simply occurs during development, it could be shifted forward or back in the sequence, so that animals hatch at different developmental stages (Fig. 1A, D). Where hatching stage is regulated in response to environmental conditions, such cases are also strong candidates for adaptive plasticity.

Since hatching is both a developmental and a life history event, the above scenarios define extremes, and hatching plasticity may often include a mixture of rate and sequence heterokairy in different traits. Elucidating this variation can reveal traits or processes that are functionally important for the plasticity. Traits that covary with hatching across environments, especially if their developmental timing differs from that of other traits, should be tested for a potential role in allowing and/or limiting hatching plasticity. Identifying such traits will require detailed studies of development, beyond simple application of standard staging tables that assume a uniform, fixed developmental sequence.

3.2. Mechanisms allowing expression of a range of phenotypes

Hatching plasticity that takes the form of sequence heterokairy requires a developmental separation between the point at which hatching becomes possible and the point at which it becomes necessary (Fig. 2). Such separation depends on overlap between the periods when embryos can continue developing under the conditions within the egg and when they are physiologically competent to survive and develop in the environment outside the egg. It also depends on flexibility in the mechanism of hatching itself, so that embryos can leave the egg at different developmental stages. Thus hatching timing may be constrained by different traits and processes in different organisms.

For instance, the aquatic eggs of the streamside salamander, *Ambystoma barbouri*, delay hatching in response to chemical cues from flatworms and fish that prey on larvae, but not eggs (Sih and Moore, 1993; Moore et al., 1996). The latest hatching animals have depleted their yolk supplies, thus energetic constraints may limit further development in the egg (Sih and Moore, 1993, Warkentin pers. obsv.). Yolk supplies also limit the delayed hatching period for terrestrially incubated fish eggs that normally hatch after flooding. Embryos do not hatch without the cues that indicate an environment suitable for larval life, but instead die within the egg when their energetic reserve is depleted (Martin, 1999; Snyder and Martin, 2002).

The aquatic eggs of American toads, *Bufo americanus*, hatch early in response to pathogenic water molds (Gomez-Mestre et al., 2006; Touchon et al., 2006). The earliest hatchlings are at the tailbud stage of development (Gosner, 1960 stage 17), incapable of muscular movement. This timing corresponds to that of enzymatic degradation of the vitelline membrane, a critical element of the hatching process, in *Bufo japonicus* (Yamasaki et al., 1990), suggesting that the hatching mechanism itself may constrain timing in this case.

In general, the extent to which hatching can be accelerated in response to cues indicating egg-stage risk, and/or delayed in
response to cues indicative of larval risk (or in the absence of cues indicating suitable conditions for larvae) depends on when hatching would otherwise occur in relation to the factors that limit hatching timing (Fig. 2). Thus some species are capable of shifting hatching timing in both directions (e.g. Latham and Just, 1989). Others may be capable only of shifts in one direction, if they typically hatch as soon or as late as possible. An initial evolutionary stage of adaptive plasticity in hatching may be the coupling of hatching timing to environmental cues, within a pre-existing period of overlap between competence for embryonic and larval development. Opposing selection pressures on hatching timing under different conditions could act to broaden the period of hatching plasticity, and potentially to shift spontaneous hatching to one extreme within it.

3.3. Respiration as a constraint on hatching timing

Oxygen consumption increases developmentally, as embryos convert relatively inert yolk into metabolically active tissue, and hypoxia can slow development or even kill embryos (e.g. Bradford and Seymour, 1988; Booth, 1995; Cohen and Strathmann, 1996; Seymour et al., 2000). Hypoxia has also been suggested to be a general trigger of hatching (Petranka et al., 1982). It is the direct trigger of hatching in several terrestrial fish, frog, and salamander eggs that normally hatch when flooded, as aquatic boundary layers limit O₂ diffusion into the egg, although hydration is also necessary in some cases (DiMichele and Taylor, 1980, 1981; Bradford and Seymour, 1988). Oxygen stress can stimulate hatching earlier in development in fully aquatic eggs as well, and in some cases supplemental O₂ delays hatching, suggesting that embryos may hatch when their oxygen demand surpasses O₂ diffusion into the egg (Latham and Just, 1989; Seymour et al., 2000; Czerkies et al., 2001). Fully terrestrial eggs of amphibians and reptiles can be killed by flooding at some developmental stages, but some species hatch prematurely if flooded later in development (A. callidryas, Pyburn, 1970; Warkentin, 2002; Anolis sagrei, Losos et al., 2003; Agalychnis spurrelli, Gomez-Mestre and Warkentin, in press). Hypoxia is the likely stimulus to hatch in at least the amphibians, since exposure to hypoxic gas mixtures also induces premature hatching (Warkentin, 2002).

