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ONTOGENY OF CARDIOVASCULAR AND RESPIRATORY PHYSIOLOGY IN LOWER VERTEBRATES

Warren W. Burggren

Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01003-0027

Alan W. Pinder

Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1 Canada

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INTRODUCTION

The ontogeny of cardiovascular and respiratory physiology in vertebrates has been, and continues to be, an area of intense study (45, 87, 124, 28, 105, see *Annual Review of Physiology* 1984. 46:617–703). While clinicial benefits accrue from a knowledge of the developmental physiology of fetal mammals, much of the motivation for general vertebrate research on this topic stems from a fascination with the inherent complexity of developmental transitions of these systems. Indeed, some of the most radical developmental transitions of any organ system occur in heart, vasculature, and gas exchange organs of animals as they make the transition from embryonic/larval/fetal life to that of free-living animals.

This review examines the cardiovascular and respiratory transitions that accompany development in fishes, amphibians, and reptiles. In many cases these processes are very similar to those occurring in birds and mammals and reflect the strong commonalities of vertebrate development. There are also fascinating differences, however, that deserve emphasis. Unfortunately, all

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too often the critical experiments remain to be performed, and therefore a major purpose of this review is to emphasize particular areas for fruitful future research.

WHY STUDY DEVELOPMENTAL PHYSIOLOGY IN LOWER VERTEBRATES?

Many aspects of the developmental transitions in cardiovascular and respiratory physiology are now well understood for mammals (although this must be qualified by recognizing that in reality it is primarily the late fetus rather than the mammalian embryo that is routinely investigated). The cardio-respiratory transitions in birds are also well appreciated, especially since the embryos of birds currently serve as the major model for investigation of the mammalian embryonic circulation (for reviews see 34, 35, 87). Far less is known about the developmental changes in cardio-respiratory physiology of fishes, amphibians, and reptiles, and virtually none of the tremendous diversity of physiology typical for these vertebrates has been described in a developmental context.

There are several important reasons for expanding this fragmentary knowledge of the developmental changes in the cardio-respiratory physiology of lower vertebrates. First, many lower vertebrates provide excellent experimental models that allow the investigator to distinguish physiologic changes associated with organogenesis, the differentiation of tissue and production of new tissues and organs, from processes associated with simple growth, in which tissue mass can increase without the qualitative change associated with tissue differentiation. "Immature animals are small—mature animals are big" is a truism, but the implications for studies of the development of cardio-respiratory physiology are often not recognized (or, if recognized, are often avoided). A vast body of literature deals with the considerable influence of body mass on physiologic processes in all vertebrates (32, 127, 112). Because the adults of a given species of bird and mammal almost always have a body mass that is far greater than that of embryos, larvae, and fetuses, and because developmental transitions usually occur at approximately the same body mass in all individuals, it is often difficult to distinguish between physiologic changes that occur simply because an animal has grown to a larger body mass from those that reflect true tissue differentiation. In many species of lower vertebrates, however, major developmental changes can occur at any of a variety of body masses. For example in the bullfrog, *Rana catesbeiana,* larval bullfrogs undergo metamorphosis to the juveniles when body mass ranges anywhere from about 5 g up to 50 or more g (similar situations arise in other amphibians and some fishes). Figure 1 shows heart mass (which is correlated with physiologic variables such as stroke volume)

as a function of body mass. The effects of body mass on heart mass can be assessed independently for larvae and adults from an examination of the slope of the line for larvae and for post-metamorphic adults. In each of these two broad developmental groups, heart mass increases approximately in proportion to body mass. In the area of body mass overlap between the two developmental groups, however, metamorphosis to the adult body form *(vertical arrows)* results in an increase in the intercept of the line describing the relationship between heart mass and body mass. Thus metamorphic climax at constant body mass results in an increased heart mass in *Rana catesbeiana*—a pure developmental effect unrelated to scaling. By choosing species and designing similar experiments that emphasize rather than minimize variation in body mass, the specific effects of growth can begin to be separated from those of organogenesis (17).

A second compelling reason for studying the development of physiologic

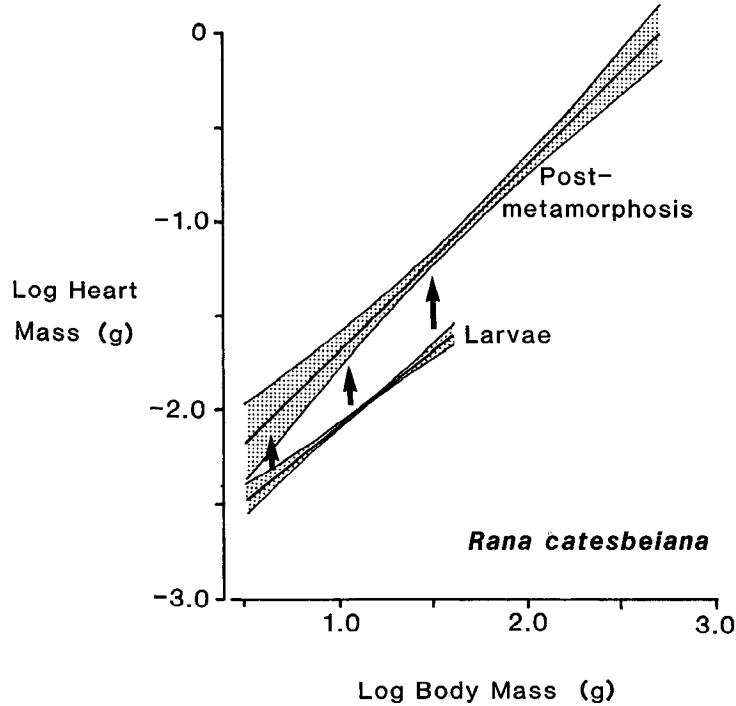

Figure 1 The relationship between heart mass and body mass in larvae and post-metamorphic juveniles and adults of the bullfrog, *Rana catesbeiana*. Linear regressions and 95% confidence intervals are provided. *Vertical arrows* from larvae to adults indicate the effect on heart mass of metamorphosis without change in body mass (essentially an upward shift in the Y-intercept of the relationship between these two variables). (W. Burggren, unpublished)

processes in lower vertebrates is that potentially they can provide important insights into the evolution of such processes (13, 14, 16–18). Of course, the concept that "ontogeny recapitulates phylogeny" is now widely recognized as overly simplistic and even anachronistic, but this does not mean that evolutionary biologists are uninterested in ontogeny. For example, the development of respiratory physiology in animals undergoing the developmental transition from water to air breathing can provide insights into feasible evolutionary steps towards air breathing and terrestriality (see 16, 18).

Finally, the study of immature stages of mammals or birds provides neither better nor worse insights into vertebrate development than does the study of the embryos and larvae of fishes, amphibians, and reptiles. There is no single vertebrate embryo that is truly representative of development in all vertebrates. For example, the current important role of the chick embryo is based as much on convenience and accessibility to material as on how representative it may be. The point is that we now need to expand our studies to understand the full extent and types of variation within physiologic development throughout vertebrates if we are to claim a general knowledge of vertebrate ontogeny.

