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Lung ventilation in salamanders and the evolution of vertebrate air-breathing mechanisms

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Functional analysis of lung ventilation in salamanders combined with historical analysis of respiratory pumps provides new perspectives on the evolution of breathing mechanisms in vertebrates. Lung ventilation in the aquatic salamander *Necturus maculosus* was examined by means of cineradiography, measurement of buccal and pleuroperitoneal cavity pressures, and electromyography of hypaxial musculature. In deoxygenated water *Necturus* periodically rises to the surface, opens its mouth, expands its buccal cavity to draw in fresh air, exhales air from the lungs, closes its mouth, and then compresses its buccal cavity and pumps air into the lungs. Thus *Necturus* produces only two buccal movements per breath: one expansion and one compression. *Necturus* shares the use of this two-stroke buccal pump with lungfishes, frogs and other salamanders. The ubiquitous use of this system by basal sarcopterygians is evidence that a two-stroke buccal pump is the primitive lung ventilation mechanism for sarcopterygian vertebrates. In contrast, basal actinopterygian fishes use a four-stroke buccal pump. In these fishes the buccal cavity expands to fill with expired air, compresses to expel the pulmonary air, expands to fill with fresh air, and then compresses for a second time to pump air into the lungs. Whether the sarcopterygian two-stroke buccal pump and the actinopterygian four-stroke buccal pump arose independently, whether both are derived from a single, primitive osteichthyian breathing mechanism, or whether one might be the primitive pattern and the other derived, cannot be determined.

Although *Necturus* and lungfishes both use a two-stroke buccal pump, they differ in their expiration mechanics. Unlike a lungfish (*Protopterus*), *Necturus* exhales by contracting a portion of its hypaxial trunk musculature (the *m. transversus abdominis*) to increase pleuroperitoneal pressure. The occurrence of this same expiratory mechanism in amniotes is evidence that the use of hypaxial musculature for expiration, but not for inspiration, is a primitive tetrapod feature. From this observation we hypothesize that aspiration breathing may have evolved in two stages: initially, from pure buccal pumping to the use of trunk musculature for exhalation but not for inspiration (as in *Necturus*); and secondarily, to the use of trunk musculature for both exhalation and inhalation by costal aspiration (as in amniotes).

ADDITIONAL KEY WORDS:—Respiration – respiratory mechanics – aspiration – functional morphology – lungfish – *Necturus* – *Amphiuma* – *Ambystoma* – *Dicamptodon* – *Siren*.

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INTRODUCTION

Lung ventilation mechanisms in vertebrates are diverse (Liem, 1985). Basal actinopterygian fishes, lungfishes, and amphibians use the oral cavity as a compressive pump to force air into the lungs (Fig. 1). Basal amniotes use a suction pump in which costal movements increase the volume of the body cavity to draw air into the lungs. This distribution of breathing mechanisms is evidence that aspiration breathing is more derived than buccal pumping (Gans, 1970a, b; Liem, 1985). With currently available information, however, more detailed resolution of the evolution of respiratory pumps has not been possible.

In this paper we investigate the evolution of breathing mechanisms by combining historical analysis with experimentally based, functional analysis of lung ventilation in salamanders. The protocol for historical analysis of a function (such as breathing) requires mapping functional information from a range of taxa onto an independently established cladogram depicting the relationships between the taxa (Lauder, 1981). For this process to yield useful information, functional data must be available for as broad a range of taxa as possible. Lung ventilation has been studied in basal actinopterygian fishes, lungfishes, frogs and many amnioties: little is known, however, about the breathing mechanisms of lunged salamanders and caecilians. Caecilians are derived in many respects including elongation of the body and loss of limbs (Duellman & Trueb, 1986); because many salamanders have retained a more primitive body form, we chose to concentrate our functional studies on lung ventilation in salamanders.

Knowledge of salamander lung ventilation potentially offers insights into both the primitive sarcopterygian breathing mechanism and the primitive tetrapod breathing mechanism. Identification of ventilation characteristics shared by lungfishes, frogs and salamanders has the potential to reveal some aspects of the primitive condition for the Sarcopterygii. Comparison with basal actinopterygian fishes may indicate whether these features are primitive for sarcopterygians alone or for all osteichthyians (Fig. 1). Similarly, identification of those characteristics of lung ventilation shared by salamanders, frogs and

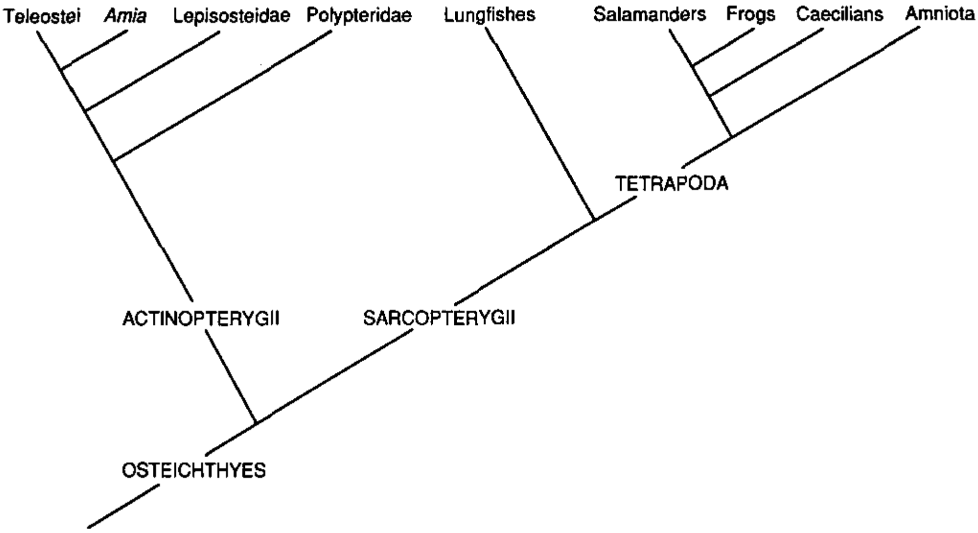


Figure 1. Phylogenetic hypothesis showing the relationships of extant, air-breathing vertebrates (actinopterygian relationships from Lauder & Liem, 1983; lissamphibian relationships from Milner, 1988; Trueb & Cloutier, 1991; but see Larsen & Wilson, 1989 for an alternative view of lissamphibian relationships). For simplicity, non-air-breathing forms have been omitted.

amniotes has the potential to reveal aspects of the primitive tetrapod breathing mechanism. Whether these features are unique to tetrapods may be determined by outgroup comparison with lungfishes. Thus, by combining our results from *Necturus* with lung ventilation data from basal actinopterygian fishes, lungfishes, frogs and amniotes, and by mapping these breathing traits onto a vertebrate phylogeny, we hope to improve our understanding of the evolution of breathing mechanisms.

MATERIALS AND METHODS

Pressure, radiographic, and electromyographic results are reported for five individuals of *Necturus maculosus* (16.8–18.8 cm snout-vent length, SVL). For individual identification, these specimens are designated as N1–N5. Buccal and pleuroperitoneal pressures were measured in three individuals (N1, N4 and N5), X-ray films or videos were taken of three individuals (N1, N2 and N5), and electromyograms of hypaxial musculature were recorded from two individuals (N3 and N5).

Pressure recordings

Pressures were recorded with Millar Microtip SPR-407 pressure transducers (Houston, TX) guided into the buccal and pleuroperitoneal cavities through cannulae. The two transducers were threaded through the guide cannulae so that the pressure sensing tip emerged from the end of the cannula in the pleuroperitoneal cavity and was within 2 mm of the cannula opening in the buccal cavity. Pressures were amplified through a Tektronix 5A18N oscilloscope amplifier in DC mode, recorded on a TEAC MR-30 cassette data recorder and played back onto a Graphtek Mark-10A thermal array paper recorder.

