Little variation in the morphology of the atria across 13 orders of birds.

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Abstract

Hearts of the endothermic mammals generate much greater blood flows and pressures than the hearts of ectothermic reptiles that represent the mammalian ancestral setting. Among mammals, the variation in atrial morphology is greater than in ectothermic reptiles. Possibly, the transition from ectothermy to endothermy and high cardiac performance associated with greater variation in cardiac structure. To test this association, we investigated the variation in cardiac morphology among the endothermic birds, as birds evolved from ectothermic reptiles and their hearts generate greater blood flows and pressures than the hearts of mammals. Hearts were assessed by gross morphology and histology, and we focused on the atria as they have multiple features that lend themselves to quantification. We found the bird hearts to have multiple features in common with ectothermic reptiles (synapomorphies), for instance three sinus horns. Convergent features were shared with crocodylians and mammals, such as the cranial offset of the left atrioventricular junction. Other convergent features like the compact organization of the chamber myocardium were shared with mammals only. Some features were distinctively avian (apomorphies), including the left atrial antechamber, and a ventral merger of the left and right atrium was found in parrots and passerine birds only. Most features, however, exhibited little variation. For instance, there were always receives three systemic veins and two pulmonary veins, whereas among mammals the number of veins are 2-3 and 1-7, respectively. Our findings suggest that the transition to high cardiac performance does not necessarily lead to greater variation in cardiac structure.

1 | INTRODUCTION

Mammals and birds evolved independently from reptile-like ancestors and the two vertebrate classes are characterized by high metabolic rates and endothermy (Warren, 2008; Green., et al., 2014; Tattersall, 2016). When the hearts of mammals and reptiles are compared, it is evident that mammalian hearts are remodeled by incorporation of systemic vein myocardium to the right atrium (Jensen, Boukens, Wang, Moorman, Christoffels, 2014a; Carmona, Ariza, Cañete, Muñoz-Chápuli, 2018). Also, mammals develop and absorb pulmonary vein myocardium, the number of venous orifices to the left atrium can vary between 1 (dugongs) and 7 (armadillos), and the myocardial sleeve of the veins may be extensive (mouse) or all but gone (harbour porpoise) (Rowlatt, 1990; Mommersteeg et al.,2007). The number of orifices and the extent of myocardium even varies within the species (Nathan, Eliakim, 1966; Calkins et al., 2007, Rowlatt, 1990). In human, for instance, the left atrium typically receives 4 pulmonary veins, but 3 and 5 veins are also frequently observed, 2 and 6 can occur but are rare (Mansour et al., 2004). Similar variation is not found in reptiles (Jensen, Moorman, Wang, 2014b), suggesting that the transition from ectothermy to endothermy, and the associated rise in cardiac pumping (Crossley et al, 2016), associates with greater variation in the building plan of the heart. We therefore hypothesized that birds will have hearts that exhibit similar degrees of variation as mammals.

The extensive studies of the chicken heart, its development in particular, sharply contrasts the comparatively meagre number of anatomical studies on hearts of other birds (Hamburger, Hamilton, 1951; Van Mierop, 1967; Poelmann, Mikawa, Gittenberger-De Groot, 1998; Sedmera, Pexieder, Vuillemin, Thompson, Anderson, 2000; Lincoln, Alfieri, Yutzey, 2004; van den Berg et al., 2009; Bressan, Lui, Louie, Mikawa, 2016). Literature on the venous-atrial region in avian species is limited (Jensen et al., 2014a). In Chicken, there is a myocardial sleeve surrounding the systemic and pulmonary veins that are within the pericardial cavity (Endo, Kurohmaru, Nishida, Hayashi, 1992; van den Hoff, Kruithof, Moorman, Markwald, Wessels, 2001), but similar studies have, to the best of our knowledge, not been extended to other avian species. Older studies made gross anatomical comparisons of multiple species (Gasch, 1888; Benninghoff, 1933) but a general paucity of images and quantifications makes it difficult to verify these findings, let alone to make comparisons to other clades of vertebrates. For instance, the walls of the atria are described as thin with thick bundles of muscle forming muscular arches (Whittow, 1999), but how this setting compares to other vertebrates remains difficult to assess.

We therefore undertook a study to assess the variation in the morphology of the avian heart. We focused on the atria and the base of the ventricle, to assess the 15 features schematized in Figure 1 across 13 orders of birds.

2 | MATERIALS AND METHODS

2.1 Isolated hearts

One heart has been isolated from one specimen of the Ostrich (*Struthio camelus*, Struthioniformes) taken from a previously published model (Jensen et al., 2013), Mallard (*Anas platyrhynchos*, Anseriformes), Chicken (*Gallus gallus*, Galliformes), Collared dove (*Streptopelia decaocto,* Columbiformes), Common swift (*Apus apus*, Apodiformes), Eurasian coot (Fulica Atra, Gruiformes), Common snipe (*Gallinago gallinago*, Charadriiformes), Sparrowhawk (*Accipiter nisus*, Accipitriformes), Barn owl (*Tyto alba,* Strigiformes), Green woodpecker (*Picus viridis,* Piciformes), Common kestrel (*Falco tinnunculus*, Falconiformes), Budgerigar (*Melopsittacus undulatus,* Psittaciformes), Barn swallow (*Hirundo rustica,* Passeriformes) Lesser redpoll (*Acanthis cabaret*, Passeriformes) and Hawfinch (*Coccothraustes coccothraustes*, Passeriformes).

2.2 Tissue preservation

The adult Mallard, Chicken HH42, and Lesser redpoll were fixed in 4% PFA for 24 hours and then stored in 70% ethanol (the Mallard was obtained from a culling program and the Lesser redpoll was found dead on a bike path). The other samples came from hearts used in anatomy classes and were therefore stored in formalin and subsequently washed with water before use and then stored again. This process happened multiple times with the exact amount unknown to us.

2.3 Histology and immunohistochemistry

The hearts from Barn swallow, Green woodpecker, Collared dove, Chicken, Budgerigar,

Common snipe, Common swift, Grey heron, Barn owl, Common kestrel and Eurasian coot were embedded in paraplast and cut in 10 µm transverse sections, apart from the Collared dove which was cut in 4-chamber view. The principal staining was picro-sirius red (muscle is stained orange, collagen red with 1 min differentiation in 0.01 M HCL). The atrial region of each heart may yield several hundreds of sections and we selected, at a fixed distance, 20 sections to represent the entire atrial region from below the atrioventricular junction to the roof of the atria. Using immunohistochemistry, we detected myocardium with cTnI mouse antibodies (Invitrogen dilution 1:200) or cTnI rabbit antibodies (Hytest 1:200) visualized by a fluorescently labelled secondary donkey-anti-mouse antibody (Invitrogen, dilution 1:200) or donkey-anti-rabbit antibody (Invitrogen, dilution 1:200) respectively coupled to Alexa 488. The sinuatrial node was detected with Isl1 goat antibodies (Neuromics, dilution 1:200) visualized by a fluorescently labelled secondary donkey-anti-goat antibody coupled to Alexa 647 (Invitrogen, dilution 1:200). Nuclei were stained with sytox orange (Invitrogen, dilution 1:500).

In situ hybridization Chicken

The cardiac troponin I (*cTnI*) probe from Somi and colleagues was used to visualize myocardium. The bone morphogenetic protein 2 (*Bmp2)* probe of Somi and colleagues was used to visualize the sinuatrial node (Somi, Buffing, Moorman, van den Hoff, 2004).

Imaging

Imaging of the picrosirius red stained slides was done with the Leica DM5000 light microscope. The slides were photographed and later merged if necessary. For the immunohistochemistry slides were viewed and photographed with the Leica DM6000B fluorescent microscope.

Analyzed structures

The following ten structures were analyzed (Figure 1): Presence of myocardium in the sinus venosus (1), If the sinus node resides at the base of the right sinuatrial leaflet (2), the presence of a sinoatrial valve and the number of leaflets it contains (3), the existence of a left sinus horn (4), the presence and size of trabeculation in the atrial wall (5), the number of pulmonary veins that entered the left atrium (6), the extent of pulmonary venous myocardium in relation to the

pericardium (7), the presence of a dorsal myocardial ridge in the antechamber of the left atrium and its development (8), the existence of a muscular shelf in the left atrial roof (9), whether or not the left and right atrial walls merged ventrally of the pulmonary artery (10)

3 | RESULTS

3.1. Gross morphology

The structures of the bird atria were described in the sequence in which the blood courses through the heart. Three large systemic veins entered the pericardial cavity, the right anterior vena cava, the left anterior vena cava, and the posterior vena cava. Their walls contained myocardium and were therefore considered to be derivatives of the sinus venosus, the chamber upstream of the right atrium in ectotherms, and the three vessels will be referred to as the posterior sinus horn (posterior vena cava), and the left and right sinus horn. The opening of the sinus venosus to the right atrium always had a myocardial valve, comprising a left and a right leaflet. The luminal side of the wall of the right atrium had multiple trabeculations of varying sizes. The trabeculations converged cranially in a large muscular arch that spanned the roof of both atria from right to left, the so-called transverse arch. At the bottom of the right atrium, the entrance to the right ventricle was guarded by the large muscular flap valve, which, on the ventricular side, is made up of thick ventricular wall, and on the atrial side is made up of much thinner atrial muscle.

The left atrium received two pulmonary veins in an antechamber before opening into the body of the left atrium. This antechamber consisted of the myocardial walls of the pulmonary veins and a dorsal ridge of myocardium which was situated immediately ventral to the esophagus. A muscular shelf in the roof of the atrial cavity partly separated the antechamber from the body of the left atrium. The walls of the antechamber were smooth. The wall of the body of the left atrium was trabeculated like the wall of the right atrium. An atrial septum, without a fossa ovalis, separated the left and right atrium. The right atrium appeared larger than the left atrium, due to a somewhat left-ward position of the atrial septum in combination with a greater caudo-cranial height of the right atrial cavity. The entrance to the left ventricle was guarded by the mitral valve consisting of thin fibro-membranous leaflets. The outflow tract of both ventricles, the aorta and pulmonary artery, were situated on the ventral side of the atria.

3.2 The sinus venosus

Myocardium was found in the left sinus horn (LSH), right sinus horn (RSH), and posterior sinus horn (PSH) (Figure 2). The amount of myocardium varied between sinus horns. In all birds in which histology was performed, the myocardium of all three sinus horns stopped in proximity of the pericardium. In the birds analyzed with Amira (Table 1, Supplementary Figure 1), the volume of the sinus venosus was compared to the volume of the right atrium (right atrial wall and right atrial trabeculations) and was 11% in the Mallard (N=1), 12% in the Barn swallow(N=1), and 13% in the Green woodpecker (N=1).

A left sinus horn was present in all sectioned bird hearts. Proximal to its entry to the right atrium, its myocardial sleeve was thick and fully surrounded the lumen (Figure 2). As the left sinus horn opened into the right atrium some muscle protruded into the lumen as a leaflet (Figure 3). Such a leaflet was observed in all birds apart from the Common kestrel where the leaflet was thin and membranous. Compared to the total volume of myocardium in the sinus venosus, the myocardium of the left sinus horn comprised 60% in the Barn swallow, 59% in the Green woodpecker and 61% in the Mallard (Table 1).

The entrance of the right sinus horn to the right atrium was separate from those of the posterior sinus horn and left sinus horn. Only in the Green woodpecker was the right sinus horn part of a larger sinus venosus. The orifice of the right sinus horn was guarded by the sinuatrial valve, the left leaflet of which was most developed in this region of the right atrium. Cranially, the left leaflet had a thick muscular margin that merged with the transverse arch in the Common swift, Eurasian coot, Green woodpecker, and Budgerigar (Supplementary Figure 2). Caudally, the right leaflet would be more developed in the Mallard, Common kestrel and Barn swallow, whereas the left leaflet appeared more developed in the Common swift, Eurasian coot and Budgerigar. The position of the orifice of the right sinus horn and posterior sinus venosus varied between species. We measured these positions relative to a dorsal-ventral axis that was established from the position of esophagus, atrial septum, aorta and pulmonary vein (Supplementary Figure 3).

3.3 Sinuatrial node in bird hearts

Detection of markers of the sinuatrial node was successful in adult Mallard, Chicken HH42, and Lesser redpoll, the best-preserved specimens (Figure 4). In the Mallard, Isl1 detection was

confined to a small oval-shaped structure at the base of the right leaflet of the sinuatrial valve (Figure 4a-c, Supplementary Figure 4). At its most expansive, the sinuatrial node cross-section was approximately 700 µm wide and 900 µm long, it was detected on 8 sections each 300 µm apart, giving a total volume of approximately 1,5 mm^3. A large coronary artery, identified by the expression of smooth muscle actin in the arterial wall, was found within the Isl1 positive domain (Figure 4b). This domain was relatively rich in collagen compared to the surrounding myocardium of the sinus venosus and right atrium (Figure 4a). In Chicken HH42, the sinuatrial node was less distinct anatomically than in the Mallard. The base of the right leaflet of the sinuatrial valve had less expression of *cTnI* than the surrounding muscle (Figure 4d-e), and it was the only muscle that expressed *Bmp2* (Figure 4f) and Isl1 (Figure 4g). In the Lesser redpoll, there was no anatomically identifiable node (Figure 4h). The base of the right leaflet of the sinuatrial valve was thicker than the surrounding walls, and this region expressed Isl1 (Figure 4i-j) and had a large coronary artery (Figure 4i). In most specimens, however, Isl1 could not be detected, likely due to poor epitope preservation and the sinuatrial junction was damaged in several specimens. On the picro-sirius red stained sections with reasonably good to good tissue presentation, a mallard-like sinus node was found in the budgerigar only (Supplementary Table 1). In the swift, woodpecker, and kestrel, there was no mallard-like sinus node, but rather thickened myocardium on the sinus-side of the right leaflet of the sinuatrial valve which resembled the Isl1 positive domain of the Lesser redpoll (Supplementary Table 1). These three species are phylogenetically in between the mallard and budgerigar, and there was no obvious phylogenetic trend concern the anatomy of the putative sinus node.

