CARDIORESPIRATORY RESPONSES OF THE TOAD (*BUFO MARINUS*) TO HYPOXIA AT TWO DIFFERENT TEMPERATURES

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Summary

Central vascular blood flows and ventilation were measured in conscious toads (*Bufo marinus*) at 15 and 25 °C. The animals were exposed to hypoxia (F_{IO_2}=0.10 and 0.05, where F_{IO_2} is the fractional oxygen concentration of inspired air) at both temperatures. In addition, the cardiorespiratory responses to hypercapnia (F_{ICO_2}=0.05) and atropine injection (5 mg kg^{-1}; 7.4 μmol kg^{-1}) were studied at 25 °C. At 25 °C, systemic blood flow ($\dot{Q}_{sys}$) exceeded pulmocutaneous blood flow ($\dot{Q}_{pc}$), indicating a large net right-to-left shunt ($\dot{Q}_{pc}/\dot{Q}_{sys}$ was 0.39). $\dot{Q}_{pc}/\dot{Q}_{sys}$ was reduced significantly to 0.22 at 15 °C. At both temperatures, $\dot{Q}_{pc}$ increased significantly during hypoxia (from 26.2 to 50.8 ml min^{-1} kg^{-1} at 25 °C and from 11.2 to 18.9 ml min^{-1} kg^{-1} at 15 °C), whereas $\dot{Q}_{sys}$ changed little (from 77.2 to 66.2 ml min^{-1} kg^{-1} at 25 °C and from 54.3 to 50.1 ml min^{-1} kg^{-1} at 15 °C). As a result, the net right-to-left shunt was greatly reduced, while total cardiac output remained almost unaffected. The ventilatory response was more pronounced during hypercapnia but, since $\dot{Q}_{pc}$ and $\dot{Q}_{sys}$ were affected similarly, there was no change in the shunt pattern. In undisturbed toads at 25 °C, atropine injection increased $\dot{Q}_{pc}$ and eliminated the net right-to-left shunt. This is consistent with the known vagal innervation of the pulmonary artery.

The present study shows that the cardiac right-to-left shunt that prevails in undisturbed and resting toads is reduced with increased temperature and during hypoxia. These findings are consistent with the general view that the cardiac right-to-left shunt is regulated and reduced whenever oxygen delivery is compromised or metabolic rate is increased.

Introduction

In the heart of anuran amphibians, the undivided ventricle receives oxygen-poor systemic blood from the right atrium and oxygen-rich blood returning from the lungs through the left atrium. During systole, blood is ejected from the ventricle through the conus arteriosus and distributed to the carotid and aortic arteries, which supply the systemic circulation, and the pulmocutaneous artery supplying the lungs and, to a minor extent, the skin. Systemic and pulmonary blood can mix both within the ventricle and within the conus arteriosus, but dye injections and direct measurements of blood oxygen levels demonstrate that there normally is a high degree of separation of blood flows (e.g. de Graaf, 1957; Simons, 1959; Haberich, 1965; Johansen and Ditadi, 1966, Tazawa et al., 1979; cf. Foxon, 1955). Although the exact mechanism for this selective distribution of blood flows remains to be elucidated (Morris, 1974; Langille and Jones, 1977; Shelton, 1976, 1985), it is well

established that vagal innervation of the pulmonary artery actively regulates pulmonary vascular resistance and, thus, pulmonary blood flow (Emilio and Shelton, 1972; Langille and Jones, 1977; Smith, 1978; de Saint-Aubain and Wingstrand, 1979). Increased vagal tone on the pulmonary artery reduces pulmonary blood flow and increases systemic recirculation of oxygen-poor blood (right-to-left, R–L, cardiac shunt).

The ability to alter pulmocutaneous blood flow ($\dot{Q}_{pc}$) independently of systemic blood flow ($\dot{Q}_{sys}$) effectively enables anurans to control arterial blood gas composition by altering pulmonary ventilation and/or by changing the magnitude of the R–L cardiac shunt (Wang and Hicks, 1996). Whenever an efficient oxygen delivery is needed (e.g. when metabolic rate is elevated or during hypoxia), an increase in $\dot{Q}_{pc}$ and a reduction in the R–L cardiac shunt maximise systemic oxygen delivery ($\dot{Q}_{sys}$ × arterial O$_2$ content) (Wang

and Hicks, 1996, 1999). In turtles, pulmonary blood flow is indeed increased and the R–L cardiac shunt decreased during exercise, hypoxia and anaemia (for a review, see Wang et al., 1997). Similar data are not available for amphibians and, with the notable exception of the study by West and Smits (1994), virtually all measurements of central vascular blood flows in anurans have been performed on anaesthetised or pithed animals (Shelton, 1970; Tazawa et al., 1979; West and Burggren, 1984).

On this background, the main purposes of the present study were (1) to characterise the cardiovascular and ventilatory responses of chronically instrumented and conscious toads (*Bufo marinus*), and (2) to investigate the general hypothesis that the R–L cardiac shunt is reduced whenever the demands on oxygen transport are intensified. We therefore measured the effects of hypoxia on blood flows at 15 and 25 °C; 25 °C is the preferred body temperature of unfed and resting *B. marinus*, while 15 °C represents the behaviourally selected temperature during hypoxia (e.g. Wood and Malvin, 1991). In addition, the effects of cholinergic blockade (atropine injection) and hypercapnia on cardiorespiratory variables were examined to elucidate further the effects of ventilation and parasympathetic tone on central vascular blood flows.

Materials and methods
Animals

Cane toads (*Bufo marinus*; 420–680 g) were purchased from Charles D. Sullivan Co. (Nashville, TN, USA) and maintained at Simon Fraser University (Burnaby, British Columbia, Canada) in large polypropylene containers (69 cm×123 cm×65 cm) filled with sand to a depth of 10 cm. The toads had free access to water and were force-fed beef liver (sprinkled with Biotin Stress Pak, Solvay Animal Health, Inc.) once a week. The animals gained weight during captivity and appeared healthy. Temperature was maintained at 21 °C, and photoperiod was 12 h:12 h light:dark. Toads were kept for at least 4 months before being transported to the University of British Columbia, where they were kept in small tanks, supplied with running water, for approximately 1 week prior to experimentation.

Anaesthesia and surgery

Toads were anaesthetised in dechlorinated tap water containing $2\,g\,l^{-1}$ tricaine methane sulphonate (MS-222, Sigma Chemical Co., St Louis, MO, USA) buffered to neutral pH with $2\,g\,l^{-1}$ $NaHCO_3$. When the corneal reflex was no longer present, the animals were placed in a supine position on a surgical table, and anaesthesia was maintained by covering the toad in paper towels soaked in the MS-222 solution.

