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Unique role of skeletal muscle contraction in vertical lymph movement in anurans

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Summary

Electromyographic (EMG) activity of skeletal muscles that either insert on the skin or are associated with the margins of subcutaneous lymph sacs was monitored for two species of anurans, *Chaunus marinus* and *Lithobates catesbeiana* (formerly *Bufo marinus* and *Rana catesbeiana*). Our hypothesis was that contraction of these muscles varies the volume, and hence pressure, within these lymph sacs, and that this pressure is responsible for moving lymph from ventral, gravitationally dependent reaches of the body to dorsally located lymph hearts. EMG activity of M. piriformis, M. gracilis minor, M. abdominal crenator, M. tensor fasciae latae, M. sphincter ani cloacalis, M. cutaneous pectoris and M. cutaneous dorsi was synchronous with pressure changes in their associated lymph sacs. These muscles contracted synchronously, and the pressures generated within the lymph sacs were sufficient to move lymph vertically against gravity to the lymph hearts. The pressure relationships were complex; both negative and positive pressures were recorded during a contractile event, a pattern consistent with the addition and loss of lymphatic fluid to the lymph sacs. Severing the tendons of some of the muscles led to lymph pooling in gravitationally dependent lymph sacs. These data are the first to: (1) describe a function for many of these skeletal muscles; (2) document the role of skeletal muscles in vertical lymph movement in anurans; and (3) reinterpret the role of the urostyle, a bony element of the anuran pelvic girdle.

Key words: lymph heart, *Chaunus marinus*, *Lithobates catesbeiana*, skeletal muscle, anuran, urostyle.

Introduction

The mid-18th Century saw the beginning of 40 years of successive effort by three distinguished German anatomists, Alexander Ecker, Robert Weidersheim and Ernst Gaupp, of the University of Freiburg on a massive, detailed, illustrated anatomy of the edible frog of Europe, *Rana esculenta*, with various sections published between 1864 and 1904. *Die Anatomie des Frosches* (Ecker, 1864) is the only work of this detail and magnitude on anuran anatomy and remains the standard today. The edible frog of Europe is related and outwardly similar to two common North American species, the leopard frog, *Rana pipiens*, and the bullfrog, *Lithobates* (formerly *Rana*) *catesbeiana.* As a consequence of their ready availability in the field and *Die Anatomie des Frosches*, a published anatomical 'roadmap', these three species became the most widely dissected anuran amphibians in the northern hemisphere for both experimental and educational purposes. Phylogenetically, however, it has since become clear that the genus *Rana* is no more 'typical' of the order than any other genus; neither are these locally common species – the bullfrog, the leopard frog and the edible frog – without significant differences both from each other and from other frogs (see Frost et al., 2006).

Amphibians are unique among terrestrial vertebrates in possessing an integument that is highly permeable to water and thus to loss by evaporation. Moreover, blood volume is challenged by a circulatory system that is profoundly 'leaky' (Hancock et al., 2000); up to ten times more lymph is produced per unit of frog tissue than in mammals. Normal blood volume cannot be sustained without an effective system for lymphatic return to the circulatory system (Zwemer and Foglia, 1943; Baustian, 1988).

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 nexus of the subvertebral, lateral, iliac and pubic lymph sacs. Some anurans, especially some ranid frogs, have more than one pair of posterior lymph hearts (Kampmeier, 1969). The hearts contract rhythmically, driven by spinal motor center stimulation of the muscle fibers (Flindt, 1966) *via* cholinergic synapses (Greber and Schipp, 1986). The fibers seem to be modified skeletal muscle fibers, based on their embryogenesis (Greber and Schipp, 1990), polynucleation and absence of intercalated discs (Schipp and Flindt, 1986) and the occurrence of satellite-like cells (Rumyantsev and Shmantzar, 1967).

The anuran lymphatic system consists of interconnected subcutaneous sacs separated by connective tissue walls that have one-way valves. The valves appear to be controllable rather than being simply passive flaps (Jolly, 1946). The various lymph sacs have been generally described and can vary interspecifically (Carter, 1979).

Two physiological characteristics determine the movement of lymph; first is the compliance of the various lymphatic sacs and second is the pressure within each lymphatic sac. Compliance (Δ volume/Δ pressure) is the product of lymph sac volume and the distensibilty of the lymph sac. Since each sac is surrounded by the highly collagenous dermis, which is not distensible, the major variable in determining lymph sac compliance is probably the initial volume of the lymph sacs. Increased mass-specific lymphatic sac compliance in the direction of lymph flow is one potential mechanism for creating a pressure difference to drive lymph flow between lymph sacs in series based on the formation of lymph, and we call this a compliance pump mechanism (Hillman et al., 2005).

A compliance pump dictates that mass-specific lymphatic fluid influx to each sac would create a higher pressure in more distal sacs as a consequence of their lower compliance, and lymph would flow towards the lymphatic hearts from the distal reaches of the hindlimb. The extensive sinus structure of anuran lymphatic sacs would infer a very compliant system as a consequence of both the obvious significant volume and relative ease at which the skin can separate from the underlying musculature. We have evaluated this mechanism in *Chaunus marinus* and *Lithobates catesbeiana* and reached the following three conclusions (Hillman et al., 2005). First, the compartmentalization of the lymph sacs allows for the creation of a sequential hydrostatic pressure head with the formation of lymph. Second, the pressure is higher in the more distal sacs, creating a series of pressure differences that would move fluid from the distal reaches toward the lymph hearts as a compliance pump. Finally, since the pressure necessary to move the lymph up to the dorsally located hearts is about 0.1–0.2 kPa, the passive pressure created by lymph addition to the subcutaneous sacs is insufficient to accomplish this vertical lymph transport (Hillman et al., 2005).

