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Exploring the Role of *Sonic Hedgehog*
in the Lymph Heart Development of *Xenopus laevis*

by

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Honors Thesis

in

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in the Department of Biology.

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Introduction

Xenopus as a Model Organism in Evolutionary Developmental Biology

Xenopus laevis, the African clawed frog, is an important model organism in the field of evolutionary developmental biology research. These tongue-less aquatic frogs are basally branching anuran amphibians that display marked sexual dimorphism and have webbed hind feet that sport several small, sharp claws. This species is a useful model organism despite its long generation time and genomic complexity, for historical and practical reasons. The animal adapts well to housing in a laboratory setting, and its reproduction can be induced simply through a single injection of human chorionic gonadotropin into a pair of adults. The great number of embryos generated, in this manner, are each large and easy to manipulate. Additionally, *Xenopus* embryos are simple to stage throughout development following external fertilization, as the appearance of anatomical characteristics is highly predictable, varies linearly with temperature, and has been well-documented (Nieuwkoop and Faber 1956). The tadpole stages of albino *X. laevis* are almost entirely transparent, allowing for non-invasive inspection of internal tissues and organs of live animals. These characteristics, in combination with its phylogenetic position in a basal tetrapod lineage, makes it a valuable and interesting experimental subject to study in the context of an evolutionary developmental laboratory.

Lymphatic Hearts and Their Apparent Evolutionary Development

Lymphatic hearts are paired, pulsatile organs that regulate the return of interstitial fluid to venous circulation in some animals (Kampmeier 1922). These organs have been documented in organisms from many vertebrate clades. Lymphatic hearts have been identified in sarcopterygiian bony fish (Laurent *et al*. 1978), amphibians, and sauropsid amniotes – the reptiles and birds (Kampmeier 1958). Notably, lymphatic hearts are absent in synapsida, the mammals. In this clade, the rhythmic contraction of skeletal muscles has likely replaced the lymphatic heart as the functional regulator of lymph's return to circulation (Kampmeier 1958). These animals' jugular lymph sac anlagen are thought to be homologous to lymphatic hearts (Kampmeier 1958). The distribution of lymphatic hearts among extant tetrapods and their vertebrate sister group suggests that these organs may be symplesiomorphic for tetrapods, and that the loss of these organs is a mammalian synapopmorphy. If you examine this distribution in more detail, it would appear that lymphatic hearts reach their evolutionary high water mark of development and utilization in amphibians. In anamniotic tetrapods, lymphatic hearts are found in both the highest numbers and the greatest specialization.

In the lungfish *Protopterus aethiopicus*, "numerous contractile cisternae" - presumably homologous structures to the tetrapod lymphatic heart - are "interposed between intercellular channels and veins" and "function as

micropumps that collect fluid from intercellular epithelial spaces and inject it into the venous circulation" (Laurent *et al*. 1978). At least ten of these cisternae were found in one individual specimen. The lungfishes are modern members of the Dipnoi subclass of bony fish, largely recognized as a sister group to crown group terrestrial tetrapods (Brinkmann 2004). The presence of lymphatic hearts among the Dipnoi indicates that the development of the lymphatic heart is not a stem group tetrapod innovation, but an ancestral trait that appeared in obligate aquatic species. The meristic nature of the organ in modern lungfish is also a presumably ancestral characteristic.

Among amphibians, the number and anatomical distribution of lymphatic hearts found throughout development vary from Order to Order, progressively decreasing in number as one moves from stem group amphibians to highly derived lineages. Among the caecilians of the Order Gymnophiona – superficially worm-like, limbless amphibians – lymphatic hearts are present in an extensive, lateral meristic series. *Hypogeophis alterans* and *H. rostratus*, possess over 100 pairs (Kampmeier 1958, Kampmeier 1969). The axolotl, the neotenic salamander *Ambystoma mexicanum*, belonging to the Order Caudata, possesses twenty pairs of lymphatic hearts in series along the length of its trunk between the pectoral and pelvic girdles (see Fig. 1) (Kampmeier 1958). In the related species *Salamadra maculosa*, fifteen hearts are present; the first lies lateral to the third and fourth myomeres, and the last lies lateral to the seventeenth and eighteenth.

In the pre-metamorphic larvae of Anurans – frogs and toads – a single pair of anterior lymphatic hearts is present, along with a variable number of pairs of posterior lymphatic hearts (one to six). This number is subject to change throughout the animals' lifespans, given the radical morphological changes that occur during metamorphosis in this group of amphibians. In all stages, however, the localization of anterior and posterior lymphatic hearts in the trunk roughly maps to the medial origins of the anterior and posterior limbs (see Figs. 1 and 2).

Anterior Lymphatic Hearts and their Gross Anatomical Development in Anurans

In *Xenopus laevis*, an anuran, six pairs of lymphatic hearts develop and regress at different points throughout the life of the animal. The anterior lymph hearts are the first to form, appearing in late embryonic development (see Fig. 3). This pair of lymph hearts develops bilaterally, lateral to somites 3 and 4 (as in *S. maculosa*), dorso-caudal to the embryonic kidneys and just below the skin at Nieuwkoop and Faber stage thirty-two (Nieuwkoop and Faber 1956). They are clearly visible to the eye under magnification at stage 40, and begin to beat at stage 43 (Smith *et al*. 2007). At this stage of development, lymphatic hearts can be visualized with the aid of magnification *in vivo* or when histochemically stained or probed *in situ* for skeletal muscle markers (see Fig. 4). As in other anurans like *Bufo sp.*, the organs are globular in shape and are stratified into three layers of

