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Lung ventilation contributes to vertical lymph movement in anurans

Michael S. Hedrick[1,*], Robert C. Drewes[2], Stanley S. Hillman[3] and Philip C. Withers[4]

[1]Department of Biological Sciences, California State University, East Bay, Hayward, CA 94542, USA, [2]Department of Herpetology, California Academy of Sciences, San Francisco, CA 94103, USA, [3]Department of Biology, Portland State University, Portland, OR 97207, USA and [4]Zoology, School of Animal Biology M092, University of Western Australia, Crawley, Western Australia 6009, Australia

*Author for correspondence (e-mail: michael.hedrick@csueastbay.edu)

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Summary

Anurans (frogs and toads) generate lymphatic fluid at 10 times the rate in mammals, largely as a consequence of their very 'leaky' vasculature and high interstitial compliance. Lymph is ultimately pumped into the venous system by paired, dorsally located lymph hearts. At present, it is unclear how lymphatic fluid that accumulates in central body subcutaneous lymph sacs is moved to the anterior and posterior lymph hearts in the axillary regions and how lymph is moved, against gravity, to the dorsally located lymph hearts. In this study, we tested the hypothesis that lung ventilation, through its consequent effects on lymph sac pressure, contributes to the vertical movement of lymphatic fluid in the cane toad (*Chaunus marinus*) and the North American bullfrog (*Lithobates catesbeiana*). We measured pressure in the dorsal, lateral and subvertebral lymph sacs of anesthetized cane toads and bullfrogs during artificial lung inflation and deflation. We also measured pressure in the subvertebral lymph sac, which adheres to the dorsal surface of the lungs, simultaneously with brachial (forelimb) and pubic (posterior) sac pressure during ventilation in freely behaving animals. There were highly significant (P<0.001) relationships between lung pressure and lymph sac pressures (r^2=0.19–0.72), indicating that pulmonary pressure is transmitted to the highly compliant lymph sacs that surround the lungs. Subvertebral sac pressure of resting animals was not significantly different between *L. catesbeiana* (518±282 Pa) and *C. marinus* (459±111 Pa). Brachial sac compliance (ml kPa^{-1} kg^{-1}) also did not differ between the two species (33.6±5.0 in *L. catesbeiana* and 37.0±9.4 in *C. marinus*). During expiration (lung deflation), reductions in expanding subvertebral sac pressure are communicated to the brachial lymph sac. Changes in brachial and pubic lymph sac pressures were correlated almost entirely during expiration rather than inspiration. The change in brachial sac pressure during expiration was 235±43 Pa for *C. marinus* and 215±50 Pa for *L. catesbeiana*, which is of sufficient magnitude to move lymph the estimated 0.5–1.0 cm vertical distance from the forelimb to the vicinity of the anterior lymph hearts. We suggest that lymph is moved during expiration to the subvertebral sac from anterior and posterior lymph sacs. During lung inflation, increased lymph sac pressure moves lymph to axillary regions, where lymph hearts can return lymph to the vascular space. Consequently, pulmonary ventilation has an important role for lymph movement and, hence, blood volume regulation in anurans.

Key words: *Chaunus marinus*, *Lithobates catesbeiana*, blood volume, respiration, lymph sac, lymph heart.

Introduction

Pulmonary ventilation in vertebrate animals is usually only considered as a means of establishing homeostatic gas exchange. Anurans (frogs and toads) are an enigma from a respiratory perspective because their ventilation is only loosely coupled to blood oxygen partial pressure (P_{O_2}) and carbon dioxide partial pressure (P_{CO_2}) (Coelho and Smatresk, 2003; Gargaglioni and Milsom, 2007) whereas in other vertebrate classes it is tightly coupled.

Anurans produce lymph at about 10 times the rate of mammals (Jones et al., 1997), so mobilization of lymph by lymph hearts is central to the maintenance of blood volume (Hillman, 1987; Hillman and Withers, 1988). In support of this hypothesis, lymph heart ablation or stoppage results in an inability to regulate blood volume and causes death within a few days (Zwemer and Foglia, 1943; Baustian, 1988; Baldwin et al., 1993).

