

Lymph heart in chick – somitic origin, development and embryonic oedema

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The lymph heart is a sac-like structure on either side of avian tail. In some adult birds, it empties the lymph from the copulatory organ; however, during embryonic development, it is thought to circulate extra-embryonic lymph. Very little is known about the origin, innervation and the cellular changes it undergoes during development. Using immunohistochemistry and gene expression profiling we show that the musculature of the lymph heart is initially composed solely of striated skeletal muscle but later develops an additional layer composed of smooth myofibroblasts. Chick-quail fate-mapping demonstrates that the lymph heart originates from the hypaxial compartments of somites 34–41. The embryonic lymph heart is transiently innervated by somatic motoneurons with no autonomic input. In comparison to body muscles, the lymph heart has different sensitivity to neuromuscular junction blockers (sensitive only to decamethonium). Furthermore, its abundant bungarotoxin-positive acetylcholinesterase receptors are unique as they completely lack specific acetylcholinesterase activity. Several lines of evidence suggest that the lymph heart may possess an intrinsic pacing mechanism. Finally, we assessed the function of the lymph heart during embryogenesis and demonstrate that it is responsible for preventing embryonic oedema in birds, a role previously thought to be played by body skeletal muscle contractions.

KEY WORDS: Lymph, Heart, Avian, Embryo, Oedema, Disease, Skeletal muscle, ACh receptor, Immobility, *crooked neck*, *Tailless*, *Rumpless*, *Araucana*

INTRODUCTION

Avian embryos have one pair of lymph hearts on either side of the first free tail vertebrae, situated caudally to the pelvic bones (Romanoff, 1960). In the chick, the lymph hearts are functional only in ovo, returning the lymph from the extraembryonic membranes (Wilting et al., 1999), and after hatching they partially degenerate (Bischof and Budras, 1993). In some birds, e.g. duck and emu, the lymph assists copulatory organ erection of male adults and lymph hearts function postnatally to return lymph from the lymphatic erectile phallus to the venous system (Budras and Berens von Rautenfeld, 1984). Unlike birds, amphibians have several pairs of lymph hearts along the vertebral column which remain functional into adult life. Mammals do not have lymph hearts as their extra-embryonic membranes and placenta are drained by uterine circulation. Propulsion of lymph in adult mammals is achieved by smooth muscle in lymph collectors, contraction of adjacent skeletal muscles and through the action of respiratory pressure changes (Jeltsch et al., 2003).

The avian lymph heart contains endothelial, smooth and striated muscle layers. Its striated musculature has ultra-structural features of both cardiac and smooth muscle so a separate histological category has been proposed (e.g. Budras et al., 1987). Controversy remains regarding its origin with some researchers suggesting it develops either from local mesenchyme or from myotomes (Waldeyer, 1864; Schipp and Flindt, 1968), or is derived from the

embryonic venous system (Fedorowicz, 1913; Jolly and Lieure, 1934). A general agreement is that the lymph heart is mesodermal in origin like all other musculature.

Striated skeletal musculature of the body originates from the somites (Christ and Ordahl, 1995) which give rise to the epaxial muscles that remain locally in the back and to the hypaxial muscles which shift to a more ventral position and form the ventral trunk and limb musculature. Cardiac muscle develops from specialised splanchnic mesoderm (Brand, 2003). Smooth muscle develops locally from mesodermal and mesectodermal cells (Le Douarin, 1982; Wilting et al., 1995).

We re-investigated the composition and development of the lymph heart and for the first time experimentally evaluated its function. We found that it is initially made exclusively of striated skeletal muscle with no smooth musculature. Using the chick-quail lineage tracing, we have shown that cells of the lymph heart originate from somites 34–41. Furthermore we show that the lymph heart has unique properties regarding its innervation profile and its response to neuromuscular blockers. Finally, we demonstrate that simple mechanical obstruction of the lymph heart or complete surgical ablation of the tissue or its genetic absence all resulted in gross oedema formation.

MATERIALS AND METHODS

Chick-quail chimeras

Fertilised White Leghorn chick and Japanese quail eggs were incubated at 38°C and 80% humidity. One of the five most newly formed somites (I–V) (Christ and Ordahl, 1995) with the overlying ectoderm (to facilitate graft orientation) was removed from quail donors (stage HH17–18) (Hamburger and Hamilton, 1951) and used for non-orthotopic transplantations.

Chick recipient embryos were manipulated in ovo at HH17–21. After windowing the shell the embryo was floated with PBS and antibiotics (Hara, 1971) and reflective aluminium foil was temporarily placed under the tail for better accessibility. Somite I and its surface ectoderm were removed with tungsten needles and microcapillary suction. Viral marking of interlimb

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somites was carried out at this stage with retrovirus expressing heat-resistant placental alkaline phosphatase, facilitating future exact localisation of operation site (Valasek et al., 2005). The quail somite was transferred with a thin glass pipette and positioned by glass needles. Albumin (2 ml) was removed, the window sealed using surgical tape and embryos re-incubated for 6–7 days until HH35–36.

In the case of dermomyotomal transplants, only dorsomedial or ventrolateral thirds of the dermomyotome with ectoderm (somites V–X) were substituted at the position of somite 37.

Tissue fixation, processing and histology

Embryos at embryonic day (E) 10 and E18 were decapitated and cut transversally below the thorax. The caudal part of the embryo was fixed in Serra's fixative (Serra, 1946), dehydrated in ethanol, CNP30 and embedded in Paraplast. Serial 10 µm transverse sections were deparaffinised and processed for immunohistochemistry. Pre-incubation in 10% heat-inactivated goat serum in PBS was followed by primary antibody incubation (overnight at 4°C). PBS washes included a 10 minute wash with H₂O₂ (0.3% v/v in PBS) for endogenous peroxidase inactivation. Secondary rabbit anti-mouse biotinylated antibody (DAKO E0354; 1:200) was applied for 1 hour followed by ABC signal amplification and DAB colour substrates (Vector). Alternatively, goat anti-mouse-AP (DAKO D0486; 1:1000) with alkaline phosphatase colour substrates (Roche) were used. Sections were counterstained with Eosin or Alcian Blue (0.5% w/v in 0.5% v/v glacial acetic acid in water), before dehydration and mounting with DPX (VWR, BDH 360292F, UK).

