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.

Keywords: *Solea senegalensis*, myogenesis, temperature, muscle, myogenic genes, epigenetics.

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; Morais et al., 2006), as well as determining amino acids requirements (Aragão et al., 2004; Pinto et al., 2010) and applying different feeding strategies to larvae and post-larvae (Engrola et al., 2010; Engrola et al., 2009a; Engrola et al., 2005; Engrola et al., 2009b; 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 are 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 ºC 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 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, 2002; Bird, 1986). 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 influences the promoter DNA methylation and expression of the *gonadal aromatase* gene, which is implicated in temperature-dependent sex ratio shifts (Navarro-Martin et al., 2011).

3.2. MiRNAs

MicroRNAs (miRNAs) are a class of 18–24 nucleotide endogenous non-coding RNAs, that are repressive post-transcriptional regulators of gene expression. They are involved in most, if not all, physiological processes, including stem cell differentiation, cell lineage specification, neurogenesis, myogenesis and immune responses (Chang and Mendell, 2007). MiRNAs are evolutionary conserved across broad phylogenetic distances (Berezikov et al., 2005; Lagos-Quintana et al., 2001) and mutations in proteins required for miRNA function or biogenesis have shown to impair animal development (Chang and Mendell, 2007).

Transcription of miRNAs occurs in the nucleus as ~ 80 nt primary transcripts (primary miRNA), which are cleaved by ribonuclease III endonuclease Drosha and its binding partner DGCR8 into a miRNA precursor or pre-miRNA (Lee et al., 2003). Following transport to the cytoplasm, the endonuclease Dicer1 cuts the pre-miRNA into a miRNA duplex (Lee et al., 2003). Generally, one strand is degraded (the passenger strand) while the guide strand produces the mature miRNA. The strand with less-stable pairing at its 5'end usually becomes the mature miRNA and is incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC), which enables the identification and binding to the target mRNA (Khvorova et al., 2003; Schwarz et al., 2003). However, there is increasing evidence that both -3p and -5p strands are functional, particularly if both are highly expressed (Guo and Lu, 2010; Tsang and Kwok, 2009).

The miRNA-mediated gene regulation involves repression and blocking of translation initiation, mRNA degradation, and sequestration of mRNA by miRNA/RISC complex in the cytoplasmic processing bodies (reviewed by Valencia-Sanchez et al., 2006). In vertebrates, most miRNAs pair imperfectly with the 3' untranslated regions (3' UTRs) of their targets. The 5' end of miRNAs provides the most consistent base pairing, particularly the nucleotides 2-7, which have been termed the 'seed' region (Brennecke et al., 2005). Each miRNA is predicted to have multiple targets, and each mRNA may be regulated by more than one miRNA (Brennecke et al., 2005).

Some miRNAs, such as miR-1, miR-133 or miR-206 are preferentially expressed in muscle and known to interact with the transcriptional networks involved in myogenesis (Rao et al., 2006). For instance, miR-206 down-regulates the p180 subunit of DNA polymerase-α, which inhibits DNA synthesis and also indirectly down-regulates the MyoD inhibitors *Id1-3* and *MyoR*, (Kim et al., 2006). In teleosts, miRNA populations have been recently associated with the regulation of muscle growth. In Nile tilapia (*Oreochromis niloticus*), miR-206 was shown to directly target the *igf-I* 3'UTR and inhibition of miR-206 significantly increased Igf-I

levels *in vivo* (Yan et al., 2012a). Also in Nile tilapia skeletal muscle, different miRNAs were found to be differentially regulated in fast or slow-growing strains and miR-133 expression was positively correlated with growth (Huang et al., 2012). In the common carp (*Cyprinus carpio*), miRNAs such as miR-1, miR-21, miR-26a, miR-27a, miR-133a, miR-206, miR-214 and miR-222 were differentially expressed during skeletal muscle development (Yan et al., 2012b). Furthermore, miRNA expression was found to differ between hyperplasic hypertrophic muscle phenotypes during zebrafish development (Johnston et al., 2009).

