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Review

Comparative cardiovascular development: improving the conceptual framework☆

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Abstract

Immature vertebrates—either as an embryo in an egg, as free-living larva, or as an in utero fetus, are clearly not just small versions of adults. Their cardiovascular physiology (and doubtlessly other aspects of physiology) differs from that of adults both qualitatively and quantitatively. Yet, comparative cardiovascular physiologists have been relatively conservative in constructing a new (or at least modified) conceptual framework for the understanding of developmental cardiovascular physiology. We recommend that this framework rely less on the established cardiovascular truisms for adult cardiovascular physiology that are proving to be less useful and in instances even inaccurate for interpreting development of the heart and vasculature. We have suggested that three methodologies in particular be incorporated to a greater extent in studies of comparative cardiovascular development: (a) emphasis on multivariate approaches; (b) differentiation between absolute (extrinsic) and relative (intrinsic) time for development, and; (c) employment of time lines for both intra- and interspecific comparisons of the ontogeny of cardiovascular processes. While certainly none of these approaches are novel and others have previously dwelt at length on their importance in other contexts, we feel that the emerging framework for investigating cardiovascular physiological development would benefit from incorporating these and other approaches into experimental design as well as data analysis. Failing to do so results in a heavy dependence on analytical approaches typically used for adults, and thus under-appreciates the novelty and complexity of the developing vertebrate cardiovascular system.

Keywords: Development; Embryo; Cardiovascular system; Heart; Chick embryo; Zebrafish; Oxygen transport

1. What's wrong with the traditional cardiovascular conceptual framework?

The current conceptual framework for the study of developmental cardiovascular physiology depends heavily on time-honored concepts developed for adult animals that have then been imported by physiologists for application to the growing, developing heart and circulation. In part, this is a reasonable—or at least practical—approach because we know so much about the adult cardiovascular system relative to the developing system. Indeed, many basic concepts that have emerged through the study of adult systems are better viewed as ubiquitous physiological laws of nature rather than characteristics that develop only upon maturation. Yet, the fit of adult characteristics into immature systems is sometimes imperfect, as the title of this article implies. To substantiate this

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opinion, we will not provide a detailed, heavily referenced review of embryonic cardiovascular physiology, for such reviews are available in abundance (see, for example, Clark, 1991; Reller et al., 1991; Burggren and Keller, 1997; Thornburg and Minette, 1998; Phoon, 2001; Noble, 2002). Instead, the intent is to identify a few truisms in cardiovascular physiology—the type of statement that is engrained in every student of animal physiology—and then show how such truisms are at best only loosely applicable to developing animals or, at worst, are quite inaccurate. Such truisms thus prove, ironically, to be false.

1.1. False truism example #1—'The embryonic heart begins to pump blood for oxygenation of tissues'

Built upon the foundation provided by the discoveries of William Harvey and his contemporaries, physiologists have been taught for centuries that the heart beats to generate convective flow for respiratory gas and nutrient transport (some would argue to produce pressure, but pressure is a secondary product of producing a flow against a resistance). However, this classic concept does not necessarily extend so conveniently from adult's back to an embryo's early hours or days when its heart first begins to function. While the oxygen transport function of the heart in well-established embryos is indisputable, and it has been known for decades that convective blood flow is required for normal embryonic development (e.g. Waddington, 1937; Flynn et al., 1991; Copp, 1995; Männer et al., 1995), the heart's role in the early embryo is currently in dispute. Indeed, several distinct lines of evidence suggest that the beat of the embryonic heart is not required for adequate tissue oxygenation and nutrient delivery acutely in early embryonic development. The evidence, emerging from several laboratories, ranges from highly invasive experiments to simple descriptive measurements of physiological performance.

1.1.1. Evidence for non-essential nature of convective blood flow

The first and most invasive line of evidence involves complete surgical ablation of the heart in early larval Ambystomid salamander (Mellish et al., 1994). Not only does the larval salamander rendered heartless actually live, but it continues to consume oxygen at similar levels to intact, control animals for several days following heart ablation. Just short of removing the heart, Flynn et al. (1991) completely severed the truncus in stage 12–13 chick embryos. They reported that the embryo survived for 24 h (although normal development of cervical flexure was impaired by this massive intervention).

A more subtle approach, but one no less disruptive to blood flow, is the complete surgical ligation of the ventricular outflow tract of the otherwise intact heart (Burggren et al., 2000), eliminating what would otherwise be a vigorous blood flow throughout the young embryo. This procedure in Day 3 and Day 4 chicken embryos caused no significant change in either oxygen consumption or embryonic growth measured by eye diameter. Furthermore, unlike the prior experiments by Flynn et al. (1991), cervical flexure continued on a normal pace during the 4-h period of measurement after outflow tract ligation. These experiments on chicken embryos, in which the circulation is morphologically intact but not generating convective flow, corroborate those observations on mutant amphibian larvae whose hearts form normally anatomically but never begin to beat, with no repercussions on oxygen uptake in the short term (Lemanski, 1973; Justus, 1978; Fransen and Lemanski, 1989; Mellish et al., 1994)

More subtle still as an experimental approach is elimination of the convective oxygen transport ability of embryonic blood without the interruption of blood flow per se. Inducing red blood cell lysis in early zebrafish larvae by exposure to phenylhydrazine (1–2 mg l^{-1}) has no significant effect on either whole animal oxygen consumption or the intraventricular pressures generated during the highly aerobic phase of isometric contraction (Pelster and Burggren, 1996). Similarly, poisoning the hemoglobin in vivo in larval *Xenopus* (stages NF1 to NF 63) by exposing them to 2% carbon monoxide has no significant effect on whole animal oxygen consumption (Burggren and Territo, 1995; Territo and Burggren, 1998). These experiments speak volumes for the effectiveness of diffusive transport of oxygen in early vertebrate embryos (and presumably for the diffusive supply of nutrients and other materials vital to organogenesis).