Hedrick et al. have demonstrated that lung inflation forces lymph in the lateral, ventral and subvertebral lymph sacs anteriorly and posteriorly to the vicinity of the dorsal lymph hearts dorsally and to the axillae of the fore and hindlimbs ventrally (Hedrick et al., 2007). Here, we are concerned with the vertical movement of lymph pooling in sacs in the posterior ventral and inguinal regions of the frog up to the vicinity of the lymph hearts.

Since neither the passive pressure head generated by the formation of lymph nor the reported diastolic pressures within lymph hearts are sufficient to move the lymph to the lymph heart, we were left to consider another mechanism, a skeletal muscle pump mechanism. Muscles within, adjacent to or closely associated with lymph sacs or lymph sac septa likely play a prominent role in the vertical movement of lymph in anurans. Pressure could be varied in lymph sacs by direct or indirect volume changes of the lymphatic sacs as a result of contractions of these muscles. Such contractions would be independent of postural and activity changes and would involve varying lymph sac volumes.

If skeletal muscles act to change the volume, and hence the pressure, within lymph sacs to move lymph to the dorsally located lymph hearts, then we would make the following predictions:

(1) A skeletal muscle pump would act on lymph sacs where the compliance pump cannot account for vertical lymph movement.

(2) Contraction of these muscles, measured by electromyographic (EMG) activity, should be synchronous with pressure changes in these lymph sacs.

(3) Contractile events and pressure changes should be coordinated between lymph sacs.

(4) Actual pressure changes recorded in lymph sacs should be complex, since lymph is moving both into and out of these lymph sacs at any given time.

(5) Pressure changes should be sufficient to move lymph to the dorsally located lymph hearts.

In mammals, there appears to be little or no involvement of skeletal muscles contributing to the pressure changes associated with lymph movement. Smooth muscle in mammalian lymph vessels generates the pressure for lymph movement, and one-way valves ensure lymph movement from peripheral to central lymph vessels (Roddie, 1990; Drake et al., 1996). The anuran subcutaneous lymph sacs do not possess smooth muscle to vary their volume. Here, we argue that there are errors in our attributions of function to some fundamental frog systems and structures, and these include the urostyle and various skeletal muscles inserting on the skin in anurans. We have identified and evaluated the following skeletal muscles in *Chaunus marinus* and *Lithobates catesbeiana* with respect to their role in lymph movement: the M. piriformis (P), the M. gracilis minor (G) and M. abdominal crenator (A), the M. tensor fasciae latae (T), the M. cutaneous dorsi (CD) the M. cutaneous pectoris (CP) and the M. sphincter ani cloacalis (S). Little is known or inferred about some of these muscles, but they are unified by inserting on the skin and/or functioning in a position to vary the volume of a lymphatic sac. We measured EMGs from these muscles in various combinations and measured pressures in corresponding lymphatic sacs to evaluate both the synchrony between the EMG activity of muscles and pressure changes in the lymph sac and the synchrony of contractions between the various muscles.

Materials and methods

Animals

Cane toads (*Chaunus marinus*) and bullfrogs (*Lithobates catesbeiana*) were purchased from commercial suppliers. Frost et al. (Frost et al., 2006), in their morphologically and genetically based analysis of the Amphibia, have provided a strong argument for the recognition of the genera *Lithobates*, which includes the traditional *Rana pipiens* and *R. catesbeiana*, and *Chaunus*, containing the former *Bufo marinus*. In spite of the familiarity of the experimental community with the older nomenclature, the genera *Lithobates* and *Chaunus* for *R. catesbeiana* and *B. marinus* are utilized here to reflect our most recent understanding of the phylogenetic relationships within the Order Anura.

Animals were maintained at 23–26°C with access to water and fed mealworms *ad libitum* 2–3 times per week. All experiments were conducted at 21–23°C under IACUC

protocols approved at Portland State University and California State University, East Bay.

Experimental protocols

Animals (*N*=34 for *C. marinus*, *N*=24 for *L. catesbeiana*) were anesthetized using buffered 0.3% tricainemethane sulfonate (MS 222), and pressure cannulae or EMG electrodes were placed in appropriate sacs or muscles. The cannulae or electrodes were sutured to the skin with 4-0 silk to stabilize their position. EMG and reference leads were constructed from 42 G stranded stainless steel wires. The output of these electrodes was amplified using Lafayette (Lafayette, IN, USA) and A-M Systems (Carlsborg, WA, USA) AC amplifiers, and the integrated signal recorded using a Powerlab (Milford, MA, USA) data acquisition system. Pressure probes were generally Millar (Houston, TX, USA) Mikro-Tip 3-3.5 French sensor attached to 2.2 French catheters. The outputs of these sensors were amplified using Millar Pressure Control Units and recorded using Powerlab data acquisition units. Impedence electrodes were secured to the urostyle and within the cloaca, with EMG electrodes also implanted in the M. piriformis to determine if the urostyle moved with activity of the M. piriformis. Animals were continuously monitored for 8–12 h the day after surgery (12–24 h recovery) in an unrestrained state within a plastic container, and only those time intervals where the animals were quiescent were used for analysis.

