Impact of rearing temperatures on Tilapia Oreochromis mossambicus growth, muscle morphology and gene expression

Abstract

Aim: Oreochromis mossambicus, tilapia is a fast growing fish able to adapt to a range of environmental conditions. The study was conducted with the aim to understand the effect of rearing temperatures on juvenile tilapia growth, muscle cellularity and expression of myoD and myostatin genes.

Methodology: Tilapia larvae were reared at 25°C, 30°C and 34°C for 60 days. Fish growth was measured in terms of body weight, white muscle fibre frequency through HE staining and qRT-PCR based expression of myoD and myostatin gene.

Results: At 60 day, tilapia juveniles reared at 30°C grew significantly higher than 25°C and 34°C, the frequency distribution of white muscle fibres in diameter class <25 µm was similar at 25°C and 30°C, but was significantly higher for fish reared at 34°C and of white muscle fibre of diameter 25-50 µm was significantly higher at 30°C in comparison to 25°C and 34°C. MyoD gene expression was significantly higher at 34°C than 25°C and 30°C. Whereas, myostatin expression was similar at all three rearing temperature.

Interpretation: The present investigation suggests that rearing temperature affects fish growth, muscle cellularity and gene expression in juvenile tilapia. Increase in water temperature to 30°C is beneficial for achieving maximum body growth and hypertrophic muscle growth in O. mossambicus.
Introduction

Abiotic factors such as change in habitat temperature, decrease in dissolved oxygen, increase in load of pollutants and their toxicity, change in hydrological regimes, hydrologic variability, eutrophication and so on affect fish and fishery adversely (Ficke et al., 2007). Water temperature and dissolved oxygen affect the growth rate, physiology and reproduction in fish and during the early life stages directly affects the rate of myogenesis, muscle fibres cellularity, expression of genes involved in muscle developmental pathway and growth pattern in adults (de Assis et al., 2004; Johnston, 2006; Alami-Durante et al., 2007; Macqueen et al., 2008; Campos et al., 2013a). Fish skeletal muscles are made up of 70% white myotomal muscle which exhibit glycolytic metabolism and are composed of fast contracting fibres constituting the bulk of the fish body size (Driedzic and Hochachka, 1976; de Paula et al., 2014; Zhang et al., 1996). In fish, growth is manifested by recruitment and hypertrophy of muscle fibres. The number of muscle fibres recruited to reach a certain girth of fish is influenced by environmental factors including diet, exercise, light and temperatures regimes (Johnston, 1999). The genes and pathways regulating the development of fish muscle are the myogenic regulatory factors. The myogenic regulatory factors (MRFs) are a family of four basic helix-loop-helix transcription factors which are highly conserved between mammals and fish and are responsible in determining muscle lineage (MyoD, Myf5, Myf4) and for initiation and stabilization of muscle differentiation (Hasty et al., 1993; Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). Expression of myoD and myf5 occurs before segmentation in adaxial cells in many teleost species (Tan and Jun Du, 2002; Temple et al., 2001; Weinberg et al., 1996). Growth in fish is also regulated by myostatin, also known as growth differentiation factor 8 (Mstn), member of transforming growth factor gene family and is a negative regulator of muscle growth (McPherron et al., 1997). Myostatin is expressed in skeletal muscle in mice (McPherron et al., 1997). It is hypothesized that myostatin may have functions other than muscle growth regulation in tissues other than muscle (Acosta et al., 2005; Ostbye et al., 2001; Lee and McPherron, 2001; Palurro et al., 2008; Rodgers et al., 2001).

