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RESEARCH ARTICLE

Regulation of blood flow in the pulmonary and systemic circuits during submerged swimming in common snapping turtle (*Chelydra serpentina*)

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ABSTRACT

Blood flow patterns and heart rate have rarely been investigated in freely swimming turtles and their regulation during swimming is unknown. In this study, we investigated the blood flow patterns and heart rate in surfacing and during graded, submerged swimming activity in common snapping turtles. We further investigated the effects of beta-adrenergic and cholinergic receptor blockade on blood flow and heart rate during these activities. Our findings illustrate that surfacing is accompanied by an increase in heart rate that is primarily due to beta-adrenergic stimulation. During swimming, this mechanism also increases heart rate while vagal withdrawal facilitates a systemic to pulmonary (left to right) shunt. The results indicate there may be important taxonomic effects on the responses of cardiac function to activity in turtle species.

KEY WORDS: Autonomic regulation, Exercise, Cardiac shunts

INTRODUCTION

The cardiac ventricle of squamate and chelonian vertebrates lacks anatomical septation, which motivated studies of the possible significance and functional consequences of changes in pulmonary and systemic blood flow in different species (Hicks and Wang, 2012). While blood flow patterns have been investigated in response to ventilatory state (Frische et al., 2000; Shelton and Burggren, 1976; Burggren, 1977; Wang and Hicks, 1996a) and during anoxic exposure (Hicks and Wang, 1998), an in-depth study of blood flow and its regulation at different swimming speeds in turtles has yet to be conducted. In addition, cardiovascular function in many turtle species, including the common snapping turtle (*Chelydra serpentina*), remains poorly understood. Given the diversity of life history traits in the chelonian lineage, differences in cardiovascular regulation may exist.

Studies on submerged swimming in red-eared slider turtles (*Trachemys scripta*) have revealed an increase in blood flow to the pulmonary circulation above that to the systemic circulation, defined as a left-to-right (L–R) shunt (Krosniunas and Hicks, 2003). Increased blood flow to the pulmonary circuit in submerged, swimming turtles may appear to be physiologically maladaptive because an increased L–R shunt could result in reduced oxygen delivery to active muscles. However, Wang and Hicks (2008) found that if demands on convective flow are minimal, an increase in pulmonary blood flow is not required to maintain resting metabolic rate in turtles (Wang and Hicks, 2008). Although blood flow to the lungs increased during submerged activity in *T. scripta* (Krosniunas and Hicks, 2003), the impact of increased swimming intensity on cardiac output distribution in turtles remains unexplored. The effects of incremental increases in swimming speed on cardiovascular physiology have been investigated only in the green sea turtle (*Chelonia mydas*), and the distribution of cardiac output was only assessed at a single swim speed (Butler et al., 1984; West et al., 1992). To date, changes in blood flow to the pulmonary and systemic circulation and the regulation of these patterns during swimming in turtles is unknown.

Autonomic control of cardiac shunts in turtles has been previously studied and there is general consensus that right-to-left (R–L) shunts are under cholinergic or vagal control, whereas L–R shunts are regulated by sympathetic output (Hicks, 1994; Burggren, 1985). In addition, in apneic turtles, there is an associated bradycardia which is mediated by increased vagal output (Hicks, 1994). The importance of vagal control of cardiac function was clarified by Hicks and Wang (1998), who showed that cholinergic blockade doubled heart rate (f_H), as well as increased systemic blood flow (by 40%) and pulmonary blood flow (by 150%) in turtles. These findings demonstrate that tonic vagal output depresses f_H while increasing pulmonary artery constriction, triggering a R–L shunt in control conditions (Hicks and Wang, 1998). The Hicks and Wang (1998) study also showed that injection of adrenaline (epinephrine) increased f_H and pulmonary blood flow, resulting in a L–R shunt of blood (Hicks and Wang, 1998). While these prior studies have been instrumental in clarifying the control of cardiac shunts in turtles, the primary turtle species investigated has been the red-eared slider and the degree to which these findings translate to other species in the diverse chelonian lineage remains uncertain. Although it is plausible that the features of cardiac function are conserved in the chelonian lineage, possible differences between turtle species that exhibit different life strategies have yet to be investigated.

Unlike the red-eared slider, the common snapping turtle (*C. serpentina*) is primarily an aquatic, sit-and-wait predator found in deep pools that will extend its neck to the surface, protruding only its nares to the water level for ventilation, and that infrequently ventures onto terrestrial habitats (Ernst and Barbour, 1989). Given these differences in natural history, our goal was to broaden the understanding of blood flow and cardiac shunts in the chelonian lineage by investigating the common snapping turtle. We monitored cardiac output and f_H in snapping turtles when their nares protruded above the water line, during periods of swimming at increasing graded speeds, and after blockade of cholinergic and beta-adrenergic regulation. We predicted that when the animals surface, f_H and pulmonary blood flow would increase as a result of a

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List of abbreviations

LAo	left aorta
LBA	left brachiocephalic artery
LPA	left pulmonary artery
$\dot{Q}_{LAo}$	left aortic blood flow
$\dot{Q}_{LBA}$	left brachiocephalic artery blood flow
$\dot{Q}_{LPA}$	left pulmonary artery blood flow
$\dot{Q}_{pul}$	pulmonary output
$\dot{Q}_{pul}/\dot{Q}_{sys}$	shunt fraction
$\dot{Q}_{RAo}$	right aortic blood flow
$\dot{Q}_{sys}$	systemic cardiac output
RAo	right aorta
$V_{S,pul}$	pulmonary stroke volume
$V_{S,sys}$	systemic stroke volume

decrease in cholinergic tone, ultimately driving increases in f_H and pulmonary blood flow to facilitate cardiorespiratory coupling. We further predicted that during submerged swimming, adrenergic stimulation would mediate a L–R shunt and cholinergic tone on f_H would be withdrawn, resulting in an increase in f_H to meet any demands on the system.

MATERIALS AND METHODS

Animals

Sixteen common snapping turtles, *Chelydra serpentina* (Linnaeus 1758), raised from hatchlings, were studied at 2–3 years of age. Turtles (n=16; mean±s.e.m. mass 4.79±0.29 kg; carapace length 25.1±0.6 cm) were used in two separate studies: study 1 (N=10) examined the effects of increasing swim speeds on blood flow patterns and f_H; study 2 (N=6) examined the role of adrenergic and cholinergic regulation on blood flow patterns and f_H at a fixed swim speed. Snapping turtles were raised and maintained at approximately 28°C in tanks (50–500 l) partially filled with fresh water, allowing turtles to voluntarily and fully submerge. All turtles were fed (Mazuri® Crocodilian Diet, Mazuri®, PMI Nutrition International, Brentwood, MO, USA) *ad libitum* 2–4 times per week, and kept on a constant 12 h:12 h light:dark cycle (light 08:00 h–20:00 h).

