Reptiles as a Model System to Study Heart Development

Bjarke Jensen and Vincent M. Christoffels

Department of Medical Biology, Amsterdam Cardiovascular Sciences, University of Amsterdam, Amsterdam UMC 1105AZ, Amsterdam, The Netherlands

Correspondence: b.jensen@amsterdamumc.nl

A chambered heart is common to all vertebrates, but reptiles show unparalleled variation in ventricular septation, ranging from almost absent in tuataras to full in crocodilians. Because mammals and birds evolved independently from reptile lineages, studies on reptile development may yield insight into the evolution and development of the full ventricular septum. Compared with reptiles, mammals and birds have evolved several other adaptations, including compact chamber walls and a specialized conduction system. These adaptations appear to have evolved from precursor structures that can be studied in present-day reptiles. The increase in the number of studies on reptile heart development has been greatly facilitated by sequencing of several genomes and the availability of good staging systems. Here, we place reptiles in their phylogenetic context with a focus on features that are primitive when compared with the homologous features of mammals. Further, an outline of major developmental events is given, and variation between reptile species is discussed.

Reptiles evolved from amphibians and gave rise to mammals and birds independently (Fig. 1; Warren et al. 2008; Zhang et al. 2014). This central position in the phylogeny of land-living vertebrates makes reptiles very attractive in evolutionary studies; they feature prominently in comparative anatomical and physiological works (Randall et al. 2002; Kardong 2006). Studies on (heart) development of reptiles, however, are few when compared with studies on development of zebrafish, *Xenopus* frogs, chicken, and mouse (Koshiba-Takeuchi et al. 2009; Jensen et al. 2013a; Poelmann et al. 2017). The paucity of studies likely reflects that reptiles have some internal development (until the beginning of heart chamber development in many species,

see below) and they have less anatomical and physiological properties in common with human as compared with other animal models such as mouse. From the perspective of heart development, reptiles are very attractive nonetheless, because they show more variation in ventricular septation than any other vertebrate class and they may suffer from ventricular septal defects (Brockman and Kennedy 1962; Sklansky et al. 2001; Jensen and Wang 2009). In human, congenital defects to the ventricular septum can have profound impact on ventricular function (Hoffman and Kaplan 2002). Nevertheless, much remains unknown of the development of the ventricular septum (Bruneau 2008; Bellmann et al. 2016). Any line of evidence

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Figure 1. Phylogeny of extant tetrapod vertebrates. Amniotes evolved from amphibians. These stem amniotes are likely to have been ectothermic. Their ventricles are likely to have had a partial septum, like the muscular ridge of extant reptiles, because this septum arise from the gestational incorporation of the outflow tract, which happens in all amniotes except amphibians. From these stem amniotes evolved the synapsid lineage, which gave rise to mammals, and the sauropsid lineage, which gave rise to reptiles (ectothermic sauropsids), and later birds (endothermic sauropsids). Endothermy evolved independently in mammals and birds, which is reflected in the convergent evolution of compact chamber walls and specialized conduction systems. The phylogenetic tree is based on Green et al. (2014). (All photos are reprinted from Wikipedia courtesy of Creative Commons Licensing.)

that can improve our understanding of septation is, therefore, important with respect to modeling human septal defects.

WHAT ARE REPTILES?

The extant reptiles comprise some 10,000 species that are grouped into the crocodilians, turtles, lizards, and snakes (Squamata), and tuataras (lizard-like nonlizards) (Fig. 1; Kardong 2006; Green et al. 2014). From the point of view of cladistics, “reptiles” also comprise birds (Aves) in the clade Sauropsida (Fig. 1; Kardong 2006; Zhang et al. 2014). Birds, however, are endothermic, or warm-blooded, as they have much higher metabolic rates than reptiles (Geiser et al. 2017) and their hearts have multiple anatomical and physiological specializations (Smith et al. 2000; Kroneman et al. 2019). Herein, “reptiles” will be used to mean ectothermic sauropsids. Reptiles and their descendant mammals and birds can lay their eggs out of the water, in contrast to amphibians, because of the amniotic membrane and a sturdy egg shell (Kardong 2006) (only monotreme mammals lay eggs, and in some reptiles all development of the egg occurs inside the female, a phenomenon known as ovoviviparity). In the heart, the most pronounced difference between amphibians and reptiles is that the myocardial outflow tract, or conus, persists in the formed hearts of amphibians, whereas it is incorporated into the ventricle of reptiles, birds, and mammals during development (Greil 1903; Jensen et al. 2013a). The incorporation of the myocardial outflow tract into the ventricle is associated with the formation of the partial ventricular septum known as the muscular ridge, from which most of the ventral part of the full ventricular septum seems to have evolved (Greil 1903; Jensen et al. 2013a; Poelmann et al. 2014).

