Computerised Modelling for Developmental Biology

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CHAPTER 2. A VISUAL MODEL: 3D RECONSTRUCTIONS OF CARDIAC DEVELOPMENT IN THE TURTLE *EMYS ORBICULARIS*

> The scientist gave a superior smile before replying, "What is the tortoise standing on?" "You're very clever, young man, very clever," said the old lady. "But it's turtles all the way down!"
>
> - Stephen Hawking, *A brief history of time*

We begin our series of case studies by looking at a visual model of developmental anatomy: a series of 3D reconstructions of the turtle heart. There are significant benefits to using a computer-assisted technique in studying development and the development of the turtle heart is particularly suited to illustrate these benefits; the embryonic turtle heart is very small, necessitating a high level of magnification, and is spatially complex. While schematic drawings, based on histological sections, can be misleading, 3D computer reconstructions closely resemble reality and allow us to get a better understanding of the spatial properties of the structure. Furthermore, by providing the models online with our TDR-viewer applet, the reader is able to interactively explore the reconstructions as well as the corresponding histological sections, which optimizes the communication of information by these models.

Based on: Bertens L.M.F., Richardson M.K., Verbeek F.J., 'Analysis of cardiac development in the turtle *Emys orbicularis* (Testudines: emidydae) using 3-D computer modelling from histological sections', *Anatomical record* 293:7 (2010), 1104-1114.

LIST OF ABBREVIATIONS

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.

2.1 INTRODUCTION

Many features of cardiac development in turtles correspond to those in birds and mammals (Goodrich, 1958; Holmes, 1976; Quiring, 1933; Shaner, 1962; Greil, 1903). 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 chapter 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 computerised high-resolution 3D reconstructions of three different developmental stages, produced with our in-house software environment, TDR-3Dbase (Verbeek *et al.*, 1995; Verbeek and Huijsmans, 1998; Verbeek, 1999; Verbeek and Boon, 2002).

The second aim of this chapter is therefore to underscore the indispensable nature of high-resolution 3D 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 3D 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 3D reconstructions along with additional images on a public website: http://bio-imaging.liacs.nl/galleries/. Here the models can be viewed interactively using our 3D model browser, TDR-viewer, a Java applet which complements our TDR-3Dbase software package and enables online viewing of 3D 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, a review of the available literature on the adult chelonian heart is provided, followed by a detailed description of the developmental series, using both histological studies and 3D 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 3D reconstruction techniques in supporting biology.

2.2 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. 2.1). The sinus venosus is partly absorbed into the right atrium and receives the systemic veins, (Fig. 2.2; 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

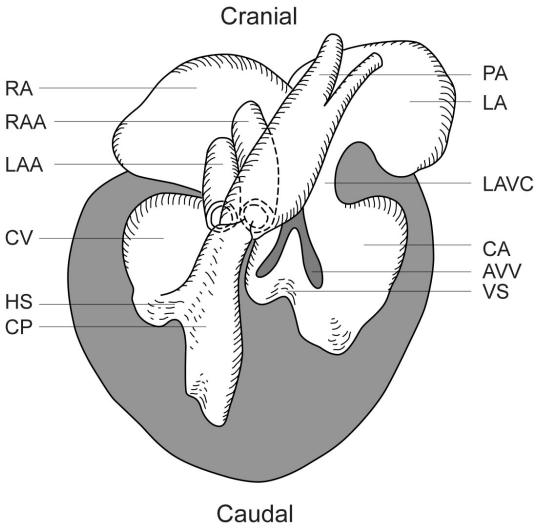

Figure 2.1. Schematic drawing of the adult testudine heart, shown in ventral view (drawing based on Hicks and Wang, 1996). For the meaning of abbreviations, see List of abbreviations.

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).

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 (Fig. 2.1, 2.2 and 2.3). 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; Burggren, 1988; Hicks and Wang, 1996; Van Mierop and Kutsche, 1984; Van Mierop and Kutsche, 1985). 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.

