

SHORT COMMUNICATION

Phenotypic plasticity of muscle fiber type in the pectoral fins of *Polypterus senegalus* reared in a terrestrial environment

Trina Y. Du¹ and Emily M. Standen^{2,*}**ABSTRACT**

Muscle fiber types in the pectoral fins of fishes have rarely been examined, despite their morphological and functional diversity. Here, we describe the distribution of fast and slow muscle fibers in the pectoral fins of *Polypterus senegalus*, an amphibious, basal actinopterygian. Each of the four muscle groups examined using mATPase staining showed distinct fiber-type regionalization. Comparison between fish raised in aquatic and terrestrial environments revealed terrestrially reared fish possess 28% more fast muscle compared with aquatically reared fish. The pattern of proximal–distal variation in the abductors differed, with a relative decrease in fast muscle fibers near the pectoral girdle in aquatic fish compared with an increase in terrestrial fish. Terrestrially reared fish also possess a greater proportion of very small diameter fibers, suggesting that they undergo more growth via hyperplasia. These observations may be a further example of adaptive plasticity in *Polypterus*, allowing for greater bursts of power during terrestrial locomotion.

KEY WORDS: Amphibious fishes, Hyperplasia, Fast muscle fibers, Slow muscle fibers, Muscle regionalization

INTRODUCTION

The muscles of fishes are composed of three main fiber types: red, white and pink. These can be visually distinguished by color but also correspond to a suite of functional characteristics. Red muscle fibers are typically slow contracting and fatigue resistant, and have a high degree of oxidative activity. White muscle fibers have fast-contracting, easily fatigued fibers with little oxidative activity. Pink fibers have characteristics intermediate to red and white. Whereas the categories of red, white and pink fibers are general descriptions, fiber types are specifically defined by their composition of myosin isoforms, which can confer a spectrum of functional properties between the extremes of red and white. A number of less common fiber types also exist, including tonic, red muscle rim, and scattered dorsal and ventral fibers (Sänger and Stoiber, 2001).

Different muscle fiber types are engaged in different modes of locomotion, with slow-contracting fibers used at slower swimming velocities, and fast-contracting fibers used during bursts of maximum speed, such as in startle responses (Bone, 1966; Jayne and Lauder, 1994; Rome et al., 1988). Accordingly, the proportions of fast and slow fibers reflect the evolutionary ecology of fishes,

with continuously swimming pelagic fishes possessing a greater proportion of slow fibers compared with fishes using primarily burst swimming behaviors (Greek-Walker and Pull, 1975). As in mammals, fish muscle responds plastically to exercise training, with endurance training at slow steady speeds generally leading to increases in the proportion of slow fibers (Davison, 1989, 1997).

The pectoral fins of fishes are morphologically and functionally diverse. They can be used as wings or oars for thrust, as foils and rudders for steering, and even as appendages for ‘walking’ on land. However, the study of fiber-type diversity and distribution in pectoral musculature has been limited to a relatively small pool of narrowly distributed marine fishes (Chayen et al., 1993; Davison, 1994; Davison and Macdonald, 1985; Devincenzi et al., 2009, 2015; Fernandez et al., 2000; Hernández et al., 2016; Kundu and Mansuri, 1991; Kundu et al., 1990; Kryvi and Totland, 1978; Walesby and Johnston, 1980). Fiber type in the context of development has also been studied in zebrafish (Patterson et al., 2008; Thorsen and Hale, 2005).

Furthermore, very little is known about the muscle fiber types of the 200+ species of amphibious fishes, which live in a variety of aquatic and terrestrial conditions with diverse life histories and locomotory strategies (Graham, 1997; Wright and Turko, 2016). The muscles of these fishes are of particular interest, as they face the enormous challenge of being functional in both the water and on land. Among amphibious fishes, muscle fiber type has only been examined in the axial and jaw musculature of Hawaiian climbing gobies (Cediél et al., 2008). It was found that species with different climbing strategies have different proportions of fast and slow fibers; species using ‘power-burst’ locomotion have a greater proportion of white muscle, whereas those using an ‘inching’ locomotion have a greater proportion of red muscle.

