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

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

[1]Zoophysiology, Department of Bioscience, Aarhus University, 8000 Aarhus C, Denmark
[2]Department of Medical Biology, Amsterdam UMC, University of Amsterdam, Amsterdam 1105AZ, the Netherlands
[3]Aarhus Institute of Advanced Studies, Aarhus University, Aarhus, Denmark
[4]Department 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. Across other tetrapod clades, cardiac smooth muscle might not associate with diving. Anat Rec, 00:000–000, 2019. © 2019 The Authors. *The Anatomical Record* published by Wiley Periodicals, Inc. on behalf of American Association for Anatomy.

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

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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; Gault, 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* (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), 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 (Pleurodira), *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 considered the possible broader distribution of atrial smooth muscle in other vertebrates, including amphibians and mammals.

MATERIALS AND METHODS

The majority of the turtle species (*Pelomedusa subrufa* [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 hermanii* [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 gerrardii* [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 (body mass unknown) at the Amsterdam University Medical Center (UMC) (Amsterdam, the Netherlands). One caecilian (*Idiocranium* 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 paraformaldehyde (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

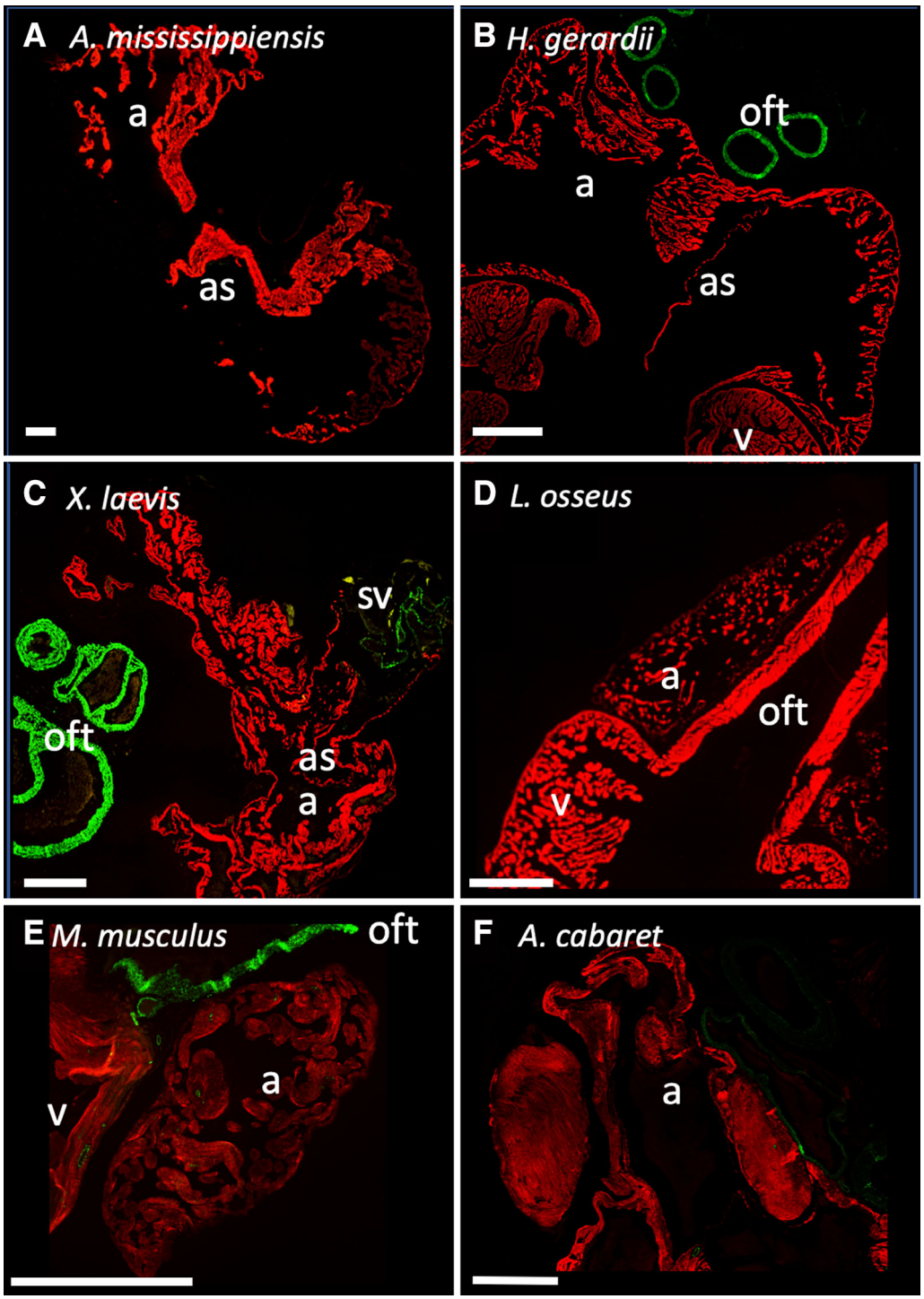

Fig. 1. Luminal atrial smooth muscle was not detected in most vertebrates. Red represents cTnI and green represents SMA, as detected by fluorescent immunohistochemistry. (**A**) American alligator, (**B**) pink-tongued skink, (**C**) African clawed frog, (**D**) longnose gar, (**E**) mouse, and (**F**) 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.

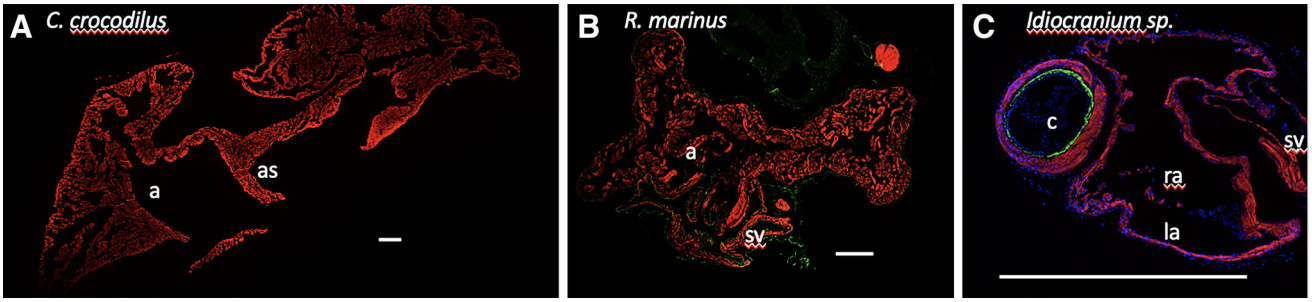

Fig. 2. Luminal smooth muscle was not detected in atrium from spectacled caiman (**A**), cane toad (**B**), or caecilian (**C**). Red represents cTnI and green represents SMA, as detected by fluorescent immunohistochemistry. Scale bars are 1 mm. (r/l)a, (right/left) atrium; as, atrial septum; sv, sinus venosus; c, conus.

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. serpentina* (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