Three *C. marinus* (mean mass 96 g) were anesthetized, and the origins of the M. abdominal crenator, M. gracilis minor and M. sphincter ani cloacalis and the insertion of M. piriformis were severed. The cutaneous openings were sutured and the animals allowed to recover. Ten days later, the animals were anesthetized and lymph was collected from a slit in the interfemoral sac by capillary tube in both tendon-ablated and three control toads (mean mass 102 g). Lymph volume was determined from mass (1 g=1 ml).

Morphological materials

Fresh specimens of both *C. marinus* and *L. catesbeiana*, including those used in the experimental protocols, were dissected under a stereo dissecting microscope; limits and configuration of lymph sacs and lymph channels were established by blunt probe. The origins and insertions of the various skeletal muscles discussed below were exposed by dissection. All pertinent observations made with fresh specimens were correlated with examination of the same structures in preserved specimens deposited in the collections of the Department of Herpetology, California Academy of Sciences. Lymph sac nomenclature follows Carter (Carter, 1979); muscle terminology follows Haslam's translation (Haslam, 1889) of Ecker's *Anatomie* (Ecker, 1864) except where otherwise indicated. Our interpretation of the origin and insertion of some of the skeletal muscles described below is non-traditional and will be justified in the Discussion.

The muscles and their lymph sac associations

M. piriformis

This muscle is present and similar in size and configuration in both *C. marinus* and *L. catesbeiana* (see Fig. 1). So far as is known, the M. piriformis is present in all extant anuran species except in members of the pelobatid genus *Pelobates* and some members of the wholly aquatic Pipidae, in which it is either reduced or absent (Cannatella, 1985) (present study). This muscle appears to have been the focus of more attention than any of the others delineated below, especially with respect to the urostyle (coccyx), to which it is attached, and its functional role in the pelvic girdle as a whole. For instance, Green considered the M. piriformis to be analogous to one of the urodelan tail muscles (Green, 1931) and, with virtually all other workers, indicated its origin to be on the urostyle. He suggested that the M. piriformis and urostyle play an important role in limiting the flexibility of the anuran pelvis in leaping. Mahendra and Charan also cite these structures as providing a rigid fulcrum for the movement of the hindlimbs, as well as having a role in the absorption of landing shock (Mahendra and Charan, 1972).

We interpret the M. piriformis as *originating* on the dorsal surface of the femur near its proximal head and *inserting* on the dorsolateral surface of the distal end of the urostyle. In *L. catesbeiana* and *C. marinus*, the insertion of M. piriformis is wholly within the dorsal portion of the pubic lymph sac (Fig. 1B,D). Contraction of the M. piriformis depresses the distal end of the urostyle, changing the volume, and hence pressure, in the pubic sac. In *C. marinus*, volume and pressure changes in the intermuscular lymph sac (Hillman et al., 2004) also occur (see Results). Thus, our observations suggest that the primary function of the M. piriformis is to depress the distal end of the urostyle, in contrast to traditional interpretations such as 'straightening the back' (Emerson and De Jongh, 1980).

M. gracilis minor

Present in both *L. catesbeiana* and *C. marinus*, this muscle is known in all extant anurans except *Phyllobates* (Dunlap, 1960). In *L. catesbeiana*, the M. gracilis minor is a long, narrow, thin, undifferentiated strap-like muscle running the length of the posteromedial aspect of the thigh just beneath the skin (Fig. 1); it is moderately firmly attached to the skin along its posterior aspect by numerous minute fibers of connective tissue. The M. gracilis minor shares a common insertion with the gracilis major on the posterior surface of the knee and originates on thick connective tissue near the posterior apex of the pelvis. The M. gracilis minor runs the length of the interfemoral and femoral lymph sacs but does not insert directly into the integument. However, as it is closely adherent to the skin of the posterior surface of the thigh, any change in configuration of this muscle in *L. catesbeiana* is likely to cause changes in volume and pressure in these sacs.

In *C. marinus*, the shape and configuration of the M. gracilis minor at the knee is similar to *L. catesbeiana*, except that *in C. marinus* a large slip of the M. gracilis minor separates from the main muscle and fans out anteromedially [termed 'M. gracilis minor anterior' by Winokur and Hillyard (Winokur and Hillyard, 1992)] so that bundles of fibers insert broadly and directly on the dermis of the skin of the ventral surface of the thigh (Fig. 1B). At insertion, these muscle fibers span an area at least 2.5 times the width of the muscle slip at its separation point and intercalate with fibers of the

