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THE EFFECTS OF ARTIFICIAL LUNG INFLATION ON PULMONARY BLOOD FLOW AND HEART RATE IN THE TURTLE *TRACHEMYS SCRIPTA*

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Summary

As for most ectothermic vertebrates, the breathing pattern of turtles is episodic, and pulmonary blood flow ($\dot{Q}_{pul}$) and heart rate (f_H) normally increase several-fold during spontaneous ventilation. While some previous studies suggest that these cardiovascular changes are caused by stimulation of pulmonary stretch receptors (PSRs) during ventilation, it has been noted in other studies that blood flows often change prior to the initiation of breathing. Given the uncertainty regarding the role of PSRs in the regulation of central vascular blood flows, we examined the effect of manipulating lung volume (and therefore PSR stimulation) on blood flows and heart rate in the freshwater turtle *Trachemys scripta*.

Turtles were instrumented with blood flow probes on the left aortic arch and the left pulmonary artery for measurements of blood flow, and catheters were inserted into both lungs for manipulation of lung volume. In both anaesthetized and fully recovered animals, reductions or increases in lung volume by withdrawal of lung gas or injection of air, N_2, O_2 or 10 % CO_2 (in room air) had no effect on blood flows. Furthermore, simulations of normal breathing bouts by withdrawal and injection of lung gas did not alter $\dot{Q}_{pul}$ or f_H. We conclude that stimulation of PSRs is not sufficient to elicit cardiovascular changes and that the large increase in $\dot{Q}_{pul}$ and f_H normally observed during spontaneous ventilation are probably caused by a simultaneous feedforward control of central origin.

Key words: reptile, turtle, cardio-respiratory interaction, ventilation, cardiovascular, blood flows, heart rate, pulmonary stretch receptors, lung inflation, *Trachemys scripta*.

Introduction

In most ectothermic vertebrates that exhibit an episodic breathing pattern, the ventilatory periods are associated with a large increase in pulmonary blood flow ($\dot{Q}_{pul}$) and heart rate (f_H) (e.g. White and Ross, 1966; Johansen *et al.* 1970; Shelton, 1970; White, 1970; Shelton and Burggren, 1976; Burggren and Shelton, 1979; White *et al.* 1989; Lillywhite and Donald, 1989; West *et al.* 1992; Wang and Hicks, 1996). In turtles, the increase in f_H and $\dot{Q}_{pul}$ during ventilation arises primarily from a reduction of vagal tone on the heart and a vagally controlled dilation of smooth muscle surrounding the extrinsic pulmonary artery (Burggren, 1977; Milsom *et al.* 1977; Hicks, 1994). Although the efferent vagal control of these changes is relatively well described, much less is known about the afferent inputs to the central nervous system that elicit changes in the vagal tone of the cardiovascular system (for a review on turtles, see Wang *et al.* 1997).

Like all other air-breathing vertebrates, turtles possess pulmonary stretch receptors (PSRs) that are innervated by the vagus nerve and convey information regarding lung volume to the central nervous system (Milsom and Jones, 1976; for a review, see Jones and Milsom, 1979). Denervation of these receptors by vagotomy consistently results in large increases in tidal volume and changes in breathing pattern (Milsom and Jones, 1980; Milsom and Chan, 1986; Milsom, 1990). The PSRs are also believed to be involved in the control of the cardiovascular system, and it seems plausible that PSR stimulation during breathing provides a primary stimulus for the characteristic increases in $\dot{Q}_{pul}$ and f_H during ventilation. However, existing studies on the role of PSRs in cardiovascular regulation in turtles have reached discordant conclusions. In recovered turtles, Burggren (1975) found that changing the lung volume by injection or withdrawal of air did not affect f_H, and he therefore concluded that PSRs are of no importance for the development of tachycardia during ventilation. In line with this observation, numerous studies have noted that the tachycardia and the increase in $\dot{Q}_{pul}$ often precede the initiation of lung ventilation and, presumably, the stimulation of PSRs (e.g. White and Ross, 1966; Burggren,

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1975; Shelton and Burggren, 1976; West *et al*. 1992; Wang and Hicks, 1996). Nevertheless, Johansen *et al.* (1977) showed that tidal inflations and deflations of the lungs elicited large increases in $\dot{Q}_{pul}$ and f_H in turtles and that the magnitude of these changes resembled those occurring during normal voluntary ventilation. As both $\dot{Q}_{pul}$ and f_H were closely correlated with lung pressure and the associated lung volume, it was hypothesized that, as turtles ascend in the water column, expansion of the lungs explains the anticipatory cardiovascular changes observed in other studies (e.g. West *et al*. 1992; Wang and Hicks, 1996).