4. Does respiratory plasticity facilitate predation-sensitive hatching in A. callidryas?

A. callidryas embryos have four potential gas exchange organs: skin, internal gills, external gills, and lungs. Lungs can contribute to oxygen uptake only after hatching, when larvae have access to air. Internal gills are likely of limited respiratory value within the egg, although well developed embryos sometimes buccal pump, circulating perivitelline fluid through their branchial chambers. Cutaneous respiration is sufficient to support embryonic development in some anamniotes (Pelster and Burggren, 1996; Territo and Burggren, 1998); this is particularly likely for small, singly laid eggs at cool temperatures. The eggs of A. callidryas, however, are large (~3 mm diameter at oviposition, ~5 mm at hatching) and develop rapidly in their warm tropical environment, both factors that increase oxygen demand. Moreover, they are packed tightly together in egg masses, reducing their surface area exposed to air for gas exchange. Embryos, however, develop normally and in synchrony in eggs with air-exposed surface area ranging from 15% to over 70% (Warkentin et al., 2005). The external gills of A. callidryas are large, compared to those of many anuran embryos. They extend beyond the length of the body (2.5 mm
long gills on an embryo 10.5 mm in total length, Warkentin, 1999b), with more than hemispherical freedom of position, and are extensively branched. The morphological elaboration of the gills suggests that they are functionally important.

4.1. The form of heterokairy in hatching plasticity

Development of *A. callidryas* is different for animals that hatch early and spend the plastic hatching period in the water, compared to those that hatch late and spend the same period in the egg (Warkentin, 1999b). Embryos progress synchronously through the standard stages of anuran development until they become hatching-competent, in Gosner (1960) stage 23, then remain in that stage until they hatch. This suggests that hatching plasticity is based on rate heterokairy, with developmental stasis or slowed development during the plastic hatching period (as in Fig. 1A, C). Staging tables, however, focus on a specific subset of developmental events. If those events show sequence heterokairy, such tables are inadequate to describe developmental progress. In fact, in *A. callidryas* there is substantial development of external and internal mouthparts, branchial baskets, lungs, gastrointestinal tract, tail musculature, and pigmentation, as well as a ∼25% increase in total length of embryos through the plastic hatching period. These events proceed in the same sequence in early- and late-hatched animals; however, both hatching and gill regression can occur either before or after various other developmental events. A subset of these events is shown in Fig. 3, revealing that hatching plasticity in *A. callidryas* includes two kinds of heterokairy. If hatching occurs early (sequence change), then gill regression also occurs early (sequence change), while other aspects of development proceed in the same sequence, but faster (rate change).

While hatching early allows escape from egg predators, the continued embryonic development of hatching-competent *A. callidryas* improves tadpole survival with aquatic predators after hatching (Warkentin, 1995, 1999a). Several developmental changes contribute to tadpole performance. For instance, lung growth enables buoyancy control, oral disk development improves ability to adhere to the water surface, hatchlings become more behaviorally responsive to predators, and tail development may improve swimming performance (Parichy and Kaplan, 1995; Warkentin, 1999b).