The following primary antibody dilutions were used: MyHC (anti-myosin heavy chain A4.1025; DSHB; supernatant 1:4, biotinylated 1:1000; required citrate antigen retrieval), SMA (anti-smooth muscle alpha actin; Sigma, clone 1A4, 1:5000), QCPN (anti-quail nuclei; DSHB; supernatant 1:1), QH1 [anti-quail endothelia (Pardanaud et al., 1987); DSHB; supernatant 1:10], 3A10 (anti-neurofilament; DSHB; supernatant 1:50).

Whole-mount in situ hybridisation

For good penetration of probes, embryos E7 and older were skinned during methanol dehydration. In situ hybridisation (Nieto et al., 1996) was performed with the following digoxigenin-labelled probes: *Myf5* (1.1 kb; a gift from Dr Anthony Graham, King's College London, UK) *MyoD* (1518 bp probe; Dr Bruce M. Paterson, NIH, Bethesda, MD), *Pax3* (645bp; Dr Martyn D. Goulding, Salk Institute, San Diego, CA) and *Pax7* (582bp; Dr Susanne Dietrich, King's College London, UK).

Innervation studies

E10, 15 and 18 chick embryonic pelves were fixed in 4% PFA-PBS for 2–4 hours, followed by washes in PBS and cryoembedding. 20-µm-thick cryostat sections were mounted on poly-L-lysine-coated slides and air-dried (1 hour).

For immunocytochemistry, sections were preincubated with 1% BSA, 5% normal donkey serum, and 0.5% Triton X-100 (for 1 hour). After rinsing in Tris-buffered saline (TBS; pH 7.4) mouse anti-neurofilament (Chemicon; mAb1592, 1:4000), mouse anti-tyrosin hydroxylase (Chemicon; mAb318, 1:2000) or rabbit anti-CGRP (Peninsula; T-4032, 1:5000) antibodies were applied overnight in a humid chamber at room temperature. Following three washes with TBS, donkey anti-rabbit Alexa Fluor 488 and/or donkey anti-mouse Alexa Fluor 555 (Molecular Probes, A21206 and A31570, 1:1000) were applied for 1 hour.

Some sections were additionally incubated with Alexa-Fluor-594-labelled alpha-bungarotoxin (Molecular Probes B-13423, 1:1000) diluted in TBS. The sections were rinsed, mounted in TBS-glycerol (1:1; pH 8.6) and a coverslip added.

For NADPH-diaphorase activity, sections were washed in 0.1 M phosphate buffer pH 7.4 and then incubated with 0.3% Triton X-100, 0.2 mg/ml nitrobluetetrazoliumchloride (NBT) and 1.0 mg/ml beta-NADPH (1–2 hours in a humid chamber at 37°C).

For the demonstration of AChE in motor endplates we used acetylthiocholiniodide solutions for 30–105 minutes as described by Karnovsky and Roots (Karnovsky and Roots, 1964) with modifications by Gruber and Zenker (Gruber and Zenker, 1978). Iso-OMPA (tetraisopropylpyrophosphoramidate, 0.1 mM; Sigma T-1505) was used as an inhibitor of non-specific cholinesterases.

Whole-mount immunofluorescence detection

Whole embryos were processed as for in situ hybridisation, followed by pre-incubation with 10% goat serum in PBS-0.5% Triton X-100 (for 24 hours) and incubation with primary antibody (MyHC-biotin or 3A10) for 24 hours. Several washes in PBS-0.5% Triton X-100 were followed by overnight incubation with Avidin-Cy3 (Amersham, 1:200) or secondary goat-anti-mouse-Alexa Fluor 488 (Invitrogen Molecular Probes, 1:200). Extensive washes were followed by confocal imaging.

Electron microscopy

Chick embryonic pelves at E10, 15, 19 were skinned, fixed [4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 hours], and washed in 0.2 M cacodylate buffer (30 minutes). Lymph hearts were dissected with adjacent tissue and fixed for an additional 1 hour in 1% osmium tetroxide in 0.1 M cacodylate buffer. Dehydration in graded ethanol and infiltration with propylene oxide was followed by embedding in Epon resin. Ultrathin (70 nm) sections were cut on a Leica Ultracut, stained with uranyl acetate, contrasted with lead citrate and viewed on a JEOL JEM-1011 microscope.

Mechanical obstruction of lymph hearts

Chick E9 embryos were exposed in ovo, the amnion cavity opened and lymph hearts were injected, using a microcapillary, with freshly prepared Mercor blue resin (Norwald, Hamburg, Germany) before polymerisation. Eggs were re-sealed and incubated for 24–28 hours.

Chick tailless/rumpless models

Tail ablations were performed by removing the tailbud at HH20–22. Unilateral tail ablations were carried out at HH22–25. Natural rumpless embryos were from Araucana club (www.araucana.org.uk). E10–HH36 embryos were photographed for presence of oedema and processed for *MyoD* in situ hybridisation.

Pharmacological immobilisation

Decamethonium bromide (Sigma D-1260) 0.2% (w/v) in PBS with antibiotics was applied to the chorioallantoic membrane in 200 µl doses on E6 and repeated on E8 through a windowed eggshell. Pancuronium bromide (Sigma P-1918, 100 µl of 8 mg/ml in PBS) was applied on E8 or E9. Embryos were examined on E10.

Photography

Whole embryos were photographed on a Nikon SMZ1500 stereomicroscope with a Nikon Coolpix digital camera, and sections were photographed on a Nikon Eclipse 400. A Leica SP2 confocal microscope was used for Fig. 1E and Fig. 3A. Image processing was performed with Adobe Photoshop 5.0LE.

RESULTS

Chick lymph heart anatomy and histology

The lymph heart of the chick embryo at E10 lies directly underneath the skin in a triangular region between the *musculus (m.) levator caudae*, *m. lateralis caudae*, *m. transversus cloacae*, *m. flexor cruris lateralis* and *m. iliotibialis lateralis* (Fig. 1A,D), adjacent to vertebrae LS12 to LS15 (i.e. LS12–Co3) and cranially reaching the caudomedial margin of the ilium. The heart usually has a few trabeculae-intraluminal bridges. Three layers can be readily recognised: endothelial, connective and muscular tissues.