Surgical procedures

Turtles were fasted for at least 10 days before the study. Turtles were induced into anesthesia by placing a plastic container containing isoflurane-saturated cotton gauze over the head of the animal. Turtles were then intubated, turned ventral-side up and ventilated at a rate of 5–7 breaths min^{-1} at a volume of 20 ml kg^{-1} of 1.5% isoflurane/room air gas mix to maintain anesthesia (Harvard Apparatus 665 ventilator, Harvard Apparatus, Holliston, MA, USA; FluTec vaporizer, FluTec, Ohmeda, OH, USA). Reflexes were regularly monitored to ensure turtles were at a surgical plane of anesthesia.

Once pedal reflexes were absent, a 3 cm×5 cm section of the plastron, immediately ventral to the major outflow vessels, was carefully removed using a cast saw (941 Cast Cutter, Stryker Instruments, Kalamazoo, MI, USA). The excised section of plastron was placed in sterile 0.9% saline to prevent desiccation. For both studies, the major outflow vessels of the heart were isolated by blunt dissection from the surrounding connective tissue, taking care to avoid the pericardium and any perivascular nerves. Blood flow probes (PS Series Flow probes, 2–6 mm cuff diameter, Transonic Systems Inc., Ithaca, NY, USA) were then placed around the left pulmonary artery (LPA; 2–4 mm), left aortic arch (LAo; 3–4 mm), left brachiocephalic trunk (i.e. the left subclavian artery and left carotid artery, LBA; 2–4 mm) and combined right aorta, subclavian, carotid (RAo; 4–6 mm). Leads from the probes were externalized through an incision medial to the left front limb, through the pectoral girdle and between the carapace and plastron. These incisions were sutured (4.0 silk with tapered needle) closed, and the positioning of the probes around the vessels was checked before the exiting leads were carefully sutured to the skin. Once in place, flow probes were bathed in acoustic coupling gel. For study 2 only, a small arterial branch off the left subclavian artery was occlusively catheterized with a polyethylene 50 (PE 50) tube filled with heparinized (50 U ml^{-1}) saline (0.9% NaCl) that was advanced into the subclavian artery; this was used to deliver drugs during the swim trials for study 2. The catheter was externalized through a puncture in the skin underneath the cut made for flow probe leads. The pectoral muscle was then sutured together and the excised plastron was replaced to cover the surgical site. Cyanoacrylic glue gel (Gorilla Glue, Inc.) was used to seal the edges of the plastron, making a watertight seal and quick-setting epoxy (Epoxy Mix 5 Minute, Gorilla Glue, Inc.) was used to further secure the plastron. Turtles were injected with 2.3 mg kg^{-1} (i.m.) of antibiotic (Enroflox®, Norbrook Labratories, Newry, Northern Ireland, UK) and 1.25 mg kg^{-1} analgesic (FluMeglumine, Flunixin Meglumine, Clipper Distributing Company, St Joseph, MO, USA) in the hindlimb. The probe leads and the catheter were anchored to the carapace using a zip tie. Turtles were allowed to recover in plastic 50 l containers for 3 days at 30°C on a 12 h:12 h light:dark cycle. After 24 h, water was added to the containers to prevent dehydration but food was withheld throughout the 3 day recovery period.

Swim flume

On the day of the study, individual turtles were transferred to a custom-built swim flume (see Joyce et al., 2018, for specifics). The water level was maintained at 35 cm, which allowed the turtle to surface and ventilate freely. A titanium 1000 W aquarium heater (Catalina RF-1000T, Catalina Aquarium, Inc., Lakeport, CA, USA) maintained water temperature at 29.4±0.08°C while ambient air temperature was maintained at approximately 28°C. Water flow was generated with a 12 V trolling motor (Endura C2-30, Minn Kota, Mankato, MN, USA) and water velocity was determined using a portable flowmeter (Flo-Mate Model 2000, Hach, Loveland, CO, USA). Laminar flow was achieved with a baffle (35 cm total height) constructed from 4.5 cm diameter polyvinyl chloride (PVC) pipes (15 cm length) glued together.

The blood flow probes were connected to T402 and T403 blood flow meters (Transonic Systems, Inc.). All probe leads and the arterial catheter were attached to helium balloons that suspended them above the swim tunnel. The outputs from the transonic meters were connected to a PowerLab® 16/35 data recording system which was connected to a computer running LabChart Pro® software (v8.2, ADInstruments, Colorado Springs, CO, USA), sampled at 100 Hz. The recording station was set up behind an opaque barrier to allow continuous monitoring of the turtle without disturbing the animal. A 40 W bulb lamp was set up at the opposite end of the swim flume to that of the recording station to limit the turtles' ability to observe the investigator. In all cases, measurements were conducted with the room lights off with the only light emitted from the lamp.

Study 1: blood flow and f_H response to surfacing and activity

Individual turtles (n=10) were placed in the swim flume and left to habituate overnight. After 16 h of habituation, control blood flow

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and f_H were recorded until they remained stable for a minimum of 1 h. Once stabilized, the turtles were visually monitored for periods of surfacing that were assumed to be periods of ventilation. This was based on the anticipatory tachycardia evident in the recording and visual verification. A minimum of five surfacing events were allowed prior to the initiation of the swim study. The power of the motor was adjusted to produce a water velocity of 14 cm s^{-1} equivalent to 0.5 carapace lengths s^{-1}. Turtles swam fully submerged for 5 min, at which point the trial was terminated and the motor was turned off. Swimming was either initiated spontaneously or induced by gentle lifting of the probe leads. Preliminary studies indicated the 5 min period was the maximal sustainable period for repeated swim trials and a prior study of swimming turtles used a similar time frame (West et al., 1992). Successful completion of a swim trial was defined as the ability of the animal to maintain its position in the water flow during the swim bout. Turtles were then given a 1 h recovery period to allow f_H and blood flow to return to control values. Two additional swim trials at 0.5 carapace lengths s^{-1} were conducted with a recovery period (1 h) in between to allow a return to control values. The swim procedures described above (three bouts of swimming interspersed by 1 h rest) were then repeated at a water velocity of 26 cm s^{-1}, equivalent to 1.0 carapace lengths s^{-1}. Following this, we attempted to swim turtles at 37 cm s^{-1}, equivalent to 1.5 carapace lengths s^{-1}. However, only 5 turtles could swim at 1.5 carapace lengths s^{-1}. At the end of the study, turtles were euthanized by an overdose of isoflurane followed by cranial pithing. Turtle swimming speed (carapace lengths s^{-1}) was calculated by dividing the average flow rate (cm s^{-1}) by carapace length (cm).

