Computerised Modelling for Developmental Biology

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CHAPTER 3. A VISUAL MODEL AT HIGH RESOLUTION: OUTFLOW TRACT DEVELOPMENT IN THE TURTLE SPECIES *EMYS ORBICULARIS*

> 'What *is* the use of repeating all that stuff,' the Mock Turtle interrupted, 'if you don't explain it as you go on? It's by far the most confusing thing *I* ever heard!'
>
> - Lewis Carroll, *Alice's adventures in Wonderland*

The next case study is an elaboration on the study of the turtle heart development presented in the previous chapter. The transition can be considered as zooming in to a higher level of magnification. Again a series of 3D models has been constructed, in order to study the spatial aspects of developmental anatomy, but while the previous chapter was based on models of the entire heart, the current study focuses on reconstructions of one particular element of the heart: the outflow tract. To this end, high resolution section images were acquired and used as the basis for the reconstructions, enabling a detailed study of the development of this structure. In this way new insights were gained concerning the development of the *cavum pulmonale*, the outflow tract and the outflow tract cushions. The case study exemplifies the use of high resolution modelling for developmental biology.

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3.1 INTRODUCTION

Numerous studies have examined development of the mammalian and chicken hearts. However, far less is known about the development of the reptilian heart (*i.e.* diapsids, excluding the birds). While several studies have addressed the anatomy and physiology of the adult reptilian heart, they often arrive at conflicting conclusions, mostly regarding ventricular septation (Webb *et al.*, 1971; Holmes, 1975; Johansen and Burggren, 1980; White, 1986; Burggren and Warburton, 1994; Bertens *et al.*, 2010). Studying the development of the anatomy and physiology is important in this respect because it can shed new light on these issues. Furthermore, with the current interest in phylogenetic relationships between reptiles, birds and mammals, and particularly the placement of the turtles (Chelonia) within reptiles, (Webb, 1979; Farmer, 1999; Lyson, 2011) studies in evolutionary development are highly relevant.

In the previous chapter a series of 3D reconstructions was presented of different developmental stages in the cardiogenesis of the turtle *Emys orbicularis*. The early looping of the turtle heart was described, along with the finding that the double loop gave rise to the *cavum pulmonale*. This hypothesis has implications for the positioning of the valves derived from the distal cushions in the outflow tract, the septation of this tract and the roles of the endocardial cushions and aortopulmonary septum in this process. Furthermore, in the adult heart the ventricle is functionally three-chambered and this has led to the conventional assumption that the presumptive ventricle becomes partially divided into three cavities. Interestingly, this arrangement of three ventricular *cava* strongly resembles the ventricular morphology of Squamata (snakes and lizards) and differs substantially from the partitioned ventricle of Archosauria (crocodilians and birds). However, our findings raise the possibility that the turtle ventricle may not be three-chambered in origin, but in fact two-chambered, with the *cavum pulmonale* arising from the outflow tract. This would represent a type of cardiac septation unknown in any other vertebrate, meriting further investigation of the outflow tract development.

In the current chapter, the development of the outflow tract of the turtle heart is examined using 3D reconstructions, assembled from histological sections of the turtle *Emys orbicularis* (the European pond turtle), at different stages of embryonic development. Subsequently, the findings are discussed in relation to current theories on chelonian and reptilian outflow tract development.

3.1.1 Current knowledge of outflow tract development in reptiles

Chapter 2 provides an overview of the developmental anatomy of the turtle heart (section 2.2). Here, further aspects of ouflow tract (OFT) development in reptiles are

discussed. Note that 'reptiles' is used here with the knowledge that it is a paraphyletic group, and that the placement of the turtle is controversial. However, it is clear that the crocodile and chick (Archosauria) among reptiles have completely septated ventricles, while the Chelonia (turtles and tortoises) and the Squamata (lizards and snakes) have a partially septated ventricle with three cavities (*cavum arteriosum*, *venosum* and *pulmonale*). We will now further discuss the similar pattern of cardiac development between Chelonia and Squamata.

In the adult heart of these species, three arterial trunks leave the ventricle: right and left aortae and the pulmonary trunk. These arise from the septation of a common OFT. At the root of each of the trunks in the formed heart, semilunar valves, with two leaflets, are present. The brachiocephalic artery branches off from the right aortic arch and gives off the subclavian and carotid arteries.

The connection of these arterial trunks to the ventricular outlet arises from the septation of the OFT and the development of valves, guarding the roots of the trunks. These developmental processes are not yet fully understood in the reptilian heart. It is, however, clear that these processes differ significantly from the mammalian situation. In both the mammalian and the reptilian heart, a curvature arises in the developing OFT, described for the human heart as a 'bayonet bend' by Orts Llorca *et al.* (1982) or a 'dog-leg bend' by Webb *et al.* (2003). This bend demarcates the transition between proximal and distal components of the OFT. Previous researchers have referred to these structures as: (i) conus or bulbus and (ii) truncus, respectively. In the turtle, the proximal component of the OFT comes to lie ventrally to the ventricle and forms the *cavum pulmonale* (Bertens *et al.*, 2010). This has important implications for the development of the valves of the arterial trunks and the septation of the OFT.