Reptiles are poikilothermic ectotherms, meaning that their body temperature follows that of the environment (Tattersall 2016). In contrast, almost all mammals and birds are homeothermic endotherms, meaning that their body temperature is constant across a temperature range (the thermoneutral zone). The metabolic rate of reptiles is then greatly influenced

by the environmental temperature, level of activity, reproductive cycle, state of digestion, etc. (Randall et al. 2002). Metabolic rate relies on adequate blood perfusion of the tissues (Crossley et al. 2016). Accordingly, cardiac output, the volume of blood pumped by the heart per minute, is tightly related to metabolic rate. Because change in heart rate is the major determinant of cardiac output in reptiles, like it is in mammals and birds, heart rate is a proxy measure for metabolic rate (Lillywhite et al. 1999; Du et al. 2010) and, in effect, rate of development (Du et al. 2011). Nevertheless, even if the body temperature of reptiles is raised to 35°C–40°C, their metabolic rate will still be some 5–10 times lower than that of equally warm mammals and birds (Else and Hulbert 1987; Randall et al. 2002).

WHY STUDY REPTILES?

In what is now known as the August Krogh principle, it is stated that for “a large number of problems, there will be some animal of choice or a few such animals on which it can be most conveniently studied” (Krogh 1929). Convenience can be a favorable combination of the ease of experimentation and the presence of intriguing biological attributes. Among the intriguing biological attributes of reptiles, we find the ability to instantaneously lose and regenerate a large body part such as the tail in geckos (Sanggaard et al. 2012), to digest huge meals during which metabolic rate increases above that of exercise (Secor and Diamond 1998), and to produce potent venom (Vonk et al. 2013). Concerning the heart, within reptiles there is an unparalleled variation in ventricular septation (Jensen et al. 2014). For example, there are two instances where septation has evolved to such an extent that the ventricle is functionally separated into a low-pressure right side and a high-pressure left side, namely, in monitor lizards and python snakes (Burggren and Johansen 1982; Wang et al. 2003). In these animals, there is a small luminal continuity between the left and right side (which closes in systole) and some mixing of bloodstreams, or shunting, is inevitable (Ishimatsu et al. 1988; Jensen et al. 2010). Although the variation in

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ventricular septation makes the hearts of reptiles particularly interesting, reptilian heart development offers insight into the evolution of a number of important processes. For instance, the pattern of electrical activation of the chambers, the cardiac conduction system, atrial septation, the atrioventricular valve apparatus of four-chambered hearts, chamber walls of predominantly compact myocardium, and outflow tract septation.

CHAMBERS OF REPTILE HEARTS

The formed reptile heart has two atria and one ventricle (Goodrich 1930; Benninghoff 1933; Holmes 1975; Webb 1979). Only crocodilians have a full ventricular septum and two ventricles (Webb 1979; Farrell et al. 1998; Cook et al. 2017). An additional chamber is found upstream of the right atrium, namely, the sinus venosus (SV) (Keith and Flack 1907). The electrocardiogram (ECG) of reptiles consequently reveals three distinct chamber activations; the SV wave followed by a sinuatrial delay, P wave (atria), followed by an atrioventricular delay, and then the QRS complex followed by the T wave (Mullen 1967; Valentinuzzi and Hoff 1972). Facilitated by the sinuatrial delay in electrical activation, the SV activates and contracts before the atria and thus functions as a separate chamber (Jensen et al. 2017b). In mammals, the embryo develops an SV, which later becomes the caval vein myocardium that activates together with the atrial myocardium (Spach et al. 1972; Opthof 1988; Steding et al. 1990; Faber et al. 2019). The walls of the atrial and ventricular chambers of reptiles are readily distinguishable from the chamber walls of mammals and birds by their very extensive layer of fine trabecular muscle (Jensen et al. 2016a, 2018; Boukens et al. 2019; Kroneman et al. 2019). Because the chamber walls are largely trabecular, much of the myocardium can be nourished by the blood of the chamber lumens (Ostadal et al. 1999; Sedmera and Ostadal 2012). The luminal nourishment is likely the cause of the significant tolerance of reptiles to ischemia arising from coronary artery ligation (Kohmoto et al. 1997; Hagensen et al. 2008).

VESSELS OF REPTILE HEARTS

The pulmonary circulation converges to a single pulmonary vein connecting to the left atrium (Jensen et al. 2014). There is little or no myocardium in the pulmonary venous walls (Joyce et al. 2019). Most mammals and all birds have myocardium in the intrapericardial part of the pulmonary veins (Nathan and Gloobe 1970; Rowlatt 1990; Kroneman et al. 2019). In reptiles, there are three caval veins connected to the right atrium as is the case in most tetrapods (Benninghoff 1933; Farrell et al. 1998). These veins, however, have myocardial walls (extending to the pericardial reflection) and are also referred to as the SV (Jensen et al. 2017b). There is a single pulmonary artery and two aortas. Each of the three arteries is guarded by a bicuspid valve (Poelmann et al. 2017). The coronary circulation begins in the valvular sinuses of either aorta, or both aortas spreading throughout the entire heart and the coronary veins connect to the SV (Grant and Regnier 1926; MacKinnon and Heatwole 1981; Jensen et al. 2016b).

