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SHORT COMMUNICATION

Visualising lymph movement in anuran amphibians with computed tomography

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ABSTRACT

Lymph flux rates in anuran amphibians are high relative to those of other vertebrates owing to 'leaky' capillaries and a high interstitial compliance. Lymph movement is accomplished primarily by specialised lymph muscles and lung ventilation that move lymph through highly compartmentalised lymph sacs to the dorsally located lymph hearts, which are responsible for pumping lymph into the circulatory system; however, it is unclear how lymph reaches the lymph hearts. We used computed tomography (CT) to visualise an iodinated contrast agent, injected into various lymph sacs, through the lymph system in cane toads (*Rhinella marina*). We observed vertical movement of contrast agent from lymph sacs as predicted, but the precise pathways were sometimes unexpected. These visual results confirm predictions regarding lymph movement, but also provide some novel findings regarding the pathways for lymph movement and establish CT as a useful technique for visualising lymph movement in amphibians.

KEY WORDS: *Rhinella marina*, Lymph hearts, Lymph sacs, Imaging, CT

INTRODUCTION

The lymphatic system of anuran amphibians is composed of interconnected subcutaneous spaces separated by one-way valves (Kampmeier, 1969). These large spaces create a high interstitial compliance that exceeds that of any other group of vertebrate (Hillman et al., 2004). The rate of lymph formation through capillary filtration is also higher in anurans than in any other vertebrate owing to relatively 'leaky' capillaries, and this, in combination with the large interstitial compliance, creates a situation where Starling forces cannot account for fluid balance at the capillary level (see Hillman et al., 2004; Hedrick et al., 2013). Blood volume homeostasis in anurans, therefore, depends on the ability to mobilise lymph from lymphatic sacs to the lymph hearts, which actively return the lymph to the venous side of the circulation (Hillman et al., 2004; Hedrick et al., 2013). Lymph hearts are critical for fluid balance in anurans and their destruction results in immediate haemoconcentration and the inability to compensate for haemorrhage (Baustian, 1988).

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The major challenge to fluid homeostasis for anurans is to move the newly formed lymph against gravity towards the dorsal lymph hearts (Hillman et al., 2004). Anurans have solved the problem of vertical lymph movement by two distinct mechanisms. Firstly, specialised skeletal lymph muscles actively contract and change the compliance of lymph sacs, thus moving lymph dorsally toward the lymph hearts (Drewes et al., 2007). The lymph muscles are skeletal muscles that have been described previously (see Kampmeier, 1969; Winokur and Hillyard, 1992), but there was no definitive function ascribed to these muscles until it was shown that they are directly involved in lymph movement (Drewes et al., 2007; Hillman et al., 2010). A unique feature of some of these muscles is that they insert on the skin, and are thus in position to directly change the compliance and pressure of subcutaneous lymph sacs (Drewes et al., 2007; Drewes et al., 2013). Secondly, lung ventilation, working in concert with lymph muscles, assists the vertical movement of lymph (Hedrick et al., 2007). The primary hypothesis for the role of the lungs is that, during exhalation, the subcutaneous lymph sac, which is located dorsally to the lungs, expands and creates a negative pressure that draws lymph from ventral locations into the subcutaneous sac (Hedrick et al., 2007). Skeletal lymph muscle ablation or interference with normal lung ventilation significantly reduces lymph flux rates (Hillman et al., 2010), supporting the hypothesis that specialised lymph muscles and lung ventilation are required for vertical lymph movement in anurans.