Both the proximal and distal components of the OFT contain endocardial cushions. The distal endocardial cushions give rise to the leaflets and sinuses of the semilunar valves, which are located during development at the dog-leg bend. The number of cushions in the distal and proximal components of the OFT has been shown to differ between taxonomic groups and no consensus exists as yet concerning the relations between the cushions in different species (Qayyum *et al.*, 2001; Webb *et al.*, 2003; Okamoto *et al.*, 2010). Goodrich (1958), Shaner (1962) and Hart (1968) all give accounts of OFT development in the reptilian heart. The authors agree that four cushions are found in the distal component of the OFT and two in the proximal component. Both in descriptions and 3D reconstructions we follow the numbering of the cushions used by Shaner (1962) and Hart (1968); one of the distal cushions is markedly bigger and this cushion is designated number DC1. Numbering continues in clockwise fashion when looking at a rostral view. The proximal cushions are designated by Hart (1968) as PCA and PCB (corresponding to proximal ridges 1 and 4 in Shaner, 1962).

Hart (1968) states that DC1 and DC3 arise at a later stage than DC2 and DC4. This temporal differentiation between the cushions is relevant in relation to the different distal and intercalated cushions observed in mammals and birds. DC1 and DC4 are said to merge very early in development at their proximal end with PCA and PCB, respectively (Shaner, 1962; Hart, 1968). The location of the proximal cushions and their fusion with the distal cushions in the reptilian heart, as described by Shaner (1962) and Hart (1968), differs from the situation observed by Qayyum *et al.* (2001) in the chick heart; in early development three distal cushions arise in the avian heart, later followed by two intercalated cushions. Of the three initial cushions the first two to develop correspond to DC1 and DC3 in reptiles. Furthermore, DC1 is seen to merge with PCA, but unlike in the reptilian OFT, DC3 does not merge with PCB.

In addition to the endocardial cushions, the aortopulmonary septum (AP septum) is said to play an important role in septating the distal end of the OFT and the allocation of the cushions between the resulting arterial trunks (Goodrich, 1958; Shaner, 1962; Hart, 1968). The AP septum is formed by migratory neural crest cells, and is visible as dense ectomesenchymal tissue on histological sections (Hart, 1975; Bartelings and Gittenberger, 1989; Qayyum *et al.*, 2001; Webb *et al.*, 2003). The main body of the septum runs through the distal end of DC1 in the direction of the heart. It gives off two lateral prongs and is described by Shaner (1962) as being Y-shaped. One of the prongs extends from DC1 in between DC2 and DC3, the other one in between DC3 and DC4. In this way each of the developing arterial trunks receives one complete distal cushion and a fragment of DC1. These cushions form the valve leaflets providing each of the three arterial trunks with a bicuspid semilunar valve (Shaner, 1962). So far this pattern of reptilian septation has been illustrated only in schematic drawings of histological sections (Goodrich, 1958; Shaner, 1962; Hart, 1968; Holmes, 1975). No clear 3D visualisation has been presented in literature. Our 3D reconstructions of the turtle OFT provide a more informative view of the septum and the cushions in the dividing distal OFT.

3.2 MATERIALS AND METHODS

Preparation of the embryos

Five developmental stages were selected, Yntema stages 8, 15, 16+/17, 19 (Yntema, 1968) and an adult stage (18 months). Of these, the histological sections of the stage 8 and stage 15 embryos have also been used in reconstructing models presented in chapter 2; here the same histological sections have been used, but new reconstructions have been made, of different structures.

The embryos were selected for the presence of significant developmental aspects of the OFT. Gravid females were collected under license from the French government. Standard injection with oxytocin was used to induce laying. Eggs were places 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. The material examined is summarized in table 3.1.

Yntema (1968) stage	Embryo code	Plane of section	Shown in figure
8	CP245	transverse	3.2A; 3.3E
15	CP46	transverse	3.1A-C; 3.2B,C
16+/17	CP450	transverse	3.4F,G
19	CP436	transverse	3.1D,F; 3.4A-E
adult, 18 months	CP434	coronal	3.3A-D; 3.1G

Table 3.1. Overview of embryos used in the study.

Acquisition of section images

Sections containing the outflow tract were digitized using the microscope and camera setup described in 2.3. Section images were captured with our inhouse software 3D acq, version 2.0 (Verbeek and Boon, 2002); using this software an image stack was created for each of the specimens. Using the video overlay function 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.

Using the metadata database, a second high resolution image stack was obtained for two of the hearts. 3D acq allows the user to construct section images of higher resolution and at a higher microscopic magnification from separate image tiles which are digitally stitched together. Using the frame grabber coordinates stored in the metadata for the original images, the software can automatically relocate the right position of a previously captured image on a slide. Subsequently, a set of image tiles (number, magnification and overlap between tiles are set by the user) is acquired which, when stitched together, form a high resolution image of the original image. In this way high resolution images were obtained at 16 x magnification, constructed of 4 x 4 image tiles, with 32 pixels overlap. Metadata for these high resolution images was added to the original XML acquisition database and the images were stored in PNG format.

Construction of 3D models

From the obtained section images 3D reconstructons were produced with the inhouse software TDR-3Dbase (Verbeek and Boon, 2002; Bertens *et al.*, 2010). All relevant anatomical structures in the images were traced and labeled with different colors and names. Using these annotations the structures were visualised in surface models, derived through automated triangulation (details on the method can be found in 2.3). The models are provided via internet using our 3D model browser application TDR-viewer (Potikanond and Verbeek, 2012) and can be found here: http://bio-imaging.liacs.nl/galleries/. The viewer allows the user to inspect the annotations and visualisations both in 2D (*i.e.* the histological section images) and 3D. The 3D views are interactive and allow for active exploration of the model information; the user can select anatomical structures to be displayed and a 3D visualisation of this selection is instantaneously rendered.

