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INVITED REVIEW

Comparative cardiovascular physiology: future trends, opportunities and challenges

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Abstract

The inaugural Kjell Johansen Lecture in the Zoophysiology Department of Aarhus University (Aarhus, Denmark) afforded the opportunity for a focused workshop comprising comparative cardiovascular physiologists to ponder some of the key unanswered questions in the field. Discussions were centred around three themes. The first considered function of the vertebrate heart in its various forms in extant vertebrates, with particular focus on the role of intracardiac shunts, the trabecular ('spongy') nature of the ventricle in many vertebrates, coronary blood supply and the building plan of the heart as revealed by molecular approaches. The second theme involved the key unanswered questions in the control of the cardiovascular system, emphasizing autonomic control, hypoxic vasoconstriction and developmental plasticity in cardiovascular control. The final theme involved poorly understood aspects of the interaction of the cardiovascular system with the lymphatic, renal and digestive systems. Having posed key questions around these three themes, it is increasingly clear that an abundance of new analytical tools and approaches will allow us to learn much about vertebrate cardiovascular systems in the coming years.

Keywords cardiovascular, comparative physiology, heart.

The legacy of Kjell Johansen

On 15 March 2012, the Zoophysiology Department of Aarhus University, Denmark, held the inaugural *Kjell Johansen Lecture*, the first in an annual physiology lecture series established to commemorate Professor Kjell Johansen's seminal contributions to comparative physiology, and to bring comparative physiologists to Aarhus to discuss current trends and future challenges. The inaugural lecture, held almost exactly 25 years after Kjell Johansen's death, was presented by Professor Warren Burggren. The lecture provided a personal account of Kjell Johansen's career, from his graduate studies at the University of Oslo, through his faculty position at the University of Washington, Seattle (USA), before he was called to Aarhus University where he created the Department of Zoophysiology and presided as head of department until his untimely and tragic death in 1987 (Linzen 1987).

Among Kjell Johansen's many remarkable attributes was his legendary ability to generate complex ideas and hypotheses in rapid-fire fashion. Many who

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worked with him recall discussions that left the discussant with an overwhelming sense of how little we know (and especially how little the discussant knew!) and how much experimentation and interpretation there was yet to be done. The senior author recalls how one of Kjell Johansen's long-standing 'pet projects' suddenly appeared in a journal, published by another research group. While a lesser scientist might have shown distress at being 'scooped', Kjell Johansen instead looked almost delighted and commented '*Good! I now know the answer, and that is one less experiment I have to do*!' Kjell Johansen was intent on sharing his ideas, seeding comparative physiology much like a farmer seeds a field, hoping and anticipating that new ideas would rise from the fertile minds with which he surrounded himself.

Here, we review current concepts regarding the evolution of the vertebrate heart and pose a series of *key questions* that we feel are ripe for study. These ideas and questions were discussed during the course of a focused workshop immediately after the Kjell Johansen Lecture. In the spirit of Kjell Johansen, we hope that the ideas and proposed experiments will result in fewer experiments we have to do ourselves.

Strategies to study cardiovascular evolution

A variety of investigative strategies at all levels of biological organization, in conjunction with a comparative and evolutionary approach, are required to understand the major evolutionary transitions of the vertebrate heart. Such approaches – some time-honoured, others new – are outlined below and represented in Figure 1.

Phylogenetic approaches

A comparative analysis of the cardiovascular characters can when placed in a phylogenetic context – be used to identify when a given character evolved. Additionally, the fossil record and other palaeontological evidence can provide insight into cardiovascular structure through speculation on heart–head distances, etc. (although direct fossil evidence of cardiovascular structure in dinosaurs is lacking – see Cleland *et al.* 2011). From these, it is possible to construct likely evolutionary scenarios to explain links between physiological or behavioural traits.

Developmental physiology

Natural selection drives evolutionary change in morphological structures and physiological processes in developing organisms, as in adults (Burggren & Warburton 1994). Consequently, as Burggren (1992) notes, '*Developmental studies of physiological traits… should be included wherever possible in studies of physiological adaptation*'. The most comprehensive understanding of the evolution of physiological process will thus come from a study of the evolutionary change in the entire life cycle of animal lineages, not just by studying the adults (Burggren 1991).

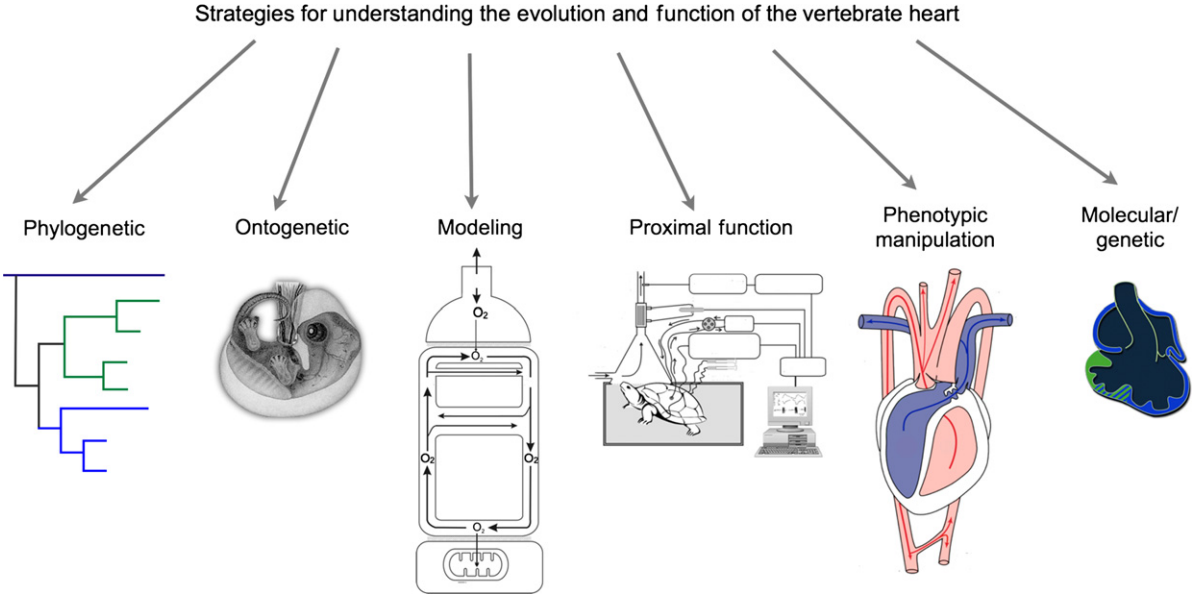

Figure 1 Interdisciplinary strategies for investigating cardiovascular function. Embryonic turtle is modified from Agassiz (1857); oxygen transport model is modified from Wang & Hicks (2002); proximal function original drawing by J.W. Hicks, schematic heart is modified from Eme *et al.* (2010) and the molecular/genetic heart is modified from Zina Deretsky, National Science Foundation after Benoit Bruneau, the Gladstone Institute of Cardiovascular Disease.

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Mathematical modelling

The vertebrate oxygen transport system is accurately described by a series of mass transfer equations as well as hemodynamic relationships. The resulting mathematical models are instructive for quantitative evaluations of the functional significance of particular cardiovascular traits.

Proximal function

Most physiological studies have largely been restricted to the laboratory, where complex multifactorial, environmental stressors are difficult to accurately reproduce, and thus the functional benefits of given traits are difficult to quantify. The recent advent of miniaturized electronics and computer-assisted data acquisition (biologging; telemetry) provides the potential to correlate cardiovascular performance with natural feeding, physical activity, basking, diving, etc. in unrestrained and even free-ranging animals.

Phenotypic manipulation

By surgical or pharmacological manipulations of given cardiovascular traits, it is possible to quantify how relevant performance variables are affected and hence provide insight into the functional significance of the trait (Sinervo & Basolo 1996). A challenge of such studies is to perform the phenotypic manipulation and then expose the animal to relevant environmental conditions. This approach benefits from being combined with field measurements.

Molecular genetics

It is now clear that cardiac development is coordinated by a set of very similar gene regulatory networks in all vertebrates, and the common developmental patterns will allow for an understanding of *how* given traits, such as the conduction system, the left atrium or the ventricular septum, appeared in specific lineages (Moorman & Christoffels 2003, Olson 2006, Koshiba-Takeuchi *et al.* 2009, Jensen *et al.* 2012). As the genomes of more and more species are being sequenced (e.g. Shaffer *et al.* 2013), it will be much easier to design probes for *in situ* hybridization and generate antibodies for expression and molecular studies.