Lymph moves through numerous subcutaneous lymph sacs in anurans (Kampmeier, 1969; Carter, 1979). A basic problem is how lymphatic fluid, which would normally pool in the dependent reaches of the limbs and compliant ventral lymphatic sacs, can then be moved vertically, against gravity, to the dorsally located lymph hearts in the low-pressure lymphatic system (Hillman et al., 2004; Hillman et al., 2005). Because anuran lungs are large and occupy a central location in the abdominal cavity, we hypothesize that changes in lung pressure during ventilation should be transmitted to the surrounding lymph sacs, including the dorsal, ventral, lateral and

subvertebral sacs (see Carter, 1979). The subvertebral lymph sac is particularly significant because it is directly connected to the brachial (forelimb) sac *via* a one-way valve that allows fluid to move in the ventral to dorsal direction. It adheres directly to the dorsal surface of the lungs and is likely to have its volume, and hence intrasac pressure, directly changed by lung ventilation cycles. The subvertebral sac is also directly connected posteriorly with the pubic sac, which is located at the end of the urostyle near the posterior pair of lymph hearts (Hillman et al., 2004).

In a companion study (Drewes et al., 2007), we proposed a role for cutaneous skeletal muscle insertion in the axillary hindlimb region contributing to vertical lymph movement in *Chaunus marinus* and *Lithobates catesbeiana* (see Frost et al., 2006). In the present study, we examine the role of pulmonary ventilation as a mechanism that contributes to the vertical movement of lymph in anurans. We investigated this problem of vertical movement of lymph and the role of pulmonary ventilation in two ways. First, we examined the relationship between lung pressure and lymph sac pressures to determine if pulmonary pressure is transmitted to the highly compliant lymph sacs. We then measured pressures in the subvertebral lymph sac simultaneously with brachial or pubic sacs in freely behaving animals to determine if pressure changes in these sacs could contribute to lymph movement. These data have been previously published in abstract form (Hedrick et al., 2006).

Materials and methods

Chaunus marinus (*N*=16; 125–260 g, mean 195 g) and *Lithobates catesbeiana* (*N*=10; 195–310 g, mean 247 g) were used in these experiments. Animals were obtained from commercial suppliers (Charles Sullivan, Nashville, TN, USA and Damon Corrie, Wildey, St Michael, Barbados). All experiments were conducted at 21–23°C under IACUC protocols approved at California State University, East Bay and Portland State University.

The first series of experiments (*N*=6 *C. marinus*; *N*=5 *L. catesbeiana*) was done with animals under MS-222 (tricaine methanesulfonate; Sigma, St Louis, MO, USA) anesthesia. The lungs were cannulated *via* the glottis with a 20 cm length of large-bore vinyl tubing for changing lung volume and recording lung pressure. The glottis was sutured closed around the cannula to prevent gas from escaping during volume changes. The dorsal, lateral and subvertebral lymph sacs were cannulated with saline-filled PE 10 tubing (i.d.=0.28 mm, o.d.=0.61 mm) for measurement of lymph sac pressures (see Hillman et al., 2004). At the end of the experiment, the anesthetized animals were euthanized by double pithing.