VALVES OF REPTILE HEARTS

The entry of the SV to the right atrium is regulated by the myocardial sinuatrial valve, which has two leaflets (Keith and Flack 1907; Faber et al. 2019). Between the atria and the ventricle(s), there is a single atrioventricular junction divided into left and right halves by the atrial septum (Benninghoff 1933; Webb 1979; Farrell et al. 1998). The atrial septum ends in a membranous parachute-shaped atrioventricular valve. It has free margins laterally and is attached to atrioventricular muscle dorsally and ventrally. Small cushions can be found opposite the free margins. Morphologically, the atrioventricular junction of (noncrocodilian) reptiles resembles a mammal atrioventricular junction with a very large membranous septal defect and with very poorly developed parietal leaflets. In crocodilians, the atrioventricular valve apparatus resembles that of birds and monotreme mammals (Van Mierop and Kutsche 1985b; Jensen et al. 2013a).

FUNCTION OF REPTILE HEARTS (VENTRICLES)

The low metabolic rate of reptiles compared with mammals and birds is reflected in their heart rates, which are several-fold lower (Lillywhite et al. 1999; Boukens et al. 2019). Also, the ventricular stroke volume is smaller, under 1 mL $\times$ kg⁻¹ body weight, compared with the $\sim$ 1 mL $\times$ kg⁻¹ body weight in mammals and 1.4 mL $\times$ kg⁻¹ body weight in birds (Seymour and Blaylock 2000; Jensen et al. 2014). Further, mean arterial systemic blood pressure is low, often some 30–40 mmHg (Burggren et al. 1998; Jensen et al. 2014), much lower than the $\sim$ 90 mmHg observed in most mammals (Poulsen et al. 2018). Therefore, a reptile ventricle performs less work per beat and the heart weight to body weight ratio is approximately one-half that of mammals, or one-quarter percent of body weight (Seymour 1987; Seymour and Blaylock 2000; Jensen et al. 2014). Hence, the work performed per beat per gram ventricle is approximately the same in ectotherms and endotherms (Hillman and Hedrick 2015). Because the (noncrocodilian) ventricle does not have a full septum, blood will preferentially be ejected to the pulmonary circulation or the systemic circulation dependent on the relative resistance of the two circulations (Hicks and Wang 2012; Joyce et al. 2016). The relative resistances are strongly influenced by the autonomic nervous system (Hicks and Wang 2012; Leite et al. 2013; Taylor et al. 2014).

THE NONCROCODILIAN REPTILE HEART LIKELY RESEMBLES THE ANCESTRAL MAMMAL HEART

That reptile hearts share some features with the (developing) hearts of mammals provides rationale for use of reptiles as a model organism of heart development. Within comparative anatomy, shared features have been recognized over many centuries (Gegenbaur 1878; Keith and Flack 1907; Goodrich 1930; Benninghoff 1933; Harvey 1995). Before homeothermic endothermy evolved within the mammal lineage, these protomammals likely had a heart quite like

those of squamate reptiles (Jensen et al. 2019). This conjecture is based on four observations. First, the sinuatrial valve is found in amphibians, reptiles, birds, and monotremes (Benninghoff 1933; Jensen et al. 2017b; Kroneman et al. 2019). Second, amphibians, reptiles, birds, monotremes, and marsupials develop their atrial septum from the primary septum only (Röse 1889, 1890; Runciman et al. 1995; Jensen et al. 2019). Third, only in the development of reptiles, birds, and mammals will the myocardial outflow tract incorporate to the ventricle (Greil 1903; Goor et al. 1972; de la Cruz et al. 1977; Zaffran et al. 2004; Rana et al. 2007; Grimes and Kirby 2009). Fourth, the atrial and ventricular chambers are much more trabecular, or spongy, in ectotherms (Benninghoff 1933; Van Mierop and Kutsche 1984; Boukens et al. 2019). All four features only occur in reptile hearts.

REPTILE GENOMES

Sequencing of genomes allows for the direct comparison of DNA of different species (Tollis et al. 2014) and it facilitates, for instance, the development of messenger RNA (mRNA) probes for the detection of transcripts by in situ hybridization (Koshiba-Takeuchi et al. 2009; Jensen et al. 2012; Poelmann et al. 2014) and the analysis of evolutionary conservation of gene regulatory elements (Kvon et al. 2016). Several genomes have been sequenced of reptiles that, from the perspective of variation in cardiac (mainly ventricular) anatomy and physiology, represent typical or specialized features. This includes the green anole (Alföldi et al. 2011) (typical lizard ventricle [Jensen et al. 2014]), the central bearded dragon (Georges et al. 2015) (typical lizard ventricle [Hanemaaijer et al. 2019]), the garter snake (Perry et al. 2018) (typical snake ventricle [Jensen et al. 2014]), the painted turtle (Badenhorst et al. 2015) (typical turtle ventricle, personal observation), the Burmese python (Castoe et al. 2011; Duan et al. 2017) (functionally divided ventricle, unique trait of pythons among snakes [Jensen et al. 2014]), the Komodo dragon (Lind et al. 2019) (functionally divided ventricle, unique trait of monitor lizards among lizards [Jensen et al. 2014]), and the American

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alligator (Green et al. 2014) (fully divided ventricle [Jensen et al. 2014]).

