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VENTILATORY MECHANICS AND THE EFFECTS OF WATER DEPTH ON BREATHING PATTERN IN THE AQUATIC CAECILIAN *TYPHLONECTES NATANS*

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Summary

The breathing pattern in the aquatic caecilian *Typhlonectes natans* was investigated by recording airflow *via* a pneumotachograph under unrestrained normal physiological conditions. Ventilatory mechanics were assessed using airflow and pressure measurements from the buccal cavity and trachea. The breathing pattern consisted of an expiratory phase followed by a series of 10–15 small buccal pumps to inflate the lung, succeeded by a long non-ventilatory period. *T. natans* separate the expiratory and inspiratory gases in the buccal cavity and take several inspiratory pumps, distinguishing their breathing pattern from that of sarcopterygians. Hydrostatic pressure assisted exhalation. The tracheal pressure was greater than the water pressure at that depth, suggesting that pleuroperitoneal pressure as well as axial or pulmonary smooth muscles may have contributed to the process of exhalation. The frequency of lung ventilation was 6.33±0.84 breaths h^{-1}, and ventilation occurred *via* the nares. Compared with other amphibians, this low ventilatory frequency suggests that *T. natans* may have acquired very efficient pulmonary respiration as an adaptation for survival in their seasonally fluctuating natural habitat. Their respiratory pathway is quite unique, with the trachea separated into anterior, central and posterior regions. The anterior region serves as an air channel, the central region is attached to the tracheal lung, and the posterior region consists of a bifurcated air channel leading to the left and right posterior lungs. The lungs are narrow, elongated, profusely vascularized and compartmentalized. The posterior lungs extend to approximately two-thirds of the body length. On the basis of their breathing pattern, it appears that caecilians are phylogenetically derived from two-stroke breathers.

Key words: breathing pattern, ventilatory mechanics, pneumotachography, amphibian, *Typhlonectes natans*.

Introduction

Amphibians are the major transitional group of sarcopterygian bimodal breathers and have recently received the most attention. Most ventilatory studies on amphibians have utilized anurans (frogs and toads), the most derived group of the lissamphibians (Boutilier, 1988; Boutilier and Toews, 1977; Brainerd et al., 1993; de Jongh and Gans, 1969), while ventilatory control in urodeles (salamanders) and Gymnophiona (caecilians) has been largely ignored. Without a better understanding of breathing mechanics and ventilatory control in these two groups, further progress in understanding the evolution of air breathing may be severely limited.

In urodeles, a handful of studies have described respiratory mechanics (Brainerd, 1994; Brainerd et al., 1993; Brainerd and Monroy, 1998; Guimond and Hutchison, 1973a,b, 1976; Martin and Hutchison, 1979), while only three studies (Bennett et al., 1999; Smits and Flanagin, 1994; Toews and Macintyre, 1978) have addressed breathing in the Gymnophiona. None of these studies assessed mechanics and ventilatory pattern. Although Carrier and Wake (1995) described the mechanics of lung ventilation in the terrestrial caecilian *Dermophis mexicanus*, they were unable to determine whether body muscle activity assisted in exhalation in *D. mexicanus*. Recently, Bennett et al. (1999) concluded that the transverse abdominis muscle does not participate in exhalation. As it is not clear from the available literature whether urodeles or Gymnophiona are the most primitive extant group of Lissamphibia, studies of their respiratory mechanics are needed to make further inferences concerning the evolution of ventilatory control and mechanics among lissamphibians.

Aquatic caecilians are restricted to tropical environments, with the majority confined to South America (Duellman and Trueb, 1986). Currently, there are only three recognized

species of aquatic caecilians in the genus *Typhlonectes*: *Typhlonectes compressicauda*, *Typhlonectes eiselti* and *Typhlonectes natans* (Nussbaum and Wilkinson, 1989; Wilkinson, 1989).

The present study examined the breathing pattern and ventilatory mechanics of the aquatic caecilian *Typhlonectes natans*. It was hypothesized that *T. natans* would use a four-stroke buccal pump or a highly modified version of a two-stroke pump, since it expires first and then inhales air. In addition, since its aquatic habitat provides hydrostatic challenges, it was hypothesized that *T. natans* would utilize hydrostatic pressure to force gas from its lungs.

Materials and methods

Animal acquisition and care

Typhlonectes natans (Duellman and Trueb) were obtained from a licensed commercial supplier and housed in 75 l glass tanks filled with 35 l of dechlorinated tap water. Each tank contained a polyvinyl chloride (PVC) tube to give the animals cover. From the PVC tube, the animal was able to extend its head to the surface to breathe. Animals were kept on a 12 h:12 h L:D photoperiod in a room maintained at a constant water temperature of 28±1 °C, a pH of 7.0 (the preferred pH for these tropical amphibians), and fed commercial fish wafers (Hikari) twice a week. Tanks were cleaned twice a month, and one tablet of erythromycin (200 mg), an antibiotic, was added to each tank after cleaning to avoid bacterial infections.

Breathing pattern and ventilatory mechanics

Experimental arrangement

The ventilatory apparatus used was similar to that of Funk et al. (1986). It consisted of a ventilatory chamber, a custom-made pneumotachograph connected to the chamber and a resting tube also attached to the chamber, all submerged in a tank of water such that a portion of the ventilatory chamber was above the water so that the caecilian could ascend and breathe (Fig. 1). The resting tube had a 0.5 cm×20 cm long slot in the center through which catheters could be placed. It rested 5 cm below the water's surface, which allowed less than one-third of the animal's body to be oriented vertically while breathing. The pneumotachograph was connected to a Validyne differential pressure transducer (DP-103-45, Validyne Engineering Corp., Northridge, CA, USA). Signals from the transducer were amplified by a Validyne carrier demodulator (CD-15), converted to a digital signal (A/D convertor, DI-205, Dataq Instrument Inc., Akron, OH, USA), and displayed and stored on a computer using a data-analysis program (Windaq 200) for later analysis. Sampling rate was 50 Hz per channel.