3.4 The right atrium

In all sectioned bird hearts, extensive trabeculations were found in the right atrial wall. The smallest birds, Barn swallow and Lesser redpoll, had the fewest trabeculations, 4 and 5 respectively were found across the atria, whereas the much larger Mallard had approximately 18. Most trabeculations were nodular in cross section and much thicker than the atrial wall (Figure 5). In Budgerigar, a large trabeculation was 0.56 mm while the atrial wall next to it was 0.021 mm this means the trabeculation was 27 times thicker than the atrial wall next to it. In Mallard, a large trabeculation was 1.79 mm while the atrial wall next to it was 0.22 mm this means that the trabeculation was 8 times thicker than the atrial wall next to it (Figure 5). The trabeculations connected together in a large muscular transverse arch in the roof of both atria.

In the birds analyzed with Amira (Table 1), half of atrial muscle was trabeculated muscle (transverse arch, left atrial trabeculation, right atrial trabeculation and dorsal ridge) in Barn swallow, Green woodpecker and Mallard. The atrial septum was thin and we never observed a shallow depression akin to the foramen ovale of eutherian mammals.

3.5 Position of the atrioventricular orifices

Inspected cranially, the atrioventricular and arterial orifices were nestled together (Figure 6). The aortic valve was almost medial and relative to its position, the right atrioventricular junction was dorsal and right, the left atrioventricular junction was dorsal left, and the pulmonary arterial valve was ventral (Figure 6). The right atrioventricular junction was configured as a C, with the medial margin provided by the interventricular septum and the parietal margin provided by the large muscular flap valve. Ventrally, the flap valve merged with the septal surface. The left atrioventricular junction was always rounded and guarded by a valve of connective tissue (Figure 6). When we inspected the sections from cranial to caudal, the leaflets of the left atrioventricular junction always appeared before the flap valve of the right atrioventricular junction (Supplementary Table 2), showing there was always an caudo-cranially offset between the two atrioventricular junctions (Supplementary Figure 5). The left atrioventricular junction appeared 1,65mm before the right atrioventricular junction averaged over 9 species. In this the Eurasian coot had the largest offset at 4,4mm and the Common snipe had the smallest with 0,3mm.

3.6 The pulmonary artery, aorta, and coronary arteries

All investigated pulmonary arterial valves had three leaflets of approximately equal size (Figure 6). The leaflets were anchored in 2 dorsal commissures, and one ventral commissure (Figure 6j). The aortic valve was approximately of the same size as the pulmonary arterial valve and also with 3 leaflets and commissures (Figure 6). In contrast to the pulmonary arterial valve, the position of the commissures of the aortic valve showed some variation (Figure 6i), such that the dorsal right commissure could be dorsal right (e.g. Ostrich and Chicken, Figure 6a,c), lateral (e.g. Kestrel, Figure 6f), or ventral right (e.g. Budgerigar, Figure 6g). The aorta always gave rise to two coronary arteries, with the right coronary artery originating from the sinus to the right of the ventral commissure, and the left coronary artery originating from the sinus to the

left of the ventral commissure (Figure 6). Immediately outside the sinus, the right coronary artery gave off a branch that descended into the ventricular septum (Figure 6d), where the flap valve of the right atrioventricular junction merged with the ventricular septum.

3.7 The left atrium

In all species, only two pulmonary veins, the left and the right, entered the pericardial cavity (Figure 7). They were of equal size and symmetrical when entering the heart. A sleeve of myocardium was found around both pulmonary veins (Figure 7). With immunohistochemistry, it was confirmed that the myocardium always stopped in the vicinity of the pericardium and the myocardium never extended into the lungs (Figure 7, Supplementary Figure 6). As the myocardial sleeve stopped, the thickness of the wall of the pulmonary vein decreased with around 50% to 75%. The myocardial sleeves around the pulmonary veins, the dorsal ridge and the left atrial shelf formed an antechamber before the body of the left atrium. The prominence of the dorsal ridge was different between species (Table 1). The left atrial shelf formed the ventral boundary of the antechamber and was mostly muscular with some collagen. The free margin of the shelf pointed towards the atrioventricular junction. While the atrial shelf was found in all birds sectioned, the development of the shelf varied between species (Table 1). The left atrial wall was thin, dominated by a few large trabeculations, like the right atrium.

3.8 Ventral merger of the atria

In the Budgerigar and the Barn swallow, we found that the walls of the left and right atrium had merged ventral to the pulmonary artery (Figure 8), over a span of approximately 1mm from cranial to caudal. In the Hawfinch, we sampled sections for every 200µm and found only one section where the walls of the left and right atrium had merged. In this section, the point of merger appeared as a band of collagen. In the Lesser redpoll, we sampled sections for every 200µm, and did not find merger on any section. However, the two atria were closely juxtaposed, if not merged, as a part of the left atrium was ventral to the pulmonary artery in sections 461-501, and a part of the right atrium was ventral to the pulmonary artery in section 521. In other orders, the atria were further apart ventral to the pulmonary artery. Only in *F. tinnunculus* (Common kestrel) were the atria close to each other, but were separated by a pad of fatty tissue (Figure 8). The Common kestrel is part of the order Falconiformes which is phylogenetically

the closest group that was sectioned to Psittaciformes (Budgerigar) and Passeriformes (Barn swallow, Hawfinch, and Lesser redpoll).

4 | DISCUSSION

In the first subsections below (4.1-4.4), we place our findings in an evolutionary context, leading to the final subsection that argues that the mammalian heart exhibits much more variation than the avian heart. On this basis, we reject the hypothesis that the transition from ectothermy to endothermy and high cardiac performance associated with greater variation in cardiac structure.

4.1 Gross morphological synapomorphies of the bird heart

Birds are grouped in the archosaur clade, which also includes crocodylians, and hearts of birds share several gross morphological features with crocodylians, and to a lesser extent with other ectothermic reptiles. We show that birds, together with ectothermic reptiles (Jensen et al., 2014b; Cook et al 2017), always have 3 sinus horns (Gasch, 1888; Quiring, 1933), an atrial septum without a foramen ovale (Röse, 1890). As in ectothermic reptiles, the right atrium is more voluminous than the left atrium (Whittow, 1999; Jensen et al., 2014b). In birds and crocodylians, but not reptiles at large, the left atrioventricular valve comprise membranes anchored in ventricular papillary muscles (Van Mierop, Kutsche, 1985; Lincoln et al., 2004; Cook et al 2017). Birds also have features that appear to be present in crocodylians, only they are much more developed in birds. This includes the large muscular flap valve in the right atrioventricular junction (Jensen et al., 2014b), the myocardial shelf between the antechamber and the left atrial body, which is a meagre flap coming in crocodiles (Webb, 1979; Cook et al 2017), and the offset between left and right atrioventricular junctions (Cook et al 2017), which we confirm is much more pronounced in birds.

4.2. Gross morphological apomorphies of the bird heart

The bird heart is readily distinct from the crocodylian heart, and hearts of other reptiles (Jensen et al., 2014b; Cook et al 2017), by the opening of 2 pulmonary veins with myocardial sleeves to the left atrium. The pulmonary veins empty to a voluminous antechamber with a dorsal ridge

of myocardium. A similar antechamber is not seen in crocodylians despite the presence of the muscular shelf (Webb, 1979; Cook et al 2017). In birds, the myocardial shelf is at some distance from the orifices of the pulmonary veins and the left atrioventricular junction. It could prevent regurgitation (Benninghoff, 1933), but given the distances to any orifice, it may simply guide blood towards the left ventricle. The myocardial shelf bears some resemblance to the membrane that divides the left atrium in the human congenital malformation of cor triatriatum (Bharucha et al., 2015; Benninghoff 1933) suggested that the myocardial shelf of birds develops from the left pulmonary ridge of the early embryonic atrium. A recent mouse model recapitulates cor triatriatum as seen in some patients, but it is not clear whether the setting is the outcome of aberrant development of the left pulmonary ridge (Muggenthaler et al., 2017).

Birds have one aorta with a tricuspid valve, ectothermic reptiles have 2 aortae with bicuspid valves. The atrial walls of birds are dominated by a few, large pectinate muscles coming together in the massive transverse arch in the atrial roof, whereas in ectothermic reptiles the atrial walls consist of a thick meshwork of innumerous tiny trabeculations (Boukens et al[1]; Sedmera et al., 2000). This architecture in birds, parallels the compact organization of their ventricular walls. Functionally, the compact wall architecture likely offers less impedance to the tremendous blood flows that characterize birds and may further allow for higher heart rates by enabling fast electrical activation of the chambers (Boukens et al[1]).

4.3 Convergent gross morphological features

The hearts of birds and mammals exhibit convergent features. Early anatomists observed that birds and mammals have a single aorta, rather than 2 as in ectothermic reptiles, and the aortic and pulmonary arterial valve is tricuspid, rather than bicuspid (Benninghoff, 1933; Bartyzel, 2009a; Bartyzel, 2009b). The total number of arterial valve leaflets is thus similar between mammals (6 leaflets), birds (6 leaflets), and ectothermic reptiles (6 leaflets), despite ectothermic reptiles having a left aorta (2 leaflets), a right aorta (2 leaflets), and a pulmonary artery (2), reflecting evolutionary conserved morphogenetic processes (Poelmann et al., 2017). Like crocodylians and mammals (Cook et al 2017), there is a full ventricular septum, and the atrioventricular junctions have an offset whereby the left is cranial to the right. Our findings indicate the offset is substantially greater in birds than in crocodylians and mammals. The pronounced offset in birds, may reflect in part that the membranous septum of the ventricle completely myocardializes, whereas in crocodylians the membranous septum exhibits less

myocardialization (Jensen et al., 2018) and in mammals the membranous septum is always present in the adult heart (Rowlatt, 1990). We confirm the constant presence of large left atrioventricular junction guarded by membranous leaflets anchored to papillary muscles, and this feature is shared by crocodylians, birds, and mammals (Jensen et al., 2013; Cook et al 2017). The ventricular walls of mammals and birds are much less trabeculated than the ventricles of ectothermic vertebrates (Jensen et al, 2016). Similarly, the atrial walls comprise a thick meshwork of trabeculations in ectotherms, whereas in birds there are some 10 trabeculations only, as previous described in chicken (Sedmera et al., 2000).

4.4 Variation between bird hearts

The ventral merger of the atria represents the clearest case for a feature that is not shared by all birds, and it may occur only within passerines and parrots. This feature has not been described previously to the best of our knowledge. In mammals, the left and right atrium can be identified by expression of Pitx2 and Bmp10 respectively (Kahr et al, 2011), but the relative contribution of the left and right atrium to the ventral merger remains to be shown. Due to the state of the tissue in our samples the expression Pitx2 and Bmp10 was not preformed, future studies could examine the origin of the ventral merger in the bird heart and test the electrical conductivity to examine reentry.

Isl1 could not be detected in most specimens, presumably due to the state of tissue preservation, but the Isl1 expressing myocardium had substantial variation in morphology. One extreme was the mallard, where Isl1 was confined to a nodal structure, not unlike in mammals (Chiodi, Bartolomew, 1967). In contrast, in chicken and the Lesser redpoll, Isl1 was detected in myocardium that at best, could be distinguished as being slightly thicker than the surrounding walls of the sinus venosus and right atrium. This anatomically poorly defined setting has been recognized previously (Davies, 1930; Chiodi, Bartolomew, 1967; Lamers, De Jong, De Groot, Moorman, 1991) and strongly resembles the pacemaker region of ectothermic reptiles (Jensen et al., 2017). The mammalian sinuatrial node resembles a horse shoe shape at the base of the superior vena cava (Keith, Flack, 1907; Davies, 1942; Opthof, 1988; Boyett, Honjo, Kodama 2000; Chandler et al. 2009). Comparative anatomy placed the sinus node of birds at the base of the right leaflet (Davies F, 1930; Chiodi, Bartolomew, 1967), which has been confirmed in chicken by electrophysiological and molecular studies (Moore, 1965; Bressan, Lui, Louie, Mikawa, 2016). Extending those studies, we show in chicken the co-localization of *Bmp2* and

Isl1 which is also seen in the pacemaker tissue of Zebrafish (Tessadori et al., 2012) and Anole lizards (Jensen et al., 2017). Also, we confirm the observation by Keith and Flack in the original description of the sinus node, that the sinus node associates with a large coronary artery (Keith, Flack, 1907).

The state of development of the sinuatrial valve appeared to vary between specimens, and while this was a difficult feature to assess from histology, the thick valvar margin found in the Green woodpecker was unusual. We found a very similar extend of myocardium in the pulmonary veins of all specimens, but the extend of pulmonary venous myocardium may vary in the chicken (Endo et al., 1992). In the Common kestrel, as in other birds of prey (Gasch, 1888), the left leaflet of the sinuatrial valve leaflet was very thin and membranous. In the birds examined with Amira, we found the size of the sinus venosus to be 20% for Mallard and Barn swallow and 30% for Green woodpecker relative to the right atrium. This corresponds to the size relation of the sinus venosus to the atrium in fishes, which is 45% for the White sturgeon and 20% for the Mako shark 20% (Gregory et al., 2004). Between specimens, there was variation in the number of trabeculations of the atrial wall. This variation could reflect phylogeny, but size of the heart is likely another factor as we found the hearts of the smallest investigated birds, e.g. barn swallow and lesser redpoll, to have particularly few trabeculations. Generally, the position of atrioventricular junctions and arterial bases were fixed, but the aorta did exhibit some rotation in the transverse plane (but much less so than in mammals (Rowlatt, 1990)).

4.5 The mammal heart is exceptional varied

Most features of the bird heart, exhibit less variation than the same features in mammal hearts. Birds always have 3 sinus horns, whereas mammals may have 2, if the left sinus horn is regressed, or 3. In mammals, the sinuatrial valve can be well-developed and reptile-like as in monotremes, well-developed only around the inferior caval vein and coronary sinus, or almost completely regressed (Rowlatt, 1990; Jensen et al., 2014a). In contrast, in birds both leaflets are always present although their state of development varies as we show here (Benninghoff, 1933).

