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REVIEW

Development of Cardiac Form and Function in Ectothermic Sauropsids

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ABSTRACT Evolutionary morphologists and physiologists have long recognized the phylogenetic significance of the ectothermic sauropsids. Sauropids have been classically considered to bridge between early tetrapods, ectotherms, and the evolution of endotherms. This transition has been associated with many modifications in cardiovascular form and function, which have changed dramatically during the course of vertebrate evolution. Most cardiovascular studies have focused upon adults, leaving the development of this critical system largely unexplored. In this essay, we attempt a synthesis of sauropsid cardiovascular development based on the limited literature and indicate fertile regions for future studies. Early morphological cardiovascular development, i.e., the basic formation of the tube heart and the major pulmonary and systemic vessels, is similar across tetrapods. Subsequent cardiac chamber development, however, varies considerably between developing chelonians, squamates, crocodilians, and birds, reflected in the diversity of adult ventricular structure across these taxa. The details of how these differences in morphology develop, including the molecular regulation of cardiac and vascular growth and differentiation, are still poorly understood. In terms of the functional maturation of the cardiovascular system, reflected in physiological mechanisms for regulating heart rate and cardiac output, recent work has illustrated that changes during ontogeny in parameters such as heart rate and arterial blood pressure are somewhat species-dependent. However, there are commonalities, such as a β-adrenergic receptor tone on the embryonic heart appearing prior to 60% of development. Differential gross morphological responses to environmental stressors (oxygen, hydration, temperature) have been investigated interspecifically, revealing that cardiac development is relatively plastic, especially, with respect to change in heart growth. Collectively, the data assembled here reflects the current limited morphological and physiological understanding of cardiovascular development in sauropsids and identifies key areas for future studies of this diverse vertebrate lineage. J. Morphol. 270:1400–1412, 2009.

KEY WORDS: cardiovascular; development; regulation; reptilian; chelonian; crocodilian; squamate; ectothermic; sauropsids

INTRODUCTION

Ectothermic sauropsids or "reptiles"[1] have long been of great significance in understanding evolutionary transitions from early tetrapods to endotherms. Perhaps reflecting the fundamental importance of the heart, the study of cardiac form and function in particular has been the subject of studies for comparative morphologists and physiologists for centuries (Bojanus, 1819; Panizza, 1833). As adults, reptiles represent a distinctive cardiovascular transition between the single circulation of fishes and the double circulation with systemic and pulmonary circuits of birds and mammals (e.g., White, 1968; 1970; Burggren, 1978; Johansen and Burggren, 1985; Wang et al., 1998; Hicks, 2002; Fisher and Burggren, 2007). Briefly, turtles, non-varanid lizards and snakes possess two separate atria that eject blood into ventricular chamber consisting of the cavum pulmonale, cavum venosum, and cavum arteriosum. In lepidosaurs, intracardiac blood flow patterns reflect both left-to-right (systemic bypass) and right-to-left (pulmonary bypass) shunts. Although considerable spatial separation of internal blood streams can be maintained, there is little or no pressure separation between the ventricular cava (White, 1968; Shelton and Burggren, 1976; Hicks and Malvin, 1992; Hicks, 2002). In some snakes (Wang et al., 2003) and varanid lizards (Burggren

[1]"Ectothermic sauropsids" is the taxonomically accurate term, but we use the paraphyletic term "reptiles" as a descriptor due to its familiarity across biological disciplines and its simple conversion into a frequently used modifier throughout the text.

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and Johansen, 1982), a functional ventricular separation is evident in the pressure differences between different cava through part of systole. Crocodilians and birds possess a structurally separate left and right ventricle, resembling that of mammals. This structural arrangement eliminates the capacity for intracardiac mixing of oxygenated and deoxygenated blood. However, the presence of a left aorta arising from the right ventricle, and the foramen of Panizzae connecting the aortic bases, enables right-to-left shunting in the Crocodilia (White, 1968; Nilsson, 1994). Despite intensive study, both the mechanisms behind intra- and extracardiac shunting in adult reptiles, as well as the functional importance of such shunting, remains intensely debated, with explanations ranging from the optimization of maximal metabolic rate to aiding digestion (Farmer and Carrier, 2000; Syme et al., 2002; Wang and Hicks, 2002; Skovgaard and Wang, 2006; Farmer et al., 2008).

Even as the comparative cardiovascular anatomy and physiology of adult reptiles is compelling and the focus of much ongoing study, this topic remains poorly explored in their embryos. Certainly, there are strictly anatomical studies dating back to the 19th century, which we shall review below. However, fundamentally lacking is the integration of morphological and physiological findings to create a comprehensive synthesis of how cardiovascular form actually dictates function in developing reptilian embryos. The lack of such an integrative approach is somewhat surprising, given the extensive investigations of cardiovascular form and function in bird embryos, larval amphibians, larval fishes, and fetal mammals (e.g., Johansen and Burggren, 1985; Hoar and Randall, 1988; Burggren and Just, 1992; Burggren and Keller, 1997). There is a burgeoning interest in comparative developmental morphology and physiology (Burggren and Warburton, 2005), based on the combination of a desire to understand both genomic and nongenomic aspects of human development through appropriate animal models, as well as the interest in understanding the interrelationship of evolution and development. Yet, until, we know more about cardiovascular development in reptiles, we are unlikely to assemble a complete understanding of cardiovascular development in vertebrates as a whole.

The purpose of this essay, then, is to generate a synthesis based on functional, cardiovascular development in reptiles, aided by the few detailed morphological studies. Given the paucity of data on most aspects of reptilian morphological and physiological development, we present less a comprehensive survey and more a call to action for future additional studies integrating morphology and physiology.

