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EFFECT OF TEMPERATURE ON CENTRAL CHEMICAL CONTROL OF VENTILATION IN THE ALLIGATOR *ALLIGATOR MISSISSIPPIENSIS*

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Summary

Central chemoreceptor function was assessed in unanesthetized alligators, *Alligator mississippiensis*, at body temperatures of 15, 25 and 35˚C. Two experiments were performed. In the first experiment, the fourth ventricle was perfused with mock cerebrospinal fluid (CSF) solutions of different pH values (7.1–7.9). Changes in pulmonary ventilation were evaluated with a pneumotachograph and arterial pH (pHa) was measured. Perfusion with low-pH solutions increased ventilation and arterial pH. Perfusion with high-pH solutions decreased ventilation and arterial pH. Mock CSF pH had a greater effect at higher temperatures. In the second experiment, the relative contributions of central and peripheral chemoreceptor drive to breathing were evaluated using hypercapnic gas mixtures to stimulate both central and peripheral chemoreceptors. Hypercapnia caused an increase in ventilation which was larger at higher temperatures. To stimulate only the peripheral chemoreceptors, the same hypercapnic gas mixtures were applied while the CSF pH of the fourth ventricle was kept constant by perfusion with a mock CSF solution. This reduced significantly the ventilatory response induced by hypercapnia. These data indicate that, regardless of the temperature, central chemoreceptors play a major role in the ventilatory regulation of the alligator. The change in pHa with temperature is compatible with the alphastat hypothesis.

Introduction

In reptiles, changes in ventilation can be elicited by peripheral arterial chemoreceptors (Benchetrit and Dejours, 1980), intrapulmonary receptors (Gatz *et al.* 1975; Milsom and Jones, 1979) and central chemoreceptors (Hitzig and Jackson, 1978). The relative contributions of central and peripheral chemoreceptors to the CO_2-induced ventilatory response are unclear. Moreover, the receptors involved in the control of breathing in reptiles have not been completely identified. Douse and Mitchell (1992) studied the role of vagally mediated peripheral chemoreceptors in hypercapnia-induced hyperventilation

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in alligators. They found that non-vagal mechanisms were also involved in the ventilatory response to CO_2 and suggested the presence of chemosensitive areas. Such areas have also been reported in a turtle (Hitzig and Jackson, 1978; Hitzig, 1982) as well as in amphibians (Smatresk and Smits, 1991; Branco *et al.* 1992) and birds (Milsom *et al.* 1981).

In many reptiles, the ventilatory response to hypercapnia is temperature-dependent (Shelton *et al.* 1986). However, the effect of temperature on the central chemoreceptor drive to breathing has only been evaluated in the turtle *Pseudemys scripta elegans* (Hitzig and Jackson, 1978), where the sensitivity of the central chemoreceptors remains constant at different body temperatures (Hitzig, 1982). Interestingly, the ventilatory sensitivity to changes in arterial P_{CO_2} is higher at higher body temperatures in another freshwater turtle, *Chrysemys picta* (Funk and Milsom, 1987).

The objective of the present study was to test the hypothesis of Douse and Mitchell (1992) that central chemosensitive areas are involved in the hypercapnia-induced ventilatory response of alligators. To characterize the receptor system further, the relative contributions of central and peripheral chemoreceptors to ventilation were measured at three different body temperatures.

Materials and methods

Animals

Experiments were performed on juvenile alligators, *Alligator mississippiensis*, weighing 669±38g (mean ± S.E.), obtained from an alligator farm in Louisiana. They were housed indoors at 25°C with free access to water and a 12h:12h light:dark photoperiod and were fed canned cat food until 7 days before surgery.

