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Lymph flux rates from various lymph sacs in the cane toad *Rhinella marina*: an experimental evaluation of the roles of compliance, skeletal muscles and the lungs in the movement of lymph

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

[1]Department of Biology, Portland State University, Portland, OR 97207-0751, USA, [2]Department of Biological Sciences, California State University East Bay, Hayward, CA 94542, USA, [3]Department of Herpetology, California Academy of Sciences, San Francisco, CA 94118, USA and [4]Zoology, School of Animal Biology, University of Western Australia, Crawley, Western Australia, Australia 6009

*Author for correspondence (Hillmans@pdx.edu)

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SUMMARY

A new method for quantitatively determining lymph flux from various lymphatic sacs of an anuran, the cane toad, was developed. This method used the dye dilution principle of $C_iV_i=C_fV_f$ following injection of Evans Blue into specific lymph sacs and measuring its appearance in the venous circulation. The apparent lymph volume was 57 ml kg^{-1}. The greatest rate of lymph return (0.5–0.8 ml kg^{-1} min^{-1}) and best linear fit of Evans Blue appearance in the circulation with time followed injections into the subvertebral lymph sac, which has direct connections to both the anterior and posterior pairs of lymphatic hearts. Rate of lymph flux from the pair of posterior lymph hearts was three times greater than the anterior pair. Rates of lymph flux were only influenced by injection volume in the crural lymph sacs, implicating lymph sac compliance as the source of the pressure for lymph movement from these sacs. Femoral lymph sac fluxes were decreased by 60% following ablation of the tendons of the sphincter ani cloacalis, abdominal crenators and piriformis. This supports a role for these muscles in generating the pressure for vertical lymph movement. Femoral lymph sac fluxes were also decreased by 70% by the insertion of a coil in the subvertebral lymph sac, preventing normal compression and expansion of this sac by the lungs. This supports a role for lung ventilation in generating the pressure for vertical movement of lymph. Contrary to previous hypotheses, fluxes from the brachial sac were not influenced by insertion of the coil into the subvertebral sac. A haemorrhage equivalent to 50% of the blood volume did not change lymph flux rates from the femoral lymph sacs. These data provide the first experimental evidence that actual lymph fluxes in the cane toad *Rhinella marina* depend on lymph sac compliance, contraction of specific skeletal muscles and lung ventilation to move lymph laterally and vertically to the dorsally located lymphatic hearts.

Key words: Anurans, lymph flux, lymph sacs.

INTRODUCTION

Anurans have a high whole body interstitial compliance and a high transvascular filtration coefficient (Hancock et al., 2000; Hillman et al., 2010). These physical characteristics are essentially unique to anurans in comparison with all other vertebrates, and lead to both high rates of plasma turnover and an inability to mobilise interstitial fluid *via* a Starling-mediated transcapillary route (see Hillman et al., 2010). Lymphatic return to the venous circulation is the principal avenue for fluid balance in maintaining blood volume and circulatory function in anurans (Zwemer and Foglia, 1943; Baustian, 1988; Baldwin et al., 1993; Hillman, 1987; Hillman et al., 2009).

The anuran lymphatic system generally consists of both an anterior and posterior pair of lymph hearts, which generate sufficient pressures during contraction to move lymph from the heart to the venous circulation through the one-way valves that separate the heart and veins. These lymph hearts are dorsally located just below the suprascapular cartilage and lateral to the distal end of the urostyle. The iliac sac is located at the nexus of iliofibular, lateral, pubic, subvertebral lymph sacs. The lymph heart contractions occur as a consequence of stimulation *via* a cholinergic synapse (Greber and Schipp, 1986) from motor neurons driven by a central spinal motor centre (Flindt, 1966). Hormones can also modify the rate and strength of the lymph heart contractions (DeGrauw and Hillman, 2004). The lymph first moves through interconnected lymph sacs, separated from each other by connective tissue walls that have one-way valves (see Jolly, 1946). The anatomy of the lymph sacs can vary interspecifically and has been generally described by Carter (Carter, 1979). The uncertainty in understanding lymph movement in anurans lies in the identification of the source(s) of the forces that move lymph from both distal regions of the body as well as vertically to the dorsally located lymph hearts. The dorsal location of the hearts creates difficulties for lymph return, because it will tend to pool in ventral body areas due to gravity (Hillman et al., 2004). Given the high rates of lymphatic return necessary to maintain blood volume, identification and analysis of the forces involved in lymph movement from distal regions of the body where it is formed back to the lymph hearts is crucial to our understanding of anuran vascular fluid balance.

The rates of lymphatic flux have been estimated from a variety of perspectives: (1) the rate that plasma is replaced following haemorrhage (Baustian, 1988); (2) the rate that plasma is lost from the circulation following anaesthetic stoppage of lymph hearts (Baldwin et al., 1993); (3) the rate of plasma protein turnover (Hillman et al., 2010); and (4) stroke volume estimates of the posterior lymph hearts (Jones et al., 1997; Malvin et al., 1995). These anuran lymph flux rates range from 0.5 to 5.8 ml kg^{-1} min^{-1}, which is one order of magnitude greater than mammalian values for adult sheep of 0.08 ml kg^{-1} min^{-1} (Brace and Power, 1981).