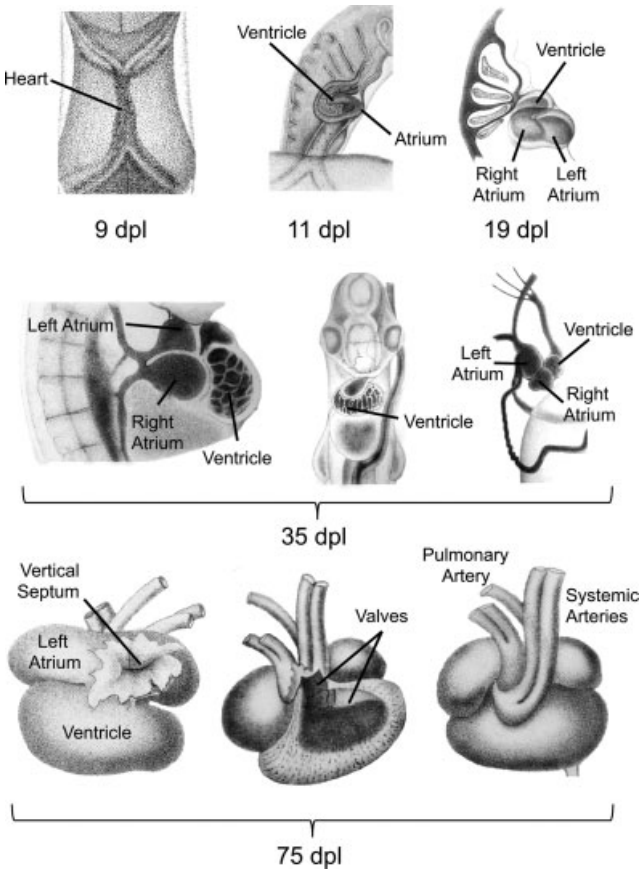

Fig. 1. Development of the heart of *C. serpentina* from 9 to 75 days post-laying (dpl). From Agassiz (1857) with rearrangement and relabeling. Embryonic size at the time of description was not given.

DEVELOPMENTAL MORPHOLOGY OF REPTILIAN HEARTS

Early Cardiovascular Development

In the earliest stages of vertebrate development, differences between clades in both cardiovascular anatomy and physiology are minimal (Burggren and Crossley, 2002), reflecting the commonality of the underlying morphogenic processes involved in heart and vessel formation. Thus, not surprisingly, the early morphological development of the squamate, chelonian, and crocodilian heart follows the ancestral vertebrate pattern and shows no substantial variation between these reptilian clades. In essence, early development of the chelonian heart progresses as in birds and mammals, with first the right and left heart anlagen appear as two lateral masses of primordial splanchnic mesoderm situated on either side of the embryo's central axis (Laale, 1984; Driever, 2000). These two anlagen migrate to the dorsal midline where they eventually merge to form a thin endocardial tube of flattened cells.

The heart tube is relatively straight in early embryonic chelonians (Agassiz, 1857), squamates

(Griel, 1903), and crocodilians (Clarke, 1891). The posterior and anterior ends of the heart tube bifurcate (Fig. 1). The developing atrium and sinus venosus appear at the posterior fork and gradually merge through remodeling of these newly formed cardiac tissues. Anteriorly, the fork of the heart tube quickly develops into the branching patterns of the aortic arches. As detailed by Agassiz (1857) for the developing turtle, *Clemmys guttata*, "In the beginning the heart is a simple straight tube connecting posterior to the transverse channel and anterior with two other channels that pass left and right." "It is within the boundaries of the central propeller of the circulation that the blood first makes its appearance, surging backward and forward." The basic pattern of heart tube formation described by Agassiz (1857) for the *Clemmys guttata* is largely repeated for heart development in lizards such as the sand lizard (*Lacerta agilis*) (Griel, 1903).

With respect to function of the early embryonic heart, weak and irregular contractions powered by cardiac muscle begin to appear at the heart tube stage between approximately Stage 6–7 for the chelonians *Chelydra serpentina* and *Lepidochelys olivacea* (Yntema, 1968; Crastz, 1982). Extrapolating from this observation of early cardiac function in chelonians relative to the overall embryonic development, heart contraction in crocodilians may occur prior to actual egg deposition (Clarke, 1891; Ferguson, 1985). Initially, cardiac contractions are in the form of peristaltic movement propelling the developing blood, which at this stage is a clear plasma nearly devoid of formed elements (red and white blood cells). Effective anterograde transport of arterial blood is concurrent with the development of one way valves in the arterial and venous circulations, which in *C. serpentina* occurs by at least Stage 8 (Yntema, 1968).

Surprisingly, effective convection of blood is not a requisite for early embryonic survival and growth in many vertebrates, where simple diffusion suffices for respiratory gas and nutrient exchange long after the heart beats and begins to move blood about the body (Burggren, 2004). However, blood convection, through complex feedback mechanisms, likely plays a role in the actual morphological development of the embryonic heart. Heart tube shaping involves both genetic and epigenetic factors, at least in chicken embryo models (Taber, 1998). Some aspects of cardiac morphogenesis (e.g., bending) involve intrinsic factors and will occur even in hearts that have been experimentally prohibited from contracting. Other cardiogenic events depend on forces external to the heart (e.g., dextral torsion) or on mechanical stress caused by heart contraction itself (e.g., looping; Ramasubramanian et al., 2008). Whether these findings in birds are representative of reptiles and other ectothermic vertebrates awaits additional study.

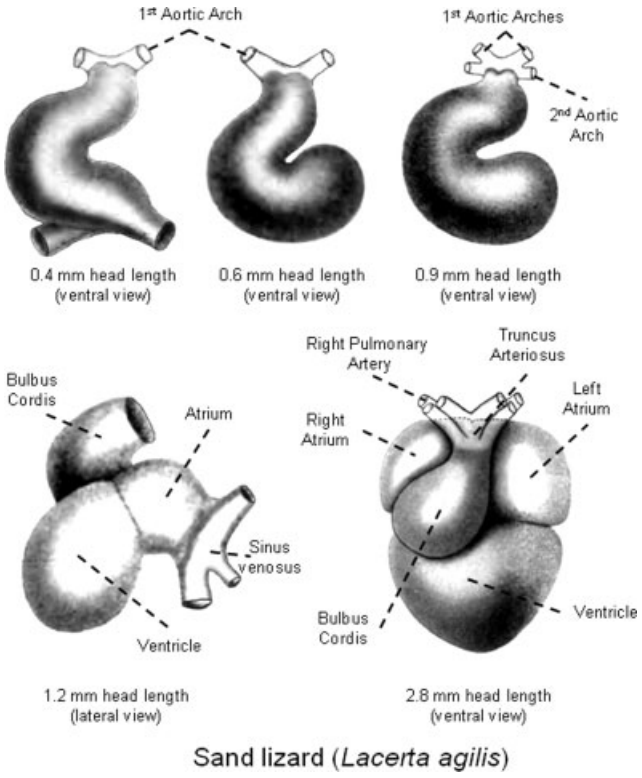

Fig. 2. Development of the heart of embryonic sand lizards, *Lacerta agilis* from 0.4 to 2.8 mm in head length, in ventral or lateral orientation. Taken from Griel (1903) with rearrangement and relabeling.

