

## BREATHING IN *RANA PIFIENS*: THE MECHANISM OF VENTILATION

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### Summary

The mechanism and pattern of ventilation in unrestrained *Rana pipiens* were investigated by simultaneous measurements of pulmonary pressure, buccal pressure and air flow at the nostrils. The buccal cavity was ventilated continuously at a rate of  $90 \pm 3.2$  oscillations  $\text{min}^{-1}$  by low-amplitude pressure swings above and below atmospheric. The lungs were ventilated intermittently by the buccal pump at a rate of  $6.3 \pm 0.8$  breaths  $\text{min}^{-1}$ . Expiration of gas from the nostrils occurred on two occasions during a lung ventilation. Ventilation of the lungs was achieved by precise timing of two valves, the nostrils and glottis. The timing of the valves determined the volume of expiratory flow on these two occasions and its relationship to inspiratory flow. Thus, the breathing movements could cause inflation, deflation, or no change in the lung volume. Periodically the lung was inflated by a sequence of successive breaths. During inflations the nostrils closed simultaneously with glottal opening and almost no gas was expired during the first expiratory phase. This caused a complete mixing of buccal contents and pulmonary gas and this mixture was pumped back into the lung. Deflations were characterized by a delay in nostril closing that resulted in a large outflow of gas from the lung and buccal cavity during the first phase of expiration. More gas left the system than was pumped into the lungs. The results suggest that coherent air flow from glottis to nostrils, as required by the 'jet stream' hypothesis of Gans *et al.* (1969), is not likely to occur.

### Introduction

Amphibians and lungfish ventilate their lungs by means of the pumping action of the buccal cavity musculature. This can generate pressures both above and below ambient so that gas can be moved between the pump and the outside air through the nostrils, or between the pump and the lungs through the glottis. The direction of gas flow depends on the coordinated action of nostrils and glottis in relation to

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the volume changes of the buccal pump. The timing of pump and valves differs from species to species and is responsible for the considerable variety in breathing patterns of these animals (Shelton *et al.* 1986).

Several breathing patterns have been described in anurans. In the totally aquatic toad *Xenopus*, for example, there are no buccal oscillations between intermittent lung ventilations. During the latter, the toad first empties both lungs and the buccal cavity through the open glottis and nostrils. It then draws fresh air into the buccal cavity through open nostrils with the glottis closed, and finally pumps this air into the lungs with the glottis open and the nostrils closed (Brett and Shelton, 1979). *Rana* and *Bufo*, in contrast, produce continuous buccal oscillations that pump air in and out of the buccal cavity with the glottis closed. The lungs of these animals are ventilated intermittently in a cycle of activity that begins when the glottis opens and the lungs deflate into the expanded buccal cavity with some gas emerging from the open nostrils. The nostrils then close and the buccal cavity contents are pumped into the lungs, after which the glottis closes, the nostrils open, and the buccal oscillations begin again (Cherian, 1956; de Jongh and Gans, 1969; West and Jones, 1975; MacIntyre and Toews, 1976; Jones, 1982). This sequence of events apparently leads to fresh air in the buccal cavity being mixed with gas from the lungs, the mixture being pumped back into the lungs.

Gans *et al.* (1969) suggested that the obvious shortcomings of such a breathing pattern could be overcome if lung gas was maintained in a coherent, dorsal air flow, separate from the fresh air held in the ventral and posterior region of the buccal cavity. Lung gas could then be expired through the nostrils with minimum contamination of the fresh air that would subsequently be pumped into the lungs. The evidence for this widely quoted hypothesis is based on experiments in which the rate of washout of an introduced gas (argon in an argon–oxygen mixture) from buccal cavity and lungs was followed after the animal began breathing air. Argon concentrations, measured at the nostrils by a mass spectrometer, increased rapidly during lung emptying and then declined slowly as the ventilation cycle continued. The authors suggest that this slow fall was due to pockets of fresh air in the buccal cavity becoming incorporated in the coherent flow of lung gas during the final stages of ejection. Other interpretations are possible, however, since the nostrils close during this period of falling concentration and the lungs are reinflated. Absence of flow through the nostrils could be responsible for the slow fall in argon concentration in the gas being sampled by the mass spectrometer.

The evidence for coherent flow is not wholly convincing, therefore, and many other features of anuran ventilation are also incompletely understood. A complete analysis would require measurements of pressure in buccal cavity and lungs, flow rates at nostrils and glottis, and instantaneous determination of gas concentrations in buccal cavity and lungs. Pressures in the system can be measured (de Jongh and Gans, 1969; West and Jones, 1975; MacIntyre and Toews, 1976; Brett and Shelton, 1979; Jones, 1982), though with some difficulty in unrestrained animals. Air flow at the nostrils is more difficult to determine. West and Jones (1975), using a thermistor system sensitive only to flow direction, and Glass *et al.* (1978) and Jones

(1982), using potentially restrictive face masks fitted with pneumotachograph screens, have made some direct measurements. Flow rates at the glottis and concentrations of gas in the buccal cavity and lungs have, so far, been impossible to determine. In the present study we have not been able to solve all these problems. However, we report on pressures and flows measured in freely moving frogs and show that, even in a single species, an extensive repertoire of breathing patterns exists as part of normal behaviour, suggesting that interpretations of breathing and gas exchange should not be restricted to single ventilatory cycles.

### Materials and methods

Frogs (*Rana pipiens*) were obtained from a commercial supplier (Xenopus Limited) and housed in large fibreglass tanks at approximately 25°C. The animals were fed blowfly larvae once a week. Two weeks before experiments, animals were acclimated to an ambient temperature of 20–22°C and feeding was suspended. Experiments were conducted on 21 animals with a mean mass of  $26.5 \pm 5.8$  g (s.e.).

#### *Buccal cavity and lung cannulation*

Frogs were anaesthetized in a solution of MS-222 ( $300 \text{ mg l}^{-1}$ ) and became immobile in approximately 15 min. A buccal cannula was made from a 4 cm length of PP190 tubing. One end of the tube was heat-flared and then bent through 90° at a point 1 cm from the heat flare. The other end was cut to a sharp bevel. A hole smaller than the bore of the cannula was made in the tympanic membrane. The bevelled end was pushed through this hole by approaching the tympanic membrane from the Eustachian tube (Jones, 1970). The heat-flared end was snugly positioned against the inside of the tympanic membrane while the 90° bend allowed the cannula to be securely sutured against the body wall. A 30 cm length of silicone rubber tubing (1.0 mm i.d.; 2.0 mm o.d.) was attached to the bevelled end of the buccal cannula. The silicone tubing was soft enough to allow the animal to move about in the experimental chamber without placing strain on the cannula.

A lung cannula was made from a 1.5 cm length of PP190 tubing which was heat-flared at one end. A short length of PP90 tubing, which also ended in a slight heat-flare, was pushed into the PP190 so that its flared end was within the cup at the end of the wider tube. This arrangement prevented the tip of the PP90 tube from contacting the lung wall, minimizing injury and fluid accumulation in the tip of the cannula. A small incision (1 cm) was made along the midline of the flank just anterior to the pelvic girdle. The posterior tip of the lung was located and withdrawn through the incision. A small hole was cut into the tip of the lung through which the cannula was inserted (Jones, 1970). The cannula was secured to the lung with a purse-string suture. The incision was closed and the cannula fastened firmly to the body wall musculature with several sutures. A 30 cm length of silicone rubber tubing was attached to the lung cannula as described for the

buccal cannula. After surgery the animals were allowed to recover for 6–24 h before any measurements were taken.

