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GILL AND LUNG VENTILATORY RESPONSES TO STEADY-STATE AQUATIC HYPOXIA AND HYPEROXIA IN THE BULLFROG TADPOLE

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Abstract. Gill ventilation frequency (f_G), the pressure amplitude (PBC) and stroke volume (Vs) of buccal ventilation cycles, the frequency of air breaths (f_L), water flow over the gills ($\dot{V}_W$), gill oxygen uptake ($\dot{M}_{G_{O_2}}$), oxygen utilization (U), and heart frequency (f_H) have been measured in unanaesthetized, air breathing *Rana catesbeiana* tadpoles (stage XVI–XIX). The animals were unrestrained except for ECG leads or cannulae, and were able to surface voluntarily for air breathing. They were subjected to aquatic normoxia, hyperoxia, and three levels of aquatic hypoxia, and their respiratory responses recorded in the steady state. The experiments were performed at 20 ± 0.5 °C.

In hyperoxia there was an absence of air breathing, and f_G, PBC and $\dot{V}_W$ fell from the normoxic values, while U increased, resulting in no significant change in $\dot{M}_{G_{O_2}}$. Animals in normoxia showed a very low f_L which increased in progressively more hypoxic states. $\dot{V}_W$ increased from the normoxic value in mild hypoxia (P_{O_2} = 96 ± 2 mm Hg), but fell, associated with a reduction in PBC, in moderate (P_{O_2} = 41 ± 1 mm Hg) and severe (P_{O_2} = 21 ± 3 mm Hg) hypoxia in the presence of lung ventilation. Gill $\dot{M}_{G_{O_2}}$ was not significantly different from the normoxic value in mild hypoxia but fell in moderate hypoxia, while in severe hypoxia oxygen was lost to the ventilating water from the blood perfusing the gills. There was no significant change in f_H from the normoxic value in either hypoxia or hyperoxia.

These data indicate, that in the bimodally breathing bullfrog tadpole, aquatic P_{O_2} exerts a strong control over both gill and lung ventilation. Furthermore, there is an interaction between gill and lung ventilation such that the onset of a high frequency of lung ventilation in moderate and severe hypoxia promotes a suppression of gill ventilation cycles.

Air breathing — Gill respiration
Aquatic respiration — Skin respiration
Bimodal breathing — Tadpole
Control of breathing — Ventilation

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Bullfrog tadpoles (stage XVI–XIX) rely on the lungs, gills and skin for gas exchange, and are obligate air-breathers in normoxia at 20 °C (Burggren and West, 1982). They may remain in the larval stage, breathing both air and water, for up to three years in the northern part of their range (Oliver, 1965). Bullfrog tadpoles typically live in ditches and pools rich in aquatic vegetation, where photosynthetic activities during all but winter days result in a net production of oxygen and uptake of carbon dioxide. Conversely, at night, the P_{O_2} falls, with a concomitant increase in P_{CO_2} due to the aerobic respiration of both plants and animals. Thus, afternoon P_{O_2}'s in excess of 450 mm Hg have been recorded for such pools in June, falling to 10 mm Hg in the early hours of the morning (Jones, 1961; Dejours, 1976). If bullfrog tadpoles are to maintain aerobic homeostasis under these conditions of large diurnal changes in aquatic P_{O_2}'s, they must then make appropriate integrated adjustments to the ventilation of both the gills and lungs.

Nothing is known of the chemical control of ventilation in larval anuran amphibians, such as the bullfrog tadpole, although recent evidence supports older work in suggesting that peripheral chemoreceptor sites, sensitive to O_2, are present in the carotid arteries of adult bullfrogs (Smyth, 1939; Ishii *et al.*, 1966; Lillo, 1980). A demonstration of respiratory chemo-sensitivity in a larval anuran would not only shed light on the respiratory adaptations of these animals to a diurnally fluctuating respiratory environment, but would also contribute substantially to our knowledge of the control of anuran respiration during ontogeny. Amphibian metamorphosis may additionally be useful as a model for the changes in respiratory control mechanisms which may have occurred in the evolution of terrestrial vertebrates.

This paper represents an initial step in our attempts to elucidate the chemical control of respiration in the larval anuran by investigating the respiratory responses to steady state hypoxia and hyperoxia in bimodally breathing bullfrog tadpoles.