Heart Chamber Formation

Chamber development of the reptilian heart, likely driven by hyperplastic cardiomyocyte growth as in other vertebrates, begins after the typical vertebrate pattern of heart tube S-folding as described above for chelonians (Fig. 1) and squamates (Fig. 2). Areas of greater cell division, combined with rings of constriction representing the endocardial cushions, identify the expansion of the cardiac chambers. The endocardial cushions undergo continuing hyperplastic growth to form cardiac valves at the base of the aortic arches and pulmonary artery, which are distinctive and well formed in all reptiles studies to date (Figs. 2 and 3). Cardiac grow continues with the folding of the heart and formation of distinctive atrial and ventricular chambers in chelonians (Agassiz, 1857; *C. serpentina* and *C. guttata*), squamates (Griel, 1903; *Lacerta agilis* and *Tripodonotus natrix*), and crocodilians (Griel, 1903).

The gross morphology of heart chamber formation in late reptilian embryos has been described in detail (Agassiz, 1857; Griel, 1903). Figures 1 and 2 drawn from these accounts reveal key stages of cardiac and great vessel growth in a chelonian and squamate species. We caution that, while these early accounts are both extensive and

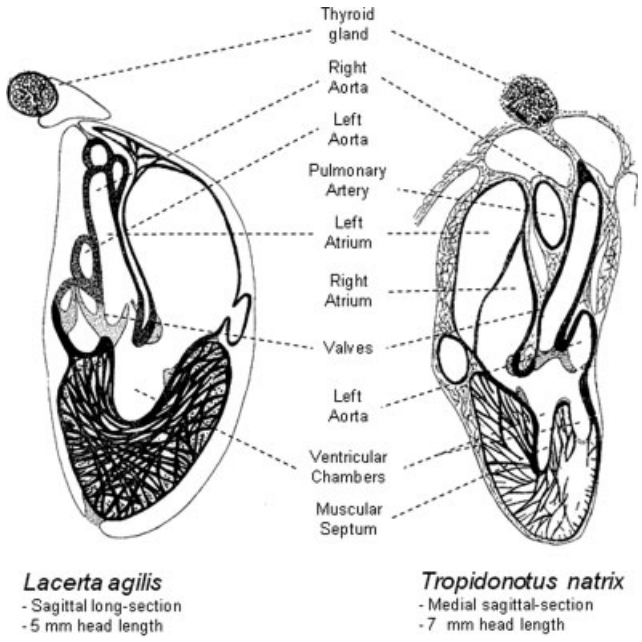

Fig. 3. A comparison of a lizard and snake heart (sagittal section) at similar stages of development. From Griel (1903) with rearrangement and relabeling.

detailed, key aspects of incubation (e.g., temperature, humidity) were not provided, which obfuscates quantitative interpretation of the rates of cardiovascular development and growth. Essentially, the basic appearance of the great vessels, the separation of the atrium into left and right chambers, the appearance of cardiac valves and other key features are qualitatively similar in chelonians and squamates. Crocodilian heart development as described by Griel (1903) also follows these same basic patterns with the further development of full ventricular separation into left and right chambers, and the distinctive arrangement of the arterial arches originating from either the left or right ventricular chamber. A particular focus for future studies should be an attempt to determine the following: 1) the mechanisms driving the different developmental pathways for crocodilian and non-crocodilian hearts; 2) what functional differences might occur in the key developmental stages spanning the transition from simple heart tube to the fully developed heart at hatching; and 3) mapping how functional changes (e.g., excitation-contraction coupling, blood pressure generation, cardiac shunting and its control) are linked to morphological changes. Such studies will necessarily involve an examination of the specific patterns of cellular differentiation and myocyte growth. Techniques such as neural crest cell ablation (e.g., Hutson and Kirby, 2007; Snider et al., 2007) and fate mapping (e.g., Schoenebeck and Yelon, 2007), which have been used in embryos of zebrafish and chickens, will greatly aid future examinations of the developing reptile heart.

As a final note on heart development, congenital cardiovascular defects have been noted in a few instances in reptiles, either as a structural developmental defect (e.g., interventricular septal defect in *Alligator mississippiensis*; Brockman and Kennedy, 1962) or as failed closure of an adult structure (e.g., patent ductus arteriosus in the tortoise *Testudo graeca*; Burggren, 1974). The functional implications of these congenital morphological defects are unknown, but are unlikely to have the same potentially profound affects seen in birds and mammals where intracardiac shunting is inevitably pathological.

Aortic Arch Development

Blood vessels in reptiles begin developing from an aggregation of mesenchymal cells, independently from heart formation, as in all vertebrates. Shortly after the appearance of cardiac pulsation, isolated proto-vessels appear in the form of the aortic arches and major veins. These vessels rapidly merge and assemble into a simple but continuous interconnected blood circuit (Shaner, 1921; Johnson, 1922). The appearance of one-way cardiac valves (described above), followed by venous valves in the peripheral circulation, enables the establishment of a definitive one-way circulation through the growing heart.

Little is known about the detailed development of the embryonic aortic arches in reptiles other than chelonians, a curious omission in the literature given that these same vessels and their impacts on systemic and pulmonary perfusion have been the focus of such intense study in adult reptiles. Accounts of chelonian and squamate heart development typically diagram the arteries and veins themselves only in shadowy outline, focusing instead on details of cardiac structure. One detailed account of reptilian aortic arch development examined embryos of the loggerhead turtle, *Caretta caretta* (Suzuki and Kasai, 1990). Notably, this study focused on the subclavian vein, revealing its formation through connection of the primary subclavian artery with a caudally extending artery arising from the aortic sac. This process differs from that in the chicken embryo, where the "secondary" subclavian artery is derived from an outgrowth of the primary subclavian artery. The functional implications of this difference remain to be determined.

