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RESEARCH ARTICLE

# Contraction of atrial smooth muscle reduces cardiac output in perfused turtle hearts

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## ABSTRACT

Unusual undulations in resting tension (tonus waves) were described in isolated atria from freshwater turtles more than a century ago. These tonus waves were soon after married with the histological demonstration of a rich layer of smooth muscle on the luminal side of the atrial wall. Research thereafter waned and the functional significance of this smooth muscle has remained obscure. Here, we provide evidence that contraction of the smooth muscle in the atria may be able to change cardiac output in turtle hearts. In *in situ* perfused hearts of the red-eared slider turtle (*Trachemys scripta elegans*), we demonstrated that activation of smooth muscle contraction with histamine (100 nmol $kg^{-1}$ bolus injected into perfusate) reduced cardiac output by decreasing stroke volume (>50% decrease in both parameters). Conversely, inhibition of smooth muscle contraction with wortmannin (10 µmol $l^{-1}$ perfusion) approximately doubled baseline stroke volume and cardiac output. We suggest that atrial smooth muscle provides a unique mechanism to control cardiac filling that could be involved in the regulation of stroke volume during diving.

**KEY WORDS: Stroke volume, Diving, Cardiac filling, Tonus waves**

## INTRODUCTION

In the late 19th century, Fano provided the first description of slow 'tonus waves' in isolated atrial preparations from freshwater turtles (*Emys orbicularis*) (Fano, 1887; Fano and Bodano, 1900; Fano and Fayod, 1888). The slow changes in atrial tone were clearly distinct and independent from the normal rapid contractions of the cardiac muscle (Fano, 1887). Shortly thereafter, a dense layer of smooth muscle lining the luminal side of the atrial wall was described in this species and other members of the family Emydidae (Laurens, 1913; Rosenzweig, 1903; Shaner, 1923). This smooth muscle originates in the sinus venosus and pulmonary veins and continues into the ventricle, where its distribution becomes much more sparse (Shaner, 1923). It was also shown that the conspicuous tonus waves were sensitive to smooth-muscle-specific pharmacological substances (Snyder and Andrus, 1919), providing conclusive evidence that the unusual waves could be attributed to smooth muscle.

The pharmacology and basic contractile properties of the atrial smooth muscle were well detailed in the early investigations. For instance, the smooth-muscle-dependent tonus contraction is potentiated by vagal stimulation, histamine and pituitary extract, but inhibited by sympathetic stimulation with adrenaline (Bottazzi and Grünbaum, 1899; Dimond, 1959; Fano, 1887; Fano and Fayod, 1888; Gault, 1917; Gruber, 1920a,b, 1921, 1927, 1934; Gruber and Markel, 1918a,b; Sollmann and Rossides, 1927). However, little consensus was reached regarding its functional role, although implications for electrical conduction or strengthening atrial contraction were suggested (Meek, 1927; Shaner, 1923). Indeed, Shaner (1923) resoundingly concluded 'it is hardly worthwhile to hazard a guess as to the function of smooth muscle in such a peculiar position'. Nonetheless, Gesell (1915) earlier observed that cardiac output decreased when atrial tonus spontaneously increased in perfused turtle hearts. This indirectly suggests that atrial smooth muscle may be able to alter ventricular filling.

Beyond an otherwise unpublished conference abstract (Gannon et al., 2003) and our incidental observations (Galli et al., 2006; Joyce et al., 2014), there has been little recent research into atrial smooth muscle in turtles, despite the fact that it is very active in isolated atrial preparations. In the present study, we adopted the *in situ* perfused heart model to experimentally investigate the effects of manipulating atrial smooth muscle contraction in the absence of confounding changes in vascular smooth muscle. Specifically, we address the hypothesis that endocardial smooth muscle contraction can change cardiac filling and cardiac output in turtles.

## MATERIALS AND METHODS

### Animals

Red-eared slider turtles [*Trachemys scripta elegans* (Wied-Neuwied 1839)] of both sexes were obtained from Lemberger Reptiles (Oshkosh, WI, USA) and maintained at Aarhus University (Denmark). The turtles were housed in 1000 litre tanks containing 800 litres of heated water (25–28°C) with free access to basking platforms. Food [commercial pellets (ReptileMin, Tetra, Germany), mussels and fresh vegetables] was provided twice weekly and the photoperiod was 14 h:10 h light:dark. Prior to each series of experiments, turtles were anaesthetised with propofol (20 mg $kg^{-1}$) injected into the supravertebral sinus (Ziolo and Bertelsen, 2009) followed by decapitation and pithing. A cast-cutting saw (Orthopaedic Frame Company, MI, USA) was then used to remove the anterior portion of the plastron and expose the heart and major blood vessels. All experiments were performed in accordance with Danish animal care legislation.

### Ringer's solution

All of our experiments employed a Ringer's solution composed of (mmol $l^{-1}$ concentrations): NaCl (95), $NaHCO_3$ (30), KCl (2.5), $MgSO_4$ (1), $NaH_2PO_4$ (1), glucose (5) and $CaCl_2$ (2). Ringer's solution was equilibrated with 2% $CO_2$ and air prepared by a Wöstoff gas-mixing pump to achieve a pH of 7.7. All chemicals were obtained from Sigma-Aldrich, Denmark, except where stated otherwise.