tissue; a middle layer of cardiac-like striated muscle cells is sandwiched between inner and outer layers of elastic connective tissue (Kampmeier 1922). The anatomical development and larval presentation of lymph hearts in species related to *X. laevis*, including *Bufo sp.*, *Ascaphus truei*, and *Rana palustris*, has been described in detail, and likely proceeds similarly. At the time that contractile motion begins, the hearts of *R. palustris* and *Bufo sp.* have separated from the venous plexus of intersegmental veins that is established dorsal to the pronephric kidney (Kampmeier 1917, Knower 1908). In young *Bufo sp.* tadpoles, each anterior lymphatic heart is continuous dorsally with an afferent lymphatic longitudinal vessel, the *lymphatica jugularis*, which drains the lymphatic sinuses of the trunk and head of the tadpole (see Fig. 5) (Kampmeier 1922). The number of afferent ports from the lymphatic vessel increases during metamorphosis. Ventrally, each heart is connected via an efferent vessel to an extension of the developing anterior vertebral vein and pronephric sinus (see Fig. 5) (Kampmeier 1922). In *Ascaphus truei* tadpoles, four afferent ports exist between each of the anterior lymphatic hearts and extensions of the jugular lymphatic vessel, and the fluid is discharged into the vein medial to the pronephros through two efferent ports, one anteriorally and the other posteriorally (Kampmeier 1958). While some species, like *Bufo sp.*, retain their anterior lymphatic hearts functionally into adulthood, the anterior lymphatic hearts of *X. laevis* are thought to regress in the adult.

The development of anuran posterior lymph hearts is delayed compared to that of the anterior pair. In *X. laevis*, four pairs of posterior lymphatic hearts appear in the later stages of tadpole development, as metamorphosis begins in earnest (Nieuwkoop and Faber 1956). By the time the posterior lymphatic hearts form at stage 51, the hindlimb and forelimb buds have already appeared – at stages 46 and 48, respectively (Nieuwkoop and Faber 1956). Interestingly, the temporal sequence in which anterior and posterior lymphatic hearts develop is the inverse of that of the limbs. The anterior lymphatic hearts appear first, followed by the posterior hearts, while the hindlimbs begin to develop before the forelimbs.

The development of the posterior organs has been best characterized in *A. truei* tadpoles. In this anuran, six pairs of these lymph hearts begin to develop bilaterally, in series, lateral to the eleventh through eighteenth myotomes, which constitute the junction of the trunk and tail of the animal at the level of the hindlimb bud (Kampmeier 1958, Kampmeier 1969). Like their anterior counterparts, these organs appear just below the skin, apparently arising from a plexus of lymphatic vessels. When fully formed, they are connected at their afferent port(s) to a lateral lymphatic vessel and to a lateral vein at their efferent port (see Fig. 6) (Kampmeier 1958). During the late stages of metamorphosis, some of these hearts are lost as they are assimilated into the dorsal caudal lymphatics of the adult frog.

There is no definitive consensus on which specific embryonic tissue gives rise to the musculature of the lymphatic hearts in anurans. One general review of *X. laevis* gross anatomy did not distinguish the origin of the muscular layer from the inner and outer layers; it was assumed to have been derived, along with the rest of the organs' tissues, from the venous endothelium, which is in turn derived from the intermediate mesoderm (Smith *et al.* 2007). The authors of more focused and comprehensive studies have claimed otherwise. One postulated that the cells of the muscular middle layer are derived relatively late in embryonic development from myoblasts of the skeletal myotome found adjacent to the intersegmental venous plexus (Knower 1918). Alternately, another has suggested that this tissue differentiates earlier from mesenchymal cells of the "mesodermal elements", or somites (Kampmeier 1922). In any case, it can be said that the lymphatic heart muscle cells must be derived from the somites, whorls of mesoderm, which contain mesenchymal cells and give rise to venous endothelium as well as myoblast-containing myotome.

Molecular Factors Affecting Lymphatic Heart Development

While the gross anatomical development of the lymphatic system in *X. laevis* and other anurans has been well documented in the twentieth century, and some studies have focused on the embryonic development of lymphatic vasculature (Ny *et al.* 2005), very little research has been directed at elucidating the

molecular mechanisms by which lymphatic heart development – specifically that of the muscular layer – is directed. The main body of research has focused on sonic hedgehog as a candidate. The role of *shh* and other transcription factors in the development of lymphatic hearts has only just recently begun to be explored.

Sonic hedgehog (*Shh*) is a highly conserved globular protein morphogen employed multiple times throughout vertebrate embryonic development to pattern axes and induce cell differentiation (Gilbert 2006). As a toolkit pathway activator, *Shh* often works in concert with other activators, like Wnt and FGF proteins, to guide the fate of cell populations (Gilbert 2006). *Shh* secreted by one set of cells acts in a concentration-dependent fashion on nearby cells (paracrine signaling) to transduce a signal that leads to the transcription of downstream target genes (Gilbert 2006). In the early embryo, *shh* is produced by the cells of the notochord along the entire length of the anterior-posterior axis. The well-characterized, textbook role of *shh* in this context is to diffuse upward from below in a gradient to pattern the dorsal-ventral axis of the neural tube in concert with BMP4 and other TGF-β family factors originating from the dorsal ectoderm (see Fig. 7) (Gilbert 2006). The concentrations of *shh* and TGF-β that a cell of the neural tube is exposed to will direct its fate in one dimension (dorsal-ventral) while other factors, such as the Hox gene products, will specify its fate in another (anterior-posterior) (Gilbert 2006). The case of *shh*'s role in neural tube patterning shows how a simple set of paracrine factors acting along differential gradients in three

dimensions can specifically induce a cell to transcribe genes that determine what tissue type it is destined to become. It is reasonable, based on the proximity of the notochord to the cells of the somites that are thought to give rise to the muscular layer of the anterior lymphatic hearts, to hypothesize that *shh* plays a part in their specification and development (see Fig. 8).

In other vertebrate model systems, some sub-populations of cells in the somites are sensitive to *shh* signaling during myogenic differentiation. A specific set of motile cells that originate in the epaxial myotome, a somite derivative, are thought to be homologous to the cells that give rise to lymphatic heart musculature in *X. laevis* (Peyrot *et al.* 2010). In zebrafish and chickens, these cells are known as muscle pioneers. Muscle pioneers are marked by expression of a protein called *engrailed-1* (*en-1*), which is regulated by *shh* exposure originating in the notochord (Peyrot *et al.* 2010). *En-1* is a homeodomain-containing transcription factor (Ingham and McMahon 2001). Classically, *en-1* first acts to delineate the polarity of meristic segments of tissue, and then works in concert with other factors expressed in gradients along other axes to activate the homeotic selector genes of the *Hox* family that specify the identity of the segment (Gilbert 2006).

