

Do Prostaglandins Regulate External Gill Regression in Anurans?

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ABSTRACT Although the endocrinological mechanism controlling regression of the internal, larval gills of anurans (frogs and toads) is well understood, the mechanism regulating loss of the external, embryonic gills is not known. Based on the homology of the mammalian ductus arteriosus with a portion of the amphibian branchial arches, and the regulation of blood flow in the mammalian ductus by prostaglandins of the E family (PGEs), we hypothesized that anuran external gill loss is also regulated by PGEs. To test this hypothesis, we topically applied both PGE2 and a synthetic analogue of PGE1, misoprostol, to embryos and young hatchlings of the red-eyed treefrog, *Agalychnis callidryas*. Both agents accelerated external gill regression. Furthermore, misoprostol overrode the inhibitory effect of hypoxia on gill regression in hatchlings and induced rapid loss of external gills in embryos, which normally maintain the gills until hatching. These observations support the hypothesis that PGEs regulate anuran external gill loss. The specific site of action for prostaglandins within the gills is not known; however, PGEs are secreted in the oral mucus of tadpoles, and this could be a natural topical source for these agents. PGEs offer a tool for manipulation of external gills and should facilitate tests of the physiological importance of these structures. *J. Exp. Zool.* 289:366–373, 2001. © 2001 Wiley-Liss, Inc.

A defining feature of amphibian larvae is gills. In most anurans (frogs and toads) gills first appear in the embryos and are of two sorts. External gills are ostensibly embryonic structures that regress at hatching or shortly thereafter. Internal gills are retained much longer, until metamorphosis. The difference in longevity of internal and external gills suggests that different hormonal pathways control their fate. Internal gill loss is one of many metamorphic events regulated by thyroid hormones (Shi, 2000, and references therein). However, what controls external gill regression is not known.

Mammalian development includes an event parallel to anuran external gill regression that suggests a possible hormonal mechanism for its regulation. The ductus arteriosus in the mammalian fetus is evolutionarily derived from the dorsal (efferent) portion of the left sixth aortic arch of lower vertebrates (Warwick and Williams, '73). Whereas in the aquatic ancestor of mammals this channel carried blood from the gills to the body, in mammalian fetuses it shunts blood away from the pulmonary trunk and into the descending aorta. A similar shunt, also called the ductus arteriosus, is present in neotenic salamanders be-

tween the fifth aortic (gill) arch and the pulmonary artery (sixth arch) and carries the bulk of blood flow to the lungs (Malvin and Heisler, '88). In anurans, a series of shunts connect the afferent and efferent gill arteries, allowing blood to bypass the gill capillaries (De Saint-Aubain, '81).

Closure of the mammalian ductus arteriosus occurs at birth and is linked to lung inflation. Rapid closure of the ductus is critical for proper perfusion of the lungs and normal pulmonary function. Before birth dilation of the ductus arteriosus is maintained by prostaglandins of the E family (PGE), and patent ductus arteriosus, a clinical problem in premature neonates, is treated with inhibitors of prostaglandin synthesis (Olley et al., '76; Gersony et al., '83). The normal closure of the ductus in mammals and the regression of external gills in anurans are both developmentally rapid events that can occur on a time scale of min-

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utes to hours. Both events are part of the abrupt shift in respiratory function associated with the transition from embryonic to free-living existence. On the basis of these similarities, as well as the structural homology, we hypothesized that the maintenance of anuran external gills and the circulation through them is under prostaglandin control.

The mammalian condition, in which PGE maintains patency of the ductus, suggests that PGE might also maintain anuran external gill circulation. However, the anatomy and blood flow patterns in amphibian larvae suggest that instead the opposite may occur. Both gilled salamanders and anuran larvae have shunts that allow blood to bypass the gills or sections of them (De Saint-Aubain, '81; Malvin and Heisler, '88). Dilation of such shunts by PGE would reduce blood flow to the gills and could thereby initiate their regression.

We tested for PGE regulation of anuran external gill loss using embryos and hatchlings of the red-eyed treefrog, *Agalychnis callidryas*, selected because of their exceptionally long and unpigmented external gills and rapid gill regression (Pyburn, '63; Warkentin, '99, 2000b). *A. callidryas* occur in lowland wet forests from the Yucatan through Panama. They lay their eggs in clumps on leaves overhanging water, into which hatched tadpoles fall. Blood circulation in the external gills is easily visible under low magnification, both in arboreal embryos and in newly hatched tadpoles (Warkentin, 2000b).