4. Embryonic temperature effect on Senegalese sole larvae growth

4.1. Muscle growth

A recent study addressed the thermal-plasticity of somatic and muscle growth in Senegalese sole larvae. The results obtained by Campos et al. (2013d) showed that incubating sole embryos at 15 ºC, 18 ºC or 21 ºC and rearing larvae at 21 ºC promotes somatic growth at 30 days post hatch (dph) relatively to incubating embryos at 15 ºC and rearing larvae at 21 ºC. An increase in weight of 25 % and 27 % was observed for the 18 ºC and 21 ºC groups, respectively, relatively to the 15 ºC one. However, it was also found that the use of 15 ºC during embryonic development does not decrease hatching rate or increases mortality of larvae and therefore its effects were exclusively observed at a growth level, indicating that 15 ºC is not an extreme temperature for Senegalese sole embryos.

Embryonic temperature influenced the muscle phenotype during Senegalese sole larvae development (Campos et al., 2013d). Muscle hyperplastic growth during metamorphic stages was promoted by an incubation temperature of 18 ºC. Also by 30 dph larvae from this group presented a higher number of fast fibres than larvae from 15 ºC. Moreover, both 18 ºC and 21 ºC groups present a higher fibre diameter relatively to the 15 ºC one. However, at this age the number of fibres between the 15 and 21 ºC groups and the total muscle cross-sectional area amongst all treatments did not differ. Such results indicate that even if a temperature of 21 ºC during embryo development promotes a faster development and a good somatic growth, rearing all larvae at the same temperature attenuates the effect of embryonic temperature. And given that by 30 dph the 18 ºC group presented the highest number of fast fibres, it was suggested that this might have positive implications on muscle growth potential of this group relatively to the 15 and 21 ºC ones. Interestingly, Dionísio et al. (2012) showed that Senegalese sole larvae initially incubated at 21 ºC showed an increased number of skeletal deformities when compared to lower embryonic temperatures (15 and 18 ºC). This also instigates the idea that 18 ºC is indeed an optimal temperature to incubate *S. senegalensis* embryos.

4.2. Molecular basis of thermal-plasticity of muscle growth

4.2.1. Myogenic genes

Myogenesis is mediated by numerous genes, namely the previously mentioned and highly conserved *MRFs* (Rescan, 2001). *MRFs* and other genes such as *myHC* and *mylc2* have been previously identified in Senegalese sole (Campos et al., 2010) and some were found to be correlated with growth and nutrient utilisation indices in juveniles. Campos et al. (2013d) investigated the expression profile and thermal-plasticity of 16 genes involved in development and growth during Senegalese sole embryonic and larval development up to 30 dph. It was found that embryonic temperature promoted a transient differential gene expression at several stages of *S. senegalensis* development (see Table 1 for a summary of the results). For instance, *myf5*, *myod2* and *follistatin* (*fst*) were highest at 21 ºC during gastrulation and/or 20S stage. It also seemed that the 15 ºC treatment (which delayed embryogenesis) prolonged the expression of *MRFs* into later developmental stages compared to higher embryonic temperatures. It was also found that *myHC* and *mylc2* transcript levels were highest at 18 or 21 ºC in late embryos and/or hatchlings, which points to a more advanced state of muscle differentiation relatively to the 15 ºC ones. Long-term effects of embryonic temperature were found for *mrf4* expression, which was highest at 18 ºC during mouth-opening and in the 15 ºC group during metamorphosis (Campos et al., 2013d). The latter seems to indicate an effort of this group towards muscle compensatory growth at this stage. A similar trend was found in genes such as *myHC*, *igf-I*, *ifg-II* or *igf1r* (Campos et al., 2013d).

The existence of two *myod* paralogues, *myod1* and *myod2*, which show different expression profiles during Senegalese sole early development, is consistent with some degree of gene subfunctionalisation. During early stages of Atlantic halibut somitogenesis, Galloway et al. (2006) showed that *myod2* has a transient left-right asymmetric expression, whereas *myod1* always presents a symmetric expression in presomitic and somatic adaxial cells (Andersen et al., 2009) thus hypothesizing that *myod2* could be somehow related to the development of external asymmetry in this flatfish species.

Pax7 was found to have a peak in expression during hatching, probably associated with the mitotic division of active *pax7*-expressing myoblasts cells. However, no thermal-induced effect was found on *pax7* expression during Senegalese sole embryogenesis except during blastulation (higher transcript levels at 18 ºC). Such results are consistent with the fact that no significant differences in fast fibre number were found between 15 ºC and 21 ºC at 30 dph.

Nevertheless, by 30 dph mRNA levels for most genes were very stable across temperature groups. These results indicate that, in this range of embryonic temperatures (15 ºC - 21 ºC), effects on gene expression in Senegalese sole larvae may be mostly transient.