An incision (approximately 2–3 cm long) was made in the skin just posterior to the sternum, and the sternum was freed from the underlying musculature and connective tissue, and elevated by clamping it to an overhead holder. This exposed the ventral side of the heart and the major vessels through a 2.5 cm×3 cm oval opening. The pericardium was opened, and

the connective tissue around the left truncus arteriosus and pulmocutaneous artery was removed to allow for placement of Transonic blood flow probes (Transonic Systems Inc., Ithaca, NY, USA). The pulmocutaneous artery was isolated and cleared for 0.5–0.7 cm distal to the truncus arteriosus; in some cases, this required that the thymus be dissected free of the vessel surface. After placing flow probes on the truncus arteriosus (model 3R or 4SB) and the pulmocutaneous artery (model 2R or 2SB), the probe windows were filled with acoustic coupling gel, and the flow probe leads were brought out of the incision. After securing the sternum to the underlying musculature and connective tissue, and sprinkling antibiotic powder (Cicatrin, Welcome Inc., Quebec, Canada) within the surgical area, the skin was closed using intermittent sutures (3-0 gauge silk). The leads from the probes were tied to the skin at a location just posterior to the incision and at several positions on the toad's back. The signal strength of Transonic flow probes is reduced if small air bubbles are present between the probe and blood vessel. In the present experiments, a good signal from the implanted flow probes was normally attained within 3–4 days post-surgery. For sampling of arterial blood and measurements of systemic arterial blood pressure, the anal artery was occlusively cannulated with PE 50 tubing (Clay Adams; 80 cm in length) filled with heparinised saline $(100\,i.u.\,ml^{-1})$.

Following surgery, which lasted 1.5–2.5 h, the toads were placed under running water to recover and then transferred to covered plastic boxes (8.5 cm×20 cm×11 cm) containing 2–3 cm of dechlorinated tap water. To ensure that the animals were not hypoxic during surgery, a small piece of Masterflex tubing was inserted into the glottis, and the lungs were inflated by mouth every 2–3 min. Following surgery, toads were given intramuscular injections of the antibiotic enrofloxacin (Baytril; 2–$3\,mg\,kg^{-1}$) to prevent infection.

Experimental protocols

3–4 days after surgery the toads were equipped with a face-mask for ventilation measurements (see below) and placed in a water-jacketed experimental chamber (25 cm×15 cm×12 cm) at 25 °C for at least 18 h before experiments began. The experimental chamber contained 1 cm of dechlorinated water and was located behind a black opaque partition to reduce visual disturbance. The chamber lid was sealed with vacuum grease, and humidified air was continuously supplied to the chamber. All six animals were subjected to hypoxia (fraction of oxygen in the inspired air, F_{IO_2}, of 0.10 and 0.05) at 25 °C (day 1) and 15 °C (day 2) as described below. In addition, four of these toads were subjected to hypercapnia and cholinergic blockade (atropine injection) on the third day.

Effects of hypoxia at 25 and 15 °C

Blood flows, ventilation and blood pressure were recorded for 2–3 h on resting and undisturbed animals to obtain control values in normoxia (F_{IO_2}=0.21). Thereafter, F_{IO_2} was reduced to 0.10 for 2 h and then further reduced to 0.05 for 1 h. Ventilation, blood flows and systemic blood pressure were

measured continuously, and an arterial blood sample (0.8 ml) was withdrawn for analysis of oxygen levels and haematocrit during the last 10 min of each exposure. Following the hypoxic exposures, F_{IO_2} was returned to 0.21 and the temperature was reduced to 15 °C over a period of 2–4 h. An identical regime of hypoxic exposures was repeated on the following day, at 15 °C. One to two hours after the toad had been returned to air (F_{IO_2}=0.21), the temperature in the chamber was gradually increased to 25 °C.

Hypercapnia and atropine injection

On the third day (12–16 h after rehabituation to 25 °C), blood flows, ventilation and blood pressure were recorded for 2–3 h on resting and undisturbed animals to obtain control values. Thereafter, toads were exposed to hypercapnia (F_{ICO_2}=0.05, balance air) for 45 min, followed by a recovery period of 2 h in normocapnic air (F_{ICO_2}=0). Animals then received an intra-arterial injection of the cholinergic antagonist atropine sulphate (5 mg kg^{-1}; 7.4 μmol kg^{-1}; Sigma Chemical Co., St Louis, MO, USA) in saline (0.9 % NaCl), and physiological variables were measured continuously for 2 h following atropine injection. Injection of saline alone had no effect on ventilation or cardiovascular variables.

Blood gas analysis

Immediately after withdrawal, the 0.8 ml blood sample was analysed for P_{O_2}, oxygen content and haematocrit. Arterial P_{O_2} (Pa_{O_2}) was measured using a Radiometer O_2 electrode (model E5046-0) maintained at the same temperature as the experimental animal. The zero setting of the O_2 electrode was verified daily, and the electrode was calibrated with humidified air prior to each measurement. Arterial O_2 content ($[O_2]_a$) was measured on 30 μl samples according to the method described by Tucker (1967). Haematocrit was determined in duplicate following 2 min of centrifugation at 10 000 g. The remaining blood was reinfused into the animals following analysis to reduce blood loss.

Measurements of ventilation, blood pressure and blood flows

Pulmonary ventilation was measured directly using pneumotachography (Glass et al., 1978; Wang, 1994). For these measurements, a light-weight mask, constructed of a flexible polymer (Dreve Dentamid, Unna, Germany), was glued over the nostrils using a two-component adhesive glue. All respiratory air flows passed through a Fleisch tube incorporated in the mask, and the resulting pressure differences across the Fleisch tube were measured using a differential pressure transducer (Validyne DP45-14, Northridge, CA, USA). Assuming laminar flow, ventilatory flow rate is proportional to the pressure gradient across the Fleisch tube. The relationship between the integrated flow signal and tidal volume was determined by injecting appropriate gas volumes through a catheter implanted in a cast (made from plaster of Paris) of a toad head fitted with the mask. Pulmonary ventilation was distinguished from buccal movements by a biphasic flow profile during expiration (Jones, 1982).

Blood pressure measurements were made by connecting the

anal artery catheter to a Statham (model P23Dd) pressure transducer kept at the same level as the animal's heart. Pressure calibrations were performed daily against a static water column. The blood flow probes were connected to a dual-channel flowmeter (Transonic Systems Inc., Ithaca, NY, USA; model T201). All blood flow probes were calibrated at the factory at 25 °C and verified by generating known flows of degassed 0.9 % NaCl through polyurethane tubing.

Calculation of blood flows

The left and right sides of the truncus arteriosus and the pulmocutaneous arteries in *Bufo marinus* are of similar diameter, and blood flows are similar when probes are placed in ipsilateral or bilateral positions (West and Burggren, 1984). Total cardiac output ($\dot{Q}_{tot}$) was therefore calculated as twice left truncus arteriosus blood flow, while total pulmocutaneous blood flow ($\dot{Q}_{pc}$) was obtained by doubling measured values in the left pulmocutaneous artery. If the blood flows in the two trunci are different, our numerical estimation will be in error, but this is unlikely to affect the relative changes in blood flows or the directional changes in cardiac shunt patterns. Blood flow to the systemic circulation ($\dot{Q}_{sys}$) was calculated as $\dot{Q}_{sys}=\dot{Q}_{tot}-\dot{Q}_{pc}$. The net R–L shunt flow ($\dot{Q}_{shunt}$) was calculated as the difference between $\dot{Q}_{sys}$ and $\dot{Q}_{pc}$. Heart rate (f_H) was calculated on a beat-to-beat basis from the pulsatile blood flow profile in the truncus. Total stroke volume (V_s, pulmocutaneous + systemic) was calculated as total cardiac output divided by f_H ($\dot{Q}_{tot}/f_H$). All blood flows are expressed relative to body mass except when raw data are given (e.g. Figs 1, 2).

