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Thermal epigenetic regulation of muscle growth and development in the Senegalese sole (*Solea senegalensis* Kaup, 1858)

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Summary

In teleosts, embryonic and larval stages are particularly sensitive to environmental conditions, since survival and potential to grow can be severely affected if, for example, water temperature and feeding conditions for larvae are not adequate. In fish species intended for aquaculture production and commercialisation, a suitable early environment is particularly important regarding growth efficiency and quality at harvest. Temperature during early stages of development is amongst the most important factors affecting somatic and muscle growth of fish and a long-term thermal plasticity has been observed in many species.

The flatfish Senegalese sole (*Solea senegalensis* Kaup, 1858) is a species that is naturally exposed to high temperature fluctuations (12 – 28 °C) in the wild, mainly due to its life cycle, which is predominantly estuarine during larval and juvenile phase and marine during adult phase. Farming of this species has largely improved in the past years; however, great fluctuations of temperature during production are still found and can contribute to variations on growth and muscle cellularity. Such thermal plasticity must arise through changes in a multitude of physiological and molecular pathways, in which epigenetic gene regulation plays an essential role.

Embryonic and larval periods are critical regarding survival and growth potential but so far temperature effect during early stages has not been investigated in Senegalese sole. Therefore, the aim of this Thesis is to provide new knowledge on the epigenetic thermal regulation of muscle growth in Senegalese sole larvae by addressing molecular, physiological and morphological aspects of the thermal plasticity of muscle growth. It should contribute to identify and select the most favourable conditions to improve Senegalese sole larval rearing in aquaculture production.

A general overview of Senegalese sole biology and development, the myogenic process in teleosts and aspects of thermal plasticity of muscle growth and epigenetic regulation of growth-related genes are presented in **Chapter 1**.

The first part of this dissertation (**Chapters 2 and 3**) examined the effect of three different embryonic temperatures (15, 18 or 21 °C) and posterior transfer to a common temperature (21 °C), on sole embryo and larval development, gene expression and muscle cellularity until 30 days post-hatch (dph).

Higher embryonic temperatures (18 and 21 °C) promoted a faster development and increased size of larvae by 30 dph relatively to the 15 °C treatment (**Chapter 2**). Since hatching rate and survival of newly-settled were not affected by temperature, this range of

embryonic temperatures only produced visible effects on growth. Muscle cellularity at equivalent developmental stages was affected by embryonic temperature throughout larval development and by 30 dph the 18 °C group had the highest number of fast fibres, which instigates the idea that 18 °C can be an optimal temperature to incubate *S. senegalensis* embryos. However at this age, the 15 °C and 21 °C groups did not differ on fibre number and total muscle area was similar across all treatments, which may have been due to rearing all larvae at a common temperature. In **Chapter 2**, the expression profile and thermal plasticity of 16 genes involved in development and muscle growth were also investigated. A transient differential gene expression was observed at several stages of development. *Myf5*, *fst*, *mylc2*, *myHC* and *mrf4* transcript levels were highest at 18 and/or 21 °C at specific points both during embryonic or larval development, which might have contributed to the general more pronounced growth of these groups. However, higher expression of several genes at 15 °C during metamorphosis may have contributed to even muscle growth across treatments. Nevertheless, results from **Chapter 2** indicate that in this range of embryonic temperatures (15 °C – 21 °C), effects on gene expression in Senegalese sole larvae are mostly transient.

Chapter 3 examined microRNAs (miRNAs) populations in Senegalese sole embryos and larvae incubated either at 15 or 21 °C and transferred to 21 °C after hatching. miRNA transcriptomes were obtained by SOLiD high-throughput sequencing and the expression of specific miRNAs validated by RT-qPCR. A total of 320 conserved miRNAs were identified, of which 47 have not been previously described in teleost species. Highest expression of specific miRNAs was found during pre-metamorphosis, which may be associated with a high growth rate and with the preparation for the metamorphic process. Embryonic temperature affected the expression of several miRNAs, and a higher incubation temperature (21 °C) promoted expression of miRNAs positively related with muscle growth such as miR-17, miR-26a, miR-181a or miR-206, at least in some developmental stages, which might have potential implications on thermal gene regulation. miRNA target prediction revealed some possible miRNA targets related with muscle development and MAPK or mTOR pathways, which were consistent with the results observed in the expression patterns across the two temperatures.

The second part of the dissertation (**Chapters 4, 5 and 6**) examined the effects of three different rearing temperatures (15, 18 or 21 °C) applied only during Senegalese sole larval pelagic phase on growth, gene expression and DNA methylation status, muscle cellularity and protein metabolism.

Embryos were all incubated at 20 °C and newly-hatched larvae were then transferred to three different temperatures (15, 18 or 21 °C) (**Chapters 4, 5 and 6**) with

posterior transfer to a common temperature (20 °C) until 121 dph. Larvae reared at 15 °C took more than twice the time (35 dph) to acquire a benthic lifestyle than larvae from 21 °C (16 dph) (**Chapters 4 and 6**). Weight, condition factor and relative growth rate (RGR) were remarkably low in pelagic larvae reared at 15 °C and were highest at 21 °C. 15 °C negatively affected survival measured in newly-settled larvae, indicating that exogenous feeding larvae were more sensitive to this lower temperature than embryos (**Chapters 4 and 6**). In pelagic larvae sampled at equivalent developmental stages, muscle growth increased with the increment of water temperature and there were concomitant changes in gene expression, particularly in myogenic genes (**Chapter 4**). Nevertheless, a mechanism of compensatory growth was activated in the 15 °C group after transfer to 20 °C and an inversion of RGR values between temperature groups was observed (**Chapters 4 and 6**). The up-regulation of *mrf4* and *myHC* and down-regulation of *mstn1* in skeletal muscle of 83 dph early juveniles from 15 °C were consistent with the proposed compensatory growth. Furthermore, muscle cellularity of early juveniles with 83 and 100 dph differed amongst temperature groups and by 100 dph the 18 °C group favoured fibre hypertrophy whereas fast fibre hyperplasia seemed a major mechanism of muscle growth at 15 °C and 21 °C, which can have implications on growth potential (**Chapter 4**).

Chapter 5 describes the DNA methylation status of the putative proximal *myogenin* (*myog*) promoter in skeletal muscle of sole pelagic larvae reared at 15, 18 or 21 °C and relates it with *myog* expression and muscle cellularity. Additionally, it was investigated the expression of *dnmts*, which are the methyltransferases responsible for methylation of cytosines in DNA. The *myog* putative promoter identified in sole was found to be highly conserved among teleost species; therefore, it is plausible that it plays a similar role in controlling *myog* expression. It was also found that 15 °C increased DNA methylation at specific CpG sites of the putative *myog* promoter, which coincided with the lowest *myog* expression levels and muscle growth. Therefore, the status of cytosine methylation in critical regulatory regions is likely to interfere with the transcriptional activity of *myog* promoter. Moreover, *dnmt1* and *dnmt3b* expression were also consistent with the observed methylation patterns across temperatures. These results provided the first evidence of an epigenetic mechanism that may be underlying the temperature-induced phenotypic plasticity of muscle growth in teleosts.

The effect of rearing temperature on protein metabolism of larvae and post-larvae was investigated in **Chapter 6** using ¹⁴C-labelled *Artemia* as feed. A first feeding trial was performed on pelagic larvae reared at 15, 18 or 21 °C at equivalent developmental stages and a second trial was conducted on post-larvae after transfer to a common rearing temperature (20 °C). Protein absorption and retention were reduced in pelagic larvae from 15 °C when compared to fish reared at higher temperatures, but transfer to 20 °C

enhanced their feed intake and protein absorption, which became higher than in the 18 and 21 °C groups. This was consistent with the proposed compensatory growth of the 15 °C treatment. The putative poorer developed digestive system at 15 °C showed no long-term consequences and seemed to be quickly recovered once larvae were moved to 20 °C. By 121 dph, no differences in size were found between the 15 °C and 21 °C treatments, demonstrating the compensatory growth of the 15 °C group, which reached a larger size than the 18 °C one. Nevertheless and considering the commercial production of Senegalese sole, one should keep in mind the initial lower survival of larvae from the 15 °C group.

This Thesis ends with a general discussion in **Chapter 7**. The effects of temperature during embryonic and larval phases in relation to muscle growth and gene regulation are discussed. The main conclusions of the present work and future research direction are also presented. Overall, the present Thesis shows that different temperatures applied during specific frames of ontogeny (embryo or larvae) can have different outcomes on muscle development of Senegalese sole larvae and early juveniles. Growth-related gene expression and methylation status also showed thermal plasticity, which may contribute to the observed phenotypic variation and adaptation to environmental temperature in early developmental stages.

Resumo

Os estádios embrionários e larvares dos peixes são particularmente sensíveis às condições ambientais, dado que a sobrevivência e o potencial de crescimento podem ser severamente afectados se, por exemplo, a temperatura da água e alimentação disponível para as larvas não forem as mais adequadas. Em espécies destinadas à produção em aquacultura é imperioso que o cultivo nos estádios mais precoces do desenvolvimento seja altamente controlado com vista a potenciar o crescimento máximo e a proporcionar uma elevada qualidade do peixe aquando da comercialização. A temperatura da água durante estes estádios precoces é um dos fatores mais importantes que podem afectar o crescimento somático e muscular dos peixes, e em numerosas espécies observa-se que o crescimento muscular apresenta uma plasticidade que advém da temperatura aplicada inicialmente.

O linguado do Senegal (*Solea senegalensis* Kaup, 1858) é uma espécie de peixe plano marinha que no seu ambiente natural é exposta a elevadas flutuações de temperatura da água (12 – 28 °C), o que se deve principalmente ao seu ciclo de vida, que é predominantemente estuarino durante a fase larvar e juvenil, e marinho durante a fase adulta. A produção em aquacultura desta espécie conheceu um enorme avanço nos últimos anos; no entanto, regista-se frequentemente uma enorme dispersão de tamanhos em cada lote de larvas que obriga a calibrações frequentes. Esta dispersão pode estar relacionada com flutuações de temperatura em estádios precoces que podem contribuir para variações de crescimento e celularidade muscular. Esta plasticidade induzida pela temperatura deve-se a alterações em diversos processos fisiológicos e moleculares, nos quais a regulação epigenética de genes desempenha um papel essencial.

Apesar da reconhecida importância da temperatura na sobrevivência e no potencial de crescimento dos peixes, o seu efeito nos estádios precoces de desenvolvimento do linguado do Senegal nunca foi investigado. Deste modo, o objectivo geral da presente Tese é o de proporcionar uma maior compreensão dos mecanismos associados à regulação epigenética induzidos pela temperatura no crescimento muscular de larvas de linguado. Diversos aspetos moleculares, fisiológicos e morfológicos serão abordados. Deste modo, esta Tese deve contribuir para identificar condições favoráveis para o cultivo do linguado durante estádios precoces do desenvolvimento.

No **Capítulo 1** descrevem-se os aspectos gerais da biologia e do desenvolvimento do linguado do Senegal. Adicionalmente, é feita uma revisão do estado do conhecimento do processo miogénico em peixes, bem como de aspetos de plasticidade induzida pela

temperatura e da regulação epigenética de genes relacionados com o crescimento e desenvolvimento.

A primeira parte experimental da presente Tese compreende os **Capítulos 2 e 3** e debruça-se sobre o efeito de três diferentes temperaturas de incubação (15, 18 ou 21 °C) e posterior transferência das larvas para uma temperatura comum (21 °C) onde foram mantidas até aos 30 dias após eclosão (dae), no desenvolvimento dos embriões e larvas, na expressão de genes e em aspectos da celularidade do músculo branco das larvas.

Verificou-se que as temperaturas mais elevadas induziram um desenvolvimento mais rápido e aumentaram o tamanho das larvas aos 30 dae (**Capítulo 2**). No entanto, as taxas de eclosão e a sobrevivência das larvas não foram afectadas pela temperatura de incubação, pelo que os seus efeitos foram apenas visíveis ao nível do crescimento. A celularidade muscular em estádios de desenvolvimento equivalentes foi afectada pela temperatura durante o período estudado, e aos 30 dae observou-se que o grupo inicialmente incubado a 18 °C apresentava o maior número de fibras musculares. No entanto, aos 30 dae as larvas inicialmente incubadas a 15 ou 21 °C não diferiram no número total de fibras, sendo que a área total do músculo foi também semelhante em todos os tratamentos. Este resultado deve-se provavelmente ao facto de todos os grupos terem sido mantidos à mesma temperatura após a eclosão. No **Capítulo 2** foi igualmente investigada a expressão de 16 genes envolvidos no desenvolvimento e crescimento muscular. Genes como *myf5*, *fst*, *mylc2*, *myHC* e *mrf4* apresentaram uma expressão pontualmente mais elevada a 18 e/ou 21 °C durante o desenvolvimento embrionário e/ou larvar, o que pode ter contribuído para o maior crescimento destes grupos. Contudo, a expressão mais elevada de alguns genes durante o período metamórfico no tratamento de 15 °C pode ter contribuído para equilibrar o crescimento muscular entre grupos. No geral, os resultados apresentados no **Capítulo 2** indicam que dentro destas temperaturas de incubação (15 °C – 21 °C), os efeitos na expressão de genes são maioritariamente transitórios.

No **Capítulo 3** foram estudadas as populações de microRNAs (miRNAs) em embriões de linguado que foram incubados a 15 ou 21 °C sendo posteriormente todas as larvas mantidas a 21 °C até 30 dae. Foi utilizada a tecnologia “SOLiD” de sequenciação em larga escala a qual foi validada por PCR em tempo real, e foram identificados 320 miRNAs conservados, dos quais 47 não estavam descritos previamente em peixes. Diversos miRNAs mostraram uma maior expressão durante a pré-metamorfose, o que pode estar associado a uma elevada taxa de crescimento nesta fase bem como a uma preparação para o processo metamórfico. A temperatura de incubação afectou a expressão de diversos miRNAs, e pontualmente observou-se que a temperatura mais

elevada promoveu uma maior expressão de miRNAs positivamente relacionados com crescimento muscular tais como miR-17, miR-26a, miR-181a ou miR-206. Este fato pode ter potenciais implicações na regulação de genes induzida pela temperatura. A previsão da regulação de genes por miRNAs em linguado revelou possíveis genes alvos envolvidos no processo de desenvolvimento muscular e nas vias metabólicas MAPK e mTOR. Estes resultados foram também consistentes com os padrões de expressão observados entre as duas temperaturas.

Na segunda parte desta Tese foram examinados os efeitos de três temperaturas (15, 18 ou 21 °C) no cultivo de larvas durante a fase pelágica, sendo todas transferidas posteriormente para uma temperatura comum (20 °C) até atingirem os 121 dae. Foram investigados aspectos do crescimento, expressão de genes e estado de metilação de DNA, celularidade do músculo esquelético branco e metabolismo proteico (**Capítulos 4, 5 e 6**).

Os embriões foram incubados a 20 °C e as larvas recém-eclodidas foram então transferidas para uma de três diferentes temperaturas (15, 18 ou 21 °C) (**Capítulos 4, 5 e 6**). Verificou-se que as larvas cultivadas a 15 °C demoraram mais do dobro do tempo (35 dae) a adquirirem um modo de vida bentônico do que as larvas cultivadas a 21 °C (16 dae) (**Capítulos 4 e 6**). O peso, índice de condição e taxa de crescimento relativa (RGR) foram muito baixos em larvas pelágicas cultivadas a 15 °C e apresentaram os valores mais elevados nas cultivadas a 21 °C. Além disso, verificou-se que uma temperatura de 15 °C afetou negativamente a sobrevivência das larvas, o que indica que larvas com alimentação exógena são mais sensíveis a esta baixa temperatura (**Capítulos 4 e 6**) do que os embriões (**Capítulo 2**). Em larvas pelágicas amostradas em estádios de desenvolvimento equivalentes, o crescimento muscular aumentou paralelamente ao incremento de temperatura, e a expressão de diversos genes também revelou um padrão semelhante, principalmente de genes envolvidos no processo miogénico (**Capítulos 4**). Contudo, depois da transferência para 20 °C foi iniciado um mecanismo de crescimento compensatório no grupo inicialmente cultivado a 15 °C e observou-se uma inversão nos valores de RGR entre tratamentos (**Capítulos 4 e 6**). No músculo dos juvenis com 83 dae inicialmente cultivados a 15 °C houve um aumento da expressão de genes como *mrf4* e *myHC* e uma diminuição de *mstn1*, o que se mostra consistente com o crescimento compensatório observado neste grupo. Além disso, a celularidade muscular de juvenis aos 83 e 100 dae diferiu entre temperaturas, sendo que aos 100 dae os juvenis inicialmente cultivados a 18 °C revelaram uma maior hipertrofia das fibras musculares relativamente aos grupos de 15 e 21 °C onde a hiperplasia se mostrou preponderante, o que pode ter implicações no potencial de crescimento.

O **Capítulo 5** descreve o estado de metilação de ADN do promotor putativo da *miogenina* (*myog*) no tecido muscular de larvas de linguado cultivadas a 15, 18 ou 21 °C e relaciona-o com a expressão de *myog*, celularidade muscular e com a expressão de *dnmts*, que são as metiltransferases responsáveis pela metilação de citosinas no ADN. O promotor putativo da *myog* em *S. senegalensis* foi identificado e verificou-se que está bem conservado entre diversas espécies de peixes, de modo que é altamente plausível que desempenhe funções semelhantes no controlo da expressão da *myog*. Também foi verificado que uma temperatura de 15 °C durante o cultivo larvar aumentou o estado de metilação do promotor, particularmente em sítios CpG específicos, o que coincidiu com a menor expressão da *myog* a esta temperatura e com o menor crescimento muscular. Deste modo, o estado de metilação das citosinas em determinadas regiões regulatórias irá provavelmente interferir com a actividade do promotor da *myog*. Além disso, o padrão de expressão das metiltransferases *dnmt1* e *dnmt3b* também se revelou consistente com a metilação observada entre temperaturas. Estes resultados evidenciaram pela primeira vez um mecanismo epigenético que pode estar subjacente à plasticidade fenotípica induzida pela temperatura no crescimento muscular de peixes.

O efeito da temperatura de cultivo no metabolismo proteico de larvas e pós-larvas de Linguado foi investigado no **Capítulo 6**, utilizando *Artemia* marcada com ¹⁴C como alimento. Um primeiro ensaio foi conduzido em larvas pelágicas cultivadas a 15, 18 ou 21 °C e em estádios de desenvolvimento equivalentes, e um segundo ensaio foi realizado em estádios pós-larvares depois da transferência para uma temperatura comum (20 °C). A absorção e retenção de proteínas foi menor nas larvas pelágicas cultivadas a 15 °C, mas a transferência para 20 °C aumentou o consumo de alimento e absorção de proteínas deste grupo, cujos valores superaram os registados nos restantes tratamentos. Estes resultados mostraram-se consistentes com o crescimento compensatório proposto para o grupo inicialmente cultivado a 15 °C. O presumível efeito negativo no desenvolvimento do sistema digestivo que esta temperatura exerceu não revelou efeitos a longo prazo e provavelmente foi facilmente recuperado após a transferência para 20 °C. O facto de em juvenis com 121 dae não se encontrarem diferenças no tamanho entre os grupos 15 °C e 21 °C demonstra a existência de um crescimento compensatório nos linguados previamente cultivados a uma temperatura mais baixa. Curiosamente, ambos os grupos (15 e 21 °C) atingiram um tamanho superior aos inicialmente cultivados a 18 °C.

A presente Tese termina no **Capítulo 7** com uma discussão geral dos resultados obtidos. Os efeitos da temperatura durante as fases embrionárias e larvares do linguado do Senegal em relação ao crescimento muscular são debatidos e as principais

conclusões e possíveis linhas futuras de investigação são apresentadas. Globalmente, a presente Tese mostra que diferentes temperaturas aplicadas durante fases específicas da ontogenia (embriões e larvas) podem produzir diferentes resultados no desenvolvimento muscular de larvas e juvenis. A expressão de genes relacionados com desenvolvimento e crescimento muscular e o seu estado de metilação também mostraram plasticidade induzida pela temperatura, o que deverá contribuir para as variações fenotípicas observadas e para a adaptação desta espécie a diferentes temperaturas nos seus estádios iniciais de desenvolvimento.

List of abbreviations

Akt	Protein kinase B
bHLH	Basic-helix-loop-helix
CpG	Cytosine-Guanine dinucleotides
DDIT4	DNA-damage-inducible transcript 4
Dnmt	DNA (cytosine-5)-methyltransferase
E-box	Enhancer Box
EIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular signal-regulated kinases
Fgf	Fibroblast growth factor
Fst	Follistatin
Hsp	Heat-shock protein
Igf	Insulin-like growth factor
Igf1r	Insulin-like growth factor receptor
MAPK	Mitogen-activated protein kinases
MEF	Myocyte enhancer factor
MiRNA	MicroRNA
Mstn	Myostatin
Myf5	Myogenic factor 5
MyHC	Myosin heavy chain
Mylc2	Myosin light chain 2
Myod	Myoblast determination protein
Myog	Myogenin
mTOR	Mammalian target of rapamycin
MRF	Myogenic regulatory factor
Mrf4	Myogenic factor 6
Pax	Paired box protein
PI3K	Phosphatidylinositol 3 kinase
RGR	Relative growth rate
RISC	RNA-induced silencing complex
SDH	Succinic dehydrogenase
SOX6	SRY (sex determining region Y)-box 6
TAF	Putative TATA box binding protein associated factor
TSS	Transcription start site
TGF	Transforming growth factor
UTR	Untranslated region

Chapter 1

General introduction

1.1. General aspects of Senegalese sole (*Solea senegalensis*) biology and production

The Senegalese sole (*Solea senegalensis* Kaup, 1858) (order Pleuronectiformes, family Soleidae) is a marine flatfish that inhabits sandy or muddy bottoms along the coasts and estuaries of North Africa and western Mediterranean up to the Gulf of Biscay (Quéro 1984; Whitehead et al. 1986).

In its natural environment, Senegalese sole feeds essentially on invertebrates living in the sediment, such as polychaetes, bivalves and small crustaceans, and can attain a maximum length of 60 cm (Cabral 2000). Its life cycle can be divided between the juvenile phase, which is predominantly estuarine, and the adult phase, which is mainly marine (Cabral 2003). It is a gonochoric species and sexual maturity of females is achieved at 3 years and 32 cm total length (Dinis 1986). The main breeding season is between March and July (Dinis 1986) with occasional spawning during autumn, both occurring in natural environment (Cabral and Costa 1999; Cabral 2000) and in wild-caught soles kept in captivity and under a naturally fluctuating temperature regime (Anguis and Cañavate 2005).

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 its natural habitat, Senegalese sole juvenile growth rates have been found higher at lower latitudes, where waters are warmer (Cabral 2003).

Senegalese sole is nowadays under the scope of researchers regarding the improvement of its production in the aquaculture industry, particularly in Southern-European countries such as Portugal or Spain (Dinis 1992; Ribeiro et al. 1999; Imsland et al. 2003), mainly due to market saturation with seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*). Furthermore, the decline of wild catches allied with its highly appreciated flesh and high market value make Senegalese sole a credible candidate for aquaculture production (Imsland et al. 2003).

In aquaculture and laboratory conditions, Senegalese sole eggs are normally obtained from natural spawning of wild broodstock kept in captivity (Ribeiro et al. 1999; Anguis and Cañavate 2005). Spawning takes place at a wide range of temperatures, reportedly from 13 to 23 °C, with higher fecundities between 15 and 21 °C (Anguis and Cañavate 2005), although Dinis et al. (1999) observed no egg release below 16 °C. The eggs are planctonic, with a diameter between 0.87-1.00 mm, have a reduced perivitellin space and a high number of small oil droplets (Lagardère 1979; Ribeiro et al. 1999). Larvae normally hatch up to 48 h post-fertilisation, depending on the water temperature (Dinis and Reis 1995). Newly hatched larvae measure around 2.4 mm, are pelagic and

possess bilateral symmetry (Lagardère 1979; Ribeiro et al. 1999). The first exogenous feeding occurs as soon as mouth and anus open, at 2 days post-hatch (dph) and around 3 mm of total length (Ribeiro et al. 1999).