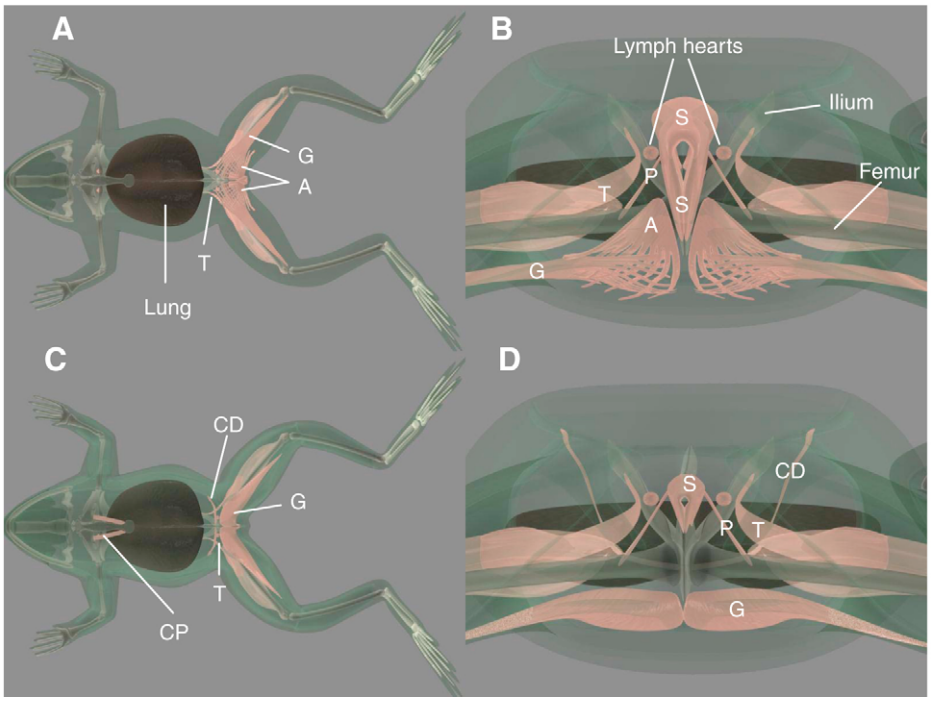

Fig. 1. Stylized models representative of morphological differences in the skeletal muscle presented in this work. (A,B) *Chaunus marinus*; (C,D) *Lithobates catesbeiana*. A and C represent ventral views; B and D represent posterior views. Key: A, abdominal crenator; G, gracilis minor; T, tensor fascia latae; P, piriformis; S, sphincter ani cloacalis; CP, cutaneous pectoris; CD, cutaneous dorsi.

M. abdominal crenator, discussed below (Winokur and Hillyard, 1992) (Fig. 1). The proximal, undifferentiated portion of the M. gracilis minor originates on the pelvis as in *L. catesbeiana*. Noble noted that '*fossorial forms do tend to have greater expansion and attachment of the M. gracilis minor to the skin than do terrestrial or aquatic genera*' (Noble, 1922). The portion of the M. gracilis minor inserting on skin is mostly within the interfemoral lymph sac, thus shortening of these fibers should affect volume and pressure in the interfemoral sac.

M. abdominal crenator [termed 'accessory head of M. gracilis minor' by Cannatella (Cannatella, 1985)]

This muscle was first described in detail by Winokur and Hillyard (Winokur and Hillyard, 1992); it is absent in *L. catesbeiana* and present in *C. marinus* (Fig. 1A,B). The M. abdominal crenator of *C. marinus* is a large fan-shaped muscle, fairly thick at its origin within the pelvic lymph sac on the posterior apex of pelvis; muscle fibers course distally and antero-distally, meeting and overlapped at nearly right angles by the fibers of the differentiated, anteromedially directed M. gracilis minor [M. gracilis minor anterior of Winokur and Hillyard (Winokur and Hillyard, 1992)], forming a latticework along their insertion on the dermis of the ventral skin (Fig. 1B); the insertions conform closely with the anterior and medial margins of the pelvic patch and are mostly within the interfemoral and femoral lymph sacs. Contraction of the M. abdominal crenator, especially in conjunction with the M. gracilis minor, should result in direct effects on the interfemoral and femoral lymph sacs and possibly the pubic sac.

M. sphincter ani cloacalis

First described by Ecker in *Rana esculenta* (Ecker, 1864), this muscle has not, to our knowledge, been described in the literature since, nor has any function been ascribed to it. It is present but reduced in *L. catesbeiana* and thick and well-developed in *C. marinus* (Fig. 1B,D). In *L. catesbeiana*, the paired, flat M. sphincter ani cloacalis originates on the posterior-most apex of the pelvic rim and, tightly adherent to the dermis, passes dorsally to insert on the fibers of the M. compressor cloacalis on both lateral surfaces of the cloaca (Fig. 1D). This muscle is contained within the upper part of the pubic sac; its contraction may serve to depress the cloaca and affect the volume of the pubic lymph sac. In *C. marinus*, the M. sphincter ani cloacalis is very large, rounded and longer than in *L. catesbeiana*. The origin is ventral on connective tissue near the pubic symphysis (within the interfemoral lymph sac), from which it passes dorsally, deep to the origin of the M. abdominal crenator (absent in *L. catesbeiana*), to insert on the compressor cloacalis, as in *L. catesbeiana* (Fig. 1B). This muscle runs along the lateral margins of the entire pubic lymph sac and tightly adheres to the skin on either side of it. Contraction of this paired muscle should directly affect volume and pressure in the pubic lymph sac, and perhaps in the interfemoral sac at its origin.

M. tensor fasciae latae

This muscle is present in both *L. catesbeiana* and *C. marinus*; in both species it originates on the ventrolateral surface of the ileum, approximately at its mid-point; the muscle passes posteriorly at an angle to insert on the anterior fascia of the M. cruralis and M. gluteus magnus of the thigh; in *L. catesbeiana*, insertion is within the proximal third of the length of the thigh; in *C. marinus*, insertion is in the middle third (Fig. 1A–D). Noble (Noble, 1922) noted that this muscle is reduced in ranids and bufonids but well-developed in *Ascaphus* and *Hymenochirus*, less so in other primitive genera, but he did not ascribe a function for it. Drewes (Drewes, 1984) noted its absence in the monotypic

hyperoliid *Kassinula wittei*. In both the bullfrog and cane toad, the M. tensor fasciae latae passes through the dorsal part of the iliac lymph sac and inserts within the femoral lymph sac; contraction of this muscle should affect the volume and pressure in both sacs. The iliac sac may be confluent with the dorsal extension of the intermuscular sac that runs along the dorsal surface of the femur and was first described by Hillman et al. (Hillman et al., 2005). Consequently, the volume of the intermuscular sac might also be varied by postural changes associated with the contraction of the thigh and calf musculature or the M. piriformis contracting and is measurable by simultaneous pressure changes in the iliac sac.