In tadpoles that hatch at a normal stage of development (6 and 7 days after oviposition under natural conditions in Panama and Costa Rica, respectively; Warkentin, '95, 2000a) and fall into normoxic water, 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 (Warkentin, 2000b). Newly hatched tadpoles maintain their external gills longer when in hypoxic water and denied access to air, and tadpoles that hatch 2 days prematurely can maintain external gill circulation for a day post-hatching (Warkentin, 2000b).

We tested whether prostaglandin E2 and a potent and more stable synthetic analogue of prostaglandin E1, misoprostol, affected the rate of gill reduction in embryos and newly hatched tadpoles of *A. callidryas*. As expected, prostaglandins of the E family did affect gill size and perfusion. The direction of the effect was congruent with the dilation of shunts allowing blood to bypass the gills; PGEs accelerated gill regression.

METHODS

To obtain adequate samples of embryos, all experiments were performed in the field in Costa Rica and Panama. However, the logistical constraints of the field sites and the delicate nature of *A. callidryas* embryos and hatchlings limited us to topical application of drugs. Preliminary tests gave no indication that PGEs delayed gill regression, thus we tested if PGE accelerated gill regression. Experimental protocols complied with NIH guidelines.

PGE2 treatment of hatchlings

Sibling pairs of *A. callidryas* hatchlings were exposed to PGE2 (Sigma, St. Louis, MO) and control solutions, and the condition of their gills was monitored for up to 4.25 hr. Five young egg clutches were collected from a pond near Sirena Biological Station, Corcovado National Park, Costa Rica. The eggs were maintained in the shade in an open-air laboratory, and misted occasionally with stream water to prevent desiccation. At age 6 days, eggs were placed individually in 10-ml plastic Petri dishes containing 5 ml of either PGE2 in local stream water, serially diluted from concentrated ethanol to make a 2.83×10^{-5} mol/l solution, or a control solution of dilute ethanol matched in concentration to the PGE2 carrier (0.4%). All embryos hatched spontaneously upon immersion (as occurs if rising water submerges egg clutches in nature). Experimental treatments and paired sibling controls were run simultaneously ($N = 10$ pairs, 2 pairs/sibship). Animals were observed under a dissecting microscope at 30 \times . External gill perfusion and gill length were recorded immediately at hatching and at 4 additional post-hatching periods, as follows (mean \pm SE [range]): (i) 12.3 ± 0.9 [3–16] min; (ii) 28.7 ± 1.0 [20–35] min; (iii) 62.8 ± 2.5 [47–78] min; (iv) 236.2 ± 5.2 [175–255] min.

The regular structure of *A. callidryas* external gills, consisting of a gill trunk with a single row of simple branch filaments extending from it (Pyburn, '63; Warkentin, '99) allowed us to define easily distinguishable levels of perfusion. External gill perfusion was categorized, and ranked for statistical tests, as follows: (0) none = no movement of blood in external gills; (1) minimal = some circulation in main vascular trunk and two or fewer branches; (2) reduced = circulation in more than two branches, but missing in some branches or branch sections, or limited to corpuscles traveling single file in branch vessels; (3) full = good circulation through-

out the entire vascular field, with corpuscles traveling in parallel in branch vessels.

The external gills of *A. callidryas* hatchlings emerge immediately anterior to the yolk sac and extend caudally beside it. Gill length was thus estimated in relation to yolk length, in increments of $\frac{1}{8}$, following Warkentin ('98, 2000b). This method of measuring gill length relative to yolk sac size allowed rapid assessment of gill size in active tadpoles with minimum manipulation. Gill lengths were converted to mm using average yolk sac length from 207 hatchlings (see Warkentin, '98).

Misoprostol treatment of young hatchlings

Sibling pairs of hatchlings were exposed to misoprostol (Searle, Skokie, IL) and a carrier control, and their gill condition was monitored for at least 50 min. Eggs were collected and maintained as above. At age 5 days individual eggs were placed into 10-ml plastic Petri dishes containing 5 ml of either 2.60×10^{-6} mol/l misoprostol solution or a carrier control (0.08% ethanol) in stream water. Some embryos that did not hatch spontaneously within 1 min were induced to hatch by prodding the egg with a probe; in a few cases egg membranes were artificially ruptured. Tadpoles were observed at 30 \times at the time of hatching and at three subsequent post-hatching periods: (1) 14.4 ± 0.5 [10–17] min; (2) 30.5 ± 1.2 [22–39] min; (3) 115.7 ± 9.7 [51–154] min. At each observation the length of the external gills and the extent of their perfusion with blood were recorded, as above. Experimental treatments and paired sibling controls were run simultaneously ($N = 10$ pairs, 2 pairs/sibship).