Surgery

Animals were anesthetized with a mixture of 5% halothane and 95% oxygen using a small animal anesthesia machine (Summit Hill Laboratories). Anesthesia was maintained throughout the operation using a mixture of 3% halothane and 95% oxygen. A catheter (PE-50) was filled with heparinized saline solution and placed in the femoral artery. The fourth ventricle was cannulated by removing the skin covering the caudal half of the skull and drilling a hole through the occipital bone in the caudal direction starting 2–3mm from the space between the occipital bone and the first vertebra. This hole provided access to the cephalic portion of the fourth ventricle. An assembly of two needles was stereotaxically placed inside the hole with the needle tips protruding into the CSF of the fourth ventricle. This allowed flow-through perfusion with mock CSF solutions, which were injected and removed from the same ventricular site. The skull opening was sealed with bone wax, followed by application of dental acrylic. Experiments began at least 24h after surgery. At the end of the experiment, the perfusion site was checked by adding Evans Blue (0.3%) to the mock CSF solution. The fourth ventricle, part of the cerebellum and adjacent portions of the medulla were stained by this procedure to show that the fourth ventricle and adjacent structures had been effectively and specifically perfused.

Ventilation

A pneumotachograph method was used to measure ventilation (Glass *et al.* 1978). To construct the face mask, the alligator was cooled to 4°C. Negative impressions of the head were made using alginate resin and plaster models were constructed. Mouthguard resin (Healthco) was molded using the plaster models. Signals from a differential air transducer (Validyne, model MP45-1) were displayed on a paper recorder (Lafayette, model 76102B).

Arterial pH and P_{CO_2}

Arterial pH and P_{CO_2} were measured with a Radiometer blood gas analyzer (BMS3 Mk2). The pH electrode was calibrated at the experimental temperature with Radiometer precision buffer solutions (S1510 and S1500). The CO_2 electrode was calibrated by means of Radiometer CCS standard gas mixtures. All blood samples were analyzed immediately after being withdrawn.

Mock cerebrospinal fluid

Solutions used to perfuse the fourth ventricle (Table 1) were prepared according to the method of Loeschke *et al.* (1958), which has also been applied in turtles (Hitzig and Jackson, 1978) and toads (Branco *et al.* 1992). Modifications were made to comply with reptile ionic concentrations (Heisey, 1968).

Bicarbonate concentration was calculated from the Henderson–Hasselbalch equation. Dissociation constant (pK′) and solubility of CO_2 were calculated according to Nicol *et al.* (1983). The solution pH was checked in the perfusate reservoir and, when necessary, the pH was adjusted by the addition of a small amount of a solution with an identical ionic composition except for bicarbonate and chloride, i.e. (in mequiv l^{-1}): Na^+ 128.5; K^+ 2.6;

Table 1. *Ionic composition of mock cerebrospinal fluid for the experiments performed at 15, 25 and 35°C*

Temperature (°C)	pH	Na^+ (mequiv l^{-1})	K^+ (mequiv l^{-1})	Ca^{2+} (mequiv l^{-1})	Mg^{2+} (mequiv l^{-1})	Cl^- (mequiv l^{-1})	HCO_3^- (mequiv l^{-1})
15	7.9	128.5	2.6	3.6	3.4	93.6	34.9
15	7.7	128.5	2.6	3.6	3.4	97.6	30.9
15	7.5	128.5	2.6	3.6	3.4	115.7	12.8
15	7.3	128.5	2.6	3.6	3.4	120.6	7.9
15	7.1	128.5	2.6	3.6	3.4	123.6	4.9
25	7.9	128.5	2.6	3.6	3.4	83.5	45.0
25	7.7	128.5	2.6	3.6	3.4	101.5	27.0
25	7.5	128.5	2.6	3.6	3.4	112.0	16.5
25	7.3	128.5	2.6	3.6	3.4	118.3	10.2
25	7.1	128.5	2.6	3.6	3.4	122.2	6.3
35	7.9	128.5	2.6	3.6	3.4	77.0	51.5
35	7.7	128.5	2.6	3.6	3.4	97.7	30.8
35	7.5	128.5	2.6	3.6	3.4	109.7	18.8
35	7.3	128.5	2.6	3.6	3.4	116.9	11.6
35	7.1	128.5	2.6	3.6	3.4	121.3	7.2