Much of our knowledge of how lymph moves in anurans is inferred from anatomical descriptions of the lymph sacs (Kampmeier, 1969), and more recent physiological measurements of lymph muscle activity, lymph sac pressure and lymph flux rates (Drewes et al., 2007; Hedrick et al., 2007; Hillman et al., 2010). However, the actual pathways of lymph movement between various lymph sacs to the lymph hearts remain unclear owing to uncertainties about how the physiological mechanisms provide a driving force for lymph movement through specific channels. We report here the first observations of lymph movement in an amphibian using computed tomography (CT). Although CT technology and higher resolution microCT have been used to describe anatomical detail in a variety of invertebrate and vertebrate species (Lauridsen et al., 2011), there are no available descriptions of lymph movement or the lymphatic system in vertebrates other than mammals (Zhang et al., 2011). Because the anatomical arrangement of the lymphatic system and lymph turnover rates of amphibians differ significantly from those of the mammalian lymphatic system (Hedrick et al., 2013), it is important and instructive to directly examine lymph movement in amphibians. This study was designed to use direct observations of lymph movement in the cane toad, *Rhinella marina* (Linnaeus 1758), with CT to test predictions about lymph movement that were previously inferred from anatomical and physiological studies.

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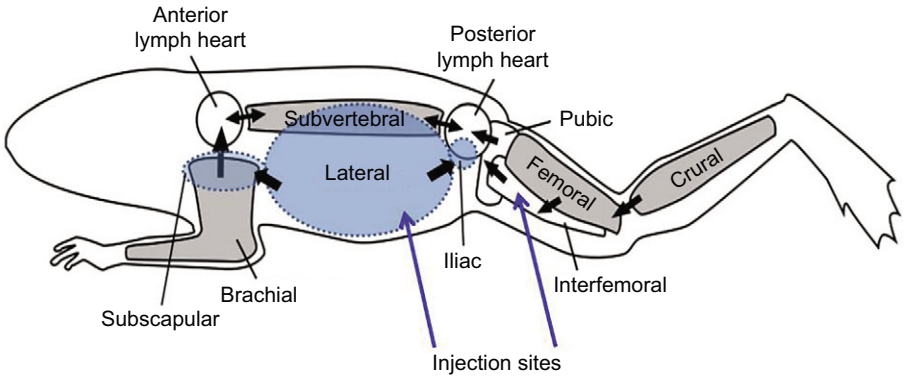

Fig. 1. Schematic diagram and approximate relationships of various lymph sacs in the cane toad. Lymph sacs or sinuses are labelled along with potential routes of lymph movement between lymph sacs (bold arrows). Injection sites of contrast agent into the lateral sac and interfemoral sac for two of the six animals described in this study are indicated (for details, see Fig. 2).

RESULTS AND DISCUSSION

Iodinated contrast agent was injected into a total of six lymph sacs in six toads. Each toad received one injection of contrast agent into a single lymph sac. The lymph sacs injected have been described previously for anurans (Kampmeier, 1969) and were designated as hindlimb sacs (femoral, interfemoral and crural), a forelimb sac (brachial), a large lymph sac lying between the musculature of the body wall and skin (lateral sac), and a large lymph sinus, the subvertebral lymph sac (i.e. cardinal lymph sinus) (Kampmeier, 1969), which is a large sinus ventral to the vertebral column and dorsal to the lungs (Kampmeier, 1969). The general relationship between the various lymph sacs examined in this study is illustrated in Fig. 1. In all cases, contrast agent moved from the point of injection through different lymph sacs to the lymph hearts. Thereafter, the contrast agent was diluted in the blood circulatory system, providing a small increase in whole-body image contrast, but could no longer be used for lymphatic or cardiovascular imaging.