The variation in development rate, in contrast, probably reflects a constraint on embryonic metabolism. These animals benefit from hatching both more developed and earlier, which should select for rapid embryonic development. Embryo development is not constrained by resources, since even the latest-hatched *A. callidryas* have substantial yolk reserves. Nor does feeding contribute to the immediate increase in development rate upon hatching, since these tadpoles do not begin feeding until at least a day later (Fig. 3). The developmental coupling of hatching and external gill loss suggests two things. First, the constraint on embryonic development rate may be respiratory. Second, maintenance of external gills may be required for hatching-competent embryos to continue developing in the egg, and/or hatching may be necessary for gill loss.

4.2. Timing of gill regression and hatching

Under normal environmental conditions, hatching-competent *A. callidryas* embryos have large, well perfused external gills, while recently hatched tadpoles do not (Fig. 4). The process of gill regression begins immediately upon hatching, and occurs rapidly. Tadpoles hatched into normoxic water and allowed access to air reduce gill perfusion within 4 min of hatching, and their gills shrink to about half of pre-hatching length within 10 min (Warkentin, 2000a). This occurs in animals hatched at the modal hatching age, as well as those hatched one or two days prematurely. Further regression of the gills is faster for older hatchlings, but tadpoles of all ages are gillless, or nearly so, within 24 h after hatching (Warkentin, 2000a). Although few comparably detailed data are available, in many anurans external gill regression appears to be a more gradual process, and occurs some time after hatching in a stable respiratory environment (reviewed in Warkentin, 2000a). Rapid gill regression immediately following hatching has, however, been reported in at least one other anuran, *Gastrotheca riobambae* (del Pino and Escobar, 1981).

Fig. 3. Developmental trajectories for *Agalychnis callidryas* hatched early (upper) and late (lower) within the plastic hatching period (hatched). The timing of standard developmental stages in the period before hatching competence (stippled) does not differ between embryos that will hatch at different stages: (a) onset of circulation in external gills (Gosner, 1960, stage 20), (b) corneas transparent (Gosner 21), and (c) opercular fold covers base of gills (Gosner 23). Subsequent developmental trajectories depend on hatching timing, in two ways. Some traits show rate heterokairy in which early hatched tadpoles develop faster than embryos, but events occur in the same sequence: (d) first gut coils formed and jaw sheaths keratinized, (e) mature tooth row ridge configuration, with some keratinization of denticles, (f) onset of feeding. Both hatching and external gill regression show sequence heterokairy, occurring at different times relative to other developmental events, but with gill regression following immediately upon hatching.
Despite their normally tight association, hatching is neither necessary nor sufficient for external gill regression in *A. callidryas*. Instead, this process depends more on oxygen availability, as also occurs in the toad *Stephophaeas anotis* (Channing, 1993). At the modal hatching age, tadpoles hatched into hypoxic water and denied access to air maintain gill circulation, and shorten their gills only slightly. It is also possible to induce gill regression prior to hatching by removing eggs from their clutches and hanging them individually in netting, to maximize the surface exposed to air. Gill regression is more gradual in these embryos than in hatchlings, but some completely lose their gills and remain in the egg until the modal hatching age (Warkentin, 2000a). Given the natural association of gill loss with hatching in *A. callidryas*, the dependence of this process on oxygen availability suggests that hatching improves gas exchange. Like the pattern of heterokairy, above, oxygen-dependent gill loss is also consistent with a functional role for the gills in hatching plasticity.

To directly test the hypothesis that external gills are necessary for hatching-competent *A. callidryas* embryos to delay hatching I manipulated gill regression in two ways (Warkentin, 2002). First, in egg clutches exposed to a hyperoxic gas mixture, as in separated eggs, embryos gradually lose their gills. When these clutches are returned to air (normoxia) before they would normally hatch, many embryos hatch immediately. Control clutches in air or left in the hyperoxic gas hatch later (Fig. 5C). Gill regression is variable, however, among eggs in hyperoxia-exposed clutches. Some individuals retain gills, or are able to increase gill size and perfusion after their return to air, confounding the test of gill function. Prostaglandin treatment offers a second, more consistent method of inducing gill regression. A PGE1 analog, misoprostol, induces rapid, local loss of perfusion and shortening of the external gills, similar to the natural post-hatching process (Warkentin and Wassersug, 2001; Warkentin, 2002). These misoprostol-treated embryos hatch shortly after regressing their gills, while carrier controls remain in the egg (Fig. 5D). Concurrent exposure to hyperoxia (35 or 42% O2 gas mixtures) reduces, but does not fully rescue, the misoprostol effect on hatching.