Anatomical conventions

For the left and right aortic arches leaving the ventrical, two different nomenclatural conventions have arisen, one based on the position at the base of the arterial arches, *i.e.* the point where the arches connect to the ventricle (Shaner, 1962), and the other based on their position further away from the ventricle, after the point of spiralling (Langer, 1894; Hochtstetter, 1901; Goodrich, 1958). Inconsistent identification of structures, as a result of these different terminologies, is exemplified by the description of Holmes (1975), who follows the naming convention according to Goodrich (1958) in his written account and his illustrations of the entire heart, but includes transverse section drawings taken from Shaner (1962), in which left and right aortae are reversed, resulting in inconsistencies within the publication itself. Here (as in the previous chapter), we follow the convention of naming the structures based on their position further away from the heart, since this coincides with the naming of *e.g.* the venae cavae and the left and right pulmonary vein. Therefore, the carotico-systemic arch is identified here as the right aorta and the systemic arch is identified as the left aorta.

3.3 RESULTS

Based on histological sections, we have reconstructed the outflow tract structures in five hearts of the turtle *Emys orbicularis*, at different developmental stages. Using these reconstructions we have examined 1) the way in which septation of the OFT is related to the looping of the heart, 2) the development and fusion of the cushions and 3) the growth and shape of the three-pronged AP septum. We provide all 3D reconstructions discussed in this chapter on the following website: http://bio-imaging.liacs.nl/galleries/. There, the reader can use our 3D-browser application, TDR-viewer (Potikanond and

Verbeek, 2012) to study the modelled anatomy, both as a whole and in separate structures.

First, we address the developmental relation between outflow tract and *cavum pulmonale*, and the implications this has for the positioning of the cushions and the septation of the proximal OFT. This is followed by a detailed description of the development and fusion of the distal, proximal and atrioventricular cushions. Finally, a description is given of the arterial arches and the separation of their roots by the AP septum.

3.3.1 Developmental origin of the cavum pulmonale and its relation to the OFT cushions

In the previous chapter, we described the early looping of the turtle heart and suggested that this double looping gave rise to the *cavum pulmonale*. This finding has implications for the positioning of the valves derived from the distal cushions. It has been proposed that in the mammalian heart the proximal cushions take part in partitioning the ventricular outflow tract, while the distal cushions develop the aortic and pulmonary roots and give rise to the valves guarding these vessels (Van Mierop, 1979). However, theories stating that the valves arise from distal cushions have to account for the shifting position of these valves from their location in the OFT to the arterial roots. Several solutions have been proposed for the mammalian heart, including migration of the aortic root towards the left ventricular outlet (Goor *et al.*, 1972) and absorption of the proximal component of the OFT into the ventricle, resulting in relocation of the aortic root (Anderson *et al.*, 1974; Webb *et al.*, 2003).

The situation in the turtle heart is significantly different. Due to looping of the heart tube at an early developmental stage, the proximal OFT comes to lie ventral to the ventricle (see 2.4). The dorsal wall of the proximal OFT fuses with the ventral wall of the ventricle, forming the horizontal septum (Fig. 3.1C). Through this looping, the proximal component of the OFT develops into the *cavum pulmonale* (CP) and comes to lie in front of the right cavity in the dorsal part of the ventricle, the *cavum venosum* (CV). The observation that the CP derives from looping of a component of the OFT is supported in the current study by the fact that both proximal cushions were found in the CP (Figs. 3.1A and B). The 3D reconstructions provide us with a clear insight into the spatial positioning of these cushions. At Yntema (1968) stages 8 and 15 one of the proximal cushions was seen straddling the horizontal septum, while the other was located in the cranial roof of the CP (Figs. 3.1B and 2). Therefore, we confirm the proximal outflow tract origin of the *cavum pulmonale* and its positional identity as being derived from the proximal component of the OFT.