#### *Pressure measurements in air*

The first set of experiments consisted of recording the pressure relationships between the lungs and buccal cavity during breathing in unrestrained animals in air. Measurements were made on seven animals with a mean mass of  $28.0 \pm 4.0$  g (s.e.). Cannulated animals were placed in a 3 l opaque container and allowed to move freely and breathe without restriction. Fresh air was supplied through a hole in the lid at a rate of  $300 \text{ ml min}^{-1}$ . The animals were allowed to acclimate to the container for several hours to days before recordings were made. The lung and buccal cannulae were air-filled and each connected to a Hewlett Packard 267 BC pressure transducer in these and subsequent experiments. The outputs of the transducers were amplified with Sanborn 1100 carrier pre-amplifiers and recorded on a two-channel Lectromed pen recorder. The Hewlett Packard 267 BC transducers were calibrated with static columns of water and the dynamic characteristics of the system (cannula plus transducer) were determined by a Hansen's 'pop-test' (McDonald, 1974). The resonant frequency of the system was determined to be 60–70 Hz with relative damping of 0.27–0.35 of critical. Since the expected frequency of the events to be recorded was between 1 and 2 Hz, the decrease in the natural frequency of the system and the increase in relative damping due to the cannula did not significantly distort the pressure signal.

#### *Air flow measurements*

Air flow at the nostrils was measured simultaneously with lung and buccal pressures. The lung and buccal cavity of frogs were cannulated as described above and the animals were placed in a tank containing water to a depth of 15 cm with a surface area of  $759 \text{ cm}^2$ . The water was aerated and maintained at  $20^\circ\text{C}$ . A plastic funnel perforated with many holes of 1 cm diameter was placed over the animal. The funnel had a base diameter of 15 cm and tapered to an opening of 3 cm just below the surface of the water. A blowhole chamber with a volume of 21 ml and a diameter of 14 mm (surface area =  $1.54 \text{ cm}^2$ ) was placed directly over the cone opening (Fig. 1). The blowhole chamber was provided with an outlet containing a low-resistance pneumotachograph. Fresh air was introduced into the blowhole chamber at a rate of  $200 \text{ ml min}^{-1}$  through an inlet port.

An animal was placed in the apparatus and allowed to recover from surgery and handling for at least 6 h. There was sufficient room in the apparatus for the animal to dive and to move around the cone. The animal quickly found the blowhole and began to breathe. Differential pressures across the pneumotachograph screen were measured with a Validyne DP45 differential pressure transducer whose output was recorded on a Racal Thermionic Store 4D four-channel tape recorder. Pressures in the buccal cavity and lung were measured simultaneously with air flow and also stored on magnetic tape. The magnetic tapes were played back at a later date through a Medelec FOR 4.2 recording oscilloscope. This allowed the

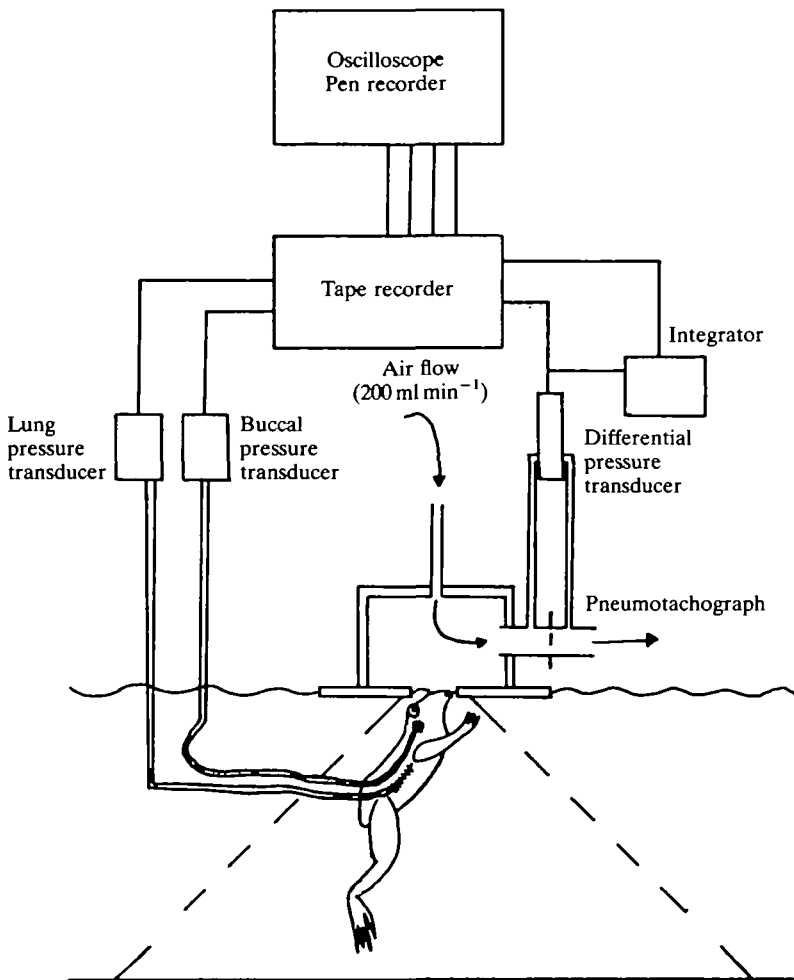


Fig. 1. Schematic diagram showing a frog in the blowhole apparatus.

superimposition of the buccal and lung pressure when the zero levels and the gains for the two transducers were set to identical levels.

The pneumotach screen was calibrated for flow by passing air through the blowhole chamber at rates measured by flow meters. The differential pressure produced across the pneumotachograph screen used in the experiments was  $4.0 \text{ Pa } 100 \text{ ml}^{-1} \text{ min}^{-1}$  and the screen was linear over the range of flow rates measured experimentally. The flow signal was taken to an integrator whose output was calibrated for volume by injecting and withdrawing known amounts of air (0.2, 0.5 and 1.0 ml) into and out of the chamber with a 1 ml tuberculin syringe.

Simple static calibration of the flow measuring system is not sufficient to confirm its accuracy in experiments in which flow rates may change quickly, as for example when the nostrils close. The frequency capabilities of the blowhole system are determined by two factors, the screen resistance and the surface area of the