In *X. laevis*, *en-1* is expressed level with the notochord in the outermost layer of a subset of trunk somites, known as the dermomyotome, at stages 28 through 39

(see Fig. 9) (Grimaldi *et al.* 2004, Peyrot *et al.* 2010). *En-1* gene expression in this region during these stages is specifically associated with the development of lymph heart myoblasts and musculature at stages 42-45. Embryos injected at the two-cell stage with *en-1* mRNA morpholinos, which post-transcriptionally knock down translation of the protein *en-1*, specifically fail to develop lymphatic heart muscle layers (Peyrot *et al*. 2010). *En-1* transcription is positively regulated by *shh* in *X. laevis* embryos (Peyrot *et al*. 2010). The inhibition of *shh* by cyclopamine before stage 26 results in significant loss of *en-1* expression in stage 37 embryos and is correlated with the specific lack of normal lymph heart musculature in these individuals at stages 42-45 (see Fig. 10). These results support the hypothesis that *shh* signaling in the anterior somites is permissive and is necessary for the induction of *en-1* transcription in the *Xenopus* muscle pioneer homologues that later differentiate to form the muscular layers of the anterior lymphatic hearts.

While interesting, these results do not illustrate whether or not *shh* signaling is sufficient to induce lymphatic hearts from somites alone, nor do they illuminate other possible factors that may influence or direct lymphatic heart localization. It is important to remember that *shh* is produced and secreted by the notochord along the entire length of the embryo. Its expression is not localized medial to somites 3 and 4, as one might expect if it were the sole determinant of lymphatic heart localization and muscular layer differentiation (see Fig. 11). One of the

open questions in this field of interest in evolutionary developmental biology is what factor(s) restrict the localization of lymphatic hearts in anurans to the limb girdles. What aspect of the embryonic signaling cascade has changed between the Anura and the Caudata that has resulted in the restriction of lymphatic hearts to two locations in the trunk in the former, when so many more of these organs are found along the entire length of the trunk in the latter? Much research remains to be done before the historically controversial ontogeny of lymphatic heart development in amphibians is understood in modern terms.

On a related note, it appears from a recent student study that the overall development of functional lymphatic hearts in *X. laevis* embryos is partly regulated by retinoic acid. Retinoic acid (RA) is known to have a primary function in patterning the anterior-posterior axis very early in development, in stages 1 through 10. Correlative data shows that when high concentrations (20µL) of exogenous RA are administered, this axis is cranially truncated (Angeles 2010), likely by the modification of cell fate downstream of the peptide growth factors signals that trigger identity-specifying homeobox gene expression in the anterior region of the embryo (Ruiz i Altaba and Jessell 1991). Among the notable defects observed in the RA treated tadpoles was a lack of an organized lymphatic system and no lymphatic hearts (Angeles 2010).

Objective

The objective of this research was to determine whether the introduction of exogenous *shh* to isolated *X. laevis* somatic tissue cultures during the reported critical period of this signaling molecule's action as activator of *en-1* transcription (prior to stage 26) would induce ectopic lymph hearts *in vitro*. Cultured chicken somites have been used to study the effects of molecular treatments outside of the complex embryonic environment, without some of the limitations of reductionist cell culture methods (Krull and Tosney 2008). The advantage of experimenting on tissue culture preparations is that it is a simplified *in vivo* system with *in vitro* accessability to molecular manipulation.

Hypothesis

It was hypothesized that *shh* signaling originating in the notochord is sufficient to induce *en-1* transcription in the cells of the anterior somites that later differentiate to form the muscular middle layer of the anterior lymphatic hearts in *X. laevis*. The null hypothesis, given the results reported by Peyrot *et al.* in their 2010 publication, was that *shh* signaling is a necessary but not individually sufficient component of the signal lymph heart myoblasts receive to differentiate from the somites.

It was predicted that, if *shh* signaling is sufficient to induce the differentiation of lymphatic heart musculature, then the addition of a high concentration of *shh* to isolated and cultured somites would result in the over-development of functional lymph heart muscle anatomy and the production of ectopic beating lymph hearts. If *shh* signaling is not sufficient to induce the differentiation of lymphatic heart musculature, then the addition of a high concentration of *shh* to cultured somites would not result in the over-development of functional lymph heart muscle anatomy and the production of ectopic beating lymph hearts. At least one heart was expected to develop per somite in the untreated controls, as would occur in *vivo* in these tissues. Assuming that lymphatic heart development would occur in both the treatment and control somites, the pulsing organ would be observable to the naked eye by the second or third day following somite isolation, at a tissue age of three and a half days (corresponding to stage 43).

Materials and Methods

X. laevis embryos were generated using standard protocols; adult male and female pairs of albino frogs were injected with approximately 250IU/mL human chorionic gonadotropin (Sigma) by tuberculin syringe to induce breeding. Pairs were housed overnight in covered bins to mate, shed, and fertilize eggs. The egg masses were collected 12-24 hours later, at approximately stage 14. The jelly layers of the embryo were removed by repeated washings with De-Jelly Solution

(Kay and Peng 1991), and the denuded embryos were spread in 0.1X Marc's Modified Ringer (MMR) Solution (Kay and Peng 1991). The embryos were allowed to reach stage 23, as assessed using the Normal Table (Nieuwkoop and Faber, 1956).