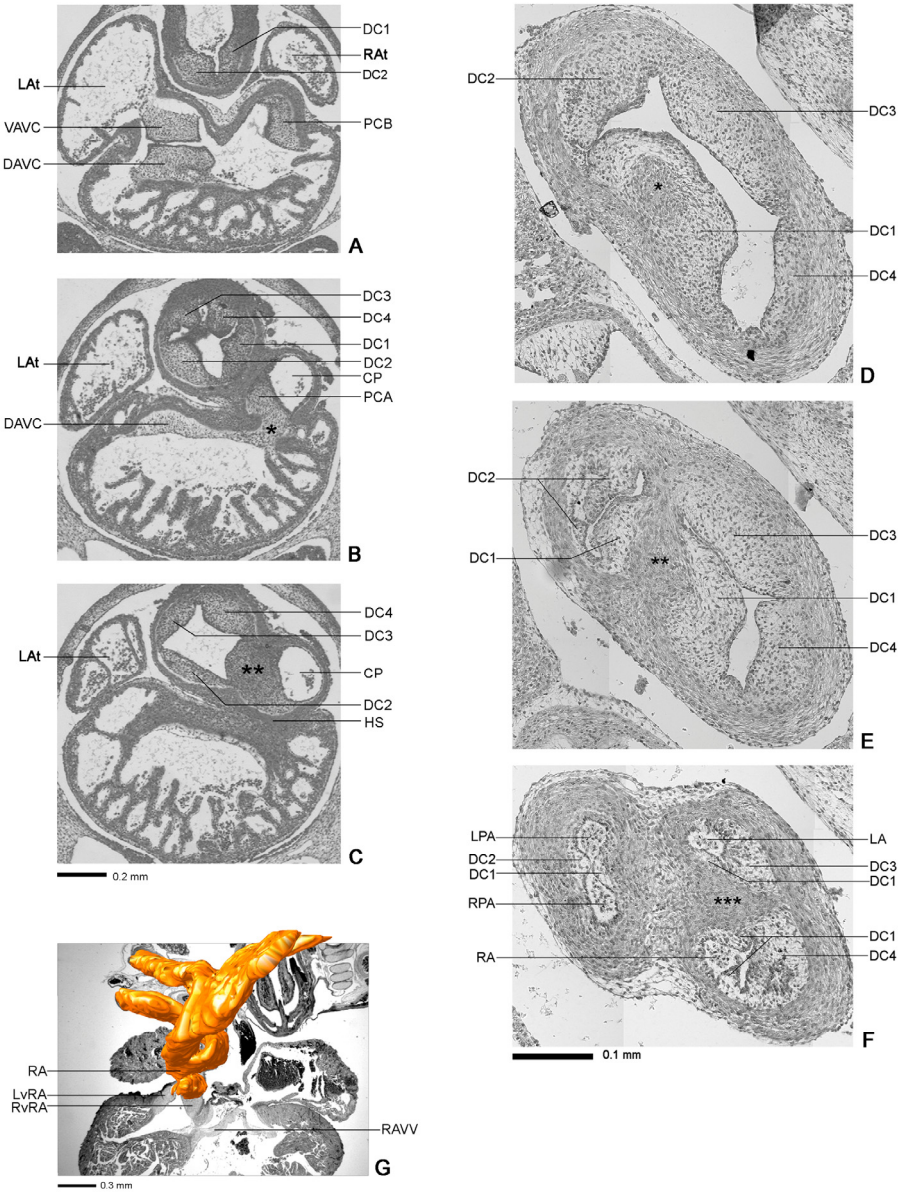

Figure 3.1. Section images of Yntema (1968) stages (A-C) 15, (D-F) 19 and (G) the adult stage (including the 3D reconstruction of the right aortic arch). Abbreviations: CP, *cavum pulmonale*; DAVC, dorsal atrioventricular cushion; DC, distal cushion; HS, horizontal septum; LA, left aorta; LAt, left atrium; LPA, left pulmonary artery; LvRA, left valve of right aorta; PC, proximal cushion; RA, right aorta; RAt, right atrium; RPA, right pulmonary artery; RvRA, right valve of right aorta; VAVC, ventral atrioventricular cushion. The fusion of PCA and the dorsal AV cushion is indicated in B by *; the fusion of DC1 and PCA is indicated by ** in C. The prongs of the AP septum are indicated in D (*, main root), E (**, prong between DC2 and DC3) and F (***, prong between DC3 and DC4).

