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Analysis of Cardiac Development in the Turtle *Emys orbicularis* (Testudines: Emidydae) using 3-D Computer Modeling from Histological Sections

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ABSTRACT

In this article we present a 3-D modeling study of cardiac development in the European pond turtle, *Emys orbicularis* (of the reptilian order Testudines). The study is aimed at elucidating the embryonic development of the horizontal septum in the ventricle and underscoring the importance of 3-D reconstructions in studying morphogenesis. Turtles possess one common ventricle, partly divided into three cava by a vertical and a horizontal septum, of which the embryonic origins have so far not been described. We used serial sectioning and computerized high-resolution 3-D reconstructions of different developmental stages to create a chronological overview of cardiogenesis, in order to study this process. This has yielded a new understanding of the development of the horizontal septum and (directly related) the looping of the heart tube. This looping is found to be markedly different from that in the human heart, with the turtle having two clear bends in the part of the heart tube leaving the primitive ventricle, as opposed to one in humans. It is this particular looping that is responsible for the formation of the horizontal septum. In addition to our findings on the ventricular septation this study has also yielded new insights into the developmental origins of the pulmonary vein. The 3-D reconstructions were built using our platform TDR-3-D base and enabled us to study the developmental processes in specific parts of the turtle heart separately and in three dimensions, over time. The complete 3-D reconstructions have been made available to the reader via internet using our 3-D model browser application, which allows interactive viewing of the models. The browser application can be found on bio-imaging.liacs.nl/galleries/emysorbicularis/TurtleGallery.html, along with additional images of both models and histological sections and animation sequences of the models. By allowing the reader to view the mate-

Abbreviations used: A = atrium; AA = aortic arch; ACV = anterior cardinal vein; AVC = atrioventricular canal; AVV = atrioventricular valve; CA = cavum arteriosum; CAVV = cushion tissue forming the atrioventricular valve; CP = cavum pulmonale; CCV = common cardinal vein; CV = cavum ventrale; DC = distal cushions; F = foramen; HS = horizontal septum; HSt = heart stalk; IAS = interatrial septum; IHC = inner heart curve; IVC = intraventricular canal; LAA = left aortic arch; LA = left atrium; LAVC = left atrioventricular canal; MC = mesenchymal cap; OFT = outflow tract; PA = pulmonary artery; PC = proximal cushions; PCV = posterior cardinal vein; PM = pectinate muscle; PV = pulmonary vein; RAA = right aortic

arch; RA = right atrium; SV = sinus venosus; TC = trabeculae carneae; V = ventricle; VS = vertical septum; VV = possible venous valve of the superior cardinal vein.

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rial in such an interactive way, we hope to make optimal use of the new 3-D reconstruction techniques and to engage the reader in a more direct manner. *Anat Rec*, 293:1101–1114, 2010. © 2010 Wiley-Liss, Inc.

Key words: *Emys orbicularis*; horizontal septum; cardiogenesis; heart development; TDR-3-D base; 3-D reconstruction

Many features of cardiac development in turtles correspond to those in birds and mammals (Greil, 1903; Quiring, 1933; Goodrich, 1958; Shaner, 1962; Holmes, 1976). These shared features include cardiac looping, ventricular trabeculation, formation of an inner heart curvature, and presence of a major pair of atrioventricular endocardial cushions that give rise to the atrioventricular valves. Other aspects of the turtle heart, however, differ greatly from the avian and mammalian heart. The septation of the ventricle for instance is remarkably different in the turtle and the developmental origins of this septation are still not fully understood. As opposed to the fully separated hearts of mammals and birds, the turtle heart is only partially separated by two incomplete septa. An attempt at explaining the development of these horizontal and vertical septa (see below) has been made by Holmes (1976), but no conclusive study of this process exists.

The aim of this article is twofold; the main biological purpose is to elucidate the embryonic development of the horizontal septum in the turtle, which is shown to be closely linked to the looping of the heart. To this end we present a detailed developmental series of cardiogenesis in *Emys orbicularis*, the European pond terrapin, clarifying the development of the horizontal septum and the looping of the heart tube. Because of the intricate 3-dimensional nature of cardiogenesis and especially the looping, studying histological sections in merely two dimensions does not suffice. For that reason we have complemented the histological studies with computerized high-resolution 3-D reconstructions of three different developmental stages, produced with our in-house software environment, TDR-3-D base (Verbeek et al., 1995; Verbeek and Huijsmans, 1998; Verbeek, 1999; Verbeek and Boon, 2002).

The second aim of this article is therefore to underscore the indispensable nature of high-resolution 3-D reconstructions in studying embryonic morphology. Our software is particularly well suited for solving complex biological problems in three dimensions, as it enables the construction of high-resolution 3-D models, directly from histological sections, and allows us to distinguish between different structures in the heart and study them separately and from every viewpoint. To offer the reader full access to the material we provide the 3-D reconstructions along with additional images on a public website: bio-imaging.liacs.nl/galleries/emysorbicularis/TurtleGallery.html. Here, the models can be viewed interactively using our 3-D model browser, a Java applet which complements our TDR-3-D base software package and enables online viewing of 3-D reconstructions. This ensures optimal insight into the obtained results and serves as an easy reference for anyone studying cardiogenesis. To the best of our knowledge, this is the first

application of these modern techniques to the study of the chelonian heart, enabling the study of all structures separately.

Besides the findings on the ventricular septation we have, in the course of this study, also come across an interesting finding regarding the developmental origin of the pulmonary vein, which adds to the current debate on the origin of this vein in mice, humans, and chicks and is described in the conclusions.

Below we provide a review of the available literature on the adult chelonian heart, followed by a detailed description of the developmental series, using both histological studies and 3-D reconstructions. The construction methods of the models are explained and finally the results of the study are discussed, considering both new insights into the ventricular septation of the turtle heart and the relevance of using 3-D reconstruction techniques in supporting biology.

CURRENT KNOWLEDGE OF THE ADULT TURTLE HEART

The reptilian order Testudines includes the aquatic and semi-aquatic “turtles”, as well as the terrestrial “tortoises” (Family Testudinidae). We are concerned here with a species of the former group, the European pond turtle, *Emys orbicularis* L. For a long time, researchers considered the turtle heart a transitional stage between the single circulatory system, found in most fish, and the double circulatory system of lungfish and tetrapods, including mammals and birds. As such it was considered inefficient and “unfinished”, only a stepping stone in the evolution from using gills (i.e. branchiate respiration) to using lungs (i.e. pulmonate respiration) for gaseous exchange. This idea originated from the observation that most reptiles have only partly separated ventricular *cava*, whereas mammals and birds have completely separated ventricles. At present it is commonly agreed that the turtle heart is in fact a highly specialized organ, adapted to its typical function in turtles—animals that possess lungs, but spend much of their time under water.

Like other tetrapods, the adult turtle possesses two thin-walled atria lined with pectinate muscles and separated by an interatrial septum (cf. Fig. 1). The sinus venosus is partly absorbed into the right atrium and receives the systemic veins, (Fig. 2; Gasch, 1888; O'Donoghue, 1918; Rau, 1924). The pulmonary vein opens into the left atrium. The atria open through the atrioventricular canal. This canal is divided into right and left atrioventricular junctions by the free margin of the atrial septum (Johansen and Burggren, 1980; Burggren and Warburton, 1994), from which hang two leaflets with rudimentary chordae tendinae. Nayak et al. (1995)

suggest that the two leaflets correspond functionally to the septal leaflet of the human right atrioventricular valve and the aortic leaflet of the human left atrioventricular valve. The valve leaflets are single-flapped and membranous and develop from endocardial cushions which meet and fuse along the free end of the developing interatrial septum (Goodrich, 1958). The valves project into the ventricular opening (Goodrich, 1958).

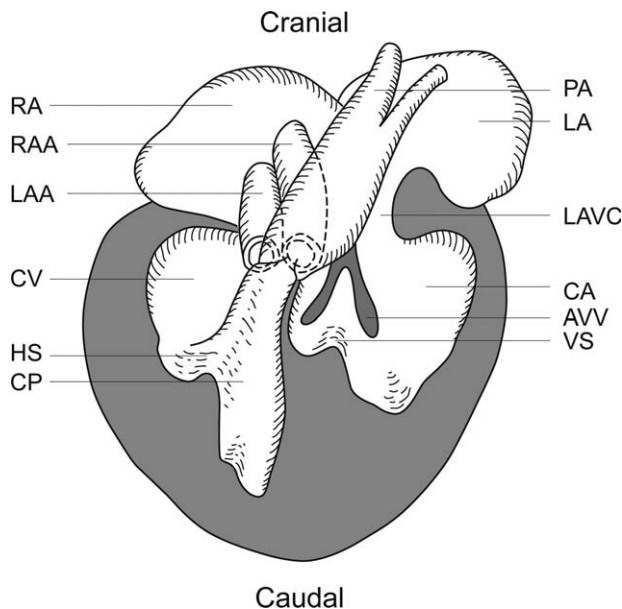


Fig. 1. Schematic drawing of the adult testudine heart, shown in ventral view (drawing based on Hicks and Wang, 1996). For the meaning of abbreviations, see list of abbreviations.

