Lymphatic regulation in nonmammalian vertebrates

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Hedrick MS, Hillman SS, Drewes RC, Withers PC. Lymphatic regulation in nonmammalian vertebrates. J Appl Physiol 115: 297–308, 2013. First published May 2, 2013; doi:10.1152/japplphysiol.00201.2013.—All vertebrate animals share in common the production of lymph through net capillary filtration from their closed circulatory system into their tissues. The balance of forces responsible for net capillary filtration and lymph formation is described by the Starling equation, but additional factors such as vascular and interstitial compliance, which vary markedly among vertebrates, also have a significant impact on rates of lymph formation. Why vertebrates show extreme variability in rates of lymph formation and how nonmammalian vertebrates maintain plasma volume homeostasis is unclear. This gap hampers our understanding of the evolution of the lymphatic system and its interaction with the cardiovascular system. The evolutionary origin of the vertebrate lymphatic system is not clear, but recent advances suggest common developmental factors for lymphangiogenesis in teleost fishes, amphibians, and mammals with some significant changes in the water-land transition. The lymphatic system of anuran amphibians is characterized by large lymphatic sacs and two pairs of lymph hearts that return lymph into the venous circulation but no lymph vessels per se. The lymphatic systems of reptiles and some birds have lymph hearts, and both groups have extensive lymph vessels, but their functional role in both lymph movement and plasma volume homeostasis is almost completely unknown. The purpose of this review is to present an evolutionary perspective in how different vertebrates have solved the common problem of the inevitable formation of lymph from their closed circulatory systems and to point out the many gaps in our knowledge of this evolutionary progression.

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THE LYMPHATIC SYSTEM OF VERTEBRATES is generally defined from a mammalian perspective as a vessel system draining the interstitial space into the venous circuit but with no connection to arterial circuit of the cardiovascular system. It transports a fraction of the interstitial fluid, known as lymph, that originates from the net filtration of plasma across the capillaries of the cardiovascular system. This definition incorporates both anatomical and functional features of the mammalian lymphatic system. In nonmammalian vertebrates, however, there is considerable variation in lymphatic structures, thus lymphatics is best defined from a functional perspective. The lymphatic vasculature of mammals has several essential physiological functions, including the maintenance of fluid balance between the plasma and interstitial compartments of the extracellular space by returning protein and fluid filtered out of the capillaries to the vascular system, involvement in immune defense, and the distribution of diet-derived fat (see Refs. 2, 3, 35, 62, 69). However, the lymphatics have historically been given short shrift as an organ system. In the general medical school curriculum, it is the system that frequently is ignored or perhaps mentioned only in passing (20, 96, 115). Why this should be the case is unclear given the extremely important role that the lymphatic system plays in fluid volume homeostasis. This attitude has changed recently, with an ever-increasing number of publications and journals devoted to lymphatics, reflecting the obvious importance of this system when something goes wrong. Genetically aberrant or damaged lymphatic vessels lead to lymphedema (excess swelling of the extremities). Lymphatic damage is a common postsurgical problem with limited therapeutic solutions. Approximately 120 million people worldwide currently suffer from lymphatic filariasis (extreme lymphedema, commonly known as “elephantiasis”) caused by a filarial worm that inhabits the lymphatic vessels and prevents lymph return to the circulation by narrowing the diameter of lymph vessels (53, 90). In addition, lymphatic vessels promote metastatic spread of cancer cells to distant organs—a leading cause of death in patients with cancer and a major obstacle in the design of effective therapies (20).

The field of mammalian lymphatics has recently seen tremendous strides in our understanding of the molecular control of lymph development, modeling of lymph function, regulation of lymphatic pumping, immune function, and metastasis (2, 3,
The physiological role of the lymphatic system in plasma volume regulation

The lymphatic system has many different functions, but paramount among these is its role in removing accumulating protein and fluid from the interstitial space and returning it to the vascular space. The net rate of filtration of fluid (plasma) from the vascular to the interstitial space, the transvascular fluid flux, and vascular and interstitial compliance are common to all vertebrates, and thus a discussion of the various modes of lymph flux regulation is important for understanding the diversity of vascular fluid regulatory capacities in vertebrates. Burggren et al. (14) have reviewed the structure, function, and evolution of the vertebrate circulatory system. The reader is also referred to Kampmeier’s (67) excellent monograph on the evolution and morphology of the lymphatic system.

THE PHYSIOLOGICAL ROLE OF THE LYMPHATIC SYSTEM IN PLASMA VOLUME REGULATION

The lymphatic system has many different functions, but paramount among these is its role in removing accumulating protein and fluid from the interstitial space and returning it to the vascular space. The net rate of filtration of fluid (plasma) from the vascular to the interstitial space, the transvascular fluid flux, and vascular and interstitial compliance are common to all vertebrates, and thus a discussion of the various modes of lymph flux regulation is important for understanding the diversity of vascular fluid regulatory capacities in vertebrates. Burggren et al. (14) have reviewed the structure, function, and evolution of the vertebrate circulatory system. The reader is also referred to Kampmeier’s (67) excellent monograph on the evolution and morphology of the lymphatic system.

Extracellular Fluid Volume Regulation

Interstitial Volume

Transcapillary Fluid

Lymphatic Fluid

Drinking → Plasma Volume

Hemorrhage

Interstitial Volume

Fig. 1. Pathways for interstitial fluid addition (black arrows) and loss from (gray arrows) the plasma volume; drinking additions to and hemorrhagic loss from the plasma volume are also included.

\[
J = F_{cap} \left( P_{cap} - P_{ist} \right) - \sigma \left( \Pi_{cap} - \Pi_{ist} \right)
\]

where \( J \) is net transvascular fluid flux (ml·min\(^{-1}\)·kg\(^{-1}\)), \( F_{cap} \) is filtration coefficient of the capillaries (ml·min\(^{-1}\)·kg\(^{-1}\)·kPa\(^{-1}\)), \( P_{cap} \) is mean capillary hydrostatic pressure (kPa), \( P_{ist} \) is interstitial hydrostatic pressure (kPa), \( \sigma \) is reflection coefficient for plasma proteins, \( \Pi_{cap} \) is colloid osmotic pressure of the plasma (kPa), and \( \Pi_{ist} \) is colloid osmotic pressure of the interstitial fluid (kPa).