We have previously proposed three mechanisms for generating the pressures required for lymph movement from distal body regions to lymph hearts: lymph sac compliance differences, pulmonary inspiration/expiration and skeletal muscle contraction. These sources of pressure are based on measurements of lymph sac compliance and estimates of rates of lymph formation (Hillman et al., 2005) and measurement of the correlation of skeletal muscle electromyographic (EMG) activity and ventilatory events with pressure changes in various lymph sacs (Hedrick et al., 2007; Drewes et al., 2007). These mechanisms contrast with the previous hypothesis that the lymph hearts themselves create the pressure differences necessary to move lymph (Baldwin et al., 1990; Toews and Wentzell, 1995).

There has been no direct measurement of rates of actual lymph flux back into the circulation from specific lymph sacs or measurement of how these rates of lymph flux into the circulation are modified by interfering with the proposed sources of energy for lymph movement to the lymph hearts. The objectives of this research were to: (1) describe the rates of lymph flux into the circulation from anterior and posterior body regions; (2) describe the effects of lowered blood volume on these fluxes; (3) test the hypothesis that skeletal muscles are involved in generating pressure differences in the lymph sacs by comparing rates of lymph flux following tendon ablation with control rates with intact tendons; and (4) test the lungs' role in compression and expansion of the subvertebral sac on lymph flux rates. The unique contributions of these experiments were to provide: quantitative estimates of lymph volume, quantitative estimates of lymph flux rates from anterior and posterior lymph hearts, a test of the relative contributions of parallel lymphatic pathways in the hindlimb, and the first experimental tests of hypotheses developed about the sources of energy (compliance, skeletal muscle contraction and lung emptying) on lymph flux rates.

MATERIALS AND METHODS

Experiments were conducted at 19–22°C on adult cane toads (114–418 g, mean 166) purchased from Florida commercial suppliers (Mark Lucas, Titusville, FL, USA). The cane toad previously known throughout most of the extant literature as *Bufo marinus*, was recently reclassified as *Chaunus marinus* (Frost et al., 2006) and most recently as *Rhinella marina* Linnaeus (Chapparo et al., 2007). Animals were maintained with continuous access to water at 20–25°C, except during the experiment. The animals were fed mealworms 2–3 times per week. All maintenance and experiments were conducted with approval of PSU-IACUC approvals psu01.02.12.1 and psu09.07.02.1.

Anaesthesia and surgeries

Animals were anaesthetised with a buffered ($NaHCO_3$) tricaine methane sulphonate solution (0.5%) until the corneal reflex was lost. A small incision (1 cm) was made along the ventral midline in both the skin and musculature to expose the ventral abdominal vein. A cannula of PE 90 Intramedic tubing (Becton Dickinson and Co., Sparks, MD, USA) filled with heparinised 0.8% saline was inserted in the vein, and the muscle and skin sutured separately with 4-0 silk. In some animals the insertional tendons of the piriformis (N=8) were cut, in others the origin tendons of the sphincter ani cloacalis and abdominal crenators (N=12) were cut, and in others all three sets of tendons were cut (N=13). In one group of animals (N=12) a polyethylene coil of 1.6 cm diameter and approximately 6 cm length constructed of 2 mm flexible tubing was inserted in the subvertebral lymph sac *via* a 0.2 cm hole, which was separately sutured closed. The coil created an air pocket and ventral depression within the subvertebral sac to interfere with lungs' ability to compress and expand this lymph sac. The animals were given a day to recover and experiments were performed once per day for the following 2–3 days post surgery. Consequently each individual participated in 2–3 different flux measurements. Sham surgery animals had identical incisions made in the skin and musculature as tendon-ablated (sham N=8) and coil-implanted animals (sham N=8), and the incisions closed with silk sutures. Because these sham surgery results were not different from control animals, they were considered part of the control group in subsequent analyses. Animals had access to water at all times except during the 20–30 min experimental period.

Experimental protocols

The general experimental design was based on the dilution principle of $C_1V_1=C_2V_2$ and $C_3V_3=C_4V_4$ in two steps. V_2 and C_2 were determined by injecting *via* a known volume (V_1) and concentration (C_1) of Evans Blue in a solution of 0.8 mg ml^{-1} of bovine serum albumin and 0.8% NaCl into the desired lymph sac of toads being anaesthetised (see below). From the relationship of $C_1V_1=C_2V_2$, the volume of lymph (V_2) in the sac was determined by measuring its Evans Blue concentration (C_2) by spectrophotometry at 512 nm. The mean concentration of lymph (C_2) was then used in the following calculation $C_3V_3=C_4V_4$ as C_3. The rate of lymph flow was determined by sampling venous blood (0.15 ml) from the implanted cannula every 5 min for 25–30 min, and determining the Evans Blue concentration of the plasma, which was separated from the blood cells by centrifugation. The slope of the linear regression of plasma Evans Blue concentration with time was C_4. V_4 was the plasma volume for this species [61 ml kg^{-1}; measured by Hillman et al. (Hillman et al., 2010)]. The V_3 was then the volume of lymph that entered the plasma from the lymph hearts per time.