The interior surface of the apical region of the ventricle is heavily trabeculated and the ventricular cavity is partially divided by two incomplete septa into three cavities (Figs. 1, 2, and 3). A large muscular septum, the "horizontal septum" demarcates the cavity known as the *cavum venosum* from the *cavum pulmonale*, while a smaller "vertical septum" separates the *cavum venosum* from the *cavum arteriosum*; in the figures the cavities are referred to as CV, CA and CP.

The horizontal septum (in the horizontal plane, from left to right sides of the ventricle) is complete on the caudal end of the ventricle, but incomplete on the cranial end. It is sometimes called "Muskelleiste" in German, or "muscular ridge" in English (Webb et al., 1971; Van Mierop and Kutsche, 1984; Van Mierop and Kutsche, 1985; Burggren, 1988; Hicks and Wang, 1996). However, like Holmes (Holmes, 1976) we consider these terms rather confusing, since "Muskelleiste" can be translated as "muscular ridge", which in turn is a term used for both vertical septum and in general for all trabeculae carneae. The septum is also referred to as interventricular septum, but this is equally misleading, since this term is also commonly used for the vertical septum. Therefore we will call this septum, as do many other authors, the horizontal septum.

The vertical septum runs dorso-ventrally, in the same sagittal plane as the interatrial septum (which means it runs vertically when the animal is on its feet). It is complete at the caudal apex of the ventricle, but incomplete at the cranial end of the ventricle, leaving a passage open between the *cavum venosum* and the *cavum arteriosum*. The septum is thought to develop from one of the trabeculae carneae and has a similar structure as the trabeculae (Holmes, 1976). Its size differs between species (Webb et al., 1971; Holmes, 1976). Its relation to the interventricular septum in chick and mouse has

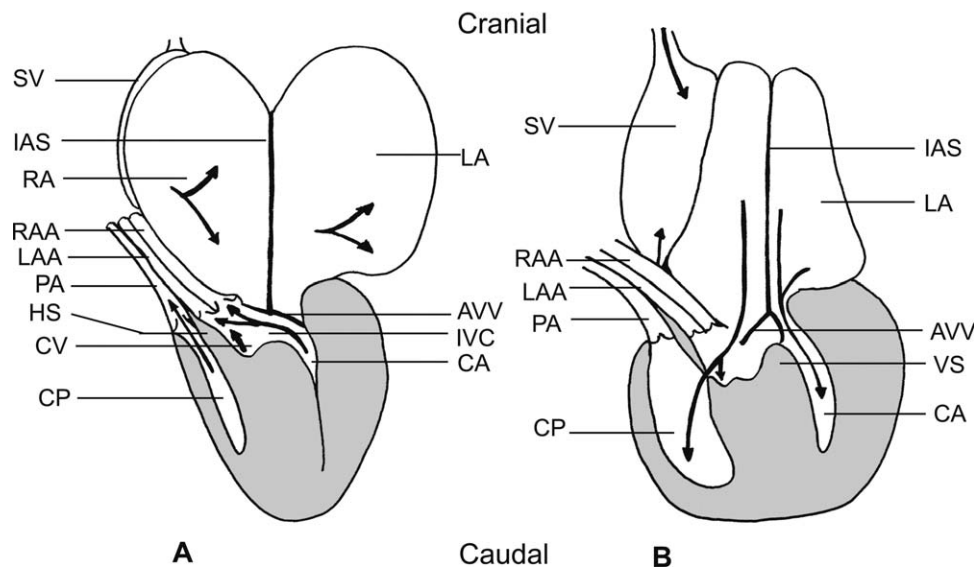


Fig. 2. Schematic drawing of the bloodflow in the chelonian heart shown in ventral view, during atrial systole (A) and ventricular systole (B); note that in order to illustrate the bloodflow, the *cavum pulmonale* has been schematically drawn next to the *cavum venosum*, as opposed to ventro-laterally (drawing based on Webb, 1971).

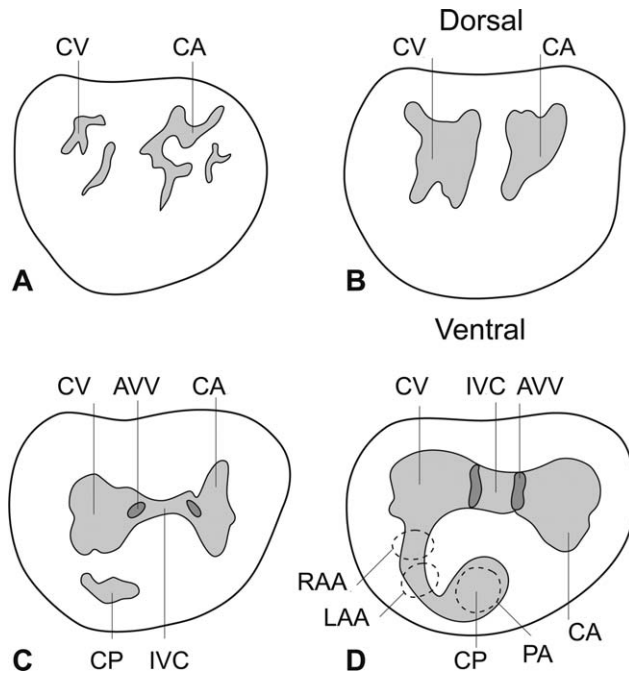


Fig. 3. Schematic drawings of transverse sections through the adult testudine heart (shown in cranial view), at different planes of section along the craniocaudal axis; starting near the ventricular apex, at the caudal end of the heart, in **A**, and progressing in the cranial direction up to the intraventricular canal, in **D** (drawing based on White, 1968).

recently been studied on the gene level by Koshiba-Takeuchi et al. (2009); they found a significant difference in the gradient formation of *Tbx5* in the ventricle between reptiles on the one hand and chick and mouse on the other, suggesting that a steep left-right gradient early in development is essential for ventricular septation. In reptiles this gradient is delayed and less pronounced, resulting in varying degrees of partial septation by the vertical septum.

The *cavum arteriosum* lies on the left side of the vertical septum. This cavity receives blood from the left atrium through the left atrio-ventricular canal. Over the free margin of the vertical septum, we find a passage from the *cavum arteriosum* on the left to the adjacent cavity, the *cavum venosum*, on the right. This passage is situated under the atrioventricular valves and is called the intraventricular canal (Johansen and Burggren, 1980). The canal was first described by White (White, 1968), but there, in our opinion erroneously, called the interventricular canal. Using the term “interventricular” implies the existence of two completely separated ventricles, which is the case in mammals, birds and crocodilians, but not in turtles. This intraventricular canal is the only exit from the *cavum arteriosum*, since this cavity does not connect directly to any arteries leaving the heart. During atrial systole the intraventricular canal is closed by the atrio-ventricular valves, which are forced caudally, into the ventricle, by the pressure of the blood flowing from atria into the ventricle. In this way all the blood from the left atrium is pumped into the *cavum arteriosum* (White, 1968; Johansen and Burggren, 1980; Hicks and Wang, 1996). This process is illustrated in Fig. 2A.

The *cavum venosum* lies to the right of the atrio-ventricular canal and according to Webb (Webb et al., 1971)

it is actually more of a canal than a cavity for retention of blood, but others claim that this is in fact the largest cardiac cavity (Burggren, 1988). Together the *cavum arteriosum* and the *cavum venosum* are sometimes called the *cavum dorsale* (White, 1968), since they occupy the most dorso(-lateral) part of the ventricle (cf. Fig. 3). As can be seen in Fig. 2A blood from the left atrium is pumped into the *cavum venosum* during atrial systole and continues into the *cavum pulmonale* (described below). The *cavum venosum* is separated from the *cavum arteriosum* by the atrio-ventricular valves, which are forced caudally (White, 1968; Johansen and Burggren, 1980; Hicks and Wang, 1996).

The third ventricular cavity is the *cavum pulmonale*. This cavity is also referred to as the *cavum ventrale* (White, 1968). It lies deeper (i.e. protrudes more apically) than the other cavities, lies ventro-laterally (on the right) of the other *cava* and is connected to the *cavum venosum*. The *cavum pulmonale*, separated from the rest of the heart by the horizontal septum, has a peculiar location, since neither of the atrio-ventricular canals is directly connected to it. Therefore, the only way blood can reach the *cavum pulmonale* is through the *cavum venosum*, over the free edge of the horizontal septum (Holmes, 1976), as seen in Fig. 2A.

In turtles and squamates, three arterial trunks arise from the ventricle, a right and a left aortic arch and a pulmonary trunk (Van Mierop and Kutsche, 1984). Each of these trunks possesses a pair of bicuspid semilunar valves (Fig. 2; Goodrich, 1958; Burggren, 1988), originating from endocardial cushion tissue in the outflow tract. The right aortic arch begins at the *cavum venosum*, dorsal to the horizontal septum (Fig. 3). The left arch begins somewhat to the right and slightly more ventrally, near the free margin of the horizontal septum, but still originating from the *cavum venosum*. The pulmonary trunk arises ventrally to the horizontal septum from the *cavum pulmonale* (Holmes, 1976).