Normally, the balance of the transcapillary hydrostatic forces favors efflux of fluid from the capillary, whereas balance of the colloid forces favors influx. On average, the net hydrostatic force dominates and there is a consequential net efflux of fluid from the vasculature into the mammalian interstitium; ∼15% filtered out of the capillaries remains in the interstitial space, and 85% is returned to the capillaries (38). This interstitial fluid, which is filtered from the capillaries, is returned as lymph to the circulation via the lymphatic system (Fig. 1). It is important to recognize that the lymph and interstitial fluid are chemically identical, and it is essentially impossible to quantify the relative volume fraction of the interstitial volume that represents lymph volume. The fluid that comprises lymph is present in both the interstitial space and lymph vessels, and some fluid volume is always present. The fraction of this fluid volume returned is quantified as the rate of fluid added to the plasma volume from the lymphatic system. Consequently, lymphatic return is generally characterized as a fluid volume flux rather than a volume. If fluid is added to the plasma space, then \( P_{cap} \) will increase and \( \Pi_{cap} \) will decrease; both changes lead to greater efflux and consequently a return toward the original plasma volume. If fluid is lost from the plasma space, then \( P_{cap} \) will decrease and \( \Pi_{cap} \) will increase, both changes favoring increased absorption of fluid from the interstitium and a return toward the original plasma volume. Consequently, there is an inherent mechanism in Starling’s equilibrium that promotes homeostatic redistribution of blood and interstitial fluid independent of a regulated negative feedback loop. Because \( F_{cap} \) and \( \sigma \) vary in different organs (108), the use of a capillary-focused equation to describe blood volume regulation would require an integration of both the blood flow and Starling variables for every organ in the body, which is an impractical approach. Furthermore, the relationship between the plasma filtration and both \( P_{cap} \) and \( P_{ist} \) is also determined by both the vascular compliance and the interstitial compliance of different organs (107). For instance, organs encased in inelastic connective tissue or bone show large increases in \( P_{ist} \) in response to any filtration (108), which would immediately prevent further hydrostatic filtration. A low \( C_{ist} \) and high reflection coefficient may explain why lymphatic vessels are absent in specific tissue compartments of mammals, including the brain, spinal cord, retina, cartilage, and bone (20). Consequently, the Starling fluid flux equation is an excellent model for describing fluid fluxes across an individual capillary in a particular organ, but it has limited capacity for interpreting transcapillary fluid redistribution at an organismal level.

A more useful model for understanding the short-term effects of volume loading and hemorrhage on plasma volume in mammals is a derivation of the Starling fluid flux equation that emphasizes whole body compliance and its interaction with fluid volume and pressure (107). The pressure in a fluid space is determined by the volume of fluid and the elastic compliance (capacitance) of the space (i.e., \( P = V/C \)). We can incorporate the compliances of the vascular and interstitial spaces and a whole body filtration coefficient to examine the kinetics of fluid flux (\( J \)) between the vascular and interstitial spaces, by the following equation:

\[
J = \frac{P_{cap} - P_{ist}}{C_{ist}} - \sigma \left( \frac{\Pi_{cap} - \Pi_{ist}}{C_{ist}} \right)
\]
J = FC (P_vas − P_cap) = FC [(V_vas/C_vas) − (V_int/C_int)]

where FC is the whole body filtration coefficient (ml·kg⁻¹·kPa⁻¹·min⁻¹), C_vas is the vascular compliance (ml·kg⁻¹·kPa⁻¹), C_int is the whole body interstitial compliance (ml·kg⁻¹·kPa⁻¹), V_vas is the vascular blood volume (ml/kg), and V_int is the interstitial fluid volume (ml/kg).

Starling models emphasizing a transcapillary mechanism of fluid flux are well appreciated and appear to adequately explain plasma volume maintenance in mammals (Fig. 1; Ref. 95). Mammals, when dehydrationally or hyperthermally challenged, decrease the efflux of plasma protein by increasing lymphatic flow (resulting in increased plasma protein concentration and decreased interstitial protein concentration) and, in some species by decreasing the filtration coefficient (11, 55–60, 98, 99). This combination of effects increases the driving force for transcapillary uptake. Compliance models are generally not necessary to invoke for mammals because plasma turns over slowly, hence lymphatic flux is a relatively small fraction of the total plasma volume (see below) and can be ignored in normal physiological states. This partially explains why the lymphatic system is poorly popularized as a mechanism for plasma volume maintenance in mammals. However, pathological situations where lymphatic flux is compromised, or compliance changes, are a different story. As we point out below, such circumstances of high capillary filtration rates and overall plasma protein turnover are in the normal circumstance for nonmammalian vertebrates, and application of the modified compliance form of the Starling equation as developed by Tanaka (107) is usually more appropriate.

PLASMA TURNOVER AND LYMPH FORMATION

Measurement of the turnover rate of labeled plasma proteins (Fig. 2) indicate that the net rates of fluid exchange between the circulation and interstitium vary dramatically among vertebrates. Plasma turnover values are very high for anurans, 3–5% of plasma volume per minute in bullfrogs (Lithobates catesbeianus = Rana catesbeiana) and cane toads (Rhinella marina = Bufo marinus) (52), compared with 0.9% per minute for fish (82,101) and 0.03–0.1% per minute for mammals (5, 8).