Gas mixtures and temperature control

Chamber temperature and the temperature of incoming gases were maintained at either 25±1 °C or 15±1 °C using a Lauda recirculating water bath. The chamber received a continuous flow of air that had passed through a water column placed in the water bath to ensure the appropriate temperature and high humidity. The hypoxic gases were prepared by mixing pure N_2 and air, whereas the hypercapnic gas mixture (F_{ICO_2}=0.05) was supplied from a premixed cylinder. The gas flow rates were controlled by means of precision needle valves, and the P_{O_2} of the mixed gases leaving the chamber was continuously monitored using a Radiometer O_2 electrode (model E5046-0; Radiometer, Copenhagen, Denmark) thermostatted to the same temperature as the gas mixtures.

Data acquisition, data analysis and statistics

Signals from the blood pressure transducer, the differential pressure transducer and the blood flow meter were recorded using an AcqKnowledge MP 100 (BioPac Systems, Inc., Santa Barbara, CA, USA; version 3.2.3) data-acquisition system at 20 Hz. In addition, several recordings of shorter duration were collected at 100 Hz. For each gas mixture/temperature combination, a continuous recording of 5–40 min (most often 10–15 min) was analysed for ventilation, mean blood flows, heart rate and mean blood pressure. Only periods of stable blood flows and ventilatory patterns within the last 40 min of
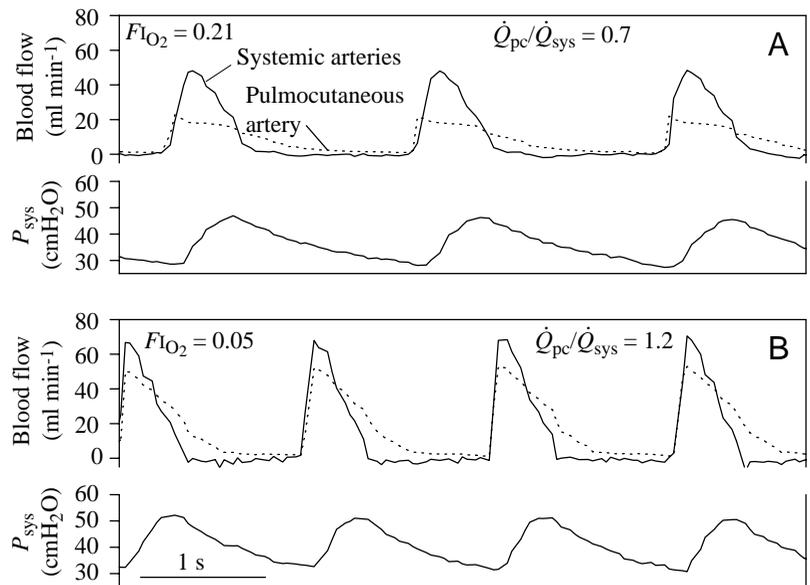
Fig. 1. Blood flows in the pulmocutaneous (broken line) and systemic (solid line) arteries, and systemic arterial blood pressure P_{sys}, in a conscious toad (*Bufo marinus*) during normoxia (A) ($F_{I_{O_2}}=0.21$) and hypoxia (B) ($F_{I_{O_2}}=0.05$) at 25 °C. Animals were equipped with blood flow probes on one pulmocutaneous artery and one truncus arteriosus, and systemic blood flow ($\dot{Q}_{sys}$) was calculated as $\dot{Q}_{tot}$ minus $\dot{Q}_{pc}$ (where $\dot{Q}_{tot}$ is total cardiac output and $\dot{Q}_{pc}$ is pulmocutaneous blood flow; see text for further explanation). 1 cmH$_2$O=98 Pa.

each gas mixture were chosen for analysis. For data on atropine injection, a continuous recording of 30 min, recorded 45–90 min following injection, was analysed. All recordings were analysed using AcqKnowledge data analysis software (version 3.2.3).

A two-way analysis of variance (ANOVA) for repeated measures was employed to identify significant effects of hypoxic gas mixtures and temperature on the reported variables. Differences among means were subsequently assessed using a Student–Newman–Keuls test. A paired *t*-test was used to determine whether atropine or hypercapnia significantly altered any variables. All statistical analyses were performed using SigmaStat statistical software (Jandel Scientific, version 1.0), and a fiducial limit for significance of $P<0.05$ was applied. All data in the figures and tables and throughout the text are presented as means ±1 S.E.M.

Results

Relationship between $\dot{Q}_{sys}$ and $\dot{Q}_{pc}$

Examples of recordings of the phasic blood flows in the pulmocutaneous and systemic arteries during normoxia and hypoxia at 25 °C are shown in Fig. 1. Blood flow in both vessels approached zero by the end of diastole, and there was an almost simultaneous ejection of blood during systole. However, once maximal blood flow had been attained, blood flow declined much more slowly in the pulmocutaneous artery. This pattern was maintained during hypoxia when $\dot{Q}_{pc}$ increased relative to $\dot{Q}_{sys}$ (see Fig. 1B).

Correlation between blood flows and ventilation

The breathing pattern during normoxia consisted of single or multiple breaths interspersed between short non-ventilatory periods, and there were no changes in vascular blood flows during ventilation. During hypoxia, the breathing pattern became clearly episodic, with well-defined ventilatory bouts composed of several breaths separated by non-ventilatory periods. Each ventilatory bout invariably commenced with several expirations and ended with several consecutive inspirations. This breathing pattern, normally termed lung inflation cycles (West and Jones, 1975), became more pronounced during hypoxia and, although all toads exhibited this response at both temperatures, it was most conspicuous at 25 °C. At the end of a lung inflation cycle and for 10–30 s into the non-ventilatory period, $\dot{Q}_{pc}$ increased while $\dot{Q}_{sys}$ decreased (Fig. 2). As a consequence, $\dot{Q}_{pc}/\dot{Q}_{sys}$ doubled following lung inflation (Fig. 2). These blood flow changes were associated with a small increase in systemic blood pressure following lung inflation.