Important commercial species of tilapia are Oreochromis mossambicus, O. aureus, O. niloticus, O. hornorum, and O. zillii. O. mossambicus is an exotic fish introduced to India in 1952 and has spread throughout the country soon after its introduction due to it being a prolific breeder and its ability to adapt to different environmental conditions which resulted in loss of local fish biodiversity and fishery in several reservoir fisheries in Tamil Nadu, Kerala and Rajasthan. Though O. mossambicus is banned in India since 1959, it is propagating rapidly in wild and constitutes bulk fishery in many reservoirs namely Vaigai, Krishnagiri, Amaravati, BhavaniSagar, Tirumooler, Uppar and Pambaram reservoirs in Tamil Nadu, Walayar, Malampuzha, Pothundy, Meenkara, Chulliar and Pechi reservoirs of Kerala, Kabini reservoir of Karnataka and Jaisamand Lake of Rajasthan (Anonymous, 2015). In biofloc system, O. mossambicus average daily growth was 0.405g day⁻¹ (Day et al., 2016), growth and specific growth rates were inversely proportional to stocking density (Shubha and Reddy, 2011) and showed significantly higher growth when fed with earthworm meal diet (Bag et al., 2012). Specific growth rate of triploid was 1.097 but hatchability and survivability was low, whereas in diploid O. mossambicus specific growth rate was 0.931 with higher egg hatchability and survivability (Nwachi and Esa, 2016). Information on muscle morphology and expression of MyoD and myostatin is meagre.

Demand for fish in India is ever increasing to feed its growing population and tilapia is becoming an important fish species in inland capture fishery. The Ujani reservoir in Maharashtra, India is also heavily populated with tilapia and constitutes a dominant fishery in the reservoir. With increase in demand, tilapia may serve to increase the inland capture fishery production. Thus, the aim of the study was to understand the impact of different rearing temperature on tilapia body growth, muscle growth characteristics and expression of MyoD and myostatin genes.

Materials and Methods

Fish growth experiment was conducted for a period of 60 days. Tilapia larvae (n = 108, 0.017±0.001 g weight,) were distributed into nine aquarium tanks with water temperatures maintained at 25°C, 30°C and 34°C (triplicates for each group). The fish were fed ad libitum with a commercial feed containing 35% crude protein throughout the experimental period. Daily, 50% water was replaced by fresh canal water. The fish were euthanized with MS222 at day 60 and were weighed (gram) and measured (centimeter). Epaxial white muscles below the dorsal fin from fish were collected for muscle histology and gene expression analysis.

White muscle samples were fixed in 10% buffered formalin and preserved in 70% ethanol. The samples were dehydrated with a series of ethanol concentration. Transverse sections (4 µm) were obtained and stained using hematoxylin-eosin method. The individual fibre area was measured using a stereo zoom microscope attached with a computerized image analysis system. In this experiment six fish per experimental group were used to measure 200 fibres in random fields of image samples of each fish per experimental group. White muscle fibre diameter (D) was measured from the fibre area (A) by the formula D = 2A π /π (Valente et al., 1999). For each group, the muscle fibre diameter were grouped into three classes <25µm, 25-50 µm and >50 µm (de Almeida et al., 2008). Epaxial white muscle was sampled and total RNA was extracted using Tri reagent (Sigma, T9424) according to the manufacturer’s protocol. The RNA was quantified using MultiSKan-Go spectrophotometer (Thermo Scientific). RNA quality was assessed using 260/280 nm OD ratio of approximately 2.0. Purified total RNA was reverse transcribed using random hexamer primers and a reverse transcription kit (Thermo fisher). Verso cDNA synthesis kit (Thermo Scientific,
Fig. 1: Transverse section of 25°C, 30°C and 34°C reared tilapia, *Oreochromis mossambicus* mosaic pattern of small and large (arrow) white muscle fiber diameter observed (100µm bar)

Fig. 2: qRT-PCR quantification of myoD and myostatin mRNA expression in white muscle of tilapia *Oreochromis mossambicus* reared at 25°C, 30°C and 34°C
temperatures. At 60 days, the MyoD mRNA level was significantly higher in 34°C than 25°C and 30°C and expression pattern was similar at 25°C and 30°C. The myostatin gene expression did not vary at all the three rearing temperatures (Fig. 2).