Methods

Experiments were performed on 14 *Rana catesbeiana* tadpoles at stage XVI–XIX, according to the staging system of Taylor and Kollros (1946). These animals possessed functional gills and lungs. The tadpoles were collected in western Massachusetts, and maintained at 20 ± 0.5 °C in aerated, running water for at least two weeks before the experiments were performed. The tadpoles were fed boiled spinach *ad libitum*.

For experimentation the tadpoles were divided into 2 groups of seven animals. All animals were anesthetized in MS222 (1 : 10,000) and weighed. In animals of both groups, a cannula of PE10 tubing was introduced into the buccal cavity *via* one narial opening. The end of the cannula was heat-flared to form a flange which was pulled snug to the interior narial opening. The cannula was water-filled and buccal pressure and respiratory frequency were measured using a Statham P23AA pressure transducer, zeroed to the water surface of the experimental chamber. In

addition to the narial cannula, in one group of animals fine insulated wires were sewn into the ventral abdomen in order to record the ECG, while in the other experimental group the opercular spout was cannulated with flexible silicone rubber tubing (O.D., 1.5 mm; I.D., 1.2 mm), as described by Gradwell (1970).

Both groups of animals were allowed to recover from the anesthesia and were then transferred to individual glass experimental chambers containing 600 ml of conditioned, aerated Amherst tap water, at 20 ± 0.5 °C. In animals of the first group the signal from the pressure transducer was amplified by a Grass 7P1B DC amplifier, while the ECG wires were led to a Grass 7P4A amplifier. Signals were recorded on a Grass 79 2-channel pen recorder, writing on curvilinear coordinates.

In both types of preparation, the tadpoles could surface to breathe air at will, and were unrestrained except for the cannulae and/or ECG leads. An opaque screen separated the tadpole from the experimenters.

The P_{O_2} of the water in the experimental chamber was controlled by bubbling appropriate mixtures of air and nitrogen, or oxygen, into the chamber through an airstone. Gas mixtures were carefully adjusted by means of a pair of calibrated flow meters. Five levels of aquatic P_{O_2} were used, ranging from hyperoxia, $P_{O_2} = 651 \pm 12$ mm Hg, to severe hypoxia, $P_{O_2} = 21 \pm 3$ mm Hg. All tadpoles were subjected to all five levels presented in random order over the course of a day, with a return to normoxia between any two consecutive conditions. The animals were exposed to each experimental level, including normoxia, for 1 hour and to an intervening normoxic condition for 0.5 hour before the conditions were changed. This ensured a return to steady state normoxic conditions between consecutive experimental conditions, as judged by the measured variables. Only those parts of the recordings in which the measured variables represented steady-state responses to the experimental conditions were analysed, typically starting after 0.25 hour of exposure to any condition.

The P_{O_2} of chamber water (PI_{O_2}) and of expired water (PE_{O_2}) was measured with either an Instrumentation Laboratory 113, or Micro 13 blood gas analyzer regulated to 20 ± 0.1 °C. Water samples were collected anaerobically in 1-ml glass syringes, in the case of the inspired sample from directly in front of the jaws of the respiring tadpole, and in the case of the expired sample from the spout cannula. The flow of water over the gills, ($\dot{V}W$), was calculated by collecting expired water in a 5-ml pre-weighed beaker for 3 min. Oxygen consumption ($\dot{M}G_{O_2}$) of the gills was calculated on a per body weight basis from the difference in PI_{O_2} and PE_{O_2}, using the solubility constant for oxygen in water at 20 °C. Utilization of oxygen by the gills (U) was calculated from the relationship:

$$U = \frac{PI_{O_2} - PE_{O_2}}{PI_{O_2}} \times 100$$

Five determinations were made in each experimental period.

All data were analyzed to provide means and standard errors. ANOVA was used to test for treatment effects while differences between specific experimental

means and the normoxic mean were assessed with Student's '*t*'-test. $P < 0.05$ was regarded as the fiducial limit of significance.

Results

1. GILL AND LUNG VENTILATION

Normoxia and hyperoxia

Gill ventilation frequency (fG) was 60 ± 4 cycles/min in *Rana catesbeiana* tadpoles under normoxic conditions (P_{O_2} = 140 ± 1 mm Hg), while the amplitude of the pressure waveform recorded from the buccal cavity in the gill ventilation cycle (PCB) was 1.1 ± 0.1 cm H_2O. Both frequency and pressure amplitude fell significantly ($P < 0.05$) in all animals in hyperoxia (P_{O_2} = 597 ± 8 mm Hg, to 47 ± 3 cycles/min and 0.5 ± 0.1 cm H_2O, respectively (fig. 1).