Form and function of vertebrate hearts

The hearts of the earliest chordates were, in all likelihood, merely contractile vessels where peristaltic movements, initiated by simple pacemakers, accounted for the propulsion of blood through a vascular tree with minimal endothelial function (see Burggren & Johansen 1986, Burggren & Reiber 2007, Xavier-Neto *et al.* 2010, Farmer 2011 for reviews). The cardiovascular systems of vertebrates therefore have undergone marked anatomical and functional changes with formation of proper cardiac chambers, evolution of a pulmonary circulation and, finally, the emergence of the four-chambered heart among the endothermic vertebrates.

The basic heart plan

In all of the ectothermic vertebrates (hagfish through fishes, amphibians and reptiles), the cardiac ventricle is a trabecular, sponge-like chamber. This contrasts with the single-lumen ventricles composed of compact walls of the endothermic mammals and birds. It is tempting to correlate this transition in ventricular anatomy with the rise in cardiac output and blood pressures that are characteristics of endothermic birds and mammals, but the advantages of single-lumen ventricles with compact walls are not obvious. For example, ventricular ejection fraction of mammals and birds is approximately 50%, whereas the typical ejection fraction of the trabecular ectothermic ventricles is close to 100% (Franklin & Davie 1992, Burggren *et al.* 1997). Because the shortening of each cardiomyocyte is around 20% in all vertebrates (e.g. Shiels & White 2008), it is possible that the architectural arrangement of the cardiomyocytes within a trabecular ventricle allows for the higher ejection fraction because most blood resides in the many miniscule cavities of the trabecular walls, allowing for a more efficient contraction according to the law of Laplace (Johansen & Burggren 1980, Van Mierop & Kutsche 1985).

Some ectotherms such as tunas, varanid lizards, pythons and crocodiles have high mammalian-like blood pressures (70–100 mmHg; Burggren & Johansen 1982, Jones *et al.* 1993, Hicks 1998, Brill & Bushnell 2001, Wang *et al.* 2003), and their stroke volume resembles that of mammals (around 1 mL kg^{-1}; Farrell 1991, 1996, Korsmeyer *et al.* 1997, Hicks *et al.* 2000, Secor *et al.* 2000, Seymour & Blaylock 2000, Brill & Bushnell 2001, Clark *et al.* 2005). These high-performance ectotherms have retained a trabecular layer within the ventricle, but their maximal heart rates are considerably lower than similar-sized mammals (rarely above 120 min^{-1}; e.g. Wang *et al.* 1997, Lillywhite *et al.* 1999, Hicks *et al.* 2000, Brill & Bushnell 2001, Clark *et al.* 2005). A key, unanswered question, then, is:

Did the evolution of high heart rates driven by the high endothermic metabolism favour a compact ventricle?

A scenario for the rise in cardiac output by elevated heart rate to accommodate the high endothermic

metabolism is illustrated in Figure 2. The tachycardia shortens the time available for filling and emptying, and we hypothesize that the transition from a trabecular to a single-lumen compact-walled ventricle yields a lower viscous resistance to inflows and outflows of the ventricle. Supporting this conjecture, the human and murine pathological condition of non-compaction with heavily trabeculated ventricles often leads to heart failure (e.g. Dyson *et al.* 1995, Freedom *et al.* 2005, Engberding *et al.* 2007). At the same time, the elevation of cardiac output, needed to sustain the rise in metabolism, was associated with a rise in blood pressure, adding additional work on the heart and requiring a thicker ventricular wall.

Figure 2 also includes a number of other changes in cardiac structure and physiology resulting from the rise in heart rate, including the need for a specialized cardiac conduction system to ensure fast and coordinated contractions of the cardiac chambers (Jensen *et al.* 2012, 2013a), improved calcium handling by the sarcoplasmic reticulum to ensure swift activation and deactivation of the contractile apparatus (Galli & Shiels 2012), increased coronary supply for the thicker and more compact myocardium, and finally a fully divided ventricle.

The evolutionary scenario outlined above is not readily investigated through testable hypotheses, but a detailed comparison of cardiac function and anatomy in birds and mammals with their reptilian ancestors would provide evidence for convergent evolution of functional solutions resulting in both higher heart rate and blood pressure.

The four-chambered heart and the functional role of cardiac shunts

The formation of the four-chambered heart with a fully divided ventricle appeared at least twice independently:

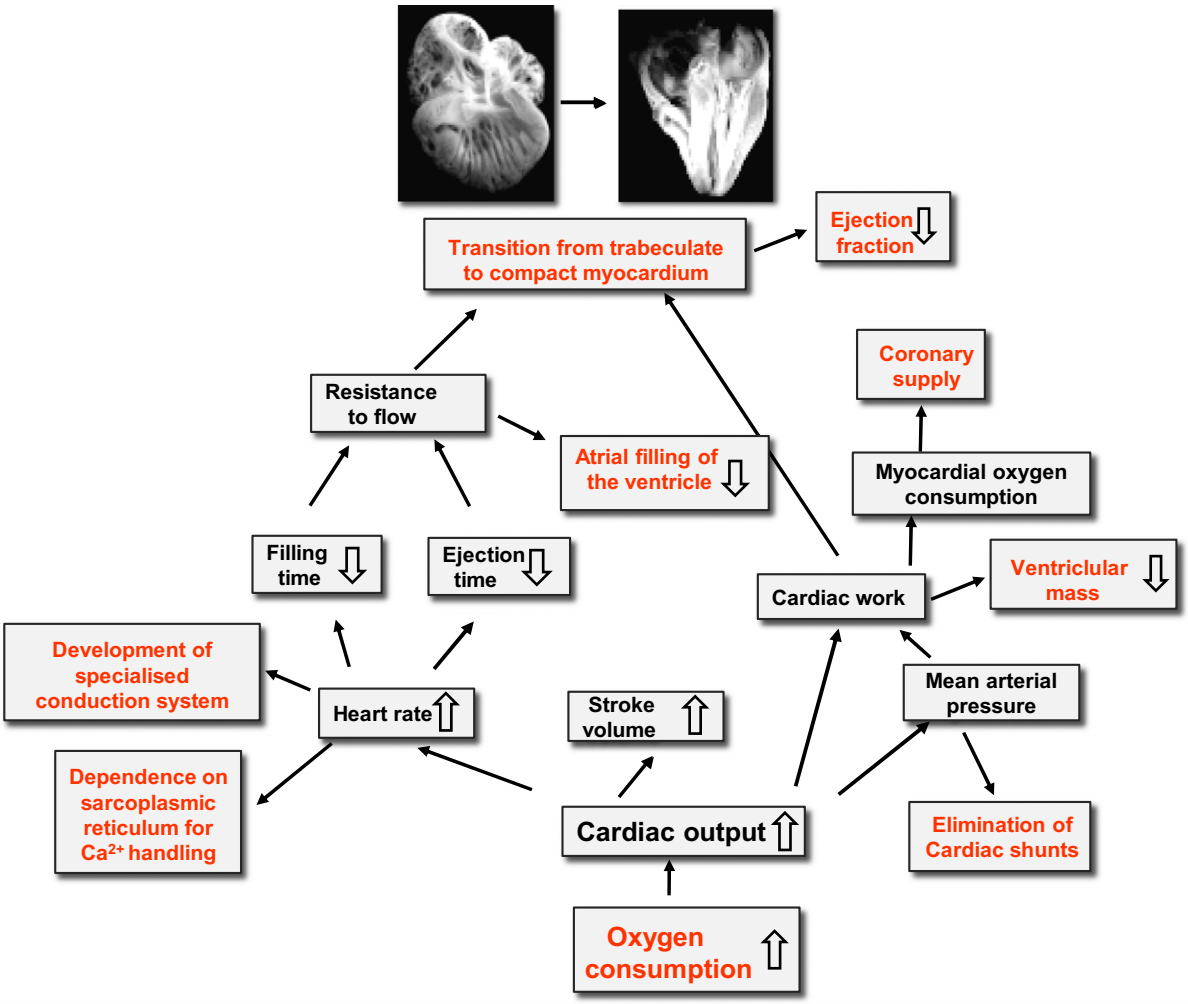

Figure 2 The complex inter-relationships of factors determining cardiac function and their potential relationship to the evolution of a greater dependence on compact rather than trabecular myocardium.

once in the vertebrate group that gave rise to crocodilians and birds (archosaurs) and once in the ancestral group of mammals (synapsids) (Goodrich 1930, Jensen *et al.* 2010b, 2014). The fully divided ventricle allows for high systemic arterial blood pressures, while keeping low blood pressures within the pulmonary circulation, thus allowing for a thinner blood–gas barrier (e.g. West 2009). A fully divided ventricle also avoids admixture of oxygen-rich and oxygen-poor blood within the heart and hence provides for a more effective oxygen transport cascade. Collectively, these characteristics are necessary to support the increased metabolism associated with endothermy that appears directly linked with the rapid expansion and success of mammals and birds (Burggren *et al.* 1997, Hicks & Wang 2012).