Embryos were transferred using 5 ¾ inch Pasteur pipettes into small black clay-filled Petri dishes filled with sterile 0.1X MMR for surgery. The embryos were manipulated using the molded end of a fire-polished glass pipette. Vitelline membranes were removed using tungsten needles. These needles were used to make transverse cuts to remove the cephalic and post-anal sections of each embryo, as well as to remove the ventral gut region (ectoderm) of the embryo via coronal section. The remaining portion of the embryo was cut sagittally, generating two sets of somites. Tungsten needles and a fire-polished glass pipette were also used to loosen and remove the dermal tissue (ectoderm) and notochord from each set of somites. Surgical tools, including forceps, tungsten needles, and black clay-filled Petri dishes were sterilized at the conclusion of each surgical session by exposure to UV light in a biosafety cabinet.

A first set of replicates (n=6) were exposed to a 0.2 mg/mL collagenase solution for five minutes at this point in order to loosen the dermal tissue and make the removal of the skin and notochord easier. Kay and Peng (1991) recommend a more aggressive enzymatic treatment in the preparation of muscle cultures or

somite isolates, consisting of a 30-minute exposure to 1mg/mL collagenase in Steinberg's Solution. Surprisingly, even the adjusted, mild process caused the somites to disintegrate. This dissociation occurred shortly before the cultures dried out and were lost, which occurred within 24 hours of excision. The remainder of the replicates (n=38) were not exposed to collagenase and were maintained under high-humidity conditions to combat these technical difficulties.

The isolated somites were transferred by micropipette from the sterile 0.1X MMR to a small sterile Petri dish containing modified L-15 amphibian culture media (80% Leibovitz L-15 medium, 5% Gibco heat-inactivated fetal bovine serum, and 5% Cellgo MediaTech Inc. antibiotic/antimycotic). The somites were then transferred in 1 μL of culture media via micropipette into every other well in an empty, sterile twelve well plate. For treated somites, 1 μL of 12.5μg/mL recombinant *sonic hedgehog* in 10% BSA PBS was added to the 1 μL of modified L-15 amphibian culture media, generating a final concentration of 6.25 μg/mL *shh* (n=32). For control somites, 1 μL of PBS was added to the 1 μL of culture media. In order to combat evaporation in this small-volume culture, the empty wells between each of the occupied wells was filled with water, and the closed plate was placed in a tape-sealed plastic box lined with moist paper towels.

The presence or absence of beating lymph hearts in each somite culture was assessed at twenty-four hour increments following surgery and shh treatment, until the cultures dried out, disintegrated, or became clouded with debris. Using an Olympus SZX12 dissection scope and ProgRes MacCapture Pro software, images were generated to document and monitor the progression of somite culture development.

Results

A total of thirty-eight somites were cultured. Thirty-two somites were treated with ectopic shh, and six were held as controls. With a single exception, no evidence of normal, functional beating lymph heart formation was evident in any treated or control somite at any point during the periods of observation photographically documented. These findings were unexpected.

In a single instance, a small, rhythmically pulsing region was observed in a *shh*-treated somite culture. This pulsing region was observed over a period that lasted at least forty-eight hours and no more than seventy-two hours. The movement began when the somite was eight days old (corresponding to embryonic stage 48) – seven days after the somite had been excised from a one day old stage 23 embryo – and continued until the somite was ten days old (stage 48-49). This small region that had displayed repetitive movement ceased

to generate movement by inspection on day ten of culture, when the tissue was eleven days old (stage 48-49). It was notable that this particular somite contracted globally at random intervals during this period of time. Neither small pulsing nor global motion was found in any of the other thirty replicates.

Discussion

The results of the *in-vitro* examination of the effect of exogenous *shh* on somites did not support the hypothesis that *shh* is sufficient to induce lymphatic heart muscle differentiation and organization. At least 31 of 32 *shh*-treated somite cultures did not develop lymphatic heart beats (an indication of a properly specified and developed muscle layer). One somite culture may have developed a lymphatic heart with functional musculature, as a small, globular, rhythmically pulsating structure was noted. These results, combined with those reported by Peyrot *et al.*, support the null hypothesis that *shh* signaling is necessary, but not sufficient, to induce differentiation of lymphatic heart myoblasts from somitic tissue.

The prediction that one lymphatic heart would develop per control somite was also not supported by the results of the experiment; zero of six *shh*-free somite cultures developed beating lymphatic hearts. In retrospect, considering the postulated roles of *shh* proposed in the hypothesis and the anatomical origin of

shh in the embryo *in vivo*, it is not surprising that no lymphatic hearts developed in the isolated controls. Even if *shh* alone was sufficient to activate an intracellular signal cascade leading to the transcription of *en-1*, without the notochord in close medial proximity to the somites, no endogenous *shh* would have been diffusing or being transported to the cells of the somite that are lymphatic heart muscle precursors responsive to that signal. No lymphatic heart musculature would differentiate and no beating lymphatic hearts would develop. Therefore, while not predicted, these results do not necessarily contradict the hypothesis that shh is a sufficient activating factor. However, they do not provide evidence to support *shh*'s sufficiency over necessity directly.

The global contractions of the one *shh*-treated somite that developed a beating organ was unexpected, but is easily rationalized. It is possible that the somite isolation surgery was imperfect and, in consequence, some neural tube cells persisted to generate nervous tissue that established neuromuscular junctions and fired spontaneously, causing the contractions. If this were a case of imperfect surgical isolation, then the unique development of a beating lymphatic heart in this somite among the treated cultures could be discounted as the result of endogenous signaling (*shh* and/or other factors) from non-somite tissue, such as the notochord, neural tube, endoderm, or ectoderm that may have been present residually in the excised tissue.

Overall, the results of this experiment support the null hypothesis. The evidence provided by Peyrot *et al.* showed that *shh* is not just permissive to lymphatic heart myogenesis, but that it is very likely a necessary factor for this developmental process in *X. laevis*. The results of the original research reported here show that *shh* is not sufficient to activate lymph heart specific myogenesis in the trunk somites of *X. laevis* when the tissue is isolated from the potential sources of endogenous signaling present in the intact embryo, such as the ectoderm, endoderm, notochord, and neural tube.

