Morphological Analysis of the Hagfish Heart. II. The Venous Pole and the Pericardium

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ABSTRACT The morphological characteristics of the venous pole and pericardium of the heart were examined in three hagfish species, *Myxine glutinosa*, *Eptatretus stoutii*, and *Eptatretus cirrhatus*. In these species, the atrioventricular (AV) canal is long, funnel-shaped and contains small amounts of myocardium. The AV valve is formed by two pocket-like leaflets that lack a papillary system. The atrial wall is formed by interconnected muscle trabeculae and a well-defined collagenous system. The sinus venosus (SV) shows a collagenous wall and is connected to the left side of the atrium. An abrupt collagen-muscle boundary marks the SV-atrium transition. It is hypothesized that the SV is not homologous to that of other vertebrates which could have important implications for understanding heart evolution. In *M. glutinosa* and *E. stoutii*, the pericardium is a closed bag that hangs from the tissues dorsal to the heart and encloses both the heart and the ventral aorta. In contrast, the pericardium is continuous with the loose periaortic tissue in *E. cirrhatus*. In all three species, the pericardium ends at the level of the SV excluding most of the atrium from the pericardial cavity. In *M. glutinosa* and *E. stoutii*, connective bridges extend between the base of the aorta and the ventricular wall. In *E. cirrhatus*, the connections between the periaortic tissue and the ventricle may carry blood vessels that reach the ventricular base. A further difference specific to *E. cirrhatus* is that the adipose tissue associated with the pericardium contains thyroid follicles. J. Morphol. 000:000–000, 2016.

KEY WORDS: sinus venosus; Myxine glutinosa; Eptatretus stoutii; Eptatretus cirrhatus; atrium; atrioventricular canal; thyroid follicles

INTRODUCTION

Hagfishes are the most ancestral of the extant vertebrates. They are eel-like fishes living in demersal, temperate marine waters in both hemispheres. There are about eighty species of hagfish (Knapp et al., 2011; Zintzen et al., 2015) grouped into seven genera of which *Eptatretus* and *Myxine* include more than 90% of all the species.

Because of its basal evolutionary position and the presence of several unusual features such as a partial cranium, the absence of vertebrae and the production of slime, the biology and morphology of hagfishes have been the subject of numerous studies (e.g., Jorgensen et al., 1998). In this regard, the circulatory system of hagfishes is quite special because it includes several accessory hearts that act as propelling pumps, a branchial heart (equivalent to the heart of other vertebrates) and a secondary circulation formed by extensive subcutaneous blood sinuses (see Johansen, 1963; Lomholt and Franko-Dossar, 1998; Kardong, 2006; Farrell, 2007, 2011). While the cardiovascular system of hagfishes has been subject to significant functional research (see Chapman et al., 1963; Satchell, 1986; Davie et al., 1987; Jorgensen et al., 1998; Farrell, 2007, 2011; Wilson et al., 2013; Davison et al., 2015), many aspects of the morphology of the hagfish heart remain poorly understood.

We have recently started a systematic study of the morphology and structure of the hagfish heart in three different species (*Myxine glutinosa*, *Eptatretus stoutii*, and *Eptatretus cirrhatus*), describing new features and revealing the presence of species-specific differences (Icardo et al., 2016). For instance, we have confirmed the absence of an outflow tract (OFT). In all gnathostomes, the OFT is the portion of the heart interposed between the ventricle and the ventral aorta (for morphological characteristics, see Icardo, 2006; Grimes et al., 2010; Jones and Braun, 2011; Icardo et al., 2016). Also, a dorso-caudal relationship between the atrium and the ventricle was described, which suggests an incomplete looping and may be related to the absence of an OFT. Further findings to date include differences in the ventral

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aorta, which is elongated in *M. glutinosa* and *E. stoutii* and sac-like in *E. cirrhatus* (Icardo et al., 2016). This indicates different developmental relationships at the level of the branchial arteries. Furthermore, granulocytes with rod-shaped granules share the subendocardial space with chromaffin cells, and the myocardiocytes of *M. glutinosa* have a high content of lipid droplets, suggesting specific metabolic requirements (Icardo et al., 2016).

In this study, we analyze the morphology of the components of the venous pole of the heart, from the atrioventricular (AV) canal to the sinus venosus, and the pericardium. To date, nothing is known regarding the structure and composition of the AV canal in hagfishes. The atrium of hagfishes has been characterized as large in size (Farrell and Jones, 1992), the cytoplasm of the atrial endocardial cells has been shown to contain numerous vesicles and vacuoles (Dvorak and Aird, 2015), and the presence of subendocardial chromaffin cells has been noted (Bloom et al., 1961; Yamauchi, 1980). However, little else is known regarding the morphology of this chamber. To date, knowledge of the sinus venosus in hagfishes is largely restricted to reports that the wall of the sinus venosus contains myocardial cells (Yamauchi, 1980). With respect to the pericardium, there is contrasting information. For instance, the connection between the pericardial and the coelomic cavities has been considered to be closed (Randall and Davie, 1980) or to be very wide (Satchell, 1991). The limits of the pericardium in relation to the arterial pole are also under discussion (Grimes and Kirby, 2009; Jones and Braun, 2011). The existence of contradictory information is explained by data extrapolation between the two taxa of Cyclostomata and by the lack of systematic studies. Consistent with the outcomes of our previous report (Icardo et al., 2016), this study reveals both common morphological patterns and several interspecific differences in the venous pole of the heart and the pericardium of hagfishes.

MATERIAL AND METHODS

This study was performed on six specimens of the Atlantic hagfish *M. glutinosa* (Linnaeus, 1758) (40–48 g of body weight; 37- 38 cm long—head to tail—), six specimens of the Pacific hagfish *E. stoutii* (Linnaeus, 1758) (25–54 g, 29–37 cm) and six specimens of the New Zealand hagfish *E. cirrhatus* (Forster, 1801) (750–850 g, 70–80 cm).

A recent study (Zintzen et al., 2015) appears to indicate that *E. cirrhatus* is indeed a complex of two species, *E. cirrhatus* and *E. cryptus*. However, morphological differences are minimal, that is, small variations in the number of prebranchial slime pores and body color, and a clear distinction is only recognized by molecular techniques. Thus, we are confident that the findings reported here describe accurately the phenotype of *E. cirrhatus*.

Sample Collection

The specimens of *M. glutinosa* and *E. stoutii* were anesthetized with 3 ml of a 1:9 clove oil to 95% ethanol solution dissolved in 3 l of artificial saltwater, and killed with a blow to the head. All housing and feeding conditions for these two species were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #2519). The specimens of *E. cirrhatus* were directly euthanized by anaesthetic overdose (1 g/l 3-aminobenzoic acid ethyl ester, buffered to pH 8). All procedures on *E. cirrhatus* were approved by the University of Canterbury Animal Ethics Committee (application number 2013/02R), following collection under the Ministry of Fisheries special permit 509. Following a ventral body wall incision the hearts of all species were excised and fixed in 3% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.3). The hearts were photographed with an Olympus digital 800 camera (Olympus Imaging Corp., Japan) before processing.

Conventional Histology

For conventional light microscopy, tissue blocks, fixed in 3% glutaraldehyde were dehydrated in graded ethanol, embedded in Paraplast (Sherwood, St. Louis, USA), and serially sectioned at 8 μm. Dewaxed sections were stained with either haematoxylin and eosin for a general assessment of tissue structure, Martin's trichrome and the Hematein-indigo carmine stain for connective tissue and Sirius red for collagen (see: Martoja and Martoja-Pierson, 1970; Sheehan and Hrapchak, 1980). Observations were made with a Zeiss Axioskop 2 *plus* microscope equipped with an AxioCam HRc digital camera.