Although cardiac shunts in reptiles and amphibians reduce arterial oxygen saturation, the mixing of oxygen-rich and oxygen-poor blood is a normal, functional characteristic of their hearts (see Johansen & Burggren 1985, Burggren & Bemis 1990, Farmer 1999, Hicks & Wang 2012), and it is relevant to ask whether there are distinct benefits to such an arrangement. Such shunting may allow for putative energy savings by reducing overall cardiac work and may enhance ventricular oxygenation in species lacking a coronary circulation. Yet, in adult birds and mammals, cardiac shunts that result from congenital cardiac defects are invariably detrimental and reduce performance. Given that cardiac shunting has been regarded as beneficial in ectothermic vertebrates, but detrimental in endothermic vertebrates, a major question in comparative cardiovascular physiology is:

Are cardiac shunts an adaptive trait – that is, a trait that conveys unique physiological advantages to ectothermic vertebrates?

Despite more than a century of anatomical and physiological studies, there is no convincing evidence that the lack of the ability to shunt blood reduces physiological performance and/or reproductive fitness (see Wang *et al.* 1998, Farmer *et al.* 2008, Hicks & Wang 2012). Thus, it is equally plausible that the unique features of the reptilian heart are embryonic or ancestral characters that, with no negative impacts on overall animal fitness, have simply not been selected against. Quantifying fitness in reptiles or any of the other vertebrates with cardiac shunts is very difficult.

Phylogenetic studies often require broad surveys of closely related species and given the substantial technical demands required to quantify cardiac shunts in a single species, such an approach would seem implausible. As an alternative, morphological studies on the ventricular anatomy may predict the ability of a given species to alter their cardiac shunts. Specifically, the size of the muscular ridge, the partial ventricular septum that separates the pulmonary and systemic cava in the ventricle, may be an important indicator of the capacity for cardiac shunting in reptiles. In some reptiles, the muscular ridge is relatively small and not well developed (Hicks 1998) and the ventricle functions as a single pressure pump during the entire cardiac cycle (Burggren 1986). Under these conditions, the size and direction of the cardiac shunts are influenced by changes in pulmonary and systemic vascular resistances (Hicks 1998, Wang & Hicks, 2002). In some reptiles (varanids and pythons), the muscular ridge is enlarged, resulting in a dual pressure pump during systole (Burggren, 1986; Hicks 1998, Jensen *et al.* 2010b,c). In these animals, mixing of deoxygenated and oxygenated blood can occur during diastole. Recently, Jensen *et al.* (2014) standardized morphological procedures to quantitate the muscular ridge and ventricular cava. By applying such techniques to a number of vertebrate species with different ventricular anatomies, it will be possible to target physiological experimentation in particular species of interest.

There have been few studies on cardiac function during development in reptiles (Eme *et al.* 2011a,b,c). A more detailed understanding of cardiac function during embryonic development may reveal whether cardiac shunts are critical for normal development. In addition, an understanding of the developmental origins of cardiac shunts may also reveal whether cardiac shunts play different roles (quantitatively or qualitatively) in hatchlings, juveniles or adult animals.

The effects of cardiac shunting on oxygen transport are easily modelled using well-known mass transfer equations. Such approaches provide testable hypothesis for the role of shunts on VO_2 max, blood oxygen and carbon dioxide regulation, aerobic dive limitations and temperature regulation (Wood & Hicks 1985, White & Hicks 1987, Wang & Hicks 1996, Wang *et al.* 1997).

Recent studies on alligators and rattlesnakes have exploited phenotypic manipulation to investigate the functional role of right-to-left shunts (Eme *et al.* 2009, 2010, Leite *et al.* 2013). These approaches reveal that eliminating the capacity for R-L shunts in both species did not influence growth rates relative to normal animals. Functional studies of animals with cardiac malformations may provide similar insight. For example, pythons with septal defects did not appear to behave or grow in an abnormal fashion (Jensen & Wang 2009).

Coronary circulation and O_2 consumption of the vertebrate heart

Proper cardiac function requires continuous ATP production and consequently most vertebrate hearts need

a continuous oxygen supply. Cardiac oxygen supply and consumption are tightly coupled with cardiac work, that is, cardiac output and arterial blood pressure (Farrell 1987, 2002, Farrell & Stecyk 2007, Duncker & Bache 2008). Although there is comprehensive understanding of the importance of cardiac oxygen supply in mammalian and avian hearts (Duncker & Bache 2008), a key area for future experimentation is to understand the evolution of the various forms of cardiac oxygen supply among vertebrates and to correlate these forms to the habitats, behaviours and metabolic status of animals.

The array of cardiac designs is remarkably diverse across vertebrates, but none are as dependent on coronary circulation as adult mammals and birds, where a coronary circulation supplies a compact wall with its dense arrangement of cardiomyocytes. This is in contrast to the ancestral vertebrate heart (possibly represented in extant cyclostomes) where the trabecular arrangement of cardiomyocytes receives no coronary circulation, and where cardiac oxygen supply is provided entirely by the oxygen-poor venous blood returning to the heart (Davie & Farrell 1991, Farrell *et al.* 2012). Certain teleosts and all amphibians also have an entirely trabecular ventricular myocardium, while others wrap the trabecular myocardium to varying degrees with a layer or layers of condensed myocardium with a distinct coronary circulation (Santer 1985). Even so, myocardial compaction and associated coronary vessels apparently appeared at the outset of vertebrate evolution, because all elasmobranchs studied have a coronary circulation (Tota 1989, De Andres *et al.* 1990, 1992, Farrell *et al.* 2012). Other teleosts, and perhaps all reptiles, have compact myocardium, but never as much as mammals (Farrell *et al.* 2012). Some teleosts even have coronary vessels in their ventricular trabeculation, as in elasmobranchs, birds and mammals. Clearly, the phylogeny of the coronary circulation is far from simple (Fig. 3). The importance of cardiac work rate and hypoxia in coronary development seems intuitive, their relative importance has still to be defined, and the selection pressures that result in the diversity of coronary arrangements remains unknown.

The phylogenetic and ontogenetic shifts from the trabecular heart to compact heart are relevant to the patterns observed in the coronary circulation. A key question is:

What is the evolutionary advantage of retaining a partially trabecular heart, the ancient, pre-vertebrate solution to cardiac oxygen supply?

Finding answers to this and other related questions requires a three-pronged experimental approach. Foremost, the diversity of cardiac anatomy must be studied in a much larger number of species in a wide phylogenetic context to reveal whether phenotypic responses relate to natural selective pressures. For example, we still lack a clear understanding of the exact distribution of the coronary circulation in elasmobranchs, lungfishes and reptiles, and it remains very uncertain whether the poor coronary supply in anuran amphibians represents an evolutionary loss. Future studies should estimate capillary density, a determinant of oxygen supply, and hence an indirect proxy for cardiac work.

The second prong of the experimental approach is to determine how anatomy of the coronary arterial tree and the venous oxygen supply relate to the environmental conditions and behaviours of a given species. This will require comprehensive measurements of relevant routine and maximum physiological variables including cardiac output, arterial blood pressure, myocardial oxygen consumption and coronary blood flow. These measurements are currently feasible, but have been made in relatively few species and concurrently in even fewer species.

The molecular building plan of the heart and the evolution of the conduction system

The high heart rates of mammals and birds require an electrical conduction system to generate and conduct the electrical impulses that ensure proper activation of the cardiac chambers. Errors in development or function of the chambers, septa or conduction system frequently result in congenital defects or arrhythmias in humans (e.g. Christoffels & Moorman 2009).

Comparative development is instructive because the profound differences between the fully formed hearts of ectotherms and endotherms are not evident in their embryonic stages. For example, the trabecular heart of ectotherms has a much simpler conduction system than birds and mammals, although the electrical activation pattern of the hearts of ectotherms and endotherms is strikingly similar (see Burggren 1978). Therefore, by comparing the hearts of endotherms and ectotherms, the basic building blocks of the conduction system can be revealed, and cardiac specializations evolved in the endotherms to support their high metabolic state and physically separated blood flows can be deduced. Generally, in vertebrates, an adult type of electrocardiogram can be detected in the early stages of chamber formation, even though a morphologically distinct conduction system has not yet developed (Christoffels *et al.* 2010). This electrical pattern indicates a shared cardiac building plan among vertebrates.

