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Molecular regulation of muscle development and growth in Senegalese sole larvae exposed to temperature fluctuations

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ABSTRACT

The Senegalese sole (*Solea senegalensis*) is a marine flatfish that is naturally exposed to high temperature fluctuations (12–28 ºC) in the wild, with a life cycle predominantly estuarine during larval and juvenile phases. Farming of this species has largely improved in the past years but marked fluctuations of temperature during production still contribute to variation on growth and muscle cellularity, particularly if they occur during early stages of development. Such thermal plasticity of muscle growth must arise through changes in a multitude of physiological and molecular pathways, in which epigenetic gene regulation is likely to play an essential role. In the present work, we review recent studies addressing molecular, physiological and morphological aspects of the thermal plasticity of somatic growth in Senegalese sole larvae and early juveniles, thus aiming to improve sole rearing in aquaculture production. The present study shows that temperature during specific time frames of ontogeny has both short- and long-term effects on growth and muscle cellularity of Senegalese sole. Nevertheless, Senegalese sole also seems to rapidly adapt to environmental temperature through a set of molecular mechanisms and physiological responses such as regulation of feed intake, even at early developmental stages.

1. Introduction

The Senegalese sole is a marine flatfish that has been under the scope of researchers regarding the improvement of its production, particularly in aquaculture industries of Southern-European countries such as Portugal or Spain (Imsland et al., 2003). Over the last years there has been a large effort in optimising feeding conditions of larvae and post-larvae, including manipulating live feed enrichments (Morais and Conceição, 2009; Morais et al., 2004, 2006), as well as determining amino acid requirements (Aragão et al., 2004; Pinto et al., 2010) and applying different feeding strategies to larvae and post-larvae (Engrola et al., 2005, 2009a,b, 2010; Gamboa-Delgado et al., 2011). Production of high quality fry is an important target for a successful and competitive expansion of aquaculture industry. Understanding the mechanisms that control early development and muscle growth is critical for the identification of time windows in development that introduce growth variation, and improve the viability and quality of juveniles (Valente et al., 2013). However, variability of survival rates and high growth dispersions of fish larvae, including Senegalese sole, is not completely overcome; moreover, procedures like fine tuning of water temperature concerning the optimisation of growth conditions in these early stages has not be targeted as priority so far. Its investigation is thus required to improve growth of juveniles up to commercial size.

Senegalese sole can be exposed to high temperature fluctuations throughout its life, which in the wild can range between 12 °C and 28 °C (Cabral and Costa, 1999; Vinagre et al., 2006). In aquaculture and laboratory conditions, Senegalese sole eggs are normally obtained from natural spawning of wild broodstock kept in captivity, and spawning takes place at a wide range of temperatures, reportedly from 13 to 23 °C but with higher fecundities between 15 and 21 °C (Anguis and Cañavate, 2005). Since water temperature during critical developmental windows of ontogeny can significantly influence the muscle growth patterns of fish by modulating the rates of hypertrophy and hyperplasia of muscle fibres (Johnston, 2006), it is imperative to identify and evaluate the developmental windows where the action of temperature might exert a long term effect. The study of the interaction between developmental stage and temperature will contribute to improve larval and juvenile growth and survival and to identify optimal conditions for muscle growth.

The thermal plasticity often observed in teleost growth arises through changes in a multitude of physiological and molecular pathways, in which epigenetic gene regulation is likely to play an essential role, namely DNA

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methylation and miRNA expression. In Senegalese sole, recent studies have investigated the impact of temperature on somatic growth and muscle development, gene regulation and protein metabolism during early stages of development. This paper reviews these new findings, as well as their potential future applications towards the improvement of Senegalese sole larval growth performance.

2. Muscle development and growth in fish

The formation of muscle (myogenesis) is a complex process, which involves cellular specification of stem cells to a myogenic lineage (myoblasts), proliferation, cell cycle exit, differentiation, migration and fusion (Buckingham, 2001; Sabourin and Rudnicki, 2000). Myogenesis is mediated by the action of numerous genes, namely the highly conserved basic/helix-loop-helix (bHLH) *myogenic regulatory factors* (*MRFs*), which include *myoD*, *myf5*, *myog* (*myogenin*) and *mrf4*, and play essential functions in myogenic lineage determination and muscle differentiation (Rescan, 2001). *MRFs* activate muscle-specific transcription through binding to the enhancer-box (E-box), a short consensus sequence present in the promoter of numerous muscle genes. *MyoD* and *myf5* are expressed in mesodermal cells committed to a myogenic fate, playing redundant roles in establishing myoblast identity, whereas *myog* and *mrf4* are involved later, initiating and maintaining the muscle differentiation programme (Rescan, 2001).

In fish embryos, the somites along the body axis will give rise to distinct cell lineages. In particular, the skeletal muscle will arise from the dermomyotome, which is a transient epithelial structure of the somites and the predominant source of myogenic cells in the embryo (Devoto et al., 2006). During zebrafish embryonic development, the somites undergo a rotation and a subset of adaxial cells expressing *myod* will differentiate into slow fibres that migrate through the embryonic myotome, across the medial fast fibres to form the most superficial layer of the myotome (slow fibres) (Devoto et al., 1996). As a result of this migration, fast skeletal muscle is now located medially. In addition, *pax7* positive cells colonise the myotome to form a second wave of fast fibres (Marschallinger et al., 2009). Most of these cells are proliferative, but quiescent *pax7* positive cells are also found between myofibres, constituting a potential reserve of myogenic progenitor cells (Buckingham and Vincent, 2009).