Similarly to other flatfish, Senegalese sole larvae undergo a dramatic metamorphic process, which starts around 8-12 dph and involves a 90° rotation of the body position and the migration of the left eye to join the other one on an ocular upper side (Fernández-Díaz et al. 2001). Body length rather than age seems to be essential to initiate metamorphosis and sub-stages of eye translocation are usually defined from stage 0 (corresponding to pre-metamorphosis) to stage 4 (corresponding to late metamorphosis) (Fernández-Díaz et al. 2001). Following such great anatomical transformation, larvae settle in the substratum and acquire a benthic lifestyle, lying on the blind side of the body. The body rotation and settlement usually is completed by 19-20 dph (Ribeiro et al. 1999; Fernández-Díaz et al. 2001). During metamorphosis, there is a rearrangement of the internal organs and digestive tract, with migration of the anus towards the pelvic fin. Only around 30 dph the digestive system completes its maturation (Ribeiro et al. 1999; Ribeiro et al. 1999).

Rotifers are usually preferred to *Artemia* nauplii as first prey of Senegalese sole larvae (Ribeiro et al. 1999; Conceição et al. 2007), and the later are normally introduced as feed around 4-5 dph. Enriched *Artemia* metanauplii are included at 9 dph and its quantity is gradually increased (Conceição et al. 2007). During the phase prior to weaning, Senegalese sole post-larvae are fed with live or frozen *Artemia*, generally enriched with live microalgae or commercial emulsions (Ribeiro et al. 1999; Engrola et al. 2005). Weaning post-larvae from live feed to artificial diets has been considered a major bottleneck in Senegalese sole production. Over the last years there has been a large effort in optimizing feeding conditions of larvae and post-larvae, including manipulating live feed enrichments (Morais et al. 2004; Morais et al. 2006; Morais and Conceicao 2009), 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. 2005; Engrola et al. 2009; Engrola et al. 2009; Engrola et al. 2010; Gamboa-Delgado et al. 2011).

However, variability of survival rates and high growth dispersions of young Senegalese sole is not completely overcome; moreover, procedures like fine tuning of water temperature concerning the optimization of growth conditions in these early stages has not been targeted as priority so far, and its investigation is thus required to promote further growth of juveniles and into commercial size.

1.2. Muscle development and growth in fish

The axial muscle of most teleost species consists mainly of a deep bulk of fast-contracting white fibres with glycolytic metabolism and rapidly fatigue, and a superficial thin strip of slow-contracting red fibres that are fatigue-resistant and have an oxidative metabolism (Johnston et al. 1977; Van Raamsdonk et al. 1982). Fast muscle constitutes the major part of the myotomal musculature (Van Raamsdonk et al. 1982). Intermediate fibre types can be found between these two main muscle domains, varying considerably from species to species, both in quantity and fibre composition, and are designated pink fibres because of their colour (Mascarello et al. 1986). Pink muscle has intermediate levels of oxidative and glycolytic activity between those found in red and white muscle (Johnston et al. 1974).

Directly or indirectly, growth-hormone (GH) participates in all major physiological processes in fish, including the regulation of osmotic balance, lipid, protein and carbohydrate metabolism, skeletal and muscle tissue growth, reproduction and immune function, and also aspects of behaviour, including appetite or aggression (Reinecke et al. 2005; Wood et al. 2005; De-Santis and Jerry 2007). The growth-hormone receptor (GHR) mediates the biological actions of GH on target cells by transducing the GH signal across the cell membrane and subsequently inducing transcription of many genes, including *insulin-like growth factor-I (Igf-I)* (Wood et al. 2005; De-Santis and Jerry 2007). GH action is largely (but not completely) mediated by the somatotrophic (or pituitary)/hepatic axis of GH/Igf-I, and this indirect pathway leads to the production and release of Igf in the liver and other tissues (Reinecke et al. 2005; Wood et al. 2005). Muscle growth and protein synthesis are largely regulated by the Igf system, which comprises Igf-I, Igf-II, Igf receptors (IgfR) and six Igf binding proteins (Igfbps) (Wood et al. 2005). Igf-I exerts its effects through the Igf-I receptor (Igf1R), which recognises Igf-II as well (Mendez et al. 2001; Pozios et al. 2001; Wilson and Rotwein 2006). Igfbps have distinct physiological roles and influence Igf action by guiding its delivery to specific tissues and enhancing or inhibiting Igf effects; in salmonids, over 99% of total circulating Igf-I is bound to Igfbps (Shimizu et al. 1999). In Atlantic salmon (*Salmo salar*) skeletal muscle, switching to fast growth after starvation and refeeding involves a local up-regulation of Igf-I, Igfbp-5.2 and Igfbp-4, and a down-regulation of Igfbp-2.1 (Bower et al. 2008). During the process of protein synthesis in muscle, Igf-I and -II activate the phosphatidylinositol 3'-kinase (PI3K)–protein kinase B–Akt/mTOR (mammalian target of rapamycin) signalling pathway through binding to the Igf1R in the sarcolemma and triggering a phosphorylation cascade that results in an increase of the *Myogenic Regulatory Factor (MRF) myoD* (myoblast determination protein) translation and protein synthesis (Bodine et al. 2001; Wilson and

Rotwein 2006). The Akt/mTOR pathway was shown to be up-regulated during muscle hypertrophy and down-regulated during atrophy (Bodine et al. 2001). The signalling pathway initiated by Igf-II in the sarcolemma through Igf1r targets the co-activators p300 and P/CAF, which are essential co-factors for *myoD* (Wilson and Rotwein 2006). In fish, Igf-I structure, regulation and function are generally similar to those in mammals (Reinecke et al. 2005). In mammals, Igf-I stimulates muscle growth through myoblast proliferation (Engert et al. 1996). Also in cultured rainbow trout (*Oncorhynchus mykiss*) muscle cells, Igf1R increases during differentiation (Castillo et al. 2002), and both insulin and Igf-I were shown to activate the essential MAPK (Mitogen-activated protein kinase) and PI3K/Akt/mTOR pathways (Castillo et al. 2006). In salmonids and carp muscle, there is a significantly greater abundance of Igf1R than insulin receptors (IR) (Parrizas et al. 1995), in contrast to what is observed in mammals. This indicates that Igf-I may contribute more to the regulation of muscle growth than insulin in fish. Both zebrafish and salmonids have at least two forms of the Igf1R (Igf1Ra and Igf1Rb), which show different patterns of expression during development (Maures et al. 2002) and are differently regulated by nutritional status (Montserrat et al. 2007; Valente et al. 2012).

The formation of muscle (myogenesis) is mediated by numerous genes, namely the highly conserved basic/helix-loop-helix (bHLH) *MRFs*, which include the previously mentioned *myoD*, plus *myf5*, *myog* (*myogenin*) and *mrf4*, which play essential functions in myogenic lineage determination and muscle differentiation (reviewed by 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 (Rescan 2001). *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; Buckingham and Vincent 2009). The activity of MyoD was shown to require acetylation by the co-activating proteins p300/CBP and PCAF, since acetylated MyoD displays an increased affinity for its DNA target (Sartorelli et al. 1999). The myocyte enhancer factor-2 family *MEF2* does not induce myogenesis *per se* but when co-expressed with *myoD* or *myog* increases the extent of myogenic conversion far above that seen with MRF's alone (Molkentin et al. 1995). In zebrafish embryos, muscle-specific expression of *myog* is controlled by multiple regulatory elements in its promoter, including two non-canonical E-boxes and the MEF2 and MEF3 binding sites (Du et al. 2003). Also in striped bass (*Morone saxatilis*), a 0.6 kb sequence of the *myog* promoter containing regulatory elements was enough to drive muscle-specific *myog* expression (Tan et al. 2002). In fish, genes often occur in pairs as the result of an ancient whole-genome duplication after the Actinopterygian/Sarcopterygian divergence (Jaillon et al. 2004)a). In

teleost, two paralogues of *myoD* (*myoD1* and *myoD2*) have been found (Macqueen and Johnston 2006; Andersen et al. 2009).

Another main factor that controls muscle growth is Myostatin (Mstn or Gdf-8), which belongs to the transforming growth factor- β (TGF- β) superfamily (Thomas et al. 2000). Myostatin functions by controlling the proliferation of muscle precursor cells (Thomas et al. 2000) and was first identified in *mstn*-null mice which had a dramatic increase in skeletal muscle mass (McPherron et al. 1997). Manceau et al. (2008) found that Mstn regulates the balance between proliferation and differentiation of embryonic muscle progenitors by promoting their terminal differentiation through the activation of the cyclin-dependent kinase inhibitor *p21* and *myoD*. Although in mammals the expression of *mstn* is almost entirely restricted to muscle, in fish there is a ubiquitous expression of *mstn*, suggesting different physiological functions (Rescan et al. 2001; Delgado et al. 2008; Funkenstein et al. 2009). Similarly to some *MRFs*, two *mstn* paralogues, *mstn1* and *mstn2*, have been found in several fish species (Østbye et al. 2001; Rescan et al. 2001).

Follistatin (Fst) is an activin-binding protein that antagonises the activity of activin (Nakamura et al. 1990). Fst has also been shown to bind to other members of the TGF- β superfamily, including Mstn (Lee and McPherron 2001; Amthor et al. 2004). In the presence of Fst, the inhibition of *pax3* and *myoD* expression promoted by Mstn was shown to be blocked in chicken limb buds (Amthor et al. 2004). Further lines of evidence support a role for Fst in muscle growth. For example, *fst* knockout mouse showed muscle deficiency (Matzuk et al. 1995) whereas transgenic mouse over-expressing *fst* showed enhanced muscle mass (Lee and McPherron 2001). In seabream, recombinant Fst inhibited Mstn activity in a similar way to that reported in mammals (Funkenstein et al. 2009). Transgenic rainbow trout over-expressing *fst* exhibited a doubled-muscle phenotype almost exclusively caused by hyperplasia, although it was not clear if this was solely due to an inhibition of *mstn* or also by binding of Fst to other growth-factors, such as activin or growth differentiation factor-11 (Medeiros et al. 2009). *Fst* transcripts are found in early embryos, suggesting its involvement in embryo development. For example, during seabream ontogeny *fst* mRNA can be detected as early as 12 hours post-fertilisation (Funkenstein et al. 2009).

The fibroblast growth-factor family (Fgf) comprises a large number of polypeptides which display a variety of biological activities during embryogenesis and in adult tissues, including cell proliferation, differentiation, migration, survival and apoptosis (Ornitz and Itoh 2001). Some members of the Fgf family and particularly Fgf6 are potent moderators of critical phases of muscle development. During mouse embryo development, *fgf6* transcripts are found only in the myotomal compartment of the somites (deLapeyriere et al. 1993). Proliferating mouse skeletal muscle cells express *fgf6* but its levels are reduced

in differentiated myofibres (Hannon et al. 1996). In fish, *fgf6* has also been associated with muscle development. In rainbow trout, *fgf6* transcripts are found up to the adult stage, suggesting that it may participate in the continuous generation of muscle fibres within the myotomal musculature (Rescan 1998).

1.2.1. Embryonic myogenesis in fish

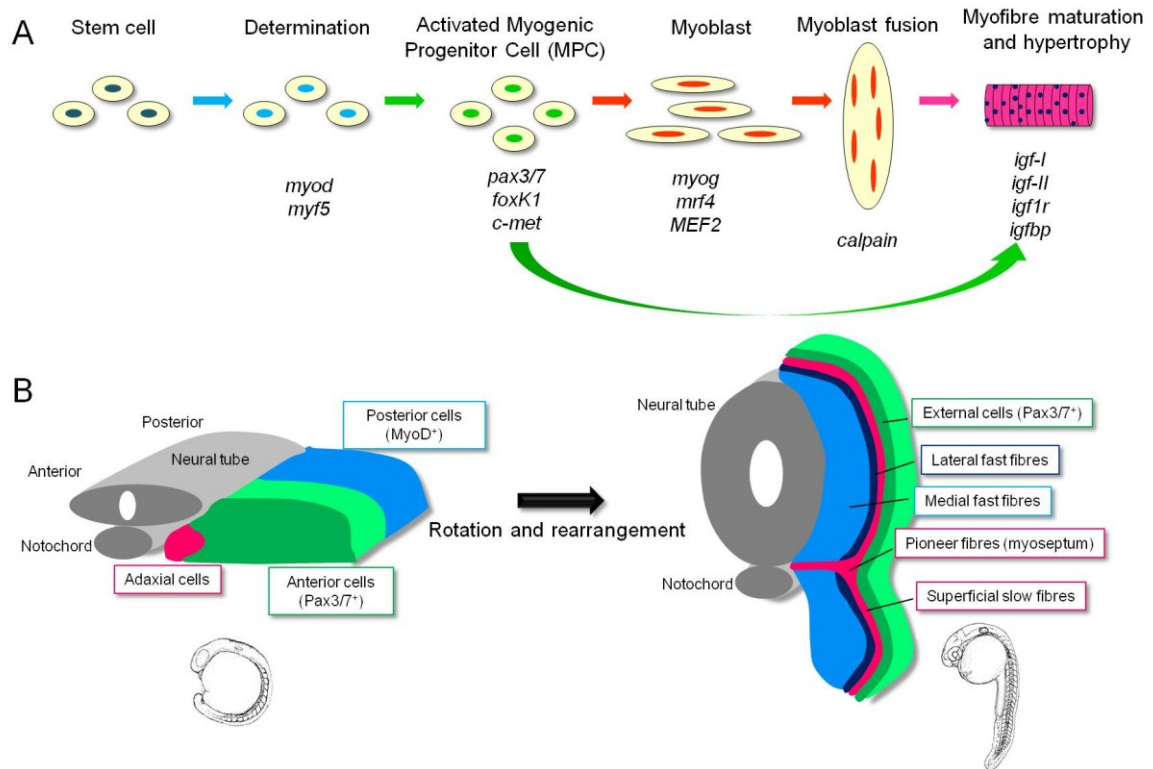
From fertilisation to hatching, fish embryos go through well defined and characterised periods of cellular cleavage, tissue differentiation and organogenesis which have been extensively studied in zebrafish (*Danio rerio*) (Kimmel et al. 1995). Myogenesis is a complex process but in all vertebrates it involves cellular specification of stem cells to a myogenic lineage (myoblasts), proliferation, cell cycle exit, differentiation, migration and fusion, all controlled by numerous signalling and effector molecules (Sabourin and Rudnicki 2000; Buckingham 2001; Buckingham and Vincent 2009).

The somites are segmental structures that are formed along the anterior/posterior axis of the embryo by segmentation of the embryonic paraxial mesoderm on either side of the neural tube and notochord (Buckingham 2001). They will differentiate into distinct compartments that subsequently give rise to distinct cell lineages: the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome. The sclerotome will give rise to the axial skeleton and the dermomyotome to the skeletal muscle and dorsal dermis. As the somite matures, cells delaminate from the dermomyotome edges and migrate underneath to form the myotome, which contains the skeletal muscle progenitors (Brent and Tabin 2002). Progenitor cells present in somites committed to a myogenic fate express the transcription factor *myoD* (Weinberg et al. 1996). In zebrafish, an array of 4 x 5 adaxial *myoD* expressing cells can be identified on either side of the notochord prior to segmentation, which later will originate slow muscles fibres (Kimmel et al. 1995; Weinberg et al. 1996). Concomitantly, cells in the anterior epithelial somite itself express *pax3* first and then *pax7*, whereas posterior cells of the epithelial somite already express *myoD* and will contribute to the medial fast fibres (Figure 1). Even if *myoD* is restricted to the posterior region in newly formed somites, by late somitogenesis it encompasses the entire extent of the somite (Hollway et al. 2007). During zebrafish development, the somites undergoes rotation and rearrangement (Hollway et al. 2007) and by 24 hours post-fertilisation (hpf) *pax3/7* positive cells appear in a dermomyotome (DM)-like position (Buckingham and Vincent 2009) (Figure 1). The DM, which is a transient epithelial structure of the somite and the predominant source of myogenic cells in the embryo, was firstly observed in amniotes (Buckingham 2006; Stellabotte and Devoto 2007), but Devoto

et al. (2006) demonstrated its existence in fish as well. During somite rotation, a subset of adaxial cells expressing *myod* differentiate into slow pioneer fibres that form the myoseptum, whereas others 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) under the regulating signal of the Hedgehog (Hh) pathway (Devoto et al. 1996; Barresi et al. 2001). As a result of lateral migration, fast skeletal muscle, which forms independently of Hh, is now located medially. In addition, *pax7* positive cells colonize the myotome to form a second wave of fast fibres (Buckingham and Vincent 2009; 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 (MPCs), analogous to those identified in amniotes (Buckingham and Vincent 2009).

The MRF *myf5* is also involved in the determination of the slow adaxial fibres and of the fast medial fibres (Buckingham and Vincent 2009). Moreover, *mrf4*, which is normally expressed in differentiated cells, can rescue myogenesis in zebrafish embryos in the absence of *myf5* and *myoD*, showing that it has a potential role as a determination factor (Hinitz et al. 2009). *Myog* has been regarded as playing an essential role in the specification and differentiation of myoblasts during embryonic myogenesis (Sabourin and Rudnicki 2000). In zebrafish, *myog* expression follows that of *myoD* in developing somites and it is only observed in cells expressing *myoD* (Weinberg et al. 1996). *Myog* expression patterns are similar in zebrafish (Weinberg et al. 1996) and brown trout embryos (Steinbacher et al. 2007): it is firstly observed in *myoD*-expressing medial cells of the forming somite and then, during somite maturation, *myog* expression extends from the medial to lateral regions of the somite. In seabream embryos, *myog* expression also gradually disappears from the medial region (designated to differentiate early into slow muscles) of the anterior somite (Codina et al. 2008). Fusion of myoblasts further seems to involve the action of calpain (Barnoy et al. 1996).

Maintaining the balance between self-renewal and differentiation is critical and in mammals, the inhibition of the Notch signalling pathway, which regulates processes of cell fate decisions, results in premature differentiation of myogenic progenitor cells, leading to a deficit in skeletal muscle later in development as well as an absence of satellite cells (Vasyutina et al. 2007). Notch signalling is therefore required to promote the self-renewal of myogenic progenitor cells. Mutation of the *forkhead transcription factor* *FoxO1*, like that of *Notch1*, leads to up-regulation of *myoD* expression (Kitamura et al. 2007) and to an increased formation of fast fibres.



Adapted from Johnston (2006) and Buckingham et al. (2009)

Figure 1. Illustration of embryonic myogenesis in fish. A) Stem cells are committed to form the Myogenic Progenitor Cell (MPC) population, which involves the expression of *myod* and *myf5* (blue). Activated MPCs express *pax3/7*, *foxK1* and *c-met* (green). The MPCs initiate the differentiation programme involving the expression of *myog*, *mrf4* and *MEF2* (red). Myoblasts in fast muscle can fuse to form short myotubes in a myoblast–myoblast fusion event, which probably involves calpain. The regulation of fibre mass is controlled by signalling pathways involving Igf family members. MPCs can fuse with existent muscle fibres in the process of nuclear accretion at any time (green arrow). B) Slow muscles are derived from the adaxial cells (red). In the epithelial somite, anterior cells (green) express first *pax3* and then *pax7*, whereas posterior cells (blue) already express *myod*. During development, the somite undergoes rotation. As a consequence, the *pax3/7* positive cells are now in a dermomyotome-like position. During somite rotation, adaxial cells differentiate into slow pioneer fibres that form the myoseptum and into slow fibres that migrate laterally across the medial fast fibres to form the most superficial layer of the myotome (red). After formation of the embryonic myotome, *pax7* positive cells colonize the myotome in order to form a second major wave of fast fibres (lateral fast fibres, dark blue) and resident progenitor cells within the muscle.

1.2.2. Second phase myogenesis – Stratified hyperplasia

In the late fish embryo/early larvae, a new wave of muscle growth contributes to the increase in size of the myotome. This second phase of muscle growth takes place in discrete germinal domains situated mainly at the dorsal and ventral regions of the myotome (Rowlerson and Veggetti 2001), and it has been termed stratified hyperplasia. This growth mechanism gives rise to a characteristic gradient in fibre diameter from the superficial to the deep layers of the developing myotome (Rowlerson and Veggetti 2001). In pre-hatching brown trout embryos with their final number of somites, expression of *myoD* and *myog* begins to decrease in the deep fibres of the myotome but persists at the dorsal and ventral extremes and in some small cells in the centre and along the lateral border of the fast muscle domains (Steinbacher et al. 2007). In this case, the appearance of small muscle cells at the myotome extremes begins when the slow fibres have not yet finished their lateral migration. Moreover, Marschallinger et al. (2009) proposed a model for post-hatching stratified fast muscle growth in teleost larvae in which *pax7*⁺ proliferative myogenic precursor cells migrate from the posterior lip of the DM to the underlying posterior fast muscle surface, where they continue to replicate and to generate daughter cells that subsequently spread out in an anterior direction before differentiation into fast muscle cells.

Both slow and fast twitch fibres are generated by stratified hyperplasia. However, stratified hyperplastic growth of slow muscle occurs even in zebrafish mutants lacking the Hh pathway (Barresi et al. 2001), thus seeming that stratified hyperplasia of slow muscle fibres in late embryonic stages is independent of the specification mechanisms described during early myogenesis.

In certain families of Antarctic notothenioid, fish show a dramatic reduction in the number of fast muscle fibres and a corresponding increase in fibre size compared to species like the Atlantic salmon, in order to reduce energy spend which is required for the antifreeze production necessary in very cold environments (Johnston et al. 2003). Hyperplastic mechanisms are also reduced in some notothenioids, where stratified hyperplasia is the only post-embryonic mechanism of fast fibre production and no mosaic hyperplasia is found (Johnston et al. 2003).

1.2.3. Third phase myogenesis – Mosaic hyperplasia

In teleost, the hyperplastic process can continue far into adulthood by mosaic hyperplasia, where scattered myogenic precursor cells are activated throughout the myotome giving a typical mosaic appearance to muscle cross-sections (Rowlerson and

Veggetti 2001). In most species, mosaic hyperplasia is the main mechanism for expanding fast fibre number in juvenile and adult stages, continuing until approximately 40% to 50% of the maximum fish length (Weatherley et al. 1988; Johnston et al. 2009). The timing of occurrence of mosaic and stratified hyperplasia depends largely on the species (see review of Valente et al. 2013). Mosaic hyperplasia can occur concomitantly with stratified hyperplasia, as in brown trout, where mosaic hyperplasia has a precocious onset and contributes to a fast somatic growth (Steinbacher et al. 2007). New cells arising at this phase fuse to form additional fibres or are absorbed by existing fibres as they expand in diameter (hypertrophic growth). The maximum diameter attained by a muscle fibre is set by cellular diffusional constraints that vary with body mass, activity patterns and metabolism (Johnston et al. 2003; Johnston et al. 2004). During mosaic hyperplasia in the common carp (*Cyprinus carpio*), the immature fibres transiently express a developmental distinct *myosin heavy chain* gene (Ennion et al. 1995), and also in zebrafish, five fast muscle *myosin heavy chain* genes were down-regulated at the cessation of mosaic hyperplasia (Johnston et al. 2009). *FoxK1*, which is a member of the forkhead/winged helix family (*Fox*) of transcription factors is a marker of MPCs alongside with *pax7*, and is expressed in mononuclear myogenic progenitor cells in adult fast muscle of tiger pufferfish (*Takifugu rubripes*), indicating that it may play a role in hyperplastic growth (Fernandes et al. 2007). Furthermore, more than 6800 transcripts are significantly up-regulated in the superficial hyperplastic zones of the myotome of late embryonic rainbow trout compared to adult muscle, amongst them *pax7* and the *MRFs* (Rescan et al. 2013).

Once the maximum fibre number (FN_{max}) is reached, myotube formation is inhibited unless the muscle is injured, suggesting the existence of a mechanism that inhibits myotube formation in undamaged muscle in fish that are greater than ~ 40 % of their maximum length (Rowlerson et al. 1997). In fast muscle of tiger pufferfish, Fernandes et al. (2005) identified genes that were differentially expressed between hyperplastic and hypertrophic phenotypes, from which four were 5 to 25-times up-regulated concomitantly with the inhibition of myotube formation, having one of them similarities with fibronectin. In zebrafish, the two most abundant categories of down-regulated genes in the hypertrophic phenotype were shown to encode contractile proteins and sarcomeric structural/cytoskeletal proteins (Johnston et al. 2009).

1.3. Environmental effects on growth of fish

In nature, teleost eggs are usually at the mercy of all types of constraints like temperature variations, oxygen availability, salinity or pH, which can be the cause of considerable embryonic mortality. Furthermore, fish larvae are also generally more sensitive to the surrounding environment than juveniles or adults since their development is not complete.