A major focus in cardiovascular development is to elucidate the common mechanisms that underlie the developmental control of the growth and patterning of the vertebrate heart. Major questions include:

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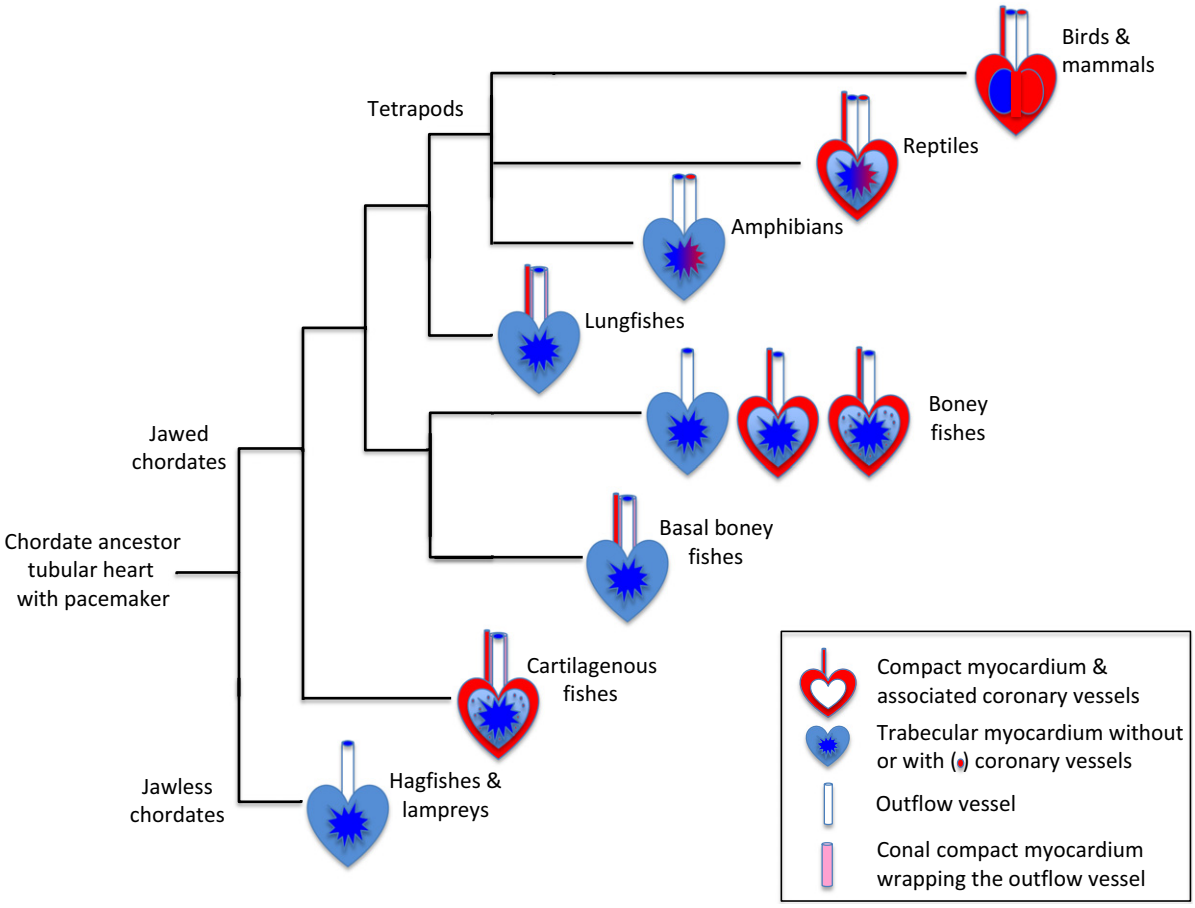

Figure 3 A schematic representation and generalization of the oxygen supply routes with the chordate phylum. The archetype avascular ventricle that relies on oxygen contained in venous blood is exemplified by cyclostomes. A coronary oxygen supply to the conal myocardium and a variable amount of compact and trabecular myocardium is seen in the elasmobranchs. Cardiac oxygen supply is highly variable among teleosts. The exact distribution of the coronary circulation in lungfish is unknown. Reptiles have a variable proportion of compact myocardium, and again the exact distribution of the coronary capillaries is unknown. The ventricle of birds and mammals is almost entirely compact myocardium, and the few trabeculations that are present contain coronary capillaries. These generalizations for lower vertebrates are based on anatomical studies for a relatively small number of the over 50 000 species.

How do the cardiac precursor cells become organized into an evolutionary conserved cardiac tube?

What controls the formation of cardiac chambers, and which components are minimally required to establish the conserved electrical pattern?

Which precursor populations and molecular signalling systems have been evolved to support the specific features of the hearts of endotherms?

We now know that the primary heart tube evident in embryonic vertebrates grows by recruitment of precursor cells. Chamber differentiation and expansion occurs at specific locations within the tube, which develop fast depolarization and strongly increased intercellular conductivity (Fig. 4). This allows the chambers to rapidly propagate the electrical impulse, which is seen as the P wave (atrial excitation) and QRS complex (ventricular excitation) in the electrocardiogram (Fig. 4). The sinus venosus, which contains the principal pacemaker in all vertebrate hearts (Burggren *et al.* 1997), the atrioventricular canal and the outflow tract initially do not differentiate into chamber-type myocardium. Rather, they retain the slow proliferative and conductive properties found in the original embryonic heart tube (Christoffels *et al.* 2010). The delay in impulse propagation in the atrioventricular canal is seen between the P wave and QRS complex in the electrocardiogram.

Heart development seems to be conserved across species and is driven by transcription factors including T-box, homeobox and GATA zinc-finger transcription factors (Olson 2006). In all species examined to date (lampreys, fish, frog, birds and mammals), Tbx2 is

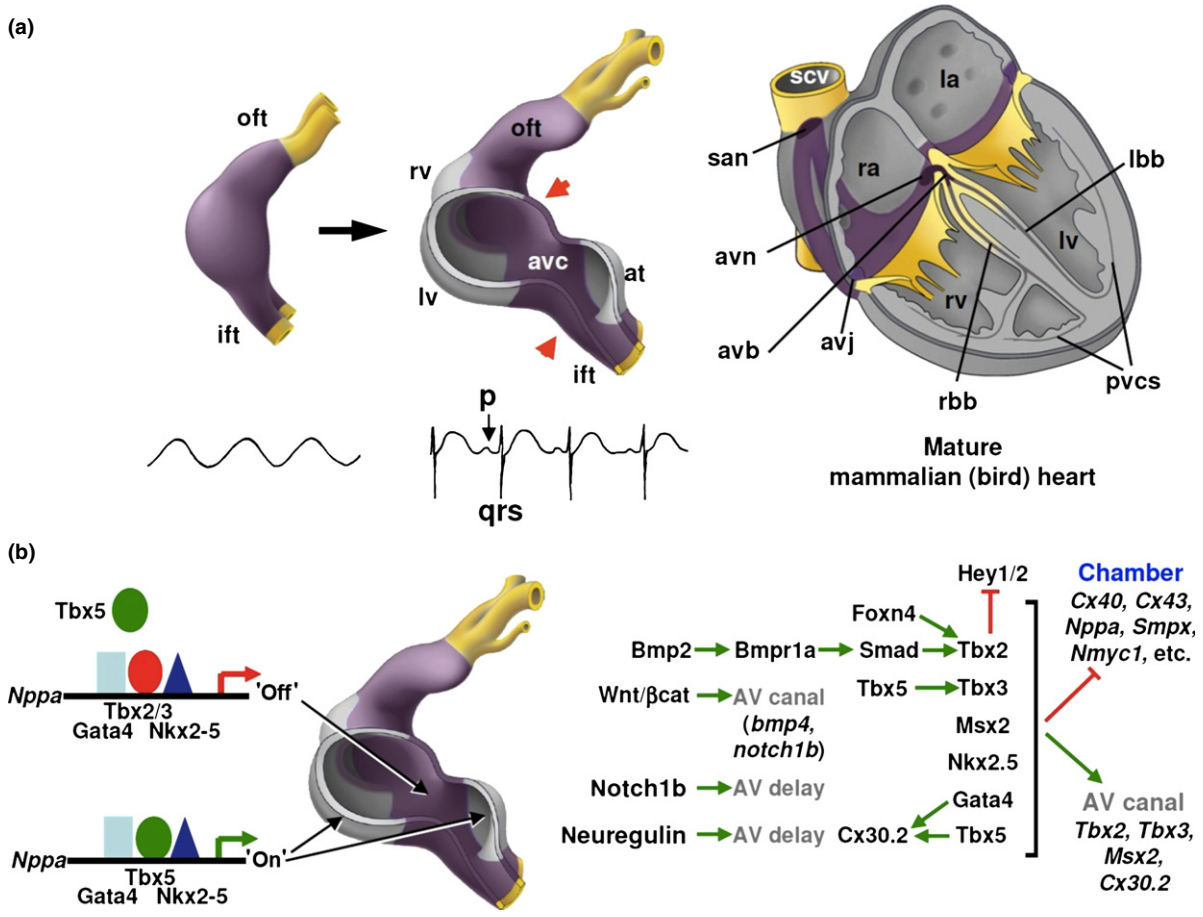

Figure 4 Cardiac development and gene expression in vertebrates. (a) Embryonic heart tube (purple), local development of chambers (grey). Chambers are characterized by fast activation and an adult-like electrocardiogram can be monitored (p, atrial activation; qrs, ventricular activation). The mature configuration of the cardiac conduction system, as seen in mammals and birds only, has still to develop. (b) Conserved transcriptional programmes including broadly expressed activators and locally expressed suppressors determine the position of chamber formation, atrioventricular development and electrical patterning. Modified from Christoffels *et al.* 2010.