There are two principal reasons for the higher plasma turnover of anurans compared with fishes and mammals: 1) a much higher interstitial compliance (higher C_int) and 2) a “leakier” vasculature (lower σ). The whole body FC determined for amphibian vascular systems (25 ml·min⁻¹·kg⁻¹·kPa⁻¹; 41, 46) is ~3- to 6-fold higher than for mammals (5, 107) but is lower than for trout (88). Interstitial compliance is more than an order of magnitude higher for anurans (1.5–3 ml·kg⁻¹·Pa⁻¹; 52) than for either the fish (88) or mammals (107), reflecting the low resistance pathway for fluid flux into an anuran’s interstitial space. Fish data (Fig. 2) present an interesting example of the interplay between FC and C_int. Trout have a high capillary permeability (42) and a higher FC (37 ml·min⁻¹·kg⁻¹·kPa⁻¹; 88) than anurans that would predict a higher plasma turnover than anurans, but trout plasma turnover is actually lower. This is because trout have a much lower C_int (0.09 ml·kg⁻¹·Pa⁻¹; 88) than anurans, which prevents fluid loss from the circulatory system because P_int increases dramatically with any filtration, even with a higher FC. The high C_int of anurans favors storage of extracellular fluid volume in the interstitium without increasing P_int or P_cap but entails the penalties of high filtrational losses and plasma turnover and requires a substantial non-Starling mechanism (lymphatic return) for fluid uptake. It is important to recognize that anurans are unique relative to the few other vertebrate groups that have been studied in having such a high C_int, and storage of lymph may be a specialized adaptation of this highly derived vertebrate lineage for handling the blood volume stress of dehydration in a terrestrial environment.

One way to assess how variation in the factors involved in transvascular flux and lymph formation affect transvascular fluid flux is to perform a sensitivity analysis based on Tanaka’s (107) equation (Eq. 2) to determine which factors have the greatest contribution to filtration. This analysis (Fig. 3) reveals a very different pattern for anurans compared with either fish or mammals, largely as a consequence of their high C_int. Anurans have a net efflux from the vascular to the interstitial space under all modeled conditions, regardless of how each variable changes (Fig. 3A). This indicates that transcapillary uptake of interstitial fluid based on Starling balance is impossible and points out the enormous importance of their lymphatic system in returning interstitial fluid to the circulatory system. This conclusion is consistent with the finding that lymph heart ablation in toads causes hemoconcentration and death within a few days (124) and an inability to compensate for hemorrhage (7). Reducing C_vas and V_int have the biggest effects (increase and decrease, respectively) on fluid loss from capillaries. Changes in V_int and C_int have relatively small effects, reflecting their initially high values. The pattern for trout under normal conditions indicates zero flux with no net driving force (100%: Fig. 3B). Thus any variation in FC will have no effect on flux as there is no net driving force, but changing the other parameters in this analysis increases or decreases flux rates between the vasculature and interstitium. Reductions in C_vas or V_int increase flux to the interstitium whereas reductions in V_b or C_int increase flux from the interstitium to the vasculature. For example, during exercise in fish, increased arterial pressure (hence V_b) drives fluid out of the capillaries, owing to the high FC, resulting in hemoconcentration and increased hematocrit (70, 104, 105). The direction of fluid flux is determined by the relative difference in pressure between the vasculature and

![Fig. 2. Comparison of plasma turnover in a variety of vertebrate species.](image-url)
Cardiovascular variables are the whole body filtration coefficient ($F_C$), blood volume ($V_b$) indicates gain of fluid from the intersitium to the vascular space. However, once fluid loss from the vascular system to return fluid. Howevet, once fluid loss from the vascular space reaches or exceeds atmospheric pressure, interstitial compliance increases dramatically (39). Changing vascular and interstitial variables do not markedly affect the net outward flux during this edematous state (Fig. 2), which is consistent with the difficulty in correcting excess interstitial fluid retention during situations that produce edema, such as lymphedema or congestive heart failure (37, 39).

LYMPHATIC FUNCTION IN FISHES

The roles of, and indeed the very existence of, a lymphatic system in fishes are controversial (87, 111–113). Until about 1980, it was thought that the lymphatic system of fish was similar in structure and function to that of mammals, but early descriptions of “lymphatic” vessels in fish may have been, in fact, descriptions of a secondary circulatory system (67, 103, 111, 117). Vogel (111, 113) first described the secondary circulatory system of teleost fishes and argued that this secondary circulation is not a lymphatic system owing to a direct connection (i.e., interarterial anastomoses) between the primary and secondary vasculature (112). This mammalian definition of lymphatics has confused the issue of lymphatics in teleost fishes that we describe below.

There are two fundamental questions to be addressed with regard to lymphatic regulation in fishes: 1) do fishes have a lymphatic system? and 2) is their secondary circulatory part of the lymphatic system? In our view, the answer to the first question, based upon several recent studies with zebrafish outlined below, is that teleost fishes do have a lymphatic system that shares many molecular and morphological features with the mammalian lymphatic system. With regard to the second question, it is still unclear whether the secondary circulation is connected to, or a component of, the lymphatic system. More studies are needed to resolve this question.

There is growing use and acceptance of zebrafish as a model for lymphatic system development in vertebrates, stressing the potential importance of comparative vertebrate model systems for studying lymphatic function. This is based on several studies showing that highly specific molecular markers for lymphangiogenesis in mammals are also expressed in zebrafish vessels that are clearly not primary circulatory vessels (20, 61). These vessels have been interpreted as lymphatic in nature, based on molecular expression data, but whether they are also part of the secondary circulation is unclear (see below). The thoracic duct was the first lymphatic vessel identified in zebrafish (71, 122), and several other lymphatic vessels have been subsequently identified in various regions, including the head and gastrointestinal tract (21). During development, a population of lymph endothelial cell (LEC) precursors sprout from the cardinal veins and form embryonic lymph sacs, as occurs in mammals. These LEC precursors are marked by the transcription factor Prox1, a mammalian homolog of the Drosophila protein Prospero, is confined to LEC nuclei in both zebrafish and mammals (100). Targeted inactivation of Prox1 completely arrests lymphatic vasculature development without affecting angiogenesis (118), and proxi knockout inhibits formation of the thoracic duct in zebrafish (29). Knockdown of delta-like-4 (DII4) or its receptors Notch-1b or Notch-6 in zebrafish also impair lymphangiogenesis, including formation of the thoracic duct (36). Vascular