Here, we describe muscle fiber type distribution and plasticity in the pectoral fin of *Polypterus senegalus*. *Polypterus*, also known as bichirs, are the basal-most acinopterygians. *Polypterus* are labriform swimmers at slow speeds, meaning they rely primarily on the oscillation of their pectoral fins to create thrust in the water. *Polypterus senegalus* are also capable of surviving on land for prolonged periods of time, where their pectoral fins can be used in a contralateral pattern during terrestrial locomotion to support the body and potentially contribute to propulsion (Standen et al., 2014, 2016). We hypothesized that the pectoral musculature of *P. senegalus* will exhibit adaptive phenotypic plasticity in response to prolonged exposure to a terrestrial environment. In particular, we propose that the proportion of fast-twitch, or white, muscle fibers may increase as a result of the use of ‘burst-like’ movements to locomote overland, mirroring the adaptations of other fishes using burst swimming or climbing behaviors.

MATERIALS AND METHODS**Animals**

Polypterus senegalus Cuvier 1829 (male and female) were obtained from the pet trade (Mirido Importations Canada, Inc., Montreal, QC,

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Canada) and reared in aquariums for 2 months in either an aquatic or terrestrial habitat. All fish were kept in a single recirculating aquarium system divided into nine individual tanks. Terrestrial tanks contained gravel-covered platforms submerged under less than 2 mm of water. Aquatic fish were kept in similar aquariums without gravel platforms and with a water depth of 20 cm. All fish were given plants and cover to reduce stress levels. Five aquatic fish and five terrestrial fish were examined. Fish varied in body size, with aquatically reared fish being larger on average (Table S1). Fish were euthanized via immersion in a lethal concentration of MS-222 in water (417 mg l^{-1}) according to animal care protocol BL-1934.

Tissue preparation

Pectoral fins were dissected off the fish immediately after euthanasia and tissue was flash frozen using isopentane in a bath of liquid nitrogen. Fin samples were stored in a -80°C freezer overnight and sectioned the following day. Sections were made using a Leica CM3050 S cryostat at $10 \mu\text{m}$, dried overnight and stored at -80°C . Between 10 and 40 sections were taken along the proximal–distal axis of each fin, ranging from the scapulocoracoid proximally and the radials distally.

Histology

Fast and slow fibers were distinguished via a mATPase staining procedure modified from Guth and Samaha (1970). Tissue was preincubated in an alkaline solution of pH 10.1, which selectively deactivates slow muscle fibers. Preincubation at several other pH levels was also attempted, but pH 10.1 was determined to give the best distinction between muscle types. Some slides were also counter-stained with a 0.25% Eosin solution to better visualize the shape of unstained cells. It should be noted that fast and intermediate fibers were probably stained similarly, and future mATPase stains at different pH along with oxidative enzyme stains could be used to distinguish between them.

Image analysis

To visualize the gross muscle morphology and quantify the proportion of fiber types, slides were photographed using a Leica M60 dissection microscope and MC170 HD camera. The resulting images were white-balanced and converted to an 8-bit grayscale image using ImageJ (Schneider et al., 2012) by splitting color channels and retaining only the red channel. Fast muscle fibers were selected by using the automatic thresholding tool to generate binary images. This fast muscle area was then measured and divided by the total muscle area. Between 4 and 34 sections were analyzed for each fin, depending on fin size, quality of the section and stain. To measure cell size, photographs were taken using a Nikon Eclipse E600 light microscope with a Canon EOS Rebel T5 camera. Individual fibers within a subregion of the adductor muscle were outlined by hand using ImageJ. Equivalent diameter, the diameter of a circle of the same area as the fiber cross-section, was used as the measure of cell size. Prior to statistical analysis, cells with outlying sizes due to human error in segmentation were removed.

