Smooth Muscle in Cardiac Chambers is Common in Turtles and Extensive in the Emydid Turtle, *Trachemys scripta*

WILLIAM JOYCE 1,2*, DANE A. CROSSLEY II, TOBIAS WANG, 1,3 AND BJARKE JENSEN 4

1Zoophysiology, Department of Bioscience, Aarhus University, 8000 Aarhus C, Denmark
2Department of Medical Biology, Amsterdam UMC, University of Amsterdam, Amsterdam 1105AZ, the Netherlands
3Aarhus Institute of Advanced Studies, Aarhus University, Aarhus, Denmark
4Department of Medical Biology, University Medical Center Amsterdam, Amsterdam, The Netherlands

ABSTRACT

A prominent layer of smooth muscle lining the luminal side of the atria of freshwater turtles (Emydidae) was described more than a century ago. We recently demonstrated that this smooth muscle provides a previously unrecognized mechanism to change cardiac output in the emydid red-eared slider (*Trachemys scripta*) that possibly contributes to their tremendous diving capacity. The purpose of the present immunohistochemical study was firstly to screen major groups of vertebrates for the presence of cardiac smooth muscle. Secondly, we investigated the phylogenetic distribution of cardiac smooth muscle within the turtle order (Testudines), including terrestrial and aquatic species. Atrial smooth muscle was not detected in a range of vertebrates, including *Xenopus laevis, Alligator mississippiensis, and Caiman crocodilus*, all of which have pronounced diving capacities. However, we confirmed earlier reports that traces of smooth muscle are found in human atrial tissue. Only within the turtles (eight species) was there substantial amounts of nonvascular smooth muscle in the heart. This amount was greatest in the atria, while the amount in proportion to cardiac muscle was greater in the sinus venosus than in other chambers. *T. scripta* had more smooth muscle in the sinus venosus and atria than the other turtles. In some specimens, there was some smooth muscle in the ventricle and the pulmonary vein. Our study demonstrates that cardiac smooth muscle likely appeared early in turtle evolution and has become extensive within the Emydidae family, possibly in association with diving. Anat Rec, 303:1327–1336, 2020. © 2019 The Authors. The Anatomical Record published by Wiley Periodicals, Inc. on behalf of American Association for Anatomy.

*Correspondence to: William Joyce, Department of Zoophysiology, Aarhus University, Aarhus, Denmark E-mail: william.joyce@bios.au.dk

Received 24 May 2019; Revised 24 June 2019; Accepted 28 June 2019.

DOI: 10.1002/ar.24257

Published online 10 October 2019 in Wiley Online Library (wileyonlinelibrary.com).
In the late 19th century, slow wave contractions (the so-called tonus waves) were observed in isolated atrial preparations from European pond turtles (Emys orbicularis) (Fano, 1887; Fano and Fayod, 1888; Bottazzi, 1897). The tonus waves were soon attributed to the conspicuous amounts of smooth muscle in the atria (Rosenzweig, 1903; Bottazzi, 1906), but despite a few decades of relatively intense research into its pharmacological properties (Fano, 1887; Fano and Fayod, 1888; Bottazzi and Grünbaum, 1899; Gautl, 1917; Gruber and Markel, 1918a, 1918b; Gruber, 1920a, 1920b, 1921, 1927; Sollmann and Rossides, 1927; Gruber, 1934; Dimond, 1959), the scientific interest in the atrial smooth muscle waned after the 1920s. As part of other studies, we also observed the tonus waves (Galli et al., 2006; Joyce et al., 2014), and with a revived curiosity into their functional role, we recently demonstrated that the atrial smooth muscle may provide a powerful means to regulate ventricular filling and hence cardiac stroke volume (Joyce et al., 2019). We predicted that atrial smooth muscle enables fine-tuning of ventricular filling and thus stroke volume during the characteristic rapid transitions from slow heart rates in apnea to the tachycardia associated with intermittent lung ventilation (Wang and Hicks, 1996; Joyce et al., 2018). Our previous experiments (Joyce et al., 2018) were designed with the atria in mind and we could not assess the presence and impact of smooth muscle in the sinus venosus or veins.

