Enzyme activities of pharyngeal jaw musculature in the cichlid *Tramitichromis intermedius*: implications for sound production in cichlid fishes

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Summary

Owing to its high degree of complexity and plasticity, the cichlid pharyngeal jaw apparatus has often been described as a key evolutionary innovation. The majority of studies investigating pharyngeal muscle behavior and function have done so in the context of feeding. Analysis of enzyme activities (citrate synthase, 3-hydroxyacyl-CoA dehydrogenase and L-lactate dehydrogenase) of pharyngeal muscles in the Lake Malawi cichlid *Tramitichromis intermedius* revealed differences between pharyngeal jaw muscles and between males and females. Therefore, these muscles have different performance characteristics, resulting in different functional characteristics of the muscles within the complex. Furthermore, the differences between muscles of males and females represent fundamental differences in muscular metabolic processes between sexes. This study is the first to demonstrate that the pharyngeal anatomy is not only used for food processing but is possibly responsible for sound production, in turn influencing sexual selection in cichlid fish.

Key words: Cichlidae, pharyngeal jaw, citrate synthase, 3-hydroxyacyl-CoA dehydrogenase, lactate dehydrogenase, sexual dimorphism, muscle, sound production, stridulation.

Introduction

The pharyngeal jaws of cichlid fish have been hypothesized to be the mechanism responsible for sound production (Lanzing, 1974; Lobel, 2001), with the pharyngeal muscles used to maneuver the pharyngeal jaws together, facilitating stridulation of the pharyngeal teeth. Courtship and feeding sounds in the cichlid *Tramitichromis intermedius* have the same dominant frequency of 517 Hz (Lobel, 2001). As the two sound types have similar characteristics, this indirect acoustic evidence suggests that the same mechanism might be responsible for both sounds.

The pharyngeal jaw complex of cichlid fishes has been studied from several different perspectives, such as evolutionary biology (e.g. Casciotta and Arratia, 1993; Kaufman and Liem, 1982), feeding biology (e.g. Aerts et al., 1986; Claes and De Vree, 1991; Liem, 1973, 1978, 1979) and biomechanics (e.g. Galis, 1992; Galis and Drucker, 1996; Lauder, 1983). Beginning with Liem (1973), most of these studies have examined and compared the anatomy and function of these different muscles and their behavior during feeding. To the best of our knowledge, no study has yet compared pharyngeal jaw muscles from a cellular perspective. The importance of such an approach is that it allows an insight into new patterns unseen by potential limitations of previously applied techniques (Galis, 1992).

Within any organism, there is a diversity of muscle types and functions (see Rome and Lindstedt, 1997), with capabilities resulting from a balance of the cellular components (Lindstedt et al., 1998). One way of determining differences in function between muscles is by comparing levels of enzyme activity (Bass et al., 1969). This enables discrimination of muscle type based on extrinsic factors (Josephson, 1975). By assaying key metabolic enzymes within muscle cells, one can determine the main energetic pathway used (aerobic *versus* anaerobic) within these muscles and compare performance capabilities quantitatively (e.g. Bass et al., 1969; Bevier, 1995; Taigen et al., 1985). By exploring the enzyme activities of a suite of muscles, it is then possible to survey and compare differences in performance capabilities and deduce differences in their function.

Elucidation of the functional properties of these muscles allows us to address three main questions: (1) are there differences in function between the different pharyngeal jaw muscles; (2) are there differences in pharyngeal jaw muscle physiology between males and females; and (3) are the pharyngeal jaws involved in sound production?

Materials and methods

Animals

The cichlid used in this study was *Tramitichromis intermedius* (=*Lethrinops intermedia*; Trewavas, 1935), a sexually dimorphic haplochromine from Lake Malawi, Africa.

In the wild, *T. intermedius* feeds primarily on insect larvae and soft invertebrates (Konings, 1990). As an insectivore, its pharyngeal apparatus is often considered to be the 'generalized form' (Fryer, 1959; Greenwood, 1973). Male *T. intermedius* build and defend nests (McKaye et al., 1993) and use courtship sounds to advertise for mates (Lobel, 1998). Only the males are known to produce specific sounds in either courtship or agonistic interactions (Lobel, 1998; Ripley, 2001).

Captive-raised male and female *T. intermedius* were kept in a 550 l aquarium and fed a mixed diet consisting of flake food, small pellets and brine shrimp several times each day.

