

Functional Roles for the Compartmentalization of the Subcutaneous Lymphatic Sacs in Anuran Amphibians

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ABSTRACT

Compliance of the subcutaneous lymph sacs of the hindlimbs increases from distal to proximal, as does limb segment mass (and presumably rate of lymph formation), for the semiaquatic bullfrog *Rana catesbeiana* and the cane toad *Bufo marinus* but not the aquatic clawed toad *Xenopus laevis*. Subcutaneous lymph-sac compliances vary interspecifically. The distal-to-proximal increase in lymph-sac compliance and estimates of lymph formation rate in the various hindlimb segments indicate that partitioning of hindlimb subcutaneous lymphatic sacs establishes a differential decrease in the intra-lymph-sac pressure for *R. catesbeiana* and *B. marinus*. These pressure differentials constitute a “compliance pump” that drives distal-to-proximal intersac lymph flow. The compliance pump alone explains lymphatic return for the aquatic frog *X. laevis* but does not explain how lymph would reach the dorsally located lymph hearts for terrestrial anurans, so we hypothesize that skeletal muscle pumps return lymph from the femoral and pubic lymph sacs to the lymph heart. This is a fundamentally different role of the subcutaneous lymph-sac system than has been previously proposed. We suggest that the more proximal subcutaneous lymph sacs are important for fluid storage because they have a relatively high compliance.

Introduction

The anuran lymphatic system consists of numerous subcutaneous sacs that are separated and compartmentalized by connective tissue septa but are interconnected by one-way valves. These one-way valves are not simple passive flaps but have an associated musculature (Jolly 1946); increased tension of these muscles would presumably tighten the valve, which would increase pressure and retain fluid in the lymph sacs. The general arrangement of the various subcutaneous lymph sacs has been well described (Ecker 1881; Toews and Wentzell 1995) but does vary interspecifically (Carter 1979). Lymph-sac compartmentalization is thought to alleviate lymph pooling that would occur if the lymph space were a single sac (Toews and Wentzell 1995).

In anurans, lymph is generally returned to the venous circulation by two pairs of lymph hearts: the anterior pair lateral to the third vertebra under the suprascapular cartilage and the posterior pair located lateral to the urostyle at the anterior limit of the pubic sac. Some anurans, especially some ranid frogs, have more than one pair of posterior lymph hearts (Kampmeier 1969). The fundamental importance of anuran lymph hearts in returning lymph to the circulation has been experimentally documented by both ablation and anesthesia. If its lymph hearts are destroyed (cauterized), then a cane toad cannot compensate for hemorrhagic stress (Baustian 1988) and within a few days dies of hemoconcentration due to lost plasma (Zwemer and Foglia 1943). If lymph hearts are stopped by anesthesia in frogs, there is also a hemoconcentration resulting from filtered plasma not being returned to the circulation (Baldwin et al. 1993). All lymph flow must pass through the four lymph hearts, so their combined output is the total lymphatic flow. Recent estimates of flow for all four lymph hearts are about 0.9–5 mL kg⁻¹ min⁻¹ (Baustian 1988; Baldwin et al. 1993; Malvin et al. 1995; Jones et al. 1997). The magnitude of these fluxes is remarkable in comparison to mammalian values of 0.083 mL kg⁻¹ min⁻¹ (for adult sheep; Brace and Power 1981).

The current hypothesis for lymph movement in anuran amphibians is that “lymph is presumably redistributed within and passed through the lymph sacs by postural movements and by the continuous pumping of (and the probable slight negative pressure created by) the lymph hearts” and that the “directional movement must be implemented by the modest aspiration of the lymph hearts” (Toews and Wentzell 1995, pp. 204, 205). This hypothesis is predicated on there being a negative lymph heart pressure that aspirates lymph to the heart from the lymph

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sacs, supplemented by postural movements. However, direct measurements of lymph heart pressures (Jones et al. 1992, 1997) provide little evidence for a negative pressure inside lymph hearts. Consequently, we believe that other mechanisms have to be involved in returning lymph along lymph sacs to the lymph hearts. Postural movements may create pressures for lymph return in active anurans, but lymph is also returned in quiescent anurans; therefore, physical movement per se is not a prerequisite for lymph flow.

We hypothesize that lymph-sac compartmentalization is another mechanism that assists lymph return to the lymph hearts because the compartments have a sequentially graded compliance that creates differential pressures for unidirectional lymph return from the extremities to the lymph hearts. Lymph-sac compliance is the change in volume per unit pressure ($C = \Delta V \Delta P^{-1}$) for the lymph-sac space. It is the product of lymph-sac volume and lymph-sac distensibility (change in volume per change in pressure per lymph-sac volume; $D = \Delta V \Delta P^{-1} V^{-1}$). The extensive and dilated sinus structure of anuran lymphatic sacs suggests a very compliant system as a consequence of both their significant initial volume and the relative ease at which the skin can separate from the underlying musculature to allow increases in volume.