For implantation of guide cannulae, animals were anaesthetized by immersion in an aqueous solution of tricaine methanesulphonate. Implantation procedures were similar to those used by Lauder & Shaffer (1986). For implantation of the buccal cannula, a small hole was drilled in the most anterior paired bones of the skull, the frontal bones (*Necturus* lacks nasal bones). A plastic cannula (polyethylene tubing, PE-90, 1.27 mm outer diameter, Becton Dickinson & Co, Parsippany, NJ) was threaded into the hole and flange formed by heating and flattening the plastic on one end of the cannula was pulled flush against the roof of the mouth. This placement of the buccal cannula was chosen to avoid any damage to the brain or sensory systems, and post-mortem dissection of cannulated animals confirmed that the cannula was positioned anterior to the brain and eyes and medial to the olfactory sacs. The pleuroperitoneal cannula was introduced into the body cavity through a 16 gauge hypodermic needle. The needle was then removed, and the cannula was sutured securely to the skin. Animals were allowed to recover from anaesthesia for at least 2 hours, and in some cases for as long as two days, before pressure recordings were made.

Recordings were made in both shallow water (3–6 cm deep) and in deeper water (15–25 cm deep) at 24–26°C. In well-oxygenated water the animals were never observed to breathe air and presumably obtained oxygen through skin and gills (Guimond & Hutchison, 1972). Accordingly, in order to induce frequent ventilation, nitrogen gas was bubbled through the water to reduce the dissolved oxygen. Under these conditions *Necturus* rose to the surface and took a breath approximately every 5 to 10 minutes.

Cineradiography and video fluoroscopy

X-Ray films of *Necturus* were taken at a rate of 100 frames s^{-1} using a Siemens cine X-ray apparatus with a Sirecon image intensifier attached to an Eclair GV-16 camera. X-Ray videos were recorded at 60 images s^{-1} on a Panasonic AG7300 video cassette recorder. Film sequences were analysed with a Lafayette analysing projector. All quantitatively analysed sequences were filmed in lateral projection. Dorsoventral films were also obtained for qualitative comparison. Mouth opening (gape) was measured with a digital caliper directly from projected film or video images. It was not possible to measure volume changes of the lungs and buccal cavity directly from our two-dimensional X-ray films and videos. Instead we used the laterally projected areas of the lungs and buccal cavity as an indicator of air transfer between atmosphere, buccal cavity and lungs (in lateral projection the lung outlines overlap completely and the equivalent area of only one lung is measured). Outlines of the buccal cavity and lungs were traced onto clear acetate sheets. Areas were obtained by transferring the outlines to a Macintosh II computer via a Summagraphics Bit Pad graphics tablet.

In order to synchronize pressure recordings with the air-flow pattern observed in radiographic images, video images from the fluoroscope were mixed (genlocked) with the video graphics from a computer configured to act as a real-time digital oscilloscope (Fig. 2). A Macintosh IIcx computer was equipped with a GW Instruments (Cambridge, MA) MacADios II analog-to-digital conversion board and MacADios Manager software. A Mass Microsystems (Sunnyvale, CA) ColorSpace IIi genlock video board was used to overlay (genlock) the real-

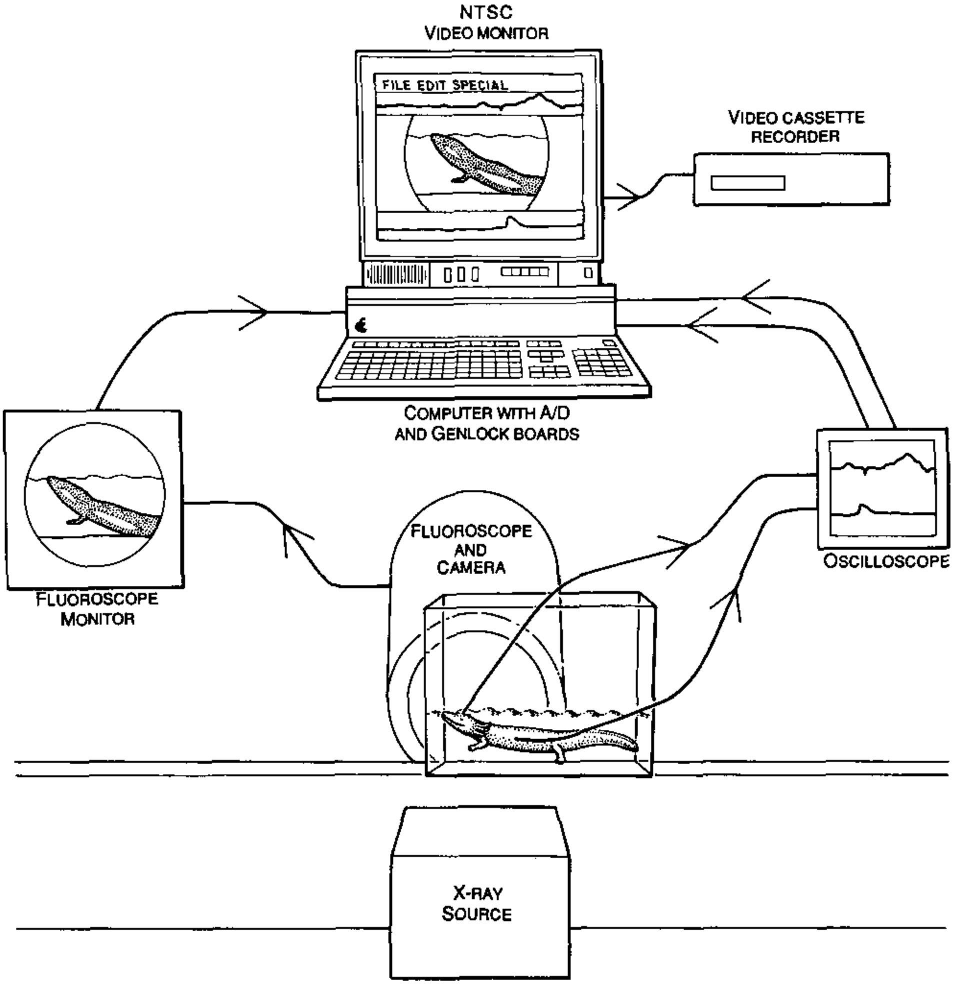


Figure 2. Apparatus for synchronizing buccal and pleuroperitoneal pressure traces with X-ray video images. Pressure transducer signals are displayed on an oscilloscope, sent to an analog to digital (A/D) conversion board in a Macintosh II computer, digitized, and plotted to screen in real time. The video signal from the fluoroscope is fed into a genlock video board. The genlock video board overlays the real time pressure traces onto the live, incoming video signal, displays the result on an NTSC monitor, and sends the mixed images to a video cassette recorder.

time pressure onto the live, real-time video from the fluoroscope and the combined data were recorded on a Panasonic AG7300 video cassette recorder. The videos were played back for field-by-field analysis (60 images s^{-1}) on a Panasonic AG1730 video cassette recorder.

Electromyography of Necturus *trunk musculature*

Four layers constitute the hypaxial trunk musculature of *Necturus*: the *m. obliquus externus superficialis*, the *m. obliquus externus profundus*, the *m. obliquus internus*, and the *m. transversus abdominis* (Fig. 3). The fibres of both the deep external oblique and the internal oblique are oriented nearly longitudinally, while those of the transverse abdominal layer are more circumferential. The