In the second series of experiments (*N*=10 *C. marinus*; *N*=5 *L. catesbeiana*), animals were anesthetized with MS-222 for implantation of cannulae for measurement of lymph sac pressures. Subvertebral sac pressures were measured with a micro-tip pressure transducer (SPR-249 and SPC 330; Millar, Houston, TX, USA) that was inserted into the subvertebral lymph sac *via* a small incision in the body wall. In five of the 10 *C. marinus*, a PE 10 saline-filled cannula was also inserted into the brachial sac *via* an 18 g needle and connected to a Statham P23db pressure transducer (Hato Rey, Puerto Rico, USA). In the remaining five *C. marinus*, pressure in the pubic lymph sac was measured with a saline-filled PE 10 cannula inserted directly into the pubic sac *via* an 18 g needle. Calibration of the cannulae was *via* a static water column. Following surgery, animals were allowed to recover with access to water 12–24 h prior to experiments. Following the recovery period, animals were placed into plastic containers for pressure measurements under quiet, unrestrained conditions for 8–12 h. Pressure recordings were acquired at 10 Hz using an A-D converter (Powerlab, Milford, MA, USA) and stored on a computer for off-line analysis. The presence of one-way valves between the brachial sac and the anterior portion of the subvertebral sac was determined by dissection. We estimated the distance from the bottom of the foot (lowest point of the brachial sac) to the valve separating the brachial and subvertebral lymph sacs (in direct communication with anterior lymph hearts) by dissection. Although this distance changes with posture and body size, it is approximately 0.5–1.0 cm, equivalent to 50–100 Pa gravitational pressure. The subvertebral sac also has a direct connection with the pubic sac posteriorly through small fenestrations that couple the two sacs.

The compliance of the brachial sac was measured in the second group of animals (*N*=5) following completion of the lymph sac pressure measurements. Compliance measurements were done using methods described previously (Hillman et al., 2005). Briefly, physiological saline (0.8% NaCl) was infused into the isolated brachial sac while measuring the resulting change in sac pressure. Saline was infused at a rate of 1% of body mass per minute, and the slope of the change in pressure was used to calculate lymph sac compliance.

The relationship between lymph sac pressures and lung pressure was quantified by linear regression analysis. Interspecific comparisons for measured variables were analyzed with an unpaired *t*-test. All statistical tests were done with GraphPad Prism (v. 5.0; San Diego, CA, USA).

Results

For anesthetized *C. marinus* and *L. catesbeiana*, changes in lung pressure were significantly correlated with pressures in the dorsal, lateral and subvertebral lymph sacs (Fig. 1) ($P<0.001$; $r^2=0.19–0.72$), indicating that pulmonary pressure was transmitted directly to the surrounding lymph sacs.

The subvertebral sac was confirmed to communicate directly with the ventral brachial (forelimb) sac through a one-way valve that allows lymph to flow from the brachial to the subvertebral sac. The subvertebral sac also communicates directly with both the anterior and posterior lymph hearts. Subvertebral lymph sac pressures were measured simultaneously with brachial lymph sac pressures in freely behaving *C. marinus* and *L. catesbeiana* to determine the relationship with lung ventilation, its associated effects on subvertebral lymph sac pressure, brachial pressure and pubic sac pressure. Mean subvertebral sac pressure was 459±111 Pa in *C. marinus* and 518±282 Pa in *L. catesbeiana* ($P>0.05$). Mass-specific compliance of the brachial lymph sac did not differ between the two species and was 37.0±9.4 ml kPa^{-1} kg^{-1} in *C. marinus* and 33.6±5.0 ml kPa^{-1} kg^{-1} in *L. catesbeiana* ($P>0.05$).

Simultaneous measurements of subvertebral sac pressure and brachial sac pressure revealed changes in subvertebral sac