STAGING OF DEVELOPING REPTILES

Organs develop in different rates between species (de Bakker et al. 2016) and although the early development of vertebrate hearts can be quite similar, the timing of the completion of the primary atrial septum, for example, may differ between species (Sissman 1970). A standardized staging of the developmental stages is then important. The early stages of reptile (heart) development can be staged much like chicken is staged (Hamburger and Hamilton 1992; Poelmann et al. 2017). Ontogeny, however, does not fully recapitulate phylogeny (Richardson 1999) and staging systems have been developed for multiple species of reptiles, including for crocodilians (Ferguson 1985), the green anole (Sanger et al. 2008), the common garter snake (Zehr 1962), the African rock python (Boughner et al. 2007), the African house snake (Boback et al. 2012), the striped sand snake (Khan Noon and Zahradnick 2017), the spiny softshell turtle (Greenbaum and Carr 2002), the common snapping turtle (Yntema 1968), the red-bellied short-necked turtle (Werneburg et al. 2009), the leopard gecko (Wise et al. 2009), and the mangrove monitor (Gregorovicova et al. 2012). In many species of lizards and snakes, there is substantial internal development before the eggs are laid. Stages younger than equivalents of Hamburger–Hamilton stage 15 of chicken (initial chamber development) have to be removed from the pregnant females (Zehr 1962). Embryos of crocodilians have between 9 and 20 pairs of somites when the eggs are laid (Ferguson 1985), by which measure they equal chicken Hamburger–Hamilton stage 10–13 (initial looping of the heart tube). The eggs of the common snapping turtle and the spotted turtle are laid when the embryos are very immature and most stages of heart development can be studied (Agassiz 1857; Yntema 1968). In the spiny softshell turtle and red-bellied short-necked turtle, the eggs are laid around the time of looping of the heart tube (Greenbaum and Carr 2002; Werneburg et al. 2009).

REPTILE HEART DEVELOPMENT

The early embryonic reptile heart is a tube that subsequently loops, like it does in most vertebrates (Agassiz 1857; Greil 1903; Ferguson 1985; Crossley and Burggren 2009; Bertens et al. 2010; Jensen et al. 2013a; Manner and Yelbuz 2019). The atrial and ventricular compartment then balloon out from the heart tube and the formation of trabeculae begin simultaneously (Fig. 2A; Moorman and Christoffels 2003). Two non-chamber parts, the atrioventricular canal and the myocardial outflow tract, are proportionally large (Fig. 2A). The development of the SV is revealed as myocardialization of the intrapericardial walls of the cardinal veins (Jensen et al. 2017b). Total myocardial mass increases throughout development because the ventricular and atrial compartments grow rapidly (Fig. 2B). In the formed heart, ~80% of the mass is ventricular and ~15% of the mass is atrial, which resembles the situation in many other vertebrates (Jensen et al. 2013a). The atrioventricular canal and the myocardial outflow tract, in contrast, are proportionally smaller over time because of low levels of proliferation (Fig. 2B; Jensen et al. 2012, 2013a). Further, the myocardial outflow tract becomes incorporated to the ventricle whereby it comes much shorter (Greil 1903; Bertens et al. 2010; Jensen et al. 2013a). In mammals and birds, similar developmental changes happen to the outflow tract (Goor et al. 1972; de la Cruz et al. 1977; Zaffran et al. 2004; Rana et al. 2007; Grimes and Kirby 2009).

Heart development in vertebrates rely on a core network of transcription factors that is evolutionarily conserved (Olson 2006; Kokubo et al. 2010; Bakkers 2011; Kelly et al. 2014; Diogo et al. 2015; Poelmann and Gittenberger-de Groot 2019). Transcription factors such as *Tbx20*, *Tbx5*, and *Nkx2.5* are required for the formation of chambers in mouse (Bruneau et al. 1999; Kraus et al. 2001; Stennard et al. 2003) and are broadly expressed in the heart of reptiles (Fig. 3; Jensen et al. 2017b). Genes that are chamber specific in mammals, such as *Nppa*, *Gja5*, and *Bmp10*, are also restricted to chambers in reptiles (Koshiba-Takeuchi et al. 2009; Jensen et al. 2018). The formation of junctions, such as the