Airflow was measured on the basis of the pressure gradient created across the resistance grid of the pneumotachograph (Hobbes, 1967; Glass et al., 1978). The ventilatory chamber was supplied with a constant flow of air (50 ml min^{-1}). From this baseline flow, the pneumotachograph was calibrated by manually injecting and withdrawing known volumes of air *via* the calibration port. The ranges of volumes and frequencies used for calibration were chosen to encompass the range of frequencies and volumes used by the animals as obtained from the preliminary studies. Calibration volumes of 0.1 and 0.2 ml given over 0.7 and 1.0 s were used to simulate inspiratory cycles, while volumes of 1.0, 1.5 and 2.0 ml withdrawn over 1.0 and 1.8 s were used to simulate expiratory cycles. Calibration data were recorded on a computerized data-acquisition system (Windaq 200). Expiratory and inspiratory durations were measured, and the measured volume (V_M) was obtained by integrating the area under the airflow *versus* time curve. The actual volumes (V_A) injected or withdrawn were

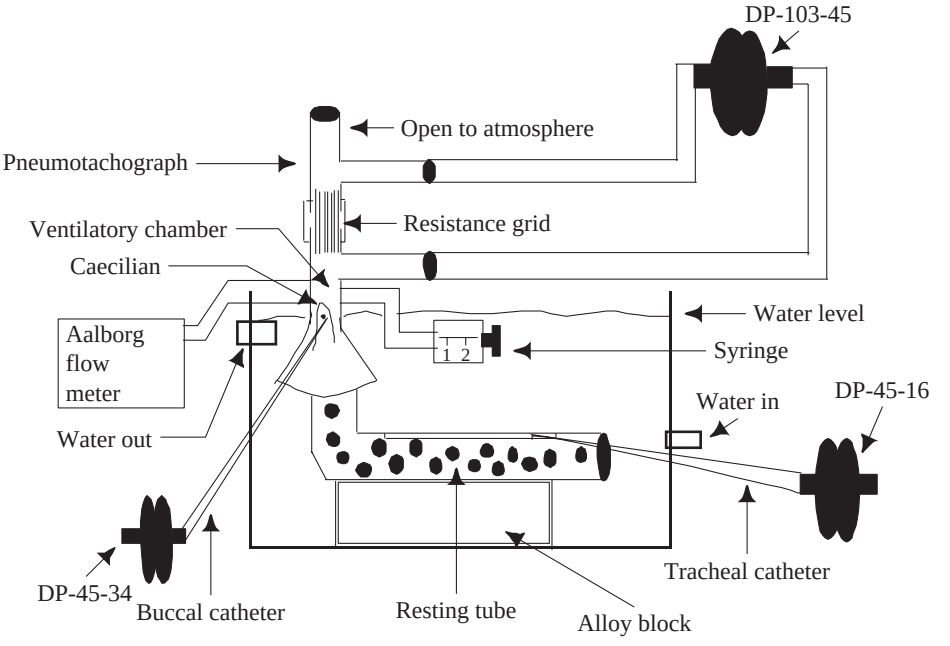

Fig. 1. Schematic drawing of the ventilatory apparatus. This arrangement was used to measure buccal pressure, tracheal pressure and ventilatory airflow. DP, differential pressure transducer.

compared with the measured volumes. The measured volume showed frequency-dependence. V_M increased as injection duration increased. Thus, V_A *versus* V_M was plotted for each injection frequency, and the resulting regression equations were used to obtain corrected volumes. For inspiration and expiration, the relationships between V_M and V_A were best described by the equations: $V_M=0.66V_A+0.01$ ($N=68$, $r^2=0.95$, $P<0.05$) and $V_M=0.82V_A-0.002$ ($N=68$, $r^2=0.87$, $P<0.05$), respectively. These equations were used to convert the raw values of V_M to V_A to obtain the best possible estimate of tidal volume.

Surgery

Caecilians were not fed for at least 3 days prior to surgery and were removed from their home tank and placed into the experimental apparatus 1 day before experimentation. They were anesthetized in a 0.06 % solution of tricaine methane sulfonate (MS-222) in dechlorinated tap water, buffered with sodium bicarbonate to a pH of 7.0. They were then weighed before being transferred to the surgery table, where they were kept moist with filter paper soaked in the MS-222 solution. A buccal catheter (flared PE-50 tubing bent to 90 ° at a point 0.6 cm from the flared end) was inserted 0.4 cm caudal to the tip of the snout. The catheter was secured with 0 gauge silk thread and positioned so that the animal could move easily without becoming entangled in the resting tube. The free end of the buccal catheter was connected to a Validyne differential pressure transducer (DP-45-34). The catheter was filled with water and cleared regularly with water during the experiment.

A custom-made T-tube was used to measure tracheal pressure. The T-tube was constructed from polyethylene tubing. At both ends of a 1 cm long PE-205 tube, a piece of PE-160 tubing (0.5 mm long) was inserted, and PE-90 tubing (0.5 mm long) was inserted at both ends of the PE-160 tubing. PE-50 tubing (0.5 mm long) was then inserted at both ends of the PE-90 tube. A hole slightly smaller than the external diameter of the PE-50 tubing was made in the center of the PE-205 tubing, into which a piece of PE-50 tubing was inserted. All joints were secured with superglue and tested for airtight seals. A mid-ventral incision was made approximately 3 cm caudal from the tip of the lower jaw to expose the trachea. Tissue and blood vessels were cleared from the anterior part of the trachea, ensuring that the nerves were left intact. A small incision was made in the trachea, into which one end of the T-tube was inserted. The tube was secured with 3-0 gauge silk thread. Another small incision was made approximately 1 cm caudal to the first incision, and the other end of the T-tube was inserted and secured with 3-0 gauge silk thread. The T-tube connections were tested for leaks and sealed if necessary. The skin was sutured and superglued to prevent water from entering the body cavity. To prevent the catheter from tangling, a fish knot was made with 0 gauge silk thread around the cannula, and the same thread was tied loosely around the animal's body.