The atrial septum of monotreme and marsupial mammals develop from the primary septum only, like in birds and ectotherms. Only in eutherian mammals is the atrial septum formed by the merger of the primary and secondary atrial septum, a process that is revealed in the adult heart by the foramen ovale (Röse, 1890; Runciman, Gannon, Baudinette, 1995; Jensen

et al.[2]).

The right atrioventricular junction in monotreme mammals is dominated by a large parietal flap valve, much like in birds and crocodylians, except there is very little if any myocardium in the monotreme valve (Dowd, 1969). In marsupial and eutherian mammals, the right atrioventricular valve is of connective tissue only and typically has 2 or 3 leaflets anchored by papillary muscle (Rowlatt, 1990; Runciman, Baudinette, Gannon, 1992). The right atrioventricular valve is without papillary muscle in the bird heart.

In monotreme mammals, the left atrium receives a single pulmonary vein, like in ectothermic reptiles, whereas between genera of marsupial and eutherian mammals, the number of pulmonary veins can vary between 1 and 7 and myocardial sleeves may be absent, short or extensive (Rowlatt, 1990). In contrast, 2 pulmonary veins connecting to the left atrium is a "konstant" feature of birds (Benninghoff, 1933). We confirm this observation and further show that the pulmonary veins have myocardial sleeves that extend to the pericardial boundary. In mammals, the myocardial sleeves are often the cause of atrial fibrillation through automaticity and reentry (Haïssaguerre et al., 1988; Chen et al., 1999; Tsuneoka, Koboyashi, Honda, Namekata, Tanaka, 2012), and it remains to be shown whether the pulmonary veins of birds are similarly arrhythmogenic.

The degree of trabeculated atrial myocardium ranges from nearly smooth-walled (fruit bat) to heavily trabeculated atria (lar gibbon) in mammals (Rowlatt 1968), whereas birds, except the smallest species (Barn swallow and Lesser redpoll), show a similar number of trabeculation.

In mammals, there is always a ventricular membranous septum which varies in size between genera and may be covered by a layer of myocardium (Rowlatt, 1990). Whereas birds do form a membranous septum in develop, it undergoes myocardialization and the formed avian heart is without a membranous septum.

The relative position of the atrioventricular junctions and the base of the pulmonary artery and the aorta, is not fixed across mammals (Rowlatt, 1990). The left and right atrioventricular junctions may be juxtaposed as in tree squirrels or far apart as in pygmy sperm whales. This in turn appears to reflect aortic wedging, the extent to which the aorta has moved from its embryonic right-ward position and towards the left ventricle (Cook et al 2017). In contrast, we found in all birds the same relative position of the atrioventricular junctions and the base of the pulmonary artery and the aorta. Only the commissures of the aortic valve appeared to exhibit some relative rotation, but such rotation, again as an outcome of aortic wedging, appears much

greater in mammals (Rowlatt, 1990).

The bird heart has several features that set it apart from ectothermic reptiles and these features do not exhibit much variation. In contrast, the features that set mammals apart from their fellow amniotes, exhibit substantial variation. The hypothesis that the ectothermy-endothermy transition led to greater variation in cardiac design, is not supported by our data. While its clear there is a common building plan to the amniote heart (Olson, 2006; Jensen, Wang, Christoffels, Moorman, 2013), the basis of the morphogenetic plasticity of mammals remains to be explored.

5 | CONCLUSION

We assessed 15 features of and around the atria of hearts from 13 orders of birds. Most features were surprisingly constant in appearance between orders, even those that were not shared with ectothermic reptiles and thus considered apomorphic. This suggests, in contrast to what was hypothesized, that the transition from ectothermy to endothermy and the concomitant evolution of a high-performance heart, does not necessarily lead to the extraordinary degree of variability in cardiac design that is observed in mammals.

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AUTHOR CONTRIBUTIONS

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REFERENCES

Bartyzel B.J., (2009a). Morphology of the pulmonary valve (valva trunci pulmonali) in chosen species of domestic and wild birds using imaging methods. *Bulletin of the Veterinary Institute in Pulawy 53*: 303-308

Bartyzel B. J., (2009b). The aortic valve and other heart structures of selected species of sea birds in a morphological and imaging scope. *Electronic journal of polish agricultural universities 12*.4

Benninghoff, A., (1933). Das Herz. In L. Bolk, E. Goppert, E. Kallius and W. Luhosch (Eds), *Handbuch der Vergleichenden Anatomie der Wirbeltzere, 6:* 467-556. Berlin and Wien: Urban und Schwarzenberg.

Bharucha T., Spicer D. E., Mohun T. J., Black D., Henry G. W., Anderson R. H., (2015). Cor triatriatum or divided atriums: which approach provides the better understanding? *Cardiology in the Young, 25*(2):193-207

Boyett M. R., Honjo H., Kodama I., (2000). The sinoatrial node, a heterogeneous pacemaker structure. *Cardiovascular Research, 47*(4): 658–687.

Bressan M., Lui G., Louie J. D., Mikawa T., (2016) Cardiac pacemaker development from a tertiary heart field. In: Nakanishi T., Markwald R., Baldwin H., Keller B., Srivastava D., Yamagishi H. (eds) *Etiology and Morphogenesis of Congenital Heart Disease*. Springer, Tokyo. pp 281-288

Calkins H., Brugada J., Packer D. L., Cappato R., Chen S.-A., Crijns H. J. G., Damiano Jr. R. J., (...), Shemin R. J., (2007). HRS/EHRA/ECAS Expert Consensus Statement on Catheter and Surgical Ablation of Atrial Fibrillation: Recommendations for Personnel, Policy, Procedures and Follow-Up. *Heart Rhythm, 4*(6): 816-861.

Carmona, R., Ariza, L., Cañete, A., Muñoz-Chápuli, R., (2018). Comparative developmental

biology of the cardiac inflow tract. *Journal of Molecular and Cellular Cardiology, 116,* 155-164

Chandler N. J., Greener I. D., Tellez J. O., Inada S., Musa H., Molenaar P., Difrancesco D., Baruscotti M., Longhi R., Anderson R. H., Billeter R., Sharma V., Sigg D. C., Boyett M. R., Dobrzynski H., (2009). Molecular architecture of the human sinus node: insights into the function of the cardiac pacemaker. *Circulation, 119*(12): 1562–1575.

Chen S.A., Hsieh M. H., Tai C. T., Tsai C. F., Prakash V. S., Yu W. C., Hsu T. L., Ding Y. A., Chang M. S., (1999) Initiation of atrial fibrillation by ectopic beats originating from the pulmonary veins: Electrophysiological characteristics, pharmacological responses, and effects of radiofrequency ablation. *Circulation, 100*(18):1879–1886.

Chiodi, V., Bortolami, R., (1967) The conducting system of the vertebrate heart. *Calderine, Bologna*.

Cook A. C., Tran V. H., Spicer D. E., Rob J. M. H., Sridgaran S., Taylor A., Anderson R. H., Jensen B., (2017) Sequential segmental analysis of the crocodilian heart. *Journal of Anatomy, 231*(4): 484-499.

Crossley G. H., Sorrentino R. A., Exner D. V., Merliss A. D., Tobias S. M., Martin D. O., Augostini R., Piccini J. P., Schaerf R., Li S., Miller C. T., Adler S. W., (2016) Extraction of chronically implanted coronary sinus leads active fixation vs passive fixation leads. *Heart Rhythm, 13*(6):1253-1259. doi: 10.1016/j.hrthm.2016.01.031.

Davies, F., (1930). The Conducting System of the Bird's Heart. *Journal of Anatomy, 64*(Pt 2), 129–146.7.

Davies, F., (1942). The conducting system of the vertebrate heart. *British Heart Journal 4,* 66–76

Dowd, D. A., (1969). The coronary vessels and conducting system in the heart of monotremes. *Cells Tissues Organs, 74.*4: 547-573

Endo H., Kurohmaru, M., Nishida, T., Hayashi, Y. (1992) Cardiac musculature of the cranial and caudal venae cavae and the pulmonary vein in the fowl. *Journal of Veterinary Medical Science, 54,* 479–484

Gasch F. R., (1888) Beiträge zur vergleichenden Anatomie des Herzens der Vögel und Reptilien. *Archiv für Naturgeschichte, 54*(1):119-154.

Green R.E., Braun E.L., Armstrong J., Earl., Nguyen N., Hickey G., … Ray D.A., (2014). Three crocodilian genomes reveal ancestral patterns of evolution among archosaurs. *Science, 346*.6215.

Gregory J. A., Graham J. B., Cech J. J. Jr., Dalton N., Michaels J., Chin Lai N., (2004). Pericardial and pericardioperitoneal canal relationships to cardiac function in the white sturgeon (Acipenser transmontanus). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology , 138*(2): 203–213.

Haïssaguerre M., Jaïs P., Shah D. C., Takahashi A., Hocini M., Quiniou G., Garrigue S., Mouroux A., Métayer P., Clémenty J., (1998). Spontaneous initiation of atrial fibrillation by extopic beats originating in the pulmonary veins. *The New England Journal of Medicine, 339*(10):659-666.

Hamburger V., Hamilton H.L., (1951) A series of normal stages in the development of the chick embryo. Journal of Morphology 88:1 49-92.

Jensen B., Agger P., de Boer B. A., Oostra R. J., Pedersen M., van der Wal A. C., Planken N. R., Moorman A. F., (2016). The hypertrabeculated (noncompacted) left ventricle is different from the ventricle of embryos and ectothermic vertebrates. *Biochimica et Biophysica Acta Molecular Cell Research, 1863* (7 Pt B): 1696-1706 https://doi.org/10.1016/j.bbamcr.2015.10.018.

Jensen B., Boukens B. J. D., Wang T., Moorman A. F. M., Christoffels V. M., (2014). Evolution of the sinus venosus from fish to human. *Journal of Cardiovascular Development and Disease, 1*:14-28.(a)

Jensen B., Boukens B. J. D., Crossley D. A., Conner J., Mohan R. A., van Duijvenboden K., Postma A. V., Gloschat C. R., Elsey R. M., Sedmera D, Efimov I. R., Christoffels V. M., (2018). Specialized impulse conduction pathway in the alligator heart. *eLife 7*: e32120

Jensen B., Moorman A. F. M., Wang T., (2014). Structure and function of the hearts of lizards and snakes. *Biological Reviews of the Cambridge Philosophical Society, 89*(2): 302–336.(b)

Jensen B., van den Berg G., van den Doel R., Oostra R. J., Wang T., Moorman A. F. M., (2013). Development of the Hearts of Lizards and Snakes and Perspectives to Cardiac Evolution. *PLoS One, 8*(6): e63651.

Jensen B., Vesterskov S., Boukens B. J. D., Nielsen J. M., Moorman A. M. F., Christoffels V. M, Wang T., (2017). Morpho-functional characterization of the systemic venous pole of the reptile heart. *Scientific reports, 7* DOI: 10.1038/s41598-017-06291-z.

Jensen B., Wang T., Christoffels V. M., Moorman A. F. M., (2013). Evolution and development of the building plan of the vertebrate heart. *Biochimica et Biophysica Acta Molecular Cell Research, 1833*(4): 783-794.

Kahr P. C., Piccini I., Fabritz L., Greber B., Schöler H., Scheld H. H., Hoffmeier A., Brown N. A., Kirchhof P., (2011). Systematic analysis of gene expression differences between left and right atria in different mouse strains and in human atrial tissue. *PLoS One, 6*(10).

Keith A., Flack M., (1907), The form and nature of the muscular connections between the primary divisions of the vertebrate heart. *Journal of Anatomy and Physiology, 41*(Pt 3), 172.

Lamers, W. H., De Jong F., De Groot I. J., Moorman A. F., (1991). The development of the avian conduction system, a review. *European journal of morphology 29*.4: 233-253.

Lincoln J., Alfieri C. M., Yutzey K. E., (2004) Development of heart valve leaflets and supporting apparatus in chicken and mouse embryos. *Developmental Dynamics 230*.2: 239-250

Mansour M., Holmvang G., Sosnovik D., Migrino R, Abbara S., Ruskin J., Keane D., (2004). Assessment of pulmonary vein anatomic variability by magnetic resonance imaging: implications for catheter ablation techniques for atrial fibrillation. *Journal of Cardiovascular Electrophysiology, 15*(4): 387–393

Mommersteeg M. T. M., Brown N. A., Prall O. W. J., de Gier-de Vries C., Harvey R. P., Moorman A. F. M., Christoffels V. M., (2007). Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. *Circulation Research, 101*(9): 902–909.

Moore, E. N. (1965). Experimental electrophysiological studies on avian hearts. Annals of the New York Academy of Sciences, 127(1), 127-144

Muggenthaler M. M. A., Chowdhury B., Hasan S. N., Cross H. E., Mark B., Harlalka G. V., Patton M. A., (...), Chioza B. A., (2017). Mutations in HYAL2, Encoding Hyaluronidase 2, Cause a Syndrome of Orofacial Clefting and Cor Triatriatum Sinister in Humans and Mice. PLoS Genetics, 13(1) art. no. e1006470.

Nathan H., Eliakim M., (1966). The junction between the left atrium and the pulmonary veins. An anatomic study of human hearts. *Circulation, 34*(3): 412–422.

Olson, E. N., (2006). Gene regulatory networks in the evolution and development of the heart. *Science 313*.5795: 1922-1927

Opthof, T., (1988). The mammalian sinoatrial node. *Cardiovascular drugs and therapy 1*.6: 573-597

Poelmann, R. E., Gittenberger-de Groot, A. C., Biermans, M. W. M., Dolfing, A. I., Jagessar, A., van Hattum, S., … Richardson, M. K., (2017). Outflow tract septation and the aortic arch system in reptiles: lessons for understanding the mammalian heart. *EvoDevo, 8*, 9. http://doi.org/10.1186/s13227-017-0072-z

Poelmann, R. E., Mikawa T., Gittenberger-De Groot, A. C., (1998). Neural crest cells in outflow tract septation of the embryonic chicken heart: differentiation and apoptosis. *Developmental Dynamics 212*.3: 373-384

Quiring D. P., (1933). The development of the sino-atrial region of the chick heart. *Journal of Morphology, 55*(1):81-118.