An example of lymph movement from ventral to dorsal and between lymph sacs is illustrated from injection of contrast agent into the left lateral lymph sac. At 9 min post-injection, contrast agent is concentrated in the ventral part of the sac (Fig. 2A). We did not observe contrast agent moving from the lateral to the more ventral abdominal lymph sac, presumably owing to the ventral septum, which separates the lateral and abdominal lymph sacs (Kampmeier, 1969). We did, however, observe contrast agent moving in a dorsal and posterior direction, against gravity, toward the posterior lymph hearts, including labelling of the left posterior lymph heart 39 min after injection into the lateral sac (Fig. 2B). At this point in time, a significant amount of contrast agent also moved dorsally and anteriorly, and became concentrated in the anterior portion of the lateral sac and subscapular sinus (see supplementary material Fig. S1). The lateral lymph sac is bounded anteriorly by the maxillary septum and this also separates the lateral sac from the subvertebral sac (Kampmeier, 1969). The lateral sac is bounded posteriorly by the inguinal septum and the wall of the iliac sinus (Kampmeier, 1969), but contrast agent clearly moved from the lateral sac, through the inguinal septum, to the iliac sinus, which is contiguous with the posterior lymph hearts (Fig. 2B). There was an overall movement of contrast agent from ventral to dorsal and in the anterior–posterior direction (see supplementary material Fig. S1 and Movie 1).

A second example of ventral to dorsal lymph movement upon injection of contrast agent into the interfemoral sac is shown in Fig. 2C,D. The interfemoral sac drains the femoral sac and provides a direct pathway to the posterior lymph hearts via an interfemoral to pubic sac pathway (see Hillman et al., 2004). Within 1 min of the contrast agent being injected in the interfemoral sac, it had moved to a ventral location and, from a posterior view, appeared as a thin layer of contrast agent (Fig. 2C). The contrast agent moved dorsally through the interfemoral–pubic sac pathway (Fig. 2D) and 30 min following injection, contrast agent had filled both posterior lymph hearts (Fig. 2D).

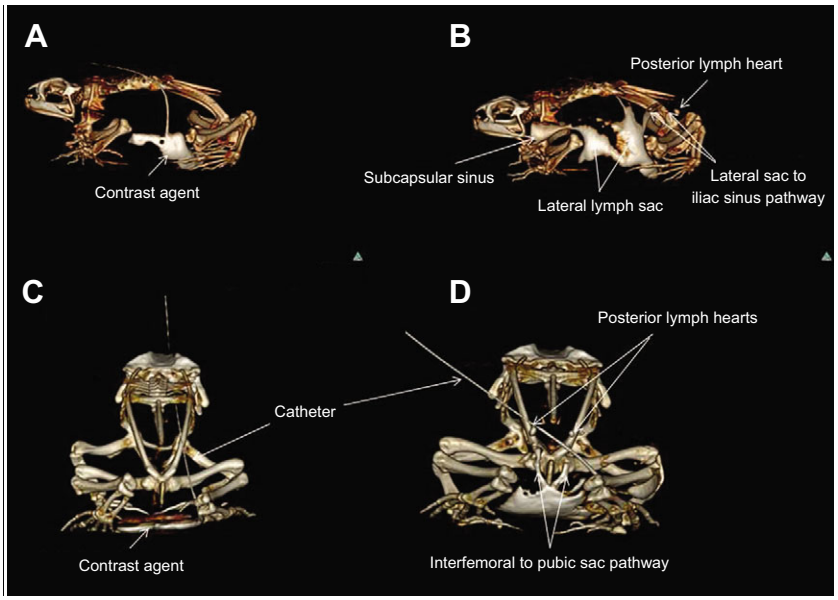

Fig. 2. Movement of iodinated contrast solution injected into the left lateral lymph sac and interfemoral lymph sac over time in two cane toads. (A) Computed tomography (CT) 9 min after injection of a 0.5 ml bolus of contrast solution into the left lateral lymph sac. The contrast-filled catheter is also visible. (B) CT 39 min after injection of contrast solution. Contrast agent moved in anterior, dorsal and posterior directions. Movement of contrast agent from the point of origin occurred in the anterior direction from the lateral sac to the subscapular sinus; movement occurred dorsally within the lateral lymph sac; and there was a pathway from the lateral sac to the posterior lymph heart via a small channel through the iliac sinus. (C) CT 1 min after injection of a 0.5 ml bolus of contrast agent into the interfemoral sac. (D) CT 30 min after injection of contrast agent. The contrast agent moved in a ventral to dorsal direction through an interfemoral sac to pubic sac pathway where it filled the posterior lymph hearts.