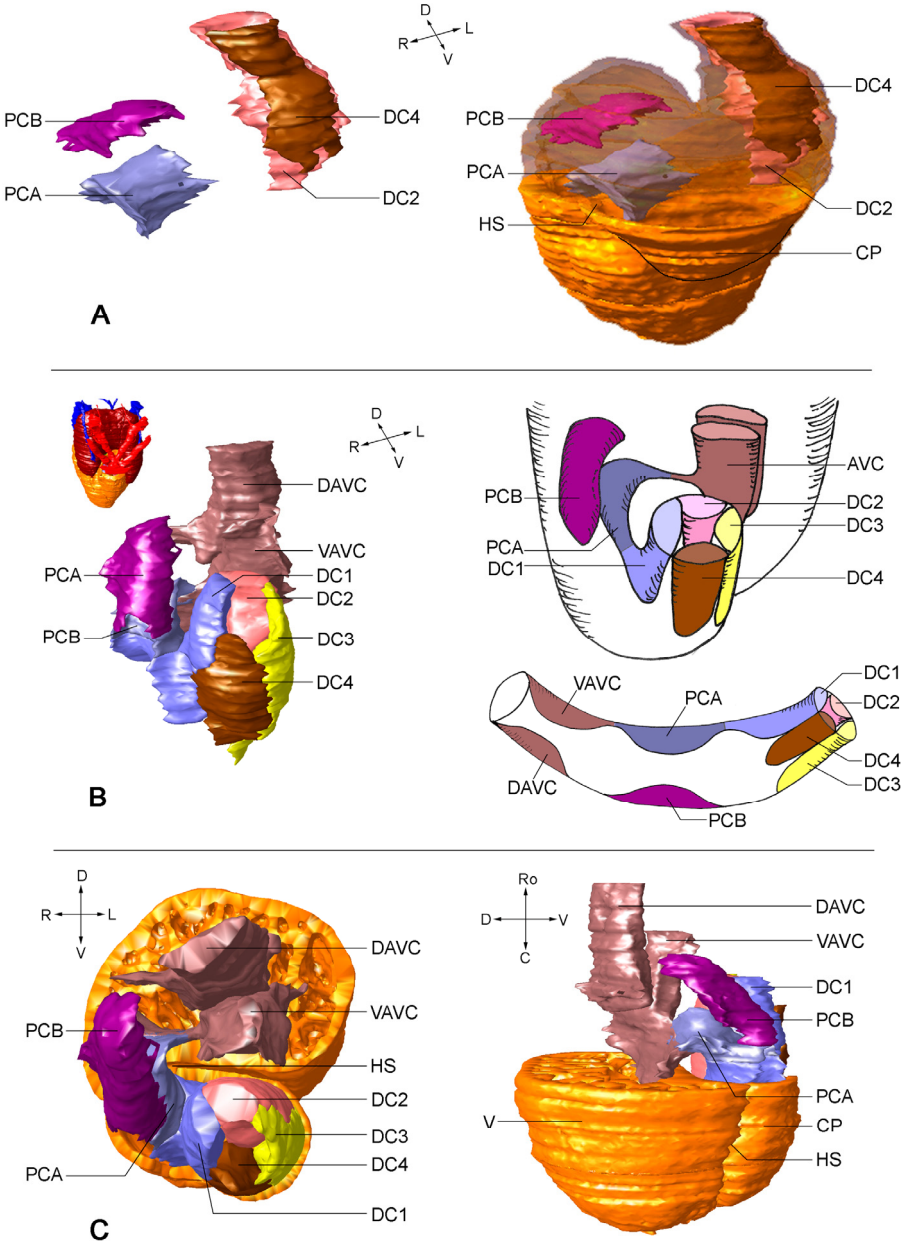

Figure 3.2. Positioning of the distal and proximal OFT cushions. (A) stage 8 in ventrolateral view, isolated (left) and *in situ* with OFT and ventricle, partially transparent (right); (B) stage 15 in ventrolateral view, isolated (left) and schematically represented to clarify the fusion of DC, PC and AVC; (C) stage 15, *in situ* in the caudal half of the ventricle, in rostral view (left) and right lateral view (right). Abbreviations: CP, *cavum pulmonale*; DAVC, dorsal atrioventricular cushion; DC, distal cushion; HS, horizontal septum; PC, proximal cushion; V, ventricle; VAVC, ventral atrioventricular cushion.

The developmental origin of the CP from the proximal OFT has two major implications for the OFT and its cushions. The first concerns the positioning of the distal cushions in the OFT. Unlike the mammalian situation, the distal cushions do not need to be actively relocated within the OFT in order to become aligned with the ventricular outlet. Due to the inclusion of the proximal OFT in the functional ventricle, the distal cushions, and therefore the arterial valves arising from them, come to lie at the outlet of the functional adult ventricle, *i.e.* the distal border of the CP. Meanwhile, the proximal cushions come to lie on the junction between *cavum pulmonale* and the rest of the ventricle, *i.e.* within the functional ventricle. The relative shift in position of the distal cushions, due to the looping, is very clear when comparing the reconstructions of the OFT at stages 8 and 15 (Fig. 3.2). This constitutes a major difference between outflow tract development in mammals and birds on the one hand, and reptiles on the other.

The second implication concerns the septation of the proximal OFT. The separate ventricles of the mammalian heart each become connected to a separate OFT, requiring complete septation of the initial proximal OFT. We found that the proximal component of the turtle OFT does not become divided, since it develops into the undivided CP. The developmental origin of the CP from the OFT, and the two discussed implications, constitute important new insights into the general theory of reptilian heart development.

3.3.2 Development and fusion of distal and proximal cushions

While Hart (1968) states that DC2 and DC4 arise earlier in reptilian development than the other cushions, Qayyum *et al.* (2001) show that DC1 and DC3 develop first in the avian heart. Our findings correspond with those of Hart (1968). At Yntema stage 8, two proximal cushions were found; only two distal cushions were present, and neither of these could be identified as DC1, due to their position in relation to PCA (Fig. 3.2A). We identify these latter two distal cushions as DC2 and DC4, confirming the reported difference between avian and reptilian OFT development. At Yntema stage 15, DC1 and DC3 had developed; at stage 19, DC1 was more prominent than the other three cushions. Several authors describe the fusion of two of the distal cushions with the two proximal cushions in the mammalian heart (Shaner, 1962; Icardo, 1990; Okamoto *et al.*, 2010), as well as in the avian and reptilian heart (Shaner, 1962). At Yntema stage 8, we found the distal cushions and proximal cushions to be clearly distinct. These cushions stayed distinct from the proximal cushions at all stages examined. This is in accord with previous descriptions of DC2 and DC4 (see 3.1), neither of which is described to fuse with a proximal cushion. At Yntema stage 15, we observed a fusion between DC1 and PCA, but no fusion of PCB with any of the distal cushions was found (Figs. 3.2B and C). At stage 16+/17 the fusion between PCA and DC1 was such that the individual cushions

could no longer be distinguished; PCB and DC4 lay much closer than at earlier stages and were nearly touching, but not fusing. At stage 19, PCB and DC4 seemed to fuse with each other, but this could not be confirmed with certainty and will need to be examined further.