Thus the study of cardio-respiratory ontogeny in lower vertebrates is compelling for the insights it can provide into both general vertebrate development as well as evolutionary questions. We now turn to the ontogeny of these physiologic processes in circulation and gas exchange in lower vertebrates.

THE CIRCULATION AND ITS REGULATION

The heart is the first organ to function in any vertebrate embryo, and the cardiovascular system is the first organ system to operate. This, plus the fact that the circulation in lower vertebrate embryos is prominent and easily observed through the embryonic body wall under the microscope, has made the embryonic circulation of lower vertebrates a source of fascination for anatomists for more than a century (see 66 for early literature). The ontogenetic changes in morphology of the cardiovascular system of amphibians (and, to a lesser extent, fishes) have been extensively described (see 11, 94–96, 103, 124). Yet, despite a substantial anatomical literature on the cardiovascular anatomy of lower vertebrate embryos, we know comparatively little about the hemodynamics and neural/hormonal regulation of the embryonic cardiovascular system of fishes, amphibians, and reptiles.

Heart Rate

RESTING HEART RATE Perhaps because it is the most easily quantified, developmental changes in heart rate (f_H) and the ontogeny of the cardiac regulatory system have received most attention. Invariably, resting f_H changes

as development proceeds from embryo through larva to adult. The pattern of change, however, varies between species and does not correlate with vertebrate class or even with families. In larval rainbow trout, resting f_H at 10°C increases from around 60 beats/min at hatching to about 70 beats/min 3 days later, but then declines again to about 50 beats/min 21 days after hatching (75). A similar pattern of change in resting f_H during larval development occurs in the brown trout *Salmo trutta* (68), and f_H also decreases during the first 50 days of larval development in the Arctic char *Salvelinus alpinus* (102). Resting f_H at 18°C in the embryos of the skate *Raja erinacea* is about 40 beats/min when the heart first begins to beat in embryos (about 30 days after fertilization at body mass about 20–50 mg) (109a). With further growth, heart rate rises to 60 beats/min and then falls again to 35–40 beats/min before birth (about five months after fertilization and 5 g body mass). Unfortunately the mechanism(s) behind these changes in resting f_H in embryonic and larval fishes remains unknown. Possible explanations include developmental changes in the membrane permeability of the cardiac pacemaker (i.e. a change in the intrinsic rate of the heart) as well as the onset of sympathetic and parasympathetic cardiac tone.

Developmental changes in resting f_H also occur in anuran amphibians. In the bullfrog *Rana catesbeiana,* resting f_H in newly hatched larvae (about 40 mg body mass—stage I in the Taylor-Kollros (TK) staging system) is approximately 135 beats/min at 20–23°C, but falls sharply with further larval development (19). From TK stages IV–VII to metamorphic climax (body mass range 2–20 g), resting f_H remains at about 40–50 beats/min, but then shows a final development change by dropping to 20–30 beats/min in the mature bullfrog (400 g). When log heart rate is plotted against log body mass for *Rana catesbeiana,* the data for all animals, regardless of developmental stage, fall along a single line with the equation $y = -0.23x + 1.85$ (W. Burggren, unpublished). Resting heart rate thus scales with body mass to the exponent −0.23, compared with a value based on interspecific comparisons between adults of about −0.25 (32, 112, 127). This suggests that changes in resting f_H between newly hatched larvae and mature bullfrogs can be correlated almost entirely on the basis of allometry (scaling) rather than organogenesis or other qualitative changes associated with ontogeny.

Resting f_H in the direct developing frog *Eleuthrodactylus coqui* shows a particularly complex pattern of change with development (25). The first heart beats, recorded in intact 3 mg embryos by visual observation through the transparent egg capsule, occur at a frequency of about 50 beats/min at 24–25°C, but increase sharply to about 100 beats/min with a slight body mass increase and further embryonic development (Figure 2). During most of the remainder of embryonic development, which sees large increases in both body mass and tissue differentiation, resting f_H shows a relatively modest further increase to about 110–120 beats/min immediately before hatching as a

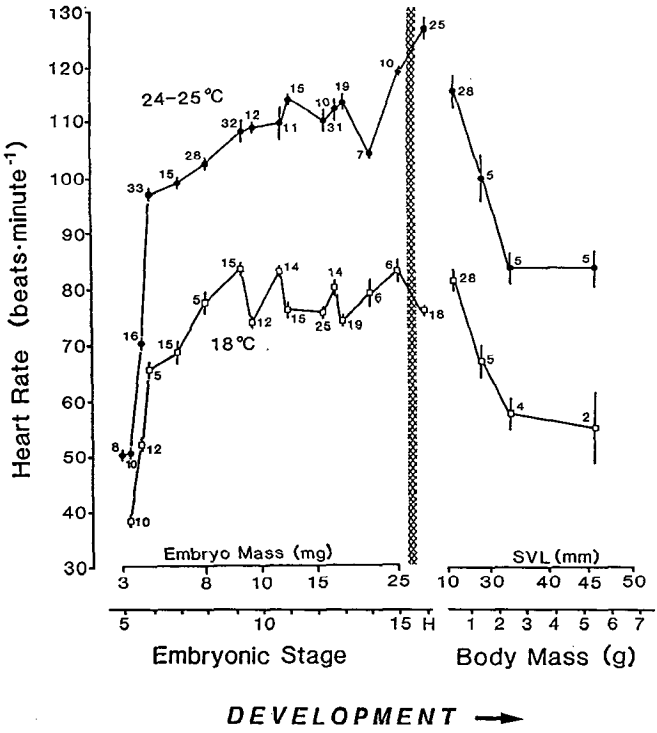

Figure 2 Changes in resting heart rate (mean ± 1 sec) during development in the Puerto Rican anuran frog *Eleutherodactylus coqui*. Heart rate data begin with the developmental stage in which the heart first beats. The *vertical hatched bar* indicates the point of development of the air-breathing adult morph (from Burggren et al, 25).

miniature adult. After hatching, resting f_H decreases from about 130 beats/min in hatchlings (<100 mg) to about 85 beats/min in the largest adults (6 g). Although at 18°C f_H is lower, this general pattern of development change in f_H persists. While changes in body mass alone no doubt influences f_H in *E. coqui*, especially after hatching, the sharp increase during early development with very little change in body mass indicates that heart rate is responding to undetermined developmental changes unrelated to simple changes in body mass.

The mechanism underlying these varying patterns of developmental changes in resting f_H in larvae of anuran amphibians awaits complete description, as it does for larval fishes. A vagal tone at rest appears in mid-larval development in *Rana catesbeiana* and results in a resting f_H about 10 beats/min lower than the intrinsic rate determined after combined cholinergic and

beta-adrenergic blockade (19). Interestingly, after metamorphosis, resting f_H is actually the intrinsic heart rate, since the sequential administration of atropine (a muscarinic cholinergic blocker) and propranolol (a beta-adrenergic blocker) to resting adult bullfrogs produces no change in f_H.