Study 2: autonomic control during surfacing and activity

Individual turtles (n=6) were placed in the swim flume to monitor surfacing and then subjected to a similar protocol to that for the 0.5 carapace lengths s^{-1} swim trials described above. After a 1 h recovery, a single injection of saline (1 ml kg^{-1}) was given through the arterial catheter to serve as a vehicle control that was equal in volume to the drug injections, and parameters were monitored for 1 h. Turtles were then given a 1 ml kg^{-1} injection of propranolol (3 mg ml^{-1}; a non-specific beta-adrenergic receptor antagonist) and allowed a minimum of 1 h for blood flow and f_H to stabilize. Following at least three surfacing events (ca. 1 h), the turtle was exercised once at 0.5 carapace lengths s^{-1} for 5 min. The turtle was allowed 1 h to recover from exercise. Turtles were then given a 1 ml kg^{-1} injection of atropine (3 mg ml^{-1}; a cholinergic receptor antagonist) and allowed a minimum of 1 h for blood flow and f_H to stabilize. Following at least three surfacing events (ca. 1 h), turtles were exercised once again at 0.5 carapace lengths s^{-1}. Turtles were allowed 1 h to recover after exercise and then the trial was terminated. At the end of the study, the turtle was deeply anesthetized with isoflurane followed by cranial pithing. Turtle swimming speed (carapace lengths s^{-1}) was calculated by dividing the average flow rate (cm s^{-1}) by carapace length (cm).

Calculations and statistics

Blood flow and f_H were calculated from a minimum 30 s trace prior to swimming, during the 5 min exercise bout, at the peak of the response to surfacing and after parameters had stabilized following saline or drug injections. In the case of the swim trials, the response was taken as the mean value of the individual trials at a given speed. Beat-to-beat f_H was derived from RAo blood flow to calculate mean f_H. Mean blood flow was determined through the LAo, RAo, LPA and LBA blood flow ($\dot{Q}_{LAo}$, $\dot{Q}_{RAo}$, $\dot{Q}_{LPA}$ and $\dot{Q}_{LBA}$, respectively). Systemic cardiac output ($\dot{Q}_{sys}$) was calculated as $\dot{Q}_{LAo}+\dot{Q}_{RAo}+\dot{Q}_{LBA}$ and pulmonary output ($\dot{Q}_{pul}$) as $2\times\dot{Q}_{LPA}$, assuming that flow through the left and right pulmonary arteries is identical (Comeau and Hicks, 1994). Systemic stroke volume ($V_{S,sys}$) and pulmonary stroke volume ($V_{S,pul}$) were also calculated as $\dot{Q}_{sys}$ and $\dot{Q}_{pul}$, respectively, divided by f_H (Wang and Hicks, 1996a). Shunt fraction ($\dot{Q}_{pul}/\dot{Q}_{sys}$) was also calculated at each time point (Crossley et al., 1998). All flow and volume data were normalized by body mass (ml min^{-1} kg^{-1} and ml kg^{-1}, respectively).

Statistical tests were performed using Statistica version 13.3 (TIBCO Software Inc., Palo, Alto, CA, USA). All data were checked for normality (P<0.05) prior to performing *post hoc* tests. A paired Student's t-test was used to analyze all parameters prior to, and at the peak of, each swimming speed. Differences in parameters between swim speeds were not conducted because not all turtles were able to swim at the highest speed. A paired Student's t-test was used to analyze all parameters before and after the saline, propranolol and atropine injections. A repeated-measures (RM) ANOVA with a Fisher's LSD test was used to make comparisons of all parameters for study 2 swimming velocity (0.5 carapace lengths s^{-1}) before and after the injection of each drug. All data are presented as means±s.e.m.

RESULTS

Control values

For all animals (n=16) prior to swimming, control values were as follows: f_H 26±2 beats min^{-1}; $\dot{Q}_{sys}$ 21.9±2.7 ml min^{-1} kg^{-1}; $\dot{Q}_{pul}$ 19.1±2.2 ml min^{-1} kg^{-1}; $V_{S,sys}$ 0.86±0.09 ml kg^{-1}; $V_{S,pul}$ 0.73±0.06 ml kg^{-1}; shunt fraction 1.01±0.14.

Surfacing response

In this study, 'surfaced' was defined as the animal's nares being observed above the water line. When at the surface, compared with submergence, f_H (paired t-test, P<2.99e^{-5}), $\dot{Q}_{sys}$ (paired t-test, P<1.79e^{-5}) and $\dot{Q}_{pul}$ (paired t-test, P<0.00056) all increased significantly. $\dot{Q}_{sys}$ increased approximately 26% while $\dot{Q}_{pul}$ increased approximately 70% during surfacing, resulting in a significant increase (paired t-test, P<0.016) in $\dot{Q}_{pul}/\dot{Q}_{sys}$ from approximately 1 to nearly 1.3 (Table 1). Surfacing also resulted in a decrease in $V_{S,sys}$ (paired t-test, P<0.009) and an increase in $V_{S,pul}$ (paired t-test, P<0.02) (Table 1).

Swim response (study 1)

Swimming resulted in a general tachycardia that increased both $\dot{Q}_{sys}$ and $\dot{Q}_{pul}$ (Fig. 1A). These cardiovascular changes appear to be a response to exercise and not a general 'stress' response because increasing water speed in the absence of swimming (Fig. 1B) was not associated with similar increases in cardiovascular parameters.

Table 1. Blood flow and heart rate in non-swimming submerged turtles (control) and turtles at the surface (study 1)

Measure	Control	Surface
f_H (beats min^{-1})	28±4	40±1*
$\dot{Q}_{sys}$ (ml min^{-1} kg^{-1})	24.24±3.51	30.13±3.70*
$\dot{Q}_{pul}$ (ml min^{-1} kg^{-1})	19.87±2.64	33.50±3.04*
$V_{S,sys}$ (ml kg^{-1})	0.90±0.12	0.78±0.11*
$V_{S,pul}$ (ml kg^{-1})	0.71±0.07	0.84±0.07*
$\dot{Q}_{pul}/\dot{Q}_{sys}$	0.96±0.14	1.26±0.15*

f_H, heart rate; $\dot{Q}_{sys}$, systemic cardiac output; $\dot{Q}_{pul}$, pulmonary cardiac output; $V_{S,sys}$, systemic stroke volume; $V_{S,pul}$, pulmonary stroke volume; and $\dot{Q}_{pul}/\dot{Q}_{sys}$, shunt fraction. Data are presented as means±s.e.m. (n=10). Asterisks indicate significant differences based on a paired t-test with P<0.05.

