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ONTOGENY OF CENTRAL CHEMORECEPTION DURING FICTIVE GILL AND LUNG VENTILATION IN AN *IN VITRO* BRAINSTEM PREPARATION OF *RANA CATESBEIANA*

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Summary

An isolated brainstem preparation of the bullfrog tadpole, *Rana catesbeiana*, displays coordinated rhythmic bursting activities in cranial nerves V, VII and X *in vitro*. In decerebrate, spontaneously breathing tadpoles, we have previously shown that these bursts correspond to fluctuations in buccal and lung pressures and to bursts of activity in the buccal levator muscle H3a. This demonstrates that the rhythmic bursting activities recorded *in vitro* represent fictive gill and lung ventilation. To investigate the ontogeny of central respiratory chemoreception during the transition from gill to lung ventilation, we superfused the isolated brainstems of four larval stage groups with oxygenated artificial cerebrospinal fluid at various levels of P_{CO_2}. We measured shifts in the pattern of fictive respiratory output and the response to central hypercapnic stimulation throughout development. At normal P_{CO_2} (2.3 kPa), stage 3–9 tadpoles displayed rhythmic neural bursts associated with gill ventilation, while stages 10–14 and 15–19 tadpoles produced oscillating bursting activity associated with both gill and lung respiration, and tadpoles at stages 20–25 displayed neural activity predominantly associated with lung ventilation. In stage 3–9 tadpoles, variations in P_{CO_2} of the superfusate (0.5–6.0 kPa) caused almost no change in fictive gill or lung ventilation. By contrast, stage 10–14 tadpoles showed a significant hypercapnic response ($P<0.05$) in the amplitude and frequency of fictive gill ventilation, which was accompanied by a significant increase ($P<0.05$) in the burst amplitude and respiratory output of cranial nerve X over that occurring at all other stages. The amplitude and frequency of fictive gill ventilation in stages 15–19 increased significantly ($P<0.05$) in response to pH reduction, but became insensitive to hypercapnia at stages 20–25. The frequency of fictive lung ventilation was unresponsive to hypercapnia in stage 10–14, increased significantly by stage 15–19 ($P<0.05$) and became maximal ($P<0.05$) in stages 20–25. Overall, we describe the ontological development of central respiratory chemoreceptors driving respiratory output in the larval amphibian, demonstrating transfer in central chemoreceptive influence from gill to lung regulation during metamorphic stages. In addition, we provide novel evidence for the stimulatory influence of central chemoreceptors on fictive gill ventilation in response to CO_2.

Key words: ontogeny, central chemoreception, gills, lung, ventilation, control of breathing, medulla, rhythm generation, tadpole, *Rana catesbeiana*.

Introduction

The evolution of terrestriality in vertebrates required changes in the form and function of the respiratory system associated with the transition from water to air environments (for reviews, see Gans, 1970; Dejours, 1988; Randall *et al.* 1981; Little, 1983; Shelton *et al.* 1986). The respiratory systems of amphibians manifest a diversity of successful adaptations to the demands imposed by the differences in the physical properties of these two environments, and the ontogeny of respiratory gas exchange in amphibian larvae reflects these evolutionary developments.

Tadpoles exchange respiratory gases at multiple sites (skin, gills and lungs), and the interdependence and balance among exchange sites changes with ontogeny (Burggren and West, 1982; Burggren, 1984). Immediately after hatching, respiratory needs are met by diffusion of both O_2 and CO_2 through well-perfused skin, constituting the dominant pathway for gas exchange until metamorphosis (Taylor and Köllros, 1946; Burggren and Doyle, 1986). However, increases in body mass and metabolic rate require the progressive development of more efficient respiratory structures (gills and lungs). The internal gills provide supplementary gas exchange (DeJongh, 1968) with rhythmic dorso-ventral movement of the buccal

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floor moving a stream of water unidirectionally into the mouth, through the gills and out of the opercular spout (Gradwell, 1972*a*,*b*). Ventilation of the paired unicameral lungs is achieved using the same buccal musculature to draw air in through the mouth (rather than through the nares as in adults) and to force it into the lungs (Burggren, 1984). Early in development (stages 4–5), the lungs are ventilated infrequently and contribute little to respiratory gas exchange. However, O_2 uptake *via* the gills and lungs increases at the onset of metamorphosis such that they each account for 20 % of the total O_2 exchange at stage 16 (Burggren and West, 1982). At this stage of development, amphibian larvae demonstrate ventilatory patterns similar to those described for lungfishes: regular branchial movements pumping water over the gills, interspersed with lung breaths (McMahon, 1969; Smatresk, 1990). During metamorphic climax (stages 18–19), the gills and tail degenerate and the lungs become the dominant site of O_2 exchange.

Such complex development of the structure and function of respiratory gas-exchange organs necessitates the simultaneous development and coordination of respiratory controllers. In early anuran larvae (stages 5–15), aquatic hypoxia stimulates gill and lung ventilation, hyperoxia induces apnoea and hypercapnic challenge evokes no ventilatory response (Burggren and Doyle, 1986; Infantino, 1992). During metamorphosis (stages 16–19), the gill and lung hypoxic responses persist, and a response to changes in CO_2 levels appears in both (Infantino, 1992). Postmetamorphic larvae (stages 20–25) display lung, but not gill, responses to hypoxia and hypercapnia (Burggren and Doyle, 1986). Such responses are apparently mediated reflexly by peripheral mechano- and chemoreceptors intimately associated with lung and gill function (West and Burggren, 1983) and, further, by central medullary chemoreceptors in the adult (Smatresk and Smits, 1991; McLean *et al.* 1995*a*,*b*). Collectively, these data indicate that progressive larval development is associated with a shift in the site of ventilatory responses from the gills to the lungs and is accompanied by the emergence of CO_2 as a source of respiratory drive.

Despite recent advances, the mechanisms of neurorespiratory control are not fully understood. Although most investigations of respiratory rhythmogenesis have been carried out in mammals (for reviews, see Richter, 1982; Ezure, 1990; Feldman and Smith, 1989; Feldman *et al.* 1990), their complexity makes amphibian models of respiratory control an attractive alternative. Recent studies of amphibians have explored the central neuronal substrate of respiratory rhythm generation by relating global changes in the execution of respiratory motor acts to brainstem neural phenomena (Kogo *et al.* 1994; Kogo and Remmers, 1994; McLean *et al.* 1995*a*,*b*). By elucidating the neural mechanisms of respiratory rhythm generation and central chemoreception in amphibians, it is possible to relate evolutionary changes in respiratory behaviour with the neural basis of such behaviour. No systematic study has yet described the developmental transitions of central respiratory chemoreception in amphibians.

The aim of the present investigation was to examine the ontogeny of central respiratory chemoreception in amphibians during the transition from gill to lung ventilation. The bullfrog *Rana catesbeiana* was chosen for this study because it undergoes extreme morphological and physiological changes during metamorphosis and because the respiratory behaviour in the adult has been well documented. In order to compare central mechanisms controlling breathing during successive stages of development, we have investigated fictive gill and lung ventilation in an isolated *in vitro* brainstem preparation from *Rana catesbeiana* tadpoles. This segment of the brain retains the necessary neural circuitry to generate the complex rhythmic neural output responsible for each type of breathing, thereby allowing us conveniently to trace the ontogeny of central respiratory chemoreception.