Scanning Electron Microscopy

Tissue blocks fixed in 3% glutaraldehyde were used. Small samples were dehydrated in a series of graded acetone, dried by the critical point method and coated with gold, following routine procedures. Samples were observed with an Inspect S microscope (FEI Company) working at 15 kV.

Semithin Sections and Transmission Electron Microscopy

Tissue blocks fixed in 3% glutaraldehyde were used. Small samples were postfixed in 1% osmium tetroxide, dehydrated in graded acetone and propylene oxide, and embedded in Araldite (Fluka, Buchs, Switzerland), following routine procedures. Semithin sections were cut with an LKB III ultratome, stained with 1% toluidine blue, and observed with the Zeiss microscope. For transmission electron microscopy (TEM), ultrathin sections cut with a Leica ultracut UCT were stained with uranyl acetate and lead citrate and examined with a Philips EM 208.

RESULTS

The Atrioventricular Canal

The AV canal was the segment interposed between the ventricle and atrium. In the three species studied, the AV canal was funnel-like, started in the caudal and dorsal side of the ventricle (Fig. 1a), showed a collagenous structure (Fig. 1b) and ended in continuity with the atrial musculature (Fig. 1c). Semithin sections revealed that the wall of the AV canal contained discrete amounts of myocardium surrounded by dense connective tissue (Fig. 1d). The muscle was often discontinuous and the connective tissue was denser on the epicardial side and under the endocardium (Fig. 1d). The AV canal also contained numerous chromaffin cells that often formed subendocardial cords (Fig. 1e). These cells were surrounded by thin cell projections or by a thick basement membrane (Fig. 1e, inset). Additionally, the AV canal showed fibroblast-like cells with

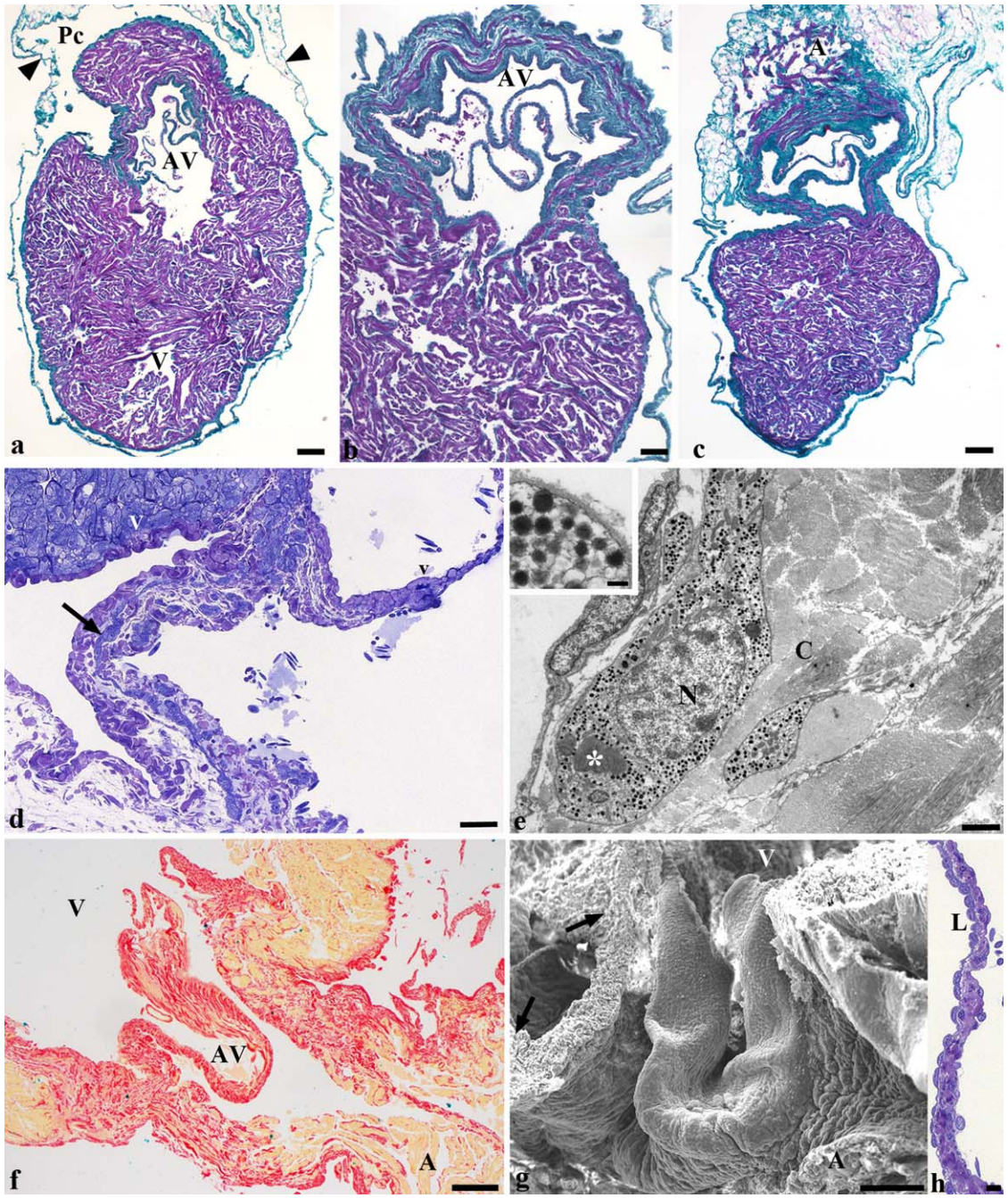

Fig. 1. Atrioventricular (AV) canal and AV valve. **a–c**: *Eptatretus stoutii*. Martin's trichrome. Serial, nonconsecutive sections of the same specimen at cranial (a), medial (b) and caudal (c) levels of the AV canal. a: Cranially, the leaflets attach to the ventricular muscle. The pericardium (arrowheads) runs dorsally to meet the loose connective tissue located dorsal to the heart. Pc, pericardial cavity. b: The wall of the AV canal contains few muscle cells and large amounts of collagen. c: Caudally, the AV muscle is continuous with the atrial muscle. Note the caudal end of the leaflets. **d**: *Myxine glutinosa*. Semithin section. The elongated AV canal contains discrete amounts of myocardium (arrow) embedded in connective tissue. **e**: *Myxine glutinosa*. TEM. Subendocardial chromaffin cells are surrounded by dense collagen (C). Cytoplasmic inclusions of moderate density may be very large (asterisk). N, nucleus. Inset of e: *Myxine glutinosa*. TEM. Chromaffin cell. Detail of granules and basement membrane. **f**: *Eptatretus cirrhatus*. Sirius red. Collagen is an important structural component of the AV canal and AV valve leaflets. Note pocket-like leaflet. **g**: *Eptatretus stoutii*. Scanning Electron Microscopy (SEM) The AV canal has been opened and one of the AV leaflets is exposed. The leaflet is pocket-like and shows a predominant cranial orientation. Arrows indicate the length of the AV canal. **h**: *Eptatretus stoutii*. Semithin section. The leaflets show a collagenous core lined by endocardium. Endocardial cells are prominent on the luminal side (L). Scale bars: a, 200 μm; b, 100 μm; c, 200 μm; d, 50 μm; e, 2 μm; inset of e, 200 nm; f, 250 μm; g, 200 μm; h, 10 μm. A, atrium. V, ventricle.

dilated rough endoplasmic reticulum and a few granulocytes with rod-shaped granules similar to those described previously (Icardo et al., 2016).