Figure 2. Morphological heart development of the corn snake. (A) At 2 days postoviposition, the heart tube has just begun to develop the common atrium (Atr) and the single ventricle (Ven). The atrioventricular canal (AVC) and the myocardial outflow tract (OFT) are proportionally large compared with later stages. By 10 days postoviposition, the left and right atrium can be seen (LA and RA, respectively), the ventricle is proportionally quite large, and the myocardial OFT is substantially shorter (because of it being incorporated into the ventricle). By 20 days postoviposition, the proportions of the heart are almost mature. (B) The volumetric and proportional changes of the major compartments of the corn snake heart in development. By 20 days postoviposition, the heart still has to grow much more (*upper graph*), but the proportions are almost the same as those of older hearts. (C) Internal morphology of the developing ventricle, showing the relation between the AVC and the bulboventricular fold (orange dot, 2 and 10 days) and later the muscular ridge (orange dot, 42 days). Notice that it is only in late stages (42 days) that some of the ventricular trabeculae (vertical septum [VS]) are much closer to the AVC than the neighboring trabeculae. (SV) sinus venosus, (d) days. (All images in Fig. 2 are reprinted from Jensen et al. 2013a under the terms of the Creative Commons Attribution License for open-access articles.)

atrioventricular junction, relies on repression of the chamber program mediated by transcription factors such as *Tbx2* and *Tbx3* and growth factors such as *Bmp2* (Yamada et al. 2000; Habets et al. 2002), which are also expressed in reptile hearts (Jensen et al. 2012). Figure 3 shows key elements of this network, the genes of which have been investigated in reptiles (text in red). The sinuatrial junction, which harbors the dom-

inant pacemaker, expresses *Tbx3* and *Bmp2* together with *Isl1*, a transcription factor that acts upstream of *Tbx3* (Hoffmann et al. 2013; Vedantham et al. 2015; Burkhard et al. 2017; Jensen et al. 2017b; van Eif et al. 2018). Whereas *Isl1* is expressed in a ring-like domain in reptiles and fish (Tessadori et al. 2012; Jensen et al. 2017b), it is confined to a small subset of the sinuatrial junction in adult mammals and birds, which,

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Figure 3. Molecular mechanisms that likely underlie reptile atrioventricular canal development. The gene-network diagram is based on studies in mouse and shows the interaction of key genes of heart development. (Diagram adapted from van Eif et al. 2018.) Genes that have been studied in reptiles, principally by in situ hybridization and examples of which are shown on the right (in late gestational anole lizard hearts), are shown in red text. The images of the sections stained with in situ hybridization are created from data in Jensen et al. (2017b) (top row) and Jensen et al. (2012) (bottom row). (RA) right atrium, (SV) sinus venosus, (Ven) ventricle, (LA) left atrium. Scale bars, 100 μm.

at least in birds, has a highly variable anatomy (Kroneman et al. 2019). One outcome of chamber repression is low expression levels of gap junction proteins such as *Gja5* and ion channels such as *Scn5a*, both of which promote fast electrical activation (Delorme et al. 1995; Remme et al. 2009; Park and Fishman 2017), and these transcripts are not detected in the atrioventricular canal of reptiles (Jensen et al. 2012, 2017b, 2018). In contrast, these genes are enriched in the chambers, including the SV. This patterning of the heart imposes the electrical conduction properties that are revealed by the ECG (Boukens and Christoffels 2012), namely, fast activation of the chambers (SV, P, and QRS deflections) and slow propagation in the chamber junctions (SV-P and PR delays).

In the atrioventricular canal, valve formation initiates from a dorsal and a ventral cushion and two much smaller cushions laterally (Greil 1903; Bertens et al. 2010; Jensen et al. 2013a). Before the completion of the atrial septum, the dorsal and ventral cushions will have merged. They will give rise to the parachute-like atrioventricular valve. Only in crocodilians, which have a full ventricular septum, will the lateral cushions

give rise to significant parietal leaflets (Van Mierop and Kutsche 1985b; Cook et al. 2017). In crocodilians, birds, and mammals, the left and right atrioventricular junction have a similar offset and they all develop a membranous septum (Greil 1903; Shaner 1962; Cook et al. 2017). In the American alligator, the membranous septum acquires some myocardium and in birds the membranous septum is completely myocardialized (Jensen et al. 2018; Kroneman et al. 2019).

Although the chamber walls of reptiles are highly trabecular, the reptile ventricle has a thin outer layer of compact muscle that expresses *Hey2* and thus resembles the compact wall of the embryonic mouse heart (Koibuchi and Chin 2007; Stefanovic et al. 2014; Jensen et al. 2018). In *Hey2* mutant mice, the trabecular marker *Nppa* becomes ectopically expressed in the compact wall (Koibuchi and Chin 2007). In the American alligator, the compact wall expresses *Hey2*, whereas *Nppa* is confined to the trabecular layer (Jensen et al. 2018). Additionally, the embryonic trabecular wall of both reptiles and mammals expresses *Bmp10* (Koshiba-Takeuchi et al. 2009) and *Gja5* (Jensen et al.