During ventricular systole deoxygenated blood from the *cavum pulmonale* enters the pulmonary trunk, whereas oxygenated blood from the *cavum arteriosum* enters the aortic arches, as shown in Fig. 2B (White, 1968; Johansen and Burggren, 1980; Hicks and Wang, 1996). However, during periods of apnea an increase in the pulmonary vascular resistance results in a shunt, which causes a decrease of pulmonary bloodflow. This means that when the turtle is under water (and will therefore not benefit from pulmonary circulation) most of the blood in the *cavum venosum* will end up in the aortic arches, as opposed to the pulmonary trunk (White, 1968; Hicks and Wang, 1996).

In animals with complete interventricular septa a similar shunting of blood can be found in crocodilians; like other reptiles crocodilians possess two aortic arches, of which the left emerges from the right ventricle (along with the pulmonary arch), which allows for a pulmonary bypass. Communication between the aortic arches is established through a foramen between the two arches, the foramen of Panizza, and an aortic anastomosis (Van Mierop and Kutsche, 1984; Van Mierop and Kutsche, 1985; Axelsson et al., 1996; Axelsson, 2001; Eme et al., 2009). Through this communication the shunting can be controlled, as happens in turtles through the pulmonary vascular resistance.

We return to the turtle and its outflow tract. During embryonic development, the outflow tract initially possesses

two proximal and two distal cushions; two additional distal cushions arise later (Hart, 1968; Langer, 1894; Van Mierop and Kutsche, 1984). Similarly, in the mammalian heart two proximal cushions and four distal ones are observed (Webb et al., 2003); however, the development and fusion of these cushions differs from that seen in reptiles (Van Mierop and Kutsche, 1984). In the avian heart we find two proximal and three distal cushions (Qayyum et al., 2001). The distal and proximal cushions in the turtle form endocardial ridges along the tract which grow towards the middle part of the outflow tract and fuse (Hart, 1968). While extending towards each other, these ridges undergo spiraling. The subdivision of the outflow tract begins at the distal end and proceeds towards the ventricle. It is brought on by fusion of opposing cushions and growing of an additional septum, the aorticopulmonary septum, from the distal end of the tract towards the cushions. This septum resembles the aorticopulmonary septum in birds and is possibly derived from neural crest (Lopez et al., 2003). As this septum grows towards the ventricle, it spirals and connects to the distal cushions, thereby dividing the aortae from the pulmonary artery (Hart, 1968). A second septum, the aortic septum, separates the left and right aortae. Semilunar valves form on the proximal faces of the distal cushions (Hart, 1968). The aorticopulmonary and aortic septa form a common mass which is Y-shaped in section. The three limbs of the Y divide the distal lumen of the outflow tract into three channels and divide the biggest of the four distal cushions into three parts. Each of these parts forms a pair of valve leaflets with one of the remaining distal cushions, thereby creating six valve leaflets divided across three lumens (Langer, 1894).

MATERIALS AND METHODS

The process of constructing the 3-D models can be described in three main steps. First, suitable specimens were prepared and sectioned. Subsequently, the sections were digitized to build an acquisition database. Finally, this acquisition database was used to construct a database containing delineations of the anatomical domains that we wanted to visualize. This database contains the 3-D model of the heart and was used to generate the visualizations. For each model a database was created.

Preparation of the Embryos

A total of 12 embryos were studied. Three embryos were used to construct the 3-D models, of stages 8, 10, and 15 (staging according to Yntema, 1968). These stages were chosen because they span the period in which the basic plan of the heart is appearing, but has not fully differentiated yet. In addition to these three stages, serial histological sections of nine embryos were also studied of the following Yntema stages: 6, 8, 9, 10, 11, 12, 14, 15, and 16. Stages 6 to 11 correspond to the following stages in chick (HH stages; Hamburger and Hamilton, 1951), mouse (Theiler stages; Theiler, 1989) and human (Carnegie stages; O'Rahilly and Müller, 1987): Yntema 6 corresponds to HH 9, Theiler 13, Carnegie 10. Yntema 8 corresponds to HH 16–17, Theiler 14, Carnegie 11. Yntema 9 corresponds to HH 18, Theiler 14, Carnegie 11. Yntema 10 corresponds to HH 19–20, Theiler 15, Carnegie 12. And Yntema 11 corresponds to HH 20, Theiler 13, Carnegie 10.

Gravid females were collected under license from the French Government. Standard injection with oxytocin was used to induce laying. Eggs were placed on a layer of sand in an incubator at 25–30°C. Embryos were fixed in Bouin's fluid for 2 days and embedded in Fibrowax according to standard protocols, serially sectioned at 7 µm and stained with Haematoxylin, Eosin and Alcian Blue.

Acquisition of Section Images

Images of the sections containing the heart (of the three embryos used for the 3-D modeling) were made using a Zeiss Axioskop microscope (Zeiss, Jena Germany), equipped with a JAI M10-RS CCD camera (JAI, Denmark) and a Marzhauser stage controller (Marzhauser, Germany) using the MAC 4000 (Marzhauser, Germany). The MAC 4000 connected to the computer through the RS-232 interface. The CCD camera in combination with a PCVision frame grabber (Imaging Technology, MA) was used for image digitization. Our acquisition software, 3-D acq, versus 2.0 (Verbeek and Huijsmans, 1998; Verbeek, 1999; Verbeek and Boon, 2002), controlled both the MAC 4000 and the PCVision frame grabber. Using the video overlay option of the PCVision frame grabber, images were aligned prior to acquisition. The metadata was stored in an XML acquisition database referring to the section images which were stored in PNG format.

Construction of 3-D Models

The annotation and subsequent visualization of the resulting 3-D models was realized with a software suite developed in our group for this purpose, the TDR-3-D base program (Verbeek et al., 1995; Verbeek and Huijsmans, 1998; Verbeek, 1999; Verbeek and Boon, 2002). The acquisition database with the section images and other relevant information is seamlessly integrated in TDR-3-D base; all relevant information is directly transferred. In each of the section images the relevant anatomical structures, labeled with separate colors and names, were traced using a WACOM LCD tablet (PL series, WACOM, Europe). Each contour was stored as an annotation to the section image in a TDR-3-D base database. This database was then used in TDR-3-D base to visualize the anatomical structures of the heart, using surface models (Verbeek et al., 1995). These surface models were derived through an automated triangulation procedure using the contour models in the database.

The TDR-3-D base software allows working in different geometrical representations; the storage is optimized for each representation and allows easy publication on internet. The data do not have to be transformed as is the case with other software environments (Ruthensteiner and Hess, 2008). The 3-D models are made available via internet using our 3-D model browser application (Verbeek et al., 2002). This browser is a java applet and requires that java 3-D is installed on the local host, that is the user's computer. Instructions and links are provided from the gallery pages. The browser is able to use all the available geometrical model information so that the user has a maximal freedom in inspecting the 3-D models. The browser opens with a 2D view (Fig. 4C) while on request the 3-D view is loaded. Selections of the separate anatomical domains can be

made from the interface and will be instantaneously altered; the 3-D views are interactive (Fig. 4A) and allow for active exploration of the 3-D model information (Fig. 4B).

RESULTS AND DISCUSSION

The 3-D models, together with the histological studies, provide a developmental overview of the heart of *Emys orbicularis*. In this section a full description is given of this developmental series, with a focus on the looping of the heart and the development of the horizontal septum. Several figures illustrate the major findings; all material has been made available on a website, bio-imaging.liacs.nl/galleries/emysorbicularis/TurtleGallery.html. Here, one can find additional figures and animation sequences and view the 3-D reconstructions interactively using the 3-D model browser.

Below a description of the developmental features is given for each of the studied stages, some based on histological sections, others on 3-D models. This is followed by some technical comments on the different models and the morphological structures presented in them.

Developmental Anatomy

In this section, we discuss the morphological findings from our histological sections and, for stages 8, 10, and 15, the 3-D models. From laying till hatching 26 stages have been described by Yntema (1968), which can be divided in three periods: presomite period (stages 0–3), somite period (stages 4–10) and limb period (stages 11–26). In order to allow comparison of the described data to development in other animals, some defining characteristics have been given for each described stage; complete descriptions can be found in Yntema (1968).

Stage 6

At this stage eight pairs of somites can be seen and a small cranial neuropore persists.

The cardiac endocardium tubes have just fused. The dorsal mesocardium of the atrium is present, but mesenchyme cannot yet be found between its layers. The pericardial coelom can be seen, as well as small amounts of cardiac jelly. No other developmental characteristics were discernible at this stage.

Stage 8 (Figs. 5A, 6A)

Fourteen pairs of somites are present at this stage and the neural folds are completely closed cranially.