specifically expressed in, and controls the formation of, the atrioventricular regions (Christoffels *et al.* 2010, Kokubo *et al.* 2010). The development of the sinus venosus, which includes the primary pacemaker, is under control of Tbx18 in mouse, a process that may be evolutionary conserved (Christoffels *et al.* 2010). The transcription factor Isl1 is expressed in the developing and mature pacemaker in both mammals and zebrafish (see Tessadori *et al.* 2012), and pacemaker function is disrupted in the absence of Isl1. Isl1 expression, for the first time, reveals the presence of a specialized conduction system tissue in endotherms as well as ectotherms.

The following are key specific features of mammalian and avian hearts: the development of a right ventricular component and interventricular septum, formation of the compact ventricular wall, the development of atrioventricular connective tissue and appearance of discrete conduction system components. Markers for these components or their progenitors have been identified in the mouse and chicken (Fig. 4b). Studying these markers in species in which specific features are not present or present in a less-developed form allows us to identify the origin and development of these features and their required pathways. For example, ventricular septation has been studied in the anole lizard (no interventricular septum) and a freshwater turtle (controversially described as having a small interventricular ridge) using the pattern of Tbx5 during embryonic development (Koshiba-Takeuchi *et al.* 2009). Similarly, mammals and birds express family member Tbx3 in the conduction system (Hoogaars *et al.* 2004). Using Tbx3 and other conserved genetic markers to identify conduction system components, we found that the conduction system design of lizard (*Anolis*), frog (*Xenopus*) and zebrafish

adults is strikingly similar to that of embryos of mammals and chicken (Jensen *et al.* 2012, 2013a). Understanding of the evolution and development of these specializations can be improved by comparisons to hearts of ectotherms – particularly to species that have anatomically and physiologically exceptional hearts such as tunas, varanid lizards, pythons and crocodiles (Burggren *et al.* 1997, Jensen *et al.* 2010a,b,c, 2014).

Control of the cardiovascular system

Autonomic control

Neural control of the vertebrate cardiovascular system is primarily achieved through the autonomic nervous system (Taylor *et al.* 1999). A coordinated regulation of visceral functions to maintain homeostasis must have been of paramount importance already in the earliest vertebrates, and the autonomic nervous system has an ancient evolutionary history. A phylogeny depicting cardiac control in extant vertebrates and two chordate sister groups (tunicates and amphioxus) is shown in Figure 5. Within hagfishes and lampreys, the autonomic nervous system is rudimentary, such that some organs are devoid of innervation, while others lack dual innervation. The typical inhibitory action of the vagus on the heart did not appear until the evolution of cartilaginous fishes, while the opposing role of an excitatory sympathetic innervation of the heart evolved later with the emergence of bony fishes (Taylor & Butler 1982).

In all vertebrates, heart rate and consequent blood flow are integrated with ventilation of the respiratory organs to optimize respiratory gas exchange. Fine control of these cardiorespiratory interactions is integrated in the brainstem (Fig. 6). Optimization of respiratory gas exchange requires matching of cardiac output to ventilation, which in fish may culminate in

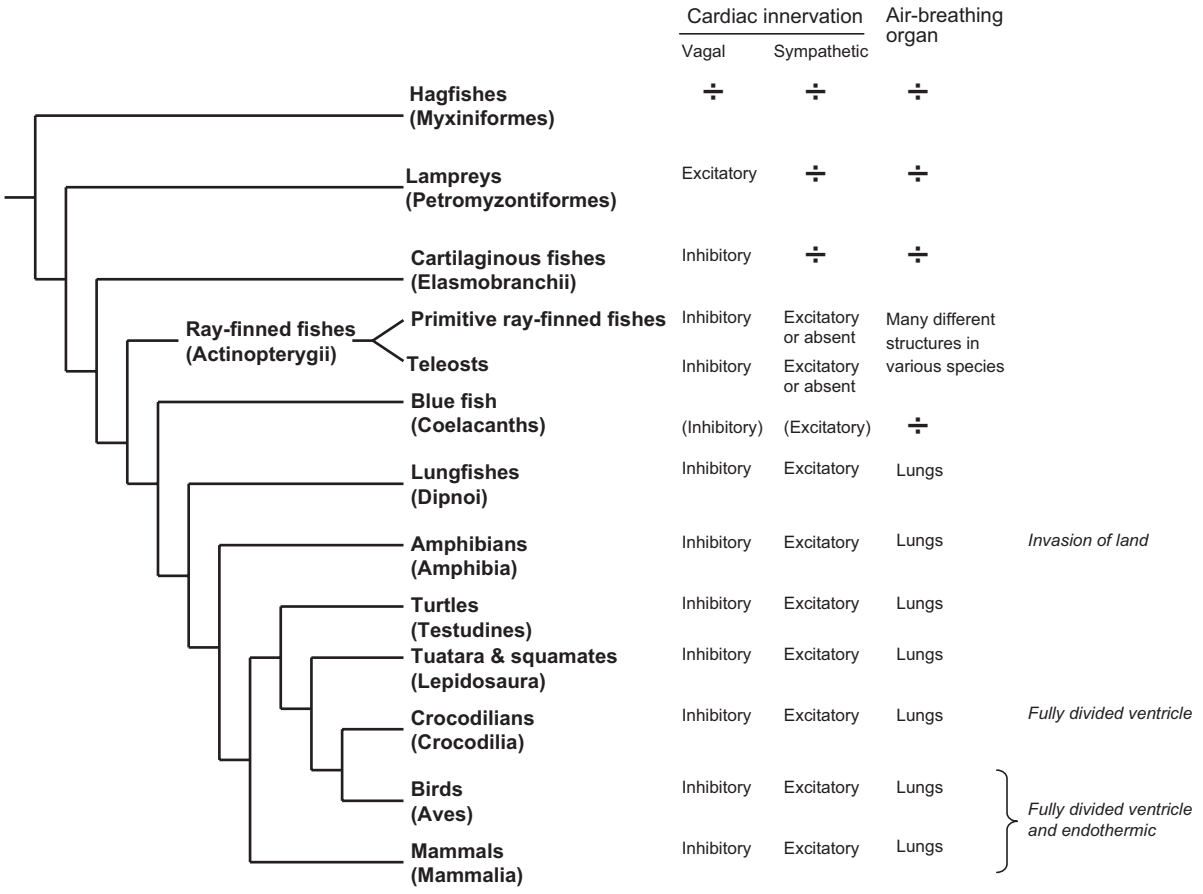

Figure 5 Phylogeny of the cardiac innervation and air-breathing organs in the major groups of living craniates. The heart of hagfishes is aneural, while the heart of lampreys receives an excitatory vagal innervation. An inhibitory vagal innervation that relies on the activation of muscarinic receptors on the heart appeared in cartilaginous fishes and was retained in all other vertebrates. The excitatory sympathetic innervation is present in most, but not in all ray-finned fishes and all other higher vertebrates. Air-breathing organs evolved independently in several groups of ray-fined fishes, while true lungs first appeared within lungfishes and are found in all tetrapods. Endothermy evolved independently in birds and mammals, possible from their reptilian ancestors (from Taylor & Wang 2009).