Exposure of mature hatchlings to misoprostol and hypoxia

Because oxygen availability affects gill regression, a further experiment was conducted to explore the interaction of oxygen concentration and misoprostol. At 7 days post-oviposition, when most undisturbed *A. callidryas* in the Corcovado study population naturally hatch (Warkentin, '95), sibling sets of hatchlings were exposed to three treatments: misoprostol in a hypoxic solution and hypoxic and normoxic carrier solutions.

A stock solution of misoprostol, or its ethanol carrier, was mixed with hypoxic water (2.0 ± 0.2 mg/l O_2 , $23.3 \pm 0.5^\circ\text{C}$) or normoxic water (7.2 ± 0.06 mg/l O_2 , $23.0 \pm 0.3^\circ\text{C}$) immediately before testing. Hypoxic water was obtained by boiling stream water for at least 10 min and then cooling it overnight in a sealed container. Oxygen levels

and temperatures were measured with a Nester Instruments Model 8500 portable oxygen meter just prior to mixing solutions. The passive rise in PO_2 of hypoxic solutions over the course of the experiment was not measured.

Misoprostol was mixed to a nominal concentration of 5.20×10^{-6} mol/l using stock solution that had been refrigerated for 9–13 days. [Storing it frozen was not possible under field conditions.] Preliminary tests showed effects of this solution on 5-day hatchlings equivalent to fresh misoprostol at 2.60×10^{-6} mol/l. Eggs were collected and maintained as above. At 7 days, individual eggs were placed in 10-ml plastic Petri dishes filled to the brim with the solution and capped without an air space to reduce rise in PO_2 of hypoxic treatments. Embryos hatched spontaneously immediately after immersion. Gill length and perfusion were monitored as above for 20 min, after which hatchlings were transferred to normoxic water. All three treatments were run simultaneously for each sibship ($N = 10$ sibships, 3 hatchlings each).

Misoprostol treatment of embryos

The effect of misoprostol on embryos was assessed in two ways. First, a series of intact egg clutches were randomly assigned to misoprostol or control treatments. Second, clutches were divided and paired sibling sets of seven embryos each were treated. The egg clutches used in these experiments were collected from Ocelot Pond, 2 km south of Gamboa, Panama, and the experiments were conducted in an open-air laboratory at the Smithsonian Tropical Research Institute in Gamboa.

Ten intact egg clutches (26 ± 2.4 eggs, range 13–37) were hung in individual plastic cups and maintained as above. At age 5 days, the five experimental clutches were sprayed with 2 ml of 2.61×10^{-4} mol/l misoprostol to completely wet the clutch surface. All run-off solution was removed from the cup and replaced with fresh water. Control clutches were sprayed with the carrier solution; i.e., 2 ml of 0.8% ethanol in rainwater. The perfusion and length of the external gills was observed periodically with a dissecting microscope at 30 \times for 2–4.5 hr after treatment.

For divided clutches, eggs were separated at 3 days, i.e., 1 day before the embryos were capable of hatching and 3 days before the peak of spontaneous, undisturbed hatching in this population (Warkentin, 2000). Two sets of seven eggs were removed from each of 20 clutches, and each set was arranged in a 10-ml plastic Petri dish as a

central egg surrounded by six other eggs. This gave two levels of exposed surface area, equivalent to interior and peripheral eggs in natural clutches. [The experiment was designed to test the effect of PGE under a range of natural exposures, not to examine the interaction between PGE and exposure.] Eggs were misted lightly with rainwater, placed in a plastic tub over wet paper towel to maintain humidity, and covered with screening to exclude insects. At age 5 days, a 0.03-ml drop of 2.61×10^{-4} mol/l misoprostol or a 0.8% ethanol carrier control was placed on the center egg and gently spread to the peripheral eggs. Fifteen min later a naive observer examined the embryos at 30 \times , estimated gill length in increments of $\frac{1}{4}$ yolk length, and scored the gill perfusion as above for the peripheral embryo with the greatest circulation (a conservative test for PGE-induced gill regression) and for the central embryo.