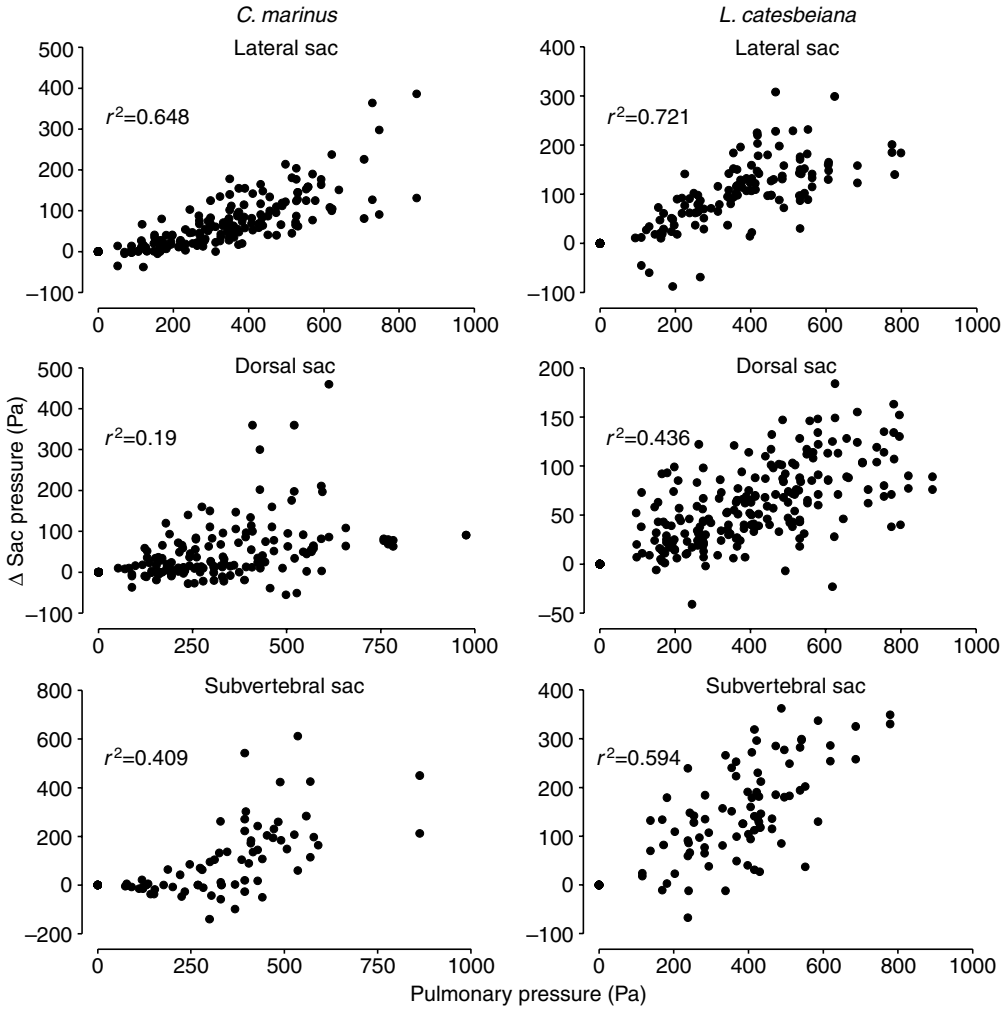

Fig. 1. Influence of pulmonary pressure on lymph sac pressures. Changes in dorsal, lateral and subvertebral lymph sac pressure as a function of pulmonary pressure in anesthetized *C. marinus* (left column) and *L. catesbeiana* (right column). All relationships were highly significant (P<0.001, N=11; linear regression) with r^2 ranging from 0.19 to 0.72.

pressure resulting from lung ventilation (e.g. Fig. 2). Subvertebral sac pressure normally increased during lung inflation (inspiration) and decreased during lung deflation (expiration), suggesting an increase in subvertebral lymph sac volume during expiration. During expiratory events, rapid changes in brachial pressure occurred (Fig. 2, Fig. 3A). Brachial pressure events were complex, with both increases and decreases observed; however, these changes occurred almost exclusively during expiration. Changes in subvertebral sac pressure during expiration were also associated with rapid reductions in pressure in the posterior pubic lymph sac (Fig. 3B). Thus, expiration, through its effects on subvertebral sac pressure, affected both anterior (brachial) and posterior (pubic) lymph sac pressures during expiration.

In *C. marinus*, rapid changes in brachial sac pressure occurred during expiration 96.4±0.1% of the time compared with inspiration (P<0.001, N=10), and rapid changes in pubic sac pressure occurred 98.8±0.01% of the time during expiration compared with during inspiration (P<0.001, N=10). Fig. 4 depicts the combined data for brachial and pubic sac pressure changes during inspiration and expiration for *C. marinus*. For *L. catesbeiana*, rapid changes in brachial sac pressure occurred during expiration 99.3±0.01% of the time compared with during inspiration (P<0.001, N=5).