The specific aim of the present study was to evaluate the role of afferent feedback from pulmonary stretch receptors (PSRs) in the regulation of blood flows and f_H in turtles. We report on the effect of both static and dynamic stimulation of PSRs on $\dot{Q}_{pul}$, systemic blood flow ($\dot{Q}_{sys}$) and f_H in both anaesthetized and conscious turtles.

Materials and methods

In this study, we report two separate series of experiments. The experiments on anaesthetized turtles were conducted at the University of Texas (Arlington), whereas the experiments on recovered and free-moving turtles were conducted at the University of California (Irvine). Because these two series of experiments differ in both protocol and experimentation, we have described each series separately below.

Animals

For both studies, turtles *Trachemys scripta* (Gray), mass 1.2–2.0 kg, were purchased from commercial suppliers and housed at 20 °C in large aquaria with free access to water and terrestrial environments, and were kept on a 12 h:12 h L:D photoperiod. Food was given twice weekly (Purina trout chow or goldfish), but was withheld for 5–7 days prior to experiments.

Instrumentation for experiments on anaesthetized turtles

The turtles were anaesthetized by an intramuscular injection of Ketamine HCl (140 mg kg^{-1}). Ketamine was chosen because it inhibits cortical function but leaves hypoxic and hypercapnic reflexes intact (Marshall and Wollman, 1980). This level of Ketamine provided sufficiently deep anaesthesia for the surgical procedures without eliminating spontanous ventilation.

Following induction of anaesthesia, a rectangular (3 cm×5 cm) hole was cut into the plastron above the heart, using a dremel tool, for placement of Doppler flow transducers (Triton Technology, Inc. San Diego, CA, USA). A transducer (2.0–2.8 mm i.d.) was placed around the left pulmonary artery close to the bifurcation of the common pulmonary artery to ensure that the pulmonary branch of the vagus was not disrupted. In addition, a transducer (2.4–2.8 mm i.d.) was placed around the left aortic arch. The leads from the flow transducers were passed through an incision cut into the skin at the left dorso-lateral margin of the neck and then through a hole drilled into the cranial border of the carapace. The square piece of the plastron was then secured back into place with rapid-setting epoxy resin, and cyanoacrylate adhesive was used to create a water-tight seal on all incisions in the skin. To cannulate the lungs, the turtle was returned to a prone position and two holes were drilled on each midpoint of the lateral border of the third dorsal scute. The lung tissue on each side was exposed, and a heat-flared PE 205 cannula with side holes was inserted into each lung air channel and tied into place using a purse-string suture (2-0 gauge surgical silk). Prior to placement, the cannulae were fitted through a hole in a rubber stopper that was then seated into the hole in the carapace and sealed with rapid-setting epoxy resin to ensure an air-tight seal. The two cannulae were connected by a T-piece and the free end was attached to another T-piece; one arm was connected to a pressure transducer (TDN-R disposable transducer, Spectramed, Oxnard, CA, USA) and the other arm was used for injections and withdrawal of air.

Blood flow transducers were connected to a three-channel directional pulsed Doppler flow meter (Bioengineering, University of Iowa, USA; model 545C-4), and the signals were converted to a digital signal using a Coulbourn Instruments conversion board and recorded at 25 Hz using CODAS data acquisition software (DATAQ Instruments, Akron, OH, USA). f_H was determined from the peak-to-peak interval of the left aortic blood flow. Following the experiments, the flow probes were calibrated at five different flow rates with the animal's own blood by cannulating the appropriate vessel. The pressure transducer was calibrated using a static water column prior to each experiment.