Fig. 3. Sensitivity analysis of the effect of individually increasing and decreasing each variable in Tanaka’s (107) analysis by 50% (Eq. 2 in text) on plasma flux. Each point is a mean value calculated from Eq. 2. Positive flux values indicate loss of plasma from the vascular space to the interstitium, and negative values indicate gain of fluid from the interstitium to the vascular space. Cardiovascular variables are the whole body filtration coefficient ($F_C$), blood volume ($V_b$), intravascular volume ($V_{ist}$), vascular compliance ($C_{vas}$) and interstitial compliance ($C_{ist}$). [Figure modified from Hillman et al. (52) with permission from the University of Chicago Press.]

interstitial spaces, and the fluxes in either direction are very high owing to the very high $F_C$. Therefore, just as for anurans, Starling equilibrium driving transcapillary flux appears to play little role in determining transvascular fluid flux in trout (15, 88). The normal mammalian pattern (Fig. 3C) is a net fluid uptake with a driving force of about −3.2 kPa, which is approximately the value predicted from vascular and interstitial colloid osmotic differences. Lowering $C_{ist}$ has the greatest effect on net fluid uptake, illustrating its importance in determining transvascular flux. Decreases in $V_{ist}$, $F_C$, and $C_{vas}$ reduce the inward flux of fluid. Increased $C_{ist}$ and decreased $V_{ist}$ with continuous angiotensin II infusion in dogs (110) shift transvascular fluid flux to an outward flux, which is very similar to the normal situation for the toad. Interstitial fluid pressure in mammals is normally negative (37, 39, 40) and represents a significant “safety factor” for preventing edema (37) in conjunction with the variable capacity of the lymphatic system to return fluid. However, once fluid loss from the vascular system to return fluid. Howevet, once fluid loss from the vascular space reaches or exceeds atmospheric pressure, interstitial compliance increases dramatically (39). Changing vascular and interstitial variables do not markedly affect the net outward flux during this edematous state (Fig. 2), which is consistent with the difficulty in correcting excess interstitial fluid retention during situations that produce edema, such as lymphedema or congestive heart failure (37, 39).
endothelial growth factor C (VEGFC), which is critical for lymphangiogenesis in mammals (68), is also necessary for development of the lymphatic system in zebrafish (71). The expression of Prox1 and neuropilin-2 (Nrp-2), which play a role in the development of small lymphatic vessels in mammals (123), were found in presumptive lymphatic vessels in zebrafish (122). However, a more recent study with transgenic zebrafish expressing the lymphatic endothelial hyaluronan receptor 1 (LYVE1) promotor, identified previously uncharacterized lymphatic vessels in the head, intestine, and superficial areas of the lateral trunk (85). LYVE1 is one of the most specific and widely used mammalian lymphatic endothelial markers (106). The general consensus from these studies is that molecular pathways controlling lymphangiogenesis are highly conserved from zebrafish to mammals.

In addition to molecular evidence for lymphatic vessel structure, functional studies also point to these zebrafish vessels as being lymphatic. For example, subcutaneous injection of fluorescin dextran or rhodamine dextran into the trunk muscle of zebrafish resulted in dye taken up by the putative lymphatic vessels, which accumulated in the venous circulation as would be expected of lymphatic vessels (71). A genetic screen identified an edematous zebrafish mutant, full of fluid, which lacked previously identified lymphatic vessels, including the thoracic duct, intersegmental lymphatic vessels, and dorsal longitudinal lymphatic vessels, but retained normal vasculature (54). Mutants developed severe edema in the lower intestine and around the eyes, suggesting the presence of lymphatic vessels in these regions. The mutation was localized to a single gene, ccbe1 (collagen and calcium binding EGF domain 1), which involved a single amino acid substitution. Interestingly, this finding in zebrafish led to the discovery of a link between the ccbe1 mutation and lymphatic hypoplasia in a human disorder (1). Knockdown of proxlβ, which prevents thoracic duct formation, also results in an edematous phenotype (29). Morpholino-mediated knockdown of Prox1 and VEGF-C in Xenopus tadpoles also produces lymph vessel defects and lymphpheda (83), indicating a similar genetic program for lymphangiogenesis in zebrafish and anurans. Finally, rapamycin, a specific inhibitor of the mammalian target of rapamycin (mTOR) that exhibits antilymphangiogenic properties in mammals, suppresses rostral and trunk lymphangiogenesis without affecting blood vessels in zebrafish (34). Taken together, the morphological and functional studies strongly suggest that zebrafish, and presumably other teleosts, have a lymphatic system that presumably functions similarly to that of other vertebrates.

The “secondary vascular system” of teleost fishes is in parallel with, and distinct from, the primary vasculature. This secondary vascular system (also known as the secondary circulation) arises from a very large number of interarterial anastomoses from the primary arterial vessels, particularly in regions with external contact with the environment, e.g., skin, gills, fins (14, 103, 111). An alternative view of the interarterial anastomoses that emerge from the primary arteries is that they are artifacts of the casting methods used to fill the vascular system (61), although this seems unlikely. These secondary arteries, similar in size to arterioles, coalesce to form secondary arteries that supply a capillary network of their own that drains into secondary veins and ultimately empty into the primary veins. The secondary vascular system is thus defined because it has direct connections with both the arterial and venous vascular of the cardiovascular system. It has been argued that the secondary circulation cannot be lymphatic because it does not fit the mammalian definition of a lymphatic system (112). Cyclostome and elasmobranch fishes do not appear to have a secondary circulation, but they do have some vessels that have been described as “venolymphatic,” with no clear distinction between the lymphatic and venous system (103). The fluid contained within the secondary circulation of teleosts appears to be composed of plasma skimmed from the primary circulation and contains very few red blood cells (RBCs) (103, 111, 113). The function of the secondary circulation is unknown, but it has been suggested to be a primitive lymphatic system (67) or have nutritive or immune sensing functions (87, 94). Given the location of the secondary circulation in regions where injuries might occur, an immune function for the secondary circulation seems a likely possibility (94) but this hypothesis has not been tested. The near absence of RBCs in the fluid of the secondary circulation would rule out a significant role in oxygen transport; however, a study with zebrafish and glass catfish found that hypoxia promoted nitric oxide-regulated perfusion of the interarterial anastomoses (63). These anastomoses were termed “arterial-lymphatic conduits” because the hypoxia-induced perfusion of interarterial conduits allowed RBCs to enter the lymphatic vessels (which expressed Prox-1), indicating there are direct connections between the primary arterial system and the lymphatic system of zebrafish (63) if the secondary circulation is considered lymphatic in function. This experimental evidence is important because it establishes a direct connection between the primary circulation and the presumptive lymphatic system (Fig. 4). The direct connection may also be important as a potential driving force to propel lymph through the lymphatic system and back to the primary circulation. The small anastomoses would siphon some of the pressure and use the energy generated from cardiac contraction to create a pressure head to propel the lymph. In the absence of lymphatic pumps (see below) this arrangement may have been necessary and possibly represents the original mechanism for lymphatic flow and its return to the circulatory system. Given the current evidence, our view is that the secondary circulation in teleost fish likely represents a “primitive” lymphatic system. Whether these secondary vessels are identical to the lymphatic vessels described for zebrafish is unclear, and more studies are needed to ascertain the connection, if any, between the lymphatic system and the secondary circulation in teleosts.

