

MECHANICS OF LUNG VENTILATION IN A POST-METAMORPHIC SALAMANDER, *AMBYSTOMA TIGRINUM*

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Summary

The mechanics of lung ventilation in frogs and aquatic salamanders has been well characterized, whereas lung ventilation in terrestrial-phase (post-metamorphic) salamanders has received little attention. We used electromyography (EMG), X-ray videography, standard videography and buccal and body cavity pressure measurements to characterize the ventilation mechanics of adult (post-metamorphic) tiger salamanders (*Ambystoma tigrinum*). Three results emerged: (i) under terrestrial conditions or when floating at the surface of the water, adult *A. tigrinum* breathed through their nares using a two-stroke buccal pump; (ii) in addition to this narial two-stroke pump, adult tiger salamanders also gulped air in through their mouths using a modified two-stroke buccal pump when in an aquatic environment; and (iii) exhalation in adult tiger salamanders is active during aquatic gulping breaths, whereas exhalation appears to be passive during terrestrial breathing at rest. Active exhalation in aquatic breaths is indicated by an increase in body cavity pressure during exhalation and associated EMG activity in the

lateral hypaxial musculature, particularly the *M. transversus abdominis*. In terrestrial breathing, no EMG activity in the lateral hypaxial muscles is generally present, and body cavity pressure decreases during exhalation. In aquatic breaths, tidal volume is larger than in terrestrial breaths, and breathing frequency is much lower (approximately 1 breath 10 min^{-1} versus 4–6 breaths min^{-1}). The use of hypaxial muscles to power active exhalation in the aquatic environment may result from the need for more complete exhalation and larger tidal volumes when breathing infrequently. This hypothesis is supported by previous findings that terrestrial frogs ventilate their lungs with small tidal volumes and exhale passively, whereas aquatic frogs and salamanders use large tidal volumes and exhale actively.

Key words: salamander, Caudata, Urodela, Amphibia, ventilation, buccal pump, transversus abdominis, hypaxial musculature, *Ambystoma tigrinum*.

Introduction

Recent studies have documented the lung ventilation mechanics of aquatic salamanders (Brainerd et al., 1993; Brainerd, 1998; Brainerd and Monroy, 1998), but little is known about the breathing mechanics of terrestrial salamanders. In other groups of amphibians, terrestrial breathing mechanics have been described for frogs (de Jongh and Gans, 1969; Vitalis and Shelton, 1990; West and Jones, 1974) and caecilians (Carrier and Wake, 1995; Bennett et al., 1999). Terrestrial frogs and caecilians both show stereotyped oscillations of the buccal cavity in which air is moved in and out of the nares while the glottis is closed. These buccal oscillations (*sensu* de Jongh and Gans, 1969) are punctuated by periodic ventilation of the lungs. In the present study, we document the ventilation mechanics of adult (post-metamorphic) *Ambystoma tigrinum* and discuss patterns of breathing mechanics in the three extant orders of amphibians (Anura, Caudata and Gymnophiona).

Larval *Ambystoma tigrinum* are completely aquatic. In

contrast, post-metamorphic adults are predominantly terrestrial, returning annually to the water for weeks or months to breed (Petranka, 1998). This amphibious life history presents an opportunity to examine the breathing behavior of *A. tigrinum* in both terrestrial and aquatic environments.

Among amphibians and air-breathing fishes, lung ventilation mechanisms may be classified as either two-stroke or four-stroke buccal pumps (Brainerd et al., 1993; Brainerd, 1994). In two-stroke breathing, both inhalation and exhalation occur within a single buccal cavity expansion and compression cycle (Fig. 1). In a single buccal expansion, both fresh air and expired gas from the lungs flow into and are mixed in the mouth. Then, as the buccal cavity is compressed, a portion of the mixture is pumped into the lungs. This two-stroke breathing mechanism is the primitive condition for amphibians (Brainerd et al., 1993; Brainerd, 1994). It has been observed in lungfishes (*Lepidosiren paradoxa* and *Protopterus aethiopicus*, Brainerd, 1994), frogs (*Rana* spp., de Jongh and

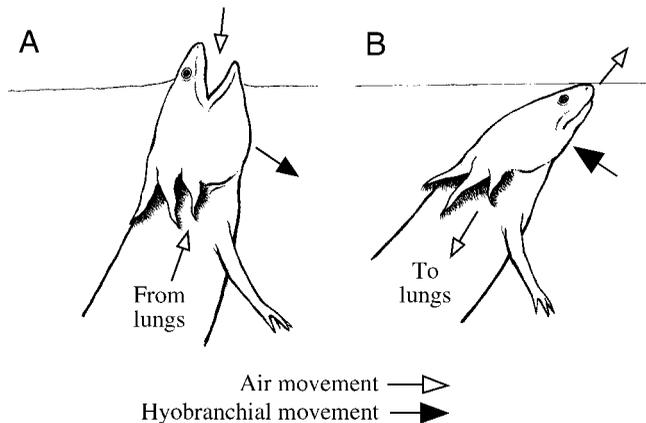


Fig. 1. Two-stroke buccal pump, as exemplified by lung ventilation in a larval tiger salamander, *Ambystoma tigrinum* (Brainerd, 1998). In two-stroke lung ventilation, air from the atmosphere and expired gas from the lungs flow into the buccal cavity and mix together during the first stroke of the pump, buccal expansion (A). In the second stroke of the pump, buccal compression (B), mixed gas in the buccal cavity is pumped into the lungs, and excess gas is pumped out through the mouth or nares.

Gans, 1969; Vitalis and Shelton, 1990; West and Jones, 1974), a toad (*Bufo marinus*, Jones, 1982; MacIntyre and Toews, 1976), a caecilian (*Dermophis mexicanus*, Carrier and Wake, 1995) and some aquatic salamanders such as *Siren lacertina*, *Necturus maculosus* and larval *Ambystoma tigrinum* (Brainerd et al., 1993; Brainerd and Monroy, 1998; Brainerd, 1998). We predict that terrestrial adult *A. tigrinum* will also use two-stroke breathing, as has been observed in other terrestrial amphibians and in aquatic larval *A. tigrinum* (Brainerd, 1998).

Although the majority of amphibians use a two-stroke buccal pump for lung ventilation, a few aquatic amphibians use a four-stroke pump (*Amphiuma tridactylum*, Brainerd et al., 1993; Martin and Hutchison, 1979; *Xenopus laevis*, Brett and Shelton, 1979; Boutilier, 1984; *Cryptobranchus alleganiensis*, E. L. Brainerd, personal observation). During four-stroke breathing, the buccal cavity expands and compresses twice for each respiratory cycle: first, the buccal cavity expands and fills with expired air from the lungs (Fig. 2A; lung exhalation); second, the floor of the buccal cavity is raised and the expired air is pushed out of the mouth or nostrils (Fig. 2B, buccal compression); third, the buccal cavity expands again and fresh air is drawn into the mouth (Fig. 2C; buccal expansion); and fourth, the buccal cavity compresses and the fresh air is pushed into the lungs (Fig. 2D; lung inflation). Four-stroke breathing in salamanders is also found in air-breathing, ray-finned fishes such as *Amia* and *Lepisosteus* (Brainerd et al., 1993; Brainerd, 1994). Phylogenetic analysis of breathing patterns in ray-finned fishes, lungfishes and amphibians indicates that the four-stroke mechanisms seen in amphibians are secondarily derived. Two-stroke breathing is the primitive condition for amphibians, and four-stroke breathing evolved independently in *Amphiuma tridactylum*, *C. alleganiensis* and *X. laevis* (Brainerd, 1999).