Materials and methods

Experiments were performed on 30 *Rana catesbeiana* tadpoles of either sex obtained from a commercial supplier (Charles D. Sullivan Co. Inc., Nashville, TN, USA). The animals were assigned to four groups according to the staging of Taylor and Köllros (1946) as follows: stages 3–9 (N=8), stages 10–14 (N=9), stages 15–19 (N=7) and stages 20–25 (N=6). For at least 5 days before experimentation, animals were housed in aerated and filtered water at 5–8 °C in a 55 l aquarium with a 12 h:12 h light:dark photoperiod.

Surgical preparation

Prior to surgery, the tadpoles were anaesthetized in tricaine methane sulphonate (1:10 000), then weighed. Once unresponsive, the dorsal cranium was removed and, with the aid of a dissecting microscope, the cranial nerves were severed at the cranial ostia. After removing the dura and arachnoidia dorsally and ventrally, the brainstem was transected just caudal to the level of the second spinal (hypoglossal) nerve and just rostral to cranial nerve (CN) V. Throughout the dissection, which required 40–50 min, the brainstem was superfused with a bicarbonate-containing artificial cerebrospinal fluid (CSF) of the following composition (in mmol l^{-1}): NaCl, 104; KCl, 4; $MgCl_2$, 1.4; D-glucose, 10; $NaHCO_3$, 25 and $CaCl_2$, 2.4, bubbled with 98 % O_2 and 2 % CO_2, pH 7.8, corresponding to the plasma pH of anuran amphibians (West *et al.* 1987).

Recording chamber

The brainstem was transferred to a superfusion recording chamber (total volume 0.50 ml) at room temperature (22–24 °C). Two superimposed disks (outer diameter 2.5 cm, thickness 1 mm) with central elliptical holes (2.0 cm×0.5 cm) partitioned the chamber into upper and lower compartments. Fine netting (1.0 mm×1.0 mm) spanning the holes was attached to the outer surface of the upper and lower discs, thereby creating a space between the netting for the brainstem. The brainstem was positioned between the two nets, ventral side

up. Artificial CSF was used as superfusate after being equilibrated with CO_2/O_2 gas mixtures in a tonometer at room temperature and was delivered into the chamber through an inflow aperture in the lower compartment at a rate of 10 ml min^{-1}. The superfusate was conducted from the opposite end of the upper compartment *via* a paper wick. Thus, the brainstem suspended between the upper and lower chambers was uniformly superfused both ventrally and dorsally. The atmosphere overlying the superfusate in the recording chamber was purged continuously with the same gas mixture as that flowing through the reservoir tonometer, thereby minimizing differences in gas mixture composition at the air–water interface. Measurements of the brainstem of stage 16–19 tadpoles (N=9) indicated medullary widths of 4.34±0.57 mm (mean ± S.D.) between CN V and X, with maximum dorsal–ventral thicknesses of 1.31±0.22 mm, thereby producing diffusion distances no greater than 655 μm.

Recording

Neural recordings of fictive gill and lung ventilation were obtained from the roots of cranial nerves V, VII and X using suction electrodes. The pipettes were made from 1 mm outer diameter, thin-walled borosilicate glass, pulled to a fine tip with a horizontal micropipette puller (Brown-Flaming, model P80), then broken and bevelled (Shöhli, Lapp-Technik) to achieve various inner tip diameters ranging from 90 to 350 μm. Action potentials were amplified (AM Systems no. 1700, Tektronix AM 502), filtered (100 Hz to 1 kHz) and recorded on video tape using a pulse code modulator (Neurodata no. 890). The signals were simultaneously averaged with a Paynter time averager, displayed on a polygraph (Gould) and digitized and analyzed on a Pentium PC (Datapac II software).

Experimental protocol

The central respiratory chemoresponsiveness of each of the four groups was evaluated by varying the P_{CO_2} of the superfusate. The superfusate was equilibrated in a tonometer with gas having a P_{CO_2} of 6.0, 4.7, 2.3, 1.3 or 0.5 kPa (balance O_2) to produce pH values of 7.4, 7.6, 7.8, 8.0 and 8.4, respectively. In preliminary experiments, superfusate pH in the tonometer measured using a macro-pH electrode (Cole-Parmer, E-05591-10) was compared with simultaneously measured superfusate pH in the recording chamber using a small glass electrode (Cole-Parmer, E-05991-60). The P_{CO_2} was adjusted until a stable measurement of the desired superfusate pH was recorded in the tonometer, and 2 min later, a shift in the recording chamber pH was observed, after which the recording chamber pH equalled reservoir tonometer pH to within ±0.01 pH units.

The experimental protocol began after the brainstem had been superfused in the recording chamber for at least 60 min at pH 7.8 and when recordings of nerve activities exhibited rhythmic bursting. Stable nerve activities were recorded for 10 min during randomly administered test solutions of pH 7.4, 7.6, 8.0 and 8.4 interspersed with 10 min baseline recordings at pH 7.8. After each 10 min recording, a 5 min equilibration period ensued to allow equalization of tonometer and recording chamber pH and stabilization of the brainstem response to the new target pH.

Analysis

For each of the four developmental groups, we analyzed the effects of superfusate pH on gill and lung ventilatory motor output from CN V, VII and X. The mean burst frequency and peak integrated amplitude for gills and lungs were measured for each 10 min recording period (pH 7.4, 7.6, 7.8, 8.0 and 8.4) with baseline recordings (pH 7.8) further averaged within each animal. Baseline recordings were made between each test period in order to quantify any drift that may have occurred during the course of the experiment. Mean values of fictive gill burst amplitude, frequency and respiratory output (frequency × amplitude) at pH 7.4, 7.6, 7.8 and 8.4 were then expressed as the percentage change from pH 8.0, and group means ± S.E.M. were calculated. A reference value of pH 8.0 was chosen since preliminary inspection of the data revealed linear increases in frequency, amplitude and respiratory output with reductions in pH from this point. The mean gill and lung frequency motor output values for each animal were used to calculate group means ± S.E.M. for each pH level. A two-way analysis of variance (ANOVA) was used to test for the significance of pH effects at different developmental stages. A Tukey test of pairwise multiple comparisons was utilized to test significant differences between individuals within treatment groups when statistically significant (P<0.05) interactions between pH and developmental stage occurred. The significance of pH effects within each developmental group was examined using a one-way ANOVA with repeated measures, with the criterion of statistical significance at P<0.05. When ANOVA revealed significant treatment effects, differences between individuals means within a developmental group were assessed for significance using the Dunnett's method of multiple comparisons.