The parallel results of two methodologically different tests provide strong support for the hypothesis that, under natural incubation conditions, hatching-competent *A. callidryas* embryos do not remain in the egg without their external gills. Gilless, hatching-competent embryos can remain in fully exposed eggs in air. Under hyperoxia, they can also remain in a subset of eggs within clutches. Nonetheless, even strong hyperoxia is insufficient to prevent most fully gillless embryos in natural clutches from hatching (Warkentin, 2002). Delaying external gill regression thus appears critical to the ability of *A. callidryas* to continue embryonic development substantially beyond the onset of hatching competence.

### 4.3. Respiratory environment of *A. callidryas* embryos

Several lines of evidence, above, suggest constraints on oxygen availability to red-eyed treefrog embryos. We used fiberoptic microprobes to measure oxygen levels inside *A. callidryas* eggs with developmentally normal embryos, in the approximate center of the perivitelline space (Warkentin et al., 2005). As expected, PO2 varied with both egg development and surface exposure. There was more oxygen in more exposed eggs and, regardless of exposure, PO2 declined with development until embryos became hatching-competent, at age 4 d (Fig. 6). Presumably the latter result reflects a developmental increase in metabolic rate, as embryos convert relatively inert yolk into metabolically active tissue. After embryos were hatching-competent, perivitelline PO2 stabilized or, for poorly exposed eggs, even increased slightly during the plastic hatching period.

We also found surprisingly low levels of oxygen in normally developing eggs with hatching-competent embryos that were,
nonetheless, not hatching. At the onset of hatching competence, across eggs less than 50% exposed to air, PO2 was 2.2±0.3 kPa (mean±SE, throughout). This is within the range that retards development, induces hatching and even kills some other terrestrial frog eggs (e.g., Bradford and Seymour, 1988). From individual hatching-competent eggs developing in synchrony we recorded mean PO2 ranging from 0.5–12.5 kPa. Hypoxia induces hatching in A. callidryas, naturally when clutches are flooded by rising pond water and experimentally when clutches are exposed to hypoxic gas mixtures (Fig. 5A, B, Warkentin, 2000b, 2002). It is therefore surprising that embryos tolerate such low PO2 without hatching and that moreover, at least based on external morphology, this large variation in PO2 does not appear to affect development rates.

How do A. callidryas embryos in poorly exposed, low PO2 eggs maintain rapid development and refrain from hatching, while even fully exposed individual eggs hatch if submerged in water? At least part of the answer may lie in the combination of embryo behavior and the PO2 variation within terrestrially incubated eggs. We found both strong spatial gradients and rapid temporal transients of PO2 within A. callidryas eggs (Fig. 6, Warkentin et al., 2005). In hatching-competent eggs we found an average oxygen gradient of 9±0.8 kPa from just inside the egg membrane to deeper within the egg. Even in poorly exposed eggs, with little oxygen in the center of the egg, PO2 is relatively high just under the air-exposed surface (9.6±1.1 kPa). The constant ciliary circulation of the perivitelline fluid is, therefore, far from sufficient to homogenize oxygen levels throughout the egg. Furthermore, oxygen levels at a single measurement point within eggs varied as much over a period of a few minutes as average levels varied among eggs across the range of surface exposures or developmental stages (Fig. 6). For hatching-competent eggs, PO2 in the center of the egg varied as much as 11.3 kPa during recordings (mean 3.5±0.2 kPa). Most of the rapid oxygen transients were clearly associated with movements of the embryo, which must move patches of well- and poorly-oxygenated fluid within the egg.

