

Sexually differentiated, androgen-regulated, larynx-specific myosin heavy-chain isoforms in *Xenopus tropicalis*; comparison to *Xenopus laevis*

Laura A. Baur · Brian T. Nasipak · Darcy B. Kelley

Received: 3 January 2008 / Accepted: 14 April 2008 / Published online: 13 June 2008
© Springer-Verlag 2008

Abstract We have shown that the sarcoplasmic myosin heavy-chain (MyHC) isoform *xtMyHC-101d* is highly and specifically expressed in the larynx of the aquatic anuran, *Xenopus tropicalis*. In male larynges, the predominant MyHC isoform is *xtMyHC-101d*, while in females, another isoform, *xtMyHC-270c*, predominates. The *X. tropicalis* genome has been sequenced in its entirety, and *xtMyHC-101d* is part of a specific array of *xtMyHC* genes expressed otherwise in embryonic muscles (Nasipak and Kelley, *Dev Genes Evol*, in press, 2008). The administration of the androgen dihydrotestosterone increases the expression of *xtMyHC-101d* in juvenile larynges of both sexes. Using ATPase histochemistry, we found that in adults, *X. tropicalis* male laryngeal muscle contains only fast-twitch fibers, while the female laryngeal muscle contains a mix of fast- and slow-twitch fibers. Juvenile larynges are female-like in fiber type composition (44% slow twitch, 56% fast twitch); androgen treatment increases the percentage of fast-twitch fibers to 86%. *xtMyHC-101d* predominates in larynges of dihydrotestosterone-treated juveniles but not in larynges of untreated juveniles. We compared the larynx-specific expression of *xtMyHC* genes in *X. tropicalis* to the MyHC gene expressed in *X. laevis* larynx (*xlMyHC-LM*) by sequencing the entire

xlMyHC-LM gene. The androgen-regulated *xtMyHC* that predominates in the male larynx of *X. tropicalis* is not the gene phylogenetically most similar to *xlMyHC-LM* at the nucleotide level but is instead a similar isoform found in the same MyHC array and expressed in the embryonic muscle.

Keywords Gene duplication · comparative genomics · evolution · *Xenopus* · Myosin heavy chain

Introduction

Myosin, the molecular motor of muscle fibers, includes two heavy chains (MyHCs) that utilize adenosine triphosphate (ATP) to produce the mechanical energy needed for contraction. MyHC expression parallels changes in fiber type composition, oxidative capacity, rate of ATP hydrolysis, and maximum shortening velocity (Lutz and Lieber 2000). The vertebrate genome contains several copies of the sarcoplasmic type II MyHC genes, which code for the MyHC proteins found in muscle tissue. The expression of different MyHC genes contributes to the functional properties of specific muscles. For example, the fiber type composition and related MyHC expression of the highly specialized leg muscles of *Rana pipiens* facilitates jumping (Lutz et al. 1998).

Laryngeal muscles of the African clawed frog, *Xenopus laevis*, are sexually dimorphic in fiber type composition (Sassoon et al. 1987), contractile properties (Tobias and Kelley 1987), and MyHC gene expression (Catz et al. 1992). These muscles express a laryngeal-specific MyHC isoform (laryngeal myosin or *xlMyHC-LM*) at much higher levels in males than in females or juveniles (Catz et al. 1992). The entirely fast-twitch composition of male laryngeal muscle (Sassoon et al., 1987) supports the rapid (>70 Hz)

Communicated by T. Hollemann

Laura A. Baur and Brian T. Nasipak contributed equally to this work.

L. A. Baur · B. T. Nasipak · D. B. Kelley
Department of Biological Sciences, Columbia University,
New York, NY 10027, USA

B. T. Nasipak (✉)
Department of Cell Biology,
University of Massachusetts Medical School,
55 Lake Ave. North,
Worcester, MA 01655, USA
e-mail: Brian.Nasipak@umassmed.edu

production of tension transients that produce click sounds (Kelley 2002). This male-typical click rate is much faster than that required for female vocalizations (Kelley 2002) and much higher than the stimulation rate at which the female muscle tetanizes (Tobias and Kelley 1987). The ability to produce these rapid contractions develops during the first 6 months of postmetamorphic development (Tobias et al. 1991a). Masculinization is halted by castration (Tobias et al. 1991b), and greatly accelerated by the administration of exogenous androgens to juveniles (Tobias et al. 1993).

The evolutionary origins of sexually differentiated expression of this laryngeal-specific MyHC have not been studied. *X. laevis* belongs to the family Xenopodinae containing at least 22 known species found in central and southern Africa. The phylogenetic relationships of extant species have been described using both mitochondrial and nuclear deoxyribonucleic acid (DNA; Evans et al. 2004). All species produce calls via contraction of the laryngeal muscles to pull apart a pair of cartilaginous disks, thus producing a “click” (Yager 1992); the temporal patterns of trains of clicks (trills) and click rates are unique to each species. While the mechanism of sound production is highly conserved, the rates at which these clicks are produced and thus the rate at which laryngeal muscles contract vary considerably among species, from two clicks per second in *X. borealis* (Yager 1992) to 70 in *X. laevis* (Kelley 2002). This variation in muscle contraction rates provides an opportunity to examine how MyHC isoform expression changes under selection for the trill rates of species-specific calls.

The genome of the single diploid *Xenopus* species, *Xenopus tropicalis*, is being sequenced in its entirety (<http://genome.jgi-psf.org/Xentr3/Xentr3.home.html>) and has greatly facilitated the study of molecular genetics in the Xenopodinae. The inspection of the *X. tropicalis* genome reveals three MyHC arrays, two of which show the conservation of synteny with MyHC arrays in the human genome (Nasipak and Kelley 2008). A third *X. tropicalis* MyHC array has no human homolog and differs from the other arrays in that it contains two types of the MyHC gene: three isoforms expressed during embryonic and larval stages and one expressed only in the laryngeal muscle (Nasipak and Kelley 2008). Whether this isoform is androgen regulated is, however, not known. The presence of an androgen-regulated, laryngeal-specific MyHC isoform in *X. tropicalis* would suggest that this isoform evolved before the split between the parent taxa ~50 mya (Evans et al. 2004).