Results

Patterns of respiratory motor output at pH 7.8

All animals (N=30) displayed rhythmic bursts of action potentials in CN V, VII and X when superfused with artificial CSF having a P_{CO_2} of 2.3 kPa and a pH of 7.8. While simultaneous recordings of all nerves were not successful in all animals, rhythmic bursts were observed in one or more of the nerves. All four developmental groups displayed two distinct patterns of cranial nerve activity. As shown in Fig. 1, typical integrated neurograms from CN VII and CN X of a stage 16 tadpole, oscillating slightly out of phase, demonstrate a high-frequency, low-amplitude bursting rhythm punctuated by high-amplitude, low-frequency bursts. According to the criterion of Gdovin *et al.* (1996), the high-frequency, low-amplitude bursting pattern was classified as fictive gill ventilation and the high-amplitude, low-frequency lung ventilation pattern as fictive lung ventilation on the basis of correlations between neural activity and respiratory

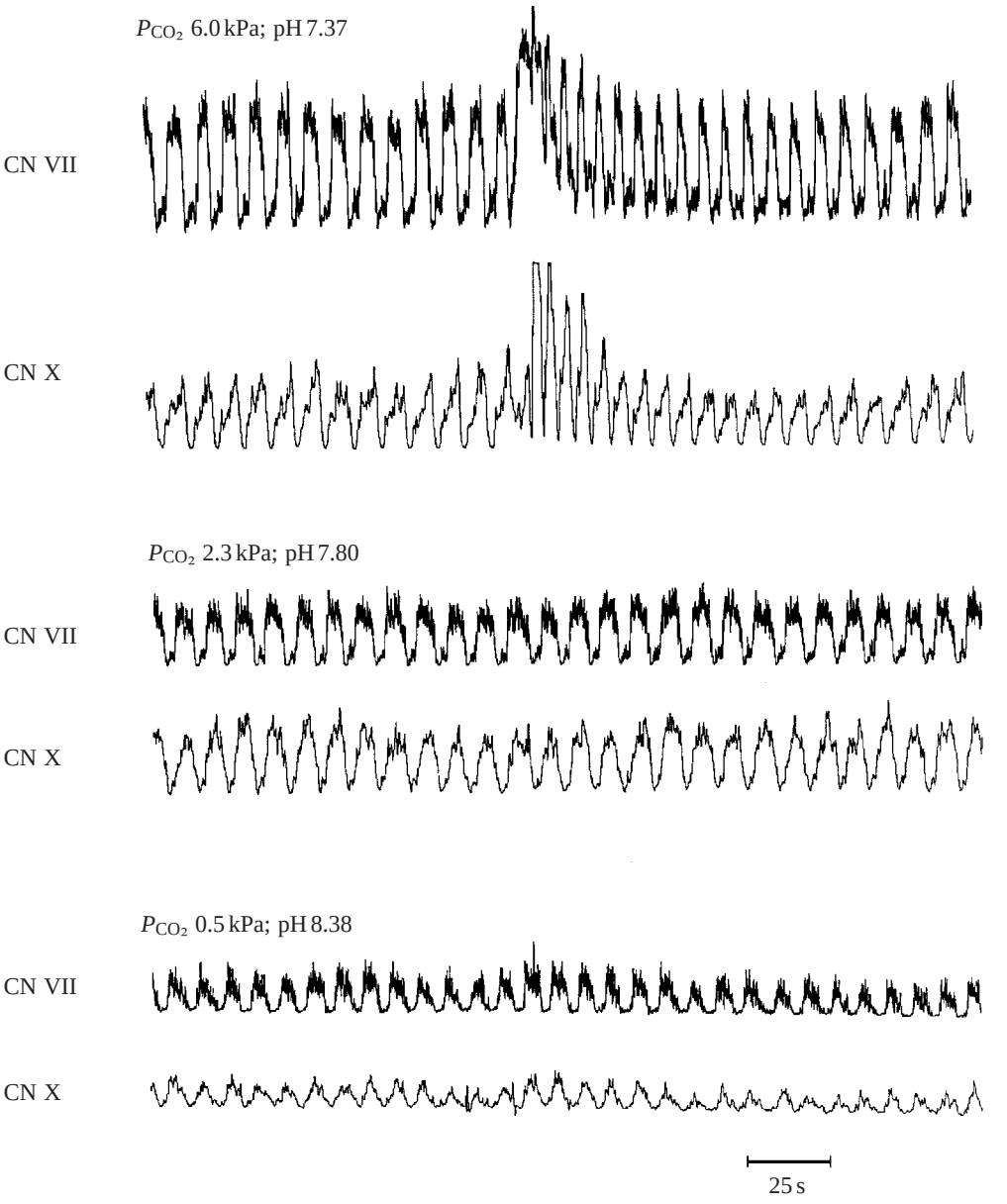

Fig. 1. Moving time average of cranial nerve (CN) VII and X gill and lung ventilatory motor output from a stage 16 *Rana catesbeiana* tadpole in response to changes in superfusate pH and P_{CO_2}. The height of the respiratory bursts was measured using arbitrary units.

mechanical events in the spontaneously breathing decerebrate tadpole.

The prevalence of fictive gill and lung respiratory activity under baseline conditions (pH 7.8) was contingent upon the developmental stage of the tadpole. As shown in Table 1, stages 3–9, 10–14 and 15–19 displayed rhythmic neural gill bursts with infrequent lung bursts occurring at irregular intervals. However, neural lung respiratory activity increased significantly (P<0.05) in tadpoles of stage 20–25 at lower pH values. Fictive lung ventilation commonly occurred in stages 3–9 and 10–14 as isolated bursts, whereas in older larvae (stages 15–19, 20–25) clusters of lung bursts appeared.

Effects of pH/P_{CO_2} on fictive gill ventilation

The response of fictive gill ventilation to increases in CO_2 varied with developmental stage. Figs 2–4 plot, respectively, the amplitude, frequency and respiratory output (amplitude × frequency) of CN VII and X gill ventilatory activity as a function of superfusate pH in four developmental groups. In the early developmental stages (3–9) (N=8), fictive gill ventilation showed no response in any output variable to reductions in pH when compared with pH 8.0, with one exception: fictive gill amplitude in CN X increased slightly, but significantly (P<0.05), at pH 7.6 and 7.4 (Fig. 2). In contrast, fictive gill ventilation in larvae of stage 10–14 (N=9) responded significantly in amplitude, frequency and respiratory output to hypercapnic superfusion, as illustrated in Figs 2–4. All these output variables in CN VII and X increased significantly (P<0.05) with reductions in pH to 7.6 and 7.4. Furthermore, CN X gill amplitude and respiratory output in stage 10–14 tadpoles increased significantly (P<0.05) at pH 7.4 and 7.6 compared with all other stages.

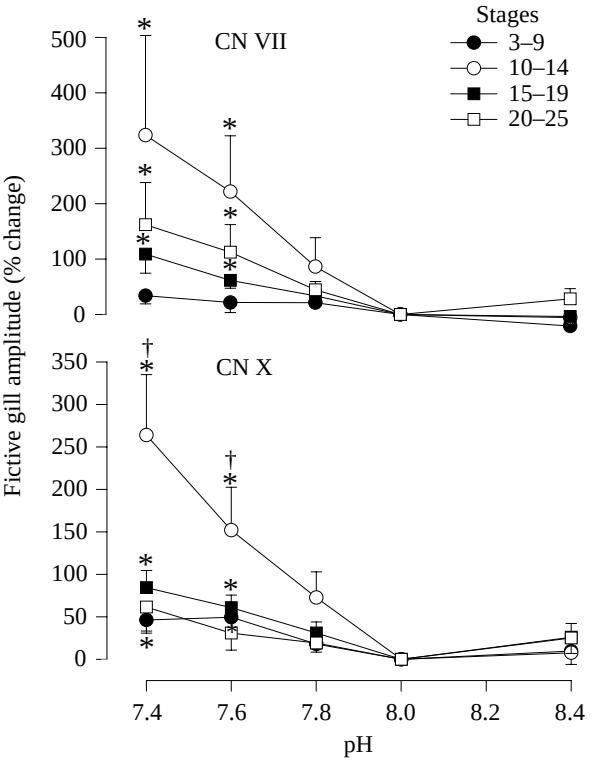

Fig. 2. Fictive gill amplitude of CN VII and X in response to changes in superfusate pH as a function of developmental stage. Values are expressed as mean relative change (%) from the value at pH 8.0. Data are shown as mean ± S.E.M., *N*=6–9 *Significantly different from the value at pH 8.0 (*P*<0.05); †significantly different from all other stages at the same pH level (*P*<0.05).