The mean frequency of lung ventilation (fL) was very low (1 ± 1/h) in normoxia, while no lung ventilation occurred in hyperoxic conditions (fig. 1).

Hypoxia

Lung ventilation frequency rose significantly ($P < 0.01$) in the hypoxic states from 12 ± 4/h in mild hypoxia (P_{O_2} = 82 ± 1) to 51 ± 27/h in severe hypoxia (P_{O_2} = 21 ± 4 mm Hg) while the mean amplitude of the pressure waveform associated with air-breathing (4.4 ± 0.6 cm H_2O) was significantly ($P < 0.05$) greater then the mean amplitude of air breaths in normoxia (3.2 ± 0.9 cm H_2O).

Moderate (P_{O_2} = 43 ± 5 mm Hg) and severe hypoxia resulted in a reduction of the mean amplitude of the gill ventilation cycle pressure waveform (PCB) from the normoxic value (fig. 1). However, the degree of reduction shown is obscured in the pooled data by the fact that although five individuals showed a dramatic reduction in gill cycle pressure, particularly in severe hypoxia (fig. 2), hypoxia was accompanied by an increase in pressure in two individuals. Interestingly, individuals showing a more marked reduction in gill ventilation cycle amplitude in hypoxia also showed a more marked reduction in hyperoxia, suggesting a higher sensitivity to oxygen at both ends of its environmental range (fig. 2).

Typically, in moderate and severe hypoxia PBC and fG were lowest after an air breath, and rose progressively until the next air breath was triggered (fig. 1, 2). Three or four low frequency gill ventilation cycles were often made just after the air breath, each with a smaller pressure excursion than the one preceeding, before the minimum amplitude was attained. Gill ventilation cycle frequency and pressure then progressively increased until the next air breath occurred, when the cycle repeated itself (figs. 1, 2).

In two individuals the pressure changes due to gill ventilation virtually disappeared in severe hypoxia, although rhythmical fluctuations at the breathing frequency could still be observed in the pressure trace (fig. 2). There was no inter-

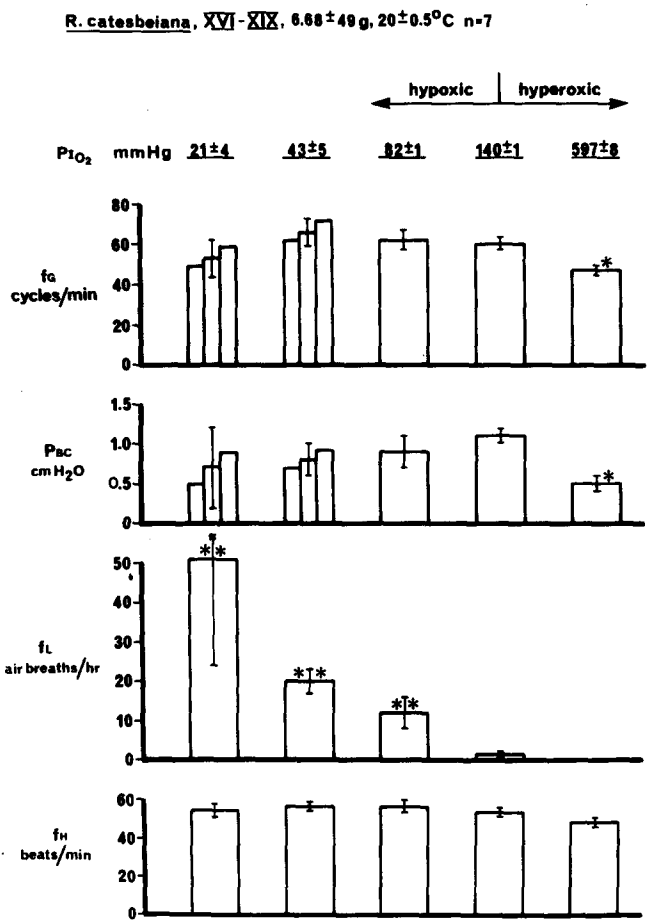

Fig. 1. Gill ventilation frequency (f_G), gill ventilation cycle pressure amplitude (PBC), the frequency of air breathing (f_L), and heart frequency (f_H) in 7 *R. catesbeiana* tapoles (stage XVI–XIX, 6.68 ± 0.49 g) exposed to aquatic normoxia, hyperoxia and hypoxia at 20 ± 0.5 °C. The mean frequency and amplitude of gill ventilation cycles are indicated for the first, middle and last third of the time period between consective air breaths in moderate (P_{O_2} = 43 ± 5 mm Hg) and severe (P_{O_2} = 21 ± 4 mm Hg) hypoxia. The vertical lines indicate the standard error of the mean values. One asterix indicates a significant difference from the normoxic value ($P < 0.05$), two ($P < 0.01$).