Ca^{2+} 3.6; Mg^{2+} 3.4; HCO_3^- 4.9; Cl^- 123.6 or Na^+ 128.5; K^+ 2.6; Ca^{2+} 3.6; Mg^{2+} 3.4; HCO_3^- 51.5; Cl^-, 77.0. Carbon dioxide concentration was kept constant at 2% in the experiment at 15˚C, 3% at 25˚C and 4% at 35˚C in all experiments. The flow was kept at 100 $\mu l\,min^{-1}$ by adjusting the elevation of the perfusate reservoir in relation to the animal's head.

Oxygen consumption

O_2 uptake was measured by flow-through respirometry using a gas-mixing pump (H. Wösthoff, Bochum, Germany) and an oxygen analyzer (Applied Electrochemistry Inc., Sunnyvale, CA 94086, model S-3A). Air flow through the animal chamber was measured with a flow meter (Gilmont, model F11).

Experimental protocol

Cannulated alligators, equipped with face masks, were isolated in a sealed polyvinyl chloride pipe (length 62cm, diameter 12cm) kept inside an environmental chamber (Lab Line, Inc.) for at least 24h at the experimental temperature. Experiments were performed at 15, 25 and 35˚C. There was no difference between the temperature in the polyvinyl chloride pipe and the environmental chamber. In the first set of experiments, the animal chamber was continuously flushed with air. The fourth ventricle was perfused with mock CSF solutions of pH7.9, 7.7, 7.5, 7.3 and 7.1 in a random order. Perfusion was maintained continuously for 1h before the data were collected. Ventilation was recorded and blood was sampled during the last 30min of each experimental condition.

A second set of experiments was performed to measure the relative contribution of central and peripheral chemoreceptor drive to breathing. Following a control period, hypercapnic gas mixtures were flushed through the animal chamber in the sequence F_{CO_2}=0.02, 0.04, 0.06 and 0.08 (see Table 2 for values in partial pressure). Ventilation was recorded and blood samples were analyzed after at least 3h in each experimental condition. The fourth ventricle was then perfused with mock CSF solution of pH7.6 at 15˚C, 7.45 at 25˚C and 7.3 at 35˚C. Different pH values were used to get as close as possible to the values assumed as controls. Time schedules for ventilation recordings and blood sampling were maintained as before.

Calculations and statistics

Values are given as mean ± S.E. (unless otherwise stated). Analysis of variance was used to test for statistical significance. Multiple comparisons of group means were tested

Table 2. *Inspired CO_2 values at 15, 25 and 35°C (altitude 1600m)*

	P_{CO_2} (kPa)		
% CO_2	15°C	25°C	35°C
2	1.6	1.6	1.6
4	3.3	3.2	3.1
6	4.9	4.9	4.7
8	6.6	6.5	6.3

using a step-down multiple-stage *F*-test (REGW; Ryan, 1960). *P* values of less than 0.05 were assumed to be significant.

Results

Experiment 1

Fig. 1 shows the ventilatory responses to the perfusion of the fourth ventricle with mock CSF solutions of different pH values (from 7.1 to 7.9) at 15, 25 and 35˚C. The increase in ventilation due to the acidification of the perfusate response was abolished at 15˚C. At 35˚C ventilation increased approximately fivefold. Slopes from linear regression of the curves were (mean ± S.D. of residuals): −2.05±0.28, −10.03±1.38 and −21.57±6.53ml BTPS $min^{-1} kg^{-1}$ pHunit^{-1} These values are significantly different from each other (*P*<0.001). The control values for ventilation were (mean ± S.E.): 5.58±0.83 (at 15˚C, *N*=6); 8.21±1.43 (at 25˚C, *N*=8); and 10.05±2.49 (at 35˚C, *N*=6); in ml BTPS $kg^{-1} min^{-1}$. The hyperventilation caused a significant (*P*<0.05) temperature-dependent increase in arterial pH (Fig. 2).