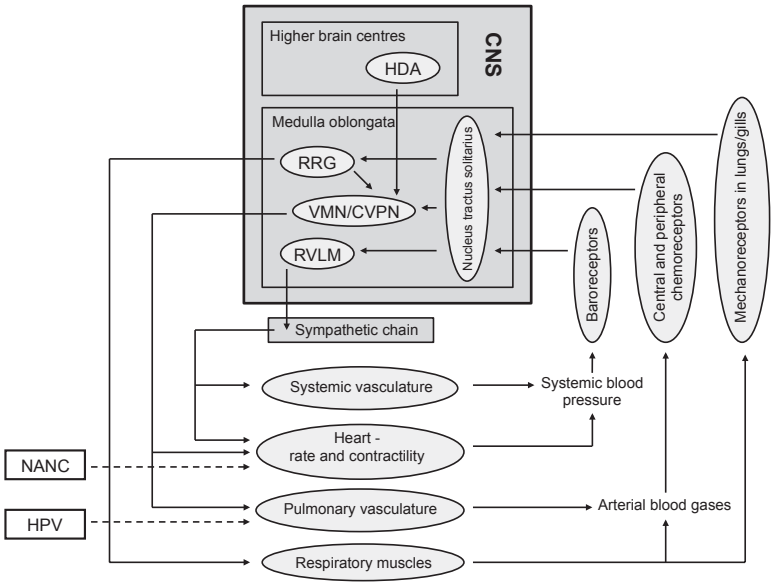

Figure 6 A generalized diagram of the major components mediating cardiorespiratory interactions in vertebrates. The components are defined, and their interactions are described in the text. CNS, central nervous system; HDA, hypothalamic defence area; HPV, hypoxic vasoconstriction; NANC, non-adrenergic, non-cholinergic, RRG, respiratory rhythm generators; RVLM, rostro-ventro-lateral medulla; VMN/CVPN, vagal motor nucleus/cardiac vagal preganglionic neurones.

synchrony between heart beat and ventilation and in mammals in respiratory sinus arrhythmia (RSA), with a rise in heart rate during inspiration (Hayano *et al.* 1990, 1996, Taylor & Wang 2009). RSA presents a useful phenomenon to explore neural elements of cardiovascular control, and it is well established that cardiac vagal preganglionic neurones (CVPN) located in the nucleus ambiguous are particularly important to slow the heart during expiration in mammals (Jordan & Spyer 1987). Their activity is influenced by the activation of the hypothalamic defence area and gated (switched off) by activity in neighbouring inspiratory neurones and mechanoreceptor inputs from the lungs (Fig. 6).

Cardio–respiratory interactions, such as RSA and the reflex changes in heart rate associated with periodic breathing, have a long evolutionary history. An understanding of the putative roles for the specific location and consequent activity of cardiac vagal preganglionic neurones in generating cardiac vagal tone and cardio–respiratory interactions in vertebrates is a central topic within comparative cardiorespiratory physiology, the overarching question being:

Is fine control of heart rate including cardio–respiratory interactions, dependent upon dual locations for cardiac vagal preganglionic neurones within the brainstem, with one population showing respiration-related activity?

More neuroanatomical data are required, but we will not be able to substantiate their functional roles until we undertake direct neurophysiological investigations of activity in vagal preganglionic neurones and the factors that influence it. To tackle this topic requires a combination of neuro-anatomical and neurophysiological approaches, including location and characterization of preganglionic neurones in the brainstem. Location of central neurone cell bodies is typically arduous and yields sparse data. However, some useful progress may be achieved without invasion of the central nervous system, as recording from and stimulating peripheral nerves, in combination with neuroanatomical studies, can reveal important aspects of central control, particularly if it includes measurement of their conduction velocities and the use of anodal block to differentiate their influences on target organs such as the heart (e.g. Barrett & Taylor 1985a,b,c, Jones *et al.* 1995, O'Leary & Jones 2003, Taylor *et al.* 2009a,b).

Ontogeny of the autonomic control

There is an ontogeny as well as a phylogeny to patterns of cardiac regulation in vertebrates (see Crossley & Burggren 2009). For example, the increased heart rate variability of the axolotl *Ambystoma mexicanum* after metamorphosis was associated with the appearance of a ventro-lateral group of CVPNs (Taylor *et al.* 2001), and similar changes appear to occur late in incubation or immediately after hatching in both reptilian and bird embryos. In human babies, respiratory arrhythmia appears at about 85% of gestational age (Taylor *et al.* 2001). Thus, a key question remains:

What is the functional relationship between developmental morphological changes taking place in the central nervous system and the onset of autonomic control of the cardiovascular system?

This question is being addressed by a combination of neuroanatomical and electrophysiological techniques applied to reptilian embryos that represent a key group in the evolution of tetrapods (Taylor & Wang 2009, Taylor *et al.* 2010). The important maturational changes that result in central control of cardio–respiratory interactions may be migration of vagal preganglionic neurones within the brainstem, myelination of peripheral nerves and completion of their innervation of target organs such as the heart.

Developmental phenotypic plasticity in cardiovascular control

Phenotypic plasticity is the capacity for a given genotype to give rise to multiple phenotypic traits dependent on the internal or external environmental milieu (Bateson *et al.* 2004, Garland & Kelly 2006, Pigliucci *et al.* 2006). The basic evolutionary concept of 'plasticity' or phenotypic flexibility within a genotype represents a powerful means of adaptation, maximizing advantageous traits for a given environment (Dewitt & Scheiner 2004, Pigliucci *et al.* 2006), but plasticity during the developmental period may also produce maladaptive traits, such as the increased incidences of cardiovascular diseases through the mechanism of 'foetal programming' hypothesis underlying adult onset human diseases (Barker 2000). Normal mammalian foetal development may require a relatively narrow range of abiotic conditions, but the effects of altered environmental conditions for non-mammalian vertebrates and the potentially deleterious consequences of cardiovascular developmental plasticity are poorly understood. This leads to two key unanswered questions:

In natural environments, does developmental cardiovascular plasticity convey advantageous or deleterious phenotypic changes in non-mammalian vertebrates?

Does developmental cardiovascular plasticity in non-mammalian vertebrates provide insight to the evolutionary constraints on developmental biology?

Egg-laying reptiles are useful models to understand the impact of the developmental environment on cardiovascular maturation because it is easy to manipulate the environment where they develop and to relate these environmental perturbations and the natural environment. Manipulation such as changes in gas composition, temperature or water content can be used to investigate phenotypic plasticity. Growth rates of several reptilian species are related to food availability, which is an advantage when conducting longitudinal studies of cardiovascular performance. Reptiles also differ in the embryonic onset, importance and magnitude of autonomic or central nervous system control. This feature makes reptiles a potentially rich group to investigate cardiovascular homeostasis in an embryonic system and assess the plasticity of regulatory control mechanisms. To date, studies of developmental plasticity in non-mammalian amniotes have been restricted primarily to the embryonic phase (Chan & Burggren 2005, Crossley & Altimiras 2005, 2012, Eme *et al.* 2011a,b, Crossley *et al.* 2012, Eme *et al.* 2012).

Hypoxic responses of systemic and pulmonary vessels

In addition to autonomic regulation, the peripheral circulation is affected by numerous local factors, including metabolites and oxygen levels. Physiologists have generally been interested in understanding how organisms sense and respond to oxygen. Comparative approaches reveal that hypoxia constricts the pulmonary vasculature, an intrinsic phenomenon termed 'hypoxic pulmonary vasoconstriction' (von Euler & Liljestrand 1946, Sommer *et al.* 2008). This response is thought to be adaptive in that it diverts pulmonary blood flow from inadequately ventilated and hypoxic parts of the lung to more highly ventilated areas. Thus, hypoxic pulmonary vasoconstriction is important for local matching of blood perfusion to ventilation by improving pulmonary gas exchange efficiency and, consequently, maintaining arterial oxygenation (Skovgaard & Wang 2006, Sylvester *et al.* 2012). Hypoxic vasoconstriction is an ancient and highly conserved response expressed in the respiratory organs of all vertebrates, including lungs of mammals, birds and reptiles, amphibian skin and fish gills.

In contrast to the pulmonary circulation, hypoxia is well known to elicit vasodilation in the systemic vasculature of most vertebrates, an equally adaptive response that allows for increased perfusion of oxygen-deprived regions in the various organs. While it may seem intuitive that the vasodilatory response precedes the constriction in evolutionary terms, a number of recent studies document that hypoxia causes constriction of vascular smooth muscles in cyclostomes, a phylogenetically early group of vertebrates, and that this response is intrinsic to the vasculature (Olson *et al.* 2001, 2008, Russell *et al.* 2008). Hypoxic vasoconstriction thus appears to be an ancient vascular response to hypoxia that has been embellished with secondary regulatory factors as vertebrates evolved to be more responsive to hypoxia. Thus, hypoxic pulmo-

nary vasoconstriction in tetrapods has evolved to become a multifactorial process associated with several signalling pathways. This leads to a key question:

What are the signalling pathways involved in hypoxic vasomotor activity?