The European-based discoverers of the atrial smooth muscle universally employed the Emydid turtle E. orbicularis (formerly Emys europaea) (e.g., Fano, 1887; Bottazzi and Grünbaum, 1899; Fano and Bodano, 1900; Rosenzweig, 1903; Oinuma, 1910), but were soon followed by North American studies on a wealth of other turtle species in the Emydid family, including Trachemys scripta (snapping turtles; Chelydridae); very little detail is given by Pereira (Pereira, 1924, 1927; Sollmann and Rossides, 1927; Robb, 1952; Dimond, 1959). There are additional vague descriptions of tonus waves in atria from Caiman crocodilus (Laurens, 1913; Gruber and Markel, 1918a, 1918b; Pereira, 1924; Sollmann and Rossides, 1927; Robb, 1952; Dimond, 1959). There are additional vague descriptions of tonus waves in atria from Chelydra serpentina (snapping turtles; Chelydridae); very little detail is given by Pereira (Pereira, 1924, 1927), where data are indiscriminately combined with findings in Emydid turtles, while Blinks and Koch-Weser (1963) cite their own unpublished observations about this species. Gaskell (1900) did not observe tonus waves in the atria of the land tortoise (Testudinidae), Testudo graeca. In a more recent conference abstract, Gannon et al. (2003)) did not locate atrial smooth muscle in a side-necked turtle (Pleurodra), Emydura macquarii, although it has been detected in the sinus venosus, the chamber upstream of the right atrium in other reptile hearts (Jensen et al., 2014, 2017). If atrial smooth muscle is functionally related to cardiovascular regulation during diving, we predict it would be prevalent in aquatic species that exhibit large changes in heart rate and cardiac output during ventilation, but absent or less conspicuous in terrestrial species where the cardiorespiratory interactions are smaller (Glass et al., 1978; Taylor and Wang, 2009).

Bottazzi (1897) observed tonus waves in atrial preparations of anuran amphibians (frogs and toad), but atrial smooth muscle has not been detected histologically (Laurens, 1913; Blinks and Koch-Weser, 1963). Also, there are numerous reports of endocardial smooth muscle in human (Nagayo, 1909; Blinks and Koch-Weser, 1963; Park et al., 2013; Okada et al., 2015) and sheep (Terasaki et al., 1993). Possibly, this reflects contributions of the Isl1-positive second heart field to the venous pole of the heart and the common origin of smooth muscle and cardiac muscle in mesodermal progenitors that express Isl1 (Douglas et al., 2006, 2009; Moretti et al., 2006).

The primary aim of this study was to unravel the evolutionary history of atrial smooth muscle in Testudines. To test the hypothesis that the smooth muscle may be linked to diving capacity, we predicted that atrial smooth muscle would be absent in terrestrial tortoises, but more developed in aquatic species. We also took the opportunity to describe smooth muscle in other parts of the heart, including the sinus venosus (Carmona et al., 2018), pulmonary veins, and ventricle. We finally predicted the possible broader distribution of atrial smooth muscle in other vertebrates, including amphibians and mammals.