Finally, under the category of descriptive studies of normal, intact systems, numerous experiments have shown that cutaneous gas exchange is a primary site for gas exchange in resting, normoxic

larval fishes. In numerous species the vascularized gills play a very small roll in early larval gas exchange (for example, Liem, 1981; Wells and Pinder, 1996; Rombough and Moroz, 1997; Rombough, 1998), which mostly occurs across the body wall, parts of which are not well vascularized.

1.1.2. What is the purpose of the embryonic heart beat?

Collectively, these data suggest that convective blood oxygen transport by the embryonic heart is not required for tissue oxygenation (or supply of nutrients and waste removal, apparently). Thus, the 'adult-based' truism that the heart must beat for tissue oxygenation is somewhat simplistic, and we should evoke a more complex picture of oxygen transport events during early development. Filling in this picture requires answering the question 'What is the purpose of the early embryonic heart beat?' The experimental demonstration of the optional role of convective blood flow described above could be interpreted in two ways. First, it could be that the embryonic heart beats to begin convective blood oxygen transport, but that tissue oxygenation (at least in the resting animal) can be completely satisfied by diffusive transport in the absence of convective transport. The second interpretation might be that the embryonic heart begins to beat for some purpose other than convective blood oxygen transport, and that the respiratory function begins later in development.

Validating one of these two disparate views of the role of early circulation will require further experimentation. Exploration of respiratory gas transport during conditions of activity or hypoxic exposure, both of which might challenge the efficacy of diffusion, would improve our understanding of the function of early heart activity. Awaiting these experiments, we can nonetheless make some assertions. We know that a point in growth is quickly reached when distances become too large for simple diffusive transport (e.g. Day 5 in chick embryos and zebrafish larvae). This transition from a period when diffusion is adequate to when convective transport is required is illustrated in Fig. 1. A traditional view (Fig. 1a) is based on the truism that the heart beats to provide convective oxygen transport—that is, the heart starts to beat at the point when such convective transport is required. We have called this hypothesis 'synchronotropy' (Burggren and Territo, 1995). Yet, the various lines of experimental evidence suggest

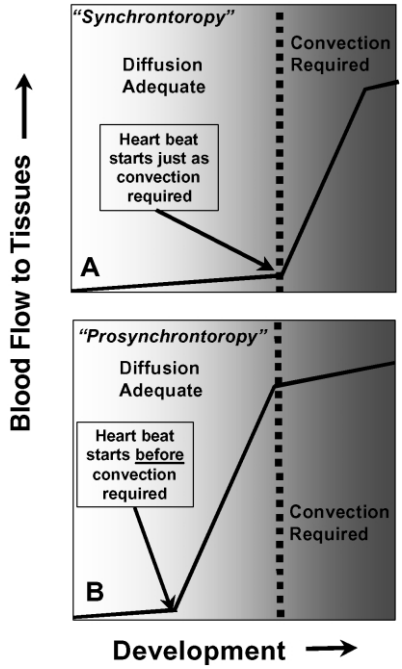

Fig. 1. In the view of heart beat onset described by 'synchronotropy', the heart begins to beat and generate flow concurrent with the need for the embryo to supplement diffusive with convective tissue oxygen supply. In 'prosynchronotropy, the heart begins to beat and generate blood pressure and flow well in advance of the need to generate a convective supply of oxygen to the tissues. From Burggren and Territo (1995).

that, instead, the heart actually begins to beat well before the absolute need for convective oxygen transport (Fig. 1b)—a hypothesis we call 'prosynchronotropy.

As evidence supporting prosynchronotropy in birds and amphibians continues to mount, it is tempting to ask the question 'Why does the embryonic heart begin to beat so early if, indeed, convective transport eventually proves not to be the role of the embryonic heart beat?' One possibility is that early beating of the heart and the modest pressures, as well as flows generated accordingly, aid in angiogenesis. Our working hypothesis, which we are testing using chick embryos, suggests that the waxing pulsatility produced by early heart contractions creates sheer and strain forces on endothelial cells along the sites of the presumptive blood vessel tracks. Sheer and strain causes secretion of VEGF and endothelin-1 by endothelial cells and endothelin-1 both in cell culture and in the intact cardiovascular system. These peptides further stimulate through a paracri-

ne effect endothelial cell growth and proliferation (see Tomanek and Ratajska, 1997; Zheng et al., 1999; Cruz et al., 2001; Meirer et al., 2001; Tomanek et al., 2001). This being the case, we predict early embryos lacking pulsatile blood flow should show reduced peripheral vascularization. Both surgical interventions and mutants with dysfunctional cardiovascular systems should prove useful in exploring this hypothesis.

1.2. False truism example #2—'Allometry explains the difference in cardiovascular performance between small (immature) and large (mature) animals'

Physiological allometry has a rich and deep history. While such allometric studies have primarily focused on metabolic rate and factors that affect it (e.g. locomotion), there have been numerous observations of scaling related to cardiovascular physiology (see references in Schmidt-Nielsen, 1985; Goldberger and West, 1987; West et al., 1997; Ar and Tazawa, 1999; Pennati and Fumero, 2000; Seymour and Blaylock, 2000; Zamir, 2000; Li, 2000; Dawson, 2001). Heart rate has been the primary index of cardiovascular function because of its relative ease of measurement. Indeed, the so-called 'mouse-to-elephant curve' is well exemplified by heart rate, which in interspecific studies of mammals, birds and many other animals scales to body mass to the power -0.25.