M. cutaneous dorsi

This muscle is absent in *C. marinus.* In *L. catesbeiana*, the M. cutaneous dorsi has no skeletal attachments; it originates on ventral connective tissue superficial to the pelvic disk and passes anteriorly and dorsally between the belly musculature and thigh to a fan-shaped insertion at the dorsal margin of the lateral lymph sac, at or near its junction with the dorsal iliac and femoral lymph sacs (Fig. 1C,D). In *L. catesbeiana*, the fan-shaped insertion runs anteriorly along the margin of the lymph sac and is approximately three times wider than the muscle at its origin. Dugés (Dugés, 1834) described this thin muscle as a '*tensor of the skin of the back*'; the greater part of this muscle and its insertion are within the lateral lymph sac; shortening of this muscle should affect the configuration of lateral, iliac and femoral lymph sacs and, to some degree, that of the dorsal sac in the posterior axillary region.

M. cutaneous pectoris

This ventral muscle is absent in *C. marinus*. In *L. catesbeiana*, the flat, paired, strap-like cutaneous pectoris originates near the third inscription of the M. rectus abdominus of the belly, from the anterior edge of the cartilaginous posterior-most extent of the metasternum of the pectoral girdle (Fig. 1C); uniform in width, it passes at a slight angle anteriorly to insert on the ventral skin along the posterior margin of the pectoral lymph sac septum. To our knowledge, Gaupp is the only author to suggest that contraction of the M. cutaneous pectoris, together with the M. pectoralis abdominus, might affect movement of the lymph of the abdominal and pectoral lymph sacs (Gaupp, 1896). Tyler suggested that the M. cutaneous pectoris and the pectoral lymph septum together constrain the distensibilty of the male vocal sac, i.e. the M. cutaneous pectoris must be reduced or lost in order to have a highly inflatable vocal sac (Tyler, 1971). Drewes established the absence of this muscle in all hyperoliid frogs, but noticed its presence in both males and females of all ranid and rhacophorid species that he examined, except *Arthroleptis* (Drewes, 1984). Contraction of the M. cutaneous pectoris should pull the central portion of the pectoral lymph septum, simultaneously affecting pressures in the pectoral and abdominal lymph sacs.

Data analysis and statistics

Correlations of pressure between pairs of lymph sacs, or correlations between EMG signals between pairs of muscles, were analyzed as the percentage of times that one event (i.e. pressure or EMG) occurred with a second event of the same type. For example, we counted the number of times a muscle was active during the recording period and determined the number of times a second muscle was also active simultaneously with the first muscle. We expressed this correlation as 'percent of events' for each muscle pair or each pair of lymph sacs that were analyzed. All percentage changes were converted to their arcsine values prior to analysis with a one-way analysis of variance (ANOVA).

For each change in lymph sac pressure we determined the pressure prior to (P_{pre}) and following (P_{post}) the pressure change event. This pressure difference (P_{pre}–P_{post}) was calculated for each lymph sac pressure 'event'. The actual pressure event was given as ΔP and was calculated as the mean pressure of the integrated signal minus P_{pre}. Correlations between (P_{pre}–P_{post}) and the mean event ΔP were analyzed by linear regression. All data analyses were done with Graphpad Prism (v. 5.0; San Diego, CA, USA).

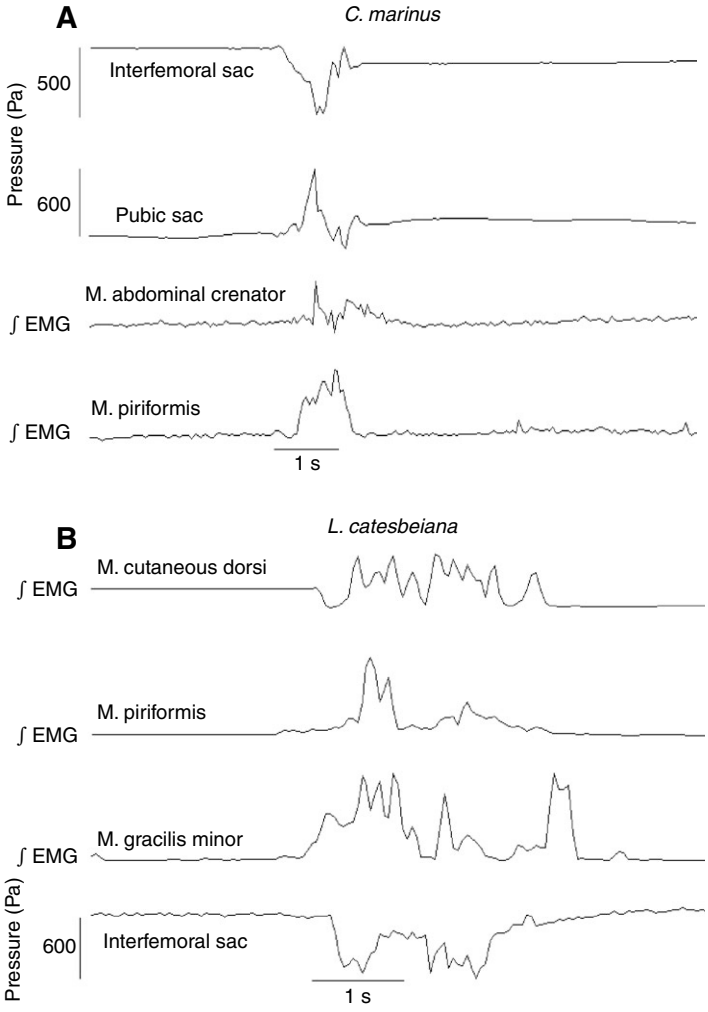

Fig. 2. Representative traces of the synchrony between integrated EMG activity of the skeletal muscles indicated and their synchrony with pressure changes in the lymphatic sacs in (A) *C. marinus* and (B) *L. catesbeiana.*

Data are reported as means ± s.e.m. and *N* (numbered animals) or *n* (number of observations).