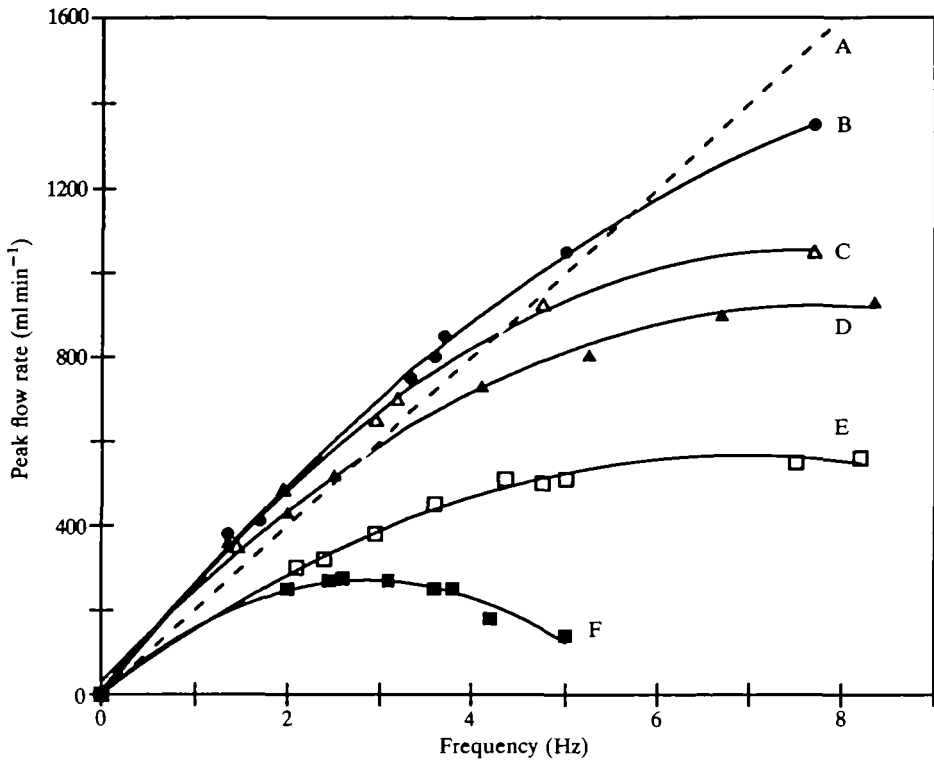


Fig. 2. Frequency response curves of the pneumotach apparatus for various combinations of blowhole diameters and screen resistances. Peak flow rates recorded by the pneumotachograph are shown as a function of the frequency of a sinusoidal pump with a stroke volume of 1.18 ml. Theoretical peak flows (A) calculated from static calibrations. Experimentally determined peak flow rates using (B) direct connection of pump to pneumotachograph screen (resistance=4.0 Pa 100 ml<sup>-1</sup> min<sup>-1</sup>), (C) pump connected to 1.54 cm<sup>2</sup> blowhole with pneumotach screen from B above, (D) direct connection of pump to pneumotachograph screen (resistance=2.0 Pa 100 ml<sup>-1</sup> min<sup>-1</sup>), (E) pump connected to 1.54 cm<sup>2</sup> blowhole with pneumotachograph screen from D above, and (F) pump connected to 4.91 cm<sup>2</sup> blowhole with pneumotachograph screen from D above. The experiments on frogs were carried out with the combination of screen and blowhole shown at C.

blowhole. A combination of blowhole diameter and screen resistance that least affected a frog's breathing pattern yet gave the best frequency response was determined by experiment. The frequency response of the blowhole apparatus was tested by applying a forced oscillation of air to the blowhole chamber using a sinusoidal pump. The pneumotachograph screens were also tested by direct connection to the pump. The effects of screen resistance, blowhole diameter and direct connections to the screens are plotted in Fig. 2. A screen resistance of 4.0 Pa 100 ml<sup>-1</sup> min<sup>-1</sup> and a blowhole surface area of 1.54 cm<sup>2</sup> gave a linear response to increasing dynamic flow up to a frequency of 4 Hz. This combination was chosen for the experiment. Smaller blowholes impeded the animal's access to

air and lower resistances gave flow signals that were too small. The highest frequencies measured during experiments were of the order of 3 Hz and were generally less than 2 Hz.

### *Cinematography*

A Bolex H16 camera was used to film the opening and closing of the nostrils in spontaneously breathing frogs while lung and buccal pressures were simultaneously recorded. Animals were cannulated as described above and placed in a 3 l container filled with water. A cone that narrowed to a 2.25 cm<sup>2</sup> opening at the water surface was placed over the animal. This opening was just large enough to allow animals to fit their snout through during ventilation. The camera was positioned so that this breathing aperture filled the entire viewfinder of the camera. The aperture was illuminated by two fibre optic lights. A neon bulb was provided in one corner of the field of view and a small rotating disc with half white and half black sectors was placed in the opposite corner. The neon bulb was triggered to flash once for every revolution of the disc and the triggering pulses were recorded on tape so that the film and pressure records could be synchronized. The filming rate was 32 frames s<sup>-1</sup> on Ilford FP4 black and white film.

## **Results**

### *Patterns of breathing in air*

Undisturbed frogs breathing air in the 3 l tank showed several types of breathing movements. The simplest were continuous oscillations of the floor of the buccal cavity (Fig. 3A). They caused low-amplitude pressure changes (10–40 Pa) around atmospheric at a mean rate of 90 min<sup>-1</sup> (Table 1). The nostrils were open throughout the whole oscillation and air moved in and out of the buccal cavity.

Buccal oscillations were interrupted periodically by a second type of movement, the lung ventilation, characterised by a large, rapid increase in buccal pressure ranging from 100 to 450 Pa followed by a rapid return to a pressure 10–40 Pa below atmospheric, after which buccal oscillations resumed (Fig. 3A). In resting animals, lung ventilations occurred at an overall frequency of 6.3 min<sup>-1</sup> (Table 1) though the pattern of their appearance was varied. Sometimes they occurred singly and sometimes in groups, always being separated by periods of variable duration during which buccal oscillations were usually produced (Fig. 3A). The amplitude of buccal oscillations varied during these periods, often decreasing gradually (Figs 3B and 4), sometimes to the extent of causing short periods of apnoea. Lung pressures did not change substantially with single lung ventilations and a mean level of 225 Pa was maintained in the lung (Table 1). However, lung ventilations were often grouped together to produce sequences of lung inflation and deflation, causing pressures to fluctuate considerably (Fig. 3B). Such sequences occurred at a mean frequency of 0.6 min<sup>-1</sup>, with a mean pressure after inflation of 394 Pa and after deflation of 116 Pa (Table 1). The pattern in these sequences consisted of three or more ventilations in succession causing stepwise

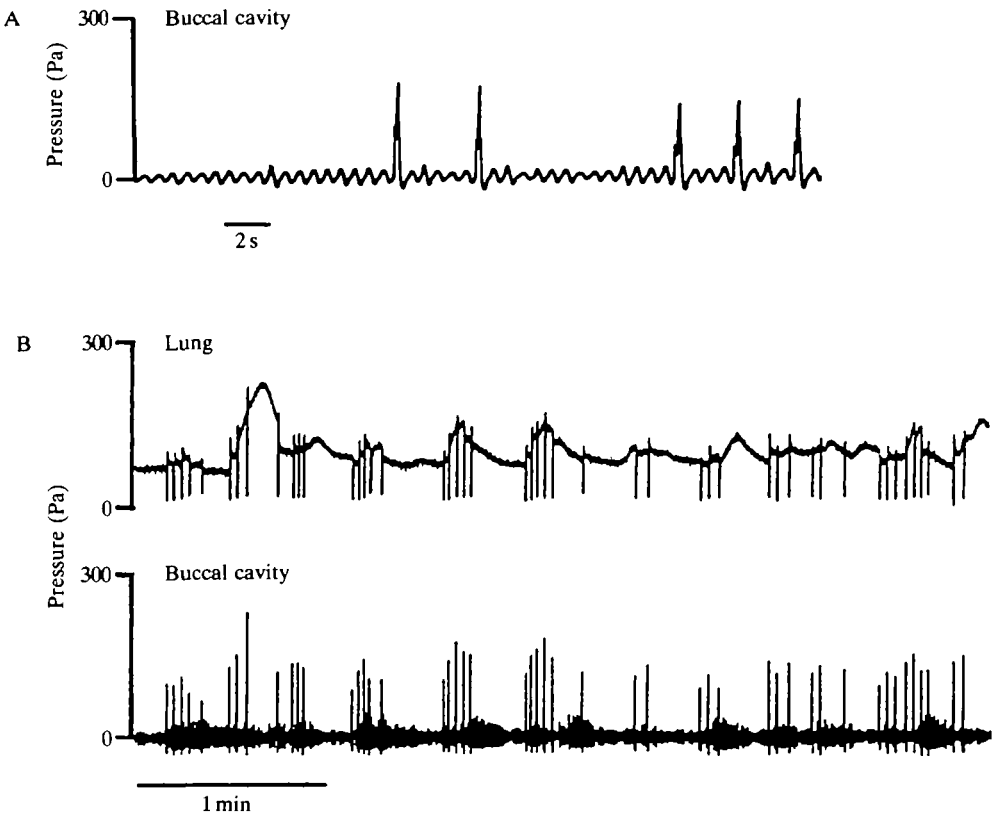


Fig. 3. Breathing patterns recorded from frogs in air. (A) Buccal pressures from a 23.1 g frog showing continuous buccal oscillations and five lung ventilations. (B) Buccal and lung pressures from a 32.4 g frog showing inflation–deflation sequences and maintenance ventilations.