Experiment 2

The results from the experiments combining hypercapnia and fourth ventricle perfusion are shown in Fig. 3. Two relationships between arterial P_{CO_2} and ventilation were plotted at each temperature: one with hypercapnia application only and the other with hypercapnia combined with perfusion of the fourth ventricle with mock CSF

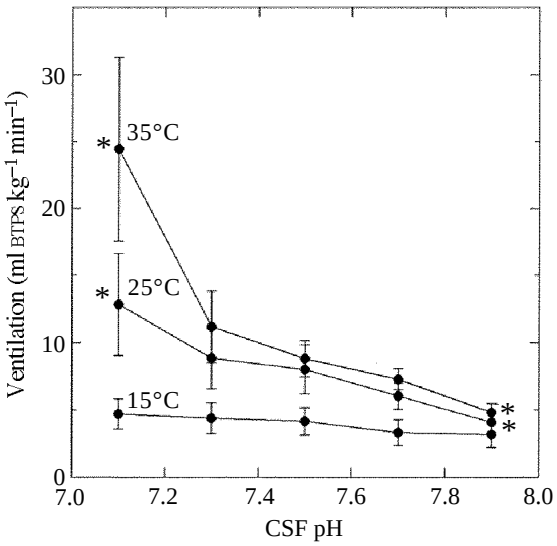

Fig. 1. Effect of perfusion of the fourth ventricle using mock CSF solutions with different pH values on pulmonary ventilation. The slopes of the linear regression of each curve are significantly different from each other (*P*<0.001). Mean values ± S.E., *N*=6, 8 and 6, respectively, for the curves at 15, 25 and 35˚C. * indicates a significant difference at the given temperature from the control value at that temperature.

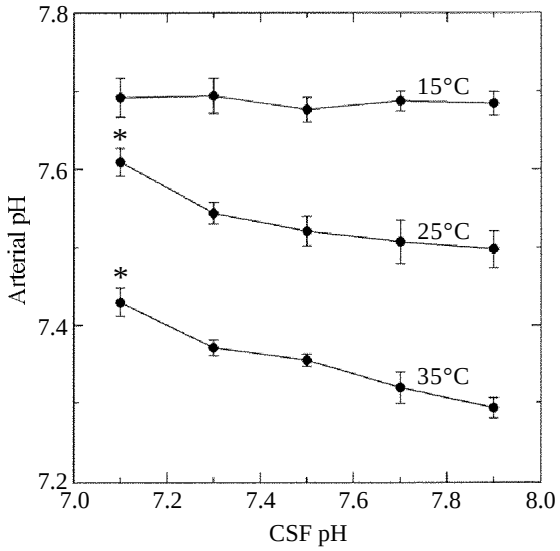

Fig. 2. Relationship between CSF pH and arterial pH. The slopes of the linear regression of each curve are significantly different (P<0.001). Mean values ± S.E., N=6, 8 and 6, respectively, for the curves at 15, 25 and 35˚C. * indicates a significant difference at the given temperature from the control value at that temperature.

solution of pH7.6 at 15˚C, 7.45 at 25˚C and 7.3 at 35˚C. These values are based on the data shown in Fig. 1. No change in ventilation was found when perfusion was performed using solutions of pH equal to control values. Ventilation increased during inspiration of hypercapnic mixtures in a temperature-dependent matter. This response was greatly attenuated by ventricular perfusion at constant pH. The magnitude of the blockage was not temperature-dependent, being 50–60% in each case (at $P_{a_{CO_2}}$=4kPa). Slopes of the curves without ventricular perfusion are (mean ± S.D. of residuals): 0.87±0.15, 2.12±0.35 and 2.85±0.07mlkg^{-1}min^{-1}kPa^{-1} at 15, 25 and 35˚C respectively. There is a significant difference between these values (P<0.001), indicating an increased sensitivity of the system with increasing temperatures. The slopes of the curves with ventricular perfusion are (mean ± S.D. of residuals): 0.39±.03, 0.44±0.14 and 0.74±0.12mlkg^{-1}min^{-1}kPa^{-1} at 15, 25 and 35˚C respectively.