Unfortunately, there have been relatively few intraspecific studies of cardiovascular allometry during development (indeed, of the allometry of any physiological variable). What the relatively few intraspecific studies tend to show is that immature individuals of a species perform not only differently than the mature adults, but that the changes are in some instances counterintuitive based on our broad knowledge derived from interspecific studies (Burggren, 2000). For example, consider resting heart rate in the mouse, *Mus musculus* where heart rate declines with increasing body mass in post-weaning individuals at a rate that would be predicted from interspecific allometric equations (literally, in this case, the 'mouse-to-elephant curve'). However, examination of resting heart rate in newborns prior to weaning reveals large heart rate increases as body mass increases, completely the opposite of what conventional allometric knowledge would predict (Hou and Burggren, 1989). Similar findings of nonlinear, and sometimes counterintuitive increases in heart rate during early development can be found in a variety of developing animals including insects, fishes, amphibians, birds and mammals (e.g. Burggren and Warburton, 1994; Rombough, 1997a; Tazawa et al., 1999; Pearson et al., 1999, 2000 Fig. 2). In one of the few studies that attempted to look at the role of heart rate in tissue oxygen consumption, Pearson et al., (1999) conclude that ''$\ldots f_H$ contributes more than other factors towards supplying the metabolic demands of the embryo during the middle of incubation and the final pipping phase, but less during the intervening period of late incubation''.

Of course, heart rate is only one factor, along with stroke volume, that determines cardiac output and, ultimately, oxygen consumption in developing animals. Numerous studies show that cardiac output increases in vertebrate embryos as 'body mass' increases (for references see Faber et al., 1974; Clark and Hu, 1990; Keller et al., 1990; Wagman et al., 1990; Hou and Burggren, 1995; Burggren and Fritsche, 1995; Keller, 1997; Jacobsson and Fritsche, 1999). However, few of these studies have accurately measured the mass of the metabolically active true embryonic tissue (borrowing instead on previously published embryonic masses that are notoriously difficult to make) for the calculation of mass-specific cardiac output that could be compared with similarly calculated data derived for fetuses or adults. In fact, studies on the early chick embryo (Faber et al., 1974) suggest that stroke volume and hence cardiac output may be most highly correlated with not body mass but rather blood volume, which changes in very complex ways during development with the growth and subsequent degeneration of the chorioallantoic membranes (see Romanoff, 1967). Collectively, these cardiovascular data suggest that simple allometric relationships derived from adult physiological studies on birds can be overwhelmed by more discrete changes associated with cell, tissue and organ formation during embryonic development, especially in very early embryos.

A lesson learned from these intraspecific physiological analyses of cardiovascular function is that allometry should not be automatically evoked when predicting, and explaining, cardiovascular changes during development in vertebrates. In fact, it is possible that developmental physiologists may have to develop a new set of tools for dealing

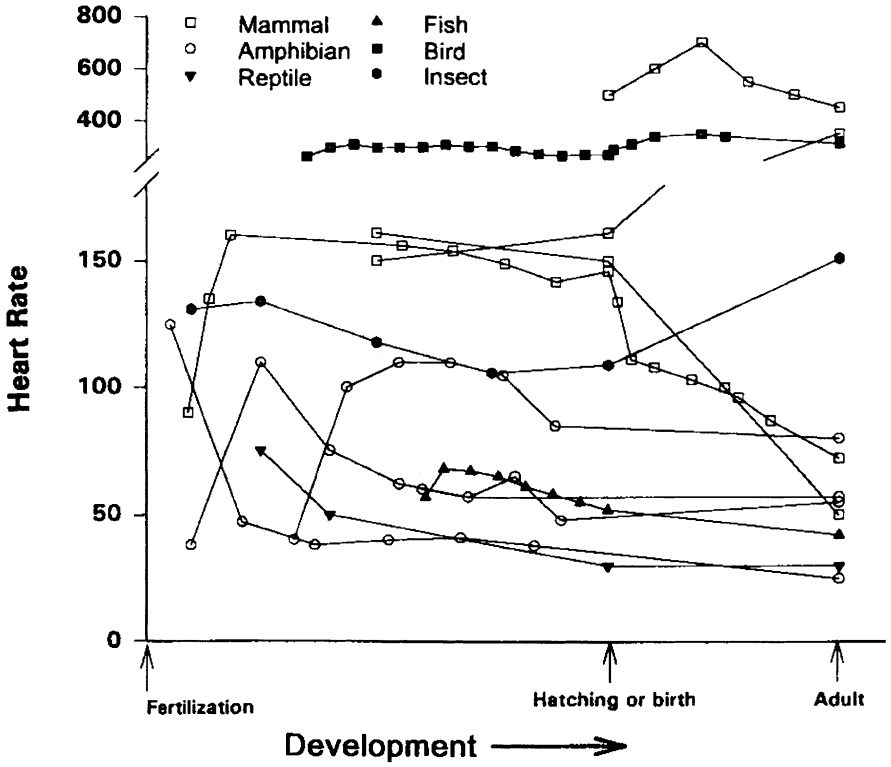

Fig. 2. Examples of complex patterns of heart rate change during development in a variety of ectotherms and endotherms. Data are normalized to a common initial fertilization, a hatching/birth event, and attainment of adulthood. (See Burggren and Warburton, 1994 for literature sources.)

with size change in developing animals. One of the basic tenets of allometry is that different sized animals be compared only when in a similar physiological state. Since most papers in developmental physiology seek specifically to identify differences in physiological state, or at least acknowledge that such differences exit, these same studies should not then evoke allometry to explain the findings of different physiological rates or processes in small, immature and large, mature animals. Indeed, the relevancy of 'classic' allometry to developmental physiology is being questioned (Hunt von Herbing, 2002). Certainly, new statistical procedures and perhaps a new conceptual framework may need to be developed to deal with intraspecific, developmental studies.