The AV canal contained an AV valve formed by two leaflets (Fig. 1a-c). The leaflets kept an oblique position with respect to the dorsoventral axis, being directly attached to the ventricular muscle cranially (Fig. 1a) and to the lateral wall of the AV canal caudally (Fig. 1b). Only the most caudal portion of the leaflets attached to the atrial myocardium (Fig. 1c). Due to the elongated shape of the AV canal the AV valves were pocket-like (Fig. 1f–g), the wall of the valvular sinuses being formed by the lateral walls of the AV segment. It is also important to remark that, because there is a craniocaudal relationship between the ventricle and the atrium, the ventricular side of the leaflets was mostly oriented cranially (Fig. 1f–g) whereas it shows the opposite orientation in the rest of the vertebrates. Papillary muscles and chordae tendineae were absent (Fig. 1g). The structure of the AV leaflets consisted of a collagenous core (Fig. 1f,h) lined by endocardium (Fig. 1g–h). Endocardial cells were more prominent on the luminal than on the parietal side of the leaflet (Fig. 1h). Ultrastructurally, the AV leaflets contained interstitial cells with a few cytoplasmic filaments and a band of extracellular filaments under the endocardial basement membrane (not shown). Species-specific differences were not observed.

The Atrium

The atrium occupied a caudal and dorsal position with respect to the ventricle and the AV canal (see Icardo et al., 2016). The atrial wall was thin (Fig. 2a) and appeared to be composed of slender, roughly rounded, loosely interconnected muscle trabeculae (pectinate muscles). The trabeculae appeared mostly oriented from the AV orifice towards the periphery (Fig. 2b). Curiously, the most external myocardiocytes were irregularly distributed forming a discontinuous muscle layer (Fig. 2c). At the areas of discontinuity, the atrial wall was only formed by the external epithelium and by collagen (Fig. 2c). Conversely, subendocardial wavy collagen bundles were a prominent component of the atrial trabeculae (Fig. 2c). Muscle cells in the atrium appeared to have a low amount of myofibrillar material and showed numerous rounded mitochondria and specific granules (Fig. 2d). Like in the AV canal (see above) and in the ventricle (Icardo et al., 2016), numerous chromaffin cells, and granulocytes with rod-shaped granules, were distributed under the atrial endocardium (not shown). No differences were observed between species.

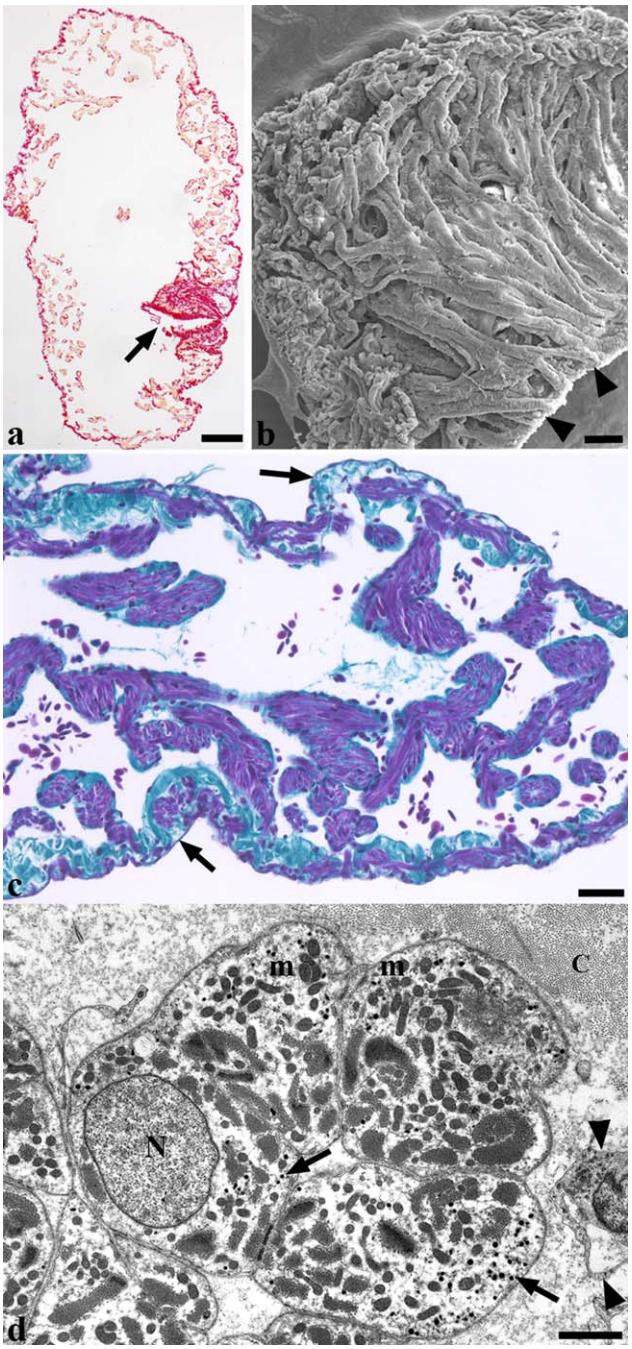

Fig. 2. Atrium. a: *Eptatretus stoutii*. Sirius red. Panoramic view. Note thin wall and the distribution of collagen. Arrow, sinoatrial region and valve. **b**: *Eptatretus stoutii*. SEM. Most trabeculae (pectinate muscles) are oriented (arrowheads) from the AV orifice towards the periphery. **c**: *Myxine glutinosa*. Martin's trichrome. The external muscle layer is often interrupted (arrows). Then, the atrial wall is supported only by collagen. **d**: *Myxine glutinosa*. TEM. Trabecular muscle cells show little myofibrillar content and numerous specific granules (arrows). Arrowheads indicate fibroblast. m, mitochondria; N, euchromatin nucleus. Scale bars: a, 400 μm; b, 250 μm; c, 50 μm; d, 2 μm.

The Sinus Venosus

The sinus venosus was a roughly tubular structure that was oriented in a craniocaudal direction and opened into the left side of the atrium (see Icardo et al., 2016). The sinus venosus showed a collagenous wall (Fig. 3a) in direct continuity with

the atrial musculature. An abrupt line at the sinoatrial junction indicated the boundary between the two chambers (Fig. 3a). The sinus venosus wall was formed by the endothelium, a thin, subendothelial dense collagenous layer and a thick, loose connective tissue layer which contained dispersed collagen bundles and adipose tissue (Fig. 3a,b). The wall of the sinus venosus also contained a variable amount of muscle cells that were more abundant closer to the atrium (Fig. 3b). Chromaffin cells and ganglion-like cells were also present. Nerve fibers were not observed.

A sinoatrial valve formed by two, thin, flap-like leaflets guarded the sinoatrial orifice (Fig. 3a,c). The valve extended between the sinus venosus opening and the atrial wall (Fig. 3c). The leaflets were mostly formed by collagen at the sinus venosus end and by muscle continuous with the atrial musculature at the atrial end (Fig. 3d). No significant differences were observed between species.