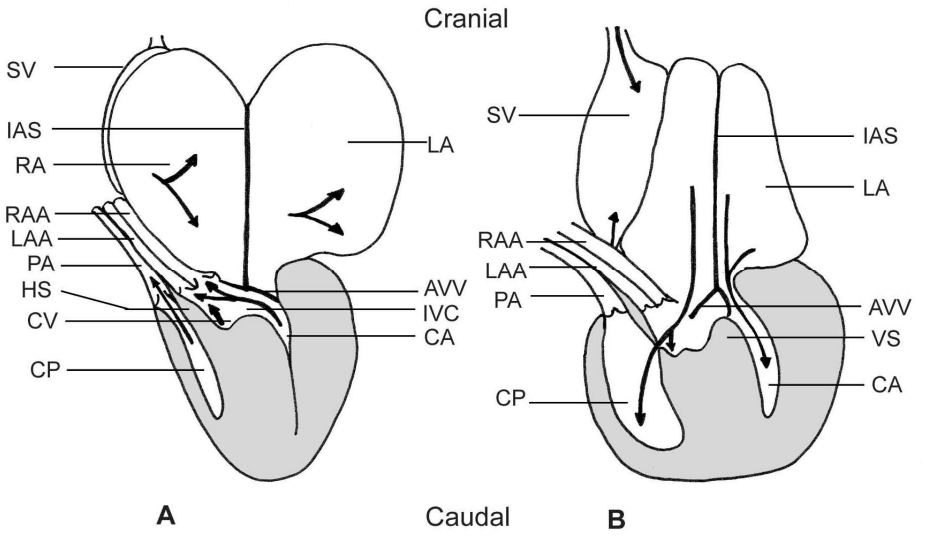

Figure 2.2. Schematic drawing of the blood flow in the chelonian heart shown in ventral view, during atrial systole (A) and ventricular systole (B); note that in order to illustrate the blood flow, the *cavum pulmonale* has been schematically drawn next to the *cavum venosum*, as opposed to ventro-laterally (drawing based on Webb, 1971).

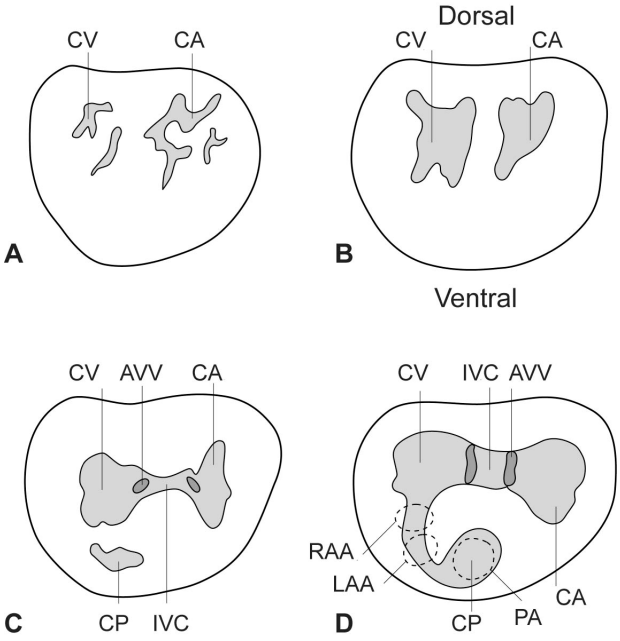

Figure 2.3. Schematic drawings of transverse sections through the adult testudine heart (shown in cranial view), at different planes of section along the craniocaudal axis; starting near the ventricular apex, at the caudal end of the heart, in A, and progressing in the cranial direction up to the intraventricular canal, in D (drawing based on White, 1968).

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 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 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 Figure 2.2A.

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. 2.3). As can be seen in Figure 2.2A 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 Figure 2.2B.

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; a thorough description is given in 3.3.3, along with several visualisations of the arches in Fig. 3.3). Each of these trunks possesses a pair of bicuspid semilunar valves (Fig. 2.2; Goodrich, 1958; Burggren, 1988), originating from endocardial cushion tissue in the outflow tract. The carotico-systemic aortic arch, *i.e.* the right aorta, begins at the *cavum venosum*, dorsal to the horizontal septum (Fig. 2.3). The systemic arch, *i.e.* the left aorta, 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 Figure 2.2B (White, 1968; Johansen and Burggren, 1980; Hicks and Wang, 1996). However, during periods of apnoea an increase in the pulmonary vascular resistance results in a shunt, which causes a decrease of pulmonary blood flow. 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 crocodilians, possessing complete interventricular septa, a similar shunting of blood can be found; like other reptiles, crocodilians possess two aortic arches, of which the left emerges from the right ventricle (along with the pulmonary arch). Communication between these aortic arches is possible 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 shunting can arise, resulting in a pulmonary bypass; this resembles the shunting found in turtles, due to changes in pulmonary vascular resistance.

During embryonic development, the turtle 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). A full account of these differences is given in chapter 3, in which we will look at the development of the cushions and the septation of the distal component of the outflow tract.