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This is the first study to use CT technology to visualise the lymphatic system of any non-mammalian vertebrate. Toads proved to be an ideal model for this technology primarily because they remain quiescent for long periods of time, which enhanced our ability to obtain clear images from non-anaesthetised animals without movement interference. The use of CT technology yielded detailed images of the contrast agent as it moved toward the lymph hearts from the various lymph sacs where it was initially injected. This provided, in some cases, clear pathways through which the contrast agent moved from the injected lymph sac to the lymph hearts (Fig. 2; supplementary material Fig. S1). However, there were two major limitations to the methodology. Firstly, the technique is primarily qualitative. Although we have made quantitative measurements of lymph flux rates using a dye-dilution technique (Hillman et al., 2010), the current CT measurements are strictly qualitative. While it is possible to perform quantitative evaluation on CT data including volumes and permeability using appropriate procedures (Miles and Kelley, 1997; Zhang et al., 2011), we did not do so in the current study because the scanning required prioritising a protocol with non-anaesthetised animals that changed position and posture between sequential scans, and was not feasible for measurements of the disappearance rates of the contrast agent. Because we have previously obtained lymph flux rates using a dye-dilution technique (Hillman et al., 2010), the scope of the current study was to visualise the lymph sacs and possible interconnecting pathways for lymph movement. Secondly, despite the lymph sacs being discrete compartments separated by septa, there is no delineation of individual lymph sacs with CT. We could accurately place cannulae into specific lymph sacs based on anatomical descriptions (Kampmeier, 1969) and previous pressure measurements (Drewes et al., 2007), but once the contrast agent left the injected sac we could not be certain when it passed into specific adjacent or downstream lymph sacs on its route toward the lymph hearts. Nevertheless, contrast agent clearly passed between lymph sacs through known septa en route to the lymph hearts (e.g. Fig. 1). Many of the septa have ostia between contiguous lymph sacs that may be large, irregular gaps, or smaller, circular pores (Kampmeier, 1969). This allows direct movement of lymph between sacs. Our previous work has inferred the movement of lymph through specific pathways based on lymph sac pressures, electromyographic activity of specific lymph skeletal muscles (Drewes et al., 2007) and measurements of lung ventilation (Hedrick et al., 2007). Our visualisation of the pathways for lymph movement confirms our previous hypotheses (e.g. Hillman et al., 2004; Drewes et al., 2007). However, the observations that contrast agent moved in the anterior direction toward the subscapular sinus, which communicates directly with the anterior lymph hearts, when injected into the lateral lymph sac (Kampmeier, 1969), was unexpected.

Based upon our previous work (Drewes et al., 2007; Hedrick et al., 2007; Hillman et al., 2010), our present study now provides a reasonably good picture of how lymph moves from the hindlimbs and posterior aspect of the animal to the posterior lymph hearts. However, lymph movement in the anterior portion of the animal to the anterior lymph hearts is less clear. Vertical lymph movement requires the creation of pressure gradients between lymph sacs to move lymph in the ventral to dorsal direction. Injection of contrast agent into the lateral sac clearly demonstrates that a pressure gradient was created to move the contrast in the anterio-dorsal direction to the subscapular sinus (Fig. 2A,B; supplementary material Fig. S1). It is likely that lung ventilation and the creation of negative pressures in the subcutaneous lymph sac, which communicates with the anterior lymph hearts and the subscapular sinus, are primarily responsible for the creation of the pressure gradient to move lymph anteriorly, but we cannot be certain of this. The results may suggest a role for some of the musculature that is associated with the lateral lymph sac including the internal and external oblique muscles, the rectus abdominis or perhaps musculature associated with the scapular region.