increases in lung pressure followed by two or more ventilations reducing the pressure again. Sometimes lung pressure continued to change after an inflation or deflation sequence had been completed (Fig. 3B). This was probably due to changes in posture and muscle tone in the flank musculature.

#### *Breathing in the blowhole apparatus*

The breathing patterns of animals in the blowhole apparatus were in most ways similar to those recorded from animals breathing in air, as the comparisons in Table 1 show. The basic form of buccal and lung ventilations was unchanged. However, the fact that animals in the blowhole apparatus were free to dive introduced some important differences into the breathing behaviour. Some animals spent the entire experimental period at the blowhole, breathing in a manner that was indistinguishable in frequency, pressure and pattern from that of the group breathing in air. Inflation–deflation sequences occurred in the animals in the blowhole apparatus as they did in the air tank. Many animals dived for



Table 1. Comparison of breathing behaviour variables as recorded from animals in air and in the blowhole apparatus

	In air	In apparatus
<i>N</i>	7	21
Mass (g)	28.0±4.0	35.0±3.1
Frequency of buccal oscillations (min <sup>-1</sup> )	90.2±3.9	80.2±3.5
Frequency of lung ventilation (min <sup>-1</sup> )	6.3±0.82	5.9±0.84
Time spent breathing (%)	100	61.2±7.5
Frequency of long ventilation in breathing period (min <sup>-1</sup> )	6.3±0.82	13.6±2.27
Frequency of inflation sequences (min <sup>-1</sup> )	0.6±0.09	0.6±0.07
Number of breaths per inflation sequence	3.5±0.33	4.3±0.36
Lung pressure after inflation sequence (Pa)	394±42	618±82
Lung pressure after deflation (Pa)	116±25	308±38
Lung pressure after single breaths (Pa)	225±40	442±48

Numbers given are means±s.e.

Student's *t*-test showed no significant differences between the variables except for the lung pressures, in which the differences were due to water pressure.

varying lengths of time so that breathing stopped and the patterns changed. If dives were short and the period at the surface prolonged, breathing soon settled to the type seen in air. Fig. 4 shows the development of a continuous air-breathing pattern after the animal surfaced from a brief dive and began a lengthy period at the water surface. Shorter periods at the surface led to more profound changes in the breathing patterns, with lung ventilations occupying the entire breathing period and no buccal oscillations developing (Fig. 5). When diving animals came to the surface, the frequency of lung ventilations increased to levels that were above those seen in non-divers or in the air-breathing group of Table 1, so that the overall frequency was not significantly different in the two groups. Lung inflations invariably occurred as an animal breathed immediately after a dive and deflations usually preceded a dive. Between dives, the inflation–deflation sequences occurred in patterns similar to those observed in animals in air. Lung pressures were significantly higher in the animal in the blowhole apparatus, owing entirely to the position of the lungs below the water line.

Typical breathing behaviour in an animal surfacing from a short dive is shown in Fig. 4. Upward movement to the blowhole caused a reduction of hydrostatic pressure in lungs and buccal cavity. As the animal broke the surface of the water, it caused a rapid flow of air out of the chamber, which was seen as a spike in the flow record. Breathing movements then began. The animal pumped its buccal cavity twice, as shown in the two initial large pressure changes in the buccal cavity. The pressure became negative after the first large buccal oscillation and air was drawn into the buccal cavity as indicated by the flow trace. Buccal pressure then rapidly

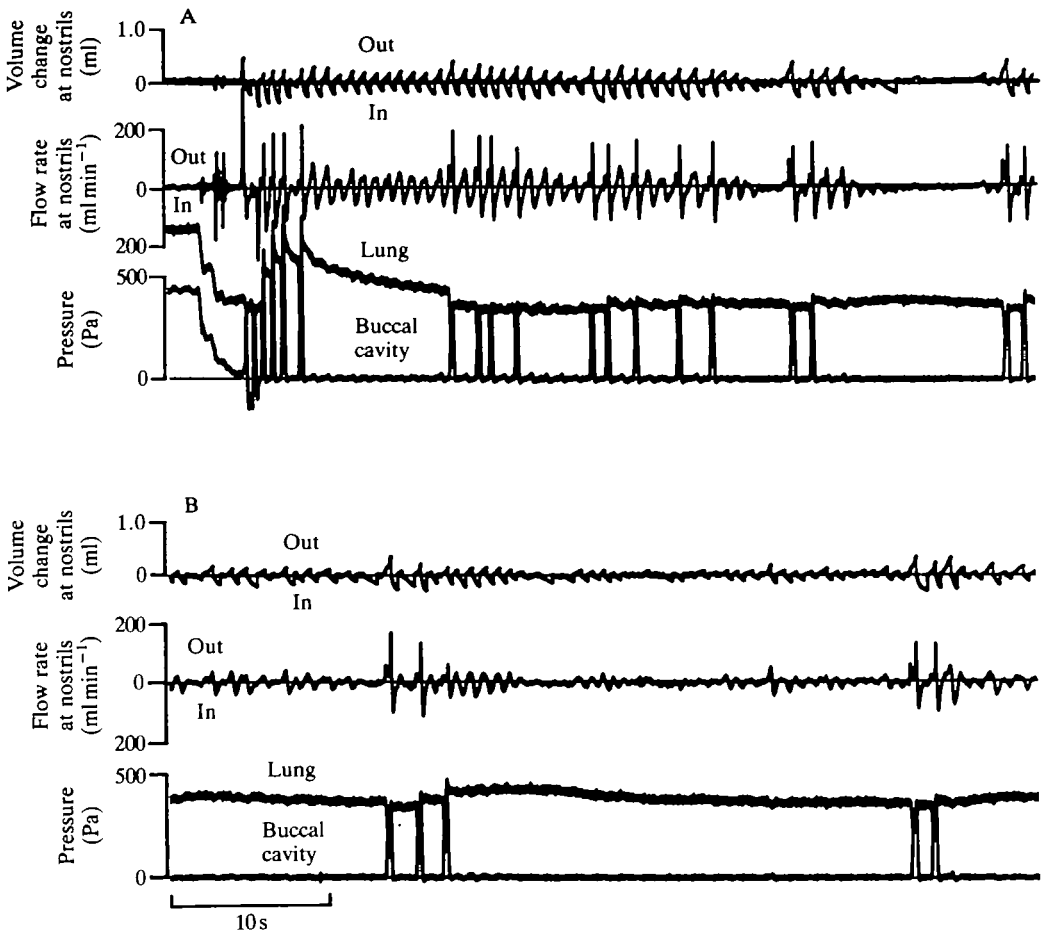


Fig. 4. Lung pressure, buccal pressure, air flow and volume change at the nostrils recorded from an 18.5 g frog surfacing to breathe at the blowhole after an 8 min dive. Initial pressure changes preceding lung ventilations are caused by the animal surfacing. Record B follows immediately on record A and is representative of the resting pattern in frogs breathing for long periods at the blowhole.