To our knowledge (and surprise), measurements of resting heart rate have yet to be made in intact reptile embryos, perhaps in part because of the opaque and generally more robust nature of the reptilian egg shell. Considerable literature exists on the metabolism and hydric relations of reptile embryos in their eggs (e.g. 135, 109), and physiologic observations of the ontogeny of the cardiovascular system should allow a much more integrated view of reptilian development prior to hatching.

HEART RATE AND ENVIRONMENTAL FACTORS Hypoxia challenges any cardiovascular and respiratory system and can occur in embryos within the specialized environments of the egg, oviduct, or uterus, which impose an additional diffusional boundary for respiratory gas transfer. Additionally, frequent and severe environmental hypoxia can characterize the freshwater habitats in which embryonic and larval fishes and amphibians develop. As adults, many lower vertebrates respond to environmental hypoxia with a bradycardia of varying magnitude (see 83, 106, 129). This response, however, appears to be absent in larval salmonid fishes (60, 75, 102) as well as in larval anuran amphibians beyond TK stage IV (50, 51, 56, 120, 143). Newly hatched larval *Rana catesbeiana* do show a sharp decrease in f_H when ambient PO_2 falls below about 40–50 mmHg, but this probably represents a direct cardiac depression caused by tissue hypoxia rather than a reflex slowing of heart beat (11).

The lack of a hypoxic bradycardia in lower vertebrate embryos does not, however, mean that the embryonic or larval heart is a metronome that is unresponsive to changes in the internal or external environment. Indeed, hypoxia results in a mild tachycardia in larval rainbow trout (75) and Arctic char (102). Voluntary locomotor activity produces a mild tachycardia even in one-day old larval trout (75). Voluntary activity has no effect on f_H in one-day old bullfrog larvae, but in all later developmental stages spontaneous movement is accompanied by a tachycardia (19). The mechanism behind exercise tachycardia has been investigated in the bullfrog. Experiments involving saline infusion into the central venous circulation of bullfrog larvae (i.e. increasing pre-load) produces a tachycardia in control larvae and also in larvae with confirmed cholinergic and beta-adrenergic blockade (19). This suggests that the tachycardia during exercise can result in part from direct effects of stretch on the cardiac pacemaker produced by enhanced venous return during activity. In the adult bullfrog, however, the activity tachycardia appears to result primarily from beta-adrenergic cardiac stimulation.

An increase in f_H occurs during the stress of hatching in the direct developing frog, *Eleuthrodactylus coqui* (25). Prior to hatching, the resting f_H in undisturbed animals within the egg is about 120 beats/min. The act of hatching, which is an explosive event requiring a few seconds of intense locomotor activity, results in an increase in f_H to nearly 160 beats/min within a min or less of hatching, and which persists for at least two hr following hatching. Because direct developing anurans like *E. coqui* hatch as miniature adults that are active predators, the hatchlings probably require all of the cardiovascular reflexes evident in the larger, mature adults.

CARDIO-RESPIRATORY COUPLING Interactions between cardiac and ventilatory events are commonplace in adult fishes, amphibians, and reptiles (83, 129, 142). Such interactions include phase locking between the ventilation cycle and the cardiac cycle, as well as diving bradycardia (or, alternatively, ventilation tachycardia) in intermittent air breathers. In some instances interactions between cardiac and ventilatory events are manifestations of reflex connections between peripheral chemo- and/or mechanoreceptors; in other instances they may represent the direct communication within the brain between cardiac and respiratory centers (see 57).

Cardio-respiratory phase coupling has been reported in larvae of the anurans *Xenopus laevis, Pachymedusa dacnicolor,* and *Rana berlandieri* (140), but its distinct absence has also been reported in larval *Rana catesbeiana* and *Rana berlandieri* (24, 144). Phase locking between cardiac and ventilatory cycles has not been reported in early larval stages of fishes or amphibians. This may reflect the fact that, at least in normoxia, the early buccal pumping movements that force water over the developing internal gills have an intermittent, erratic nature (e.g. 75, 102).

Ventilation tachycardia has been described repeatedly in adult air-breathing fishes (see 26, 27). Since air breathing starts relatively early in some air-breathing species, it would be of considerable interest to know if cardio-respiratory interactions develop equally early. Unfortunately, little is known about the ontogeny of the respiratory physiology of any air-breathing fish (see below). Virtually nothing can be said about the physiologic development of the complex cardiovascular system of air-breathing fishes, which generally have both gills and accessory gas exchange organs (lung, gas bladder, or modified branchial chamber). A noteworthy observation, however, involves the strictly aquatic larvae of the air-breathing fish *Monopterus albus* (93). In the early larval stages prior to establishment of internal gill ventilation, the pectoral fins produce a flow of water from anterior to posterior over the surface of the body wall. This in itself is not remarkable, and indeed water currents over the body surface have been observed in several fish larvae (see 124). In *Monopterus albus,* however, the flow of blood in the capillaries of

the skin is from posterior to anterior. Thus cutaneous gas exchange operates as a countercurrent mechanism, which allows *Monopterus* to remove 40% of the available O_2 from the stream of water passing over the body surface. Whether such a mechanism occurs in other fishes, or indeed in larval amphibians where the ciliated surface of the embyro can generate a coordinated convective flow of perivitilline fluid over the body surface (12), begs further investigation.

In some larval amphibians, f_H is unaffected during bouts of intermittent lung ventilation, with ventilation tachycardia occurring only after metamorphosis to the final adult form (19, 143). Recent measurements on the giant larvae (up to 24 cm, 100 g) of the South American frog *Pseudis paradoxus* have shown that a mild ventilation tachycardia occurs as the larvae float at the water surface and take intermittent air breaths (W. Burggren, M. Glass, A. Abe, E. Bicudo, unpublished). Moreover, when the larvae are prevented from taking air breaths, f_H falls below the normal f_H recorded during the voluntary interbreath interval. Arterial blood PO_2 in unrestrained *Pseudis* varies from a low of 30 mmHg during voluntary breath holding up to 100 mmHg immediately following an air breath, but whether the cardiac reflexes are mediated by these changes in blood PO_2 (or the associated changes in blood O_2 content) awaits further investigation. Ventilation tachycardia has also been noted in larvae of the tiger salamander, *Ambystoma tigrinum* (71).

Pharmacology of the Embryonic and Larval Heart

The cholinergic and adrenergic sensitivity of the cardiac pacemaker changes with development in the bullfrog *Rana catesbeiana*. Dose-response curves for ACh using in situ preparations indicate that the pacemaker's cholinergic sensitivity increases progressively during larval development (20). At metamorphosis, however, there is a sharp decrease in cholinergic sensitivity to levels comparable to the earliest larval stages examined. Dose-response curves for atropine correspondingly indicate that cholinergic blockade of the chronotropic response requires higher dosages in both early larval and postmetamorphic stages than in late larval stages. In vitro studies, using isolated, spontaneously active atria from *Rana cartesbeiana,* indicate that both larval and adult hearts begin to show a heart rate acceleration at similar physiologic doses (about 10^{-7} M) of norepinephrine, but the atrial tissue of adults requires greater doses than does larval tissue to produce maximal adrenergic stimulation (86).