4.2.2. Thermal-plasticity of the miRNA transcriptome

A parallel study investigated the thermal plasticity of the Senegalese sole miRNA transcriptome during development by high-throughput SOLiD sequencing technology, to determine potential changes in embryos that were subjected to two different incubation temperatures (15 ºC or 21 ºC) and larvae that were reared at a common temperature of 21 ºC (Campos et al., 2014). This work identified 320 conserved miRNAs in Senegalese sole, of which 48 have not been previously described in any teleost species. A large proportion of miRNAs had a peak of expression at pre-metamorphic and/or metamorphic stages. Since Senegalese sole larvae display a high growth rate and accumulate a huge amount of energetic compounds until metamorphosis onset (Yufera et al., 1999), it is plausible that the very high expression of several miRNAs at a pre-metamorphic stage can be associated with a high growth rate and/or preparation for the metamorphic process. Validation of specific miRNAs' expression by qPCR showed that miR-26a, miR-181a-5p and miR-206-3p, which are known to be positively related with muscle growth, had higher levels at 21 ºC than at 15 ºC during embryogenesis and/or at hatching (Table 1), indicating a higher activation of the myogenic process at a higher temperature.

Skeletal muscle growth is strongly stimulated by Igf-I (Wood et al., 2005), which promotes both proliferation and differentiation of myoblasts (Coolican et al., 1997), as well as myotube hypertrophy (Rommel et al., 2001). These functions are mediated by the Igf-I receptor (Igf1R) through activation of two major intracellular signalling pathways: the mitogen-activated protein kinases (MAPKs), and the mTOR (mammalian target of rapamycin) through phosphatidylinositol 3 kinase (PI3K)/Akt (Nave et al., 1999). The MAPK/ERK (extracellular signal-regulated kinases) pathway is a key signalling in skeletal muscle, since it is essential for muscle cell proliferation (Jones et al., 2001). mTOR mediates signalling in response to nutrient availability, cellular energy, mitogenic signals, and various types of stress signals. In Senegalese sole, *igf-I* transcripts levels were very low in embryos, but greatly increased after hatching, whereas *igf-II* as well as *igf1r* levels were high throughout development (Campos et al., 2013d), indicating that these genes are developmentally regulated. It is likely that *S. senegalensis igfs* had contributed to the observed growth thermal-plasticity. MiRNA target prediction in Senegalese sole revealed possible target mRNAs related with myogenesis, MAPK and mTOR pathways (Campos et al., 2014), which indicate a possible increased myogenic differentiation occurring at 21 ºC. Moreover, these results are consistent with the observed differences in muscle phenotype

between groups, namely the larger fast muscle fibres at 21 ºC than at 15 ºC during hatching (Campos et al., 2013d). Our data suggest the involvement of miRNAs in temperature-induced phenotypic plasticity of muscle growth in Senegalese sole but further studies are required to ascertain their precise role in epigenetic regulation of myogenesis in fish.

5. Rearing temperature effect on Senegalese sole larvae and juvenile growth

5.1. Muscle growth and gene regulation in Senegalese sole pelagic larvae

The effects of rearing temperature on Senegalese sole larvae were investigated by Campos et al. (2013b). Eggs incubated at 20 ºC and larvae reared at 15 ºC, 18 ºC or 21 ºC during pelagic phase showed striking differences regarding size and development. Larvae reared at 15 ºC took more than twice the time to acquire a benthic lifestyle than larvae from 21 ºC (35 dph and 16 dph, respectively). During metamorphosis, larvae from 21 ºC had a similar total length (6.9 ± 0.8 mm) to larvae from 18 ºC (6.7 ± 0.8 mm); however both had a larger body length than larvae from 15 ºC (5.5 ± 0.8 mm).

Weight of pre-metamorphic and metamorphic larvae at comparable developmental stages was highest at 21 ºC and lowest at 15 ºC. This was reflected on condition factor, which was significantly lower at 15 ºC and indicates poorer nutritional status and also in relative growth rate (RGR) values, which were considerably lower at 15 ºC (15.3) compared to 18 (31.3.) and 21 ºC (46.4). Furthermore, a temperature of 15 ºC during the pelagic phase negatively affected survival of larvae, indicating that exogenous feeding larvae are more sensitive to low temperatures than embryos (Campos et al., 2013d).