2012, 2018). The embryonic ventricular (and atrial) walls are highly similar in reptiles and mammals; however, a key difference is whether the trabecular component is the most proliferative (leading to a reptile-like ventricle) or the compact component is the most proliferative (leading to a mammal-like ventricle) (Benninghoff 1933). The endocardium has important roles in regulating the contributions of the trabecular and compact components (D'Amato et al. 2016; Del Monte-Nieto et al. 2018). A recent systemic endothelial knockout of the chromatin remodeler *Ino80* (*Tie2Cre; Ino80 fl/fl*) in mouse resulted in arguably the most reptile-like ventricular wall of any mouse model (Rhee et al. 2018). In the human heart, an excessive amount of trabecular myocardium allows for the diagnosis of noncompaction cardiomyopathy (Finsterer et al. 2017; Towbin and Jefferies 2017). This lesion has been considered a persistence of the evolutionarily old wall architecture (Angelini et al. 1999; Freedom et al. 2005). In contrast to reptile trabeculae, the trabeculae during noncompaction do not express the trabecular markers CX40 (*GJA5*) and ANF (*NPPA*); the trabeculae are much fewer, orders of magnitude larger, and are vascularized by the coronary circulation (Jensen et al. 2016a, 2017a). Noncompaction may then be better defined as an aberration of normal mammalian cardiogenesis (Anderson et al. 2017).

CARDIOMYOCYTES

The cardiomyocytes are only ~5–10 μm wide in reptiles, other ectotherms, and birds. In mammals, the ventricular cardiomyocytes in particular are much wider by comparison, ~25 μm (Hirakow 1970; Shiels and White 2008; Hirose et al. 2019). Simulations suggest that the electrical propagation through wider mammalian cardiomyocytes will be faster than through narrower reptile-like cardiomyocytes (Jensen et al. 2013b), consistent with the relatively fast chamber activations in mammals (Boukens et al. 2019). The size difference also yields a comparatively low surface-to-volume ratio in mammals, which is partly compensated for by a comparatively elaborate T-tubule system (Hirakow 1970;

Shiels and Galli 2014). In reptiles, the excitation–contraction coupling relies to a great extent on extracellular calcium ions, whereas in endotherms more calcium comes from intracellular stores (Bers 2002; Shiels and Galli 2014).

Cardiomyocyte proliferation underlies the formation of chambers from the embryonic heart tube (Sedmera et al. 2003). Cell-cycle activity and possibly proliferation of heart cells has been assessed by the detection of BrdU of pulse-labeled nuclei, or by the use of antibodies against PCNA and pHH3 located in the nucleus (Riquelme et al. 2011; Jensen et al. 2013a; Jacyniak and Vickaryous 2018). That said, it is difficult to assign correctly a cell cycle-marker-positive nucleus to its native cytoplasm whereby cardiomyocyte identity can be established by the detection of sarcomeric proteins (Ang et al. 2010; Leone et al. 2015). In reptiles, this difficulty is likely aggravated by the small size of the cardiomyocytes.

SEPTATION OF THE REPTILE HEART

Septa develop in the atrium, ventricle, and outflow tract. Atrial septation is initially revealed as a ridge of mesenchyme in the atrial roof (Bertens et al. 2010; Jensen et al. 2019). The myocardial atrial septum then grows as a sheet from the roof and it carries on its leading edge the ridge of mesenchyme as a cap. This mesenchymal cap will eventually merge with the atrioventricular cushions and a dorsal protrusion of *Isl1*-expressing mesocardium, also known as the vestibular spine (Jensen et al. 2019). Although this merger of mesenchymal tissues closes the primary foramen, secondary perforations develop in the cranial part of the atrial septum that allow oxygen-rich blood to reach the left atrium. After hatching, the perforations are likely closed by growth of the surrounding tissues (myocardium, endocardium, fibroblasts). Reptiles do not form a secondary foramen or a secondary septum as seen in placental mammals (Röse 1889, 1890; Jensen et al. 2019). Nonetheless, many reptiles have a large aggregate of trabeculae in the same position as the *crista dividens* of the secondary septum, which becomes the superior rim of the oval fossa after birth (Jensen et al.

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2019). The inferior rim of the oval fossa consists of myocardialized mesenchyme of the mesenchymal cap and the dorsal mesenchymal protrusion. In reptiles, the dorsal mesenchymal protrusion has some capacity to myocardialize (myocardialization also happens in the contiguous sinus septum) and, at least in monitor lizards, the mesenchymal cap also develops myocardium (Jensen et al. 2019). The most pronounced difference between reptiles (and non-placental mammals) and placental mammals with regard to the secondary septum, then, may be the extent of its growth.