Interestingly, the pattern of developmental change for cholinergic inotropic responses of isolated ventricular strips from *Rana catesbeiana* differ markedly from the chronotropic responses of the intact heart of this species, since the ventricle of the adult bullfrog rather than of the larva is more sensitive to the inhibitory inotropic effects of ACh (S. Petrou, I. Walhquist, W. Burggren,

unpublished). Complicating the situation still further, the pattern of developmental change for adrenergic inotropic responses of isolated ventricular strips in vitro is the opposite of inotropic responses produced by ACh—the adult heart is the least sensitive to the inotropic effects of epinephrine (S. Petrou, I. Walhquist, W. Burggren, unpublished).

Developmental changes in chronotropic and inotropic responses to acetylcholine and catecholamines may result from (*a*) changes in the number of receptors in the pacemaker cell membranes, (*b*) changes in affinity of each receptor site, or (*c*) both of these changes. Future investigations of developmental changes in cardiac pharmacology of lower vertebrate embryos should prove to be particularly rewarding.

Central Hemodynamics

The very small size of the heart and central vessels of vertebrate embryos has until recently precluded detailed physiologic measurements of central hemodynamics. The development of new microtechnologies such as pulsed-Doppler and laser blood flow monitoring and microelectrodes for measurement of blood pressure and blood gases has greatly expanded our ability to investigate cardiovascular physiology in vertebrate embryos, larvae, and fetuses. While most of this emerging technology has been applied to the investigation of the central hemodynamics in the chick embryo (see 34, 35, 87), the results of the relatively few measurements that have been made in lower vertebrate embryos are rather intriguing. Ventricular systolic blood pressure has been measured with a microelectrode recording technique in the embryos of the skate *Raja erinacea* (109a). Pressure increases from about 1 mmHg 30 days after fertilization (body mass 10 mg) to about 13 mmHg 144 days after fertilization (body mass 4.4 g), which is close to the end of the five to six month period of embryonic development at 18–20°C. Microelectrode measurements of blood pressure in the ventricle and conus arteriosus have also been made in pithed, immobilized larvae of the bullfrog *Rana catesbeiana* (109b). Marked seasonal differences may occur in the cardiac performance of these anurans. Measurements made during the fall months in early developmental stages (e.g. TK stage II) show that contraction of the conus rather than the ventricle produces the highest arterial pressure at systole. With further development, conal systolic pressures decrease while ventricular systolic pressures rise. By TK stage X–XIII of larval development, the central arterial hemodynamics are essentially the same as those of the post-metamorphic adult. Overall, there is a rise in systolic arterial pressure during larval development, from about 2 mmHg at TK stage II (about 0.3–0.4 g) to about 12 mmHg at TK stage XIV (about 10 g) (all values measured during the fall and winter months).

A similar rise in arterial systolic pressure with larval development has been

confirmed using an indwelling sciatic artery cannula in intact, conscious larvae of the frog *Pseudis paradoxis* (W. Burggren, M. Glass, A. Abe, E. Bicudo, unpublished). In this species, however, larval body mass decreases rather than increases with larval development. Systolic pressure also increases by 50% from a mean pressure of about 20 mmHg to about 30 mmHg at metamorphosis, even though there is no body mass change during this final stage of metamorphosis in *Pseudis*. Changes in peripheral resistance, perhaps associated with changes in body mass, as well as developmental changes in gas exchange organs and their blood perfusion pattern may all interact in complex and as yet undetermined ways to alter blood pressure during development.

Finally, cardiac output has been measured in the larvae of the salamander *Ambystoma tigrinum* (76). To our knowledge, this is the only such measurement for lower vertebrate embryos. Cardiac output in conscious, restrained larvae is about 100 ml/kg/min at 20°C, which is within the range reported for adult *Rana*, *Xenopus*, and *Amphiuma*. Inter-individual variation in cardiac output in larval *Ambystoma tigrinum* results primarily from changes in stroke volume rather than heart rate. How cardiac output varies during locomotor activity, or in response to changes in environmental temperature or oxygen availability, in this, or other lower vertebrate species, awaits further research.

The Pheripheral Circulation

Compared even with our fragmentary knowledge of the ontogeny of central vascular hemodynamics in lower vertebrates, physiologic development of the peripheral circulation is practically unexplored. Many anecdotal observations of capillary recruitment, backwards surging during early diastole, and cessation of arterial flow during diastole have been made, especially for fish embryos and larvae (e.g. 75, 102). Blood flow in the capillary loops of the external gills of larval fishes and amphibians is particularly amenable to observation under the microscope, and velocity can even be measured in individual capillaries using pulsed-Doppler crystals (W. Burggren, unpublished), but few systematic studies have been attempted as yet.

In the larval bullfrog, *Rana catsbeiana*, catecholamines dilated the branchial vasculature of the larvae as early as stage III (86). The branchial vessels of the larvae of the anuran *Litoria ewingi* have an extensive network of shunt vessels (103), but the small size of the larvae is not conducive to pharmacological or physiologic investigation. The much larger size of the larvae of the salamander *Ambystoma* has permitted considerable investigation of the pharmacology of the peripheral vessels, however, especially those in the external gills (39, 94–96). The circulation to gas exchangers in *Ambystoma* is extremely complex (94, 95, 98). The lungs are both in parallel (through the pulmonary artery) and in series (through the ductus arteriosus) with the gill

circulation. The gill circulation includes shunts around the gas-exchanging surfaces. The cutaneous circulation is in parallel with the systemic circulation and thus receives at least partially oxygenated blood. In larval *Ambystoma*, lung perfusion is primarily via the ductus arteriosus, in series with gill arches three; when the gills (but not the aortic arches originally supplying the gills) disappear at metamorphosis, the pulmonary artery becomes the source for pulmonary blood (98). In larval *Ambystoma*, severe aquatic hypoxia increases lung perfusion and decreases perfusion of the first gill, perhaps increasing pulmonary oxygen uptake and reducing oxygen loss through the gill. After metamorphosis, pulmonary perfusion is unaffected by aquatic hypoxia (98).

Branchial and proximal pulmonary arteries are under both cholinergic and adrenergic regulation in larval *Ambystoma* (94–96). Vagal stimulation and acetylcholine both vasoconstrict the branchial vessels. The pulmonary artery is constricted by acetylcholine (94, 96) and vagal stimulation (39), but is unaffected by catecholamines. Reciprocal changes in blood flow between the pulmonary and branchial vascular beds can be produced by changes in circulating levels of catecholamines, or neural stimulation, and by those effects on peripheral vascular resistance, as observed in adult amphibians (13, 53). Whether there is a capacity for autoregulation in the circulation of larval *Ambystoma* is unclear. Malvin (95) reports that local alterations in CO_2 and pH do not affect vasomotion in the branchial circulation, in contrast to Figge's (59) earlier studies on this species. Larval *Ambystoma* are obligate air breathers (cf. 13) and possess well-developed lungs even as quite small larvae.