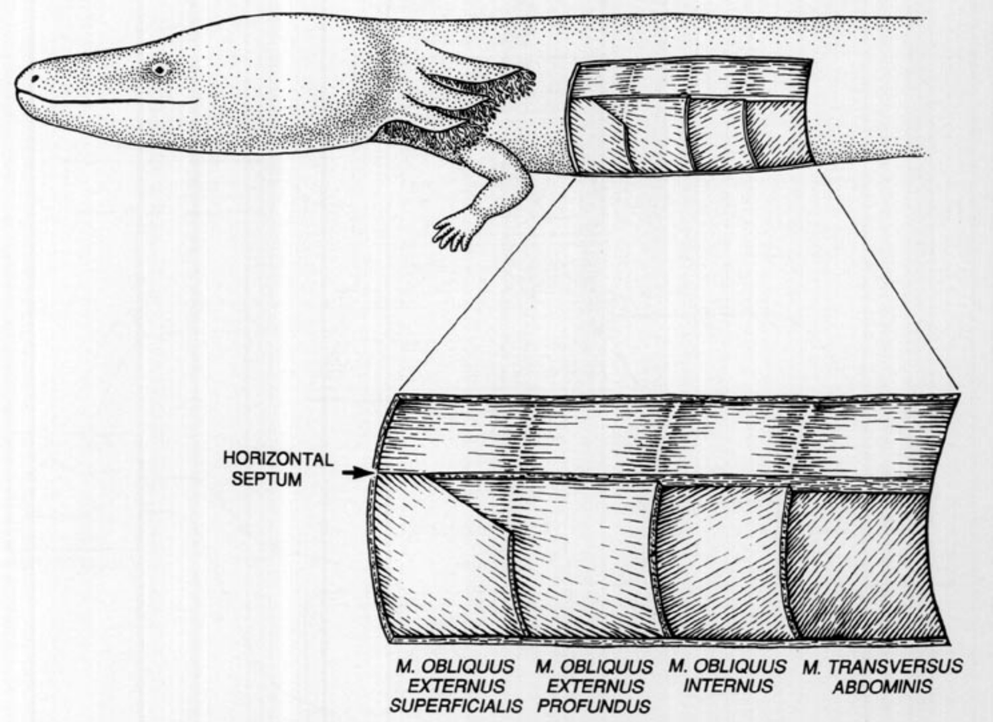


Figure 3. Hypaxial musculature of *Necturus maculosus*. Successive layers of hypaxial musculature were removed from anterior to posterior to show deeper layers. From superficial to deep the layers shown are the *m. obliquus externus superficialis*, the *m. obliquus externus profundus*, the *m. obliquus internus*, and the *m. transversus abdominis*. The acute angles of the muscle fibres relative to the horizontal septum were measured for determination of fibre angles.

superficial external oblique is a thin, superficial layer of fibres with an intermediate orientation. To calculate the mean fibre angle for each layer, we drew the orientations of 12 equally spaced fibres with a camera lucida and used a protractor to measure the angle of each fibre relative to the horizontal septum.

The ypsiloid apparatus with its associated muscles and the *m. rectus abdominis* have been suggested to play a role in buoyancy regulation in some lunged salamanders (Whipple, 1906). *Necturus*, however, completely lacks the ypsiloid apparatus, and thus there was no possibility of testing this hypothesis as part of our study. *Necturus* does have a well-developed rectus abdominis muscle, in which the fibres run longitudinally. Given this fibre orientation, it is unlikely that the rectus contributes to exhalation in *Necturus*. However, we did not implant electrodes in the rectus abdominis, and thus cannot contribute information on any possible role of the rectus in lung ventilation.

Electromyography was performed using the standard procedures described by Schaffer & Lauder (1985). We anaesthetized the animals by immersion in an aqueous solution of tricaine methanesulphonate and implanted bipolar, fine-wire electrodes (stainless steel wire, 0.051 mm diameter, bared tips 0.5 mm long, *c*. 1 mm apart in muscle) percutaneously at several depths in the hypaxial muscle mass along the mid-lateral line. Recordings were made during lung ventilation and during lateral body undulation induced by touching or holding an animal's tail. Signals were amplified 5000 to 10 000 times through Grass

P511J amplifiers set to a bandpass of 30–10 000 Hz, recorded on a TEAC MR-30 cassette data recorder at a tape speed of 35 cm s^{-1} (effective frequency response DC-10 000 Hz), and played out on a Graphtek Mark-10A thermal array paper recorder. After making recordings we killed the animals by immersion in a euthanasia solution of tricaine methanesulphonate and dissected out the electrode tips to determine in which of the hypaxial layers they had been implanted. Only results for which the placement of the electrode tips was confirmed by dissection are presented.

We had difficulty obtaining electromyographic results from *Necturus*: the animals had a tendency to rotate along their long axis thus tangling the cable and pulling out the electrodes. This was especially problematic during the process of euthanasia for the purpose of dissecting out the electrode tips. In our early attempts the animals dislodged most of their electrodes when we immersed them directly in the euthanasia solution. In later attempts we gradually chilled the animals by adding ice to their water and then subsequently added tricaine methanesulphonate. This procedure was more successful, but our sample sizes are nonetheless small due to the animals dislodging electrodes at all stages of the recording process.

Observations of other species

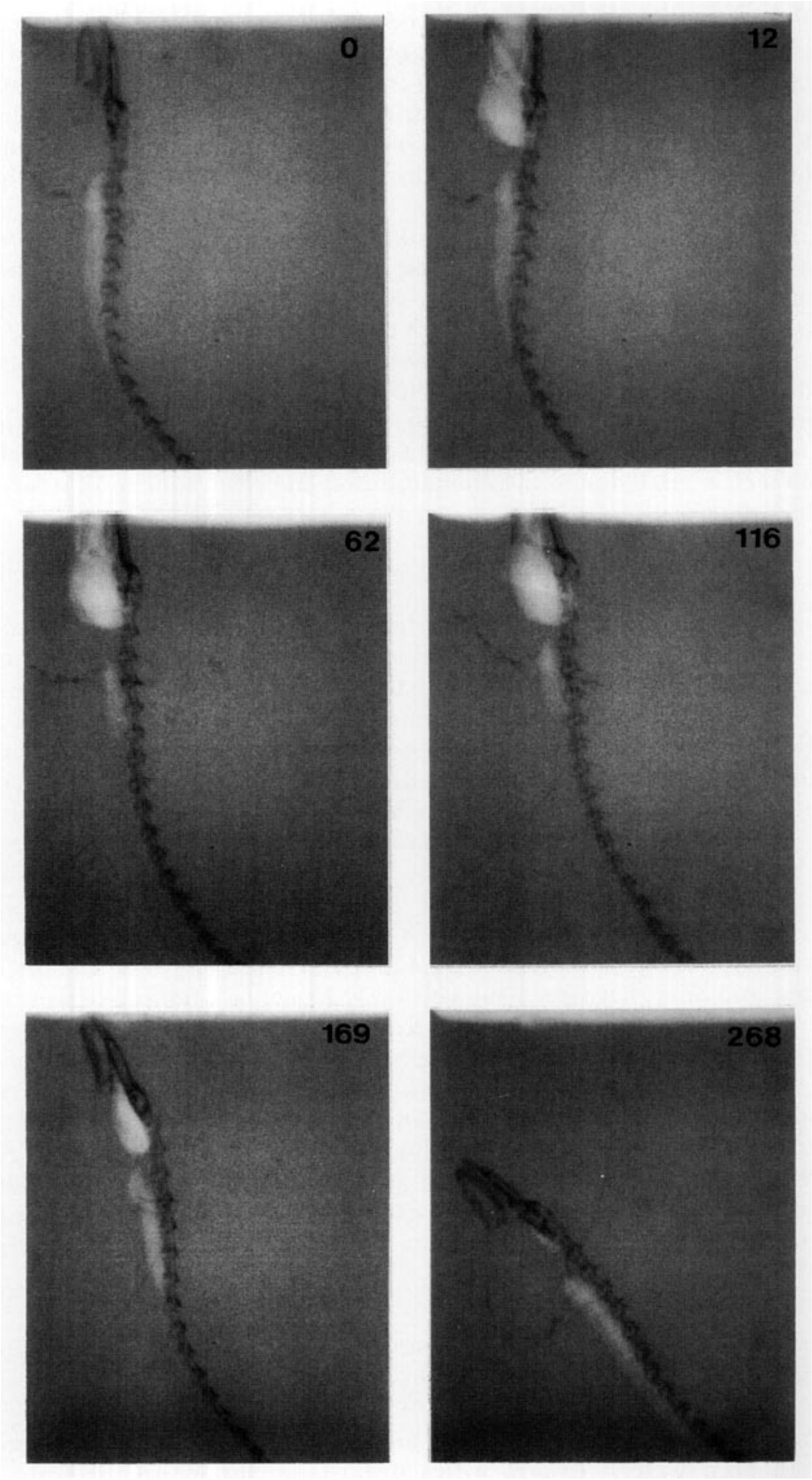
We initially chose to study *Necturus* because large specimens were easily and inexpensively obtained from biological supply houses. On theoretical grounds, however, *Necturus* may not have been the best possible choice because it is a highly aquatic, paedomorphic salamander possessing reduced lungs which contribute little to gas exchange under normoxic conditions (Guimond & Hutchison, 1972). In order to test whether *Necturus* uses an unusual lung ventilation mechanism, we took X-ray videos of lung ventilation in four other species of aquatic and terrestrial salamanders: two aquatic adult *Amphiuma tridactylum* (59.5 and 69.6 cm SVL), three aquatic adult *Siren intermedia* (*c*. 19–23 cm SVL), one aquatic larval *Ambystoma tigrinum* (11.7 cm SVL), three terrestrial adult *Dicamptodon* sp. (11.8, 12.6 and 13.6 cm SVL), and two terrestrial adult *Ambystoma tigrinum* (12.4 and 13.2 cm SVL). The aquatic adults, *Amphiuma* and *Siren*, have been shown to rely heavily on lungs for gas exchange under normoxic conditions (Guimond & Hutchison, 1973, 1974). *Ambystoma* and *Dicamptodon* are particularly interesting because they possess both aquatic larval and terrestrial adult stages and thus exhibit what is thought to be the primitive life history for lissamphibians (Duellman & Trueb, 1986; Carroll, 1988).