Muscle preparation

Sexually mature *T. intermedius* individuals were euthanized with an overdose of ethanol-buffered MS-222 (MBL Animal Care Protocol No. 01-13). Under a stereomicroscope, the muscles responsible for the principal movements of the pharyngeal jaws from both left and right sides were dissected (*sensu* Liem, 1973): levator externus II (LE2), levator externus III (LE3), levator externus IV (LE4), levator posterior (LP), protractor pectoralis (PP), pharyngeohyodius (PH), pharyngeohyodius cleithralis externus (PHCE), pharyngeohyodius cleithralis internus (PHCI) and retractor dorsalis (RD) (Fig. 1). A small sample of axial muscle (Ax) just anterior to the caudal peduncle was also dissected to serve as an example of fast-twitch glycolytic muscle (Greer-Walker and Pull, 1975; Mosse and Hudson, 1977; Rome et al., 1988). Muscles were weighed and then homogenized on ice using a glass–glass homogenizer in 10 volumes of cold buffer (7.5 mmol l^{-1} Tris; 1 mmol l^{-1} EDTA; pH 7.6). Samples were

Fig. 1. Schematic representation of the musculature of the pharyngeal jaws of male *Tramitichromis intermedius*. Abbreviations: levator externus 2 (LE2), levator externus 3 (LE3), levator externus 4 (LE4), levator posterior (LP), protractor pectoralis (PP), pharyngeohyodius (PH), pharyngeohyodius cleithralis externus (PHCE), pharyngeohyodius cleithralis internus (PHCI), retractor dorsalis (RD), lower pharyngeal jaw (LPJ), and upper pharyngeal jaw (UPJ).

then put through two freeze–thaw cycles to ensure rupturing of mitochondria and stored at 0°C until analysis.

Analysis of enzyme activity

Muscle samples were kept on ice immediately before analysis to prevent denaturation of the enzymes. Supernatant was drawn from the crude homogenate and added to the reaction mixture. Enzyme activities were determined using the protocols outlined below and assayed using a Perkin-Elmer Lambda 3B UV/Vis spectrophotometer.

Citrate synthase (CS): 0.25 mmol l^{-1} 5,5′-dithiobis(2-nitrobenzoic acid); 0.3 mmol l^{-1} acetyl-CoA; pH 8.0. The reaction was catalyzed with 0.5 mmol l^{-1} oxalacetic acid and assayed at 412 nm.

3-Hydroxyacyl-CoA dehydrogenase (HOAD): 100 mmol l^{-1} triethanolamine; 5 mmol l^{-1} EDTA; 0.15 mmol l^{-1} NADH; pH 7.0. The reaction was catalyzed with 0.1 mmol l^{-1} acetoacetyl-CoA and assayed at 340 nm.

L-lactate dehydrogenase (LDH): 50 mmol l^{-1} triethanolamine; 5 mmol l^{-1} EDTA; 0.15 mmol l^{-1} NADH; pH 7.6. The reaction was catalyzed with 2.4 mmol l^{-1} pyruvate and assayed at 340 nm.

Enzyme activities were calculated as µmol product min^{-1} g^{-1} tissue (Bergmeyer, 1974). In addition to the determination of individual enzyme activities within tissues, relative levels of β-oxidation (CS activity divided by HOAD activity; Olson, 2001) and the relative anaerobic capacity (LDH activity divided by CS activity; Bass et al., 1969) were determined to distinguish muscle type.

Statistics

Intermuscular and intersexual activities were compared using a two-way analysis of variance (ANOVA). Statistical tests were performed using StatView Software (SAS Systems, Cary, NC, USA), with significance set at $P<0.05$.

Results

T. intermedius pharyngeal jaw muscles have different capacities for aerobic activity, fatty acid oxidation and anaerobic capacity (N=8 males and 8 females). CS activities (Fig. 2A) were significantly different among muscles ($P<0.0001$, F=6.698, d.f.=9) and significantly different between males and females (P=0.0097, F=6.873, d.f.=1). Differences in CS activity in females were found between LE2 and Ax, LE3 and Ax, LE2 and LE4, LE2 and LP, LE2 and PH, LE2 and PHCE, LE2 and PHCI, LE2 and PP, LE2 and RD, LE3 and LP, LE3 and PHCI, and LE3 and PP (using a *post-hoc* Student–Newman–Keuls test, $P<0.05$). No significant differences in activity were found among the muscles of males. CS activities of LE2 were significantly different between males and females (Student–Newman–Keuls test, $P<0.05$).