Statistical analysis

Differences in fiber-type proportion were compared between treatment groups with a linear mixed effects model using the lme function in the R package nlme (v3.1-131, <https://CRAN.R-project.org/package=nlme>). Treatment (aquatic/terrestrial) and muscle group were treated as fixed main effects, and individual was included as a random effect to account for non-independence between sections taken from fins of the same individual.

RESULTS AND DISCUSSION

Fiber-type regionalization in *Polypterus*

Four muscle groups were examined: the abductors, including the abductor superficialis and profundus, the adductor, coracometaptyrgialis I and II, and the zonopropterygialis (Wilhelm et al., 2015). The abductors contained a superficial layer of slow fibers, with a deep region composed mainly of fast fibers interspersed with some slow fibers (Fig. 1A). In some sections, a thin layer of slow fibers was also visible between the fast zone and the radials on the ventral (post-axial) third of the fin. Fiber-type distribution in the adductor was similar to that in the abductor, also being composed of a zone of fast fibers sandwiched between two thinner layers of slow fibers (Fig. 1A). However, the compartmentalization of slow and fast fibers appeared to be more distinct in the adductor. In both the abductors and adductor, regionalization of fiber types was stronger towards the ventral edge of the fin, and a more mosaic distribution was found towards the dorsal (pre-axial) edge. The coracometaptyrgialis muscles originate ventrally on the distal scapulocoracoid and insert on the ventro-medial surface of the metapterygium. A uniform layer of slow muscle fibers surrounded two-thirds of the muscle boundary, from its medial insertion on the metapterygium, along its border with the adductor, stopping when it reached its border with the abductors (Fig. 1B). The interior of this muscle was composed mainly of fast fibers, with a few interspersed slow fibers. The zonopropterygialis lies on the dorsal edge of the fin between the abductors and adductor, originating on the pectoral girdle skeleton and inserting on the propterygium. Like the other muscles, slow

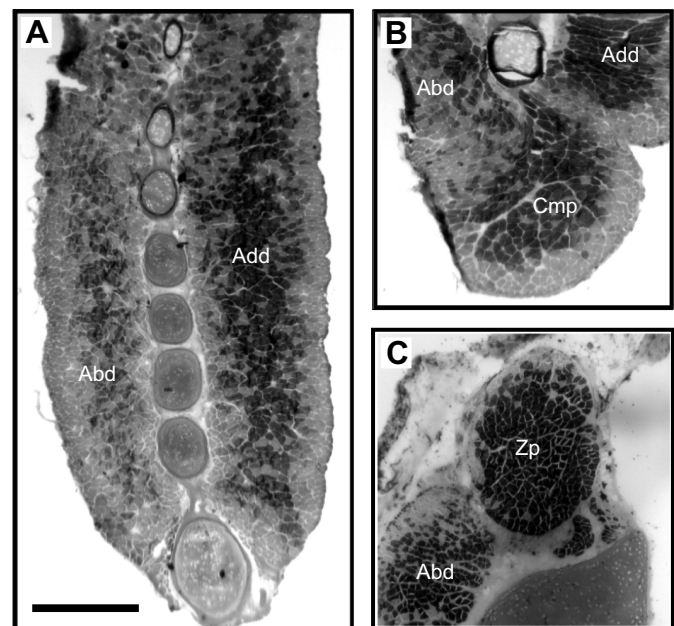


Fig. 1. Representative cross-sections of the pectoral fin musculature of *Polypterus senegalus* showing slow and fast fiber types after alkaline mATPase staining. (A) Section taken from the distal fin. (B) Section taken from the middle fin. (C) Section taken from the proximal fin. Fast-contracting fibers appear more darkly stained than slow-contracting fibers. Images that best highlight boundaries between muscle groups and fiber-type regions are displayed. A and B are from aquatic fish and C is from a terrestrial fish. There was no observable difference in the qualitative pattern of fiber-type distribution between treatment groups. All images are of left fins oriented with the lateral surface to the left, medial surface to the right, pre-axial up and post-axial down. Scale bar is 0.5 mm for all images. Abd, abductor; Add, adductor; Cmp, coracometaptyrgialis; Zp, zonopropterygialis.

fibers were confined to a thin superficial layer with few isolated cells in the main body (Fig. 1C).