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### Atrial strips

Atria were dissected from *T. s. elegans* hearts ($n$=6, body mass: 0.32±0.07 kg, mean±s.d.) and each heart provided one strip preparation from the right atrium. The upper end of the strip was tied to a metal rod suspended from a force transducer (Fort 10, World Precision Instruments, Sarasota, FL, USA) and the lower end was secured to one of two silver electrodes. The second silver electrode was held in parallel approximately 5 mm from the strip. The signals from the force transducer were digitised using a Biopac MP100 (Biopac Systems, Goleta, CA, USA) data-acquisition system. The preparation was then lowered into a water-jacketed organ bath containing 50 ml of Ringer's solution and the temperature was maintained at 25°C during the experiment. The preparations were stretched to remove slack and allowed to rest for 20 min. Then, electrical stimulation was started at 0.5 Hz, which is within the *in vivo* heart rate range (15–40 beats $min^{-1}$) for this species (Joyce et al., 2018b; Wang and Hicks, 1996) and similar to the intrinsic heart rate exhibited in our perfused heart experiments (~40 beats $min^{-1}$). The two electrodes were connected to a Grass SD9 stimulator (Quincy, MA, USA) providing 5 ms pulses at 150% the threshold voltage required to elicit contraction. The preparations were then stretched to attain maximum force of contraction and allowed to stabilise for a further 20 min before the experimental protocol commenced. At the end of the experiment, the length (5.5±0.7 mm, mean±s.d.) and mass (2.8±1.0 mg, mean±s.d.) of the strips were measured, although the force data were not normalised to cross-sectional area because the composition of smooth and cardiac muscle was not known; therefore, it was not possible to reliably estimate the contribution of each muscle type to the forces generated.

During the experimental protocol, histamine concentration in the Ringer's solution was increased in a concentration-dependent manner from 1 to 100 to 1000 nmol $l^{-1}$. The baths were then twice washed with fresh Ringer's solution before the $H_1$-receptor antagonist diphenhydramine (10 µmol $l^{-1}$; Drescher et al., 1998) was applied. The preparations were incubated with diphenhydramine in Ringer's solution for 30 min before the histamine dose–response protocol was repeated. Twitch force and resting tension were measured before histamine and after each incremental addition. When smooth muscle oscillations were evident, the force measurements were taken at the troughs of the tonus waves, in order to avoid overestimating resting tension during spontaneous smooth muscle contractions.

### *In situ* perfused hearts

The double-perfused *in situ* preparation was based on that previously described (Farrell et al., 1994; Franklin, 1994; Joyce et al., 2016) in turtles ($n$=9, body mass: 0.38±0.08 g, mean±s.d.). Perfusion of the left and right atria was achieved with cannulae inserted into the left pulmonary vein and sinus venosus, respectively, which required the pericardium being opened. Opening the pericardium allowed for direct observation of the atria. To limit the contribution of smooth muscle in the sinus venosus, the inflow cannulae were placed near the sinoatrial junction, although care was taken not to advance the cannulae far enough to interfere with valve function and the tips of cannulae did not enter the atria themselves. The cannulae were tied in place with 4-0 surgical silk and cardiac perfusion was initiated at low input pressure with Ringer's solution. The right aortic arch and pulmonary artery were then cannulated, and all other central vessels were ligated with 4-0 silk. Cannulations were performed with custom-made double-bore stainless steel cannulae (Franklin and Axelsson, 1994), allowing pressures to be measured at the tip of insertion. The preparation was then transferred to a chamber containing 0.9% NaCl solution. Experiments were conducted at room temperature (21–22°C).

Inflow cannulae were connected to adjustable filling pressure units that supplied Ringer's solution from a reservoir at a constant pressure. Outflow cannulae were connected to an output pressure head, whilst output flows were recorded with flow-through Transonic flow probes (4 mm diameter; 4NRB) connected to a flow meter (Transonic; T206). Polyethylene pressure cannulas were connected to disposable pressure transducers (PX600; Baxter Edwards, Irvine, CA, USA) that were calibrated against a static water column before each experiment. The signals from the pressure transducer and flow meter were collected using a Biopac MP100 data-acquisition system.

After 10 min of initial stabilisation, the filling device was set to provide a filling water column of 10 cm. The output pressure column was adjusted to provide a physiological arterial pressure (approximately 2.5 kPa) (Joyce et al., 2018b). The preparation was then allowed to stabilise for a further 10 min before the experimental protocol commenced.

We examined the effects of bolus injections of histamine (100 nmol $kg^{-1}$), delivered into the perfusate via the filling unit supplying the right atrium. This dose was selected based on pilot experiments and previous studies (e.g. Skovgaard et al., 2009). Histamine injections were performed in eight hearts, thereby excluding one with negligible baseline cardiac output (which we later established to be due to spontaneous smooth muscle contraction).

In six preparations, the myosin light chain kinase inhibitor wortmannin (Hölzel Diagnostika, Germany) was then added to the perfusate reservoir to achieve a final concentration of 10 µmol $l^{-1}$, to inhibit smooth muscle contraction (Burke et al., 1996). When stable effects of wortmannin were established (after at least 20 min of perfusion), the bolus injection of histamine (100 nmol $kg^{-1}$) was repeated ($n$=5).