The null hypothetical necessity and insufficiency of *sonic hedgehog* as an activator of *engrailed-1* is – most importantly – not contradictory to what is observed in the normal *in vivo* development of the lymphatic hearts in *X. laevis* embryos. The specific localization of *en-1* positive future lymphatic heart myoblasts to two regions of the trunk anterior-posterior axis in wild type *Xenopus* (see Figs. 9 and 10) is not consistent with the hypothesis that notochord-secreted *shh* is sufficient in and of itself to trigger differentiation of these cells from the adjacent somites. If *shh* were necessary and sufficient, *en-1* positive cells would be found in a continuous lateral line along the trunk dermomyotome, shadowing *shh* expression (see Fig. 11), and a longitudinal suite of lymphatic hearts would probably develop in anurans as in caudates as a result (see Fig. 1).

Future Directions

The effect of exogenous shh application on cultured somites could be further examined in a repeat experiment by conducting *in situ* hybridizations of *en-1* mRNA on treated and control samples. This would indicate whether or not *en-1* is activated in isolated *X. laevis* somites under either condition, and if any differences in the expression pattern can be discerned.

While shh overexpression studies have been conducted in *X. laevis* embryos *in vivo*, and no ectopic hearts were reported to have developed, the results of such an experiment have not been published comprehensively (Peyrot *et al.* 2010). Thus, an independent study of the effect of exogenous *shh* application on the expression of *en-1* and the subsequent development of functioning lymphatic heart musculature in whole-animals is also in order. Light microscopic inspection for lymphatic heart beats, 12/101 Ab whole-mount staining for differentiated muscle, and whole-mount *in situ* hybridization of *en-1* mRNA are methods that could be used to detect natural and ectopic lymphatic heart that may develop as a result of such an experiment.

Much still remains to be determined; the field is wide open. While it seems clear that in anurans *sonic hedgehog* signaling plays an integral role in the medial-lateral patterning that specifies future lymphatic heart muscle tissue from the rest

of the somite, the other factors affecting lymphatic heart musculature development in this and other axes have not been described. Obviously, these other factors, perhaps from the ectoderm and likely from the toolkit, play an important and necessary role – just as the TGF-β family of proteins from the ectoderm works in concert with *shh* from the notochord to specify the fates of neural tube cells. Some of these unknown signals may co activate *en-1* in the cells of the anterior somites, but it is just as likely that some repress activation of *en-1* in the intervening somites of the trunk, preventing these somite cells from becoming lymphatic heart myoblasts. The window of time during development in which these factors must act has been roughly established (prior to stage 26), and their target is known (*en-1*), so it is now a matter of deciding which toolkit protein to investigate and pursuing different options for inhibition/knockdown and up-regulation/ ectopic expression studies *in vivo*.

As of yet, none of the molecular aspects of lymphatic heart muscle development have been explored in caudates. *A. mexicanum*, a caudate model organism, could be the subject of a comparative study of lymphatic heart molecular developmental biology that mirrors the work that has been done (and will be done in the future) on *Xenopus*. A wonderful starting point would be to determine if *en-1* is a lymphatic heart myoblast marker in this species. Then, cyclopamine-inhibition and morpholino knockdown experiments could be conducted on albino embryos to determine if *shh* is necessary but not sufficient to induce *engrailed-1*

expression in this species as well. In this way, differences and similarities between the homologous but divergent physiology can be noted and considered. These types of comparisons between *A. mexicana* and *X. laevis* could provide the raw material for a theoretical model that describes the evolution of the molecular regulatory networks that specify lymphatic heart development in caudate and anuran amphibians. Such a theory would enrich our understanding of this interesting and primal aspect of tetrapod evolution.

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Figures

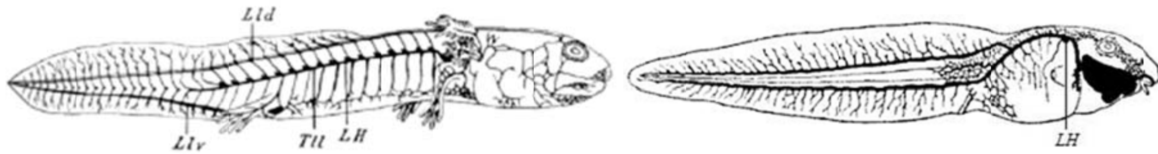

Figure 1. A lateral view (anterior right) of salamander and anuran larvae, injected with ink to trace the vessels, sinuses, and hearts of the lymphatic system (after Kampmeier 1969 (after Hoyer and Udziela 1912, Hoyer 1905)). Not to scale.

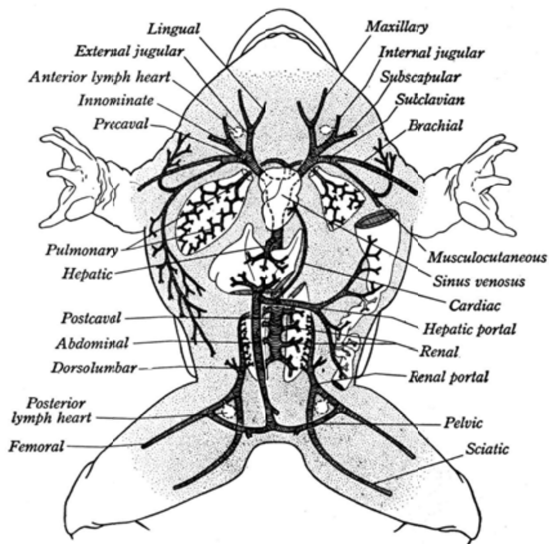

Figure 2. A generalized representation of the anuran venous system, showing the anatomical location of the anterior and posterior lymphatic hearts (after Getty Images, Inc.).