The hyperplastic mechanisms responsible for increasing the number of muscle fibres in embryos, larvae and juveniles can be of two types: stratified hyperplasia, where discrete germinal zones are found in the lateral margins of the myotome (Rowlerson and Veggetti, 2001), and mosaic hyperplasia, where new myotubes form on the surface of fast muscle fibres throughout the myotome, giving rise to a mosaic of fibre diameters (Weatherley et al., 1988). Mosaic hyperplasia is mainly responsible for expanding fast fibre number in juvenile and adult stages of the majority of the species, continuing until approximately 40% of the maximum fish length (Weatherley et al., 1988). Subsequent growth exclusively involves an increase in the length and diameter (hypertrophy) of the fibres (reviewed by Johnston et al. (Johnston et al., 2011)).

3. Epigenetics

3.1. DNA methylation

The development of different organs and tissues in an organism requires heritable, self-perpetuating changes in the programming of gene expression (Goldberg et al., 2007; Lindeman et al., 2011; Reik, 2007). These epigenetic changes occur without changes to the underlying DNA sequence and include covalent and non-covalent modifications of DNA and histones, as well as their influence on chromatin structure, which can be inherited within chromosomes (Goldberg et al., 2007). Epigenetic mechanisms can also change genome function under exogenous influence, and environmental constraints can cause epigenetic alterations that can be transmitted transgenerationally (Anway et al., 2005).

DNA methylation is a covalent modification that is heritable by somatic cells after cell division (Goll and Bestor, 2005). In mammals, nearly all DNA methylation occurs on cytosine residues of CpG (Cytosine–Guanine) dinucleotides and is often associated with a repressed chromatin state and inhibition of transcription, or so-called epigenetic gene inactivation (Bestor, 2000). DNA methylation cooperates with histone modifications to perform this repressive function (Bird and Wolffe, 1999). Acetylation of histone 3 at lysine 9 is known to be linked to active transcription, whereas methylation of H3K9 with associated with repressed transcription (Fuks, 2005).

DNA methylation is found throughout the genome with the conspicuous exception of unmethylated regions called CpG islands, which have a high frequency of CpG dinucleotides (Bird, 1986, 2002). Most CpG dinucleotides in CpG islands are normally constitutively unmethylated, irrespective of expression (Walsh and Bestor, 1999; Warnecke and Clark, 1999). However, a portion of CpG islands in mammals undergoes cytosine methylation during development and differentiation (Reik, 2007). In the genomes of vertebrates, including some fish and amphibians, the 5′ ends of some genes are associated with CpG islands (Cross et al., 1991; Stancheva et al., 2002).

The correct pattern of cytosine methylation in CpG dinucleotides is required for normal development in vertebrates. In zebrafish (*Danio rerio*), the sperm genome is hypermethylated relative to the genome of the oocyte; however, a demethylation of the embryonic genome occurs post-fertilisation, but re-methylation increases rapidly and is re-established by the gastrula stage (Mhanni and McGowan, 2004). The apparent conservation of this demethylation/re-methylation process across vertebrate species implies that it is a necessary part of the normal development. DNA cytosine methylation is carried out by a group of DNA (cytosine-5)-methyltransferase proteins, known as Dnmts (Goll and Bestor, 2005). Dnmt1 is the most abundant Dnmt and is involved in maintaining existing methylation patterns and has a direct role in histone methylation (Detich et al., 2001; Rai et al., 2006). Interestingly, zebrafish Dnmt1 morphants exhibited dramatic reductions of both genomic cytosine and genome-wide histone H3K9 methylation levels (Rai et al., 2006), suggesting that Dnmt1 activity helps direct histone methylation during terminal differentiation of particular tissues, such as skeletal muscle. Dnmt3a and Dnmt3b are two functionally related proteins that are essential for *de novo* methylation (Chen et al., 2003; Goll and Bestor, 2005; Li et al., 2007). Although DNA methylation patterns are stably maintained in differentiated mitotic cells, new patterns arise during embryonic cell differentiation and germ line specification throughout development (Reik, 2007). Dnmt3a and Dnmt3b are required for this process, and the inactivation of both genes causes a complete failure in the genome-wide methylation (Chen et al., 2003; Li et al., 2007). In zebrafish, four *dnmt3b* and two *dnmt3a* paralogues have been identified and it was suggested that they may play different roles in thermal epigenetic regulation of gene expression during early embryo development (Campos et al., 2012). Moreover, *dnmt3a* paralogues are highly and ubiquitously expressed in zebrafish adult tissues, whereas *dnmt3b* are differentially expressed, further indicating that *dnmt3a* and *dnmt3b* are diverging (Campos et al., 2012).

Correct DNA methylation patterns are essential for normal myogenesis. The demethylation of regulatory regions in myogenic genes at the beginning of the differentiation program is essential to the commitment of cells towards the muscle lineage. For instance, the *myogenin* (*myog*) promoter is initially methylated but becomes demethylated in myogenic cell cultures at the onset of muscle differentiation (Lucarelli et al., 2001). In zebrafish, muscle phenotypic abnormalities derived from DNA methylation inhibition have been observed in the organisation of fibres of trunk musculature and on the somites ability to form correctly shaped myotomes (Martin et al., 1999).

There is some evidence that water temperature directly influences DNA methylation levels on teleosts. Polar fish exhibit higher global methylation levels than tropical and temperate fish (Varriale and Bernardi, 2006). Also in the European sea bass (*Dicentrarchus labrax*), temperature