Röse C., (1890). Beitrage zur vergleichenden Anatomie des Herzens der Wirbelthiere. *Gegenbaurs Morphologisches Jahrbuch, 16:* 27-96

Rowlatt, U., (1968). Functional Morphology of the Heart in Mammals. *Integrative and Comparative Biology, 8*;2(1):221–229.

Rowlatt, U., (1990). Comparative anatomy of the heart of mammals. *Zoological Journal of the Linnean Society, 1*;98(1):73-110.

Runciman S. I. C., Baudinette R. V., Gannon B. J., (1992). The Anatomy of the Adult Marsupial Heart - an Historical Review. *Australian Journal of Zoology, 40*:21-34

Runciman S. I. C., Gannon B. J., Baudinette R. V., (1995). Central cardiovascular shunts in the perinatal marsupial. *Anatomical Record, 243*:71-83

Sedmera D., Pexieder T., Vuillemin M., Thompson R.P., Anderson R.H., (2000) Developmental patterning of the myocardium. *Anatomical Record, 1*; 258(4): 319–337.

Somi S., Buffing A. A. M., Moorman A. F. M., van den Hoff M. J. B., (2004). Dynamic patterns of expression of BMP isoforms 2, 4, 5, 6, and 7 during chicken heart development. *The Anatomical Record 279*.1, 636-651.

Tattersall G. J., (2016). Reptile thermogenesis and the origins of endothermy. *Zoology 119.5*: 403-405

Tessadori F., van Weerd J. H., Burkhard S. B., Verkerk A.O., de Pater E., Boukens B. J., Vink A., Christoffels V. M., Bakkers J., (2012). Identification and Functional Characterization of Cardiac Pacemaker Cells in Zebrafish. *PLoS One, 7*(10).

Tsuneoka Y., Kobayashi Y., Honda Y., Namekata I., Tanaka H., (2012). Electrical activity of the mouse pulmonary vein myocardium. *Journal Pharmacological Science, 119*:287–292.

van den Berg G., Abu-Issa R., de Boer B. A., Hutson M. R., de Boer P. A., Soufan A. T., Ruijter J. M., Kirby M. L., van den Hoff M. J., Moorman A. F., (2009). A caudal proliferating growth center contributes to both poles of the forming heart tube. *Circulation research 104*.2: 179-188.

Van den Hoff M. J. B., Kruithof B. P. T., Moorman A. F. M., Markwald R. R, Wessels A., (2001). Formation of myocardium after the initial development of the linear heart tube. *Developmental biology, 240*.1, 61-76.

Van Mierop L. H. S., (1967). Location of pacemaker in chick embryo heart at the time of initiation of heartbeat. *American Journal of Physiology-Legacy Content 212*.2: 407-415

Van Mierop L. H. S., Kutsche L. M., (1985). Development of the ventricular septum of the heart. *Heart and Vessels, 1*(2): 114-119. https://doi.org/10.1007/BF02066358

Warren, W. C., Hillier, L. W., Marshall Graves, J. A., Birney, E., Ponting, C. P., Grützner, F., … Wilson, R. K., (2008). Genome analysis of the platypus reveals unique signatures of evolution. *Nature*, *453*(7192), 175–183.

Webb, G., (1979). Comparative cardiac anatomy of the Reptilia. III. The heart of crocodilians and an hypothesis on the completion of the interventricular septum of crocodilians and birds. *Journal of Morphology, 161*(2): 221-240.

Whittow G. C., (1999). *Sturkie's Avian Physiology (Fifth Edition).* Academic Press; Cambridge, Massachusetts.

Wyneken J., (2009). Normal reptile heart morphology and function. *Veterinary Clinics of North America: Exotic Animal Practice, 12*(1): 51-63.

Boukens et al. in press[1] and Jensen et al., in press, Anat Rec[2] are unpublished at this time and

therefore not full references.

FIGURE LEGENDS

Figure 1 Scheme of the analyzed structures of the avian hearts. 1) myocardium in the sinus venosus, 2) the sinuatrial node, 3) sinuatrial valve and leaflets, 4) left sinus horn, 5) trabeculations of the atrial wall, 6) position of right atrioventricular junction and muscularity of the valve, 7) position of the pulmonary artery and valve leaflets, 8) number of pulmonary veins, 9) the extent of pulmonary venous myocardium relative to the pericardium (dashed line), 10) dorsal ridge of the antechamber of the left atrium, 11) muscular shelf in the roof of the left atrium, 12) position of left atrioventricular junction and muscularity of the valve, 13) position of the aorta and valve leaflets, 14) position of the orifices of the coronary arteries, 15) presence of ventral merger of the atrial walls.

Figure 2 Myocardium in the sinus venosus of the Budgerigar (a,c,e) and the Mallard (b,d,f). Picro-sirius red stained 10µm histological sections. (a,b) The right sinus horn (RSH) contains myocardium (black arrows). (c,d) The left sinus horn (LSH) contains myocardium (black arrows). The myocardial wall can be quite thick proximal to the atria, but tapers off distally. At the pericardial border, the vessel wall may be without myocardium. (e,f) The posterior sinus horn (PSH) contains myocardium (black arrows). Ao, aorta; LA, left atrium; LAM, left atrial muscle; PA, pulmonary artery; RA = right atrium. In the images from the Budgerigar, blood has been painted over with white for clarity.

Figure 3 Leaflet of the valve of the left sinus horn in the Green woodpecker (a) and the Common kestrel (b). Birds generally have a prominent valve (black arrow) guarding the orifice of the left sinus horn (LSH), here exemplified by the Green woodpecker. It was found on sections representing 0.7mm out of the total height of the atria of 6.8mm. In the Common kestrel, the valve was very thin membranous leaflet and it was found on sections representing 0.7mm out of the total height of the atria of 6.6mm. Ao, aorta; Eso, esophagus; LA, left atrium; LV, left ventricle; LSH, left sinus horn; PA, pulmonary artery; PSH, posterior sinus horn; RA, right atrium. In the images, blood has been painted over with white for clarity.

Figure 4 Sinuatrial node in Mallard (a-c), Chicken HH42 (d-g), and Lesser redpoll (h-j). (a-c) In the Mallard, there was a nodal structure at the base of the right leaflet of the sinuatrial

valve (e) which expressed Isl1 (f-g) and which had a large coronary artery (white arrowhead in (f)). (d-e) In the Chicken HH42, the sinus venosus (SV) expressed the myocardial marker *cTnI* and this expression was relatively weak at the base of the right leaflet of the sinuatrial valve (black arrowhead in (e)). (f-g) The base of the right leaflet of the sinuatrial valve expressed *Bmp2* (red arrowheads in (f)) and Isl1 (g). (h-j) In the Lesser redpoll, Isl1 was expressed in the base of the right leaflet of the sinuatrial valve. There was no nodal structure but the Isl1 expressing wall was thicker than the surrounding walls and there was a large coronary artery (white arrowhead in (i)). Ao, aorta; LA, left atrium; PA, pulmonary artery; PSH, posterior sinus horn; RA, right atrium; SAJ, sinuatrial junction.

Figure 5 Atrial trabeculations in the Barn swallow and the Mallard. Picrosirius red stained section of Budgerigar (a,b) and Mallard (c,d). The position of the of the trabeculations imaged in (a,c) are indicated by black squares in the overviews (b,d). Red arrows indicate trabeculations, black arrows point to the atrial wall in between the trabeculations. Note the several-fold greater thickness of the trabeculations to the atrial wall. Ao, aorta; E, epicardium; LA, left atrium; P, pericardium; PA, pulmonary artery; RA, right atrium. In the images from the Budgerigar, blood has been painted over with white for clarity.

Figure 6 Major arteries. (a) Gross morphology of the ventricular base of the with indication of the valve commissures (green arrowheads) and the right coronary artery (R). (b-d) In the Ostrich, the left coronary artery (L) originates from the sinus of the ventral-left leaflet of the aortic valve (b) and the right coronary artery (R) originates from the sinus of the ventral-right leaflet of the aortic valve (c) (arrow heads indicate leaflet commissures). (d) The 3D reconstruction of the lumen of the major arteries shows the right coronary immediately splits into a branch that runs in the interventricular septum (IVS), next to the ventral merger of the right atrioventricular valve and the interventricular septum. The right coronary gives rise to the right circumflex artery (rc) leading to the dorsal descending artery (dd) in the interventricular sulcus. The left coronary splits into the ventral descending artery (vd) in the interventricular sulcus and the left circumflex artery (lc). The images of (b-d) are derived and modified from the Ostrich 3D model published previously (Jensen et al., 2013). In the Common kestrel (e-f) and Budgerigar (g-h), the origin of the coronary arteries from the aorta was like in the Ostrich, and the intraseptal branch of the right coronary artery was always found next to the ventral

merger of the right atrioventricular valve with the interventricular septum. The cartoons (i-j) show the distribution of the commissures of the valve leaflets between all sectioned birds. Ao, aorta; LA, left atrium; LAVV, left atrioventricular valve; PA, pulmonary artery; RA, right atrium; RAVV, right atrioventricular valve.

Figure 7 Pulmonary vein myocardium. The first column, picrosirius red stained section. Black arrows point to the distal-most extent of myocardium. The second and third column shows immunohistochemical detection of cTnI at the cites indicated by the arrows. In the Kestrel, only the right pulmonary vein is shown since the left pulmonary vein was damaged during sectioning. Eso, esophagus; LA, left atrium; lpv, left pulmonary vein; RA, right atrium; rpv, right pulmonary vein. In all picrosirius red images, apart from Mallard, the blood has been painted over with white for clarity.

Figure 8 Ventral merger of the atria in budgerigar and barn swallow. (a-d) In the Budgerigar, the atria were merged ventral to the pulmonary artery (PA), by connective tissue (a-b) and myocardium (M) of the left (LA) and right (RA) atrium (c-d). The section in (c) is caudal to (a). In the Barn swallow, the myocardium of the left and right atrium is merged ventral to the pulmonary artery. (g-h) In the Common kestrel, the myocardium of the left and right atrium was not merged ventrally. Ao, aorta; F, fat; LAAC, left atrial antechamber; LSH, left sinus horn; PSH, posterior sinus horn; RSH, right sinus horn. In all images, the blood has been painted over with white for clarity.

TABLES

Table 1 Proportions of atrial structures, estimated from areas in Amira.

Structure\Species	Mallard	Green woodpecker	Barn swallow
Total weight (g)	0,7	0.07	0,005
Sinus venosus	3%	5%	5%
Left sinus horn	5%	7%	7%
RA wall	20%	21%	29%
RA trabeculations	8%	11%	8%
Transverse arch	32%	20%	26%
Dorsal ridge	1%	4%	5%
LA shelf	2%	0,2%	0,5%
LA wall	17%	15%	13%
LA trabeculations	8%	15%	7%

Values are rounded to whole numbers, except for values less than 1.

SUPPLEMENTARY FIGURES

Supplementary Figure 1 Amira example slides of Mallard and Green woodpecker. (a) fully labeled mallard section in Amira. (b) picrosirius red section of the labeled section in a. (c) fully labeled Green woodpecker section in Amira. (d) picrosirius red section of the labeled section in d.

Supplementary Figure 2 Thick margin of the sinuatrial valve in the Green woodpecker. The red arrow points to the thick margin of the valve which persists for 800µm out of a total of 6800 µm for the whole atria. Ao, aorta; Eso, esophagus; LA, left atrium; LSH, left sinus horn; PA, pulmonary artery; RA, right atrium.

Supplementary Figure 3 Variation in the position of the orifice of the sinus horns to the right atrium. The colored ovals indicate the approximate size of the orifice to the size of the atria.

Supplementary Figure 4 Identification of the sinuatrial node in the Mallard. Picro-sirius red stained section the black square highlights a part of the sinuatrial junction(SAJ) while the black arrow points at the sinuatrial node(a). Cartoon of the sinus venosus with section lines marking the region that was examined(b). Immunohistochemistry sections of the area marked in panel a with the black square and the section lines in panel b. The TNI signal is marked white, Isl1 signal is marked red and nuclei signal blue this way co-localization of Isl1 and nuclei turns purple(c-f). Magnification of the area's marked with the white square in panel e and f respectively (e ', f'). Ao, aorta; Eso, esophagus; LA, left atrium; LSH, left sinus horn; PA, pulmonary artery; PSH, posterior sinus horn; RA, right atrium; RSH, right sinus horn; V, ventricles.

Supplementary Figure 5 Offset in atrioventricular junctions in the Collared dove. The left atrioventricular junction (LAVJ) is cranial to the right atrioventricular junction (RAVJ). LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

Supplementary Figure 6 Example examination of pulmonary vein myocardium in Collared dove. Picrosirius red figures (a, d) of the section show general morphology of the atria and surrounding tissue. The black boxes in these images represent the locations of the immunohistochemistry images(b-c, e-f). Green marks the cTnI domain while gray marks nuclei. Eso, esophagus; LA, left atrium; LAAC, left atrial antechamber; LPV, left pulmonary vein; RA, right atrium; RPV, right pulmonary vein.