RESULTS

All hatchlings emerged from the egg with long (≥ 1.9 mm), fully perfused gills and gill lengths were indistinguishable between experimental and control animals (Figs. 1–2; 5-day hatchlings, Friedman test statistic = 0.4, $P = 0.53$, $N = 10$ pairs; 7-day hatchlings, Kruskal–Wallis test statistic = 0.65, $P = 0.70$). However, by the first post-hatching observation period both PGE2 and misoprostol treatments induced changes in gill size and perfusion.

Hatchlings treated with PGE2 showed reduced gill length and perfusion compared to controls (Fig. 1). We used randomization tests based on repeated measures ANOVA to assess the effect of treatment, time, and interaction on gill length and, separately, on gill perfusion (Manly, '91). Both gill length and perfusion differed significantly across treatments and over time, but the treatment by time interaction was non-significant (length–treatment, $F_{1,18} = 6.9$, $P = 0.03$; time, $F_{4,72} = 120.6$, $P < 0.0001$; interaction, $F_{4,72} = 4.4$, $P = 0.9$; perfusion–treatment, $F_{1,18} = 14$, $P = 0.002$; time, $F_{4,72} = 12.9$, $P < 0.0001$; interaction, $F_{4,72} = 4.5$, $P = 0.19$; 5,000 repetitions).

Hatchlings treated with misoprostol at 5 days lost blood circulation in their external gills, and the gills shortened even more rapidly than with PGE2 (Fig. 2). After a ca. 15-min exposure to misoprostol, gill length and level of perfusion were significantly lower in experimental tadpoles than controls (Friedman tests: both test statistics = 10, both $P = 0.0016$, $N = 10$ pairs) and the difference was maintained over time (Fig. 2).

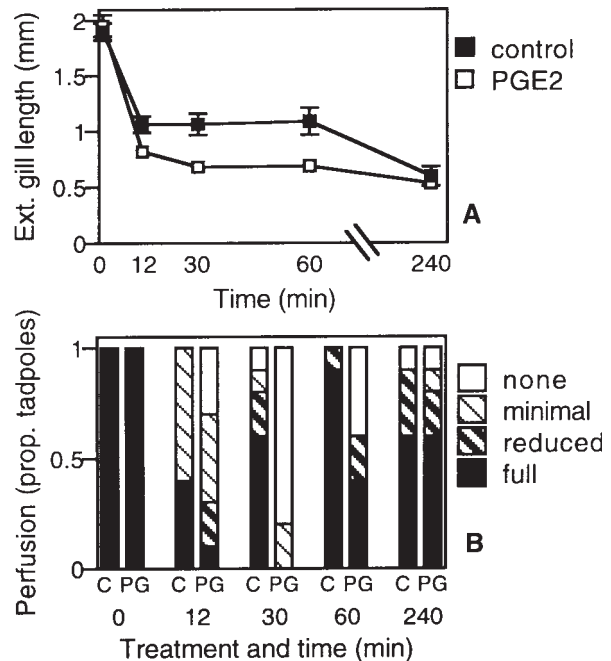


Fig. 1. Effect of prostaglandin E2 on the length (A) and perfusion (B) of transient external gills in *A. callidryas* hatchlings at several times post-treatment. Gill lengths are means \pm SE. Perfusion is proportion of animals showing each level of circulation in control (C) and experimental (PG) treatments; see Methods for definitions of levels. Times are approximate since different animals were measured at slightly different times post-treatment (see Methods for time ranges). PGE2 reduced gill size and perfusion.

The 7-day hatchlings in normoxic water rapidly lost external gill circulation and reduced gill size. In hypoxic water without PGE fewer tadpoles lost circulation, and gill length was not reduced as much as under normoxia. In contrast, hatchlings exposed to misoprostol in hypoxic water exhibited greater reduction in gill size and perfusion than even those in normoxic water without PGE (Fig. 3). After 20 min in hypoxic water, both external gill length and perfusion were significantly lower in misoprostol-treated hatchlings than in their sibling controls (Friedman tests: length, test statistic = 8.1, $P = 0.004$; perfusion, test statistic = 4.9, $P = 0.027$, $N = 10$ pairs).