In farmed fish species, there has been a great effort over the years to provide the best conditions for successful development of embryos and small larvae, since environmental circumstances present during early stages and often short and specific developmental frames can be reflected later, frequently in an irreversible way (Johnston 2006). Environmental conditions during these periods can therefore influence the subsequent muscle growth potential, animal welfare and final flesh quality.

1.3.1. Thermal plasticity of growth-related gene expression and muscle growth

Physiological mechanisms in fish are highly modulated by thermal conditions, since their body temperature is very close to that of the surrounding water (reviewed by Angilletta et al. 2002) and growth-related gene expression has been shown to be affected by temperature. For example, in rainbow trout fry fed *ad libitum*, a higher rearing temperature led to increased *igf-I* mRNA levels in liver and higher plasma Igf-I, which correlated with growth rate (Gabillard et al. 2003). In tiger pufferfish embryos, the peak of *myog* expression is higher and occurs at a later developmental stage at a higher incubation temperature (Fernandes et al. 2006). In Atlantic salmon, *myod1a* and *myog* show similar expression patterns with respect to somite stage at incubation temperatures of 2, 5 and 8 °C until the “eyed stage”, in spite of different rates of somite formation, but a delay in the expression of *myf5*, *mrf4* and *mlc1* with respect to somite stage at 2 °C compared to 8 °C was observed (Macqueen et al. 2007). Such heterochronies in *MRFs* are a potential source of variations in adult muscle phenotype deriving from changes in embryonic temperature (Macqueen et al. 2007; Macqueen et al. 2008). Heterochronies are usually regarded as important adaptive responses, but that is not always the case, as for example different embryonic temperatures in Atlantic cod failed to show heterochronies and phenotypic plasticity, indicating that many occurrences of heterochrony might not bring any adaptive response but rather be unfavourable to the organism (Hall and Johnston 2003).

Water temperature has also the ability to greatly affect larval and juvenile growth (Johnston and Cole 1998; Lopez-Albors et al. 2003; Alami-Durante et al. 2007;

Albokhadaim et al. 2007). Several studies reported temperature effects on the number of muscle fibres in larvae (Galloway et al. 1998; Galloway et al. 1999; Carey et al. 2009; Silva et al. 2011) and adults (Macqueen et al. 2008). However, temperature impact on muscle growth depends largely on the species and the temperatures to which they are naturally exposed in the wild. In warmer water and/or active species, the maximum fibre diameter reaches a limiting value at a smaller body length compared to very cold-adapted and sedentary teleost species, where the relaxation of diffusional constraints due to the low temperature allows fibre diameter to increase further and reach higher maximum values (Johnston et al. 2003; Johnston et al. 2003). It is therefore not expectable that similar variations in water temperature lead to similar muscle growth on every species. For example, incubating and rearing seabass larvae at 15 °C promotes the highest number of fibres at yolk sac resorption and the best growth of juveniles compared to higher or lower temperatures, whereas in rainbow trout optimal fibre number during yolk sac resorption and the best growth of juveniles is observed at 4 °C compared to 8 °C or 12 °C (Wilkes et al. 2001).

1.3.2. Metabolism and temperature

By virtue of temperature effects on molecular interactions, temperature affects rates of cellular processes and the metabolic and physiological activities of tissues and organs. For example, in fish muscle, response to cold leads to the adjustment of membrane phospholipids and fatty acid composition to maintain the dynamic phase behaviour of the membrane, which in turn affects the activities of the membrane proteins that establish basal metabolic rate (reviewed by Guderley 2004). Cold-acclimation can significantly increase the density of mitochondria and capillary supply to skeletal fish muscle, which reduces diffusion distances and increase the capacity for aerobic ATP production relative to fish acutely exposed to a low temperature (Johnston and Dunn 1987; Guderley 2004). Interestingly, in fast muscle of goldfish (*Carassius auratus*), cold-acclimation promotes an increase in functional oxidative capacity, which is due to higher rates of mitochondrial biogenesis, but without producing mitochondrial uncoupling (Dos Santos et al. 2013).

Changing temperature has further biological repercussions. In Senegalese sole juveniles, acclimation to different temperatures induces changes in plasma metabolites and increasing rearing temperature from 18 °C to 26 °C raises their metabolic activity (Costas et al. 2011). It has also been shown that sole adjusts its osmoregulatory system to compensate the effects of temperature on electrolyte transport capacity and that thyroid hormones are implicated in temperature acclimation (Arjona et al. 2010). In zebrafish, decreasing temperature decreases the metabolic rate; interestingly, in response to

exercise at 18 °C, carbohydrates accumulate in the liver but are not adequately addressed to respond to power swimming activity due to the low metabolic rate at 18 °C comparing with higher temperatures (Vergauwen et al. 2013). Furthermore, amino acid metabolism has been shown to be influenced by temperature. For instance, rearing larvae of the African catfish (*Clarias gariepinus*) at 28 °C or 31 °C increases the absorption and depletion rates of amino acids and lead to a higher retention efficiency of yolk nutrients compared to those reared at 25 °C (Conceição et al. 1998).

1.4. Epigenetic gene regulation in fish

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; Reik 2007; Lindeman et al. 2011). These epigenetic changes occur without changes to the underlying DNA sequence and include covalent and non-covalent modifications of DNA and histone proteins and their influence on chromatin structure, which can be inherited with chromosomes (Goldberg et al. 2007). Epigenetics is therefore a bridge between genotype and phenotype. 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 also plays an important role in the maintenance of genome integrity by transcriptional silencing of repetitive DNA sequences and endogenous transposons (Bestor 1998). It is found throughout the genome with the conspicuous exception of unmethylated regions called CpG islands (Bird 1986; Bird 2002). The generally accepted definition of a CpG island is a stretch of non-methylated DNA longer than 200 bp with over 50 % G + C content and a CpG frequency of at least 0.6 (Gardiner-Garden and Frommer 1987). 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 mammals, DNA methylation is essential for embryogenesis, during which methylation patterns change dynamically and prepare embryos for further differentiation (Reik et al. 2001). Two main waves of genome-wide epigenetic reprogramming characterise zygote and primordial germ cell formation during mammalian development (Reik et al. 2001). In fish, there is also a dynamic change in methylation of the embryonic genome. In zebrafish, the genome of the sperm 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). The preference of Dnmt1 for hemimethylated CpG sites is thought to form the basis of high-fidelity maintenance of the epigenetic code during replication (Vilkaitis et al. 2005). 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. When zebrafish embryos are treated with methylation inhibitors, phenotypic abnormalities are observed in somites and muscle development (Martin et al. 1999). In particular, the trunk musculature contains poorly organised fibres and somites fail to form appropriately shaped myotomes. 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. The first connection between DNA methylation and the activation of the myogenic

program came through the observation that treatment with 5-azacytidine, a methyltransferase inhibitor, converted embryonic fibroblasts to muscle (Taylor and Jones 1982). Furthermore, *myoD* was expressed and the *myoD* CpG island demethylated in embryonic myogenic cells derived from the mouse 10T1/2 cell line treated with 5-azacytidine (Jones et al. 1990). Similar effects occur after the transfection of fibroblasts with an antisense RNA against Dnmt1 (Szyf et al. 1992). Another regulatory region in *myoD*, the distal control element, was found to be specifically demethylated during somitogenesis in mice and preceded *myoD* transcription, suggesting that demethylation of the distal enhancer is essential for *myoD* activation (Brunk et al. 1996). Furthermore, the *myog* promoter is initially methylated but becomes demethylated in myogenic cell cultures at the onset of muscle differentiation (Lucarelli et al. 2001). The *myog* promoter has a relatively low density of CpG residues (Fuso et al. 2010) but methylation of cytosine nucleotides within its promoter appears to play a role in the negative regulation of *myog* transcription (Fuso et al. 2010; Palacios et al. 2010).

In teleost, there are some evidences that water temperature directly influences DNA methylation levels. Polar fish exhibit higher global methylation levels than tropical and temperate fish (Varriale and Bernardi 2006). Also in the European sea bass, 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). Nevertheless, it is still unknown whether temperature can directly influence methylation patterns of myogenic genes.

1.5. MicroRNAs

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 (Lagos-Quintana et al. 2001; Berezikov et al. 2005) and mutations in proteins required for miRNA function or biogenesis have shown to impair animal development (reviewed by 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

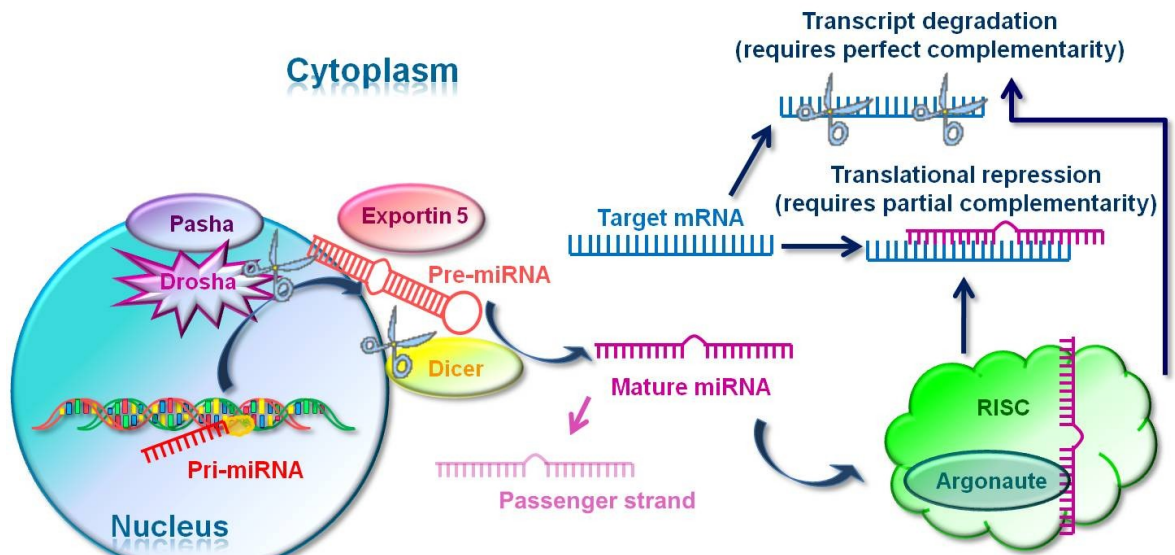
duplex (Hutvagner et al. 2001; Lee et al. 2003) (Figure 2). Generally, one strand is degraded (the passenger strand) while the guide strand confers the mature miRNA. The selection of the appropriate strand is determined by the strength of base pairing at the ends of the miRNA duplex (Figure 2). The strand 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) (Figure 2). However, there are increasing evidences that both -3p and -5p strands are functional, particularly if both are highly expressed (Tsang and Kwok 2009; Guo and Lu 2010).

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 (P-bodies) (reviewed by Valencia-Sanchez et al. 2006). There is a good correlation between miRNA-mediated mRNA translation repression and accumulation of mRNAs in the P-bodies; however, this process can be reversed indicating that P-bodies can function as temporary storage sites for mRNAs which do not participate in protein synthesis (reviewed by Pillai et al. 2007). In vertebrates, most miRNAs pair imperfectly with the 3' untranslated regions (3'UTRs) of their targets. This imperfect base pairing prevents cleavage by RISC, supporting instead the translational repression of the mRNA. 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 many targets, and each mRNA may be regulated by more than one miRNA (Brennecke et al. 2005). Interestingly, it has been found that mechanisms like DNA methylation can also regulate the expression of miRNAs (reviewed by Sato et al. 2011).

Some miRNAs, such as miR-1, miR-133 or miR-206 are strongly expressed in muscle and known to interact with the transcriptional networks involved in myogenesis (Rao et al. 2006). miR-1 and miR-133 originate from the same polycistron and are transcribed together. However, miR-1 promotes skeletal myoblast differentiation by suppressing histone deacetylase (HDAC-4), which results in increased Mef2 activity (Lu et al. 2000; Chen et al. 2006). In contrast, expression of miR-133 inhibits the progress of myogenic process by repressing *serum response factor* that in turn keeps myoblasts in a proliferative state (Chen et al. 2006). 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). miR-206 also induces myoblast differentiation by down-regulating *pax7*, which is mainly expressed during proliferation (Dey et al. 2011). Some ubiquitously expressed miRNAs also have myogenic functions.

For example, miR-214 reduces the histone methyltransferase Ezh2 protein (suppressor of skeletal muscle cell differentiation) and promotes muscle differentiation (Juan et al. 2009).

In teleosts, miRNAs populations have been recently associated with the regulation of muscle growth. In Nile tilapia, 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. 2012). 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, 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. 2012). Furthermore, miRNAs expression was found to differ between the hyperplasic muscle phenotype and the hypertrophic muscle phenotype during zebrafish development (Johnston et al. 2009). Still, in spite of the increasing number of studies on miRNA-mediated regulation of teleost myogenesis, the effect of temperature on miRNAs populations and consequent regulation of muscle growth was never investigated.



Adapted from Mack (2007)

Figure 2. MiRNA biogenesis and post-transcriptional gene silencing. A primary miRNA (pri-miRNA) transcript is encoded in the DNA and transcribed in the nucleus. Then, it is processed by Drosha and exported into the cell's cytoplasm where it is further processed by the enzyme Dicer. After strand separation (-5p and -3p), the mature miRNA represses protein production either by blocking translation or causing transcript degradation. Depending on the miRNA, passenger strand can be either degraded or not and also have a role in mRNA regulation.

1.6. Objectives

This Thesis aims to contribute to a better knowledge on the effects of water temperature on growth and its regulation during the early life stages of Senegalese sole development. Critical developmental windows during ontogeny where the action of temperature might exert a long term effect will also be identified and evaluated. This Thesis addresses molecular, physiological and morphological aspects of the thermal plasticity of muscle growth in sole larvae. This knowledge may contribute to identify and select the most favourable conditions to improve Senegalese sole larvae growth in aquaculture.

Two temperature experiments are described. One comprises the manipulation of embryonic temperature with larval rearing at a single common temperature (**Chapters 2 and 3**). In **Chapter 2**, the effects of embryonic temperature on development, growth, growth-related gene expression and fast muscle phenotype of sole embryos and larvae until 30 dph are evaluated. **Chapter 3** investigates how miRNAs populations, in particularly those related with muscle development and growth, are affected by embryonic temperature. The second experiment involves changes in rearing temperature only during larval pelagic phase and transfer of benthic post-larvae to a common temperature (**Chapters 4, 5 and 6**). **Chapter 4** characterises the thermal-plasticity of growth, fast muscle development and growth-related gene expression of Senegalese sole larvae, post-larvae and early juveniles. **Chapter 5** determines how DNA methylation status of a myogenic gene such as *myogenin* in muscle of sole larvae is affected by rearing temperature, concomitantly with gene expression and muscle cellularity. In **Chapter 6**, Senegalese sole larvae and post-larvae protein metabolism is characterised in relation to different rearing temperatures during the pelagic phase.

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Chapter 2

Incubation temperature induces changes in muscle cellularity and gene expression in Senegalese sole (*Solea senegalensis*)

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Abstract

Fertilised eggs of Senegalese sole were incubated at 15, 18 or 21 °C, and after hatching all larvae were reared at 21 °C until 30 days post-hatch. By this point larvae from the 18 or 21 °C temperature groups had 11 and 9 % more muscle fibres than those from 15 °C, respectively. Hyperplastic growth during metamorphosis was higher in larvae from 18 °C. Embryonic temperature induced gene expression changes, albeit with a variable pattern throughout development. *Myf5*, *myod2*, *myHC* and *fst* mRNA levels were significantly higher at several stages prior to hatching in embryos incubated at 21 °C, whereas *hsp90AB* and *hsp70* transcripts were present at higher levels in the 15 °C group. *Myf5*, *myod1*, *myod2*, *pax7*, *myog*, *fst*, *igf-II*, *igf1r*, *hsp90AA* and *hsp90AB* were expressed at higher levels during early development, particularly during somitogenesis. In contrast, *mrf4*, *myHC*, *mylc2*, *igf-I*, *mstn1* and *hsp70* were up-regulated at later stages of larval development, namely during and after metamorphosis. This study is the first example of thermal plasticity of myogenesis with prolonged effect in a flatfish.

Keywords: *Solea senegalensis*, Epigenetics, Development, Muscle cellularity, Thermal plasticity, Myogenesis

2.1. Introduction

In teleosts, thermal conditions during early stages of development can produce different phenotypes regarding muscle fibre composition and growth patterns in the adults (Johnston 2006). Muscle formation is the consequence of a series of complex, concerted events (Steinbacher et al. 2006), and changes in temperature during critical developmental windows have the potential to irreversibly alter muscle cellularity. In Atlantic salmon (*Salmo salar*, Linnaeus), thermal conditions until the eyed-stage of embryogenesis were found to program the adult muscle phenotype (Macqueen et al. 2008), and the relative timing of epiboly in relation to segmentation was related with muscle cellularity at hatching (Hall and Johnston 2003). Changes in timing and intensity in expression of genes like *myogenin* (*myog*), *forkhead/winged helix transcription factor*

(*foxK1*) and other *myogenic regulatory factors* (*MRFs*) with temperature have been implicated in plasticity of fish muscle phenotypes (Fernandes et al. 2006; Macqueen et al. 2007; Fernandes et al. 2007a; Fernandes et al. 2007b).

The ultimate size of a fish species is shaped by the balance between recruitment and enlargement of muscle fibres (Johnston et al. 2011). In zebrafish (*Danio rerio*, Hamilton), a 4 x 5 array of cuboidal cells adjacent to the notochord expresses the *MRFs* *myoD* and *myf5* prior to the formation of somites (Devoto et al. 1996; Daggett et al. 2007). These genes are involved in specifying the skeletal muscle lineage, as well as in regulating the balance between cell proliferation and differentiation (Buckingham 2001). Expression of the *MRFs* *myog* and *myogenic factor 6* (*mrf4*) occurs later, initially in adaxial cells and then in the developing somite (Rescan 2008). The dermomyotome, a transient epithelial sheet on the external surface of the somite, expresses *paired box protein 7* (*pax7*) and seems to be the predominant source of myogenic precursors that account for the expansion of the primary myotome in the embryo (Marschallinger et al. 2009). The second phase of myogenesis - stratified hyperplasia - leads to an expansion of the embryonic myotome, and involves the formation of new muscle fibres at the junction between slow and fast fibres and next to the horizontal septum of the myotome (Rescan 2008), which positively stain for *pax7* and *myog* (Steinbacher et al. 2006). The hyperplastic process can continue far into adulthood by mosaic hyperplasia, where scattered myogenic precursor cells are activated throughout the myotome and express markers such as *myosin heavy chain* genes (Johnston et al. 2009), *foxK1* (Fernandes et al. 2007b) or *pax7* (Seale et al. 2000). These new cells fuse to form additional fibres or are absorbed by fibres as they expand in diameter (hypertrophic growth).

In fish, as in other vertebrates, there are a large number of molecular and physiological pathways involved in cell proliferation, differentiation and death. The insulin-like growth factors I and II (Igf-I and -II) are pivotal regulators of growth, and in *ad libitum* fed rainbow trout (*Oncorhynchus mykiss*, Walbaum) temperature seemed to promote growth by stimulating plasma Igf-I levels (Gabillard et al. 2003). The transforming growth factor- β (TGF- β) superfamily of proteins includes factors like *myostatin* (*mstn*), which is known for its potent ability to negatively regulate mammalian skeletal muscle growth, as first shown in *mstn*-null mice which had a dramatic increase in skeletal muscle mass (McPherron et al. 1997). In mammals, expression of *mstn* is almost entirely restricted to muscle but in teleosts the ubiquitous expression of *mstn* suggests additional physiological functions (Delgado et al. 2008; Funkenstein et al. 2009; Campos et al. 2010). *Mstn*-mediated inhibition of myogenesis was shown to be directly antagonized by follistatin (Lee and McPherron 2001; Amthor et al. 2004). Transgenic rainbow trout overexpressing *follistatin* (*fst*) exhibited the doubled-muscle phenotype, though it was not clear if this was

due to an inhibition of *myostatin* or other growth-factors (Medeiros et al. 2009). Heat shock proteins (HSPs) comprise constitutive and inducible isoforms and may be differently induced in response to a variety of metabolic insults, thus playing a central role in cellular homeostasis as they protect newly synthesized proteins and ensure protein proper folding (Lanneau et al. 2008; Stenslokken et al. 2010). In fish, HSPs have shown to accumulate in response to heat stress, but also in response to cold treatments (Stenslokken et al. 2010).

Conditions that influence muscle growth assume particular significance when it comes to species intended for aquaculture production, where size and flesh quality have major importance. The flatfish Senegalese sole (*Solea senegalensis*, Kaup) is an attractive species for marine farming, and a great effort has been done in the past years regarding production improvements. This species shows a promising growth potential during larval and post-larval stages (Conceição et al. 2007) but myogenesis has not been studied in Senegalese sole and neither have the effects of different temperatures on its muscle growth dynamics. In aquaculture conditions, Senegalese sole eggs are normally incubated within 16 to 22 °C but it is unknown whether embryonic temperature has a long-term effect on growth. In the present study, the effects of three incubation temperatures (15, 18 and 21 °C) on muscle fibre recruitment were studied, concomitantly with the expression of genes related with muscle development in embryos and larvae of Senegalese sole.

2.2. Materials and methods

All animal handling protocols were conducted according to the European Economic Community animal experimentation guidelines directive of November 24th, 1986 (86/609/EEC).

2.2.1. Fish rearing

The Senegalese sole incubation experiment took place at the LEOA facility, University of Algarve, Faro, Portugal. Senegalese sole embryos, from a single batch, were obtained by natural spawning of wild-caught broodstock (3 males and 3 females), which have been maintained at the LEOA for 3 years. Eggs were placed in the incubation tanks *circa* 3 h after spawning. They were randomly split in three groups, placed in plastic bags containing water from the breeding tank (approximately 21 °C) and allowed to equilibrate to the different incubation tanks (15 °C, 18 °C and 21 °C). Senegalese sole embryos were

then distributed in three fibreglass conical tanks (100 L) per temperature group, at a density of 100 eggs L⁻¹, and incubated at three different temperatures (15.2 ± 0.3 °C, 18.4 ± 0.4 °C or 20.9 ± 0.3 °C). After hatching all larvae were reared at the same temperature (20.8 ± 0.1 °C) following an acclimation period as above, until 30 days post-hatching (dph). Larvae were initially reared in 100 L conical tanks at a density of 40 larvae L⁻¹, and after settling (18 dph) transferred into flat-bottom tanks. Water temperature, O₂, salinity, pH and nitrogenous compounds were monitored regularly during the entire trial, and larvae were exposed to an artificial photoperiod of 12 h light:12 h dark. Estimations of hatching rate were performed by randomly counting 20× the number of hatched larvae in 5 mL of water from a known volume of the incubation water for each tank; survival of newly settled larvae was determined by counting all the larvae that were transferred to the flat-bottom tanks.

At mouth opening, larvae were fed rotifers (rots) (*Brachionus* sp.) enriched with Red Pepper (Bernaqua, Olen, Belgium), at an initial density of 3 rots·mL⁻¹. *Artemia* nauplii (na) (Inve, Dendermonde, Belgium) were introduced at 5 dph and their density was gradually increased from 4 to 8 na·mL⁻¹, becoming the only prey offered at 8 dph. *Artemia* enrichment was done at 250 000 nauplii/metanauplii L⁻¹, with 0.4 g L⁻¹ in two doses of a 1:1 mixture (weight basis) of Easy DHA Selco® (Inve) and Micronised Fishmeal® (Ewos, Bathgate, UK). Live *Artemia* meta-nauplii (M24) were then introduced at 9 dph, increasing from 12 to 20 M24·mL⁻¹ until 18 dph. Frozen meta-nauplii with the same enrichment were offered when larvae settled at the bottom (around 18 dph) until 30 dph.