Musculature of the lymph heart is striated skeletal type

To determine the composition of the developing lymph heart we examined its make-up from E10 to E18. We found that at E10 it is composed almost exclusively of striated skeletal muscle cells (myosin heavy chain antibody positive in Fig. 1E,F,H). Smooth muscle protein (smooth muscle actin antibody positive) was distinctly detected only at E18 (Fig. 1I). Skeletal muscle fibres were thin and relatively sparse with frequent branching creating a mesh appearance (Fig. 1E). The over-bridging trabeculae also contained

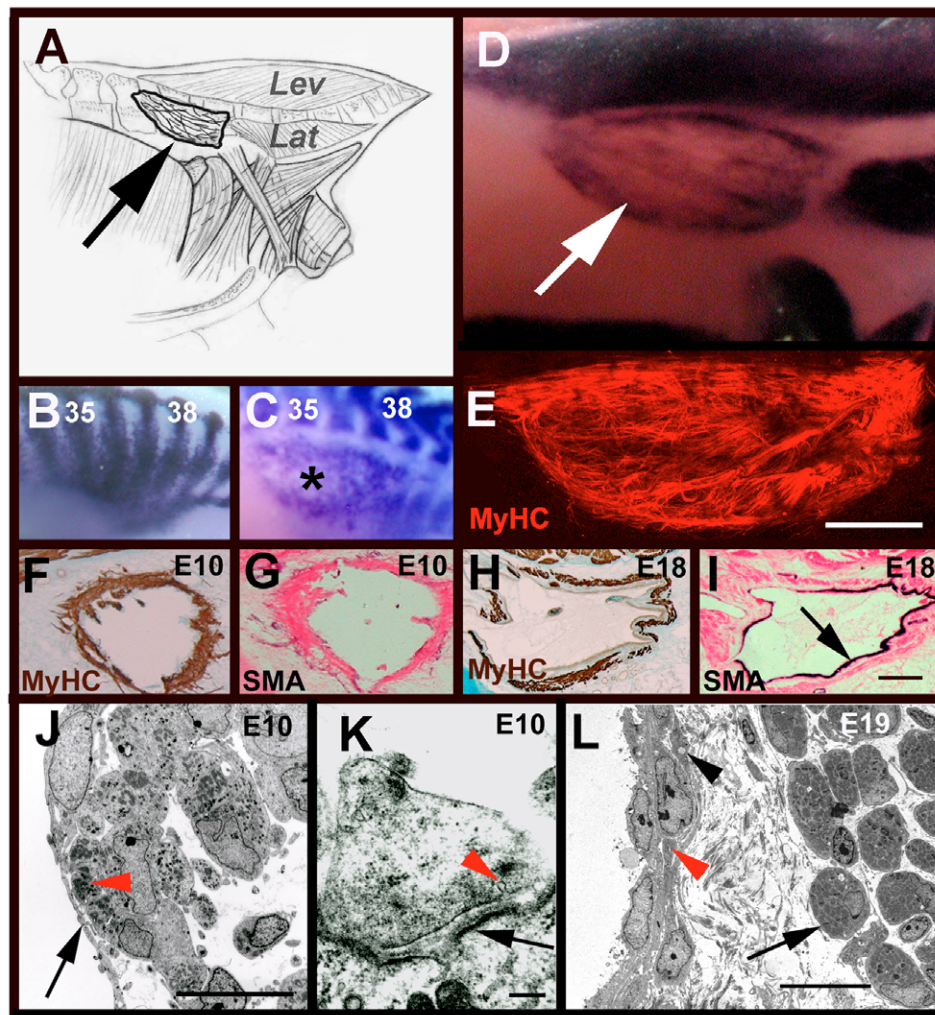


Fig. 1. Early lymph heart has only striated musculature. (A) Schematic representation of musculature in the caudal region of E10 chick embryo. Lateral view showing the lymph heart (arrow) and selected labelled muscles: Lev, *musculus levator caudae*; Lat, *m. lateralis caudae*; for other muscles see Valasek et al. (Valasek et al., 2005). (B–D) *MyoD* whole-mount in situ hybridisation; lateral views below the hind limb bud. (B) E6 showing single myogenic cells detaching from myotomes into the more superficial layers. Myotomes 35 and 38 are labelled. (C) At E8, the cells coalesce into the anlage of the lymph heart (asterisk). (D) By E10, the lymph heart musculature (arrow) is well organised. (E) E10: confocal immunofluorescence with pan-myosin heavy chain (MyHC) shows the branching structure of the muscle fibres. Scale bar: 500 μ m. (F–I) Longitudinal sections through the lymph heart with adjacent epaxial muscle. At E10 there is robust MyHC expression (F; brown) and absence of SMA expression (G; counterstaining with eosin, pink). At E18 there is robust MyHC expression (H; brown) and distinct SMA expression adjacent to the endothelium (I; arrow. Counterstained with eosin, pink). Scale bar: 500 μ m. (J–L) Electron micrographs of lymph heart wall. (J) At E10, myoblasts containing mostly immature contractile myofilaments (red arrowhead) with few Z-disc striations are directly underneath the endothelial lining (arrow). They have many adherent contacts with each other. Scale bar: 10 μ m. (K) Motor endplate at E10 with distinct postsynaptic density (arrow), a shallow postsynaptic fold, basal lamina in the synaptic cleft, discrete presynaptic dense projections (spike-like electron dense areas) and an omega-shaped profile (red arrowhead) suggesting exocytotic release of transmitter. Scale bar: 200 μ m. (L) At E19, the subendothelial layer is occupied by cells with extensions containing secretory vesicles (black arrowhead) and darker cytoplasm with smooth muscle proteins (red arrowhead). Lymph heart striated muscle fibres situated in a more peripheral region have more mature myofilaments with Z-discs (arrow) compared to those at E10. Loose connective tissue separates smooth and striated muscle layers. Scale bar: 10 μ m.

striated musculature (not shown). Similarly, when we examined the expression of muscle determining factors *MyoD* (Fig. 1B–D) and *Pax3*, *Pax7*, *Myf5* (not shown) during earlier development (HH18–36) we observed a clear distinction from both smooth and cardiac musculature (Table 1). However, at E18, a distinct layer of smooth muscle had developed in the subendothelial layer, well separated from the striated muscle (Fig. 1I,L) by loose connective tissue.