Embryos exposed to misoprostol also exhibited reduced gill perfusion and gill shortening (Figs. 4 and 5). In groups of embryos exposed to misoprostol in Petri dishes, both central embryos, with little exposed surface, and peripheral embryos, with higher exposed surfaces, differed in gill length and perfusion from controls 15 min after treatment (Friedman tests: central, length, test statistic = 12.8, $P = 0.0003$; perfusion, test statistic = 5, $P = 0.025$; peripheral, length, test statis-

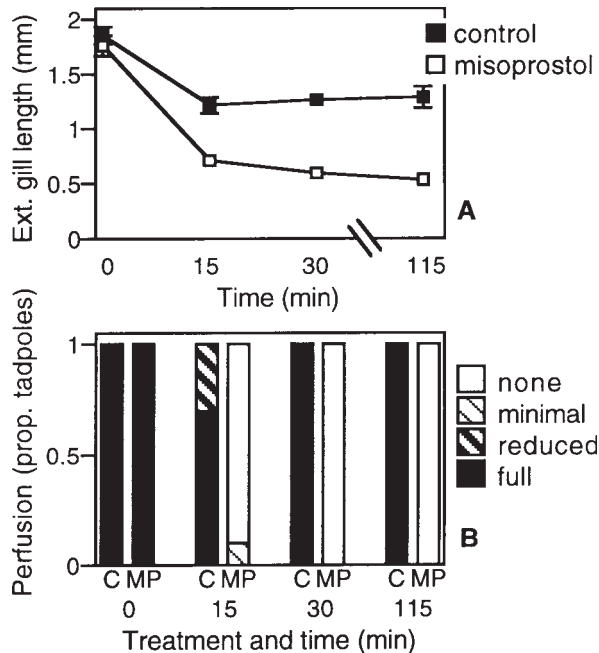


Fig. 2. Effect of a PGE1 analogue, misoprostol, on the length (A) and perfusion (B) of transient external gills in *A. callidryas* hatchlings at several times post-treatment. Gill lengths are means \pm SE. Perfusion is proportion of animals showing each level of circulation in control (C) and misoprostol (MP) treatments. Perfusion levels are defined in the methods, as are exact time ranges; times given here are approximate. Misoprostol reduced gill size and perfusion even more strongly than PGE2 (Fig. 1).

tic = 8.5, $P = 0.0037$; perfusion, test statistic = 7.2, $P = 0.0073$, $N = 20$ pairs). In natural clutches, control embryos maintained large (≥ 1.9 mm long), fully perfused gills throughout the observation period; no change in gill condition was observed. In contrast, both gill size and gill perfusion were dramatically reduced in misoprostol-treated clutches within minutes of treatment (Fig. 5). Although the extent of the reduction varied among embryos, in every experimental clutch some embryos had completely lost external gill circulation and reduced gill length by at least half within 9 min.

Although not quantified, no effects of the PGE treatments on heart rate or on blood flow to other parts of the body were observed. Specifically, no changes were observed in blood flow in the tail fin or other parts of the skin, which is well vascularized and functions as a respiratory surface in anuran embryos and larvae (Burggren and Just, '92).

DISCUSSION

Prostaglandin E2 and the PGE1 analogue misoprostol both affect external gill size and circulation in *A. callidryas*, as suggested by homol-

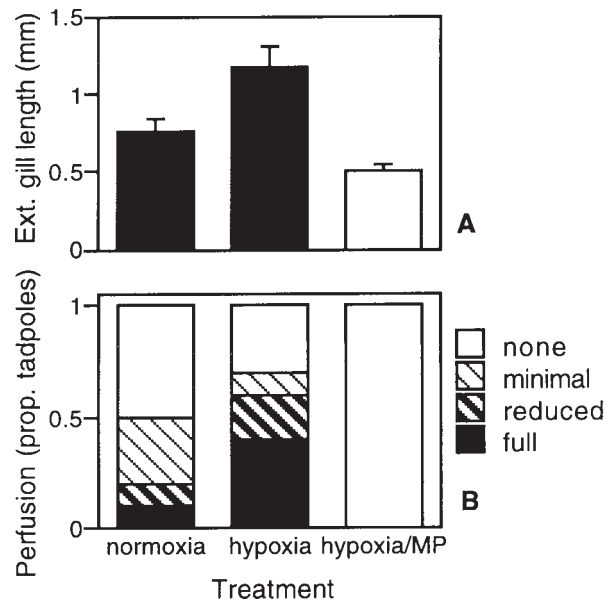


Fig. 3. Length (A) and perfusion (B) of the transient external gills of *A. callidryas* 20 min after hatching for animals exposed to a hypoxic solution of misoprostol (MP) and hypoxic and normoxic controls. Gill lengths are means \pm SE; perfusion levels are defined in the methods. Misoprostol overrode the inhibitory effect of hypoxia on gill regression.