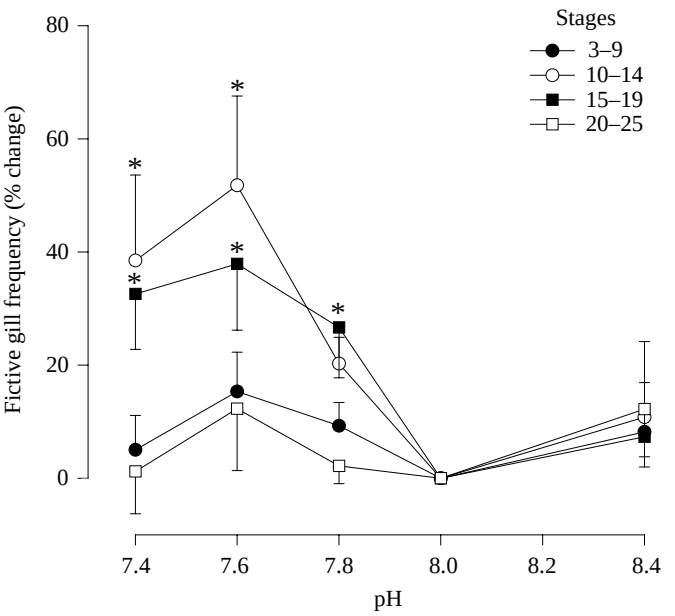

Fig. 3. Fictive gill frequency of both CN VII and X in response to changes in superfusate pH as a function of developmental stage. Values are expressed as mean relative change (%) from the value at pH 8.0. Data are shown as mean ± S.E.M., *N*=6–9. *Significantly different from the value at pH 8.0 (*P*<0.05).

Fictive gill ventilation in metamorphic larvae, stages 15–19 (*N*=7), also responded significantly to hypercapnic stimulation. As illustrated in Fig. 1, reduction of pH from 7.8 (P_{CO_2}=2.3 kPa) to 7.37 (P_{CO_2}=6.0 kPa) increased the amplitude of fictive gill respiration and induced the appearance of fictive lung bursting, while increasing pH to 8.38 (P_{CO_2}=5.3 kPa) decreased gill amplitude. Figs 2 and 3 demonstrate significant increases (*P*<0.05) in gill burst amplitude and frequency in both CN VII and X when pH was reduced to pH 7.6 and 7.4 and compared with pH 8.0. At pH 7.8, significant increases (*P*<0.05) in both CN VII and X were observed in fictive gill ventilation frequency only (Fig. 3). Correspondingly, fictive gill respiratory output increased significantly (*P*<0.05) at pH 7.8, 7.6 and 7.4 in CN VII and at pH 7.6 and 7.4 in CN X. Despite this stimulatory effect, however, fictive gill amplitude and respiratory output from CN X were significantly smaller (*P*<0.05) than that those observed in stages 10–14 at pH 7.4 and 7.6.

Stage 20–25 tadpole larvae (*N*=6) showed no fictive gill ventilatory response of any output variable to central hypercapnic challenge in CN X (Figs 2–4). By contrast, CN VII demonstrated small, but significant, increases (*P*<0.05) in amplitude and respiratory output at pH 7.6 and 7.4 compared with pH 8.0. The values for gill burst frequency of CN VII and X, listed in Table 1, further reflected developmental shifts in

Table 1. *Gill and lung ventilatory motor output frequency from cranial nerves VII and X in response to superfusate pH as a function of development*

	Developmental stage							
	3–9	10–14	15–19	20–25	3–9	10–14	15–19	20–25
pH	Fictive gill frequency (min^{-1})				Fictive lung frequency (min^{-1})			
8.4	45.15±3.14	30.31±5.47	37.64±3.92	41.85±10.65	0.20±0.09	0.18±0.07	0.03±0.03	0.03±0.03
8.0	43.04±3.37	29.04±6.32	34.67±2.63	38.40±10.92	0.30±0.14	0.16±0.06	0.06±0.04	0.03±0.03
7.8	46.50±3.29	33.94±6.21	42.97±2.74	38.46±9.79	0.31±0.11	0.17±0.06	0.10±0.06	1.69±1.17
7.6	48.68±3.54	39.84±7.59*	46.80±3.60*	42.95±9.04	0.28±0.13	0.18±0.08	0.31±0.22	5.13±2.79*,†
7.4	45.21±4.58	39.44±8.00*	45.29±3.90*	36.36±7.69	0.38±0.14	0.69±0.23	0.71±0.17*	8.23±2.92*,†

Data are shown as mean ± S.E.M., *N*=6–9.
*Significantly different from the value at pH 8.0 (*P*<0.05); †significantly different from all other stages at the same pH level (*P*<0.05).

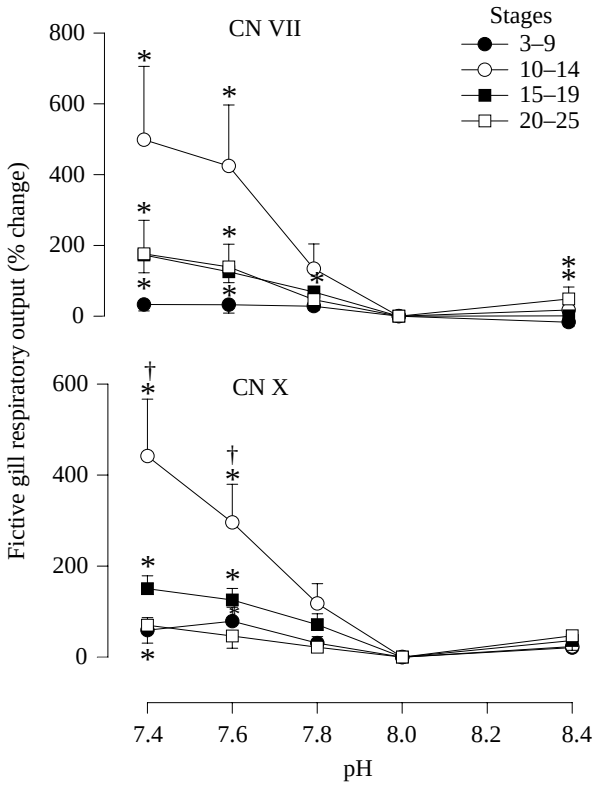

Fig. 4. Fictive gill respiratory output of CN VII and X showing the combined response of frequency and amplitude to changes in superfusate pH. Values are expressed as mean relative change (%) from the value at pH 8.0. Data are shown as mean ± S.E.M., N=6–9. *Significantly different from the value at pH 8.0 (P<0.05); †significantly different from all other stages at the same pH level (P<0.05).