Fast muscle growth was also affected by rearing temperature (Campos et al., 2013b). Between pre-metamorphosis and metamorphosis, the muscle total cross-sectional area had a 2.7, 3.0 and 4.2-fold increase and fibre diameter showed a 1.3, 1.1 and 1.6 fold-increase at 15, 18 and 21 ºC, respectively. This indicates that within these developmental stages, a rearing temperature of 21 ºC directly promotes general growth and fibre hypertrophy relatively to lower temperatures.

In pre-metamorphic larvae, mRNA levels of all *MRFs* except those of *myod1* were highest at 21 ºC, as well as *myosins* and *igf-I*, which supports the muscle growth results (Table 1). However, since whole larvae were used in the qPCR analysis, it remains to be seen if *mstn1*, which was also up-regulated at 21 ºC, played other functions than regulating muscle growth of pre-metamorphic larvae, since in Senegalese sole (Campos et al., 2010), as in other teleost (Funkenstein et al., 2009; Zhong et al., 2008), *mstn1* might have additional functions other than just regulating muscle growth.

During metamorphosis, the majority of *MRFs* as well as *igf-I*, *myosins* and *fgf6* were also significantly up-regulated at 21 ºC (Table 1). Igf-I is mainly produced in the liver but is also found in other tissues, including skeletal muscle of Senegalese sole (Campos et al., 2010). As referred above, Igf-I can induce proliferation and differentiation of myoblasts, and the thermal-induced plasticity of muscle cellularity is coherent with the expression profile of *igf-I* in sole larvae. In muscle, both Igf-I and –II can activate the PI3K–Akt–TOR pathway *via* binding to the Igf1R in the sarcolemma and trigger an increase in *myoD* translation and protein synthesis (Bodine et al., 2001; Wilson and Rotwein, 2006). Nevertheless, expression patterns of *igf-I*, *igf-II* and *igf1r* were remarkably different in pre-metamorphic and metamorphic larvae, which suggest that these genes can be differentially regulated. Interestingly, the increased *mstn1* expression at 21 ºC was no longer observed during metamorphosis. Moreover, *pax7* mRNA levels did not differ amongst temperatures, thus seeming that, in this regard, post-hatch stages may be less susceptible to changes in the number of *pax7* expressing cells.

5.2. Thermal-plasticity of *myog* epigenetic regulation in Senegalese sole

The *myog* promoter has a relatively low density of CpG residues (Fuso et al., 2010) but methylation of cytosine nucleotides within its promoter seems to play a role in negative regulation of its transcription (Fuso et al., 2010; Palacios et al., 2010). Since *myog* expression in sole larvae was affected by rearing temperature, its influence on methylation levels of the *myog* putative promoter in muscle was further examined (Campos et al., 2013a). Overall cytosine methylation (including CpG and non-CpG sites) was highest at 15 ºC (Table 1). Furthermore, three CpG sites were significantly hypermethylated at 15 °C compared with 21 °C (and a similar trend occurred in other CpGs) and their location in the promoter seems relevant for the regulation of *myog* transcription (Faralli and Dilworth, 2012), since they are in the vicinity of TAF and MEF2 binding sites. Results from sole *myog* methylation levels are consistent with the highest muscle growth at 21 ºC in addition to the up-regulation of *myog* expression, suggesting that thermal-plasticity of an epigenetic mechanism can promote differential gene expression and modulate muscle growth in Senegalese sole.

Campos et al. (2013a) observed that *dnmt3b* mRNA levels in Senegalese sole were highest at 15 ºC (Table 1) but those of *dnmt3a* were very stable across temperatures, which might indicate a subfunctionalisation of *dnmt3* genes as previously suggested for zebrafish (Campos et al., 2012). Zebrafish *dnmt3a* and *dnmt3b* paralogues may play different roles in thermal epigenetic regulation of gene expression during embryonic development since for instance, at several stages of development there was an up-regulation of *dnmt3b1* at the

lowest incubation temperature (23 ºC), whereas *dnmt3a1* and *dnmt3a2* were up-regulated at higher temperatures (27 ºC or 31 ºC) (Campos et al., 2012).