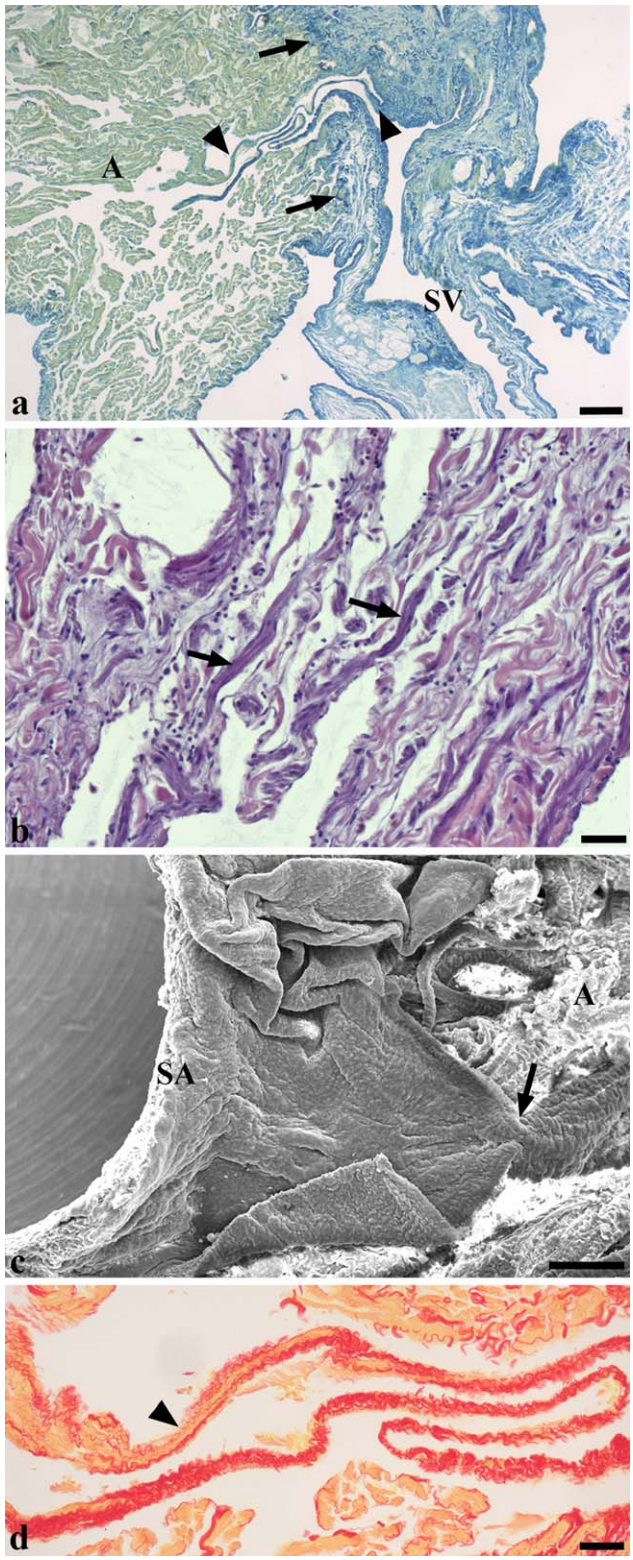

Fig. 3. Sinus venosus and sinoatrial valve. **a**: *Eptatretus cirrhatus*. Hematein trichrome. Panoramic view. Note distinct boundary (arrows) between the atrium (A) and the sinus venosus (SV). The sinoatrial valve leaflets extend (arrowheads) between the sinus venosus and the atrium. **b**: *Eptatretus cirrhatus*. Haematoxylin-eosin. Detail of SV wall near the atrium. Muscle cells (arrows) intersperse with collagen bundles. Note the loose structure of the SV wall. **c**: *Eptatretus stoutii*. SEM. The sinoatrial (SA) aperture and valve are exposed. The valve extends between the opening of the sinus venosus and the atrial musculature. Arrow indicates leaflet attachment. **d**: *Eptatretus cirrhatus*. Sirius red. The atrial part of the sinoatrial valve (arrowhead) is mostly formed by myocardium. Scale bars: a, 500 μm; b, 50 μm; c, 200 μm; d, 100 μm.

The Pericardium

In the three species studied, the pericardium consisted of a visceral layer or epicardium and a parietal layer or pericardium. The parietal layer covered the ventral and lateral walls of the ventricle and the AV canal. However, the left and right portions of the pericardium, instead of fusing behind the ventricle and AV canal to envelop the heart, extended dorsally to join the connective and adipose tissues situated dorsal to the heart (Figs. 1a and 4a). Caudal to the AV canal, the pericardium fused with the sinus venosus on the left side of the atrium and directly with the atrial surface on the right side (Fig. 4a–c). Fusion occurred on the two sides at approximately the same left-right level (Fig. 4a–c). This arrangement closed the pericardial cavity caudally but excluded most of the atrium from the cavity (Fig. 4a–c). The relation between the pericardium and the atrium was also clearly seen when the pericardium was pulled (Fig. 4d). The part of the atrium not contained within the pericardial cavity was covered by a mesothelium.

In relation to the ventral aorta, the pericardium was different in the three species studied. In *M. glutinosa* and *E. stoutii*, the pericardium extended cranially (Fig. 4e) covering the ventral and lateral aspects of the aorta (Fig. 4f), up to the level where the aorta bifurcates, at the cranial end of the gill pouches. As it occurred at the level of the ventricle and AV canal, the left and right sides of the pericardium passed behind the aorta (Fig. 4e,f) to become continuous with the