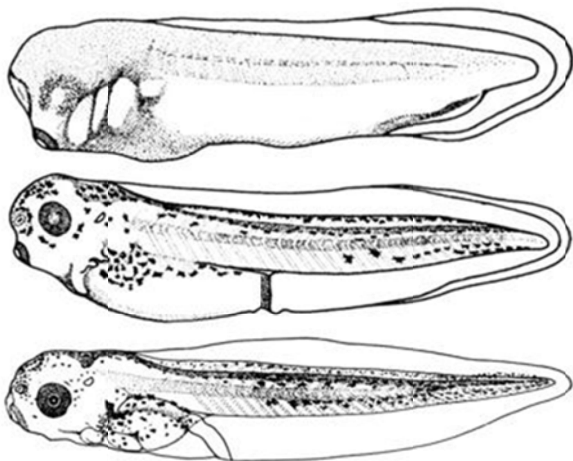

Figure 3. Lateral view (anterior left) of stage 32 (top), 40 (middle) and 43 (bottom) *X. laevis* (after Nieuwkoop and Faber 1956). Not to scale.

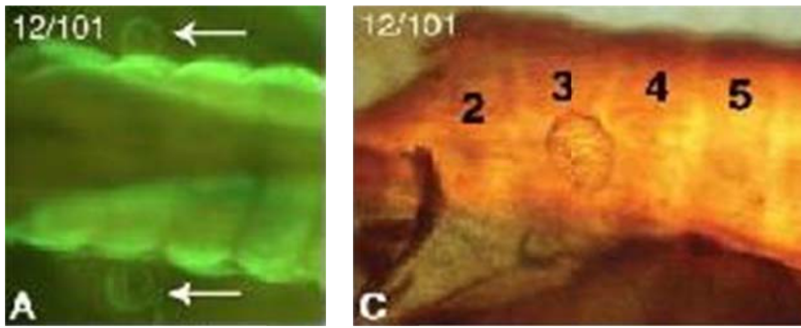

Figure 4. Anterior lymphatic hearts labeled with 12/101 antibody in stage 42 tadpoles (anterior left) (after Peyrot *et al*. 2010). White arrows in A, a dorsal view, indicate the location of the lymphatic hearts. A lateral view shows the animal's left lymphatic heart.

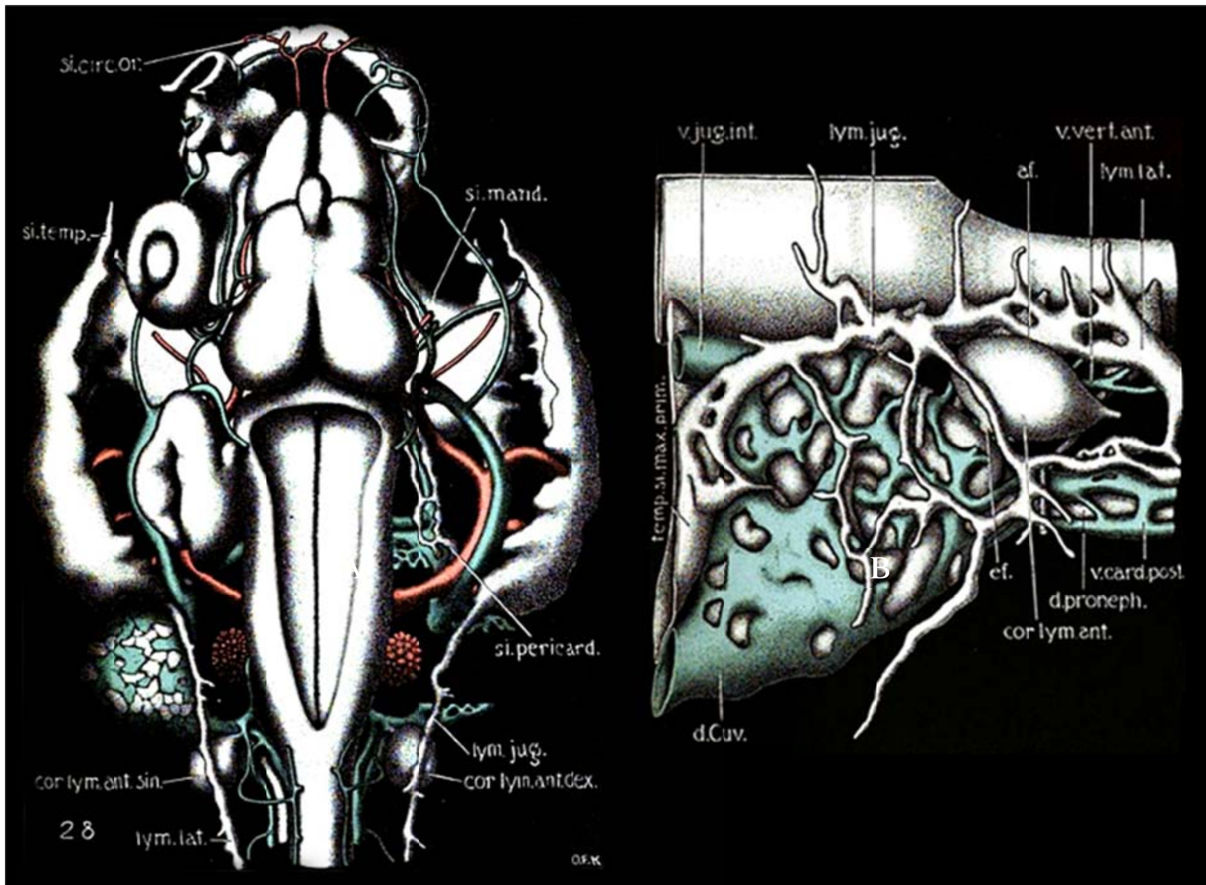

Figure 5. Dorsal (anterior up) and left lateral (anterior left) views of the lymphatic and hemocirculatory vessels and sinuses in relation to the anterior lymphatic heart in *Bufo sp.* (after Kampmeier 1922). Note that lymphatic fluid gathered in the *sinus maxillarus primigenus* (si temp.) drains bilaterally caudally to the dorsal afferent duct (af.) of the anterior lymphatic hearts (cor. lym. ant.) via the *lymphatica jugularis* (lym. jug.). The lymphatic hearts pump lymph ventro-medially via and efferent duct (ef.) into the complex kidney plexus of the developing anterior vertebral vein (v. vert. ant.).