Experimental protocol for experiments on anaesthetized turtles

All experiments were conducted within 24 h following surgery with the turtle still under light anaesthesia (head resting on the substratum, but intact pinch and ciliary reflexes). We performed three different manipulations of lung volume. First, step-wise changes in lung volume were imposed by injecting or removing 10 ml of air in a step-wise fashion to give a total change in lung volume of up to 60 ml. These injections were performed at less than 6.0 ml s^{-1} as faster injection rates often induced the turtle to open its glottis and release the injected air. Second, 60 ml of air was injected at different rates (5–20 s per injection) to create 'ramp' changes in lung volume. These inflations were repeated using a hypercapnic gas mixture (10 % CO_2, balance air), 100 % N_2 and 100 % O_2 in random order. The turtles were allowed to recover for 20 min between gas treatments. Third, an artificial breathing bout was simulated by performing five tidal ventilations with a volume of 10 or 20 ml $breath^{-1}$ at frequencies of 15 and 30 breaths min^{-1}. These volumes and frequencies are within the range reported for spontaneously breathing turtles (e.g. Milsom and Chan, 1986; Funk and Milsom, 1987). To ensure a uniform degree of lung inflation, intrapulmonary pressure was adjusted at 0 cmH_2O (1 cmH_2O=98.1 Pa) by opening the cannula to the atmosphere before the tidal ventilations commenced. Breathing bouts were

always initiated by withdrawing lung gas to simulate an exhalation.

Instrumentation for experiments on fully recovered turtles

Turtles were prepared for instrumentation as described previously (Wang and Hicks, 1996). Briefly, a piece of soft rubber tubing was inserted through the glottis for artificial ventilation (8–15 breaths min^{-1} at 10–20 ml kg^{-1}) with a gas mixture consisting of 30 % O_2, 3 % CO_2 (balance N_2). This gas mixture was passed through a halothane vaporizer (Dräger, Lubeck, Germany) initially set at 3–4 % to induce anaesthesia; thereafter, the halothane level was reduced to 0.5–1 % for the remaining surgery. The heart and central blood vessels were exposed by removing a 4 cm×5 cm portion of the plastron with an electric bone saw, and 2R transit-time ultrasonic blood flow probes (Transonic System, Inc., Ithaca, NY, USA) were placed around the left pulmonary artery and the left aortic arch. Both lungs were cannulated as described above. Finally, the excised pieces of plastron were glued in place with silicone and two-component epoxy glue, and the leads from the probes were externalized ventrally and strapped to the carapace. Following surgery, artificial ventilation was continued until the turtle started breathing on its own; it was then placed in a small holding tank. Experiments were conducted following at least 72 h of recovery from surgery.

The flow probes were connected to a Transonic T201 dual-channel blood flow meter from which the signals were continuously recorded at 15 Hz (Acknowledge data-acquisition systems, Biopac Systems). The Transonic blood flow probes were calibrated at the factory at 25 °C, and we have periodically verified the calibration by generating known flows through either excised vessels or polyurethane tubing.

During experiments, turtles were permitted to move freely within an experimental chamber (30 cm×30 cm×60 cm) which was covered in dark plastic to minimize visual disturbance. Ventilation was measured as described by Glass *et al.* (1983). Briefly, the water surface was covered by a grid, except at a funnel-shaped breathing hole (7 cm in diameter), and a pneumotachograph (0-5 LPM, Hans Rudolph), connected to a Validyne differential pressure transducer (DP45-14), was placed at the gas inlet to the breathing funnel. The air flow leaving the funnel was maintained at 500 ml min^{-1}, and the air flow through the pneumotachograph therefore increased during inhalation and decreased during exhalation. The signal from the differential pressure transducer was recorded at 15 Hz (Acknowledge data-acquisition system).

Experimental protocol for experiments on fully recovered turtles

Turtles were placed in the experimental chamber and left undisturbed for 3–4 h. Resting blood flows and ventilation were then measured for a minimum of 1 h, after which artificial manipulations of lung volume were performed. These manipulations consisted of (1) injection of 60 ml of air, (2) withdrawal of 60 ml of air and (3) rapid withdrawal and injection of 20–30 ml of air to simulate a breathing bout. All experiments were conducted at 25 °C.

Data analysis and statistical analysis

Data on anaesthetized turtles were analyzed using WINDAQ (DATAQ Instruments, Inc. version 1.13), whereas data obtained on recovered turtles were analyzed using Acknowledge data-analysis software (version 3.0). A repeated-measures analysis of variance (ANOVA) followed by a Scheffé test was used to analyze the effects of breathing bouts, lung volume/gas changes and incremental changes in lung volume on blood flows and f_H immediately prior to and following manipulation. All statistical tests were performed using Statistica (StatSoft, Inc., version 4.5). The level of significance was set at $P<0.05$, and the data are presented as means ±1 S.E.M.