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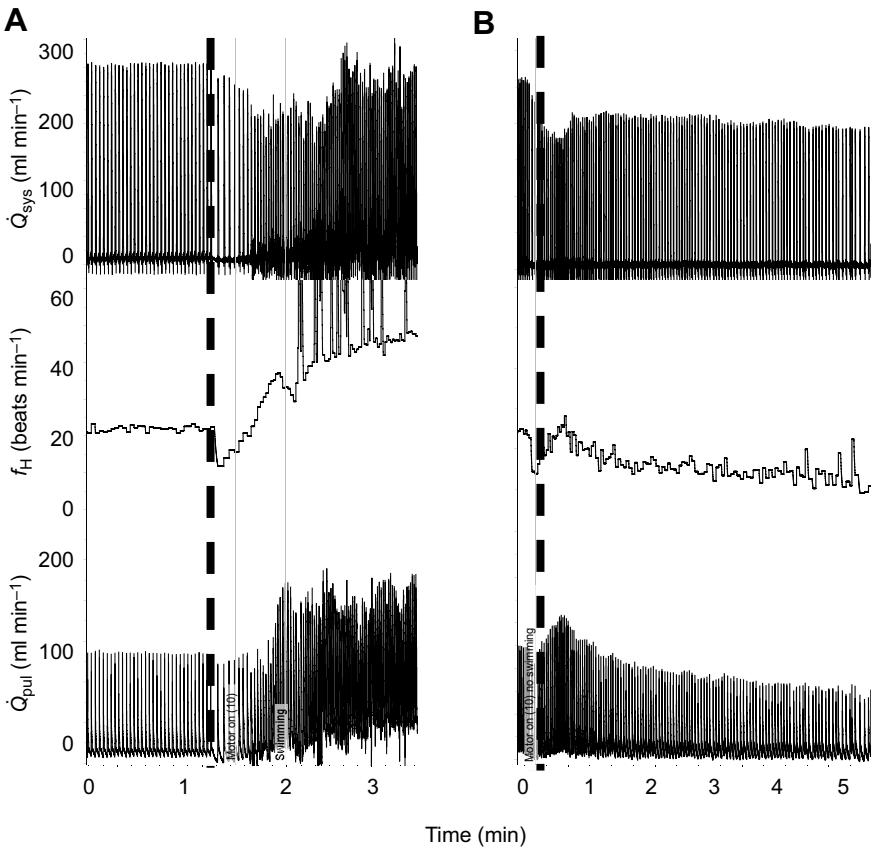

Fig. 1. Swim response. (A) Representative trace of systemic cardiac output ($\dot{Q}_{sys}$), heart rate (f_H) and pulmonary cardiac output ($\dot{Q}_{pul}$) for a turtle swimming at 0.5 carapace lengths s^{-1}. The dashed line indicates the onset of increased water speed. (B) The same animal with water flow at the same speed as in A, but with the animal not swimming. Note the lack of increase of f_H and small changes in $\dot{Q}_{pul}$ and $\dot{Q}_{sys}$ when the animal was not swimming.

Swimming bouts resulted in significant increases of 13%, 19% and 26% in $\dot{Q}_{sys}$ at swimming speeds of 0.5 carapace lengths s^{-1} (paired *t*-test, $P<7.05e^{-6}$), 1.0 carapace lengths s^{-1} (paired *t*-test, $P<0.0005$) and 1.5 carapace lengths s^{-1} (paired *t*-test, $P<0.02$), respectively (Fig. 2A). The significant increase in $\dot{Q}_{pul}$ was 62% higher than the change of $\dot{Q}_{sys}$ in animals swimming at 0.5 carapace lengths s^{-1} (paired *t*-test, $P<0.0018$) and 57% higher than the change in $\dot{Q}_{sys}$ for animals swimming at 1.0 carapace lengths s^{-1} (paired *t*-test, $P<0.0003$). However, $\dot{Q}_{pul}$ at 1.5 carapace lengths s^{-1} was not significantly elevated (Fig. 2B).

Swimming caused significant decreases in $V_{S,sys}$ of 27% and 19% at 0.5 carapace lengths s^{-1} (paired *t*-test, $P<0.001$) and 1.0 carapace lengths s^{-1} (paired *t*-test, $P<0.004$), respectively (Fig. 2C). Unlike $V_{S,sys}$, $V_{S,pul}$ was not affected by swimming at any speed (Fig. 2D).

Swimming caused a significant increase in f_H at 0.5 carapace lengths s^{-1} (paired *t*-test, $P<7.05e^{-06}$), 1.0 carapace lengths s^{-1} (paired *t*-test, $P<0.0003$) and 1.5 carapace lengths s^{-1} (paired *t*-test, $P<0.0026$) (Fig. 2E). Interestingly, all swim speeds resulted in similar peak f_H of 38, 39 and 38 beats min^{-1} at 0.5, 1.0 and 1.5 carapace lengths s^{-1}, respectively, similar to the f_H of surfaced animals (Fig. 2E).

Swimming caused a significant increase in $\dot{Q}_{pul}/\dot{Q}_{sys}$ in animals swimming at 0.5 carapace lengths s^{-1} (paired *t*-test, $P<0.0095$) and at 1.0 carapace lengths s^{-1} (paired *t*-test, $P<0.023$), denoting an overall left to right shunt of blood to the pulmonary circulation (Fig. 2F). This relationship was absent at 1.5 carapace lengths s^{-1} as $\dot{Q}_{pul}/\dot{Q}_{sys}$ was unaffected by swimming at this speed (Fig. 2F).

Drug treatment response (study 2)

None of the control saline injections affected blood flow or f_H (data not shown). Injection of the non-specific beta-adrenergic receptor antagonist propranolol (3 mg kg^{-1}) significantly decreased both f_H (paired *t*-test, $P<0.02$) and $V_{S,sys}$ (paired *t*-test, $P<0.05$) without affecting any other parameter (Table 2). The propranolol injection and subsequent swim trial were followed by injection of the cholinergic antagonist atropine (3 mg kg^{-1}). Atropine caused a significant increase in $\dot{Q}_{pul}$ (paired *t*-test, $P< 0.0034$), $V_{S,pul}$ (paired *t*-test, $P<0.003$), f_H (paired *t*-test, $P<0.012$) and $\dot{Q}_{pul}/\dot{Q}_{sys}$ (paired

Table 2. Blood flow and heart rate before (control) and after propranolol or atropine injection in submerged animals (study 2)

Measure	Control	Propranolol	Control	Atropine
f_H (beats min^{-1})	24±2	19±2*	20±2	23±1*
$\dot{Q}_{sys}$ (ml min^{-1} kg^{-1})	16.47±1.65	15.44±1.46	15.72±1.07	16.22±1.85
$\dot{Q}_{pul}$ (ml min^{-1} kg^{-1})	21.07±5.86	13.10±3.47	15.12±2.70	28.82±3.65*
$V_{S,sys}$ (ml kg^{-1})	0.71±0.07	0.81±0.06*	0.82±0.09	0.73±0.11*
$V_{S,pul}$ (ml kg^{-1})	0.83±0.06	0.65±0.14	0.73±0.09	1.25±0.12*
$\dot{Q}_{pul}/\dot{Q}_{sys}$	1.32±0.38	0.80±0.14	0.99±0.20	1.98±0.40*

f_H, heart rate; $\dot{Q}_{sys}$, systemic cardiac output; $\dot{Q}_{pul}$, pulmonary cardiac output; $V_{S,sys}$, systemic stroke volume; $V_{S,pul}$, pulmonary stroke volume; and $\dot{Q}_{pul}/\dot{Q}_{sys}$, shunt fraction. Data are presented as means±s.e.m. (n=6). Asterisks indicate significant differences based on a paired *t*-test with $P<0.05$.