The objective of this study was to characterize the compliance of the lymph sacs of the hindlimb for the aquatic clawed toad *Xenopus laevis*, the semiaquatic bullfrog *Rana catesbeiana*, and the terrestriofossorial cane toad *Bufo marinus*. These lymph-sac compliance data and estimates of lymph flow enable us to determine whether distal-to-proximal lymph-sac compliance differences are consistent with the appropriate pressure gradient for lymph return.

Material and Methods

Bufo marinus (121–255 g, mean 162 g), *Rana catesbeiana* (213–385 g, mean 302 g), and *Xenopus laevis* (38–81 g, mean 67 g) were purchased from commercial suppliers. They were maintained, and experiments were conducted at 20°–22°C.

The relative proportion of hindlimb mass and limb segment mass was measured by blunt dissection of the segments and limb for a separate group of six *R. catesbeiana*, six *X. laevis*, and seven *B. marinus*.

Compliance Measurement

The anurans were anesthetized in 2 g L⁻¹ MS222 buffered with NaHCO₃ to pH 7. Lymph sacs were isolated sequentially by external ligation at their junctions. The plantar sac was isolated at the talocrural joint, the crural sac by ligation at the tibiofemoral joint, and the femoral sac at the coxal joint. In a separate set of measurements for *B. marinus* and *R. catesbeiana*, the interfemoral sac was isolated by ligation at the coxal joint; the pubic sac measurements followed with no further ligation.

This division of interfemoral and pubic lymph sacs is anatomically accurate in *B. marinus* and *X. laevis* but is an artificial division for *R. catesbeiana* because its interfemoral sac is freely contiguous with the pubic region; however, we ligated between these regions to measure separately the interfemoral and pubic lymph portions of the contiguous sac.

Three 21-gauge needles were inserted in the isolated sac—two attached to pressure transducers and the third attached to an infusion pump filled with 0.8% NaCl. The saline was infused at 1% of body mass per minute, while pressure was measured and stored with a MacLab data acquisition system (model 8e). The slope of pressure change with time was determined with the average linear slope function of the MacLab data pad from just after the start of infusion until the pressure started to increase in a nonlinear manner. Anuran lymph sacs are normally in a deflated (low-volume) state rather than distended, so we have focused on the physiologically relevant part of the compliance curve. In some cases, the pressure trace was erratic for one of the pressure probes (which would indicate an air bubble), so only the nonerratic trace was analyzed; otherwise, both compliance values were averaged. Pressure transducers were calibrated with a column filled with anuran physiological saline.

Differences in body mass for the three species mean that it is necessary to normalize absolute compliance measurements (mL kPa⁻¹) to make interspecific comparisons. One way to normalize compliances is to express them as mass-specific units (mL kg⁻¹ kPa⁻¹). However, there are also differences in the relative proportion of body mass that is hindlimb and differences in the relative proportions of the three limb segments for the hindlimb; therefore, we also present compliance data normalized to hindlimb segment mass (mL kg_{seg}⁻¹ kPa⁻¹).

Model of Lymph-Sac Flow

We estimated lymphatic pressures for the hindlimb lymph sacs of our three species from measurements of lymph-sac compliance by estimating the rate of lymph formation in each hindlimb segment based on the following three assumptions. First, we assumed that total lymph flow for one hindlimb of *B. marinus* is 0.4 mL kg⁻¹ min⁻¹ (if total flow is 1.6 mL kg⁻¹ min⁻¹ and each of the four lymph hearts contributes 25% of the total lymph flow). This is probably a reasonable estimate for *B. marinus* given that direct measurement of flow from one posterior lymph heart was 0.43 mL kg⁻¹ min⁻¹ (Jones et al. 1997), which was 25% of total lymphatic return following hemorrhage (1.6 mL kg⁻¹ min⁻¹; Baustian 1988). This is equivalent to 3.48 mL kg_{seg}⁻¹ min⁻¹ for *B. marinus*. For *R. catesbeiana* and *X. laevis*, we estimated hindlimb lymph flow corrected for the proportion of hindlimb mass compared with *B. marinus*, that is, 0.58 mL kg⁻¹ min⁻¹ for *R. catesbeiana* and 0.61 mL kg⁻¹ min⁻¹ for *X. laevis*. Second, we assumed that the total lymph pumped by the posterior lymph hearts originates only from its ipsilateral

Table 1: Hindlimb segment masses and body mass of cane toads *Bufo marinus* ($n = 7$), bullfrogs *Rana catesbeiana* ($n = 6$), and clawed toads *Xenopus laevis* ($n = 6$)

Hindlimb Segment	<i>Bufo marinus</i>	<i>Rana catesbeiana</i>	<i>Xenopus laevis</i>
Percent of body mass: ^a			
Foot ^a	4.6 ± .3 ^A	6.1 ± .3 ^B	8.1 ± .4 ^C
Calf ^b	5.5 ± .4 ^A	7.4 ± .3 ^B	11.0 ± .6 ^C
Thigh ^a	12.8 ± .8 ^A	19.8 ± .5 ^B	16.2 ± 1.0 ^C
Total ^a	23.0 ± 3.4 ^A	33.4 ± 2.2 ^B	35.2 ± 1.8 ^B
Percent of hindlimb mass:			
Foot	20.2 ± .6 ^A	18.3 ± .5 ^B	22.9 ± .4 ^C
Calf	24.2 ± 1.3 ^A	22.3 ± .5 ^A	31.2 ± .6 ^C
Thigh	55.6 ± 1.4 ^A	59.4 ± .6 ^B	45.9 ± .8 ^C
Body mass (g)	99.8 ± 4.9 ^A	431.5 ± 31.8	66.9 ± 6.3