Arterial pH decreased by approximately 0.16 units for an increase in temperature of 10˚C (Fig. 4). Neither pHa nor P_{CO_2} was significantly affected by the perfusion of the fourth ventricle with mock CSF solution of normal pH.

Discussion

The present study provides the first evidence that central chemoreceptors have a role in the control of breathing in crocodilians. Central chemoreceptors have been extensively studied in mammals (Schlaefke, 1981). In ectothermic vertebrates, the first evidence of central chemoreceptor function was reported by Hitzig and Jackson (1978), who perfused fourth ventricles of turtles with mock CSF solutions of different pH values, as had

originally been carried out in mammals (Loeschcke *et al.* 1958). The same technique was later used in amphibians (Smatresk and Smits, 1991; Branco *et al.* 1992), where there is evidence of a major role for central chemoreceptors in the control of ventilation.

Effects of temperature on resting ventilation

There was a 2.8-fold increase in ventilation with increasing temperature from 15 to 35°C. Similar results have previously been reported for another reptiles without fourth ventricle cannulae (Davies, 1978; Davies *et al.* 1982; Funk and Milsom, 1987), which implies that cannulation of the fourth ventricle did not affect ventilation. In turtles, Funk and Milsom (1987), using *C. picta*, reported a threefold increase in pulmonary ventilation