Intramuscular regionalization is relatively common in vertebrates though not well understood (Kernell, 1998). Regionalization may originate as a consequence of development (Narusawa et al., 1987), as patterns can appear even in the absence of innervation, indicating that initial fiber type may be developmentally determined (Condon et al., 1990). Evidence for this in fish pectoral fins can be seen in zebrafish, where the configuration of peripheral slow fibers surrounding deeper fast fibers appears early in development (Patterson et al., 2008). However, examples of disparate adult muscle patterns arising from similar embryonic phenotypes suggest that a developmental origin of regionalization probably does not apply in all cases (Wang and Kernell, 2001). Regardless of the origin of intramuscular regionalization, it is likely that compartmentalization of fiber types reflects functional compartmentalization within muscles (Higham and Biewener, 2011). This is certainly the case in axial musculature of fishes, where slow and fast fibers are recruited for different behaviors. The presence of distinctive regionalization in the fins of *P. senegalus* probably further contributes to behavioral flexibility, allowing the same muscle to be used for a variety of functions.

When muscles have broad skeletal attachments, such as in the pectoral fins of *Polypterus* (Wilhelm et al., 2015), multiple lines of force with different contraction properties can be produced in the same muscle. In the fins, the attachment points of the slow fibers located on the periphery of the muscle body may need to exert proportionally less force to move the fin compared with fibers near the center of the fin. To illustrate this effect, imagine the fin as a lever with its fulcrum at its base (articulation with the pectoral girdle) and its load distributed across the fin. When effort (force) is applied further away from the fulcrum, as in the case of the more peripheral and distal insertion points of the slow fibers, the mechanical advantage is greater than when effort is applied closer to the fulcrum, as in the case of the fast fibers, which have more insertion points along the middle sections of the basal radials. Such a mechanical

advantage may serve to compensate for the lower force production and contraction speed (though higher endurance) of slow fibers.

The pattern of regionalization seen in *P. senegalus* appears to be a common configuration among fishes, including the white croaker (Devincenti et al., 2009), striped whitefish (Devincenti et al., 2015) and some nototheniids (Fernandez et al., 2000; Sanger et al., 2005). These species of fishes also rely primarily on their pectoral fins for propulsion during swimming, and the pattern of fast fibers localized to the superficial regions and slow fibers in the deep muscle may confer a general functional advantage. However, this is certainly not the case for all labriform swimmers, as the rabbit fish (Kryvi and Totland, 1978) and the nototheniid *Trematomus bernacchii* (Davison and MacDonald, 1985) appear to have a complete mosaic distribution of fast and slow fibers, with no regionalization of fiber type.

Muscle fiber-type plasticity under terrestrial conditions

Compared with *P. senegalus* raised in an aquatic habitat, terrestrially reared fish had a significantly greater proportion of fast fibers in all four muscle groups ($P=0.0015$, Fig. 2A). Muscle group also had a significant effect ($P=0.0019$), with model contrasts showing a lower proportion of fast fibers in the abductors than in the other muscle groups ($P=0.0018$). This marked increase in the proportion of fast muscle fibers is another example of a phenotypically plastic response to the requirements of life on land (Standen et al., 2014; Wright and Turko, 2016). Compared with aquatic locomotion, which is facilitated by buoyancy in water, terrestrial locomotion requires greater force to move the fins. The increased proportion of fast fibers thereby probably provides a functional advantage during terrestrial locomotion. Changes in fiber-type proportion between aquatic and terrestrial fish also reflect the altered role of the pectoral fin during terrestrial locomotion, with *Polypterus* engaging their pectoral fins as supportive structures during short bursts of terrestrial locomotion (Standen et al., 2016).