O_2 sensing by vascular tissues has been intensely studied in mammals for more than 50 years. Numerous mechanisms have been proposed to explain how blood vessels sense low PO_2 and transduce this signal into dilation or constriction, but none have received unequivocal support and the O_2-sensor and sensor/transduction cascade(s) remain unresolved (Ward 2008, López-Barneo *et al.* 2010, Clanton *et al.* 2013). Nevertheless, there is now evidence that a mechanism involving hydrogen sulphide (H_2S) metabolism may explain both hypoxic vasoconstriction and hypoxic vasodilatation (Olson *et al.* 2006, 2010). The mechanisms of the vascular oxygen sensor are more likely to be unravelled in phylogenetically ancient groups of vertebrates without the secondary regulatory factors. Accordingly, a comparative approach is likely to provide novel insights. Studies of respiratory vessels from primitive (i.e. early) air-breathers, such as lungfish, as well as reptiles and mammals, may reveal an evolutionary progression in complexity of the oxygen sensor and mechanisms underlying hypoxic vasoconstriction.

Cardiovascular interactions with other organ systems

Physiologists have tended to focus on individual systems (e.g. cardiovascular, gas exchange, ion exchange, endocrine, neural systems), viewing homeostasis through the eyes of regulation of the system they study. While there is, of course, broad appreciation that all of these systems operate in an integrated fashion to achieve homeostasis, studies specifically designed to look at the *interactions* between systems are relatively uncommon. Below we explore as examples three sets of interactions – with the lymphatic, renal and digestive systems – and how the comparative approach will provide model approaches to investigate these interactions.

Cardiovascular–lymphatic interactions

Maintenance of plasma volume is critical for cardiovascular homeostasis. There is tremendous diversity in both plasma volume as a fraction of body mass and the regulatory mechanisms that maintain plasma volume, with no clear phylogenetic pattern (Takei 2000). Plasma volume regulation entails balancing fluid exchange between the vascular and interstitial compartments. Regulation of plasma volume (V_p) and the fluid exchange between vascular and interstitial compartments are governed by a combination of short-term and long-term effector feedback loops. Long-term regulation of V_p is accomplished by renal mechanisms. Short-term, transient changes in plasma volume occur through transcapillary fluid flux or through changes in lymphatic fluid flux. Compared with mammals, ectothermic vertebrates have substantially greater transcapillary fluid flux rates, which promote a high rate of lymph formation (Hedrick *et al.* 2013).

Recent investigations on anurans have shed some light on this process. Plasma turnover rates in anurans are 3–5% of V_p min^{-1} and much higher compared with either fish (0.8–0.9%) or mammals (<0.1%) (Hillman *et al.* 2004). The reason for the much higher plasma turnover in anurans is 10-fold higher interstitial compliance (C_{ist}) in anurans, where the extensive subcutaneous lymph sacs provide considerable lymphatic storage capacity with little interstitial pressure development to counterbalance the hydrostatic pressure in the capillary. Thus, the net balance of forces favours the loss of plasma from the vascular space to the interstitium under most physiological conditions, even including dehydration and haemorrhage (Hillman *et al.* 1987). Filtered plasma is then returned to the vascular space via the lymphatic system. This comparison points out the extreme importance of the interactions between cardiovascular–lymphatic function in anurans, but the synergy between these two systems is almost completely unstudied in fish, reptiles and birds. The extremely high rates of lymph formation and plasma turnover in anurans beg the question:

How do anurans maintain cardiovascular homeostasis given their unusual lymph and plasma dynamics?

Anurans have two pairs of spinally innervated lymph hearts that actively pump lymph into the venous renal portal system (Crossley & Hillman 2010). These lymph hearts are under feedback control of arterial baroreceptors (Crossley & Hillman 1999), but are also under hormonal control (De Grauw & Hillman 2004) and through the volume of lymph provided by the various lymph sacs (Hillman *et al.* 2010). Lymph hearts are also found in some reptiles and a few birds, but their role in cardiovascular homeostasis is unknown. The critical issue for anurans is that any lymph formed moves gravitationally to the ventral regions of the animal, but must be moved to the dorsally located lymph hearts before it can be returned to the vascular space (Hillman *et al.* 2004).

Anurans overcome this morphological challenge using a variety of mechanisms including differential lymph sac compliance, contraction of specialized

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skeletal muscles and lung ventilation to move lymph (Hillman *et al.* 2005, 2010, Drewes *et al.* 2007, 2013, Hedrick *et al.* 2007, 2011). The finding that lung ventilation is involved in lymph movement (Hedrick *et al.* 2007), in addition to its traditional role in gas exchange, is a novel finding and may explain the evolutionary origin of the well-known link between blood pressure and ventilation in mammals (McMullan & Pilowsky 2010).

A summary of the various factors involved in moving lymph in anurans, which may be applicable to other vertebrates, is shown in Figure 7. Whether these processes to move lymph are unique to anurans is unclear and so comprise key areas for future study. In this regard, imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) should prove very useful for visualizing pathways and providing non-invasive techniques for evaluating lymphatic function.

Other taxa in addition to amphibians may offer interesting insights that can help us understand cardiovascular–lymphatic interactions. For example, fish have a 'secondary circulation' that is connected to the arterial system via small anastomoses referred to as arterial-lymphatic conduits (Jensen *et al.* 2009). Whether the secondary circulation represents a primitive lymphatic system has been the source of much speculation and controversy (Isogai *et al.* 2009, Vogel 2010, Hedrick *et al.* 2013). Molecular control of lymphatic vessel generation (lymphangiogenesis) is conserved in fish and mammals (Yaniv *et al.* 2006), suggesting that common molecular mechanisms are involved in development of the lymph system in vertebrates. The use of molecular tools will continue to be important in lymphatic studies, but should be applied more broadly to examine unique or conserved features in a comparative context.

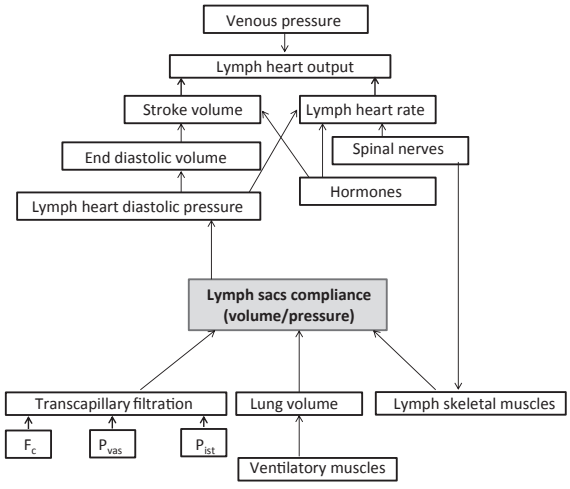

Figure 7 Schematic diagram of the various forces involved in moving lymph in anurans. Dorsally located lymph hearts pump lymph into the venous circulation. The various lymph sacs are combined into a single box (shaded) for clarity. Lymph sac compliance (ΔVolume/ΔPressure) in the various lymph sacs is affected by transcapillary filtration, ventilation acting on lung volume and a variety of skeletal lymph muscles. Transcapillary filtration is influenced by the whole body filtration coefficient (F_c), vascular pressure (P_{vas}) and interstitial pressure (P_{int}) as described by Tanaka (1979). See Hillman *et al.* (2004) for additional details.

Birds also present a particularly interesting physiological problem, having mammalian-like arterial blood pressures coupled with a low plasma oncotic pressure. This situation should favour high transcapillary filtration rates, yet birds tolerate dehydration and haemorrhage to a much greater extent than mammals (Djojusugito *et al.* 1968, Carmi *et al.* 1994). This differential capacity remains unexplained, but a role for lymph mobilization is implied within this problem. Clearly there is much investigation to be completed in the future to determine the proximate and evolutionary aspects of cardiovascular–lymphatic interactions in vertebrates.

Cardiovascular–renal interactions during development

As in adults, a developing animal increasingly functions as a collective of highly interactive systems that respond to environmental challenge in a tightly integrated fashion, rather than as distinct units. Consequently, a major question in physiology is:

When does the co-dependency of major organ systems first develop and how does it then mature?

The nexus of the cardiovascular and renal system provides a logical starting point for beginning an intersystems approach in developmental physiology. The adult cardiovascular system depends upon the osmoregulatory system to maintain blood pressure and flow, as well as blood fluid volume, via hormonal regulation. Likewise, changes in cardiovascular function, particularly blood pressure, can influence blood supply to the nephrons of the kidneys, heavily influencing renal function and therefore salt and water balance through the renin-angiotensin system (RAS) pressure and act through negative feedback, inhibiting further renin release (Vander 1980).