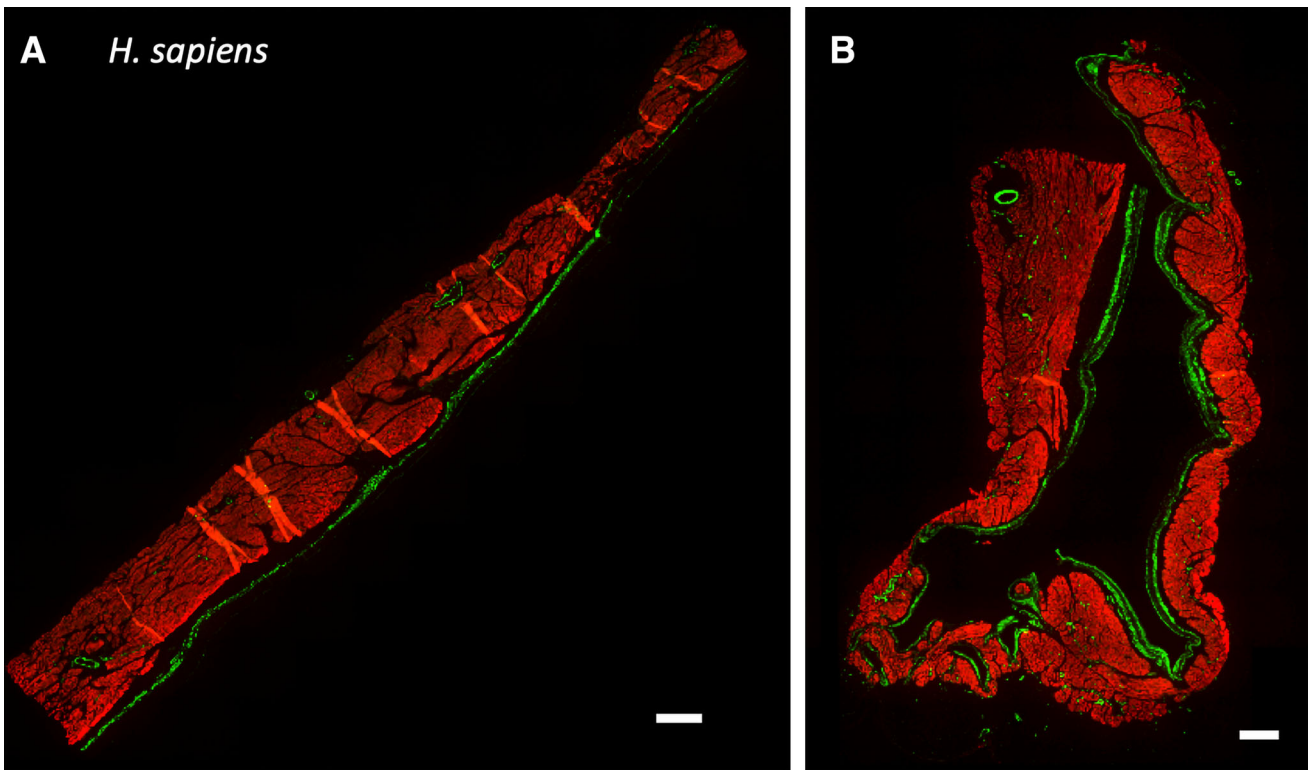

Fig. 3. Smooth muscle in human atrial wall. Red represents cardiac cTnI and green represents SMA, as detected by fluorescent immunohistochemistry. (**A**) left anterior atrium and (**B**) left atrial appendage. Scale bars are 1 mm.