In this paper, we examine differences in MyHC expression in adult male and female *X. tropicalis* larynges, as well as the effects of exogenous androgens on laryngeal muscle fiber types. We found that the MyHC isoform *xtMyHC-101d* is expressed in the larynx of *X. tropicalis* and its expression is sexually differentiated and androgen

regulated. However, while the physiological process of androgen-regulated fiber type switching appears to be conserved in the two species, phylogenetic analysis revealed that the *X. laevis xtMyHC-LM* is not the homolog of *xtMyHC-101d* but of the physically adjacent isoform *xtMyHC-101c*, an isoform not expressed in the larynx of *X. tropicalis*.

Materials and methods

Animals and hormone treatment

Adult males and females and stage 65 (Nieuwkoop and Faber 1956) tadpoles were obtained from Nasco (Ft. Atkinson, WI) and kept in 10-L Rubber Maid™ tanks filled 6 in. deep with standing tap water (26°C) on a 12-h light/dark cycle. Adult and fully metamorphosed juveniles were fed frog chow (Nasco), and tadpoles were fed larval Z (Zeigler) and tadpole mash (Nasco). The end of metamorphosis was determined by the disappearance of the tail.

Juveniles 0–7 days after the end of metamorphosis received implants of 0.25 mg of dihydrotestosterone (DHT, Sigma) mixed in a 1:10 ratio with medical grade silicon and allowed to solidify for 24 h. Control animals received 2.5 mg silicon pellets. The pellet was inserted through a 1-mm incision into the dorsal lymph sac posteriorly and positioned behind the ocular orbits. Animals were killed 14 days after implantation by immersion in MS-222, followed by the dissection of the heart. Juvenile animals of both sexes were used with no difference in the androgen response observed; thus, male and females were pooled.

Histochemistry

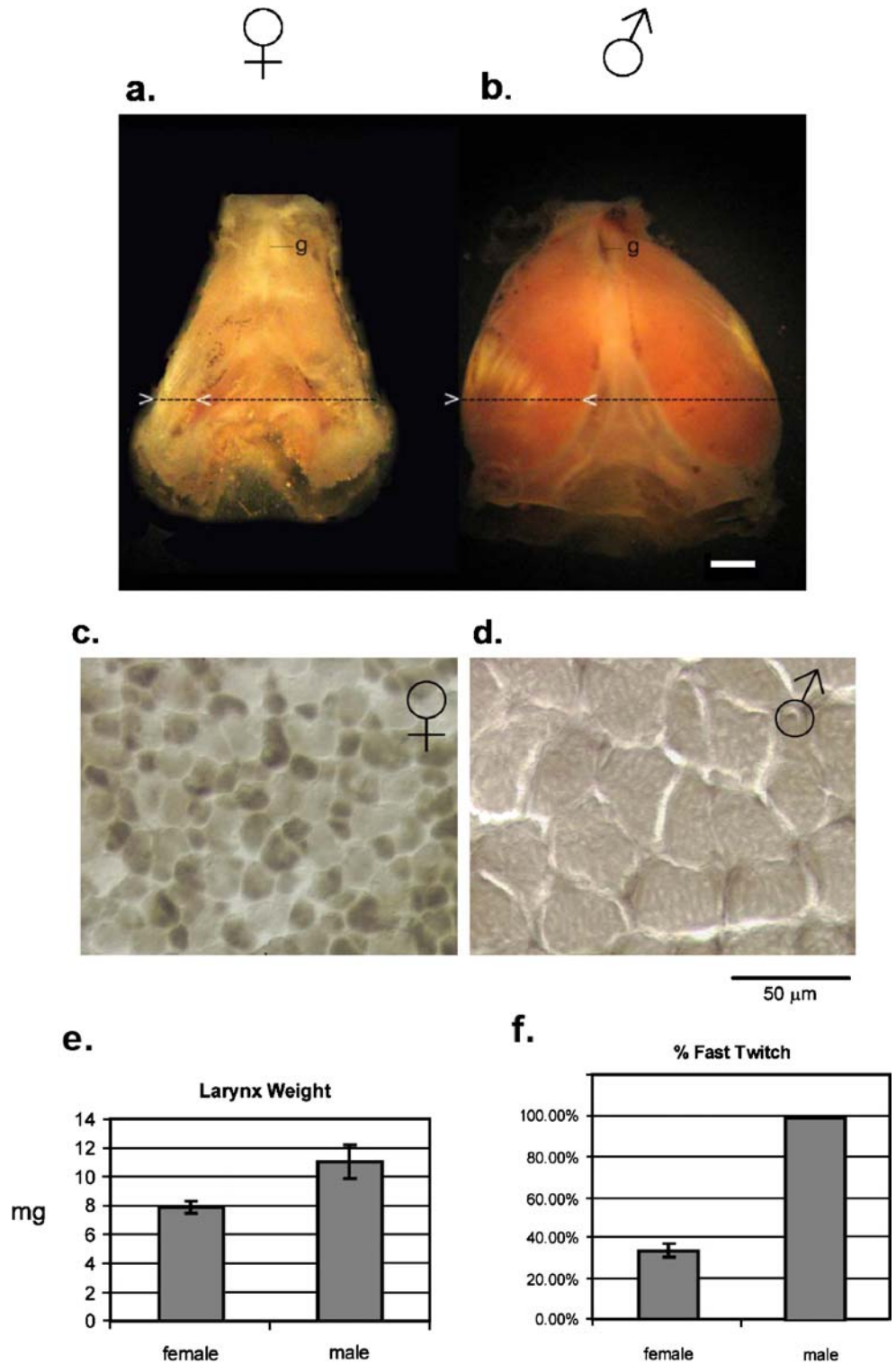
Frogs were deeply anesthetized in MS-222 (ethyl-*m*-amino benzoate, methane sulfonic acid, Sigma; immersion in a 0.1% solution), and larynges were removed and frozen in dry ice in 60% OCT (O.C.T 4583, Tissue Tek) and transversely sectioned (30 μm) using a Bright cryostat at –20°C. Sections were allowed to dry for 4 h before being stored at –80°C. To distinguish between slow- and fast-twitch fibers, we used a modification of Guth and Samaha (1970). Slides were incubated for 15 min in acid buffer (50 mM potassium acetate, pH 4.35) and then for 30 min in ATPase solution (100 mM adenosine monophosphate, 2.7 mM ATP, 50 mM KCl, 18 mM CaCl₂) at 30°C, washed with 1% CaCl₂, placed for 3 min in cobalt chloride (2% in water), washed in water, and finally placed for 3 min in an ammonium sulfide solution (2% in water) before being washed and coverslipped in Crystal Mount (Biomed). Using this procedure, slow-twitch fibers stain intensely due to a black precipitate, while fast-twitch fibers stain less intensely (Sassoon et al. 1987).