At Yntema stages 8 and 15, PCA was seen cranially to PCB and both were in the same horizontal and sagittal planes. At stage 8, neither PCA nor PCB were continuous with the AV cushions, whereas at stage 15 PCA had become continuous with the ventral AV cushion (Figs. 3.2B and C). At the adult stage, tissue at the base of the right AV valve could be seen touching the base of the right aortic leaflet deriving from DC1 (Fig. 3.1G). At all stages examined, PCB was clearly distinct from the AV cushions, which is in conflict with the description by Shaner (1962).

Thus, according to our findings, in the turtle *Emys orbicularis* DC2 and DC4 develop before DC1 and DC3. DC1 fuses with PCA while PCB remains separate from the distal cushions at least until Yntema (1968) stage 16+/17. PCA fuses with the ventral AV cushion between stage 8 and 15, while PCB remains distinct from the AV cushions. This fusion of one distal cusion, one proximal cushion and one AV cushion into a long ridge is also seen in the chick heart (Qayyum *et al.*, 2001). However, the distal cusion that contributes to this fused mass is early-developing in the chick (Qayyum *et al.*, 2001), but develops later in the turtle.

3.3.3 Development of the arterial arches and semilunar valves by means of the aortopulmonary septum

The following description is based on observations in the reconstruction of the adult heart (CP434). The pulmonary artery is positioned ventrally, overlying the *cavum pulmonale*. It bifurcates close to the ventricular outlet into left and right pulmonary arteries. Dorsal to this, the left aortic trunk overlies the *cavum venosum*, and dorsal to this, the right aorta is also continuous with the *cavum venosum*. Close to its origin, the right aorta bifurcates into the aorta proper and the brachiocephalic artery. The latter splits into four smaller arteries: the left and right subclavian arteries ventrally, and dorsal to these, the left and right carotid arteries (Figs. 3.3A-D). The early development of these aortic arches can be seen in the reconstruction of stage 8 (Fig. 3.3E).

Semilunar valves develop at the roots of the arterial trunks, and are derived from the distal endocardial cushions. The allocation of these distal cushions over the arterial roots is brought about by the aortopulmonary septum growing proximally along the OFT and separating the cushions. Up to now, no clear illustrations have been presented in literature of the three-dimensional structure of the reptilian AP septum. Accounts of the AP septum in reptiles have been limited to descriptions, accompanied

by images of histological sections and schematic 2-dimensional drawings (Goodrich, 1958; Shaner, 1962; Hart, 1968).

We have constructed 3D models of this septum at two relevant developmental stages, 16+/17 and 19. These clarify the spatial distribution of what we assume to be neural-crest mesenchyme, of which the prongs of the septum are constituted. For birds and humans the division between the aorta and pulmonary artery has been illustrated as a trouser shape (Bartelings and Gittenberger, 1986; Qayyum *et al.*, 2001), *i.e.* a ridge of tissue from which two prongs extend proximally. However, in agreement with Webb *et al.* (2003) we do not find a clear bar-shaped septal ridge distal to the prongs. The neural-crest derived tissue can be seen to form two wedges between the vessels at the points of their bifurcation. Distal to this point, the arteries are separated by myocardial tissue which is seen to move in between the arches from the outer myocardial sleeve (Figs. 3.1E and F).

As can be seen at stage 19 (Figs. 3.1D-F and 3.4A-E) the mesenchymal tissue extends in three directions; the main root invades DC1 from its distal end and gives off two further prongs. Shaner (1962) describes a Y-shaped septum, but does not specify the direction of the three legs of this Y-shape. From our reconstructions, both secondary prongs can be seen attaching to the main root in a perpendicular direction, *i.e.* in the transverse plane (Figs. 3.1D-F). As such, the tissue resembles a three-pointed caltrop, with each of the prongs perpendicular to the other two (Figs. 3.4D and E). One of these prongs splits off more proximally and this prong divides the aortic arches from the pulmonary arch; together with the main root this constitutes the so-called aortopulmonary septum. It extends from the root between DC2 and DC3, and thereby splits off the pulmonary artery, which therefore contains a segment from DC1 and the complete DC2. It corresponds to the prong extending between DC2 and DC3 in the avian OFT (Qayyum *et al.*, 2001). The position of this split leaves the pulmonary artery furthest removed, both in the ventrodorsal and in the rostrocaudal planes, from the transition between OFT and ventricle, over the free margin of the horizontal septum. This represents the definitive conformation of the adult turtle arterial trunks (Figs. 3.3A-D).

The second prong splits off more distally. It extends between DC3 and DC4 and splits the common aortic channel into left and right aortic arches. This constitutes the so-called aortic septum, which is not found in the avian OFT (Shaner, 1968). The position of the split locates the root of the left aorta just dorsal and to the right of the pulmonary artery; the left aorta contains a segment from DC1 and the complete DC3. The right aorta in turn is located dorsally and to the left of the left aorta and contains a segment from DC1 and the complete DC4.