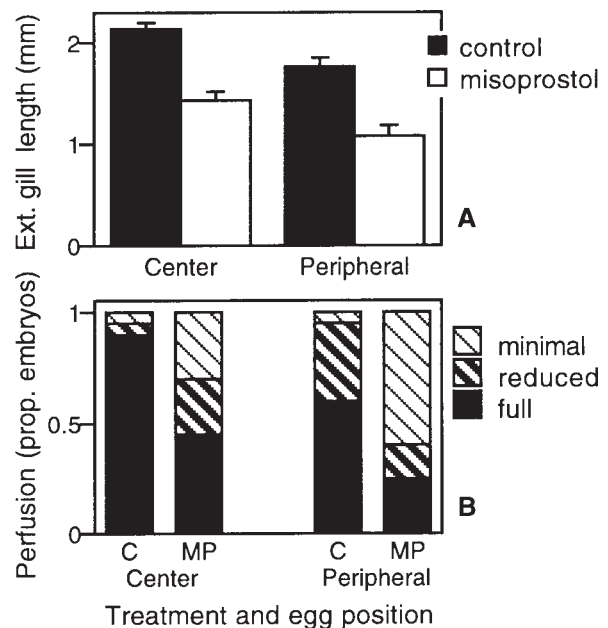


Fig. 4. Effect of misoprostol on the length (A) and perfusion (B) of the transient external gills of *A. callidryas* embryos for eggs surrounded by other eggs (center) and at the edge of a cluster (peripheral). Gill lengths are means \pm SE; perfusion levels are defined in the methods. In embryos, as in hatchlings (see Fig. 2), misoprostol reduced gill size and circulation.

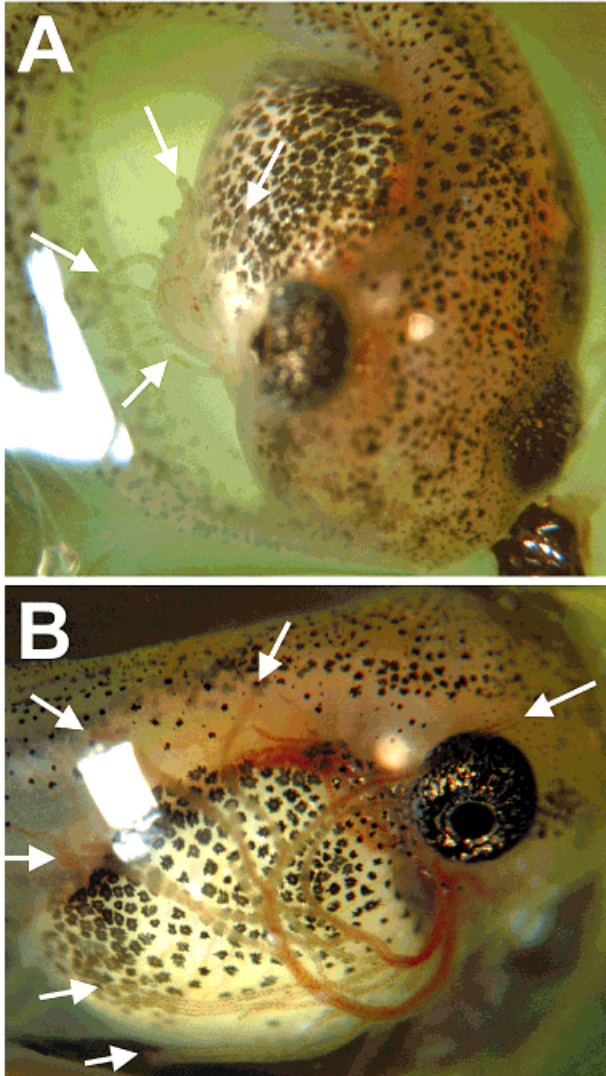


Fig. 5. Embryo of *A. callidryas* treated with misoprostol (A) and a control embryo (B). The ends of several gill filaments are marked with arrows, demarcating the extent of the external gills. Note the large, well-perfused gills of the control embryo and the reduced, blanching external gills of the misoprostol-treated embryo. For scale, eye diameter is 0.6 mm. Images were digitally processed in Photoshop to improve contrast and brightness and, where possible, to remove reflections from the egg capsule.