2.2.2. Embryo and larva sampling

Samples of embryos and larvae were taken for gene expression analysis and histology. All samples were snap-frozen in liquid nitrogen and conserved at -80 °C until further analysis. Embryos were collected according to developmental stages shown by Lagardère (1979). Larval metamorphic stages were evaluated according to the eye-translocation stage (Fernandez-Diaz et al. 2001). The sampling points for gene expression were blastula (BL), 75% epiboly (75EP), 20 somites (20S), final somitogenesis (FS), hatching (0 dph), mouth opening (MO), pre-metamorphosis (stage 0, PreM, 8 dph for larvae from 18 and 21 °C, 9 dph for larvae from 15 °C), metamorphosis climax (stage 3, Met, 14 dph for larvae from 18 and 21 °C, 15 dph for larvae from 15 °C) and 30 dph. Larvae were removed from the tanks with a Pasteur pipette, observed under a dissecting microscope to ensure that they were at the correct stage, killed by over-anaesthesia with MS-222 (400 mg·L⁻¹), rinsed with water, placed in plastic tubes and snap-frozen in liquid nitrogen. Larvae were not fed prior to sampling and any *Artemia* or rotifer remains were

eliminated during the rinsing step. For muscle cellularity analysis, samples were taken at 0 dph, mouth opening, pre-metamorphosis (stage 0), metamorphosis (stage 3), post-metamorphosis (stage 4, 22 dph) and 30 dph. Larva total length (L_T) (n=30) was measured at each sampling point, and dry weight (mg) was determined at the end of the experiment (30 dph).

2.2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from three pools of Senegalese sole embryos and larvae per temperature (40 embryos, 15 larvae per pool). Embryos/larvae were placed into Lysing Matrix D tubes (Medinor) containing lysis buffer, homogenised for 40 s at 6000 rpm using the MagnaLyser instrument (Roche) and total RNA obtained using the mirVana miRNA isolation kit (Ambion, Austin, Texas, USA), according to the manufacturer's instructions. Assessment of RNA quality was performed by agarose gel electrophoresis on a 1.2% (w/v) agarose gel containing SYBR® safe (Invitrogen, Carlsbad, CA, USA) DNA gel stain. RNA samples were then quantified with a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). Absorbance ratios (260/280 nm) were greater than 1.9, indicating high purity RNA. Firefly luciferase mRNA (Promega, Fitchburg, Wisconsin, USA) was used as exogenous standard for qPCR quantification the embryos and larvae RNA. One microgram of total RNA was used to synthesise cDNA with the QuantiTect reverse transcription kit (Qiagen, Venlo, Netherlands), as reported elsewhere (Fernandes et al. 2008).

2.2.4. Pax7 cloning and sequencing

BLAST similarity searches against the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) were performed to identify orthologues of *pax7* in other teleost species. Degenerate primers (Fw 5'→3' CBCTGCCAAATCAYATYMGAC, Rev 5'→3': CSGCKGTGAADGTGGTRC) were designed against the most conserved regions of the sequences. Cloning and sequencing were performed as previously reported (Campos et al. 2010).

2.2.5. Quantitative real-time PCR (qPCR)

Specific primers for qPCR were used (see Table 1 for primer sequences and GenBank accession numbers). Whenever possible, primers that span at least one intron/exon border were designed to avoid amplification of potential contaminating

genomic DNA, as detailed elsewhere (Fernandes et al. 2008). Quantification of gene expression was performed by qPCR with SYBR Green chemistry on a LightCycler® 480 (Roche, Basel, Switzerland) (Campos et al. 2010). Specificity of the qPCR reaction and the presence of primer dimers were checked by examining the melting generated curve with a dissociation protocol from 65 to 97 °C. Five-point standard curves of a 5-fold dilution series (1:1–1:625) of pooled RNA were used for PCR efficiency calculation. All samples were run in duplicate and minus reverse transcriptase and no template controls were included in all plates. A positive plate control was also used. CT values were determined using the fit-point method using the LightCycler® 480 software with a fluorescence threshold arbitrarily set to 0.3. Target gene data was normalised with exogenous luciferase (Campos et al. 2012), and the expression of target genes in each temperature and along development was evaluated as previously reported (Fernandes et al. 2006).

2.2.6. Morphometry

Two fish per tank were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) in phosphate buffered saline (PBS tablets, Sigma-Aldrich, St. Louis, Missouri, USA) for 6 h (0–8 days) or 12 h (14–30 days), and washed in PBS. Samples were then dehydrated in a graded ethanol (AGA, Prior Velho, Portugal) series, cleared in xylol (Prolabo, VWR International LLC, Radnor, PA, USA) and finally included in paraffin Histosec® (Merck, Whitehouse Station, NJ, USA). Larvae were sectioned (10 µm) transversely to the body axis at a post-anal level, mounted on slides coated with aminopropyltriethoxysilane (APES) (Sigma-Aldrich, St. Louis, Missouri, USA), to improve section adhesion and then stained with haematoxylin–eosin (Merck, Whitehouse Station, NJ, USA) before placing a cover slip. For succinic dehydrogenase (SDH) activity analysis, whole fish (3 per tank) were snap-frozen in isopentane (Sigma-Aldrich, St. Louis, Missouri, USA) at –80 °C. Before freezing, small individuals were combined in composite blocks and sandwiched with two slices of pig liver as previously described ((Silva et al. 2008). Sections were cut at 7 µm in a cryostat CM 1950 (Leica Microsystems GmbH, Wetzlar, Germany), mounted on slides treated with APES and histochemically stained for SDH activity, which is a marker for mitochondrial content and oxidative metabolism, and distinguishes between slow and fast fibres.

Morphometric variables were measured in transversal body sections of individual fish, at a post-anal location. Total cross-sectional muscle area [A (mm²) (muscle)], the total number of fibre [N (fibres)] and the fibre cross-sectional area [\bar{a} (µm²) (muscle fibre)] were measured. The total cross-sectional muscle area [A (fast muscle)] was computed

after tracing the physical limits of interest of the section on the monitor, at a 200 x magnification. Muscle fibre outlines were traced using a 400 x magnification. In smaller larvae the maximum possible number of fibres was actually measured in total cross-sectional area. The fibre diameter (μm) was estimated indirectly, as the diameter of a circle having the same area of a fibre in a perfect cross-section. The sampling location selected was shown to be bilaterally symmetrical regarding fibre number and mean cross-sectional area so at 30 dph only one side of the myotome was measured concerning fibre area, but in both sides fibre number and muscle total area were measured. This study was performed using an Olympus BX51 microscope (Olympus Europa GmbH, Hamburg, Germany) with the Cell[^]B Basic imaging software.

The relative contribution of hypertrophy and hyperplasia towards the increase in the cross-sectional area was estimated as reported elsewhere (Valente et al. 1999), as follows: $\Delta A (\mu\text{m}^2) = Nm\Delta\bar{a} (\mu\text{m}^2) + \bar{a}m (\mu\text{m}^2) \Delta N$, where Δ is calculated between two consecutive sampling times (t and $t+1$) and Nm and $\bar{a}m$ refer to the mean total number of fast fibres and fibre area at t , respectively. Probability density functions were determined at metamorphosis and 30 dph using the statistical program for the analysis of muscle fibre populations *FibreA* (Johnston et al. 1999).

2.2.7. Statistical analysis

Statistical analyses followed previously reported methods (Zar 1996). All data were tested for normality and homogeneity of variance and log transformed when required. Overall influence of temperature and developmental stage in muscle cellularity was modelled in a two-way ANOVA using temperature and developmental stage as independent factors using the software STATISTICA (version 8.0; StatSoft, Tulsa, OK, USA). When these tests showed significance, individual means were compared using the Tukey HSD *post-hoc* test. When data did not meet the normality or homogeneity of variance requirements, a Kruskal–Wallis ANOVA followed by a Median test was applied instead. At each particular developmental stage, the effects of the temperature were analysed for covariance (ANCOVA), in which temperature was set as the independent variable while the total length was set as a covariate. The effects of temperature in the percentage of small fibres was analysed by a one-way ANOVA. Significance levels were set at $P < 0.05$. Gene expression data were further subjected to unsupervised hierarchical cluster analysis with Babelomics 4 Suite software (<http://www.gepas.org/>) using Pearson's correlation coefficient as a similarity measurement.

Table 1.

List of specific primers used for real-time qPCR.

Gene	Fwd sequence (5'→3')	Rev sequence (5'→3')	Accession no (GenBank)	Size (bp)	E(%)
<i>myf5</i>	GAGCAGGTGGAGAACTACTACG	CCAACCATGCCGTCAGAG	FJ515910	89	96
<i>mrf4</i>	GAGAGGAGGAGGCTCAAGAAG	CAGGTCCTGTAATCTCTCAATG	EU934042	137	100
<i>myog</i>	GTCACAGGAACAGAGGACAAAG	TGGTCACTGTCTTCCTTTTGC	EU934044	118	99
<i>myod1</i>	CTCCTCCTCCCCGTCATC	TTGGTGGTCTTCCGCTTG	FJ009109	144	96
<i>myod2</i>	ACAGCCACCAGCCCAAAC	GTGAAATCCATCATGCCATC	FJ009108	194	92
<i>pax7</i>	CTTCTTATCACAGTCATCCTCGTC	CAGTTCCTTCAGGTGAGGCTTC	JQ001819	98	100
<i>mstn1</i>	GGGAGATGACAACAGGGATG	TGGATCCGGTTCAGTGGC	EU934043	91	96
<i>fst</i>	CATCAAAGCTAAGTCGTGTGAG	CACCGCCTCCTCTGTCTTG	EU934045	133	93
<i>myHC</i>	GAAAAATCTGACAGAGGAAATGG	CCTTGGTGAGAGTGTTGACTTTG	FJ515911	143	87
<i>mylc2</i>	GTACAAGGAGGCGTTCACAATC	CCAGCACGTCCCTAAGGTC	FJ515912	77	90
<i>igf-I</i>	CAGGCTATGGCTACAACACAC	CACAGTACATCTCCAGGCG	AB248825	93	95
<i>igf-II</i>	GCAGAATGAAGGTCAAGAAGATG	CGAGACCACTTCCACAGC	AB248826	89	94
<i>igf1r</i>	GCTGTAAAAATAGGAGATTTTCGG	GGAGCAAACCCTTACCACC	FJ515914	82	100
<i>hsp90AA</i>	AGCAGTACATTTGGGAGTCTG	GGTGAAGGATGACTTTTGTGC	AB367526	96	91
<i>hsp90AB</i>	GAAATAACAAGATGCCTGAAG	TGTTGGAATAGAAAGTGTGATG	AB367527	125	87
<i>hsp70</i>	CTGTATTTGATGCTAAGCGTTTG	GACCTCTACTTTGGGTTTGGA	AB513855	119	91
<i>luciferase</i>	TCATTCTTCGCCAAAAGCACTCTG	AGCCCATATCCTTGTCGTATCCC	EU684088	198	93

For each gene, its GenBank accession number, amplicon size (bp) and amplification efficiency (*E*). The annealing temperature of all primer pairs is 60 °C.

2.3. Results

2.3.1. Zootechnical performance

Senegalese sole embryos developed faster at higher temperatures. Embryonic life lasted 34 h at 21 °C, 42 h at 18 °C and 62 h at 15 °C. Mouth-opening occurred at 2 dph in larvae from 18 and 21 °C and at almost 3 dph in larvae from 15 °C. Hatching rate and survival of newly settled larvae were not significantly affected by temperature (Table 2). Embryonic temperature influenced weight at 30 dph, since larvae from 18 and 21 °C were 25 and 27.2% significantly heavier than larvae from 15 °C, respectively ($P < 0.05$) (Table 2). Significant differences were seen in L_T at hatching, MO, Post-met and 30 dph, with larvae from the 15 °C group being significantly smaller ($P < 0.05$) than the 21 °C counterparts. At 30 dph larvae from 21 °C and 18 °C were 6.7% and 6% longer than larvae from 15 °C, respectively ($P < 0.05$) (Figure 1).

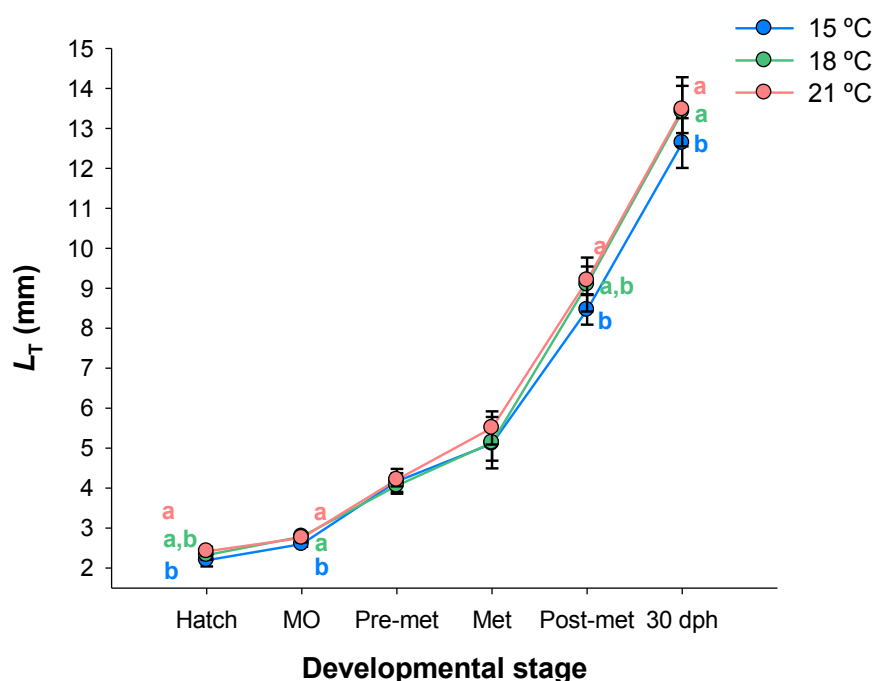


Figure 1. Total length L_T (mm) in Senegalese sole larvae incubated at 15, 18 and 21 °C and reared at 21 °C until 30 dph. Larvae were measured at Hatch (Hatching), MO (mouth-opening), Pre-met (pre-metamorphosis), Met (metamorphosis), Post-met (post-metamorphosis) and 30 dph (30 days post-hatching). (^{a,b,c}) Different letters indicate significant differences between 15 and 21 °C ($P < 0.05$) at the same developmental stage. Hatch (Hatching), MO (mouth-opening), PreM (pre-metamorphosis), Met (metamorphosis), PostM (post-metamorphosis), 30 dph (30 days post-hatching).

Table 2.Growth and survival for *S. senegalensis* incubated at different temperatures

	15 °C	18 °C	21 °C
Hatching (%)	52.9 ± 23.3	46.8 ± 6.8	44.2 ± 15.8
Survival (%)	23.4 ± 3.4	20.3 ± 4.7	20.2 ± 0.7
DW 30 dph (mg)	2.94 ± 0.35 ^a	3.32 ± 0.52 ^b	3.42 ± 0.31 ^b
L_T 30 dph (mm)	12.6 ± 0.6 ^a	13.4 ± 0.9 ^b	13.5 ± 0.6 ^b

 L_T - total length (mm)^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)**2.3.2. Embryonic temperature effect on larvae muscle growth**

The SDH activity used in this study to trace red and white fibre cross-sectional contours in developing larvae allowed discrimination between these fibre types since hatching. A superficial monolayer (SM) was identifiable in all temperatures (data not shown) allowing the clear discrimination between red and white fibre types. The general trend was that 15 °C during incubation produced larvae with less fibres (N), as well as smaller diameter and total muscle cross-sectional area (A) than the 18 or 21 °C groups (Figure 2). However, by the end of the experiment the number of fibres was not significantly different between the 15 and 21 °C treatments (Figure 2). The relative contribution of hypertrophy/hyperplasia to muscle growth changed as larvae grew (Figure 3). Between hatching and mouth opening, the increase of fibre size, reflecting hypertrophic growth, was the dominant process in increasing fast muscle area, and no significant differences were seen between temperatures ($31.6 \pm 11.5\%$, $39.6 \pm 13.7\%$ and $44.3 \pm 5.1\%$, for 15, 18 and 21 °C, respectively) (Figure 3). From mouth opening until metamorphosis, hyperplasia became more predominant. At pre-metamorphosis, stratified hyperplasia began to be clearly observed, as a slight gradient in fibre size started to be visible in the extreme apical regions until deep in the myotome. At this point, the contribution of hyperplasia to increasing muscle area was of $88.3 \pm 5.8\%$ in fish from 18 °C, which was significantly higher than hyperplasia in fish from 15 ($67.1 \pm 8.3\%$) ($P < 0.001$) and 21 °C ($76.6 \pm 5.1\%$) ($P < 0.05$). Mosaic hyperplasia was scarcely observed in post-metamorphic larvae (around 22 dph), but evident at 30 dph, with scattered small new fibres spread throughout the myotome. Again, 18 °C was the temperature that favoured hyperplastic growth, though without statistical importance.

Both temperature and developmental stage significantly affected ($P < 0.0001$) fast muscle cellularity but the interaction between these two factors was only significant for

muscle fibre diameter. At hatching, significant differences were seen in fibre diameter, as larvae from 15 °C produced smaller fibres than those from 18 or 21 °C ($P < 0.001$), but this difference was not significant regarding N or A despite the trend for higher values for 18 and 21 °C (Figure 2). By pre-metamorphic and following stages there was a great increase in fast muscle growth in mostly measured parameters (Figure 2), and differences between incubation temperatures became more apparent. The fast muscle cross sectional area (A) at 21 and 18 °C was larger than at 15 °C ($P < 0.001$ and $P < 0.05$, respectively). The N in fish from 21 °C was significantly higher than in the 18 and 15 °C counterparts ($P < 0.05$). The fish from 18 °C had significantly larger fibre diameter than 21 °C ($P < 0.05$) and 15 °C ($P < 0.001$), and 21 °C was also significantly higher than 15 °C ($P < 0.05$) (Figure 2).

At metamorphosis, A was larger at 18 and 21 °C than at 15 °C ($P < 0.05$), and N was significantly higher in the 18 and 21 °C treatments than at 15 °C ($P < 0.05$), with the highest value for 18 °C. The fibre diameter was larger at 18 and 21 °C than at 15 °C ($P < 0.001$) (Figure 2). Probability density function distribution (PDFs) of fast muscle fibre diameters in fish from the 15 °C treatment had a left-skewed distribution, reflecting the higher percentage of smaller fibres observed at this temperature ($P < 0.05$) (Figure 4a). In particular, the percentage of fibres smaller than 10 µm in diameter was $83.6 \pm 11.3\%$, $70.9 \pm 7.1\%$ and $67.6 \pm 7.1\%$ in fish from 15, 18 and 21 °C, respectively.

In the post-metamorphic stage A was significantly higher in 18 and 21 °C treatments than at 15 °C ($P < 0.05$). Significant differences were found in N between 15 and 21 °C ($P < 0.05$), with higher values for the later (Figure 2). There were also significant differences between fibre diameters ($P < 0.001$). By 30 dph A did not differ significantly between treatments, but the largest areas were found in the 18 °C and 21 °C larvae. N was significantly higher in fish from 18 °C than in the ones from 15 °C ($P < 0.05$) (Figure 2). A similar pattern but with significant differences was found for diameter ($P < 0.001$). PDFs of fibres showed that the 15 °C treatment had more fibres in the left-hand tail of the distribution, albeit to a lesser extent than during metamorphosis (Figure 4b).

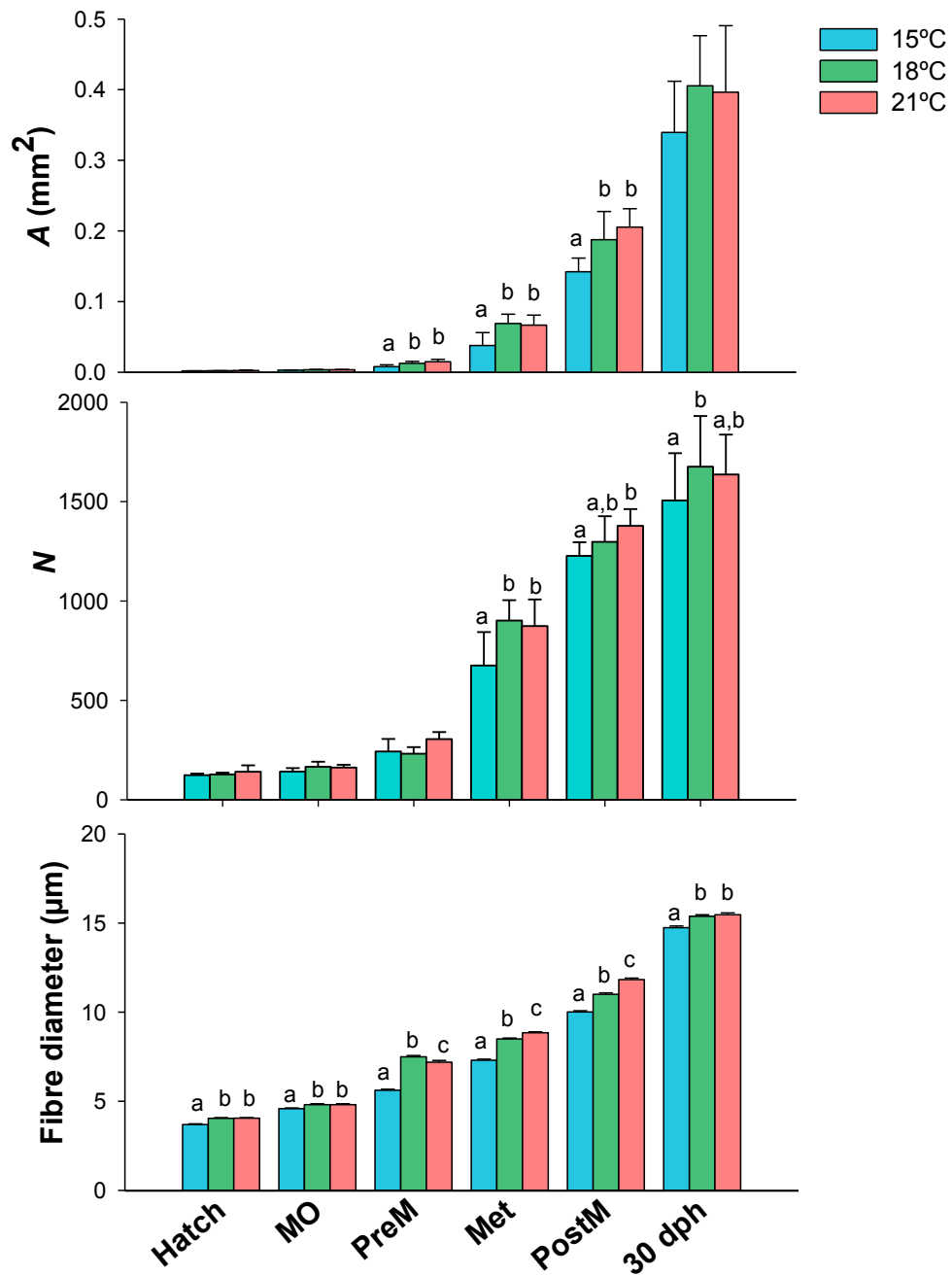


Figure 2. Total cross-sectional muscle area A (mm^2), total number of fibres N and fibre diameter (μm), in fast muscle during development in Senegalese sole larvae incubated at different temperatures (15 °C, 18 °C and 21 °C). (^{a,b,c}) Different letters indicate significant differences between temperatures ($P < 0.05$) at the same developmental stage. Hatch (Hatching), MO (mouth-opening), PreM (pre-metamorphosis), Met (metamorphosis), PostM (post-metamorphosis), 30 dph (30 days post-hatching).