The study showed that rearing temperatures affect the fish growth, muscle growth characteristics and expression of muscle myogenic regulatory factor MyoD and myostatin gene in juvenile tilapia, Oreochromis mossambicus. Temperature is one of the most important extrinsic factor modulating growth affecting developmental processes throughout the life of fish (Johnston et al., 2011), affects somatic growth trajectory and the number and size distribution of muscle fibres in juvenile and adult fish (de Paula et al., 2014; Johnston et al., 2009; Macqueen et al., 2008; Stickland et al., 1988). It was observed that higher temperature enhance fish metabolism and feed consumption which results in higher growth (Kieffer et al., 2014). Similarly in the present study, fish exhibited greater growth at rearing temperature of 30°C than at 25°C and 34°C. It suggests that temperature of 30°C enhances metabolism in tilapia resulting in highest growth of fish. Rearing temperature of 34°C induced thermal stress resulting in lower growth of tilapia.

De Paula et al. (2014) subjected pacu fish (Piaractus mesopotamicus) to 24°C, 28°C and 32°C for a period of 60 days and observed that the body weight was higher at 32°C but lower at 24°C. The frequency distribution of white muscle fibres in the diameter class <25 µm was significantly higher at 24°C than 28°C and 32°C, and was similar between 28°C and 32°C. The frequency distribution of 25-50 µm diameter fibres were similar at all three temperatures and in class >50 µm, 32°C showed higher percentage than 24°C. de Assis et al. (2004), observed that pacu eggs incubated at 25°C, 27°C and 29°C until hatching and reared at room temperature exhibited increase in fibre diameter with increase in temperature. New fibre recruitment was more at 29°C than at 25°C and 27°C. Muscle fibre hypertrophy was from hatching to 60 day and the interaction of muscle hypertrophic and hyperplastic growth processes at 29°C produced largest fish in the experimental period. Similarly, in this experiment, at 60 days of rearing of O. mossambicus, frequency of muscle fibres of class <25 µm showed highest frequency at 34°C suggesting that thermal stress at 34°C induced muscle fibre recruitment. Frequency distribution of 25-50 µm muscle fibre class was highest at 30°C suggesting that this temperature condition induces muscle hypertrophy in which fibres expand and absorb satellite cell nuclei to maintain a relatively constant ratio between

### Table 1: Oligonucleotide primers used for qRT-PCR amplification of the genes MyoD, myostatin and reference gene β-actin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
<th>Annealing temperature, °C</th>
<th>Size of amplified fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyoD</td>
<td>Forward: TCA GAC AAC CAG AAG AGG AGG ATG TTT</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGT TCT GCG AGG GTG ATC GTA CTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myostatin</td>
<td>Forward: TGT GGA CTT CGA GGA GGT TGT TGG</td>
<td>58</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGG CTT TGT AGT GTC TTG GT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: CCA CAC AGT GCC CAT CGA</td>
<td>58</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCA CGC TCT GTC AGG ATC TCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#AB-1453) was used to prepare cDNA, in which RT enhancer was included to remove the contaminating DNA, eliminating the need for DNAse I treatment. It degraded double stranded DNA during transcription of RNA and was inactivated after 2 min at 95°C. MyoD and myostatin gene expression was detected by quantitative real time PCR using BioRad CFX 96 real time system C1000 Touch thermal cycler. A sample without cDNA template (NTC) was used to verify that the master mix was free from contaminants. Two microliters of cDNA (20 nm µl-1) were amplified with DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Scientific, Thermo Scientific, #F-416) and 400 nm of gene specific primer (Table 1) to yield a final volume of 20 µl. The real time conditions were as follows: initial denaturation at 95°C for 3 min followed by 39 cycles of denaturation at 95°C for 10 sec, and annealing/extension at 58°C for 30 sec. The β-actin gene was used as a reference to normalize the quantification of mRNA target. Primer pairs for MyoD, myostatin and β-actin were synthesized based on (Nebo et al., 2013; Qiang et al., 2012).