Tracheal pressure was measured using a Validyne differential pressure transducer (DP-45-16) and fed into an open channel of the data-acquisition system. Air was pumped into the animal's lungs, and the syringe was then removed to determine patency. If the lungs did not collapse, the system was checked and corrected for obstructions. Once corrected, the catheter was filled with water to a point 2 cm from the T-tube; subsequently, the water level never changed by more than 0.1 cm during the rest period. The catheter never became blocked during the experiments.

Experimental protocol

The breathing pattern was recorded for 2 h prior to any surgical intervention and after at least 4 h of habituation to the experimental apparatus. Following the initial 2 h recording of the breathing pattern, the animal was anesthetized and implanted with buccal and tracheal cannulae as described above. On recovery from surgical procedures (35–45 min after surgery), the animal was replaced into the ventilatory apparatus and allowed to habituate for a further 4 h prior to recording. Buccal pressure (P_b), tracheal pressure (P_t) and airflow were then recorded for a 3 h period.

Data analysis

The following ventilatory variables were measured: breathing frequency, expiratory volume, inspiratory volume, the number of inspiratory buccal oscillations per breath, expiratory duration and total breath duration. Buccal pressure, tracheal pressure and airflow were plotted and examined graphically using the Windaq data-acquisition software to assess the timing relationships for the opening and closing of the nares and glottis and their relationship to the buccal expansion and compression cycles. Post-acquisition data analysis was used to analyze buccal and tracheal pressures. Raw values for airflow and for buccal and tracheal pressures were exported from the Windaq data-acquisition software into a spreadsheet (Microsoft Excel) and graphics programs (Freelance, Sigmaplot) to describe ventilatory mechanics graphically.

Effects of deep versus *shallow water on ventilatory pattern*

The effects of water depth on breathing pattern were studied. In experiments in which buccal pressure was measured, a buccal cannula was implanted as described above. Airflow and buccal pressure were measured using the experimental arrangement described above. To assess the effects of water depth on ventilatory variables, the resting tube was positioned at two depths: 16.5 cm for deep-water experiments and 3.5 cm for shallow-water experiments.

Experimental protocol

Unanesthetized animals, on which surgery had not been performed, were allowed to habituate at each water depth for at least 2 h before the breathing pattern was recorded for 2 h. After implantation of the buccal catheter, the animal was allowed to recover from the anesthetic before being placed back into the ventilatory apparatus to continue recovery for a minimum of 15 h before the deep- and shallow-water measurements were repeated.

Data analysis

After the experiments, the data were analyzed to determine expiratory volume (V_{te}; ml), inspiratory volume (V_{ti}; ml), breathing frequency (f; breaths h^{-1}), the number of inspiratory buccal oscillations per breath (I; oscillations $breath^{-1}$), expiratory duration (T_e; s), total inspiratory duration ($T_{i,tot}$; s), the duration of a single inspiratory buccal oscillation (T_i; s), total breath duration (T_b; s) and peak expiratory flow ($\dot{V}_{pe}$; $ml\,s^{-1}$) for the deep- and shallow-water experimental recordings. Paired *t*-tests were used to compare the mean values of variables between deep- and shallow-water experiments. In addition, expiratory and inspiratory buccal pressures (P_{be} and P_{bi}; kPa) for the deep- and shallow-water experiments were compared. Results were considered significant at $P<0.05$. All measurements are presented as means ± S.E.M.

Morphology of the respiratory structures of Typhlonectes natans

Experimental individuals were killed at the end of the experiment using an anesthetic overdose followed by an intracardiac injection of saturated potassium chloride. The following morphometric measurements (using calipers, to the nearest 1 mm) of respiratory structures were taken: total body length, the distance between the glottis and the point where the tracheal lung began, the length of the tracheal lung, the length of the left posterior lung and the length of the right posterior lung. From the above measurements, the ratios of the lengths of the trachea and of the right and left posterior lungs to body length were calculated.

Results

Six caecilians were used to describe breathing pattern and ventilatory mechanics, ranging in body mass from 22.6 to 52.4 g.

Breathing pattern

In order to breathe, the animal ascended the water column with its head directed in line with its vertebral column, broke the water's surface with the tip of its head and exposed 0.3–0.4 cm of its snout, which included the external nares. Expiration occurred *via* the nares and was represented by a positive deflection on the airflow traces. Expiratory volume was 1.31±0.35 ml (N=79) and expiratory duration was 1.49±0.13 s (N=79). The expiratory phase was followed immediately by an inspiratory phase during which air was inhaled through the nares as the buccal cavity expanded. The buccal cavity was then compressed, transferring air into the lungs. These inspiratory buccal pumping actions were repeated in series 10–15 times (12.18±1.85, N=926). Although not statistically significant ($P>0.05$), inspiratory volume was generally larger (1.51±0.47 ml, N=79) than expiratory volume. In the present study, a single breath is defined as an expiration followed by a series of inspiratory oscillations (Fig. 2) and lasted for 8.89±0.45 s (N=79). The small upward deflection in the airflow trace before the expiratory phase is due to movement of the animal's head when it comes out of the water to breathe.

On completing its ventilation, the animal submerged, maintaining a head-up orientation. The mouth remained closed throughout the ventilatory cycle. Each breath (ventilatory period) was succeeded by a long non-ventilatory period (Fig. 2). The breathing frequency was 6.33±0.84 breaths h^{-1} (N=79).

Ventilatory mechanics

Fig. 3 shows the airflow and pressure events for the expiratory phase and for a portion of the lung-filling phase for a typical breath.