Because input and output pressures were controlled in tandem, input pressure was calculated as the average of right and left atrial input pressures and output pressure was calculated as the average of pulmonary artery and right aortic pressures (Farrell et al., 1994; Joyce et al., 2017). Total cardiac output was defined as the sum of pulmonary and right aortic blood flow and normalised to body mass. Stroke volume was calculated from the quotient of cardiac output and heart rate, the latter of which was automatically derived from the pulsatile flow traces. Cardiac power, normalised to body mass, was calculated from the difference between filling and output pressure, multiplied by mass-specific cardiac output, and multiplied by 0.0167 to convert to mW.

### Statistical analyses

For the *in vitro* strip experiments, two-way repeated-measures ANOVA and Sidak's *post hoc* tests were used to compare the effect of increasing histamine concentration with and without diphenhydramine on the change in twitch force (cardiac muscle contraction) or resting tension (tonic smooth muscle contraction) from pre-treatment values.

For perfused hearts, a linear regression was performed to investigate the relationship between input pressure and cardiac output under baseline conditions. Paired $t$-tests were used to compare the effects of histamine and wortmannin on each cardiovascular parameter (input pressure, output pressure, cardiac power, cardiac output, stroke volume and heart rate). Paired $t$-tests were also used to compare the change in

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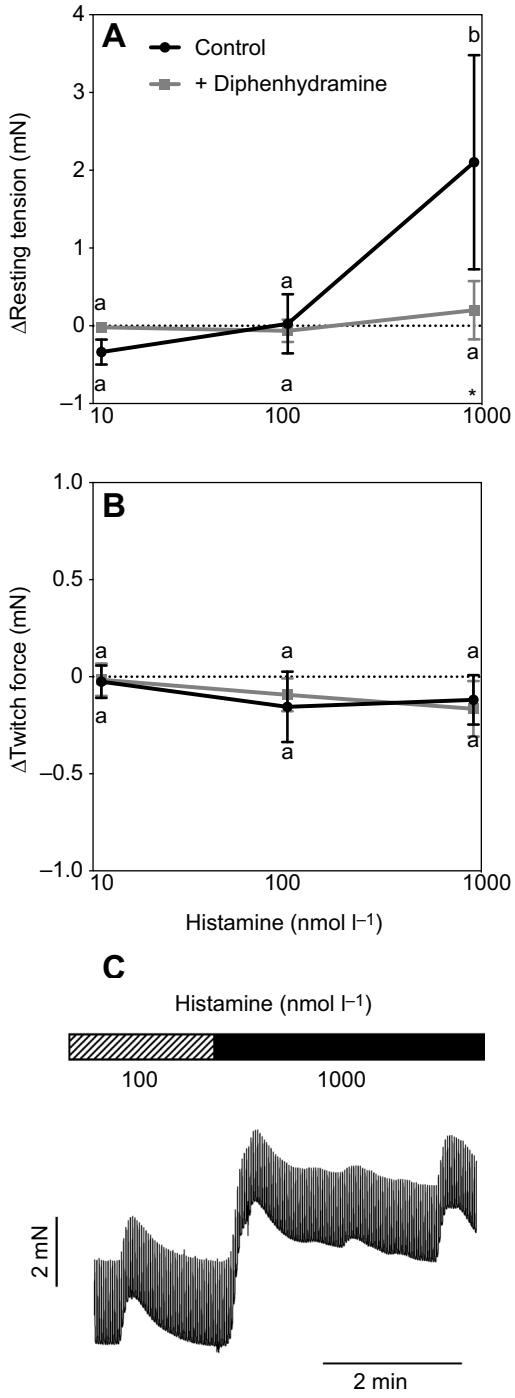


**Fig. 1. The effect of histamine treatment (10–1000 nmol $l^{-1}$), before and after treatment with the $H_1$ receptor antagonist diphenhydramine (10 μmol $l^{-1}$), on smooth muscle and cardiac muscle contraction in atrial strips from *Trachemys scripta elegans*.** Data represent the change (Δ) in resting tension and twitch force relative to pre-treatment values. Dissimilar letters represent significant changes within a variable with increasing histamine concentration. Asterisk indicates a significant difference ($P$<0.05) between control and diphenhydramine treated preparations at a given histamine concentration (repeated-measures two-way ANOVA). (A) Histamine increased resting tension, suggesting it contracted atrial smooth muscle, although this effect was blocked by diphenhydramine. (B) Histamine did not significantly affect cardiac muscle twitch force. $N$=6. Values are means±s.d. (C) An original and representative trace depicting the effect of increasing histamine concentration (100–1000 nmol $l^{-1}$) on twitch force and resting tension in an atrial strip.

each parameter invoked by histamine in control conditions and during perfusion with wortmannin.

All statistical analysis was performed in GraphPad Prism 7.0d (GraphPad Software, La Jolla, CA, USA) and changes were considered statistically significant when $P$<0.05. All data are presented as means±s.d.