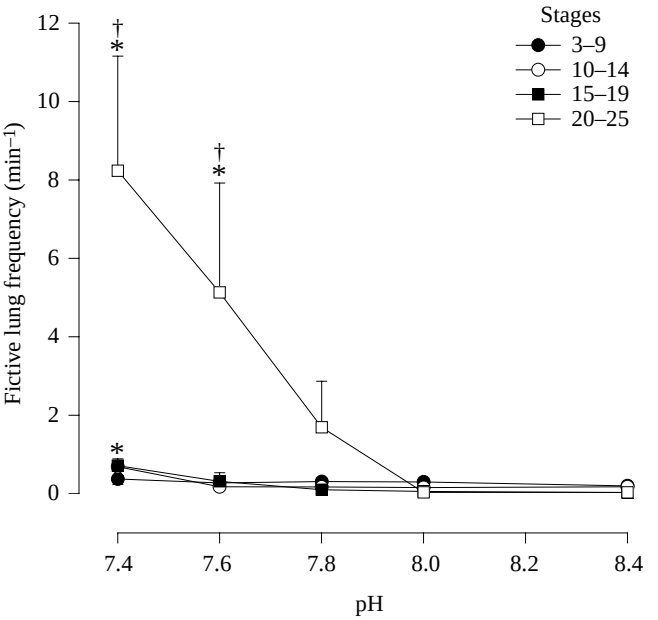

Fig. 5. Fictive lung frequency of both CN VII and X in response to changes in superfusate pH as a function of developmental stage. Data are shown as mean ± S.E.M., N=6–9. *Significantly different from the value at pH 8.0 (P<0.05); †significantly different from all other stages at the same pH level (P<0.05).

chemosensitivity, increasing significantly (P<0.05) at pH 7.6 and 7.4 in stages 10–14 and 15–19 compared with values at pH 8.0.

Effects of pH/P_{CO_2} on fictive lung ventilation

Fictive lung respiration, like fictive gill ventilation, exhibited a developmentally dependent response to decreases in superfusate pH. Fig. 5 and Table 1 illustrate the effects of pH variation (7.4–8.4) on the frequency of fictive lung ventilation in all four developmental stage groupings. Fictive lung frequency in stage 3–9 and 10–14 tadpoles was insensitive (P>0.05) to hypercapnia, while fictive lung burst frequency in stages 15–19 demonstrated a significant increase (P<0.05) at pH 7.4. Fictive lung frequency in stages 20–25 responded significantly (P<0.05) to pH reduction at pH 7.6 and 7.4 and was significantly (P<0.05) greater than fictive lung burst frequency in all other stages at these pH levels. In contrast, fictive lung amplitude remained relatively constant during development and appeared insensitive to pH reduction.

Discussion

In the phylogenetic progression from fish to birds and mammals, central respiratory chemosensitivity has been clearly established in amphibians (for reviews, see Smatresk, 1990; Milsom, 1995; Shelton *et al.* 1986). Comparative studies have documented the regulation of lung ventilation by central CO_2 chemoreceptor stimulation in adult frogs and toads

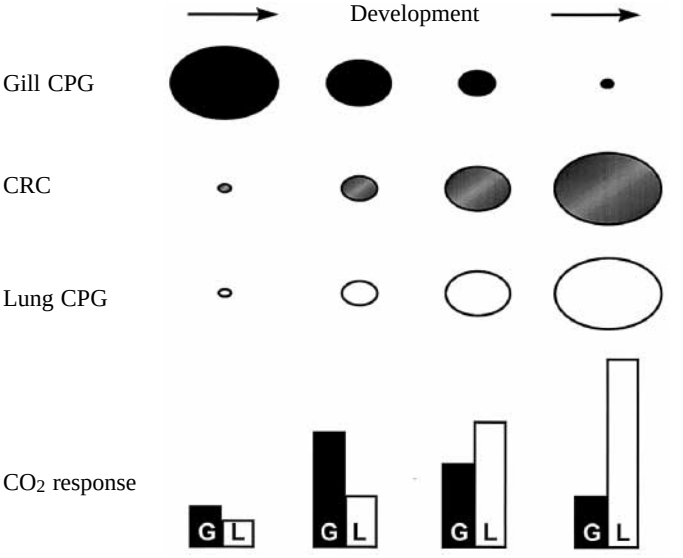

Fig. 6. A diagram of a possible model for the ontogeny of neural respiratory function. Concomitant with a developmental increase in the role of central respiratory chemoreceptors (CRCs), gill central pattern generator (CPG) functionality gradually decreases in strength while lung central pattern generator function increases. Differential responses of these ventilatory rhythmicities to CO_2 predict a maximal gill (G) response at intermediate stages and a peak response in lung (L) ventilatory output at the oldest stages.

(Branco *et al.* 1992; Smatresk and Smits, 1991; Kogo *et al.* 1994; Kogo and Remmers, 1994; McLean *et al.* 1995*a*,*b*). The influence of central chemoreceptive function on gill ventilation, however, is largely unknown. The present investigation has demonstrated the existence of central respiratory chemoreceptors in the *in vitro* brainstem of anuran tadpoles that exert a developmentally dependent influence on fictive gill and lung ventilation. Our results show that hypercapnia has a significant influence on fictive gill ventilation in tadpoles of intermediate developmental stages (10–14 and 15–19) and that significant hypercapnic stimulation of fictive lung ventilation occurs in older animals (stages 15–19 and 20–25).

In the isolated *in vitro* brainstem preparation from *Rana catesbeiana*, we have used whole nerve recordings of fictive gill and lung motor output from the trigeminal (Vth), facial (VIIth) and vagal (Xth) nerve roots to evaluate the ontogeny of central chemoreception. These nerves have previously been shown to innervate antagonistic muscle groups of the buccal and pharyngeal cavity involved in amphibian gill and lung ventilation (Gradwell and Walcott, 1971; DeJongh and Gans, 1969; Sakakibara, 1984; West and Jones, 1975) and to display bursts of coordinated rhythmic activity corresponding to fluctuations in buccal pressure, lung pressure and bursts of buccal levator EMG activity in decerebrate, spontaneously breathing tadpoles (Gdovin *et al.* 1996).

Regulation of fictive gill ventilation

For the brainstems of immature larvae (stages 3–9), reductions in the pH of the superfusate had no general effect on gill ventilatory motor output; fictive gill burst frequency and amplitude of CN VII were statistically unresponsive to hypercapnia. Gill amplitude recorded from CN X, however, showed small but significant increases in response to a reduction in superfusate pH (7.6, 7.4). This finding agrees with the results of Infantino (1990, 1992), who showed that hypercapnia had no effect on gill frequency in freely swimming, stage 4–7 tadpoles. Thus, it appears that central or peripheral respiratory chemoreceptors in stages 3–9 play little role in driving gill ventilatory output. This finding may be attributed to a sparse distribution of medullary respiratory chemoreceptors in early larvae, thereby decreasing the overall drive to respiratory pattern generators or reducing chemoreceptive synaptic connectivities. Despite the apparent lack of central or peripheral CO_2 regulation of gill ventilation in stage 3–9 larvae, previous studies by Burggren and Doyle (1986) and Infantino (1992) on intact larvae indicate strong hypoxic gill ventilatory drive during this period as well as apnoea following hyperoxic exposure.