Results

Effect of manipulating lung volume in anaesthetized turtles

In anaesthetized turtles (N=7), both f_H and $\dot{Q}_{LPA}$ increased during spontaneous ventilation (from 18.9±3.5 to 22.1±3.3 beats min^{-1}, P=0.035, and from 6.4±2.1 to 8.7±2.8 ml min^{-1} kg^{-1}, P=0.014, respectively). Step changes in lung volume did not elicit significant changes in $\dot{Q}_{LPA}$, $\dot{Q}_{LAo}$ or f_H, and the effects of altering lung volume by bolus injections of 60 ml of different gas compositions are presented

Table 1. *The effect of increasing lung volume with a bolus injection of 60 ml of different gases on left pulmonary blood flow and heart rate in anaesthetized turtles* Trachemys scripta

	$\dot{Q}_{LPA}$			f_H		
Gas Mixture	Initial (ml min^{-1} kg^{-1})	Final (ml min^{-1} kg^{-1})	Relative change	Initial (beats min^{-1})	Final (beats min^{-1})	Relative change
Air (N=7; n=27)	4.1±0.8	4.0±0.6	4±8	17.9±2.2	19.2±2.2	9±4
N_2 (N=7; n=10)	3.6±0.8	3.8±0.7	14±10	18.5±3.4	19.8±3.3	9±5
O_2 (N=5; n=7)	5.3±1.6	4.4±0.8	–11±9	21.3±4.1	22.6±3.2	14±12
CO_2 (N=7; n=10)	4.1±0.8	3.7±0.5	–2±8	18.1±2.5	17.8±2.8	–2±6

$\dot{Q}_{LPA}$, left pulmonary blood flow; f_H, heart rate.
N, number of animals; n, number of observations.
Values are means ± S.E.M.

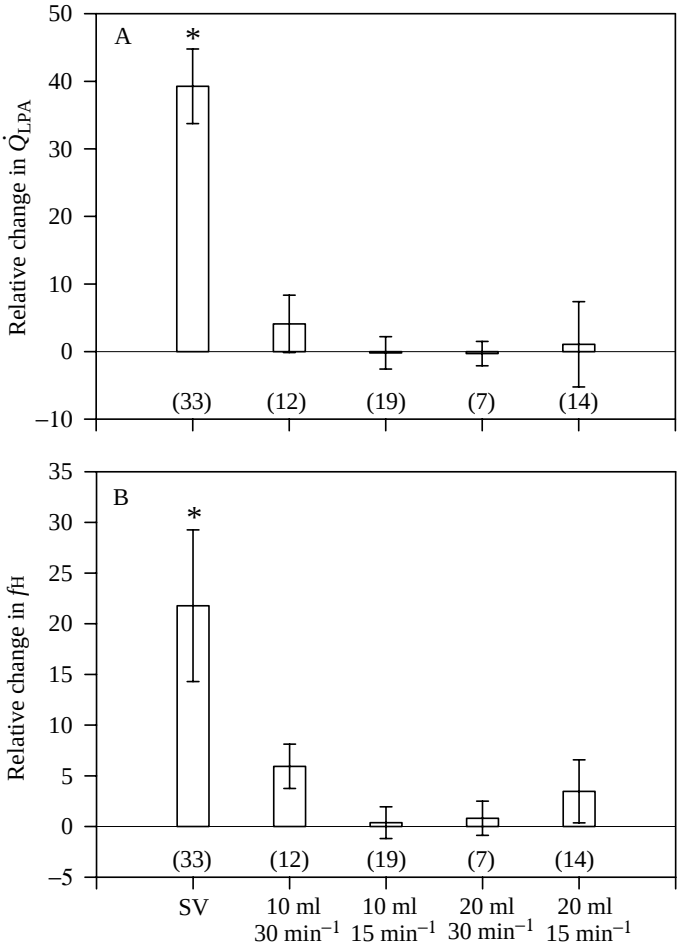

Fig. 1. Relative changes in left pulmonary artery blood flow ($\dot{Q}_{LPA}$) and heart rate (f_H) during spontaneous (SV) and artificial ventilation in anaesthetized turtles. Only spontanous ventilation elicited a statistically significant effect ($P \leqslant 0.05$) on these parameters (marked with an asterisk). The number of observations (N) are indicated in parentheses. Values are means ± 1 S.E.M.