Fiber counting and measurement

Three sections from each animal were taken from the midpoint of the larynx (see Fig. 1), and fast- and slow-twitch fibers were identified using differential ATPase staining.

For each section, at least 300 intact fibers were counted using ImageJ to ensure statistical significance. Photomicrographs of stained laryngeal sections were taken at $\times 20$ magnification using a Spot RT slider camera (Diagnostic Instruments).

Fig. 1 Adult female and male *X. tropicalis* larynges (**a**). Adult female larynx, dorsal view. **b** Adult male larynx, dorsal view. The position of the glottis (*g*) is indicated. Sections for ATPase histochemistry were taken from the region of the muscle indicated by the *dashed line*. The medial–lateral extent of laryngeal muscles in males and females is indicated by *arrowheads*. *Scale bar*=1 mm. **c**, **d** ATPase-treated female and male *X. tropicalis* laryngeal muscle sections (*scale bar*=50 μ m). **e** Male and female laryngeal weights and **f** percent fast twitch-type muscle fibers



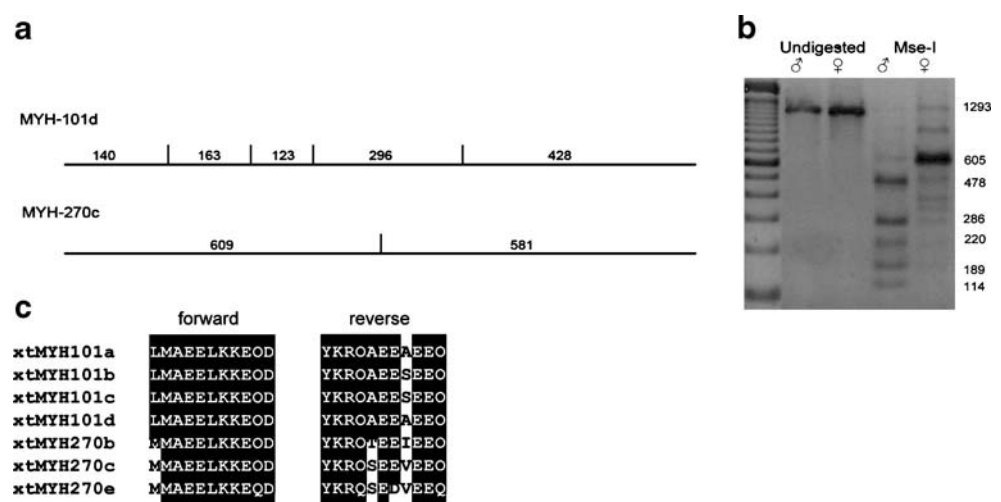
RNA isolation and cDNA synthesis

Frogs were deeply anesthetized in MS-222, and the larynx was removed. The muscle—from adult male and female *X. tropicalis* as well as juveniles treated for 2 weeks with either DHT implants or empty silicon implants—was separated from the cartilage. Tissues were homogenized using Trizol (Invitrogen), and ribonucleic acid (RNA) was isolated per manufacturer's protocol and reconstituted in 20 μ L of water. First-strand complementary DNA (cDNA) synthesis was carried out using 0.25 μ g of RNA in 20 μ L of reaction using a Superscript RT kit (Invitrogen).

Degenerate PCR and restriction digests

We used degenerate primers directed to a conserved region of the MyHC sequence and polymerase chain reaction (PCR) to determine the MyHC content of male and female laryngeal muscles (protein sequences: MMAEELKKEQD, YKRQAEAEAEQ; Primer sequences: atgatggcngargaryt naaraargarcargay, atrttyctygtcgnctyctcgnctyctycty). The reaction yielded a 1,300-bp PCR product from the reverse-transcribed cDNA sample, which, when digested with the restriction enzyme *Mse*I, yields a specific pattern of subfragments (Fig. 2a) for each of the 16 identified *xt*MyHC genes (Nasipak and Kelley 2008). The PCR was performed using Ready *Taq* DNA polymerase (Sigma) in 20- μ L reactions with 500 nM primer concentrations (1 min at 95°C, 2 min at 52°C, 1 min at 72°C [eight times]; 1 min at 95°C, 1 min at 60°C, 1 min at 72°C [20 times]). Restriction digests were carried out for 2 h at 37°C before being run on a 2% agarose gel. A digital photograph was taken of the gel, and the sizes of the resulting bands were determined by taking a vertical cross-section of the pixels and plotting the peaks against the ladder.