In pre-metamorphic larvae (stages 10–14), gill ventilation displayed a vigorous response to central hypercapnic stimulation. Gill frequency and amplitude increased significantly when the brainstem preparation was exposed to a superfusate pH of 7.6 and 7.4. Furthermore, gill amplitude and respiratory output at pH 7.4 and 7.6 in CN X were significantly greater than at all other stages. By contrast, ontogenetic studies of intact stage 9–14 tadpoles by Infantino (1992) showed that mild hypercapnia (P_{CO_2}=1.6 kPa) produced no significant difference in the frequency of gill ventilation compared with baseline P_{CO_2} levels (<0.3 kPa) before metamorphosis (stage 16), while the response of gill frequency to severe hypoxia (P_{O_2}=4.0 kPa) was maximal (Burggren and Doyle, 1986). The discrepancies between the results of Infantino (1992) and those of the present investigation may be due to differences in the level of CO_2 challenge (the *in vitro* preparation also showed no significant difference in fictive gill frequency when exposed to artificial CSF at a P_{CO_2} of 2.3 kPa) or the differences may reflect dissimilarities in intact and isolated preparations. During this period of development, the gills are the second major site of oxygen uptake and CO_2 elimination (approximately 40 %) after the skin (Burggren and West, 1982), so it is not surprising that buccal motor output, which ultimately produces ventilation of the gills, can be regulated in response to central chemoreceptor stimulation.

By stage 16 of development, tadpoles begin to undergo metamorphosis, reaching climax by stage 18–19. During this time, the gills, lung and skin all contribute to O_2 and CO_2 exchange and the pattern of ventilation shifts from gills to lungs as gill regression begins and pulmonary respiratory efforts become regular (Burggren and West, 1982). In metamorphic stages 15–19, we observed significant increases in the amplitude and frequency of gill ventilation. This response contrasts with the results generated in previous studies by Infantino (1992). Using intact metamorphic tadpoles, Infantino (1992) demonstrated that aquatic hypercapnia produced significant depression of gill ventilation frequency, concluding that such a response must serve to reduce the uptake of CO_2 across the gills. These discrepancies may reflect differences in the level of hypercapnic stimulation, differences between intact and *in vitro* preparations or differences between central and peripheral CO_2 chemoreceptor influences on gill ventilation.

Although a significant hypercapnic influence on gill ventilation was observed in stage 15–19 brainstem preparations in the present study, gill amplitude and respiratory output in CN X responded significantly less than that observed in pre-metamorphic animals (stages 10–14). These results suggest a progressive decrease in central hypercapnic chemoreceptive regulation of gill ventilation prior to reabsorption of the gills. Similarly, Burggren and Doyle (1986) found gill frequency unresponsive to hypoxia after stage 16, indicating a reduction in hypoxic reflex regulation of gill ventilation as branchial regression was initiated. Together, these studies imply that, as metamorphosis proceeds, reflexes regulating CO_2 and O_2 levels are shifted away from buccal pattern generators.

In stage 20–25 post-metamorphic tadpoles, fictive gill ventilatory output was insensitive to central hypercapnic challenge with one exception: the amplitude of gill motor output in CN VII showed small, significant increases during reductions in the pH of the superfusate. After stage 20, rapid regression of the internal gill and operculum occurs, with

complete regression by stage 24 (Taylor and Köllros, 1946; Atkinson and Just, 1975). Thus, the overall reductions in frequency and amplitude observed in the present study are not surprising and confirm similar studies in intact tadpoles (Infantino, 1992), which concluded that post-metamorphic gill ventilatory reflexes are insignificant.

Although the response of gill ventilation to CO_2 has been previously described in water- and air-breathing fishes and larval amphibians, the role of central chemoreceptors has not been defined (Shelton *et al.* 1986; Milsom, 1995). Gill ventilation of intact water- and air-breathing fishes has been shown to respond modestly to aquatic hypercapnia, although the specific site of action has not been identified (Johansen, 1970; Randall and Jones, 1973; Janssen and Randall 1975; Eddy, 1976; Hargis, 1976; Dively *et al.* 1977; Cameron, 1978; Thomas and LeRuz, 1982; Thomas, 1983; Heisler *et al.* 1988; Wood *et al.* 1990; Aoto *et al.* 1990; Kinkead and Perry, 1991). Acidic superfusion of the isolated brains of carp, lamprey and lungfish has also been shown to produce no significant change in gill ventilation (Hughes and Shelton, 1962; Rovainen, 1977). Walker *et al.* (1990) showed in an *in vitro* tadpole brainstem–spinal cord preparation displaying fictive gill and lung ventilation that gill frequency was insensitive to hypercapnic superfusion (pH 7.2–7.8), suggesting a lack of central chemoreceptors. Thus, the present study provides the first unequivocal demonstration of central CO_2 chemoreceptors influencing gill ventilation in fish or larval amphibians.

Regulation of fictive lung ventilation

Despite the presence of lungs by stage 3 of development (Atkinson and Just, 1975; Burggren and Mwalukoma, 1983; Burggren, 1989), pulmonary ventilation in normoxic water at 25 °C occurs irregularly until metamorphic stages are reached (Burggren, 1984; Helff, 1932; Infantino, 1992; Burggren and Doyle, 1986; Burggren and Infantino, 1994). Previous studies by Helff (1932) indicated that the lungs are not essential prior to metamorphosis in *Rana pipiens* and *Rana catesbeiana* (Just *et al.* 1973). Significant stimulation of fictive lung frequency in our *in vitro* brainstem preparation by exposure to strong central hypercapnic challenge (pH 7.4) did not occur until developmental stages 15–19. These results contrast directly with those documented by Walker *et al.* (1990), who found, in an isolated metamorphic tadpole brainstem–spinal cord preparation, complete insensitivity of fictive lung ventilation to central hypercapnic stimulation. Since the tadpole developmental stage and P_{CO_2} level were not reported in this study, the source of the discrepancy is not apparent. Our results accord with studies by Infantino (1992) showing that the frequency of lung ventilation in intact tadpoles was significantly increased by increases in CO_2 levels during metamorphosis. An investigation of amphibian pulmonary O_2 and CO_2 exchange by Burggren and West (1982) showed that during stages 16–19 the lungs account for 20 % of total O_2 uptake, increasing to 70–80 % after metamorphic climax. Metamorphosis, therefore, marks the initiation of regular pulmonary ventilation, which becomes increasingly dominant with development (Burggren and Infantino, 1994).

During post-metamorphic stages (20–25), fictive lung frequency in the *in vitro* brainstem preparation increased significantly in response to hypercapnia and differed significantly from hypercapnic responses at all other stages. This response confirms previous studies by Infantino (1992) in intact tadpoles at a similar stage, as well as in adult bullfrogs (Smyth, 1939; Jackson and Braun, 1979; McLean *et al.* 1995*a*). Overall, it appears that central chemoreceptive reflexes regulating CO_2 are shifted towards the regulation of fictive lung ventilation once metamorphosis begins.