Changes in subvertebral sac pressure during expiration were clearly transmitted to the brachial sacs. The changes in subvertebral sac pressure were predominantly negative pressures, consistent with the hypothesis that during expiration the subvertebral sac volume increased, resulting in negative pressures. The magnitude of pressure change in the subvertebral sac was higher than those in the brachial sac. The absolute change in brachial sac pressure (i.e. positive or negative) during expiration averaged 235±43 Pa in *C. marinus* and 215±50 Pa in *L. catesbeiana* (P>0.05, N=8) (Fig. 5). The changes in brachial sac pressure that occur during expiration in both species are of sufficient magnitude to move lymph the estimated 50–100 Pa against gravity to the dorsally located lymph hearts (Fig. 5, shaded box).

Discussion

These data support the hypothesis that lung ventilation, through its effects on subvertebral and brachial lymph sac pressure, is linked to the vertical movement of lymph in the anterior region of *C. marinus* and *L. catesbeiana*. We hypothesized that lung deflation increased subvertebral sac volume, resulting in a rapid reduction in subvertebral sac pressure sufficient to overcome the gravitational force to aspirate lymph from the ventral reaches of the forelimb to the

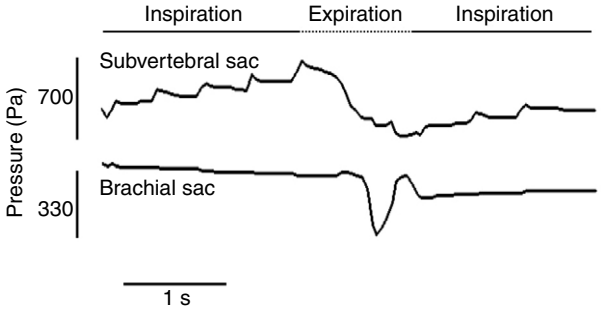

Fig. 2. Subvertebral and brachial sac pressures measured for one *C. marinus* during a single breath. Inspiration (solid horizontal line) and expiration (broken horizontal line) are indicated above the subvertebral pressure trace. Note that subvertebral sac pressure (top trace) increases during inspiration with little or no change in brachial sac pressure (bottom trace). During expiration there is a decrease in subvertebral sac pressure that is rapidly transmitted to the brachial sac.

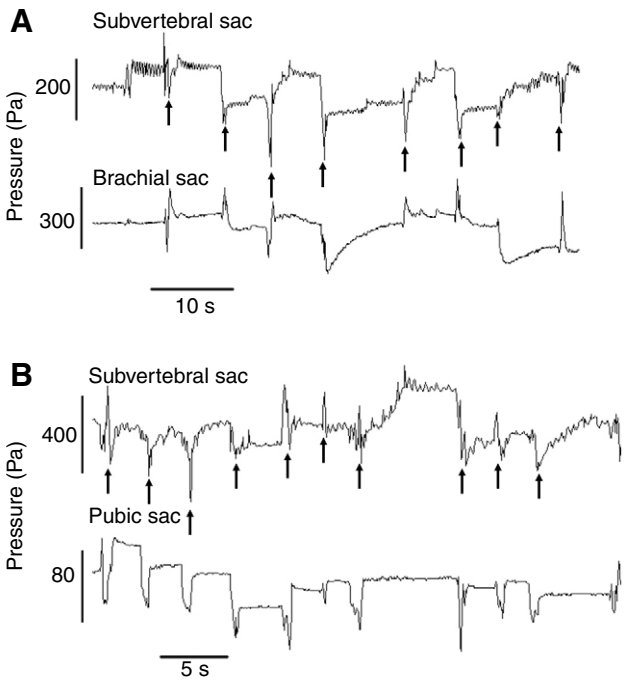

Fig. 3. (A) Subvertebral sac pressure and brachial sac pressure during a breathing sequence in one *C. marinus*. Arrows indicate expiration events recorded in the subvertebral trace; note that rapid increases and decreases in brachial lymph sac pressure occur, but are predominantly during expiration. (B) Subvertebral sac pressure and pubic sac pressure during a breathing sequence in one *C. marinus*. Arrows indicate expiration events recorded in the subvertebral trace; note the rapid decreases in pubic sac pressure that occur during expiration.