Chorioallantoic Membrane Vasculature Development

Embryos of oviparous reptiles also develop an extensive chorioallantoic membrane (CAM) lining the eggshell. The CAM serves as the sole respiratory gas exchange organ until hatching. Deoxygenated afferent and oxygenated efferent blood sup-

plies for the CAM are derived from chorioallantoic artery and vein, respectively. General patterns of growth and differentiation in the CAM and heart have been described for the European pond turtle, *Emys orbicularis* (Nechaeva et al., 2007). Increase in mass of both the CAM and heart are closely correlated with oxygen consumption prior to day 50 (total incubation time $\sim$ 100 days at 25°C; Ewert, 1985). The surface area of the CAM has also been investigated in the veiled chameleon (*Chamaeleo calyptratus*), where peak CAM growth and development occurs in three phases, with the middle phase of rapid CAM growth correlating with growth and differentiation of the embryo per se (Andrews, 2007). Observations of the CAM have also been extended to the fetal membranes of viviparous squamates (Knight and Blackburn, 2008 for earlier literature), showing cytological specializations for gas exchange and absorption in the specialized intrauterine environment. The specialized vasculature of the CAM has yet to be pharmacologically categorized in reptiles and may not be representative of the embryonic vascular beds with which it interconnects.

Environmental Effects on Cardiovascular Developmental Morphology

A developing vertebrate embryo is, of course, far more than the product of its genes—epigenetic, nongenomic phenomena introduce a developmental plasticity that potentially plays major roles in eventual morphological and physiological phenotypes (cf. Gilbert and Epel, 2008). The influence of environment (especially incubation temperature) on sex determination is perhaps the best example of this in reptiles (e.g., Janzen and Phillips, 2006). Once again, however, few studies address the role of thermal or other environmental factors in the development of cardiovascular structures and their subsequent function. Reflecting the relative ease of access to extraembyronic vasculature (especially to vessels of the CAM), most of the scant literature in reptiles focuses on environmental effects on these vessels. Chronic temperature change has been considered as an environmental stressor affecting CAM vascular density in the snapping turtle, (*C. serpentina*; Birchard and Reiber, 1995). While not surprisingly incubation time is longer at 24°C than 30°C but CAM vascular density (as well as peak oxygen consumption and embryonic mass) is unaffected by temperature. Incubation temperature in the veiled chameleon, *C. calyptratus*, influences the extent of CAM vascularization along with embryo growth and development (Andrews, 2007). Interestingly, this study revealed a nonlinear Q_{10} for important morphological aspects of development, with the greatest impact occurring as temperature was increased from 25 to 28°C, but little subsequent change accompanying a 28 to 30°C increase. Birchard and Reiber (1996), focusing exclusively on the heart mass across development in the snapping turtle (*C. serpentina*), reported a sigmoid rate of increase in cardiac mass at both 24 and 29°C. These investigators concluded that temperature-induced developmental plasticity is greatest in early development. Thus, key aspects of development are differentially affected by environmental temperature, and interspecific differences also appear evident. Additional experiments not only documenting temperature-induced morphological changes but also any physiological implications of these experiments are called for.

Oxygen, or more precisely the lack of oxygen, is also a stressor that has been implicated in affecting CAM development. In the American alligator, experimental reductions in permeability of the eggshell (created by painting a region of the eggshell with a gas-impervious paint) creates an underlying, localized hypoxia has been shown to stimulate vascularization of the underlying CAM (Corona and Warburton, 2002). Interestingly, this is in contrast to similar experiments in chicken eggs, where hypoxia depressed the growth of underlying CAM vasculature (Wagner-Amos and Seymour, 2003).

Many reptilian eggs are highly permeable to water and can both gain and lose water to the environment in which they are incubated (e.g., Ji and Du, 2001; Brown and Shine, 2005). In an intriguing study of common snapping turtles (*C. serpentina*), Packard and Packard (2002) reported that raising embryos in a dehydrating environment (-100 kPa vs. -700 kPa) resulted in accelerated heart growth. These authors ascribed these changes to heart enlargement associated with hypovolemia and increased blood viscosity. Similar morphological changes are exhibited by turtles (Kam, 1993) and American alligators incubated under chronic hypoxic conditions (Crossley et al., 2005). Collectively, these data suggest that cardiac hypertrophy may be a common response to increased cardiac demands in developing reptiles.

Having provided an overview of developmental morphology of the reptilian heart and vasculature, we now focus on developmental cardiovascular physiology, emphasizing linkages between function and form.

DEVELOPMENTAL CARDIOVASCULAR PHYSIOLOGY

The functional changes that accompany the various anatomical stages of cardiovascular maturation described above in reptiles are only poorly understood. For example, we are currently unable to match key physiological changes (e.g., generation of blood pressure, development of anterograde blood flow) to key morphological developmental events (e.g., valve and cardiac chamber formation).

Oviparous reptiles produce an egg that can vary tremendously in shell composition, ranging from a highly calcified to a parchment like shell with limited calcification (Packard et al., 1982). These reptilian eggshell characteristics greatly complicate invasive cardiovascular experimentation by not universally providing a solid substrate on which to attach instrumentation such as catheters (Warburton, 1997). Moreover, the typical state of CAM adhesion to the overlaying shell membrane, and the tendency of CAM vessels to constrict upon being touched, makes blood vessel catheterization or Doppler measurements of blood flow challenging in these embryonic animals (for embryonic measurement techniques see Burggren and Fritsche, 1995; Schwerte and Fritsche, 2003; Burggren and Blank, 2009). Despite these challenges, cardiovascular physiological data has been collected from representative reptilian species; American alligator, common snapping turtle, desert tortoise (*Gopherus agassizi*), and African Brown house snakes (*Lamprophis fuliginosus;* Birchard et al., 1984; Birchard and Reiber, 1996; Crossley II, 1999; Crossley et al., 2003b; Crossley II and Altimiras, 2005). Information about the maturation of blood pressure, heart rate, and regulatory mechanisms emerging from these studies can be used to illustrate how ontogeny impacts cardiovascular development. However, additional representative species and different reptilian clades need to be studied to provide a comprehensive comparative overview of how basal cardiovascular function matures and is regulated in developing reptiles.