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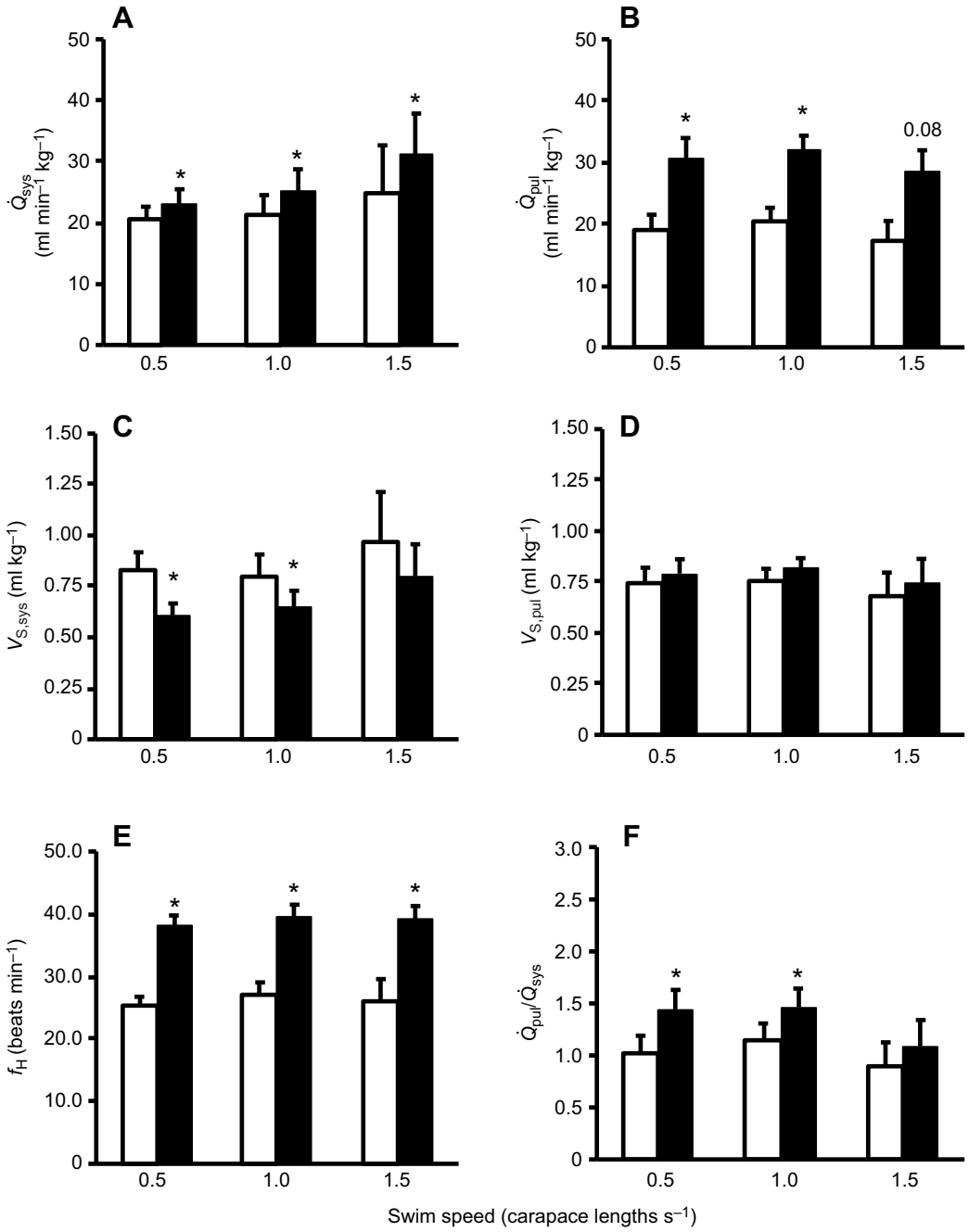

Fig. 2. Effect of swim speed on f_H and blood flow. (A) $\dot{Q}_{sys}$, (B) $\dot{Q}_{pul}$, (C) systemic stroke volume ($V_{S,sys}$), (D) pulmonary stroke volume ($V_{S,pul}$), (E) f_H and (F) shunt fraction ($\dot{Q}_{pul}/\dot{Q}_{sys}$) in stationary animals (open columns) and in animals swimming at 0.5, 1.0 and 1.5 carapace lengths s^{-1} (filled columns). Data are presented as means±s.e.m. Asterisks indicate a significant difference based on a paired *t*-test with $P<0.05$. In the case of $\dot{Q}_{pul}$ at 1.5 carapace lengths s^{-1} the (non-significant) *P*-value is presented. Sample size: $n=10$ turtles swimming at 0.5 and 1.0 carapace lengths s^{-1} and $n=5$ swimming at 1.5 carapace lengths s^{-1}.

t-test, $P<0.015$), and a significant decrease in $V_{S,sys}$ (paired *t*-test, $P<0.016$) without changing $\dot{Q}_{sys}$ (Table 2).

Surfacing response after drug injection (study 2)

Pre-drug injection responses to surfacing were similar to those reported for the animals used for the swim study only (study 1), with the exception of $V_{S,sys}$ and $\dot{Q}_{pul}/\dot{Q}_{sys}$, which were unaffected by surfacing, possibly as a result of the reduced sample size of six animals (Table 2). After propranolol injection, surfacing had no impact on $\dot{Q}_{sys}$ or $V_{S,sys}$ (Fig. 3A,C); however, surfacing significantly increased $\dot{Q}_{pul}$ (RM ANOVA, $P<6.25e^{-7}$), but to a level that was significantly lower (LSD *post hoc*, $P<0.00028$) than that prior to propranolol injection (Fig. 3B). Importantly, the percentage increase in $\dot{Q}_{pul}$ that accompanied surfacing after propranolol injection was approximately 112% (Fig. 3B). Surfacing after injection of propranolol resulted in a significant increase in $V_{S,pul}$ (RM ANOVA, $P<3.7e^{-07}$) to an absolute value that was significantly higher (LSD *post hoc*, $P<0.03$) than that without propranolol (Fig. 3D). After propranolol injection, f_H increased significantly when animals surfaced (RM ANOVA,