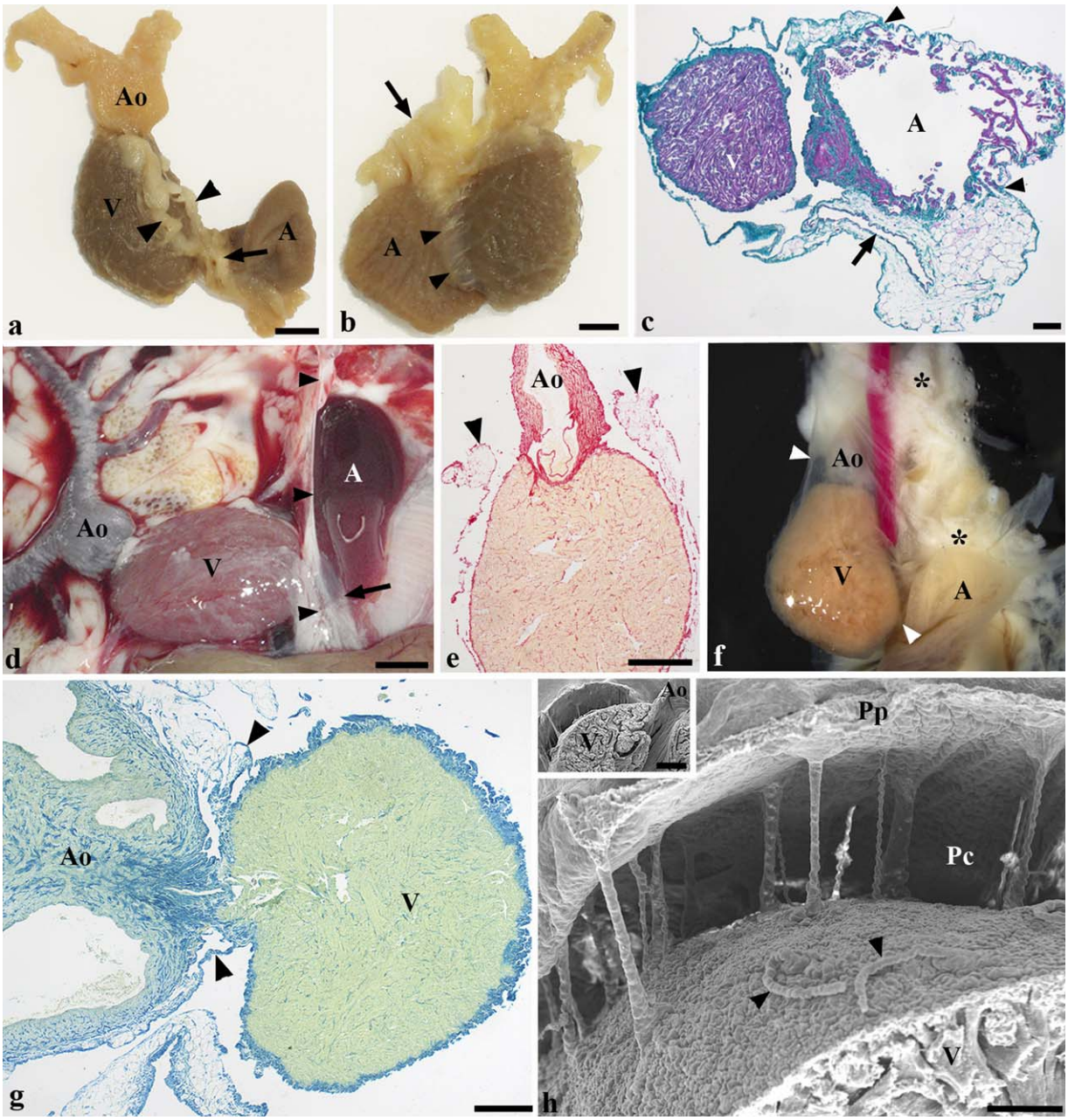

Fig. 4. The pericardium. **a**: *Eptatretus cirrhatus*. Panoramic view. Left heart side. The left and right sides of the pericardium (arrowheads) are cut. Arrow indicates the sinus venosus and the limit of the pericardial cavity. **b**: *Eptatretus cirrhatus*. Panoramic view. Right heart side. Arrowheads indicate the fusion of the pericardium with the atrial surface. The sinus venosus (arrow) emerges from above the left side of atrium. **c**: *Eptatretus stoutii*. Martin's trichrome. Transverse section. The pericardium fuses (arrowheads) with the atrial surface. The sinus venosus (arrow) is on the left side of atrium. **d**: *Eptatretus cirrhatus*. Panoramic view. The pericardium (arrowheads) has been cut and the pericardial cavity open. The pericardium fuses with the sinus venosus (arrow) excluding most of the atrium from the pericardial cavity. **e**: *Eptatretus stoutii*. Sirius red. Frontal section. The left and right sides of the pericardium (arrowheads) pass the aorta to fuse with dorsal structures. **f**: *Eptatretus stoutii*. The ventral aorta, the ventricle and the AV canal are contained within the pericardial cavity and are suspended in the coelomic cavity due to the dorsal attachment of the pericardium. A red probe has been inserted in the pericardial cavity from above. It runs along the aorta and ventricle, reaching the AV canal. Arrowheads indicate the limits of the pericardial bag. The atrium is excluded from the pericardial cavity. Asterisks indicate the location of the digestive tube, which is surrounded by adipose and connective tissue. **g**: *Eptatretus cirrhatus*. Hematein trichrome. Tangential section. The loose periaortic tissue is continuous with the pericardium (arrowheads). **h**: *Myxine glutinosa*. SEM. Ventricle. Numerous finger-like bridges attach the parietal layer (Pp) of the pericardium to the ventricular surface and delimit the pericardial cavity (Pc). Ruptured bridges lay on the ventricular surface (arrowheads). Inset of h: *Myxine glutinosa*. SEM. Panoramic view of the same specimen. Scale bars: a, 500 μm; b, 500 μm; c, 200 μm; d, 500 μm; e, 500 μm; f, 500 μm; g, 600 μm; h, 200 μm; inset of h, 500 μm. A, atrium. Ao, ventral aorta. V, ventricle.

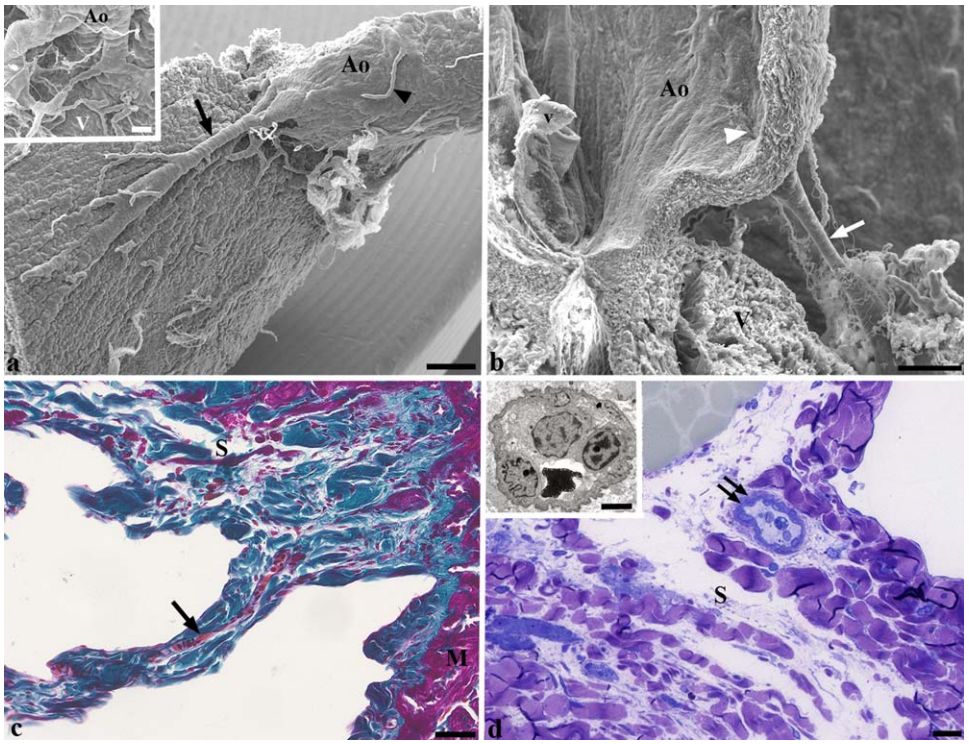

Fig. 5. Atypical features of the pericardium. **a**: *Eptatretus stoutii*. SEM. A thick bridge (arrow) extends between the aorta and the ventricular surface. The bridge ramifies before becoming continuous with the epicardial tissue. Arrowhead indicates ruptured connective bridges attaching the two layers of the pericardium. Inset: *Eptatretus stoutii*. SEM. The bridges are shorter, more ramified and appear interconnected. **b**: *Eptatretus stoutii*. SEM. A thick bridge (arrow) arises from the outer aortic surface. Note depression (arrowhead) in the inner aortic surface at the point of origin of the bridge. **c**: *Eptatretus cirrhatus*. Martin's trichrome. An extension of the periaortic tissue reaches the ventricle. The extension contains a large blood vessel (arrow). M, ventricular myocardium. S, subepicardial tissue. **d**: *Eptatretus cirrhatus*. Toluidine blue. Ventricular base. The subepicardial tissue (S) shows a small blood vessel (double arrow) filled with blood cells. Inset: *Eptatretus cirrhatus*. TEM. In the same location, a capillary contains erythrocytes and other blood cells. Scale bars: a, 300 μm; inset of a, 100 μm; b, 200 μm; c, 50 μm; d, 20 μm; inset of d, 3 μm. Ao, aorta. V, ventricle.

tissue located dorsally. Thus, the long ventral aorta was included within the pericardial cavity.

The dorsal attachment of the pericardium created a membranous bag which contained most of the heart and the aorta (Fig. 4f). The area of attachment corresponded to the tissue located ventral to the digestive tube (Fig. 4f). Thus, the heart remained suspended from this dorsal attachment in the coelomic cavity. Of note, the parietal pericardium was an epithelial, transparent layer which was easily torn during dissection (Fig. 4f).

In contrast, in *E. cirrhatus*, the ventral aorta was covered by a loose connective tissue in continuity with the parietal pericardium (Fig. 4g) (See also fig. 7c of Icardo et al., 2016). Thus, no pericardial cavity surrounded the aorta in the New Zealand species. The loose periaortic tissue contained variable amounts of adipose tissue, collagen bundles, fibroblasts and small vessels. In addition, the pericardium dorsal to the heart was infiltrated by adipose tissue in *E. cirrhatus* (Fig. 4a). In the three species studied, large amounts of adipose tissue were located dorsal to the heart and ventral aorta, between the gill pouches and around the digestive tube (Fig. 4f).

In the three species studied, the visceral and parietal layers of the pericardium surrounding the heart were connected by numerous, finger-like, connective bridges (Fig. 4h). The connections tended to be shorter and wider near the ventricular apex. The length of the bridges limited the size of the pericardial cavity (Fig. 4h, inset), which was widest in the New Zealand species. Finger-like bridges were also frequent between the parietal pericardium and the initial portion of the ventral aorta in *M. glutinosa* and *E. stoutii*, being practically absent at most cranial levels. In addition, the bridges and the subepicardial connective tissue contained ganglion-like cells in *E. cirrhatus* but not in the other two species.