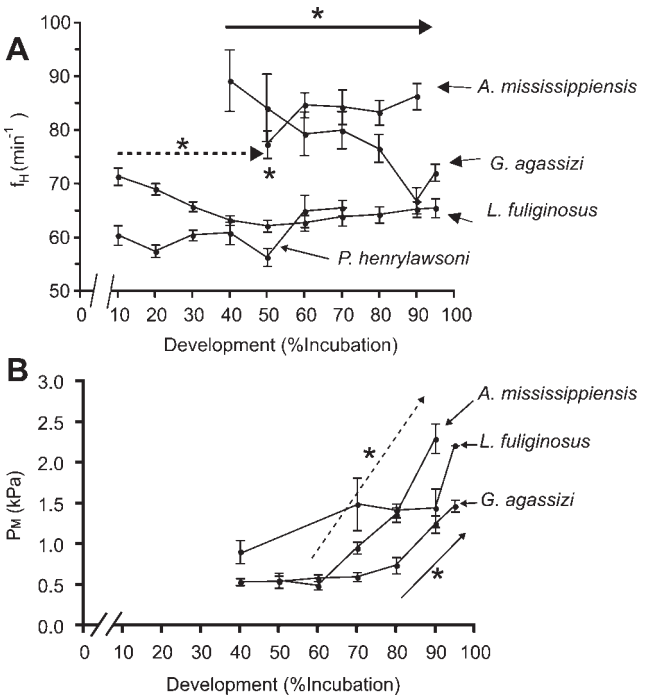

Fig. 4. Changes of cardiovascular parameters during development of different reptiles (**A**) Heart rate (f_H) of embryonic American alligators, Desert tortoise, African brown house snakes and Lawson's Dragons at 10% intervals of incubation. (**B**). Mean arterial pressure (Pm) of embryonic American alligators, Desert tortoise, and African brown house snakes at 10% intervals of incubation. Data are presented as the mean ± SE. The numbers for all species are a minimum of 5. An asterisk above an arrow (dashed for the house snake and solid for the desert tortoise) indicates a significant change. A single asterisk indicates differences from a given incubation point from the remaining points in development.

Basic Cardiovascular Function

Embryonic heart rate (f_H) is the most readily measured cardiovascular parameter and can be reliably monitored via minimally invasive methods over an extensive period of development. Given that changes in f_H typically alter cardiac output and convective transport of materials in the blood, f_H alone provides important information about cardiovascular function in the embryonic animal. The pattern of change in f_H during development differs across reptilian taxa. In the American alligator, f_H is constant at ~80 beats·min^{-1} over the final 40% of incubation (Crossley et al., 2003b; Crossley et al., 2005). Additional measurements at 40% (Warburton et al., 1995) and 50% (Fig. 4A) of incubation suggest that f_H may increase during the initial portion of embryonic American alligator development, a pattern that is similar to that in other vertebrate species (Van Mierop and Bertuch, 1967; Burggren and Warburton, 1994; Tazawa and Whittow, 1994; Tazawa et al., 1994; Tazawa and Hou, 1997; Crossley et al., 2003a). If heart mass is used as an indicator of stroke volume changes, as has previously been validated in embryonic common snapping turtles (Birchard and Reiber, 1996), then embryonic American alligators exhibit a relatively constant stroke volume over the final 50% of development (Crossley and Altimiras, 2005). Given that cardiac morphology and chamber structure is established during the first 50% of development (Figs. 1 and 2), chamber volume may then also be constant over late development. Together with the constant f_H for the final 40% incubation, these findings suggest that cardiac output also remains constant during this period. While additional crocodilian species should be investigated, the association of the crocodilian and avian lineages implies that this may be an inherent pattern to archosaurian vertebrates during ontogeny (Webb, 1979).

Changes in f_H during development have been studied in two chelonian species, the common snapping turtle (Birchard and Reiber, 1996) and the desert tortoise (Fig. 4A; Crossley and Reiber, unpublished data). While the small number of chelonian species prohibits generalizations, it appears that both species developing at 29°C (*C. serpentina*) and 30°C (*G. agassizi*) exhibits a progressive fall in f_H from 50% of incubation until hatching.

This pattern suggests that without an accompanying increase in stroke volume, overall cardiac output would be decreasing (Birchard and Reiber, 1996). Unless blood oxygen content increases sharply over the second half of development, these chelonian species would be subjected to a developmentally related reduction in O_2 transport. A developmentally related hypoxemia has been previously suggested in common snapping turtles (Birchard and Rieber, 1995). In that study, an index of cardiac output (the product of f_H and heart mass) was used to demonstrate that common snapping turtles might experience a reduction in O_2 delivery as ontogeny progresses. The authors do point out that this index relies on a strong correlation between heart mass and stroke volume, as well as a constant O_2 carrying capacity (Birchard and Reiber, 1996). Given a potential decrease in stroke volume coupled to a constant or falling f_H, either the O_2 carrying capacity or the tissue O_2 extraction must increase if O_2 delivery is to remain constant. Embryonic chickens exhibit an increase in blood O_2 carrying capacity toward the end of incubation (Tazawa, 1980), which suggests that developmental changes in O_2 transport can occur during vertebrate development. While measurement of O_2 carrying capacity has yet to be conducted in embryonic chelonians, data from *Crocodylus porosus* indicates that O_2 affinity does change with development (Grigg et al., 1993), so important changes in carrying capacity may well occur in reptilian ontogeny.

The pattern of f_H development in squamate reptiles differs between taxa. In the embryonic African brown house snake (*L. fuliginosus*), f_H falls over the first 50% of development, and then stabilizes (Fig. 4A). In contrast, in Lawson's dragon (*Pogona henrylawsoni*), f_H is relatively stable during incubation (Fig. 4A; Crossley and Burggren, unpublished data). Similarly, the montane lizard (*Bassiana duperreyi*), also exhibits a relatively constant f_H ($\approx$50 min^{-1}) over the final 65% of incubation (Radder and Shine, 2006). It is difficult to determine whether these patterns are species-specific or reach deeper into the sauropsid phylogeny. However, both f_H patterns differ from those seen during development in either crocodilians or chelonians. While the implications of this pattern difference are unclear, when combined with change in heart mass the early drop in heart rate seen in embryonic African brown house snakes (Fig. 5) indicates that a marked reduction in cardiac output may occur early in snake ontogeny. As previously stated, direct measures of stroke volume are needed in this species to determine if embryonic development is accompanied by a relative reduction in cardiac output and a decrease in O_2 transport and if such changes might be compensated for.