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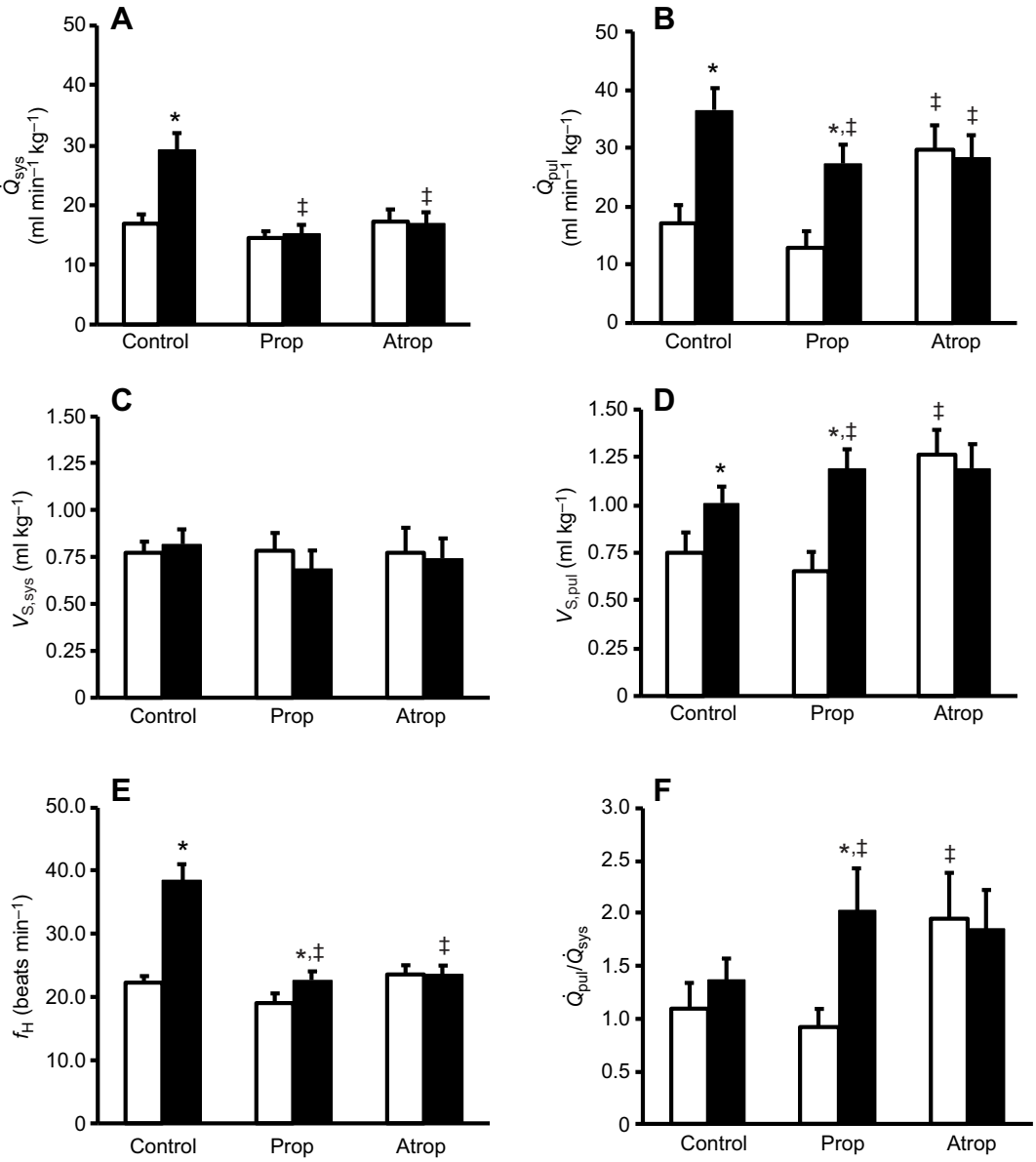

Fig. 3. Effect of drug injection on the surfacing response. (A) $\dot{Q}_{sys}$, (B) $\dot{Q}_{pul}$, (C) $V_{S,sys}$, (D) $V_{S,pul}$, (E) f_H and (F) $\dot{Q}_{pul}/\dot{Q}_{sys}$ in submerged animals (open columns) and animals at the surface (filled columns), without drug injection and following injection of propranolol (Prop) or atropine (Atrop). Data are presented as means ±s.e.m. (n=6). Asterisks indicate significant differences between submerged and surfaced values based on a repeated measures (RM) ANOVA with P<0.05. Double daggers indicate significant differences in the measured parameters across drug treatments based on a RM ANOVA with P<0.05.

P<0.025), reaching a value significantly below that prior to propranolol injection (LSD *post hoc*, $P<4.4e^{-10}$) (Fig. 3E). Finally, surfacing after injection of propranolol resulted in an increase in $\dot{Q}_{pul}/\dot{Q}_{sys}$ (RM ANOVA, P<0.0016) (Fig. 3F). After injection of atropine, surfacing had no effect on any of the parameters measured (Fig. 3A–F).

Swimming response after drug injection (study 2)

Swimming at 0.5 carapace lengths s^{-1} prior to drug treatment resulted in similar responses for all measured parameters to those determined during the swim study (study 1). The notable exception was the significant increase in $V_{S,pul}$ (RM ANOVA LSD *post hoc*, P<0.027). After propranolol injection, $\dot{Q}_{sys}$ no longer changed during swimming at 0.5 carapace lengths s^{-1} (Fig. 4A). After propranolol injection, swimming at 0.5 carapace lengths s^{-1} significantly increased $\dot{Q}_{pul}$ (RM ANOVA, LSD *post hoc* P<0.00027), but to a level that was significantly lower than that prior to propranolol injection (Fig. 4B). Swimming after injection of propranolol resulted in a significant decrease in $V_{S,sys}$ (RM ANOVA LSD *post hoc* P<0.006) and an increase in $V_{S,pul}$ (RM ANOVA LSD *post hoc*, P<0.0007) (Fig. 4C, D). After propranolol injection, f_H increased significantly in animals swimming at 0.5 carapace lengths s^{-1} (RM ANOVA LSD *post hoc*, P<0.005) but the increase was less than that prior to propranolol injection (Fig. 4E). Lastly, swimming after propranolol injection resulted in an increase in $\dot{Q}_{pul}/\dot{Q}_{sys}$ (RM ANOVA, P<0.0015) (Fig. 4F), while swimming after atropine injection had no effect on any of the measured parameters (Fig. 4A–F).

DISCUSSION

Cardiorespiratory synchrony, blood flow patterns and the role of cardiac shunts in chelonians have been extensively investigated (Hicks and Wang, 1996, 2012, 1998; Hicks, 2002). Our study was