## RESULTS

### Effect of histamine on atrial smooth and cardiac muscle *in vitro*

To inform our perfused heart studies, we first studied isolated atrial strip preparations. Histamine was classically identified as a contractor of atrial smooth muscle in turtles (Blinks and Koch-Weser, 1963; Gruber, 1927) and its cardiovascular effects have recently been studied in reptiles (Skovgaard et al., 2009, 2018). In our atrial strip preparations, pre-treatment resting tension was 8.0±5.7 mN and active twitch force was 3.4±1.1 mN. We confirmed that histamine increased atrial tonus, as evidenced by an increase in resting tension of 2.1±1.4 mN (Fig. 1A), an effect that was blocked (i.e. the change in tension was significantly reduced; $t$=5.9, $P$<0.05) by the $H_1$ receptor antagonist diphenhydramine (Gannon et al., 2003). We also observed that histamine had no significant inotropic effect on cardiac contractions (Fig. 1B,C), indicating that histamine was a suitable agent to probe atrial smooth muscle regulation in working hearts.

### Smooth muscle contraction decreases cardiac output in perfused hearts

Baseline flows were established that were similar to previous perfused heart studies (Bundgaard et al., 2018; Farrell et al., 1994; Joyce et al., 2016) and *in vivo* blood flow measurements (Joyce and Wang, 2014; Wang and Hicks, 1996) in this species. Under control conditions, there was a significant negative relationship between input pressure and cardiac output ($R^2$=0.63, $P$=0.01; Fig. 2), and the regression through all individual data points predicts a filling pressure of 1.03 kPa when cardiac output is zero (i.e. the $x$-intercept in Fig. 2).

Histamine (100 nmol $kg^{-1}$ bolus injection into perfusate) resulted in an elevation of input pressure (0.57±0.28 to 0.81±0.23 kPa; $t$=5.2, $P$<0.05; Fig. 3A) whilst output pressure (2.45±0.27 to 2.10±0.22 kPa; $t$=4.2, $P$<0.05; Fig. 3B), cardiac power (1.28±1.09

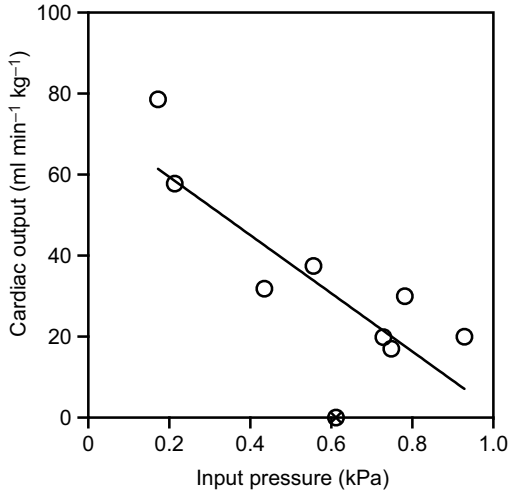


**Fig. 2. The relationship between input pressure and cardiac output under baseline conditions ($R^2$=0.63, $P$=0.01).** The crossed symbol represents the heart excluded from the histamine experiments owing to low baseline flow. $N$=9.



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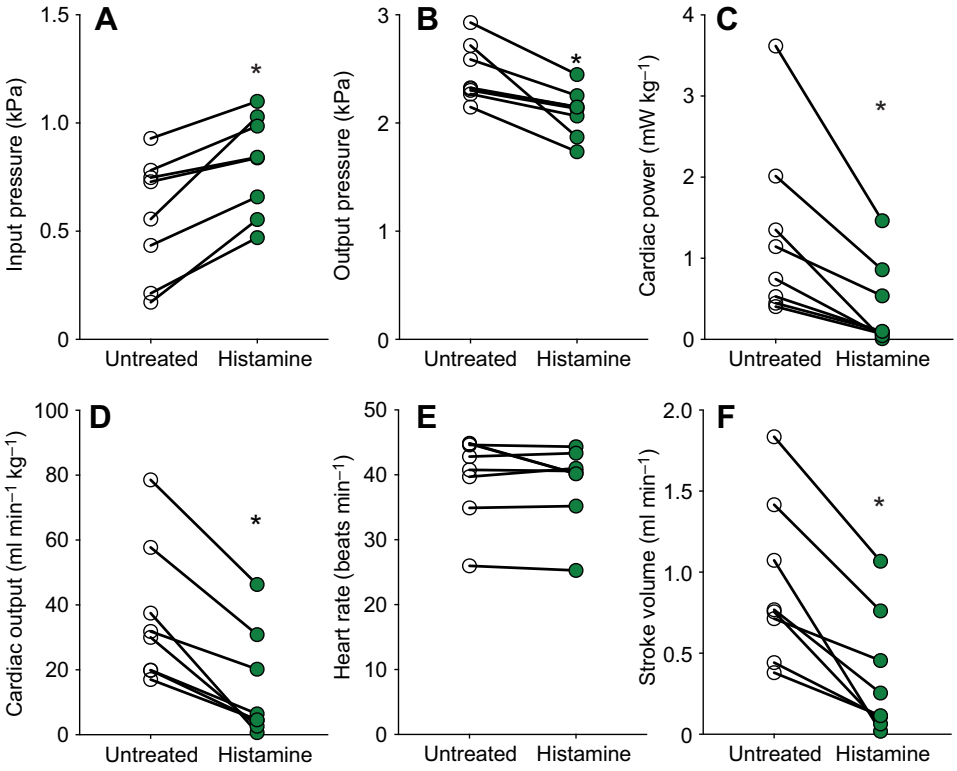


**Fig. 3. The effect of histamine (100 nmol kg$^{-1}$ bolus into perfusate) on cardiac performance in perfused *Trachemys scripta elegans* hearts.** (A) Input pressure. (B) Output pressure. (C) Cardiac power. (D) Cardiac output. (E) Heart rate. (F) Stroke volume. Asterisks indicate significant differences ($P$<0.05) following histamine injection (paired $t$-tests). $N$=8. Individual values are presented.