relationship between the frequency of air breathing and the frequency and amplitude of gill ventilation: those individuals showing the greatest increase in air breathing frequency in hypoxia did not show the greatest concomitant reduction in gill ventilation.

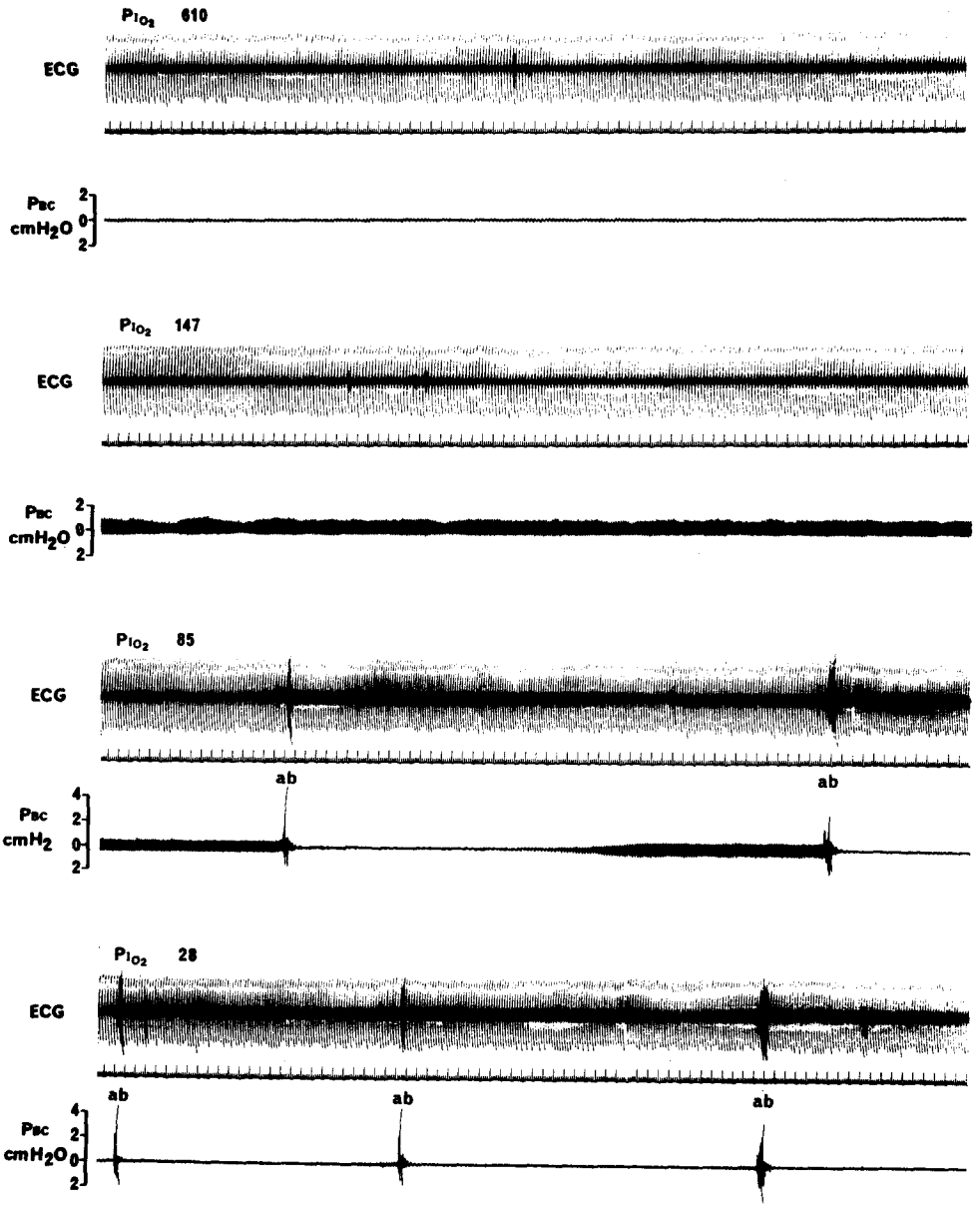

Fig. 2. Heart frequency (ECG) and respiratory responses of a single *R. catesbeiana* tadpole (Stage XVI–XIX) exposed to steady-state aquatic hyperoxia, normoxia, and moderate and severe hypoxia at $20 \pm 0.5\,^{\circ}C$. The upper trace of each set is the ECG; the middle, a one second time marker; and the lower is a buccal pressure trace recorded *via* the narial cannula. Air breaths are denoted by ab above the event. Note the marked reduction in buccal pressure amplitude in both hyperoxia and in hypoxia after an air breath.

2. HEART FREQUENCY

Mean heart rate (f_H) was 53 ± 3 beats per minute in normoxia, and did not change significantly in either hypoxia or hyperoxia in spite of the large increases in lung ventilation frequency under these conditions (fig. 1). No tachycardia was associated with air breaths, and experimental emersion of two tadpoles did not promote a bradycardia.

3. GILL WATER FLOW, GILL OXYGEN UTILIZATION AND UPTAKE

Seven tadpoles with both narial and opecular spout cannulations showed little or no air breathing in normoxia ($P_{O_2} = 141 \pm 1$ mm Hg) or mild hypoxia ($P_{O_2} = 96 \pm 2$ mm Hg), but commenced air breathing in moderate hypoxia ($P_{O_2} = 41 \pm 1$ mm Hg) at a rate not significantly different from that of the animals described in section 1. This behavioral difference between the two groups was possibly related to the greater drag of the opecular spout cannula compared to the ECG leads, which would have been experienced by these animals upon surfacing to ventilate the lungs.