The standard protocol was to inject *via* a syringe a volume of either 0.1% or 1% of body mass of stock Evans Blue solutions (either 5 or 0.5 mg ml^{-1}) into a selected lymph sac (Fig. 1). If the lymph sacs were bilateral (brachial, femoral, crural), half of the total volume was injected in each sac. This allowed us to vary the injection volume, yet keep a constant absolute quantity of Evans Blue injected. By varying the volume we were ostensibly varying the initial pressure in specific lymph sacs in proportion to their compliance (Hillman et al., 2005). Consequently if volume influenced the lymph flux rates, we would conclude that pressures resulting from compliance were important in moving lymph from that lymph sac. If a compliance effect was noted we could further validate a compliance driven mechanism by using additional Evans Blue solutions (1.66 or 0.83 mg ml^{-1}) where the volume injected was adjusted (0.3 and 0.6% body mass) to maintain a constant quantity of Evans Blue injected. This allowed us to determine whether there was a linear relationship of flux and pressure.

The volume of lymph present (V_2) and the mean Evans Blue concentration (C_2) following injection were determined after the lymph flux measurements were completed on an individual. A body-mass-adjusted volume of Evans Blue was injected into a specific lymph sac, and the animal was then placed into the anaesthetic solution. When anaesthetised (*ca*. 15 min) the animal was removed and lymph was sampled (100–200 μl) by capillary tube by opening specific lymph sacs (subvertebral or pubic) from the region closest to the lymph heart from the injection site. This allowed the determination of the lymph concentration (C_2 and C_3 of the calculations) at the midpoint of the 25–30 min Evans Blue sampling period for lymph flux measurements.

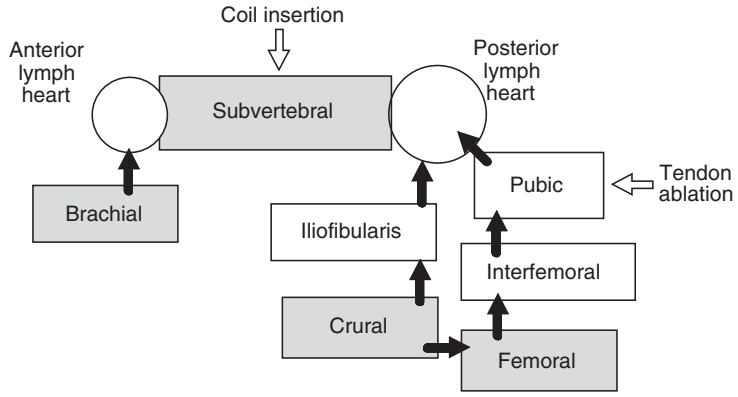

Fig. 1. A diagrammatic representation of the lymph sacs, lymph pathways and their positions in relation to the anterior and posterior lymph hearts. Shaded boxes indicate where Evans Blue injections were made.

Haemorrhage experiments

The animals used in the haemorrhage experiments had their urinary bladders drained and were bled from the ventral abdominal vein cannula a total volume equivalent to 3% of their bladder empty body mass. The 3% loss was achieved by three separate 1% body mass bleeds in succession, spaced 10 min apart. This was followed by injection of the Evans Blue into the femoral lymph sac and a standard experimental protocol followed.

Statistics

All calculations assumed an isometric relationship of the variable to body mass. For comparisons of two treatments two-tailed *t*-tests were utilised; for comparisons of three or more treatments one-way or two-way analyses of variance (ANOVA) were used. Linear regression was used when computing results with four or more measurements with time or pressure. Significance was set at a $P<0.05$.

RESULTS

Lymph dilution volumes

There were no significant differences between the calculated volume of lymph present with Evans Blue injected into the brachial sac with lymph collected from the subvertebral sac or Evans Blue injected into either the crural or femoral sacs with lymph collected from the pubic sac (Fig. 2). The mean lymph volume by dilution ($N=32$) from all three regional injections was $56.6\pm3.5\,ml\,kg^{-1}$ (±s.e.m.).

Lymph flux rates

Absolute lymph flux rates varied with lymph sac injection site ($P<0.0001$, two-way ANOVA). The linear regression with time of Evans Blue (mean $R^2=0.87$) and absolute lymph flux rates ($0.8\pm0.07\,ml\,kg^{-1}\,min^{-1}$) were greatest with Evans Blue injections into the subvertebral lymph sac compared with femoral ($P<0.001$), crural ($P<0.001$) and brachial lymph sacs ($P<0.0001$, Fig. 3). Injection volume did not influence lymph flux rates in any lymph sac, except in the crural lymph sac ($P<0.0013$). The appearance of Evans Blue varied linearly with time (mean $R^2=0.70$), and the absolute lymph flux rates were the lowest ($0.1\pm0.05\,ml\,kg^{-1}\,min^{-1}$) in the brachial sac. The appearance of Evans Blue following injection into the femoral lymph sac varied with time (mean $R^2=0.83$) and the lymph flux rates ($0.65\pm0.06\,ml\,kg^{-1}\,min^{-1}$), and were not different from the 1% crural lymph sac flux rates ($0.56\pm0.05\,ml\,kg^{-1}\,min^{-1}$) but were greater than the 0.1% crural lymph sac rates ($0.15\pm0.06\,ml\,kg^{-1}\,min^{-1}$). The Evans Blue appearance from crural lymph sac injections varied linearly with time (mean $R^2=0.73$). Lymph flux rates of the crural sacs were proportional to injection volumes, and hence pressure ($P<0.013$, Fig. 4).