Key words: cardiac function; diving physiology; stroke volume; heart anatomy

MATERIALS AND METHODS

The majority of the turtle species (Pelomedusa subrubra [n = 3; 20–35 g], Chelodina mccordi [n = 3; 14–15 g], Pelodiscus sinensis [n = 2; 5 g], Cyclanorbis senegalensis [n = 2; 0.2–0.45 kg], Testudo hermannii [n = 3; 25–27 g], Chelonoidis carbonaria [n = 3; 2.4–4.8 kg], and T. scripta [n = 10; 0.3–1.7 kg], a skink, Cyclodomorphus gerrardi [n = 1; 0.44 kg], a spectacled caiman, Caiman crocodilus [n = 1; 4 kg], African clawed frogs, Xenopus laevis [n = 2; 50 g], cane toads, Rhinella marinus [n = 2; 100–200 g] and Longnose gar (Lepisosteus osseus [n = 1]) were obtained from commercial sources or donated from private collections and maintained at the Aarhus University (Aarhus, Denmark). C. serpentina (n = 3; 30–35 g) and Alligator mississippiensis (n = 1; 1 kg) hearts were obtained from animals maintained at the University of North Texas (Denton, Texas). Mouse (Mus musculus [n = 1]), and a bird, the lesser redpoll (Acanthis cabaret [N = 1]), sections were obtained from archived samples (mass unknown) at the Amsterdam University Medical Center (UMC) (Amsterdam, the Netherlands). One caecilian (Idioecranium sp.) section was taken from unpublished data associated with an earlier study (de Bakker et al., 2015). Healthy human (Homo sapiens) cardiac samples were provided from the Department of Pathology, Amsterdam UMC, AMC (Amsterdam, the Netherlands).

For the hearts used for immunohistochemistry, the animals were euthanized with an overdose of pentobarbital (200 mg kg⁻¹) before the brain was destroyed. All experiments were performed in accordance with local animal care regulations.

Immunohistochemistry

Hearts were fixed for 24 hr in paraformldehyde (4% in phosphate-buffered saline [PBS]) and stored in 70% ethanol. The hearts were then embedded in paraffin (Paraplast,
Sigma P3558) and cut into 10-μm transverse or coronal sections. A standard immunohistochemistry protocol was followed as described elsewhere (Jensen et al., 2016; 2017), where we demonstrated specific detection of cardiac muscle and smooth muscle in anole lizards, the Ball python, and the American alligator. Briefly, cardiac muscle was detected with rabbit polyclonal antibody to cardiac Troponin I (cTnI: 06/02-IV-4T21/2, HyTest Ltd, dilution 1:600) which was

Fig. 1. Luminal atrial smooth muscle was not detected in most vertebrates. Red represents cTnI and green represents SMA, as detected by fluorescent immunohistochemistry. (A) American alligator, (B) pink-tongued skink, (C) African clawed frog, (D) longnose gar, (E) mouse, and (F) lesser redpoll bird (all detected SMA in B, E, F was within arterial walls). Scale bars are 1 mm. a, atrium; as, atrial septum; pv, pulmonary vein; sv, sinus venosus; v, ventricle; oft, outflow tract.
detected with a secondary donkey anti-rabbit antibody conjugated to the fluorophore Alexa 647 nm (Mol Prob A31573, dilution 1:250). Smooth muscle was detected with mouse monoclonal antibody to smooth muscle actin (SMA, Sigma A2547, 1:600) which was detected with a secondary donkey anti-mouse antibody conjugated to the fluorophore Alexa 555 nm (Invitrogen A31570, 1:250). Images were acquired with a Leica DM6000 microscope under the control of LAS X software (Leica Microsystems, Wetzlar, Germany).

**Statistical Analyses**

The number of pixels containing myocardium (red) and smooth muscle (green) in composite images were determined by splitting the red and green colors using the “Color Threshold” function of ImageJ (NIH, Bethesda, MD, Version 1.51k), and then measuring the area on the split colors allowing us to calculate relative smooth muscle area as a percentage of the total muscle area (smooth and cardiac muscle). To maintain standardization, only transverse images of the atria were used for this quantification, thus the final sample sizes in this analysis were as follows: *P. subrufa* (*n* = 2), *C. mccordi* (*n* = 2), *P. sinensis* (*n* = 1), *C. senegalensis* (*n* = 2), *C. serpenitia* (*n* = 2), *T. hermanii* (*n* = 2), *C. carbonaria* (*n* = 3) and *T. scripta* (*n* = 6). For each heart, the % area of smooth muscle was averaged from three or four equidistant sections from across the atria, although in some *C. mccordi* (*n* = 1), *P. sinensis* (*n* = 1), and *C. carbonaria* (*n* = 2) only two representative sections could be used. Due to the low...
animal sample sizes available for most species, no statistical comparisons were made between species. A linear regression was performed to investigate the relationship between body mass and atrial smooth muscle coverage in *T. scripta*. To investigate whether there were chamber differences in the proportion of the detected SMA relative to all detected SMA and cTnI, we used the Plugin function “RGB Measure” of ImageJ after having delineated the sinus venosus, atria, or ventricle with the Freehand selections tool (epicardial vessels were excluded from this