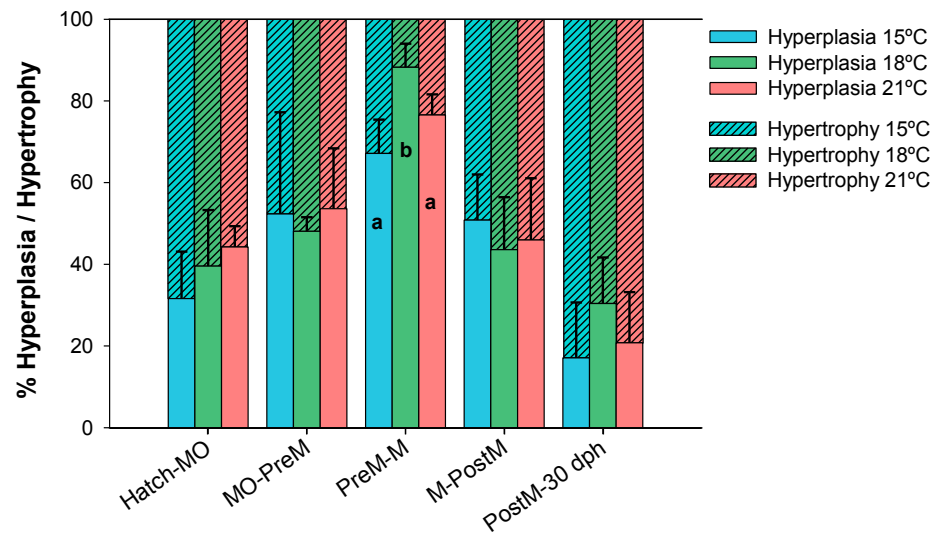


Figure 3. Contribution of hyperplasia to white muscle growth in Senegalese sole larvae incubated at different temperatures (15, 18 and 21 °C). (^{a,b,c}) Different letters indicate significant differences between temperatures ($P < 0.05$) at the same developmental stage. Hatch (Hatching), MO (mouth-opening), PreM (pre-metamorphosis), M (metamorphosis), PostM (post-metamorphosis), 30 dph (30 days post-hatching).

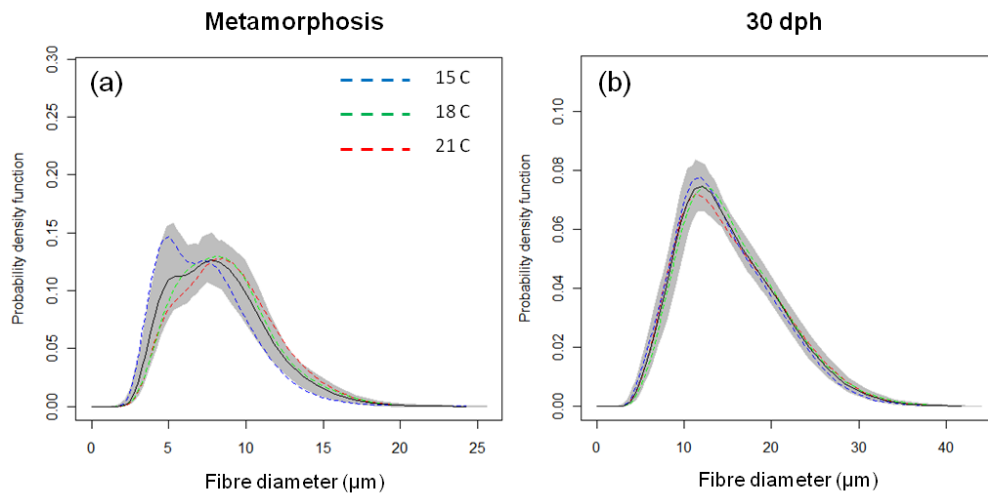


Figure 4. Embryonic temperature affected Senegalese sole larvae muscle fibre distribution. Probability density function (PDFs) of muscle fibre diameter and percentage of fibres smaller than 10 µm in diameter in larvae during (a) metamorphosis climax, and (b) 30 dph ($n=6$ per temperature group). The dashed lines show the average PDFs (a,b), and the solid line central to the shaded area is the average PDF for combined groups (blue — 15 °C, green — 18 °C and red — 21 °C). The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

2.3.3. Developmental expression and thermal plasticity of growth-related genes

Hierarchical clustering of gene expression was performed to identify common expression patterns amongst genes. Genes were clustered vertically according to similarity in expression during development. Two major groups of genes: one cluster of genes that were up-regulated during embryonic and hatching stages (*pax7*, *hsp90AB*, *igf1r*, *myog*, *myod1*, *fst*, *myf5*, *hsp90AA* and *myod2*), and a set of genes that were up-regulated in later larval stages (myosin light chain 2 (*mylc2*), *igf-I*, myosin heavy chain (*myHC*), *hsp70*, *igf-II*, *mstn1* and *mrf4*) (Figure 5, Supplementary Figures 1, 2).

All genes examined displayed a dynamic expression pattern throughout development and several differences in gene expression between temperatures were observed both in embryonic and larval phases (Figure 5, Supplementary Figures 1, 2). The effect of temperature on *MRF* transcript levels during the embryonic phase depended on the developmental stage.

The *MRFs* involved in muscle lineage specification (*myod1*, *myod2* and *myf5*) were expressed early in development, during gastrula phase, and high levels of *myod2* transcripts were even found during blastulation (Figure 5). During epiboly, *myf5* showed a higher expression at 21 °C than at 15 °C ($P < 0.05$). *Myod2* expression was higher at 21 °C during 20 somites stage ($P < 0.05$). Their expression peaked during somitogenesis until hatching. *Mrf4* and *myog* transcripts were firstly found during somitogenesis. *Myog* had a similar expression pattern to the other *MRFs*, whereas *mrf4* transcript levels increased during development. *Pax7* mRNA was detectable in very low amounts at the blastula stage, had an expression peak during hatching, and then decreased until 30 dph (Figure 5, Supplementary Figure 1).

After hatching, *MRF* transcript levels between temperatures were quite similar, but at pre-metamorphosis and metamorphosis, larvae incubated at 15 °C showed higher amounts of *myod1*, *myod2*, *myf5*, *mrf4* and *myog* mRNA (Figure 5, Supplementary Figures 1, 2). At the end of the experiment, the fish from 21 °C had significantly higher *mrf4* expression than fish from 15 ($P < 0.05$) or 18 °C ($P < 0.05$). *Pax7* expression with temperature followed a similar trend to that of *MRFs* during somitogenesis (Figure 5, Supplementary Figure 1), but decreased with embryonic temperature in larvae at mouth-opening, pre-metamorphosis and metamorphosis stages, although without statistical significances.

The earliest detectable expression of *myosins* (*myHC* and *mylc2*) was observed at 20 somites, and these genes showed a marked increase during development and particularly after hatching (Figure 5, Supplementary Figures 1, 2). Both genes were relatively stable across temperatures, nevertheless significant differences between

treatments were found at the end of somitogenesis and at hatching for *myHC* (higher at 21 and 18 °C, respectively) ($P < 0.05$), and at mouth opening for *mylc2* (higher at 18 °C) ($P < 0.05$). During metamorphosis, *myHC* and *mylc2* expression was 2.8- and 2.2-fold higher in the 15 °C group relatively to 21 °C, respectively, albeit not significant.

Fst was first detected at the blastula stage and its expression increased until the end of segmentation. Its transcripts levels decreased from the hatching stage until pre-metamorphosis, and then remained relatively stable until 30 dph (Figure 5, Supplementary Figure 1). *Fst* was significantly more expressed at 21 °C than at 15 °C during epiboly and 20 somite stages ($P < 0.05$) (Figure 5, Supplementary Figure 1). *Msnt1* expression increased from the mouth opening stage until 30 dph (Figure 5, Supplementary Figure 1) and it did not differ between temperatures during sole embryonic phase. By 30 dph the values were quite similar across temperatures in both genes.

Igf-I, which was found in very low amounts until hatching, unlike *igf-II* and *igf1r*, had a relatively high expression throughout Senegalese sole development (Figure 5, Supplementary Figures 1, 2). *Igf-I*, *igf-II* and *igf1r* expression with temperature was highly dependent of the developmental stage. Again, during metamorphosis, expression of these genes was higher in larvae from the 15 °C treatment (for example, 4.2-fold relatively to 21 °C in *igf-II*) but not significant due to large standard deviations.

Hsps (70, 90AA and 90AB) were differentially expressed during sole development (Figure 5, Supplementary Figure 1). *Hsp70* expression increased until hatching, greatly decreased during mouth opening, and again increased until 30 dph. *Hsp90AA* had the highest expression during segmentation, decreased at hatching and mouth opening, and remained relatively stable until 30 dph. *Hsp90AB* was highly expressed throughout ontogeny, with maximum levels during somitogenesis, hatching and at 30 dph. *Hsp70* was often found to be more expressed in the 15 °C group than in the 18 or 21 °C counterparts, before hatching ($P < 0.05$), while at post-hatching no significant differences were observed. *Hsp90AA* was fairly stable across temperatures. *Hsp90AB* expression with temperature depended on the developmental stage, and the only significant difference was observed in the blastula stage, with a 1.9-fold higher expression in the 15 °C treatment relatively to the 21 °C one ($P < 0.05$).

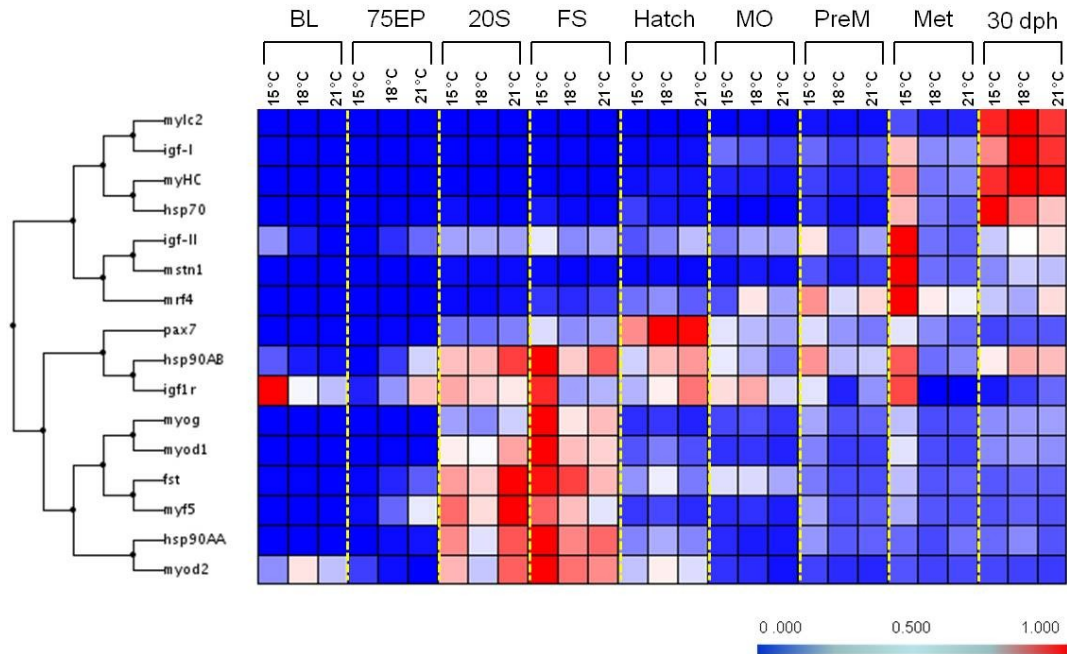


Figure 5. Heat map representing the unsupervised hierarchical clustering analysis of the genes according to similarity in expression trend during Senegalese sole development ($n=3$ per treatment, mean values are presented). Expression data were obtained by real-time qPCR and normalised with exogenous *luciferase* RNA. Each row represents the expression pattern of a single gene across development and each column corresponds to a treatment. Expression levels are represented by a colour tag, with red representing the highest levels (1) and blue the lowest levels of expression (0). BL (blastula), 75EP (75% epiboly), 20S (20 somites), FS (final somitogenesis), Hatch (Hatching), MO (mouth-opening), PreM (pre-metamorphosis), Met (metamorphosis), 30 dph (30 days post-hatching).

2.4. Discussion

In the present work we demonstrate that differences in embryonic temperature were sufficient to produce differences in growth, muscle phenotype and gene expression in Senegalese sole larvae up to 30 dph, even if the larvae were reared at the same temperature after hatching. Generally, incubation of embryos at 15 °C produced smaller larvae with less and smaller fibres throughout growth compared to those incubated at 18 and 21 °C. During hatching and at first feeding no evident gradient in deep fibre size across the muscle quadrant in any direction was observed, suggesting that stratified hyperplasia of fast fibres was still incipient in all temperature groups. A great increase in fast fibre number was seen particularly after mouth opening, and the highest percentage of hypertrophic growth was found by 30 dph. The high recruitment of new fibres during

pre-metamorphic and metamorphic stages corresponded to stratified hyperplasia, since scattered small fibres throughout the myotome (mosaic hyperplasia) were seen only in a post-metamorphic phase. In a related species, the common sole (*Solea solea*, Linnaeus), stratified hyperplasia was firstly detected by 12 dph, and mosaic hyperplasia was seen at 2.5 months old (Veggetti et al. 1999). In our study it is noteworthy that fast fibre hyperplastic growth between pre-metamorphosis and metamorphosis was significantly higher in larvae from 18 °C. Changes in growth and muscle cellularity with temperature in teleosts have shown to be highly dependent on the species. In the European sea bass juveniles that originated from lower embryonic temperatures showed a higher capacity to recruit new fibres than juveniles from higher temperatures (Alami-Durante et al. 2007), whereas in Atlantic salmon an incubation temperature of 5 °C significantly increased the fibre final number relatively to a lower or higher temperature (Macqueen et al. 2008), and individuals with a sustained hyperplastic growth attained larger sizes (Albokhadaim et al. 2007). Moreover, incubation temperature was also suggested to influence later juvenile fish appetite and feeding behavior, with direct effects on growth (Albokhadaim et al. 2007). In the Clyde herring (*Clupea harengus*, Linnaeus) embryonic temperature affected the number of undifferentiated myoblasts and also had persistent effects on juvenile adult hyperplastic growth (Johnston and Cole 1998). Interestingly, in blackspot seabream (*Pagellus bogaraveo*, Brünlich) larvae, the axial musculature showed a variable response to incubation temperature that depended on spatial localisation (Silva et al. 2011), stressing the complexity of muscle growth.

The ability of a species to recruit muscle fibres may compromise its ultimate size, and the extent of hyperplastic growth seems to rely on the capacity of the stem cell self-renewal system to form new fibres (Perrot et al. 1999; Devoto et al. 2006). Recent studies proposed that the precursor cells in the posterior lateral fast muscle derived from the dermomyotome form a mitotically active population and are responsible for the intense stratified hyperplasia during teleost larval life (Marschallinger et al. 2009). In the present work we found that *pax7* had a peak in expression during hatching, probably associated with the mitotic division of active *pax7*-expressing myoblasts cells. On the other hand, *MRFs* such as *myod1*, *myod2* and *myf5* were expressed at higher levels during embryo somitogenesis. At hatching their expression was considerably down-regulated, and then slightly increased throughout ontogeny. In vitro studies showed that *MRFs* and *pax7* are reciprocally inhibited (Olguin et al. 2007). *Pax7* can act through protein–protein interactions with *Myod* transcriptional complexes resulting in inhibition of myogenesis, and *Myog* was found to directly affect *pax7* expression in differentiating cells (Olguin et al. 2007). Moreover, in brown trout (*Salmo trutta*, Linnaeus), *myog* and *myod* expression was found to decrease in pre-hatched embryos that have acquired their final number of

somites but persisted in zones at the dorsal and ventral extremes and next to the horizontal septum, which seemed related with the second-phase myogenesis (Steinbacher et al. 2007). In our study *myod1*, *myod2* and *myf5* were expressed in a presomitic stage, probably related with the early commitment of the myoblasts to a myogenic progenitor cell population also found in other fish species (Cole et al. 2004; Fernandes et al. 2007a). The higher expression of *myod2* in blastula than in late epiboly indicates that this is a maternal transcript and it is gradually degraded until zygotic expression starts. This expression pattern was markedly different to that of *myod1*, suggesting that these two *myod* paralogues are undergoing subfunctionalisation. We also found that *mrf4* had a different expression when compared with the other *MRFs*. In zebrafish, *mrf4* is dynamically expressed in terminally differentiated muscle fibres but not in early somitic cells lacking *myosin*, and reduced *myosin* expression coincide with a decrease in *mrf4* transcript levels (Hinits et al. 2007). Similarly, we also did not see *mrf4* mRNA before the appearance of myosin transcripts (20 somites stage) and *mrf4* up-regulation in later stages of development supports its involvement in fibre differentiation as well as specification. Moreover, the marked increase in *myHC* and *mylc2* transcript levels during development is likely related with the increase in fibre cross-sectional area.

Within some developmental stages, differences in sole *MRF* expression were observed between incubation temperatures, and until hatching some genes had similar expression patterns, reflecting their concerted involvement in myogenesis. Thermal-induced changes in expression have been found in other species, as for example *myog* expression in embryos of several pufferfish species showed a consistently higher expression at higher temperatures (Fernandes et al. 2006). In our work there were no differences in the timing of expression of *MRFs* and *myHC* transcripts between temperature groups, which corroborates a previous report in another pleuronectiforme (Galloway et al. 2006). In the Atlantic salmon, *myod1a* and *myog* showed similar expression patterns with respect to somite stage at incubation temperatures of 2 °C, 5 °C and 8 °C until the “eyed stage”, in spite of different rates of somite formation, but a delay in the expression of *myf5*, *mrf4* and *mhc1* with respect to somite stage at 2 °C compared to 8 °C (Macqueen et al. 2007). Nevertheless, an incubation temperature of 5 °C until the “eyed stage” significantly increased the muscle fibre number relatively to a lower or higher temperature in three years adults (Macqueen et al. 2008).

In Senegalese sole juveniles, *mstn1* is known to be nutritionally regulated in muscle and liver, with higher transcript levels in the liver of fish that showed reduced growth due to a diet with higher lipid content (Campos et al. 2010). The present study indicates that *mstn1* is involved in embryonic development, since its transcripts were found from the 20 somite-stage until 30 dph. It may be involved in thermal phenotypic plasticity, as during

metamorphosis it was up-regulated *circa* 5-fold at 15 °C compared to the other temperature groups. *Fst* is known to inhibit the activity of the Mstn C-terminal dimer (Lee and McPherron 2001), and in Senegalese sole it was found to be highly expressed during development, particularly during somitogenesis, and in some cases its expression was higher in embryos from the 21 °C group. In Atlantic salmon embryos, *fst1* plays a role in regulating anterior somite muscle progenitors (Macqueen and Johnston 2008) and it may have a similar function during somitogenesis in Senegalese sole.

Igf-I is known to be mainly synthesised in the liver, but a high expression in Senegalese sole skeletal muscle has been reported (Campos et al. 2010). In the Chilean flounder (*Paralichthys adspersus*, Steindachner), expression of *igf-I* and *igf1r* was associated with development of the nervous system, as well as muscle and bone structures (Escobar et al. 2011). In the present study, *igf-I*, *igf-II* and *igf1r* transcripts were observed since blastulation, and *igf-II* and *igf1r* showed a high expression throughout development. In zebrafish, *igf-1b* and *igf-2a* were detected since the 1-cell stage, and Igf signalling has been proven to be involved in midline formation and expansion of the notochord (Zou et al. 2009). Our results support the involvement of Igf signaling in controlling embryo and larvae development; however *igfs* and *igf1r* did not show a coordinated expression pattern with temperature. This is in line with a previous report in rainbow trout, which suggest that temperature does not control muscular growth through regulation of *igf* receptor (Gabillard et al. 2003).

The cytosolic proteins Hsp90AA (inducible form) and the Hsp90AB (constitutive form) comprise 1–2% of cellular proteins under non-stress conditions (Sreedhar et al. 2004). Hsp90AA has been associated with cell cycle regulation and response against thermal-induced stress and Hsp90AB is more involved in with early embryonic development, microtubule formation and long-term cell adaptation (Sreedhar et al. 2004). In Senegalese sole these two genes were found in all developmental stages examined and *hsp90AB* was generally highly expressed than *hsp90AA*, highlighting the importance of constitutive expression. In zebrafish embryos, *hsp90α* was strongly expressed in developing somites and it was found to be required for myofibril assembly in skeletal muscle (Sass et al. 1999; Du et al. 2008), which suggests a similar role in Senegalese sole, since *hsp90AA* was predominantly expressed during somitogenesis. Nevertheless, no perceivable induction of this gene with temperature was observed, perhaps because the temperature range used in this study is well within the physiological limits for Senegalese sole (Dinis et al. 1999). Expression of *hsp70* increased during Senegalese sole ontogeny and was generally higher in embryos incubated at 15 °C than in other temperature groups. In two intertidal sculpin species (*Oligocottus maculosus* and *O. snyderi*) it was found that the levels of *hsp70* were higher in the sculpin that was more

exposed to natural fluctuations in water temperature, and those levels were only slightly influenced by induced changes in water temperature (Nakano and Iwama 2002). It was suggested that the less thermally sensitive sculpin enhanced its tolerance by having a large constitutive pool of Hsp70.

The present results show that incubation of *S. senegalensis* eggs at 15 °C produced a delay in embryonic development and smaller larvae with less and smaller fibres throughout on-growth compared to those incubated at 18 and 21 °C. Several key genes involved in growth and myogenesis were found to be differentially expressed with temperature, in a stage-dependent manner. Higher expression of *myf5*, *myod2*, *myHC*, *mylc2* or *fst* at 21 °C and 18 °C during early stages might be related with the higher muscle growth observed in these groups and to a higher recruitment of new fast fibres during stages like metamorphosis. Our study provides a clear example of thermally induced phenotypic plasticity of myogenesis in a flatfish and shows that temperature changes during a critical window of development can have a prolonged effect on gene expression and muscle growth.

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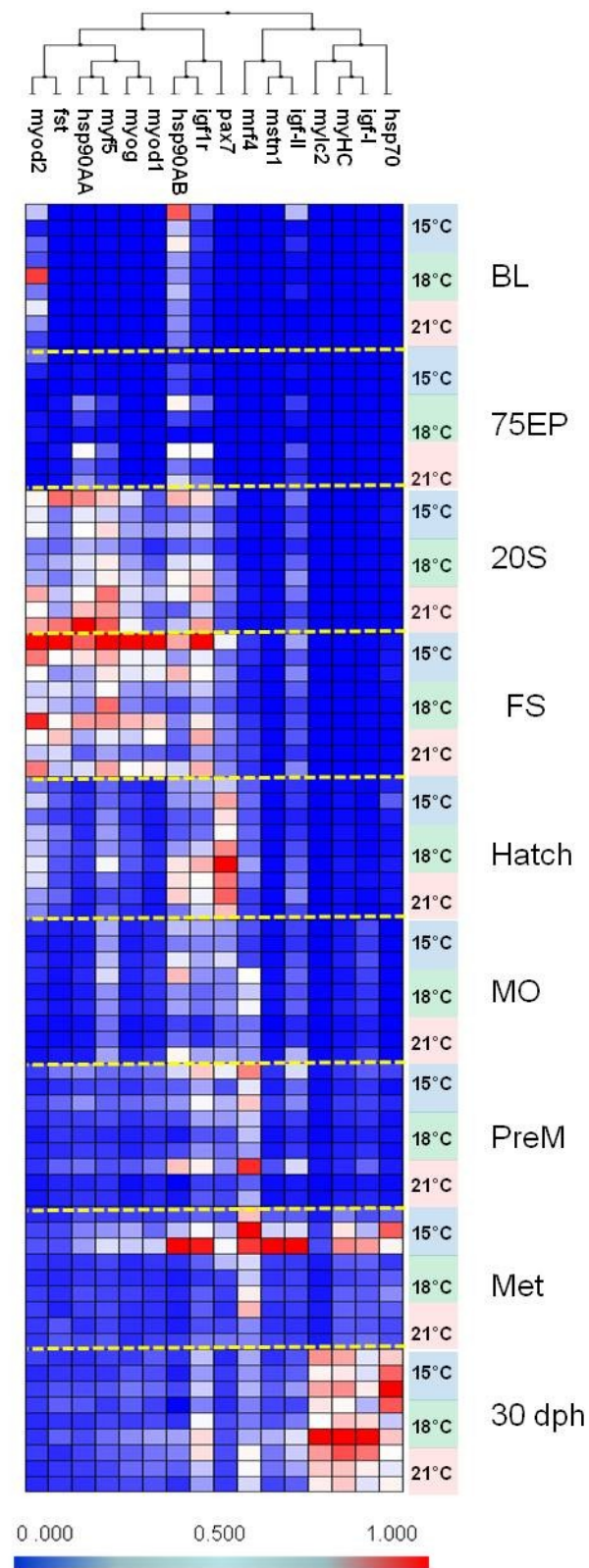
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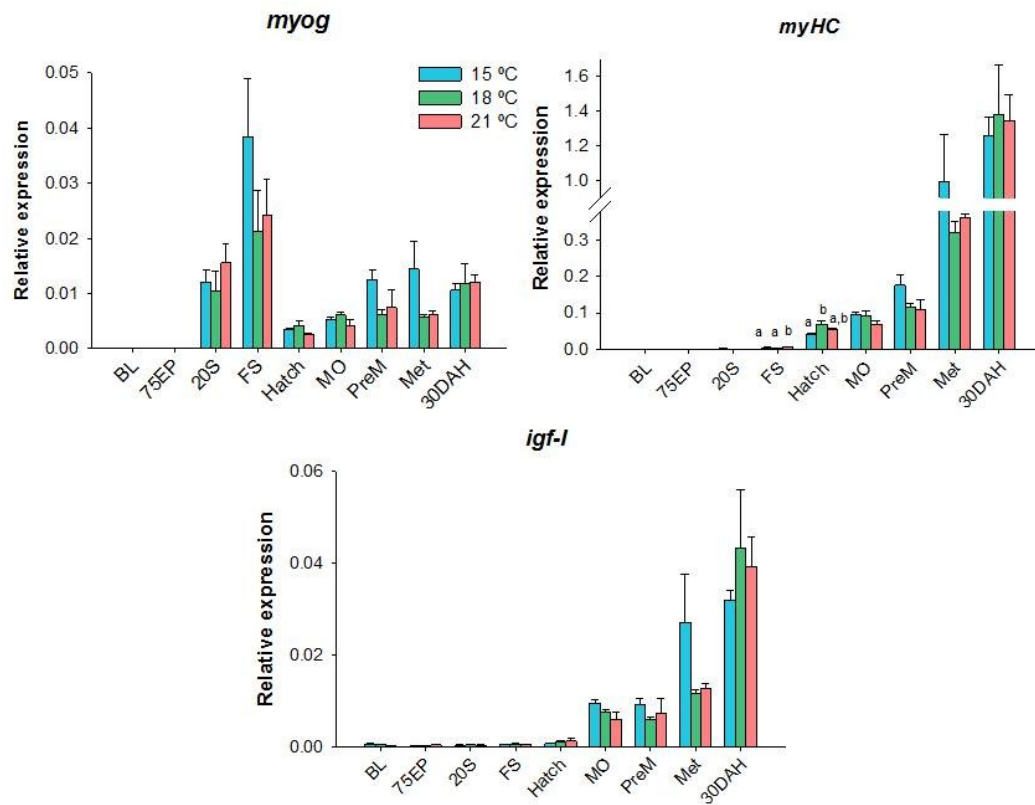
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Supplementary Figure 1.