The rate of water flow over the gills ($\dot{V}_W$) was significantly lower ($P < 0.01$) in hyperoxia (0.05 ± 0.01 ml/g/min) than in normoxia (0.21 ± 0.03 mg/g/min), and was significantly increased ($P < 0.05$) in mild hypoxia to 0.33 ± 0.05 ml/g/min (fig. 3). With the initiation of air breathing in moderate hypoxia $\dot{V}_W$ fell, and was not significantly different from the control value. A further fall occurred in the severely hypoxic condition (fig. 3).

Associated with changes in $\dot{V}_W$ were corresponding changes in f_G and the stroke volume of gill ventilation cycles (V_S). f_G was significantly ($P < 0.01$) lower in hyperoxia (43 ± 2/min) than in normoxia (71 ± 3 min), but was not significantly lower in any hypoxic condition. V_S rose from the normoxic value of 0.003 ml/g to 0.004 ml/g in mild hypoxia, but fell in severe hypoxia to 0.002 ml/g, and fell markedly in hyperoxia to 0.001 ml/g.

Utilization (U) of inspired O_2 by the gills was $43 \pm 3\%$ in normoxia, being significantly higher ($P < 0.01$) in hyperoxia ($64 \pm 4\%$) and significantly lower in mild ($33 \pm 3\%$) and moderate ($17 \pm 3\%$) hypoxia (fig. 3). O_2 uptake by the gills ($\dot{M}_{G_{O_2}}$) was 1.3 ± 0.1 μM/g/h in normoxia, and was not significantly different in either hyperoxia or mild hypoxia (fig. 3). With the advent of lung ventilation in moderate hypoxia, $\dot{M}_{G_{O_2}}$ was significantly reduced ($P < 0.01$) to 0.2 ± 0.0 μM/g/h, principally due to the low O_2 utilization in this state. In severe hypoxia the P_{O_2} of expired water leaving the spout was higher than the P_{O_2} of inspired water in all animals, indicating a reversal of normal oxygen exchange across the gills. In this condition some 0.1 μM/g/h of O_2 was lost to the water ventilating the gills (fig. 3). This loss was modest due to both the small difference in P_{O_2} between the inspired and expired water (18 ± 8 mm Hg) and the moderate flow rate of water over the