Fig. 2 Restriction digest of a conserved region of MyHC for *xt*MyHC-101d and *xt*MyHC-270c (a). The number of base pairs composing each fragment is indicated. Resulting bands from *Mse*I digestion of PCR fragments amplified from cDNA from adult male and female laryngeal muscle tissues (b). Comparison of the distance from the well to each band resulting from the digest with the migration distance of a 100-bp ladder allows the calculation of the sizes for each band, which are similar to that of *xt*MyHC-101d in the male larynx and *xt*MyHC-270c in the female larynx.



Real-time PCR

Real-time PCR reactions were performed using SYBR green (Invitrogen) in 20- μ L reactions with 0.33 μ L of cDNA and a 300-nM concentration of primers against the *X. tropicalis* MyHC genes (primer sequences in Nasipak and Kelley 2008). Samples were run in an ABI 7700 thermal light cycler under the following conditions: 15 min at 94°C (1 \times), 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C (40 \times). Premeasured concentrations of plasmids into which each gene fragment had been cloned were used to determine the relative amounts of each gene amplified. EF1 primers were used to control for absolute cDNA quantity.

Cloning *xl*MyHC-LM

RNA was isolated from an adult male *X. laevis* larynx using Trizol. To amplify the 5' end of the *xl*MyHC-LM gene, cDNA was prepared using the Ambion RLM 5' rapid amplification of cDNA ends kit. PCR was performed with the provided primer and an added primer to a highly conserved 5' MyHC sequence (agggttgccctggatgattgac). This DNA fragment was inserted into the pCR2.1 TA cloning vector (Invitrogen) following the manufacturer's protocol. Samples were sequenced by Genewiz (www.genewiz.com) using M13F and M13R universal primers. This information, along with the existing 3' sequence information (GenBank AS no. L01495), was used to generate primers to amplify the remaining ~5,000 bp of the sequence. This product was cloned into the pCR2.1 TA cloning vector and sequenced from both ends using M13 universal primers. As the sequence information was returned, new primers were designed to overlap with the existing sequence by approximately 100 bp. This sequence was submitted to GenBank (GenBank Accession Number EU621675).

Phylogenetic analysis

The 16 MyHC sequences from *X. tropicalis* (Nasipak and Kelley 2008) were analyzed using the sequence of the masticatory myosin from dog (cfMyHC16) to root the final tree. Sequences were aligned using Clustal, and poorly aligned regions were removed. These regions were as follows: the region ending at amino acid (aa) 117, the two loop regions aa 205–214 and aa 618–643, the region of non-MyHC sequence from xtMyHC-270d aa 327–351, and the 3' end beginning at aa 1920. All sequence numbers are given as the aa position relative to the hMyHC-emb (X13988, start codon).

Alignments were converted to Nexus format, and Bayesian analysis was performed using MrBayes (<http://mrbayes.scs.sfu.edu>). A four by four nucleotide model with an F81 substitution model was used with no rate variation across sites. Nucleotide state frequencies were given equal priors and followed a dirichtlet distribution. Topology was equal a priori, and branch lengths were unconstrained, following an exponential distribution. Two independent simulations were run for 1,000,000 generations with sampling every 50 generations, resulting in a standard deviation of 0.000126 between the runs. Trees were calculated after excluding the first 20,000 generations, where the remaining generations showed no trend for the marginal likelihood. The probability scale reduction factor was near 1 for all parameters, and the 99% credible set of trees contained a single tree. Although the entire set of *X. tropicalis* MyHC sequences was analyzed, only the branch containing the four xtMyHC-101a–d and x/MyHC-LM is shown. The remainder of the tree is the same as that previously obtained from a similar analysis (Nasipak and Kelley 2008).

A distance matrix was obtained by submitting the same sequences to DNAdist, and sequences were also analyzed by sequential alignment of the coding regions using BLAST2 implemented on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST).

RDP sequence analysis

The similarity of the *X. laevis* x/MyHC-LM gene to genes xtMyHC-101c and xtMyHC-101d from *X. tropicalis* was assessed using Bootscan. The settings were: window size=100 bp, step size=10 bp, program=neighbor joining.

Results and discussion

Laryngeal muscle of *X. tropicalis* is sexually dimorphic

The larynges of adult *X. tropicalis* are sexually dimorphic in morphology, size, and fiber composition (Fig. 1). In both

sexes, the larynx consists of a cartilage skeleton flanked by laryngeal dilator muscles (the area between arrowheads; Fig. 1a, b); both larynges are triangular in shape, but the male larynx is more robust with a greater muscle mass. Although female body size is larger, the weight of the male larynx (Fig. 1e) exceeds that of the female (male mean=111±11 mg [SD; N=4], female mean=79±4 mg [N=4]). Following ATPase staining, sections through female larynges reveal a mixture of small, darkly staining fibers and larger, more lightly staining fibers (Fig. 1c), while the male larynx consists of fibers with larger cross-sectional areas that stain uniformly for ATPase (Fig. 1d). Female larynges average 34±3.4% (N=4) large, lightly staining fibers, while male larynges are entirely (100%) made up of this fiber type (Fig. 1f). The staining pattern is very similar to that seen in *X. laevis* and suggests that male larynges are uniformly fast twitch while female larynges consist of a mix of fast- and slow-twitch fiber types (Sassoon et al. 1987).

In *X. laevis*, the male laryngeal muscle expresses an androgen-regulated MyHC isoform that is specific to the larynx (Catz et al. 1992). To determine whether this is also the case for *X. tropicalis*, we used restriction fragment analysis to identify which MyHC gene products are expressed in male and female laryngeal muscles. We used the genomic sequences of all MyHC isoforms in *X. tropicalis* (Nasipak and Kelley 2008) to identify restriction enzyme sites that would produce isoform-specific fragment sizes. For example, *MseI* digests were predicted to produce two fragments of approximately 630 bp from xtMyHC-270c and several smaller fragments of xtMyHC-101d (Fig. 2a).