Effects of hypoxia and temperature on haemodynamic variables and ventilation

Hypoxia evoked an increase in ventilation and a redistribution of blood flow between the systemic and pulmocutaneous arteries, and the results are presented in Fig. 3. At 25 °C, ventilation increased significantly from a normoxic value of 76.3 ± 15.1 ml min^{-1} kg^{-1} to 132.6 ± 22.7 ml min^{-1} kg^{-1} (Fig. 3A). This ventilatory response was accompanied by an approximate doubling of $\dot{Q}_{pc}$ from 26.2 ± 3.7 to 50.8 ± 6.8 ml min^{-1} kg^{-1} (Fig. 3C), while $\dot{Q}_{sys}$ tended to decrease, albeit not significantly, from 77.2 ± 11.6 to 66.2 ± 9.8 ml min^{-1} kg^{-1} (Fig. 3D). At 15 °C compared with 25 °C, ventilation was significantly reduced to 39.0 ± 16.3 ml min^{-1} kg^{-1} during normoxia (Fig. 3A), while $\dot{Q}_{pc}$ and $\dot{Q}_{sys}$ were reduced to 11.2 ± 2.5 and 54.3 ± 9.7 ml min^{-1} kg^{-1}, respectively (Fig. 3C,D). Ventilation did not increase significantly during hypoxia at 15 °C (Fig. 3A), but $\dot{Q}_{pc}$ increased to a maximum value of 18.9 ± 2.6 ml min^{-1} kg^{-1} (Fig. 3C).

The increased $\dot{Q}_{tot}$ with elevated temperature (Fig. 3B) was associated with reciprocal changes in f_H and V_s (Fig. 3D,F). During normoxia, f_H increased from 15.9 ± 1.6 min^{-1} at 15 °C to
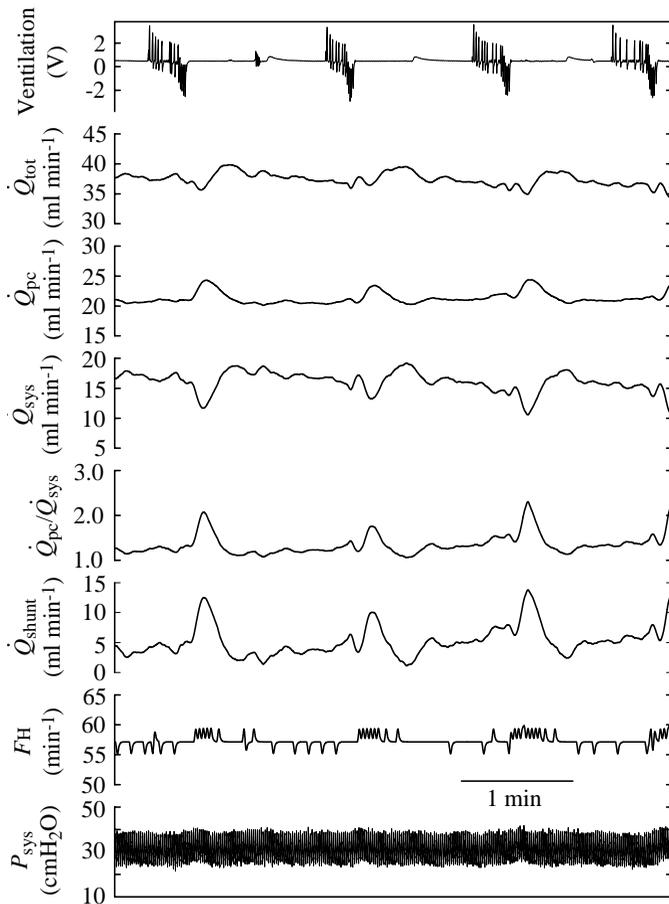
Fig. 2. Haemodynamic changes during four lung inflation cycles in *Bufo marinus* under hypoxic conditions (F_{IO_2}=0.5). Toads were equipped with blood flow probes on one pulmocutaneous artery and one truncus arteriosus, and systemic blood flow ($\dot{Q}_{sys}$) was calculated as $\dot{Q}_{tot}$ minus $\dot{Q}_{pc}$ (where $\dot{Q}_{tot}$ is total cardiac output and $\dot{Q}_{pc}$ is pulmocutaneous blood flow; see text for further explanation). Ventilation is expressed as the raw signal (in volts) from the differential pressure transducer. Positive values denote expiration. $\dot{Q}_{shunt}$, net shunt blood flow; f_H, heart rate; P_{sys}, systemic arterial blood pressure. 1 cmH$_2$O=98 Pa.

Table 1. *Arterial* P_{O_2}, *oxygen content ([O$_2$]$_a$) and haematocrit in* Bufo marinus *exposed to hypoxia at 15 and 25 °C*

Temperature (°C)	F_{IO_2}	Pa_{O_2} (mmHg)	[O$_2$]$_a$ (mmol l^{-1})	Haematocrit (%)
25	0.21	69.7±3.6	2.68±0.17	19.0±1.8
	0.10	41.6±2.4*	1.65±0.10*	19.4±2.3
	0.05	20.8±2.7*	0.83±0.06*	26.1±4.5*
15	0.21	33.0±1.9	1.78±0.25	16.6±1.6
	0.10	25.4±4.6*	1.64±0.17	16.7±1.3
	0.05	18.5±2.5*	1.01±0.22*	18.6±1.4

Values are means ± S.E.M.

Means that are significantly different from the normoxic condition (F_{IO_2}=0.21) are marked with an asterisk.

F_{IO_2}, fractional oxygen content of inspired air; Pa_{O_2}, arterial P_{O_2}. 1 mmHg=0.133 kPa.

presented in Table 1. Pa_{O_2} was higher at 25 °C than at 15 °C, but this difference was most prominent during normoxia. As Pa_{O_2} declined during hypoxia, the arterial oxygen content ([O$_2$]$_a$) was reduced. This reduction was much more pronounced at 25 °C, where [O$_2$]$_a$ was reduced to 30 % of the normoxic value, whereas [O$_2$]$_a$ remained at almost 60 % of the normoxic level at 15 °C. Haematocrit increased by 38 % during hypoxia at 25 °C, but there was no significant increase at 15 °C.

Effects of hypercapnia

On the third experimental day, ventilation and all haemodynamic variables during normoxia at 25 °C were similar to the values obtained under the same conditions on the first experimental day. Hypercapnia (F_{ICO_2}=0.05) at 25 °C elicited a fivefold increase in ventilation from 76.7±19.3 to 418.4±92.9 ml min^{-1} kg^{-1} (Fig. 4A). In addition, $\dot{Q}_{pc}$ almost doubled from 25.6±3.9 to 46.9±3.1 ml min^{-1} kg^{-1}, while $\dot{Q}_{sys}$ increased from 70.3±18.1 to 89.8±16.1 ml min^{-1} kg^{-1} (Fig. 4C,D), although this increase was not statistically significant. Although $\dot{Q}_{pc}/\dot{Q}_{sys}$ increased slightly from 0.51±0.14 to 0.62±0.14 during hypercapnia, this was not statistically significant and the net R–L cardiac shunt flow was not affected (Fig. 4E,F).