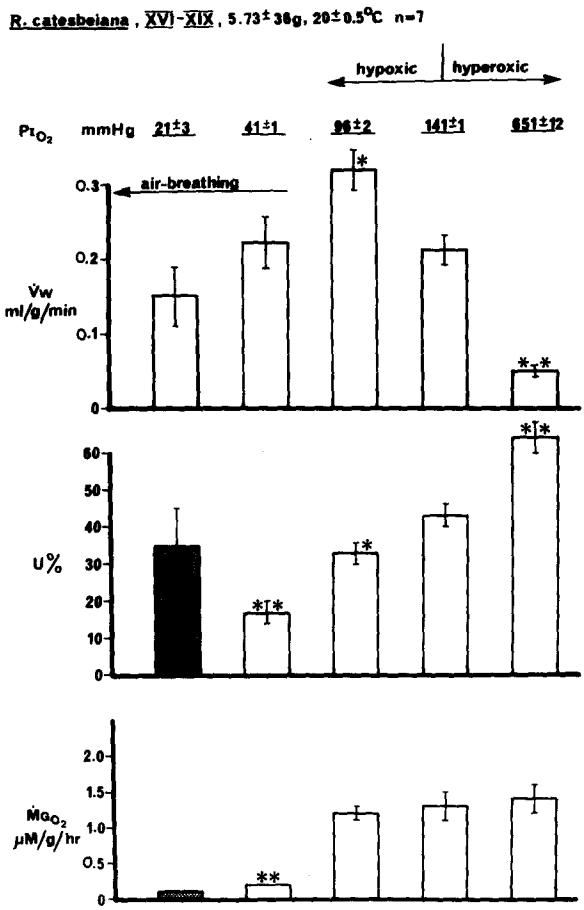

Fig. 3. Gill water flow rate ($\dot{V}w$), oxygen utilization (U) and the rate of oxygen exchange across the gills ($\dot{M}_{GO_2}$) in 7 *R. catesbeiana* tapoles (stage XVI–XIX, 5.73 ± 0.36 g) exposed to aquatic normoxia, hyperoxia and hypoxia at 20 ± 0.5 °C. These animals commenced air breathing in moderate hypoxia (P_{O_2} = 41 ± 1 mm Hg). The hatched bars in severe hypoxia indicate that in this condition 35 ± 10% of the O_2 in expired water is due to O_2 enrichment by the gills, while 0.1 μM/g/h of O_2 leaves the gills. The vertical lines indicate the standard error of the mean values. Where they are absent the standard error is not large enough to be mathematically significant. One asterix indicates a significant difference from the normoxic value ($P < 0.05$), two ($P < 0.01$).

gills 0.15 ± 0.04 ml/g/h). In this last condition 35 ± 10% of the O_2 in expired water was due to O_2 enrichment of the inspired water by O_2 leaving blood in the gills (fig. 3).

There was no significant difference in $\dot{M}_{GO_2}$ in normoxia in this study and a parallel study with animals of the same development stage in an open respirometer (Burggren and West, 1982). However, the animals in this study showed a small but significant elevation in $\dot{V}w$ and Vs, and a small but significant compensating decrease in f_G and U, compared to animals in the respirometer.

Discussion

The present study has demonstrated that in a larval anuran the P_{O_2} of inspired water is a potent factor in respiratory control, influencing ventilation of both the gills and lungs. There is a significant hypoxic component to the respiratory drive in normoxic water, as evidenced by the significant reduction in f_G and PBC, and the absence of lung ventilation, in hyperoxygenated conditions. A similar 'hypoxic drive' has been revealed in reptiles (Glass *et al.*, 1978) and bimodally breathing fishes (Burggren, 1979).