Statistical analysis: The standard length and body weight of fish were analyzed using ANOVA complemented with Multiple Comparison Test (Tukey). The muscle fibre diameters were expressed as frequency percentage. The relative gene expression data were analyzed by Kruskal-Wallis test.

Results and Discussion

O. mossambicus reared for a period of 60 days exhibited increase in body weight and standard length. At 60 day, tilapia juveniles reared at 30°C grew significantly higher (1.27 g, 153.62%) than at 25°C (1.42 g, 153.57%) and 34°C (0.82 g, 95.23%). The hematoxylin-eosin staining showed that the white muscle contributed maximum to the fish muscle mass. The white muscle consisted of round and polygonal fibres separated by endomysium, a fine septum of connective tissue. Muscle fibres were distributed into a mosaic pattern of fibres with different diameters (Fig. 1). At 60 day, the frequency distribution of white muscle fibres in the diameter class <25 µm was similar at 25°C and 30°C, but was significantly higher for fish reared at 34°C. The frequency distribution of white muscle fibre of diameter 25-50 µm was significantly higher at 30°C in comparison to 25°C and 34°C, and the larger fibres >50 µm were significantly lower at 30°C, and were similar at 25°C and 30°C reared fish (Table 2). Gene expression of muscle genes MyoD and myostatin quantified using RT-qPCR showed changes in fish reared at three different temperatures. At 60 days, the MyoD mRNA level was significantly
the nucleus and cytoplasm of fiber (Koumans and Akster, 1995). In this study, new fibre recruitment was observed in O. mossambicus to maintain the somatic growth, even at stressful rearing temperature of 34°C. Fertilized eggs of Senegalese sole and Solea senegalensis, incubated at 15°C, 18°C and 21°C till hatching, and subsequently raised the fish at 21°C until 30 days post hatch. It was observed that muscle fibers were more at 18°C and 21°C in comparison to 15°C and hypertrophic growth was higher at 18°C (Campos et al., 2013b). Similar trend was observed at 30°C where muscle hypertrophy was highest.

Skeletal muscle formation in fish involves specific control of several myogenic regulatory factors (MRFs) (Fuentes et al., 2013). These MRFs control cellular specification, activation and differentiation of myogenic cells. The committed and activated cells express myogenic factor 5 (myf5) and myoblast determination factor (myoD) (Watabe, 2001, 1999). In the present study, it was observed that at 60 days of rearing MyoD expression was significantly higher at rearing temperature of 34°C than at 25°C and 30°C. It suggests that higher temperature induces expression of MyoD which controls satellite cell proliferation (Kuang and Rudnicki, 2008; Megeney and Rudnicki, 1995, Watabe, 1999). In pacu MyoD expression was higher at 24°C and 32°C and myostatin gene expression was similar at all rearing temperatures (de Paula et al., 2014). They concluded that rearing at lower temperature alters gene expression and induces delay in muscle growth in juvenile pacu demonstrating thermally induced phenotypic plasticity. At 60 days, the myostatin gene expression did not vary at all rearing temperatures that were evaluated suggesting additional role other than muscle growth regulation in fish (Garikipati et al., 2006, 2007; Ostbye et al., 2001; Radaelli et al., 2003).

This study suggests that temperature affects the growth of O. mossambicus. Increase in water temperature to 30°C is beneficial for achieving maximum body growth and hypertrophic muscle growth. High temperature (34°C) inhibits fish growth and induces muscle hyperplasia and higher expression of MyoD gene in O. mossambicus.

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References


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Kieffer, J.D., F.M. Penny and V. Papadopoulos: Temperature has a reduced effect on routine metabolic rates of juvenile shortnose sturgeon (Acipenser brevirostrum). Fish Physiol. Biochem, 40, 551-559 (2014).