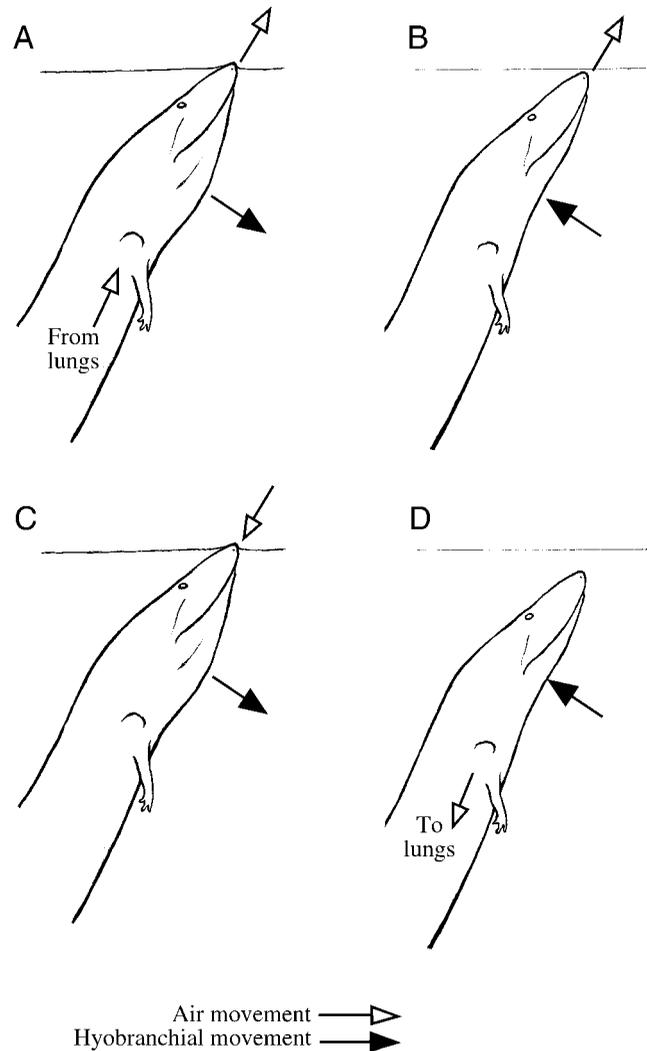


Fig. 2. Four-stroke buccal pump, as exemplified by lung ventilation in *Amphiuma tridactylum* (Brainerd et al., 1993). In the first stroke of a four-stroke breath (A), the buccal cavity expands and fills with expired gas from the lungs, and expired air flows out the nares or mouth. In the second stroke (B), the buccal cavity compresses, and expired gas is forced out of the buccal cavity. In the third stroke (C), the buccal cavity expands and fills with fresh air; in the fourth stroke (D), the buccal cavity compresses and inflates the lungs with pure fresh air (unlike two-stroke breathing, no expired air is re-inspired). Two- and four-stroke buccal pumps were named by analogy with two- and four-stroke engines (Brainerd et al., 1993; Brainerd, 1994).

In two- and four-stroke breathing, fresh air may be drawn into the buccal cavity either through the nostrils or by opening the mouth. Among salamanders, ontogenetic changes result in a shift from mouth breathing in the larva to narial breathing in the post-metamorphic adult. For example, the aquatic larvae of ambystomatids, dicamptodontids and salamandrids all breathe through their mouths, whereas terrestrial adults in these families all breathe through their nares (E. L. Brainerd, personal observation). In agreement with these observations, pedomorphic perrenibranchiate species such as *Siren lacertina* and *Necturus maculosus* breathe with their mouths as

adults (Brainerd and Monroy, 1998; Brainerd et al., 1993), whereas aquatic salamanders that undergo more complete metamorphosis such as *Amphiuma tridactylum* and *Cryptobranchus alleganiensis* use narial breathing (Brainerd and Dumka, 1995; Brainerd, 1997). Larval *Ambystoma tigrinum* use their mouths for breathing (Brainerd, 1998), but we predict that post-metamorphic terrestrial adult tiger salamanders will breathe using only their nares.

Hypaxial trunk musculature (particularly the M. transversus abdominis) is involved in the exhalation phase of breathing in aquatic salamanders including larval *Ambystoma tigrinum* (Brainerd, 1998) and in an aquatic frog (*Xenopus laevis*; de Jongh, 1972). In contrast, no hypaxial muscle activity has been observed during terrestrial breathing in frogs (de Jongh and Gans, 1969) or in caecilians (Bennett et al., 1999). What is the expectation for terrestrial salamanders? Are hypaxial muscles used to assist exhalation, as observed in the larval condition, or do terrestrial adult tiger salamanders rely on a passive mechanism for exhalation, as observed in other terrestrial amphibians?

The primary objective of this paper is to characterize and quantify the mechanics of lung ventilation in adult (post-metamorphic) *Ambystoma tigrinum* using electromyography, X-ray videography, standard videography and pressure measurements. We address the following three questions. (i) Do adult tiger salamanders use a two-stroke buccal pump as observed in the larval stage? (ii) Do they use only the nares to breathe? (iii) Do terrestrial adult tiger salamanders recruit their hypaxial muscles for pulmonary exhalation? We compare our results with previously published data from larval *A. tigrinum* (Brainerd, 1998) and compare the ventilatory mechanics of *A. tigrinum* with those of other amphibian taxa to investigate the evolution of amphibian breathing mechanics.

Materials and methods

Animals and recording conditions

Adult *Ambystoma tigrinum* (Green) were obtained from a scientific supplier (Sullivan's Amphibians, Nashville, TN, USA) and kept on moist paper towels at room temperature (22 °C). The animals were fed a diet of crickets with vitamin and mineral supplements. Seven individuals were used for ventilation measurements. Individual identification numbers and corresponding snout–vent lengths in centimeters are: 01, 13.5 cm; 02, 12.5 cm; 03, 11.0 cm; 04, 11.0 cm; 05, 12.5 cm; 06, 12.0 cm; 07, 13.0 cm. In addition, two *Salamandra salamandra* (L.) (snout–vent lengths 11.2 cm, 10.5 cm) and one *Dicamptodon tenebrosus* (Good) (snout–vent length 13.5 cm) were used for comparison with *A. tigrinum*. The *S. salamandra* specimens were acquired from a scientific supplier (Glades Herp Inc., Fort Myers, FL, USA) and maintained as described for *A. tigrinum*. The *D. tenebrosus* specimen was collected in northern California and kept in a cold room at approximately 5 °C. Terrestrial breathing measurements were made in an aquarium with no water, and aquatic breathing measurements were performed in water

6–8 cm deep (Brainerd, 1998). All experiments were performed at room temperature (22 °C). Animal care and use procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst (approval no. 19-10-07).

Pressure recordings

Buccal and pleuroperitoneal cavity pressures were measured as described by Brainerd et al. (1993) and Brainerd (1998). Salamanders were anesthetized in a solution of tricaine methanesulfonate (1 g l⁻¹) (Finquel brand, Argent), buffered to pH 7.0 with sodium bicarbonate. A small hole was drilled in the snout (at the midline of the anterior end of the frontal bone), and a polyethylene cannula (1.27 mm o.d.; PE 90), flared at the proximal end, was introduced into the buccal cavity. A 14 gauge hypodermic needle was used to introduce a similar cannula into the pleuroperitoneal cavity at mid trunk, outside the lungs. The animal was allowed to recover for 12 h.

Millar Mikro-tip SPR-407 pressure transducers were threaded into the cannulae. Pressure signals were amplified 100× using Tektronics AM502 direct current amplifiers and recorded on a Macintosh computer with a GW Instruments data acquisition board and SuperScope software. Pressure signals were sampled at 4000 points s⁻¹.

Using a Color-key graphics overlay (Televeyes Pro, Computer Eyes, Inc.), we superimposed real-time pressure traces from SuperScope onto live video recordings of animals breathing. The combined, synchronized images were then recorded onto video cassette for reference. Pressure traces were also saved as digital waveforms for quantitative analysis.