Effects of cholinergic blockade by atropine injection

Injection of the cholinergic antagonist atropine during normoxia at 25 °C caused sustained increases in $\dot{Q}_{pc}$ and f_H, while ventilation only increased transiently during the first 20 min. At 30–60 min following atropine injection, f_H had increased from 49.3±6.5 to 53.4±5.8 min^{-1} (Fig. 5D), while V_s did not change (2.67±0.54 *versus* 2.33±0.45 ml kg^{-1}, respectively). At the same time, $\dot{Q}_{pc}$ increased from 30.3±3.8 to 63.8±7.4 ml min^{-1} kg^{-1} (Fig. 5A), while $\dot{Q}_{sys}$ was unaltered (72.8±13.5 *versus* 66.5±17 ml min^{-1} kg^{-1}; Fig. 5B). As a result, the net cardiac R–L shunt was reduced from 42.6±14.5 to 2.8±20.4 ml min^{-1} kg^{-1}, and $\dot{Q}_{pc}/\dot{Q}_{sys}$ increased from 0.50±0.12 to 1.24±0.32 (Fig. 5C). In Fig. 5E, the individual values of $\dot{Q}_{pc}/\dot{Q}_{sys}$ obtained during hypoxia (F_{IO_2}=0.05; 25 °C) on day 1

44.5±5.3 min^{-1} at 25 °C (Q$_{10}$=2.65), whereas V_s decreased from 4.29±0.67 to 2.46±0.31 ml kg^{-1}(Q$_{10}$=1.9). Hypoxia elicited a small but significant increase in f_H to 51.2±3.5 min^{-1} at 25 °C, whereas there was no significant effect at 15 °C. Regardless of temperature, hypoxia did not significantly affect V_s.

Both hypoxia and temperature influenced the cardiac shunt pattern (Fig. 3G,H). At 25 °C, the net R–L cardiac shunt flow was reduced from a normoxic value of 51.0±12.1 to 15.5±12.9 ml min^{-1} kg^{-1} and $\dot{Q}_{pc}/\dot{Q}_{sys}$ increased from 0.39±0.08 to a maximum value of 0.89±0.18. $\dot{Q}_{pc}/\dot{Q}_{sys}$ was reduced to 0.22±0.04 at 15 °C, but the net R–L shunt flow was not affected by temperature. Although the effects of hypoxia on the cardiac shunt patterns were attenuated at low temperature, $\dot{Q}_{pc}/\dot{Q}_{sys}$ increased significantly to 0.45±0.09 at the most hypoxic condition.

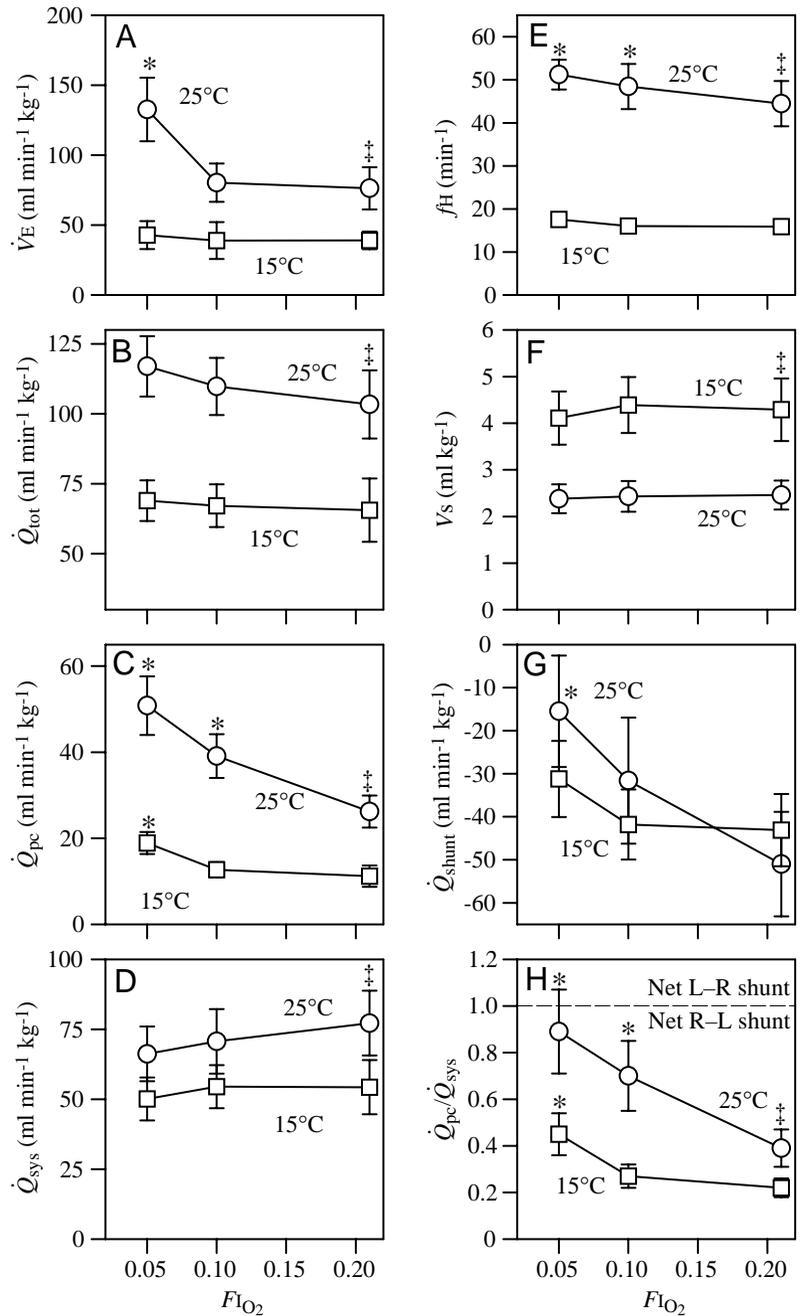Arterial P_{O_2}, blood oxygen content and haematocrit values are

Fig. 3. Effects of hypoxia on ventilation and haemodynamic variables in conscious toads (*Bufo marinus*) at 15 and 25 °C (squares and circles, respectively); $\dot{V}_E$, minute ventilation (A); $\dot{Q}_{tot}$, total cardiac output (B); $\dot{Q}_{pc}$, pulmocutaneous blood flow (C); $\dot{Q}_{sys}$, systemic blood flow (D); f_H, heart rate (E); V_s, total stroke volume (F); $\dot{Q}_{shunt}$, net R–L cardiac shunt (G) and $\dot{Q}_{pc}/\dot{Q}_{sys}$ (H). Values are presented as means ±1 S.E.M. (*N*=6). Mean values that are significantly different from the normoxic condition ($F_{I_{O_2}}$=0.21) are marked with an asterisk, and significant differences between the two temperatures during normoxia are marked with a double dagger.

are compared with those obtained on the same animals following cholinergic blockade. These values for $\dot{Q}_{pc}/\dot{Q}_{sys}$ under these two conditions appear to be linearly correlated, and a linear regression yields an r^2 of 0.84.

Discussion

This is the first description of the cardiorespiratory response to hypoxia and altered body temperature in a chronically instrumented and conscious amphibian. During hypoxia, *Bufo marinus* increased ventilation and pulmonary blood flow, which led to a reduction in the net R–L cardiac shunt. This cardiovascular response is consistent with the view that the

cardiac R–L shunt is reduced whenever the demands on oxygen transport are increased (Wang and Hicks, 1996). The present study also shows that the cardiorespiratory response to hypoxia was attenuated with decreasing temperature.