In addition to interspecific differences in f_H developmental pattern, there are also interesting f_H differences at any given point in incubation (Fig. 4A). Interspecific comparisons should only be conducted with an appreciation that the trait is an apomorphic character of a particular clade. With this in mind, the representative squamates have heart rates that are 20–30% lower than that seen in the American alligator (Fig. 4A). This difference is also present when comparing the embryonic desert tortoise to both squamate embryos (Fig. 4A). It should be noted that embryonic common snapping turtles incubated at the same temperature as the desert tortoise, 29°C, have similar heart rates to those of the African brown house snake and the Lawson's dragon (Fig. 4A; Birchard and Reiber, 1996). Interestingly, embryos of the montane lizard acutely exposed to 30°C have an f_H, well above 140 min^{-1}. Thus, incubation temperature does not account for the interspecies difference (Radder and Shine, 2006). Therefore, the phylogenetic relationship of the reptilian species studied so far may strongly affect the relationship between temperature and f_H of embryonic reptiles.

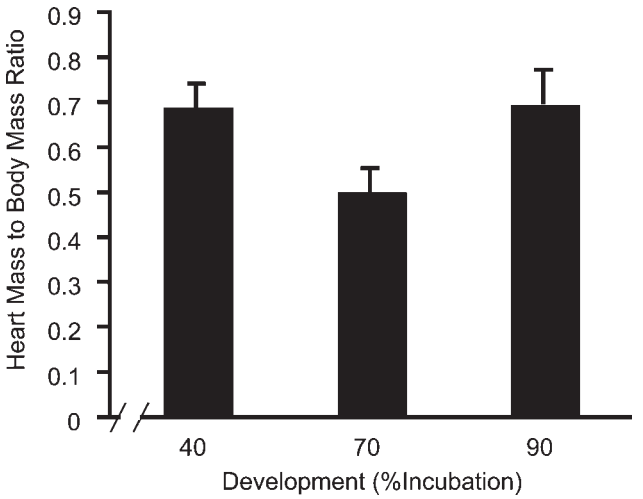

Fig. 5. Heart to embryonic body mass ratio of embryonic African brown house snakes during incubation. Data are presented as the mean ±SE. The number for each time of incubation is 5, 5, and 4, respectively.

The ontogeny of arterial pressure (Pa) in reptiles unfortunately is known for only three species—the American alligator, the desert tortoise (Crossley and Reiber, unpublished data) and the African brown house snake (Crossley and Burggren unpublished data). These species offer insights into how Pa changes in embryonic reptiles during development. As seen across amniotic vertebrates, Pa significantly increases over the final 30–40% of reptilian incubation (Fig. 4B). Interestingly, between 40 and 60% of incubation time, Pa is remarkably similar between the desert tortoise and the American alligator (Fig. 4B). Pa then becomes species-specific at 70% of incubation, after which the desert tortoise and the American alli-

gator maintain a parallel pattern of pressure development (Fig. 4B). Given the anatomical differences in ventricle structure, i.e., septated vs. nonseptated ventricle, this may reflect an increase in left ventricular development and function in crocodilians. The embryonic house snake sample size is small, however, and these limited data should be viewed only as establishing the possible range of arterial pressures in reptiles.

Stroke volume measurements are necessary to understand the maturation of cardiac output during reptilian ontogeny, but it is technically very challenging to quantify this parameter in any egg-laying amniote. Somewhat less challenging is the measure of arterial or venous blood flow in the CAM, the respiratory membranes lining the inner surface of the shell. This measure reflects changes in blood flow distribution between the systemic circulation of the animal and the vascular tree of the gas exchange structure. The CAM arterial blood flow in embryonic alligators at 80% of incubation is 1.3 ± 0.4 ml $\times$ min^{-1} or 52 ml $\times$ min^{-1} $\times$ kg^{-1} (Crossley and Altimiras, unpublished data), a perfusion rate approximately double the lung blood flow reported for adult caimans (Skovgaard et al., 2005). High CAM blood flows could be expected given the absence of functional lungs in the developing animal. The CAM blood flow in embryonic chickens has been variously estimated to be 20% (Tazawa and Takenaka, 1985) to 50% (Olszowka et al., 1988) of the total cardiac output. Thus, total cardiac output in embryonic alligators could be as high as 250 ml $\times$ min^{-1} $\times$ kg^{-1} at 80% of incubation. While this estimate must be validated, a significant portion of cardiac output passes through CAM in embryonic alligators. Thus, changes in vascular resistance of the CAM vascular tree induced, for example, by circulating hormones or changes in environmental temperature or oxygen, would have a pronounced impact on blood flow distribution and pressure profiles within the embryonic animal.

Cardiovascular Regulation

Cardiovascular regulation is typically assessed using two methodologies: the response to pharmacological treatment and the response to environmental stress (temperature, hypoxia, hypercapnia). Both approaches reveal mechanisms involved in the development of cardiovascular regulation in embryonic reptiles. Acute exposure to hypoxia (10–15% O_2) has been used to identify the developmental transition points when the embryonic cardiovascular system begins to exhibit adult-like responses to the hypoxic stress—typically a bradycardia. The onset of the typical adult hypoxic response appears to be species-dependent. Embryonic American alligators respond to hypoxia with a pronounced bradycardia early in development (40% incubation), while at a similar point in development the California kingsnake (*Lampropeltis getulus*) exhibits a tachycardia (Warburton et al., 1995; Warburton, 1997). Clearly, the onset of cardiovascular regulatory mechanisms in embryonic reptiles is complex, as suggested for other animals (Spicer and Burggren, 2003), and will require many additional cardiovascular studies across a multitude of species to illustrate commonalities during reptilian development.

Receptor-Mediated Cardiovascular Regulation

Pharmacological treatment with agonists and antagonists has been the most extensively used method to identify the onset of cardiovascular regulation in reptilian embryos. Receptor antagonists have predominately been used to delineate the presence of cholinergic and adrenergic receptor tone (continuous stimulation) acting upon the vascular tree and heart in embryonic reptiles. Embryonic American alligators lack cholinergic tone on the heart during the final 30% of incubation (Crossley et al., 2003b), a feature that is also found in the embryonic desert tortoise during the final 50% of incubation (Crossley and Reiber, unpublished data). This feature is not universal to embryonic reptiles, because common snapping turtles at 90% of development have a clear increase in heart rate following cholinergic receptor blockade (Crossley and Alvine, unpublished data) a feature also evident in African brown house snakes following cholinergic blockade at 90% of incubation (Crossley and Burggren, unpublished data). Clearly, patterns of cholinergic regulation differ both between and within the major clades of sauropsids.