We recorded buccal and pleuroperitoneal cavity pressures from two African lungfish, *Protopterus aethiopicus* (19.0 cm and 19.7 cm SVL). The procedure was the same as that for *Necturus*, with the exception that the lungfish took a breath spontaneously every 5–10 minutes, thus making it unnecessary to deoxygenate the water.

RESULTS

Lung ventilation in Necturus maculosus

Frames from an X-ray film of *Necturus* lung ventilation are reproduced in Fig. 4. In deep water, *Necturus* swims toward the surface of the water for a


0
12
62
116
169
268

breath. As the snout breaks the surface, the mouth begins to open and the buccal cavity expands to draw in fresh air (frame 12). At near maximum gape and buccal expansion, air from the lungs is rapidly exhaled into the already expanded buccal cavity. Some of this exhaled air may flow directly out the opened mouth, but some of it probably mixes with the fresh air already in the buccal cavity. The mouth then closes while the buccal cavity is still expanded and filled with a mixture of fresh and exhaled air (frame 62, tip of snout not visible because it is protruded above the X-ray frame). *Necturus* does not pump air from the buccal cavity into the lungs immediately after closing the mouth. Instead the buccal cavity remains expanded for a variable length of time, in this case for 740 ms (until frame 116; see Table 2, Comp to Glott). When compression does begin, it progresses rapidly (frame 169, compression complete). Just after compression is completed, air is concentrated in the anterior part of the lungs, but as the animal settles toward the bottom of the aquarium, air is redistributed along the length of the lungs (frame 268). Any residual air in the buccal cavity is gradually released as bubbles.

Synchronized pressure events and air flow patterns

Figure 5 shows buccal and pleuroperitoneal pressure traces synchronized with movement and air flow patterns from X-ray video. A negative buccal pressure spike occurs just as the mouth begins to open (time = 0 ms). As soon as the mouth is open, buccal pressure rises rapidly to ambient atmospheric pressure. At near maximum gape and buccal expansion, expiration occurs: pleuroperitoneal pressure rises sharply and lung area begins to decrease. Buccal cavity pressure remains at atmospheric until the gape begins to close, at which time buccal pressure begins to rise. As the gape closes, air between the jaws flows out the mouth and the projected area of air contained within the buccal cavity decreases. After this drop, however, buccal area remains roughly constant for the period from 500 to 1200 ms. Buccal pressure gradually increases during this period, and begins to drop only after the buccal cavity begins to empty and the lungs begin to fill with air. This finding indicates that peak buccal pressure is attained before the glottis opens, and that opening of the glottis releases the positive pressure in the buccal cavity. After the lung area ceases to increase (indicating that the transfer of air from buccal cavity to lungs is complete), buccal pressure drops below atmospheric, possibly as a result of the buccal cavity recoiling to its rest position after being compressed.

Figure 4. Frames from an X-ray film of a *Necturus* breath taken at 100 frames s^{-1} (individual N2). Numbers in the upper right are frame numbers. Air in the buccal cavity and lungs appears white because it is less X-ray opaque than the surrounding tissues and water. In order to improve contrast in water it was necessary to collimate the X-ray beam such that as little air as possible was included in the frame. Thus the tip of the snout is lost when it is well above the water surface (this is not necessary with video fluoroscopy). In frame zero, the animal has approached the surface and the mouth is just about to open. In frame 12, gape and buccal expansion are near maximum but exhalation has not yet begun. In frame 62, exhalation is long finished and the mouth has been closed already for 20 frames. In frame 116, the buccal cavity is still expanded and compression of air from the buccal cavity to the lungs just about to begin. In frame 169, compression of air from the buccal cavity to the lungs is complete (total length of breath, 1690 ms). Just after compression is completed, air is concentrated in the anterior part of the lungs, but as the animal settles toward the bottom of the aquarium, air is redistributed along the length of the lungs (frame 268). Excess air remaining in the buccal cavity after the lungs are filled is released as bubbles from the mouth.

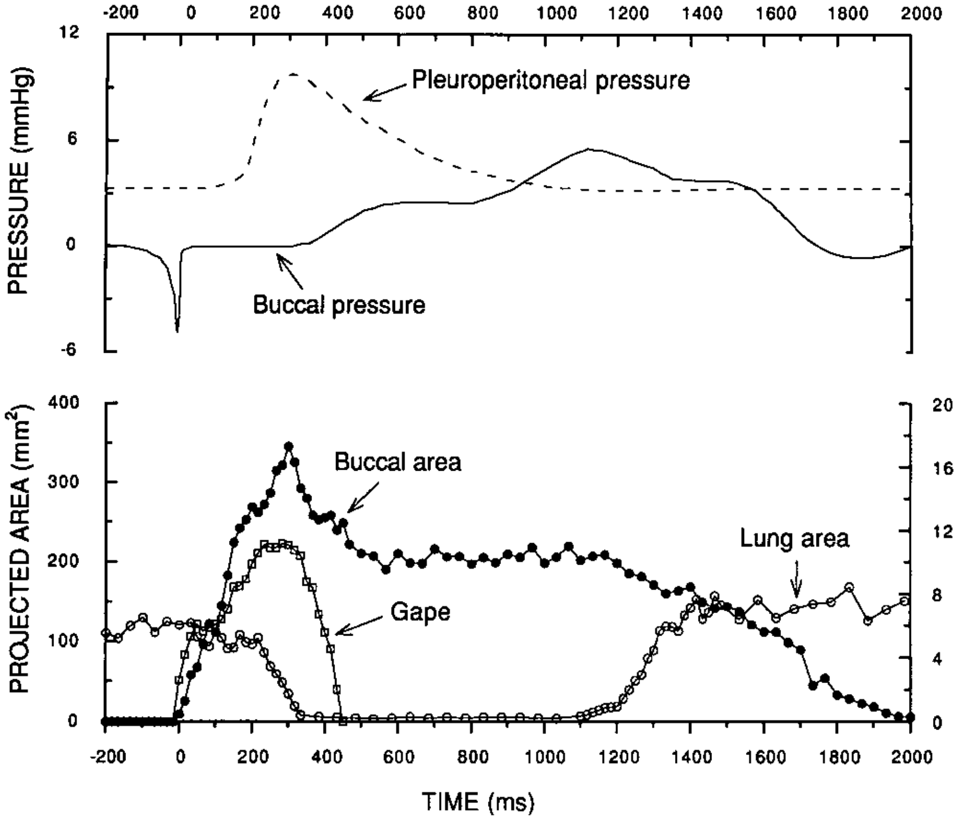


Figure 5. Buccal and pleuroperitoneal pressure synchronized with movement and air flow information from X-ray video of a single *Necturus* breath (from individual N5). Zero pressure is atmospheric pressure. The pleuroperitoneal pressure trace is offset 3.3 mmHg above atmospheric due to hydrostatic pressure. The pleuroperitoneal cavity is not pressurized above ambient hydrostatic pressure when the animal is at rest. Buccal and lung areas are the laterally projected area of the lungs and of the buccal cavity. The lateral projection areas of the buccal cavity and lungs show the pattern of air movement between atmosphere, buccal cavity and lungs.