to 0.40±0.53 mW kg$^{-1}$; $t$=4.0, $P$<0.05; Fig. 3C), total cardiac output (36.6±21.4 to 14.5±16.5 ml min$^{-1}$ kg$^{-1}$; $t$=5.2, $P$<0.05; Fig. 3D) and stroke volume (0.92±0.50 to 0.35±0.38 ml kg$^{-1}$; $t$=5.8, $P$<0.05; Fig. 3F) decreased significantly. Heart rate did not significantly change (39.8±6.5 to 38.8±6.1 kPa; $t$=1.3, $P$=0.25; Fig. 3E).

Injection of histamine was clearly associated with a visible diastolic contraction of the atria, apparently limiting cardiac filling (Movie 1). At the start of the recording, prior to the effect of histamine, both atria are distended during diastole. As the effect of histamine is gradually established, the atria clearly become tonically contracted, despite the continuation of normal sinus rhythm.

We next used the myosin light chain kinase inhibitor wortmannin (10 µmol l$^{-1}$ perfusion) to achieve specific smooth muscle inhibition. This resulted in the opposite effect to histamine: input

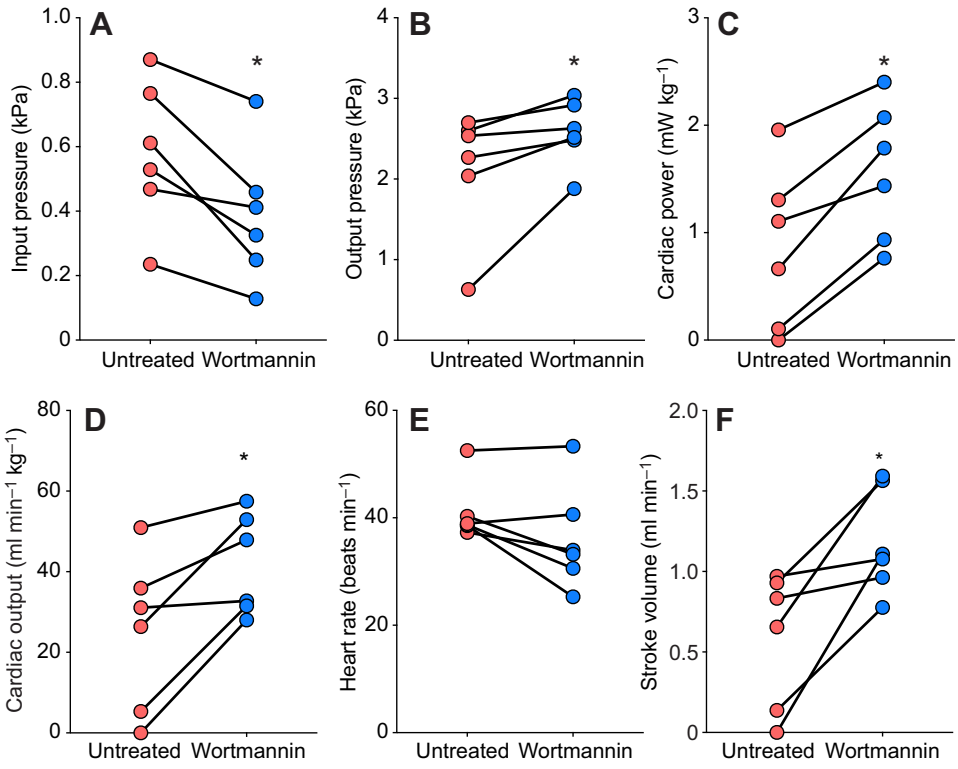


**Fig. 4. The effect of smooth muscle inhibition with wortmannin (10 µmol l$^{-1}$ perfusion) on cardiac performance in perfused *Trachemys scripta elegans* hearts.** (A) Input pressure. (B) Output pressure. (C) Cardiac power. (D) Cardiac output. (E) Heart rate. (F) Stroke volume. Asterisks indicate significant differences ($P$<0.05) following histamine injection (paired $t$-tests). $N$=6. Individual values are presented.

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pressure decreased (0.58±0.23 to 0.39±0.21 kPa; $t$=4.0, $P$<0.05; Fig. 4A), whilst output pressure (2.12±0.77 to 2.58±0.41 kPa; $t$=2.6, $P$<0.05; Fig. 4B), cardiac power (0.86±0.75 to 1.57±0.64 mW kg$^{-1}$; $t$=6.1, $P$<0.05; Fig. 4C), total cardiac output (25.0±19.2 to 41.8±12.5 ml min$^{-1}$ kg$^{-1}$; $t$=3.6, $P$<0.05; Fig. 4D) and stroke volume (0.59±0.42 to 1.18±0.33 ml kg$^{-1}$; $t$=3.5, $P$<0.05; Fig. 4F) increased significantly. Heart rate did not significantly change (41.0±5.7 to 36.2±9.8 kPa; $t$=2.1, $P$=0.09; Fig. 4E).