LYMPHATIC FUNCTION IN LUNGFISHES AND THE WATER-LAND TRANSITION

Lungfishes (Dipnoi) occupy an important evolutionary position as the sister group of modern tetrapods. This group appears to have the first recognizable tetrapod-like lymphatic system among the vertebrates. The lymphatic system of Dipnoi is characterized by the presence of lymphatic capillaries and a large number of lymphatic micropumps located throughout the body, except for the central nervous system (114). There are high concentrations of lymphatic micropumps in the fin regions, up to 100 mm⁻² in the South American lungfish (Lepidostrom paradoxa). The lymphatic micropumps are fed by thin-walled afferent lymphatic vessels that have no direct
connection with the primary circulation and contain valves at the inlet and outlet that are adjacent to a blood capillary, presumably to collect filtrate from the adjacent interstitial space. There was no evidence for the “secondary circulation” vessels characteristic of teleosts (114). No studies have examined lymphatic development, the rate of volume flux, nor measured the pressures within the lymphatic system in this key group of vertebrates.

The recently sequenced genome of the African coelacanth (Latimeria chalumnae) provides some additional insight into the evolution of the lymphatic system in the water-land transition of vertebrates (4). The data indicate that tetrapods have expansion in at least four conserved regions of the genome involved in lymphatic development and function compared with teleosts. This suggests a more elaborate lymphatic system and provides evidence for a potential elaboration of lymphatics in the water-land transition. This raises questions about the adequacy of teleost models, but also suggests that comparisons among tetrapods could be useful for correlates of lymphatic structure and function. The sequencing of the coelacanth genome opens up a myriad of potential studies to examine the evolution of the tetrapod lymphatic system.

LYMPHATIC FUNCTION IN AMPHIBIANS

The lymphatic system and lymph hearts of caecilians (order Gymnophiona) and salamanders (order Urodela) are poorly known; much more is known about lymphatics in frogs and toads (order Anura). Caecilians are legless, burrowing amphibians with a “wormlike” appearance (49). They are highly segmented, with a bilateral pair of lymph hearts between each segment; thus more than 200 pairs of lymph hearts have been found (67). Salamanders also have numerous lymph hearts, with anywhere from 8 to 23 pairs depending on the species (67). An unusual and characteristic feature of the salamanders is the presence of what has been termed the “cardial lymph propulsor” (67). This lymphatic propulsor lies tightly against the truncus arteriosus, the major outflow tract of the amphibian heart. It is in direct communication with the venous circulation, but it is not clear if it is distinct from typical lymph hearts.

Anatomically, anurans are a highly derived and specialized group of amphibians, as is their lymphatic system. The anuran lymphatic system consists of extensive subcutaneous and intrapleuroperitoneal sacs separated by connective tissue walls and interconnected by one-way valves. These valves appear to be controllable rather than being simply passive flaps (64). The various lymph sacs have been generally described for a variety of anurans, but they do vary interspecifically (18). These large subcutaneous lymph sacs account for the high Cист of anurans compared with either fishes or mammals. Owing to their very high Cист, the addition of even a volume equivalent to 4% of a toad’s plasma volume (their minute plasma turnover) will only generate an increased interstitial pressure of 1–2 Pa. This low interstitial pressure means that lymph will preferentially move gravitationally and pool in the ventral lymph sacs. The problem for anurans, then, is that this lymph must be moved from ventral regions to the dorsally located lymph hearts that pump the lymph into the venous circulation. To describe this, we have used the analogy of a “sump pump” that is placed in an attic instead of a basement to move ventrally pooled lymph (47). The pooled lymph is at ~200 Pa below the location of the

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**Fig. 4. Hypoxia-induced linearization of arterial-lymphatic conduits (ALCs), lymphatic dilation, and blood perfusion in zebrafish and glass catfish.**

**A:** ALCs appear to be secondary circulation vessels that create anastomoses between the primary circulation (segmental artery, SA) and the segmental lymphatic (SL) vessels. **B:** ALCs in glass catfish (outlined in black boxes) are linearized during hypoxia. **C:** ALCs in transgenic zebrafish tail region close to the SA and lymphatic vessel (LV) as shown by enhanced green fluorescent protein (EGFP)-positive structures that include blood and endothelial cells and lymphatic endothelial cells that express EGFP. **D:** hypoxia-induced dilation and blood perfusion in a collecting duct lymphatic vessel (CLV). Dashed lines indicate the border of the CLV. **E:** dilation of the lymphatic thoracic duct (TD) under hypoxic conditions. Dashed lines indicate the border of the TD. **F:** summary of vessel diameters for ALC, lymphatic vessel, and segmental artery under normoxia (open bars) and hypoxia (solid bars). Note that hypoxia increases diameter of ALCs and lymphatic vessels but not segmental arteries. **G:** linearization of ALCs under normoxia and hypoxia. [Figure reproduced from Jensen et al. (63) with permission from the Proceedings of the National Academy of Sciences.]
lymph hearts (sump pumps) and must be moved this vertical distance against gravity to be pumped back into the vascular circulation by the lymph hearts. Salamanders and caecilians do not have a high interstitial compliance and are more “fishlike” with tight skin. Thus the anatomical arrangement found of the lymphatic system of anurans is unique among both amphibians and vertebrates.