There are few comparable oxygen measurements from within eggs, but most of those previously published describe neither such short-term temporal variation, nor such steep spatial gradients in the perivitelline fluid. They report the level of variation across eggs, as shown in the SE in Fig. 6, and do not address any potential variation within eggs (Seymour and Bradford, 1987; Seymour and Roberts, 1995). A spatial oxygen gradient of 5 kPa has been reported in the perivitelline fluid in snail egg capsules within gelatinous egg masses, from near the
outward-facing surface of the egg to its center (Kuang et al., 2002). Without more measurements of other species that specifically report PO2 variation within eggs, however, it remains unclear if A. callidryas have unusually variable oxygen levels in their eggs or if this is characteristic of other species as well. Such variation, despite the constant mixing, suggests a strong O2 flux and may be most likely for large, rapidly developing, terrestrial eggs with only partial surface exposure. For A. callidryas, however, the strong spatial gradient in oxygen levels within eggs suggests that the location of embryonic respiratory surfaces could have a substantial effect on oxygen uptake.

4.4. Adaptive behavior of embryos: optimal orientation for O2 uptake?

Selectively positioning external gills in the high oxygen zone near air-exposed egg surface might substantially improve oxygen uptake, particularly for those embryos in the eggs with the lowest interior PO2. This would be essentially a behavioral counterpart to the development of a high density of chorioallantoic blood vessels adjacent to the air space in bird eggs (Reizis et al., 2005). Such behavior might reduce variation in metabolic rate among eggs with different exposures, offering a potential explanation for the developmental synchrony of eggs with very different central PO2 values. It could also explain the seemingly contradictory results that hypoxia induces premature hatching, but embryos refrain from hatching at PO2 as low as 0.5 kPa in the center of the egg. This mechanism depends critically on three things: (i) the availability of at least a small well-oxygenated area within the egg, (ii) the presence and functional value of external gills, and (iii) the behavioral ability to position gills within the high-O2 area.

Almost all A. callidryas eggs have some air-exposed surface, creating a high oxygen area within the egg (Warkentin et al., 2005). Consistent with the proposed mechanism, the few eggs trapped behind their clutchmates, without exposure to air, are developmentally retarded and often die. Similarly, young eggs in water die while older eggs in water or hypoxic gas hatch prematurely (Pyburn, 1970; Warkentin, 2002). Both submergence and gaseous hypoxia reduce PO2 at the exposed egg surface, presumably compromising the high-O2 patch within the egg.

The proposed mechanism is also consistent with the premature hatching of externally gillless embryos from egg clutches in air (Warkentin, 2002). Amphibian embryos can respire cutaneously. Hatchable A. callidryas, however, curl most or all of the way around the egg’s circumference, and one side of their body faces the egg interior (Figs. 4 and 7). Thus even embryos in the best position with respect to their patch of air-exposed egg surface have much of their skin in low oxygen areas. The gills provide a more spatially flexible and concentrated surface for gas exchange. Even placing egg clutches in hyperoxic gas only reduced, but did not prevent, the hatching of fully gillless embryos, suggesting that it did not restore oxygen uptake to gilled levels for many individuals (Warkentin, 2002). This is consistent with the variable extent of gill regression in hyperoxia-exposed clutches; cutaneous gas exchange may be inadequate in poorly exposed eggs even under hyperoxia. In contrast in fully exposed eggs, where embryos regressed their gills without hatching, about half of the skin would be exposed to high PO2 along the egg surface (Warkentin, 2000a).

Initial results from research currently in progress (K.M. Warkentin and J.R. Rogge) suggest both that the external gills contribute substantially to O2 uptake, and that embryos actively position their gills near the air-exposed egg surface. Based on closed-system respirometry, and one method of inducing gill regression, embryos with gills appear capable of regulating their metabolic rate at lower external PO2, compared to gillless embryos. Observations of embryo positions indicate that they are highly non-random, with gills usually near the air-exposed patch of egg surface and rarely in the interior of the egg (Fig. 7). Moreover, embryos experimentally displaced to position their gills away from the exposed surface move to return their gills to the exposed patch within seconds. We predict that embryo behavior will also vary with development and egg surface exposure, with older embryos and those in less exposed eggs being more constrained with respect to gill position. Behavioral observations of snail embryos, Helisoma trivolvis, also reveal responses to changes in oxygen availability. These embryos speed up ciliary rotation under hypoxia, and the embryos change position within their relatively large egg capsules.
moving toward the periphery of the egg mass (Kuang et al., 2002). Both of these responses should function to improve O$_2$ availability. Thus embryo behavior could play an adaptive role in oxygen uptake in diverse taxa.