Supplementary Figure 2.

Chapter 3

Thermal plasticity of the miRNA transcriptome during Senegalese sole development

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Thermal plasticity of the miRNA transcriptome during Senegalese sole development

Abstract

Fertilised eggs of Senegalese sole (*Solea senegalensis*) were incubated at 15 or 21 °C and after hatching all larvae were reared at 21 °C until 30 days post-hatch. SOLiD high-throughput sequencing was used to compare miRNA transcriptomes in Senegalese sole embryos and larvae. We have identified 320 conserved miRNAs in Senegalese sole, of which 47 have not been previously described in teleost species. mir-17a-5p, mir-26a, mir-130c, mir-206-3p, mir-181a-5p, mir-181a-3p and mir-199a-5p expression levels were further validated by RT- qPCR. The majority of miRNAs were dynamically expressed during early development, and a peak of expression was observed at pre-metamorphosis and/or metamorphosis stages for many miRNAs. Embryonic temperature affected the expression of several miRNAs. In particular, a higher incubation temperature (21 °C) promoted expression of some miRNAs positively related with growth, at least in some developmental stages. This is in agreement with previous studies reporting enhanced growth with higher embryonic temperatures in Senegalese sole. miRNA target prediction revealed possible target mRNAs related with myogenesis, MAPK and mTOR pathways, which are also consistent with the observed mRNA expression patterns at different embryonic temperatures.

Keywords: *Solea senegalensis*, miRNA, thermal plasticity, myogenesis, growth, epigenetics.

3.1. Introduction

MicroRNAs (miRNAs) are endogenous short non-coding regulatory RNAs that post-transcriptionally regulate gene expression by forming miRNA-mRNA pairs to their target genes, thus repressing and reducing mRNA stability (Berezikov et al. 2005). Mature miRNAs are often conserved across a wide range of species, and mutations in proteins required for miRNA function or biogenesis have shown to impair animal development (Chang and Mendell 2007). They are involved in the majority of physiological processes, including stem cell differentiation, cell lineage specification, haematopoiesis,

neurogenesis, myogenesis, and immune responses (Chang and Mendell 2007). miRNA-mediated gene regulation involves repression and blocking of translation initiation, mRNA degradation, and sequestration of mRNA by miRNA/RISC complex (RNA-induced silencing complex) in the cytoplasmic processing bodies (reviewed by Bartel 2004).

Several miRNAs are known to control myogenesis in vertebrates. Some of them (e.g., miR-1, miR-133 and miR-206) are specifically expressed in muscle and positively related with growth (Baskerville and Bartel 2005; Chen et al. 2006). Others, such as miR-181, have a broader tissue expression but are still involved in myoblast differentiation and in establishing the muscle phenotype (Naguibneva et al. 2006). In zebrafish, fast muscle miRNAs populations are differentially regulated during the transition from hyperplastic to hypertrophic muscle phenotype in adult fish (Johnston et al. 2009). Moreover, miR-206, miR-26a and miR-214 are differentially expressed in the process of skeletal muscle development in common carp (*Cyprinus carpio*) (Yan et al. 2012), further indicating an important role for miRNAs during fish muscle development.

Skeletal muscle growth is strongly stimulated by Igf-I (Insulin-like growth factor 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. Phosphorylation of mTOR increases levels of protein synthesis by regulating essential proteins controlling mRNA translation. In teleost such as the fine flounder (*Paralichthys adspersus*), the analysis of these signalling pathways in skeletal muscle revealed that they contribute to fish somatic growth (Fuentes et al. 2011).

Water temperature has the ability to greatly affect teleost larval and juvenile growth, and several studies reported temperature effects on muscle cellularity of larvae (Johnston 2006). The flatfish Senegalese sole (*Solea senegalensis*) is a species of commercial interest for marine aquaculture, particularly in Southern Europe which in natural and aquaculture conditions can be exposed to large thermal variations (Imsland et al. 2003). In a previous study it was shown that higher embryonic temperatures (18 and 21 °C) promoted a faster development and increased size of sole larvae by 30 days post hatch (dph) relatively to a lower temperature (15 °C) (Campos et al. 2013b). Furthermore, muscle cellularity at equivalent developmental stages was affected by temperature and a

transient differential gene expression due to incubation temperature was also observed at several stages. For example, *myf5*, *fst*, *myHC* and *mrf4* were highest at 18 and/or 21 °C at specific points (Campos et al. 2013b). However, it is unknown whether miRNAs are involved in the thermal-plasticity of myogenesis observed in fish. Hence, we have used the high-throughput SOLiD sequencing technology to determine potential changes in the miRNA transcriptome in Senegalese sole embryos and larvae that were subjected to two different incubation temperatures (15 °C or 21 °C). Moreover, we have predicted targets of miRNAs involved in myogenesis and muscle growth, where mTOR and MAPK pathways play an essential role.

3.2. Material and methods

3.2.1. Fish husbandry

The Senegalese sole incubation temperature took place at the LEOA facility, University of Algarve, Portugal. Senegalese sole embryos were incubated at two temperatures (15.2 ± 0.3 °C and 20.9 ± 0.3 °C) in triplicate groups. Eggs were randomly distributed amongst 6 fibreglass conical tanks (100 L), at a density of 100·eggs L⁻¹. After hatching all larvae were reared at the same temperature (20.8 ± 0.1 °C) until the metamorphosis climax. Water temperature, O₂, salinity, pH and nitrogenous compounds were monitored regularly during the entire trial, and larvae were exposed to an artificial photoperiod of 12h:12h light:dark. They were reared in 100 L conical tanks at a density of 40 larvae L⁻¹. At mouth opening larvae started to feed on rotifers (*Brachionus* sp.). *Artemia* nauplii were introduced around 5 days post-hatch (dph) and their density was gradually increased from 4 to 8 na·mL⁻¹ until 8 dph. Live *Artemia* meta-nauplii were then introduced at this time point on increasing from 12 to 20 M24·mL⁻¹ until 18 dph. Frozen meta-nauplii were offered when larvae settled at the bottom (around 18 dph) until 30 dph.

3.2.2. Sampling and RNA extraction

All animal handling protocols were conducted according to the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes. Embryos and larvae samples were taken in triplicate according to developmental stages shown by Lagardère (1979) and Fernández-Díaz et al. (2001). The sampling points were: 75% epiboly (75Ep), 20 somites (20S), hatching (0 dph), pre-metamorphosis (Pre-Met, stage 0 according to the eye-

translocation stage, 8 dph for larvae from 21 °C, 9 dph for larvae from 15 °C), metamorphosis climax (Met, stage 3 according to the eye-translocation stage, 14 dph in larvae incubated at 21 °C and 15 dph in larvae incubated at 15 °C) and 30 dph. The embryos were snap-frozen in liquid nitrogen, and larvae were killed by over-anaesthesia with MS-222 (400 mg l⁻¹) and snap-frozen in liquid nitrogen. All samples were stored at -80 °C until further analysis. Total RNA was extracted using the *mirVana* miRNA isolation kit (Ambion) according to the manufacturer's instructions. Assessment of RNA quality was performed by agarose gel electrophoresis on a 1.2 % (w/v) agarose gel containing SYBR safe DNA gel stain. RNA samples were then quantified with a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). Absorbance ratios (260/280 nm) were greater than 1.9, indicating high purity RNA.

3.2.3. SOLiD sequencing of Senegalese sole small RNAs

Small RNA reads were generated from eight SOLiD (Life Technologies, Austin, Texas, USA) libraries prepared from pools of triplicate samples of embryos and larvae from the following stages and at 15 and 21 °C incubation temperatures: 75% epiboly (75Ep), 20 somites (20S), hatching (0 dph) and metamorphosis climax (stage 3, as defined by Fernández-Díaz et al. (2001)). The enriched small RNA fraction (< 40 nucleotides (nt) nucleic acid fraction) was recovered by sodium acetate precipitation, assessed on Agilent 2100 Bioanalyzer with small RNA chip (Agilent Technologies), and used for the amplified small RNA library template preparation using SOLiD™ total RNA-seq kit with the barcoded SOLiD™ 3' primers (Life Sciences). The average length of the amplified cDNA template were assessed in Agilent 2100 Bioanalyzer with high sensitivity DNA chip kit (Agilent Technologies) to calculate the ratio of the desired miRNA ligation products (120 -130 bp). To quantify the precise cDNA yield of miRNA ligation products for SOLiD™ system templated beads preparation, real-time quantitative PCR (qPCR) was carried out using KAPA SYBR FAST Light Cyclers 480 qPCR kit with SOLiD™ primer mix and DNA standers (Kapa Biosystems). Each library template was clonally amplified on SOLiD™ P1 beads by emulsion PCR (ePCR) then sequenced by SOLiD4 system (Life Sciences) on a quadrant of a slide with 50 nt read length.

3.2.4. Sequencing data analysis

The SOLiD raw colour spaced reads were pre-processed using the CLC Genomics Workbench 4.9 (Aarhus, Denmark) to remove low quality reads and clip internal adaptor sequence (CTGCTGTACGGCCAAGGCC), thus leaving reads ranging between 10 and 35

nucleotides (nt) in length. Reads corresponding to small RNAs other than miRNAs (such as tRNAs, rRNA, mtRNA or snRNAs) were discarded by BLAST against ncRNA databases. Unannotated clusters and singletons were mapped against miRNAs sequences deposited in miRBase 18.0 (<http://mirbase.org/>) in order to identify conserved miRNAs. The remaining reads were mapped against *Pleurinectiformes* and *Solea* ESTs databases (NCBI) to identify possible non-conserved miRNAs. For each mappable sequence between 16 and 28 nts and with five or above counts, hairpin folding was evaluated by sequence analysis to identify the presence of a stem loop using the RNA secondary structure prediction tool in the CLC Genomics Workbench. The flanking EST sequence of each analysed read should be at least 60 nt, and the free energy of the fold-back structure (ΔG) should be ≤ -18 kCal mol⁻¹.

The number of reads in each sample was normalised by dividing the number of reads of a given miRNA in a sample by the total number of trimmed miRNA reads in the same sample. To be able to differentiate the expression of each miRNA and categorize them according to their expression pattern, a heatmap chart was drawn and hierarchical clustering with Euclidean distance and complete linkage algorithms was performed using the MeV online software (<http://www.tm4.org/mev/>).

3.2.5. Quantitative real-time PCR assay (qPCR)

Real-time PCR (qPCR) was used to validate the SOLiD profile of 7 selected miRNAs (mir-17a-5p, mir-26a, mir-130c, mir-206-3p, mir-181a-5p, mir-181a-3p and mir-199a-5p) using LNATM-enhanced microRNA qPCR specific primers (Exiqon A/S, Vedbaek, Denmark) (Table 1). These miRNAs were selected based on: 1) previous knowledge of their functions in other species, namely involvement in development and growth, muscle differentiation or response to stress, and 2) differential expression during development and/or temperature, as evidenced by the SOLiD sequencing results. Furthermore, expression analysis of these selected miRNAs was also performed in pre-metamorphic larvae (stage 0 according to the eye-translocation stage) and in 30 dph larvae, in order to have a more complete overview of these miRNA transcript levels during Senegalese sole development.

cDNA was synthesised from RNA diluted to 5 ng· μ l⁻¹ using the miRCURY LNATM Universal RT microRNA PCR Kit (Exiqon) and following the manufacturer's instructions. Reactions were performed using SYBR Green chemistry (Exiqon) on a Light Cycler 480® (Roche Applied Science, Rotkreuz, Switzerland) using white 96 well plates and the following running conditions: denaturation at 95 °C: 10 min followed by 45 amplification cycles at 95 °C: 10 s and 60 °C: 1 min. Specificity of reaction was analysed by melting

curve analysis (Campos et al. 2010). Five-point standard curves of a 5-fold dilution series (1:1-1:625) of pooled RNA were used for PCR efficiency calculation (Fernandes et al. 2006). All samples were run in duplicate. C_T values were determined using the fit-point method using the LightCycler® 480 software with a fluorescence threshold arbitrarily set to 0.3. The provided control RNA Spike-in (Exiqon) was used as exogenous reference to examine the miRNAs expression profile during development and across temperatures, using the $\Delta\Delta C_T$ method, according to Fernandes et al. (2006). Differences in the expression of the target miRNAs between temperatures were evaluated by a Student's t-test using the SigmaPlot 11.0 statistical software (Systat Software, San Jose, CA, USA).

Table 1.

List of primers used in quantification of miRNAs by qPCR. Amplicon sizes, melting temperatures (T_m) and PCR efficiencies (E) are also indicated.

MicroRNA name	Mature sequence (5' – 3')	ID qPCR primers (Exiqon)	Amplicon (bp)	T_m (°C)	E (%)
miR-26a	UUCAAGUAAUCCAGGAUAGGCU	151790-1	49	69	98
miR-17a-5p	CAAAGUGCUUACAGUGCAGGUA	151777-1	45	71	90
miR-130c	CAGUGCAAUAUUAAAAGGGCAU	151782-1	48	69	94
miR-206-3p	UGGAAUGUAAGGAAGUGUGUGG	151786-1	45	70	102
miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC	151813-1	46	70	95
miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU	151802-1	46	71	94
miR-181a-3p	ACCAUCGACCGUUGAUUGUACC	151808-1	41	71	91

3.2.6. Target prediction analysis

3'-UTRs for 3,946 annotated ESTs representing 3,397 *S. senegalensis* genes were obtained from NCBI-SRA and dbEST. MiRNA gene target prediction was performed using miRanda v3.3a (John et al. 2004) and the RNAhybrid web server (bibiserv.techfak.uni-bielefeld.de/rnahybrid) (Enright et al. 2003; Kruger and Rehmsmeier 2006). MiRanda implements a dynamic search programme for maximal local complementarity alignments between the miRNA and the reference sequence and generate scores based on the position-specific empirical models (John et al. 2004). The free energy of optimal strand-strand interaction is calculated using the ViennaRNA package incorporated within the program. Targets for *S. senegalensis* miRNAs were predicted using the following default parameters: Gap open penalty, -9.0; gap extend penalty, -4.0; score threshold, 140.0; energy threshold, 1.0 kcal·mol⁻¹; scaling parameter, 4.0. Targets predicted to have an energy threshold below -20 kcal·mol⁻¹ were selected for further analysis. The RNAhybrid web server was further used to calculate the minimum free energy of the stable duplex between miRNA and target site within the 3'-UTR.

3.3. Results

3.3.1. SOLiD sequencing of small RNAs

To identify Senegalese sole miRNAs during development, 8 small RNA libraries were subjected to high-throughput sequencing using the SOLiD platform. A total of 55,053,966 raw reads were obtained from the 8 libraries, resulting in a total of 27,639,077 reads after sequence trimming. After discarding rRNAs, tRNAs and snoRNAs by comparing with the ncRNA databases, 320 conserved miRNAs -5p and -3p were found amongst 149 miRNAs families in the 8 samples (Table 2). From these, 47 miRNAs had not been previously described in fish (Supplementary table S1). Read lengths distributions for each sample are shown in Figure1. Two peaks in reads length were found for all samples, one at 22-23 nt and another around 28-29 nt, which correspond to miRNAs/interfering miRNAs and piwi-interactions RNAs, respectively. The majority of reads were around the 22 nt peak, indicating miRNA enriched samples (Figure 1). No novel miRNAs were identified amongst the publicly available nucleotide sequences from Senegalese sole.

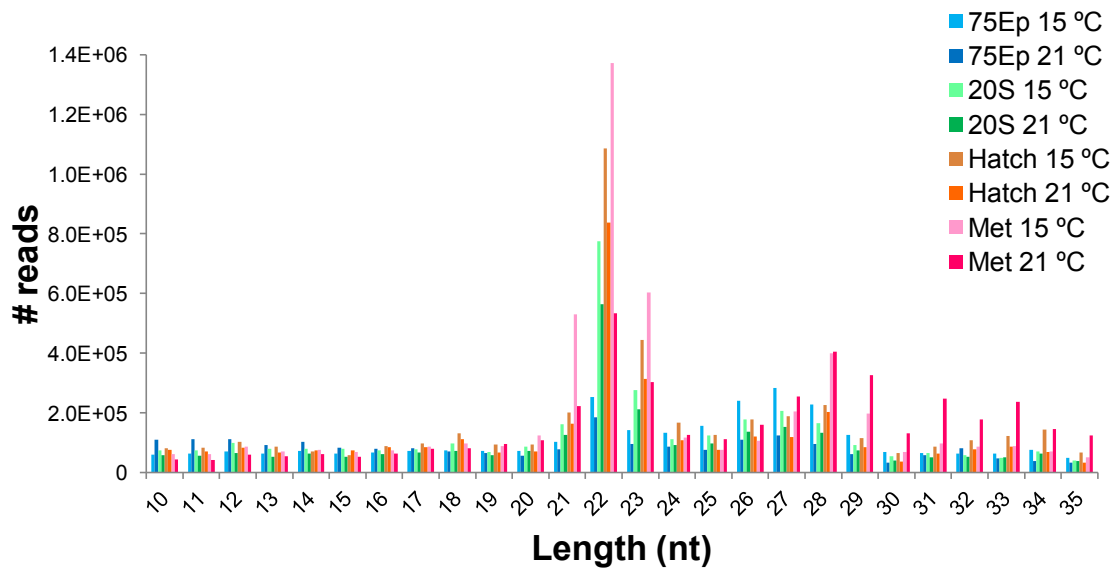


Figure1. Histogram of read lengths (nt, nucleotides) obtained by SOLiD sequencing of Senegalese sole RNA samples. After trimming, two peaks in read length were found for all samples: one at 22-23 nt and a smaller one around 28-29 nt, which correspond to miRNAs/short interfering miRNAs and piwi-interacting RNAs, respectively. The majority of reads were around the 22 nt peak, indicating miRNA enriched samples. Embryos were incubated at 15 °C or 21 °C and transferred to 21 °C after hatching. The developmental stages analysed were 75% epiboly (75Ep), 20 somites (20S), hatching (hatch) and metamorphosis (met).

Table 2.

Summary of the sequenced miRNA transcriptomes in Senegalese sole embryos and larvae incubated at 15 °C or 21 °C.

Sample	# Total reads	# Trimmed (% Total)	Average length (nt)	# Annotated miRBase (% Total)	# Conserved miRNAs
75Ep 15 °C	6 642 089	2 802 986 (42.2)	23.5	200 224 (3.0)	232
75Ep 21 °C	8 842 550	2 169 069 (24.5)	21.1	174 027 (2.0)	231
20S 15 °C	6 566 636	3 320 266 (50.6)	22.4	993 981 (15.1)	265
20S 21 °C	6 101 835	2 564 327 (42.0)	22.7	689 954 (11.3)	265
Hatch 15 °C	7 268 950	4 312 425 (59.3)	23.0	1 467 039 (20.2)	285
Hatch 21 °C	6 200 277	3 256 433 (52.5)	22.4	1 162 239 (18.7)	281
Met 15 °C	7 105 697	4 965 849 (69.9)	23.0	2 391 674 (33.7)	288
Met 21 °C	6 325 932	4 247 722 (67.2)	25.2	804 587 (12.7)	279

75Ep: 75% epiboly, 20S: 20 somites, Hatch: hatching, Met: metamorphosis stage 3.

3.3.2. Differential expression of conserved miRNAs during Senegalese sole development and with embryonic temperature

Hierarchical clustering of the 320 conserved miRNAs found with SOLiD sequencing in Senegalese sole revealed three main clusters (Figure 2): miRNAs with very low number of reads throughout development (Figure 2A), miRNAs generally highly expressed throughout development (Figure 2B) and miRNAs with variable expression during development (Figure 2C).

MiRNA diversity and expression varied amongst developmental stages (Figures 2, 3). Notably, several passenger strands showed very high expression values in one or more stages of development, such as miR-140-3p, miR-181a-3p, miR-199-3p or miR-203b-5p (Figure 2A, Figure 3E). Generally, at earlier stages of development such as 75Ep and 20S, there was less variety and lower number of miRNAs than at later stages (Figure 2). The miRNA with highest number of reads during 75Ep in both temperature groups was miR-430d (3.1 and 1.6% of the total number of reads at 15 and 21 °C, respectively) (Figure 2B), followed by miR-430a and 430c. The miR-430 family was prominent until hatching, but its expression decreased sharply after that and at metamorphosis it had almost completely ceased (Figure 2B). At 75Ep, the passenger strands with higher number of reads were miR-203b-5p, miR-202-5p and miR-20a-3p (Figure 2B, C). During the 20S stage, miRNAs like the miR-203a, miR-17a-5p or miR-130c or were found to be highly expressed (Figure 2B, C, Figure 3A, C). At hatching, miR-130c was the miRNA with the highest number of reads in both temperatures (2.4 and 6.3% of total miRNAs at 15 and 21 °C, respectively). At this point, miR-203a, miR-204, miR-301c and miR-10d were also found in large number of reads (Figure 2A). During metamorphosis, miR-192 was the miRNA with highest number of reads in the 15 °C group (4.0%) and the second highest in the 21 °C group (1.2%). miR-130c, miR-125b-5p, miR-199a-5p and miR-181a-5p were also amongst the miRNAs with higher expression at this stage (Figure 2B, Figure 3C, D, F). Also the passenger strands miR-199-3p, miR-140-3p and miR-181a-3p were expressed at high levels during metamorphosis (Figure 2B, Figure 3E). Furthermore, expression of some passenger and guide strands of the same miRNA differed during development, as the case of miR-140-3p, which was more expressed throughout development than its guide strand miR-140-5p (Figure 2A and C, respectively).