5.3. Protein metabolism is affected by rearing temperature

To examine the short- and long-term effects of three rearing temperatures (15, 18 or 21 ºC) during pelagic phase on protein metabolism and growth trajectories of Senegalese sole, two feeding trials using ^{14}C-labelled *Artemia* were performed on sole larvae and post-larvae (Campos et al., 2013c). The highest values of protein digestibility of larvae were found in the 18 ºC group, followed by the 21 ºC and the lowest were in the 15 ºC one (Table 1). This indicates a poorer digestive capacity of the 15 ºC larvae, which is consistent with their much reduced size and developmental delay (Campos et al., 2013b; Campos et al., 2013c). Moreover, retention efficiency of amino acids was higher in the 18 ºC group compared to the 15 ºC one. A lower retention efficiency of amino acids at the lowest temperature has been previously seen in other teleosts (Conceição et al., 1998). This is highly relevant, since amino acids are a major source of energy and the building blocks for protein deposition and growth in fish larvae (Conceição et al., 2010). Such results also agree with the gene expression patterns and epigenetic regulation during the pelagic phase described by Campos et al. (2013a; 2013b), since the expression of most growth-related genes was lowest at 15 ºC and highest at 21 ºC. The findings that a rearing temperature of 18 ºC promoted the highest protein absorption but a lower size and RGR values relatively to 21 ºC, point towards a good digestive capacity of the 18 ºC group but perhaps a lower feeding activity in the rearing tanks compared to the 21 ºC larvae. It would be pertinent to investigate the digestive enzyme profile and expression of genes related with the development of digestive system and digestion at different rearing temperatures.

5.4. Rearing temperature during the pelagic phase has a long-term effect on protein metabolism of post-larvae and muscle growth of early juveniles

The long-term effects of rearing temperature during the pelagic phase were investigated by Campos et al. (2013b; 2013c). Transfer of Senegalese sole larvae after completion of metamorphosis to a common rearing temperature (20 ºC) brought a number of alterations at growth, protein metabolism and gene expression levels. Particularly important is the fact that the 15 ºC group initiated a process of compensatory growth after transfer to higher temperature and overcame the initial growth limitations. The improvement of the digestive process of the 15 ºC fish, shown by the *Artemia* labelling results (Campos et al., 2013c) probably had a major contribution to this. All post-larvae had a similar growth

opportunity at 20 ºC (12-14 days) before being sampled for the tracer study and at this stage, the previously observed differences in weight were no longer significant. Interestingly, at 24 hours after feeding a single (^{14}C-labelled) meal, the 15 ºC group had much higher protein digestibility (and also feed intake and number of *Artemia* prey ingested) than the 18 or 21 ºC ones (Table 1), despite the non-significant differences in protein retention. It thus seems that post-larvae from 15 ºC were allocating more energy to somatic growth than post-larvae from the other temperatures. For example, in juvenile flounder (*Paralichthys olivaceus*) that achieved full compensatory growth after thermal manipulation, body lipids and energy content from the lowest temperature were significantly lower than at higher temperatures (Huang et al., 2008). It is not known, however, how body composition of Senegalese sole larvae and post-larvae was affected by rearing temperature, though it is likely that some changes had occurred.

When comparing the protein digestibilities at the first and second feeding trials, the 15 ºC group was the only one where digestibility was higher in post-larvae than in metamorphic larvae, giving an additional indication that this temperature clearly held back the digestive process but in a transient way (Campos et al., 2013c).

As reported by Campos et al. (2013b), fast muscle bulk greatly increases between pelagic larvae and benthic stages, such as post-larvae and early juveniles. For instance, between metamorphic larvae and early juveniles at 83 dph, muscle total cross-sectional area had an impressive fold increase of 29.3, 43.0 and 47.0 in the 21, 18 and 15 ºC groups, respectively. It is remarkable however how the 18 and 15 ºC fish presented a much higher increase than the 21 ºC ones, which was mainly due to the fact that during pelagic phase the 21 ºC group was already much larger than the other two groups. Such results agree with the increasing RGR values towards the lowest temperatures, particularly the 15 ºC one. In 83 dph early juveniles, it was also found that the expression of myogenic genes like *mrf4* and *myHC* (and positive correlations with growth for *pax7* and *myog*) in fast muscle could be related with an increased growth effort of the 15 ºC fish (Table 1), even if at this age had not attained yet the size of their counterparts reared at 21 ºC or 18 ºC. Whether the more elevated levels of *mstn1* mRNA found in fish reared at 18 ºC and 21 ºC at 83 dph are associated with higher muscle protein catabolism remains to be determined.