Ontogenetic coordination of central gill and lung ventilatory regulation

The present study of *Rana catesbeiana* has shown that stimulation of central chemoreceptors initiates changes in reflexes modulating the amplitude and frequency of respiratory motor output and that these are transferred from the gill to the lungs as development proceeds. Stimulation of fictive gill ventilation during central hypercapnia peaks in the intermediate stages of larval development (stages 10–14), declining from this point as fictive lung ventilation becomes increasingly sensitive to central pH stimulation. Furthermore, stimulation of central chemoreceptors generates significant changes in both gill and lung motor output only during a brief metamorphic period (stage 15–19). Hence, the emergence of central respiratory chemoreception of pulmonary function immediately precedes the disappearance of gill central respiratory chemoreceptor regulation. Such patterns have also been observed in ontogenetic studies of hypoxic sensitivity in intact tadpole larvae (Burggren and Doyle, 1986). A parsimonious model that may account for the complex developmental transitions of neural respiratory function in anuran larvae is shown in Fig. 6. This model is based on the parallel developmental changes in three functional entities: central respiratory chemoreception (CRC), the gill central pattern generator (CPG) and the lung CPG. Early in development, the gill CPG is highly functional and rhythmic branchial oscillations for gill ventilation dominate respiratory output, while lung central CPG output is nascent. As development proceeds, lung CPG output gradually becomes more functional and gill CPG output is reduced. In later stages of neural ventilatory development, the lung CPG function dominates while the expression of gill CPG function is minimal. Concomitant with the ontogenetic modulation of ventilatory pattern generation, central respiratory chemoreceptive drive progressively increases. Therefore, central chemoreceptive reflexes modulating gill CPGs should be most sensitive at intermediate stages of development and those regulating lung CPGs should peak at later stages.

Although clear evidence has been presented demonstrating the developmental role of central respiratory chemoreceptors in amphibian larvae, further studies are needed to determine the location, distribution, biophysiological characteristics and plasticity of such receptors during development. The *in vitro*

tadpole brainstem may represent an ideal model for such studies as well as for those investigating other mechanisms of neurorespiratory control.

References

AOTA, S., HOLMGREN, K. D., GALLAUGHER, P. AND RANDALL, D. J. (1990). A possible role of catecholamines in the ventilatory responses associated with internal acidosis or external hypoxia in rainbow trout *Oncorhynchus mykiss*. *J. exp. Biol.* **151**, 57–70.

ATKINSON, B. G. AND JUST, J. J. (1975). Biochemical and histological changes in the respiratory system of *Rana catesbeiana* larvae during normal and induced metamorphosis. *Dev. Biol.* **45**, 151–165.

BRANCO, L. G. S., GLASS, M. L. AND HOFFMAN, A. (1992). Central chemoreceptor drive to breathing in unanesthetized toads, *Bufo paracnemis*. *Respir. Physiol.* **87**, 195–204.

BURGGREN, W. W. (1984). Transition of respiratory processes during amphibian metamorphosis: from egg to adult. In *Respiration and Metabolism of Embryonic Vertebrates* (ed. R. J. Seymour), pp. 31–50. Boston, MA: Dr W. Junk Publishers.

BURGGREN, W. W. (1989). Lung structure and function: Amphibians. In *Comparative Pulmonary Physiology: Current Concepts* (ed. S. C. Wood), pp. 153–192. New York: Marcel Dekker.

BURGGREN, W. W. AND DOYLE, M. (1986). Ontogeny of the regulation of gill and lung ventilation in the bullfrog, *Rana catesbeiana*. *Respir. Physiol.* **66**, 279–291.

BURGGREN, W. W. AND INFANTINO, R. L., JR (1994). The respiratory transition from water to air breathing during amphibian metamorphosis. *Am. Zool.* **34**, 238–246.

BURGGREN, W. W. AND MWALUKOMA, A. (1983). Influence of chronic hypoxia and hyperoxia on gas exchange processes in larval and adult bullfrogs (*Rana catesbeiana*). I. Morphological responses of the lungs, skin and gills. *J. exp. Biol.* **105**, 191–203.

BURGGREN, W. W. AND WEST, N. H. (1982). Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog, *Rana catesbeiana*. *Respir. Physiol.* **47**, 151–164.

CAMERON, J. N. (1978). Regulation of blood pH in teleost fish. *Respir. Physiol.* **33**, 129–144.

DEJONGH, H. J. (1968). Functional morphology of the jaw apparatus of larval and metamorphosing *Rana temporaria*. *Neth. J. Zool.* **18**, 1–103.

DEJONGH, H. J. AND GANS, C. (1969). On the mechanism of respiration in the bullfrog, *Rana catesbeiana*. A reassessment. *J. Morph.* **127**, 259–290.

DEJOURS, P. (1988). *Respiration in Water and Air: Adaptations – Regulation – Evolution*. Amsterdam, New York, Oxford: Elsevier.

DIVELY, J. L., MUDGE, J. E., NEFF, W. H. AND ANTHONY, A. (1977). Blood P_{O_2}, P_{CO_2} and pH changes in brook trout (*Salvelinus fontinalis*) exposed to sublethal levels of acidity. *Comp. Biochem. Physiol.* **57**A, 347–351.

EDDY, F. B. (1976). Acid–base balance in rainbow trout (*Salmo gairdneri*) subjected to acid stresses. *J. exp. Biol.* **64**, 159–171.

EZURE, K. (1990). Synaptic connections between medullary respiratory neurons and considerations on the genesis of respiratory rhythm. *Prog. Neurobiol.* **35**, 429–450.

FELDMAN, J. L. AND SMITH, J. C. (1989). Cellular mechanisms underlying modulation of breathing pattern in mammals. *Ann. N.Y. Acad. Sci.* **563**, 114–130.

FELDMAN, J. L., SMITH, J. C., ELLENBERGER, H. H., CONNELLY, C. A., LIU, G. S., GREER, J. J., LINDSAY, A. D. AND OTTO, M. R. (1990). Neurogenesis of respiratory rhythm and pattern: emerging concepts. *Am. J. Physiol.* **259**, R879–R886.

GANS, C. (1970). Strategy and sequence in the evolution of the external gas exchangers of ectothermal vertebrates. *Forma. Functio.* **3**, 61–104.

GDOVIN, M. J., TORGERSON, C. S. AND REMMERS, J. E. (1996). Characterization of gill and lung ventilatory activities of cranial nerves in the spontaneously breathing tadpole, *Rana catesbeiana*. *Physiologist* **39**, 189.

GRADWELL, N. (1972*a*). Gill irrigation in *Rana catesbeiana*. I. On the anatomical basis. *Can. J. Zool.* **50**, 481–499.

GRADWELL, N. (1972*b*). Gill irrigation in *Rana catesbeiana*. II. On the musculoskeletal mechanism. *Can. J. Zool.* **50**, 501–521.

GRADWELL, N. AND WALCOTT, B. (1971). Dual function and structural properties of the interhyoideus muscle of the bullfrog tadpole (*Rana catesbeiana*). *J. exp. Biol.* **176**, 193–197.