Critique of the present study

We measured blood flow in the pulmocutaneous artery ($\dot{Q}_{pc}$) before it branched into the small cutaneous artery and the larger pulmonary artery. Thus, our measurements cannot distinguish between the blood flows in these two branches ($\dot{Q}_{cut}$ and $\dot{Q}_{pul}$, respectively). However, because $\dot{Q}_{cut}$ is only 10–15 % of $\dot{Q}_{pc}$, and since the absolute changes in $\dot{Q}_{cut}$ are small compared with those in $\dot{Q}_{pul}$ (West and Burggren, 1984), our
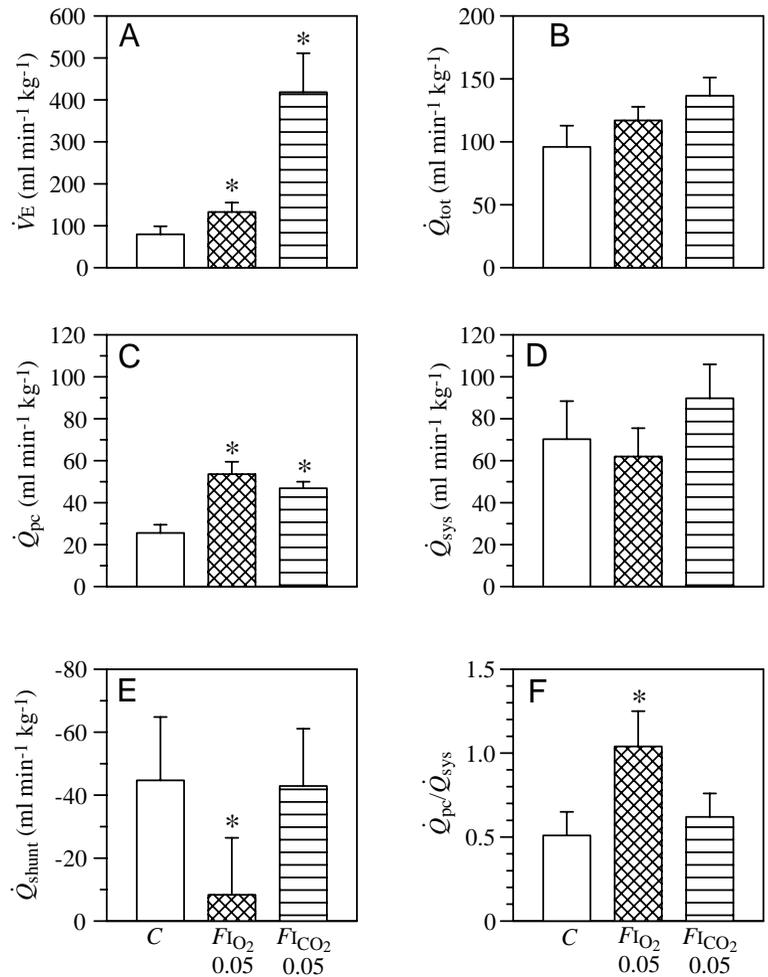
Fig. 4. Minute ventilation $\dot{V}_E$ (A), blood flows ($\dot{Q}_{pc}$, pulmocutaneous blood flow; $\dot{Q}_{shunt}$, net R–L cardiac shunt; $\dot{Q}_{tot}$, total cardiac output) (B–D) and cardiac shunt patterns (E,F) in undisturbed toads breathing room air (open columns), during severe hypoxia (cross-hatched columns) and during hypercapnia (striped columns) at 25 °C. Values are presented as means +1 S.E.M. (N=4), and means that are significantly different from the normoxic condition (C) are marked with an asterisk. $\dot{Q}_{sys}$, systemic blood flow.

measurements of $\dot{Q}_{pc}$ will largely reflect changes in $\dot{Q}_{pul}$. Thus, our calculations of net shunt flows should be qualitatively sound.

Because the toads were exposed to hypoxia on two consecutive days (first at 25 °C and then at 15 °C), it is possible that the attenuated hypoxic response measured at 15 °C partially reflects the previous hypoxic exposure. However, several observations suggest that this is not the case. First, the hypoxic ventilatory response at both temperatures in the present study was similar to that reported previously (Kruhøffer et al., 1987). Second, a significant recruitment of anaerobic metabolism, with the associated disturbances of acid–base status, is normally not present at an F_{IO_2} of 0.05 in *B. marinus* (Pörtner et al., 1991a,b; Wood and Malvin, 1991). Third, haematocrit returned to normal values on day 2. Finally, the cardiorespiratory variables measured during normoxia on day 3 at 25 °C (i.e. following two set hypoxic exposures) were virtually identical to those measured on day 1. Collectively, there did not appear to be any lasting effects of hypoxia and it is unlikely, therefore, that the hypoxic exposure on day 1 influenced cardiorespiratory responses on the following days.