The formed reptile ventricle may be viewed as having three partial septa, which only become apparent well after chamber formation has begun (Webb et al. 1971; Farrell et al. 1998; Jensen et al. 2014). One septum is the so-called vertical septum, which is the highest sheet-like aggregate of trabecular myocardium beneath the atrioventricular valve (Webb et al. 1971; Jensen et al. 2014). Early anatomical works suggested it is homologous with the full ventricular septum of mammals and birds (Greil 1903; Robertson 1913). In monitor lizards, the vertical septum is particularly high and *Tbx3* and *Tbx5* are more expressed in its vicinity (Hanemaaijer et al. 2019). This setting resembles the crest of the ventricular septum of mammals (Moskowitz et al. 2004; Bakker et al. 2008). In mammals and chicken, *Tbx5* is expressed in a left-right gradient over the ventricular septum and, in mouse, the absence of *Tbx5* results in septal defects (Bruneau et al. 1999, 2001; Koshiba-Takeuchi et al. 2009; Katano et al. 2019). A gradient of *Tbx5* was described near the vertical septum of the turtle *Trachemys* (Koshiba-Takeuchi et al. 2009). However, the turtle *Pelodiscus* has the gradient over the muscular ridge where it also is in squamates (Fig. 4A; Poelmann et al. 2014). Nonetheless, the original model (Koshiba-Takeuchi et al. 2009) appears valid for animals with a full ventricular septum because *Tbx5* expression in the American alligator is confined to the left ventricle with a gradient over the ventricular septum (Fig. 4B; Jensen et al. 2018). The gradient of *Tbx5* on the muscular ridge results from its development at the boundary between the embryonic ventricle (that expresses *Tbx5*)

and the myocardial outflow tract (Fig. 4A). Opposite to the muscular ridge is the third septum, the bulbuslamelle (Brücke 1852; Greil 1903; Jensen et al. 2014). Like the muscular ridge, it also develops at the boundary of the ventricle and the outflow tract and it too has a gradient of *Tbx5* (Fig. 4A).

The full ventricular septum of crocodilians propagates the activating current toward the apical region in a manner that closely resembles the propagation in the ventricular septum of birds and mammals (Fig. 5; Christian and Grigg 1999; Sedmera 2011; Jensen et al. 2018). The ventricular septum receives a dorsal extension of atrioventricular canal-like myocardium (Greil 1903; Swett 1923), which expresses *Tbx3* and the cutting of which leads to atrioventricular blockade (Fig. 4C,D; Jensen et al. 2018). In monitor lizards, the apical region near the vertical septum activates early (Hanemaaijer et al. 2019); in other reptiles, such as the green anole, leopard gecko, corn snake, the central bearded dragon, and turtles, the electrical propagation occurs in a primitive pattern from base to apex and atrioventricular conduction is not confined to the dorsal part like in crocodilians, mammals, and birds (Fig. 5; Mines 1913; Jensen et al. 2012; Gregorovicova et al. 2018). HNK-1, a marker of the atrioventricular conduction axis in human, chicken, and quail, also identifies the atrioventricular conduction axis in crocodilians (Wessels et al. 1992; Anderson et al. 2018; Kvasilova et al. 2019). Crocodilians appear to lack the equivalent of mammalian bundle branches and Purkinje system and the ventricular activation is long like it is in other reptiles (Fig. 5; Zhao-Xian et al. 1991; Heaton-Jones and King 1994; Jensen et al. 2018; Boukens et al. 2019).

Septation of the outflow tract has received comparatively less attention than the atrial and ventricular septation (Shaner 1962; Hart 1968, 1969; Poelmann et al. 2017). Like in mammals and birds, the wall of the early outflow tract is composed of myocardium up to the pericardial reflection and its inside is almost filled with cardiac jelly (Jensen et al. 2013a; Poelmann et al. 2017). There is only a single narrow lumen. This lumen becomes divided by two opposing mesenchymal ridges that merge. The divided lumen

Figure 4. Septum development. (A) In the developing lizard brown anole (Sanger stage 9), *Tbx5* is expressed in the ventricle but not in the outflow tract (OFT). As the OFT is being incorporated into the ventricle, two septa can be identified by a gradient of *Tbx5*, namely, the muscular ridge (MR) and the bulbus lamelle (BL). (Panel adapted from Hanemaaijer et al. 2019 with permission from Company of Biologists © 2019.) (B) In the developing American alligator (Ferguson stage 18), *Tbx5* is expressed in the left ventricle and much less so in the right ventricle, similar to chicken and mouse (Koshiba-Takeuchi et al. 2009). (C,D) In American alligators, the atria are in contact with the ventricular septum by a dorsal myocardial pathway that expresses *Tbx3*, much like the atrioventricular bundle of the cardiac conduction system of mammals and birds (Hoogaars et al. 2004). (LV) left ventricle, (RV) right ventricle, (LA) left atrium, (RA) right atrium. (The images in B–D' are reprinted from Jensen et al. 2018 under the terms of the Creative Commons Attribution License for open-access articles.)