An active β-adrenergic receptor tone elevating f_H is present during embryonic development in reptiles. Embryonic American alligators and desert tortoises (Fig. 6A; Altimiras et al., unpublished data; Crossley and Reiber, unpublished data) respond to β-adrenergic receptor blockade with a decrease in f_H during the final 40% of incubation. A similar response occurs in snapping turtles at 90% (Crossley and Alvine, unpublished data) and brown house snakes (Fig. 6A) at 70 and 80% of incubation (Crossley and Burggren, unpublished data). Therefore, reptilian embryos resemble bird embryos (Tazawa et al., 1992; Crossley and Altimiras, 2000; Burggren and Crossley, 2002; Crossley et al., 2003a) during incubation in the reliance on a β-adrenergic receptor tone to maintain elevated f_H.

The role of a β-adrenergic tone on arterial pressure differs between the reptiles studied to date. Embryonic desert tortoises appear to have a vasodilation induced by a β-adrenergic tone that appears at 80% of incubation (Fig. 6B). This feature is absent in snapping turtles at 90% of

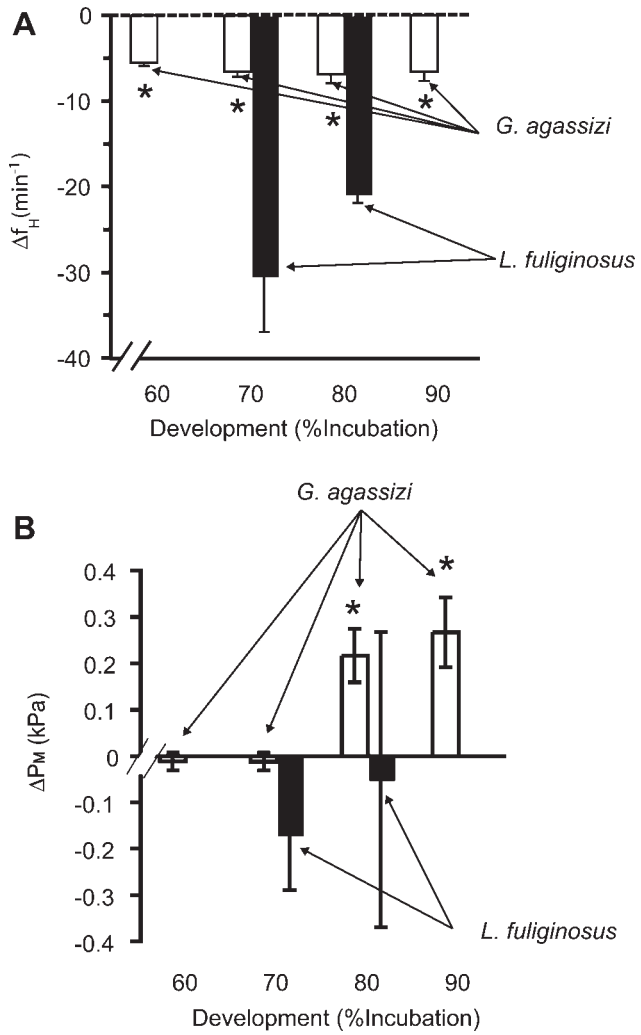

Fig. 6. Changes of cardiovascular parameters during development in the desert tortoise and African Brown house snake. (**A**). The change in heart rate (Δf_H) in response to propranolol injections (0.05 ml of 3 mg $\times$ ml^{-1}) in the desert tortoise (open column) and the African Brown house snake (closed column) during incubation (%). (**B**). The change in mean arterial pressure (Pm) in response to propranolol injections (0.05 ml of 3 mg $\times$ ml^{-1}) in the desert tortoise (open column) and the African Brown house snake (closed column) during incubation (%). Data are presented as the mean $\pm$SE. $N \geq 5$ for the desert tortoise and 2 for the African Brown house snake. An asterisk indicates a significant response to drug injection. Statistical significance was not tested for the house snake.

incubation (Crossley and Alvine, unpublished data) and in embryonic brown house snakes (Fig. 6B; Crossley and Burggren, unpublished data). While the findings for the brown house snake are based on only two animals, it is clear that the function of a β-adrenergic tone on arterial pressure can vary across species.

Collectively, the available information for cholinergic and adrenergic receptor tone on embryonic cardiovascular function in reptiles illustrates that many aspects of cardiovascular regulation remain enigmatic, and lags far behind our understanding of such regulation in the avian embryo (cf. Burggren and Crossley, 2002). Key features need to be detailed, including the timing of functional tone across species, the role of α-adrenergic tone and the origin tone (i.e., from the sympathetic nervous system or catecholamines) to clarify not only the maturation of cardiovascular physiology in reptiles, but also how modifications of cardiovascular function might alter the continuing structural development of the heart and vasculature.

Reflexive Regulation

Centrally mediated by cardiovascular reflexes are critical components of the cardiovascular response to exogenous and endogenous challenges (Kirchheim, 1976; Spyer, 1981; Bagshaw, 1985; Kumada et al., 1990; Rowell and O'Leary, 1990). These short-term modulators of cardiovascular function have evolved along with the circulatory system in vertebrates to ensure efficient transport (Bagshaw, 1985). While these responses have been well characterized in a wide variety of adult vertebrates, little is known of the maturation of these mechanisms. To date only the baroreflex (an inverse relationship between change in Pa and f_H) has been assessed during reptilian development with any detail. In embryonic American alligators, the baroreflex first becomes evident at 70% of incubation time, but is absent during desert tortoise development (Crossley and Reiber, unpublished data). While the baroreflex is functional in the American alligator embryo, it is limited to a hypertensive response that is mediated via an increase in vagal function (Crossley et al., 2003b). This indicates that while cholinergic tone lowering f_H is absent in developing American alligators, the vagus is functional over the final 30% of incubation time. Extensive studies are needed to determine why a hypotensive response is absent, but it is feasible that the sympathetic innervation of the heart is, as yet, nonfunctional. In compiling information available on cardiovascular regulation during reptilian ontogeny, it is clear that extensive characterizations must be completed to determine the capacity for regulation and the underlying basis for any regulatory ability in these developing animals.