increased and lung pressure rapidly decreased until the two became equal and thereafter increased together to a maximum pressure. When this was reached, buccal pressure fell to atmospheric while lung pressure stayed at its new level as the glottis closed. This action of inflation was repeated three more times with air moving into the buccal cavity and being pumped into the lung. Further evidence of this inflation is revealed by the volume trace, which shows that 2.09 ml of air was inspired and 0.75 ml was expired over the first four lung ventilations, causing a net increase in lung volume of 1.34 ml. The animal then continued to oscillate its buccal cavity, as shown by the low-amplitude pressure changes in the buccal cavity and the large tidal changes in flow and volume, until the next lung ventilation. The fall in lung pressure during this period may have been due to adjustments in

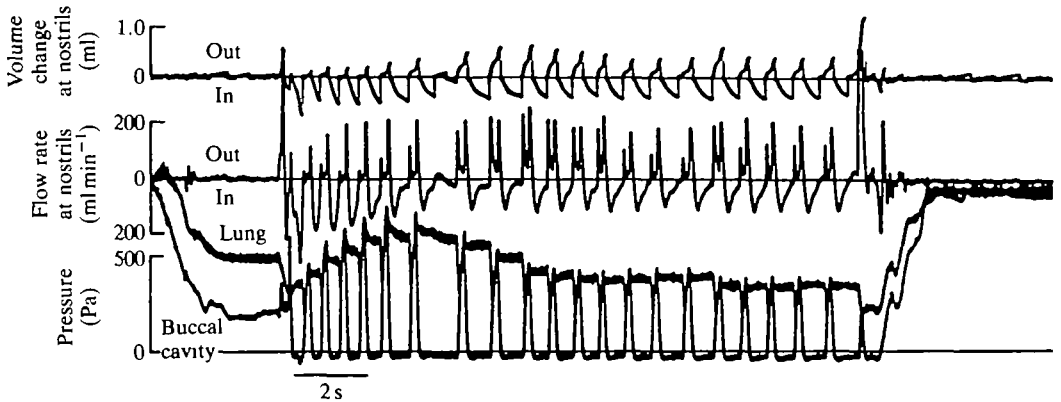


Fig. 5. Lung pressures, buccal pressures, air flows and volume changes at the nostrils recorded from a 25.3 g frog surfacing briefly to breathe at the blowhole before a further dive. Recording is representative of patterns in frogs that spent the majority of the experimental period under water.

viscous and elastic elements in the lungs and body wall, relaxation of the flank musculature, or a leaky glottis. Flow records suggest the latter is unlikely. The next lung ventilation following 10 s after the inflation sequence caused a deflation of the lung as indicated by a sudden reduction in lung pressure. The volume record shows that 0.25 ml of air was inspired into the buccal cavity but 0.43 ml was expired. Further lung ventilations served to maintain lung pressure and volume; the transition to the continuous air-breathing pattern can be seen in the rest of Fig. 4A,B. Short periods of apnoea when buccal oscillations decreased in amplitude and eventually stopped can also be seen in Fig. 4A,B as the pattern typical of continuous air-breathing developed.

The pattern of air flow during buccal oscillations was tidal and relatively simple. Air flow patterns during a lung ventilation were variable and much more complex. These complexities are illustrated in Fig. 5, which shows a continuous record of a short burst of lung ventilations produced by an animal that surfaced rapidly and dived again immediately. This pattern with no buccal oscillations was typical of active animals showing brief visits to the surface. Breathing began as the buccal pressure became negative and caused a substantial inflow of air into the buccal cavity. The lungs were inflated by the initial six lung ventilation movements (Fig. 5) then deflated over a series of 14 lung ventilations. At the end of the short breathing period the animal deflated its lungs to acquire neutral or negative buoyancy before it began another dive; there was a large fall in lung pressure and an equally large outflow of gas at the end of the breathing burst.

The details of air flow are shown more clearly in Fig. 6A,B, the two parts of which consist of the first 12 ventilations of Fig. 5 plotted on an extended time base. In a single cycle of lung ventilation, inhalation of fresh air through the nostrils occurred in a single pulse during which the buccal floor was lowered, whereas exhalation occurred in two pulses, one just before and one just after air was

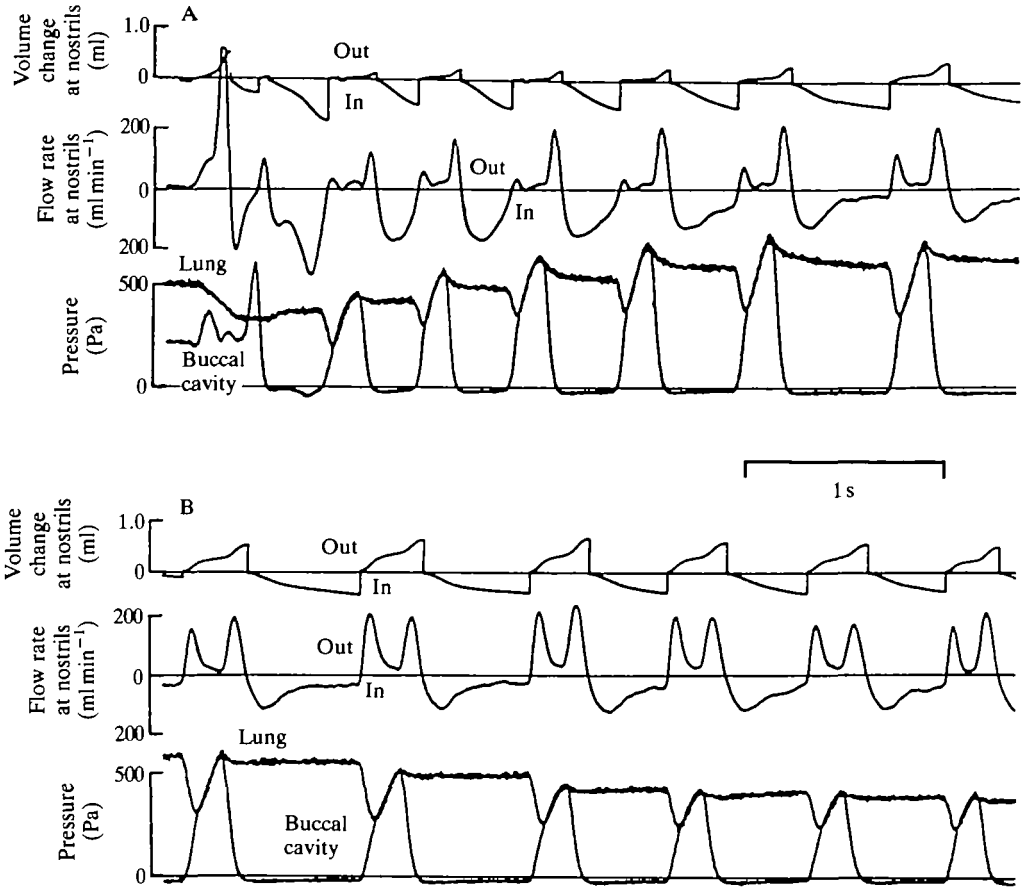


Fig. 6. Details of pressures and flows from a 25.3 g frog as it surfaced briefly at the blowhole before a further dive. Recording of the first 12 ventilations shown in Fig. 5, plotted on a faster time base. Traces A and B are continuous.

pumped into the lung through the glottis. The flow patterns associated with lung inflation were characteristically different from those associated with lung deflation. The initial inflation breaths showed large inhalations into the buccal cavity with little exhalant flow from the nostrils. As the lung inflation sequence proceeded so the volume of gas leaving the nostrils during the second flow pulse gradually increased, the initial exhalant pulse remaining small. The lung then started to deflate with the initial exhalant pulse growing in size, the inhalations diminishing in volume and pressure in the lung declining. The volume changes derived by integrating the flow rates show clearly that inhalation volume was greater during inflation and exhalation volume was greater during deflation. In total, however, more gas entered the system than left it during such an inflation-deflation sequence.