Anurans generally have two pairs of dorsal lymph hearts (67, 81). Both pairs are innervated by spinal nerves (28, 84, 92, 93) and are under feedback control of the arterial baroreceptors (23, 121) as well as hormonal control by angiotensin II and arginine vasotocin (27). Lymph hearts generate pressures of ~1.0–1.5 kPa at rates of ~40–60 beats/min (23, 27, 65, 66, 119). Lymph hearts are critical to vascular homeostasis in anurans: their destruction results in hemoconcentration, interstitial edema, and death within a few days (124). Anurans with cauterized lymph hearts are unable to replace fluids lost through hemorrhage (7). Clearly, lymph heart transport is critically important in maintaining blood volume and cardiovascular homeostasis, and anurans provide the clearest case for the necessity of negative feedback control of lymphatic flux to maintain plasma volume.

Lymph hearts consist of three tissue layers, a tunica interna intima consisting of an endothelial cell lining, a middle tunica media that contains the musculature of the lymph heart, and an outer tunica externa made up of fibroelastic tissue (89). In Xenopus, as in mammals, the endothelial tissue arises from the blood vasculature and requires the homeobox transcription factor prox1 (83). The lymph heart musculature, however, is under different developmental control and expresses the skeletal muscle marker myoD, rather than cardiac markers (89). In the absence of Hedgehog signaling and Engrailed-1 knockdown, lymph heart muscle fails to develop despite normal development of the endothelium, and embryos develop edema (89). The separate developmental pathways for endothelial and lymph muscle may provide the basis for the origin of the jugular (endothelial) lymph sac in mammals, which has the same anatomical location as the anterior lymph hearts of amphibians (89).

Lymph heart output can be rapidly modulated by both rate and stroke volume changes (65, 66). Lymph heart stroke volume is ~20% of end-diastolic volume, which is ~10 μL/kg measured by conductance manometry and up to 29 μL/kg measured by ultrasound (24). Lymph heart stroke volume varies widely (66), but little is known of the effect of physical factors, such as interstitial fluid pressure or venous pressure, on regulation of lymph heart stroke volume. Nevertheless, lymph heart stroke volume is clearly determined, in part, by lymph input (like cardiac heart stroke volume). This is apparent from the following three findings: 1) lymph heart stroke volume declines with dehydrational fluid loss, despite no change in lymph heart systolic pressure (66); 2) postural shifts in anesthetized animals that favor pooling of lymph around the posterior hearts cause a dramatic rise in stroke volume (66); and 3) lymph heart output in general has been reported to correlate with the rate of lymph formation (6). However, direct measurements of lymph heart pressure-volume (P-V) indicate that stroke volume is not correlated with preload variables, as predicted by a Frank-Starling mechanism (Fig. 5; 24). Rather, lymph heart stroke volume appears to be determined by venous (afterload) pressure, the pressure against which the lymph hearts pump (24). Interestingly, anuran lymph hearts share some common characteristics of isolated mammalian lymphatics. Both lymph hearts and lymphangions increase contractility in response to increased input pressure, and stroke volume is limited by increased afterload (24, 26, 97). Both produce similar systolic pressures (~1 kPa), but lymphangions produce greater stroke volumes in response to increased preload (i.e., Starling mechanism) whereas lymph hearts do not.

Given that lymph heart output is primarily determined by afterload, the main problem facing anurans is how lymph can be moved from ventral regions to the dorsally located lymph hearts. We found that anuran amphibians have three mechanisms to move lymph from ventral lymph sacs to the dorsally located lymph hearts. First, lymph is moved along the hindlimb in a distal to proximal direction owing to differential compliance of the lymph sacs, a mechanism that we have termed a “compliance pump” (48). As lymph enters the various hindlimb sacs (foot, calf, thigh), the lower compliance of the distal (foot) sac creates a higher pressure than the more proximal (calf, thigh) lymph sacs, thus lymph moves in the distal to proximal direction because of the pressure gradient created by the differential lymph sac compliance. However, the pressures generated are small (<20 Pa) and although able to move lymph horizontally along the hindlimb, are not sufficient to move lymph vertically; other mechanisms are necessary to move lymph from the ventral portion of the animal to the dorsally located lymph hearts, a vertical distance equivalent to ~200 Pa (see Fig. 6). This compliance pump mechanism appears to be important only in terrestrial/semiterrestrial species (e.g., cane toads and bullfrogs) because aquatic species (e.g., African clawed frogs) do not have differential hindlimb lymph sac compliance (48). This might be expected given there is no gravitational influence on lymph movement for aquatic species (see below).

The second mechanism that promotes the vertical movement of lymph is the contraction of various skeletal muscles, many of which insert on the skin (31, 32). Contraction of these muscles, which are also closely associated with lymph sacs, alter lymph sac compliance and volume and, therefore, cause
movement of lymph into or out of lymph sacs. The actual movement of lymph is difficult to quantify for individual sacs because the initial volume of each sac determines its compliance and movement of lymph is dependent upon the compliance of neighboring sacs. The skeletal lymph muscles of the posterior region of anurans were described nearly two centuries years ago (cf. Ref. 33), but in many cases the function of these muscles was unknown. Because many of these muscles insert on the skin, their function remained elusive (47). We used measurements of lymph sac pressures combined with electromyography of the putative lymph muscles to define their role in lymph movement (31). Furthermore, there are both similarities and differences in the presence, absence, and degree of development of some of these lymph muscles. This has allowed us to make phylogenetically independent determinations of form and function in these muscle groups (32). Our analysis of over 400 species of anurans, from a variety of habitats, indicate there has been bidirectional selection within these muscle groups with greater elaboration of these muscles in more terrestrial species and reduction in more aquatic species (32). The degree of development of these muscles is obviously correlated with habitat; corresponding rates of lymph flux are also correlated with both habitat and lymph muscle development in three species of anurans that span the environmental gradient from aquatic to terrestrial habitats (51).