We complemented our molecular and immunohistological profiling of the lymph heart development by transmission electron microscopy. At E10 (Fig. 1J) the subendothelial layer was adjacent

to a layer of myoblasts containing mostly immature contractile myofilaments with few Z-disc striations. These cells had many adherent contacts with each other. Several neuromuscular junctions with shallow subsynaptic folds were seen (Fig. 1K). At E19 (Fig. 1L) the subendothelial cells attained characteristics of myofibroblasts with part of their cytoplasm filled with smooth muscle myofilaments and part by secretory vesicles. An intermediate layer of loose connective tissue was readily identifiable. At this stage the outer striated muscle layer had a more differentiated appearance with many more Z-disc containing

Table 1. Properties of chick lymph heart musculature until E10

Protein/gene	LH	Striated	Heart	Smooth
SMA Ab	–	–	–	+
MyHC Ab	+	+	+	–
<i>Pax3</i>	+	+	–	–
<i>Pax7</i>	+	+	–	–
<i>Myf5</i>	+	+	–	–
<i>MyoD</i>	+	+	–	–

myofilaments. However, a few myocytes with lighter cytoplasm and less contractile myofilaments (Berens von Rautenfeld and Budras, 1981) were also detected (data not shown). We failed to find any neuromuscular junctions at this stage.

These observations suggest that the musculature of the chick lymph heart has striated skeletal muscle characteristics alone during the first half of in ovo development. Smooth muscle subsequently develops during the second half of in ovo development.

The lymph heart originates from somites 34–41

We next determined the origin of the lymph heart. The developing lymph hearts are composed of skeletal muscle and as all skeletal muscle of the vertebrate body develops from somites (Christ and Ordahl, 1995), we postulated somitic origin of this musculature. Therefore, we determined its segmental origin using the chick-quail chimeras.

Grafting experiments showed that the striated muscle of the lymph heart originated from somites 34–41 (Table 2). Neighbouring somites (33 and 42) never gave rise to this musculature despite forming adjacent local tissues. From somite 38 caudally, the proportionate contribution gradually decreased, with somite 41 contributing only a few myogenic cells to the caudal end of the lymph heart. This was consistent with the pattern observed during the early differentiation of the myogenic cells directly from the somites/dermomyotomes (Fig. 1B,C). Furthermore the transplanted quail somitic cells formed all the other cell types of the lymph heart, i.e. the connective tissue (Fig. 2B) and the endothelial cells (Fig. 2C).

These experiments resolved the issue of the developmental origin of the chick lymph heart, showing that it arises from somites 34–41.

The lymph heart is a hypaxial structure

Having established that the lymph heart is somitic in origin we next determined which specific part of the somite contributes to the lymph heart.

We substituted the dorsal portion of the newly formed somite I (at the position of somite 37) of the chick host with corresponding tissue of quail origin (Fig. 2A). These experiments showed that quail cells contributed to the lymph heart, same as following whole somite grafts ($n=10$; data not shown). The dorsal portion of somite I forms the complete dermomyotome (Christ and Ordahl, 1995).

In order to define which portion of the dermomyotome contributes to the development of the lymph heart we next substituted the dorsomedial or ventrolateral thirds (Huang and Christ, 2000) of the dermomyotome of somite 37 at older somite stages (V–X) with quail dermomyotomal tissue (Fig. 2D,F). These experiments showed that the ventrolateral third of the dermomyotome gives rise to the lymph heart and hypaxial tissues, whereas epaxial *m. levator caudae* remained unpopulated by quail cells ($n=4$, Fig. 2G). We did not observe any decrease in the quantity of quail cells in the lymph heart (not shown) compared to whole somite substitutions. The dorsomedial third ($n=4$) gave rise only to

Table 2. Somitic origin of chick lymph heart musculature

Somite	32	33	34	35	36	37	38	39	40	41	42
No. of chimeras	5	8	8	7	7	5	6	3	4	7	3
No. of positive LH	0	0	7	6	7	5	4	2	2	(4)*	0

*Only a few myogenic cells contributed to the caudal end of the lymph heart (LH).

the epaxial structures and there were no quail cells in the lymph heart (Fig. 2E). These experiments show that the ventrolateral lip is the source of somitic cells that form the lymph heart and thus lymph heart belongs to the hypaxial territory.

Innervation of the lymph heart

To complement the developmental and morphological data concerning the lymph heart musculature with more functional aspects, we investigated the innervation of this organ.

Whole-mount neurofilament immunostaining revealed five to six segmental branches in the vicinity of the lymph heart at E10 (Fig. 3A). These fibres originate from a plexus which innervates local skin, dermis and adjacent muscles. It is supplied by ventral rami of the spinal nerves 29–33 (LS9–Co1, corresponding to somites 35–39), confirming that the innervation usually reflects the segmental origin (Kida, 1997).

As the lymph heart musculature is initially only of striated skeletal muscle type, we first investigated the distribution of classical neuromuscular junctions. Fluorescently labelled alpha-bungarotoxin detected nicotinic acetylcholine receptors of the endplates in both lymph heart and body muscle fibres at E10 in a scattered pattern (Fig. 3B,C). At this stage intimate contacts with nerve fibres were scarce in both tissues (Fig. 3B,C). However, by E18 the endplates of the body skeletal muscle became more organised and formed close contacts with nerve fibres (Fig. 3E). By contrast, the lymph musculature became devoid of nerve fibres and bungarotoxin labelling remained scattered (Fig. 3D). These immunohistological findings are in agreement with our electron microscopy data that detected the presence of neuromuscular junctions in the lymph heart only at E10 (Fig. 1K).

Specific acetylcholinesterase (AChE) is classically restricted to the neuromuscular junctions, whereas non-specific activity can be detected throughout the muscle fibres. The lymph heart musculature exhibited only diffuse activity with no reaction product on the alpha-bungarotoxin positive endplates (Fig. 3F). Furthermore this activity was non-specific as it was abolished by the use of iso-OMPA – an inhibitor of pseudocholinesterases (data not shown).

The lymph heart at later stages also develops a layer containing smooth muscle protein, so we examined autonomic nervous system innervation. In the stages examined (E10–19) we could not detect any significant signal for either NADPH-diaphorase – a marker for parasympathetic efferents (Grozdanovic et al., 1992) or tyrosine hydroxylase – sympathetic efferents (Yurkewicz et al., 1981) in the wall of the lymph heart (Fig. 3H,J). These markers were robustly expressed in other parts (Fig. 3I,K) of the embryo at all stages.

Lastly we examined the distribution of sensory axons in the lymph heart using immunoreactivity against calcitonin gene-related peptide (CGRP) (Schrodl et al., 2001). The wall of the lymph heart was devoid of CGRP (Fig. 3J) in contrast to the intestinal wall (Fig. 3K).