Variation in pressure events

We recorded simultaneous buccal and pleuroperitoneal cavity pressures during a total of 48 breaths from three individuals. Five pressure events occurred in every breath (Fig. 6): (1) buccal negative pressure spike corresponding to mouth opening; (2) pleuroperitoneal positive peak corresponding to exhalation; (3) beginning of buccal positive pressure corresponding to the beginning of buccal compression; (4) buccal pressure peak corresponding to the opening of the glottis; followed by (5) a brief period of negative pressure after compression.

Although these five pressure events occurred consistently in each breath, their magnitudes and relative timings showed a great deal of variation both within and between individuals (Tables 1, 2). Single factor ANOVAs to test for differences between the individuals studied resulted in significant individual effects on four out of the seven variables measured. Because of these individual differences, means and standard deviations are reported for each of the three individuals (Tables 1, 2).

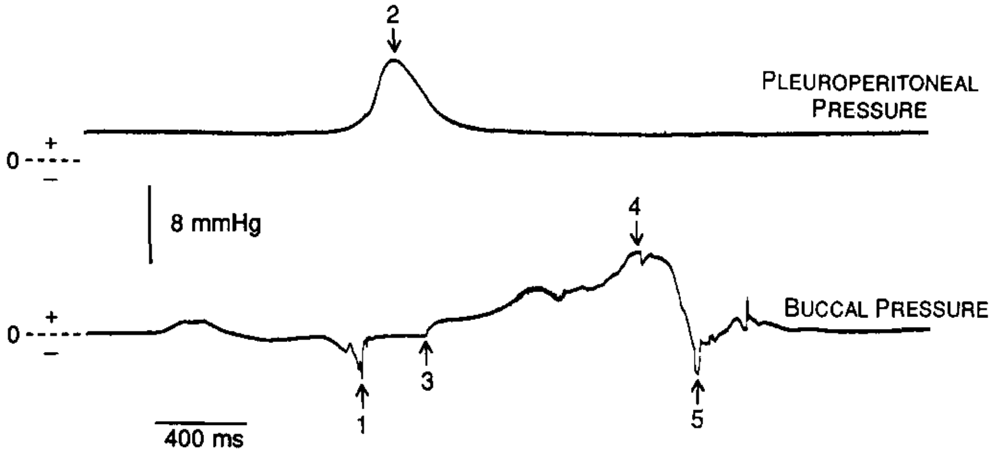


Figure 6. Consistent events of buccal and pleuroperitoneal pressure traces in *Necturus*. 1. Buccal negative spike (NegBu). 2. Pleuroperitoneal positive peak (MaxPer). 3. Beginning of compression (Comp). 4. Buccal positive peak corresponding to opening of the glottis (Glott). 5. Negative pressure dip after compression. Atmospheric pressure is marked with a dotted line labelled zero and indicating the direction of positive and negative pressure for each trace. The baseline pressure in the pleuroperitoneal cavity is the ambient hydrostatic pressure at the level of the transducer (in this case 4 mmHg above atmospheric). The buccal and pleuroperitoneal pressure traces shown here were recorded from individual N1.

Evidence of the large amounts of variation in pressure timing and magnitudes can be seen in the pressure traces depicted in Figs 5 and 6. Many of the pressure variables in these two traces are near the mean values for the individuals from which they were recorded (N5 for Fig. 5 and N1 for Fig. 6). Several variables, however, are quite far from their means. For example, the maximum buccal pressure in Fig. 6 is 8.9 mmHg, which is almost two standard deviations above the mean for N1. The time between the buccal negative spike and the maximum pleuroperitoneal pressure (events 1 to 2) shows the most extreme deviation from mean. In Fig. 5, NegBu to MaxPer is 300 ms, which is more than two standard deviations above the mean for N5 whereas in Fig. 6, NegBu to MaxPer is 150 ms, which is nearly two standard deviations below the mean for N1. Thus,

Table 1. Magnitudes of pressure events for three individual *N. maculosus*. See Fig. 6 for graphical representation of pressure events. Summary statistics include the sample mean, one standard deviation (SD) and the number of breaths analysed for each individual (N). The results of single-factor ANOVA tests for the effects of individual differences are reported for each pressure magnitude (*$P < 0.05$), **$P < 0.01$, NS, not significant). The magnitude of the maximum peritoneal pressure was measured as the increase in pressure relative to ambient hydrostatic pressure at the level of the pressure transducer, not relative to atmospheric pressure

	Negative buccal spike (Event 1), NS	Maximum peritoneal pressure (Event)**	Maximum buccal pressure (Event 4) NS
Individual N1	−3.1 mmHg	5.5 mmHg	4.3 mmHg
$N = 13$	SD = 1.2	SD = 2.5	SD = 2.6
Individual N4	−2.7 mmHg	3.5 mmHg	4.1 mmHg
$N = 19$	SD = 0.9	SD = 0.8	SD = 1.3
Individual N5	−3.4 mmHg	6.8 mmHg	4.1 mmHg
$N = 16$	SD = 0.9	SD = 1.5	SD = 1.1

TABLE 2. Relative timing of pressure events three individual *N. maculosus*. See Fig. 6 for graphical representation of pressure events. Summary statistics include the sample mean, one standard deviation (SD) and the number of breaths analysed for individual (*N*). The results of single-factor ANOVA tests for the effects of individual differences are reported for each timing variable (* $P < 0.05$, **$P < 0.01$, NS, not significant). Based on our synchronized pressure and X-ray images we interpret the timing of these events as follows: events 1 to 5, total time span of lung ventilation (Total Time); events 1 to 2, time from the beginning of mouth opening to the middle of exhalation (NegBu to MaxPer); events 1 to 3, time during which the mouth is open (NegBu to Comp); and events 3 to 4, time between mouth closing and opening of the glottis (Comp to Glott)

	Total time (Events 1 to 5), NS	NegBu to MaxPer (Events 1 to 2)**	NegBu to Comp (Events 1 to 3)**	Comp to Glott (Events 3 to 4)**
Individual N1	1700 ms	230 ms	340 ms	790 ms
$N = 13$	SD = 150	SD = 50	SD = 60	SD = 280
Individual N4	1660 ms	170 ms	260 ms	1040 ms
$N = 19$	SD = 170	SD = 20	SD = 40	SD = 190
Individual N5	1620 ms	210 ms	320 ms	840 ms
$N = 16$	SD = 330	SD = 30	SD = 40	SD = 80

although these two traces show the basic features found in all *Necturus* lung ventilation pressure traces, it is not possible to present a single, representative pressure trace because every trace will exhibit some variables close to their means while others will be more distant.

Electromyography of trunk musculature

In order to test whether contraction of one or more of the hypaxial muscle layers is responsible for the large positive pleuroperitoneal pressure observed during expiration in *Necturus*, we recorded hypaxial electromyograms simultaneously with pleuroperitoneal pressure. Consistent results were obtained from electrodes implanted in the deep external oblique and in the transverse abdominal muscles. Electrodes in the internal oblique gave inconsistent results: varying levels of activity were seen in about half of the breaths recorded; no activity at all was seen in the others. Electrodes in the deep external oblique showed virtually no activity during lung ventilation but the same electrodes recorded activity during body undulation (Fig. 7). The transverse abdominal muscle was always active during both lung ventilation and lateral undulation. During exhalation, activity in the transverse abdominal began 20 to 70 ms before pleuroperitoneal pressure began to increase (Fig. 7). Activity in the transverse abdominal ceased either shortly before or after the pleuroperitoneal pressure began to decrease. In five out of ten recorded ventilations, activity ended 10 to 25 ms before the pressure began to drop. In four breaths activity ended 10 to 30 ms after the pressure began to drop, and in one breath activity ended simultaneously with pleuroperitoneal pressure drop.