Additionally, terrestrially reared fish differed in their pattern of proximal–distal variation. The proportion of fast fibers tended to increase closer to the shoulder girdle in abductors of terrestrially

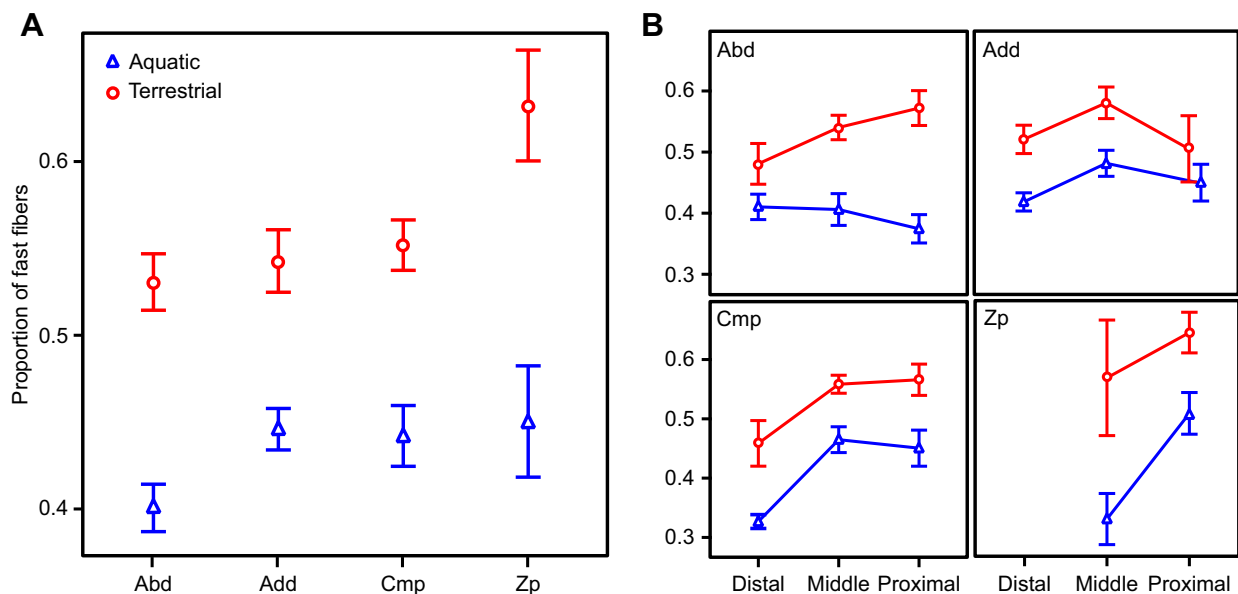


Fig. 2. Proportion of total muscle area composed of fast fibers in aquatic and terrestrially reared *P. senegalus*. (A) Significant main effects in a linear mixed model were found for rearing environment ($F=22.4874$, $P=0.0015$) and muscle group ($F=5.0681$, $P=0.0019$). (B) Differences in proximal–distal variation in the proportion of fast fibers within muscles between treatment groups. For both A and B, data are the total mean \pm s.e.m. of multiple sections in all individuals. Fiber-type proportion was measured in five aquatic and five terrestrial fish. Abd, abductor; Add, adductor; Cmp, coracometypterygialis; Zp, zonopropterygialis.

reared fish (Fig. 2B). The opposite trend was seen in aquatically reared fish, where the proportion of fast fibers tended to decrease closer to the pectoral girdle. Proximal–distal variation was similar between groups for the other muscles, with an increased proportion of fast fibers proximally in the coracometapecterygialis and zonoproterygialis, and relatively little change in the adductors. This shift may reflect specific changes in the function of the abductor muscles during terrestrial locomotion. During swimming, the abductors contribute primarily to the upstroke, which repositions the fin without contributing to forward propulsion or requiring a large amount of force production. However, the role of the abductors during terrestrial locomotion probably requires them to exert more forceful contractions, as a result of both the absence of buoyancy and their presumed participation in the load-bearing stage of the step cycle (Standen et al., 2016).