Note. Values are mean ± SE. All differences between limb segments as percent of body mass for individual species are significant by Student-Newman-Keuls (SNK) post hoc comparison (ANOVA) except *B. marinus*'s foot-calf comparison ($P = 0.252$). All differences between limb segments as percent of hindlimb mass for individual species are significant by SNK post hoc comparison (ANOVA). Superscript capital letters indicate significant differences between species by SNK post hoc test.

^a Significant difference between species at $P < 0.001$ by ANOVA (individual species) and MANOVA (all segments except total hindlimb) for foot, calf, and thigh masses.

hindlimb. This is probably an overestimate of the actual hindlimb subcutaneous lymph-sac flow because the ventral-dorsal intermuscular, lateral, and subvertebral lymph sacs also communicate to the posterior lymph hearts. Third, the rate of lymph formation in each segment of the limb is proportional to each segment's mass relative to the total limb mass. This assumes blood flow is proportional to tissue mass and that the filtration coefficients and mean capillary pressure do not vary in different limb segments or interspecifically. The last assumption is consistent with whole-animal measurements of Hancock et al. (2000).

We first calculate the relative rate of pressure change associated with lymph formation in each individual subcutaneous lymph sac of a hindlimb, assuming isolation of each lymph sac (i.e., the muscular one-way valves between the sacs are closed). The pressure developed in each sac over time is simply the rate of lymph formation in that segment divided by its lymph-sac compliance. The interfemoral and pubic sacs have no intrinsic lymph formation, and so no pressure develops.

We then use a simple steady state model to determine the relative pressures and volumes in each hindlimb segment lymph sac. This model is based on the lymph flow in any segment equaling the accumulated flow from filtration into it and flow from all of the more distal sacs. Lymph-sac pressure is determined by resistance to flow and flow rate. The pressure must decrease from proximal to distal to drive flow from distal to proximal. Resistance to flow between sacs is complex and is determined by the resistance of the intersac valve(s) and its modulation by the valve musculature and resistance to flow through the sac itself. None of these resistances are known, so

we assume that each intersac resistance is the same and that the total distal-to-proximal pressure difference is 0.02 kPa. The pressure in each sac then determines the lymph fluid volume in the sac.

The assumption of a total pressure difference of 0.02 kPa is based on a mobilizable lymph volume of 2%–4% (Hillman and Withers 1988) and a whole-body interstitial compliance of 1,000 mL kg⁻¹ kPa⁻¹ (S. S. Hillman and P. C. Withers, unpublished data), giving an approximate interstitial pressure calculation of 0.02–0.04 kPa in the absence of actual measurements. Calculated pressures and lymph volumes would vary in proportion should another pressure difference value be used; for example, if the pressure was 0.04 kPa, then all pressures and volumes would be twice as great.

Statistical comparisons were assessed by one-way ANOVA and Student-Newman-Keuls post hoc comparisons, using statistiXL version 1.3. Data were log₁₀-transformed if standard errors increased with the means.

Results

Hindlimb Mass

The proportion of the hindlimb mass segments and total hindlimb mass to body mass varied between species (Table 1). *Bufo marinus* had a significantly smaller hindlimb than *Rana catesbeiana* and *Xenopus laevis* (ANOVA, $F_{2,16} = 23.2$, $P < 0.001$). There was a significant difference between the mass of each limb segment as a percent of total body mass for each species separately (ANOVA, $P < 0.001$) and combined (MANOVA, $P < 0.001$), with foot mass < calf mass < thigh mass (except foot

mass was not significantly less than calf mass for *B. marinus*). There were also differences in limb segment mass between species, with the same pattern for foot and calf (*B. marinus* < *R. catesbeiana* < *X. laevis*) but a different pattern for thigh mass (*B. marinus* < *X. laevis* < *R. catesbeiana*). Thus, *B. marinus* has proportionally smaller hindlimb segments than *R. catesbeiana* and *X. laevis*.

These differences in hindlimb segment mass were also evident when considering the mass of each segment relative to the hindlimb mass. The proportion of the hindlimb mass segments to total hindlimb mass varied significantly between species (Table 1) by ANOVA for each species ($P < 0.001$) and MANOVA for species combined ($P < 0.001$), with foot mass < calf mass < thigh mass. There were also differences between species. *Xenopus laevis* has a relatively big foot and calf and small thigh compared with *B. marinus* and *R. catesbeiana*; that is, its hindlimb segments are more similar in size. *Rana catesbeiana* has a relatively smaller foot and calf and bigger thigh than *B. marinus*.