Cardiac looping is well underway and clearly visible in the 3-D model (Fig. 5A). The S-looping, described by Männer (2000, 2009), is in progress, but is still in the early phase, corresponding to the third phase in the chick heart; the proximal part of the outflow tract is still on the right side of the heart and the common atrium on the left. The caudal end of the original heart tube, now on the dorsal side of the heart, has ended up in a fold, which is forming the atria. In the cranial direction along the tube lie the ventricle and outflow tract, which are still continuous regions. In this part of the tube a distinct bending process is taking place. On leaving the primitive ventricle the tube first passes cranially, then bends caudally and to the left, which forms a first, proximal bend. Subsequently it bends again, cranially and to

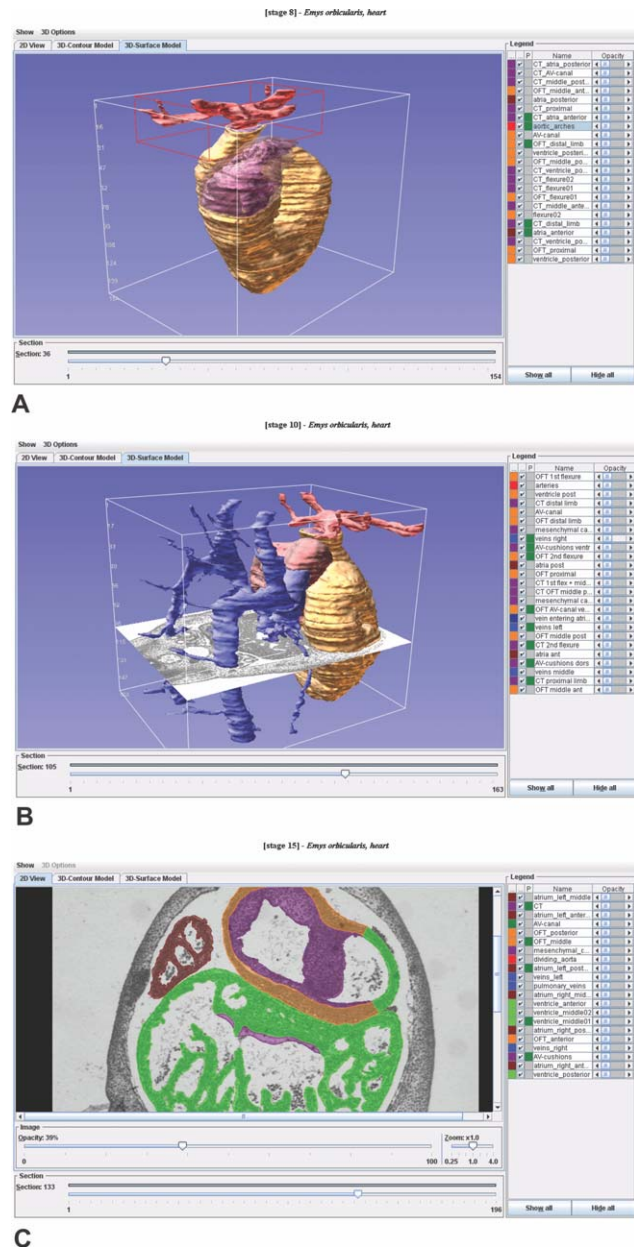


Fig. 4. Screenshots of the 3-D browser showing the three reconstructions in different modes. In (A) a surface representation of stage 8 is shown; in addition to the standard interactive visualization the opacity of each of the anatomical domains can be changed interactively. Anatomical domains can also be deselected so that internal structures come into view. In (B) a surface representation of stage 10 is shown in combination with a 2-D cursor that corresponds to the original section. And in (C) a 2D screenshot of stage 15 is shown; here, the legend indicates all anatomical domains in the entire model with the ones seen in this section image highlighted with green boxes.

the left, thereby forming a second, distal bend. The bending is starting to separate the outflow tract and *cavum pulmonale* from the rest of the ventricle and is responsible for the future development of the horizontal septum (see below).

In the outflow tract two distal and two proximal cushions are visible. Cardiac jelly is still present in the

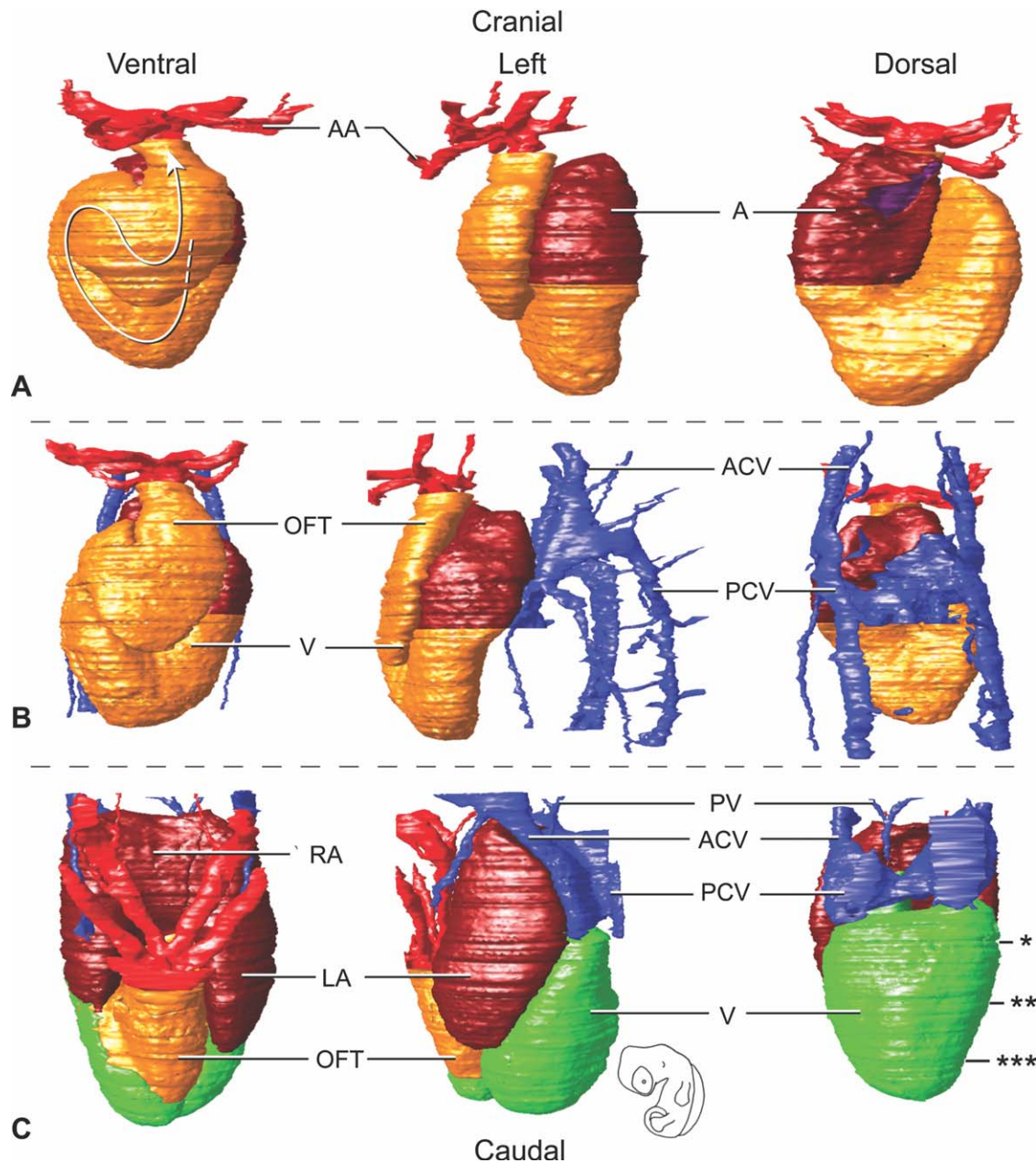


Fig. 5. Overview of the 3-D reconstructions of three *Emys orbicularis* embryos, Yntema stages 8 (A), 10 (B), and 15 (C), shown in ventral, left and dorsal view. The ventral view of A shows the direction of the looping of the heart tube (indicated by the arrow). The asterisks in the

dorsal view of C correspond to the cutaway views in Figure 10. The schematic drawing of the embryo, in a lateral left view, corresponds to the orientation of the left view of the models. The color labeling of the models is explained in the list of abbreviations.

ventricle. Small trabeculae carneae are just becoming visible. None of these trabeculae, however, can yet be identified as the vertical septum. Furthermore, the *cavum arteriosum* and *cavum venosum* are still indistinguishable.

The interatrial septum is barely indicated as a slightly thickened ridge in the ventral part of the atrial wall. This ridge, the presumptive septum primum, is covered with cushion tissue, forming the "mesenchymal cap" (Fig. 6A). Although the endocardium overlying the pulmonary pit is not yet perforated, the pulmonary ridges are thickened. The atria are still in open connection

with the ventricle. In the passage between common atrium and ventricle, two thick endocardial cushions can be seen, continuous with the mesenchymal cap, which will go on to form the atrioventricular valve leaflets at a later stage.

Stage 9 (Fig. 7)

Nineteen pairs of somites are present, the lens pit is visible and the first pharyngeal slit is open.