A haemorrhage equivalent to 3% of the initial body mass had no effect ($P=0.46$, two-way ANOVA) on lymphatic flux rates from the femoral lymph sacs in either control or with the combinations of tendon ablation (Fig. 5); hence, all haemorrhage and control data were included in the subsequent analyses. Control rates of femoral lymph flux were $0.51\pm0.06\,ml\,kg^{-1}\,min^{-1}$. The ablation of bilateral insertion tendons of the piriformis on the urostyle had no effect on the mean rates of lymphatic return ($0.50\pm0.07\,ml\,kg^{-1}\,min^{-1}$). The same was true of simultaneous ablation of the origin tendons of the abdominal crenator and sphincter ani cloacalis on femoral lymphatic fluxes ($0.59\pm0.12\,ml\,kg^{-1}\,min^{-1}$). By contrast, ablation of the tendons from all three muscles significantly reduced lymphatic flux rates ($0.22\pm0.04\,ml\,kg^{-1}\,min^{-1}$) following femoral sac injections ($P<0.01$, Fig. 5). The insertion of flexible coils in the subvertebral lymph sac significantly reduced ($P<0.0004$, Fig. 6) femoral lymph flux rates from 0.54 ± 0.06 to $0.17\pm0.05\,ml\,kg^{-1}\,min^{-1}$ but not brachial sac fluxes.

DISCUSSION

The major difficulty in the application of Evans Blue injections into specific lymph sacs to achieve quantitative estimates of a lymphatic volume flux is the estimation of the actual lymphatic Evans Blue concentration that the lymph hearts pump. Previous attempts using Evans Blue dye dilution (Bonetto et al., 1981: Baldwin et al., 1990: Malvin et al., 1995) simply measured the change in plasma Evans Blue concentration with time. This allows a determination of a change in lymph flux but, without an estimate of lymphatic dilution and plasma volume, this cannot provide a quantitative estimate of the lymphatic flux. We used an estimate of $61\,ml\,kg^{-1}$ for plasma

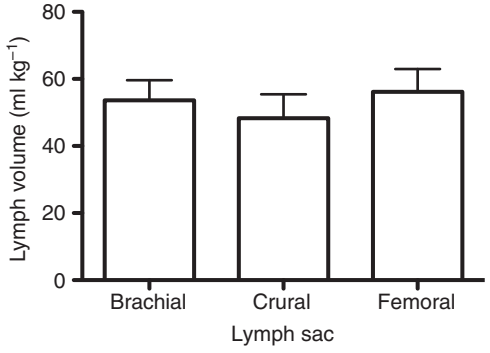

Fig. 2. The apparent lymph dilution volumes following injection into either the brachial sac collected from the subvertebral lymph sac ($N=12$) or the femoral ($N=14$) and crural ($N=7$) lymph sacs collected from the pubic lymph sac. Values are means and s.e.m.

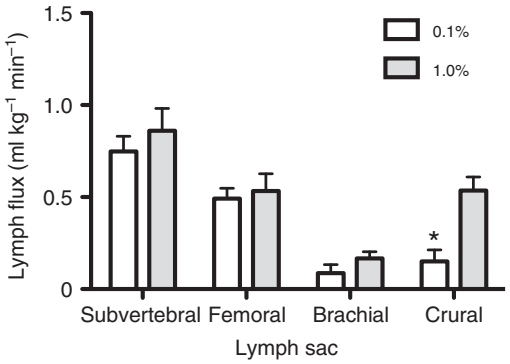

Fig. 3. The lymph flux rate estimates following Evans Blue injections of 0.1% and 1% body mass into specific lymph sacs. *N* was 16 for the subvertebral sac, 20 for the femoral sac, 16 for the brachial sac and 19 for the crural sac. Values are means and s.e.m. Asterisk indicates a significant effect of injection volume (P<0.013).

volume (Hillman et al., 2010). The apparent lymph dilution volume at 15 min (C_2) was used as the mean concentration of Evans Blue in the lymph. The 15 min time point was approximately the midpoint for the 25–30 min flux determinations.

The lymphatic flux estimates that we report here for subvertebral lymph sac injections (0.5–0.8 ml kg^{-1} min^{-1}) are within the range of previous estimates of lymph flux in toads [0.5 ml kg^{-1} min^{-1} (Baustian, 1988; Jones et al., 1997); 1.8 ml kg^{-1} min^{-1} (Hillman et al., 2010)]. Time accounted for 70–87% of the variance in the appearance of Evans Blue in the vasculature, with the highest correlations from the subvertebral sac injections central to both pairs of lymph hearts and the lowest correlation from the injection sites that were from the more peripheral lymph sacs (femoral, brachial and crural). Besides the obvious sources of error associated with measurement and dilution there are two other variables that would preclude absolute correlations with time. The first is that lymph heart rates can vary with time (Priestly, 1878a; Priestly, 1878b). The other is that once Evans Blue-labelled protein enters the circulation, it can escape from the circulatory system back into the lymphatic system. Both of these effects would lead to an underestimate of actual lymphatic fluxes. If we assume that variation in standard curves reflect measurement errors of about 1–2% of the variation with time, then the remaining 10–12% of the variation in subvertebral sac lymph flux with time reflects variation in lymph heart rate and escape of Evans Blue back into the lymphatic system during the measurement period. The potential error is small in proportion to the variance with time. We conclude that the Evans Blue dilution procedure we used provides a repeatable and reliable technique for measuring the rate of lymphatic flux into the circulation from specific lymph sacs in the toad, and the entry into the circulation with time is quite linear.