Peripheral circulation in fish and amphibian embryos and larvae will be aided by the rhythmic swimming movements of the body, especially if the veins are extensively valved (a feature of embryonic/larval anatomy that is currently unknown). Evidence for blood convection caused by locomotor activity comes from quite unrelated investigations of cardiac pacemaker physiology. Normal embryonic development of heart structure occurs in so-called cardiac lethal mutants of the salamander *Ambystoma mexicanum*, but the cardiac pacemaker fails to depolarize spontaneously because of low membrane ion permeability (see 85 for review). Interestingly, the cardiac lethal mutants can develop to the point of hatching, and the larvae actually swim and survive for several days, without the heart beat ever beginning. Clearly, in extremely small larvae, a combination of diffusion of gases, nutrients, and wastes, combined with convection generated by body movements powered by skeletal muscles, can substitute effectively for cardiac-derived blood convection. This raises the question of how much the embryonic circulation actually contributes to the transport needs prior to hatching. While the basic physiology of development of cardiac pacemakers in vertebrate hearts has been considerably advanced by studies using these embryonic mutants, their use as a model system to examine the role of the embryonic circulation has been rather neglected to date.

RESPIRATION AND METABOLISM

Respiration and metabolism change dramatically over the course of development of all lower vertebrates. These changes occur in three major categories: (*a*) organogenesis and the differentiation of structures involved in gas exchange and transport; (*b*) increase in size, affecting both metabolic rate and the relative importances of convection and diffusion in gas exchange and transport; and (*c*) transitions in respiratory medium, most dramatically seen in amphibians metamorphosing from aquatic, primarily water-breathing larvae to terrestrial, air-breathing juveniles (Figure 3). At very small body size, diffusion is adequate for gas exchange (Figure 3*a*). At larger sizes and particularly in hypoxic environments, external gas conductance may be increased by external convection generated by cilia or body movements (Figure 3, stage *b*). Small larvae depend largely on cutaneous gas exchange with internal convection for gas transport (external convection is still important) (stage *c*). At larger body sizes, specialized gas exchangers (gills) develop to provide increased surface area for gas exchange, and they must be ventilated (stage *d*). Eventually air is used as an oxygen source and less commonly as a sink for CO_2, with concomitant changes in the gas exchange organ(s) and its regulation (stage *e*). Many fish end development at stage *d*, whereas reptiles pass through stages *a* to *c* in the egg and hatch at stage *e*.

Fish, amphibians, and reptiles differ greatly in their development at hatching (Figure 4). Fish hatch before what is usually thought of as embryonic development is complete; the digestive, respiratory, and cardiovascular sys-

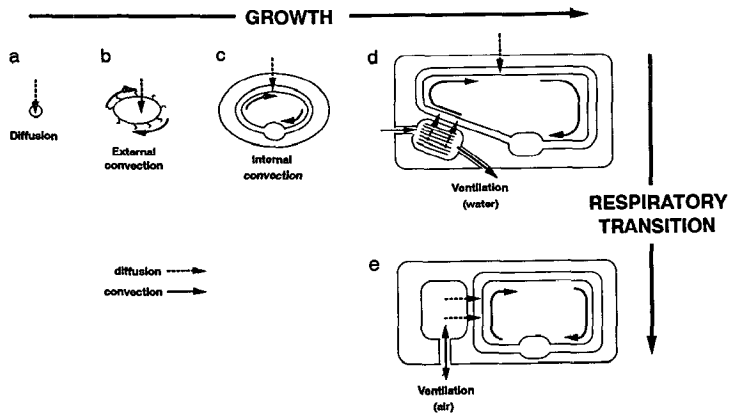

Figure 3 Overview of respiratory development in air-breathing lower vertebrates. Although innumerable variations exist (most of which have not been investigated), this common pattern of development is related to the increase in body size and (with the exception of strictly aquatic fishes and a few amphibians) the respiratory transition from an aquatic larva to an air-breathing or bimodally breathing adult.

tems are commonly not functional and the larva still carries a yolk sac. Fish and amphibians generally hatch as larvae and undergo a more-or-less radical change in body form, function, and life-style (metamorphosis). Reptiles are more completely developed, often appearing like a small version of the adult, and do not undergo metamorphosis. Cardiovascular and especially respiratory development differ greatly in fish, amphibians, and reptiles (Figure 4). Significant events include the initiation of blood circulation, appearance of hemoglobin, and organogenesis of gills and lungs. Except for the great majority of fishes and a few amphibians, air breathing becomes a significant source of oxygen before development to the adult. There is wide variation in the timing of these developmental events within each class (not shown in Figure 4), but even greater variation exists between classes. For example, most fish hatch into larvae before having a functional circulatory system; amphibians hatch into larvae with functional circulation and gills, and reptilian embryos hatch into air-breathing juveniles without any intervening larval stage. Not indicated in Figure 4 is the phenomenon of viviparity, which occurs in many lower vertebrates (see 89 for review).

Metabolism

Metabolic rate determines the rate at which respiratory gas exchange must occur. Metabolism has a number of peculiarities in rapidly growing and differentiating systems; in particular, in the rapid conversion of metabolically

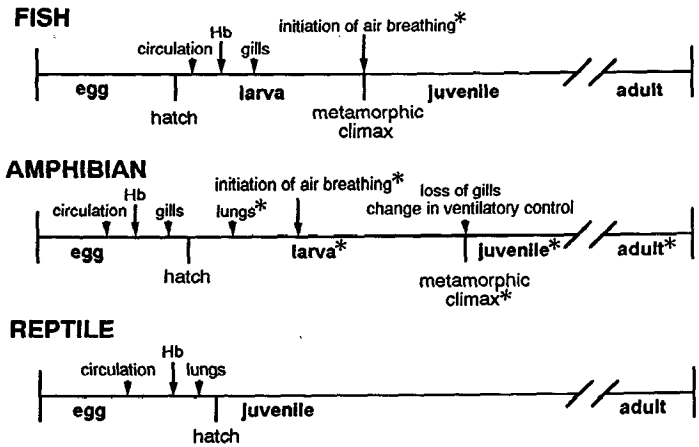

Figure 4 Cardiovascular and respiratory development in relation to life history stages in lower vertebrates. Developmental processes or stages that may or may not occur in a given species within the class are indicated by an *. As examples, most fishes remain strictly water breathers, some amphibians fail to develop lungs and breathe air, and some amphibians do not normally metamorphose to juvenile or adult forms.

inactive storage materials to metabolizing tissue, and in the large proportion of the energy budget going towards growth and biosynthesis (1). In contrast to mammalian embryos, which are closed systems only for the first few days of development and exchange materials with the mother thereafter, all materials necessary for growth and development (except water and oxygen) must be present at the time of laying in oviparous ectothermic vertebrates. Although environmental temperature has obvious effects on metabolism and development in ectotherms (6, 69, 148), this will not be considered in detail here.