Cardiac jelly is still present in both the atria and the ventricular part of the heart, with early signs of cell

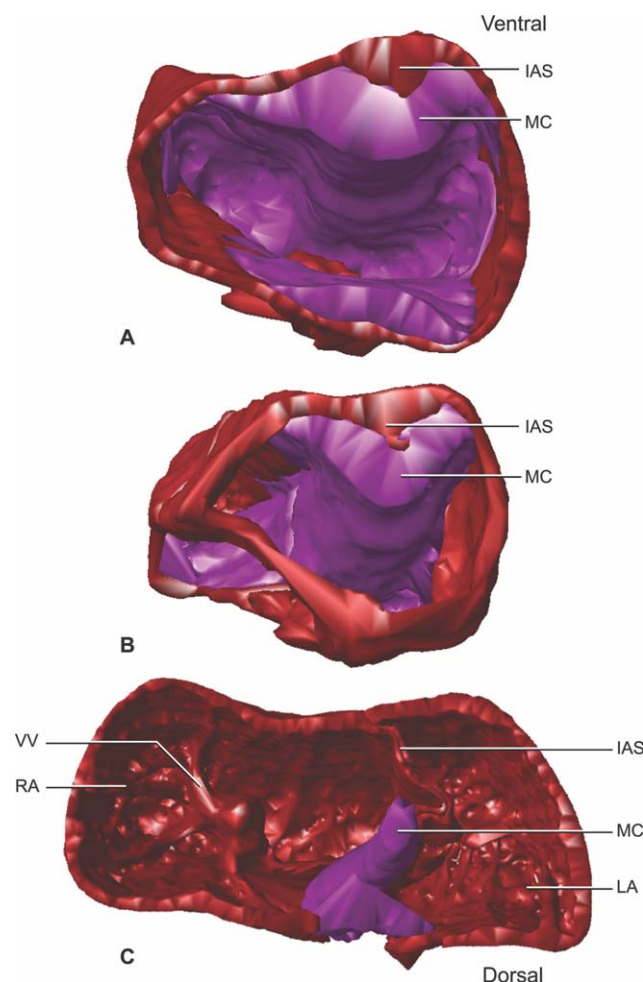


Fig. 6. Cutaway views of the 3-D reconstructions of three *Emys orbicularis* embryos, Yntema stages 8 (A), 10 (B), and 15 (C), showing (part of) the atria and mesenchymal cap in caudal view.

delamination in the future atrioventricular and ventricular region. Trabeculae carneae are clearly visible in some embryos at this stage, but still developing in others.

Stage 10 (Figs. 5B, 6B)

At this stage twenty-four pairs of somites have developed, the first two pharyngeal slits are open and all four limb buds are present.

At this stage cardiac looping is still in progress and the distal bend of the heart tube is moving towards the apex of the ventricle (Fig. 5B). The heart can still be seen as one folded tube and is in the phase of late S-looping, the fourth phase as described by Männer (2000, 2009); the outflow tract is moving to the left and the common atrium has expanded to the right. In the ventricle trabeculae are still developing and the vertical septum is not yet evident. The *cavum arteriosum* and *cavum venosum* cannot be distinguished. In some embryos trabeculae were only just arising, while in others some trabeculae were found to be two or three times as high as they were wide. In the outflow tract the two proximal and distal two cushions are very clear.

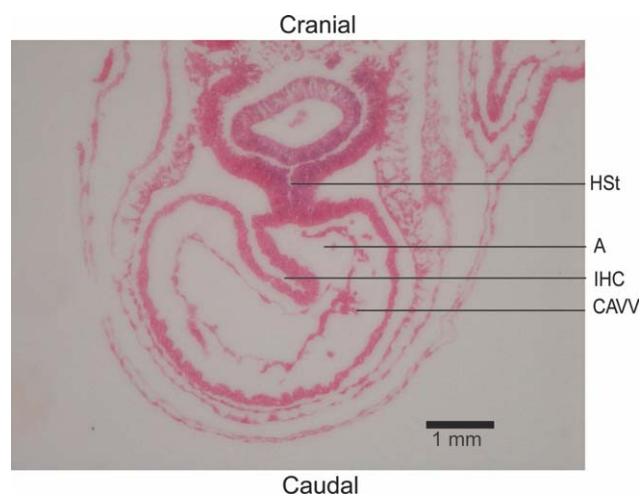


Fig. 7. Section image of the heart of a stage 9 *Emys orbicularis* embryo in caudal view.

In the common atrium the interatrial septum is forming (Fig. 6B). The dorsal mesocardium has not yet lumensized to form the pulmonary vein. Cardiac jelly is still present in both the atrium and the ventricle, but is diminishing. In the atrium pectinate muscles start to develop. Atrioventricular cushions are now distinct and will go on to form the atrioventricular valve leaflets and the atrioventricular septum. Cell delamination is still in its early stages, with only a few cells delaminating in the outflow tract and the atrioventricular cushion tissue. In one of the four embryos cushions were noted in the outflow tract. The outflow tract is still an undivided channel.

Stage 11 (Fig. 8)

Thirty-one pairs of somites are present, the first pharyngeal slit is still open, but the second is covered by the hyoid arch. The cervical flexure has increased prior to turning of the embryo onto its left side.

The atrium is now partially divided by the atrial septum, which extends approximately half the way through the common atrium. The mesenchymal cap is still present and is almost completely acellular at this stage, as can be seen in Fig. 8. The dorsal mesocardium has advanced since the previous stage and now contains vacuoles joining to form the pulmonary vein. The pulmonary vein opens through the pulmonary pit into the left atrium, immediately left of the septum primum. In the atria cardiac jelly is still very prominent and in the atrioventricular canal the cushions are even more apparent. Endocardial cell delamination is very clear in the inner parts of the cushions (Fig. 8).

In the ventricle hardly any cardiac jelly remains in the trabeculated area. The trabeculae are now up to three to four times as high as they are wide. In the distal part of the outflow tract two endocardial cushions, a ventral and a dorsal one, are clearly visible and as in the atrioventricular cushions cell delamination can be seen in the inner parts. The inner heart curvature is lined with endocardial tissue, connecting the ventral cushion in the outflow tract to the superior atrioventricular cushion. The aortopulmonary septum is not present at this stage.

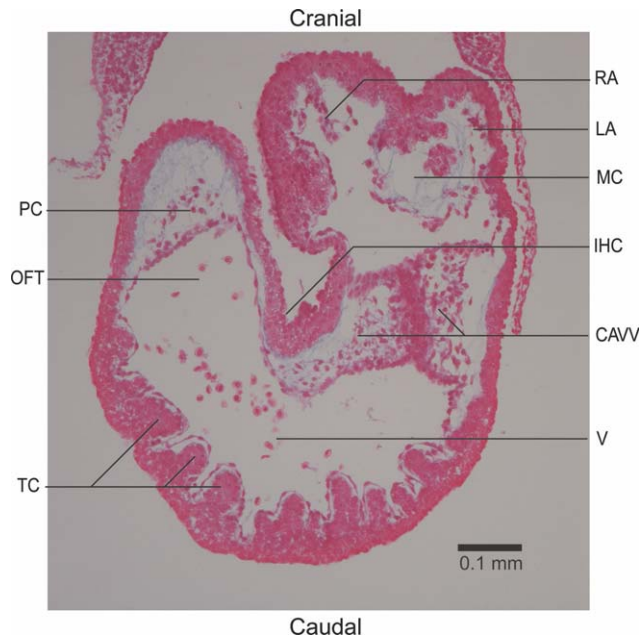


Fig. 8. Section image of the heart of a stage 11 *Emys orbicularis* embryo in caudal view.

Stage 12

The embryo now lies on its left side, the pharyngeal slits have disappeared, the retina is pigmented and the apical ridge is beginning to form in the forelimb bud.

The septum primum in the common atrium has not developed much since the previous stage and still spans only half of the length of the atrium. The pulmonary vein can be traced from both lung buds to the left atrium. In the atria small pectinate muscles can be seen. Cardiac jelly is also still present in the atria. In the atrioventricular cushions cell delamination has advanced little if any.

In the ventricle trabeculae are very prominent. The endocardial cushions in the outflow tract are more heavily populated with cells than before. The distal cushion spirals to the left as it extends proximally. The outflow tract is still undivided at this stage.

Stage 14 (Fig. 9A, B)

The maxillary and lateral nasal processes have fused and the forelimb is in early peddle stage, with the digital plate vaguely indicated.

The right and left sinus valve ridges are both present at this stage. The septum primum now spans approximately two thirds to three quarters of the common atrium. The mesenchymal cap is completely acellular. In one of the three embryos a small additional septum was noted in the atrium, possibly the venous valve of the superior cardinal vein. The pectinate muscles in the atria are now more distinct, generally as high as they are wide and the cardiac jelly has vanished. The atrioventricular cushions are prominent and completely infiltrated with cells. The cushions have not yet fused (Fig. 9A,B), but seem to be touching, although this could be due to the phase in which the cardiac activity has been arrested.