1.3. 'False truism' example #3—'Cardiac function in developing animals becomes optimized by the summation of a tonic vagal depression and adrenergic'

In most adult vertebrates, cardiac function is under a pronounced tonic parasympathetic depression originating from vagal fibers. This inhibition is balanced to some extent by the often-opposing concurrent stimulatory actions of adrenergic stimulation through circulating catecholamines and/or sympathetic cardiac stimulation. Simultaneous cholinergic and adrenergic tone affords precise regulation of cardiac function and allows the optimization of hemodynamic performance even as demand for cardiac output and distribution changes.

The obvious question is 'does this offsetting parasympathetic and sympathetic tone also characteristic of developing animals'? Given the nearly ubiquitous utilization of this dual regulatory system in adult vertebrates, is this truism inaccurate for developing animals? That the early embryonic/larval/ fetal heart operates more or less in a metronomic fashion without influence from innervation or circulating catecholamines has been long known (Pappano, 1977). Numerous studies have investigated the onset of sympathetic, parasympathetic or peptidergic cardiac innervation as well as the growing role of circulating catecholamines

in regulating blood pressure, heart rate and stroke volume in vertebrates (for recent papers giving older citations see Roberts, 1991; Segar et al., 1992; Gordon et al., 1993; Burggren and Keller, 1997; Crossley and Altimiras, 2000; Moriya et al., 2000; Crossley et al., 2002). Relatively few studies, however, have attempted to look in an integrated fashion for the relative timing of the onset of numerous cardiac regulatory mechanisms over the a wide period of development. In the absence of such broadly integrative studies, many physiologists tend to view the various regulatory systems 'coming on line' more or less concurrently and developing their influence upon the heart in an approximately linear fashion. Certainly, the anatomical presence of vagal nerves that when stimulated causes bradycardia is often interpreted to mean that a vagal tone is also developing, when in fact vagal tone may be absent for some additional period of time. In fact, several simple and non-exclusive patterns for the maturation of parasympathetic vagal as well as sympathetic tone are conceivable during nervous system maturation:

- Functional activity of either control system mirrors the pattern of morphological maturation—i.e. gradual activation associated with gradual structure development.
- Functional control of the heart by a regulatory component is delayed compared to structure development—e.g. vagal innervation of the heart occurs, but the vagus remains nonfunctional.
- Functional activation of vagal and adrenergic cardiac tones during embryonic development occurs in an asynchronous manner, reflecting a combination of the first and second of the possible patterns.

Which pattern is evident? Again, temporal changes in cardiovascular control mechanisms over wide periods of development have been determined primarily in birds, using a combination of agonists and antagonists. In embryonic chickens (*Gallus gallus*) a clear adrenergic tone on cardiovascular function is evident approximately 50% through development (day 10) of incubation (Crossley and Altimiras, 2000). The influence of this tonic adrenergic regulation is significant, since there is a lack of parasympathetic vagal tone on the cardiovascular system during chicken development despite the anatomic vagal innervation of the heart (Crossley and Altimiras, 2000), though episodic, transient vagally-induced bradycardia has been reported (Hoechel et al., 1998). Thus, it appears that embryonic chickens possess a combined strategy for the maturation of cardiac regulation exhibiting both asynchronous activation as well as delay of full function. This pattern of nonlinear development of cardiac tone has also been demonstrated in fetal sheep (*Ovis aries* Mouflon) (Thornburg and Morton, 1983, 1986; Reller et al., 1989), larvae of the African clawed frog (*Xenopus laevis*) (Fritsche and Burggren, 1996; Jacobsson and Fritsche, 1999), larvae of the bullfrog *Rana catesbeiana* (Burggren and Doyle, 1986) and embryos of the desert tortoise, *Gopherus agassizii* (Crossley, 1999). All of these species maintain basic and sufficient CV performance without a major tonic vagal input during development, a finding that is in sharp contrast to the pattern of simultaneous dual cardiac control well defined for adult vertebrates.

Only poorly understood are the likely permissive roles of the vagus or sympathetic nerves in developing cardiac reflexes, which we know play important if subtle roles in adult vertebrates (e.g. Izzo and Taylor, 1999). Moreover, developmental cardiovascular studies are typically carried out in inactive, if not anesthetized animals. Consequently, those components of the developing reflexes that might be evoked by increased metabolic demand largely remain to be identified. Another limitation to our studies is that most investigations of the onset of cardiovascular control use pharmacological probes rather than neurotomy and afferent or efferent stimulation, due to the size or difficulty of access of the developing animals. As a consequence of having only some of the full complement of arrows in our experimental quiver, we have much to learn about the developmental onset of the multiple components regulating the heart. What is clear, however, is that the physiological regulatory pathway from embryo, larva or fetus to adult is a complex one, and that both interpolation from the adult to immature forms, or extrapolation from immature forms through intermediate stages, ultimately will have to be backed up by actual measurements.