The expiratory phase

Expiration began with a rapid rise in tracheal pressure (Fig. 3B, arrow a) with peak pressures ranging from 0.5 to 1.3 kPa. This rapid increase may indicate compression of tracheal gas by the body wall muscles. Within a few milliseconds of the start of the tracheal pressure change, the pressure in the buccal cavity increased slightly (Fig. 3B, arrow b), possibly as a result of the rapid efflux of air from the lungs following glottal opening. Opening of the nares was marked by a positive deflection on the airflow trace (Fig. 3A, arrow c) as air moved from the lungs through the buccal cavity and into the ventilatory chamber. There were no buccal cavity

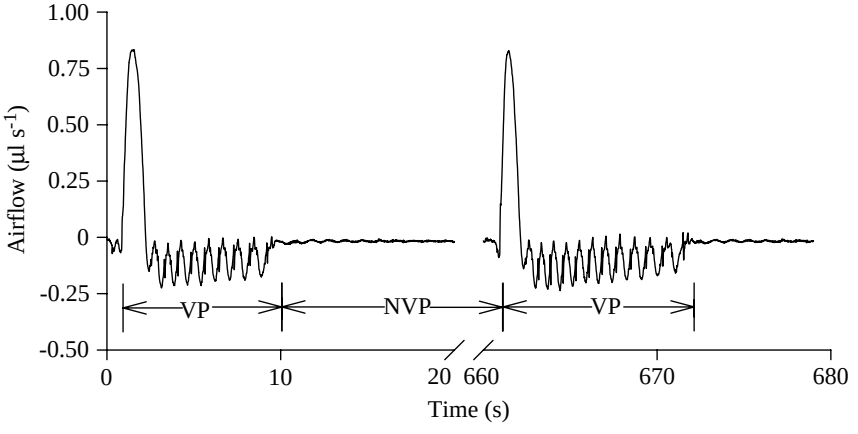

Fig. 2. Airflow recording showing the typical breathing pattern of *Typhlonectes natans* (uninstrumented) when the resting tube was 5 cm below the water's surface. The recording shows one expiratory cycle (positive deflection in the airflow trace) and several small inspiratory cycles (negative deflection in the airflow trace) followed by a nonventilatory period (NVP). VP, ventilatory period. Two sequential breaths are shown. Note the break in the time axis.

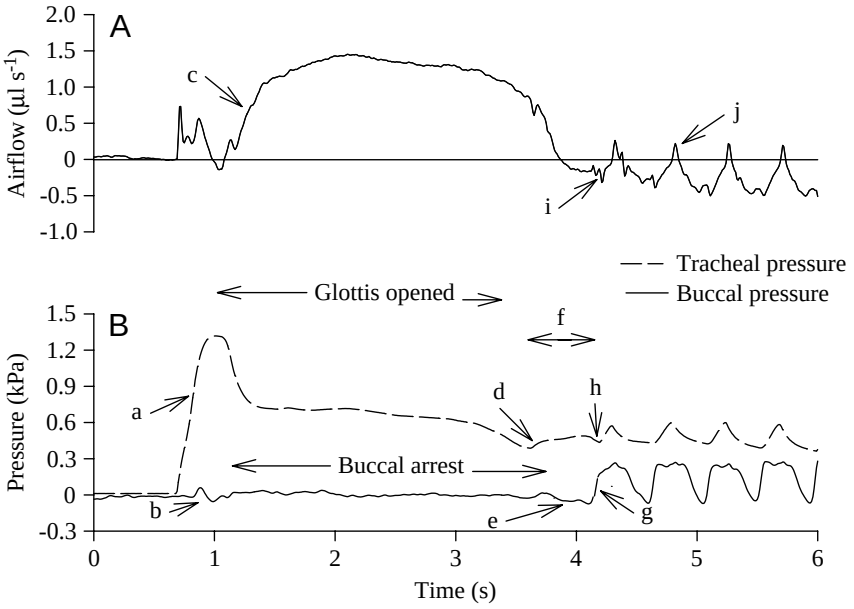

Fig. 3. Representative recordings of airflow (A) and tracheal and buccal pressures (B) used to assess ventilatory mechanics. The resting tube was 5 cm below the water's surface. The letters used to identify different regions of the traces are explained in the text. Pressures are expressed relative to ambient pressure (0 kPa).

movements during the expiratory period. As expiration continued and lung volume declined, tracheal pressure decreased, initially sharply and then slowly, throughout the remainder of the expiratory phase. Tracheal pressure never decreased to atmospheric level (0 kPa in Fig. 3B). The end of expiration was typically marked by a slight increase in tracheal pressure (Fig. 3B, arrow d) accompanied by a small increase in buccal pressure as the remaining air was expelled from the buccal cavity through the still open nares (positive deflection on the airflow trace).

The lung-filling phase

The first inspiration began with a decrease in buccal pressure (Fig. 3B, arrow e). As buccal cavity pressure decreased, air moved through the open nares (negative deflection on the airflow trace) into the buccal cavity. In most amphibians, the glottis opens during the buccal expansion phase; however, in *T. natans*, the tracheal pressure increased slightly during the buccal expansion phase, suggesting that the glottis was closed (Fig. 3B, period f). The first buccal filling cycle ended with a rapid increase in buccal pressure above ambient due to buccal compression (Fig. 3B, arrow g). Buccal pressure reached a peak, leveled off, then rose to a second peak and fell slightly to plateau before falling rapidly to subatmospheric levels. The glottis opened at the first buccal pressure peak, as marked by a sudden negative deflection in the tracheal pressure trace (Fig. 3B, arrow h). This was immediately followed by an increase in tracheal pressure as buccal compression continued. An increase in the narial resistance was marked by a depression on the airflow trace (Fig. 3B, arrow i). Airflow reversed at this point, but was still negative. It is possible that there was a head/water movement artifact that obscured the airflow at this point. The increase in narial resistance minimized air loss and facilitated gas transfer into the lungs during the buccal compression phase. Some air did escape from the nares (Fig. 3B, arrow j) near the peak of the buccal compression cycle, but the remaining air in the buccal cavity was forced into the lungs as tracheal pressure increased. The small positive airflow during the buccal compression phase was not observed in uninstrumented animals (Fig. 2) and could be related to the presence of the catheter preventing the animal from closing the buccal cavity completely. The elevation in buccal pressure above tracheal pressure was accompanied by an increase in tracheal pressure, more clearly indicating gas transfer from the buccal cavity into the lungs. Glottal closure marked the end of gas transfer into the lungs, whereupon buccal pressure rapidly decreased to subatmospheric level. The pattern described above for the first inspiratory buccal pump was repeated in series 10–15 times. A small increase in tracheal pressure was associated with each inspiratory buccal pump.