Fig. 4. The phylogenetic distribution of smooth muscle in different regions of the heart in eight turtle species. Red represents cTnI and green represents SMA, as detected by fluorescent immunohistochemistry. Scale bars are 100 μm for all species, except for *C. senegalensis* and *T. scripta* (500 μm), and *C. carbonaria* (1 mm). Phylogeny based on Crawford et al. (2015).
analysis to the extent it was possible). The output value (mean) is a composite measure of the number of pixels that contain each color and the color intensity. We only used images that contained both sinus venosus and atria (N = 73). In 25 of the 73 images, there was also ventricular tissue. Differences between the sinus venosus and atria were tested with paired $T$-test. We used the Pearson correlation test for significant relation between the proportion of SMA in the sinus venosus as compared to the atria and ventricle. Statistical analyses were performed in SPSS (IBM SPSS Statistics version 24) or GraphPad Prism (Version 8.0). Data are presented as means ± SD.

RESULTS

Cardiac Smooth Muscle in Vertebrates

To resolve the evolutionary history of smooth muscle in the heart, we selected a range of vertebrate species and performed fluorescent immunohistochemistry against SMA and cardiac muscle in the sinus venosus, atria, and ventricle. Smooth muscle was readily detected in the large arteries connected to the ventricle and coronary arteries of all investigated species. Luminal smooth muscle was not detected in cardiac chambers from American alligator (Alligator mississippiensis) (Fig. 1A), spectacled caiman (C. crocodilus) (Fig. 2A), pink-tongued skink (C. gerrardii) (Fig. 1B), longnose gar (L. osseus) (Fig. 1D), mouse (M. musculus) (Fig. 1E), or lesser red poll bird (A. cabaret) (Fig. 1F). Only in the African clawed frog (X. laevis) (Fig. 1C) and cane toad (R. marinus) (Fig. 2B) did we detect a small amount of smooth muscle in the sinus venosus, although none was observed in another amphibian, the caecilian (Idiocranium sp.) (Fig. 2C). We confirmed earlier reports (Nagayo, 1909; Park et al., 2013) that atrial smooth muscle could be detected in human atrium (Fig. 3), but most vertebrates appear to have very little, if any, smooth muscle in contact with chamber lumens.

Cardiac Smooth Muscle in Turtles

All eight turtle species exhibited both smooth and cardiac muscle in the sinus venosus (Fig. 4). In proportion to the total amount of smooth and cardiac muscle, the sinus venosus had significantly more smooth muscle than the
atria, which in turn had significantly more smooth muscle than the ventricle (Fig. 5) (concerning the ventricle, in some specimens much of the SMA signal came from the walls of coronary arteries). The proportion of smooth muscle in the atria was positively related to the proportion of smooth muscle in the sinus venosus (Fig. 5), but the proportion of smooth muscle of the ventricle was not (Pearson correlation, \( P = 0.271 \)). In \( T. \) scripta there was a great amount of smooth muscle in the sinus venosus and atria (Fig. 4) that represented 17.4 ± 7.9% (mean ± SD) of total muscle area (Fig. 6A). Smooth muscle was observed equally in the left and right atria, and appeared homogeneously within each atrium. Smooth muscle was also relatively prevalent (7.1 ± 1.5% total muscle area) in \( C. \) senegalensis, a distantly related soft-shelled turtle (Fig. 6A,C). In the other species, smooth muscle on atrial trabeculae was sparser, although positive identifications were made in all species except land tortoises (\( T. \) hermanii and \( C. \) carbonaria Fig. 6D) and one of the side-necked turtles, \( C. \) mcordi (Fig. 4). Notwithstanding, smooth muscle was identified on the atrial septum in all species except \( P. \) sinensis, in which a limited sample size may have precluded finding it, although it was minimal in \( P. \) subrufa and \( C. \) carbonaria (Fig. 4). In all species, except for \( T. \) scripta and \( C. \) senegalensis, the total contribution of smooth muscle averaged less than 2% total muscle area in the atria (including atrial septum) (Fig. 6A). In \( T. \) scripta, smooth muscle was also observed on ventricular trabeculae (Fig. 4), although this was clearly very much sparser than in the atria so was not the main focus of our study. Traces of smooth muscle were also found in the \( C. \) senegalensis and \( C. \) serpentina ventricle.