2. Some key elements of a new conceptual framework for the study of developmental cardiovascular physiology

Cumulatively, these three examples of physiological truisms related to blood oxygen transport,

allometry and cardiovascular regulation in mature animals reveal that truisms should not automatically be evoked to explain events and patterns observed during the ontogeny of vertebrates. That is, dependable truisms that have emerged for adults may be invalid in an ontogenetic context, because developing animals are not just miniature versions of the larger adults. As evident from the examples above, our most dependable (comfortable) cardiovascular truisms have limited utility in the frontier of embryonic/larval cardiovascular physiology. Consequently, a new, or at least modified, conceptual framework for cardiovascular development needs to be created. While it is beyond the scope of this article to draft this framework, below we suggest a few examples of conceptual elements that could be used as part of an emerging framework for cardiovascular developmental physiology.

2.1. Emphasize multivariate interactions

Those physiologists who have eked out physiological data sets from developing animals have almost inevitably been confronted with a complex collage involving the variable of interest (e.g. heart rate, blood pressure, cardiac output, vagal tone, metabolic rate). The complexity is then amplified by differences in time, developmental stage, body mass, body size (as distinct from body mass), nutritional state, photoperiod, gender, etc. It has long been appreciated that the interaction between body mass and development is particular important—and thorny. As Feder (1981) cautioned in the early 1980s, 'Results of most previous studies must be accepted with caution because few investigators have recognized the effects of body size, time of day, trophic state, microbial VO_2, access to air, or incidental experimental stress on larval anurans'. Despite this and more recent invitations for the use of multivariate approaches that attempt to incorporate and correct for the multitude of complicating variables, (e.g. Burggren, 1997; Klingenberg, 1998; Stern and Emlen, 1999), this approach is still underutilized. Moreover, even the sources of variation are subject to variation in different vertebrates during their growth and development. For example, in a study of anuran larvae, most (59–90%) of the variation in metabolic rate was attributable to differences in body mass, with a small but significant influence exerted by developmental stage (Feder, 1982). Not surprisingly these investigators emphasized the importance of correcting for body mass in development analyses. Yet, Spicer and Gaston (1999) have recently emphasized how little body mass explains many profound changes in physiological characteristics during an animal's life span. As an example they describe how changes in body mass explain only 1/3 of the variation in metabolic rate during the life cycle of the tree swallow (*Tachycineta bicolor*). However, they go on to point out that an interspecific analysis of metabolic rate in 22 bird species reveals that body mass accounts for almost all (95%) of the interspecific variation in metabolic rate.

Against this swirling backdrop of multiple variables, and the pervasive yet sometimes enigmatic influence of body mass, simple one-dimensional analyses are at best going to leave unrevealed important relationships between variables, and at worst are misleading. Three dimensional analysis (and thinking) can be particularly helpful when experimental perturbations are causing a dissociation of variables that we usually link (or completely blend) in our minds. For example, we too often think of body mass and developmental stage as joined 'lock-step', and we view with suspicion any developing animal that departs from normal tables of development. A typical normal table for development might provide not only a set of morphological markers (and less often, a set of physiological markers), but also provide a time at which they should occur, and an expected body mass at that time! Yet, as Starck (1993) emphasizes for bird embryos, 'Normal stages provide a simple tool for comparison and are independent from physical time and body size'. We sometimes forget or ignore this independence. Indeed, a factor as simple, and commonly measured as nutritional state, can make for smaller or larger animals at an identical developmental stage according to the staging schemes. Thus, when considering cardiovascular variables that may be dictated in part or in whole by body mass (e.g. cardiac output), it will be necessary to consider the variable of interest in the context of both body mass and development. This concern becomes all the more obvious as we now consider development in a chronological context.

2.2. Differentiate between absolute and relative time for development

Time is a major variable when considering vertebrate development, and has been pondered in

an ontogenic context by many authors for the past century (e.g. Loeb and Northrup, 1917; Pearl, 1928; Brody, 1945; Alderdice and Velsen, 1978; Reiss, 1989; Rombough, 1997b; Starck, 1993; Burggren, 1998). The semantics describing the problem of developmental timing vary, but the nature of the problem itself does not. Thus, the vast majority of developmental cardiovascular studies interpret their data using one of two time metrics. The first is absolute time for development (also called 'chronological time', 'clock time', or 'extrinsic time'). When animals are developing at a standard, constant temperature (e.g. an incubation temperature of 37.5 °C. for the chicken embryo), then absolute time is commonly equated with stages drawn from normal tables of development. Thus, a chicken embryo might be described interchangeably as a 50–55 h chick embryo or at Hamburger Hamilton stage 15. However, as we shall now explore, variations in environment may cause development to slow down or, in fewer cases, to accelerate.

Temperature is one obvious environmental factor important to the consideration of absolute vs. relative time for development. In a comprehensive consideration of developmental time, Reiss (1989) focuses on temperature (along with body size) as the major confounding factors in using chronological or clock time (which he calls 'extrinsic' time) in measuring developmental time ('intrinsic time'). Though relatively few studies have been carried out, developing vertebrates have high, and variable, Q_{10}s for metabolic rate in early development. For example, Fig. 3 illustrates how the Q_{10} for metabolic rate in the zebrafish, *Brachydanio rerio* ranges from a high of 4–5 immediately after hatching and then again at approximately 100 days of age, to a nadir of approximately 2 from 10–40 days (Barrionuevo and Burggren, 1999). Paradoxically, the Q_{10} for heart rate is relatively constant at a value of 1.6–2 over the entire period of hatching to 100 days. A more focused analysis on the larval period from hatching through day 10 reveals that these developmental changes in Q_{10} can be quite abrupt in the zebrafish. As an example, Q_{10} for oxygen consumption measured over the temperature range of 18–28 °C falls from above 3 at day 2 to approximately 1.5 just two days later at day 4 (Bagatto, 2001). Clearly, even small changes in temperature can have large and differential effects on physiological processes.