5. Conclusions and future directions

Although respiratory structures are renown for their plasticity in response to oxygen availability and demand, the development and regression of external gills in anurans have been considered canalized events, suitable as general markers of development (e.g., Nieuwkoop and Faber, 1956; Gosner, 1960). In part, this may be because the physiological role of these structures has been equivocal (Burggren and Just, 1992). Diffusion alone provides sufficient oxygen for embryos of some well-studied amniotes (Pelster and Burggren, 1996; Territo and Burggren, 1998). Clearly however, this is not the case for all species or under all incubation conditions, particularly not for larger eggs at higher temperatures, even for eggs incubated in air (Seymour, 1999). *A. callidryas* provides one example where the external gills are physiologically important. In such species, gill regression may often be environment-dependent, resulting in sequence heterokairy that would make it a poor marker of developmental progress.

In *A. callidryas*, extended maintenance of the external gills is necessary for hatching-competent embryos to continue developing in the egg and so improve their chance of survival with aquatic predators. Thus respiratory plasticity contributes to adaptive plasticity in hatching. It is not clear though, if the facultative delay in gill regression evolved under selection for later or more plastic hatching, or if pre-existing respiratory plasticity facilitated the evolution of hatching plasticity. This could be assessed by a phylogenetic analysis of developmental physiology.

In conjunction with gill maintenance, the oxygen-sensitive behavior of embryos appears critical for plasticity, and perhaps also for normal development prior to hatching competence. Embryo behavior has been studied largely for its role in the development of capabilities that will be important in later life (Bate, 1999; Grillner, 2000; Bekoff, 2001). The discovery of risk-sensitive hatching showed that, at least in some cases, embryos respond to information about their environment in an adaptive manner. The likely role of embryo behavior in oxygen uptake adds a second, earlier, adaptive function of embryo behavior. Perhaps more importantly, it raises the question: How much embryo behavior reflects adaptive responses to their current environment, and how much is simply preparation for future life stages? Embryo behavior should be examined in other species where egg development is potentially oxygen limited, and any oxygen-sensitive behavior should be tested for current utility in O$_2$ uptake.

Respiratory plasticity and embryo behavior contribute to hatching plasticity in *A. callidryas* because oxygen uptake can constrain development in this species. Potential respiratory constraints on development are widespread, but far from universal. Thus other traits and processes must limit hatching timing, and plasticity in other species. Analyses of heterokairy and developmental overlap in traits and processes that support embryonic life, post-hatching life, and the process of hatching should reveal other mechanisms allowing phenotypic flexibility in hatching.

Risk-sensitive hatching timing has evolved multiple times, across a broad range of taxa in different ecological contexts. In most cases we know little about the mechanisms underlying this plasticity; however, it is clear that different mechanisms are important in different taxa. For instance, embryonic development may be constrained by resource limitation or oxygen stress, thus increased provisioning and improved O$_2$ uptake both contribute to plasticity. Embryos use environmental information in the form of mechanical disturbance, hypoxic stress, and chemical cues, indicating that multiple sensory pathways play a role in hatching timing across, and even within species. The process of hatching is largely enzymatic in some species and behavioral in others, requiring links between sensory pathways and different response mechanisms.

Thus hatching plasticity is constructed of a number of different components, which vary among species. Some of these components may have evolved under the environmental heterogeneity and opposing selection that favor hatching plasticity. Others are likely to be ancestral traits that facilitate the evolution of plasticity. If most components of plasticity are the latter, it may be relatively easy to evolve. Phylogenetic comparative analyses of traits contributing to hatching plasticity in clades wherein this plasticity varies should clarify to what extent this plasticity depends on pre-existing and derived traits.

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