The movement of lymph through the lymphatic system of anurans is complex owing to the large number of subcutaneous sacs and deep sinuses present, and uncertainties about the connections between these spaces. Older anatomical descriptions drew a distinction between subcutaneous lymph reservoirs (i.e. under the skin) and those placed more deeply (lymph sinuses). It is estimated there are over 30 subcutaneous lymph sacs and perhaps over 40 well-defined deep lymph sinuses in anurans (Kampmeier, 1969). Kampmeier noted the difficulty of defining a precise number of sinuses because of their irregular shapes and conformity with visceral organs. Given this level of anatomical complexity, it is not surprising that lymph moves through the lymphatic system in unpredictable ways. For example, injection of contrast agent into the subcutaneous sac (cardinal lymph sinus) revealed a complex pathway through the abdominal and pelvic region as described by Kampmeier (Kampmeier, 1969) (data not shown). These pathways would be very difficult to identify through conventional techniques such as pressure measurements, given the uncertainties about the anatomical relationships between these deep sinuses. Although we have fairly precise anatomical descriptions from the literature (see Kampmeier, 1969), our present study provides a non-invasive method to visualise lymph movement in anurans that could not be determined from anatomical descriptions alone. Thus, CT technology is a powerful tool that provides a more dynamic view of lymph movement between lymph sacs towards the lymph hearts. This represents a significant advance in our ability to define the lymphatic system in anurans and may also prove useful to visualise the lymphatic system of other vertebrates.

MATERIALS AND METHODS

Cane toads (*R. marina*; *N*=6) with snout–vent lengths of ~10–13 cm (~250–350 g) were anaesthetised with buffered (pH 7.0) MS-222, and small bore cannulae (PE 50) were placed into a variety of lymph sacs (subvertebral, lateral, interfemoral, femoral, brachial, crural) for subsequent injection of contrast solution (0.25–1.0 ml Visipaque^{TM}, iodine at 320 mg ml^{-1}). Each toad was instrumented with a single catheter into a single lymph sac. The cannula was introduced through a small cutaneous incision created with an 18 gauge needle above the relevant lymph sac, whereupon the cannula was threaded into the lymph sac and secured to the skin with 4-0 silk. The procedure for placing the catheter took 10–15 min. Animals were allowed 24 h to recover with water in the container to prevent dehydration. Surgical implantation of cannulae and recovery were performed in the Department of Biosciences, Aarhus University. Following recovery, the animals were transported by vehicle, in the same containers as for recovery but without access to water, to Aarhus University Hospital, Skejby, where CT data were collected. For CT, we placed one or two toads in clear plastic containers with plastic lids containing holes for air flow. The cannula was passed through one of the holes and the plastic box was covered with a cloth to reduce visual disturbances. No anaesthesia was used during the acquisition of CT data and toads were without access to water for ~4–8 h over the course of the day when measurements were taken.

Isotropic 0.216 mm^3/voxel CT data were acquired with a 64-Slice Siemens Somatom Definition (Siemens Medical Solutions, Germany) at 80 kV, tube current 80–200 mA. Scanning time was ~12 s. To visualise the movement of contrast from the point of injection, scans were performed repeatedly in a period up to 240 min following injection. The CT images were taken as serial 'snapshots' to image contrast agent movement from the point of injection through the lymphatic system (see supplementary material Fig. S1 and Movie 1). CT data were analysed qualitatively in the DICOM viewer Osirix^{TM}, using the 3D Volume Rendering viewer. The injected

iodine agent provided good image contrast and the movement of the contrast agent was clearly visible over time, although clearance rates differed considerably between lymph sacs. Following completion of the CT scans, the animals were transported back to the Department of Biosciences where the cannulae were removed and the toads returned to the animal care facility. There was no mortality associated with these experiments. The experiments were performed with permission from the Danish Inspectorate for Animal Experimentation within the Danish Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food administration.

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Competing interests
The authors declare no competing financial interests.

Author contributions
M.S.H., K.H., T.W. and J.T. performed the experiments. M.S.H., K.H., H.L. and T.W. analysed the data and prepared the figures. All authors were involved in writing and editing the manuscript.

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