2.3 MATERIALS AND METHODS

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

Preparation of the embryos

A total of 12 embryos was studied. Three embryos were used to construct the 3D 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 9 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 7µm 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 3D modelling) 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, USA), was used for image digitization. Our acquisition software, 3Dacq, version 2.0 (Verbeek *et al.*, 1998; 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 3D models

The annotation and subsequent visualisation of the resulting 3D models was realized with a software suite developed in our group for this purpose, the TDR-3Dbase 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-3Dbase; all relevant information is directly transferred. In each of the section images the relevant anatomical structures, labelled with separate colours 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-3Dbase database. This database was then used in TDR-3Dbase to visualise 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-3Dbase software allows one to work 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 3D models are made available via internet using our 3D model browser application, TDR-viewer (Potikanond and Verbeek, 2012). This viewer is a java applet and requires that java 3D is installed on the local host, *i.e.* the user's computer. Instructions and links are provided from the gallery pages. The viewer is able to use all the available geometrical model information so that the user has maximal freedom in inspecting the 3D models. The viewer opens with a 2D view (Fig. 2.4C) while on request the 3D view is loaded. Selections of the separate anatomical domains can be made from the interface and will be instantaneously altered; the 3D views are interactive (Fig 2.4A) and allow for active exploration of the 3D model information (Fig 2.4B).

2.4 RESULTS AND DISCUSSION

The 3D 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 http://bio-imaging.liacs.nl/galleries/. Here one can find additional figures and animation sequences and view the 3D reconstructions interactively, using the TDR-viewer.

Below a description of the developmental features is given for each of the studied stages, some based on histological sections, others on 3D 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 3D 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 endocardial 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 (Fig. 2.5A and 2.6A)

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 3D model (Fig. 2.5A). The S-looping, described by Männer *et al.* (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 the left, thereby forming a

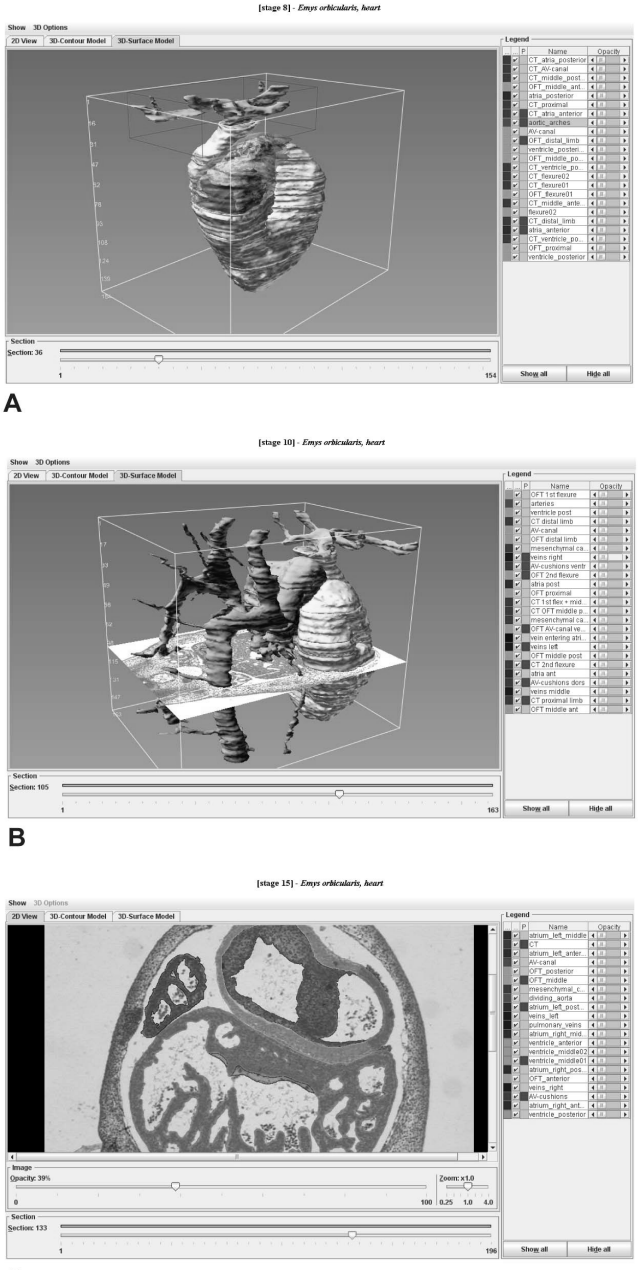

Figure 2.4. Screenshots of the TDR-viewer showing the three 3 reconstructions in different modes. In (A) a surface representation of stage 8 is shown; in addition to the standard interactive visualisation the opacity of each of the anatomical domains can be changed interactively. Anatomical domains can also be deselected so that internal structures come into view. In (B) a surface representation of stage 10 is shown in combination with a 2D 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 using green boxes.