These electromyographic results constitute evidence that *Necturus* actively exhales by contracting the transverse abdominal to increase pleuroperitoneal pressure (possibly with some contribution from the internal oblique). The main mass of the external oblique (the *profundus*) appears not to contribute to exhalation. Further evidence that the transverse abdominal is the primary expiratory muscle comes from the muscle fibre orientations of the hypaxial layers. The thin superficial external oblique has a mean fibre angle of 34±3.1°

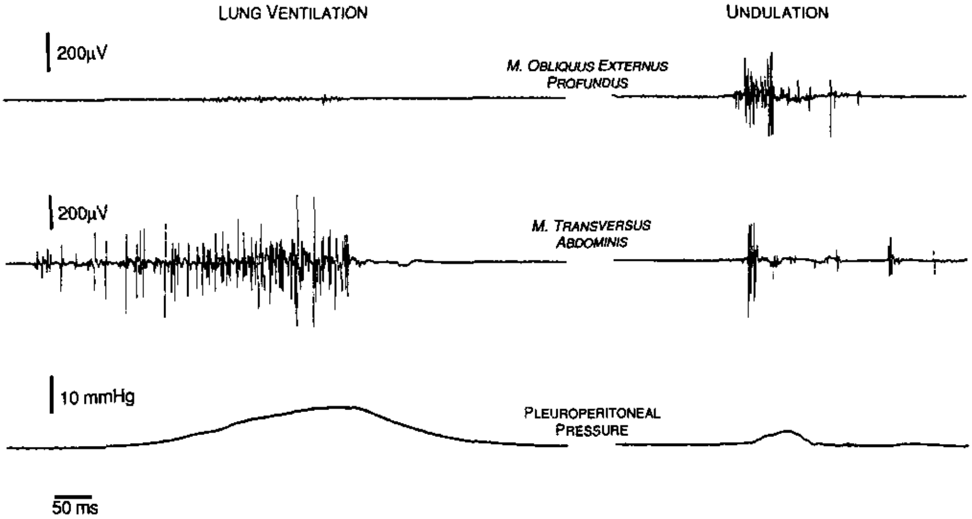


Figure 7. Electromyograms of *Necturus* hypaxial musculature with simultaneously recorded pleuroperitoneal pressure trace (from individual N3). The *m. obliquus externus profundus* is active during lateral body bending (undulation) but relatively inactive during lung ventilation (conclusion based on two electrodes implanted in individual N5: $N = 7$ undulations and $N = 9$ breaths). The *m. transversus abdominis* is active during both lung ventilation and undulation ($N = 10$ breaths, 6 from individual N3 and 4 from individual N5; $N = 10$ undulations, 7 from individual N3, 3 from N5).

to the horizontal septum (mean ± 1 standard deviation). The deep external oblique and internal oblique have mean fibre angles of 12 ± 4.5 and $22 \pm 3.4°$ to the horizontal septum, respectively, with fibres of the deep external oblique nearest the midlateral line being completely longitudinal (Fig. 3). Fibres run more circumferentially within the transverse abdominal, with a mean fibre angle of $60 \pm 2.6°$ to the horizontal. These fibre angles are evidence that the transverse abdominal is more effective than the obliques at increasing pleuroperitoneal pressure and decreasing lung volume. From the fibre architecture of the hypaxial muscles combined with our electromyographic results we conclude that the transverse abdominal is the primary expiratory muscle in *Necturus*.

Lungfish pressures

To test whether lungfishes might also use some of their hypaxial musculature for expiration, we recorded buccal and pleuroperitoneal cavity pressures during 34 breaths from two individuals of *Protopterus aethiopicus*. Buccal and pleuroperitoneal pressure recordings from one breath are shown in Fig. 8. The timing of expiration is known from seven breaths recorded with synchronized X-ray video. No increase in pleuorperitoneal pressure was observed during expiration in any of the recorded breaths, indicating that *Protopterus* does not use hypaxial muscles for breathing.

Ventilation in other salamanders

In order to determine whether the ventilation pattern observed in *Necturus* is common to other salamanders, we recorded X-ray videos of lung ventilation in

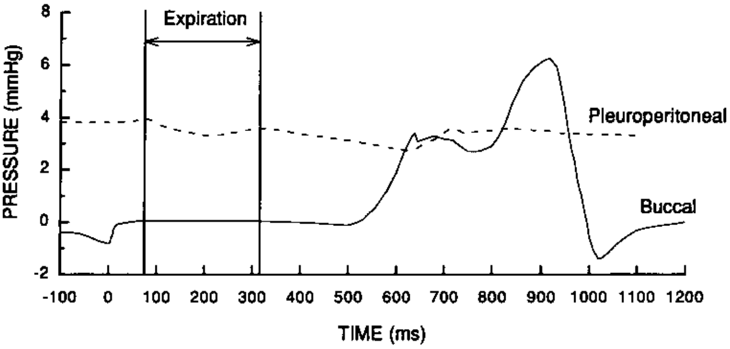


Figure 8. *Protopterus aethiopicus* buccal and pleuroperitoneal cavity pressures during lung ventilation. Expiration occurs between the vertical lines. Pleuroperitoneal pressure drops slightly during expiration rather than increasing as in *Necturus*. Zero pressure is atmospheric pressure. As in *Necturus*, the pleuroperitoneal pressure baseline is above atmospheric due to hydrostatic pressure.

two aquatic adult species (*Amphiuma tridactylum* and *Siren intermedia*), two terrestrial adult species (*Dicamptodon* sp. and *Ambystoma tigrinum*), and an aquatic larva of *A. tigrinum*. In addition to a diversity of life stages and habitats, the salamanders chosen also exhibit diversity in their status as narial or mouth breathers. We observed that *Necturus*, *Siren* and the larval *Ambystoma* all breathed through their mouths, whereas *Amphiuma* and the two terrestrial adults breathed through their nares.

With the notable exception of *Amphiuma*, all of these salamanders invariably used the same pattern of air transfer between buccal cavity and lungs as observed in *Necturus*. This shared pattern consists of just one buccal expansion-compression cycle for both expiration and inspiration: (1) the buccal cavity expands and fills with fresh air while air from the lungs is simultaneously exhaled into and through the fresh air filled buccal cavity; (2) the mouth or nares are closed, the buccal cavity compresses, and a mixture of fresh and exhaled air is pumped into the lungs.

In contrast, *Amphiuma* uses one complete buccal expansion-compression cycle for expiration and another complete cycle for inspiration. The initial stage of expiration is the same in *Amphiuma* as in other salamanders, but then the patterns diverge: (1) the buccal cavity expands and fills with fresh air while simultaneously air from the lungs is exhaled into the buccal cavity; (2) the buccal cavity then compresses and the air in the buccal cavity is expelled through the nares; (3) the buccal cavity expands again and fills with fresh air; (4) the buccal cavity compresses and fresh air is pumped into the lungs; (5) numbers (3) and (4) may be repeated one or more times, thus pumping several mouthfuls of fresh air into the lungs.