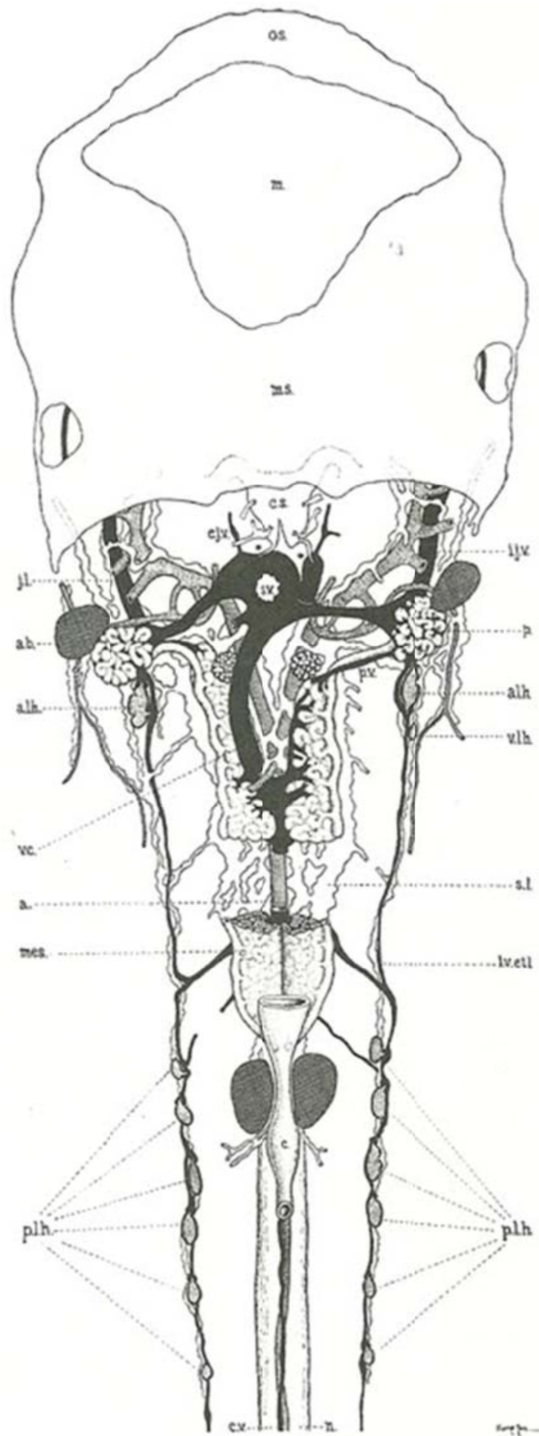

Figure 6. Ventral (anterior up) view of the lymphatic and hemocirculatory vessels and sinuses in relation to the posterior lymphatic hearts in *A.truei* (after Kampmeier 1958). Note that lymphatic fluid gathered in the caudal region of the tadpole drains bilaterally and rostrally through the lateral lymphatic vessel (l.v.et l.) to the posterior lymphatic hearts (p.l.h.). The posterior lymphatic hearts pump lymph ventrally into the lateral vein (l.v.et l.). In *X. laevis*, only four pairs of lymphatic hearts are formed instead of the six seen here.

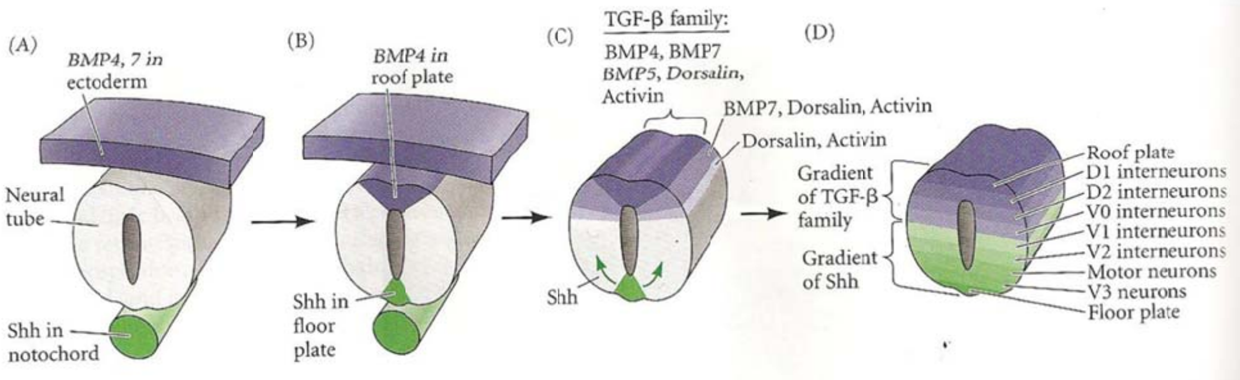

Figure 7. Transverse view of dorsal-ventral patterning of the neural tube cells in the embryo by *shh* and other factors (after Gilbert 2006).

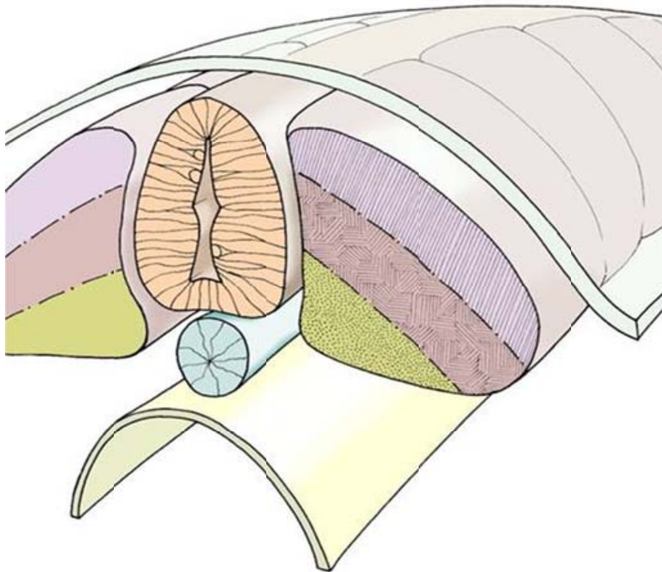

Figure 8. Transverse view of the dorsal anatomy of the embryo, showing the spatial relationships among the notochord (blue), neural tube (peach), somites (purple, pink, and yellow), ectoderm (clear), and endoderm (tan) (after Dryden 2011). Note that the myotome (pink middle layer of the somites) is well within the range of *shh* diffusion noted in Fig. 6.