Blood pressure and fluid volume regulatory systems, such as the RAS, have been extensively studied in adult vertebrates, especially mammals (for a recent review see Crowley & Coffman 2012). However, our knowledge of such intersystem physiological interactions during development of the vertebrate embryo/foetus is scarce at best and many unanswered questions remain:

Do cardiovascular and renal development and maturation parallel each other?

Does an experimental perturbation altering one system also influence the other, revealing early co-dependency?

When do the mechanisms and signals for intersystem communication first appear during development and how do they mature?

Serving as both a complicating and intriguing framework for answering the questions outlined above is the fact that the cardiovascular and renal systems have distinctive developmental timelines. For instance, the cardiovascular system develops and becomes functional earlier in time than any other major organ system, including the renal system (Bagatto & Burggren 2006, Burggren & Reyna 2011). Certainly, this differential development will influence both the strength and direction of 'crosstalk' between the systems. Using the chicken embryo as a model, initial interactions are hypothesized to be in the direction of the cardiovascular to the renal system (Fig. 8), with renal responses being biochemical (e.g. changes in renin release), physiological (e.g. changes in nephron perfusion or ultrafiltrate formation) or morphological (e.g. changes in nephron numbers or glomerular structure). However, as the renal system further develops, it will also begin to influence the cardiovascular system via endocrine control and indirect neural reflex arcs. The responses of the maturing cardiovascular system are likely to be largely physiological (e.g. change in blood pressure, heart rate) rather than morphological, because of the advanced stage of cardiovascular development and the emergence from the 'critical window' for development of this system.

Finding experimental approaches for examining intersystem interactions during vertebrate development can be more problematic than with the traditional, more narrowly focused single-system studies. Assessment of interactions across development can be approached using an environmental or pharmacological stressor, for example, that directly affects only one of the two interacting systems of interest. 'Crosstalk' would then be revealed by a response in the system not directly affected. For example, the renal system can be challenged by exposure to RAS agonists (e.g. ANG I, ANG II) and antagonists (e.g. renin inhibitors, ACE inhibitors, ANG II receptor blockers). Such stressors should have limited *direct* cardiovascular effects. Therefore, detectable changes in cardiovascular function likely result from changes in renal signals (presumably hormonal) sent to the heart – that is, reflecting communication between the two developing systems.

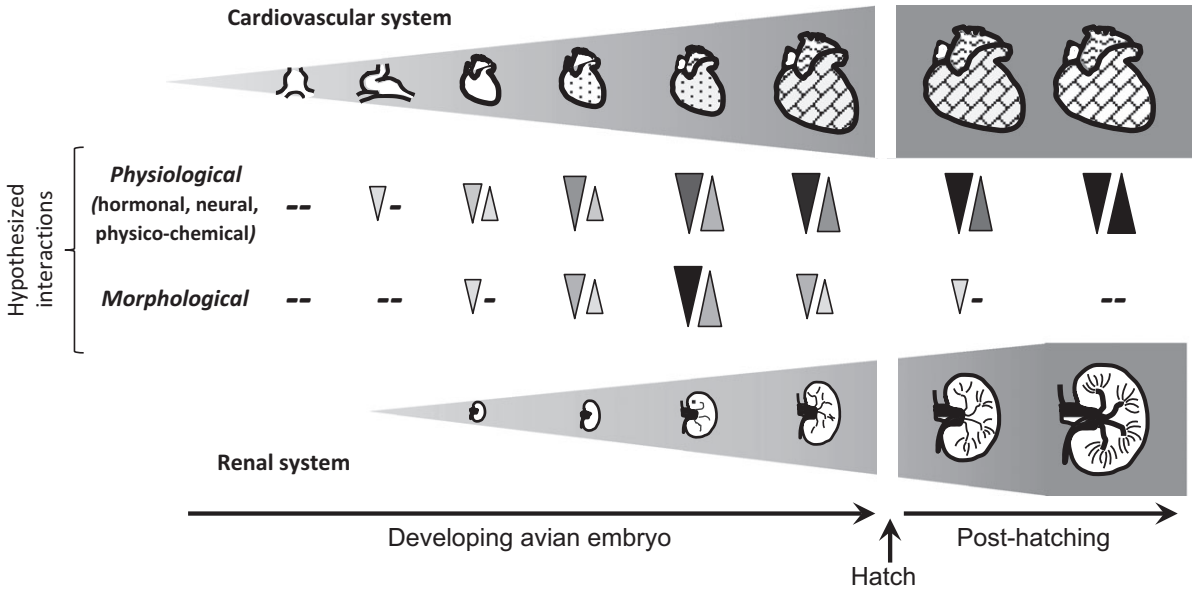

Figure 8 Hypothesized ontogenetic interactions between the cardiovascular and renal systems across avian embryonic development. Shading and size of the arrow heads represent the extent and strength of interactions between distinct systems. The cardiovascular system develops before the renal system, and renal development actually continues beyond hatching. Consequently, early developmental interactions are assumed to be primarily in the direction of cardiovascular to renal system. As the renal system develops, it will begin to influence the cardiovascular system, completing a two way 'crosstalk' between the systems that will mature as development proceeds. While physiological intersystem interactions will continue beyond development, morphological influences may only occur within a particular 'critical window' for development.

To fully understand the onset and ontogenetic changes in cardiovascular–renal interactions, we also must search for and identify 'critical windows' during development. Critical windows are those specific periods during development when a tissue or system is most sensitive to perturbation (e.g. Burggren & Fritsche 1995, Burggren 1998, Burggren & Reyna 2011). Critical windows have been employed within the framework of the development of individual organs and systems, but we can also apply this concept to cardiovascular–renal interactions. This will allow us not only to address whether intersystem communication occurs during development but also to determine when and for how long it is most important.

Cardiovascular–digestive system interactions

Comparative studies on autonomic control of the cardiovascular system have emphasized the role of adrenergic and cholinergic system as well as endothelial function, while the role of alternative factors has received considerably less attention. Metabolic rate increases during digestion in most vertebrates due to digestive and anabolic processes, and the increased oxygen demand is met by an increase in both heart rate and stroke volume (e.g. Secor *et al.* 2000), as well as redistribution of flows to the gastrointestinal organs (Farrell *et al.* 2001). This regulation is governed by the ANS, primarily by release of parasympathetic tone on the heart. However, in addition to autonomic regulation, a non-cholinergic-non-adrenergic (NANC) factor acts to stimulate heart rate during digestion in snakes (Wang *et al.* 2001, Skovgaard *et al.* 2009, Enok *et al.* 2012). There also seems to be a NANC factor that stimulates the heart during digestion in frogs and humans with transplanted hearts continue to increase heart rate in response to feeding despite the lack of cardiac innervation (Waaler *et al.* 2002). This leads to the question:

What is the importance and mechanisms behind NANC cardiovascular regulation during digestion?

One possible NANC factor is histamine, which has a direct chronotropic effect on heart rate during the initial phase of digestion in pythons (Skovgaard *et al.* 2009). Mast cells are a major store of histamine in vertebrates, and mast cells are distributed throughout the body, including cardiac tissues, and provide a likely site of release. However, other NANC factors may also contribute.

Synthesis and conclusions

Comparative cardiovascular physiology has never been at a more exciting position in the physiological sciences. New and additional conceptual frameworks, such as 'evo-devo' and comparative developmental physiology (Burggren & Crossley 2002, Burggren & Warburton 2005, Warburton *et al.* 2006), have helped define our current view of the evolution and function of vertebrate cardiovascular systems. The expansion of additional animal models, along with the continuing exploitation of existing models (Burggren 2000, Burggren & Warburton 2007), has extended our experimental reach. Experimental paradigms involving phylogenetic, mathematical and developmental strategies (Hicks & Wang 2012) are accelerating our acquisition of new understanding about how cardiovascular systems evolved, and how they currently function. Further accelerating our progress is the wide availability of expanded tools spanning from molecular levels (e.g. Moorman & Christoffels 2003, Christoffels *et al.* 2010, Jensen *et al.* 2013a,b) to whole animal levels (e.g. nuclear magnetic resonance, echocardiography and other imaging technologies (Jensen *et al.* 2010b).

In this essay, we have not tried to present an inclusive list of promising areas for future comparative cardiovascular research. Rather, we have each tried to shed light on some of the key, unanswered questions and unmet challenges in cardiovascular biology as we see them through our own lenses. We have additionally attempted to indicate how a comparative approach can help advance not just our basic understanding, but can lead to novel insights into clinically relevant malformations and cardiac anomalies, as well. This is, after all, the very approach that would be advocated by Kjell Johansen – Viking and Physiologist.

Conflict of interest

None.

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