HARGIS, J. R. (1976). Ventilation and metabolic rate of young rainbow trout (*Salmo gairdneri*) exposed to sublethal environment pH. *J. exp. Zool.* **196**, 39–44.

HEISLER, N., TOEWS, D. P. AND HOLETON, G. F. (1988). Regulation of ventilation and acid–base status in the elasmobranch *Scyliorhinus stellaris* during hyperoxia-induced hypercapnia. *Respir. Physiol.* **71**, 227–246.

HELFF, O. M. (1932). Studies on amphibian metamorphosis. X. Hydrogen-ion concentration of the blood of anuran larvae during involution. *Biol. Bull. mar. biol. Lab., Woods Hole* **63**, 405–418.

HUGHES, G. M. AND SHELTON, G. (1962). Respiratory mechanisms and their nervous control in fish. *Adv. comp. Physiol. Biochem.* **1**, 275–364.

INFANTINO, R. L. (1990). Ventilatory responses to inspired gas variation in larval bullfrogs. *Physiologist* **33**, A35.

INFANTINO, R. L. (1992). Ontogeny of ventilatory regulation in the bullfrog *Rana catesbeiana*. PhD thesis, University of Massachusetts, Amherst, MA, USA.

JACKSON, D. C. AND BRAUN, B. A. (1979). Respiratory control in bullfrogs: cutaneous *versus* pulmonary response to selective CO_2 exposure. *J. comp. Physiol.* **129**, 339–342.

JANSSEN, R. G. AND RANDALL, D. J. (1975). The effects of changes in pH and P_{CO_2} in blood and water on breathing in rainbow trout *Salmo gairdneri*. *Respir. Physiol.* **25**, 235–245.

JOHANSEN, K. (1970). Cardiorespiratory adaptations in the transition from water breathing to air breathing. *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 1118–1119.

JUST, J. J., GATZ, R. N. AND CRAWFORD, E. C., JR (1973). Changes in respiratory functions during metamorphosis of the bullfrog, *Rana catesbeiana*. *Respir. Physiol.* **17**, 276–282.

KINKEAD, R. AND PERRY, S. F. (1991). The effects of catecholamines on ventilation in rainbow trout during hypoxia or hypercapnia. *Respir. Physiol.* **84**, 77–92.

KOGO, N., PERRY, S. F. AND REMMERS, J. E. (1994). Neural organization of the ventilatory activity of the frog, *Rana catesbeiana*. I. *J. Neurobiol.* **25**, 1067–1079.

KOGO, N. AND REMMERS, J. E. (1994). Neural organization of the ventilatory activity in the frog, *Rana catesbeiana*. II. *J. Neurobiol.* **25**, 1080–1094.

LITTLE, C. (1983). *The Colonization of Land: Origins and Adaptations of Terrestrial Animals*. Cambridge; New York: Cambridge University Press.

MCLEAN, H. A., KIMURA, N., KOGO, N., PERRY, S. F. AND REMMERS, J. E. (1995*a*). Fictive respiratory rhythm in the isolated brainstem of frogs. *J. comp. Physiol.* **176**, 703–713.

MCLEAN, H. A., PERRY, S. F. AND REMMERS, J. E. (1995*b*). Two regions in the isolated brainstem of the frog that modulate respiratory-related activity. *J. comp. Physiol.* **177**, 135–144.

MCMAHON, W. K. (1969). A functional analysis of the aquatic and aerial respiratory movements of an African lungfish, *Protopterus aethiopicus*, with reference to the evolution of the lung-ventilation mechanisms in vertebrates. *J. exp. Biol.* **51**, 407–430.

MILSOM, W. K. (1995). Regulation of ventilation in lower vertebrates: role of CO_2/pH chemoreceptors. In *Advances in Environmental and Comparative Physiology* (ed. R. Gilles), pp. 1–74. Berlin: Springer Verlag.

RANDALL, D. J. AND JONES, D. R. (1973). The effect of deafferentation of the pseudobranch on respiratory response to hypoxia and hyperoxia in the trout (*Salmo gairneri*). *Respir. Physiol.* **17**, 291–301.

RANDALL, H., BURGGREN, W. W. AND HASWELL, M. S. (1981). *The Evolution of Air Breathing in Vertebrates*. Cambridge; New York: Cambridge University Press.

RICHTER, D. W. (1982). Generation and maintenance of the respiratory rhythm. *J. exp. Biol.* **199**, 93–107.

ROVAINEN, C. M. (1977). Neural control of ventilation in the lamprey. *Fedn Proc. Fedn Am. Socs exp. Biol.* **36**, 2386–2389.

SAKAKIBARA, Y. (1984). The pattern of respiratory nerve activity in the bullfrog. *Jap. J. Physiol.* **34**, 269–282.

SHELTON, G., JONES, D. R. AND MILSOM, W. K. (1986). Control of breathing in ectothermic vertebrates. In *Handbook of Physiology*, section 3, *The Respiratory System*, vol. II, *Control of Breathing*, part II (ed. A. P. Fishman, N. S. Cherniak, J. G. Widdicombe and S. R. Geiger), pp. 857–909. Bethesda: American Physiological Society.

SMATRESK, N. J. (1990). Chemoreceptor modulation of endogenous respiratory rhythms in vertebrates. *Am. J. Physiol.* **259**, R887–R897.

SMATRESK, N. J. AND SMITS, A. W. (1991). Effects of central and peripheral chemoreceptor stimulation in ventilation in the marine toad, *Bufo marinus*. *Respir. Physiol.* **83**, 223–238.

SMYTH, D. M. (1939). The central and reflex control of respiration in the frog. *J. Physiol., Lond.* **95**, 305–327.

TAYLOR, A. C. AND KÖLLROS, J. (1946). Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.* **94**, 7–24.

THOMAS, S. (1983). Changes in blood acid–base balance in trout (*Salmo gairdneri*) following exposure to combined hypoxia and hypercapnia. *J. comp. Physiol.* B **152**, 53–57.

THOMAS, S. AND LERUZ, H. (1982). A continuous study of rapid changes in blood acid–base status of trout during variations of water P_{CO_2}. *J. comp. Physiol.* B **148**, 123–130.

WALKER, R., GALANTE, R. J., FISHMAN, A. P. AND PACK, A. I. (1990). Effect of GABA on gill and lung ventilation in an *in vitro* isolated brainstem preparation in the tadpole. *Physiologist* **33**, A35.

WEST, N. H. AND BURGGREN, W. W. (1983). Reflex interactions between aerial and aquatic gas exchange organs in the larval bullfrog. *Am. J. Physiol.* **244**, R770–R777.

WEST, N. H. AND JONES, D. R. (1975). Breathing movements in the frog *Rana pipiens*. I. The mechanical events associated with lung and buccal ventilation. *Can. J. Zool.* **53**, 332–344.

WEST, N. H., TOPOR, Z. L. AND VAN VLIET, B. N. (1987). Hypoxemic threshold for lung ventilation in the toad. *Respir. Physiol.* **70**, 377–390.

WOOD, C. M., TURNER, J. D., MUNGER, R. S. AND GRAHAM, M. S. (1990). Control of ventilation in the hypercapnic skate *Raja oscellata*. II. Cerebrospinal fluid and intracellular pH in the brain and other tissue. *Respir. Physiol.* **80**, 279–298.