FIGURES AND SUPPLEMENTARY FIGURES

Figure 1

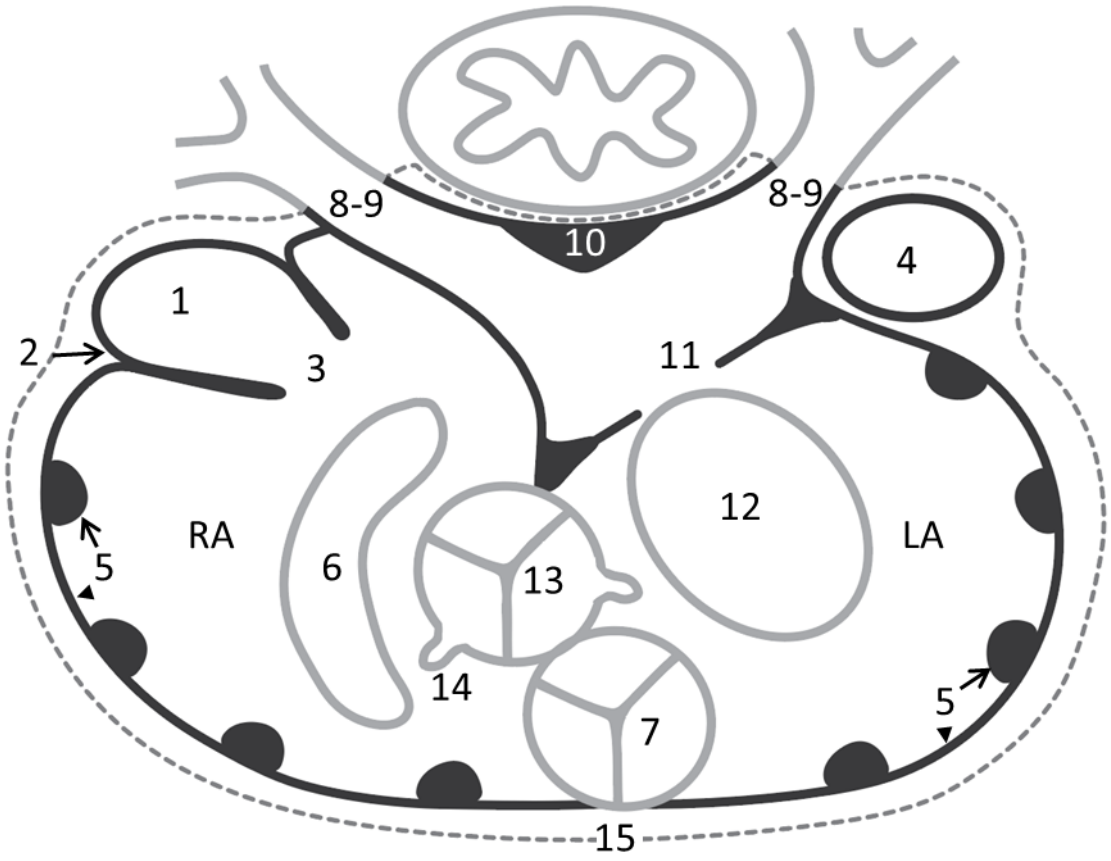

Figure 2

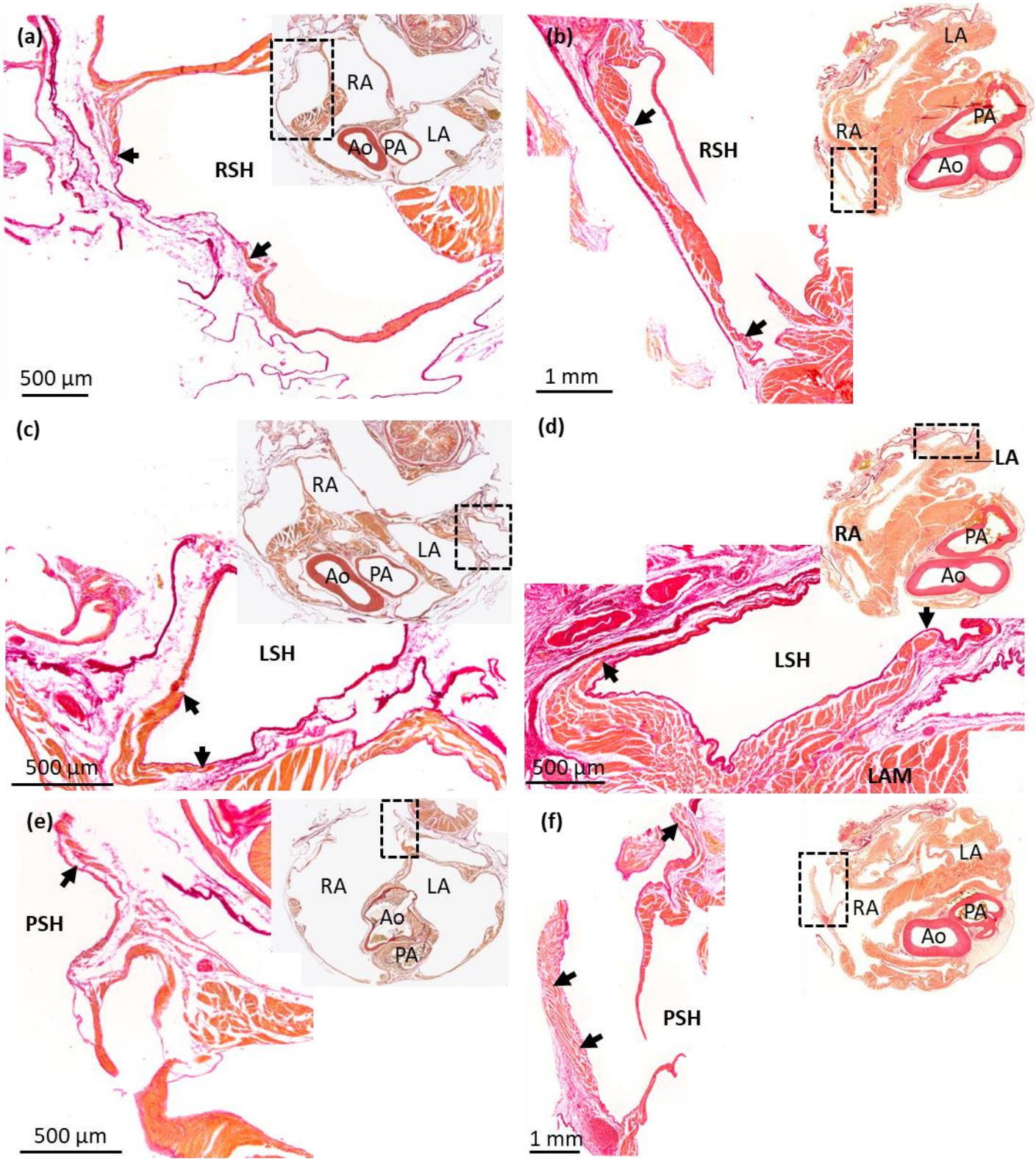
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Figure 3