The timing and levels of activation of the muscles of the buccal floor, nostrils and glottis are clearly of fundamental importance in regulating air flow during

Table 2. Comparison of inflation and deflation breaths with respect to duration of phases 1 to 4 (see Fig. 8), the rate of change of pulmonary pressure during emptying (phase 2) and filling (phase 3) of the lung, and the volume of air inspired (phase 1) and expired (phase 4 for inflation breaths and phases 2 and 4 for deflation breaths) through the nostrils

	Inflation	Deflation
Durations (s)		
Phase 1 (inspiration)	0.321±0.016	0.310±0.016
Phase 2 (lung emptying and expiration 1)	0.047±0.002	0.068±0.004*
Phase 3 (lung filling)	0.092±0.003	0.093±0.007
Phase 4 (expiration 2)	0.091±0.003	0.106±0.004*
Volume (ml 100 g <sup>-1</sup> )		
Inspiration (phase 1)	0.92±0.04	0.55±0.03*
Expiration (phases 2 and 4)	0.69±0.03	0.85±0.04*
Slope of pressure change (Pa s <sup>-1</sup> )		
Lung emptying (phase 2)	1888±157	1866±207
Lung filling (phase 3)	1700±133	975±107*

Comparisons were made using one-way ANOVA (\* shows significant differences at  $P < 0.05$ ).

Measurements were made on inflation ( $N=45$ ) and deflation breaths ( $N=34$ ) from four frogs and are expressed as means±s.e.

inflations and deflations. A lung ventilation cycle can be divided into the four phases that are shown in Fig. 8 and considered in detail in the Discussion. The durations of these phases and the volumes of gas pumped were different for inflations and deflations, as the data of Table 2 show. Significantly more gas was inspired through the nostrils (phase 1) during an inflation than in a deflation. The duration of an inspiratory phase depended on the type of breathing pattern. When an animal had just surfaced from a dive, particularly one of some duration, the lungs were inflated with a rapid succession of ventilation movements with no intervening buccal oscillations (Figs 4A, 5 and 6A). During these inflations phase 1 was substantially shorter in duration than it was in deflations (Fig. 6). However, when an animal settled to breathing at the surface, with buccal oscillations occurring between lung ventilations, the duration of phase 1 was relatively constant and there was no significant difference between inflations and deflations (Table 2). During deflations, significantly more gas was expired, with both expiratory phases (2 and 4) being of greater duration than they were in inflations (Table 2). The duration of the lung-filling phase (phase 3) did not differ significantly in inflations and deflations. However, more gas was pumped into the lung in an inflation, as suggested by the differences between inspiration and expiration volumes and confirmed by the greater rate of rise of lung pressure during phase 3 (Table 2). In the lung-emptying phase (2), the rate at which lung pressure fell was the same for both inflations and deflations but the duration of this phase was greater in deflations, allowing a greater volume of gas to flow out of the

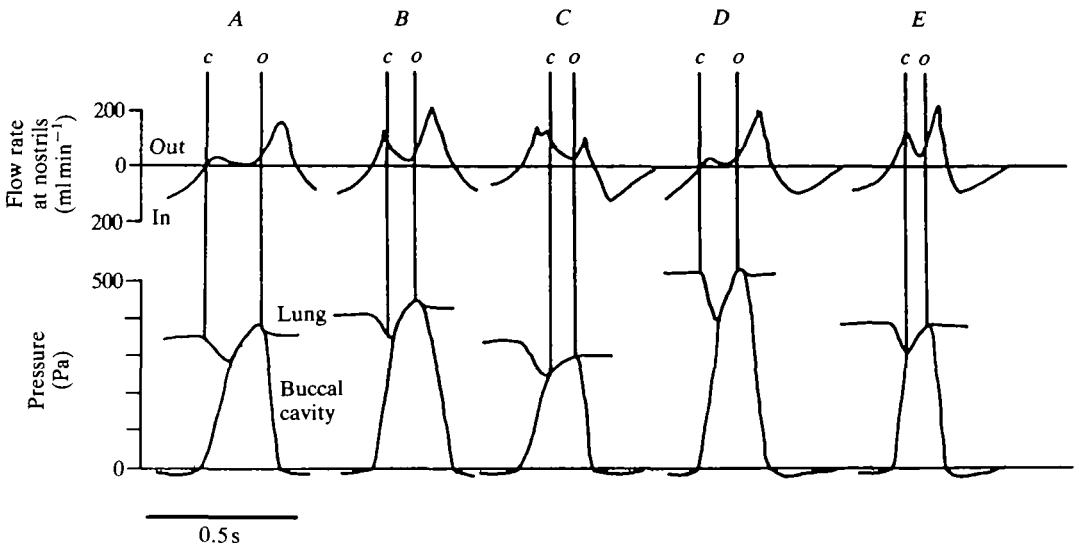


Fig. 7. Redrawn air flow profiles with associated lung and buccal pressure recordings showing variety of breaths found in frogs breathing at the blowhole. Vertical lines indicate when the nostrils close (*c*) and open (*o*) as determined by ciné film analysis. (A) Inflation breath with nostrils closing as glottis opens causing small or no initial expiration. (B) Inflation breath with nostrils closing after glottis opens, causing larger initial expiration. (C) Deflation breath with nostrils closing after glottis opens, causing maximal initial expiration. (D) Maintenance breath with nostrils closing as glottis opens, causing small initial expiration. (E) Maintenance breath with nostrils closing after glottis opens, causing larger initial expiration.

lung (Table 2). Direct observations could not be made on the glottis but the pressure records (e.g. Fig. 6) show that it must be open throughout phases 2 and 3. The duration of its opening was significantly greater in deflations.

#### *Ciné film analysis*

Analysis of ciné films established the precise timing of nostril movements in relation to the other events in the ventilation cycles. The films were analyzed on a frame-by-frame basis for a total of 78 breaths from six animals. The nostrils remained open during buccal ventilations, although, in some animals, small changes in nostril aperture could be seen. During lung ventilations, however, the nostrils were closed for a brief period so that gas could be pumped into the lung through the open glottis as the buccal floor was raised.

The measurements of gas flow at the nostrils suggested that there was some variation in nostril timing and movement. The variety of flow patterns associated with lung inflation, deflation and maintenance ventilations is illustrated in Fig. 7, with pressures and flows redrawn from original records. The timing of nostril movement, as determined from the film analysis and associated pressure records, is shown in Fig. 7.