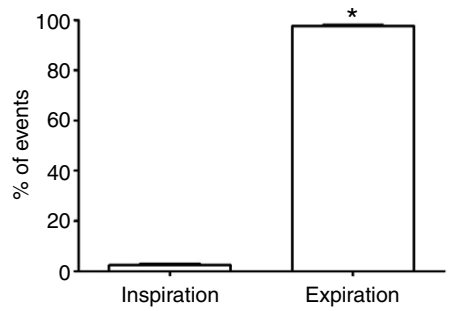

Fig. 4. Frequency of occurrence (% of events) of combined brachial and pubic sac pressure events with inspiration and expiration in *C. marinus*. Brachial and pubic sac pressure events occurred much more frequently during expiration than during inspiration (*P<0.001; unpaired t-test). Values are means ± s.e.m. (N=10)

axillary region, where the anterior lymph hearts are located. These pressures were transmitted to the forelimb brachial lymph sacs and were of sufficient magnitude to move lymph the required vertical distance (0.5–1 cm) to reach the subvertebral sac, where lymph can be directed to the anterior lymph hearts. Subvertebral pressures were also associated with reductions in the posterior pubic lymph sac, which is directly connected to the subvertebral sac. We have demonstrated that there is coordination between activity of a number of skeletal muscles and lymph sacs, including the pubic sac, that are responsible for moving lymph in the posterior region of anurans (Drewes et al., 2007). This suggests that lung ventilation, particularly expiration, is also coordinated with skeletal muscle and lymph sac pressure changes to move lymph in both the anterior and posterior region of anurans. A model depicting this hypothesis is presented in Fig. 6A. In support of this hypothesis, we confirmed that changes in brachial and pubic sac pressure associated with respiration occurred almost entirely during expiration rather than inspiration. We also propose that increased lung pressure during inspiration is transmitted to the surrounding lymph sacs and would force the accumulated lymph both anteriorly and posteriorly towards the lymph hearts (Fig. 6B). The latter hypothesis is supported by the observation that changes in lung pressure in anesthetized animals are transmitted to the highly compliant lymph sacs surrounding the lungs.

Anurans ventilate their lungs with a positive pressure pump mechanism unlike the negative-pressure aspiratory pump characteristic of reptiles, birds and mammals. Expiration by anurans is considered to be passive, relying on elastic recoil of the lungs to generate air flow out of the lungs. However, recent findings indicate that anurans use a variety of different breath types, including both inflation and deflation breaths (see Gargaglioni and Milsom, 2007). Our study would suggest that deflation breaths in anurans are associated with the functional movement of lymph. In our view, an important role for expiration in anurans, in addition to gas exchange, is to expand the subvertebral sac and aspirate lymph from ventral forelimb areas up to the dorsal lymph hearts. This may explain why lung ventilation is poorly correlated with changes in blood gases in anurans. For example, lung ventilation episodes occur in bullfrogs despite unidirectional ventilation that eliminates oscillations in blood gases (Kinkead and Milsom, 1994). In addition, minimally instrumented *Bufo* (=*Chaunus*) *marinus* exhibit very long apneas (up to 8 h) despite very low arterial P_{O_2} and stable arterial P_{CO_2} levels (Coelho and Smatresk, 2003).