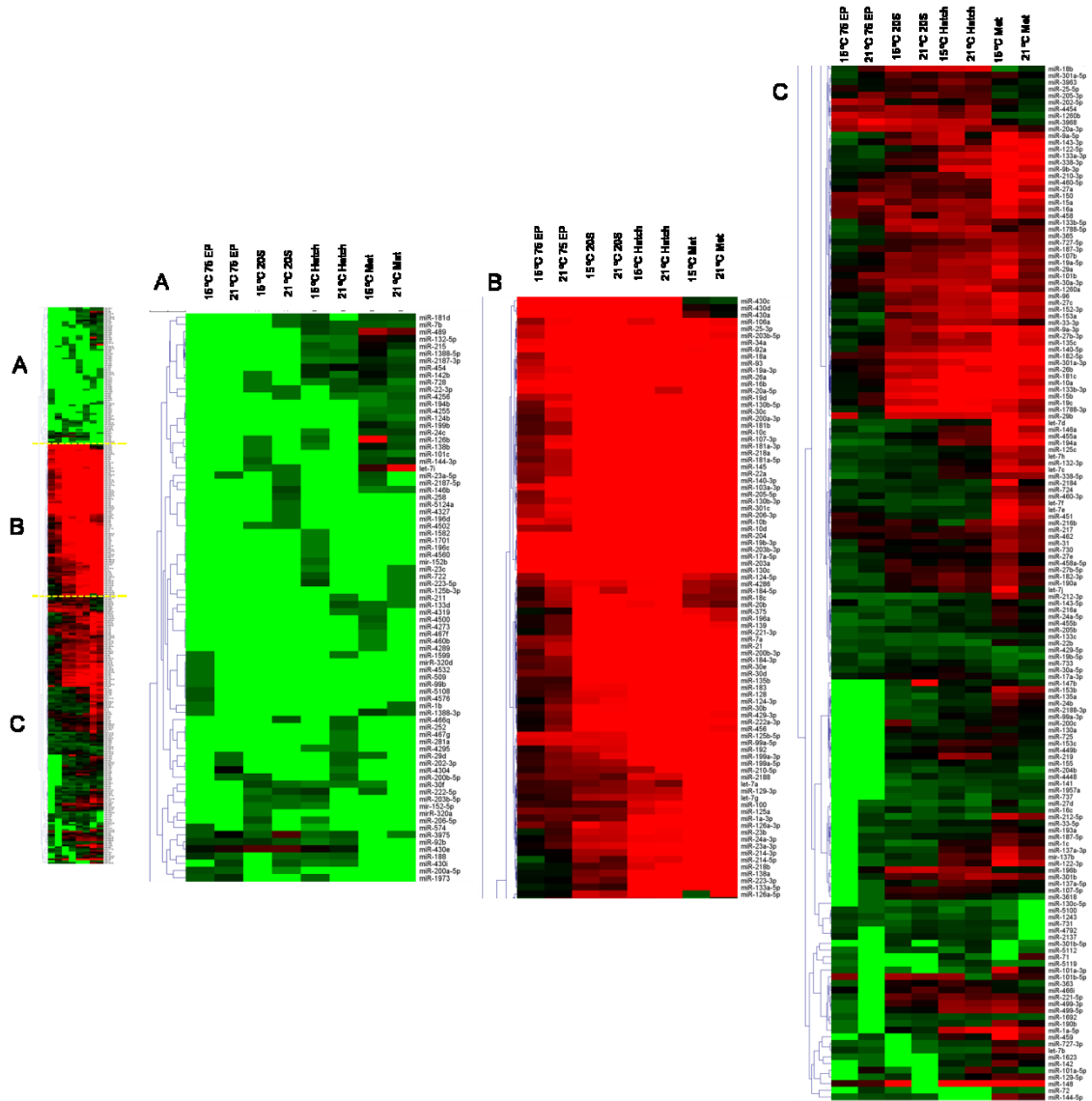


Figure 2. Heatmap of conserved miRNAs obtained by SOLiD sequencing during Senegalese sole early development. The complete heatmap (normalised to the number of trimmed reads, log2 transformed, Euclidean distance, complete linkage) has 320 rows (miRNAs) and 8 columns that correspond to 4 developmental stages (75Ep, 20S, hatching and metamorphosis) and two embryonic temperatures (15 and 21 °C). For visualisation purposes, heatmap was split in 3 parts: A) miRNAs with very low number of reads throughout development, B) miRNAs generally highly expressed throughout development and C) miRNAs with variable expression through development. Red and green indicate highest and lowest expression levels, respectively.

The digital expression profiles obtained with SOLiD sequencing in Senegalese sole were validated by qPCR of selected miRNAs ($r = 0.69, 0.76, 0.98, 0.91, 0.97, 0.98$ and 0.81 for miR-17a-5p, mir-26a, miR-130c, miR-206-3p, miR-181a-5p, miR-181a-3p and miR-199-5p, respectively). qPCR assay of selected miRNAs was performed in the same

stages as the SOLiD sequencing, but also in pre-metamorphic and 30 dph larvae (Figure 3A-G). It was found that most analysed miRNAs had the highest expression levels during metamorphosis, some of them decreasing again afterwards. Particularly high during pre-metamorphosis were the miR-181a-5p, miR-26a, miR-17a-5p, miR-130c, miR-181a-5p and miR-181a-3p (Figure 3). There were also found some significant differences in miRNAs expression between embryonic temperatures. miR-17a-5p, miR-26a, miR-130c and miR-206-3p transcripts were significantly higher at 21 °C than at 15 °C during 75Ep ($P < 0.05$) (Figure 3B, C, G). During the 20S stage, miR-130c and miR-181a-3p showed a 1.5- and 4.9-fold higher expression at 15 °C than 21 °C ($P < 0.05$), respectively (Figure 3C, E). At hatching, miR-17a-5p, miR-26a, miR-130c, miR-181a-5p, miR-199a-5p and miR-206-3p were highly expressed at 21 °C compared to 15 °C (Figure 3A, B, C, D, F, G; $P < 0.05$). No significant differences between temperatures were found after hatching.

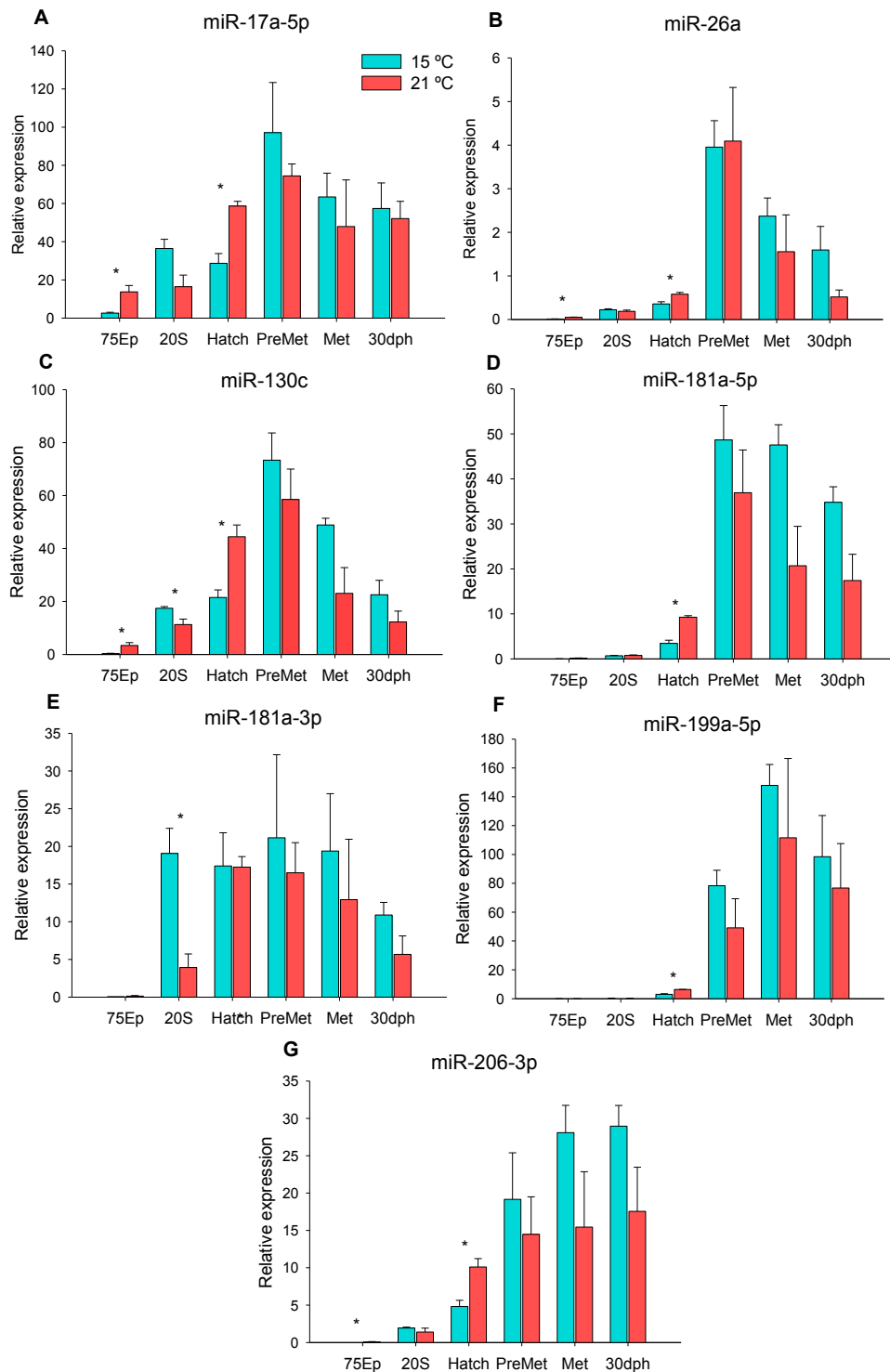


Figure 3. Relative expression of miR-17a-5p, miR-130c, miR-26a, miR-181a-5p, miR-181a-3p, miR-199a-5p and miR-206-3p during Senegalese sole early development. Embryos were incubated at 15 °C (blue bars) or 21 °C (red bars) and transferred to 21 °C after hatching. Six developmental stages were analysed (75% epiboly (75Ep), 20 somites (20S), hatching (hatch), pre-metamorphosis (pre-met), metamorphosis (met) and 30 days post-hatch (dph)) ($N = 3$). Transcript levels were determined by qPCR and Control RNA Spike-in (Exiqon) was used to examine the miRNAs expression profile during development and between temperatures. Significant differences between temperatures are indicated by asterisks ($P < 0.05$).

3.3.3. *miRNA target prediction in Senegalese sole*

Several miRNAs had possible mRNAs targets belonging and/or affecting MAPK and mTOR pathways (Figure 4, Table 3). Namely, miR-130c was predicted to regulate *EIF4E* (eukaryotic translation initiation factor 4E) and *MAPK9*, miR-17a-5p may bind to the 3'UTRs of *Sestrin3*, *MAPK3* and *MAPK13*, miR-181a-5p could target *DDIT4* (DNA-damage-inducible transcript 4) and *MAPK3*, miR-181a-3p may regulate *Calpain1* and miR-206-3p was predicted to target *Sestrin1*.

Table 3.

Predicted targets of five differentially expressed miRNAs during early development of Senegalese sole.

miRNA	Ensembl Gene ID	Gene Name	miRanda		RNAhybrid
			Score	Energy (kcal/mol)	Energy (kcal/mol)
miR-130c	ENSGACG00000005536	<i>EIF4E</i>	156	-25.65	-28.7
miR-130c	ENSGACG00000018076	<i>MAPK9</i>	142	-21.42	-24.4
miR-17a-5p	ENSGACG00000017027	<i>Sestrin3</i>	143	-24.03	-26.8
miR-17a-5p	ENSGACG00000013102	<i>MAPK3</i>	154	-21.58	-26.2
miR-17a-5p	ENSGACG00000004911	<i>MAPK13</i>	140	-22.15	-24.1
miR-181a-5p	ENSGACG00000019700	<i>DDIT4</i>	146	-20.26	-22.5
miR-181a-5p	ENSGACG00000013102	<i>MAPK3</i>	161	-20.51	-26
miR-181a-3p	ENSGACG00000018991	<i>Calpain 1</i>	156	-20.35	-31
miR-206-3p	ENSGACG00000008895	<i>Sestrin1</i>	144	-20.56	-22.7

Targets were predicted using miRanda and RNAhybrid. MiRanda score and minimum free energy ($\Delta G \text{ kcal} \cdot \text{mol}^{-1}$) calculated using miRanda and RNAhybrid are marked for each microRNA-3'-UTR target prediction.

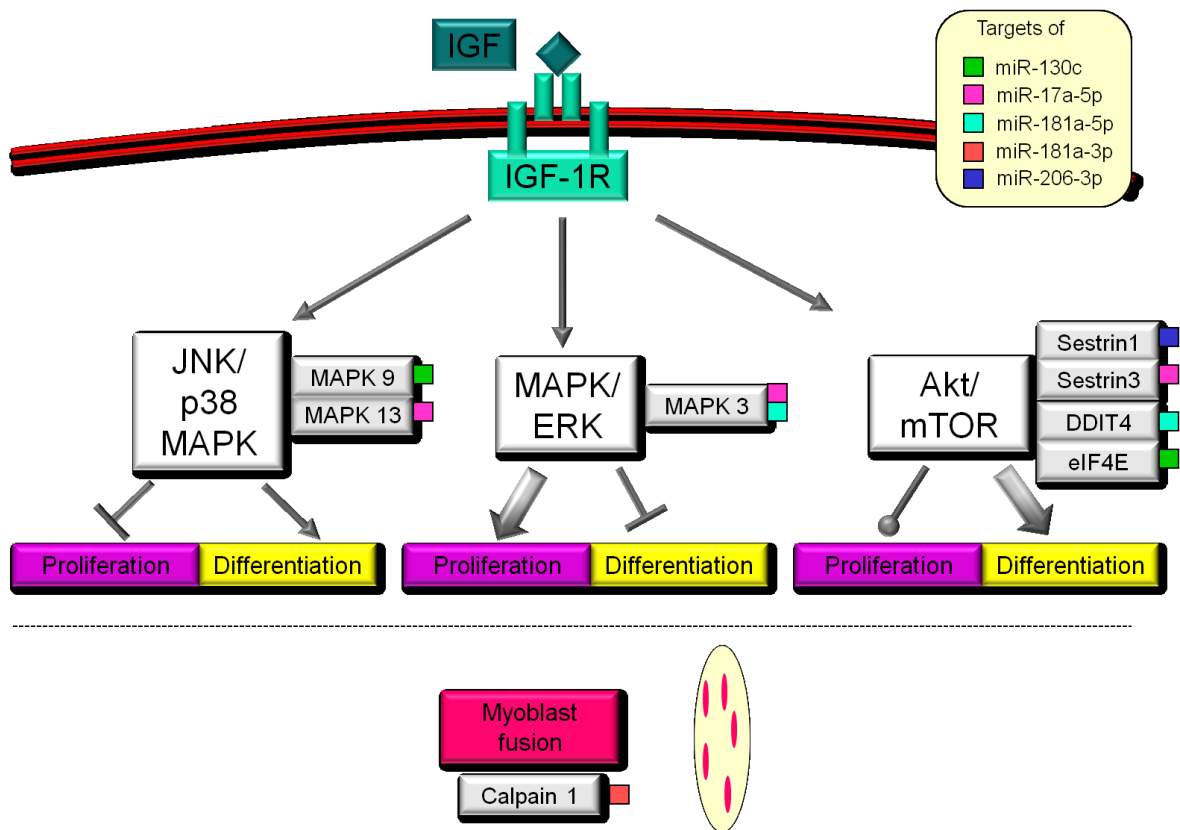


Figure 4. Simplified model of predicted miRNA targets related with JNK/p38 MAPK, MAPK/ERK and Akt/mTOR pathways. IGF functions are mediated by the IGF-I receptor (Igf1R) through activation of two major intracellular signalling pathways: MAPK and mTOR. mTOR and p38 MAPK pathways have overlapping yet distinct roles during myogenesis: both are required for myogenic differentiation and p38 MAPK, but not mTOR, also inhibits proliferation. It was also suggested that under normoxia activation of MAPK/ERK signalling pathway not only stimulates myoblast proliferation but also suppresses myogenic differentiation. miR-130c was predicted to regulate *EIF4E* and *MAPK9*, miR-17a-5p may bind to the 3'UTRs of *Sestrin3*, *MAPK3* and *MAPK13*, miR-181a-5p could target *DDIT4* and *MAPK3*, miR-181a-3p may regulate *Calpain1* and miR-206-3p was predicted to target *Sestrin1*. In Senegalese sole, some of these miRNAs were up-regulated at 21 °C during early development, which might have had implications on muscle growth. Arrow indicates activation, circle indicates little effect and horizontal line indicates inhibition.

3.4. Discussion

3.4.1. Characterisation of Senegalese sole miRNAs

Using high-throughput SOLiD sequencing, 320 conserved miRNAs guide and passenger strands were identified and their expression profiles analysed in Senegalese sole embryos and larvae subjected to two different incubation temperatures (15 °C and 21 °C). The high conservation observed between Senegalese sole mature miRNA sequences and other species may indicate that they have similar functions. From the 320 conserved miRNAs, 47 were not previously found in fish but were known in mammals, birds or nematodes. Despite the generally low number of reads of these miRNAs, most of them were found in more than one sample of small RNAs and regulated during Senegalese sole development. However, their role in embryogenesis is currently unknown. Up or down-regulation of some of them is known to be involved in cancer-associated gene deregulation, such as for miR-211 (Levy et al. 2010) or miR-509 (Zhai et al. 2012). miR-71 mediates the effects of germ cell loss on life span, and its over-expression was found to extend the life span of *Caenorhabditis elegans* lacking germ cells (Boulilas and Horvitz 2012). Nevertheless, the functions of many of these miRNAs remain unclear.

Several passenger strands were found to have a high number of reads in one or more stages of development (Figure 2). It seems that miRNAs that possess both highly conserved -5p and -3p sequences, especially for seed and anchor sequences, can originate two mature functional miRNAs, such as the case of miR-18a, miR-140 or miR-17 (Guo and Lu 2010). The present findings, where a high and dynamic expression of these and other passenger strands like that of miR-181a or miR-199 was observed, seems consistent with an active role of some miRNAs' passenger strands in Senegalese sole development.

3.4.2. Expression profile and thermal plasticity of conserved miRNAs during Senegalese sole development

Critical early events like organogenesis, hatching or metamorphosis in fish involve dramatic changes in signalling, physiology and morphology (Kimmel et al. 1995; Heisenberg et al. 2000; Stickney et al. 2000; Trikic et al. 2011). Well defined expression windows of myogenic and growth-related genes during Senegalese sole ontogeny and larval development have been previously characterised, since different genes are activated or down-regulated at different stages of development (Funes et al. 2006; Campos et al. 2013b). The differential expression observed in Senegalese sole miRNAs

during development (Table 2) highlights the importance of miRNAs in tissue and organ differentiation. miRNA differential expression has been found during the development of vertebrate species, and similarly to our work, generally an increasing number of miRNAs are observed from the earliest to the latest stages of development (Darnell et al. 2006; Bizuayehu et al. 2012). Furthermore, we found that some miRNAs had either a relatively ubiquitous or a more specific expression during development. The miR-430 family is an example of the latter (highly expressed only in earlier stages) and it is well known to accelerate the deadenylation and clearance of maternal transcripts during zygotic stages (Giraldez et al. 2006; Thatcher et al. 2007). Other miRNAs were mostly observed after hatching or metamorphosis, such as miR-199, miR-192, miR-214 and the let-7 family.

miR-26a, miR-17a-5p, miR-130c, miR-181a -5p and miR-181a-3p showed a peak of expression mainly during the pre-metamorphic stage (Figure 3). Similar results were found for some miRNAs in the olive flounder (Xie et al. 2011). It is known that Senegalese sole larvae have a high growth rate and accumulate energetic compounds until the onset of metamorphosis (Yufera et al. 1999); once metamorphosis starts, the growth rates decrease significantly (Fernández-Díaz et al. 2001). It is plausible that up-regulation of some miRNAs at a pre-metamorphic stage is associated with the preparation for the complex metamorphic process. Furthermore, miRNAs such as miR-199a-5p or miR-206-3p showed a peak during or after metamorphosis climax (Figure 3). Flatfish larvae undergo a particularly impressive metamorphosis, resulting in an asymmetrical craniofacial remodelling and lateralized behaviour (Fernández-Díaz et al. 2001; Schreiber 2006) and such modifications certainly involve regulation from a large number of miRNAs. In the olive flounder it was also suggested an involvement of miR-206a in the metamorphic process, which is mediated by the thyroid hormone (Fu et al. 2012).

Some of the miRNAs identified in Senegalese sole are known to play a positive role in general growth and specifically in muscle development. In mammals, miR-26a up-regulation is described to post-transcriptionally repress the histone methyltransferase Ezh2, which is a suppressor of skeletal muscle cell differentiation (Wong and Tellam 2008). In fish, such as the common carp, this miRNA was also associated with myogenesis (Yan et al. 2012). In our study, the higher miR-26a expression observed in the 21 °C group during hatching (and a similar trend during 75Ep and 20S) indicates activation of the myogenic process at a higher temperature. A similar conclusion can be drawn from miR-181a-5p expression in Senegalese sole embryos. miR-181 was found to be strongly up-regulated in regenerating muscle from an *in vivo* mouse model of muscle injury (Naguibneva et al. 2006) and endurance exercise was also found to significantly increase the expression of miR-181 in mice skeletal muscle (Safdar et al. 2009). These results are consistent with previous findings in Senegalese sole, where a lower incubation

temperature (15 °C) produced smaller larvae with smaller fast fibres (Campos et al. 2013b). The fact that miR-181a-3p presented high expression values and also a different thermal plasticity than its guide strand, particularly during embryo development, seems to indicate that it also plays a role in Senegalese sole development.

miR-206, known to reinforce the myogenic program by inhibiting the expression of DNA polymerase α (responsible for cell proliferation) and indirectly down-regulating Id1-3 and MyoR, which are inhibitors of MyoD (Kim et al. 2006), showed increased expression levels during embryogenesis at 21 °C compared to 15 °C, further supporting the idea that this temperature enhanced muscle differentiation. Interestingly, myoD was found to regulate the expression of miR-133 (Liu et al. 2007) and miR-206 (Rosenberg et al. 2006). Expression levels of *myod2* (and in less extension *myod1*) in Senegalese sole embryos incubated at 15 °C or 21 °C (Campos et al. 2013b) at 20S stage are consistent with the expression of miR-206 (and also miR-133, as observed from SOLiD results) observed in the present study, which is higher at 21 °C. In zebrafish and medaka (*Oryzias latipes*), miR-17 is ubiquitously expressed during embryonic development but becomes restricted to proliferative tissues later in development (Ason et al. 2006). In our work we observed a higher expression of miR-17a-5p towards the 21 °C group at some developmental stages (Figure 3A); whether this is related with increased cellular proliferation is however unknown. Interestingly, miR-214/199 cluster activation was found to coincide with the recruitment of *myoD* and *myogenin* (Juan et al. 2009). Furthermore, miR-199, together with expression of miRNAs such as miR-206 or miR-17 were positively associated with muscle regeneration and remodelling (Maciotta et al. 2012). This is consistent with the miR-199, miR-206 and miR-17 transcript levels observed in the present study, at least in some developmental stages. miR-130c expression during embryogenesis did not show a consistent trend with temperature. In zebrafish skeletal muscle, miR-130c was down-regulated in the muscle hypertrophic phenotype (Johnston et al. 2009) and it is plausible that it also has a role in the cellularity phenotypes related to temperature previously reported in Senegalese sole (Campos et al. 2013a; Campos et al. 2013b). In any case, the post-hatch transfer of all larvae to 21 °C seemed to have attenuated the differences on miRNAs levels between temperatures observed during embryogenesis.