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

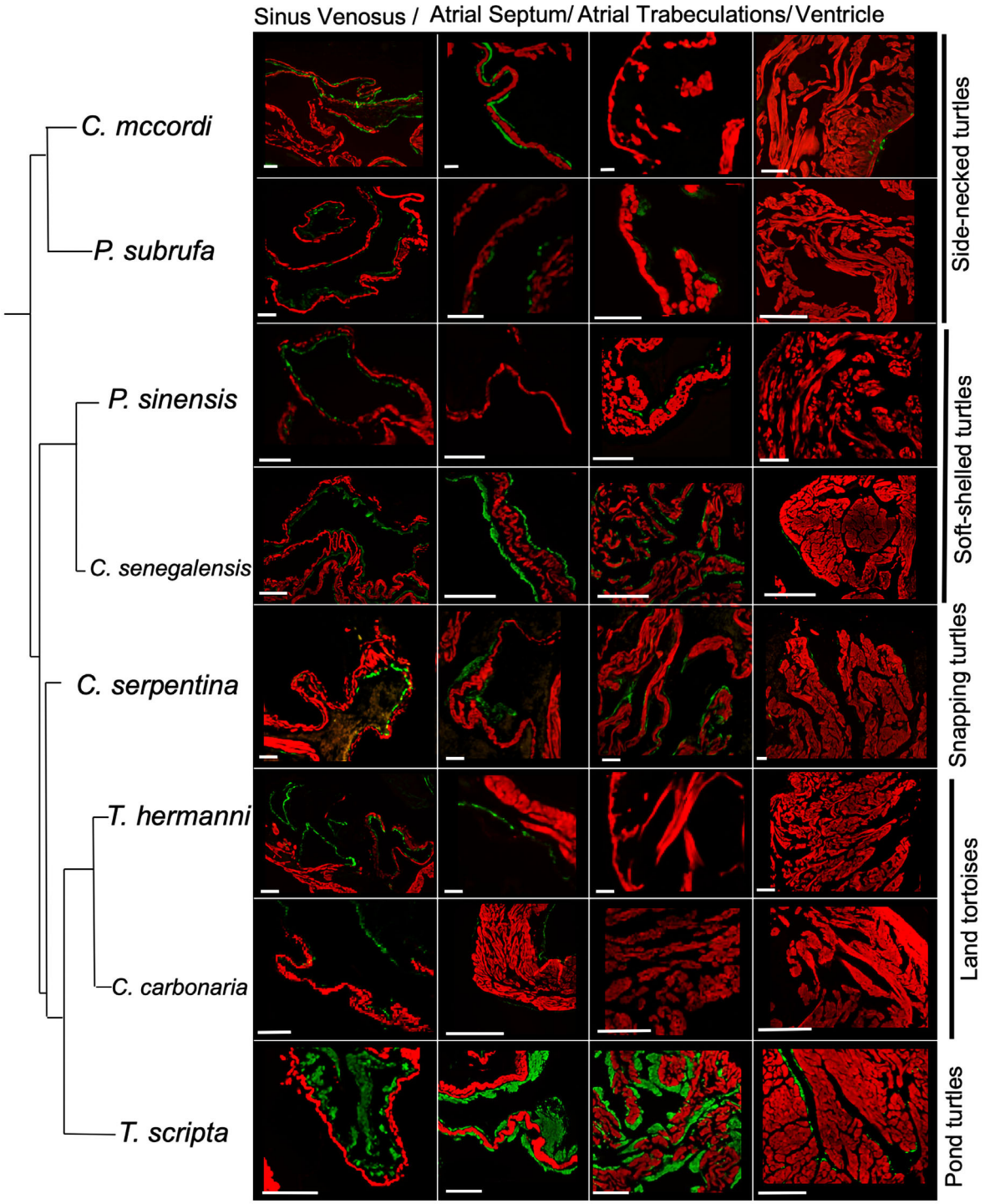

Fig. 4. The phylogenetic distribution of smooth muscle in different regions of the heart in eight turtle species. Red represents cTnI and green represents SMA, as detected by fluorescent immunohistochemistry. Scale bars are 100 μm for all species, except for *C. senegalensis* and *T. scripta* (500 μm), 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 (*Alligators mississipinesis*) (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