The apparent lymphatic volume was large (57 ml kg^{-1}) and independent of which lymph sac the Evans Blue was injected into (Fig. 2). This indicates that Evans Blue either equilibrates rapidly throughout the various lymphatic sacs or the lymph volume is proportionally equivalent in the subvertebral and femoral regions. It also means that only one dilution factor needs to be used in the calculation of absolute lymph flux rates following injection of Evans Blue into any lymph sac. The range of apparent lymph volume was large between individuals, 33–110 ml kg^{-1}, so using a mean lymph volume as we did, would give a wide range of lymph flux rates even if it was invariant. Consequently a significant source of the variation in the lymph flux rates that we report probably reflects inter-individual variation in lymphatic volume, rather than variation in lymphatic fluid mobilisation capacity.

Evans Blue injected into the subvertebral lymph sac has access to both the paired anterior and posterior lymph hearts (Baldwin et al., 1990). Injection of Evans Blue into the brachial sac would have more immediate access to the paired anterior lymph hearts, while injections into the crural and femoral lymph sacs would reach the paired posterior lymph hearts first. From this anatomical logic, subvertebral injections would represent the sum of all four hearts and define the maximal capacity for lymph return. In this regard lymph flux rates were the greatest and showed the best correlation with time following subvertebral sac injections. The combination of brachial and femoral flux rates account for 80% of the total subvertebral flux rates. The lymph flux rates from femoral (0.1 and 1%) and crural (1%) injections were three times greater than brachial injections. This indicates that return of lymph from the paired posterior lymph hearts is greater than the paired anterior lymph hearts. This may reflect the long-term lymphatic drainage demands of the hindlimbs, which are a much greater fraction of body mass

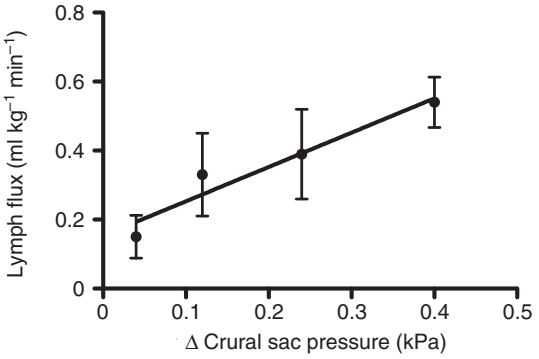

Fig. 4. The regression of pressure change in the crural sacs, calculated from their compliance and the volume injected, on the rate of lymph flux. Values are means and s.e.m.

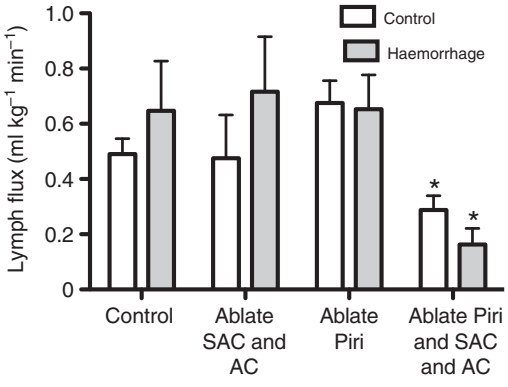

Fig. 5. Lymph flux rates from the femoral sac in control (*N*=20) following tendon ablation of the sphincter ani cloacalis (SAC) and abdominal crenators (AC, *N*=12) or piriformis (Piri, *N*=8) muscles or ablation of SAC, AC and Piri (*N*=13) in control states or following a haemorrhage of 50% of their blood volume. Values are means and s.e.m. There was no effect of haemorrhage in any treatment. Asterisks indicate significant differences from control and the two other ablation treatments (P<0.01).

than the forelimbs. Kampmeier reported that the anterior and posterior lymph hearts are the same size in toads (Kampmeier, 1969). As anterior lymph hearts beat at the same frequency as the posterior lymph hearts (Pratt and Reid, 1939), our data suggest that the stroke volumes of the anterior lymph hearts are lower than the posterior lymph hearts. This may result from contractility differences of the lymph heart musculature or the preload and afterload conditions that the lymph hearts experience. The anterior and posterior lymph hearts discharge into different veins separated by a portal system (anterior hearts into the anterior vertebral vein, and posterior hearts into the posterior vertebral vein). Consequently it is inappropriate to use the output of any one heart to estimate total lymphatic flux by direct proportionality.