Fiber size under terrestrial conditions

Absolute differences in fiber size were difficult to compare between the aquatic and terrestrial groups, as individuals varied in body size (Table S1), with which fiber diameter is known to be correlated (Kundu et al., 1990; Kundu and Mansuri, 1991). Comparison between two size-matched pairs did not reveal any consistent difference in the fiber-size distribution between treatments (Fig. 3). In one pair, the aquatically reared fish had smaller fibers on average (Fig. 3A), whereas another pair showed the opposing condition, with the terrestrially reared fish possessing smaller fibers (Fig. 3B). However, a *z*-test of equal proportions showed terrestrially reared fish do have a slightly higher proportion of very small fibers (<5 μm), with very small fibers accounting for 7.9% of all fibers in terrestrially reared fish as opposed to 5.6% in aquatically reared fish ($P=0.0341$).

The presence of disproportionately more very small diameter fibers in terrestrially reared fish suggests that the pectoral muscles of aquatic and terrestrial *P. senegalus* may grow differently. In particular, terrestrial fish may undergo more hyperplasia, growth via the origination of new fibers from satellite cells (Johnston, 1982) or division of existing fibers (Willemse and Van den Berg, 1978). It is also possible that fiber transitions, changes in the composition of myosin heavy chain isoforms within individual muscle fibers, may also occur in terrestrially reared *P. senegalus*. Reversible transitions between fast and slow muscle phenotypes can be effected by a number of conditions, including changes in neuromuscular activity and mechanical loading (Pette and Staron, 2000).

Organisms have a variety of ways to cope with changes in their environment, with few environmental gradients as drastic as the

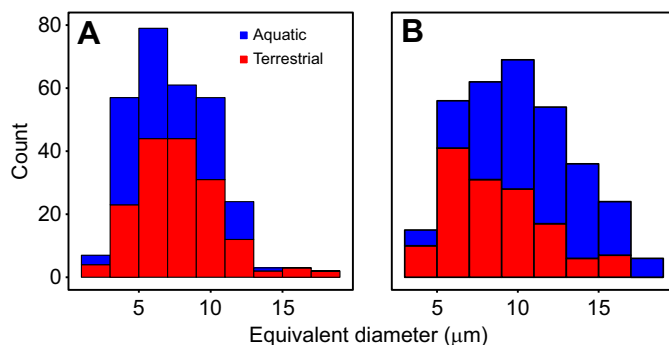


Fig. 3. Stacked frequency distributions of equivalent fiber diameter in size-matched pairs of aquatically and terrestrially reared *P. senegalus*. (A) Pair 1 – aquatic: 6.2 cm, 1.89 g; terrestrial: 6.2 cm, 1.74 g. (B) Pair 2 – aquatic: 6.9 cm, 2.39 g; terrestrial: 6.8 cm, 2.17 g.

transition between water and land. Here, we demonstrate how fin muscles plastically respond to a shift from aquatic to terrestrial life in *P. senegalus*. While an association between skeletal plasticity and improved functional performance on land was previously shown in terrestrially reared *Polypterus* (Standen et al., 2014), further work is required to test the functional properties of various muscle fibers in *Polypterus*, as well as to understand the functional consequences of pectoral fiber-type regionalization.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.Y.D., E.M.S.; Methodology: T.Y.D., E.M.S.; Investigation: T.Y.D.; Formal analysis: T.Y.D.; Validation: T.Y.D., E.M.S.; Writing – original draft: T.Y.D., E.M.S.; Writing – review & editing: T.Y.D., E.M.S.; Visualization: T.Y.D.; Supervision: E.M.S.; Resources: E.M.S.; Funding acquisition: E.M.S.

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Supplementary information

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Table S1. Specimen lengths and weights

Specimen	Length (cm)	Weight (g)
Aquatic 1	6.6	1.70
Aquatic 2	6.2	1.89
Aquatic 3	7.4	3.1
Aquatic 4	11.1	10.3
Aquatic 5	6.9	2.39
Terrestrial 1	6.2	1.74
Terrestrial 2	6.7	2.01
Terrestrial 3	6.0	1.64
Terrestrial 4	6.8	2.17
Terrestrial 5	6.1	1.64