The third mechanism that is responsible for the vertical movement of lymph is lung ventilation (Fig. 6; 43). Changes in lung volume associated with ventilation facilitate lymph movement by changing the volume of the lymph sacs surrounding the lungs, in particular the large subvertebral lymph sinus that is located in the dorsal space between the lungs and vertebral column. The subvertebral sinus has direct connections with the anterior and posterior lymph hearts in anurans. During lung inflation (inhalation), the increase in lung pressure is transmitted to the surrounding lymph sacs and this facilitates movement of lymph toward the lymph hearts by “squeezing” the lymph in the sacs. During lung deflation (exhalation), the reduction in lung volume causes an increase in the volume of the subvertebral sinus because the two structures are attached through a thin pleural membrane. This causes pressure in the subvertebral space to decrease, thus providing a “suction” effect to pull lymph into the subvertebral sinus (43). Reductions in subvertebral sinus pressure during lung deflation are of sufficient magnitude to move lymph from ventral to dorsal locations (43). It is also clear that skeletal muscle contraction and lung ventilation act in a coordinated fashion to move lymph. Cutting the insertional tendons of the m. gracilis minor, m. sphincter ani cloacalis, and m. piriformis significantly reduced lymph flux rates in cane toads (50). The reduction of lymph flux by tendon ablation required removal of all three muscles, indicating there is redundancy in the role of the lymph muscles for mobilizing lymph. In a separate experiment, inserting a plastic coil into the subvertebral sinus to prevent ventilatory-induced changes in subvertebral volume also significantly reduced lymph flux rates to the same degree as tendon ablation of the lymph muscles (50). These experiments indicate that both lymph skeletal muscles and lung ventilation have a significant impact on lymph flux rates in anurans (Fig. 7). Given the importance of the lungs for determining lymph flux rates in anurans, we hypothesized that lung morphological properties such as lung volume and lung compliance might be correlated with lymph flux rates in anurans. There are significant family level differences in lung volume and compliance, with higher values in the more terrestrial Bufonidae (toads) compared with intermediate values in semiaquatic Ranidae (frogs) and the lowest values in the totally aquatic Pipidae (clawed frogs) (44). Moreover, the values for lung volume and compliance were correlated with those species for which lymph flux rates have been measured (44, 50). These data suggest that greater lung volumes and lung compliance values

![Fig. 6. Schematic diagram of the anatomical arrangement of the hindlimb lymph sacs for the cane toad. Arrows indicate pathways for lymph flux leading to the posterior LH. Relevant pressures are indicated in hindlimb lymphatic sacs and the gravitational potential energy (PE) that must be overcome to move lymph from ventral regions to the dorsal LH. The 3 types of pumps (compliance, skeletal muscle, and respiratory) and where they act to facilitate lymph movement are shown (see text). [Figure modified from Hillman et al. (47) with permission from the University of Chicago Press.]

![Fig. 7. Lymph flux rates measured from the femoral lymph sac after tendon ablation of skeletal lymph muscles or placing a plastic coil into the subvertebral lymph sac to prevent complete filling of the lung in cane toads. Ablation of the m. sphincter ani cloacalis (SAC) and m. abdominal crenators (Ab Cr) or the m. piriformis (Piri) is required to cause a significant reduction in lymph flux. Lymph flux also decreases significantly when the lungs are prevented from fully expanding. [Figure adapted from Hillman et al. (50) with permission from the Journal of Experimental Biology.]](https://www.jap.org)
allow greater lymph flux rates in more terrestrial species that require greater lymph mobilization to maintain cardiovascular homeostasis.

The regulation of lymph flux by the coordinated action of lymph skeletal muscles and lung ventilation begs the question as to how lymph mobilization is regulated. One hypothesis is that central arterial pressure/volume homeostasis is the key to lymph mobilization and that lung ventilation and lymph muscle contraction are effectors of the baroreflex. Previous work has shown that lymph heart frequency is inversely correlated with peak arterial pressure, and this correlation is abolished by acute transection of the recurrent laryngeal nerve, a primary baroreceptor nerve, in cane toads (23). Lung ventilation can be altered by changes in mean arterial pressure, indicating that it is linked to the baroreflex (45). There have been few studies in ectothermic vertebrates of a link between blood pressure and breathing, although there is a large literature on this topic for mammals (see Ref. 80 for a recent review). The studies with amphibians would indicate there is a significant influence of blood pressure on breathing and that the proximate role is regulation of lymph flux. The loose coupling of arterial blood gases and ventilation in anurans (22) may be explained by the finding that lung ventilation provides a significant cardiovascular function of lymph mobilization. There has been speculation about the origin of the link between blood pressure and breathing in mammals (80); the amphibian studies suggest that the origin of this reflex may lie in the role of lung ventilation to mobilize lymph in anuran amphibians. Lymph hearts, lymph skeletal muscles, and lung ventilation all appear to be under control of negative feedback loops that may be linked to pressure and/or volume sensing in the vascular space. Major questions to be answered are how these various feedback loops are integrated in the central nervous system, how the effectors are coordinated to regulate lymph flux and plasma volume, and whether these feedback loops also exist in the lungfish or developed in the water-land vertebrate transition.