HOAD activities (Fig. 2B) were significantly different among muscles ($P<0.0001$, F=8.056, d.f.=9) and between males and females (P=0.0184, F=5.078, d.f.=1). Differences in HOAD activity in female muscles were found between LE2

and Ax, LE2 and LE4, LE2 and LP, LE2 and PH, LE2 and PHCE, LE2 and PHCI, LE2 and PP, LE2 and RD, LE3 and LE4, LE3 and LP, LE3 and PHCI, LE3 and PP, and LE3 and RD (Student–Newman–Keuls test, $P<0.05$). No significant differences in HOAD activity were found among the muscles of males. LE2 had significantly different levels of activity between males and females (Student–Newman–Keuls test, $P<0.05$).

LDH activities (Fig. 2C) were significantly different among muscles ($P<0.0001$, $F=4.651$, d.f.=9). For males, differences in LDH activity were found between LE2 and PH, LE4 and PH, and PP and PH (Student–Newman–Keuls test, $P<0.05$). There were no differences in LDH activity among females, nor were there any differences in anaerobic capacity between males and females.

Males have a significantly higher overall relative capacity for β-oxidation than females ($P<0.0001$, $F=18.822$, d.f.=1; Fig. 3A). For males, significant differences in HOAD/CS activities were found between Ax and LP, Ax and PP, LE2 and LP, LE2 and PP, LE3 and PP, LE3 and LP, LE4 and LP, LE4 and PP, LP and PH, LP and PHCE, LP and PHCI, LP and RD, PH and PP, PP and PHCE, PP and PHCI, and PP and RD (Student–Newman–Keuls test, $P<0.05$). There were no differences in activity among the muscles of females. HOAD/CS activities for both LP and PP were different between males and females (Student–Newman–Keuls test, $P<0.05$).

Male pharyngeal jaw muscles have a significantly higher relative anaerobic capacity than females ($P<0.0001$, $F=53.530$, d.f.=1; Fig. 3B). Significant differences for males existed between Ax and LP, LE2 and LP, LE2 and PP, LE3 and LP, LE3 and PP, LE4 and LP, LP and PH, LP and PHCE, LP and RD, PH and PP, PP and PHCE, and PP and PHCI (Student–Newman–Keuls test, $P<0.05$). There were no differences in relative anaerobic capacity among the muscles of females, but significant differences existed for both LP and PP between males and females (Student–Newman–Keuls test, $P<0.05$).

Discussion

The muscles associated with the pharyngeal jaw apparatus have different capabilities for aerobic and anaerobic performance, and, between sexes, there is a dimorphism in functional capacities of the LP and PP. This muscle complex is, in fact, a mosaic of different muscle types with different physiological properties, demonstrated by the ratios of muscle enzyme activities (i.e. LDH/CS and HOAD/CS; Bass et al., 1969). The difference in relative anaerobic capacity between males and females suggests different uses of the pharyngeal jaw complex. As the fish examined do not differ in feeding behavior, such a dimorphism in muscle physiology is probably not the result of diet.