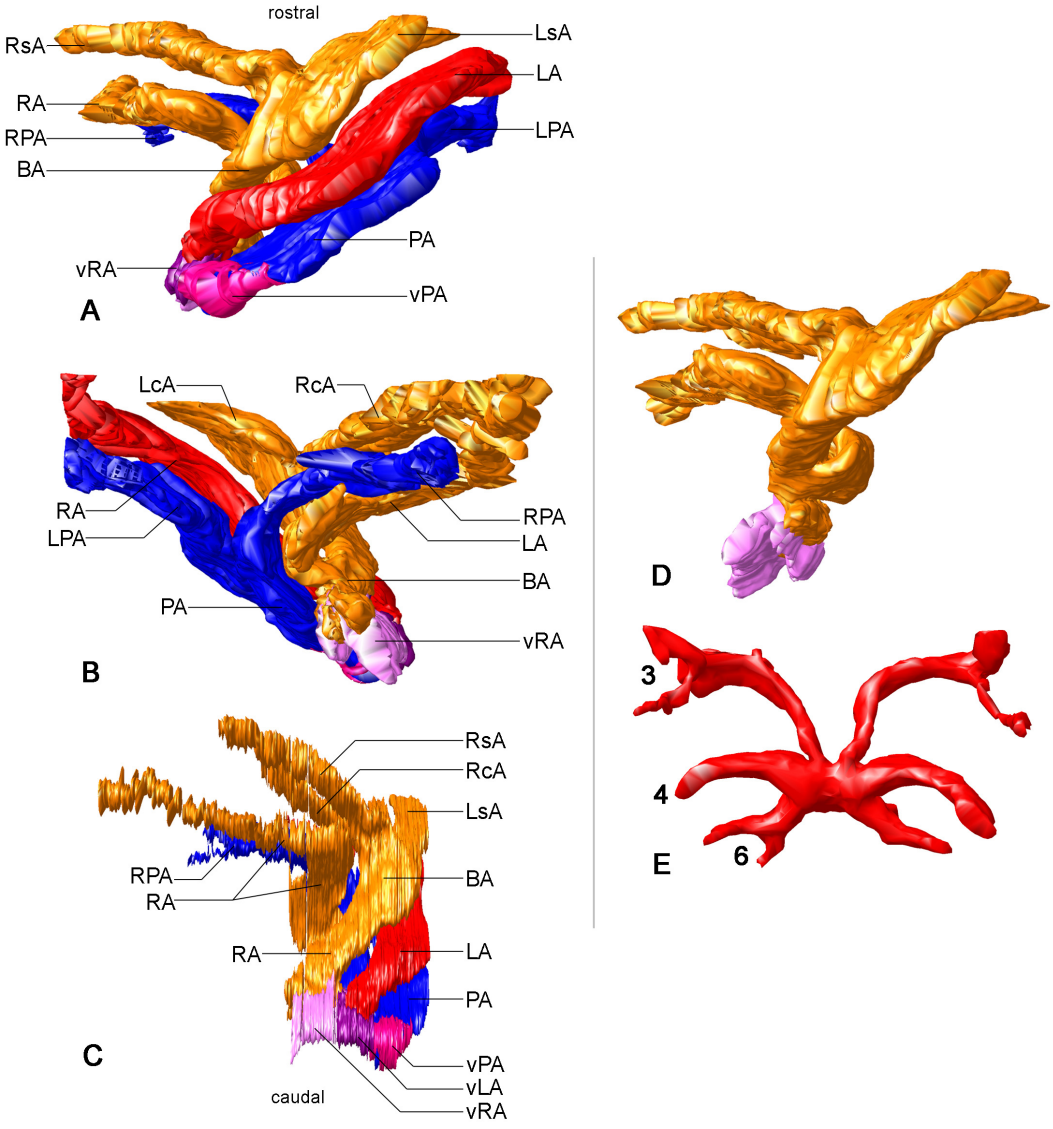

Figure 3.3. Overview of the arterial arches at their origins from the ventricle. (A-D) arterial trunks and their semilunar valves in a reconstruction of the adult heart in ventral (A and D), dorsal (B) and right lateral (C) views; (E) aortic arches 3, 4 and 6 in a reconstruction of stage 8, in ventral view. Abbreviations: BA, brachiocephalic artery; CP, *cavum pulmonale*; CV, *cavum venosum*; LA, left aorta; LcA, left carotid artery; LPA, left pulmonary artery; LsA, left subclavian artery; PA; pulmonary artery; RA, right aorta; RcA, right carotid artery; RPA, right pulmonary artery; RsA, right subclavian artery; v, valves.