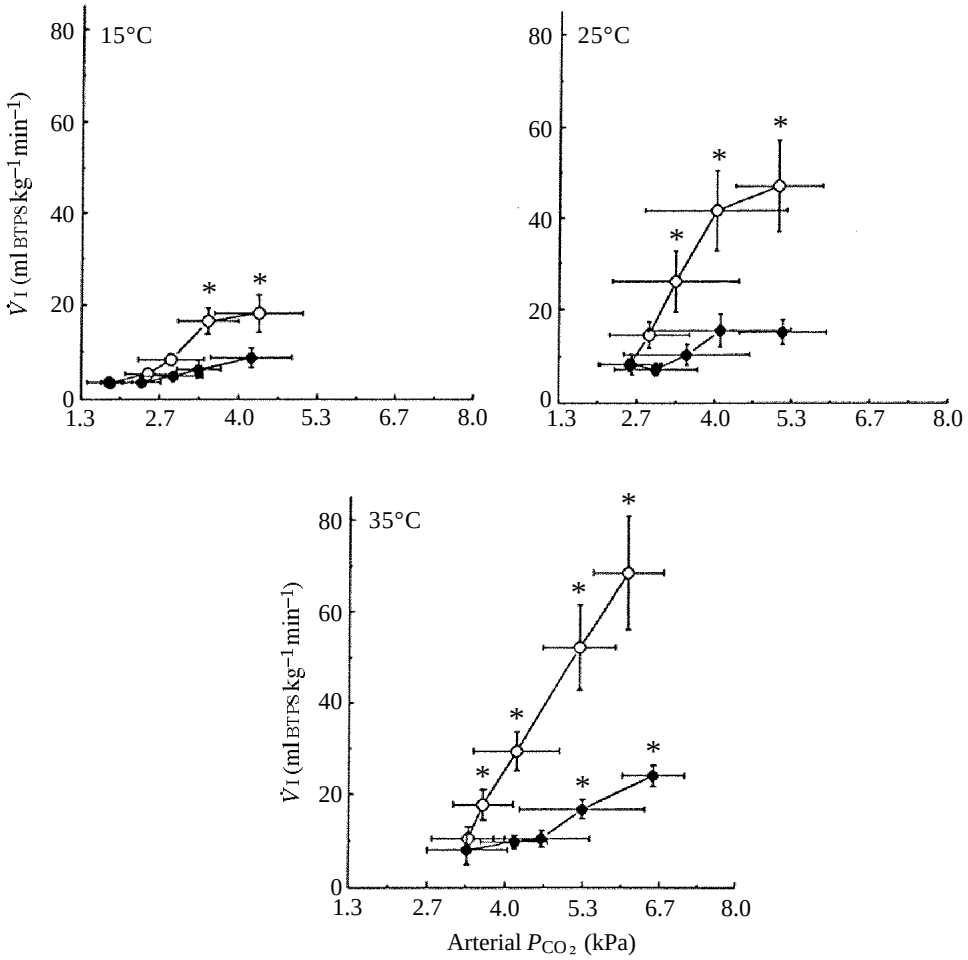

Fig. 3. Effects of hypercapnia on pulmonary ventilation ($\dot{V}_I$, inspired volume) at different temperatures. Open circles: without perfusion of the fourth cerebral ventricle. Filled circles: with perfusion of the fourth ventricle, applying mock CSF solution of pH close to the control value (see text). Mean values ± S.E., N=6, 8 and 6, respectively, for the curves at 15, 25 and 35°C. * indicates a significant difference from the control (normoxia) value.

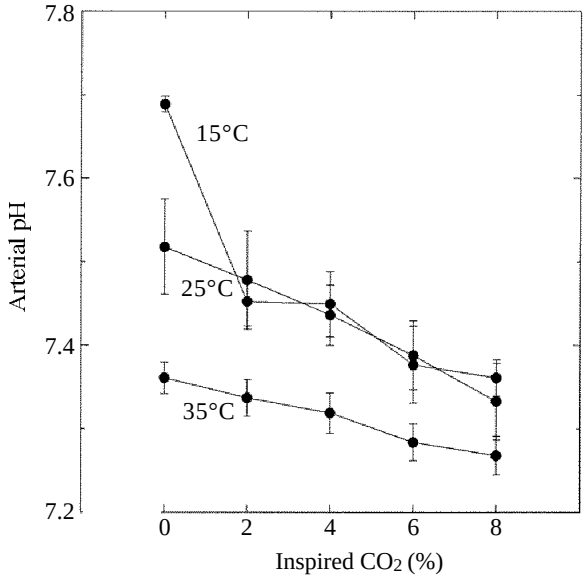

Fig. 4. Relationship between arterial pH and inspired CO_2. Mean values ± S.E., N=6, 8 and 6, respectively, for the curves at 15, 25 and 35°C. At all three temperatures, there is a significant decrease in arterial pH at inspired CO_2 values of 2, 4, 6 and 8%.

between 10 and 30°C, while Jackson *et al.* (1974) and Jackson (1971), using *P. scripta*, found no clear relationship between temperature and ventilation over the same temperature range. Kinney *et al.* (1977) also found an increase in ventilation with an increase in body temperature in the turtle *P. floridana*. The discrepancy between the two studies is probably due to the different methods used to measure ventilation. Lizards have also been shown to increase their ventilation with increasing body temperature (Bennett, 1973; Crawford and Kampe, 1971).

Changes in arterial pH can be attributed to a relative hypoventilation, since oxygen consumption increased 5.8 times between 15 and 35°C while the air convection requirement (the ratio between ventilation and oxygen uptake) decreased 1.2 times between 15 and 35°C (Fig. 5). Consequently, arterial pH changed by $-0.016\text{units}^{\circ}\text{C}^{-1}$ (Fig. 4), which is in agreement with previous studies of alligators (Davies, 1978; Davies *et al.* 1982; Douse and Mitchell, 1992). Reeves (1972) suggested that, in ectothermic vertebrates, the fractional dissociation of histidine imidazole groups is the controlled variable in acid–base balance instead of pH. One requirement for the 'alpha-stat hypothesis' (Reeves, 1972) is that $\text{dpH}/\text{d}T$ will be $-0.015\text{units}^{\circ}\text{C}^{-1}$ or more negative; our data fulfill this particular requirement. However, there is some controversy about the application of the hypothesis to alligators. Davies (1978) and Davies *et al.* (1982), on the basis of measurements of gas exchange and arterial blood acid–base status, reported that the control of breathing in alligators is indeed a physiological defense of the alpha-imidazole. However, Douse and Mitchell (1992) found that pH was not stable during prolonged exposures to reduced temperature. Moreover, the latter study also reports that