Results

Synchrony

Pressure changes in the femoral, interfemoral, pubic, iliac and intermuscular sacs were always correlated with simultaneous EMG activity of skeletal muscles in both *C. marinus* and *L. catesbeiana*. Representative traces of this synchrony are presented in Fig. 2. For *C. marinus*, the EMG activities of M. abdominal crenator/gracilis muscles were synchronous with M. sphincter ani cloacalis and M. tensor fascia latae over 75% of the time; however, M. piriformis was active with abdominal crenator/gracilis about 50% of the time (P<0.05, N=7; ANOVA). When the M. piriformis was active, then the abdominal crenator complex also contracted in concert with all the other muscles from 75% to 95% of the time. Consequently, EMG activity of the M. abdominal crenator/M. gracilis minor, M. sphincter ani cloacalis, M. tensor fascia latae and M. piriformis is coupled to pressure changes in the interfemoral, pubic, intermuscular and iliac lymph sacs. For *L. catesbeiana*, activity of the M. cutaneous dorsi, M. piriformis, M. cutaneous pectoris, M. gracilis minor and M. tensor fasciae latae were always synchronous with one another from 90% to 100% of the time. Given the universal synchrony of all the skeletal muscles measured, the synchrony of pressure changes was measured only for M. piriformis and M. cutaneous dorsi, within intermuscular and pubic pathways. Pressure changes were synchronous with EMG activity of these two muscles.

Activity of the M. piriformis was associated with changes in the impedance between the urostyle and cloaca, indicating a change in the distance between the structures. The impedance both increased and decreased, suggesting that the M. coccygeoiliacus, a urostyle elevator, also plays a role in the urostyle deflection (Fig. 3).

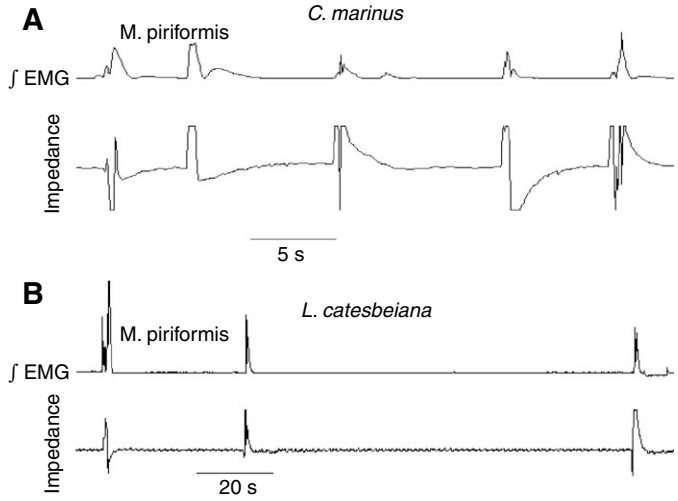

Fig. 3. Examples of correlation between EMG activity in M. piriformis and impedance change between the urostyle and cloaca in (A) *C. marinus* and (B) *L. catesbeiana*.

Pattern of pressure changes

There are two parallel pathways for the return of lymph from the hindlimb to the posterior lymph hearts in anurans: intermuscular to iliac sac and interfemoral to pubic sac. Skeletal muscle contractions led to a variety of pressure patterns in the interfemoral and pubic sacs. There are three types of pressure change patterns that occur in response to muscle contraction: a combination of an increase and decrease in pressure, a decrease in pressure alone or an increase in pressure alone. Fig. 4 illustrates two types of pressure changes that occurred in the pubic lymph sac. The most prevalent pattern of pressure change (60%) was a 'biphasic' pattern consisting of both increases and decreases of pressure (Fig. 5). For both the interfemoral and pubic sacs, there was only a pressure decrease in about 25%, and only a pressure increase in about 15%, of recorded pressure events. Following a lymphatic skeletal muscle contraction, there was an increase in lymphatic sac pressure (i.e. P_{pre}–P_{post}<0) about 50% of the time (indicating the entry of lymph) and a decrease (i.e. P_{pre}–P_{post}>0) about 50% of the time (indicating lymph efflux). In less than 10% of events, there was no change in either interfemoral or pubic sac pressure, indicating no net lymph flux.