Although we attempted to record lung pressure in addition to pleuroperitoneal (body cavity) pressure, repeated attempts to cannulate the lungs were unsuccessful. The lungs of tiger salamanders are small (3–5 mm in diameter), and introducing a cannula with a large enough diameter to measure air pressure in the lungs was impractical. It is also difficult to measure buccal pressures at the air/fluid interface in the buccal cavity. If an air-filled cannula is used, then fluid from the mouth, in the case of terrestrial breathing, or water from the environment, in the case of aquatic breathing, tends to cause a fluid block in the air-filled cannula, which attenuates pressures. If a water-filled cannula is used, then air bubbles tend to cause a blockage. We attempted to overcome these problems by using a Millar Mikro-tip pressure transducer and threading the transducer down the cannula such that the sensor rested just (0.5 mm) inside the buccal cavity. This seems to have solved the problem of blockage, because we saw no evidence of signals becoming attenuated over time (as is seen in air- or water-filled cannula systems). However, this solution created a problem with the baseline for buccal pressure. We found that the baseline for the buccal trace did not remain zeroed at atmospheric pressure, but varied by as much as 0.1 kPa above or below atmospheric pressure (with no obvious correspondence to the behavior of the animal). We are uncertain of the cause of this baseline shift, but it meant that we could only measure relative changes in buccal

pressure and not absolute pressure relative to atmospheric pressure. Therefore, all pressure traces are shown as floating traces, with a scale bar to indicate magnitude but with no indication of a zero reference.

Electromyography

Patch electrodes were manufactured and implanted as described by Carrier (1993). Patch electrodes were used to minimize cross talk between the thin sheets of lateral hypaxial musculature (Loeb and Gans, 1986; Carrier, 1990). Patch electrodes were constructed from reinforced silastic sheeting (Dow Corning, 0.25 mm thick), fine wire (silver 0.051 mm diameter; Cooner Fine Wire, CA, USA) and liquid silicone. Electromyographic (EMG) recordings were made from the four layers of lateral hypaxial musculature: Mm. obliquus externus superficialis (OES), obliquus externus profundus (OEP), obliquus internus (OI) and transversus abdominis (TA) (Fig. 3). The TA electrode was placed between the TA and OI, with the recording side facing the TA. The OI electrode was placed between the OI and OEP, facing the OI. The OEP electrode was placed between the OI and OEP, facing the OEP. The OES electrode was placed between the OEP and OES, facing the OES. EMG leads were soldered to a connector sutured to the dorsum of the salamander. The connector was attached to a shielded and insulated cable. Six-pin connectors were used to accommodate three pairs of electrode wires. Thus, signals were recorded from a maximum of three muscles simultaneously.

EMG signals were amplified 10 000 \times , using Grass Instruments P511J amplifiers, and filtered after recording with a digital bandpass filter (200–1000 Hz). Signals were sampled at 4000 Hz with a GW Instruments analog-to-digital acquisition and analysis board and Superscope software.

To record EMGs during heavy breathing after exercise, we

induced the animals to walk on a trackway for about 3 min (locomotion induced by spraying the animals with a fine mist of water). We then recorded EMGs for up to 2 min after the cessation of exercise.

X-ray videography

X-ray videography was used to observe patterns of air flow between the lungs, buccal cavity and atmosphere. Lateral views were recorded for three *Ambystoma tigrinum*. Two individuals were instrumented with buccal and pleuroperitoneal pressure transducers, and pressures were synchronized with the X-ray video recordings. The third individual remained uninstrumented. Lateral and dorsoventral views were also recorded for two *Salamandra salamandra* specimens and one *Dicamptodon tenebrosus*. A Siemens cineradiographic unit with a Sirecon image intensifier and a Sony VX1000 digital camcorder (shutter speed 1/250 s) were used to make X-ray video recordings at a time resolution of 60 fields s^{-1} .

To estimate the change in volume of the lungs and buccal cavity, we measured the area of the lungs and buccal cavity, in lateral view, from X-ray video recordings. The duration and angle of gape were also measured. Using Adobe Premiere, we made Quicktime movies of breathing from X-ray video recordings. Quicktime movies were imported into NIH Image, and the areas of the lungs and buccal cavity were then quantified.

Quantitative analysis of pressures and EMGs

Superscope software was used to measure the maxima and minima of pleuroperitoneal (body) and buccal pressure waves. Values were imported into spreadsheets in Microsoft Excel for further analysis. Generally, muscle activity was dominated by the M. transversus abdominis (TA), and we therefore quantified activity of the TA muscle only. Onset and offset of

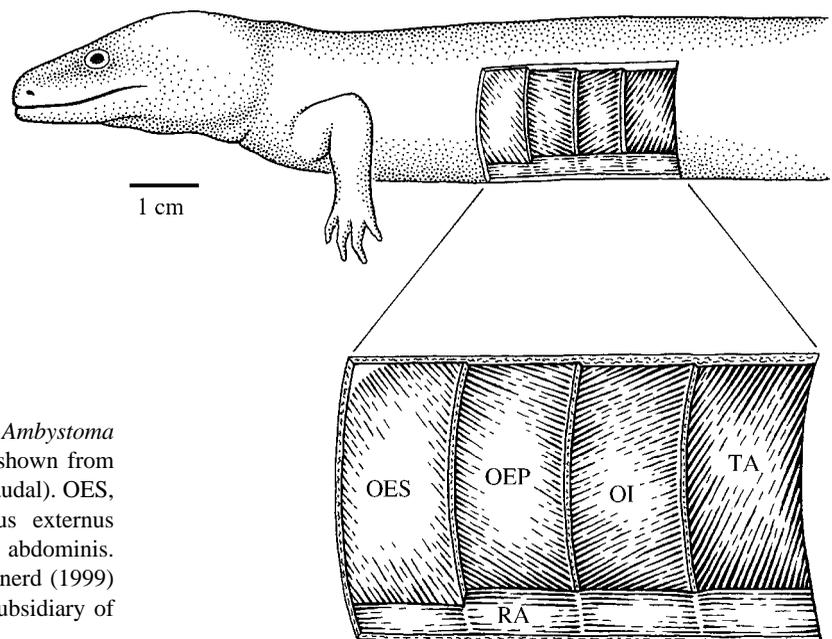


Fig. 3. The lateral hypaxial musculature of adult *Ambystoma tigrinum*. Successive, deeper layers of musculature are shown from left to right (superficial layers removed from cranial to caudal). OES, M. obliquus externus superficialis; OEP, M. obliquus externus profundus; OI, M. obliquus internus; TA, M. transversus abdominis. RA, M. rectus abdominis. Figure from Simons and Brainerd (1999) (with permission). Copyright 1999 Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

electromyographic activity of the TA were identified using Superscope, as described above for body and buccal pressure changes.

Results

Metamorphosed, adult *Ambystoma tigrinum* show two distinct modes of breathing. Adult *A. tigrinum* may breathe exclusively through their nares during 'terrestrial' breathing or they may breathe predominantly by opening their mouths and gulping air during 'aquatic' breathing. The ventilation mechanics of both terrestrial narial breaths and aquatic gulping breaths are examined below.

Terrestrial breathing

Terrestrial breathing occurs when the salamanders are in a terrestrial environment or have their bodies submerged in water but their nares at the surface. Two components make up terrestrial breathing: buccal oscillation and lung ventilation. Buccal oscillation begins as the floor of the buccal cavity is raised; the volume of the buccal cavity decreases, and gas is pushed out of the mouth through the nares (Fig. 4; 0 s). Then, the floor of the buccal cavity is depressed, expanding the buccal cavity and causing air to flow in through the nares (Fig. 4; 0.08 s). X-ray images of *Ambystoma tigrinum* reveal that, during buccal oscillation, air moves in and out of the buccal cavity while the lungs remain inflated and the lung volume remains constant (Fig. 4).