Comparison with previous studies

Total stroke volume and cardiac output at 25 °C in the present study are almost identical to those reported by McKean et al. (1997) for *in situ* perfused hearts of *B. marinus*. However, our measurement of $\dot{Q}_{tot}$ is almost twice the value reported by West and Smits (1994) in conscious and chronically instrumented *B. marinus* at 22–23 °C (103 *versus* 57 ml min^{-1} kg^{-1}). This was due to a higher f_H and V_s in the present study (f_H, 45 *versus* 30 min^{-1}; V_s, 2.56 *versus* 1.92 ml kg^{-1}). Most previous studies on *Bufo* spp. report f_H within the range 25–40 min^{-1} at 20–25 °C (e.g. Boutilier and Toews, 1977; West and Burggren, 1984; Wang et al., 1994), and the reasons for the high $\dot{Q}_{tot}$ and f_H in the present study are unknown. Because vascular injections of adrenaline evoked a significant reflex bradycardia (results not shown) and since atropine injection caused a small but significant tachycardia (Fig. 5), it seems that the vagal innervation of the heart was intact and fully functional. Similarly, the low $\dot{Q}_{pc}/\dot{Q}_{sys}$ during normoxia (Fig. 3) and the large increase in $\dot{Q}_{pc}$ following administration of atropine (Fig. 5) indicate that the innervation of the pulmonary artery was functionally intact. In anaesthetised *Rana catesbeiana*, opening of the pericardium increased V_s through an increase in end-diastolic ventricular volume (Tazawa et al., 1979), and it is possible that disruption of the pericardium during placement of the flow probes contributed to the high V_s in the present study. Furthermore, it
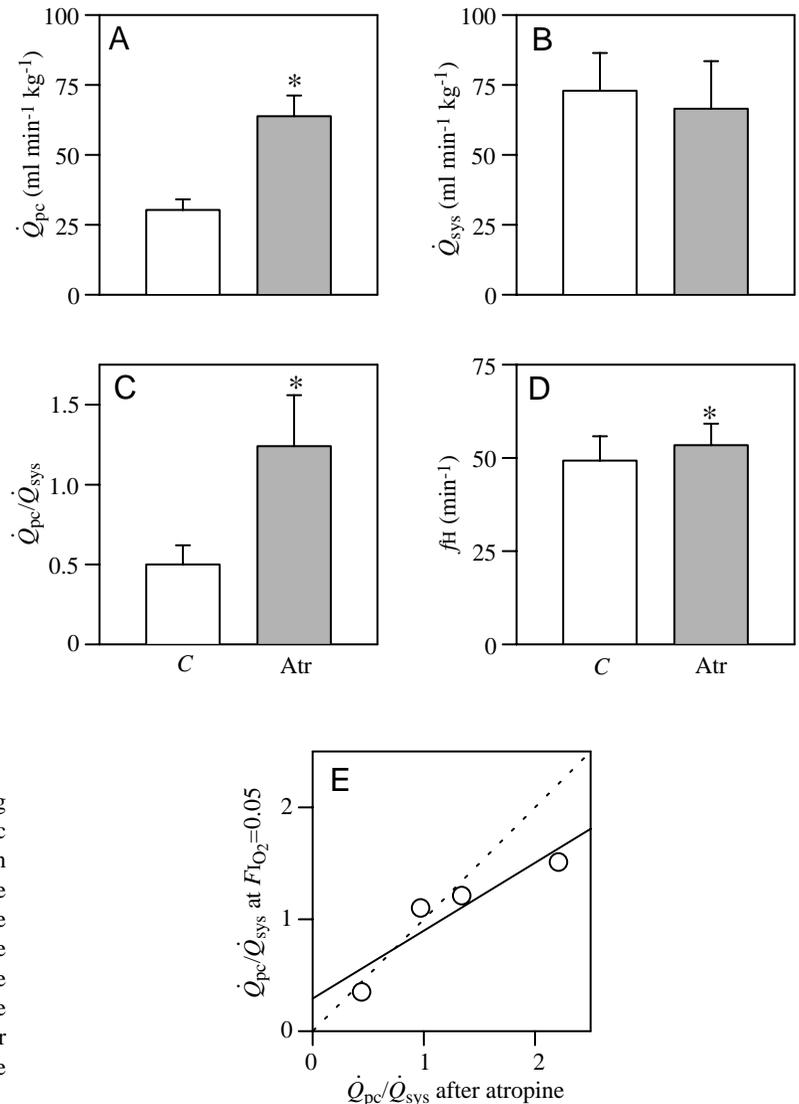
Fig. 5. Effects of atropine (Atr) injection ($5\,\mathrm{mg\,kg^{-1}}$) during normoxia on pulmocutaneous blood flow $\dot{Q}_{pc}$ (A), systemic blood flow $\dot{Q}_{sys}$ (B), $\dot{Q}_{pc}/\dot{Q}_{sys}$ (C) and heart rate f_H (D) in conscious toads (*Bufo marinus*) at $25\,^{\circ}\mathrm{C}$. Values are presented as means $+1$ S.E.M. ($N=4$), and means that are significantly different from the normoxic condition (C) are marked with an asterisk. (E) The relationship between the individual measurements of $\dot{Q}_{pc}/\dot{Q}_{sys}$ following atropine injection during normoxia and during hypoxia. A linear regression is shown by the solid line ($r^2=0.84$), whereas the dashed line depicts the 1:1 relationship).

is possible that the experimental manipulation in the present study was associated with a high degree of cardiac sympathetic stimulation. Finally, it is well established that seasonal variations have profound effects on physiological parameters such as heart rate in amphibians (Jones, 1968; Lund and Dingle, 1968; Weathers, 1975; Glass et al., 1997; Rocha and Branco, 1998). For example, f_H in *Bufo paracnemis* maintained at $25\,^{\circ}\mathrm{C}$ increases to approximate $35\,\mathrm{min^{-1}}$ in the spring compared with $25\,\mathrm{min^{-1}}$ during the winter (Glass et al., 1997).

The blood gas levels and ventilation rates were similar to those described previously for *Bufo paracnemis* at similar temperatures (Kruhøffer et al., 1987; Branco et al., 1993; Wang et al., 1998b). Kruhøffer et al. (1987) reported that pulmonary inflation cycles are an obligatory component of the breathing pattern in *B. paracnemis*. However, in the present and previous studies on *B. marinus*, pulmonary inflation cycles were not always present during normoxia, but were invariably observed during hypoxia and hypercapnia (MacIntyre and Toews, 1976; Boutilier and Toews, 1977).

Central vascular blood flows

The temporal relationship between blood flow in the truncus arteriosus and pulmocutaneous artery in *Bufo marinus* (Fig. 1) is consistent with that recorded in other anuran amphibians (Shelton and Jones, 1965a,b; Shelton, 1970; Langille and Jones, 1977). As in those studies, we found that ejection of blood into the truncus arteriosus and pulmocutaneous artery was almost simultaneous, that blood flow declined much more slowly in the pulmocutaneous artery than in the systemic artery, and that most of the blood flow during diastole was directed towards the pulmonary circulation. These differences have been attributed to a higher vascular compliance in the pulmonary circulation (Shelton, 1976; Langille and Jones, 1977).

Effects of ventilation on heart rate and blood flows

During normoxia, there were no obvious haemodynamic changes during ventilation in our experiments. In contrast, West and Burggren (1984) observed an almost twofold increase in $\dot{Q}_{pc}$ with no change in f_H during spontaneous

ventilation in a conscious specimen of *Bufo marinus*, and similar changes have been reported for mildly anaesthetised frogs (Shelton, 1970; De Saint-Aubain and Wingstrand, 1979).

The present study shows that there are temporal changes in central vascular blood flows during the lung inflation cycles that dominate the ventilatory pattern during hypoxia (Fig. 2). During the initial lung deflation, $\dot{Q}_{pc}$ either decreased slightly or remained unchanged. Following inflation, however, $\dot{Q}_{pc}$ increased transiently. Similar findings have been reported during hypoxia in *Xenopus laevis* (Emilio and Shelton, 1972) and during hypercapnia in *Bufo marinus* (West and Smits, 1994). The initial increase in $\dot{Q}_{pc}$ was partially mediated by a redistribution of blood from the systemic circulation (Fig. 2). The basis for the changes in $\dot{Q}_{pc}$ during the inflation cycles is difficult to interpret as it probably involves direct effects of lung pressure, changes in cardiac filling and neural reflexes (for reviews, see West and Van Vliet, 1992; Wang et al., 1999). It is possible that the initial increase in $\dot{Q}_{pc}$ following inflation is due to dilatation of the pulmocutaneous artery and that the subsequent increase in $\dot{Q}_{tot}$ merely reflects an increased end-diastolic filling of the heart as venous return is enhanced because of the increased intra-abdominal pressure.