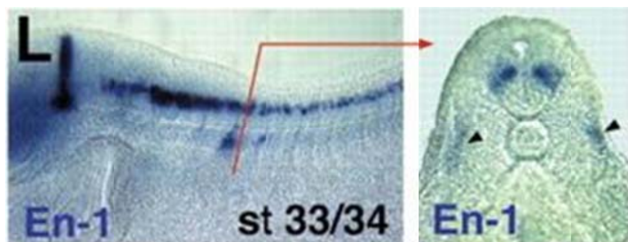

Figure 9. *In-situ* hybridization of *en-1* mRNA in the myogenic trunk tissue of a stage 33 *X. laevis* embryo (after Grimaldi *et al.* 2004). Lateral (anterior left) and transverse views show that the *en-1* expressing cells are found in the superficial, lateral portion (dermomyotome) of an anterior somite.

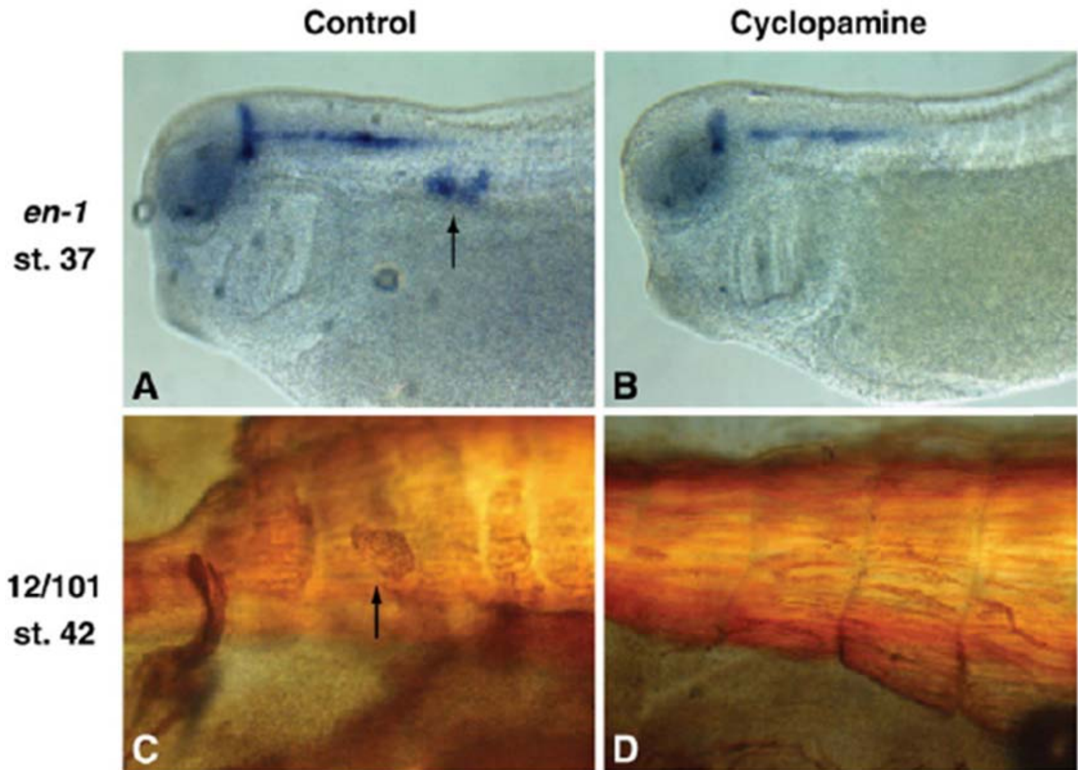

Figure 10. The effects of cyclopamine treatment to inhibit *shh* production in *X. laevis* embryos (anterior left) on later *en-1* expression and lymphatic heart development (after Peyrot *et al.* 2010). *In-situ* hybridizations at stage 37 (A, B) show the loss of *en-1* mRNA transcription in treated embryos (B), and immunohistochemical treatments with 12/101 antibody at stage 42 (C, D) show the correlated lack of lymphatic heart musculature in treated tadpoles (D).

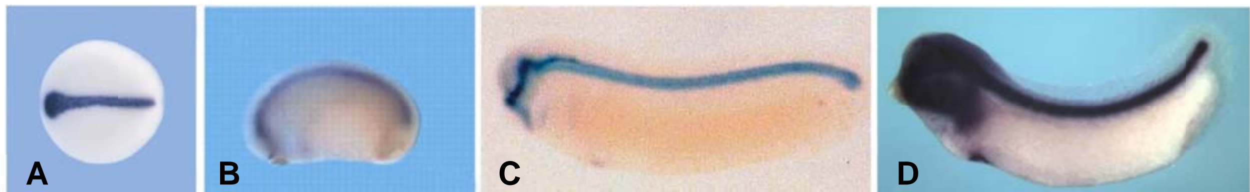

Figure 11. Dorsal (A) and lateral (B-D) views of *shh* mRNA *in-situ* hybridizations in stage 13 (A), 22-24 (B), 28 (C), and 32 (D) *Xenopus* embryos (anterior left) (after Inomata *et al.* 2008, Silva *et al.* 2003, Takabatake *et al.* 2000, and Coxhead 2011, respectively from left to right). Note that throughout the developmental stages relevant to *en-1 shh* dependency (A-C), *shh* is expressed along the entire length of the notochord.