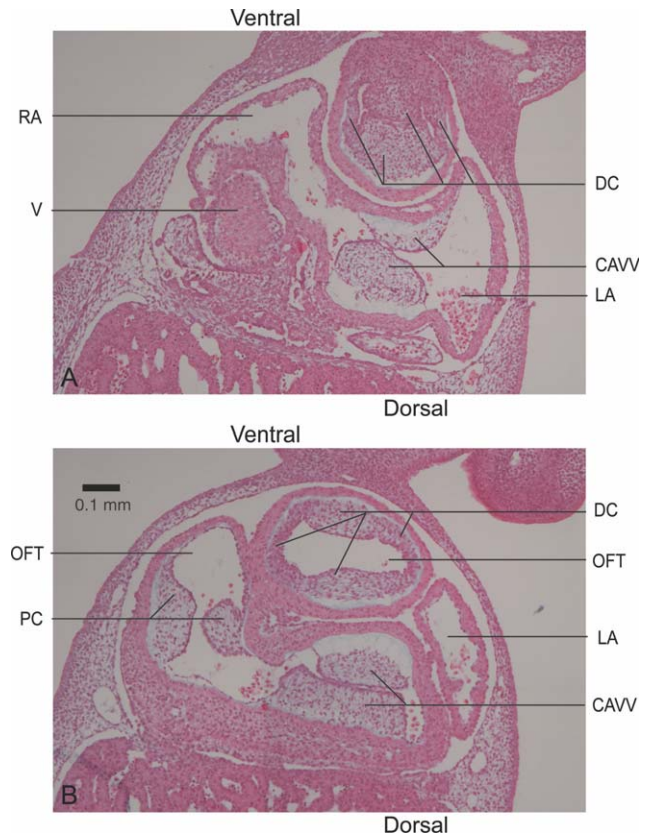


Fig. 9. Section images of the heart of a stage 14 *Emys orbicularis* embryo in caudal view; **A** lies cranial to **B**.

In the outflow tract six endocardial cushions can be seen, two proximal and four distal cushions (Fig. 9A,B). The distal cushions account for the H-shaped form of the lumen, when seen in transverse sectioning. Like the atrioventricular cushions the proximal outflow tract cushions are completely infiltrated with cells. The cushions approach each other, but are not yet touching.

Stage 15 (Figs. 10A, B, and C; 11A, B, and C)

The cervical sinus is closed and the digital plate of the forelimb is well formed with no digital grooves present.

By now the distal bend of the original heart tube has reached the apex of the ventricle and the walls of both have merged. The heart is in the fifth phase described by Manner (2000, 2009), the phase of cardiac septation. The caudal apex of the bend has formed the *cavum pulmonale*, continuous on the cranial end with the outflow tract (Fig. 5C). The merged wall between the *cavum pulmonale* and the rest of the ventricle has formed an incomplete septum, the horizontal septum. This is clearly visible in the cutaway views of the 3-D model of this stage, shown in (Fig. 12). The *cavum pulmonale* can be seen on the ventral side of the incomplete septum, at the base of the outflow tract, as a distinct cup-shaped chamber. It is almost completely separated from the rest of the ventricle, save for a small passage on the cranial side, connecting it to the *cavum venosum*. From this developmental series it can be concluded that the *cavum*

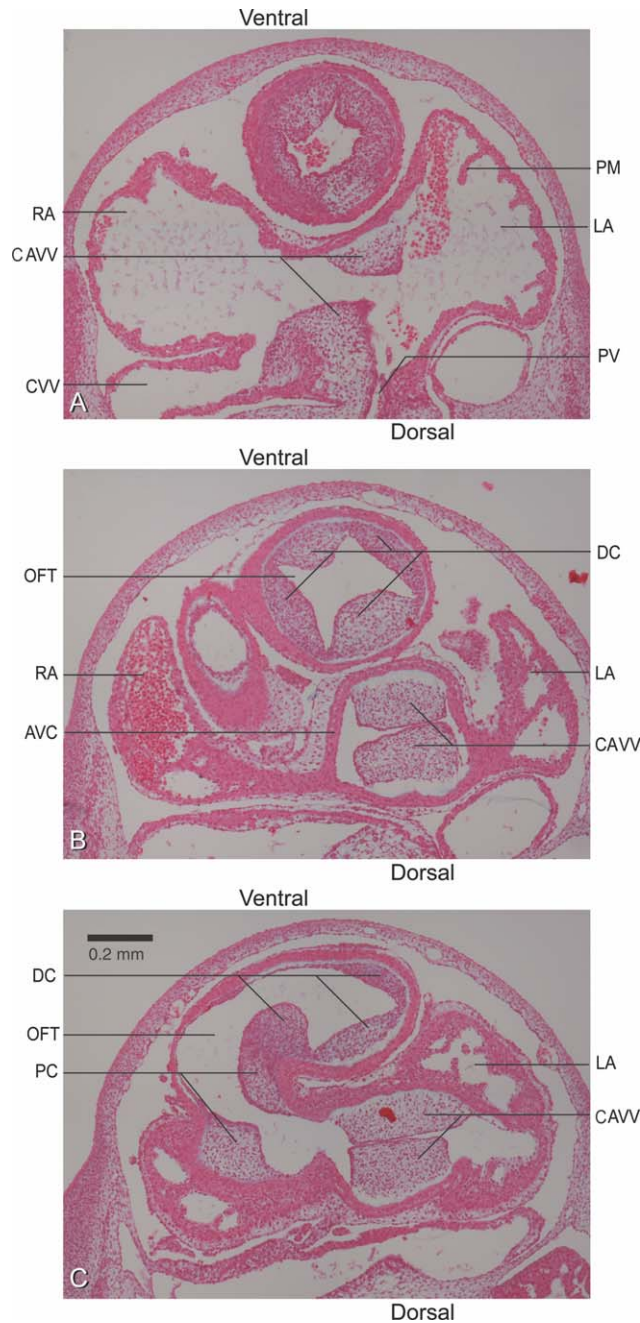


Fig. 10. Section images of the heart of a stage 14–15 *Emys orbicularis* embryo in caudal view, progressing from cranial (A) to caudal (C).

pulmonale develops at the proximal end of the outflow tract and the horizontal septum is formed through bending of the tube (see Conclusion).

It is interesting to note that the *cavum pulmonale* has almost no trabeculae at this stage, while the rest of the ventricle is heavily trabeculated, with trabeculae five to seven times as high as they are wide (Fig. 11B and C). One of these trabeculae is slightly bigger with a dorso-ventral base thereby forming a septum at the apex of the ventricle. This is the vertical septum, dividing the *cavum venosum* from the much smaller *cavum arterio-*

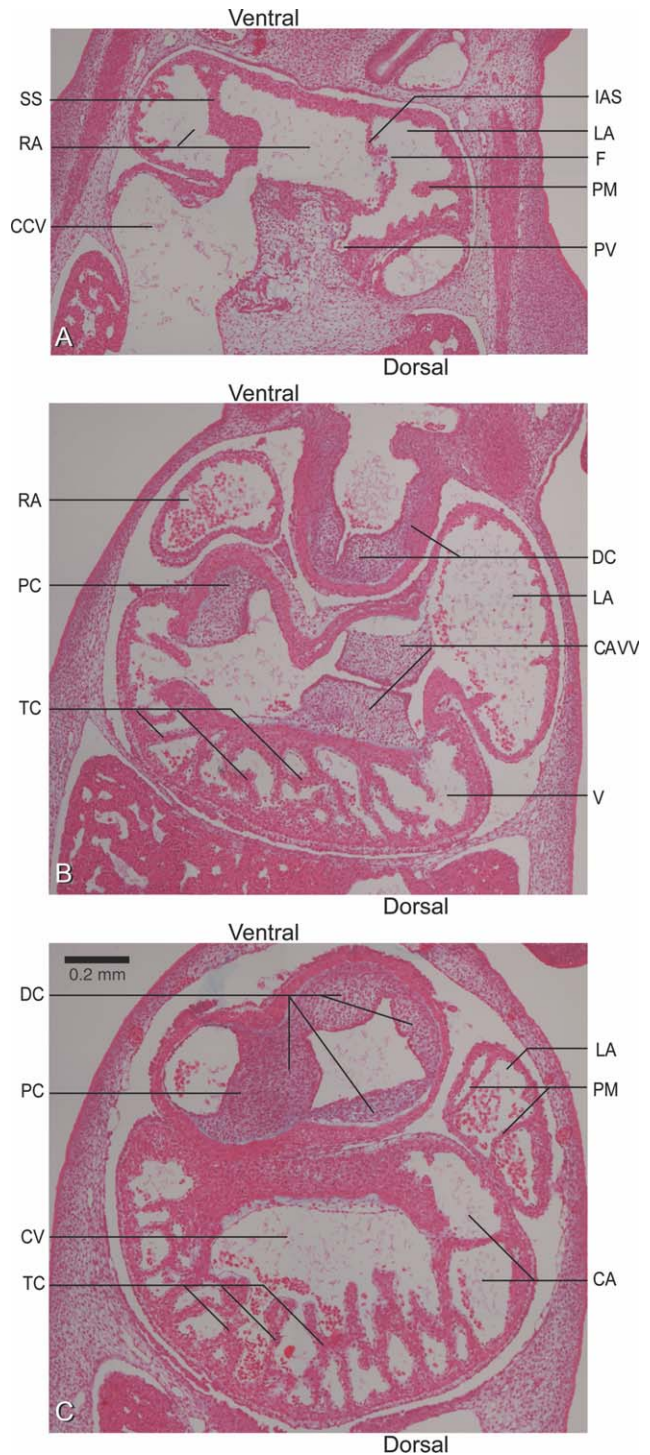


Fig. 11. Section images of the heart of a stage 15 *Emys orbicularis* embryo in caudal view, progressing from cranial (A) to caudal (C).

sum (Fig. 11C and 12). This septum is about half the size of the horizontal septum. It is not covered by a mesenchymal cap and is therefore more of a muscular ridge than a true interventricular septum.