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Figure 5. Optical mapping of the electrical activation of the ventricle of reptiles. In the developing corn snake, the ventricular activation initiates in the ventricular base, near the atrioventricular canal (AVC), similar to the activation pattern in other developing and adult squamate reptiles. In the early stages (here exemplified by 16 dpo), the ventricle is broad and the activation front sweeps rightward. In later stages (here exemplified by 32 dpo), the ventricle is elongated and the activation front again propagates from the vicinity of the AVC to the most distal parts, which are now the apex and the “shoulder” on the *left* side. The pattern of ventricular activation is then greatly influenced by the shape of the chamber. (Images in the *left* panel are adapted from Gregorovicova et al. 2018, with permission, from Company of Biologists © 2018.) In the juvenile American alligator, the ventricular activation initiates in the ventricular septum (S), which on the ventral surface, results in an apex-first activation pattern. This pattern in mammals and birds has been interpreted as resulting from a specialized conduction system. Despite the specialized manner of electrical activation, total ventricular activation as measured by the QRS duration (*bottom* graph) is slow in alligators, even at mammal-like body temperatures. (LA) left atrium, (LV) left ventricle, (OFT) outflow tract, (RA) right atrium, (RV) right ventricle, (V) single ventricle. (The images in the *right* panel are reprinted from Jensen et al. 2018 under the terms of the Creative Commons Attribution License for open-access articles.)

consists of a pulmonary channel and an aortic channel. Within the aortic channel and immediately distal to the leading edge of the primary division, an additional mesenchymal ridge divides the aortic channel into a left and a right channel (Greil 1903; Hart 1969; Jensen et al. 2013a). By these divisions, the principle layout of the pulmonary artery and the left and right aorta is established. In some snakes, the left lung is less developed than the right lung, and the left branch of the pulmonary artery develops accordingly (van Soldt et al. 2015). A contribution of neural crest cells to outflow tract septation seems likely given the presence of chondrocytes at the arterial roots (Young 1994; López et al. 2003) and the expression of the transcription factor TFAP2 α in the pharyngeal arches and

distal outflow tract (Poelmann et al. 2017). Later in development, mesenchymal-to-myocardial transformation occurs of the proximal part of the division of the pulmonary and aortic channels (Poelmann et al. 2017). This process also occurs in mammals and birds and gives rise to the so-called conal septum (van den Hoff et al. 2001; Yelbuz et al. 2003; Poelmann et al. 2017). The conal septum and the moderator band of the right ventricle resembles the muscular ridge of the reptile ventricle and they are thought to be homologous structures on the basis of anatomy and position (Gasch 1888; Van Mierop and Kutsche 1984, 1985a; Jensen et al. 2013a).

The rare human lesion of a triple outlet right ventricle resembles the reptile setting of two aortas and one pulmonary artery on the right side of

Figure 6. Remodeling (red drawings) of the ventricular base in association with full ventricular septation. The base of the single ventricle of the lizard is characterized by three arterial roots (LAo, RAo, PA) on the ventricular right. To the left of the arterial roots is the single atrioventricular canal (AVC), which is split by the atrial septum into two orifices, each guarded by a single large and medial valve (L, R). To transition in development from an embryonic lizard-like setting to the setting of mammals, the aortic channel must wedge leftward over the ventricular septum and the AVC must expand substantially, particularly on the right. (Ao) aorta, (L) left atrioventricular valve, (LAo) left aorta, (PA) pulmonary artery, (MV) mitral valve, (R) right atrioventricular valve, (RAo) right aorta, (RAVV) right atrioventricular valve. (Image based, in part, on Plate 26 of Heine 1976.)

the ventricle (Cook et al. 2017; Katewa et al. 2019). In contrast, in normal human development, the aortic root will “wedge” leftward between the pulmonary artery and the atrioventricular junction and over the ventricular septum, giving the left ventricle a dedicated outlet (Fig. 6; Asami 1969; Wessels et al. 1992; Anderson et al. 2019). Concomitantly, an atrioventricular remodeling and expansion ensures a persistent communication between the right atrium and the right ventricle (Lamers et al. 1995). It also results in large caliber atrioventricular junctions that, possibly, can better accommodate the greater blood flows associated with endothermy (Fig. 6; Jensen et al. 2013a,b). The aortic wedging and atrioventricular remodeling only occur in all animals that develop a full ventricular septum, namely, mammals, birds, and crocodilians (Hanemaaijer et al. 2019).

CONCLUDING REMARKS

The intimate phylogenetic relation between reptiles, mammals, and birds has long been recognized by comparative anatomists and physiologists and our understanding of it is now being deepened by comparisons of genomes and expression of homologous genes and proteins. The study of reptile heart development provides an important insight into evolutionary conservation, which, if present, suggests that the pro-

cess, or gene, or function in question is important. Unparalleled variation in ventricular septation means the reptiles are attractive models to study the evolutionary and developmental conditions leading to the full ventricular septum. The many similarities of the ventricular septum of crocodilians and mammals illustrate this. But the crocodilian ventricular septum also exemplifies a general challenge we face, namely, what is homology (Shubin et al. 2009)? Even if ventricular septation is by similar processes, the common ancestor of mammals and crocodilians did not have a full ventricular septum and the full septum evolved during the specific histories of mammals and crocodilians. Consequently, the ventricular septum of crocodilians shows both evolutionary conservation and divergence with regard to the ventricular septum of mammal. Ultimately, it is a fairly subjective assessment whether there is evolutionary conservation.

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