Using this approach, the undigested fragments were approximately 1,300 bp in both sexes, which is the appropriate length (Fig. 2b). The restriction digest yielded prominent fragments of approximately 478, 286, 220, 189, and 114 bp in length for the male laryngeal muscle sample, as well as a lesser band at 605 bp (Fig. 2b). These sizes are close to the expected fragment sizes of a restriction digest of the gene 101d: 477, 296, 220, 164, and 124 bp (Fig. 2a) but are not similar to the pattern predicted from any other *X. tropicalis* MyHC isoform. In the female larynx, the *MseI* restriction digest yielded one predominant 605-bp band (Fig. 2b), corresponding to gene 270c, which has only one *MseI* restriction site near the center of the amplified region (Fig. 2a). The digest also contains numerous other bands, indicating that other MyHC isoforms were present. Neither of these banding patterns is similar to that expected from the restriction digest of xtMyHC-101c, which has expected fragment sizes of 847, 287, and 16 bp.

We conclude that male laryngeal muscle expresses predominantly the MyHC gene 101d, with a lesser contribution given by MyHC 270c; MyHC-101d is not expressed in the female muscle, which instead expresses

predominantly MyHC 270c along with other isoforms. MyHC 101d expression is thus responsible for the contractile properties and fiber type composition of the male laryngeal muscle, while the female muscle reflects the contribution of several isoforms. Sex differences in myosin isoform expression in *X. tropicalis* larynx resemble those seen in *X. laevis*, with the male muscle being larger (Sassoon and Kelley 1986), having a homogenous fiber type (Sassoon et al. 1987) and expressing a male-specific MyHC isoform (Catz et al. 1992).

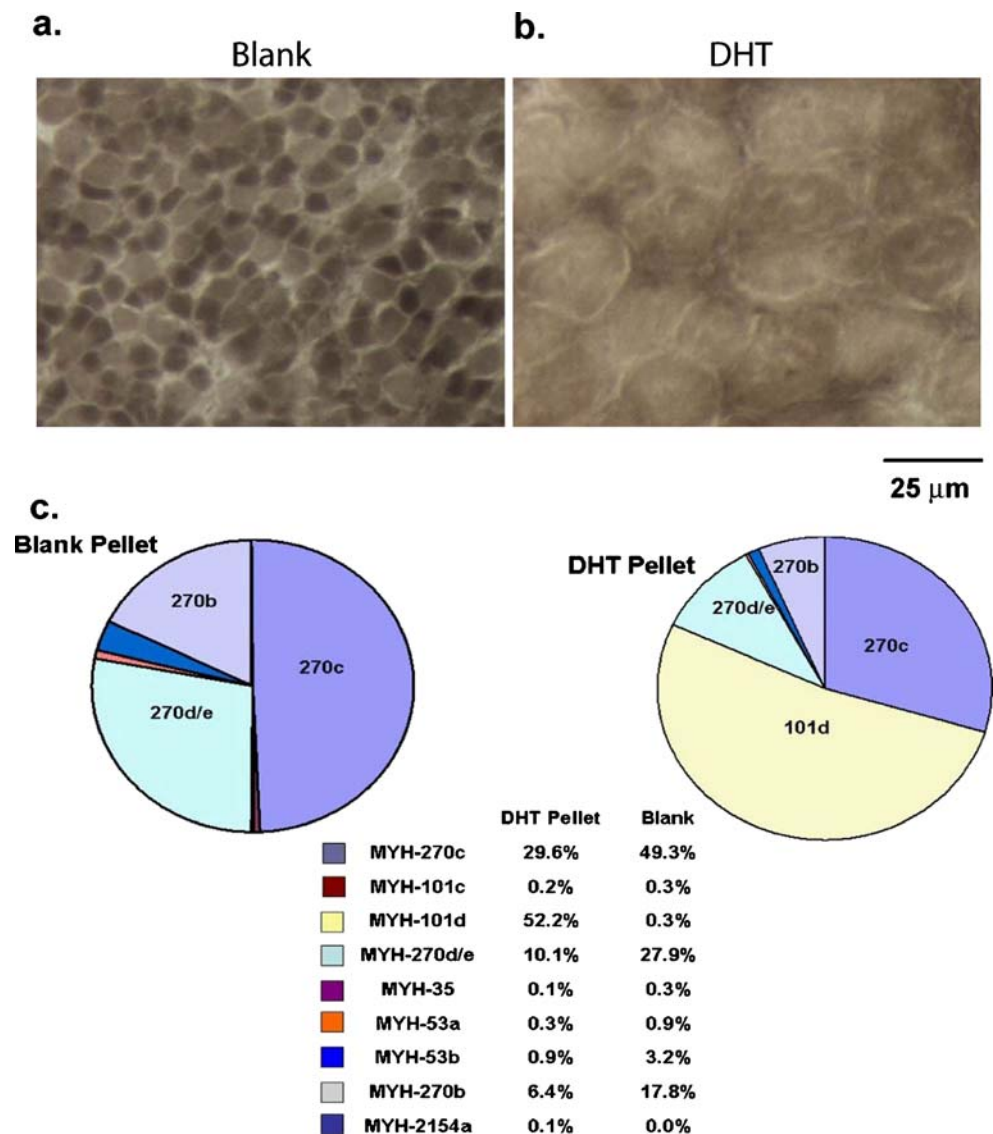
Androgen masculinizes the developing *X. tropicalis* larynx

To determine whether sex-specific MyHC expression is due to secretion of androgenic steroid hormones, newly post-metamorphic (NF st. 66) *X. tropicalis* were implanted with DHT or blank pellets for 14 days. Compared to blank-implanted controls, DHT-treated juveniles exhibited in-

creased laryngeal mass (not shown) and larger muscle fibers (compare Fig. 3b to a). Since the total body mass of the DHT-treated animals also increased slightly compared to controls, we used larynx-to-body mass ratios to analyze the larynx-specific effects of DHT. The average larynx-to-body mass ratio of DHT-implanted animals was 1.15% ($n=5$, $SD=0.21\%$), while that of controls was 0.35% ($n=5$, $SD=0.07\%$). Androgen treatments of juveniles increased the percentage of fast-twitch fibers (Fig. 3a,b). Larynges of control animals were 49.8% ($n=5$, $SD=10.3$) fast-twitch fibers, while the larynges of androgen-treated animals were 90.8% ($n=5$, $SD=6.3$) fast-twitch fibers.