Effects of temperature on cardiorespiratory variables during normoxia

As in previous studies on *Bufo paracnemis*, ventilation decreased when temperature was reduced from 25 to 15 °C (Fig. 3A; Kruhøffer et al., 1987; Branco et al., 1993). The decreased ventilation was accompanied by reductions in $\dot{Q}_{tot}$ and f_H, whereas V_s increased. A decreased f_H with reduced temperature is consistent with previous studies on *Bufo* and *Rana* (e.g. Glass et al., 1997; Weathers, 1975). This is probably due to the direct effects of temperature on the pacemaker cells of the heart (Clark, 1920), although cardiac vagal tone increases with elevated temperature (Barcroft and Izquierdo, 1931; Taylor, 1931; Taylor and Ihmied, 1985; Young, 1959; Lund and Dingle, 1968; Courtice, 1990). The effect of temperature on V_s has not been previously studied, but Weathers (1975) reported that the stroke flow in the hind limb of *Rana catesbeiana* is not affected by temperature (see Hillman, 1991). The increased V_s at low temperature in the present study may, at least partially, be due to a longer filling time at the lower heart rate and an associated augmentation of end-diastolic volume.

All toads in the present exhibited a net R–L shunt during normoxia (Fig. 3), whereas West and Smits (1994) reported almost equal blood flows in the pulmocutaneous and systemic arteries. In our study, the net shunt was greatly reduced following atropine injection, and it is possible that our toads had a larger vagal tone on the pulmocutaneous artery than in the study by West and Smits (1994). Further, the presence of an R–L shunt in undisturbed toads is supported by measurements of blood gas levels. Thus, systemic arterial blood haemoglobin O_2-saturation in *Bufo* is normally 60–80 % (e.g. Weathers, 1975; Boutilier et al., 1987; Wood and Malvin, 1991) because of admixture of O_2-poor systemic venous blood.

Decreased temperature further reduced $\dot{Q}_{pc}/\dot{Q}_{sys}$ from 0.39 to 0.22 in the present study (Fig. 3). In agreement with these changes, the R–L shunt increases with reduced temperature in several species of reptile (Heisler and Glass, 1985; Ishimatsu et al., 1988; Wang et al., 1998a).

The presence of a net R–L shunt in undisturbed animals seems to be a general phenomenon in amphibians and reptiles, but the functional significance of this shunt pattern is unknown (see Hicks and Wang, 1996). Further, in the light of the present study on *Bufo marinus* and previous studies on reptiles, it appears to be a general trend that the net R–L shunt decreases or does not change whenever the temperature is increased. A reduction in R–L shunt will, all other variables being equal, increase the arterial O_2 content and provide a means to improve systemic oxygen delivery as metabolic rate rises with temperature (see Wang and Hicks, 1996).

Effects of hypoxia on cardiorespiratory variables

The qualitative effects of hypoxia on cardiorespiratory variables were similar at 15 and 25 °C, but all responses were greatly attenuated at the lower temperature. As an example, the hypoxic ventilatory response was more rapid at the higher temperature, as shown previously for *Bufo paracnemis* (Kruhøffer et al., 1987) and other air-breathing vertebrates (e.g. Jackson, 1973; Glass et al., 1986, Rocha and Branco, 1998). Also, the effects of hypoxia on $\dot{Q}_{pc}$ were more pronounced at 25 °C than at 15 °C in the present study. An increased pulmonary blood flow during hypoxia has also been documented in the Australian lungfish (Fritsche et al., 1993) and in chelonians (Burggren et al., 1977; West et al., 1992; Wang et al., 1997). In the hypoxic toads, $\dot{Q}_{pc}$ remained elevated during the non-ventilatory periods (Fig. 2). This response may indicate a stimulation of chemoreceptors that selectively control the cardiovascular system. Consistent with this view, heart rate, but not ventilation, is increased following reductions in oxygen-carrying capacity in *B. paracnemis* (Wang et al., 1994; see also Wang et al., 1997). It is also possible that stimulation of pulmonary stretch receptors during the non-ventilatory periods contributed to the elevated $\dot{Q}_{pc}$ but, since the roles of the afferent receptors that lead to respiratory activity and cardiovascular responses are poorly understood in amphibians, a mechanistic explanation must await future studies.

In anurans, the distribution of blood flows between the systemic and pulmocutaneous arteries is determined by the resistances (R_{sys} and R_{pc}, respectively) in these circuits (Langille and Jones, 1977). R_{pc} is actively controlled by a cholinergic vagal innervation of a muscular sphincter located on the pulmonary artery proper; i.e. immediately downstream from where the cutaneous artery branches off the pulmocutaneous artery (de Saint-Aubain and Wingstrand, 1979; de Saint-Aubain, 1982). Consistent with this innervation, $\dot{Q}_{pc}$ increases after atropine injection (Emilio and Shelton, 1972; Smith, 1978; West and Burggren, 1984). In the present study, atropine injection eliminated the net R–L shunt in three of four animals. Furthermore, $\dot{Q}_{pc}/\dot{Q}_{sys}$ following atropine

injection (Fig. 5E) was similar to that observed during severe hypoxia (Fig. 4F), which may indicate that cardiovascular changes during hypoxia are achieved by reduced vagal tone. However, the pulmonary vasculature in *Bufo marinus* also receives a predominantly dilatory sympathetic adrenergic innervation that may contribute to reducing R_{pc} (Campbell, 1971a,b; see Holmgren and Campbell, 1978). Moreover, the levels of circulating catecholamines increase during severe hypoxia (J. H. Andersen, F. B. Jensen and T. Wang, unpublished results), which is likely to dilate the pulmonary vasculature and further reduce R_{pc}.

Hypoxia was also associated with an increased haematocrit at 25 °C (Table 1). This response has been observed in other amphibians and appears to be caused by reductions in plasma volume rather than by contraction of the spleen (e.g. Pinder and Smits, 1993). As a result, blood oxygen-carrying capacity increases during hypoxia, which promotes systemic oxygen delivery.

Effects of hypercapnia on cardiorespiratory variables

Hypercapnia increased ventilation approximately fivefold (Fig. 4A, a response that is mediated primarily through central chemoreceptors (Smatresk and Smits, 1991; Branco et al., 1992, 1993). Hypercapnia also increased $\dot{Q}_{tot}$ by 42 %. However, because $\dot{Q}_{pc}$ and $\dot{Q}_{sys}$ increased similarly, there were only minor changes in cardiac shunt patterns (Fig. 4E,F), which is consistent with the results of West and Smits (1994). Thus, the cardiovascular response to hypercapnia differs markedly from that during hypoxia, during which the R–L shunt is reduced. This difference may be due to the inhibitory role of CO_2 on the mechanosensitivity of pulmonary stretch receptors (Milsom and Jones, 1977) or may be because CO_2 may cause vasoconstriction of the pulmonary vasculature (Smith, 1978; West and Burggren, 1984).

In conclusion, the present study shows that undisturbed conscious toads exhibit a large net R–L cardiac shunt. However, when temperature is increased and when oxygen transport is challenged by hypoxia, the net R–L cardiac shunt is greatly reduced. These findings are consistent with the general view that the cardiac R–L shunt is reduced whenever the demands for oxygen delivery are augmented (Wang and Hicks, 1996, 1999). Furthermore, it appears that the cardiac shunt is actively regulated and may be involved in the regulation of arterial blood gas composition (Wang and Hicks, 1996). Because there were qualitative differences in the cardiorespiratory responses to hypoxia and hypercapnia, it is also clear that the changes in blood flows and cardiac shunt patterns are not entirely dependent on the ventilatory changes, and it is likely that these systems are controlled independently.

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