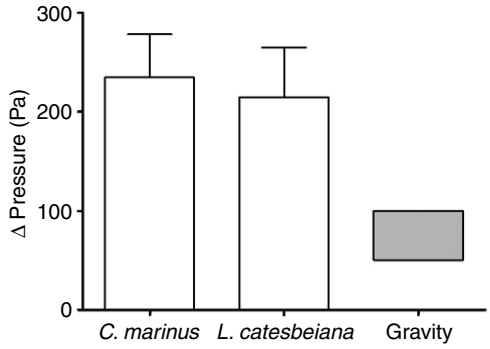

Fig. 5. Pressure changes (Pa) that develop in the brachial lymphatic sac during expiration in *C. marinus* and *L. catesbeiana* (means ± s.e.m.) compared with the estimated range of 0.5–1.0 cm (50–100 Pa; shaded box) of gravitational pressure necessary to raise lymph to the subvertebral lymph sac. Values were not different between the two species (P>0.05).

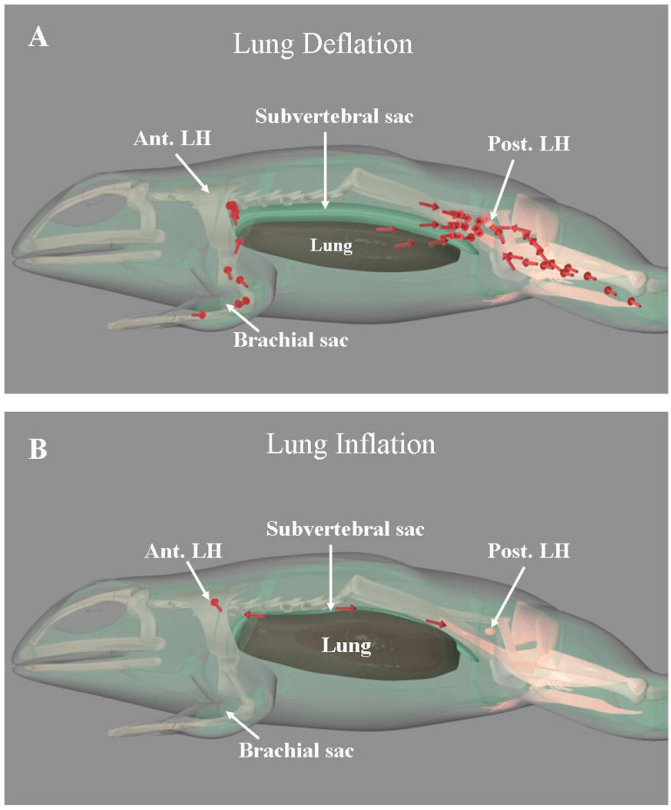

Fig. 6. Stylized models of *C. marinus* illustrating the hypothesis for the effects of lung ventilation on vertical lymph movement. (A) Lung deflation causes an increase in subvertebral lymph sac volume and a decrease in pressure, resulting in movement of lymph from the brachial lymph sac. Posterior skeletal muscles are also active during expiration, providing movement of lymph vertically in the posterior portion of the animal. Red arrows indicate the direction of lymph movement. (B) Lung inflation increases the pressure within the subvertebral, dorsal and lateral lymph sacs and forces lymph towards the anterior (Ant. LH) and posterior (Post. LH) lymph hearts. Red arrows indicate the direction of lymph movement.