in Table 1. The effects of simulating a breathing bout by consecutive injections and withdrawals of lung gas at several combinations of both frequencies and tidal volumes are presented in Fig. 1. In no case did this manipulation elicit a significant change in $\dot{Q}_{LPA}$ or f_H, whereas spontanous ventilation was associated with a significant increase in both $\dot{Q}_{LPA}$ and f_H (Fig. 1).

Effect of manipulating lung volume in recovered turtles

All four turtles examined in this experimental protocol exhibited large increases in f_H and $\dot{Q}_{LPA}$ during ventilation. As in the experiments on anaesthetized turtles, artificial manipulation of lung volume did not elicit significant changes in $\dot{Q}_{LAo}$, $\dot{Q}_{LPA}$ or f_H. Injection into the lungs or withdrawal from the lungs of 60 ml of air caused a mean increase in $\dot{Q}_{LPA}$ of 7±11 %, a mean decrease in $\dot{Q}_{LAo}$ of 4±11 % and a mean increase in f_H of 4±8 % (16 observations on four turtles, respectively).

Examples of the effects of manipulating lung volume in a 1.2 kg turtle are presented in Fig. 2. This turtle exhibited a pronounced cardio-respiratory interaction and, in these particular records, $\dot{Q}_{LPA}$ increased after the initiation of ventilation. Fig. 2A shows ventilation, lung pressure, $\dot{Q}_{LPA}$ and $\dot{Q}_{LAo}$ before and after two injections of 60 ml of air into the lungs. Shortly after the first injection, the turtle performed a series of exhalations, which were associated with an increase in $\dot{Q}_{LPA}$ and $\dot{Q}_{LAo}$. However, the second injection was not followed by a ventilatory period and, in this case, the artificial increase in lung volume was not associated with any changes in blood flows. Similar traces are shown in Fig. 2B,D; in these experiments, injections and withdrawals of 60 ml of air did not influence blood flows. However, the second injection in Fig. 2D elicited two exhalations, which were associated with a large increase in $\dot{Q}_{LPA}$.

In several cases, $\dot{Q}_{LPA}$ and f_H decreased when the turtle submerged its head, as shown for $\dot{Q}_{LPA}$ in Fig. 2C. In this example, $\dot{Q}_{LPA}$ increased immediately when the turtle extended its head out of the water into the breathing funnel.

Discussion

This study was designed to investigate whether stimulation of PSRs is responsible for the increase in heart rate and pulmonary blood flow that has been observed during ventilation in turtles and numerous other species of ectothermic vertebrates (e.g. White and Ross, 1966; Johansen *et al.* 1970; Shelton, 1970; White, 1970; Shelton and Burggren, 1976; Burggren and Shelton, 1979; White *et al.* 1989; Lillywhite and Donald, 1989; West *et al.* 1992; Wang and Hicks, 1996). Specifically, we assessed the effects of artificial lung inflation on f_H and blood flows in both conscious and anaesthetized turtles in an attempt to overcome the limitations of each experimental design. While studies on conscious and free-moving animals are not encumbered with the problems associated with anaesthesia, it is often difficult to control parameters such as lung volume and pressure because the animals are free to change their diving depth and may start to ventilate in response to changes in lung volume. In contrast, although lung volume and pressure can be controlled with great precision in anaesthetized animals, it is difficult to ascertain whether the anaesthetic depresses synaptic function in the central nervous system or receptor function.

In our experiments, lung inflation had no or little direct effect on f_H or central vascular blood flows, which is consistent with Burggren's (1975) original observation that artificial changes in lung volume do not affect f_H in non-anaesthetized turtles. However, our results are in contrast with the study of Johansen *et al.* (1977), in which tidal inflations and deflations of the lungs in restrained non-anaesthetized turtles elicited large increases in $\dot{Q}_{pul}$ and f_H. A cardiovascular effect of PSR stimulation in turtles is, furthermore, supported by the finding that electrical stimulation of the afferent cervical vagus elicited a tachycardia and increased $\dot{Q}_{pul}$ in anaesthetized turtles (Comeau and Hicks, 1994; Hicks and Comeau, 1994).