Lung ventilation begins with a larger buccal expansion than is seen during buccal oscillations. A small amount of gas is then exhaled from the lungs and mixes with the fresh air in the buccal cavity (Fig. 5; 0.47 s). The buccal cavity is then compressed, filling the lungs with the mixture of fresh and expired air (Fig. 5; 0.57 s). Note that the volume of air exchanged, the tidal volume, is only a small fraction of the total lung volume. Close examination of Fig. 5 is required to see a difference in lung volume between maximum exhalation (0.47 s) and maximum inhalation (0.57 s). Dorsoventral view X-ray videos also showed the small tidal volumes during

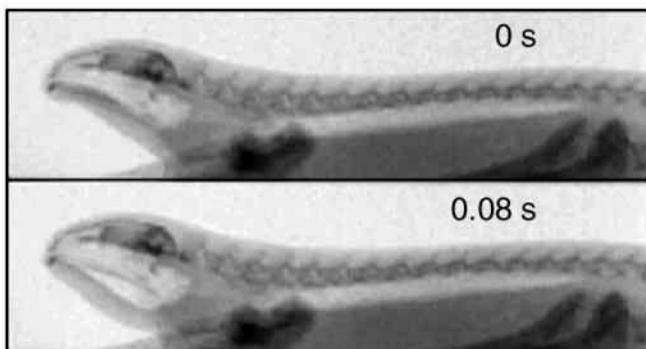


Fig. 4. X-ray positive images of buccal oscillation in an adult *Ambystoma tigrinum* in left lateral view. The buccal cavity is shown compressed (0 s) and expanded (0.08 s). Note that buccal volume changes while lung volume remains constant.

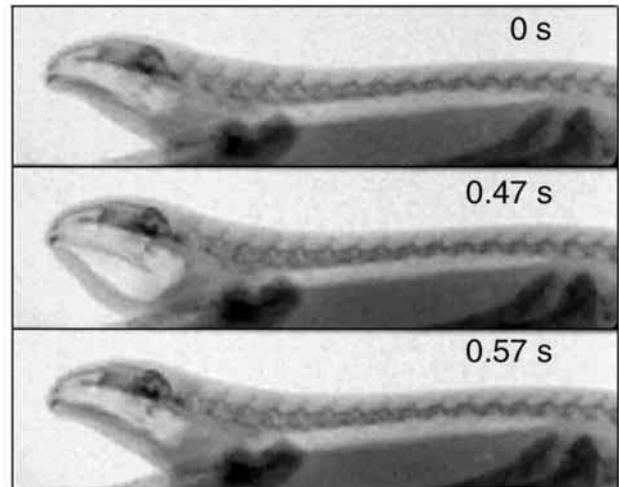


Fig. 5. X-ray positive images of terrestrial lung ventilation in an adult *Ambystoma tigrinum* in left lateral view. The buccal cavity begins to expand as air is drawn into the mouth through the nares (0 s). The buccal cavity is maximally expanded as air is exhaled into the mouth from the lungs, and lung volume reaches its minimum (0.47 s). Mixed gas then is forced from the mouth into the lungs during buccal cavity compression (0.57 s), and excess air in the buccal cavity is expelled through the nares.

breathing. Because the volume of the buccal cavity greatly exceeds the tidal volume, 'extra' mixed gas that does not fit into the lungs is expelled from the buccal cavity through the nares after the lungs have been filled.

Changes in lung and buccal areas during the course of a representative terrestrial narial breath are plotted in Fig. 6 (laterally projected areas measured from X-ray video recordings). Simultaneous pressure changes in the buccal cavity and pleuroperitoneal cavity (i.e. body pressure) are also shown (Fig. 6). Points 1–3 define one buccal oscillation. Points 2 and 3 indicate the maximum and minimum buccal pressures, respectively, during an oscillation. The mean change in the amplitude of buccal pressure is 209 ± 130 Pa (mean \pm s.d., $N=6$; Table 1). Body pressure does not change during buccal oscillation. The mean duration of a single buccal oscillation is 628 ± 100 ms (mean \pm s.d., $N=6$; Table 2).

Points 3, 4 and 5 define the lung ventilation cycle (Fig. 6). The mean change in buccal pressure amplitude (points 4–3) is 667 ± 350 Pa, while the mean change in body pressure amplitude (points c–b) is 46 ± 20 Pa (mean \pm s.d., $N=6$; Table 1). The mean duration of a terrestrial buccal ventilation cycle (points 5–3) is 1197 ± 240 ms (mean \pm s.d., $N=6$; Table 2). The mean duration of the body pressure change associated with lung ventilation (points d–a) is 645 ± 190 ms. The amplitudes and durations of buccal and body pressure changes were measured in six salamanders for a minimum of 10 breaths per individual. The mean pressure changes were calculated for each individual salamander, and these means were then used to calculate the mean and standard deviation of pressure changes for the six individuals (i.e. $N=6$ individuals, not $N>60$ breaths; Tables 1, 2).

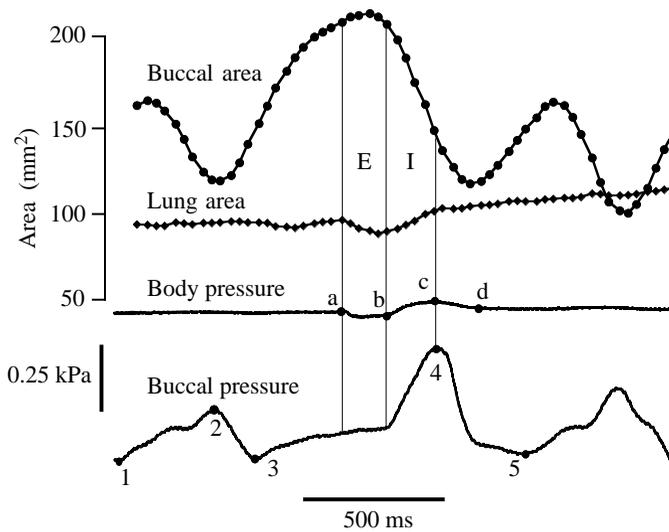


Fig. 6. Typical narial breath of *Ambystoma tigrinum* in a terrestrial environment. The upper two curves show the projected area of air in the lungs and buccal cavity, measured from lateral-view X-ray video recordings. The lower two traces show buccal and pleuroperitoneal (body) pressure changes, recorded synchronously with the X-ray video (baselines are free-floating; see Materials and methods). Numbers 1–3 define one buccal oscillation cycle: 1, start; 2, maximum; 3, end. Numbers 3–5 define one lung ventilation cycle: 3, start; 4, maximum; 5, end. Letters a–d define changes in body pressure during lung ventilation: a, start; b, minimum; c, maximum; d, end. See Tables 1, 2 for mean amplitude and duration values and standard deviations. Vertical lines delineate lung exhalation (E), as indicated by a small decrease in lung area coincident with a drop in body pressure (a–b). Lung inflation (I), buccal compression and a rise in body pressure (b–c) occur simultaneously.

The small tidal volume of terrestrial breaths is evident in Fig. 6. Expiration (E) is indicated by the small decrease in lung area and the concomitant small rise in buccal area. Simultaneously, a small drop in body pressure (points a–b) is observed. In the buccal cavity, fresh air is drawn in through the nares and mixes with the small volume of air exhaled from the lungs. Inspiration (I) occurs simultaneously with an increase in body pressure (points b–c). The expired air emptied from the lungs to the mouth is a small fraction of the fresh air drawn into the buccal cavity through the nares. Point 5 marks the end of the ventilation cycle as body pressure returns to baseline and a buccal oscillation cycle begins again.

In resting or slightly active salamanders, the frequency of buccal oscillation is approximately 50 oscillations min^{-1} and the frequency of lung ventilation is 4–6 breaths min^{-1} . Amplitudes and frequencies of both buccal oscillation and ventilation increase with the salamander's level of stress or exercise. While the adult salamander is in a terrestrial environment, buccal oscillations occur almost continuously. In contrast, buccal oscillation rarely occurs when the salamander is submerged in an aquatic environment.