Lymph-Sac Compliance

Mass-specific lymph-sac compliance (Table 2) of the three primary limb segments varied significantly between sacs for *B. marinus* and *R. catesbeiana* (ANOVA, $P < 0.001$) but not *X. laevis* ($P = 0.060$). For *B. marinus*, all lymph sacs differed in compliance except crural-interfemoral ($P = 0.676$) and femoral-pubic ($P = 0.829$), and all sacs differed in compliance for *R. catesbeiana*. There were no significant differences between species in plantar or crural sac compliance, but for femoral and pubic sacs, there was a significant difference for *X. laevis* compared with *B. marinus* and *R. catesbeiana* (but not between *B. marinus* and *R. catesbeiana*).

Segment-specific lymph-sac compliances (Table 3) of the three primary limb segments varied significantly for all three

sacs between *B. marinus* and *R. catesbeiana* (ANOVA, $P < 0.001$) but not *X. laevis* ($P = 0.204$). There were no significant differences between species in plantar or crural sac compliance, but for the femoral sac, there was a significant difference for *X. laevis* compared with *B. marinus* and *R. catesbeiana* (but not between *B. marinus* and *R. catesbeiana*).

Lymph-Sac Flow and Pressure

The calculated rates of increase in lymph-sac pressures based on intrinsic lymph formation and sac compliance are greater for the distal limb sacs than the proximal sacs (Fig. 1). This pattern of decreasing distal-to-proximal pressure is consistent with the required direction of pressure differentials necessary for lymph return and indicates that no segment is preferentially storing lymph (if any segment had a lower pressure than the more proximal segment, then it would have to accumulate lymph until its pressure was higher than that of the more proximal segment). However, these various rates of increase in pressure can only be sustained up to the point where valves between lymph sacs open to allow steady state flow to more proximal sacs.

In steady state, the actual intrasac pressures are determined by the relative flows and resistances, not compliances. For example, the interfemoral pressure is calculated to be very high on the basis of its low compliance and the high accumulated lymph flow into the sac ($P = V/C$), but whether this very high pressure is realized depends on the resistance to flow into the next sac (pubic). Because it would be highly undesirable for the interfemoral pressure to be very high (because plantar, crural, and femoral pressures would have to be even higher to maintain steady state flow, and this would promote excessive lymph storage), we suggest that either the resistance to flow from the interfemoral to the pubic sac is very low (e.g., there is no division at all in *R. catesbeiana*) or there is muscle-assisted

Table 2: Body-mass-specific compliance ($\text{mL kPa}^{-1} \text{kg}^{-1}$) of the hindlimb subcutaneous lymph sacs for cane toads *Bufo marinus*, bullfrogs *Rana catesbeiana*, and clawed toads *Xenopus laevis*

Lymph Sac	<i>Bufo marinus</i>	<i>Rana catesbeiana</i>	<i>Xenopus laevis</i>	Species Comparisons ^a
Plantar	4.2 ± 1 (13)	6.7 ± 1.6 (14)	15.7 ± 4.7 ^A (10)	NS
Crural	12.5 ± 2.3 ^A (11)	25 ± 4.2 (13)	21.8 ± 4.1 ^A (11)	NS
Femoral	76 ± 11.9 ^B (11)	113 ± 11 (14)	16.2 ± .4 ^A (11)	$P < .001$; $B = R$, $B \neq X$, $R \neq X$
Interfemoral	23.5 ± 7.5 ^A (12)	(4.1 ± .8) ^b (12)
Pubic	91.5 ± 28.5 ^B (8)	45.3 ± 9.4 (41.2 ± 9.4) ^b (9)	5.8 ± 1.8 ^A (9)	$P < .001$; $B = R$, $B \neq X$, $R \neq X$

Note. Values are mean ± SE, with n (number of sacs measured) in parentheses; NS = nonsignificant difference. $B = B. marinus$; $R = R. catesbeiana$; $X = X. laevis$. Superscript capital letters indicate compliances that are not significantly different within species (ANOVA with \log_{10} -transformed data, Student-Newman-Keuls post hoc test).

^a Species comparisons are for \log_{10} -transformed data.

^b Separate interfemoral and pubic sac compliances were measured for *R. catesbeiana* by ligating between these sacs, and these separate values are given in parentheses; however, these sacs are actually contiguous, so the combined compliance for both sacs is also given (as pubic).

Table 3: Segment-specific compliance ($\text{mL kPa}^{-1} \text{kg}^{-1}$) of the hindlimb subcutaneous lymph sacs for cane toads *Bufo marinus*, bullfrogs *Rana catesbeiana*, and clawed toads *Xenopus laevis*

Lymph Sac	<i>Bufo marinus</i>	<i>Rana catesbeiana</i>	<i>Xenopus laevis</i>	Species Comparisons ^a
Plantar	92 ± 21 (13)	110 ± 25 (14)	194 ± 58 ^A (10)	NS
Crural	228 ± 43 (11)	339 ± 57 (13)	199 ± 37 ^A (11)	NS
Femoral	594 ± 93 (11)	571 ± 56 (14)	100 ± 40 ^A (11)	$P < .001$; $B = R$, $B \neq X$, $R \neq X$

Note. Values are mean ± SE, with n (number of sacs measured) in parentheses; NS = nonsignificant difference. $B = B. marinus$; $R = R. catesbeiana$; $X = X. laevis$. Superscript capital letters indicate compliances that are not significantly different within species (ANOVA, \log_{10} -transformed data, Student-Newman-Keuls post hoc test).