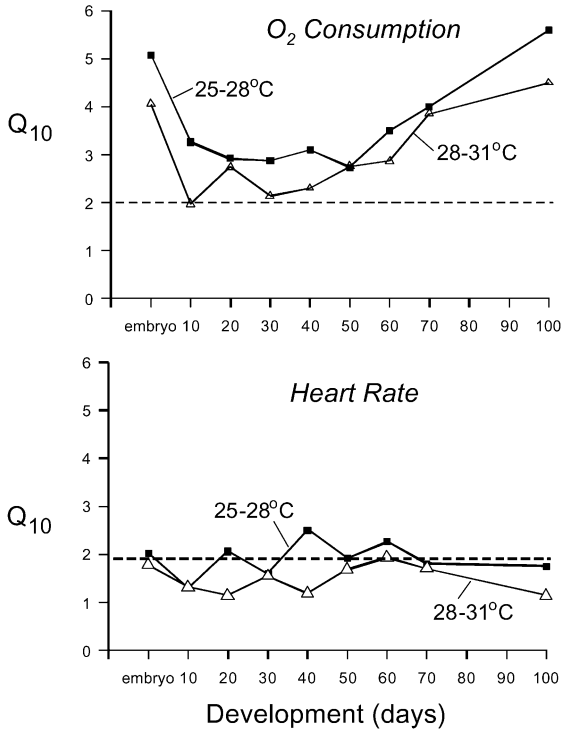

Fig. 3. Q_{10}s over two temperature ranges (25–28° and 28–31 °C) for oxygen consumption ($\dot{M}O_2$—top panel) and heart rate (lower panel) in zebrafish from hatching to day-100 of development. (From Barrionuevo and Burggren, 1999).

Generally, the Q_{10} for whole animal development in poikilotherms, measured by rate of progression through morphological developmental stages, is often both large and variable. Consequently, quite small variations in temperature during the course of incubation can lead to quite large differences between the predicted time for development based on elapsed chronological (extrinsic) time and the actual (intrinsic) time required for development.

Homeothermic endotherms (or immature homeothermic endotherms that are incubated by capable thermoregulators) might be expected to develop along an absolute time line that is invariable. However, in an experimental setting, at least, a subtle transient drop or rise in environmental temperature (inherent in all but the most expensive egg incubators, for example) can lead to significant discrepancies between absolute time and developmental time. For temporal heterotherms and ectotherms, large changes in temperature that may be typical for the environment in which they develop can, of course, have enormous effects. The eggs

of many fishes, amphibians and reptiles will develop over a fairly broad range of temperature. Investigators (especially in fish physiology and aquaculture) drew upon analytical tools such as 'degree days' (the days of development X the temperature for development) in an attempt to 'normalize' for temperature's effect on development (Blaxter, 1969). Though many heterothermic vertebrates will develop over a range of temperatures (allowing the application of the 'degree day' concept), most have an optimal temperature providing the lowest mortality, with an increase in developmental anomalies and mortality associated with temperature extremes.

As we learn more about the physiology of developing vertebrates, we are realizing that temperature fluctuations may do more than simply retard or accelerate organismal ontogeny by altering intrinsic developmental times. Rather, temperature may well have differential effects on the morphology and physiology of various organ systems. For example, in the zebrafish, *Brachydanio rerio*, the optimal temperature for reproduction and larval growth was 25 °C with mortality increasing when temperature departs in either direction from 25 °C (Bagatto, 2001). Importantly, thermal tolerances and thermal optima appear to change throughout development, being very narrow at the beginning of development and widening as development proceeds. Even maternal rearing temperature has an effect on subsequent thermal profiles for larvae. Clearly, temperature is not just retarding or accelerating development as a whole, which otherwise remains unchanged qualitatively or quantitatively. Thus, we hypothesize that animals of the same species raised at different temperatures may collectively represent a complex developmental mosaic for that species, at least during their early developmental phases.

Environmental factors other than temperature can also have profound effects that argue for multivariate analyses. Level of environmental oxygenation and food availability, for example, can influence the total amount of time required for development, with both hypoxia and undernourishment lengthening development time. Apart from the obvious effect of altering developmental time for the whole organism, the effects can be more subtle. Hypothetically, an environmental condition that might retard overall body mass and morphological appearance could actually induce a compensatory acceleration of development of a particular structure or process. Oxygen deprivation during development is interesting to consider in this regard. While chronic hypoxia is well known to retard overall development in many vertebrates, one could argue (temporarily setting aside concerns over teleology) that a developing animal facing hypoxia might accelerate growth of those systems and processes that contribute to the acquisition of oxygen (e.g. branchial, pulmonary, hematopoeitic and cardiovascular systems), perhaps at the expense of other systems that are unrelated to oxygen acquisition. Indeed, chronic mild hypoxia is known to stimulate growth of the chorioallantoic membranes (CAM) of the chick and alligator embryo when applied universally to the embryo (but not necessarily when applied regionally to areas of the CAM) [see (Corona and Warburton, 2000) for recent literature]. Thus, if one were basing development on CAM morphology, the embryo is then considered to be accelerated rather than retarded in development (thereby highlighting the importance of considering multiple features when staging animals).