Terrestrial hypaxial muscle activity

Fig. 7 shows electromyograms (EMGs) recorded from the

Table 1. Amplitude variables measured from buccal and body pressure traces of lung ventilation in *Ambystoma tigrinum*

	Points*	Amplitude‡ (kPa)
Terrestrial narial breathing		
Buccal pressure		
Oscillation	2–1	0.209±0.13
Ventilation	4–3	0.667±0.35
Body pressure	c–b	0.046±0.02
Aquatic gulp breathing		
Buccal ventilation	3–2	1.969±0.41
Body pressure	b–a	0.399±0.49

*Points from Fig. 6 for terrestrial breathing and from Fig. 9 for aquatic breathing.

‡Mean ± s.d. for six individuals. A mean for at least 10 breaths was calculated for each individual, and then the mean of those means ($N=6$), with standard deviation, was calculated to represent the mean variation between individuals within the species.

Table 2. Duration variables measured from buccal and body pressure traces of lung ventilation in *Ambystoma tigrinum*

	Points*	Duration‡ (ms)
Terrestrial narial breathing		
Buccal pressure		
Oscillation	3–1	628±100
Ventilation	5–3	1197±240
Body pressure		
Exhale	b–a	194±110
Inhale	c–b	242±80
Aquatic gulp breathing		
Buccal pressure	4–1	758±190
Body pressure	c–a	706±308

*Points from Fig. 6 for terrestrial breathing and from Fig. 9 for aquatic breathing.

‡Mean ± s.d. for six individuals. A mean for at least 10 breaths was calculated for each individual, and then the mean of those means ($N=6$), with standard deviation, was calculated to represent the mean variation between individuals within the species.

lateral hypaxial musculature and synchronously recorded pressure traces from the buccal and pleuroperitoneal cavities during several buccal oscillations and a single lung ventilation cycle. Breathing at rest (Fig. 7A) and heavy breathing recorded immediately after exercise (Fig. 7B) are shown. During quiet breathing (Fig. 7A), no hypaxial muscle activity is seen in Mm. transversus abdominis (TA), obliquus externus profundus (OEP) or obliquus externus superficialis (OES). Other recordings that include the M. obliquus internus (OI) indicate that this muscle is also silent during terrestrial breathing at rest. However, exercise-induced increase in metabolic rate or hypoxic stress induced by adding nitrogen to the air results in more frequent ventilation combined with short-duration

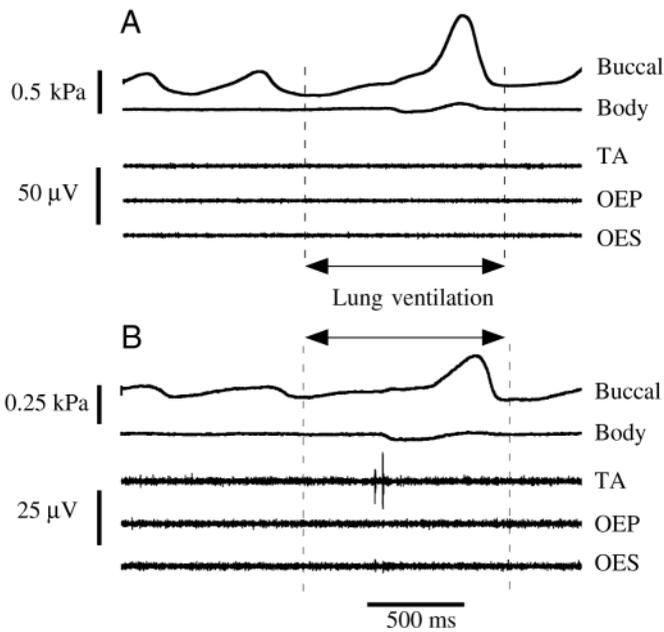


Fig. 7. Electromyograms (EMGs) of the lateral hypaxial muscles of *Ambystoma tigrinum* during narial breathing in a terrestrial environment. The upper two traces show buccal and pleuroperitoneal (body) pressure changes (baselines are free-floating; see Materials and methods), and the lower three traces show EMGs of the Mm. transversus abdominis (TA), obliquus externus superficialis (OES) and obliquus externus profundus (OEP). (A) Traces recorded at rest; (B) traces recorded immediately after exercise.

activity of the TA (Fig. 7B). This EMG activity is coincident with the onset of expiration. Although hypaxial activity during heavy breathing is dominated by the TA muscle, small spikes in the OES, OEP and OI are also seen in some individuals.

Aquatic breathing

When adult tiger salamanders are placed in water (6–8 cm depth), they generally float at the surface and continue buccal oscillations interspersed with terrestrial-type lung ventilations. After approximately 1–3 h in the water, however, we found that they usually sink to the bottom and rest there between trips to the surface for breaths. Buccal oscillation in the submerged animals ceases, and lung ventilation occurs much less frequently than lung ventilation in the terrestrial environment. Terrestrial breathing frequency is generally approximately 4–6 breaths per minute, whereas submerged animals only come to the surface for a breath approximately once every 10 min.

These infrequent 'aquatic' breaths are quite different from terrestrial lung ventilations (Fig. 8). Aquatic breathing is characterized by a rapid lifting of the head and biting at the air while expanding the buccal cavity and drawing air in through the open mouth ('gulping'). Furthermore, tidal volume is much greater in aquatic gulping breaths than in terrestrial breaths (compare the change in lung volume between full exhalation and full inhalation in Figs 5 and 8).

Fig. 8 shows a sequence of X-ray images of a typical aquatic gulping breath in an adult tiger salamander. A breath begins as

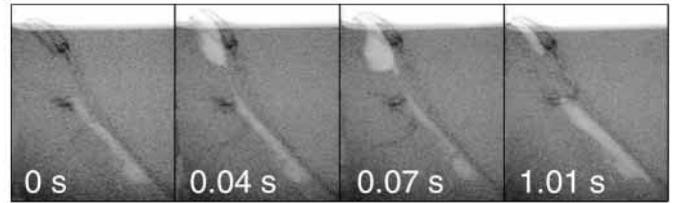


Fig. 8. X-ray positive images of an adult *Ambystoma tigrinum* in lateral view during a representative aquatic gulping breath. The salamander begins a breath by swimming to the water surface (0 s). The mouth opens and fresh air is drawn into the buccal cavity (0.04 s). The mouth closes and gas is exhaled from the lungs to mix with the air in the buccal cavity (0.07 s). Mixed gas is then pushed from the buccal cavity to the lungs (1.01 s) and 'extra' gas in the buccal cavity is exhaled through the mouth and/or nares (not shown).

the salamander swims to the surface and then tosses its head back and expands the buccal cavity. The mouth opens and air is drawn into the buccal cavity (Fig. 8; 0.04 s). Buccal inhalation is followed by closing of the mouth. Exhalation of air from the lungs generally occurs both during and after mouth closing, and expired gas mixes with fresh air in the buccal cavity (Fig. 8; 0.07 s). As the salamander completes the breath, the buccal cavity is compressed, forcing some of the mixed gas into the lungs (Fig. 8; 1.01 s). Because the volume of the buccal cavity exceeds the tidal volume of the lungs, extra gas that does not fit into the lungs remains momentarily in the buccal cavity (Fig. 8; 1.01 s) and is expelled through the mouth or nares shortly thereafter.

We observed some variability in X-ray sequences of aquatic gulping breaths. The salamanders often exhaled air from the lungs early, before reaching the surface and beginning an aquatic gulp. This early exhalation sometimes occurred long before the breath, while the animal was resting on the bottom of the aquarium, and sometimes occurred during the approach to the surface for a breath. Thus, the volume of air in the lungs at the beginning of an aquatic gulp varied from almost zero to approximately half the volume present after full inspiration. In addition, the salamanders sometimes did not noticeably open their mouths, but instead seemed to take a gulp-type breath through their nares. These breaths were otherwise very similar to the aquatic gulps; the rapid head-lift associated with buccal expansion was present, and tidal volume was large.