We used quantitative PCR to examine the effect of DHT treatment on the profile of MyHC gene expression in the juvenile *X. tropicalis* larynx (Fig. 3c,d). The predominant isoform in the untreated juvenile larynx is *xtMyHC-270c* (49.3%); the expression of *xtMyHC-270d/e* is 28.9% and of *xtMyHC-270b* is 17.8% (Fig. 3c). The MyHC expression

Fig. 3 Laryngeal sections from juvenile *X. tropicalis* implanted for 14 days with a blank silastic pellet (a) or a 3 mg DHT pellet (b). The percent myosin gene expression in the laryngeal muscle of 14-day DHT-treated and control animals (c)



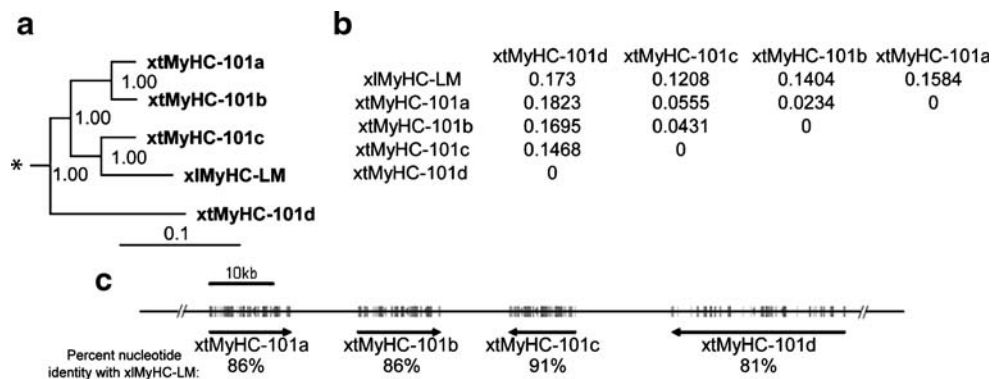


Fig. 4 Phylogenetic relations of *xtMyHC-101a-d* to *x/MyHC-LM* as determined by Bayesian analysis show *xtMyHC-101c* to be the homolog of *x/MyHC-LM* (a). Asterisk indicates the remainder of MyHC isoforms included in the analysis, including 12 of the 16 isoforms identified in Nasipak and Kelley (2008) and the *Canis familiaris* MyHC-16 (XM_546980). The DNA distance matrix between *X. tropicalis* genes *xtMyHC-101a-d* and the *X. laevis* gene

x/MyHC-LM confirms that the Bayesian analysis as *xtMyHC-101c* has the closest distance to *x/MyHC-LM* (b). The arrangement of MyHC genes *xtMyHC-101a-d* on scaffold 101 of the *X. tropicalis* genome project along with the percent nucleotide identity as identified by a BLAST2 alignment with *x/MyHC-LM* is given below each gene (c). Vertical bars represent exons

profile of DHT-treated juveniles differs substantially from the untreated profile: The predominant MyHC is *xtMyHC-101d* (52.2%); *xtMyHC-270c* (29.6%), *xtMyHC-270d/e* (10.1%), and *xtMyHC-270b* (6.4%) are also expressed (Fig. 3d). In terms of the predominant isoform, the untreated juvenile resembles the adult female (*xtMyHC-270c* predominates), while the DHT-treated juvenile resembles the adult male (*xtMyHC-101d* predominates).

These results show that androgen has a powerful masculinizing effect on the juvenile *X. tropicalis* larynx. This androgen sensitivity is very similar to that of *X. laevis*, where androgen treatment brings about an increase in the size of the larynx and the size of muscle fibers and results in all muscle fibers having acid-sensitive ATPase activity (Tobias et al. 1993) as well as the expression of *x/MyHC-LM* (Catz et al. 1995). In *X. laevis*, endogenous androgen appears responsible for male-specific laryngeal traits because castration halts masculinization (Tobias et al. 1991b; Catz et al. 1995). Both *X. laevis* and *X. tropicalis*, therefore, appear to exhibit a conserved androgen-sensitive process of masculine development leading to a change in muscle fiber properties and in MyHC isoform expression.

xtMyHC-101c rather than 101d is closer to *X. laevis* *x/MyHC-LM* in sequence

To determine to which of the *X. tropicalis* genes the *X. laevis* *x/MyHC-LM* is orthologous, we sequenced the entire *X. laevis* *x/MyHC-LM* gene and compared it to the *X. tropicalis* MyHCs (Fig. 4). Bayesian phylogenetic analysis revealed that *x/MyHC-LM* forms an extremely well-supported monophyletic clade with *xtMyHC-101c* (Fig. 4a) and is also closest to *xtMyHC-101c* using distance matrix analysis (Fig. 4b) and as reported by a BLAST alignment (Fig. 4c). Thus, the *X. tropicalis* ortholog of

x/MyHC-LM is shown with confidence to be *xtMyHC-101c*, rather than *xtMyHC-101d*. While *xtMyHC-101d* is the androgen-sensitive, laryngeal-specific MyHC isoform in *X. tropicalis* (Figs. 2 and 3), *xtMyHC-101c* is instead expressed in tadpole and embryonic muscles (Nasipak and Kelley 2008) but not in the male laryngeal muscle (Figs. 2 and 3).