Occasional events such as incomplete mouth closure and artifacts resulting from air bubbles in the catheters sometimes resulted in pressures that were not consistent with gas transfer. Buccal pressure occasionally appeared to be elevated above ambient pressure at the end of the buccal compression cycle, when airflow suggested that inspiration was still occurring. Whether this is an airflow or buccal pressure artifact is not clear from the recordings. In addition, because of the volume of air left in the tracheal catheter, pressures recorded from this location were also attenuated.

Effects of water depth on breathing pattern

Eleven animals (18.9–53.4 g) were used to examine the effects of water depth on breathing pattern and ventilatory variables. For the most part, there were no differences in breathing pattern between depths of 3.5 and 16.5 cm, except that more animals used two rather than one expiratory cycle when placed in the deeper water. The ventilatory variables, i.e. peak expiratory flow, expiratory duration, expiratory and inspiratory volume, the number of inspiratory buccal oscillations (Table 1), were compared statistically between the deep- and shallow-water experiments. In deep water, peak

Table 1. *Summary of ventilatory variables measured in* Typhlonectes natans *placed 16.5 cm (deep) or 3.5 cm (shallow) from the water surface*

Variable	Deep water	Shallow water
f (breaths h^{-1})	7.58±1.89	6.40±1.40
V_{te} (ml)	0.96±0.11	1.06±0.12
V_{ti} (ml)	0.97±0.13	1.14±0.23
P_{be} (kPa)	0.29±0.06	0.15±0.03*
P_{bi} (kPa)	0.60±0.11	0.37±0.08*
T_e (s) with two peaks	1.58±0.17	1.87±0.15
T_e (s) with one peak	1.27±0.07	1.74±0.16*
$T_{i,tot}$ (s)	9.05±0.63	9.5±0.48
T_i (s)	0.79±0.04	0.79±0.06
T_b (s)	10.63±0.73	11.37±0.53
$\dot{V}_{pe}$ (ml s^{-1})	1.10±0.11	0.89±0.09*
I (oscillations $breath^{-1}$)	11.69±0.91	12.81±1.33

Values are means ± S.E.M. (N=11).

Significant differences (P<0.05) from the deep-water value are indicated by an asterisk.

f, frequency of breathing; V_{te}, expiratory tidal volume; V_{ti}, inspiratory tidal volume; P_{be}, expiratory buccal pressure; P_{bi}, inspiratory buccal pressure; T_e, expiratory duration; $T_{i,tot}$, total inspiratory duration; T_i, the duration of a single inspiratory buccal oscillation; T_b, total breath duration; $\dot{V}_{pe}$, peak expiratory flow; I, number of inspiratory buccal oscillations.

expiratory flow was significantly greater and expiratory duration was significantly shorter (Table 1, P<0.05), perhaps because of the increased hydrostatic pressure exerted through the body wall on the lungs. Expiratory duration was not significantly different in breaths where two expiratory cycles were taken. Breathing frequency, the number of inspiratory buccal oscillations per breath, the duration of a single inspiratory cycle, and expiratory and total inspiratory volumes were not significantly affected by water depth. In this study, the total inspiratory volume was considered to be the total amount of air inspired in a breath, i.e. the sum of the volumes pushed into the lungs with each buccal movement.

Experiments were also performed to determine whether water depth altered peak buccal pressure, because greater buccal pressures may be required to overcome hydrostatic pressure with increasing depth. Peak expiratory buccal pressure was significantly greater at a depth of 16.5 cm (Fig. 4; Table 1, P<0.05). The amplitude of buccal pressure during inspiratory buccal oscillations was also greater in deep-water experiments (Fig. 4; Table 1, P<0.05), which may be related to the increased force needed to overcome hydrostatic pressure in order to force air into the lungs.

Morphology of the respiratory organ of T. natans

Nine caecilians were used to describe the gross morphology of the respiratory system. Body mass ranged from 17.9 to 61.91 g, and total body length ranged from 28.4 to 45.7 cm. Measurements of the respiratory structures are summarized in Table 2.

T. natans have very small heads and small buccal cavities. The distance between the tip of the lower jaw and the glottis ranged from 0.8 to 1.5 cm. The external nares were located on the lateral sides of the upper snout approximately 0.2 cm rostral to the eye spots. The internal nares were on the upper palate of the buccal cavity and had flaps that may allow them to be sealed to control airflow and to separate the external environment from the buccal cavity. The buccal cavity was separated from the trachea by a glottal valve.

Three regions of the trachea were distinguished: anterior, central and posterior (Fig. 5). The anterior trachea was attached to the glottis, extended from the glottal valve to the first set of respiratory tissues and led to the central tracheal region, which was attached to the respiratory tissues. The

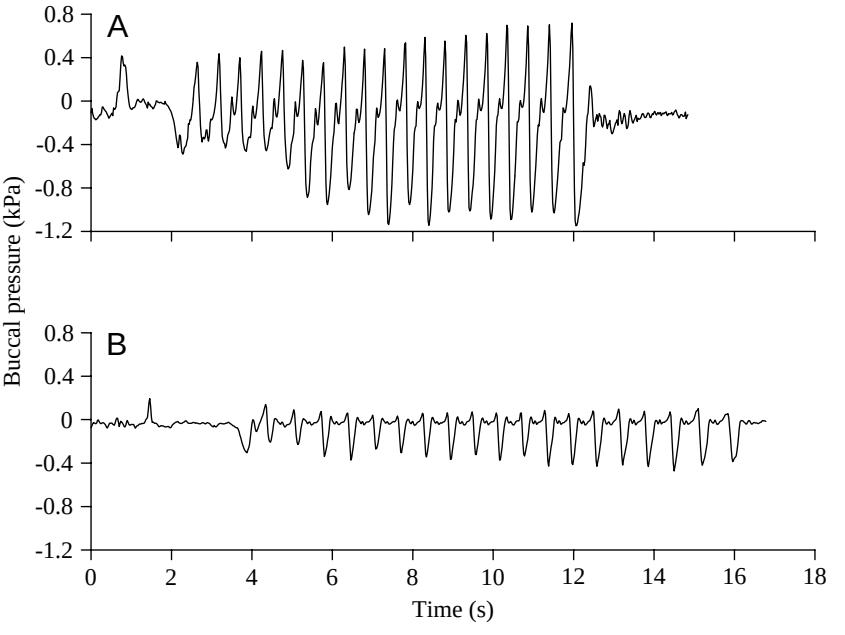

Fig. 4. Buccal pressure recordings of *Typhlonectes natans* in deep (16.5 cm) (A) and shallow (3.5 cm) water (B). Note that the number of inspiratory buccal oscillations is fewer in deep water. Pressures are expressed relative to ambient pressure (0 kPa).