LYMPHATIC FUNCTION IN REPTILES AND BIRDS

Very little is known about the physiology of lymphatics in reptiles and birds as it relates to plasma homeostasis. Reptiles show large intraclass variation in lymphatic vessel and lymph heart structure, but the basis for this variation is unknown (67). On the other hand, birds show much less variation in the lymphatic system except for the presence of posterior lymph hearts in the large ratites (e.g., ostrich and cassowary; 67). Indeed, basic cardiovascular variables such as vascular/interstitial volume and compliance have not been measured in these groups, so there is a large gap in our knowledge of the magnitude of lymphatic flux and its role in plasma volume maintenance. Lymph hearts are widespread among the reptiles and have been described in snakes, lizards, turtles, and crocodilians (19, 67, 79). In many snakes, the lymphatic system is extensive and forms perivascular lymphatic networks associated with the major arterial and venous vessels (19). Posterior lymph hearts in many reptiles are housed by modifications of the posterior ribs and pelvis. For example, in lizards and snakes the posterior vertebrae near the pelvis are distinguished from more anterior vertebrae by the absence of ribs and enlargement of the transverse processes, forming a “fork” in which the lymph heart is contained (67). In some cases transverse processes have a small “cup” indentation into which the lymph heart fits. Similar anatomical features have been found in large dinosaurs and, based upon anatomical measurements, it has been estimated that the posterior lymph heart of brontosaurus (Apatosaurus excelsus) had a volume of ~8 liters (67). Lymph hearts of the Eastern painted turtle (Chrysemys picta picta) generate a pressure of ~0.32 kPa at a rate of ~38 beats/min (116). These values are lower than in amphibians, suggesting that lymph heart output in turtles may be lower than for anurans; however, lymph stroke volume has not been measured in turtles. Given the high rate of plasma turnover in amphibians compared with other vertebrates, greater lymph heart outputs would be expected in amphibians.

Reptiles also have a remarkable capacity to maintain blood volume when hemorrhagogically stressed (73–77). A transcapillary (Starling) mechanism has been suggested for this regulation (77, 102), but this hypothesis has not been rigorously tested for reptiles. Reptiles also have reflexive ventilatory and muscular activity responses consistent with decreasing interstitial compliance during hemorrhage (73, 76), suggesting that effectors similar to those in amphibians may be present to facilitate lymph movement with hypovolemic stress. There is also evidence for differences in tail interstitial compliance consistent with interspecific tolerance of tilt-induced hypotension (74). Recent studies using lizard tail loss (autotomy, a behavioral escape mechanism) as a model for lymhangiogenesis (10, 25) have shown for a gecko (Christinus marmoratus) that, following voluntary tail loss, tail regeneration occurs without any associated lymphedema. Tails developed lymphatic vessels early in the regeneration process and the lymphatic system was highly effective during tail regeneration at removing fluid from the interstitial space. There are clear similarities between reptiles and amphibians in variation in interstitial compliance and ventilatory responses to hypovolemia, suggesting a role for lymphatics in plasma volume regulation that remains unexplored.

Similar to the situation in reptiles, lymph hearts and vessels in birds are known primarily from anatomical descriptions, and there are no studies that have characterized a role for the avian lymphatic system for maintaining plasma volume. Avian embryos have a pair of lymph hearts that return lymph in ovo from the extraembryonic membranes (120). These hearts partially degenerate after hatching (9). Flightless ratite birds apparently use a lymphatic pressure mechanism, rather than a blood vascular mechanism, for penile erection (12), and lymph hearts near the copulatory organ assist the return of lymph from the penis to the venous system (13).

Birds also have a remarkable capacity to maintain blood volume after hemorrhage or dehydration (16, 17, 30, 72, 91). They have high arterial pressures and presumably high capillary pressures coupled to low colloid forces. Thus it is difficult to imagine that a Starling-based mechanism can account for their blood volume regulatory capabilities during dehydration and hemorrhage owing to the presumably large efflux forces at the capillary level, although it has been the mechanism invoked to explain mobilization of interstitial fluid to the plasma space (16). A clear unresolved issue for both reptiles and birds is the role of lymphatic return in the maintenance of plasma volume.
CONCLUSIONS AND PERSPECTIVES

We suggest that some important lymphatic-related variables (lymphatic flux, vascular compliance, interstitial compliance, whole body filtration coefficients) have remained in the recesses of comparative cardiovascular biology. However, their measurement is central to our ability to understand the comparative role of the lymphatic system in plasma volume regulation and the evolution of the vertebrate cardiovascular system. The lymphatic system is poorly described for nonmammalian vertebrates, but developmental studies point to highly conserved molecular pathways for lymph vessel formation in zebrafish, anurans, and mammals. The evolution of the lymphatic system appears to have originated in teleost fishes, perhaps with direct connections to the primary vasculature, using the energy of the cardiac heart to propel lymph through lymph vessels. This was followed by accessory lymph hearts, originating in the Dipnoi and Amphibia to pump lymph into the venous system. Most of our knowledge of lymphatic function in nonmammalian vertebrates is from anuran amphibians, which are highly derived and specialized. The anuran body plan is also unique in having large subcutaneous lymph sacs that confer a large interstitial compliance; this anatomical feature appears to have evolved to maximize lymph storage capabilities. Recent work has revealed a unique set of effectors (specialized skeletal muscles and lung ventilation) that are coordinated to mobilize lymph to the lymph hearts. Given that anurans were the first vertebrates to successfully colonize a large variety of terrestrial habitats, a large interstitial compliance appears to have been a successful evolutionary solution to the problems associated with maintaining plasma volume in the face of dehydration in the water-land transition. By contrast, a large interstitial compliance in mammals is associated with pathological conditions that cause edema and are difficult to ameliorate owing to the imbalance of forces that maintain the edematous state. Lymph hearts are retained in reptiles and some birds, but in mammals they were replaced with contractile lymph vessels capable of intrinsic pumping of interstitial fluid throughout the lymphatic system, and the skeletal muscle pump, into the venous circulation. Perhaps a key adaptation in mammals was the reduction in net transcapillary flux that allowed lymphatic pumping confined to the lymphatic vessels. The reduction in overall lymphatic return may have allowed the lymph system of mammals to assume a greater role in immune surveillance, including the proliferation of lymphoid tissue that is far more developed in mammals compared with other vertebrates.

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