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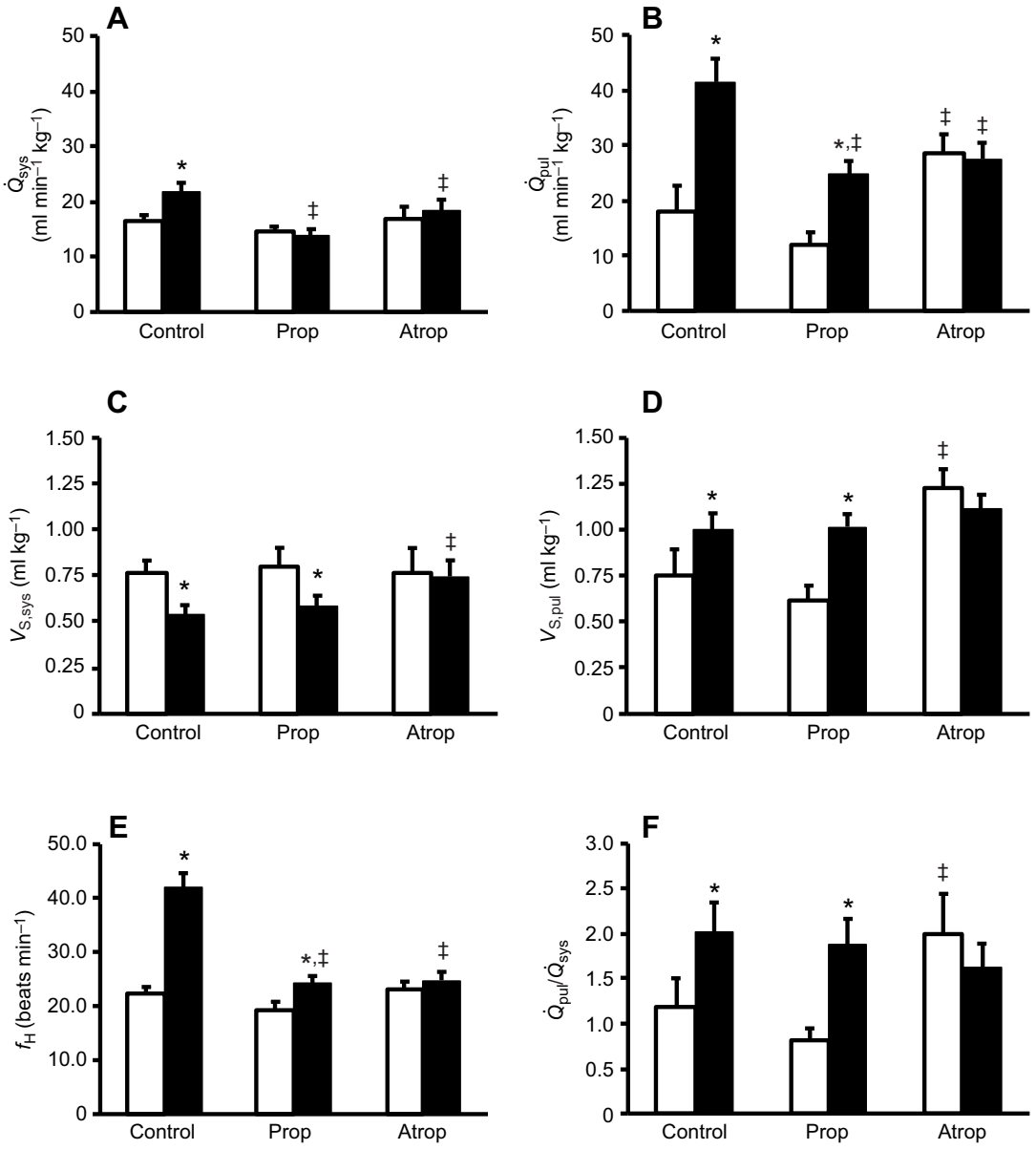

Fig. 4. Effect of drug injection on the swimming response. (A) $\dot{Q}_{sys}$, (B) $\dot{Q}_{pul}$, (C) $V_{S,sys}$, (D) $V_{S,pul}$, (E) f_H and (F) $\dot{Q}_{pul}/\dot{Q}_{sys}$ in stationary animals (open columns) and in animals swimming at 0.5 carapace lengths s^{-1} (filled columns), without drug injection and following injection of propranolol (Prop) or atropine (Atrop). Data are presented as means±s.e.m. (n=6). Asterisks indicate a significant difference between stationary and swimming values based on a RM ANOVA with P<0.05. Double daggers indicate significant differences in the measured parameters across drug treatments based on a RM ANOVA with P<0.05.

designed to expand the understanding of how blood flow patterns change and their regulation in a turtle species that primarily inhabits the bottom of the water column while submerged, during swimming and when surfacing (Ernst and Barbour, 1989). Our findings suggest adrenergic stimulation of f_H is pronounced in animals that surface, while cholinergic inhibition of pulmonary blood flow primarily decreases in surfacing and swimming animals.

Cardiovascular responses to surfacing in non-swimming turtles (study 1)

Previous studies have described the general cardiovascular pattern in surfacing and ventilating turtles which is characterized by a tachycardia and increased pulmonary blood flow prior to the ventilatory cycle in 'anticipation' of ventilation (West et al., 1992; Wang and Hicks, 1996a,b). We noted a similar sequence of cardiovascular changes prior to surfacing in common snapping turtles (data not presented). In animals that had surfaced, f_H approximately doubled and was associated with an increase in $\dot{Q}_{sys}$, $\dot{Q}_{pul}$ and $\dot{Q}_{pul}/\dot{Q}_{sys}$, denoting an increase in L–R shunt (Table 1). Similar findings have been previously reported in several turtle species (Shelton and Burggren, 1976; Johansen et al., 1977; Butler et al., 1984; West et al., 1992), indicating this is a generalized cardiovascular response that occurs with surfacing and the onset of pulmonary ventilation. Although we did not measure ventilation in this study, the similarity of our findings compared with those of other studies suggests the common snapping turtles in this study were ventilating once surfaced (Table 1).

Cardiovascular responses to swimming (study 1)

Swimming bouts impacted all flow parameters and f_H (Fig. 1A). Swimming at each speed was associated with an increase in $\dot{Q}_{sys}$, while swimming at 0.5 and 1.0 carapace lengths s^{-1} decreased $V_{S,sys}$,

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indicating the change in $\dot{Q}_{sys}$ was due to an elevation in f_H and not $V_{S,sys}$ (Fig. 2A,C,E). $\dot{Q}_{pul}$ increased at two of the three swim speeds, but the lack of significance at 1.5 carapace lengths s^{-1} was possibly due to the small sample size (Fig. 2B). These changes in cardiac function were not attributed to a sensory response to the change in water velocity in the swim flume, as verified by measurements in two animals that did not swim when water speed was increased (Fig. 1B). $\dot{Q}_{sys}$ increased between 15% and 19% during swimming, while $\dot{Q}_{pul}$ increased approximately 60% (Fig. 2A,B). Additionally, $V_{S,pul}$ was constant at all swim speeds, unlike $V_{S,sys}$ (Fig. 2C,D). Interestingly swimming turtles remained submerged during swimming bouts, which seemed initially counter-intuitive given this could compromise oxygen transport to active tissues. However, two prior studies of submerged swimming in turtles have reported increased pulmonary perfusion relative to systemic perfusion, suggesting the resulting increase in $\dot{Q}_{pul}/\dot{Q}_{sys}$ associated with swimming may be a consequence of the ventricle anatomy as well as differential vascular regulation (West et al., 1992; Krosniunas and Hicks, 2003). It should be noted that without measures of oxygen content, as Wang and Hicks (1996a,b) discussed, extrapolations of the impact of $\dot{Q}_{pul}/\dot{Q}_{sys}$ changes on systemic oxygen delivery during swimming should be made with caution. They pointed out that the capacity for simultaneous R–L and L–R shunts of the chelonian heart could result in a net increase in oxygen content in both systemic and pulmonary arterial blood (Wang and Hicks, 1996a). This possibility was verified in anesthetized turtles (Ishimatsu et al., 1996). Thus, the net increase in cardiac output distributed to the pulmonary circulation in swimming snapping turtles may result in an overall increase in oxygen delivery to active muscle. However, a recent study of free-swimming untethered turtles reported a large fluctuations in blood P_{O_2}, suggesting that blood P_{O_2} may not be tightly regulated in turtles in any active state (Williams and Hicks, 2016). Clearly, additional studies are needed to investigate the impact of L–R shunts and the increase in $\dot{Q}_{pul}/\dot{Q}_{sys}$ evident in this and other studies on oxygen transport to active tissue in swimming turtles.