Table 2. *Morphological variables of the respiratory organ of* Typhlonectes natans

Variable	
Body mass (g)	31.45±5.89
Total body length (cm)	35.47±2.55
Distance between lower jaw and glottis (cm)	1.13±0.11
Length of anterior trachea from glottis (cm)	1.63±0.17
Length of tracheal lung (cm)	5.94±0.45
Length of left posterior lung (cm)	15.21±1.02
Distance between apex of left posterior lung and tail (cm)	11.21±1.46
Distance between glottis and end of posterior trachea (cm)	7.57±0.49
Length of right posterior lung (cm)	23.89±1.80
Distance between apex of right posterior lung and tail (cm)	3.01±0.28
Ratio of tracheal lung length to total body length	0.17±0.01
Ratio of left posterior lung length to total body length	0.43±0.02
Ratio of right posterior lung length to total body length	0.67±0.01

Values are means ± S.E.M. (N=9).

respiratory tissues along with the attached trachea were termed the tracheal lung. The caudal end of the tracheal lung ended close to the middle of the heart. The central tracheal region led to the posterior tracheal region (Fig. 5).

The anterior trachea was supported by complete cartilaginous rings, whereas the central and posterior tracheal regions were supported by rings of tissues that were half cartilaginous and half connective tissue. The anterior trachea was poorly vascularized compared with the central and posterior regions. The posterior trachea bifurcated and gave rise to the left and right posterior lungs (Fig. 5). The right posterior lung was significantly longer (P<0.05) than the left (Table 2). Both were elongated and tapered near their apices. The tracheal lung and the left and right posterior lungs were attached to the body cavity by connective tissue. These lungs were highly compartmentalized, profusely vascularized and had no observable central air channel. The respiratory structures extended almost two-thirds of the body length.

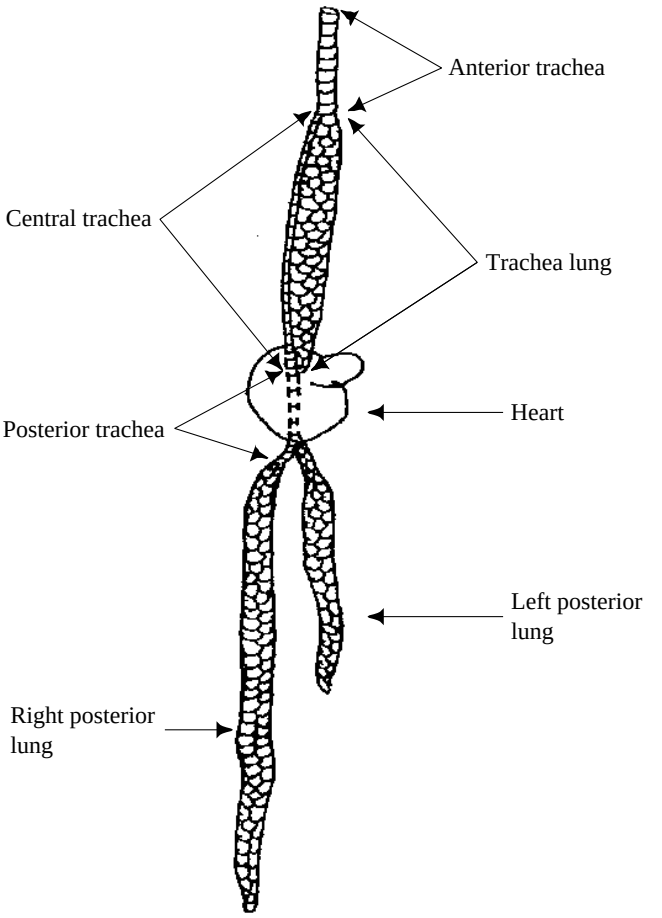

Fig. 5. Schematic drawing of the respiratory organs of *Typhlonectes natans*.

Discussion

Two-stroke breathers (most sarcopterygians) expire and inspire in a single buccal pump, and the concentration of P_{O_2} in the buccal cavity gas is therefore lower than atmospheric. They may take several breaths to increase the P_{O_2} level in their lungs. *T. natans*, *Amphiuma means*, *Siren lacertina* and *Xenopus laevis* separate the expired and inspired gases, and their breathing pattern is therefore functionally similar to that of actinopterygians (four-stroke breathers). They have modified their breathing pattern such that there is an initial expiration, and subsequent buccal pumps inflate their lungs. Since two-stroke ventilation is prevalent among most anurans, urodeles and lungfishes, it is likely that the breathing patterns of *T. natans*, *Amphiuma*, *Siren* and *Xenopus* are secondarily derived from the basal sarcopterygian pattern of breathing as an adaptation to the re-evolution of an aquatic way of life.

Recently, Carrier and Wake (1995) described the mechanics of breathing in a fossorial caecilian (*Dermophis mexicanus*); however, technical problems limited pressure measurements to buccal and pleuroperitoneal pressures only. In contrast, the present study measured airflow changes as well as buccal and tracheal pressures. Tracheal pressure was used because it was difficult to obtain reliable pressure measurements from a lung cannula since the lungs of these animals are narrow and have no observable central air channel. A custom-made T-tube was developed which allowed air to flow unimpeded through the trachea while tracheal pressure was recorded.