For a representative gulping breath in an aquatic environment, simultaneous recordings of buccal and pleuroperitoneal (body) pressure changes as well as electromyographic activity (EMG) of M. transversus abdominis (TA) are shown in Fig. 9. Point 1 indicates the start of the buccal pressure change. Point 2 is the minimum pressure attained during buccal inspiration. Point 3 is the maximum pressure achieved during buccal compression and lung inflation. Point 4 indicates the end of the breath (i.e. return to baseline pressure). Point a indicates the start of the rise in body pressure associated with the breath. Points b and c indicate the maximum and minimum body pressures, respectively, during the breath. The mean change in buccal pressure amplitude

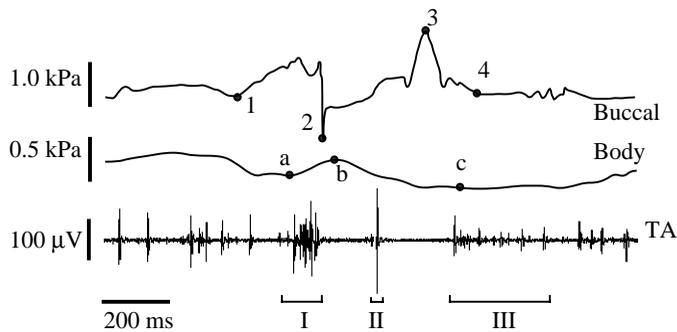


Fig. 9. Representative gulping breath of adult *Ambystoma tigrinum* in an aquatic environment. Simultaneous recordings of buccal and pleuroperitoneal (body) pressure changes as well as electromyographic (EMG) activity of *M. transversus abdominis* (TA) is shown. Pressure baselines are free-floating (see Materials and methods). Point 1 indicates the start of the buccal pressure change. Point 2 is the minimum pressure attained during buccal inspiration. Point 3 is the maximum pressure achieved during buccal compression and lung inspiration. Point 4 indicates the end of the breath (i.e. return to baseline pressure). Points a, b and c indicate the onset, maximum and minimum body pressures, respectively, during the breath. See Tables 1, 2 for mean changes in amplitude and duration values and standard deviations. Three bursts of EMG activity (I, II, III) are discussed in the text (see Table 3).

during lung ventilation is 1969 ± 410 Pa, while the mean change in body pressure amplitude is 399 ± 490 Pa (means \pm S.D., $N=6$; Table 1). The mean duration of an aquatic gulp breath is 758 ± 190 ms, while the mean duration of the change in body pressure is 706 ± 308 ms (means \pm S.D., $N=6$; Table 2). A burst of TA activity is associated with the increase in body pressure (Fig. 9, points a–b).

Pressure changes due to hydrostatic pressure were observed as the salamander moved up and down in the water column (6–8 cm water). However, during most of the ventilation sequence (Fig. 8), the salamander remained stationary at the surface of the water; therefore, changes in buccal and pleuroperitoneal (body) pressure due to changing hydrostatic pressure during lung ventilation were small.

For a typical aquatic ‘gulp’ breath, changes in the volume of the lungs and buccal cavity as well as the simultaneous changes in buccal and pleuroperitoneal (body) pressure are illustrated in Fig. 10. Buccal inspiration begins as the salamander’s snout reaches the surface and the buccal cavity expands, filling with air through the mouth or nares or through both (Fig. 10, phase I). The mouth remains open briefly (Fig. 10, gray bar; 62 ± 47 ms; mean \pm S.D., $N=7$ breaths from one individual) during buccal inhalation but generally closes before the completion of lung exhalation. During lung exhalation, air moves from the lungs into the closed buccal cavity. An increase in body pressure accompanies exhalation and a burst of hypaxial muscle activity (discussed below) precedes this rise in body pressure. The expired and fresh air mix in the buccal cavity, and the mixture is pumped back into the lungs as the floor of the mouth rises during lung inflation (Fig. 10, phase II). Note that tidal volume in aquatic breaths is

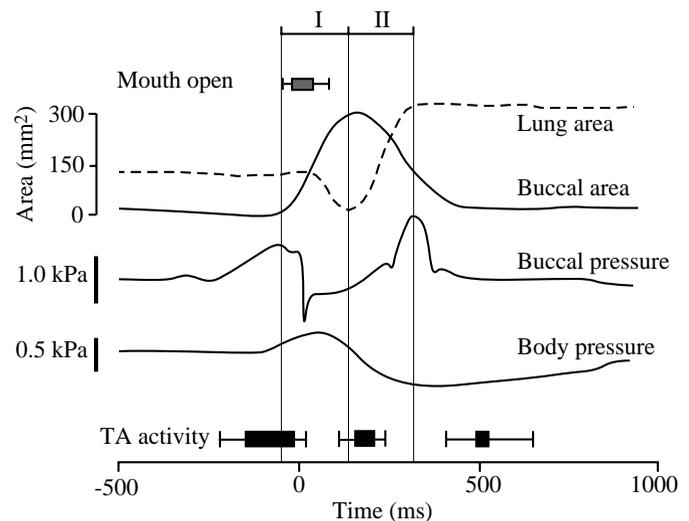


Fig. 10. Summary diagram of ‘gulp’ breathing from adult *Ambystoma tigrinum* in an aquatic environment. Buccal and lung area changes are based on lateral-view X-ray kinematic data from six oral breaths. Buccal and pleuroperitoneal (body) pressures are based on breaths recorded from five individuals (baselines shown free-floating; see Materials and methods). Black bars indicate the mean onset, offset and standard deviations of *M. transversus abdominis* (TA) activity. TA data are compiled from 50 breaths (10 breaths from each of five salamanders; means for each salamander are averaged to give an overall mean; $N=5$). Gray bar indicates when the mouth is open; based on X-ray video recordings, $N=7$ breaths. Error bars show one standard deviation. During phase I, lung area decreases (lung exhalation) while buccal area increases and buccal pressure drops (buccal inspiration). The first burst of TA activity is associated with an increase in body pressure and exhalation. During phase II, lung area increases (lung inspiration), while buccal area decreases and buccal pressure rises (buccal expiration).

large; during lung inflation, the lateral projected area of the lungs increases from nearly 0 to 300 mm^2 (Fig. 10).

Aquatic hypaxial muscle activity

During aquatic gulping breaths, some electromyographic (EMG) activity was observed in all four lateral hypaxial muscle layers (*Mm. obliquus externus superficialis*, *obliquus externus profundus*, *obliquus internus*, *transversus abdominis*). Although variability in amplitude, onset and duration occurred among muscles, the muscle activity was generally dominated by the *M. transversus abdominis* (TA). Thus, we quantified the mean and standard deviation of the onset, offset and duration of activity for the TA muscle only (Fig. 10; Table 3).

One to three distinct bursts of activity and two consistently ‘silent’ periods were observed for all gulp breaths. The first burst of TA activity began before or during buccal inhalation and was associated with a rise in body pressure (Fig. 10). The first burst occurred 100% of the time (Table 3), starting 142 ± 73 ms (mean \pm S.D. from five individuals, 10 breaths each) before the minimum buccal pressure and continued for 109 ± 81 ms (Fig. 10). Mean offset occurred 6 ± 21 ms before the minimum buccal pressure (Fig. 10).

Table 3. Occurrence of *M. transversus abdominis* activity during aquatic gulp breathing in 10 breaths from each of five adult *Ambystoma tigrinum*

Salamander identification	Burst I*	Burst II*	Burst III*
1	10	10	5
2	10	10	4
3	10	8	6
4	10	6	10
5	10	0	1
Total	50/50	34/50	26/50
Total (%)	100	68	52

*Number of times that a burst of electromyographic activity occurred in 10 breaths from each individual. Bursts are defined in Fig. 9.