The atrioventricular cushions are still developing and by now approximately two thirds or three quarters of their bulk is infiltrated by cells. The cushions are

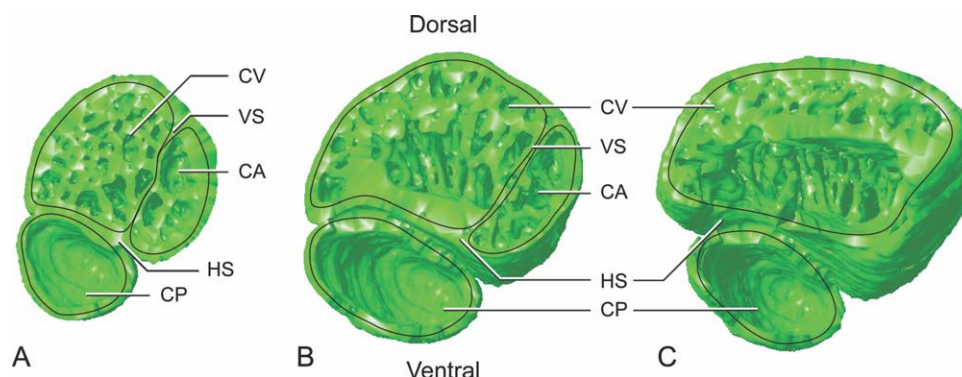


Fig. 12. Cutaway views of the 3-D reconstruction of a stage 15 *Emys orbicularis* embryos, showing only the ventricle in cranial view, at three craniocaudal levels. The asterisks in Fig. 3 correspond to the planes of section (* corresponds to A, ** to B, and *** to C).

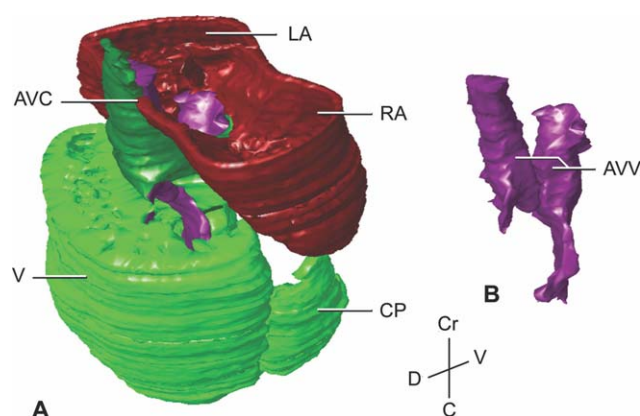


Fig. 13. Cutaway view of the 3-D reconstruction of the stage 15 *Emys orbicularis* embryo, showing parts of atria, ventricle, and atrioventricular canal in a ventrolateral view in A. B shows the atrioventricular valves separately in the same plane of view.

touching but have not yet fused (Figs. 10B, C, and 11B). The atria and ventricle are no longer in open connection, but are separated by an atrioventricular canal, in which the atrioventricular cushions are suspended (Figs. 10B, 13, and 15). The ventral atrioventricular cushion is still connected at one end to the proximal cushion tissue in the outflow tract and at the other end to the mesenchymal cap (as can be seen in the animation sequence of the cushion tissue, compare bio-imaging.liacs.nl/galleries/emysorbicularis/TurtleGallery.html). In the outflow tract the four distal and the two proximal cushions are now clearly visible (Figs. 10B and 14). Two of the distal cushions are nearly fusing and the most distal of the proximal cushions is continuous with one of the distal cushions (Figs. 10C and 11C).

The atrial septum now extends three quarters of the total distance to the atrioventricular canal and its two limbs are easily distinguishable (Fig. 6C). The mesenchymal cap is still present, but very small and is completely infiltrated with cells. In one of the four embryos perforations in the atrial septum were seen. Immediately left of the septum is the pulmonary pit, in which the lumen of the pulmonary vein is now unequivocally present (Fig. 11A).

Early Stage 16

This is the latest stage in developmental series available to us in this analysis and it resembles the preceding stage in most aspects. The septum primum remains approximately the same. The pectinate muscles have grown a little and are now two or three times as high as they are wide. The trabeculae are still about five to seven times as high as they are wide. Both the atrioventricular cushions and the outflow tract cushions are completely infiltrated with cells. The cushions are clearly touching but still have not fused.

Source Data and Annotation of the 3-D Models

In each of the 3-D models the following structures have been labeled: atria, ventricle, outflow tract, aortic arches, and endocardial cushion tissue (Fig. 5). In two models (Fig. 5B and C), the veins entering the heart have also been traced. All of the structures have been divided into smaller sub-structures enabling the user to study smaller parts separately and from the inside.

In this study, the models were based on sectioned embryos and the recognition of different tissue types was facilitated by staining methods. Alternatively, a non-invasive technique could have been used; given the size of the *Emys* heart, micro Magnetic Resonance Imaging (μ MRI) could have been a good candidate. However, a μ MRI study of an adult heart (not published) has taught us that the level of detail is significantly lower than could be achieved using invasive techniques, combined with histological staining. For this reason, physical sectioning was preferred.

The manual delineation of the anatomical domains was, for this specific section material, favored over automated methods. Given the standard staining of the sections, functional differences between parts of the same tissue type could not have been distinguished automatically; for example, by using automated tracing the atria and ventricle would all have become part of one structure, based on their tissue type. Manual delineation allowed us to distinguish between structures based on their functionality. Atria and ventricle are therefore annotated as different structures, which makes the models more useful. Furthermore, the structures are more detailed and more reliable than would have been

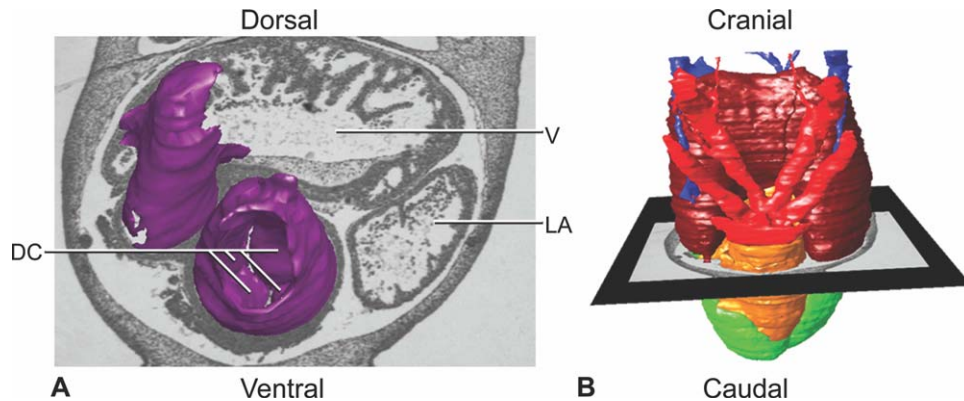


FIG. 14. Cutaway view of the 3-D reconstruction of the stage 15 *Emys orbicularis* embryo, showing the distal cushions in the outflow tract, together with a section image in **A**. The position of the section image in relation to the rest of the heart is shown in **B**.

possible using computerized tracing; irrelevant material (e.g. blood remaining in the lumina) or artefacts from staining and sectioning would have been falsely included using automatic tracing.

For the stage 8 model (Fig. 5A), 154 section images were used. The ventricle and outflow tract could not be clearly demarcated from one another at this stage, and so have been assigned the same color label (orange). The common atrium has not yet divided into right and left parts and is therefore shown as one structure. The veins entering the heart were similar to those seen at stage 10 and were not included in this model.

The stage 10 model (Fig. 5B) was constructed from 163 section images. The outflow tract and ventricle are still not completely developed and are therefore presented in orange as one structure. Although the interatrial septum is beginning to form the atria are not yet easily distinguishable and are therefore also shown as one structure. The arterial arches leaving the heart and the veins entering it are shown in detail, as well as the cushion tissue.