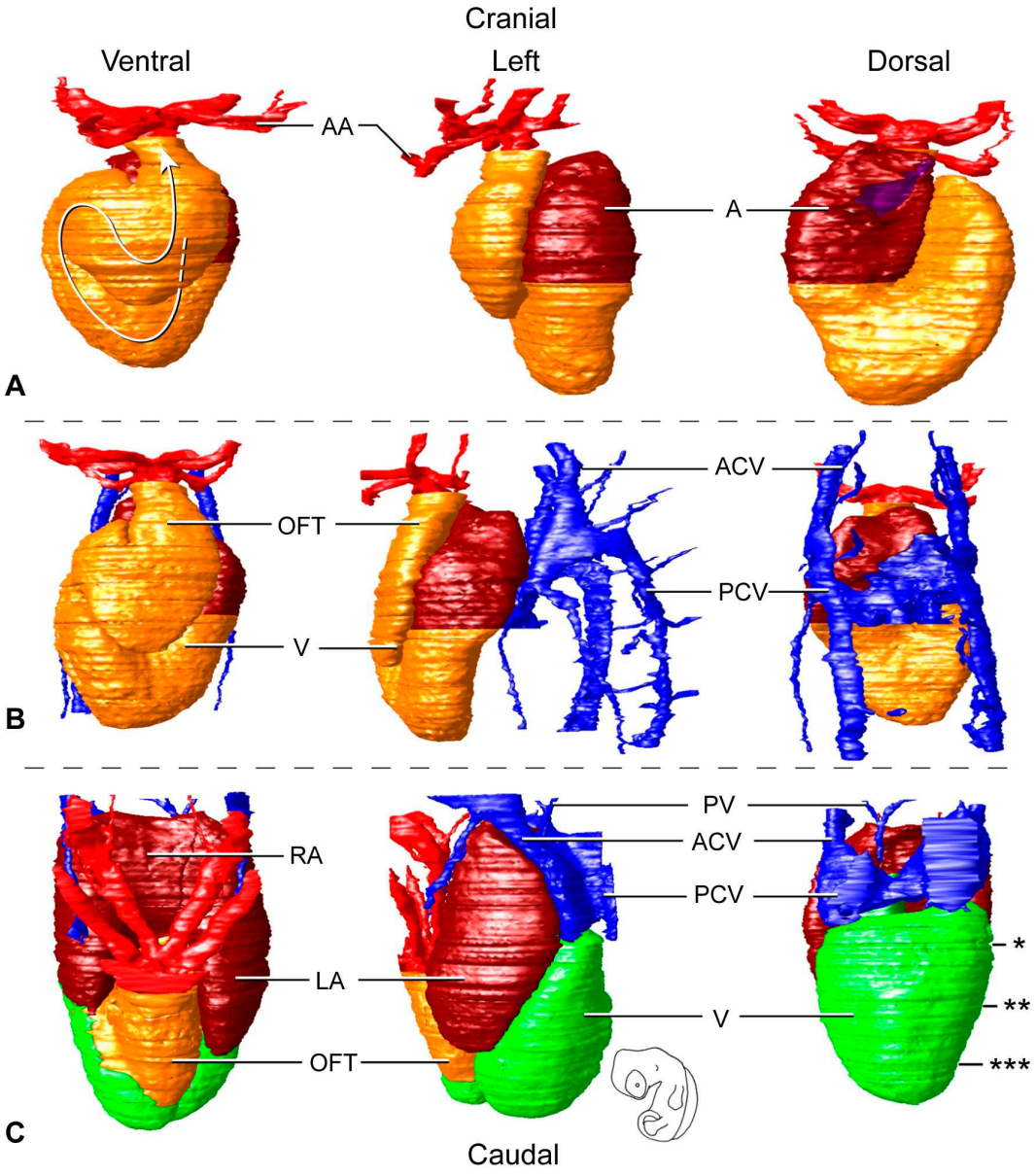

Figure 2.5. Overview of the 3D reconstructions of three *Emys orbicularis* embryos, Yntema stages 8 (A), 10 (B) and 15 (C), shown in ventral, left and dorsal view. The ventral view of A shows the direction of the looping of the heart tube (indicated by the arrow). The asterisks in the dorsal view of 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.