A silent period associated with lung exhalation and buccal expansion was followed by a short-duration burst of EMG activity in the TA. This second burst was associated with the end of lung exhalation and the beginning of lung inflation. Body pressure continued to decrease. The second burst occurred 68% of the time (Table 3; 34 out of 50 breaths; $N=5$ salamanders; 10 breaths per individual). This burst began 172 ± 40 ms after minimum buccal pressure and lasted 20 ± 4 ms (Fig. 10). Mean offset occurred 201 ± 22 ms (mean \pm s.d.) after minimum buccal pressure (Fig. 10). The second burst was followed by a second silent period associated with lung inflation.

The third burst began 497 ± 81 ms after minimum buccal pressure and continued for 62 ± 27 ms (Fig. 10). Mean offset occurred 516 ± 122 ms after minimum buccal pressure (means \pm s.d.) (Fig. 10). This third burst occurred 52% of the time (Table 3).

X-ray and standard video data reveal that air may leave the lungs and buccal cavity before the onset of buccal pressure changes, during an 'early' exhalation (Fig. 11). The onset of early exhalation was variable, occurring minutes to a fraction of a second before the beginning of a breath. Early exhalation often occurred while the salamander remained at the bottom of the aquarium. Alternatively, or in addition, the salamander exhaled as it swam towards the surface to begin the breathing sequence. Early exhalation was associated with a rise in body pressure and with hypaxial muscle activity in *Mm. transversus abdominis*, *obliquus externus superficialis* and *obliquus externus profundus* (Fig. 11). Other EMG recordings which included the *M. obliquus internus* indicated that this muscle is also active during early exhalation.

Observations of lung ventilation in other terrestrial salamanders

For comparison with lung ventilation in *Ambystoma tigrinum*, we used X-ray video recordings to study the mechanics of terrestrial breathing in two other post-metamorphic salamanders, *Dicamptodon tenebrosus* and *Salamandra salamandra*. We found that lung ventilation in these species is very similar to lung ventilation in tiger salamanders. All three species use a two-stroke buccal pump, breathe through their nares and utilize small tidal volumes in the terrestrial environment.

Discussion

Two types of breathing

In terrestrial environments, adult (post-metamorphic) *Ambystoma tigrinum* breathe through their nares, employing buccal oscillation and two-stroke lung ventilation mechanics that closely resemble the breathing mechanics of most anurans (e.g. *Rana* spp., de Jongh and Gans, 1969; West and Jones,

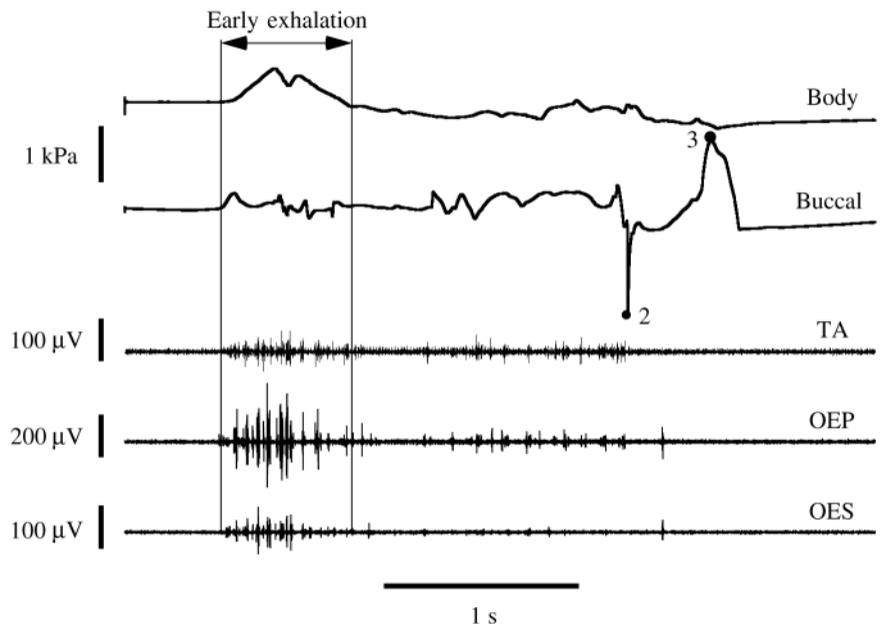


Fig. 11. Early exhalation prior to an aquatic gulping breath in *Ambystoma tigrinum*. Electromyographic (EMG) activity from *Mm. transversus abdominis* (TA), *obliquus externus superficialis* (OES) and *obliquus externus profundus* (OEP) during a typical 'gulp' breath. An increase in pleuroperitoneal (body) pressure accompanies EMG activity in all the lateral hypaxial muscle layers. Pressure baselines shown as free-floating (see Materials and methods). Vertical lines (arrow) indicate 'early exhalation' discussed in the text. Points 2, 3 are the same as in Fig. 10.

1974; *Bufo marinus*, MacIntyre and Toews, 1976; Jones, 1982). However, in addition to narial breathing, we also observed 'gulp' breathing in adult *A. tigrinum*. In an aquatic environment, adult tiger salamanders sometimes breathe by opening their mouths to gulp air from above the water surface. Our discovery of this mouth breathing was unexpected; post-metamorphic salamanders, frogs and caecilians generally use only their nares for breathing (Brainerd et al., 1993; Brainerd, 1999; de Jongh and Gans, 1969; Carrier and Wake, 1995; Bennett et al., 1999). Therefore, this study of ventilation mechanics documents two distinct types of breathing in post-metamorphic *Ambystoma tigrinum*: (i) terrestrial narial breathing and (ii) aquatic gulping breathing.

Terrestrial narial breathing consisted of buccal oscillations and lung ventilations (Figs 4–7). Buccal oscillations occur at frequencies comparable with those of frogs and toads (de Jongh and Gans, 1969; West and Jones, 1974; MacIntyre and Toews, 1976; Jones, 1982). Lung ventilation is powered by a two-stroke buccal pump, and only a small proportion of the air in the lungs is exchanged with each breath (Figs 5, 6). Unlike frogs that exhibit a series of consecutive lung inflations (Vitalis and Shelton, 1990; MacIntyre and Toews, 1976), we did not observe serial lung inflations in our tiger salamanders. However, Boynton and Smatresk (1994) observed up to three consecutive breaths in adult *Ambystoma tigrinum* and a further increase in the number of consecutive breaths associated with aquatic hypercapnia.

In aquatic environments, adult (post-metamorphic) tiger salamanders sometimes breathe by opening their mouths to gulp air from the surface of the water (Fig. 8). The salamander approaches the surface of the water with a variable amount of air in its lungs. At the water surface, the salamander opens its mouth and expands the buccal cavity to draw in fresh air. Exhalation follows, as air moves from the lungs and mixes with the fresh air in the buccal cavity. At the end of exhalation, the lungs are nearly empty (Fig. 8, 0.07 s). Lung inflation begins as the mixture of fresh and expired air in the mouth is pumped into the lungs. In the final step, often performed as the salamander submerges, extra air remaining in the buccal cavity is expelled.

Active versus passive exhalation

Our results indicate that exhalation is passive in terrestrial breaths and active in aquatic gulping breaths. During terrestrial breathing at rest, body cavity pressure decreases slightly during exhalation, and we observed no electromyographic (EMG) activity in the lateral hypaxial musculature (Fig. 7A). We conclude that exhalation is passive, probably driven by stored elastic energy in the lungs and/or body wall. Exhalation may also be assisted by the contraction of smooth muscle in the walls of the lungs (Stark-Vancs et al., 1984). However, exhalation in tiger salamanders is very rapid (194 ± 110 ms; Table 2; Fig. 6), and it seems unlikely that the slow contraction of smooth muscle could assist this rapid movement.