At 100 dph, fast fibre hyperplasia seemed a major mechanism of muscle growth at 15 ºC and 21 ºC, whereas fibre hypertrophy appeared more relevant in the 18 ºC group (Campos et al., 2013b). Furthermore, *myod1*, *myod2* and *igf1r* transcript levels in fast muscle agreed with the observed muscle phenotype. Interestingly, at this age it was found that the length of juveniles did no longer differ amongst temperatures. Moreover, juveniles from the 15 ºC treatment had the same weight as the 18 ºC ones. It could be hypothesized that the 18 ºC fish would have a lower growth, since fibre hyperplasia is a mechanism that has been

positively correlated with larger body sizes (Alami-Durante et al., 2007; Valente et al., 1999). In fact, by 121 dph the 15 ºC group had attained the same weight as the 21 ºC one and both were significantly higher than the 18 ºC juveniles (Campos et al., 2013c). It is not well understood why this intermediate temperature produced the smallest juveniles after transfer to 20 ºC since its initial performance was superior relatively to the 15 ºC ones. Nevertheless, the fact is that initially rearing larvae at 15 ºC made them overcompensate the 18 ºC ones. Perhaps the transition from 18 ºC to 20 ºC was not sufficient to induce a compensatory growth response or at least not as accentuated as at 15 ºC, and therefore these fish did not significantly change their growth curve. Considering the commercial production of Senegalese sole, one should keep in mind the initial lower survival of larvae from the 15 ºC group.

6. Conclusions

In light of these recent findings, we suggest that Senegalese sole embryos and larvae can be reared in a temperature range from 15 ºC to 21 ºC; however, exogenous feeding pelagic larvae are more sensitive to the lowest temperature (15 ºC) than embryos and these larvae present a much lower growth and survival rates than those reared at 18 ºC or 21 ºC.

Moreover, an incubation temperature of 18 ºC followed by transfer to 21 ºC promotes larval muscle hyperplastic growth, which can have positive implications on growth potential; however, at 30 dph the 15 and 21 ºC groups did not differ significantly in fibre number or muscle area, highlighting how initial temperature effects may become attenuated during ontogeny.

It is noteworthy that *myf5*, *fst*, *mrf4*, *mylc2* and *myHC* were amongst the genes most affected by embryonic temperature and thermal plasticity of miRNAs such as miR-17, miR-26a, miR-181a and miR-206 may have potential implications on thermal gene regulation. Furthermore, computationally predicted mRNA targets for several miRNAs were related with the mTOR and MAPK pathways, which are directly involved in muscle growth.

Rearing pelagic larvae at 15 ºC greatly decreases their growth and survival and delays their development, decreases protein absorption and retention, down-regulates *myog* gene expression and increases DNA methylation levels of the *myog* putative promoter in skeletal muscle compared to higher rearing temperatures - 18 and 21 ºC. Nevertheless, such negative effects are mostly transient once newly-settled larvae are transferred to 21 ºC, since Senegalese sole initially reared at 15 ºC during the larval pelagic phase undergo a mechanism of compensatory growth and were equal in weight to the 21 ºC group by 121 dph, being both groups larger than fish initially reared at 18 ºC. Muscle hyperplastic and

hypertrophic growth mechanisms linked to their previous thermal histories are likely to be related to these growth results.

In summary, this review 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 even in early ontogeny stages through a set of physiological responses ranging from adjustment of feed intake to epigenetic regulation of gene expression.

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Table 1. Summary of the general effects of water temperature on gene expression, *myog* methylation and protein metabolism in Senegalese sole embryos, larvae and post-larvae.

	Embryonic temperature [a]				**Rearing temperature** [b]					
	Gene regulation level				**Gene regulation level**				**Protein metabolism level**	
	myogenic genes [c]		**miRNAs related to myogenesis** [c]		***myog* methylation**	***dnmts*** [c]	**myogenic genes** [c]		**protein absorption**	
	Embryos	Larvae	Embryos	Larvae	Larvae		Larvae	Post-larvae/Juveniles	Larvae	Post-larvae
15 ºC										
18 ºC			n/d	n/d						
21 ºC										
Ref.	Campos et al., 2013d		Campos et al., 2014		Campos et al., 2013a		Campos et al., 2013b		Campos et al., 2013c	

[a] In the incubation temperature experiment, all larvae were reared at 21 ºC.

[b] In the rearing temperature experiment, all benthic post-larvae were reared at 20 ºC.

[c] General expression patterns for protein-coding and mRNA and miRNAs genes are indicated but it should be noted that they may vary for specific genes. Please check the corresponding reference for further details. Relative transcript and methylation levels are colour-coded as red, yellow and green for high, intermediate and low levels, respectively. n/d designates not determined.