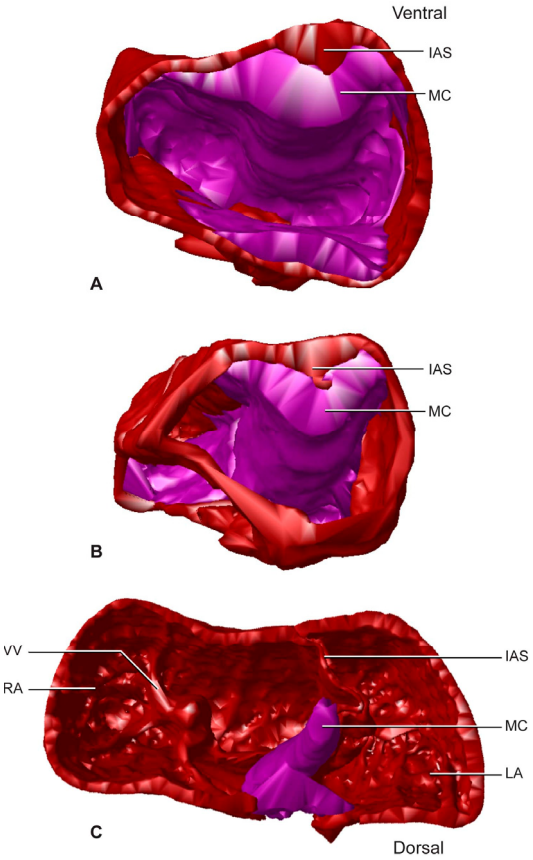

Figure 2.6. Cutaway views of the 3D reconstructions of three *Emys orbicularis* embryos, Yntema stages 8 (A), 10 (B) and 15 (C), showing (part of) the atria and mesenchymal cap in caudal view.

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 (explained below). In the outflow tract two distal and two proximal cushions are visible. Cardiac jelly is still present in the 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. 2.6A). 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.

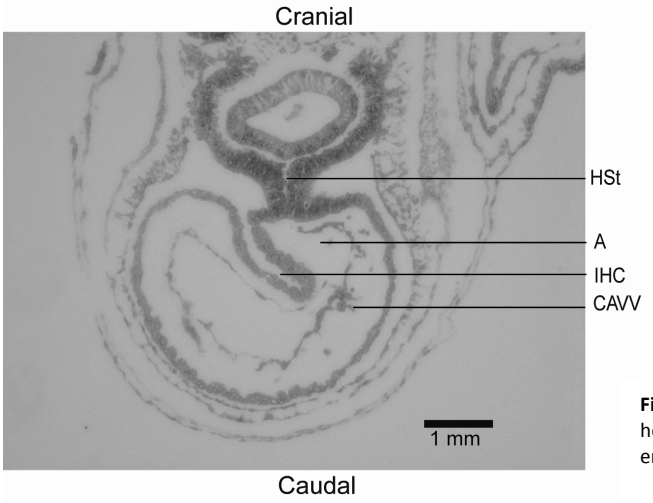

Figure 2.7. Section image of the heart of a stage 9 *Emys orbicularis* embryo in caudal view.

Stage 9 (Fig. 2.7)

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 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 (Fig. 2.5B and 2.6B)

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.2.5B). 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 two distal cushions were very clear.

In the common atrium the interatrial septum is forming (Fig. 2.6B). The dorsal mesocardium has not yet lumenised 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 have started to develop. Atrioventricular cushions are now distinct and will go

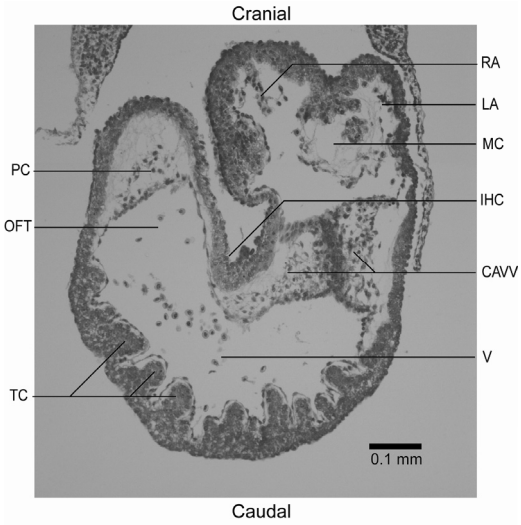

Figure 2.8. Section image of the heart of a stage 11 *Emys orbicularis* embryo in caudal view.