The crural lymph sacs were the only injection sites where lymph flux measurements were significantly influenced by the injection volume (Fig. 3). Injection volume can be converted into pressure increases from the compliance of the crural sac [12 ml kg^{-1} kPa^{-1} (Hillman et al., 2005)] and amounts to a 360 Pa pressure difference between the smallest and largest injection volumes. The significant linear relationship of pressure (volume) and lymph flux rates (Fig. 3) indicates that the primary force moving lymph from the crural sac to the iliofibularis and femoral sacs is the result of the volume/pressure of lymphatic fluid contained within the crural sacs. This volume sensitivity of the crural injection site is coupled with the most complicated and distant pathway to the lymph heart. This may provide a possible explanation for why the crural sac flux measurements had a low correlation with time (R^2=0.73). The same variation in injection volumes would only represent a 60 Pa pressure difference in the femoral sac. Consequently the lack of effect of injection volume in the femoral sac is not unexpected as the pressure change is only 16% of the femoral sac change and is insufficient to move lymph vertically to the lymph hearts. The crural sac data support our compliance pump model for lymph movement resulting from lymph formation in lymph sacs compartmentalised into a series arrangement of increasing compliance (Hillman et al., 2005).

Measurements of lymphatic flux rates from the femoral and crural lymph sacs did not differ from one another at the highest (1%) injection volumes. The crural sac connects proximally with two sacs parallel to one another, the iliofibularis and femoral sac. The iliofibularis takes a direct route to the posterior lymph hearts *via* the iliac lymph sac while the femoral lymph sac traverses a more circuitous route *via* the interfemoral then the pubic lymph sac before reaching the posterior lymph hearts (see Fig. 1). However, the principal route of lymphatic return from the crural sac appears to be *via* the femoral sac route and not the iliofibularis sac. If the iliofibularis route was a significant pathway for lymph return to the posterior lymph hearts, then the crural injections should have exceeded the femoral injection measurements at the highest injection volumes because lymph can be transported there *via* two parallel pathways not just the femoral pathway.

Muscle tendon ablation indicates that lymph mobilisation from the femoral sacs is partially dependent upon the effective contractions of three muscles: the piriformis, the sphincter ani cloacalis, and the abdominal crenators. These results are consistent with the model proposed by Drewes et al. (Drewes et al., 2007), based on EMG and lymph sac pressure changes for synchronised contractions of these muscles providing part of the pressure necessary for moving lymph from the femoral sac into the interfemoral sac, then dorsally up the pubic sac to the posterior lymph hearts. The tendon ablations of the piriformis alone or both the sphincter ani cloacalis and abdominal crenators did not influence lymphatic mobilisation from the femoral sac, indicating there is some redundancy in the ability of the muscles to generate sufficient pressure to move lymph dorsally. It is also important to recognise the potential contributions of the lungs in moving lymph (see below).

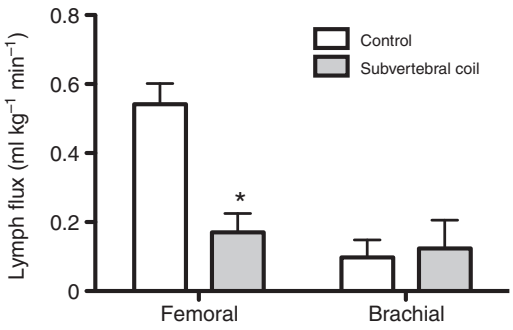

Fig. 6. Lymph flux rates from the femoral (N=18 control, N=13 coil) and brachial (N=8 control, N=5 coil) lymph sacs in control and following the insertion of a plastic tubing coil into the subvertebral sac. Values are means and s.e.m. Asterisk indicates a significant effect on femoral flux rates with coil insertion (P<0.0004).

Interestingly, subjecting the toads to a haemorrhage of 3% of their body mass (about 50% of their plasma volume) did not influence the lymphatic flux rates from the femoral lymph sacs. This volume is replaced within 20 min post-haemorrhage, determined both by volume replacement (Baustian, 1988) and the lack of compromise of maximal blood flow rates (Hillman and Withers, 1988). During this fluid mobilisation phase replacing the volume lost by haemorrhage, the rates of lymphatic fluid flux were the same as control. This indicates that the normal lymphatic lymph flux rates were not enhanced in response to lowered blood volume. A cautionary point should be raised concerning the haemorrhage results, as some lymph will have already been translocated from the lymph to plasma volume during the sequential haemorrhage. As a consequence the injection of Evans Blue into this lower lymph volume will give a higher lymph concentration of Evans Blue than the control value we used in the calculations. This variation would tend to overestimate the actual rates of lymph flux. Haemorrhage has been shown to increase lymph heart rate (Middler et al., 1968) but this conclusion was made from a comparison of control rates of 0 beats min^{-1}. Lymph heart rates decrease with dehydration (Jones et al., 1992) and in response to elevated blood pressures that result from blood volume expansions (Crossley and Hillman, 1999; DeGrauw and Hillman, 2004).

The coil implanted in the subvertebral sac compromised lymphatic flux from the femoral sac but not the brachial sac. This is partially in conflict with the model of Hedrick et al. (Hedrick et al., 2007); they postulated that the dorsal movement of lymph would be assisted by inflation and deflation of the subvertebral sac caused by pressure changes associated with expiration and inflation of the lungs. The movement of lymph from the brachial sac clearly warrants further study. Given its low compliance and complicated lymphatic pathways, it may rely on a combination of mechanisms for vertical lymph movement including activity. It is perhaps significant that the brachial sac fluxes had the lowest correlation with time. It may also be that position of the coil is crucial for demonstrating the importance of subvertebral sac volume changes in brachial lymph movement. The large effect of the coil on lymph movement from the femoral sac was unexpected. Anatomically the subvertebral sac is open to the dorsal part of the pubic and iliac lymph sacs (Baldwin et al., 1990), so pressure changes in the

subvertebral sac could be communicated to the pubic sac. In addition, pressure changes in the pubic sac are simultaneous with expiration (subvertebral sac expansion) and activity of the piriformis, sphincter ani cloacalis and abdominal crenators (Drewes et al., 2007; Hedrick et al., 2007). The combination of partial inhibition of lymphatic flux with both the subvertebral coil insertion and tendon ablation is strong experimental evidence that both mechanisms play a role in the vertical elevation of lymph from the hindlimbs. We find no support for the hypothesis that the lymph hearts themselves create the pressures necessary to move lymph vertically (Baldwin et al., 1990; Toews and Wentzell, 1995).