In some breaths the nostrils closed at the same time as the glottis opened, judging the latter by the beginning of the fall in lung pressure. These breaths were associated with lung inflation (Fig. 7A) or with maintenance of volume and pressure (Fig. 7D). In other breaths the nostrils closed much later, at or approaching the time when the buccal and lung pressures became equal following lung emptying. These were associated with lung deflations (Fig. 7C), though occasionally both lung inflations (Fig. 7B) and maintenance ventilations (Fig. 7E) were found to have this type of nostril timing. The rapid fall of buccal pressures to atmospheric levels after lung filling was always initiated by opening of the nostrils. Nostril opening and glottis closure were quite closely synchronised at the end of lung filling and no consistent timing differences could be correlated with different types of breathing pattern. Occasionally the nostrils remained open throughout the lung ventilation and this was always accompanied by a large expiration of gas from the animal.

Of the breaths analyzed, 47% resulted in inflation, with these being evenly distributed between types A and B (see Fig. 7). All deflations were of type C and represented 22% of the breaths analyzed. Maintenance breaths occurred 31% of the time and type D was twice as frequent as type E.

### Discussion

The new information concerning air flow patterns, simultaneously recorded with buccal and lung pressure, during spontaneous breathing in undisturbed *Rana pipiens* has allowed a reappraisal of the ventilatory mechanisms. There is much greater variability than previously thought, even in resting animals. In diving animals further variation is seen.

#### *Buccal oscillations*

The experiments have shown that the continuous buccal oscillations give rise to a simple tidal pattern of flow at low rates through the nostrils. The pressure events of these buccal oscillations have been also described by de Jongh and Gans (1969) in *Rana catesbeiana*, by West and Jones (1975) in *Rana pipiens*, and in *Bufo marinus* by MacIntyre and Toews (1976) and Jones (1982). The oscillations appear to be common to semi-aquatic and terrestrial anurans but are absent in the aquatic anuran *Xenopus laevis* (Brett and Shelton, 1979).

The function of buccal oscillations is not entirely clear. They certainly renew the air in the buccal cavity in preparation for a lung ventilation, but, when continued for extended periods between lung ventilations, the final oscillations must have a negligible effect. During these extended periods the amplitude of the oscillations may decrease and short periods of apnoea ensue. Foxon (1964) thought that the movements had an olfactory function and that gas exchange across the bucco-pharyngeal epithelium was of little importance. This conclusion was based on the work of Czopek (1955), who shows that less than 1% of the total length of capillaries in respiratory surfaces was to be found in the buccal cavity of *Rana*. He

suggested that capillary density in a respiratory surface gave an indication of the contribution of that surface to overall gas exchange. The frequency of buccal oscillations remains relatively constant even when activity levels change. There are substantial fluctuations in amplitude and larger-amplitude oscillations are invariably seen just before and immediately after lung ventilations. These must be important in clearing the buccal cavity of gas from the lungs in preparation for the next lung ventilation, as de Jongh and Gans (1969) suggest.

### *Lung ventilation*

In most lung ventilations, the volume of the lungs at the end of a breath was different from that at the beginning. The sequence of events for typical inflation and deflation breaths is summarized in Fig. 8. Intermediate patterns of behaviour exist and cause smaller volume changes. The pressure and flow relationships in a single breathing cycle will be described for a lung inflation before considering the differences found in a deflation breath.

#### *Phase 1. Inspiration*

A large volume of air is inspired into the expanding buccal cavity through open nostrils. Pressure in the buccal cavity during this time is slightly below atmospheric levels and pressure in the lung is held above atmospheric by the closed glottis. Volume change in the buccal cavity is large and flow rates at the nostrils high. Inspiratory muscles are clearly activated to high levels, even when the phase duration remains constant. Greater activation levels become necessary when phase duration is shortened, as it is during the ventilations immediately following a dive.

#### *Phase 2. Lung emptying and expiration 1*

The beginning of the phase is marked by glottis opening, allowing gas to leave the lung and the pressure to fall. The nostrils close rapidly so that very little gas leaves the buccal cavity. Pressure in the buccal cavity increases rapidly and the end of the phase is marked by the equilibration of lung and buccal pressures. Since a negligible volume of gas leaves *via* the nostrils, there must be complete mixing between buccal and pulmonary gas during this and the subsequent phase.

#### *Phase 3. Lung filling*

The phase begins as the lung pressure starts to rise. The pressures in lung and buccal cavity are identical throughout, though an unmeasurably small gradient must exist across the open glottis to drive gas into the lungs. The buccal floor is raised by vigorous excitation of its muscles causing high rates of lung filling. The nostrils are closed at the beginning of the phase but begin to open towards the end when pressures start to fall.

#### *Phase 4. Expiration 2*

The beginning of the phase is marked by the glottis closing so that pressure in



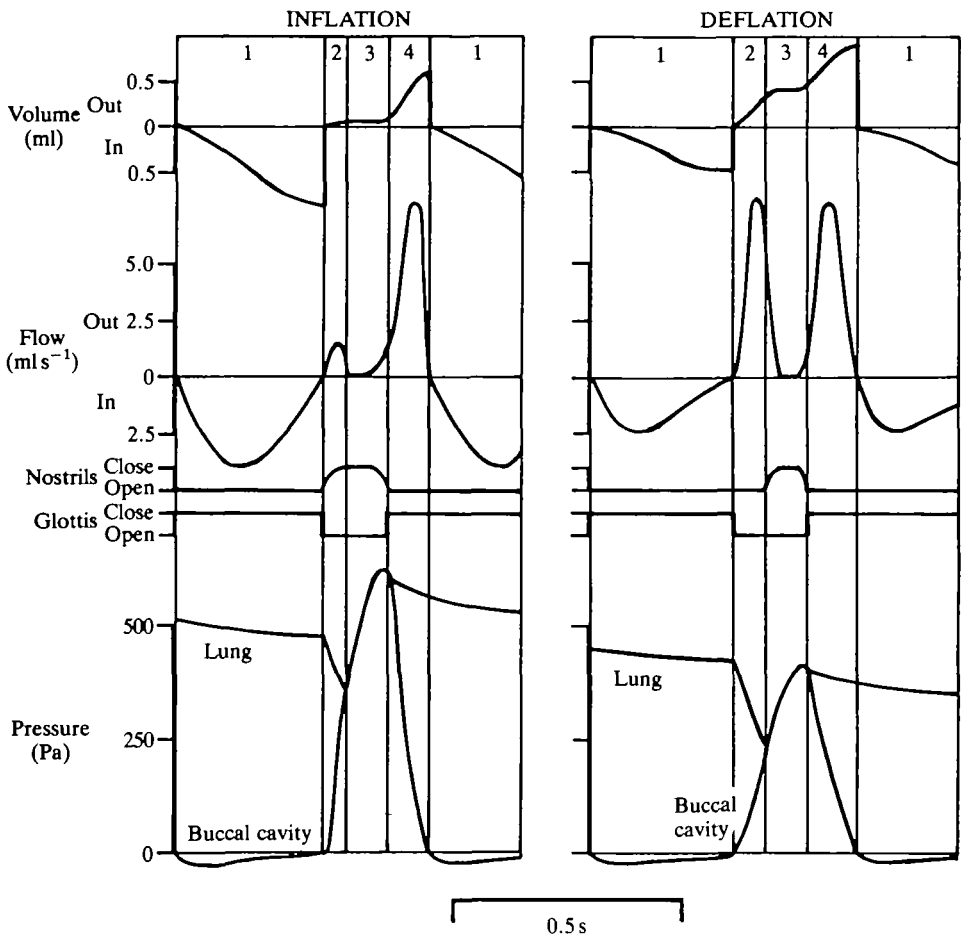


Fig. 8. Lung ventilation in *Rana pipiens*. Diagram to show volume changes and air flows at the nostrils, pressures in the buccal cavity and lungs, and the timing of nostril and glottis movement during a lung inflation and a lung deflation. The ventilation cycle is divided into four phases: (1) inspiration; (2) lung emptying and expiration 1; (3) lung filling; (4) expiration 2. For further details see text.

the lung is maintained at a higher level than at the start of phase 1. The volume of the buccal cavity continues to decrease during this phase but the buccal pressure falls to atmospheric levels because gas is expired at high flow rates through the fully opened nostrils. The phase ends and the next phase 1 begins as the pressure crosses the zero line.