Although no EMG activity of the lateral hypaxial musculature is generally associated with terrestrial breaths in

tiger salamanders at rest, some hypaxial muscle activity occurs during post-exercise breathing and under hypoxic conditions (Fig. 7B). In these circumstances, hypaxial muscle activity is characterized by short-duration bursts, often dominated by the M. transversus abdominis. It is unclear whether these short-duration bursts actually contribute to exhalation of air from the lungs. The activity occurs at the start of exhalation, but is not associated with a rise in body pressure, as would be expected for active exhalation. Instead, body cavity pressure decreases during exhalation, in the same manner as in resting breaths with no EMG activity (Fig. 7A).

In contrast to the passive exhalation seen in terrestrial breaths, adult tiger salamanders exhale actively during aquatic gulping breaths. We observed 1–3 distinct bursts of activity in the M. transversus abdominis (TA) (Figs 9, 10). The first burst of TA activity powers active exhalation; it occurs just prior to and during exhalation, and it is associated with a rise in body cavity pressure. The second and third bursts are more puzzling. The second burst is characterized by a short duration and is associated with the beginning of buccal compression and lung inflation (Figs 9, 10). The function, if any, of this extremely brief burst is unclear. The third burst of TA activity occurs after lung inflation and may function to reposition the newly inspired air. In addition, a burst of EMG activity is associated with early exhalation (Fig. 11), indicating that early exhalation, when it occurs, is also actively powered by the hypaxial muscles.

Tidal volume and active versus passive exhalation

Our finding that exhalation is passive in terrestrial breaths but active in aquatic gulping breaths is somewhat counterintuitive. One might expect that exhalation would be passive in the aquatic environment, because salamanders might be able to take advantage of hydrostatic pressure to force air out of the lungs (as in submerged crocodylians; Gans and Clark, 1976). However, our finding is also consistent with the breathing patterns of other amphibians occupying terrestrial or aquatic habitats. All the aquatic salamanders and frogs studied use active exhalation (*Xenopus laevis*, de Jongh, 1972; *Necturus maculosus*, Brainerd et al., 1993; *Siren lacertina*, Brainerd and Monroy, 1998; *Amphiuma tridactylum* and *Cryptobranchus alleganiensis*, Brainerd, 1997; larval *A. tigrinum*, Brainerd, 1998). Similarly, terrestrial frogs, toads and caecilians exhale passively (*Rana* spp., de Jongh and Gans, 1969; West and Jones, 1974; *Bufo marinus*, Jones, 1976; MacIntyre and Toews, 1976; *Dermophis mexicanus*, Carrier and Wake, 1995; Bennett et al., 1999). Why does lung ventilation in the aquatic environment require active exhalation?

We believe that the answer may lie in the difference in tidal volume between aquatic and terrestrial breaths. In adult tiger salamanders, aquatic gulping breaths occur much less frequently than terrestrial breaths, and the tidal volume of the aquatic breaths is much larger. In aquatic breaths, the lungs are almost completely emptied and refilled, whereas in terrestrial breaths, only a small fraction of the lung volume is exchanged (compare Figs 5 with 8). We have observed small tidal volumes in other

post-metamorphic terrestrial salamanders (*Dicamptodon tenebrosus* and *Salamandra salamandra*), and frogs and toads also exhibit small tidal volumes (*Rana* spp., de Jongh and Gans, 1969; West and Jones, 1974; *Bufo marinus*, Jones, 1976; MacIntyre and Toews, 1976). In contrast, aquatic salamanders, including larval *A. tigrinum*, utilize large tidal volumes; X-ray video recordings reveal that they empty their lungs almost completely with each breath (*Necturus maculosus*, Brainerd et al., 1993; *Siren lacertina*, Brainerd and Monroy, 1998; larval *A. tigrinum*, Brainerd, 1998; *Amphiuma tridactylum* and *Cryptobranchus alleganiensis*, Brainerd, 1999).

We conclude that exhalation is passive in terrestrial breaths because the volume of air exhaled is small, and lung and body wall elasticity are sufficient to produce this small-volume exhalation. In aquatic breaths, however, hypaxial muscle activity is required to empty the lungs to produce a breath with a large tidal volume. This conclusion is supported by results reported previously for an aquatic salamander, *Siren lacertina* (Brainerd and Monroy, 1998). In *S. lacertina*, the first part of exhalation is passive, and the TA then becomes active at the end of exhalation to force the remainder of the air out of the lungs.

Comparison of larval and adult gulping breaths

A typical aquatic, gulping breath for adult *Ambystoma tigrinum* resembles the breathing mechanics of aquatic larval *A. tigrinum* (Brainerd, 1998). Larval and adult gulping breaths are similar in that both larvae and adults swim to the surface, open their mouths, and ventilate their lungs using a two-stroke buccal pump (Figs 1, 8). Given this overall similarity, we conclude that the adult gulping breaths probably represent a retention of the larval breathing behavior across metamorphosis. Adult gulping breaths do, however, differ in several respects from larval breathing patterns. These differences, discussed below, indicate that this behavior is not simply retained, but is also modified by metamorphosis.

Adult tiger salamanders usually exhale some or all of the air from their lungs before reaching the surface (Fig. 11), whereas larvae rarely do this. This 'early' exhalation may increase the efficiency of breathing because the percentage of exhaled air mixing with fresh air during the subsequent two-stroke breath is reduced. The volume of this early exhalation is variable, and therefore the volume of air in the lungs prior to the exhalation phase of the subsequent two-stroke breath (Fig. 10, phase 1) is also variable. This variability in lung volume at the start of each aquatic gulp may be responsible for some of the variability in amplitudes and durations of pressure changes during aquatic gulps (Tables 1–3).

In the two-stroke portion of the adult gulping breaths, exhalation takes place slightly later than in the larval two-stroke breaths. In the adults, exhalation takes place predominantly after the mouth has been closed (Fig. 10, phase 1), whereas in the larvae, exhalation occurs while the mouth is still open (Brainerd, 1998). In the adults, mouth gape during inspiration is short (62 ± 47 ms), and the gape is not wide, compared with the larval gape (compare Fig. 8 in the present

study with Fig. 1 in Brainerd, 1998). Furthermore, among the adults, the width of the gape and the timing of mouth closing are more variable than in the larvae.

The shapes of the buccal pressure traces are similar in larval and adult gulping breaths, with the exception of the pressure drop associated with buccal inspiration (Figs 9, 11, point 2). In larval tiger salamanders, buccal pressure drops slowly and then rises suddenly to atmospheric pressure, presumably as the seal of the mouth is broken (Brainerd, 1998). In adult gulping breaths, buccal pressure drops much more rapidly and then rises slowly towards atmospheric pressure, presumably as air is drawn in through the higher resistance posed by the narrower gape of the adults (compare Fig. 9 in the present study with Fig. 6 in Brainerd, 1998).

During a gulping breath, the mean body pressure change observed for adult salamanders (0.399 ± 0.49 kPa, mean \pm S.D.; $N=6$; Table 1) is similar to the mean pressure change observed in larval breathing (0.44 ± 0.09 kPa, mean \pm S.D.; $N=6$; Brainerd, 1998). While the means are similar, the standard deviation of the mean is much greater in the adult salamanders. This interindividual variation in body pressure is consistent with the interindividual variation in the onset and duration of hypaxial muscle activity (Fig. 10; Table 3). A comparison of larval and adult gulping electromyographic activity during breathing reveals that, although both larval and adult breaths exhibit a burst of hypaxial activity associated with expiration at the beginning of the breath, the adults frequently show two additional bursts of activity during the breathing cycle (discussed above; Figs 9, 10; Table 3).

Interindividual variation

The general ventilatory mechanics are described for representative breaths (e.g. Figs 6, 9). However, we documented variations in pressure and in the occurrence of EMG activity and its timing among and within individuals (Tables 1–3; Fig. 10). The variation observed in the breathing mechanics of adult *Ambystoma tigrinum* may reflect the truly amphibious nature of its lifestyle. Moreover, this interindividual variation in breathing is consistent with variation documented for other complex behavior patterns such as breathing and feeding among amphibians (Brainerd, 1998; Shaffer and Lauder, 1985).

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