How do the estimates presented here compare with previous estimates of lymph flux and stroke volume? There are a variety of other data for toads with which our femoral flux data can be compared. The grand mean for femoral sac flux was $0.54\,ml\,kg^{-1}\,min^{-1}$. Assuming equal flow outputs from each posterior lymph heart ($0.27\,ml\,kg^{-1}\,min^{-1}$), and a lymph heart rate of $50\,beats\,min^{-1}$ (DeGrauw and Hillman, 2004), the stroke volume estimate for each lymph heart is approximately $0.005\,ml\,kg^{-1}\,beat^{-1}$. These estimates are essentially the same as reported by Parsons et al. (Parsons et al., 1994) of $0.27\,ml\,kg^{-1}\,min^{-1}$ but greater than the values reported by Jones et al. (Jones et al., 1992) of $0.1\,ml\,kg^{-1}\,min^{-1}$ and less than the values reported by Jones et al. (Jones et al., 1997) of $0.43\,ml\,kg^{-1}\,min^{-1}$ and Malvin et al. (Malvin et al., 1995) of $4\,ml\,kg^{-1}\,min^{-1}$. If the subvertebral sac injection data ($0.8\,ml\,kg^{-1}\,min^{-1}$) provide the most appropriate estimate of the total output of the four lymph hearts, then it is less than the rate ($1.8\,ml\,kg^{-1}\,min^{-1}$) calculated from Evans Blue loss from the plasma (Hillman et al., 2010) and the same as rates calculated from the replacement of plasma following haemorrhage (Baustian, 1988). Given the close correspondence of the current measurements with published estimates using other techniques, we feel these comparisons reinforce the validity of this technique for a quantitative measure of lymph flux.

The results indicate that a substantial dilution pool of fluid (lymph) exists in *R. marina*. About 1% of this volume is returned to the plasma space per minute. This rate of lymph flux is not influenced by a haemorrhagic 50% reduction of plasma volume from any of the studied lymph sacs. Lymph flux rates were greater from the posterior lymph hearts compared with the anterior lymph hearts. Ablation of the tendons of the skeletal muscles proposed for moving lymph dorsally by Drewes et al. (Drewes et al., 2007) decreases lymph return by about 60%. Insertion of a coil in the subvertebral lymph sac decreases lymph return from posterior body regions by about 70%, providing experimental support for the hypothesis developed by Hedrick et al. (Hedrick et al., 2007) that lung ventilation also contributes to vertical lymph movement. Finally, variation in injected volume only influences lymph movement from the crural lymph sac, providing the first experimental confirmation for lymph sac compliance playing a role in lymph movement from the hindlimbs (Hillman et al., 2005).

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REFERENCES

Baldwin, A. L., Rozum, J. S. and Gore, R. W. (1990). Routes of water and small solute transport between blood and lymph in frog. *Physiol. Zool.* **63**, 1141-1156.

Baldwin, A. L., Ferrer, P., Rozum, J. S. and Gore, R. W. (1993). Regulation of water balance between blood and lymph in the frog *Rana pipiens*. *Lymphology* **26**, 4-18.

Baustian, M. (1988). The contribution of lymphatic pathways during recovery from hemorrhage in the toad *Bufo marinus*. *Physiol. Zool.* **61**, 555-563.

Bonetto, R., del Castillo, E. and Uranga, J. (1981). Effect of glomerulopressin on the lymphatic hearts of the toad, probably mediated by prostaglandins. *Cell. mol. Life Sci.* **37**, 209-210.

Brace, R. A. and Power, G. G. (1981). Thoracic duct lymph flow and protein fluid dynamics: responses to intravascular saline. *Am. J. Physiol.* **247**, R800-R805.

Carter, D. B. (1979). Structure and function of the subcutaneous lymph sacs in anura (Amphibia). *J. Herpetol.* **13**, 321-327.

Chaparro, J. C., Pramuk, J. B. and Gluesenkamp, A. G. (2007). A new species of arboreal *Rhinella* (Anura: Bufonidae) from cloud forests of southeastern Peru. *Herpetologica* **63**, 203-212.

Crossley, D. A. and Hillman, S. S. (1999). The role of pulmocutaneous baroreceptors in the control of lymphatic heart rate in the toad *Bufo marinus*. *Physiol. Biochem. Zool.* **72**, 109-115.

DeGrauw, E. A. and Hillman, S. S. (2004). General function and endocrine control of the posterior lymph hearts in *Bufo marinus* and *Rana catesbeiana*. *Physiol. Biochem. Zool.* **77**, 594-600.