Our lab is currently investigating the differential effects on different organ systems and their physiological processes created by environmental perturbances such as hypoxia. In the chick embryo, for example, a 6-day period of chronic hypoxia (15% O_2) beginning at the start of egg incubation causes a slowing of body mass accumulation and a reduction in oxygen consumption, and an increase in [Hb] and Hct (Dzialowski et al., 2002). However, after return to normoxia at day 6, all measured variables have returned to control levels by day 12 and stay at control levels through hatching. Thus, hematopoesis and whole body anabolism producing tissue growth waxed and waned, respectively, during early hypoxic exposure, and then hematopoesis slowed while anabolism increased during the subsequent recovery period. The continuing investigation of differential effects of environmental perturbation on anatomy and physiology of developing animals should provide considerable insight into developmental processes, and the ontogenetic prioritization of resources.

2.3. Consider using normalized time lines for comparison

Because developmental trajectories leading to specific phenotypes are influenced by temperature,

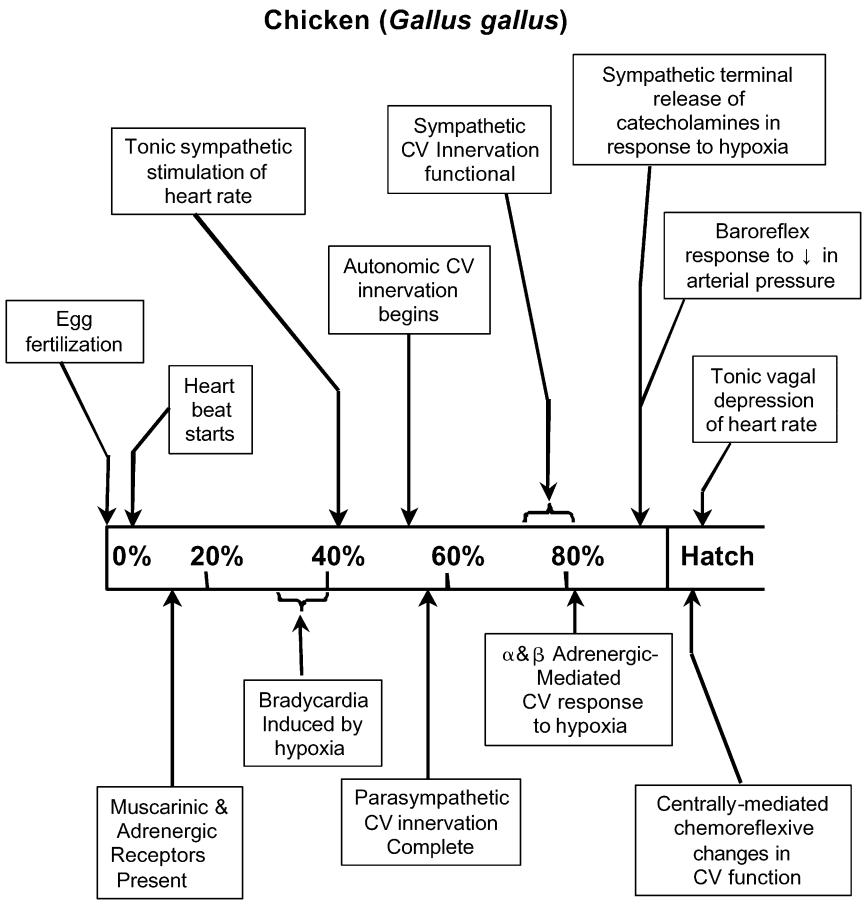

Fig. 4. Normalized time lines showing the onset of cardiovascular control in the embyros of the chicken (*Gallus gallus*) and (b) the emu (*Dromiceius novaehollandiae*). Data from Crossley and Altimiras, 2000.

oxygen, nutrition, etc., comparing events in development either between populations or between species can be problematic. We have found that the use of simple 'normalized time lines' for conveying the onset of physiological events facilitates interspecific comparisons (Burggren and Pinder, 1991). A normalized time line is a simple two dimensional, graphic covering all or a portion of a life span (thus, 'normalized'), and portraying the timing of onset (and/or disappearance) of anatomical, physiological or behavioral features (Fig. 4). Time lines are fairly common in nature history studies (e.g. Reiss, 1989), but are underutilized by developmental physiologists.

Utilizing time lines as a tool for assessing physiological function in embryonic and larval animals eliminates the difficulty inherent in comparing species with differing lengths of periods for development. A semi-quantitative comparison can then be conducted between individuals of a given species or between separate species at defined intervals of ontogeny. Thus, comparisons between diverse species can be carried out to determine patterns that may be consistent during vertebrate development.