DISCUSSION

Two principle observations may be made when our data from *Necturus* are placed in a phylogenetic and comparative context (Fig. 9):

(1) three groups of sarcopterygians (lungfishes, salamanders, and frogs) share a pattern of air transfer during breathing that is different from the pattern observed in basal actinopterygian fishes;

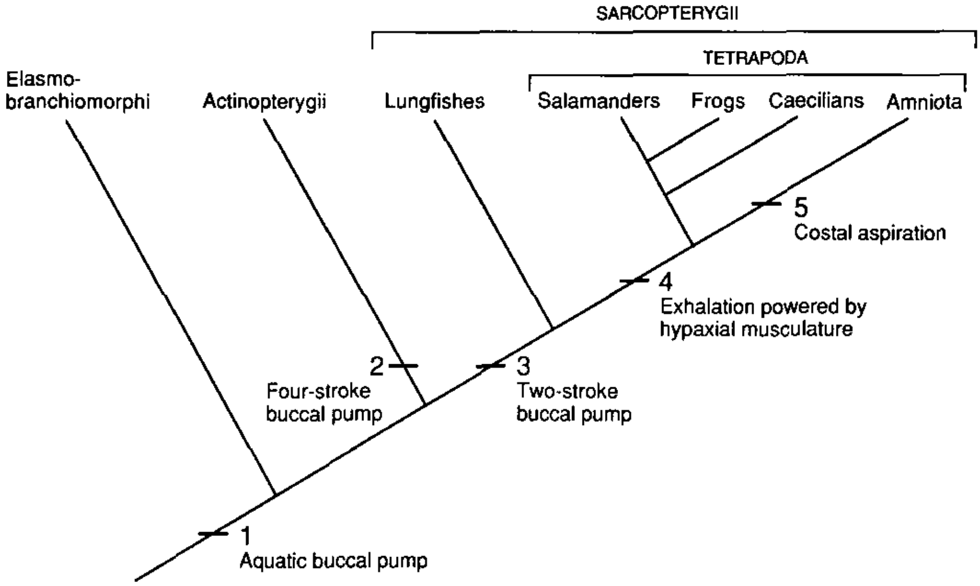


Figure 9. Functional characters relating to lung ventilation mapped onto a cladogram of air-breathing vertebrates (actinopterygian relationships: Lauder & Liem, 1983; lissamphibian relationships: Milner, 1988; Trueb & Cloutier, 1991; but see Larson & Wilson, 1989 for an alternative view of lissamphibian relationships). 1. Use of buccal pump for suction feeding and gill irrigation (Wu, 1990; Tanaka, 1973). 2. Use of a four-stroke buccal pump for lung ventilation: buccal cavity expands and fills with lung air, compresses to expel lung air, expands again to draw in fresh air, and then compresses to pump fresh air into the lungs. 3. Use of a two-stroke buccal pump for lung ventilation: buccal cavity expands and fills with fresh air, lung air is expired into the expanded buccal cavity, and then the buccal cavity compresses and pumps air into the lungs. 4. Hypaxial musculature used to power active exhalation. 5. Intercostal muscles used to power inhalation by aspiration (Carrier, 1989).

(2) in *Necturus* and amniotes, but not in lungfishes, active expiration is powered by an increase in pleuroperitoneal pressure effected by contraction of hypaxial musculature.

Air transfer pattern—Sarcopterygii

With the exception of *Amphiuma*, all of the salamanders we examined expire and inspire within one buccal expansion–compression cycle: (1) the buccal cavity expands and fills with fresh air while air from the lungs is simultaneously exhaled into and through the fresh air filled buccal cavity; (2) the mouth or nares are closed, the buccal cavity compresses, and a mixture of fresh and exhaled air is pumped into the lungs. Because the buccal cavity makes only two movements, one expansion and one compression, we call this ventilation mechanism a "two-stroke buccal pump".

This two-stroke pattern of air transfer is fundamentally the same pattern that has been observed in anurans and lungfishes (Anura: de Jongh & Gans, 1969; West & Jones, 1974; MacIntyre & Toews, 1976; Vitalis & Shelton, 1990; Dipnoi: Bishop & Foxon, 1968; McMahon, 1969). In frogs and toads, despite their use of complex inflations, deflations and non-ventilatory buccal oscillations, the basic air transfer pattern is the same as that seen in salamanders. During ventilatory cycles, anurans expand the buccal cavity to draw in fresh air through open nares, expire pulmonary air into the expanded buccal cavity, and then close the nares and pump the mixture of fresh and exhaled air into the lungs (de Jongh & Gans, 1969; West & Jones, 1974; MacIntyre & Toews, 1976; Vitalis & Shelton, 1990; but see Brett & Shelton, 1979 for a different pattern observed in *Xenopus laevis*).

Air transfer in the lungfishes *Protopterus* and *Lepidosiren* differs only slightly from the pattern in salamanders and frogs. Whereas salamanders and frogs begin expiration near maximum buccal expansion, lungfishes begin expiration early in buccal expansion (Bishop & Foxon, 1968; McMahon, 1969; Brainerd, 1993). Because expiration occurs early in buccal expansion, while the buccal volume is still small, most of the expired air flows out the mouth and a large volume of fresh air is drawn into the buccal cavity after expiration (Brainerd, 1993).

Some two-stroke buccal pumpers with large tidal volumes take one or more accessory inspirations after the first expiratory–inspiratory cycle. In these cases the buccal cavity is expanded without exhaling, and only fresh air is pumped into the lungs. *Lepidosiren* generally takes one or two accessory inspirations (Bishop & Foxon, 1968). *Protopterus* has been observed to take one accessory inspiration when heavily instrumented, but does not do so under normal laboratory conditions (McMahon, 1969). Although *Lepidosiren* uses accessory inspirations, and therefore accessory buccal expansions and compressions, we still categorize this lungfish as a two-stroke buccal pumper on the basis of the first buccal expansion–compression cycle in each breath, during which both expiration and inspiration occur.

By this definition, the large aquatic salamander *Amphiuma tridactylum* is not a two-stroke buccal pumper. Instead of expiring and inspiring within one expansion–compression cycle, *Amphiuma* uses one complete buccal pump cycle for expiration, and one or more complete cycles for inspiration. These observations of *Amphiuma* breathing differ from the observations of Martin & Hutchison (1979). The individuals that we observed always pushed their snouts above the surface before beginning buccal expansion for expiration. In contrast, Martin & Hutchison observed *Amphiuma* filling the buccal cavity with air from the lungs while still on the way to the surface. They also observed two buccal fillings associated with expiration whereas our subjects expanded the buccal cavity just once for expiration. It seems likely that *Amphiuma* is capable of breathing in several ways, perhaps depending on the water depth, the animal's size, or other individual differences. In neither of the two observed breathing patterns does both expiration and inspiration occur within the same buccal pump cycle. We therefore conclude that *Amphiuma* cannot be categorized as a two-stroke buccal pumper.

Despite the use of a different ventilation pattern by *Amphiuma*, our observation that four other salamander species share the two-stroke pattern leads us to conclude that two-stroke ventilation is the primitive pattern for salamanders (the five salamander species we examined represent five different families: Proteidae, Ambystomatidae, Sirenidae, Amphiumidae and Dicamptodontidae). Since the two-stroke pattern is also shared with lungfishes and anurans, we conclude that two-stroke buccal pump ventilation is the primitive breathing mechanism for sarcopterygian vertebrates (Fig. 9, character 3).

Air transfer pattern—Actinopterygii

In contrast to the sarcopterygian two-stroke system, basal actinopterygian fishes use a 'four-stroke buccal pump' (Fig. 9, character 2). Actinopterygian fishes use four buccal movements, two expansions and two compressions, for each breath: (1) the buccal cavity expands and fills with expired air from the lung; (2) the buccal cavity compresses to force the expired air either out the opercular openings (*Lepisosteus*, Rahn *et al.*, 1971; *Polypterus*, Brainerd, Liem & Samper, 1989) or out the slightly opened mouth (*Amia*, Deyst & Liem, 1985); (3) the buccal cavity expands again to draw in fresh air; (4) the mouth closes and the buccal cavity compresses to pump fresh air into the lungs (Brainerd, 1993).

Evolutionary relationships of air transfer patterns

The observed differences between the buccal pumps of basal actinopterygians and basal sarcopterygians raises the question of the evolutionary relationship between these mechanisms (Fig. 9, characters 2 and 3). Three hypotheses are plausible: (1) two-stroke and four-stroke buccal pumps arose independently; (2) either the two-stroke or the four-stroke buccal pump is primitive and the other is derived; or (3) two-stroke and four-stroke buccal pumps are both derived mechanisms relative to some unknown lung ventilation mechanism present in the common osteichthyian ancestor.

The simplest way to test these hypotheses would be by outgroup comparison. Unfortunately there is no extant, air-breathing outgroup for the Osteichthyes (Fig. 9; Osteichthyes as defined in Fig. 1 to include tetrapods). Alternatively, detailed study of respiratory structure and function in basal vertebrates might yield information supporting either homology or convergence in actinopterygian and sarcopterygian respiratory systems. For example, the issue of the homology of actinopterygian and sarcopterygian lungs is implicit in the above hypotheses: hypotheses (2) and (3) require the homology of actinopterygian and sarcopterygian lungs. If the common ancestor of the Actinopterygii and Sarcopterygii did not have lungs, then the breathing mechanisms must have arisen independently. It is most parsimonious to hypothesize that lungs arose just once in an osteichthyian ancester rather than twice (Liem, 1988). Anatomical and developmental data, however, do not support the homology of actinopterygian and sarcopterygian lungs (Jarvik, 1980). Further work on the histology, innervation, circulation and development of vertebrate lungs might resolve this issue.