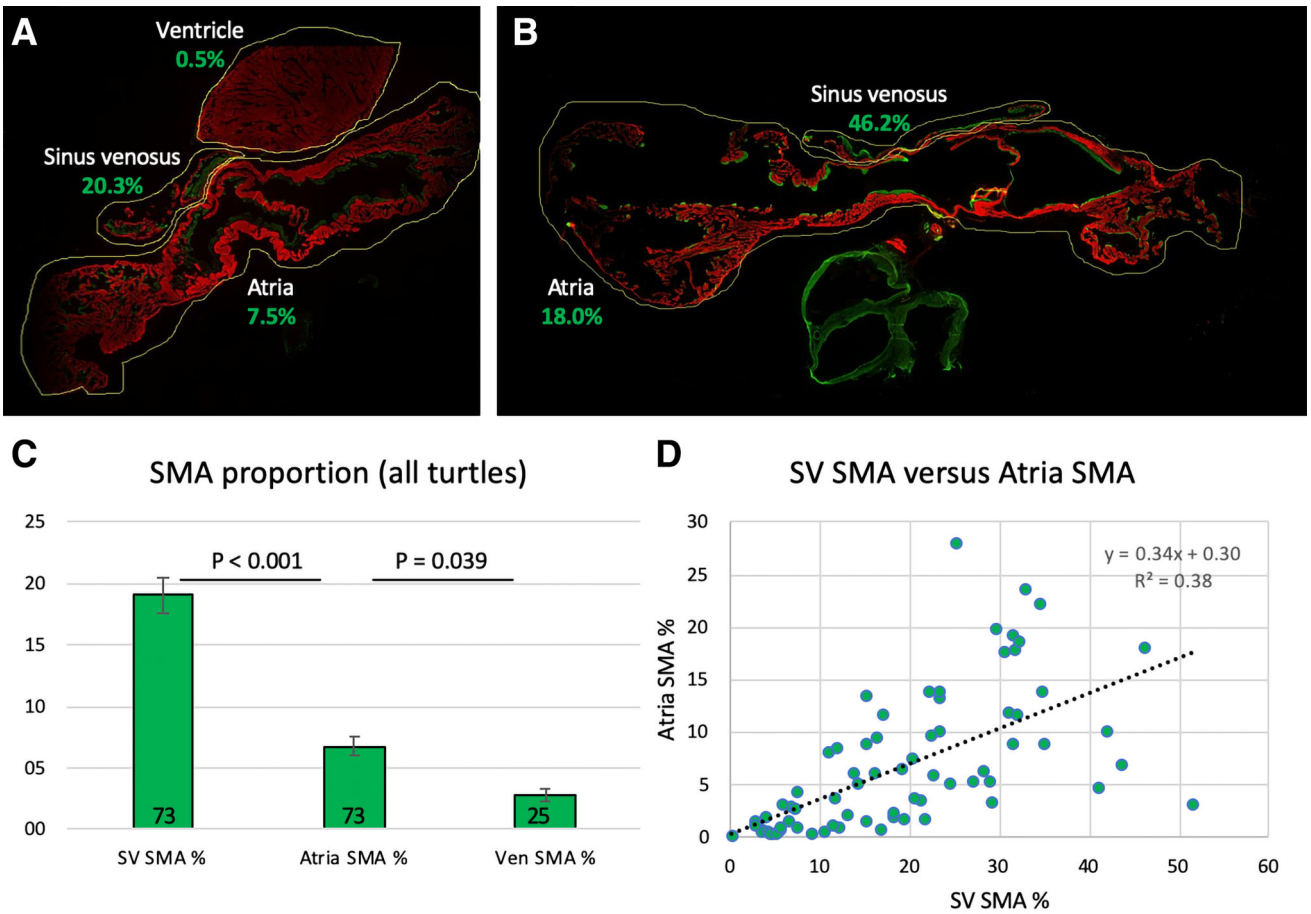

Fig. 5. Assessment across turtles of the proportion of signal from SMA (green) out the total signal from SMA and cTnI (red), measured by the ImageJ Plugin function "RGB measure." The yellow lines indicate the region of interest within which the measurements were made. Two specimens of the Pond slider with intermediate **(A)** and high **(B)** proportions (numbers in green) of SMA in the sinus venosus and atria. (**C**) Across turtles, the sinus venosus was proportionally richer in SMA than the atria, which in turn were richer in SMA than the ventricle (*P*-values of paired two-tailed *T*-tests, numbers in columns are the number of assessed sections). (**D**) The proportion of SMA in the sinus venosus was significantly positively related to the proportion of SMA in the atria (Pearson correlation, $P < 0.001$).