In contrast, then, to the conserved physiological process of fiber type switching, the gene responsible is not conserved between *X. laevis* and *X. tropicalis*. In *X. laevis*, *x/MyHC-LM* is expressed in the adult larynx but not in embryos (Edwards et al. 1999), a completely different pattern of expression from that of its *X. tropicalis* ortholog. Phylogenetic analysis indicates that *xtMyHC-101d* and *xtMyHC-101c* were both present before *x/MyHC-LM* (Fig. 4a) and before the split between *X. laevis* and *X. tropicalis* (50 mya, Evans et al. 2004). Changed expression and regulation of one of these two genes could have occurred in either the *X. tropicalis* or the *X. laevis* lineage.

xtMyHC-101c and *xtMyHC-101d* are physically linked as part of a tandem array (Fig. 4c). This close apposition of a highly similar sequence might allow for recombination and gene conversion, events common in MyHC arrays (Moore et al. 1992). We considered the hypothesis that *X. laevis* *x/MyHC-LM* derives from an ancestral MyHC with sequence similarity to *xtMyHC-101d*. In this scenario, divergence could have been the result of recombination in a common ancestor between the 3' end of the *xtMyHC-101c* precursor and the 5' end of *xtMyHC-101d* precursor. To test this idea, we examined the complete alignment of the *x/MyHC-LM* sequence with the genes of the MyHC array containing *xtMyHC-101c* and *xtMyHC-101d* using the Bootscan algorithm (Salminen et al. 1995) of the recombination detection program (RDP). This algorithm compares genes fragment by fragment, generating multiple

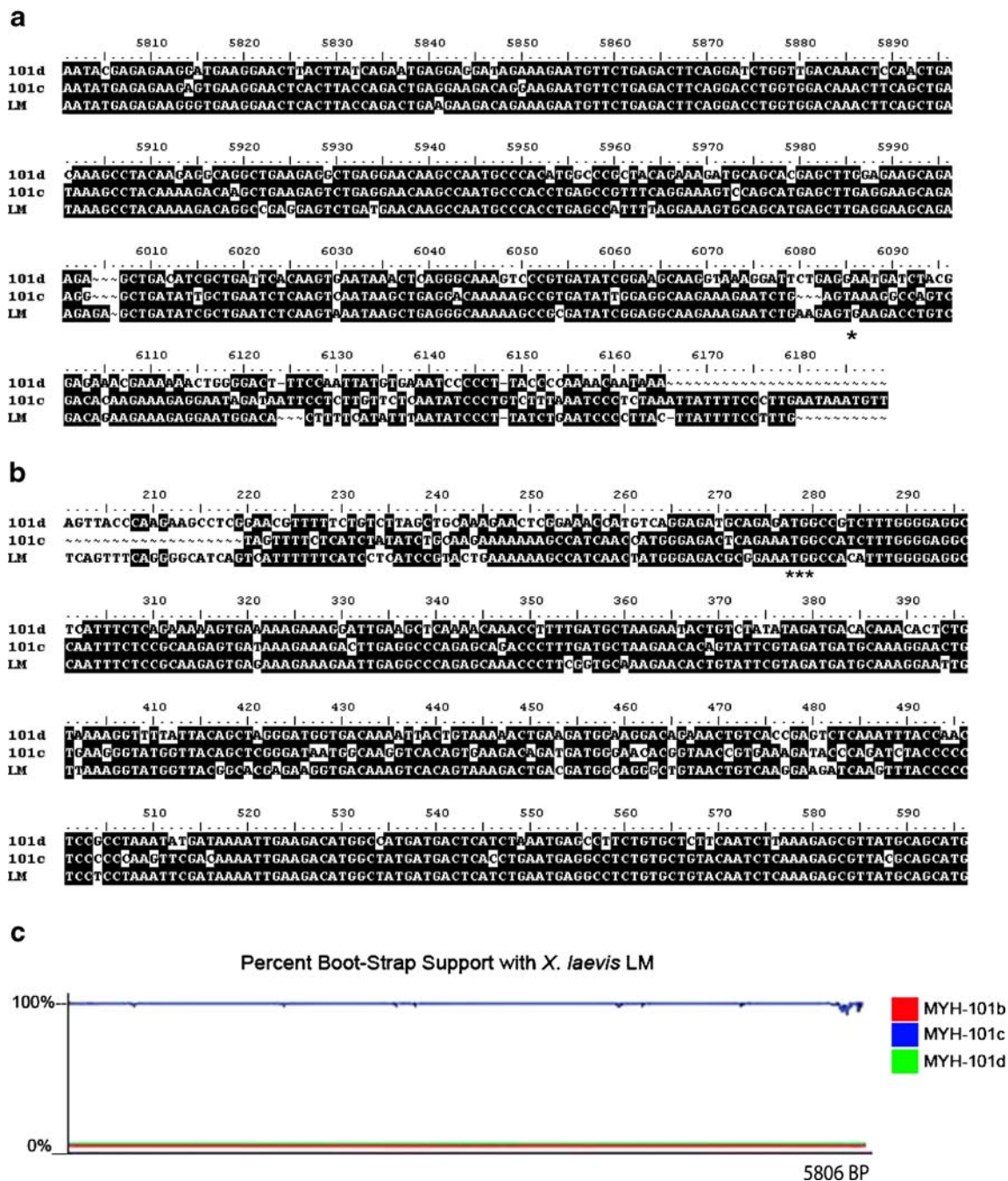


Fig. 5 The aligned 3' ends of the genes *xtMyHC-101d*, *xtMyHC-101c*, and *xtMyHC-LM* (a). A star denotes the end of the protein coding region and the start of the 3' untranslated region. The aligned 5' ends of the genes *xtMyHC-101d*, *xtMyHC-101c*, and *xtMyHC-LM* (b). Three stars denote a possible start codon. Full-gene comparison of the *X. laevis* gene *xtMyHC-LM* with the *X. tropicalis* genes *xtMyHC-101c* (blue) and *xtMyHC-101d* (green) using Bootscan (Salminen et