To summarise, we detected only somatomotor innervation of the lymph heart. This was, however, atypical, as not only the axons disappeared during the later development, but the ACh receptors were devoid of AChE activity at all stages.

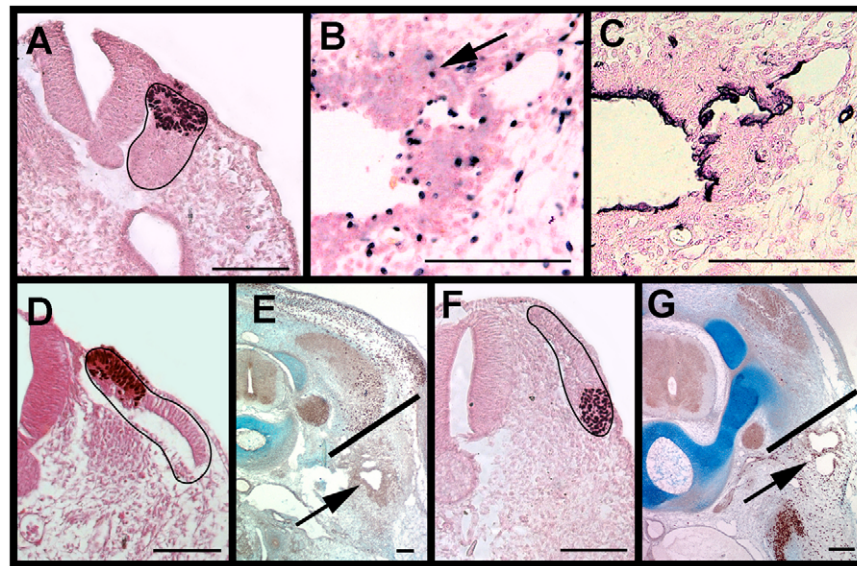


Fig. 2. Chick-quail somitic chimeras show hypaxial origin of lymph heart. (A) Immunohistochemistry with QCPN shows the localization of grafted quail cells 6 hours after transplantation of the dorsal part of a newly formed somite I (outlined). (B,C) Similar grafts of the dorsal parts of somites 34-41 give rise to lymph heart tissue after 7 days (see Table 2) including the skeletal muscle cells with darker cytoplasm (B, arrow) and the endothelial QH1-positive cells lining the lumen (C). Ectoderm, associated with some somitic tissue grafts, never contributes to the lymph heart (see Fig. S1 in the supplementary material). (D) QCPN immunohistochemistry detects quail nuclei in grafted tissue 5 hours after transplantation of the medial portion of the dermomyotome (outlined) of somite VII. (E) Grafting of the medial third of the dermomyotome gives rise only to the local epaxial *m. levator caudae* and dermis (above black line), but not to the hypaxially located tissues (below black line, including the lymph heart; arrow). (F) QCPN immunohistochemistry detects quail nuclei in grafted tissue 4 hours after transplantation of the lateral third of the dermomyotome (outlined) of somite V. (G) Transplantation of lateral dermomyotome gives rise to quail cells in the lymph heart (arrow). Scale bars: 100 μ m.

Embryonic oedema is caused by dysfunction of the lymph heart

The knowledge that the lymph heart is of somitic origin and that it is composed of striated skeletal muscle with ACh receptors allowed us to examine its role during development using surgical procedures and pharmacological reagents.

Absence of the lymph heart (microsurgical and genetical)

In order to examine the effects of absence of the lymph heart on the embryo, we removed all tissue below somite 33 – by ablating the tail bud at HH20-22. Fourteen embryos (out of 30) survived until E10. Six embryos were highly oedematous (compare Fig. 4A and 4B), and upon whole-mount examination with *MyoD* these embryos revealed not only absence of the tail, but also complete absence of the lymph hearts (Fig. 4C). The oedema was clearly evident from E8 onward and was most prominent in the region of the hind limbs reaching cranially to the base of the wings. The remaining eight embryos (as well as other embryos with more caudal ablations) had some degree of tail developed, together with small lymph hearts and less prominent or no oedema (data not shown). Similar oedemas were incidentally noticed in some cases of amniotic constrictions following interventions on the caudal end of young embryos. It appeared that constrictions just above the tail level caused oedema of the body similar to removal of the tail. Unilateral tail ablations ($n=27$) resulted in mild or no oedema.

A naturally occurring autosomal dominant chick mutant *Araucana rumpless* provided another means to examine the consequence of lymph heart absence during embryonic development. This mutant is characterised by ear-tufts of feathers and absence of the tail (parson's nose). The hatch rate of fertilised

eggs is approximately 75%. Upon examination of the unhatched eggs they usually contained very oedematous dead embryos. We examined the development of *Araucana rumpless* and found that the complete tailless phenotype had developed in some embryos by E10 and that although they were alive they all displayed gross oedema (Fig. 4D). Their lymph hearts were either absent or only minimally developed. Remaining embryos displayed varying degrees of shortened tail with lymph hearts being partially developed, accompanied by very mild or no oedema.

These experiments show that either the surgical ablation of the tail or its absence due to a genetic mutation result in oedema.

Obstruction of the lymph heart

Absence of the whole tail does cause absence of the lymph heart, but it also causes defects of other structures of the tail including lymph and blood vessels. In order to examine the role of the lymph heart pump itself without affecting the other structures, we obstructed the lumen of the lymph heart by the injection of fast hardening Mercor resin, which solidifies in less than 2 minutes, into both lymph hearts. This resulted in a gross oedema formation after 1 day (Fig. 4E) in all of the embryos that survived this procedure ($n=4/12$).

Paralysis of the lymph heart

The fact that the lymph heart contains only skeletal muscle by E10 enabled us to examine the effects of absence of its mechanical contractions by neuromuscular junction blockers.