METABOLIC DEVELOPMENT IN FISH Much of the available information on metabolic changes during development in lower vertebrates comes from fish, in which the major interest has been in establishing optimal conditions for aquaculture. Oxygen consumption ($\dot{M}O_2$) of the fish oocyte is very low; metabolism increases sharply upon fertilization. As the metabolizing cell mass increases at the expense of the mass of storage products, metabolic rate per embryo increases slightly more than proportionately (123–125, 138) so that the metabolic rate per gram of metabolizing tissue increases 20 to 50% before hatching (69). Some of this increase results from the start of muscle activity or cardiac work in the embryo (113, 138, 149). There is usually a sharp increase in metabolic rate, with hatching into a yolk sac larva, probably caused by increased spontaneous swimming (38, 42, 138, 141). Critical PO_2 increases throughout embryonic development because of the fixed resistance of the egg membranes and increasing oxygen uptake (124, 125). If environmental oxygen is lowered close to or below critical PO_2, hatching may occur sooner than normal, presumably to remove the resistance of the egg membranes to oxygen exchange and allow increased oxygen uptake (44, 92).

There may be a change in the scaling factor for metabolism between embryos, in which metabolism increases directly with body size, and adults, in which metabolism increases only as the 0.8 power of body size. In some larval fish (salmonids, mullet), routine and maximum metabolic rates increase in direct proportion to body mass (138, 147). In other fish (cyprinids, Oreochromis) mass-specific metabolic rates of larvae already decrease with size as is common in older animals (42, 148). The relative factorial scope for activity (maximum MO_2/routine MO_2) increases from two to four times during larval growth in trout, but is independent of body size in cyprinids (147, 148).

Respiratory enzyme activities and use of energy sources during development have been investigated in fish embryos (7). Of the energy stored in the yolk, 43% is catabolized; the remainder contributes to embryo mass (125). Unfertilized oocytes are glycolytic; after fertilization metabolism is supported both by glycolysis and oxidative phosphorylation of carbohydrates. Lipids are not used extensively until late in embryogenesis. Cells moving during gastrulation have much higher lactate dehydrogenase (LDH) activity than

stationary cells (7). Protein catabolism is also important during late embryogenesis and the first few days after hatching, but lipid catabolism becomes dominant later (37).

Activities of aerobic enzymes are generally high and glycolytic enzyme activity low immediately after hatching in fish, which suggests that early larval activity is largely fueled aerobically (62, 73, 74). Glycolytic enzyme activities increase later in development, correlated with increased use of white muscle and increased versatility of swimming performance (47, 62). The detailed pattern of these changes in enzyme activity varies from group to group, apparently correlated with differences in life-style and activity patterns (47, 74).

METABOLIC DEVELOPMENT IN AMPHIBIANS AND REPTILES Metabolic changes during development in amphibians and reptiles are even more poorly understood than in fishes. The main determinant of resting $\dot{M}O_2$ in embryonic stages of anurans is body mass; $\dot{M}O_2$ increases directly with mass of metabolizing tissue in salamander embryos (132) and, as in fish, there is a large increase in $\dot{M}O_2$ with hatching, perhaps due to increased spontaneous activity (A. W. Smits, personal communication). In larval anurans, $\dot{M}O_2$ increases as the 0.83 power of body mass, although in some species there are stage-specific changes as well, which are probably correlated with change of life-style (49).

Metabolic development in reptile embryos shows at least two patterns (135, 145). All embryos show an initial exponential increase in $\dot{M}O_2$ (per unit fresh egg mass), probably reflecting an exponential increase in mass of metabolizing tissue. Some reptiles hatch at the end of this exponential growth, while others show a distinct decrease in metabolic rate before hatching, which is thought to be part of a mechanism allowing eggs that develop at slightly different rates to hatch simultaneously, or to allow for a waiting period for environmental cues (135, 145). There is no post-hatching biochemical metamorphosis in reptiles as there is in fish and amphibians, although there are gradual changes in aerobic scope for activity and the balance between aerobic and anaerobic capacity due to increasing body mass (5, 150).

Developmental Changes in Gas Exchange Sites

SIZE AND DIFFUSION In early stages of development, all vertebrates rely on diffusion for both exchange and transport of respiratory gases. In amphibians this dominance of cutaneous gas exchange may persist into the adult, but in most vertebrates cutaneous exchange is eventually eclipsed by lungs or gills. This change in gas exchange site is due to both increasing body size and organogenesis of the specialized gas exchangers (Figure 3). Size increase is a much more important factor in development of lower than higher vertebrates,

since the mass of free-living lower vertebrates may increase more than six orders of magnitude between hatching and adulthood, for example in carp *(Cyprinus carpio)* from a one or two mg newly hatched larva to a >1 kg adult (108). Some amphibians increase in mass over three orders of magnitude even after metamorphosis, and this growth is correlated with changes in aerobic and anaerobic capacities and activity patterns (133). Most mammals grow less than two orders of magnitude between birth and adulthood. In general, body surface area to mass ratio and the diffusion distance for cutaneous gas exchange decreases with increasing mass while the surface area of gills increases, usually with a shorter blood-water distance than skin, which results in a switch of primary gas exchange site from skin to gills during larval development (78, 108, 119).

Diffusion is adequate for both gas exchange and transport between environment and mitochondria at body diameters of less than 1 mm (33), the approximate diameter of many fish at hatching (6). In newly hatched fish, the cardiovascular system is often rudimentary, does not extend throughout the body, and generally does not contain red cells. Fish usually hatch without gills, mouth, or opercular openings (6, 63, 107, 124). The degree of cardio-respiratory development at hatching is highly variable, probably correlated with oxygen availability in the spawning waters (124). Newly hatched fish move at low Reynolds number (Re), in which viscosity predominates, but move at progressively higher Re with growth, so that inertial effects become much more important. This affects locomotor energetics, activity patterns, optimal body shape for maximum thrust and minimum drag (4, 137, 141) and, most importantly for respiration, the relative thickness of the hydrodynamic and diffusion boundary layers surrounding the animal (54, 141). Total resistance to oxygen uptake is dominated by resistance to diffusion across the boundary layer at low Re in adult bullfrogs (117); boundary layers are likely to be of even greater importance to very small aquatic animals living at even lower Re (67). A possible reason for continuous swimming in larval anchovy *(Engraulis mordax)* is to dissipate the boundary layer and increase cutaneous gas exchange (141).

Diffusion distances between water and tissues are kept as short as possible and thus gradients as steep as possible. Larval fish swim by using a layer of red muscle fibers immediately under the epithelium that are directly supplied with oxygen by diffusion. These muscle fibers move to the midline (their normal location in adult fish) as the circulation and gills develop (4, 47, 74). *Neoceratodus* (Australian lungfish) larvae, as well as many amphibian larvae, have cilia on the body surface (21, 146). Cilia are only useful at low Reynolds number (thus small body size and low fluid velocities), but may be particularly useful in dissipating boundary layers since they move fluid very close to the body wall (137).