Based upon the fact that only male *T. intermedius* produce sounds, and both males and females were fed the same diet, the observed physiological dimorphism in the capacity for
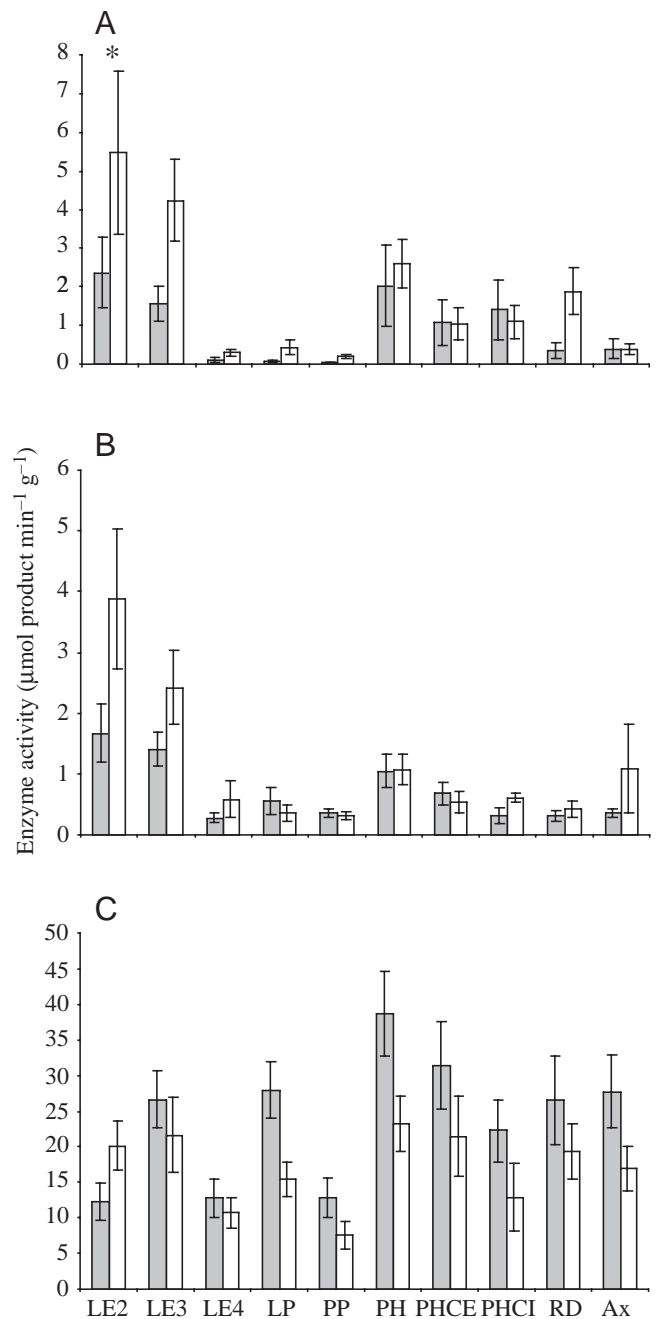
Fig. 2. Enzyme activities for pharyngeal jaw muscles of *Tramitichromis intermedius* (means ± S.E.M). (A) Citrate synthase activity. (B) 3-Hydroxyacyl-CoA dehydrogenase activity. (C) L-Lactate dehydrogenase activity. Gray bars represent males, and white bars represent females. Significant differences in activity between muscles for each sex are listed in the text. Asterisks indicate significant difference in activity for muscles between males and females ($P<0.05$). Abbreviations: levator externus 2 (LE2), levator externus 3 (LE3), levator externus 4 (LE4), levator posterior (LP), protractor pectoralis (PP), pharyngeohyodius (PH), pharyngeohyodius cleithralis externus (PHCE), pharyngeohyodius cleithralis internus (PHCI), retractor dorsalis (RD), axial muscle (Ax).
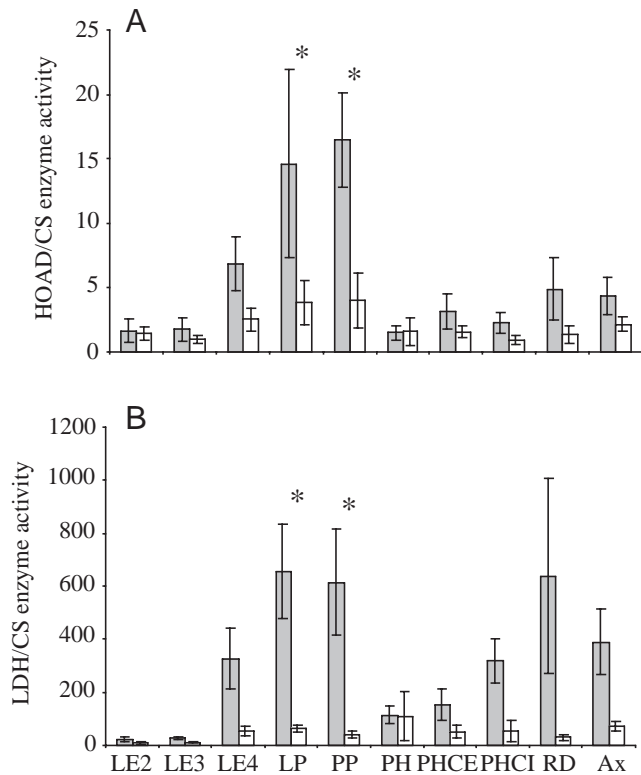
Fig. 3. (A) Relative capacity for fatty acid oxidation, expressed as a ratio of HOAD/CS activity, and (B) relative anaerobic capacity, expressed as a ratio of LDH/CS activity, of pharyngeal jaw muscles of *Tramitichromis intermedius* (means ± S.E.M). Gray bars represent males, and white bars represent females. Asterisks indicate significant difference in activity for muscles between males and females (*P*<0.05). Abbreviations: levator externus 2 (LE2), levator externus 3 (LE3), levator externus 4 (LE4), levator posterior (LP), protractor pectoralis (PP), pharyngeohyodius (PH), pharyngeohyodius cleithralis externus (PHCE), pharyngeohyodius cleithralis internus (PHCI), retractor dorsalis (RD), axial muscle (Ax), 3-hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS), L-lactate dehydrogenase (LDH).