Firstly we investigated the effect of applying a depolarising competitive blocker with a long half-life. Application of Decamethonium bromide resulted in complete paralysis (apart from the amniotic smooth muscle contractions) and there was no reaction of the embryonic body to mechanical stimuli at any point. We

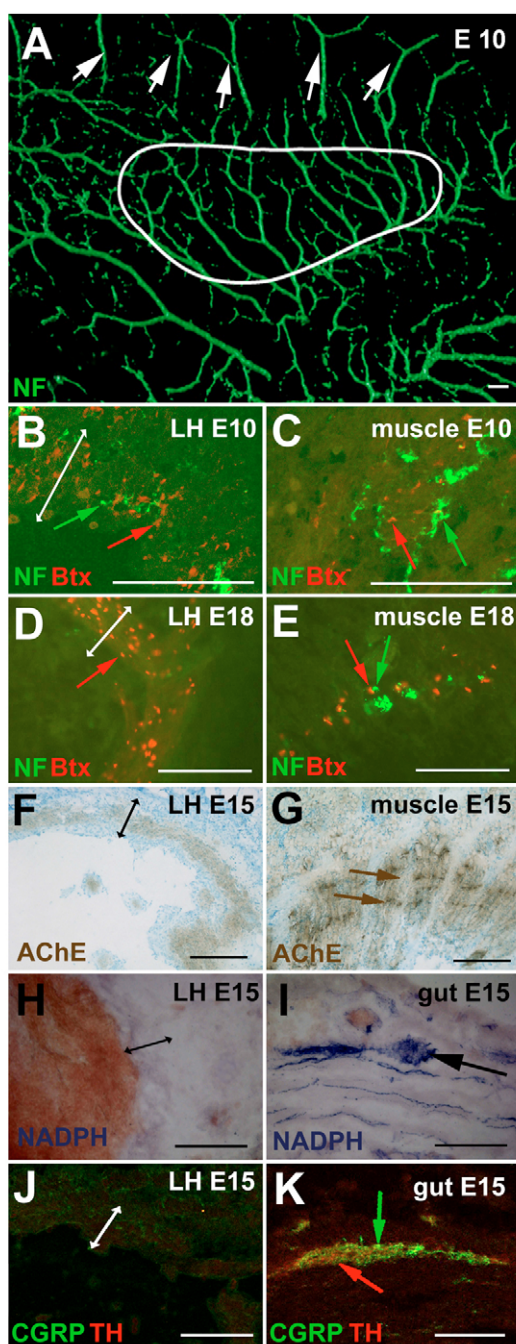


Fig. 3. Lymph heart innervation. (A) Whole-mount neurofilament immunofluorescence showing a lateral view of the tail at E10 with hypaxial branches overlying the lymph heart (white outline) that also supply local dermis and musculature. Segmental epaxial branches (white arrows) lie dorsal to the lymph heart. (B-E) Immunofluorescence for neurofilament (green) and alpha-bungarotoxin binding (red) in the lymph heart (LH) wall (thickness indicated by double-headed arrow). (B,C) Scattered bungarotoxin-positive acetylcholine receptors (red arrow) and neurofilaments (green arrow) at E10 in lymph heart and striated muscle, respectively. (D) At E18 the lymph heart is practically devoid of any neurofilaments while retaining the ACh receptors (red arrow). (E) In body muscle, neuromuscular junctions are now regularly organised in bands (red arrow) and form intimate contacts with the neurofilaments (green arrow). (F) At E15, acetylcholinesterase reaction product (brown) is diffusely seen in the striated muscle of the lymph heart wall. (G) At E15, body muscle fibres have similar diffuse activity; however, their ACh receptors are organised in bands (brown arrows), and display significantly higher activity, which is not blocked by iso-OMPA (not shown). (H) At E15, parasympathetic efferent innervation, assessed by NADPH-diaphorase histochemistry (blue reaction product), failed to give a signal in the wall of the lymph heart, with red blood cells in its lumen. (I) At E15, the wall of the hindgut has robust parasympathetic NADPH-diaphorase activity in the nerve fibres and in the ganglia of the myenteric plexus (arrow). (J,K) Sympathetic efferent innervation determined by immunoreactivity to tyrosine hydroxylase (TH; in red) and sensory axons through immunoreactivity for CGRP (green). (J) At E15, lymph heart tissue expressed neither marker. (K) At E15, robust expression of both markers was detected in the nerve tissue in the wall of the hindgut (red and green arrows). Scale bars: 100 μ m in A-J; 50 μ m in K.

From this surprising difference in sensitivity of the lymph heart and body musculature to the neuromuscular blockers we deduce that the oedema of the immobile embryos is caused mainly by the absence of lymph heart contractions.

DISCUSSION

The lymph heart is a structure not widely recognised, partly because of its transient nature, and that it does not exist in mammals. In our study we show that the musculature of the chick lymph heart is composed of striated skeletal muscle cells that originate from somites 34-41 and belong to the hypaxial muscle group. For the first time we show that its absence or dysfunction results in embryonic oedema.

The lymph heart musculature revisited

The lymph heart musculature has peculiar properties suggesting it cannot be ascribed to the classical groups – smooth, skeletal striated or cardiac striated muscle (Budras et al., 1987). Despite some species-specific differences, its histological structure is similar to the striated skeletal muscle fibres (multinucleated, with peripherally located nuclei) but thinner in diameter. Some fibres branch, although they do not anastomose like cardiomyocytes (Rumyantsev and Krylova, 1990). Also present are light-coloured cells with few filaments that resemble conductile bundles of His, found in cardiac tissue (Budras et al., 1987). Furthermore, complete automaticity has been described after denervation in amphibians (Rumyantsev and Krylova, 1990).

Our data show that the lymph heart musculature in chick is exclusively a striated skeletal muscle in the first half of in ovo development. Later a separate layer of myofibroblasts develops

observed mild oedema formation and death within 2-3 hours when applied at E10, whereas earlier application at E6 allowed further development, despite repeated doses on alternate days for sustained paralysis. These embryos displayed gross oedema from E8 and by E10 this was severe (Fig. 4F) and accentuated by degenerated oedematous skeletal muscles (Macharia et al., 2004). The character of the oedema was otherwise similar to the models above.

Pancuronium bromide resulted also in complete body movement paralysis. However, there was no obvious ($n=6$) or only mild oedema ($n=1$). The lymph hearts were observed to contract either spontaneously at three beats every 10 minutes (normal rate is 5/minute) or on a direct gentle mechanical stimulus. No response was elicited in the body musculature which was hypoplastic but not oedematous. Thus approximately 5% function of the lymph heart was sufficient to prevent oedema formation.