Given the body mass range we encountered within and between species, in \( T. \) scripta we established that there was no relationship between body mass and atrial smooth muscle area (\( R^2 = 0.15, P = 0.44 \)) (Fig. 7). Also, in \( T. \) scripta there...
was no relation between the amount of SMA in the sinus venosus and the atria (in proportion to the total amount of smooth and cardiac muscle, $R^2 = 0.02$). However, the proportional amount of SMA was greater in *T. scripta* compared to the other turtles in the sinus venosus (unpaired two-tailed T-test, unequal variance assumed, $P < 0.001$) and in the atria (unpaired two-tailed T-test, unequal variance assumed, $P < 0.001$) but not in the ventricle (unpaired two-tailed T-test, unequal variance assumed, $P = 0.939$).

**DISCUSSION**

The cardiac output of *T. scripta* is dramatically reduced when the smooth muscle of the atria is induced to contract (Joyce et al., 2019) and we demonstrate here an extensive layer of smooth muscle in atria of this species. We also reveal large amounts of smooth muscle in the sinus venosus, the cardiac chamber between the systemic veins and the right atrium (Jensen et al., 2014, 2017; Icardo, 2017), and we consider it likely that contraction of the smooth muscle of both chambers could reduce cardiac filling by impeding venous return. Cardiac filling may also be limited by reduced compliance of the chamber walls. From a functional point of view, there are at least two similar adaptations to altering venous return among vertebrates. Diving mammals have a vena caval sphincter that may impede venous return during diving bradycardia (Harrison and Tominson, 1956; Elsner et al., 1971; Blix, 2018; Lilie et al., 2018) and some terrestrial snakes have a “corkscrew” caval vein that may facilitate venous return during gravitational challenges such as during climbing (Lillywhite, 1987; Conklin et al., 2009). In consistent with earlier descriptions (Shaner, 1923; Robb, 1952), smooth muscle was sparse but consistently identified in the ventricle of *T. scripta* (and minute amounts were found in *C. senegalensis* and *C. serpentina*).

**Smooth Muscle in the Atria of Turtles**

Our results suggest that (nonvessel) cardiac smooth muscle appeared early in turtle evolution, possibly in the sinus venosus before other chambers, as it was observed, at least in small amounts, in representatives across the turtle phyl- ogeny, including side-necked turtles (*Pleurodora*) that diverged from other turtles over 150 million years ago (Crawford et al., 2015; Shaffer et al., 2017). It was not, however, observed in other reptiles, including crocodilians or birds, which as archosaurs represent the closest living relatives to turtles (Chiang et al., 2012; Crawford et al., 2012, 2015; Feng et al., 2012). This accords with our earlier observation on the lack of tonus waves in atrial strips from crocodilians, lizards, or snakes (Galli et al., 2006; Joyce et al., 2014).