^a Species comparisons are for \log_{10} -transformed data.

emptying of the interfemoral sac (see “Discussion”). So, for steady state flow, we assume that the interfemoral and pubic pressures are the same.

In our steady state model, sac pressure decreases monotonically from 0.02 kPa in a distal-to-proximal direction (Fig. 2; because each intersac resistance is assumed to be equal, except interfemoral-to-pubic, and the total pressure differential is 0.02 kPa). In *B. marinus* and *R. catesbeiana*, lymph volume is greatest in the femoral sac, then the pubic sac, as would be expected from their high compliances, but for *X. laevis*, the lymph volume is relatively low in all sacs.

Discussion

Lymph flow from the distal reaches of the hindlimb follows a subcutaneous route of plantar sac (around the foot) to crural sac (around the calf) to femoral sac (around the thigh) to interfemoral sac (a ventral subcompartment below the femoral sac that covers the pelvic patch) to the pubic sac, which connects to the posterior lymph heart (Fig. 3). Lymph moves between these sacs as a consequence of hydrostatic pressure differences. We have measured the compliance of the hindlimb lymph sacs for an aquatic anuran (*Xenopus laevis*), a semiterrestrial anuran (*Rana catesbeiana*), and a terrestriofossorial anuran (*Bufo marinus*), and we consider here the probable role for compliance of these lymph compartments in returning lymph to the lymph hearts. These species were chosen as representatives of the aquatic, semiaquatic, and terrestriofossorial groups of anurans identified by Carter (1979) based on the study of lymph sacs in a wide range of species. We suggest that our findings of substantial differences in lymph-sac compliance between these aquatic, semiterrestrial, and terrestriofossorial anurans reflect functional differences related to gravitational problems with lymph return from hindlimbs to the dorsal lymph hearts in terrestrial species, but the generality of our findings clearly requires the study of more species from each group.

Lymph-Sac Compliance

If we assume a series circuit for the plantar, crural, and femoral sacs, then the total compliance for the hindlimb is about 54,

140, and 90 $\text{mL kPa}^{-1} \text{kg}^{-1}$ for *X. laevis*, *R. catesbeiana*, and *B. marinus*, respectively. These subcutaneous lymph-sac compliance measurements for anurans are greater than whole hindlimb compliance measurements reported for mammals, which are from 29 to 47 $\text{mL kPa}^{-1} \text{kg}^{-1}$ (Guyton 1965; Brace et al. 1977), so we would expect these highly compliant lymph sacs to have an important functional role in promoting extracellular fluid formation by ultrafiltration and possibly fluid storage in the lymph sacs.

The general pattern for *R. catesbeiana* and *B. marinus* is increasing hindlimb lymph-sac compliance from distal to proximal (plantar to crural to femoral), but *X. laevis*, in contrast, has a constant (even decreasing) compliance from distal-to-proximal hindlimb (Tables 1, 2). These patterns in hindlimb segment compliance form the basis for our model of lymph return.

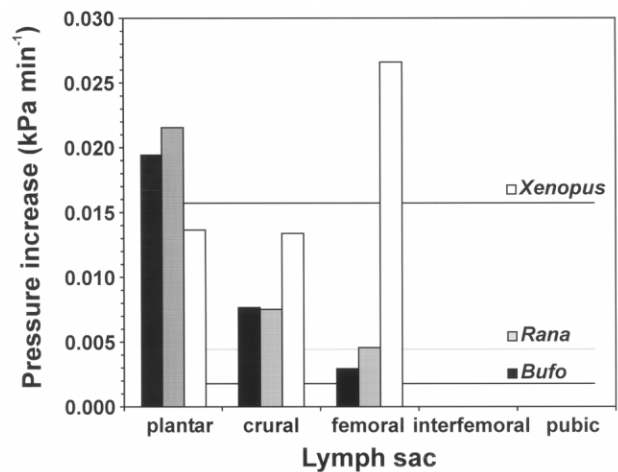


Figure 1. Rate of hydrostatic pressure increase for each hindlimb segment lymph sac (kPa min^{-1}) created by the formation of lymph into each sac with no flow between sacs for the marine toad *Bufo marinus* (black bars), bullfrog *Rana catesbeiana* (gray bars), and clawed toad *Xenopus laevis* (white bars). The average pressure is also shown for each species, assuming that lymph is distributed to equal pressure in all lymph sacs of the hindlimb.