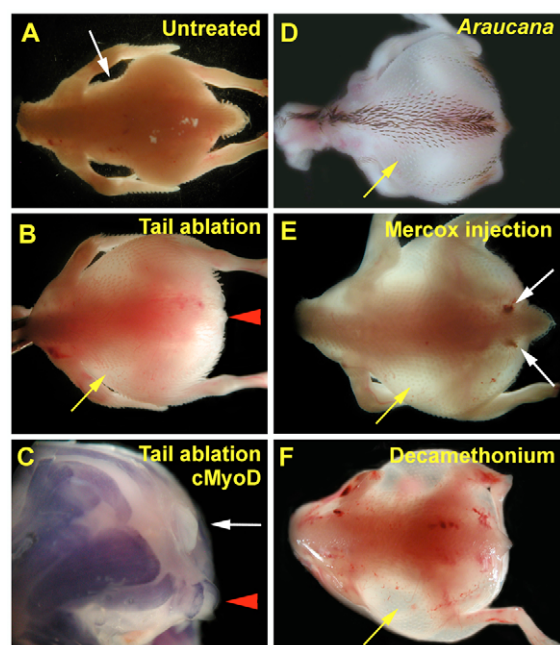


Fig. 4. Lymph heart dysfunction results in embryonic oedema.

(A) Normal E10 chick embryo (dorsal view). Arrow denotes flank skin adherent to the trunk. (B) E10 embryo following tail ablation at HH20 showing oedema (arrow). (C) *MyoD* whole mount in situ hybridisation of embryo in B showing absence of lymph heart (white arrow) and all local tail muscles. Absent tail makes the cloacal tubercle appear more prominent (arrowhead in B and C). (D) Spontaneous mutant *Araucana rumpless* chick at E10 is tailless and oedematous. (E) Mechanical blockage of the lymph hearts injected at HH36 with Mercor resin (white arrows) results in oedema (yellow arrow). (F) E10 embryos treated with decamethonium develop oedema (arrow).

around the endothelium. This may reflect the gradual involution of the outer striated muscle and a change of the heart towards a lymphatic collector. Lymphatic collectors possess a wall of smooth muscle (Baumel et al., 1993) and contract spontaneously 8–10 times/minute (Witte et al., 2006), thereby taking over the function of the lymph heart. Nevertheless some striated muscle cells have been reported in the lymph heart even in 7-month-old adult chicken (Bischof and Budras, 1993).

For the first time we described the innervation of this organ as being purely somatic and, moreover, to diminish during the second half of in ovo development. This may, however, be specific to chick as some other bird species retain a specific role of the lymph heart in copulatory organ erection (Budras and Berens von Rautenfeld, 1984) and thus the suggestion of parasympathetic innervation is relevant (Budras et al., 1987). The autonomic innervation of the smooth muscle layer of the lymphatic collectors (Witte et al., 2006) may perhaps develop only postnatally.

The patterning and development of the lymph heart

The early development of the lymph heart is rather intriguing. All three cell types forming the lymph heart (endothelial, muscle and connective tissue cells) originate from the somites. Lymphendothelial cells can be clearly demonstrated at E5.5 by their *Prox1* expression (Wilting et al., 2006), i.e. at the same time as the myogenic cells (Fig. 1B). It is therefore likely, that these cells leave the somite – or more precisely the hypaxial dermomyotome –

together. A retrovirally marked single cell (Kardon et al., 2002) in a differentiated somite (VII) at limb level gave rise to myogenic and endothelial cells in the limb periphery. These cells were often in close proximity, suggesting again a common translocation or only later local proliferation and differentiation.

The process of release of myogenic cells from the dermomyotomes 34–41 is unusual, as these cells appear as single cells (Fig. 1B), yet they already express *MyoD*. Thus this process cannot be the classical migration of single myogenic cells as in the limb, where the *MyoD* differentiation step occurs only after they reach their final position in the limb bud (Amthor et al., 1998). Furthermore, we never detected the expression of scatter factor (SF/HGF) in this region (data not shown), which would mediate the release of myogenic cells from the dermomyotome via single cell migration (Bladt et al., 1995; Dietrich, 1999). It appears that the development of the lymph heart striated muscle represents a unique process of release of single myogenic cells from maturing dermomyotomes.

Lymph heart and embryonic oedema

We show for the first time a causative relationship between the function of the lymph heart and embryonic oedema. Total absence of the lymph heart or absence of lymph heart flow, either by obstruction or by its paralysis, leads to the accumulation of lymph in the subcutaneous tissues. The limited extent of the oedema below the wings probably reflects the leakage of lymph near the caudal end and perhaps also the relative looseness of the subcutaneous tissue in the younger embryos, which allows for pooling of the lymphatic fluid. The cranial region of the embryo is drained directly to the jugular or subclavian veins (Baumel et al., 1993) independently of the lymph hearts. Lymphatic connection between the cranial and caudal territories established through the development of the thoracic duct around E10 (Romanoff, 1960) does not seem to affect the extent of oedema.

Rumplessness, oedema and further development

Certain degrees of taillessness/rumplessness occur also spontaneously. Approximately 1% of chick embryos from our egg supplier display varying degree of rumplessness at E10 and a few severe cases were also oedematous. Landauer and Baumann (Landauer and Baumann, 1943) observed similar rates of spontaneous rumplessness and noted that this can be increased by shaking eggs prior to incubation. Variations in incubation temperature and application of chemical compounds, including insulin, have been reported to result in a similar phenotype. Kaplan and Grabowski (Kaplan and Grabowski, 1967) applied Trypan Blue to E2 chick eggs and observed the formation of caudal haematoma resulting in rumplessness in 15% of embryos. They also noted oedema in 12% of the samples but did not comment on any relationship with the degree of rumplessness. Fulton et al. (Fulton et al., 1987) observed oedema in 40% of rumpless autosomal recessive *Ottawa naked* chicks.

Araucana rumpless embryos have genetically shorter tails; however, they also have superimposed inter-individual variability of further tail truncations. We speculate that embryos with severe truncations resulting in complete absence of lymph hearts become so oedematous that it prevents their further development and they die in ovo after E10.

Oedema in pharmacologically immobilised embryos

Decamethonium-induced immobility was complete for both the body and the lymph heart musculature and resulted in gross oedema formation (our observations) (Macharia et al., 2004; Hosseini and

Hogg, 1991; Sullivan, 1966). By contrast, pancuronium, which also paralysed the body musculature, left the lymph hearts contracting (although more slowly) and oedema did not form. It is probable that the lymph heart muscle also has lower sensitivity to other paralysing agents such as botulotoxin (Drachman and Sokoloff, 1966) or D-tubocurarine (Murray and Smiles, 1965). These studies used botulotoxin A and tubocurarine, respectively, alongside decamethonium but reported oedema only in the decamethonium-treated embryos. Another study using curare and succinylcholine infusion (Oppenheim et al., 1978) did not mention oedema at all and body weights suggest there indeed was no apparent oedema.