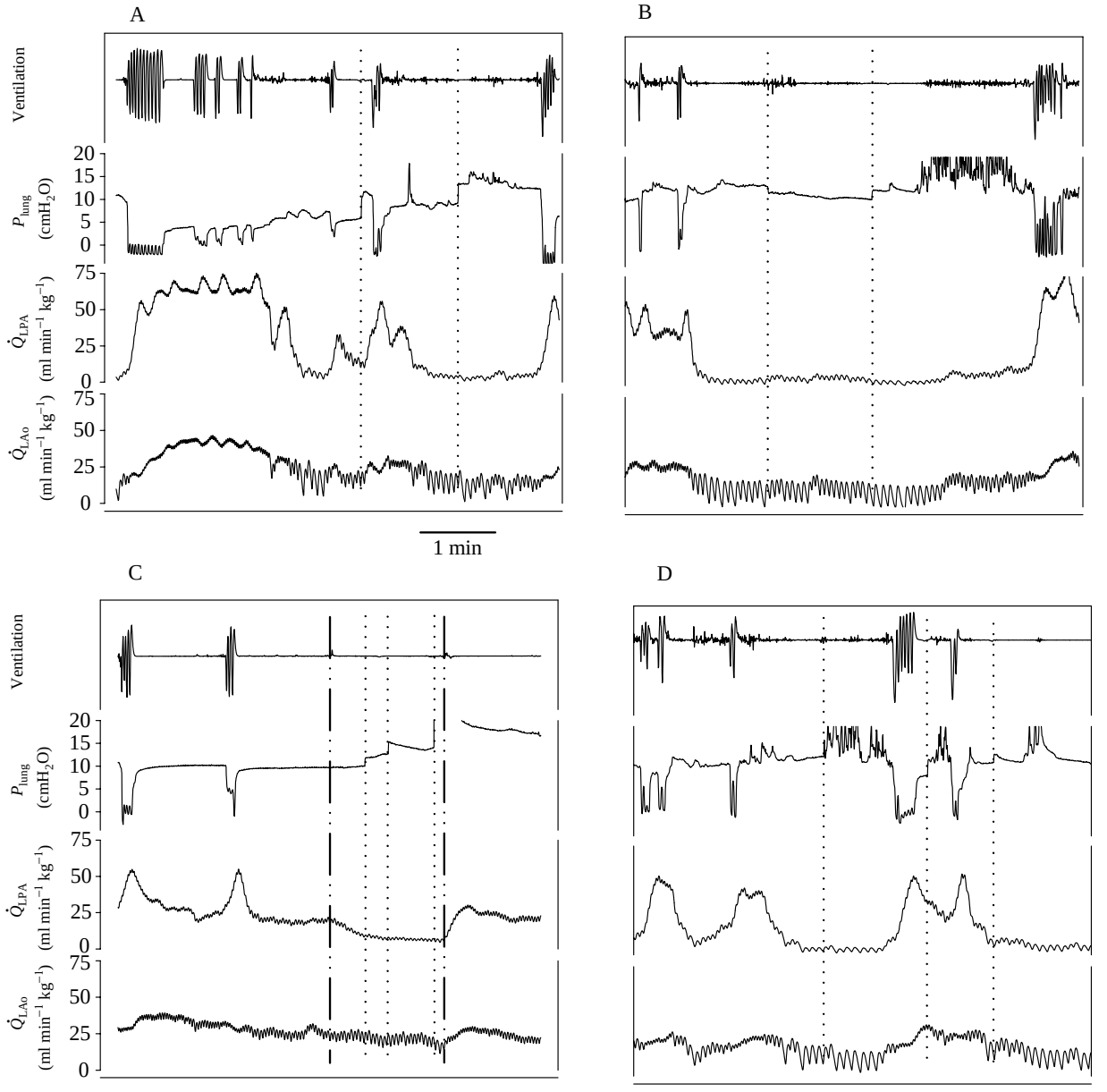

Fig. 2. Changes in blood flow in the left pulmonary artery ($\dot{Q}_{LPA}$) and in the left aortic arch ($\dot{Q}_{LAo}$) and lung pressure (P_{lung}) associated with intermittent ventilation in a fully recovered and freely moving turtle (1.2 kg). Injections of air or withdrawal of lung gases are indicated by the dotted lines. (A) Before and after two injections of 60 ml of air into the lungs (dotted lines). (B) Before and after withdrawal from the lungs and then addition of 60 ml of air to the lungs (dotted lines). (C) Before and after injections of 60 ml of air (dotted lines). The turtle submerged its head and approximately 1 min later extended its head out of the water (broken lines). (D) Before and after injections of 60 ml of air. See text for further explanation. 1 cmH_2O=98.1 Pa.

However, since the entire vagus was stimulated in these experiments, it is not possible to ascribe the observed cardiovascular effects to PSR stimulation.

Given the similarity in experimental designs, the disparity between our findings and those of Johansen *et al.* (1977) is not easily explained. First, although the animals in Johansen's study were restrained, the authors report that their observations were made on resting animals (as in the present study), and differences in the level of activity are, therefore, unlikely to explain the different findings (for information on the effects of exercise on blood flows in turtles, see Shelton and Burggren, 1976; West *et al.* 1992; Wang *et al.* 1997). Second, Johansen *et al.* (1977) observed that manipulation of lung volume elicited cardiovascular changes regardless of whether the turtle was fully submerged or was able to extent its head to the surface, whereas we could not elicit cardiovascular changes in submerged or non-submerged turtles. Thus, although stimulation of trigeminally innervated facial receptors can influence blood flows (Burggren, 1975; Fig. 2C), the degree of submergence is unlikely to explain the

opposing findings in the two studies. Third, although Johansen *et al.* (1977) did not measure lung ventilation directly, breathing movements could be assessed from the measurements of lung pressure. Thus, while it is conceivable that the artificial changes in lung volume could, at least under some circumstances, induce spontaneous ventilation and the associated increase in $\dot{Q}_{pul}$ and f_H, this factor cannot explain the discrepancy between the two studies. Nevertheless, it should be noted that, in