al. 1995) (c). The program compares the genes in 100-bp segments along the length of the entire gene. The *x*-axis denotes the percent bootstrap support that *xtMyHC-LM* is found in a monophyletic clade with each gene, and the *y*-axis denotes the nucleotide position in the alignment. Note that *xtMyHC-101c* maintains its similarity to *xtMyHC-LM* throughout the length of its sequence

phylogenetic trees. The program then plots the percent of trees in which *xtMyHC-LM* was found in a monophyletic clade with every other *X. tropicalis* gene along the length of the sequence. This analysis, however, reveals that the *xtMyHC-LM* gene maintains its similarity to *xtMyHC-101c* throughout the length of its sequence (Fig. 5c). In

addition, both the 3' and 5' untranslated regions of the *xtMyHC-LM* gene are closer in sequence identity to *xtMyHC-101c* than to *xtMyHC-101d* (Fig. 5a,b). We therefore conclude that *xtMyHC-LM* did not derive from a recombination event between ancestral *MyHC-101c* and *MyHC-101d* genes.

The finding that members of a MyHC array can exchange function during evolution demonstrates that the spatial/temporal gene expression patterns, as well as the coding sequence, can be rearranged within a tandem array of genes. Dynamic control of MyHC gene expression could contribute to species-specific optimization of muscle physiology and function. The *X. tropicalis* advertisement call is a slower trill than the *X. laevis* advertisement call (35 vs 70 Hz, respectively), and it will be of interest to determine whether sequence differences between the laryngeal myosins contribute to muscle contractile properties. Furthermore, many species of *Xenopus* exist and are available for study, each one producing calls at characteristic click rates, providing a pool of potentially diverse MyHC sequences associated with different physiological outputs. A comparison of MyHC genes across these species could also reveal which regions are most important for the evolution of these gene arrays as well the underlying mechanisms for changes in gene expression.

References

- Catz DS, Fischer LM, Kelley DB (1995) Androgen regulation of a laryngeal-specific myosin heavy chain mRNA isoform whose expression is sexually differentiated. *Dev Biol* 171:448–457
- Catz DS, Fischer LM, Moschella MC, Tobias ML, Kelley DB (1992) Sexually dimorphic expression of a laryngeal-specific, androgen-regulated myosin heavy chain gene during *Xenopus laevis* development. *Dev Biol* 154:366–376
- Edwards CJ, Yamamoto K, Kikuyama S, Kelley DB (1999) Prolactin opens the sensitive period for androgen regulation of a larynx-specific myosin heavy chain gene. *J Neurobiol* 41:443–451
- Evans BJ, Kelley DB, Tinsley RC, Melnick DJ, Cannatella DC (2004) A mitochondrial DNA phylogeny of African clawed frogs: phylogeography and implications for polyploid evolution. *Mol Phylogenet Evol* 33:197–213
- Guth L, Samaha FJ (1970) Procedure for the histochemical demonstration of actomyosin ATPase. *Exp Neurol* 28(2):365–367
- Kelley D (2002) Hormonal regulation of motor output in Amphibians: *Xenopus laevis* as a model system. In: Pfaff D, Arnold A, Etgen E, Fahrbach S, Rubin R (eds) *Hormones, brains and behavior*. Elsevier, San Diego, pp 445–468
- Lutz GJ, Bremner S, Lajevardi N, Lieber RL, Rome LC (1998) Quantitative analysis of muscle fibre type and myosin heavy chain distribution in the frog hindlimb: implications for locomotory design. *J Muscle Res Cell Motil* 19:717–731
- Lutz GJ, Lieber RL (2000) Myosin isoforms in anuran skeletal muscle: their influence on contractile properties and in vivo muscle function. *Microsc Res Tech* 50:443–457
- Moore LA, Tidyman WE, Arrizubieta MJ, Bandman E (1992) Gene conversions within the skeletal myosin multigene family. *J Mol Biol* 223:383–387
- Nasipak B, Kelley D (2008) The genome of the diploid anuran *Xenopus tropicalis* contains a novel array of sarcoplasmic myosin heavy chain genes expressed in larval muscle. *Dev Genes Evol* (in press) DOI 10.1007/s00427-008-0225-0
- Nieuwkoop PD, Faber J (1956) *Normal table of Xenopus laevis* (Daudin). Amsterdam: North Holland
- Salminen MO, Carr JK, Burke DS, McCutchan FE (1995) Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. *AIDS Res Hum Retrovir* 11:1423–1425
- Sassoon D, Kelley DB (1986) The sexually dimorphic larynx of *Xenopus laevis*: development and androgen regulation. *Am J Anat* 177:457–472
- Sassoon DA, Gray GE, Kelley DB (1987) Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. *J Neurosci* 7:3198–3206
- Tobias ML, Kelley DB (1987) Vocalizations by a sexually dimorphic isolated larynx: peripheral constraints on behavioral expression. *J Neurosci* 7:3191–3197
- Tobias ML, Marin ML, Kelley DB (1991a) Development of functional sex differences in the larynx of *Xenopus laevis*. *Dev Biol* 147:251–259
- Tobias ML, Marin ML, Kelley DB (1991b) Temporal constraints on androgen directed laryngeal masculinization in *Xenopus laevis*. *Dev Biol* 147:260–270
- Tobias ML, Marin ML, Kelley DB (1993) The roles of sex, innervation, and androgen in laryngeal muscle of *Xenopus laevis*. *J Neurosci* 13:324–333
- Yager D (1992) A unique sound production mechanism in the pipid anuran *Xenopus*. *Zool J Linn Soc* 104:1559–1567