Breathing pattern and ventilatory mechanics

The ventilatory pattern of *T. natans* consisted of an expiratory phase and an inspiratory (lung-filling) phase followed by a non-ventilatory period. The glottal valve was generally closed except when air was leaving or entering the lungs, whereas the flaps of the internal nares were normally open during expiration and inspiration but closed during lung filling and when the animal was under water.

The degree of narial opening and closing was not studied, but characteristic inflections on the airflow traces appear to represent changes in narial resistance in this and other studies (Jones, 1982; Vitalis and Shelton, 1990). The cyclical timing of narial and glottal valve opening may be tightly regulated by the central nervous system for effective gas transfer (Ito and Watanabe, 1962; West and Jones, 1975). The role of afferent feedback in this process and in the initiation and/or termination of breathing movements has not been described for any lower vertebrate. In a related study (K. C. Prabha, unpublished data), the effects of vagotomy on breathing in *T. natans* were examined, and it was found that animals became buoyant and appeared to suffer a lack of coordination between breathing movements and airflow. This suggests that afferent feedback *via* the vagus nerve may coordinate the complex sequence of valve and muscular events, and may also signal when the tracheal lung is full, thus terminating the lung-filling phase.

T. natans took 5–8 breaths h^{-1} under normal resting conditions. This low frequency of breathing, compared with other amphibians (Bennett and Wake, 1974; Gatten et al., 1992; Hutchison, 1971; Smits and Flanagin, 1994), could be due to their lower resting metabolic rates and/or increased efficiency of lung gas exchange arising from the separation of inspiratory and expiratory flows. The partitioning of aquatic and aerial gas exchange has been described in *T. natans* by Smits and Flanigan (1994), who found that pulmonary gas exchange was dominant during recovery from exercise, whereas cutaneous gas exchange was prevalent during resting. Thus, cutaneous gas exchange appears to be adequate to sustain the resting metabolic needs of these animals (Bennett and Wake, 1974; Gatten et al., 1992; Smits and Flanagin, 1994) and may have decreased their need to air breathe.

In the present study, the inspiratory volume tended to be greater (but not significantly so) than the expiratory volume. Gas lost *via* cutaneous respiration could account for this disparity, since Toews and Macintyre (1978) and Smits and Flanigan (1994) found the skin to be a primary site (88 %) for the elimination of CO_2 in *T. natans*.

T. natans first expired and then inhaled air repeatedly to inflate the lungs. This pattern eliminates mixing of expired gases in the buccal cavity and therefore represents a major improvement in the efficiency of breathing compared with animals using two-stroke breaths. A similar mechanism has evolved in two aquatic urodeles, *Amphiuma tridactylum* (Guimond and Hutchison, 1974; Martin and Hutchison, 1979) and *Siren lacertina* (Guimond and Hutchison, 1974; Martin and Hutchison, 1979), and in one aquatic anuran, *Xenopus laevis* (Brett and Shelton, 1979). Brainerd and Monroy (1998) recently reported that *Siren lacertina* has a two-stroke breathing pattern with modifications similar to those in lungfishes to improve ventilation. It appears that the separation of expired and inspired gases in these diving animals may be an adaptation to increase the efficiency of air breathing, which would decrease the number of excursions to the surface and thus reduce predation risk (Brett and Shelton, 1979). A similar phenomenon has been observed in lungfish, in which expiration occurs during the early phase of the buccal expansion cycle, with the buccal cavity continuing to expand after expiration is complete (McMahon, 1969). This suggests that most of the expired air flows out of the buccal cavity, and air is then inspired into the mouth as the expansion continues. From an evolutionary perspective, it is easy to imagine that such lung inflation cycles using two-stroke breathing mechanics could become progressively modified to the point where the first breath in the series is expiratory only and subsequent breaths are inspiratory, as observed in the present study in *T. natans*.

Carrier and Wake (1995) have described the breathing mechanics of a terrestrial caecilian, *Dermophis mexicanus*. Like *T. natans*, *D. mexicanus* exhale first. In the present study, *T. natans* were occasionally observed to use two or three expiratory pulses. However, there was no significant difference in total expiratory volume between a single expiratory pulse and multiple pulses. The reason for the use of multiple expiratory pulses is unknown; expiration was always followed by a series of inflation cycles.

It appears that *T. natans* no longer use a typical lissamphibian single breath, but instead use lung inflation cycles or 'bout' breathing patterns. The first cycle consists of a single buccal compression and expansion phase that appears to become arrested to allow the tracheal lung to empty. Subsequent cycles are devoted exclusively to lung filling, in which a two-stroke mechanism fills the buccal cavity. This pattern bears some similarity to the lung inflation cycle seen in toads and frogs and can be thought of as an extension of two-stroke breathing mechanics with specialization of individual buccal oscillations in the cycle.

The effects of water depth on breathing pattern

The regulation of lung volume is poorly understood in lower vertebrates and is especially complicated in diving animals for which changing water depth and hydrostatic pressure may interact to influence lung volume. In addition to elastic recoil of the lungs, which stores and returns the energy of the buccal force pump, there are other mechanisms that can be employed by amphibians to aid expiration. *Amphiuma means* and *Siren lacertina* use pulmonary smooth muscle to increase lung pressure further during expiration (Guimond and Hutchison, 1973b, 1974). Some terrestrial salamanders are able to use their hypaxial muscles to achieve forceful expiration (Brainerd et al., 1993). Trunk muscles are used by some toads to produce explosive exhalation as part of a defensive response. In addition to active muscular assistance, hydrostatic pressure is commonly used to aid expiration in diving vertebrates such as snapping turtles (*Chelydra serpentina*) and the spotted gar (*Lepisosteus oculatus*) (Gaunt and Gans, 1969; Smatresk and Cameron, 1982). Anurans and urodeles typically display pulmonary pressures of 0.2–0.5 kPa (Brainerd et al., 1993; de Jongh and Gans, 1969; Macintyre and Toews, 1976; Vitalis and Shelton, 1990; West and Jones, 1975). The terrestrial caecilian *D. mexicanus* maintains a resting pleuroperitoneal pressure of 1.5 kPa and uses this pressure to force air out of the

lungs (Carrier and Wake, 1995). In the present study, *T. natans* maintained resting pulmonary pressures of approximately 0.5 kPa, but could generate a tracheal pressure as high as 1.5 kPa during exhalation. The tracheal pressure on exhalation was often higher than the height of the water column, suggesting that *T. natans*, like its terrestrial counterpart *D. mexicanus*, may use its axial muscles or pulmonary smooth muscle to aid in expiration.