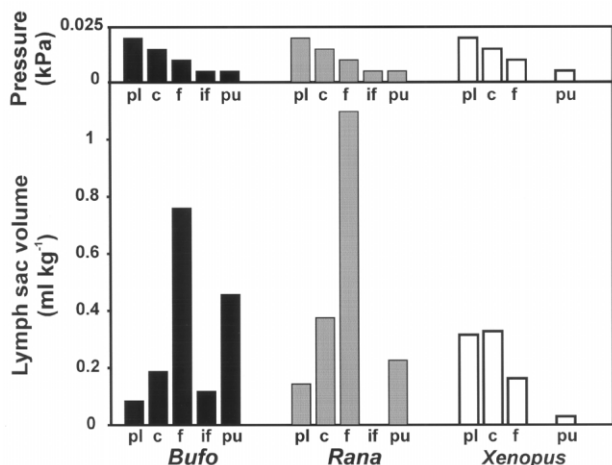


Figure 2. Volume of lymph calculated for each hindlimb segment lymph sac (mL kg^{-1}) for steady state lymph flow in the marine toad *Bufo marinus* (black bars), bullfrog *Rana catesbeiana* (gray bars), and clawed toad *Xenopus laevis* (white bars). The pressure differential is assumed to be 0.02 kPa and equally partitioned between the distal-to-proximal lymph sacs (see text).

Compliance-Assisted Lymph Return

Our hypothesis (Hillman et al. 2004) that compartmentalization of the subcutaneous lymph space into lymph sacs creates a gradient of compliance from distal (low compliance) to proximal (high compliance) is supported by our compliance measurements for the hindlimbs of both *B. marinus* and *R. catesbeiana*. The pattern of hindlimb lymph-sac compliance for *X. laevis* is different, but we believe that this is expected for an aquatic frog compared with terrestrial frogs.

Filtration of fluid from tissues into the interstitial spaces (and hence lymph sacs) will, if the fluid accumulates in that lymph sac (i.e., the intersac lymph valves are closed), cause the intrasac pressure to increase. Two characteristics determine the potential rate of increase in pressure by lymph formation within a lymph sac; the volume of lymph entering the sac and the compliance of the sac. The rate of lymph flow into a sac increases from distal to proximal as limb segment mass increases distally to proximally (Table 1), hence pressure would be expected to increase in lymph sacs from distal to proximal (if their compliance was the same). However, increased compliance of lymph sacs along the direction of flow (Table 2) would result in smaller pressures. Our model for lymph-sac pressures, in the absence of flow between lymph sacs, shows that the net effect of filtration and compliance for limb segments of *R. catesbeiana* and *B. marinus* is for the intrasac pressure to decrease distally to proximally (Fig. 1). This series of compliance-determined pressure differences is consistent with the required hydrostatic pressure differential required to drive lymph flow unidirectionally from the most distal sacs (least compliant) to the more proximal sacs (most compliant). We call this a compliance pump

for lymph return. That pressure decreases distally to proximally indicates that no segment preferentially sequesters lymph (otherwise, a segment with a lower pressure than a more proximal segment would have to accumulate lymph until its pressure increased to above that of the more proximal segment).

The pressure in the pubic lymph sac would need to increase to about 0.2 kPa to raise lymph from its ventral-dependent reaches to the dorsally located lymph heart (vertical distance $\cong 2 \text{ cm} \cong 0.2 \text{ kPa}$). Such an increase in pressure is untenable because lymph accumulation in the more distal sacs would be excessive (see “Muscle-Assisted Lymph Return”), given the high compliance of the femoral and pubic lymph sacs. So, there must be another mechanism for lymph return from the ventral reaches of the hindlimb to the lymph heart in addition to the compliance pump.

The pattern of compliance for hindlimb sacs is quite different for the aquatic *X. laevis* than the more terrestrial *R. catesbeiana* and *B. marinus*. Compliance is essentially the same for the hindlimb sacs and low for the pubic sac (Tables 2, 3). There is no distal-to-proximal pressure gradient created in the hindlimb of *X. laevis* by distal-to-proximal compliance change, and the pressure (if equalized in all sacs) is high (at 0.016 kPa after 1 min) because of the low compliance (Fig. 1). For aquatic frogs submerged under water, such as *X. laevis*, there is no gravitational hydrostatic pressure head between the lymph sacs and the dorsal lymph heart; no pressure head is needed to drive lymph return against gravity to the dorsal lymph heart. Low compliance would result in relatively substantial pressures developing in lymph sacs as a consequence of lymph formation. Aquatic frogs also presumably do not require any high compliance lymph sacs for lymph storage (e.g., for dehydration tolerance). It is therefore not surprising that the hindlimb lymph sacs of *X. laevis* have low compliance, with no distal-to-proximal differences. The femoral pressure is the highest of the limb sacs, so equalization of pressure in the plantar and crural sacs to femoral pressure could easily drive lymph flow to the pubic sac, hence lymph heart.