Cardiovascular response to surfacing after blockade (study 2)

Prior to autonomic blockade, surfacing increased $\dot{Q}_{sys}$, $\dot{Q}_{pul}$, $V_{S,pul}$ and f_H (Fig. 3A,B,D,E); however, $\dot{Q}_{pul}/\dot{Q}_{sys}$ was only slightly elevated as a result of the small elevation in $V_{S,sys}$ (Fig. 3C,F). While the basis for this difference is unclear, the reduced sample size in the drug treatment protocol may have contributed to the difference. After beta-adrenergic blockade with propranolol, only baseline $V_{S,sys}$ and f_H were affected (Table 2). During the subsequent surfacing events, snapping turtles were no longer able to increase $\dot{Q}_{sys}$ but $\dot{Q}_{pul}$ still doubled and the tachycardia associated with surfacing was significantly dampened (Fig. 3A,B). The sustained increase in $\dot{Q}_{pul}$ with a blunted tachycardia in surfacing turtles was due to an increased $V_{S,pul}$ response after treatment with propranolol (Fig. 3D). Previous studies on red-eared slider turtles have reported a pronounced cholinergic tone on f_H and have suggested that L–R shunts are the result of adrenergic stimulation (Comeau and Hicks, 1994; Hicks and Comeau, 1994; Hicks, 1994). The pronounced increase in $\dot{Q}_{pul}/\dot{Q}_{sys}$ in snapping turtles that surface after beta-adrenergic blockade suggests in this species that the L–R shunt during ventilation may be under cholinergic control (Fig. 3F).

Injection of atropine revealed a post-beta-adrenergic receptor blockade cholinergic tone on f_H of similar strength to the adrenergic tone (Table 2). A clear cholinergic tone on the pulmonary vasculature was also present, limiting $\dot{Q}_{pul}$, which was eliminated following atropine injection (Table 2). It should be noted that differences in systemic and pulmonary vascular distensibility may have influenced the magnitude of the response to atropine, as has been suggested in amphibians (Hillman et al., 2014). However, a prior study that involved a meta-analysis of several species questioned the importance of inherent vascular properties on blood flow patterns in recovered reptiles (Filogonio et al., 2017). Interestingly, while there was cholinergic tone on both f_H and $\dot{Q}_{pul}$, it differed in strength as evident in the 15% versus 90% increase in each parameter, respectively (Table 2). A prior study of anesthetized tortoises found a clear cholinergic tone inhibiting pulmonary blood flow while a tone on f_H was absent (Greunz et al., 2018). These results suggest there may be differential vagal regulation of f_H and pulmonary artery (Table 2). While this differential capacity would be experimentally challenging to test, we did find a single turtle where atropine injection had no effect on f_H but resulted in a marked increase in $\dot{Q}_{pul}$, supporting this possibility (data not shown). Atropine injection abolished the effect of surfacing on all cardiac parameters measured (Fig. 3A–F). Therefore, the increase in $\dot{Q}_{pul}$, $V_{S,pul}$ and $\dot{Q}_{pul}/\dot{Q}_{sys}$ is mediated by vagal withdraw of cholinergic tone on the pulmonary vasculature.

Cardiovascular function during swimming after pharmacological blockade (study 2)

Prior to pharmacological blockade, f_H and blood flow patterns were similar to those of turtles used for the swim study (Fig. 4A–F). Following beta-adrenergic blockade, $\dot{Q}_{sys}$ no longer changed during swimming at 0.5 carapace lengths s^{-1}, while $V_{S,sys}$ was similar to the pre-propranolol values (Fig. 4A,C). In a prior study of American alligators, it was determined that f_H secondarily contributed to increases in $\dot{Q}_{sys}$ in swimming animals (Joyce et al., 2018). Thus while, the increased $\dot{Q}_{sys}$ in swimming animals was likely attributed to f_H (Fig. 4E), beta-adrenergic receptor-mediated systemic dilation as well as cardiac contractility likely contributed to the function. $\dot{Q}_{pul}$ still increased but was dampened in swimming turtles after propranolol injection, while $V_{S,pul}$ and $\dot{Q}_{pul}/\dot{Q}_{sys}$ were similar to pre-propranolol levels (Fig. 4B,D,F). These findings suggest that during submerged swimming, beta-adrenergic stimulation primarily increases f_H and possibly increases the conductance of the pulmonary vasculature. Hicks and Wang (1996) reported that adrenaline injections in red-eared slider turtles caused a tachycardia and doubled $\dot{Q}_{pul}$ without changing $\dot{Q}_{sys}$. This supports our interpretation of the role of adrenergic stimulation on blood flow in swimming turtles. As determined for surfacing turtles, cholinergic blockade eliminated the changes associated with swimming in $\dot{Q}_{pul}$, $V_{S,pul}$ and $\dot{Q}_{pul}/\dot{Q}_{sys}$ (Fig. 4B,D,F), which demonstrates that the increase in these parameters is mediated by a change in cholinergic pulmonary vascular tone. It is important to note that while our findings of the blood flow and f_H response to injection of propranolol followed by atropine are clear, reversal of the drug order could have revealed different regulatory patterns.

Conclusion

This is the first study to examine total blood flow patterns during graded swimming and their regulation during swimming in any turtle species. Based on our findings, common snapping turtles increase f_H via a pronounced beta-adrenergic stimulation when surfacing, with vagal withdrawal having a small effect on f_H. This effect on f_H was also present in swimming animals, while vagal withdrawal contributed primarily to the increase in $\dot{Q}_{pul}$. These results differ from our prediction based on the literature.

This suggests there may be phylogenetic, natural history and anatomical factors that dictate the regulatory mechanisms in cardiac function in turtle species during ventilation and while swimming.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.R.K.; Methodology: A.R.K., B.S.; Formal analysis: B.S.; Investigation: A.R.K., D.A.C.; Resources: D.A.C.; Data curation: A.R.K., B.S., D.A.C.; Writing - original draft: A.R.K., D.A.C.; Writing - review & editing: A.R.K., B.S., D.A.C.; Supervision: D.A.C.; Project administration: D.A.C.

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