The utility of this approach in visualizing the developmental onset of key components in cardiovascular control can be illustrated by contrasting the embryos of two avian species, the chicken (*Gallus gallus*) (Fig. 4) and the emu (*Dromiceius novaehollandiae*) (Fig. 5). Emus and chickens represent distinct species in both a phylogenetic context as well as their length of egg incubation. However, constructing time lines of defined intervals (e.g. percentage of total incubation), allows a comparison between these two bird species with very different incubation periods (emus = 52 days vs. chickens = 21 days). This method illustrates

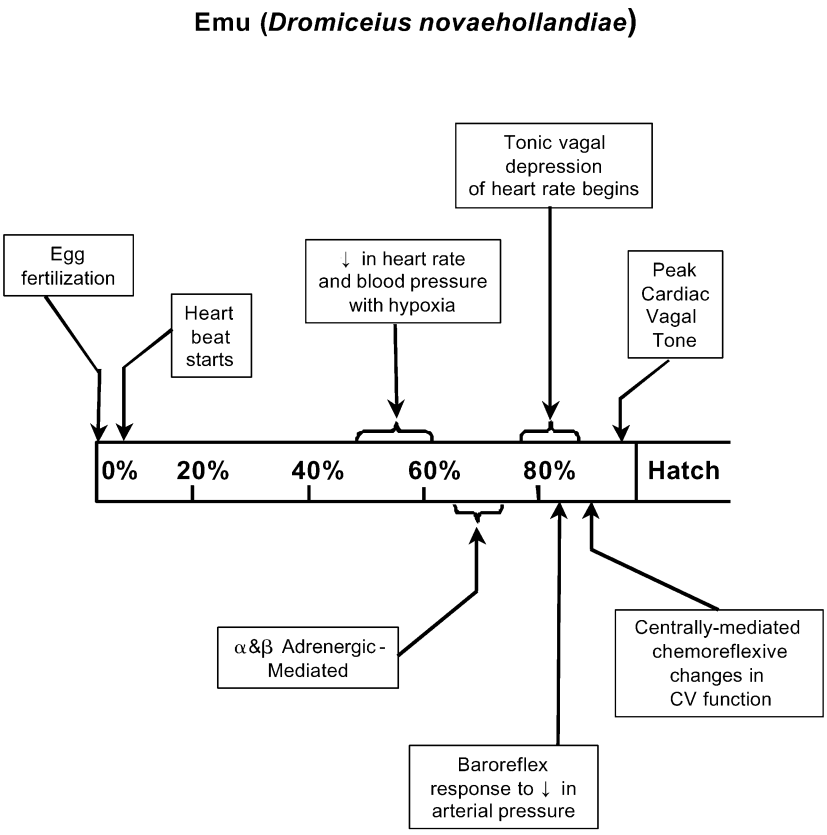

Fig. 5. Normalized time lines showing the onset of cardiovascular control in the embyros of the emu (*Dromiceius novaehollandiae*). Data from Crossley et al., 2002.

important interspecific similarities in the timing of onset during embryonic development of various sensory and motor elements of baroreceptor and chemoreceptor (oxygen-sensitive) control. For example, both emus and chickens possess a clear β-adrenergic tone on the cardiovascular system at 50–60% of incubation (Figs. 4 and 5), possibly a typical trait among avian species. Yet, important developmental differences are also clearly illustrated with a normalized time line, with emus exhibiting a functional baroreflex substantially early than embryonic chickens (Fig. 6). Therefore, the strength of time line comparisons is their ability to not only illustrate similarities between species, but also help identify characteristics unique to a particular species. Time line analysis can also be useful in looking at intra-specific changes during development that might be brought on by environmental perturbation, as discussed earlier.

3. Conclusion

The field of developmental cardiovascular physiology—especially in a comparative context—is thriving, with increasing numbers of studies focusing on the events and timing of cardiovascular control as vertebrate embryos and larvae mature. We have advocated in this essay that investigators be cautious about adopting cardiovascular truisms that are time-honored for adults, but may be misleading as basic tenets for vertebrates in early phases of their development. Life as an embryo/larvae is not just different, but it can be complex, and a conceptual framework needs to be able to explain and incorporate this complexity. Additionally, we have suggested several analytical tools—multivariate analysis, differentiating between absolute and relative time, the use of time lines for comparing species—that may assist future investigations of developmental cardiovascular

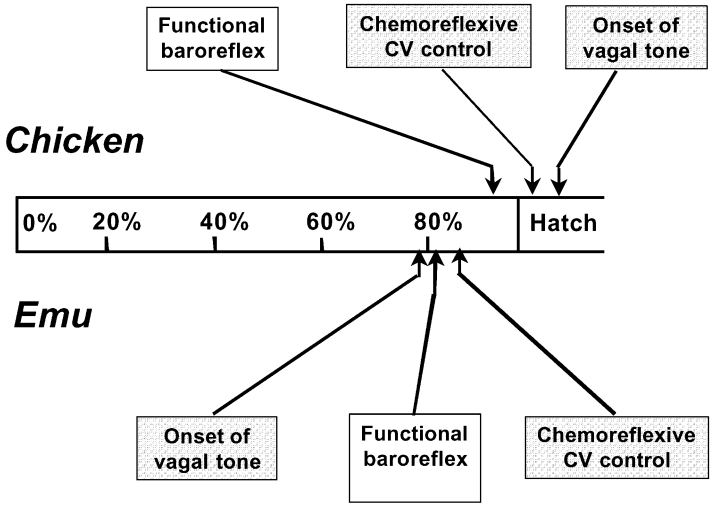

Fig. 6. A merging of normalized time lines for the chicken and emu (Figs. 4 and 5) clearly reveal dissimilarities in ontogeny of key events in cardiovascular control. Data are a composite drawn from Crossley and Altimiras (2000) and Crossley et al. (2002).

physiology. Additional aspects of a new conceptual framework for developmental cardiovascular physiology need to be clearly identified and incorporated as they prove successful in unraveling the myriad remaining mysteries of cardiovascular physiological ontogeny.

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