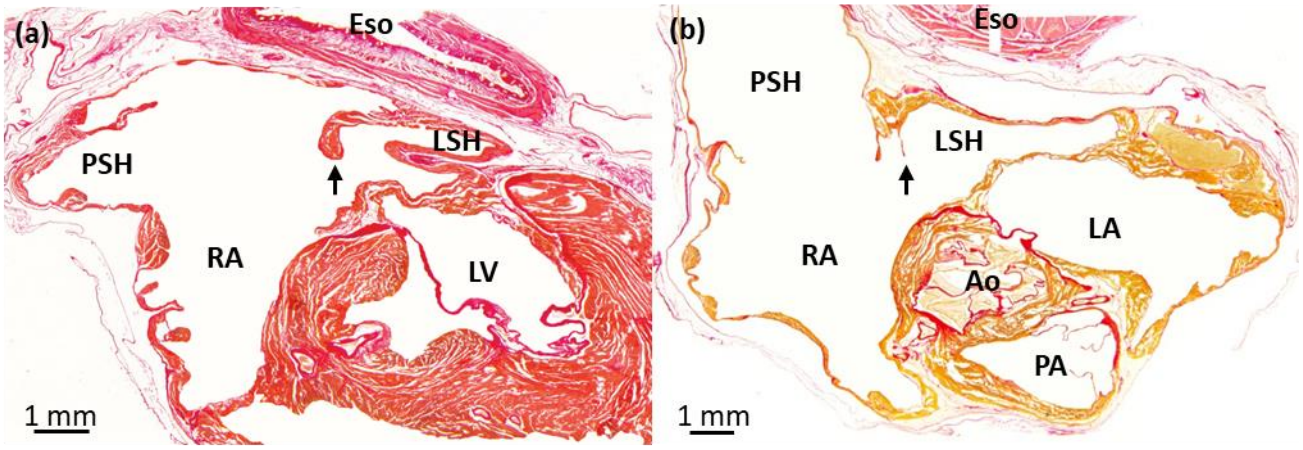


Figure 4

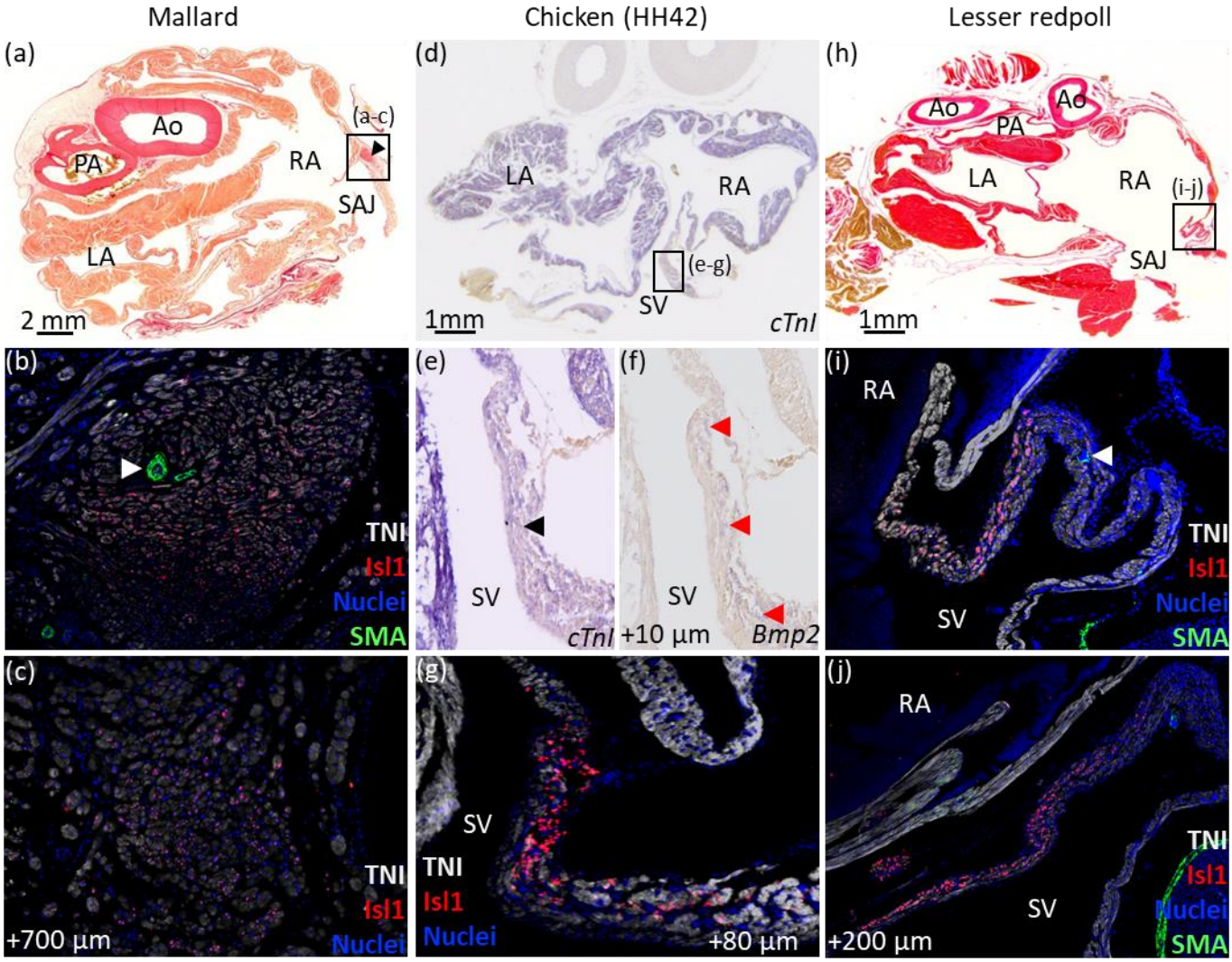


Figure 5

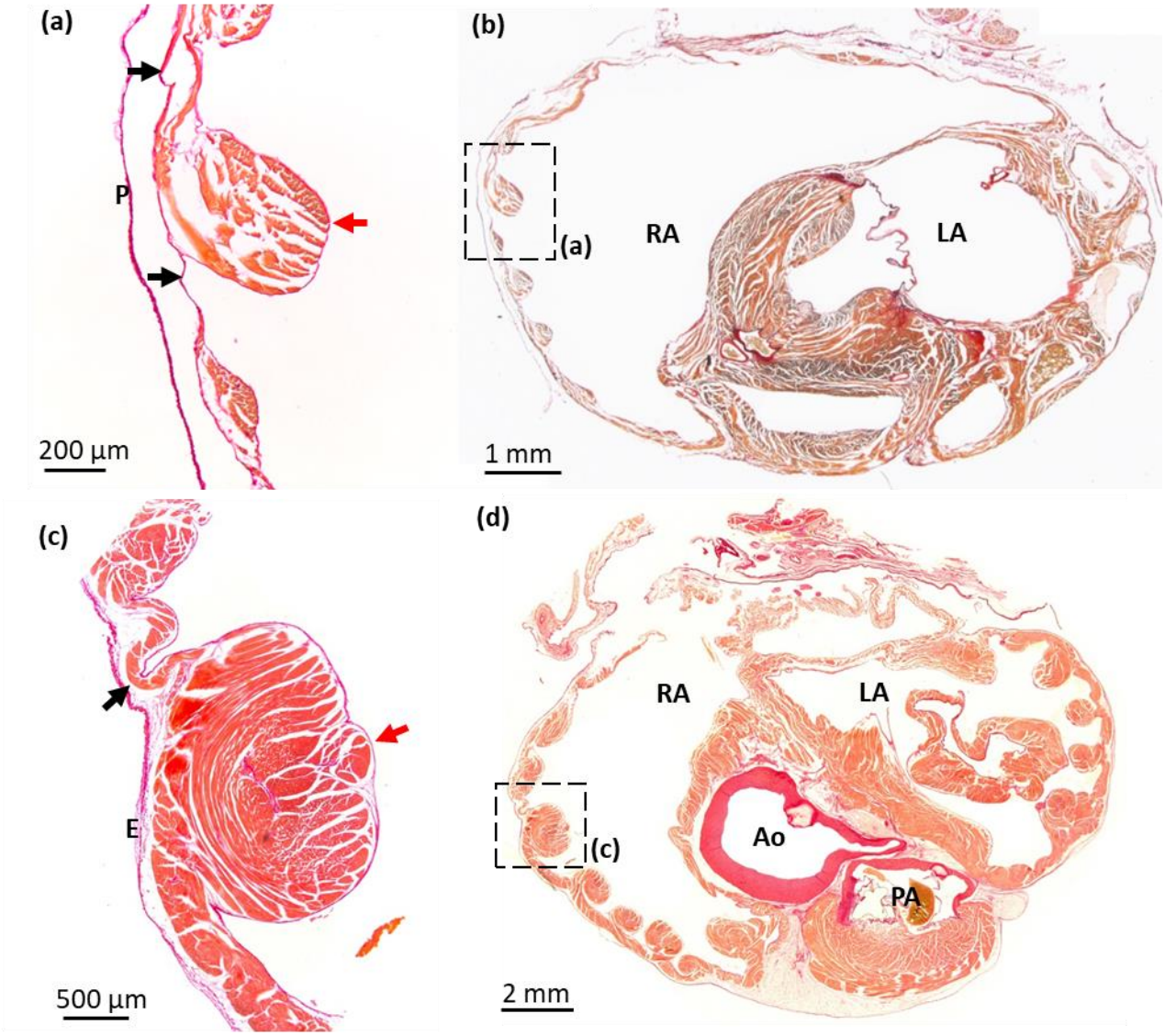


Figure 6

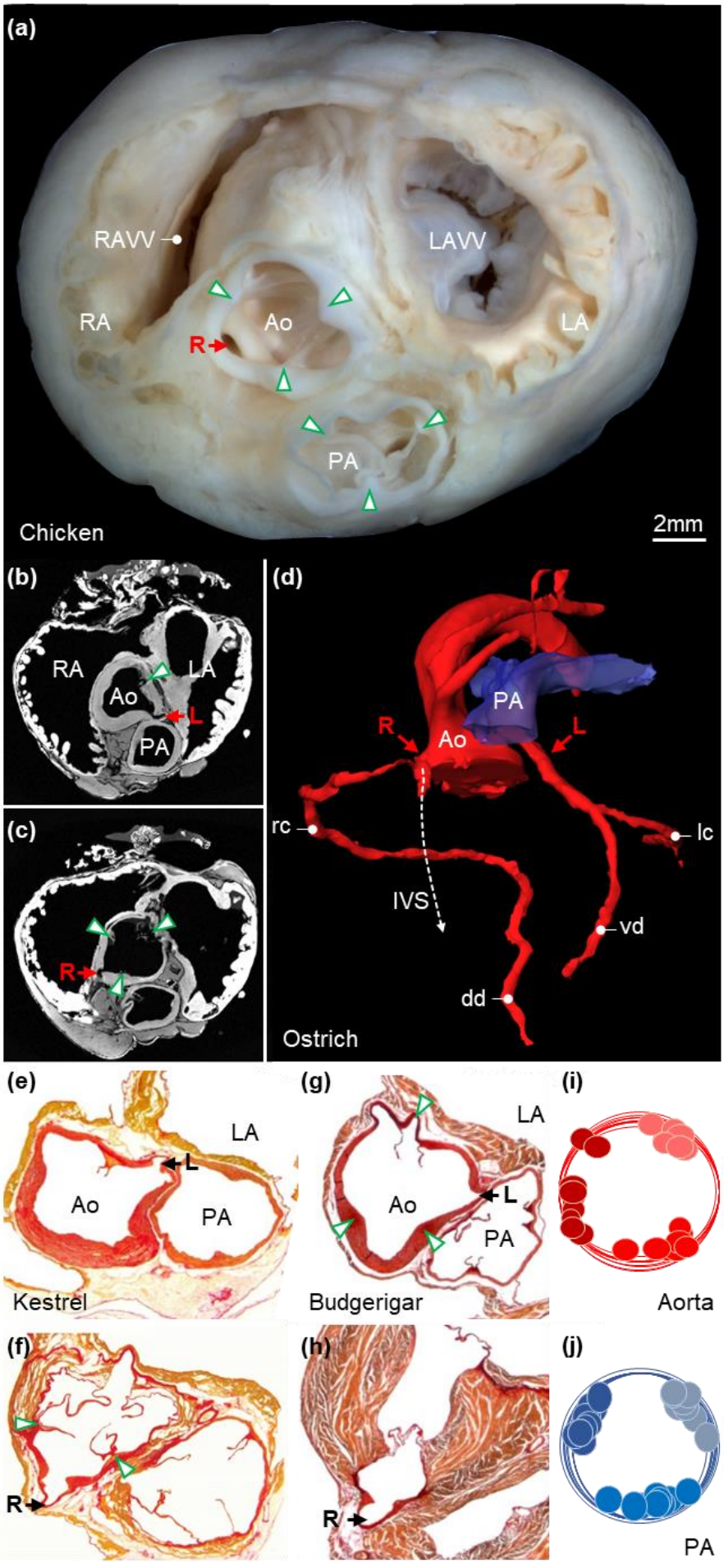

(a)
RAVV
LAVV
RA
Ao
LA
R
PA
Chicken
2mm
(b)
RA
LA
Ao
L
PA
(c)
R
(d)
PA
R
Ao
L
rc
lc
IVS
vd
dd
Ostrich
(e)
LA
L
Ao
PA
Kestrel
(g)
LA
Ao
L
PA
Budgerigar
(i)
Aorta
(f)
R
(h)
R
(j)
PA

Figure 7

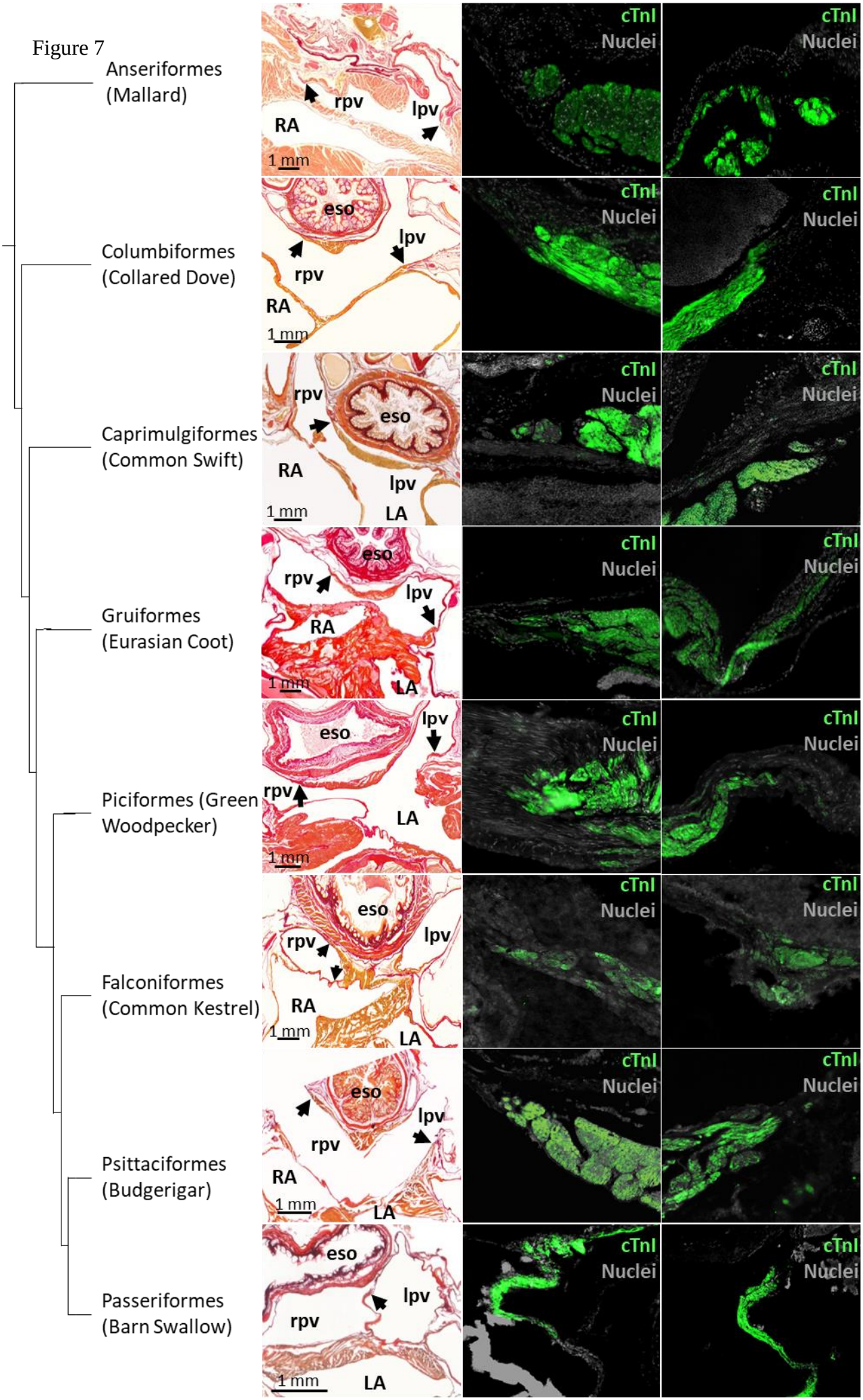
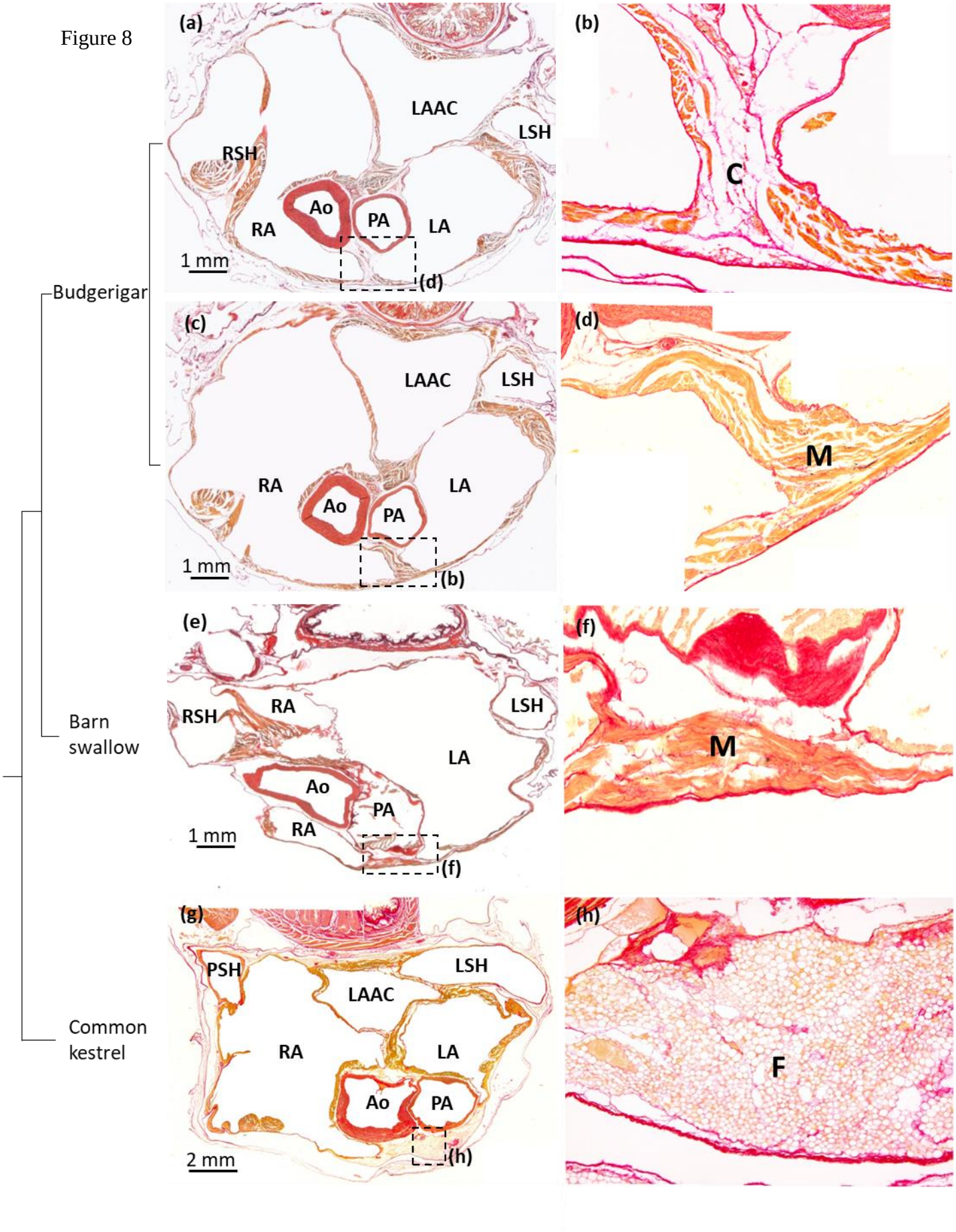
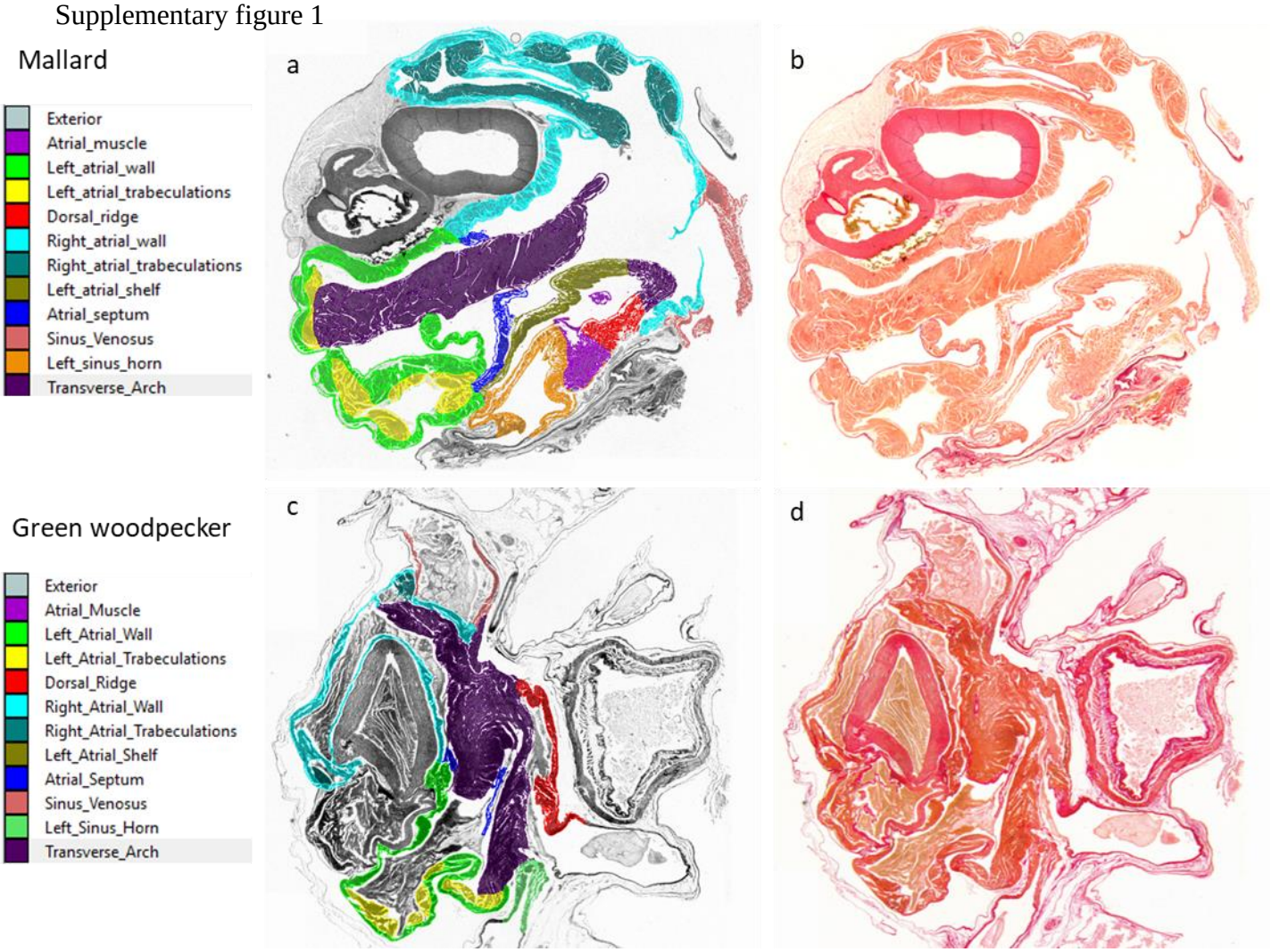