Detailed study of the breathing mechanisms themselves could also help determine the evolutionary relationship between the actinopterygian and sarcopterygian systems. In particular, studies of respiratory control mechanisms and of ventilatory motor patterns might yield evidence supporting either homology or convergence in actinopterygian and sarcopterygian respiratory systems (Smatresk, 1990). For example, it has long been recognized that the buccal pumps used in air ventilation must be derived from the basic aquatic buccal pump (McMahon, 1969). Use of a buccal pump for suction feeding and gill irrigation is primitive for gnathostome vertebrates (Wu, 1990; Tanaka, 1973; Fig. 9, character 1). Comparison of aquatic respiration and aquatic suction feeding with aerial respiration might yield information on the manner in which primitive aquatic respiratory control systems or motor patterns were modified for aerial respiration (Brainerd, 1993). If actinopterygians and sarcopterygians are found to exhibit similar modifications of the aquatic system, then the homology of the breathing mechanisms would be supported. At present, however, we are unable to distinguish between the three hypotheses of evolutionary relationship between the sarcopterygian two-stroke and actinopterygian four-stroke breathing mechanisms.

Exhalation in the Sarcopterygii

Although lungfishes and *Necturus* share the two-stroke ventilation pattern, we found that their expiration mechanisms differ. In *Necturus*, air is actively expired, driven by a large increase in pleuroperitoneal cavity pressure (Fig. 5; Table 1). Electromyograms recorded from *Necturus* indicate that the increase in pleuroperitoneal pressure is generated by activity in the hypaxial musculature (Fig. 7). Thus some of the hypaxial trunk musculature, which in fishes is used exclusively for swimming, has assumed an expiratory function in *Necturus*.

McMahon (1969) measured lung pressures in the lungfish *Protopterus* and concluded that expiration is driven by elastic recoil of their hyperinflated lungs, perhaps aided by hydrostatic pressure and contraction of smooth muscle in their pulmonary walls. Not having measured pleuroperitoneal pressure, McMahon left untested the possibility that lungfishes might use their hypaxial musculature to aid expiration. Based on our finding of a slight pleuroperitoneal pressure decrease during expiration in *Protopterus* (Fig. 8), rather than the increase seen in *Necturus*, we conclude that lungfishes do not use hypaxial muscles for lung ventilation.

Hypaxial morphology supports the hypothesis that ventral trunk musculature, which in actinopterygian fishes and lungfishes is used only for locomotion, has adopted a ventilatory function in *Necturus*. Actinopterygian fishes have just two layers of hypaxial musculature: the external oblique and the internal oblique (Maurer, 1912). Lungfishes have three hypaxial layers: the external oblique superficialis, the external oblique profundus, and the internal oblique (Maurer, 1912). *Necturus* and other salamanders have a fourth layer, the transverse abdominal, which is composed of fibres running more circumferentially than the fibres of the oblique layers (Maurer, 1892; see Results for fibre angles in *Necturus*). In amniotes, the *m. transversus abdominis* is universally used as an expiratory muscle. Lizards, snakes, turtles, crocodilians, birds and mammals have all been shown to use the transverse abdominal for expiration (Gaunt & Gans, 1969; Rosenberg, 1973; Gans & Clark, 1976; de Troyer & Loring, 1986; Fedde, 1987; Carrier, 1989). The presence and expiratory function of the transverse abdominal in both *Necturus* and amniotes is evidence that the use of hypaxial musculature for expiration is an innovation unique to tetrapods (Fig. 9, character 4).

In contrast to salamanders and amniotes, frogs and toads do not use hypaxial muscles for expiration (de Jongh & Gans, 1969). Mapping this result onto Fig. 9 suggests that it would be equally parsimonious to hypothesize that salamanders and amniotes acquired the use of hypaxial musculature for expiration independently as to hypothesize the acaquisition of this ability in the tetrapod ancestor and its loss in frogs and toads. Anurans, however, have a highly derived body form relative to salamanders and amniotes (Gans, 1970a). Anurans have almost completely lost the tail and have just two layers of hypaxial musculature (Maurer, 1895). Due to the derived body forms of frogs and toads, we prefer the hypothesis that, although lost in anurans, the use of hypaxial musculature for expiration is primitive for tetrapods (Fig. 9, character 4).

Evolution of aspiration breathing

Based on the breathing mechanisms of living vertebrates, we suggest a two stage transition from buccal pump ventilation to aspiration pump ventilation. Beginning with the use of a pure buccal pump with no involvement of the trunk musculature (lungfishes), the first stage is the use of trunk muscles for expiration and a buccal pump for inspiration (salamanders; Fig. 9, character 4), and the second stage is the use of trunk muscles for both expiration and inspiration by costal aspiration (amniotes; Fig. 9, character 5). It is somewhat difficult, however, to reconcile this scenario with the morphology of the Temnospondyli, the fossil amphibians thought to be most closely related to the Lissamphibia (Milner, 1988; Trueb & Cloutier, 1991). Some of the temnospondyls were exceptionally large (> 1 m) and had well developed, double-headed ribs (Carroll, 1988). It has frequently been argued, based on their size and rib morhpology, that forms such as these must have been costal aspiration breathers (Gans, 1970b; Romer, 1972; Packard, 1976). We present three hypotheses for the relationship between salamander breathing mechanisms and those putatively possible for temnospondyls:

(1) Temnospondyls breathed by costal aspiration and modern amphibians have returned secondarily to buccal pumping. This hypothesis implies that the similarity between lungfish and lissamphibian two-stroke buccal pumps is coincidental.

(2) Temnospondyls ventilated their lungs with a two-stroke buccal pump assisted by hypaxial expiration, and modern amphibians have simply retained this system.

(3) Larval temnospondyls breathed in the same manner as modern salamanders, but the large adults were costal aspiration breathers. Lissamphibians evolved from temnospondyls by paedomorphosis and thus retained the larval buccal pump breathing system.

In view of the distinct similarity between lungfish and salamander ventilation, hypothesis (1) is less parsimonious than either (2) or (3). Hypotheses (2) and (3) are essentially similar: both imply that the lung ventilation mechanism used by modern salamanders has been retained from the tetrapod ancestor. Hypothesis (3) is particularly appealing: it allows the adult temnospondyls to be costal aspiration breathers while the motor patterns and respiratory control mechanisms for buccal pumping are retained in the larvae and later passed on to the Lissamphibia. This hypothesis is supported by a recent cladistic analysis which proposes a sister group relationship between lissamphibians and a family of small temnospondyls, the Dissorophidae (Milner, 1988). Milner's analysis provides evidence for progressive miniaturization by paedomorphosis in the temnospondyl line, and thus supports the hypothesis that lissamphibians inherited the larval temnospondyl breathing mechanism.

CONCLUSIONS

Perhaps the greatest advantage of a cladistic-phylogeny-based, historical study such as this is not only our ability to answer some questions about the evolution of function, but also our ability to formulate more explicitly the remaining questions. Our study concluded that basal actinopterygian fishes breathe by four-stroke buccal pumping whereas basal sarcopterygians breathe by two-stroke buccal pumping. This result highlights the importance of determining whether lungs, ventilatory motor patterns and respiratory control systems are homologous in the Actinopterygii and Sarcopterygii. We also hypothesized that aspiration breathing evolved from buccal pumping in two stages: the first stage being the use of trunk musculature for exhalation, and the second stage the use of trunk musculature for both exhalation and inhalation. This hypothesis has focused our attention on the evolutionary relationships of extinct temnospondyl amphibians and the lissamphibians. Future work elucidating the evolutionary processes that have been operative in the amphibian and amniote lineages will have important consequences for our understanding of the evolution of vertebrate breathing mechanisms.

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