In the most extreme case, in one heart that had to be excluded from the histamine series of experiments owing to extremely low baseline cardiac performance, smooth muscle inhibition with wortmannin increased cardiac output from almost zero to relatively normal levels (Fig. 5). In this particular case it required an extended 'perfusion time' for wortmannin to act (>40 min) because so little perfusate entered the heart until cardiac output began to be restored. Indeed, in hearts with

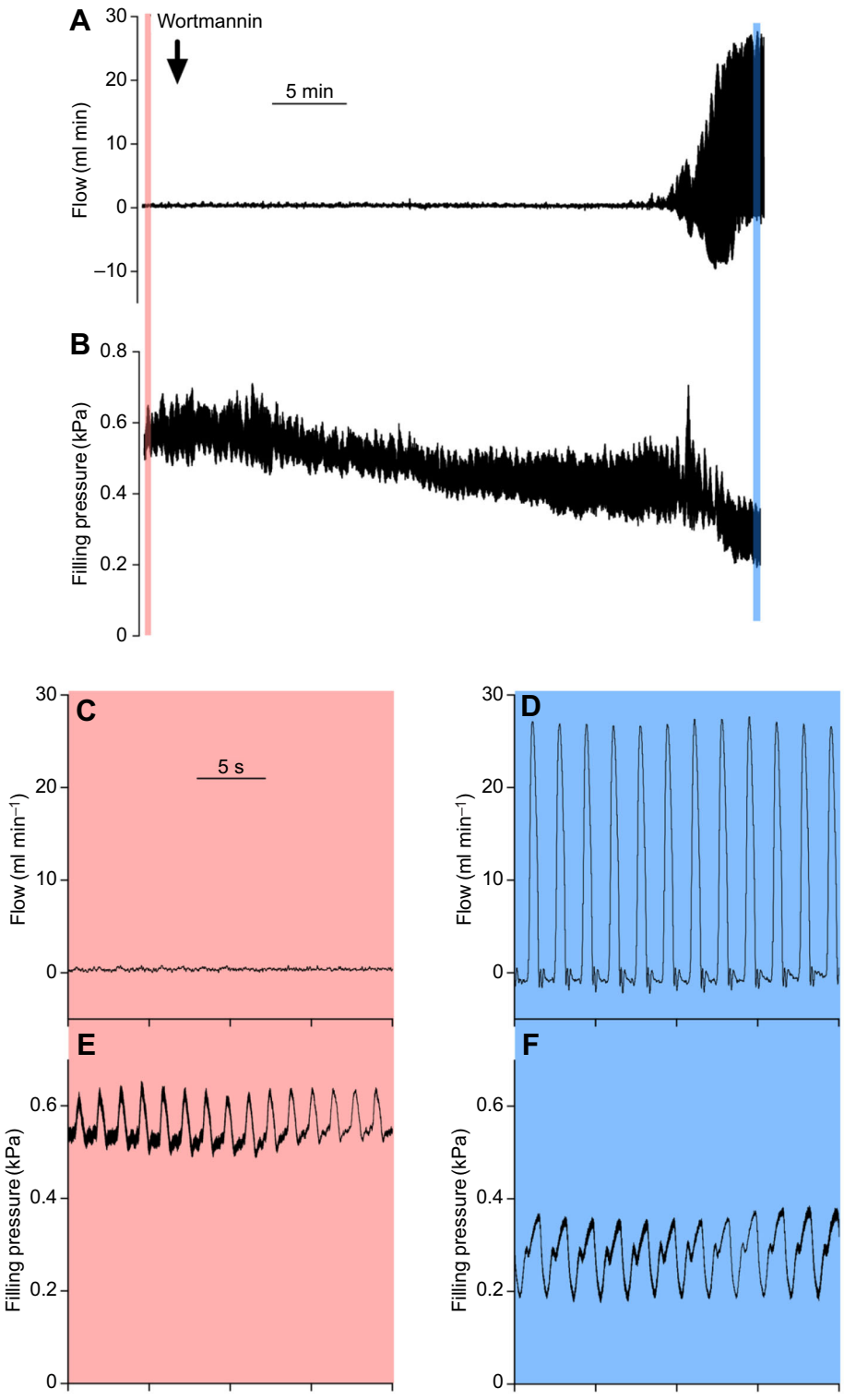


**Fig. 5. The effect of smooth muscle inhibition with wortmannin (10 µmol l$^{-1}$ perfusion) on a perfused *Trachemys scripta elegans* heart.** Flow (A,C,D) is represented by a pulmonary artery flow trace and input (filling) pressure (B,E,F) is the right atrial input pressure. Pink shading represents a pre-wortmannin measurement and blue represents the values after wortmannin had taken effect. Turtle body mass: 0.34 kg.

baseline cardiac output ≥25 ml min$^{-1}$ kg$^{-1}$, wortmannin perfusion required 20 min to take effect, but this period was substantially prolonged in the hearts with lower baseline cardiac output (Fig. S1).

There was no change (from pre-injection) in input pressure when the bolus injections of histamine were repeated during perfusion with wortmannin (0.27±0.15 to 0.05±0.04 kPa; $t$=3.2, $P$<0.05; Fig. 6A). Perfusion with wortmannin also reduced the decrease in cardiac output elicited by histamine (−24.1±11.5 to −7.0±3.3 ml min$^{-1}$ kg$^{-1}$; $t$=3.2, $P$<0.05; Fig. 6D). Although the changes in other variables did not reach statistical significance ($P$>0.05), these findings suggest that histamine largely exerts its cardiac effects through smooth muscle contraction.