In relation to the pericardium and pericardial cavity, other morphological observations are of interest. In *M. glutinosa* and *E. stoutii*, we detected the presence of numerous bridges extending between the external surface of the aortic base and the ventricular surface (Fig. 5a). These bridges were long and thick (Fig. 5a) or shorter and interconnected (Fig. 5a, inset). They always branched before attaching to the ventricular surface. The presence of the long bridges often coincided with small depressions in the inner surface of the ventral aorta (Fig. 5b). However, the

aortic wall was never perforated and the bridges were exclusively formed by loose connective tissue.

In *E. cirrhatus*, the loose connective tissue surrounding the ventral aorta was of irregular size, contained abundant blood vessels and showed numerous extensions that reached the ventricular surface (Fig. 4g). On occasions, these extensions contained blood vessels that reached the ventricle (Fig. 5c). In addition, small blood vessels were identified in the heart subepicardium at the ventricle-aorta junction (Fig. 5d). These vessels could contain erythrocytes (Fig. 5d, inset) and other blood cells.

Finally, the adipose tissue accompanying the parietal epicardium in *E. cirrhatus* contained cystic structures of variable size that appeared isolated or forming small groups (Fig. 6a). Each cyst consisted of a peripheral epithelium surrounding a central lumen (Fig. 6a). The basal surface of the epithelium was smooth and appeared surrounded by a rich vascular plexus (Fig. 6a,b). The apical surface of the epithelium was also smooth (Fig. 6b) but appeared often interrupted by cell extrusion with release of the cytoplasmic content into the cyst lumen (Fig. 6b, inset). Individual epithelial cells were cuboidal to columnar and showed basal, euchromatic nuclei (Fig. 6b). Using TEM, epithelial cells appeared joined by apical tight junctions and lateral desmosomes (Fig. 6c). The basal cell pole was smooth and rested on a continuous basement membrane (not shown). The apical cell pole showed short microvilli and multivesicular bodies projecting into the lumen (Fig. 6c, inset). The apical cytoplasm showed numerous secretory vesicles and tubular profiles of the smooth endoplasmic reticulum. However, the most distinguishing feature of the epithelial cells was the presence of dark, round inclusions of variable size, mostly accumulated in the apical cytoplasm (Fig. 6b). The inclusions were membrane-bound and showed a nonhomogeneous content and different degrees of electron density (Fig. 6c,d). Small inclusions were often seen surrounding the larger ones. Fusion of the inclusions with disappearance of the limiting membrane was also observed (Fig. 6d). The cyst cavity contained numerous inclusions and cell debris (Fig. 6a,b). In addition, the presence of flocculent material that

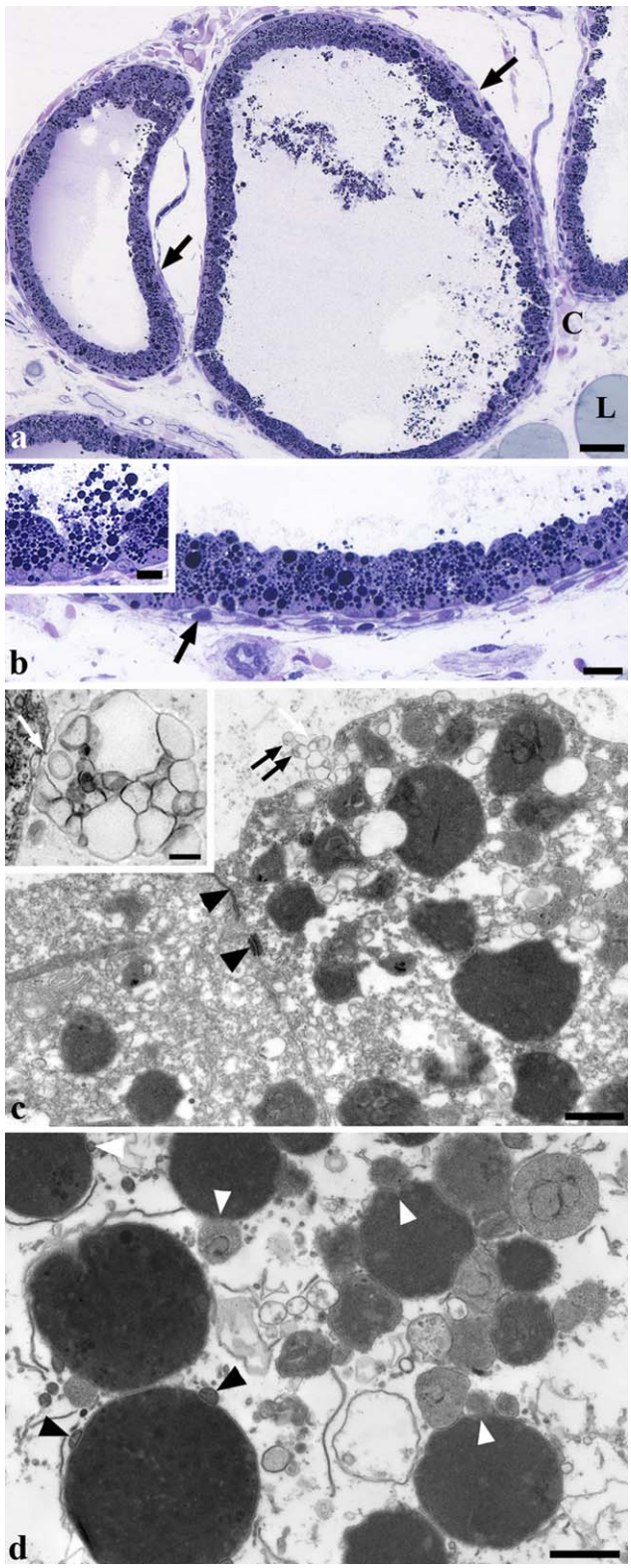

Fig. 6. *Eptatretus cirrhatus*. Adipose tissue associated with the pericardium. **a**: Semithin section. Toluidine blue. Cystic structures are formed by a peripheral epithelium. The cystic lumen contains numerous dark inclusions and a flocculent material that stains irregularly. The space between cysts is occupied by lipid droplets (L), collagen bundles (C) and blood vessels. Arrows, pericystic blood vessels. **b**: Semithin section. Toluidin blue. Detail of the epithelium. Note row of basal nuclei. The apical cytoplasm is filled with round, dark inclusions of variable size. Arrow, pericystic blood vessels. Inset of b: Semithin section. Toluidin blue. One cell is extruding the cytoplasmic content. **c**: TEM. Epithelial cells are joined by apical tight junctions and lateral desmosomes (arrowheads). Dark inclusions of variable size pack the apical cytoplasm. Double arrow, multivesicular body. Inset of c: TEM. Detail of multivesicular body. Arrow indicates membrane continuity. **d**: TEM. Apical cell pole. Note inhomogeneity of inclusions. Small, primary, membrane-bound lysosomes (black arrowheads) gather around large, secondary lysosomes. Often, the membrane of the small lysosomes is lost (white arrowheads). Scale bars: a, 50 μm; b, 20 μm; inset of b, 10 μm; c, 1 μm; inset of c, 250 nm; d, 1 μm.

stained irregularly with toluidine blue was also detected (Fig. 6a).