our experiments, manipulation of lung volume often initiated breathing and that the concomitant changes in f_H and $\dot{Q}_{pul}$ resembled those observed during spontaneous breathing. In these instances, we ascribe the cardiovascular changes to the normal cardio-respiratory interaction rather than to a change in lung volume proper. Finally, although it is possible that the different results are caused by less-traditional factors, such as seasonal (or diurnal) changes or differences in the prandial state, we find it unlikely that the expression of a basic reflex arch could vary in such a fundamental fashion.

Although our data and those from Burggren (1975) agree that PSR stimulation alone is not sufficient to elicit cardiovascular changes, it cannot be concluded that PSRs are not involved in the regulation of blood flows and f_H. In mammals, the increase in f_H during inspiration is primarily due to an inhibition of the cardiac vagal motor neurones, but the respiratory sinus arrhythmia is also influenced by other factors, such as enhanced venous return from the 'respiratory pump' (for reviews, see Daly, 1985; Richter and Spyer, 1990). Because mechanical ventilation or weak electrical afferent stimulation of the pulmonary vagal branch elicits tachycardia, the inhibition of the cardiac vagal motor neurones can, at least partly, be ascribed to PSR stimulation. However, the inhibition of cardiac vagal motor neurones is also a result of central irradiation (or overflow) from the respiratory centres, which is independent of afferent feedback manifested as a synchronous phasic activity of both the sympathetic and cardiac vagal impulses (Daly, 1985; Taylor, 1987; Richter and Spyer, 1990; Richter *et al.* 1991; Montano *et al.* 1996). Therefore, because artificial lung ventilation does not cause changes in f_H or $\dot{Q}_{pul}$ in turtles, it seems reasonable to suggest that the cardiovascular changes during ventilation in these animals are predominantly caused by central irradiation from respiratory centres. In this case, the cardio-respiratory interactions originate within the central nervous system and are not contingent on afferent input. If this is the case, specific denervation of the PSRs should diminish the $\dot{Q}_{pul}$ and f_H attained during ventilation. Such experiments are unfortunately technically difficult because the PSR afferents lie within the same nerve tract as the efferent nerves controlling pulmonary vascular impedance.

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