Magnitude of pressure changes

The mean pressure changes (ΔP) measured during a contraction event were all greater than 200 Pa (2 cm H_2O) for the pubic pathway of *C. marinus*, and 60–80 Pa for *L. catesbeiana*, whether positive or negative (Table 1). The mean pressure changes measured during a contraction event ranged from 160 to 170 Pa for the intermuscular pathway of *C. marinus* and 130–240 Pa for *L. catesbeiana* (Table 1). Consequently, mean pressure changes (either positive or negative) are sufficient to move lymph dorsally the 1–2 cm required to reach the posterior lymph hearts, even for the largest individuals of both species, whether *via* intermuscular or pubic pathways. The mean pressure change recorded during a contraction event (ΔP)

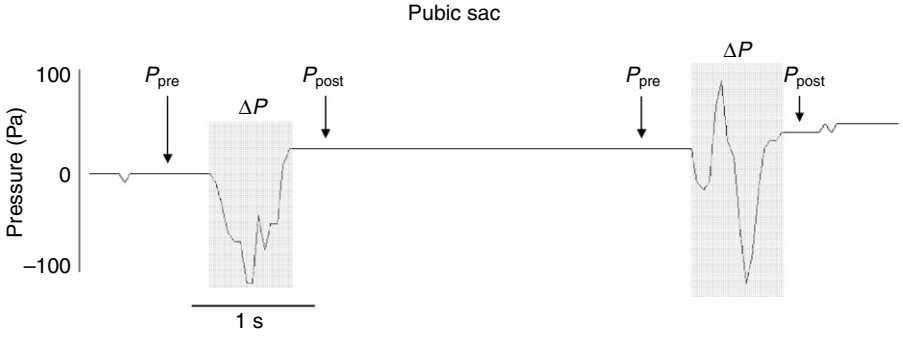

Fig. 4. Pattern of pressure change in the pubic sac during a contractile event. Two examples of the types of pressure changes recorded from lymph sacs. The first event illustrates a 'decrease only' pattern while the second event illustrates the more common 'biphasic' increase/decrease pressure pattern. Pressure before (P_{pre}) and following (P_{post}) the pressure event (shaded area; ΔP) are shown (see text).

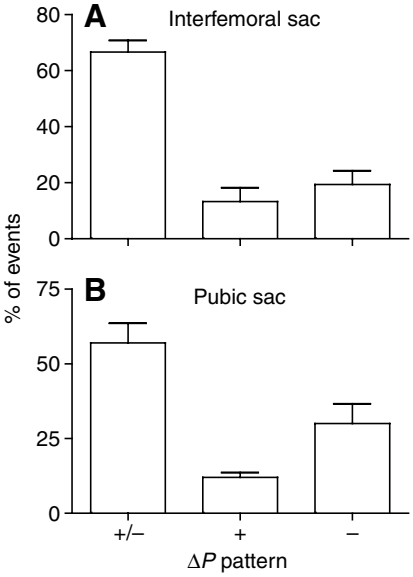

Fig. 5. Frequencies of varying patterns of pressure change for the (A) interfemoral and (B) pubic lymph sacs of *C. marinus*. The majority of pressure events are 'biphasic' [increase (+)/decrease (–)] compared with increase-only (+) or decrease-only (–). Values are means ± s.e.m.

was significantly correlated (r^2=0.47–0.88; P<0.001, n=74–200) with the P_{pre}–P_{post} contractile event pressure change in the intermuscular, iliac, interfemoral and pubic lymph sacs (Fig. 6). This P_{pre}–P_{post} pressure change will reflect the compliance of any sac and the actual net volume of lymph added or removed from that sac during the event, whereas the mean event ΔP reflects ability of a lymph sac to generate pressure (positive or negative) to move lymph. The significant correlations indicate that the mean event pressure change is strongly correlated to whether there was a net gain or loss of lymph during the contraction event. For both species, the P_{pre}–P_{post} pressure change was negative about 45% of the time and positive about 45% of the time, indicating an equal frequency of net lymph loss and gain. Only very infrequently was there no net pressure change (<5%) or change in lymph volume.

Interfemoral lymph sac volume

An average of 0.028 ml of lymph (N=3) was collected from the interfemoral lymphatic sac of anesthetized control toads, while an average of 0.62 ml of lymph (N=3) was collected from each tendon-ablated toad.

Discussion

This study provides the first experimental evidence that lymph movement in anurans involves skeletal muscle contraction and also provides the first functional description for some of these muscles. It is clear that changes in lymph sac pressures were correlated with the contractions of a number of skeletal muscles that are associated with these lymph sacs. Furthermore, the magnitude of the pressure changes is sufficient to move lymph vertically to the vicinity of the dorsally located lymph hearts. Thus, the predictions concerning the association between lymph sacs and skeletal muscles (see Introduction) were experimentally validated in this study. The skeletal muscles identified here can clearly contribute to the vertical movement of lymph from the hindlimbs to the dorsally located lymph hearts in the interfemoral/pubic pathway and the intermuscular/iliac pathway. Our description of lymphatic sac-related skeletal muscles and pressure and EMG data support our hypothesis that vertical transport of lymph must be principally accomplished by contraction of skeletal muscles rather than a compliance pump (Hillman et al., 2004; Hillman et al., 2005) or by negative pressures created by the lymph hearts alone (Toews and Wentzell, 1995). Lymphatic movement is also influenced by ventilatory events driven by skeletal muscles (Hedrick et al., 2007).

Our data also allow a reinterpretation of the function of the urostyle, suggesting that it is involved with changing the volume and pressure of the pubic sac, and hence lymph movement, rather than previous interpretations that proposed a locomotory role for the urostyle (Whiting, 1961; Gans and Parsons, 1966; Emerson and DeJongh, 1980; O'Reilly et al., 2000). In the present study, deflections of the urostyle relative to the cloaca were coincident with contractions of the M. piriformis. Because contractions of the M. piriformis cause movement of the urostyle, we interpret the action of the M. piriformis as inserting on the urostyle rather than originating on it. This is contrary to the traditional interpretation of the M. piriformis originating on the urostyle and inserting on the femur (see Emerson and DeJongh, 1980). Impedance changes of the urostyle relative to the cloaca indicate that movement in both the dorsal and ventral directions occurred. It is likely that the coccygeoiliacus, a urostyle elevator, also contracted during these events, thus providing movement in the dorsal direction.