Anseriformes
(Mallard)
Columbiformes
(Collared Dove)
Caprimulgiformes
(Common Swift)
Gruiformes
(Eurasian Coot)
Piciformes (Green
Woodpecker)
Falconiformes
(Common Kestrel)
Psittaciformes
(Budgerigar)
Passeriformes
(Barn Swallow)
rpv
lpv
RA
LA
eso
1 mm
cTnI
Nuclei

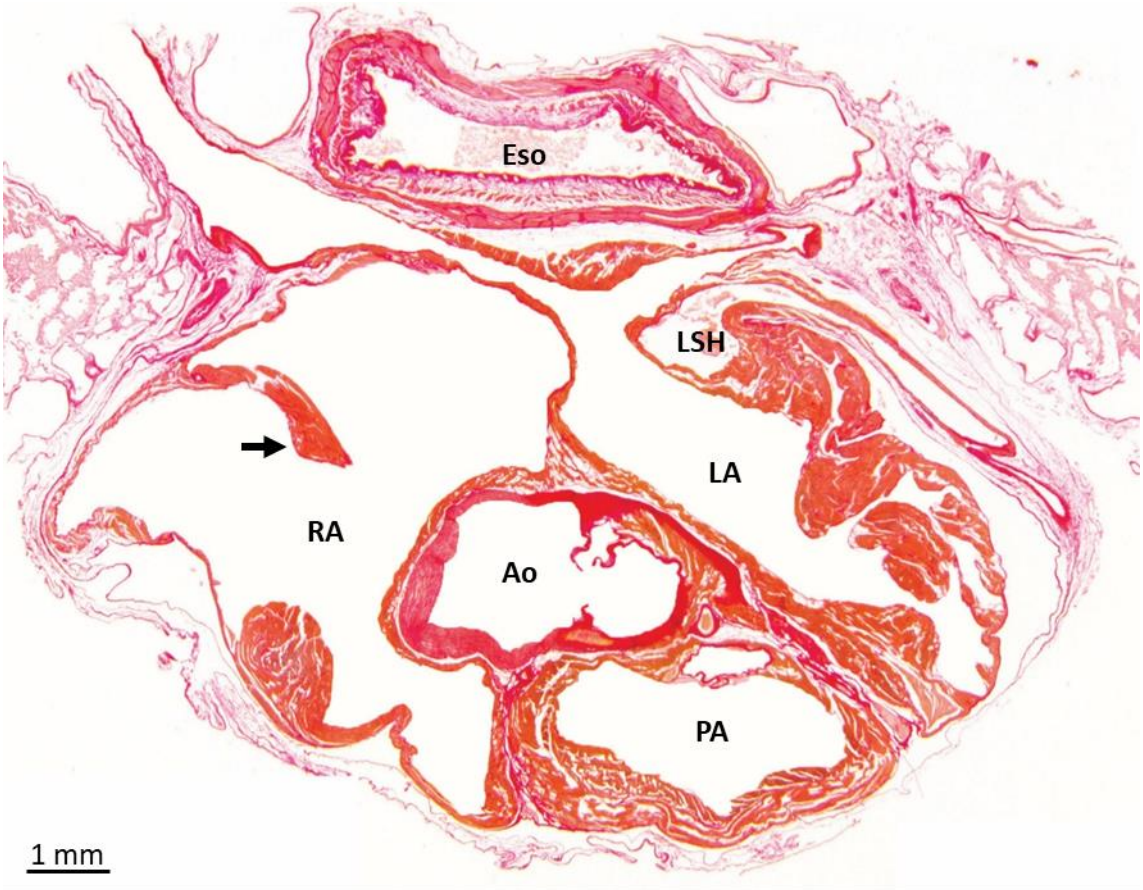
Figure 8


(a)
LAAC
LSH
RSH
Ao
PA
RA
LA
1 mm
(d)
(b)
C
Budgerigar
(c)
LAAC
LSH
RA
LA
Ao
PA
1 mm
(b)
(d)
M
(e)
RSH
RA
LSH
LA
Ao
PA
RA
1 mm
(f)
(f)
M
Barn swallow
(g)
PSH
LSH
LAAC
RA
LA
Ao
PA
2 mm
(h)
(h)
F
Common kestrel