In the model of stage 15 (Fig. 5C), built from 196 section images, the ventricle and outflow tract are clearly separated and have each been given a different color (green and orange, respectively). The interatrial septum is almost completely closed (save for several foramina) and it is now possible to distinguish between the left and right atrium. We have therefore assigned different structure labels to the atria (as opposed to one label for the common atrium in the other models), enabling the user to study the atria simultaneously and separately. Since the dividing line between the atria is very clear from the outside of the model no difference in color between the two structure labels was deemed necessary. As in the previous model, veins, arteries, and cushion tissue are visible. The atrioventricular canal, which at this stage can be distinguished from the ventricle and atria, is labeled as a separate structure.

All section images, annotated domains and 3-D representations are available on the website. Our interactive 3-D model browser application (see Fig. 4 and website) provides the user with a wealth of functionalities, which help understand the 3-dimensional structure of the hearts. The 2D view allows the user to browse through the annotated sections (Fig. 4C). In a section image annotated domains can be selected or hidden and the

opacity of selected structures can be adapted. Selecting a particular domain from the legend results in the selection of the first section image in which this domain occurs; in this manner quick inspection and exploration of specific structures is made possible. A slider below enables browsing through all consecutive section images.

The model can be inspected in 3-D using the 3-D contour or surface view. It can be moved around and inspected from all viewpoints, both as a whole and only showing selected domains. In the surface representation the opacity of each of the anatomical domains can be changed interactively (Fig. 4A). A 2-D cursor can be added, showing the original section image (Fig. 4B). Switching from 3-D to 2-D view allows inspection of the annotated areas in that particular section image.

CONCLUSION

This study yielded important new insights into the developmental origins of the horizontal septum in the turtle. From the 3-D models it becomes clear that the septation is closely linked to the looping of the heart. This looping is responsible for the formation of the horizontal septum and the demarcation of the *cavum pulmonale* and outflow tract. The looping of the turtle heart is quite distinct from that found in human development. In humans, the part of the heart tube leaving the ventricle has one clear bend, originally termed the “bayonet bend” (Orts LLorca et al., 1982) and later described as the “dog-leg” bend (Webb et al., 2003), which separates the distal part of the outflow tract from the proximal part. The proximal part of the outflow tract, leaving the ventricle, is relatively straight. The two developing ventricles lie next to each other (seen in a more or less coronal plane) and become separated by an interventricular septum, through expansion of the ventricles, while the medial walls of the ventricles grow together gradually and fuse.

The situation in the turtle is markedly different. Here, the part of the heart tube leaving the primitive ventricle does not have one but two very clear bends. The tube first passes cranially, bends caudally and to the left, forming a first, proximal bend; it then bends a second time, cranially and to the left, forming a second, distal bend. These bends are very clear in the cranial views of the 3-D reconstructions of stages 8 and 10 (Fig. 5A, B).

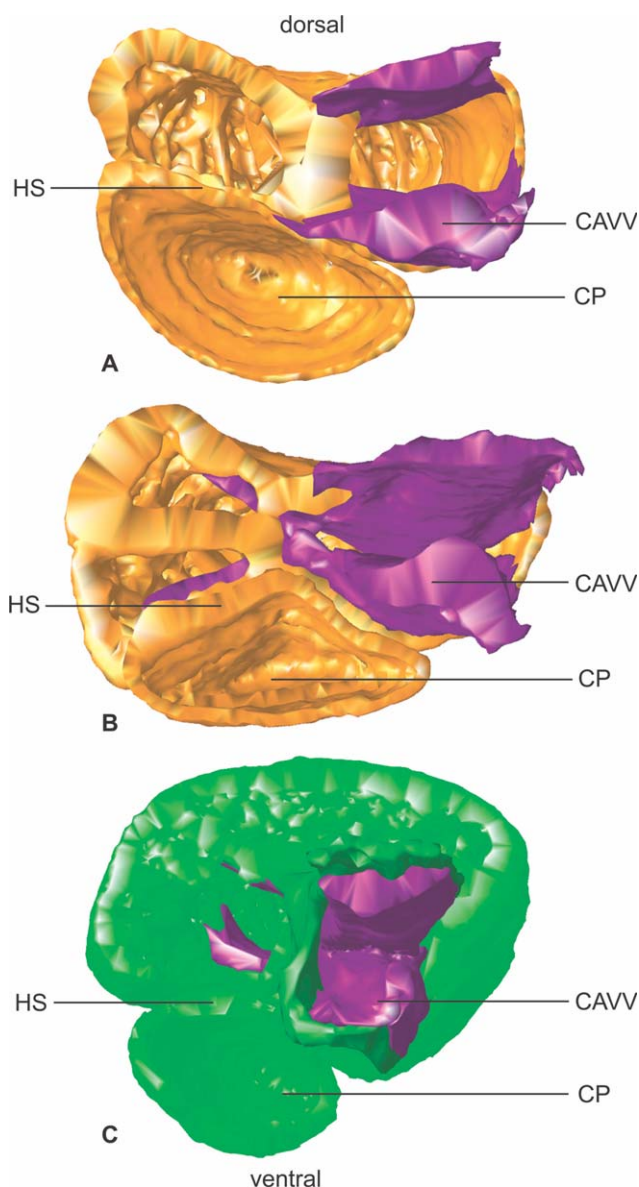


Fig. 15. Cutaway views of the 3-D reconstructions of stages 8 (A), 10 (B), and 15 (C). *Emys orbicularis* embryos, showing the caudal parts of the ventricle in a cranial view, including the ventricle and cushion tissue which will form the AV-valves. The division between the *cavum pulmonale* and the rest of the ventricle, by the developing horizontal septum, is visible.

Because of this difference in heart looping the establishment of septa in the primitive ventricle in humans and turtles occurs in a strikingly different way. In the turtle the second, distal bend of the tube comes to lie ventrally to the ventricle and where the walls of bend and ventricle touch they merge to form the horizontal septum, which can be seen in Figs. 5, 12, and 15. We can see the distal bend moving gradually in the caudal direction during development, to reach the level of the apex of the ventricle. The lumen of this bend becomes the *cavum pulmonale* and is connected to the rest of the ventricle over the free margin of the horizontal septum.

So whereas the ventricles in the developing human heart lie next to each other in the coronal plane, the *cavum pulmonale* lies in front of the rest of the ventricle in the turtle heart. And whereas the human interventricular septum forms through expansion of the ventricles, the horizontal septum in the turtle forms through merging of the ventral part of the ventricular wall with the dorsal wall of the distal (i.e. second) bend in the outflow tract. The mechanisms of heart looping and septation are therefore significantly different.

Our findings contradict the theory proposed by Holmes (1976) on the development of the horizontal septum. He suggests that the cranial part of this septum derives from the spiral fold of the conus arteriosus, while the caudal part derives from hypertrophied trabeculae that join the cranial part. Van Mierop and Kutsche (1984) also suggest that a cranial part of the septum may be formed by condensation of trabeculae. It is clear from Figs. 5, 12, and 15, however, that the septum consists of merged walls and that the walls of the *cavum pulmonale* are smooth and do not contain any trabeculae.

Another interesting biological finding concerns the developmental origin of the pulmonary vein. The development of this vein in human, mouse, and chick has been the matter of some debate (Blom et al., 2001; Webb et al., 2001). This debate centers around the relationship between the vein and the systemic venous sinus (sinus venosus). Although immunohistological studies have suggested that the human pulmonary vein originates from the sinus venosus (Blom et al., 2001), recent studies in chick and human indicate that the vein canalizes as a newly formed channel within the developing posterior mediastinum and uses the remnants of the dorsal mesocardium to establish a direct connection with the atrium (Webb et al., 2000, 2001).

Our findings in the turtle support these recent findings in chick and human. The sections show that the pulmonary vein in turtles also derives from the mediastinal myocardium and not from the sinus venosus. It forms in the pulmonary pit of the left atrium as was described for the chick (Webb et al., 2003). The sinus in reptiles is partly absorbed into the right atrium and receives the systemic veins (left and right precaval veins and postcaval veins) and coronary sinus (Gasch, 1888; O'Donoghue, 1918; Rau, 1924). The left atrium receives only the pulmonary vein or veins (Gasch, 1888; O'Donoghue, 1918; Rau, 1924).

With respect to 3-D reconstruction techniques this study illustrates the indispensable nature of these techniques when studying morphogenesis in the turtle heart. Many biological studies would benefit from high-resolution 3-D reconstructions. Our software, which allows high-resolution modeling and manual discernment between functionally separate structures (see Results and Discussion), is therefore of great use. By enabling the readers to study the models in an interactive way, using our 3-D browser application, we hope to go beyond a descriptive reporting of our findings, using merely static 2-D images, and to invite the readers to join the ongoing search for a more thorough understanding of cardiogenesis in the turtle.

In the models of stages 8 and 10, the ventricle and outflow tract together have been assigned one structure label (orange) and the common atrium has also one structure label (burgundy). In the model of stage 15, the ventricle and outflow tract can be distinguished separately and

have therefore been assigned separate structure labels (green and orange, respectively); the atria have also been assigned different structure labels, but in the same color (burgundy) and the atrioventricular canal is dark green. In all three models vessels leaving the heart are red, vessels entering are blue and cushion tissue is purple.

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