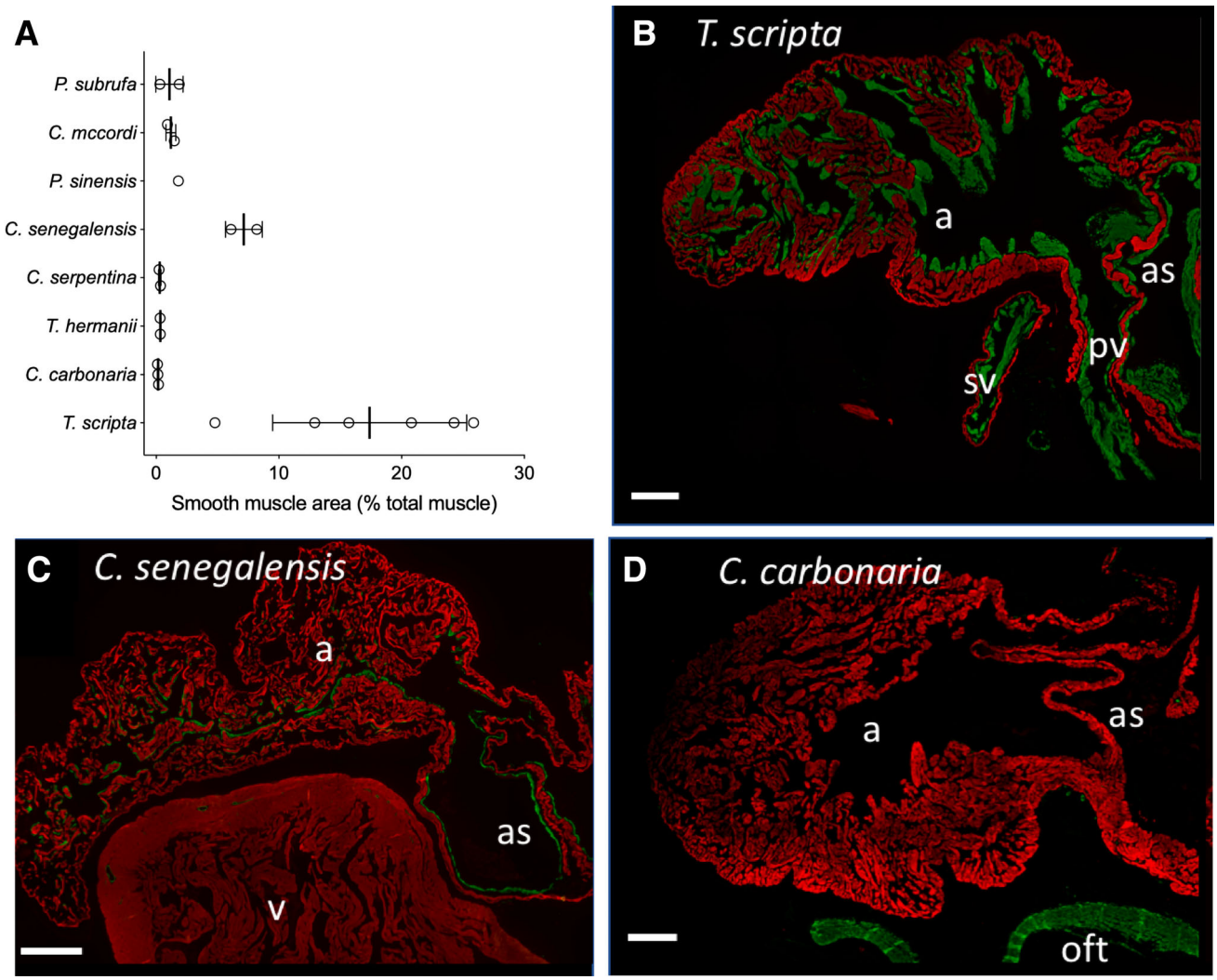

Fig. 6. The interspecific variation in atrial smooth muscle in turtles. (**A**) Mean area of smooth muscle as a percentage of total muscle (smooth + cardiac muscle) area in eight species. Values are means ± SD. (**B–D**) Red represents cardiac Troponin I and green represents smooth muscle actin, as detected by fluorescent immunohistochemistry. (B) A prominent case of atria smooth muscle in *T. scripta*. (C) Intermediate levels of smooth muscle seen in *C. senegalensis*. (D) Near absence of atrial smooth muscle in *C. carbonaria*. For B–D, scale bars are 1 mm. a, atrium; as, atrial septum; pv, pulmonary vein; sv, sinus venosus; v, ventricle; oft, outflow tract.

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 $17.4 \pm 7.9\%$ (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 ($7.1 \pm 1.5\%$ 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. mccordi* (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

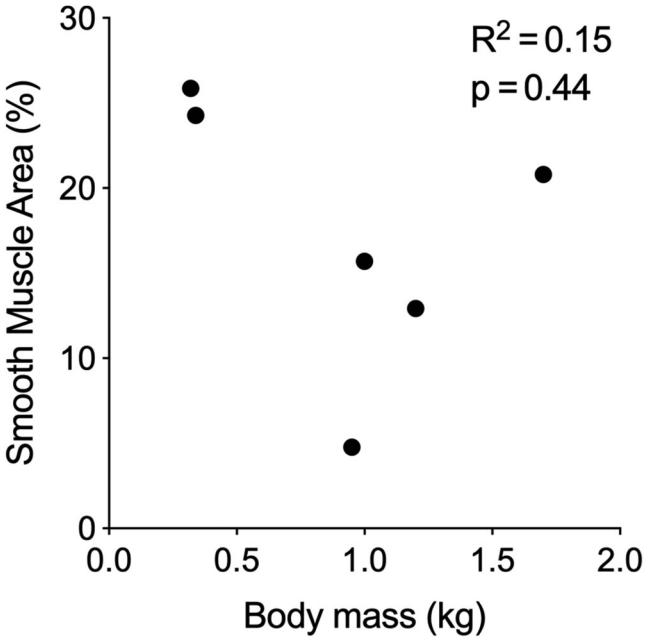

Fig. 7. There was no significant relationship between atrial smooth muscle (% area of total muscle) and body mass in *Trachemys scripta* (linear regression).

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 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 Tomlinson, 1956; Elsner *et al*., 1971; Blix, 2018; Lillie *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 phylogeny, including side-necked turtles (Pleurodira) 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 (Chiari *et al*., 2012; Crawford *et al*., 2012, 2015; Fong *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; Bottazzi, 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) (Deirochelyinae, 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, 1900).

Smooth Muscle in the Hearts of Other Vertebrates

The phylogenetic distribution of atrial smooth muscle within the hearts of nonreptilian vertebrates remains somewhat enigmatic. Bottazzi (1897)) provided a description of atrial tonus in anuran amphibians (*Bufo viridis* and *Pelophylax esculentus*), but these findings received little subsequent attention and were not independently verified (Blinks and Koch-Weser, 1963). Although we were able to see smooth muscle in the sinus venosus of both *X. laevis* and *R. marinus*, we did not locate smooth muscle in the atria. This is consistent with previous functional studies on isolated atrial preparations in these two species which did not report on tonus waves, even upon treatment with adenosine triphosphate (ATP) or acetylcholine (O'Donnell and Wanstall, 1982; Meghji and Burnstock, 1983; Minerds and Donald, 1997), which certainly activates the smooth muscle tones in atrial preparations from turtles (Fano, 1887; Joyce *et al*., 2014). Furthermore, although SMA is expressed transiently 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 atria 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

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 Gloobe, 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.

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