Breathing patterns in deep and shallow water

The airflow profiles of *T. natans* in deep and shallow water were quite similar. Breathing frequency, expiratory volume, inspiratory volume, the number of inspiratory buccal oscillations per breath and total breath duration were not significantly different between deep- and shallow-water studies (Table 1). Animals usually expired gas in one cycle, but in deep water some animals used two or even three expiratory cycles.

Expiration in deep versus *shallow water*

In the present study, less than one-third of the animal's body was vertically oriented in shallow water, whereas at least two-thirds of the animal's body was so oriented in deep-water experiments. In deep water, the animals used hydrostatic pressure to aid removal of gas from their lungs, as shown by the higher peak flow, shorter expiratory duration and higher expiratory buccal pressure (Table 1) in deep-water experiments. The expiratory volumes in deep and shallow water were not significantly different, implying that expiratory volume was regulated, probably by pulmonary mechanoreceptors.

Inspiration in deep versus *shallow water*

During inspiration, animals generated higher buccal pressures in deep water than in shallow water (Table 1), which may indicate that *T. natans* modulate their buccal pressure with water depth to regulate their inspiratory volume. Both *T. natans* and *D. mexicanus* have a high buccal pressure compared with those of other amphibians, suggesting that they have powerful buccal musculature (Carrier and Wake, 1995).

The tracheal lung is separated from the left and right posterior lungs by the posterior trachea; it would therefore be possible for *T. natans* to use the posterior lungs for both buoyancy and respiration. Because the measured pressures may have been attenuated because of the experimental conditions of the present study, it is difficult to determine the process by which air was transferred to the posterior lungs. Inspiratory buccal pressure is not great enough to force air into the posterior lungs (Fig. 6) when the animal is vertical. It is possible that inhalation may be limited to filling only the tracheal lung and that air is transferred to the posterior lungs after the animal returns to the bottom, where hydrostatic pressure will be equalized across the animal's horizontal body.

Morphology of the respiratory organ

The respiratory morphology of *T. natans* is similar to that of *Siren lacertina* (Guimond and Hutchison, 1973b) and

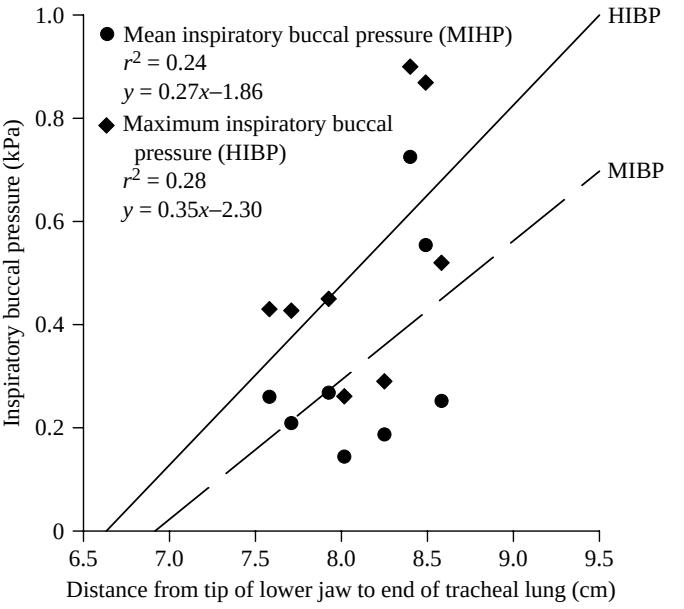

Fig. 6. The relationship between the distance from the tip of the lower jaw to the end of the tracheal lung and the mean and maximum inspiratory buccal pressure when the resting tube was 5 cm below the water's surface. At no time was the inspiratory buccal pressure great enough for air to reach the end of the tracheal lung. Each point represents an individual animal. ●, mean; ◆, maximum.

Amphiuma means (Guimond and Hutchison, 1974). *T. natans*, *A. means* and *S. lacertina* all have narrow and elongated lungs. In *A. means* and *S. lacertina*, the trachea connects the buccal cavity to the right and left lungs. In *T. natans*, the anterior trachea connects the buccal cavity to the tracheal lung, and the posterior trachea connects the tracheal lung to the left and right posterior lungs. In both *T. natans* and *A. means*, the right lung is significantly longer than the left; however, in *S. lacertina*, the lungs are of equal length (Guimond and Hutchison, 1973b).

The trachea in *S. lacertina* and *A. means* serves only as an air channel, whereas in *T. natans* it is highly modified and also serves as a respiratory structure. The trachea in *T. natans* has anterior, central and posterior regions. The anterior tracheal region is constructed of full cartilaginous rings and serves only as an air channel. The central tracheal region has lung-like tissue that has not been described in any other amphibian. The posterior tracheal region serves as an air channel leading to the posterior lungs (Fig. 5). The central and posterior tracheal regions are constructed from rings (half of each ring consists of cartilaginous tissue while the other half is well-vascularized and non-cartilaginous), suggesting an involvement in gas exchange. The central trachea is divided into an air channel portion and a respiratory portion. One possible explanation for this derived tracheal lung is that it reduces the distance between the exterior and the lung and, therefore, the buccal pressure needed to overcome the hydrostatic pressure when the animal is vertically oriented. This remains an interesting and unsolved evolutionary problem.

All three lungs are highly compartmentalized (septated) and

have extensive vascularization similar to that of *A. means* and *S. lacertina* (Guimond and Hutchison, 1973b, 1974). Numerous internal septae in the lungs increase the respiratory surface area, enabling enhanced extraction of the inspired oxygen.

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