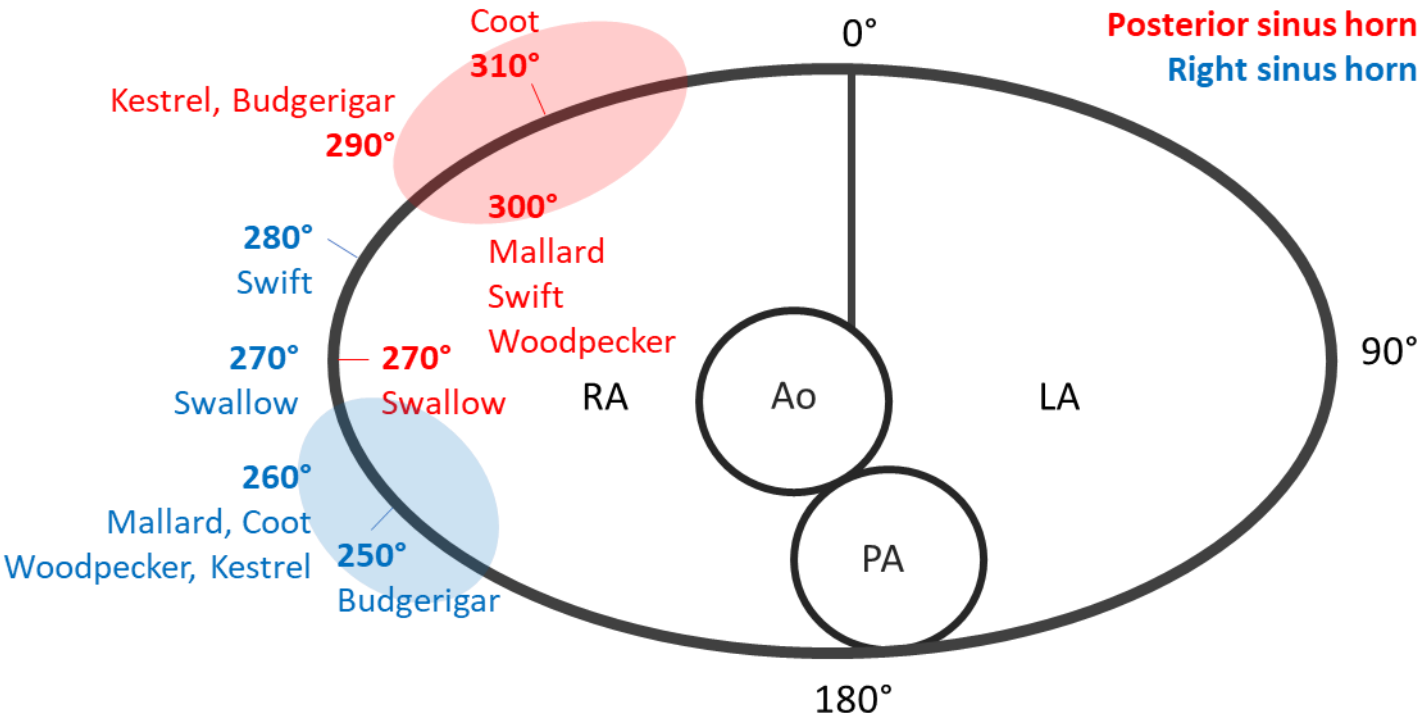
Supplementary figure 1



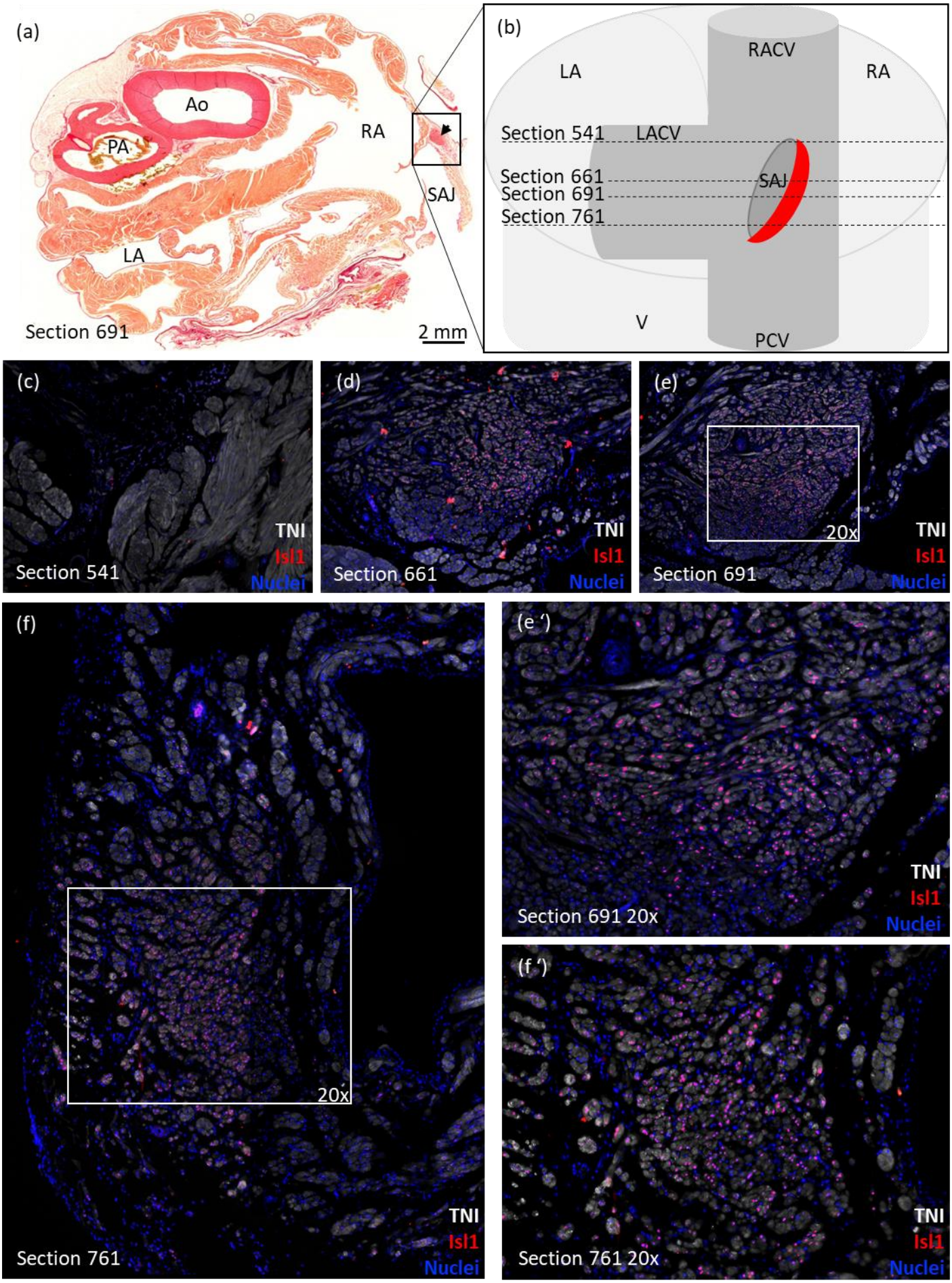
Supplementary figure 2



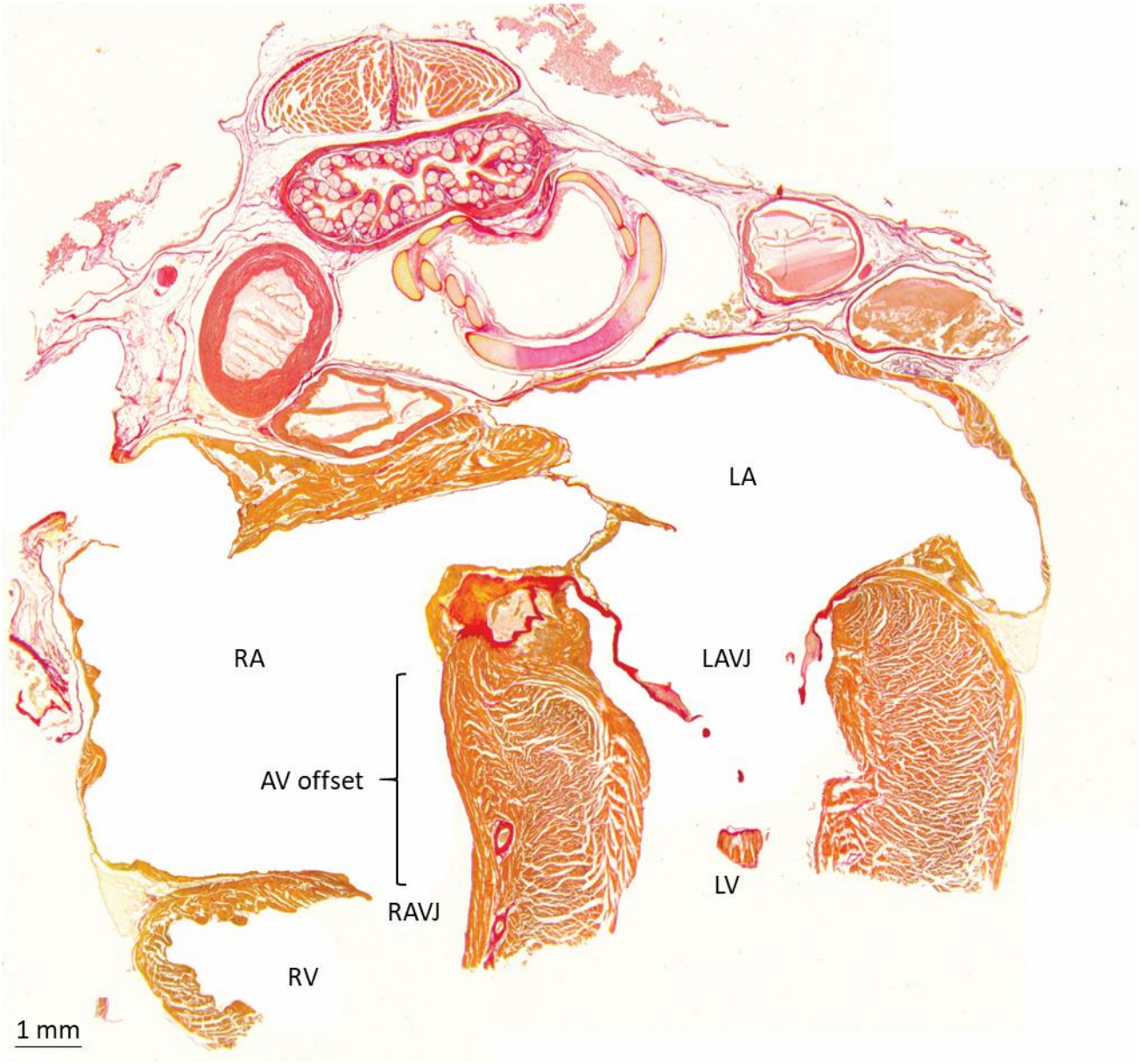
Supplementary figure 3



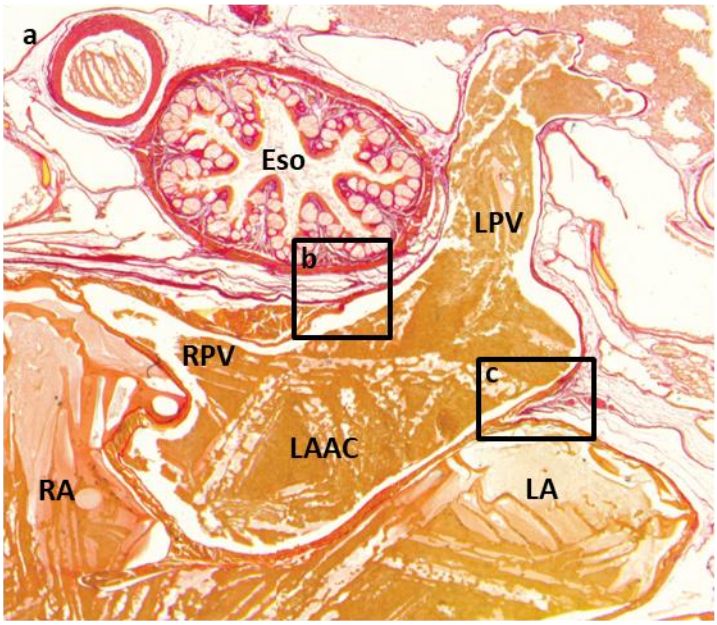
Supplementary figure 4

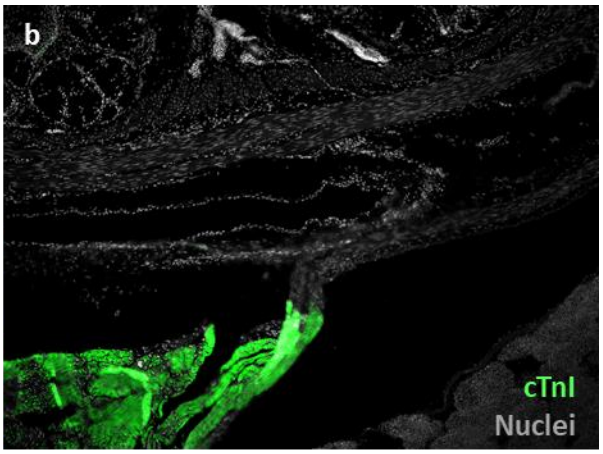


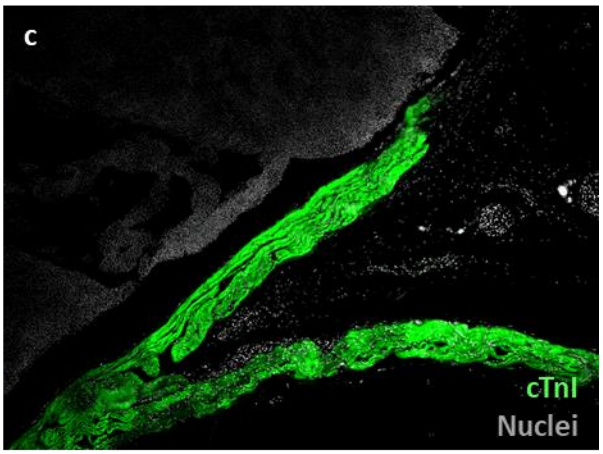
Supplementary figure 5

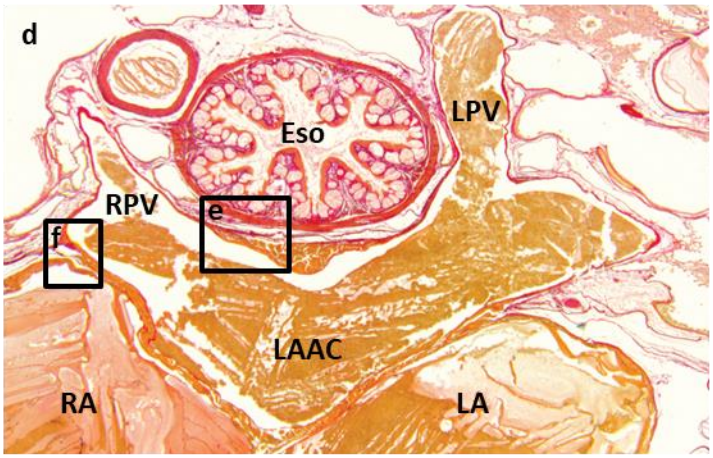


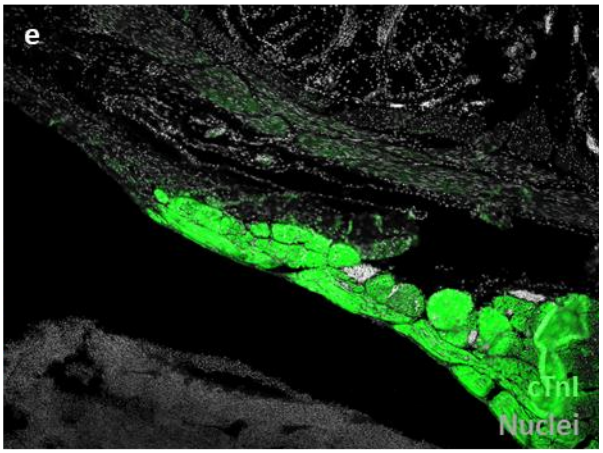
Supplementary figure 6

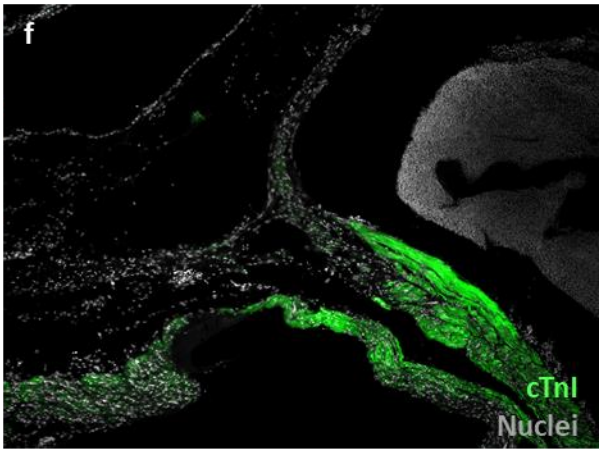












SUPPLEMENTARY TABLES

Supplementary table 1

Species\Structure	1) Myo SV	2) SAN (mol)	2) SAN (histo)	3) SAV + Leaflets	4) LSH	5) Trabeculations	6) RAVJ	7) PAV	8) NO. PV	9) Myo PV	10) Dorsal ridge	11) Shelf	12) LAVJ	13) AoV	14) Cor	15) Ventral merger
Ostrich	-	-	-	M+MRI	M+MRI	M+MRI	M+MRI	M+MRI	M+MRI	-	M+MRI	M+MRI	M+MRI	M+MRI	M+MRI	M+MRI
Mallard	Yes	Yes	Yes (on right)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Chicken	Yes	Yes	SA thickening (on right)	-	-	-	-	-	-	-	-	-	-	-	-	-
Collared Dove	Yes	-	-	Yes	Yes	Yes	-	-	Yes	Yes	Yes	Yes	-	-	-	Yes
Common Swift	Yes	-	SA thickening (on right), 541-621	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Eurasian Coot	Yes	-	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Common Snipe	Yes	-	-	Yes	Yes	Yes	D	D	Yes	Yes	Yes	Yes	D	D	D	Yes
Gray Heron	-	-	-	M	M	-	-	-	M	-	-	-	-	-	-	M
Barn Owl	D	D	-	D	D	D	D	D	D	D	D	D	D	D	D	D
Sparrowhawk	Only LSH	-	-	D	Yes	Yes	Yes	Yes	D	D	D	D	Yes	Yes	Yes	Yes
Green Woodpecker	Yes	-	SA thickening (on right), 1081-138	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Common Kestrel	Yes	-	SA thickening (on right), 801-901	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Budgerigar	Yes	-	Yes, 550-650	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Barn Swallow	Yes	-	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Lesser Redpoll	Yes	-	SA thickening (on right)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Hawfinch	Yes	-	D	D	Yes	Yes	Yes	Yes	Yes	Histo only	Yes	Yes	Yes	Yes	Yes	Yes
Macroscopic	M															
Not possible	-															
Damaged	D															
Histologic/IHC	Yes															

Supplementary table 2

	L-R offset in mm	Atrial muscle, cranial	Left AVJ	Right AVJ	Atrial muscle, caudal	total atrial muscle in mm
Species		Section	Section	Section	Section	
Mallard	1,8	271	991	1171	1201*	9,3*
Barn Swallow	1,2	120	270-330	390	540*	4,2*
Budgerigar	1,75	300	600-725	775	850*	5,5*
Common Kestrel	2,2	501	881-1101	1101	1281*	7,8*
Coot	4,4	81	361-641	801	821*	7,4*
Woodpecker	0,8	701	1061-1201	1141	1381*	6,8*
Hawfinch	>0,4	201	601	starts after 641	641*	4,4*
Redpoll	1,2	321	441-501	561	621*	3*
Snipe	0,3	480	990-1140	1020	1410*	9,3*
Swift	1,2	221	561-681	681	821*	6*
Sparrowhawk	>0,8	261	661	starts after 741	741*	4,8*
Ostrich		61	175-212	178-242	359	
Collard dove1	3,3					7,5
Barn Owl	D	D	D	D	D	D
1: The collared dove was sectioned in 4-chamber view therefore the L-R offset and total atrial muscle could be measured through the use of a scalebar rather than calculating the difference between sections.						
*the last section taken still contained atrial muscle therefore the very bottom of the atrium was unknown.						
D: damaged						