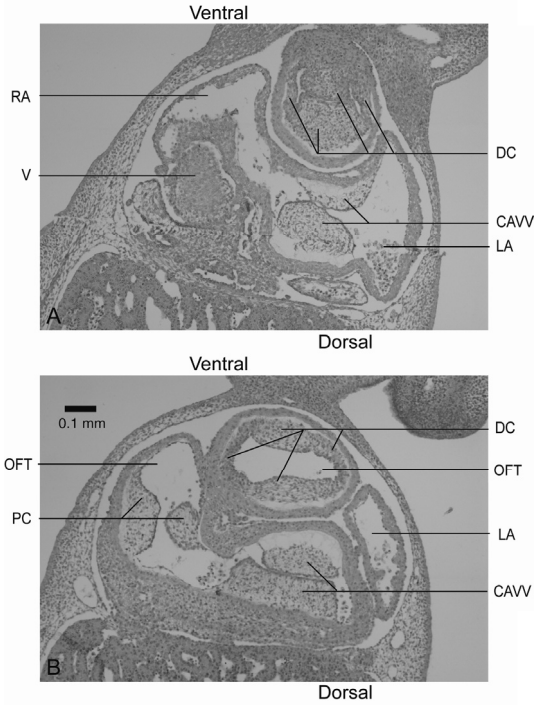

Figure 2.9. Section images of the heart of a stage 14 *Emys orbicularis* embryo in caudal view; A lies cranial to B.

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. 2.8)

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 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. 2.8. 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. 2.8).

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 aorto-pulmonary septum is not present at this stage.

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. 2.9A and 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. 2.9A and 2.B), but seem to be touching, although this could be due to the phase in which the cardiac activity has been arrested.

In the outflow tract six endocardial cushions can be seen, two proximal and four distal cushions (Fig. 2.9A and 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 (Fig. 2.10A, B and C; 2.11A, 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 Männer (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. 2.5C). 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 3D model of this stage, shown in (Fig. 2.12). 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 pulmonale* develops at the proximal end of the outflow tract and the horizontal septum is formed through bending of the tube (*cf.* 2.5).

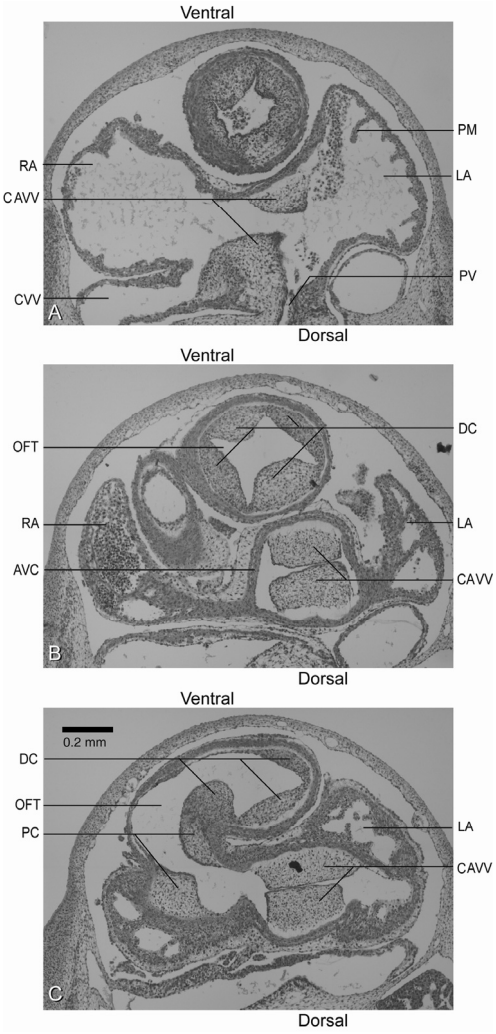

Figure 2.10. Section images of the heart of a stage 14-15 *Emys orbicularis* embryo in caudal view, progressing from cranial (A) to caudal (C).

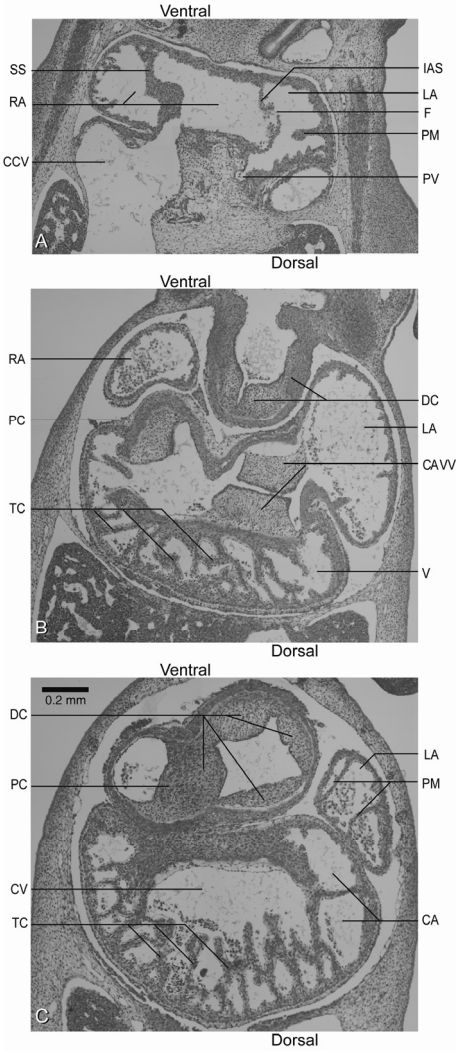

Figure 2.11. Section images of the heart of a stage 15 *Emys orbicularis* embryo in caudal view, progressing from cranial (A) to caudal (C).