DISCUSSION

The Atrioventricular Canal and the Atrium

The AV canal is a funnel-like segment that constitutes a distinct morphological component of the hagfish heart. The presence of the AV canal in jawed fish has received little attention. However, this segment of the heart can be recognized at embryonic stages in teleosts and chondrosteans (Icardo, 2006; Icardo et al., 2009a) as well as in adult specimens across groups. In teleosts, the AV canal is short and appears formed by a ring of compact, vascularized myocardium surrounded by a connective tissue ring (Icardo and Colvee, 2011). A similar arrangement is observed in elasmobranches and sturgeons (unpublished observations). However, the AV canal in hagfishes is long, lacks blood vessels and establishes a wide separation between the atrial and ventricular chambers. The muscle component of the AV canal is scarce, with sparse muscle bundles that are surrounded by connective tissue. In addition, the continuity of the AV muscle with the atrial and ventricular musculature is, at best, very poor. The segment length and the poor muscular content explain why the AV delay in the electrocardiogram is much longer in hagfishes than in other fishes (Farrell, 2007).

The AV valve contains two leaflets that adopt a pocket-like configuration. While this is a quite uncommon morphology for this region of the heart, it may just be an adaptation to the length of the canal. The AV leaflets are formed by a dense collagenous core lined by endocardium. The leaflets contain few interstitial cells, and papillary muscles and chordae tendineae are absent. This is a simple structure which contrasts with that of elasmobranches (Hamlett et al., 1996) and sturgeons (Icardo et al., 2009b), where the leaflets are thick, contain numerous cells and appear supported by a system of collagenous chordae. Thick leaflets are also observed in Holosteans (unpublished observations) whereas the AV leaflets in many teleosts show a discrete dense core formed by cells and extracellular matrix (Icardo et al., 2003; Icardo and Colvee, 2011). Of note, the structure of the AV leaflets in hagfishes is similar to that of the bicuspid (aortic) valve (Icardo et al., 2016) supporting the notion that the valve leaflets at the two ventricular ends of hagfish and jawed fish species have a comparable histology and composition (Icardo and Colvee, 2011).

The hagfish atrium is a large sac. Its wall is formed by slender trabeculae (pectinate muscles) mostly consisting of muscle cells and collagen. Atrial myocardiocytes show the typical muscle banding pattern although the myofibrillar content appears to be lower than that in the ventricular myocytes. This is consistent with findings in other osteognatostomes groups (Santer, 1985). Also, myocardial specific granules appear to be more abundant in the cytoplasm of the atrial cells than in the ventricular cells (Shibata and Yamamoto, 1976). Few lipid droplets were present in the atrial myocardiocytes of *M. glutinosa* and *E. stoutii*. This contrasts with the high number of these droplets seen in the ventricle of *M. glutinosa* in a previous study (Icardo et al., 2016), suggesting chamber-specific differences in energy use and/or storage. Conversely, collagen is a prominent subendocardial component in the muscle trabeculae. Collagen also forms a continuous layer under the epicardium, contributing to the structural closure of the atrial wall. This situation may seem striking but the existence of a discontinuous muscle envelope also occurs in the atrium of, for instance, many teleost species (unpublished observations). Unfortunately, the functional contribution of collagen to atrial physiology, other than limiting chamber expansion, is presently unknown.

The AV canal and the atrial wall contain numerous chromaffin cells. These cells have previously been described in the atrium and ventricle of the cyclostome heart (Bloom et al., 1961; Caravita and Coscia, 1966; Shibata and Yamamoto, 1976; Icardo et al., 2016). This results validate those observations. Chromaffin cells contain catecholamines and their possible role in the control of cardiac performance has previously been discussed (Bloom et al., 1961; Shibata and Yamamoto, 1976). The presence of granulocytes containing rod-shaped granules has also been reported in the hagfish ventricle (Icardo et al., 2016) and aorta (Wright, 1984). These cells appear to be blood-borne and to represent the single type of granular leukocyte existing in hagfishes (Icardo et al., 2016). However, their specific role is unclear.

From a physiological viewpoint, the length of the AV canal and its low muscle content, as well as the position of the atrium, caudal, and dorsal to the ventricle (Icardo et al., 2016), impose an increased functional burden to the heart. A strict dorsal position of the atrium and a short AV segment facilitate ventricular filling and heart pumping (see Farrell, 2007). In cyclostomes, the presence of the portal heart and other accessory hearts may overcome this difficulty by assisting venous return and facilitating cardiac filling by a *vis-a-tergo* mechanism.

The sinus venosus: An evolutionary hypothesis. The hagfish sinus venosus is a venous confluent which receives the major cranial and caudal veins, being the single connection between the heart and the hepatic veins. In other vertebrates, the sinus venosus is the place of location of the heart pacemaker (see Jensen et al., 2014). We could not demonstrate the presence of specialized nodal tissue in the sinus wall or at the

sinoatrial junction. In contrast, electrical recordings have shown the presence of a discrete negative deflection in the electrocardiogram that was related to depolarization of the sinus venosus. However, it was observed only in *E. cirrhatus* and only when the electrode was placed in the central region of the sinus venosus (Davie et al., 1987). Similar studies in *M. glutinosa* did not report on sinus venosus electrical activity (Arlock, 1975; Satchell, 1986). Nonetheless, electrical activity in the hagfish heart is quite unusual because excised myocardial fragments exhibit a high degree of automatism and many myocardiocytes throughout the atrium and ventricle show pacemaking characteristics (Jensen, 1965; Arlock, 1975). Myocardiocytes exhibit low resting membrane potentials (Jensen, 1965) and are rich in hyperpolarization-activated cyclic nucleotide-gated (HCN) ionic channels (Wilson and Farrel, 2013). In *E. stoutii*, HCN channels appear to control heart rate and chamber synchrony by allowing the spread of excitation from the atrium to the ventricle (Wilson and Farrel, 2013; Wilson et al., 2013). In this sense, the presence of a sinus venosus with a morphologically or functionally well-defined pacemaker may not be strictly necessary.

Another important morphological feature is that the sinus venosus opens into the left side of the atrium. Although the wall of the sinus venosus contains some myocardiocytes, the morphological arrangement of this chamber raises doubts on whether it may be considered a true sinus venosus. The vertebrate heart develops in series with the sinus venosus appearing at the caudal end of the atrium. In gnathostomes, the sinus venosus remains as a distinct chamber retaining its position as the most posterior chamber of the heart. In this sense, the position of the sinus venosus is quite unusual in terms of homology. However, this situation is similar to that seen in the basal amphioxus where the cardiac nature of the sinus venosus is somewhat controversial (see Simoes-Costa et al., 2005; Xavier-Neto et al., 2010).

In amphioxus, the sinus venosus, which contains muscle and is able to contract (Xavier-Neto et al., 2010), is not the most posterior portion of the heart and appears inserted between the pumping (subendostylar and subintestinal) vessels as the result of the developmental emergence of the hepatic tissues at metamorphosis (Holland et al., 2003). A similar developmental mechanism appears to occur in the larval lamprey (Baxter, 1957; Richardson et al., 2010) and a similar mechanism may, therefore, be operative in hagfishes. In all these cases, the morphological result is that the connection between the sinus venosus and the atrium looks artificial in terms of homology. Thus, the hagfish sinus venosus may be closer to the sinus venosus of amphioxus than to that of the vertebrates. In amphioxus, the sinus venosus appears to be the vestige of an ancestral pulsating chamber (Simoes-Costa et al., 2005) which is later substituted by the definitive chamber.

The general design of the circulatory system of hagfishes includes a portal heart, a sinus venosus, two muscular chambers and the ventral aorta. This layout is reminiscent of the system of contractile vessels that powers the circulation in amphioxus (Satchell, 1991; Kardong, 2006; Farrell, 2011). Are there any more similarities between the heart of amphioxus and hagfishes? The consideration of the hagfish heart as being formed of three (Farrell, 2007) or four cardiac chambers (Randall and Davie, 1980) has impeded further elaboration on this matter. We (Icardo et al., 2016) and others (Kardong, 2006; Farrell, 2007) have claimed that heart OFT components (either conus arteriosus or bulbus arteriosus) are absent in hagfishes. The anatomical position of the sinus venosus appears to indicate that this chamber does not develop in series from a cardiopharyngeal field common to the ventricle and atrium. This means that the hagfish sinus venosus may be analogous (i.e., a functional correlate) but not homologous (i.e., not of cardiac origin) to the sinus venosus of other vertebrates. If this hypothesis is true, as it appears to be in amphioxus (Simoes-Costa et al., 2005; Xavier-Neto et al., 2010), this would leave the hagfish as the single extant chordate with a two-chambered heart. Nonetheless, a complete description of the heart should also include the neglected AV connecting segment, which is particularly long in hagfishes. This segment appears to be substituted or deeply rearranged in more derived species.