Drewes, R. C., Hedrick, M. S., Hillman, S. S. and Withers, P. C. (2007). Unique role of skeletal muscle contraction in vertical lymph movement in anurans. *J. Exp. Biol.* **210**, 3931-3939.

Flindt, R. (1966). Relative coordination der lympherzbewegungen bei Anuen. *Pflugers Arch.* **290**, 28-37.

Frost, D., Grant, T., Faivovich, J., Bain, R. H., Haas, A., Haddad, H. F. E., De Sa, R. O., Channing, A., Wilkinson, M., Donellan, S. C. et al. (2006). The amphibian tree of life. *Am. Mus. Nat. Hist.* **297**, 1-370.

Greber, K. and Schipp, R. (1986). Besonderheiten dur struktur und innervation des lympherzmyocards vo Anuren-eine vergleichende elektronenmikroskopische, histochemische und fluoreszenmikroskopische untersuchung. *Verh. Dtsch. Zool. Ges.* **79**, 75-82.

Hancock, T. V., Hoagland, T. M. and Hillman, S. S. (2000). Whole-body systemic transcapillary filtration rates, coefficients, and isogravimetric capillary pressures in *Bufo marinus* and *Rana catesbeiana*. *Physiol. Biochem. Zool.* **73**, 161-168.

Hedrick, M. S., Drewes, R. C., Hillman, S. S. and Withers, P. C. (2007). Lung ventilation contributes to vertical lymph movement in anurans. *J. Exp. Biol.* **210**, 3940-3945.

Hillman, S. S. (1987). Dehydrational effects on cardiovascular and metabolic capacity in two amphibians. *Physiol. Zool.* **60**, 608-613.

Hillman, S. S. and Withers, P. C. (1988). The hemodynamic consequences of hemorrhage and hypernatremia in two amphibians. *J. Comp. Physiol.* **157B**, 807-812.

Hillman, S. S., Hedrick, M. S., Withers, P. C. and Drewes, R. C. (2004). Lymph pools in the basement, sump pumps in the attic: the anuran dilemma for lymph movement. *Physiol. Biochem. Zool.* **77**, 161-173.

Hillman, S. S., Withers, P. C., Hedrick, M. S. and Drewes, R. C. (2005). Functional roles for the compartmentalization of the subcutaneous lymphatic sacs in anuran amphibians. *Physiol. Biochem. Zool.* **78**, 515-523.

Hillman, S. S., Withers, P. C., Drewes, R. C. and Hillyard, S. D. (2009). *Ecological and Environmental Physiology of the Amphibia*, 420 pp. Oxford: Oxford University Press.

Hillman, S. S., DeGrauw, E. A., Hoagland, T., Hancock, T. and Withers, P. C. (2010). The role of vascular and interstitial compliance and vascular volume in the regulation of blood volume in two species of anuran. *Physiol. Biochem. Zool.* **83**, 55-67.

Jolly, J. (1946). Recherches sur le systeme lymphatique des batrachiens. *Arch. Anat, Microscop. Morphol. Exp.* **36**, 3-44.

Jones, J. M., Wentzell, L. A. and Toews, D. P. (1992). Posterior lymph heart pressure and rate and lymph flow in the toad *Bufo marinus* in response to hydrated and dehydrated conditions. *J. Exp. Biol.* **169**, 207-220.

Jones, J. M., Gamperl, A. K., Farrell, A. K. and Toews, D. P. (1997). Direct measurement of flow from the posterior lymph hearts of hydrated and dehydrated toads (*Bufo marinus*). *J. Exp. Biol.* **200**, 1695-1702.

Kampmeier, O. F. (1969). *Evolution and Comparative Morphology of the Lymphatic System*. Springfield, IL, USA: Thomas.

Malvin, G. M., Macias, S., Sanchez, M., Dasalla, R., Park, A. and Duran, M. (1995). Lymphatic regulation of hematocrit during hypoxia in the toad *Bufo woodhousei*. *Am. J. Physiol.* **269**, R814-R821.

Middler, S. A., Kleeman, C. R. and Edwards, E. (1968). Lymph mobilization following acute blood loss in the toads, *Bufo marinus*. *Comp. Biochem. Physiol.* **24**, 343-353.

Parsons, G. G., Wentzell, L. A., Jones, J. M. and Toews, D. P. (1994). The role of arginine vasotocin in the control of posterior lymph heart function in the toad *Bufo marinus*. *Physiol. Zool.* **67**, 515-525.

Pratt, F. A. and Reid, M. A. (1939). Synchronism of anuran lymph hearts and the integration of their spinal centers. *J. Physiol.* **95**, 345-355.

Priestley, J. (1878a). An account of the anatomy and physiology of batrachian lymph-hearts. *J. Physiol.* **1**, 1-18.

Priestley, J. (1878b). Contributions to the physiology of batrachian lymph hearts. *J. Physiol.* **1**, 19-71.

Toews, D. P. and Wentzell, L. A. (1995). The role of the lymphatic system for water balance and acid–base regulation in the Amphibia. *Adv. Comp. Environ. Physiol.* **21**, 201-213.

Zwemer, R. L. and Foglia, U. G. (1943). Fatal loss of plasma after lymph heart destruction in toads. *Proc. Soc. Exp. Biol. Med.* **53**, 14-17.