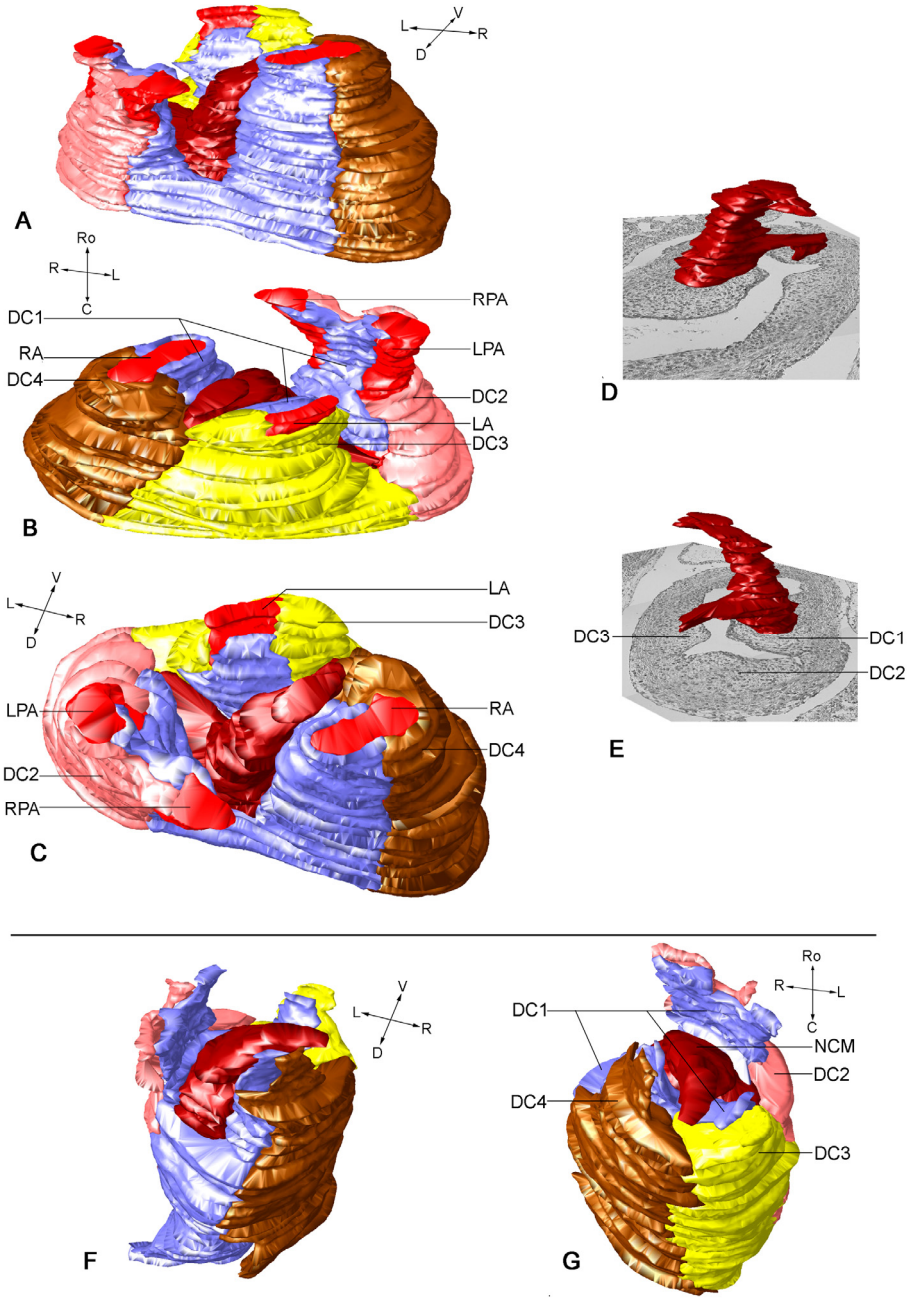

Figure 3.4. Division of the distal cushions by neural crest derived mesenchyme. (A-E) reconstruction of a stage 19 heart showing the aortopulmonary septum and the separation of the distal cushions; (F-G) reconstruction of stage 16+/17 heart showing separation of the distal cushions. Abbreviations: APS, aortopulmonary septum; DC, distal cushion; LA, left aorta; LPA, left pulmonary artery; NCM, neural crest derived mesenchyme; PC, proximal cushion; RA, right aorta; RPA, right pulmonary artery.

3.4 CONCLUSION AND DISCUSSION

We have constructed five 3D models of the outflow tract at different developmental stages in the turtle species *Emys orbicularis*. Examination of these models has yielded significant new insights into the development of the turtle outflow tract. This extends the account, presented in the previous chapter, of the origin of the ventricular cavities or *cava* in this species, by adding additional stages and using high resolution modelling.

We find that the *cavum pulmonale* arises from the proximal OFT segment in the early embryo, although in the adult it is functionally one of the three ventricular cavities. Surprisingly, therefore, this ventricular cavity is developmentally derived from part of the outflow tract that has become displaced into the ventricular region by the looping pattern of the primary heart tube. Therefore the *cavum pulmonale* is functionally, but not developmentally a ventricular structure.

This has important consequences for other aspects of cardiac development. One of these is that the proximal OFT does not become divided, in contrast to the mammalian and avian proximal OFT. Second, the distal OFT cushions do not undergo an active positional shift, but come to lie at the ventricular outlet as a result of the inclusion of the proximal OFT in the functional adult ventricle. Together, these features represent a type of cardiac development not previously described in any vertebrate.

The study presented here is based on 3D reconstructions of histological sections, using high resolution section images. These images were acquired and annotated with our in-house software, and the models are provided to the reader at http://bio-imaging.liacs.nl/galleries/. Schematic drawings can confuse due to their intrinsic level of abstraction. In contrast, 3D reconstructions are more true to nature and allow one to examine a 3-dimensional structure from different viewpoints, thereby clarifying the particular spatial characteristics of the structure. Secondly, by digitally stitching image tiles together high resolution images were obtained, which present a higher microscopic magnification with a higher resolution. Changing solely the magnification or the resolution cannot yield a higher level of detail while retaining the same field of view.

Finally, by allowing the reader full access to both the histological sections and the 3D reconstructions, we hope to optimise the knowledge gained from these models. The viewing applet, available via the website, lets the user browse through the 2-dimensional section images in the image stack, with the option to show our annotations of the relevant structures. In the 3D view, the annotated structures can be examined separately or together and from all viewpoints. In this way the user can verify the findings and further investigate aspects of the outflow tract and relate these to our 3D models of the entire heart, discussed in chapter 2.