The volume of gas pumped into the lungs during phase 3 ultimately determines whether the breath is an inflation or a deflation and clearly depends on a number of closely coordinated changes in breathing behaviour. The important determinants of this volume are the timing and force of contractions of the buccal musculature during both inspiration and lung-filling phases, together with the

timing of nostril closure in the lung-emptying phase. During deflations, the muscles lowering the buccal cavity floor contract with less force than in inflations, so that a smaller volume of air is inspired in phase 1. There is also a delay in nostril closure so that gas is expired in substantial quantity during phase 2 as the lung empties. The rate of lung emptying appears to be the same in inflations and deflations but to go on for a 45% longer period in the latter. This suggests that lung emptying may be a purely passive process relying on elastic recoil of the lungs but there has been no direct confirmation of this suggestion in the present work. The effect of the expiration of gas from the nostrils in phase 2 of a deflation breath is to reduce the total volume in the lungs and buccal cavity considerably, the latter already being at a reduced volume because of the smaller inspiration. In addition, the muscles raising the buccal floor contract with less force so that a smaller volume of gas is pumped into the lungs, even though the duration of phase 3 is the same as during an inflation breath. The final result of these manoeuvres is that the lung is at a smaller volume and lower pressure when the glottis closes than it was at the beginning of phase 1. The volume of the buccal cavity continues to decrease during a phase 4 that is slightly longer in a deflation than in an inflation, so that the volume expired is somewhat greater and the buccal cavity begins the next cycle at a lower volume.

The differences between inflation and deflation breaths are thus the result of precise adjustments in motor activity to the respiratory effectors. In a deflation, buccal cavity muscles contract less vigorously during both inspiration (phase 1) and lung filling (phase 3), and nostril closure is later by some 70 ms than in an inflation. Glottis opening lasts for 20 ms longer, though our evidence is not based on direct observation. Clearly the respiratory control system, though producing a fairly stereotyped pattern of activity, is capable of subtle variations that give rise to changes in lung volume over a wide range. These variations must have adaptive significance.

#### *Lung inflation–deflation sequences*

Inflation–deflation sequences are an integral part of the respiratory pattern of *Rana pipiens*. They are most reliably seen during diving behaviour in animals that are ventilating their lungs after periods of apnoea. After a dive, a number of lung inflations establish positive buoyancy. In addition, inflation of the lung with freshly inspired air must be important in renewing the oxygen stores depleted during the preceding dive. Deflation breaths always follow quickly on such long inflations, even though the animal remains at the surface and continues to ventilate its lungs. A substantial deflation breath usually precedes the next dive. However, inflation–deflation sequences are also seen in animals that are out of water, reinforcing the view that this type of breathing pattern is important in gas exchange and is not simply a means of buoyancy control. It seems likely that inputs from respiratory gas receptors, lung stretch receptors and some form of buoyancy receptors are involved in the control of inflation–deflation sequences.

The importance of these sequences in ventilation of the lungs as part of the

normal gas exchange process is confirmed by the fact that they increase in frequency when metabolic rate goes up or when lung ventilation is stimulated by some other means. Pinder and Burggren (1986) showed that hypoxia stimulated regular large oscillations in lung volume in *Rana pipiens*. MacIntyre and Toews (1976) found that 5% CO<sub>2</sub> increased lung ventilation and the number of inflation–deflation sequences in *Bufo marinus*. Inflation–deflation sequences similar to those described in this study have also been reported by West and Jones (1975) in *Rana pipiens*, and by de Jongh and Gans (1969) in *Rana catesbeiana*. The lung ventilation rate recorded by West and Jones (1975) in their experiments was greater than 30 min<sup>-1</sup>, probably because their animals were restrained and somewhat stressed. Both the high ventilation rate and the high frequency of the large inflation–deflation sequences were the result of stress and a high metabolic rate. The lung ventilation rate in our study was 6.3 min<sup>-1</sup>, indicative of unrestrained animals at rest, yet inflation–deflation sequences were still present. This suggests that such inflation sequences are not solely a consequence of stress but that they do increase in frequency under conditions that lead to elevated metabolism.

Study of flow patterns during inflation–deflation sequences leads us to different interpretations of the lung ventilation process from those of previous workers (de Jongh and Gans, 1969; West and Jones, 1975). Air flow measurements show that the early closure of the nostrils during phase 2 of a lung inflation allows very little air to leave the animal in this phase. The gas refilling the lung during phase 3 must be a complete mixture of the freshly inspired air taken into the buccal cavity in phase 1 and the lung gas entering the buccal cavity in phase 2. During such lung ventilations the coherent stream of lung gas that minimizes mixing, as proposed by Gans *et al.* (1969), cannot exist. A coherent stream of lung gas could only be established in lung deflations since there is a substantial flow of gas from the nostrils during phase 2 in this type of breath. However, the flow profiles show two rapid accelerations and decelerations in the gas leaving the nostrils during phases 2 and 4. Such rapid changes of flow would inevitably disrupt the laminar flow required for a coherent stream of lung gas to emerge from the nostrils.

De Jongh and Gans (1969) deduced air flow patterns in *Rana catesbeiana* from pressure–volume relationships and electromyography of the muscles controlling nostrils and glottis. West and Jones (1975) used similar techniques and measured the direction of flow at the nostrils by means of a bead thermistor. Both investigations concluded that the main outflow of gas from the nostrils occurred in phase 2 of a lung ventilation, with both nostrils and glottis open. They proposed that only small volumes of gas emerged from the nostrils at low flow rates during phase 4 after the glottis had closed, with the buccal cavity increasing in volume during this phase. The pressure gradient between the buccal cavity and the outside air is greatest at this time, however, and the nostrils are open. The fall to atmospheric pressure occurs very rapidly, suggesting high flow rates. Our measurements confirm that high flows through the nostrils during phase 4 are always found in *Rana pipiens*, with the buccal cavity still decreasing in volume

after the completion of lung filling. The gas flowing out through the nostrils during phase 4 can only be that remaining in the buccal cavity after lung filling. A central point of the hypothesis put forward by Gans *et al.* (1969) is that the coherent stream of pulmonary gas, conveyed to the nostrils and expired, bypasses pockets of air in the buccal cavity. Air from these pockets can then be pumped into the lung. It is difficult to reconcile our finding of a substantial final expiration during phase 4 with the suggestion that a refined system exists to ensure that it contains air that is least contaminated with the gas leaving the lung.

Patterns of ventilation in *Rana pipiens* are highly variable. Long-term records taken over several hours are required to understand the significance of this variability. The animals frequently inflate and deflate their lungs over several successive breaths. These sequences are separated by breaths which maintain lung pressure over a narrower range. The patterns are controlled by precise timing of the buccal pump, the nostrils and the glottis. Flow measurements at the nostrils show that complete mixing of inspired air and lung gas occurs during lung inflations and almost certainly during other breaths as well. Further experiments to measure gas concentrations in the system are required in order to evaluate the consequences of this mixing in the overall gas exchange process.

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