This hypothesis fits the predictions and scenarios for heart evolution presented in previous papers (Simoes-Costa et al., 2005; Xavier-Neto et al., 2010). Namely, the occurrence of one ancestral, single contractile vessel on which a succession of patterning events results in the progressive acquisition of chambers and valves (Hochgreb et al., 2003; Holland et al., 2003; Davidson, 2007). It would also indicate a smooth evolutionary transition between the heart of protochordates and chordates, and would explain the absence of fossil records of animals displaying a two-chambered heart (see Simoes-Costa et al., 2005). Simply, the research focus was not placed on the appropriate animal due to previous misconceptions. In this sense, the lamprey heart would most probably resemble the hagfish heart, but many morphological details are poorly known.

The Pericardium

The vertebrate pericardium is a membranous bag consisting of an inner layer or epicardium, applied to the heart surface, and of an outer layer or parietal pericardium, in contact with surrounding structures. In Gnathostomata, the parietal pericardium (or, simply, pericardium) is attached to the adjacent skeletal structures, the stiffness of

the system depending on the specific skeletal attachment (Santer, 1985; Satchell, 1991). This description also applies to the lamprey pericardium (Percy and Potter, 1991). By contrast, we show here that the hagfish parietal pericardium adopts the shape of a bag hanging from the tissues located dorsal to the heart. This includes the connective and adipose tissues that surround the digestive tube. The lack of skeletal attachments explains why the pericardial pressure of hagfishes is close to the ambient pressure (Satchell, 1991).

There has been some discussion regarding the presence (Santer, 1985; Satchell, 1991) or absence (Randall and Davie, 1980) of communications between the pericardial and coelomic cavities in Cyclostomata. For instance, adult lampreys show a peritoneal-pericardial communication (Percy and Potter, 1991). However, the pericardium is a closed pouch in hagfishes. The heart is suspended in the coelomic cavity due to the dorsal attachment of the pericardium. In most vertebrates (Männer et al., 2001; Schulte et al., 2007; Icardo et al., 2009a), development of the pericardium starts at the boundary between the sinus venosus and the liver, being in relation with the formation of the transverse septum. In lampreys, however, the epicardium develops from a paired outgrowth of cells located in the dorsal wall of the coelomic cavity, lateral to the early digestive tube (Pombal et al., 2008). A similar developmental origin in hagfishes would explain the present anatomical findings. The morphological situation is different in adult lampreys because they develop a transverse septum between the liver and the pericardium at metamorphosis (Percy and Potter, 1991). On the contrary, hagfishes show a single coelomic cavity.

The limits of the pericardium in relation to the ventral aorta have also been under discussion, mostly centered on whether the pericardium stops at the ventricle-aorta boundary (Satchell, 1992) or covers the beginning of the aorta (Grimes and Kirby, 2009). In *M. glutinosa* and *E. stoutii*, the pericardium runs along the ventral aorta, which is therefore included within the pericardial cavity. By contrast, the pericardium is continuous with the loose periaortic connective tissue in *E. cirrhatus*. Identification of this loose tissue as pericardial or arterial in origin will require further developmental studies. It is also unclear whether the vessels contained within the periaortic tissue can be considered *vasa vasorum*. In fact, small vessels practically never entered the structure of the aorta, remaining at the boundary between the arterial adventitia and the periaortic tissue.

A remarkable finding of this study is the fact that a large part of the atrium is excluded from the pericardial cavity. We do not have a clear explanation for this feature but it may be related to the spatial relationship between the atrium and the sinus venosus. It is also unclear whether the atrium is never covered by the pericardium or the exclusion is secondary to fusion of the two layers of the pericardium. Developmental studies will be necessary to answer these questions.

Other features related to the pericardium should also be highlighted. The presence of finger-like, connective bridges joining the two layers of the pericardium may serve to control the size of the pericardial cavity in the absence of an external rigid support. The fact that these connections are wider and shorter near the heart apex raises the possibility of the existence of two different types of pericardial connections. Nonetheless, the structural composition is similar in the two cases. From a comparative point of view, connective bridges arising from the heart surface are not unusual but they may have different functional implications. In lungfishes, a thick ligament attaches the ventral surface of the ventricle to the parietal pericardium (Icardo et al., 2005). It may be homologous to the *gubernaculum cordis*, a thick ligament that attaches the ventricular apex to the ventral pericardium and to the visceral peritoneum in many reptilian hearts (Wyneken, 2009). However, the role of the *gubernaculum cordis* appears to be to induce longitudinal tension in the ventricle during systolic contraction (Wyneken, 2009). Conversely, multiple connections attach the heart surface to the parietal pericardium in the teleost *Anguilla anguilla*, and at least one connection extends between the lateral surface of the conus arteriosus and the pericardium in the polypteriform *Erpetoichthys calabaricus* (unpublished observations). In these cases, the structure and the possible functional role of the pericardial connections are unknown.

The connective, ramified bridges extending between the surface of the swollen aortic base and the ventricular surface are unusual. They resemble the numerous transient, peritruncal vessels that can be observed around the base of the aorta during early coronary development, before the establishment of the definitive main coronary trunks (see Bernanke and Velkey, 2002). It is clear, however, that these bridges do not contain blood vessels. Conversely, the bridges connecting the loose periaortic tissue and the ventricular surface in *E. cirrhatus* contained blood vessels. In addition, small vessels were observed in the subepicardial tissue. Our observations contradict previous reports on the absence of blood vessels in the hagfish heart (see Farrell, 2007) but they do not support the existence of a true coronary circulation. However, they clearly illustrate that cranial vessels are able to reach the heart ventricle. On the whole, the observations in the three species of hagfish are reminiscent of the two main modes of cardiac vascularization in vertebrates: epibranchial arteries and true coronaries.

Finally, the presence of cystic structures in the adipose tissue associated with the pericardium in *E. cirrhatus* is worth of note. The cysts resemble the thyroid follicles described in both *M. glutinosa* (Waterman and Gorbman, 1963) and *E. stoutii* (Henderson and Gorbman, 1971). However, the follicular cells in *E. cirrhatus* show a higher density of cytoplasmic inclusions. The density of the pericystic blood vessels is also higher. In addition, we show here that the inclusions have the structural characteristics of the primary and secondary lysosomes involved in hydrolysis of thyroglobulin and the subsequent release of the thyroid hormones (see Bloom and Fawcett, 1994). This is consistent with the uptake of radioactive iodine in the cysts of *M. glutinosa* (Waterman and Gorbman, 1963). However, the irregularly stainable material observed in the cyst lumen is not like the colloid observed in other vertebrates (also, see Waterman and Gorbman, 1963). The present results could not discern on whether the precursors of the thyroid hormones are stored in the cyst lumen or directly in the large cytoplasmic inclusions (see Henderson and Gorbman, 1971). In this regard, the multivesicular bodies could be implicated in hormone precursor exocytosis. One of the functions of these bodies is to discharge their protein content into the extracellular space (Hanson and Cashikar, 2012). Conversely, the reason why these follicles have only been found in *E. cirrhatus* appears to depend on small differences in location. In *M. glutinosa*, the thyroid follicles are located beneath the pharynx and ventral to the aorta (Waterman and Gorbman, 1963) whereas in *E. stoutii* they are located in the adipose tissue existing between the gill pouches (Henderson and Gorbman, 1971). These tissues have not been sampled here.

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