GILL DEVELOPMENT External gills precede internal gills in lungfish and some other primitive fish (27). In most fish internal gills first develop early in larval life as primary bars, with little surface for gas exchange (Figure 4). Primary lamellae quickly appear, and finally secondary lamellae do, both of which increase in number over the larval and juvenile life of the fish (36, 108). Branchial exchange surface increases much more rapidly than body mass while secondary lamellae are appearing (6, 47, 74, 108) so that mass-specific gill surface area increases rapidly. Over the same range of growth, mass-specific skin surface area decreases with an exponent of approximately -0.33, thus suggesting that gas exchange partitioning shifts from exclusively cutaneous to primarily branchial during larval growth. After metamorphosis, gill surface area increases with approximately the same allometric exponent as metabolic rate, while mass-specific body surface area continues to decrease (108), so that gas exchange partitioning shifts further toward the gills with increasing size. The small size of most fish larvae has precluded direct measurement of gill/skin gas exchange partitioning.

The developmental shift in partitioning may be accompanied by changes in oxygen-binding properties of blood as gas exchange switches from a primarily diffusion-limited exchanger (skin) to an actively ventilated, more perfusion-limited gas exchanger (gills). There are ontogenetic changes in expression of multiple hemoglobins in salmonids (64, 65), but the functional significance of these changes is unknown.

At hatching, amphibian larvae are usually larger than fish larvae (although still only 5–25 mg) and many amphibian species hatch more fully developed, with functional cardiovascular system and circulating red cells (11, 12, 28). In larval salamanders, external gills quickly appear and persist throughout larval development. Many anurans have external gills for a few days after hatching, then they degenerate and are replaced by internal gills (11, 97). Amphibian gills are morphologically different from fish gills, with leaf-like lamellae extending from finger-like primary gill bars in urodeles and tufts of finger-like lamellae extending from the gill bars in anurans (29, 97, 103). These gills are much more irregular than the regularly spaced, plate-like lamellae of fish gills. The upstream side of the branchial basket is often adapted for filter feeding in anuran larvae; gas exchange is not the sole function of the branchial basket (55, 103).

DEVELOPMENT OF AIR BREATHING: RESPIRATORY TRANSITIONS FROM WATER TO AIR Some fish, most amphibians, and all reptiles start to breath air at some point in their ontogeny (Figure 4). Lungs appear to be a primitive feature of bony fish and are retained in lungfish and some other primitive fish (27, 121). Most modern teleosts have a swim bladder, probably derived from the primitive lung, but many genera of teleost fish have secondarily developed

other aerial gas exchangers, including modifications of the pharynx, intestine, and branchial chambers. Amphibian larvae have lungs for aerial gas exchange, although they may not contribute significantly to gas exchange until close to metamorphosis. Reptiles hatch as juveniles rather than larvae and have fully functional lungs (110). Changes may occur in aerobic and anaerobic scope (5), respiratory surface area (134), and hemoglobin isoforms (118) with growth in reptiles, but most remain primarily air breathers throughout life.

Because of the physico-chemical differences between water and air in density, capacitances for oxygen and carbon dioxide, and diffusion rates, the structures of gas exchangers for water and air are very different (40, 41). Water breathers have a much higher ventilation requirement because of the low oxygen capacitance of water. They have a low PCO_2 because CO_2 capacitance of water is 20–30 times higher than O_2 capacitance, and gas exchange is more diffusion-limited because of the much slower rate of diffusion in water and the formation of diffusion boundary layers. Regulation of ventilation usually occurs through oxygen-sensitive chemoreflexes (33, 40, 111). Air breathers, in contrast, have a lower ventilation requirement because of the relative abundance of oxygen in air. However, air breathing animals have a much higher blood PCO_2 because (*a*) they hypoventilate compared to water breathers, and (*b*) air-breathing organs (typically lungs or swim bladders) are often tidally ventilated, which is less effective than a unidirectionally ventilated system like gills. Air-breathing animals usually regulate ventilation through both CO_2/H^+-sensitive chemosensors and oxygen-sensitive chemosensors (33, 40).

AIR-BREATHING FISH Air-breathing fish are typically found in warm hypoxic waters. These fish generally use the air-breathing organ to augment oxygen uptake, but excrete almost all CO_2 through reduced gills because of the high capacitance and diffusion of CO_2 in water compared to O_2. (As a consequence the blood PCO_2 is so low that little CO_2 is excreted into air) (40, 121, 122). A few air-breathing fish can also excrete significant amounts of CO_2 into air (10, 101), but this is exceptional. Gas exchange partitioning depends on numerous factors, including gas partial pressures in air and water, ventilation of the gills and air-breathing organs, surface areas and diffusion distances in the gas exchangers, circulatory arrangement, and perfusion of the gas exchangers. No one study or series of studies has addressed all the important variables. Oxygen uptake partitioning may change rapidly during development as gas exchangers develop or degenerate. As in purely aquatic fish, newly hatched larvae respire cutaneously with both skin and gills as the gills develop. After two to three weeks, air-breathing organs appear (lungs, suprabranchial chambers, and so on), increase in surface area, and are finally

ventilated. When air breathing starts, the rate of increase of gill surface area with body size decreases (from a scaling exponent of 2.4 to 0.8), body scales develop, probably reducing cutaneous gas exchange (77, 119), and aquatic oxygen uptake decreases (131). In the air-breathing swamp eel *Monopterus cuchia,* cutaneous surface area and probably cutaneous gas exchange is reduced upon start of air breathing because the large pectoral, dorsal, and ventral fins of the larva disappear (130).

AMPHIBIAN METAMORPHOSIS The most dramatic transition from water breathing to air breathing occurs in amphibians. Some amphibians, such as the tiger salamander *(Ambystoma tigrinum),* change very little during metamorphosis, from larvae with external gills and well-developed lungs to morphologically similar but gill-less adults. At the other extreme is *Bufo americanus,* whose larvae do not have functional lungs until close to metamorphic climax, but metamorphose into almost completely terrestrial, air-breathing juveniles in only a few days.

Ambystomid salamanders are highly aquatic both as larvae and adults. Large larvae obtain approximately 65% of their oxygen from water with roughly equal contributions from skin and gills, and 35% of oxygen through lungs; adults obtain approximately 35% of their oxygen through the skin from water and 65% through the lungs. The proportion of pulmonary gas exchange increases in both larvae and adults in response to hypoxia (70). In keeping with the relatively minor morphological changes associated with metamorphosis, there is no major change in Hb-O_2 affinity or other hematological measurements (31). Blood properties do change with body mass in larvae, however; oxygen carrying capacity and Hill's n both increased with increasing body mass (22).

Immediately after hatching anuran larvae respire exclusively with water using gills and skin. *Bufo americanus* larvae remain obligate water breathers until metamorphosis, while older *Xenopus* larvae in hypoxic water can obtain 100% of their oxygen requirement from air with their lungs (56, 139). The skin is the most important site of gas exchange throughout larval growth of bullfrog larvae, by providing around 60% of oxygen uptake and excreting a similar amount of CO_2. Gills provide the remaining 40% of oxygen in early stages, but unlike fish, they become progressively less important during larval growth as lungs become more important for oxygen uptake. Since the lungs are not as important for CO_2 excretion, cutaneous CO_2 excretion increases to over 80% (30).