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. 2.11B and C). One of these trabeculae is slightly bigger and its dorso-ventral base forms a septum at the apex of the ventricle.

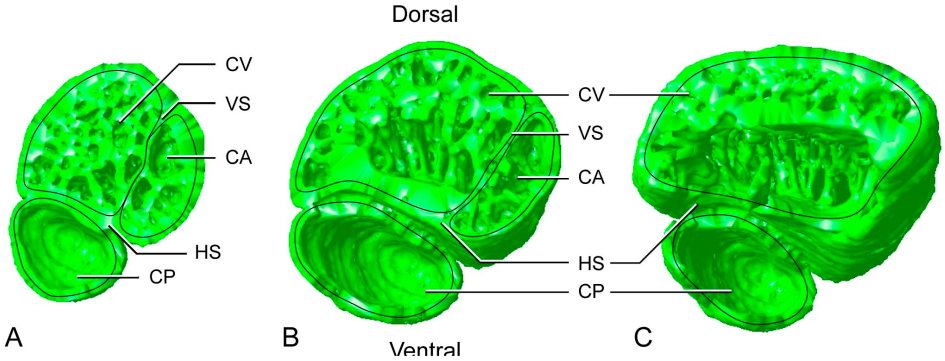

Figure 2.12. Cutaway views of the 3D reconstruction of a stage 15 *Emys orbicularis* embryo, showing only the ventricle in cranial view, at three craniocaudal levels. The asterisks in Fig. 2.3 correspond to the planes of section (* corresponds to A, ** to B and *** to C).

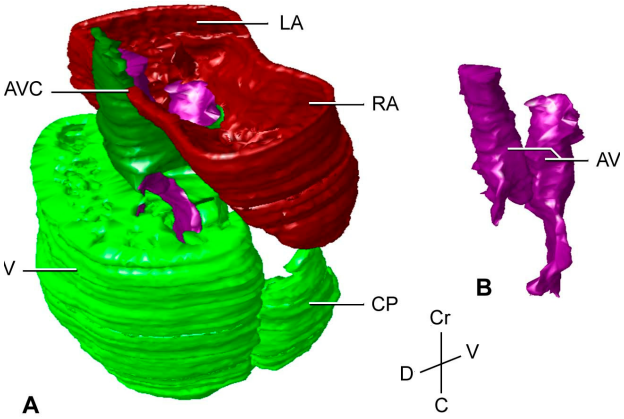

Figure 2.13. Cutaway view of the 3D reconstruction of the stage 15 *Emys orbicularis* embryo, showing parts of atria, ventricle and atrioventricular canal in a ventrolateral view in A. B shows the atrioventricular valves separately in the same plane of view.

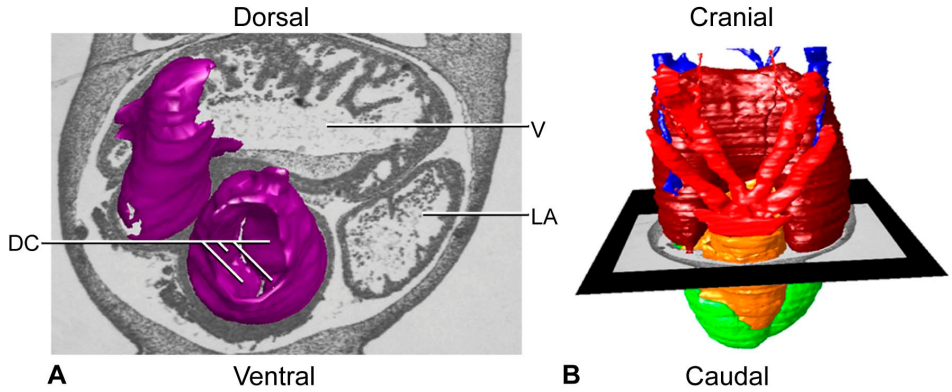

Figure 2.14. Cutaway view of the 3D reconstruction of the stage 15 *Emys orbicularis* embryo, showing the distal cushions in the outflow tract, together with a section image in A. The position of the section image in relation to the entire heart is shown in B.