We have shown that a number of skeletal muscles compress lymph sacs in the posterior region of anurans to move lymph dorsally (Drewes et al., 2007). The contraction of these muscles and lymph sacs is highly coordinated. In the present study, we showed that the posterior pubic sac pressure is also coordinated with expiration (Fig. 3B). It could be argued that skeletal muscles in the anterior region also compress lymph sacs and move lymph dorsally; however, this does not appear to be the case. First, unlike the posterior region of the animal, there are few muscles in the anterior region that insert on skin or are associated with lymph sacs. One muscle that does fit that description is the M. cutaneous pectoris, which is present in *L. catesbeiana* but absent in *C. marinus*. Contraction of the M. cutaneous pectoris should pull the central portion of the pectoral lymph septum, simultaneously affecting pressures in the pectoral and abdominal lymph sacs (Drewes et al., 2007). Because both species show changes in brachial sac pressure coincident with subvertebral sac changes, and although *C. marinus* lacks the M. cutaneous pectoris it has similar changes in lymph sac pressures during ventilation, it seems unlikely that skeletal muscles play a significant role in the movement of lymph in the anterior region of anurans, suggesting that lung ventilation is the primary mechanism for the movement of lymph in the anterior region. It is also unlikely that a 'compliance pump' mechanism (see Hillman et al., 2005) could account for the vertical movement of lymph from the forelimb to the subvertebral sac. With a brachial sac compliance of approximately 35 ml kPa^{-1} kg^{-1} (both species), it would require approximately 3.5 ml/limb to move lymph the estimated 1 cm (100 Pa) distance from the forelimb to the valve separating the brachial and subvertebral sacs *via* a compliance mechanism. There is no evidence that volumes of this magnitude are present in the brachial forelimb sacs of *C. marinus* or *L. catesbeiana*.

To our knowledge, subvertebral lymph sac pressures have never been measured in anurans prior to this study. Mean subvertebral sac pressures were approximately 450–500 Pa and did not differ between the two species, but are comparable to lung pressures that have been measured in anurans. Lung pressures range from 200 to 600 Pa during episodic ventilation in *Bufo* (*Chaunus*) *marinus* (Wang, 1994; Macintyre and Toews, 1976). Similar pressures have also been measured in *Rana* (*Lithobates*) *pipiens* (West and Jones, 1975; Vitalis and Shelton, 1990) and *Rana* (*Lithobates*) *catesbeiana* (Kinkead and Milsom, 1994). This suggests that the subvertebral sac, which adheres closely to the dorsal surface of the lung, may be a suitable location for measuring lung pressure in anurans. Lung ventilation cycles were clearly measurable from subvertebral lymph sac pressure in both species.

Our study suggests that lung ventilation in anurans is linked to blood volume status and blood pressure in anurans. Previous studies have shown that blood pressure status is directly linked to lymph heart function, but the effects of lung ventilation are unclear. Lymph hearts can be stopped by increasing blood pressure (Yamane, 1990; Crossley and Hillman, 1999) or by hypervolemia created by intravenous infusion of isotonic saline (Williams et al., 1998; DeGrauw and Hillman, 2004). Physiological doses of arginine vasotocin can increase lymph

heart pressure without changing lymph heart rate (DeGrauw and Hillman, 2004). However, stimulation of the recurrent laryngeal nerve, which causes lymph hearts to stop beating (Crossley and Hillman, 1999), also causes apnea at high electrical stimulation intensities (Van Vliet and West, 1986). Further experiments are needed to clarify the role of blood pressure and blood volume status on lung ventilation in anurans. In mammals, the link between lung ventilation and blood pressure is better defined, with hypotension causing a stimulation of lung ventilation and hypertension resulting in hypoventilation (Saupe et al., 1995; Wilson et al., 1998).

Given the apparent importance of the subvertebral sac and lung ventilation for movement of lymph in the anterior region of anurans, we would predict that 'lunglessness' is not a viable option for anurans, as it is for plethodontid urodeles and one species of caecilian, since lymph movement from the forelimb is dependent upon lung ventilation in anurans. We would also predict that ventilation should be more tightly coupled to blood volume status *via* baroreceptor input in anurans compared with urodeles. With a decline in blood volume and blood pressure, we would predict that the frequency of expiratory events should increase to assist the movement of lymph. These predictions await further experimentation.

In summary, we suggest that lung ventilation, through its effects on subvertebral lymph sac pressure, overcome the problem of moving lymph against gravity to the dorsally located lymph hearts. This unique function for the lungs as a mechanism to move lymph may explain why the control of ventilation is only loosely coupled to blood gas status and would implicate blood volume as another important input into the control of ventilation in anuran amphibians.

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