We have identified specific skeletal muscles that have a direct connection to the skin or margins of the lymphatic sacs that carry lymph to the posterior lymph hearts in two anuran species that differ in their ability to mobilize lymph following hemorrhage and dehydration (Hillman et al., 1987; Hillman and

Table 1. *Mean event pressure changes (ΔP) in various lymph sacs recorded during skeletal muscle contraction in C. marinus and L. catesbeiana*

	Lymph sac	Mean positive pressures	Mean negative pressures
C. marinus	Interfemoral	239±44 (67)	–234±45 (71)
	Pubic	218±48 (76)	–317±49 (73)
	Intermuscular	170±26 (80)	–159±23 (82)
	Iliac	71±14 (29)	–130±18 (46)
L. catesbeiana	Pubic	81±12 (31)	–64±12 (39)
	Intermuscular	244±29 (21)	–128±21 (19)

Values are means ± s.e.m. (*n*).

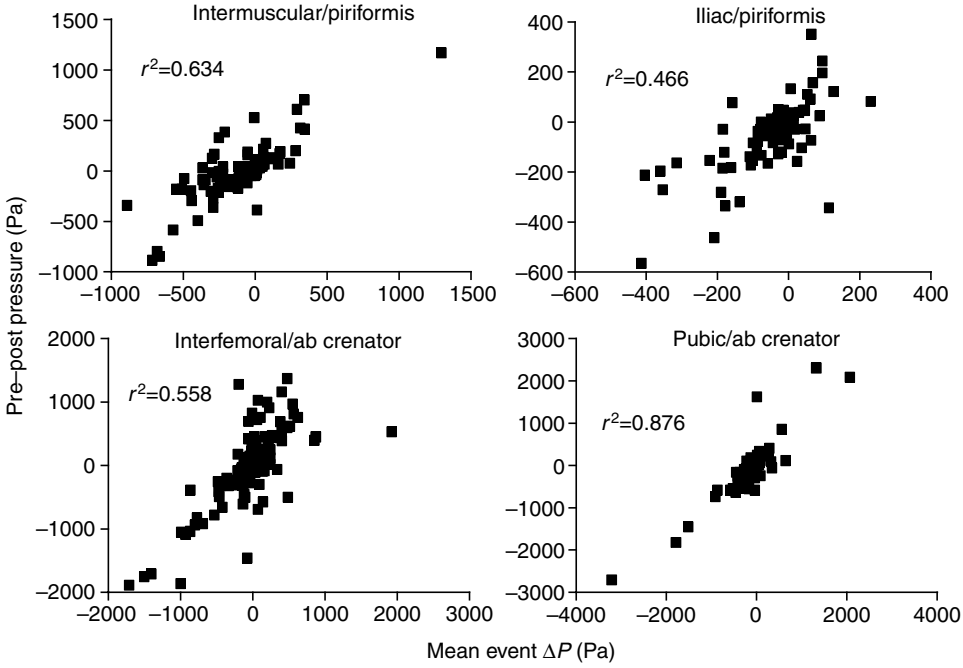

Fig. 6. Correlation between the mean event pressure (ΔP) and the net change (P_{pre}–P_{post}) for the intermuscular, iliac, interfemoral and pubic lymph sacs of *C. marinus*.

Withers, 1988). Some muscles are only found in one of the species: e.g. M. abdominal crenator in *C. marinus* and M. cutaneous dorsi and M. cutaneous pectoris in *L. catesbeiana.* Some muscles are found in both species with little interspecific variation in size or shape: e.g. M. tensor fascia latae and M. piriformis. Two muscles, M. gracilis minor and M. sphincter ani cloacalis, were present in both species but with greater development in *C. marinus* than *L. catesbeiana*. The interspecific variation in size and morphology of some of these skeletal muscles (M. gracilis minor, M. abdominal crenator, M. sphincter ani cloacalis, M. cutaneous dorsi) may provide an instructive system for delineating morphological adaptations that assists lymph movement in more terrestrial species to ameliorate the cardiovascular stresses associated with dehydration. A systematic analysis of this variation in correlation with degree of terrestriality would certainly prove worthwhile.

Intra-lymphatic sac pressure data support our hypothesis that contraction of these muscles varies the volume and pressure of these lymph sacs. This variation in pressure is sufficient to assist lymph movement from adjacent lymph sacs (femoral, crural, lateral) and also provide the force necessary for the vertical movement of lymph to the dorsally located posterior lymph hearts (Table 1). Pressure events were complicated, consisting of different patterns of pressure changes (Figs 4 and 5). This is to be expected if fluid is moving into and out of lymph sacs. This latter interpretation is supported by the observation that pressure normally increased or decreased after a change in lymph sac pressure caused by muscle contraction. In nearly all cases, net lymph sac pressure either increased or decreased, indicating that fluid entered or left the lymph sac, respectively.

Finally, the tendon-ablation experiment demonstrated that preventing the action of the M. abdominal crenator, M. gracilis minor, M. sphincter ani cloacalis and the M. piriformis led to pooling of lymph in the interfemoral lymph sac of *C. marinus*. We interpret this result to indicate that these amphibians were unable to move lymph vertically into the pubic sac; hence, lymph could not be pumped back into the circulatory system *via* the posterior lymph hearts so instead pooled in the interfemoral sac.

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