aerobic/anaerobic activity could be explained by a role for the muscle in sound production in males. Other sonic fishes, such as the toadfish *Opsanus beta* and *Opsanus tau* (family Batrachoididae), have been shown to display sexually dimorphic performance capacities of their homologous sound-producing muscles (Walsh et al., 1987, 1989). By demonstrating such sexual dimorphism in the physiology of the pharyngeal musculature in *T. intermedius*, this study corroborates acoustic and behavioral data, supporting the hypothesis that this apparatus is used in sound production in male cichlid fish.

The pharyngeal jaws play an important role in feeding in many teleost species (Lauder, 1983), and functional duality of the pharyngeal jaws for feeding and sound production has been shown in the grunt *Haemulon plumieri* (family Haemulidae; Burkenroad, 1930) and the Japanese parrot fish *Oplegnathus fasciatus* (family Oplegnathidae; Nakazato and Takemura, 1987) and suggested in the crevalle jack *Caranx hippos* (family Carangidae; Taylor and Mansueti, 1960). The highly maneuverable design of the pharyngeal jaw apparatus in the cichlids, resulting from the lower pharyngeal sling (Liem, 1973, 1986), where the jaws are principally occluded by the LE4 and LP (Liem, 1991), may contribute to the amplitude modulation in the acoustic signal (Lobel, 2001). By contrast, the pharyngeal jaws of the damselfish (*Pomacentridae*) are occluded by contraction of the PP, which raises the cleithrum (Galis and Snelderwaard, 1997). This comparatively limited movement of the pomacentrid pharyngeal jaws may result in the limitation for only pulse patterning of the acoustic signal (Lobel, 2001).

Previous studies have suggested that the RD is the sonic muscle in fish that use the pharyngeal jaws for sound production. In *O. fasicatus*, Nakazoto and Takemura (1987) identified the RD as the primary muscle responsible for stridulation of the pharyngeal jaws during sound production. However, they only implanted an electromyographic electrode in the dilator operculi and inferred the role of the sonic muscle through anatomical dissections. Lanzing (1974) also suggested that this muscle was the 'sonic muscle' in Mozambique tilapia *Oreochromis mossambicus* (=*Tilapia mossambica*) but this was inferred only from histological sections of a young juvenile individual. Both of these 'sonic muscles' originate on the vertebral column and insert on the posterior section of the upper pharyngeal jaw; both muscles match the position and description of the RD (Winterbottom, 1974). The data from the present study indicate that the *T. intermedius* RD is designed for high levels of anaerobic performance, but this muscle is not sexually dimorphic. Although the RD does contribute to the movement of the upper pharyngeal jaw in *T. intermedius*, its role as a principal muscle in sound production is questionable.

It is possible that the enzyme activities in cichlid pharyngeal musculature might differ with diet. The pharyngeal toothplates of cichlids have been show to vary with diet (e.g. Greenwood, 1973), and variation in the performance of the pharyngeal muscles might follow a similar pattern. These performance capabilities may be determined by diet (i.e. in molluscivores, insectivores and piscivores, where different properties are necessary for the demands of different food processing) or this could be a pattern seen in all cichlid pharyngeal muscles. Comparison of the pharyngeal muscle performance capabilities in other cichlid species with diverse ecologies or evolutionary histories may elucidate the universality of the aerobic–anaerobic ratios across the Cichlidae.

The relationship between the pharyngeal jaws for feeding and sound production may have profound evolutionary implications. The importance in feeding (Liem, 1973, 1991) and the adaptability of the pharyngeal jaws for processing different food types (e.g. Greenwood, 1965; Huysseune, 1995; Smits et al., 1996; Witte et al., 1990) allow cichlids to exploit different trophic niches (Sage and Selander, 1975). Furthermore, acoustic communication may be an important mechanism for sexual selection in cichlid fish (Lobel, 1998, 2001). With the pharyngeal jaw serving as a possible

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