This is the vertical septum, dividing the *cavum venosum* from the much smaller *cavum arteriosum* (Fig. 2.11C and 2.12). 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 touching but have not yet fused (Fig. 2.10B, 2.10C and 2.11B). The atria and ventricle are no longer in open connection, but are separated by an atrioventricular canal, in which the atrioventricular cushions are suspended (Fig. 2.10B, 2.13 and 2.15). 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, wich can be found at htpp://bio-imaging.liacs.nl/galleries/). In the outflow tract the four distal and the two proximal cushions are now clearly visible (Fig. 2.10B and 2.14). Two of the distal cushions are nearly fusing and the most distal of the proximal cushions is continuous with one of the distal cushions (Fig.2.10C and 2.11C); a more detailed account of this is given in section 3.3.3.

The atrial septum now extends three quarters of the total distance to the atrioventricular canal and its two limbs are easily distinguishable (Fig. 2.6C). 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. 2.11A).

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 3D models

In each of the 3D models the following structures have been labelled: atria, ventricle, outflow tract, aortic arches and endocardial cushion tissue (Fig. 2.5). In two models (Fig. 2.5B en 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 (µMRI) could have been a good candidate. However, a µMRI 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, favoured 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; *e.g.* by using automated tracing the atria and ventricle

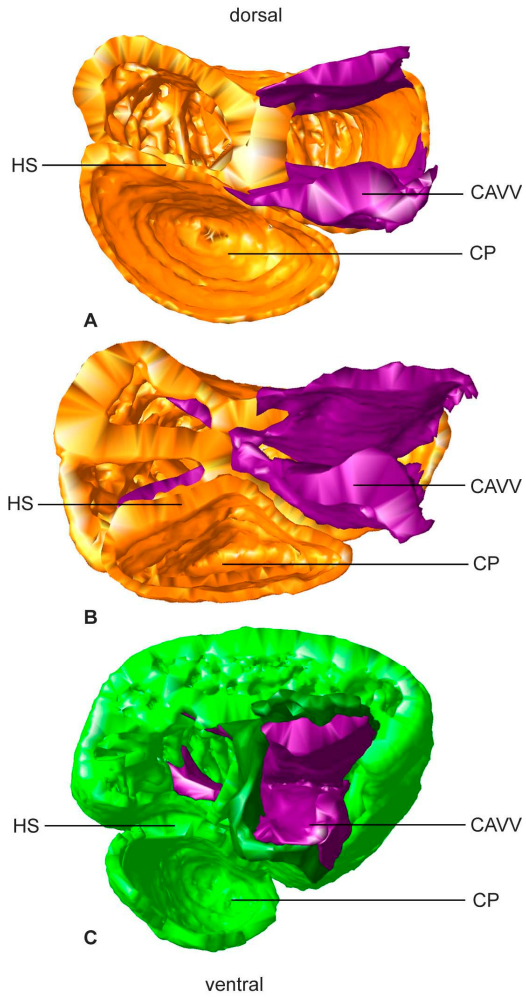

Figure 2.15. Cutaway views of the 3D reconstructions of stages 8 (A), 10 (B) and 15 (C) *Emys orbicularis* embryos, showing the caudal parts of the ventricle in a cranial view, including the ventricle and cushion tissue which will form the AV-valves. The division between the *cavum pulmonale* and the rest of the ventricle, by the developing horizontal septum, is visible.

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 possible using computerised 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. 2.5A), 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 colour 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. 2.5B) 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. 2.5C), built from 196 section images, the ventricle and outflow tract are clearly separated and have each been given a different colour (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), thereby 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 colour 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 labelled as a separate structure.

All section images, annotated domains and 3D representations are available on the website. Our interactive TDR-viewer application (see Fig. 2.4 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. 2.4C). 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 3D using the 3D 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. 2.4A). A 2D cursor can be added, showing the original section image (Fig. 2.4B). Switching from the 3D to the 2D view allows one to inspect of the annotated areas in that particular section image.

2.5 CONCLUSION

This study yields important new insights into the developmental origins of the horizontal septum in the turtle. From the 3D 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); this 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 3D reconstructions of stages 8 and 10 (Fig. 2.5A and B).

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 Figures 2.5, 2.12 and 2.15. 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.

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 Figures 2.5, 2.12 and 2.15 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 centres 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.*, 2001; Webb *et al.*, 2000).

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 3D 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 3D reconstructions. Our software, which allows high-resolution modelling and manual discernment between functionally separate structures, is therefore of great use. By enabling the readers to study the models in an interactive way, using our TDR-viewer application, we hope to go beyond a descriptive reporting of our findings, using merely static 2D images, and to

invite the readers to join the ongoing search for a more thorough understanding of cardiogenesis in the turtle.