ENVIRONMENTAL IMPACTS ON CARDIOVASCULAR DEVELOPMENTAL PHYSIOLOGY

The eggshell permeability of reptilian eggs is quite high compared with birds (Packard et al., 1979). This is of particular importance given that nest conditions typical of reptiles can subject the developing embryo to numerous acute environmental stressors including variation in oxygen and carbon dioxide, dehydration and temperature

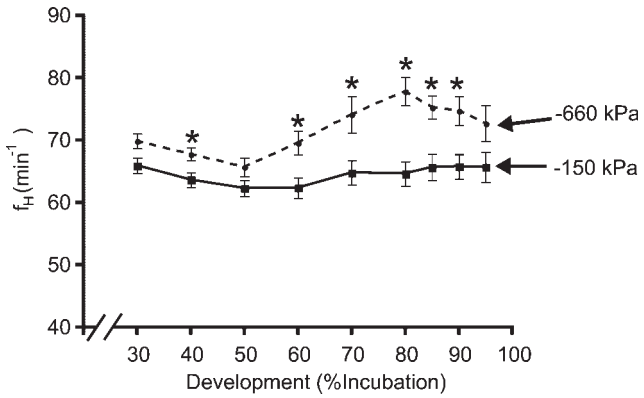

Fig. 7. The resting heart rate (f_H) of embryonic African brown house snakes incubated at a hydration of −150 and −660 kPa. Data are presented as the mean ±SE $n \geq 6$ for all points. An asterisk indicates a significant difference in f_H at a given point of incubation.

(Packard et al., 1993; Ackerman and Lott, 2004). While the developmental environment is known to fluctuate, the impact of these fluctuations on embryonic cardiovascular physiology of reptiles has been investigated on only a limited basis.

Changes in temperature, hydration and imposition of hypoxia (10–15% O_2) have been used as chronic developmental challenges to illustrate the influence of environmental factors on reptilian cardiovascular development. The impact of the thermal environment on embryonic cardiovascular physiology has been investigated only in embryonic common snapping turtles, to our knowledge. Elevated incubation temperature results in a relative increase in resting f_H, but this response disappears after 70% of incubation (Birchard and Reiber, 1996). Thus, in any given species resting f_H may be programmed to a specific rate and chronically increasing temperature simply decreases the time needed to achieve this level. Chronic incubation at different levels of hydration has also been used as an environmental stress factor in embryonic reptiles, a stimulus with ecological relevance given the high permeability of the reptilian eggshell and the effect this could have on the water economy of the developing animal. This potential stress has been investigated for its impact on cardiovascular physiology in the African brown house snake (Crossley and Burggren, unpublished data). Chronic dehydration (−660 kPa) results in an elevation in f_H from 40 to 90% of incubation when compared to a group incubated at −150 kPa (Fig. 7). While the mechanisms underlying this change in f_H are unknown, hydration status of the surrounding incubation media clearly impacts development of cardiovascular function in reptiles. Collectively, these data suggest that the critical window for perturbation of cardiovascular function is dependent on the developmental stress experienced by the embryo.

The physiological impact of incubation in 10% O_2 has been determined in the American alligator and the snapping turtle (Crossley and Altimiras, 2005; Crossley and Alvine unpublished). In the American alligator, this incubation condition results in an embryonic animal that is relatively hypotensive and bradycardic at 90% of development.

This feature does not hold true for embryonic snapping turtles at the same developmental point again illustrating differences between reptilian species (Crossley and Alvine, unpublished data). Thus, while reduction in O_2 availability differentially affects those species in which cardiovascular responses have been assessed, fundamental differences in metabolic rate could account for these findings.

FUTURE CHALLENGES AND OPPORTUNITIES

Interest in developmental morphology and physiology is burgeoning, driven by the emerging interest in the relationship between evolution and development, an appreciation of the key role of larvae/embryos in ecosystems, and a desire to understand human development through animal models (Burggren and Warburton, 2005; Warburton et al., 2006; Müller, 2007; Carroll, 2008; Burggren and Blank, 2009). Exploration of the cardiovascular system has often led the way, in part because this system is the first to function in vertebrate embryos, has distinct periods of developmental vulnerability, and shows major evolutionary modifications within vertebrates. Unfortunately, to date, the study of reptiles has not yet contributed in any substantive way to this experimental expansion of vertebrate developmental morphology and physiology. We can only speculate as to the reasons. Certainly, there are vertebrate "darlings" of the animal model world, including the relatively newly emergent zebrafish embryos and the classic embryonic chicken. The historical emergence of an animal model reflects a positive feedback process—the more that is known about an animal (good, bad or indifferent), the better a model it becomes (Burggren, 2000). We can hardly expect at this point for a reptilian developmental model, however compelling, to eclipse more established models. Yet, there is much to learn from physiological and morphological development in reptiles, which occupy a pivotal point in the evolutionary transition to birds (and thus in the evolution of endothermy, full pressure separation of the heart, hyperosmotic excretion, viviparity, etc.).

Challenges to the use of reptiles in cardiovascular developmental studies remain. Perhaps foremost among these is the irregular availability of oviparous species (and of course access to fetuses in viviparous and oviviparous species) and, related

to that, the additional burden of husbandry and the establishment of breeding colonies. In this respect, we suggest that the seasonally plentiful eggs of the highly fecund common snapping turtle and the commercially farmed American alligator make these two species good candidates for reptilian model status. New techniques and modifications of old ones are emerging that should also assist in the study of reptilian cardiovascular development, particularly in the physiological arena. Miniaturization of instrumentation and expansion of capability for in vivo measurement of blood flows, pressures and oxygenation has steadily advanced (for entry into literature see Burggren and Fritsche, 1995; Schwerte et al., 2003; Bagatto and Burggren, 2006; Burggren and Blank, 2009). In vitro "shell-less culture" of early bird embryos (e.g., Dunn, 1991; Kawashima et al., 2005) now allows access to the functional, growing cardiovascular system in ways not previously imaginable, and modification of this technique applied to reptilian species will allow expanded studies of cardiovascular morphology, physiology and their linkage.

In summary, we can at this time only assemble a tantalizing patchwork of information about how the cardiovascular system of a diverse group of reptiles differentiates, grows and functions during embryonic development. Yet, the insights achieved to date, and the promise of more to come, underscores the vital need for additional morphological and physiological data.

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