Bullfrog larvae have gas in their lungs soon after hatching (46, R. L. Infantino, personal communication), but do not obtain much oxygen through them until TK stage XVI (30, 24). The lungs are at first simple thin-walled sacs, but in later stages (TK V to XXII) develop primary, secondary, and

tertiary septa, which increase the surface area (3, 15, 46). At metamorphosis the gills involute, aquatic oxygen uptake decreases sharply, and pulmonary oxygen uptake increases from less than 20% in pre-metamorphic larvae to almost 70% in juveniles (30, 72). There is a change in hemoglobin types to lower affinity variants as metamorphosis occurs—this appears to be correlated with increased reliance on a high capacitance, highly stable gas exchange medium (9, 88, 115).

Regulation of Ventilation

Changes of ventilatory regulation with the developmental transition from water breathing to air breathing resemble these thought to have occurred during the evolutionary transition from water to air. With the shift in respiratory medium, the control system changes from being almost entirely oxygen-driven to a combination of acid-base and oxygen-driven. Acid-base status adjusts from one with low PCO_2 and low bicarbonate to high PCO_2 and high bicarbonate, with little change in pH.

Almost nothing is known of the development of ventilatory control in fish. Newly hatched dogfish (*Scyliohinus* sp.) decrease gill ventilation frequency in hypoxia, probably as a direct consequence of oxygen limitation (43), while adult fish increase ventilation during hypoxia (40, 111). It is not known when the chemoreceptor reflex responsible for this response becomes functional.

Early bullfrog larvae (TK stages I–X) are almost entirely aquatic, are insensitive to PCO_2, and increase gill ventilation in response to aquatic hypoxia (21, 79). The receptors mediating these responses are located both centrally (location unknown, but presumably in a central vascular compartment and/or the CNS) and on the gill arches. The presence of the latter has been confirmed by the extremely rapid time course (<2 sec) of ventilatory responses to step changes of PO_2 of inspired water (81) and by ablation of selected regions of the gill arches (X. Jia, unpublished).

The lungs of pre-metamorphic larvae and the air-breathing organs of bimodally breathing fish are used only as an auxiliary oxygen supply and are ventilated infrequently (21, 24, 79, 104, 121, 126). Air-breathing frequency and the proportion of aerial oxygen uptake increases during aquatic hypoxia (2, 10, 30, 58, 104, 121, 144). Unlike breathing in entirely aquatic fish, there may be a simultaneous decrease in gill ventilation in bimodal breathers, probably to reduce oxygen loss from the blood (56, 61, 142, 143). Commonly, there are also vascular shunts that reduce the amount of blood perfusing the gill during aquatic hypoxia (13, 94–96, 98).

Ventilation in bimodal breathers is complicated by non-respiratory factors. Many larvae are filter feeders, which may uncouple gill ventilation from respiratory requirements (55). Lung ventilation changes buoyancy and re-

quires movement to the surface, which may decrease locomotor efficiency; it is energetically expensive, and may expose the animal to predators (52, 90, 91, 128, 139).

Most adult anurans are still bimodal breathers, but use skin and lungs rather than gills, skin, and lungs. Lung ventilation frequency of adults also increases in hypoxia, with a concomitant increase in the proportion of oxygen taken through the lungs (82, 116). Ventilation sometimes decreases in hyperoxia, which indicates that part of the normal drive for ventilation is due to oxygen (136). Cutaneous gas exchange with water is probably also regulated, although the degree of regulation and the route, i.e. skin ventilation, capillary recruitment, or perfusion rate, is as yet uncertain (7, 23, 53, 99, 100, 114, 117).

Anuran larvae entering metamorphic climax (TK stages XX–XXIV) change from larval to adult breathing pattern. Late-stage larvae (XVI–XIX) are obligate air breathers, increase lung ventilation, but not gill ventilation, in response to hypoxia, and start to show sensitivity to CO_2 (21, 79). As the gills involute between stages XX and XXIV and the tail is resorbed, respiratory surface area is lost for aquatic CO_2 excretion. Blood PCO_2 increases from 3–5 to 13–15 mmHg, compensated by an increase in HCO_3 so that pH does not change (3, 48, 84). Although most CO_2 is excreted through the skin in adults, control of CO_2 excretion and acid-base balance is by lung ventilation (21, 30, 80, 83). The switch to adult respiration appears to be between TK stages XXI and XXII, when the larval pattern of infrequent single lung ventilations changes within one or two days to the adult pattern of much more frequent ventilations, which are often grouped into bouts (R. L. Infantino, personal communication).

Ontogenetic changes in the pattern of ventilation in newly hatched or born reptiles have not been investigated to our knowledge.

CONCLUDING REMARKS

In spite of literally centuries of anatomical study of the embryos of fishes, amphibians, and reptiles, developmental changes in the cardio-respiratory physiology of these vertebrate classes are only beginning to be investigated in detail. The recent advent of miniaturized techniques for recording hemodynamics and blood gases in very small animals is making an important impact upon the field, and more sophisticated physiologic measurements on earlier developmental stages are emerging.

These studies clearly indicate that qualitative as well as quantitative differences in cardio-respiratory physiology separate embryonic and larval forms from the terminal adult. In many instances, the earliest embryonic

stages are very simple, indeed, from both an anatomical and physiologic perspective. In contrast to the embryos of birds and mammals, however, the larvae of fishes and amphibians undergo most development only after hatching into free-living entities. This self-sufficient existence demands that, even as development progresses, the organism must be able to respond physiologically (as well as behaviorally) to the inevitable changes in both the external and internal environment. The notion that the adult is physiologically the most complex developmental stage in a species' life cycle should be discarded (or at the very least be verified on a species-by-species basis) because data on numerous fronts indicate that even quite early developmental stages may possess complex mechanisms for finely regulating cardiovascular and respiratory performance.

Major gaps exist in our knowledge of the cardio-respiratory physiology of lower vertebrates. Many of these deficiencies have been identified above, but some bear special emphasis. Ultimately, adjustments in heart rate, blood pressure, stroke flow, and so on serve to maintain oxygen transport homeostasis. Yet crucial factors in this homeostatic process—e.g. cardiac output, arterial and venous oxygen content—remain undescribed.

Another area demanding further attention involves the onset of physiologic function, rather than the continuing developmental changes. This gap exists for all vertebrates, including mammals. For example, most fetal physiology is based on relatively developed fetuses with nearly complete regulatory systems. The origins of cardio-respiratory processes in the early embryos deserve much further attention, although admittedly, these are also the most difficult stages to examine experimentally.

Finally, we are struck by the relative dirth of information on physiologic development in reptiles compared with fishes and amphibians. This is perhaps unexpected, since there is a vast literature on the physiology of adult reptiles. The embryos of some reptiles are often very large relative to those of amphibians and fishes (or even birds and mammals), which could permit physiologic measurements on embryos that might not be possible in other vertebrates. Thus an increased focus on reptilian embryos in the future would have the combined effects of adding to our specific knowledge of reptilian development as well as providing a paradigm for future investigations of basic aspects of vertebrate development.

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