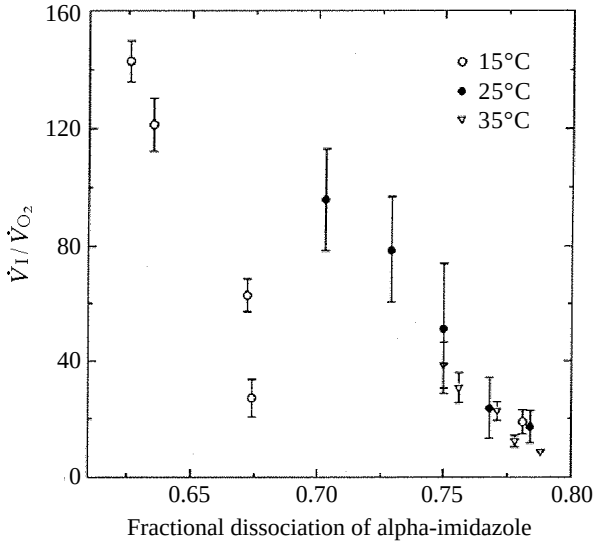

Fig. 5. Relationship between ventilation-to-metabolism ratio ($\dot{V}_I/\dot{V}_{O_2}$) and fractional dissociation of alpha imidazole at each temperature. Values are means ± S.E. for 6, 8 and 6 animals, respectively, at 15, 25 and 35°C.

the dissociation of alpha-imidazole decreased significantly with decreasing temperature based in plasma values. As noted by Douse and Mitchell (1992), the alpha-stat hypothesis might be more accurately tested if intracellular acid–base responses to alteration in body temperature were taken into consideration instead of extracellular variables. Nevertheless, Fig. 5 shows that the relationship between air convection requirement and alpha-imidazole dissociation is similar to the one that appears in Davies *et al.* (1982), where it is concluded that the control of breathing in alligators is a physiological defense of alpha-imidazole. In Fig. 5, the curve at 15°C is not parallel to the ones at 25 and 35°C because the drop in arterial pH at 15°C was greater. The reason that pH drops more steeply at 15°C than at 25 or 35°C during inspiration of 2% CO_2 is that there is no significant ventilatory response, as shown in Fig. 3 (Dejours, 1966). However, when ventilation was plotted against alpha-imidazole dissociation, no common relationships were observed, as reported for turtles (Hitzig, 1982).

Effects of temperature on central chemoreceptor function

The effects of temperature on the central chemoreceptor drive to pulmonary ventilation have previously been evaluated only in turtles. Hitzig and Jackson (1978) found no significant change in the central chemoreceptor sensitivity between 20 and 30°C. Their conclusion was based on the changes in ventilation-to-metabolism ratios during ventricular perfusion, which precludes a comparison with the present data for ventilation (Fig. 1). However, Hitzig (1982), using the same method of perfusing the brain ventricular system in conscious turtles originally described in the paper above, reported that the ventilatory response to CSF pH remains constant at body temperatures of 20 and 30°C. In contrast, the present study shows that alligators have a sharp increase in central

chemoreceptor sensitivity with increasing temperatures and also a markedly higher sensitivity to inspired CO_2 with increasing temperature in relation to pulmonary ventilation. Why such differences exist is unclear.