The relationship between air breathing and gill ventilation in the bullfrog tadpole is complex. Hyperoxia results in a complete cessation of air breathing, and also a reduction in f_G, PBC and $\dot{V}_W$. However, the increase in air breathing frequency in hypoxic conditions in most individuals was also associated with an inhibition of gill ventilation, which became more pronounced as hypoxia became more severe. This argues for a receptor whose input inhibits gill ventilation in the face of an elevated blood P_{O_2} caused by either hyperoxic water or air breathing. It seems unlikely that such a receptor would be primarily influenced by the very small P_{CO_2} changes brought about by the lungs, as only 3% of the total flux of CO_2 in normoxia is *via* the pulmonary route (Burggren and West, 1982). Furthermore, the suppression of gill ventilation in tadpoles at this developmental stage in both hyperoxic and hypoxic conditions in which air breaths are frequent, could then be explained by the same mechanism. The progressive increase in gill ventilation between air breaths would then be explained by a progressive hypoxemia until the next air breath was triggered. In both hyperoxia and severe hypoxia after an air breath, there is a reduction in PBC compared to the normoxic state, implying that either P_{O_2} at the putative receptor is higher in *both* hyperoxia hypoxia in the presence of frequent air breathing, than in normoxia, or that gill ventilation may also be inhibited by lung inflation *per se*, perhaps due to input from pulmonary stretch receptors (Taglietti and Casella, 1966, 1968). Singh and Hughes (1973) suggested that in *Anabas* in normoxia the cessation of water breathing is partly due to the presence of O_2 in the air chamber after an air breath. Furthermore, they thought that the resumption of gill ventilation before an air breath would serve to remove accumulated CO_2 from the blood before the O_2 demand was satisfied by the air breath. This may well be true for stage XVI–XIX tadpoles, in which 41% of CO_2 effluxes *via* the gills in normoxia (Burggren and West, 1982).

In normoxia, some 40% of the oxygen in the water passing over the gills is utilized by the stage XVI–XIX tadpole, which is comparable to the utilization in many fishes at rest (Shelton, 1970). As pointed out by Shelton (1970) for fish, utilization depends in part on the P_{O_2} gradient across the gills. Although O_2 utilization usually varies inversely with gill ventilation the extent of the variation depends also on the way in which the factor responsible for the change in ventilation affects the P_{O_2} gradient across the gills. Viewed in this light the constancy of gill oxygen uptake in hyperoxia, normoxia, and mild hypoxia in the absence of air breathing, due to

to the offsetting effects of changes in utilization and gill ventilation, may be serendipitous. Certainly gill oxygen uptake falls markedly in moderate hypoxia principally due to a fall in utilization, which must be affected by the fall in the aquatic oxygen tension and probably also by an elevation in $P\bar{v}_{O_2}$ due to the onset of air breathing.

No significant changes in heart rate were found in this study in any experimental condition, and air breaths did not trigger a ventilation tachycardia. Although a change in heart rate, unaccompanied by data on stroke volume, does not necessarily reflect a similar change in cardiac output, most of the available evidence from aquatic or amphibious vertebrates suggests a positive correlation between them (Randall, 1970; Satchell, 1971; Johansen, 1971). Increases in heart frequency associated with air breaths have been observed in airbreathing fishes (Singh and Hughes, 1973). The lack of a heart frequency response to air breathing during steady state hypoxia in *Rana* tadpoles implies the lack of a functional cardiac chronotropic reflex pathway from lung stretch receptors, although they are known to be present in the adult frog (Taglietti and Casella, 1966, 1968).

Finally, it is interesting to compare the respiratory movements recorded in tadpoles and those observed in the adult anuran (De Jongh and Gans, 1969; West and Jones, 1975; McIntyre and Towes, 1976; Brett and Shelton, 1979). The two distinct types of ventilation cycle in the adult anuran, termed buccal cycles and lung ventilation cycles by West and Jones (1975), appear to have their counterparts in the bimodally-breathing tadpole in gill ventilation cycles and air breaths respectively. Certainly the amplitude of air breaths observed in this study fell within the range of pressures recorded for lung ventilation cycles in adult Ranids, while the gill ventilation cycles occurred within the range of frequencies and pressures recorded for buccal cycles in *Rana pipiens* at 24 °C (West and Jones, 1975). A major difference, however, between the breathing mechanism in the larval and adult anuran is that inspiration of the respiratory medium is *via* the mouth in tadpole and *via* the nares in the adult. Narial closure at the appropriate time in the cycle, synchronised with elevation of the buccal floor and opening of the glottis, is important in the adult lung ventilation cycle (West and Jones, 1975), and presumably the jaws must close at the same stage in the cycle in order to accomplish effective lung-filling in the tadpole. It is interesting in this regard to note that elevation of the premaxilla by the action of muscles of the lower jaw has often been proposed as a mechanism of narial closure in the adult frog (Gaupp, 1896; Willem, 1919, 1920; De Jongh and Gans, 1969), manual elevation of the premaxilla causing narial occlusion in anaesthetized animals (West and Jones, 1975). This mechanical linkage may facilitiate the change in the path of the inspired medium upon metamorphosis, by timing narial closure in the adult to the action of muscles causing jaw closure in the tadpole.

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