The atrial smooth muscle was particularly extensive in *T. scripta*, a species in the family (Emydidae) in which it was first described and detailed (Fano, 1887; Bottazzii, 1906; Gruber and Markel, 1918a; Shaner, 1923). Although we only studied one Emydid species in our phylogenetic survey, the extensive functional data suggest that atrial smooth muscle is well developed across this family as tonus waves are prevalent in atrial preparations species in both major subfamilies (Emydinae, e.g., *E. orbicularis*; Fano, 1887) (Deirochelinae, e.g., *T. scripta*; Joyce et al., 2014). Even in *T. scripta*, smooth muscle was sparse in the ventricle, which concords with Shaner’s (1923) anatomical description, and the fact that Fano only observed ventricular tonus thrice in over 100 experiments (Fano, 1887). It is surprising that tonus waves have been reported in *C. serpentina* (Pereira, 1924), including in the ventricle (Blinks and Koch-Weser, 1963), given its sparse distribution, although the earlier descriptions in this species were vague. Our anatomical data consolidate the absence of atrial tonus in land tortoises (Testudinidae) (Gaskell, 1990).

**Smooth Muscle in the Hearts of Other Vertebrates**

The phylogenetic distribution of atrial smooth muscle within the hearts of nonreptilian vertebrates remains somewhat enigmatic. Bottazzii (1897) provided a description of atrial tonus in anuran amphibians (*Bufo viridis*) (Deirochelinae, e.g., *T. scripta*; Joyce et al., 2014). Furthermore, although SMA is expressed trans- siently in the embryonic heart of *X. laevis*, no expression was found in the adult *X. laevis* heart (Saint-Jeannet et al., 1992; Warkman et al., 2005; Barillot et al., 2008).

We did, nevertheless, verify that human atrium contain traces of smooth muscle (Nagayo, 1909; Douglas et al., 2006; Park et al., 2013). We cannot speculate on its possible function, but human atrium does not appear to exhibit tonus waves (Meyer et al., 1996; Maier et al., 2000), including after exposure to acetylcholine (Nadler et al., 2011), thus we do not necessarily suggest it is functionally equivalent to atrial

![Graph](image_url)
smooth muscle in turtles. It nevertheless demonstrates that atrial smooth muscle, *per se*, may not be unique to the turtle lineage.

**Pulmonary Veins of Turtles**

Extensions of left atrial myocardium that partially envelop the pulmonary veins, known as “myocardial sleeves,” have been well described in mammals and birds (Nathan and Globo, 1970; Roux et al., 2004; Kroneman et al., 2019), but were not seen in corn snakes or anole lizards (Jensen et al., 2013). Where we could clearly observe the pulmonary veins in *T. scripta* (Fig. 6B), it appeared that there may be a small quantity of myocardium surrounding the vein, but this is not well developed.

**CONCLUSIONS**

In summary, our comparative study indicates that atrial smooth muscle evolved early in the order of Testudines. The atrial smooth muscle is particularly scarce in terrestrial tortoises, but well developed in some aquatic species, which lends tentative support to our hypothesis that it may be involved in the regulation of cardiac output of turtles during diving. All of the turtles investigated exhibited both smooth muscle and cardiac muscle in the sinus venosus, which may also be able to contribute to the regulation of cardiac output. A mixture of smooth and cardiac muscle in the sinus venosus was also evident in the anuran amphibians and has previously been reported in fish (Yamauchi, 1980; Icardo, 2017), so we believe it likely represents the ancestral state in vertebrates. Although human atrial tissue also contains traces of smooth muscle, there is little indication that it is functionally homologous to that in turtles.

**ACKNOWLEDGMENTS**

We thank Dr Holly Shiels (University of Manchester) for helpful discussions, Quinn Gunst and Corrie de Gier-de Vries for assistance with immunohistochemistry, Allard van der Wal and Nicoline van den Berg for providing tissue blocks of human heart tissue (Amsterdam UMC, Academic Medical Center), and Rasmus Buchanan and Heidi Meldgaard (Aarhus University) for animal curation and maintenance. This study was funded by the Danish Research Council (Natur og Univers, Det Frie Forskningsråd). W.J. was supported by an EliteForsk travel grant from the Danish Ministry of Higher Education and Science.

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