## DISCUSSION

The functional significance of atrial smooth muscle in freshwater turtles has remained enigmatic for more than a century. Our data from *T. s. elegans* suggest that atrial smooth muscle has the potential to dramatically alter cardiac function, including, in the most extreme case, arresting cardiac output altogether. Thus, in consistency with Gesell's (1915) earlier observations, we provide experimental evidence that atrial smooth muscle may play an important role in altering cardiac filling.

At first glance, the negative correlation between input pressure and cardiac output under baseline conditions appears to be in striking contrast to the classic representation of the Frank–Starling law of the heart, where increasing filling pressure augments stroke volume (Berlin and Bakker, 2015; Starling, 1921). Indeed, the Frank–Starling law has been previously described in its conventional form in perfused turtle hearts (Farrell et al., 1994; Franklin, 1994). However, it is important to emphasise that filling pressure is typically taken as a proxy for end-diastolic volume, and that it is the stretch of the myocardial muscle fibres that underlies the molecular mechanism for the Frank–Starling law (Berlin and Bakker, 2015; Magder, 2015). Thus, in the turtle heart, smooth muscle contraction in the atria dissociates filling pressure from end-diastolic volume because it provides an additional means to regulate atrial dimensions that is independent of filling pressure. The negative correlation between input pressure and cardiac output is therefore not surprising and is in accordance with the classic view of myocardial performance: in hearts in which the atrial smooth muscle was spontaneously constricted, cardiac output was reduced but, because all of the hearts were filled by a similar pressure column, the recorded filling pressure increased. Indeed, the $x$-intercept in Fig. 1 was 1.03 kPa (10.5 cm $H_2O$), almost identical to the pressure column (10 cm) filling the heart. Thus, when the heart, positioned at the base of the static water column, is not pumping perfusate, the filling pressure will clearly be the same as the hydrostatic pressure exerted by the column.

The pronounced effect of atrial smooth muscle contraction on ventricular filling must be reconciled with our recent report on the small atrial contribution to ventricular filling in another reptile, the American alligator (*Alligator mississipiensis*) (Joyce et al., 2018a). This may be partially ascribed to species differences; the anatomy of the alligator heart differs from turtles, including possessing a complete interventricular septum (Jensen et al., 2018; Seymour et al., 2004). In other reptiles, including snakes and lizards, it has

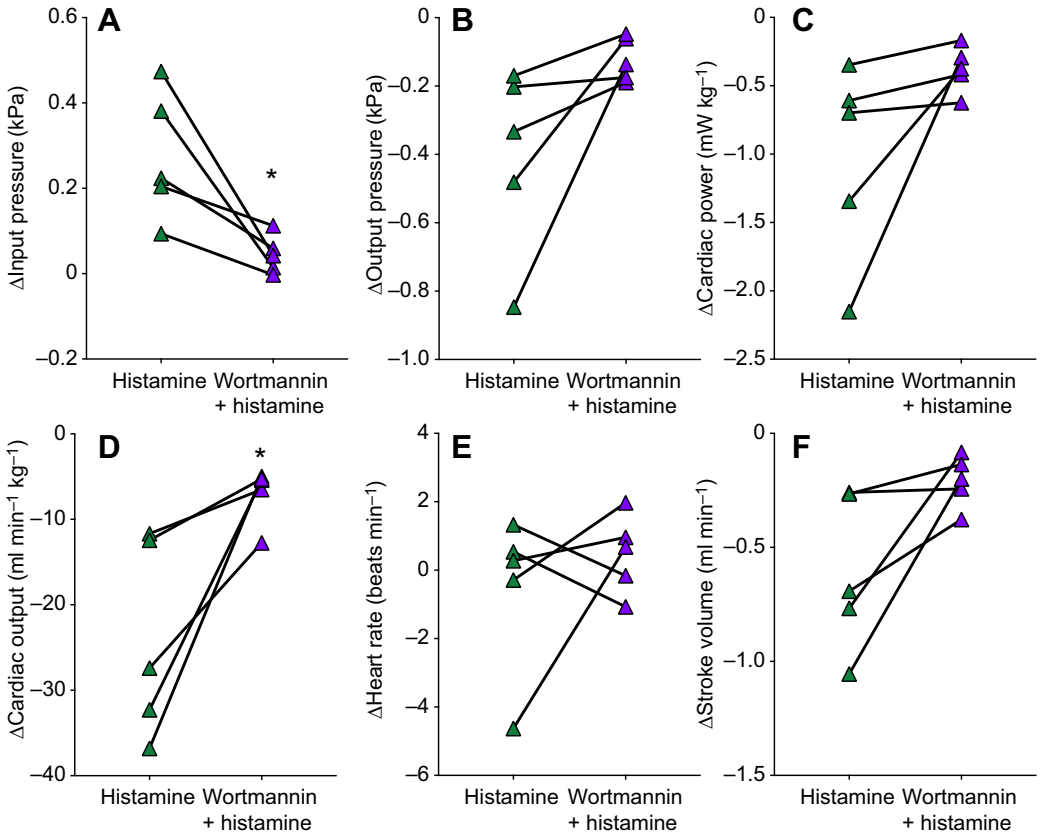


**Fig. 6. The change in cardiac parameters after the injection of histamine (100 nmol kg$^{-1}$ bolus into perfusate) in perfused *Trachemys scripta elegans* hearts before and during perfusion with wortmannin (10 µmol l$^{-1}$).** (A) Input pressure. (B) Output pressure. (C) Cardiac power. (D) Cardiac output. (E) Heart rate. (F) Stroke volume. The changes (Δ) in each of the parameters are relative to pre-histamine treatment. Asterisks indicate significant differences ($P$<0.05) in the changes in variable in control and wortmannin treated conditions (paired $t$-tests). $N$=5. Individual values are presented.