Data on the turtle *Pseudemis scripta elegans* indicate that central chemoreceptors are involved only in the control of respiratory frequency whereas peripheral receptors modulate tidal volume (Hitzig *et al.* 1985). In alligators, central chemoreceptors adjust not only respiratory frequency but also tidal volume (data not shown).

Ventilation was unchanged when ventricular perfusion was applied using mock CSF solution of pH equal to the control value under normoxic conditions. When fourth ventricle pH was kept constant and arterial pH was varied, by increasing inspired CO_2 concentration, there was a reduction of approximately 60% in the ventilatory response to the same change in arterial pH, indicating that the perfusion procedure could buffer the central chemoreceptor environment from changes in arterial P_{CO_2} (Fig. 3). The remaining 30% change in the ventilatory response can be attributed to stimulation of central chemosensitivity areas not affected by the perfusion and/or receptors other than those in the nervous system. Peripheral chemoreceptors have been identified in the turtle *Pseudemys scripta* (Frankel *et al.* 1969) and tortoise *Testudo horsfieldi* (Benchetrit *et al.* 1977). Airway CO_2 receptors have been reported in the lizard *Tupinambis negropunctatus* (Fedde *et al.* 1977), in the garter snake *Thamnophis sirtalis* (Coates and Ballam, 1989) and in the alligator *A. mississippiensis* (Douse *et al.* 1989; Douse and Mitchell, 1992).

The arterial P_{CO_2} measurements during hypercapnia were, in general, lower than the inspired P_{CO_2} values (Table 2 and Fig. 3). Davies *et al.* (1982) also reported low P_{CO_2} values corresponding to inspired CO_2 concentrations. This may be due to extrapulmonary CO_2 loss in alligators (Davies *et al.* 1982) and in turtles where, depending on the species, it can be as high as 64% of the total CO_2 loss (Jackson *et al.* 1976). It is also possible that an intracardiac right-to-left shunt might be responsible for the low arterial P_{CO_2} values (see Wood, 1984).

Central chemoreceptors in the alligator appear not to be as sensitive to changes in the acid–base status as they are in turtles and amphibians. *Pseudemys scripta elegans* (Hitzig, 1982) is extremely sensitive to very small changes in CSF pH and responds more than three times more vigorously than *Bufo paracnemis* (Branco *et al.* 1992). Essentially the same experimental protocol used in *Bufo* was applied in the present study. In *Bufo*, a significant change in pulmonary ventilation was measured when perfusing the fourth ventricle with a CSF solution differing from the control pH by 0.2 units (Branco *et al.* 1992), whereas in the alligator a change was only measured when the pH of the solution differed by 0.4 units from the control. Moreover, the slope of the relationship between pulmonary ventilation and perfusate pH in *Bufo* (mean ± S.D. of residuals), $-292.6\pm24.3 ml\,BTPS\,min^{-1}\,kg^{-1}\,pH^{-1}$ unit, is much greater than that measured in alligators, $-10.03\pm1.38 ml\,BTPS\,min^{-1}\,kg^{-1}$ pHunit (data not shown). All values mentioned above were obtained with animals equilibrated at 25°C. This difference in sensitivity may be due to the presence of intrapulmonary receptors in alligators (Douse *et al.* 1989), whereas turtles, amphibians and mammals lack such a receptor system. Some evidence suggests that central chemoreceptors play a minimal role in species with

intrapulmonary chemoreceptors (see Douse and Mitchell, 1992). However, the present study indicates that a species with intrapulmonary chemoreceptors (alligators) does have functional central chemosensory mechanisms.

In conclusion, the present data are consistent with the hypothesis that central chemoreceptors are essential for a normal ventilatory response to hypercapnia in alligators. Alligator central chemoreceptors are functionally similar to those documented in mammals (Schlaefke, 1981), birds (Milsom *et al.* 1981) and amphibians (Smatresk and Smits, 1991; Branco *et al.* 1992), indicating that they are a fundamental characteristic of respiratory control in terrestrial ectothermic vertebrates.

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