Muscle-Assisted Lymph Return

Neither the hindlimb compliance pump nor lymph heart aspiration can account for lymph movement from the subcutaneous lymph sacs of the hindlimb to the dorsally located posterior lymph heart for *R. catesbeiana* or *B. marinus* (see also Hillman et al. 2004). For the required pressure head to be generated by the formation of lymph, there would have to be excessive lymph sequestration in the more distal sacs (especially the high compliance femoral sac). The reported diastolic pressures within lymph hearts (Jones et al. 1992, 1997) are not sufficient to aspirate lymph up to the dorsally located lymph heart but will certainly contribute to filling the lymph heart.

Compliance of the interfemoral and pubic lymph sacs could be varied by direct or indirect volume changes of the sacs as

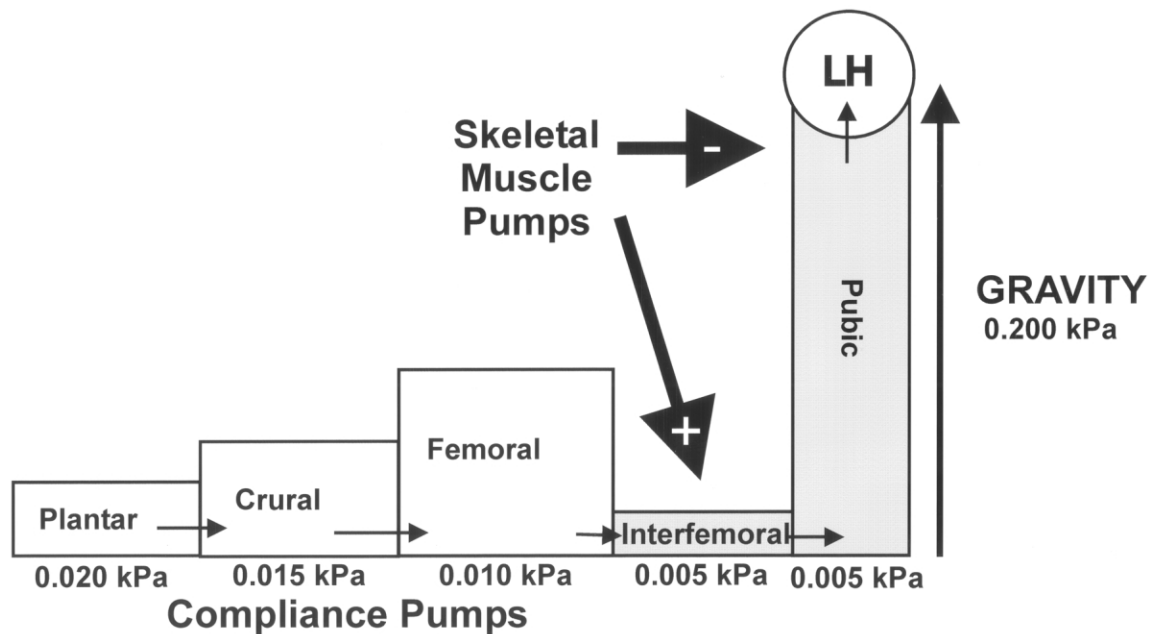


Figure 3. Schematic summary of our model for lymph return from the hindlimb of anuran amphibians. The plantar, crural, and femoral sacs provide lymph flow via the compliance pumps, whereas skeletal muscle pumps (positive and negative pressure) are required to drive lymph flow through the interfemoral and pubic sacs to the dorsally located lymph heart (*LH*) against the gravitational hydrostatic pressure head of about 0.2 kPa (see text).

a result of skeletal muscle contractions independent of postural changes and activity. Winokur and Hillyard (1992) have described muscles (*gracilis minor* and *abdominal crenator*) that insert on the dermis of the interfemoral sacs. They suggest that contraction of these muscles would decrease the volume of the femoral and interfemoral sac, increasing lymph pressure and thereby assisting lymphatic return to the dorsal lymph hearts. Our data would argue that the interfemoral sac is a low-compliance conduit of low-fluid volume and sits in the most dependent region of the most compliant sac. Hence, it is in an excellent position to collect lymph. Skeletal muscle involvement could assist this collection by expanding volume and assist pumping this fluid dorsally by decreasing the volume of the interfemoral sac. The pubic sac, in contrast, is a high-compliance chamber suitable for storing a large volume of lymphatic return at low pressure but readily emptying when compressed by external muscles. Contraction of the *piriformis* muscle (that originates on the femur and inserts on the urostyle and surrounds part of the pubic sac) could presumably depress the urostyle and expand the volume of the pubic sac, hence decreasing its pressure. Similarly, the subvertebral, ventral, lateral, and dorsal sacs would be sensitive to variations in body volume that result from lung inflation and deflation. The volume of the intermuscular sac could be varied by postural changes associated with the contraction of the thigh and calf musculature. We are currently examining the hypothesis that skeletal muscle

contraction creates appropriate pressures to move lymph through the interfemoral and pubic sacs (and other sacs) to the lymph heart.