ogy with mammalian vascular structures and their sensitivity to PGE during development. These prostaglandins accelerate gill regression both in recently hatched tadpoles, which normally lose external gills, and in embryos, which normally maintain external gills. The direction of the PGE effect on gill regression is consistent with the dilation of vascular shunts, as expected based on branchial vasculature anatomy in other anurans and in salamanders (De Saint Aubain, '81; Mc-

Indoe and Smith, '84a,b; Malvin and Heisler, '88). The effect seems to be local, within the gills, rather than systemic as no alteration in blood flow was observed elsewhere in the body.

Concentrations of prostaglandins in vertebrate tissues range from pico- to nanogram amounts per milligram (Oliw et al., '83). Topically applied misoprostol within this concentration range (i.e., 1 ng/mg) induced gill regression, suggesting that the process could be naturally induced by endogenous PGEs. If so, those PGEs appear to act downstream in the regulatory pathway from oxygen level, an environmental regulator of gill regression (Warkentin, 2000b); i.e., misoprostol overrides the inhibitory effect of hypoxia on gill regression. Environmental and endocrinological factors may interact in the regulation of gill regression, or oxygen availability may affect levels of PGE in gill tissues and so indirectly affect gill loss.

Levels of PGEs or rates of PGE synthesis in anuran external gill tissue have not been measured, nor have PGE receptors been identified in the gills. There is, however, a local upstream source of PGE available. Tadpoles secrete PGE₂ into their mouths from oral mucus glands (Wassersug and Karmazyn, '84; Wassersug, '86). This may be carried out of the mouth through the gill slits by buccal pumping and, if so, would wash over the external gills. If this route is a major regulatory pathway for PGE to reach the gills, we would expect high concentrations of PGE receptors on the external surface of the external gills. If the modulation of gill regression by PGE is, instead, primarily an autocoid process, we would expect to find PGE receptors internally in the branchial vasculature.

The effect of PGE on the mammalian ductus arteriosus is to maintain dilation of the vessel (Olley et al., '76; Gersony et al., '83). Thus, if the response to PGE were uniform throughout the branchial circulation, we might expect PGE to maintain anuran external gill perfusion. However, depending on the precise site of action, a dilatory effect could reduce blood flow to the external gills. Specifically, the dilation of alternative pathways for blood flow, such as through the internal gills or lungs, might reduce the pressure, and therefore flow, through the external gills. Shunts that allow blood to bypass the gill tufts or gill filaments have been anatomically identified in other anurans (De Saint Aubain, '81; McIndoe and Smith, '84a,b). Shunts also allow variable partitioning of blood flow between gills and lungs in neotenic salamanders (Malvin and Heisler, '88). In vitro

assays of the response of particular portions of the branchial vasculature to PGEs would clarify the specific site and mechanism by which PGE stimulates external gill regression.

The relative importance of alternative respiratory surfaces to developing anurans is controversial. The physiological role of external gills in particular is equivocal (Burggren and Just, '92). As certain fish and amphibian embryos can develop to hatching and beyond without blood flow or functional hemoglobin, diffusion alone may supply sufficient oxygen to these young animals (Humphrey, '72; Smith and Armstrong, '93; Pelster and Burggren, '96; Territo and Burggren, '98). The large size and morphological complexity of external gills in other anuran embryos suggest that these structures were elaborated under natural selection and serve an important, presumably respiratory, function (e.g., Pyburn, '63; del Pino and Escobar, '81; Kluge, '81; Warkentin, '99). Measurements of the rate of oxygen uptake by normally gilled embryos and embryos without external gills would clarify the respiratory importance of the gills separately from other respiratory surfaces. Topical PGE treatment offers a simple way to experimentally induce gill regression and does not appear to alter circulation elsewhere in the body.

The dramatic effect of topically applied PGEs on external gill regression in vivo does not definitively prove that gill regression is naturally regulated by PGE. It does, however, clearly support the hypothesis. Detection of PGE receptors in the gill shunts, and demonstration of increased PGE levels during the period when gills regress, would add substantial support to the hypothesis.

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