It is evident that the difference of lymph heart sensitivity to neuromuscular blockers cannot be explained by an additional autonomic mode of innervation. The lymph heart does have ACh receptors and is sensitive to decamethonium blocking. However, the lymph heart does not have abundant neuromuscular junctions and its ACh receptor clusters do not possess specific AChE activity, suggesting that activation of contraction may not necessarily involve classical ACh release from a nerve fibre. It is known that some AChE-positive endplates do not bind bungarotoxin (Kallmünzer et al., 2006), but in the case of the lymph heart we have the opposite situation, i.e. binding of bungarotoxin without AChE activity on the endplates. To our knowledge this is a unique situation.

Decamethonium not only binds to the acetylcholine receptor, but also depolarises the membrane, rendering the membrane of a myofibre incapable of excitation by, for example, mechanical stimulation or by an action potential spreading from adjacent cells (with possible pace-maker activity). The other neuromuscular blockers used in chick did not cause oedema and importantly these blockers affect only the ACh transmission [either by release of ACh – botulotoxin A and beta-bungarotoxin, or by mechanically blocking the receptor without depolarising the membrane (pancuronium, D-tubocurarine)] without hindering the membrane excitation by direct mechanical stimuli.

We hypothesise that application of decamethonium at E10 caused the quick death by prolonged depolarisation of fairly well developed muscle fibres, leading to a gradual release/leak of a substance (e.g. potassium) leading to cardiac arrest (Rang, 2003). To avoid these sudden metabolic/mineral changes we started the decamethonium application at E6, while the muscle fibres are less differentiated. Decamethonium itself is not known to have a direct effect on cardiac muscle (Punnen et al., 1984) and indeed when applied at E6 the blood heart continues to function normally.

Automaticity of the lymph heart

The difference in sensitivity to the immobilising agents is not inherent and only develops during ontogenesis. Initially the chick lymph heart and the local tail musculature contract simultaneously. After E8 these two muscle groups contract independently (Romanoff, 1960). Similarly the rhythmic contractions of the chick lymph heart cease to be influenced by chlorotone anaesthetic at E8, whereas younger hearts were paralysed like other body musculature (Romanoff, 1960). The differences in sensitivity to chlorotone as well as pancuronium suggest that the lymph heart musculature is to a certain extent independent of the classical neural input. This is further supported by the fact that the lymph heart has initially somatomotor but no autonomic innervation, and that the somatomotor innervation diminishes during development. The absence of AChE activity from the ACh receptor clusters is also puzzling.

However, whether the chick lymph heart possesses an intrinsic pacemaking system is as yet unclear. Witte et al. (Witte et al., 2006) found that 3% of the smooth muscle cells in the tunica media of the

contractile lymph collectors have properties of pacemaker cells. However, the observed contractions of lymph hearts in our pancuronium model at E10 are unlikely to be controlled by smooth muscle pacemaker cells, as they have not differentiated at this stage. Furthermore Berens von Rautenfeld and Budras (Berens von Rautenfeld and Budras, 1981) and Budras et al. (Budras et al., 1987) observed on electron micrographs of the striated lymph heart musculature in some birds that there are contracting-filament-poor fibres which resemble the conductive bundle of His of the blood heart. Amphibian denervated lymph hearts show automatic contractions 4 weeks after surgery (Rumyantsev and Krylova, 1990).

Taken together these observations suggest that the lymph heart musculature is specialised and has acquired different properties to body skeletal musculature despite its common embryonic origin.

Oedema in genetically immobile embryos – crooked neck

Severe oedema is also present in an immobile naturally occurring autosomal recessive *crooked neck dwarf* (*cn/cn*) chick mutant (Asmundson, 1945; Oppenheim et al., 1997). There is an indirect defect in the ryanodine receptor alpha, which controls the release of calcium from the sarcoplasmic reticulum following depolarisation of the sarcolemma (Airey et al., 1993; Ivanenko et al., 1995; Oppenheim et al., 1997). Thus there is no coupling of the nerve impulse and physical muscle contraction. It has been described that these embryos also have progressive embryonic muscle degeneration (Kieny et al., 1983; Kieny et al., 1988) and suffer from oedema of the same appearance as our decamethonium-treated embryos.

Embryonic immobility per se does not cause oedema

All the oedemas of immobility were ascribed to the absence of lymph propulsion or decreased venous return, features dependent on skeletal muscle contractions (Macharia et al., 2004). The following considerations enable us to challenge this notion and ascribe a novel role for the lymph heart.

Firstly, we showed that pancuronium-immobilised embryos, which have no body muscle movement but retain some contractility of the lymph heart, fail to develop gross oedema.

Secondly, the degree of oedema of embryos immobilised by either decamethonium or genetically is very comparable to our models with lymph heart absence or its obstruction (bearing in mind, that the muscle atrophy induced by decamethonium enhances the oedematic appearance).

Lastly, if propulsion of the body lymph was dependent on the skeletal muscle contractions, we would expect oedema to develop also in mouse embryos lacking muscle movement. However, the murine mutant of the ryanodine receptor (*RyR-1* null – related to the above chick *crooked neck*) lacks skeletal muscle contractions and yet is not oedematous (Takeshima et al., 1994) or has variable small oedema in the head and rump region (H. Takeshima, personal communication).

All these observations suggest that immobilisation on its own is not sufficient to account for an obvious oedema and point to the specific role of the lymph heart.

It is noteworthy that there are also other causes of avian embryonic oedema, for example ‘chick oedema disease’ caused by organophosphates (Gilbertson et al., 1991; Brunstrom, 1988). Their pathogenesis appears to include activation of cytochromes and effects of arachidonic acid metabolites (Rifkind et al., 1990) or direct cardiotoxicity with heart failure (Walker and Catron, 2000) whereas a direct role of lymph heart in these scenarios is unlikely.

Conclusion

Our work has resulted in three major findings: (1) lymph heart contains striated muscle from somites 34-41; (2) lymph heart musculature displays unique properties including bungarotoxin-binding endplates without AChE activity; (3) embryonic lymphoedema is prevented by the mechanical action of the lymph heart and not by mobility of the body musculature.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/24/4427/DC1>

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