Aquatic to Terrestrial-Fossorial Anurans

Carter (1979) observed that aquatic anurans have larger lymph sacs than terrestriofossorial anurans. This observation is based in part on the intimate contact of skin with underlying tissues (e.g., cranial co-ossification) for dorsal and to some extent lateral and ventral lymph sacs of terrestriofossorial species. His interpretation was that the extensive subcutaneous spaces of aquatic anurans, in conjunction with lymph hearts, renal portal veins, and the kidney, were a mechanism for rapid excretion of water that is osmotically absorbed across their skin. However, the water that is osmotically absorbed across the skin appears to pass preferentially directly into the circulation (cutaneous capillaries) rather than the subcutaneous lymph spaces (Word and Hillman 2005), and water uptake rates are correlated with cutaneous blood flow rates (Viborg and Rosenkilde 2004). The cutaneous water permeability and urine flow rates of aquatic anurans are both generally lower than for terrestrial species (Schmidt 1965; Bentley 1969; Pruett et al. 1991). Consequently, the premise that Carter (1979) presents for aquatic species' having a water uptake problem may be flawed. If larger lymph sacs were more compliant, then the larger lymph sacs of aquatic

species would lead to a lower hydrostatic pressure being developed for the same rate of lymphatic influx to that sac and would promote lymph storage. This is counterintuitive because we would expect that aquatic anurans would be less likely to need to store lymphatic fluid (e.g., for dehydration tolerance) than terrestrio-fossorial anurans.

Larger lymph sacs are not necessarily more compliant; for example, the femoral lymph sac of *B. marinus* is smaller than that of *X. laevis* but has a higher compliance (Table 2), and segment-specific compliance varies markedly, from 100 to 600 mL kPa⁻¹ kg⁻¹ (Table 3). The aquatic *X. laevis* has similar compliance for all hindlimb segments, whereas the more terrestrial *R. catesbeiana* and terrestrio-fossorial *B. marinus* have low compliance for the distal limb segments and markedly higher compliance for the proximal limb segment. This suggests that the “extensive” lymph sacs of *X. laevis* and the “less extensive” lymph sacs of *R. catesbeiana* and *B. marinus* (Carter 1979) do not correspond to more and less compliant sacs, and these species differ in their potential role for lymph storage. Lymph return seems more important than storage for *X. laevis* (extensive but low compliance sacs), whereas a functional role for lymph storage seems more important for *R. catesbeiana* and *B. marinus* (less extensive but more compliant sacs).

Aquatic frogs (such as *X. laevis*) lack myointegumental fibers, whereas terrestrio-fossorial species (such as *B. marinus*) have extensive fibers in the ventral lymph sacs (Carter 1979). Carter did not ascribe any function to these myointegumental fibers, other than perhaps to aid water uptake through the “pelvic patch.” We suggest that these myointegumental fibers would limit the compliance of the ventral lymph sacs if they become distended by lymph storage (see “Scenario for Lymph Storage”). The compliances that we measured are for the initial filling of the lymph sacs with fluid; as the sacs became progressively filled, their compliance decreased because of the inelasticity of skin. We suggest that further study of the effect of lymph-sac volume on compliance will prove fruitful, especially for comparison of sacs with and without myointegumental fibers.

Scenario for Lymph Storage

One of our conclusions from measurements of lymph space compliance is that more terrestrial anurans (*R. catesbeiana* and *B. marinus*) have a lower distal compliance and higher proximal compliance than an aquatic anuran (*X. laevis*). This differential in compliance forms a storage system that preferentially locates lymph in the proximal rather than the distal hindlimb segments. For a frog to store lymphatic fluid, it would seem an appropriate strategy to locate the bulk of the stored lymphatic fluid in the proximal segment (e.g., femoral sac) rather than the distal segments (e.g., plantar and crural sacs). It would also require a controllable segment between the storage site and the lymph heart pump to allow a controlled metering of lymph to the lymph heart dependent on blood volume status. This would

be the function of the interfemoral and pubic sacs. This would explain why lymph-sac-fluid loading does not influence lymph heart rate or pressure (Williams et al. 1998). The magnitude of this preferential proximal storage of lymphatic fluid is readily apparent from the compliance of the segments (Fig. 2). To promote fluid storage in this manner, it is only necessary to increase the intrasac pressure. This could be readily accomplished by reduced muscle action for the return of lymphatic fluid against hydrostatic pressure or by increased tonic activity of the interlymph-sac valve muscles (described by Jolly 1946).

Whether there is such subcutaneous lymphatic fluid storage is unclear. Ewer (1952) and Middler et al. (1968) have suggested that some anuran amphibians store fluid in the subcutaneous lymph sacs, lymphatic fluid is generally isosmotic (Word and Hillman 2005) or slightly hypoosmotic (Hillyard and Larsen 2001) and therefore not as good a water storage fluid as hypoosmotic urine. The lymph-sac volumes (2%–4% of body mass; Hillman and Withers 1988) are also less than the bladder volume. Therefore, the urinary bladder is clearly a more important storage organ of hypoosmotic fluid (e.g., Ruibal 1962; McClanahan 1967; Bentley 1971), but this does not discount that lymph sacs have a storage role in some species under some circumstances, such as buffering blood volume changes during hemorrhage or dehydration (Hillman and Withers 1988). This merits further study.

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