long been believed that atrial contraction provides the dominant source of ventricular filling (Burggren et al., 2014; Johansen, 1959; Johansen and Burggren, 1984), although this has not been considered in detail in turtles. Additionally, in turtle hearts, atrial smooth muscle may contract atria to such an extent (or with specificity) that it interferes with flow through the atrioventricular canal, which was explicitly avoided by ligation of the atria in alligators (Joyce et al., 2018a).

The capacity to change stroke volume via atrial smooth muscle contraction may benefit turtles during diving. When submerged, *T. scripta* exhibits a low heart rate (diving bradycardia), whilst heart rate increases twofold to threefold during the intermittent episodes of pulmonary ventilation (Burggren, 1975; Joyce et al., 2018b; Wang and Hicks, 1996). Based on intrinsic mechanisms alone (autoregulation), the bradycardia would be expected to augment stroke volume as cardiac filling time is prolonged (Altimiras and Axelsson, 2004; Joyce et al., 2018b). However, stroke volume is in fact maintained (Wang and Hicks, 1996) or even decreased (Burggren et al., 1997) despite the prevailing bradycardia. The fall in heart rate is achieved by increased vagal tone (Burggren, 1975), which also provides a powerful stimulation of atrial smooth muscle contraction (Bottazzi, 1900, 1906; Fano, 1887; Fano and Fayod, 1888; Meek, 1927). The fact that atrial smooth muscle contraction decreases cardiac filling indicates that it may be able to constrain the rise in stroke volume during dive bradycardia. This is analogous to the contraction of the vena caval sphincter as a means to decrease stroke volume in diving mammals (Blix, 2018; Elsner et al., 1971; Harrison and Tomlinson, 1956; Lillie et al., 2018). Reducing stroke volume during diving would be energetically beneficial to the heart, as we observed smooth muscle contraction decreased cardiac power (work). Moreover, it would facilitate the temporal matching of pulmonary perfusion and ventilation and hence increase gas exchange efficiency (Malte et al., 2016).

We focused on histamine as it stimulated smooth muscle with minimal effect on cardiac contraction. Its physiological role remains unclear, although histamine does constrict the pulmonary arterial sphincter in loggerhead sea turtles (*Caretta caretta*) (García-Párraga et al., 2018), and may contribute to the low pulmonary blood flow (right-to-left shunt) that prevails during diving (Wang and Hicks, 1996). Together, our data suggest that histaminergic control may be involved in the integrative cardiovascular response to diving.

Emydid turtles, including *T. s. elegans*, exhibit a remarkable capacity for hypoxic, or even anoxic, hibernation in ice-covered lakes (Jackson, 2002). Therefore, an obvious question arises regarding the potential role of atrial smooth muscle (Meek, 1927). However, smooth muscle contraction is abolished by low pH (Andrus, 1919; Gruber, 1927; Snyder and Andrus, 1919) and high levels of adrenaline (Gruber, 1920b, 1921; Sollmann and Rossides, 1927), and both acidosis and increased plasma catecholamines are characteristic of anoxic hibernation (Keiver et al., 1992; Overgaard et al., 2007; Wasser and Jackson, 1991). We therefore regard it as highly unlikely that the atrial smooth muscle is particularly active during hibernation.

We studied *T. s. elegans* because it belongs to the family (Emydidae) in which atrial smooth muscle was first detailed (Bottazzi, 1906; Fano, 1887; Fano and Fayod, 1888; Pereira, 1924; Rosenzweig, 1903; Shaner, 1923). Atrial smooth muscle has not been histologically detected in other reptiles, such as lizards (Bottazzi, 1897; Laurens, 1913), and we have never observed tonus waves in cardiac preparations from crocodilians or snakes (Galli et al., 2006; Joyce et al., 2014). A dedicated study on the phylogenetic distribution of smooth muscle within the order of turtles (Testudines) will be necessary to resolve the broader evolutionary history of atrial smooth muscle in turtles.

In summary, our results reveal a unique and novel mechanism to change cardiac output in an emydid turtle. It is possible that the atrial smooth muscle is activated by vagal stimulation (Bottazzi, 1900; Fano, 1887) during diving to reduce stroke volume, thereby accommodating the large changes in cardiac output required to match periodic lung ventilation with pulmonary perfusion.

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#### Competing interests
The authors declare no competing or financial interests.

#### Author contributions
Conceptualization: W.J.; Methodology: W.J.; Formal analysis: W.J.; Investigation: W.J.; Resources: M.A., T.W.; Data curation: W.J.; Writing - original draft: W.J.; Writing - review & editing: M.A., T.W.; Visualization: W.J.; Supervision: M.A., T.W.; Project administration: M.A., T.W.; Funding acquisition: T.W.

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#### Supplementary information
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