3.4.3. Prediction of potential targets of the differentially regulated Senegalese sole miRNAs

Several miRNAs were computationally predicted with high confidence to target mRNAs related with muscle development or within the mTOR or MAPK pathways (Figure 4, Table 3). It has been shown that mTOR and p38 MAPK pathways have overlapping yet

distinct roles during myogenesis: both are required for myogenic differentiation and p38 MAPK, but not mTOR, also inhibits proliferation (Ren et al. 2010). It was also suggested that the normoxia activation of MAPK/ERK signalling pathway not only stimulates myoblast proliferation (Jones et al. 2001) but also suppresses myogenic differentiation (Ren et al. 2010). MAPKs can be activated by a wide variety of different stimuli, but in general MAPK1 and 3 (also called ERK2 and ERK1, respectively) are preferentially activated in response to growth factors whereas the JNK and p38 kinases (which include amongst others MAPK9 and MAPK13) are more responsive to stress stimuli like osmotic shock or ionizing radiation (Pearson et al. 2001). The major direct or indirect targets of mTOR appear to be mechanisms responsible for ribosome recruitment to mRNA, such as the eukaryotic translation initiation factor 4E (eIF4E), which is activated by the phosphorylation of its repressors, the 4EBP proteins (Marcotrigiano et al. 1999; Gingras et al. 2001). Phosphorylation of 4EBP1 by mTOR stimulates translation initiation through the release of eIF4E from 4EBP1, allowing eIF4E to associate with eIF4G to enhance cap-dependent translation (Marcotrigiano et al. 1999).

miR-181a-5p was predicted to target the 3' UTR of the DNA-damage-inducible transcript 4 (*DDIT4* or *REDD1*). It has been demonstrated that REDD1 suppresses mTOR activity through the TSC1/TSC2 heterodimer (Brugarolas et al. 2004; DeYoung et al. 2008). miR-181a-5p up-regulation at 21 °C during hatching might relate with a down-regulation of such mTOR inhibitors. miR-181a-5p was also predicted to target *MAPK3*. In the present work, the up-regulation of miR-181a at 21 °C during early development may have led to higher myogenic differentiation of this group. miR-17a-5p was also predicted to target *MAPK 3* as well as *MAPK13* 3'UTR, which are involved in MAPK/ERK and JNK/p38 MAPK signalling pathways, respectively. Interestingly, in human cell lines miR-17-5p was found to target the 3'UTR of *MAPK9*, belonging to the JNK/P38 MAPK pathway (Cloonan et al. 2008). MAPK9 is a negative regulator of cellular proliferation (Sabapathy et al. 2004) and this interaction contributed to the proliferative phenotype caused by miR-17-5p (Cloonan et al. 2008). In the present study, miR-130c was predicted to target MAPK9 mRNA and might have a role in regulating proliferation at some developmental stages.

Calpains have been suggested to play a role in myogenesis, namely in promoting myoblast fusion (Dedieu et al. 2002; Honda et al. 2008). Interestingly, miR-181a-3p was highly predicted to target the 3' UTR of *Calpain 1*. The fact that miR-181a-3p was upregulated at 15 °C during somitogenesis indicates a possible repression of *Calpain 1* and a lower fusion of myoblasts to multinucleated myotubes at this temperature.

miR-206 was predicted to target *Sestrin1*, which codes for a negative regulator of mTOR signalling through activation of AMPK and TSC2 phosphorylation (Budanov and

Karin 2008). The up-regulation of miR-206 at 21 °C during early development seems to indicate a higher repression of myogenic inhibitors, which might include *Sestrin1*. Furthermore, *Sestrin3* was predicted to be regulated by miR-17a. Like *Sestrin1* and *Sestrin2*, *Sestrin3* elevates the activity of AMPK (Budanov and Karin 2008; Chen et al. 2010), and it has been shown that the induced expression of *Sestrin3* by FoxO1 transcription factor inhibits mTOR Complex 1 (mTORC1), which regulates cell growth mostly through the increase in protein synthesis (Chen et al. 2010). miR-17a up-regulation at 21 °C at hatching is consistent with a possible down-regulation of *Sestrin3* and with an increase in growth.

It should be noted however that miRNAs can have dozens of mRNAs targets (Brennecke et al. 2005) and might regulate genes involved in the most diverse functions. Furthermore, whole larvae and not only skeletal muscle were used to investigate miRNAs expression in sole; hence, it is likely that expression levels of many miRNAs (except for example those of miR-206, which is muscle specific) reflect a broader gene regulation and not only at muscle level.

3.5. Conclusions

We have identified 320 conserved miRNAs in Senegalese sole, most of them dynamically expressed during early development and some not previously described in teleost species. Embryonic temperature affected the expression of several miRNAs, and it seems that a higher incubation temperature (21 °C) promoted a higher expression of miRNAs positively related with growth, at least in some developmental stages. This is consistent with previous studies reporting increased growth of Senegalese sole larvae exposed to higher embryonic temperatures. miRNA target prediction revealed some possible mRNA targets involved in myogenesis through MAPK and mTOR pathways. Furthermore, the expression of these miRNAs amongst temperatures was consistent with the differential action of these pathways regarding muscle differentiation and proliferation.

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Chapter 4

Thermal conditions during larval pelagic phase influence subsequent somatic growth of Senegalese sole by modulating gene expression and muscle growth dynamics

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Thermal conditions during larval pelagic phase influence subsequent somatic growth of Senegalese sole by modulating gene expression and muscle growth dynamics

Abstract

In the present study, Senegalese sole eggs incubated at 20 °C were reared at three different temperatures (15 °C, 18 °C or 21 °C) during the pelagic phase, and then transferred to a common temperature (20 °C) from benthic stage until 100 days post-hatch (dph). Somatic growth, fast muscle cellularity and expression of 15 growth-related genes were compared at the same developmental stage for each temperature (pre-metamorphic larvae, metamorphic larvae, post-metamorphic larvae) and at 83 dph and 100 dph early juveniles. During pre-metamorphosis and metamorphosis, larvae from 21 °C weighed significantly more than those reared at 18 °C or 15 °C ($P < 0.001$). Relative growth rate (RGR) of pelagic larvae and survival of newly-settled larvae were also higher at 21 °C ($P < 0.05$). Furthermore, an increase in muscle growth towards the highest temperatures was observed concomitantly with an increase in gene expression, namely *myogenic regulatory factors* (MRFs), *myosins*, *igf-I* and *fgf6*. After transfer to a common temperature (20 °C), the 15 °C group initiated a process of compensatory growth, inverting relative growth rate values that became the highest, particularly between 83 dph and 100 dph ($P < 0.05$). This increased growth effort in the 15 °C group was accompanied by an up-regulation of gene expression in fast muscle, particularly in the 83 dph juveniles. *Mrf4* and *myHC* were up-regulated at 15 °C and positive correlations with growth were also found for *myog* or *pax7* ($P < 0.05$). In contrast, *mstn1* was down-regulated at 15 °C ($P < 0.05$), suggesting a boost in muscle growth. By 100 dph, the 15 °C group had already reached the same weight as the 18 °C and the same length as the 21 °C ones. At this age, the 18 °C juveniles had the largest mean muscle fibre diameter ($P < 0.001$), and thus fibre hypertrophy seems to be a major growth mechanism relatively to the other groups. Our work shows that temperature during pelagic phase greatly influences the growth trajectory of Senegalese sole juveniles.

Keywords: Myogenesis, muscle cellularity, *Solea senegalensis*, thermoregulation of myogenesis, growth-related genes

4.1. Introduction

The phenotype of myotomal muscle in juvenile and adult teleost fish is known to be related with water temperature during very early stages of development (reviewed by Johnston 2006). Temperature can significantly influence the muscle growth patterns of fish by modulating the rates of hypertrophy and hyperplasia of muscle fibres. Nevertheless, temperature effects depend largely on the species and on the temperatures to which they are exposed in their natural environment. For example in seabass (*Dicentrarchus labrax*) larvae, different rearing temperature regimes were shown to modulate the rates of hypertrophy and hyperplasia of fast muscle fibres throughout the larval, fingerling and juvenile periods (López-Albors et al. 2008), whereas in turbot (*Scophthalmus maximus*) two rearing temperatures applied only during the metamorphic period of larval development induced drastic changes on the distribution of muscle fibre types (Calvo and Johnston 1992). In seabream (*Sparus aurata*) there were persistent effects of temperature on muscle growth patterns, with fish reared at a low temperature until metamorphosis with 20% more smaller fibres than the high temperature treatment (Garcia de la serrana et al. 2012). Furthermore, in Atlantic halibut (*Hippoglossus hippoglossus*), which is a cold-water flatfish, it was shown that hypertrophy is the predominant mechanism of axial white muscle growth in yolk-sac larvae and increasing rearing temperature during this stage increases white muscle fibre hyperplasia (Galloway et al. 1999).

Similarly to embryonic myogenesis, post-embryonic muscle growth encompasses proliferation, fusion and differentiation of muscle fibres, events that involve a large number of genes (reviewed by Johnston et al. 2011). Generally, the myotome of young teleost larvae grows firstly by stratified hyperplasia, with the appearance of myogenic precursor fibres scattered throughout the myotome (mosaic hyperplasia) later in development (Johnston and Hall 2004; Valente et al. 2013). Amongst the genes controlling muscle development and growth, the genes within the somatotrophic/Igf (Insulin-like growth factor) axis, the *myogenic regulatory factors* (MRFs) (*myod*, *myf5*, *mrf4* and *myog*), the *paired-box protein pax7*, or the *transforming growth factor- β* (TGF- β) superfamily, which includes genes like *myostatin* (*mstn*) (De-Santis and Jerry 2007) are particularly important. Myostatin functions by negatively controlling the proliferation of muscle precursor cells (Thomas et al. 2000) and is antagonised by Follistatin (Fst) (Amthor et al. 2004). Also, members of the fibroblast growth-factor family (FGF) are potent moderators of critical phases of muscle development; particularly, *fgf6* participates in the continuous generation of muscle fibres within teleost myotomal musculature, as occurs in rainbow trout (*Oncorhynchus mykiss*) (Rescan 1998). Myosin is the major structural component of

skeletal muscle and in Senegalese sole (*Solea senegalensis*) juveniles, *mylc2* and *myHC* were correlated with several growth parameters in differentially growing feeding treatments (Campos et al. 2010). Heat shock proteins (HSPs) play a central role in cellular homeostasis and protein folding, and in fish can be differently induced in response to a variety of metabolic insults (Yamashita 2010). The expression of many of these genes has been shown to change with temperature and possibly underlying differential muscle growth (Wilkes et al. 2001; Hall et al. 2003; Fernandes et al. 2006; Campos et al. 2013c) .

Thermally-induced early programming can have a profound impact in the posterior growth of a fish and hence be an important tool to modulate fish phenotype. Senegalese sole is a poikilotherm animal, which in the wild can adapt to a wide range of water temperatures (13 – 28 °C) (Vinagre et al. 2006). In aquaculture conditions is it also subjected to high temperature fluctuations (Imsland et al. 2003). However, a tighter control of water temperature seems essential for the improvement of commercial production of this species. Differences in larvae size and muscle cellularity caused by temperature have been reported in a previous study, where *S. senegalensis* eggs were incubated at different temperatures throughout embryonic development (Campos et al. 2013c). Senegalese sole larvae incubated at a higher temperature (21 °C) showed better growth, but increased the number of skeletal deformities when compared to lower embryonic temperatures (15 and 18 °C) (Dionísio et al. 2012). Furthermore, in a parallel nutritional study with Senegalese sole exposed to different thermal conditions (15 °C, 18 °C and 21 °C) during larval pelagic phase and posteriorly transferred to a common temperature, *Artemia* consumption was affected by temperature and higher growth rates were associated with higher protein digestibility in both larvae and post-larvae (Campos et al. 2013a). In seabass juveniles, lower somatic growth and food consumption due to a lower rearing temperature reduced muscle fast fibre hyperplasia (Nathanailides et al. 1996). Such phenotypic plasticities are likely to be associated with changes in the expression of growth-related genes and hence muscle phenotype during ontogenic development.

In the present work, the thermal plasticity of muscle growth and expression of growth-related genes were evaluated in Senegalese sole larvae reared at 15 °C, 18 °C and 21 °C during the pelagic phase, and then transferred to a common temperature (20 °C) from the benthic stage until 100 days post-hatch (dph). Larvae were evaluated and compared throughout critical developmental stages (pre-metamorphosis, metamorphosis, post-metamorphosis) and also early juveniles with 83 dph and 100 dph.

4.2. Material and methods

4.2.1. Fish rearing and sampling

Animal handling protocols were conducted according to the directive of 22nd September 2010, (Directive 2010/63/EU) from the European Economic Community concerning animal experimentation guidelines.

Senegalese sole eggs were incubated in a 200 L cylindro-conical tank at a temperature 20.2 ± 0.5 °C. Transfer of newly hatched larvae to different temperatures was gradual. After hatch, larvae were transferred to 9 fibreglass conical tanks (100 L) in a closed recirculation system with an initial density of 60 larvae L⁻¹, where temperature rounded 20 °C. Only then, temperature from the 9 tanks was gradually adjusted to 15, 18 or 21 °C (15.2 ± 0.5 , 18.3 ± 0.6 or 21.1 ± 0.4 °C), at a rate of 1 °C per hour, resulting in triplicate groups for each temperature. Water temperature, salinity, O₂, pH and nitrogenous compounds were monitored regularly and larvae were exposed to an artificial photoperiod of 12h light: 12h dark. At mouth opening, larvae were fed rotifers (*Brachionus* sp.) enriched with DHA Selco® (Inve, Dendermonde, Belgium) for 6 hours prior to harvesting. *Artemia* was introduced according to larvae developmental stage, which corresponded to different days in the three rearing temperatures. *Artemia* AF strain nauplii (na) (Inve) were introduced in pre-metamorphic larvae between 5 dph and 8 dph and then gradually decreased as metanauplii were introduced just before the onset of metamorphosis between 9 and 16 dph. *Artemia* enrichment was done at 250 000 nauplii/metanauplii L⁻¹, with 0.4 g L⁻¹ supplementation of Easy DHA Selco® (Inve) and Micronised Fishmeal® (Ewos, Bathgate, UK) added in two doses of a 1 : 1 mixture (weight basis). Metanauplii supply was gradually changed from live to frozen *Artemia* as the larvae became benthic. Settlement occurred at 4, 7 and 12 days after metamorphosis stage 2 in the 21, 18 and 15 °C treatments, respectively. Metamorphic stages were evaluated according to the eye-translocation stage (Fernández-Díaz et al. 2001). After settling, water temperature of all treatments was gradually adjusted to ~20 °C before larvae being transferred to flat-bottom plastic tanks (10 L volume, 600 larvae per tank), and maintained all at the same temperature (20.4 ± 0.5 °C). No mortalities of larvae were observed during transfer procedure as it was as gentle as possible. Survival of newly-settled larvae regarding temperature treatments was then estimated. Weaning was initiated at 53 dph in post-larvae from 21 °C, 57 dph in post-larvae from 18 °C and 69 dph in the 15 °C ones. Post-larvae were initially hand fed, twice a day (10 h and 17 h) with a commercial inert diet AgloNorse no.1 (EWOS, Scotland) (0.2 – 0.6 mm) and co-fed with frozen *Artemia* metanauplii. The *Artemia* supply was gradually decreased and an inert diet was later

supplied by automatic feeders for 24 h a day. Survival rates of 100 dph early juveniles (relatively to the number of benthic post-larvae placed in the flat-bottom tanks) were estimated for all treatments.

Samples of larvae were taken for gene expression analysis and histology throughout critical developmental stages (Figure 1). The sampling points were: pre-metamorphosis (Pre-met, stage 0; 8 dph, 9 dph and 12 dph for larvae from 21 °C, 18 °C and 15 °C, respectively), metamorphosis (Met, stage 2; 12 dph, 15 dph and 23 dph for larvae from 21 °C, 18 °C and 15 °C, respectively), post-metamorphic larvae (Post-met; 35 dph, 39 dph and 51 dph for larvae from 21 °C, 18 °C and 15 °C, respectively), and early juveniles with 83 dph and 100 dph. Larva total length (L_T) and dry weight (DW) (mg) ($n = 20$) were measured at each sampling point and also at mouth-opening stage (MO; 2 dph, 2 dph and 3 dph for larvae from 21 °C, 18 °C and 15 °C, respectively). Growth was expressed as relative growth rate (RGR, % day⁻¹) between developmental stages. RGR was determined as: $(e^g - 1) \times 100$ with $g = [(\ln_{\text{final weight}} - \ln_{\text{initial weight}})/\text{days}]$ in DW (mg) (Ricker 1958) (Table 2). The condition factor (K) was calculated as: $(\text{fish DW}/L_T^3) \times 100$ (Table 2).

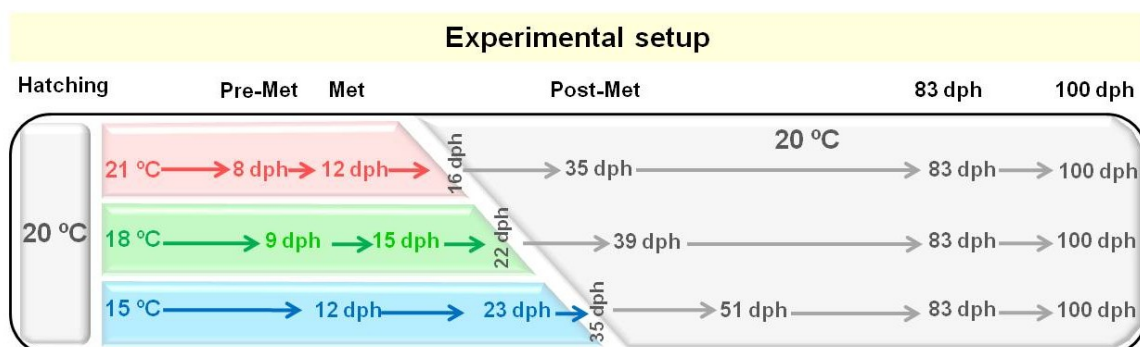


Figure 1. Experimental setup. Senegalese sole hatched larvae were reared at 21 °C (red), 18 °C (green) and 15 °C (blue) until acquiring a benthic lifestyle which occurred at 16 days post hatching (dph) in larvae from 21 °C, 22 dph in larvae from 18 °C and 35 dph in larvae from 15 °C. Newly-settled larvae were then transferred all to the same rearing temperature (20 °C, grey area) and reared until 100 dph. Samples were taken at pre-metamorphosis, metamorphosis, post-metamorphosis, 83 dph and 100 dph young juveniles.

4.2.2. RNA extraction and cDNA synthesis

Larvae were killed by over-anaesthesia with MS-222 (400mg l⁻¹) and snap-frozen in liquid nitrogen. All samples were conserved at -80 °C until further analysis. Total RNA was extracted from pools of whole larvae during pre-metamorphosis (stage 0), metamorphosis (stage 2) and post-metamorphosis stages (pools of 10-15 larvae, 2 pools per tank, 6 pools per temperature) and from fast muscle of post-larvae/juveniles with 83 dph and 100 dph (pools of fast muscle from 10 larvae, 2 pools per tank, 6 pools per group) using Qiazol (Qiagen, Hilden, Germany) and according to manufacturer's instructions. Assessment of RNA quality was performed by agarose gel electrophoresis on a 1.2% (w/v) agarose gel containing SYBR[®] safe (Invitrogen, Carlsbad, CA, USA) DNA gel stain. RNA samples were then quantified with a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). Absorbance ratios (260/280 nm) were greater than 1.9, indicating high purity RNA. Total RNA was treated with gDNA wipeout buffer supplied with the QuantiTect reverse transcription kit (Qiagen) for 5 min to remove any trace of genomic DNA contamination. One microgram of total RNA from each pool of whole larvae or fast muscle from each stage was used to synthesise cDNA with the QuantiTect reverse transcription kit, as reported elsewhere (Fernandes et al. 2008).

4.2.3. Quantitative Real-Time PCR (qPCR)

Whenever possible, primers that span at least one intron/exon border were designed to avoid amplification of potential contaminating genomic DNA, as previously described (Fernandes et al. 2008; Campos et al. 2013c). Netprimer (<http://www.premierbiosoft.com/>) was used to estimate the melting temperatures of the primers and to investigate the presence of potential dimers and hairpins. The genes investigated were: *myod1*, *myod2*, *myf5*, *mrf4*, *myog*, *pax7*, *mylc2*, *myHC*, *mstn1*, *fst*, *fgf6*, *igf-I*, *igf-II*, *igf1r* and *hsp70*. In order to examine thermal plasticity of gene expression, Senegalese sole *elongation factor 1 α* (*eef1a1*), *ribosomal protein 4* (*rpS4*) and *ubiquitin* (*ubq*) were used as endogenous reference genes. Quantification of gene expression was performed in duplicate by qPCR with SYBR Green chemistry (Roche) on a LightCycler[®] 480 (Roche), as detailed elsewhere (Fernandes et al. 2008; Campos et al. 2012). Primer sequences, amplicon sizes and qPCR amplification efficiencies are shown in Table 1.

Table 1.

List of specific primers used for real-time qPCR.

Gene	Fwd sequence (5'→3')	Rev sequence (5'→3')	Accession no (GenBank)	Size (bp)	E (%)
<i>myf5</i>	GAGCAGGTGGAGAACTACTACG	CCAACCATGCCGTCAGAG	FJ515910	89	89
<i>mrf4</i>	GAGAGGAGGAGGCTCAAGAAG	CAGGTCCTGTAATCTCTCAATG	EU934042	137	95
<i>myog</i>	GTCACAGGAACAGAGGACAAAG	TGGTCACTGTCTTCCTTTTGC	EU934044	118	92
<i>myod1</i>	CTCCTCCTCCCCGTCATC	TTGGTGGTCTTCCGCTTG	FJ009109	144	89
<i>myod2</i>	ACAGCCACCAGCCCAAAC	GTGAAATCCATCATGCCATC	FJ009108	194	94
<i>pax7</i>	CTTCTTATCACAGTCATCCTCGTC	CAGTTCCTTCAGGTGAGGCTTC	JQ001819	98	91
<i>mstn1</i>	GGGAGATGACAACAGGGATG	TGGATCCGGTTCAGTGGC	EU934043	91	98
<i>fst</i>	CATCAAAGCTAAGTCGTGTGAG	CACCGCCTCCTCTGTCTTG	EU934045	133	91
<i>myHC</i>	GAAAAATCTGACAGAGGAAATGG	CCTTGGTGAGAGTGTTGACTTTG	FJ515911	143	91
<i>mylc2</i>	GTACAAGGAGGCGTTCACAATC	CCAGCACGTCCCTAAGGTC	FJ515912	77	92
<i>igf-I</i>	CAGGCTATGGCTACAACACAC	CACAGTACATCTCCAGGCG	AB248825	93	90
<i>igf-II</i>	GCAGAATGAAGGTCAAGAAGATG	CGAGACCACTTCCACAGC	AB248826	89	90
<i>igf1r</i>	GCTGTAAAAATAGGAGATTTCCG	GGAGCAAACCCTTACCACC	FJ515914	82	91
<i>fgf6</i>	CGGTGGAGAGAGGAGTCG	AAGACTGTCGTTCCGTATAACC	FJ009110	94	96
<i>hsp70</i>	CTGTATTTGATGCTAAGCGTTTG	GACCTCTACTTTGGGTTTGGA	AB513855	119	90
<i>eef1a1</i>	ATTGGCGGCATTGGAACA	CATCTCCACAGACTTGACCTC	AB326302	117	91
<i>rps4</i>	CTGCTGGATTCATGGATGTG	GGCAGTGATGCGGTGGAC	AB291557	101	90
<i>ubq</i>	TCTGCGTGGTGGTCTCATC	TGACCACACTTCTTCTTGCG	AB291588	135	92

For each gene, its GenBank accession number, amplicon size (bp) and amplification efficiency (*E*) are indicated. The annealing temperature of all primer pairs is 60 °C, except *mstn1*, which is 61 °C.

4.2.4. Morphometry

Two larvae per tank were wholly fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) in phosphate buffered saline (PBS tablets, Sigma-Aldrich, St. Louis, Missouri, USA) for 6h (8-12 dph), 12h (15-23 dph) or 24h (35-100 dph) and washed in PBS. The 83 and 100 dph larvae were then decalcified. Samples were dehydrated in a graded ethanol (AGA, Prior Velho, Portugal) series, cleared in xylol (Prolabo, VWR International LLC, Radnor, PA, USA) and included in paraffin Histosec® (Merck, Whitehouse Station, NJ, USA). Larvae were sectioned (10 µm) transversely to the body axis at the anal opening level, mounted on slides coated with aminopropyltriethoxysilane (APES) (Sigma-Aldrich, St. Louis, Missouri, USA), to improve section adhesion, and then stained with haematoxylin–eosin (Merck, Whitehouse Station, NJ, USA) before placing a cover slip.

Morphometric variables were measured in transversal body sections of individual fish, at the level of the anal opening at all ages. In larvae and post-larvae, the total fast cross-sectional muscle area [A (mm²) (muscle)], the total number of fast-twitch fibre [N (fibres)] and the total fast-twitch fibre cross-sectional area [\bar{a} (µm²) (muscle fibre)] were measured. In juveniles with 83 dph and 100 dph, a minimum of 900 fibre cross-sectional areas were measured per fish, distributed by 6 or 7 fields (90619 µm² each) located at different parts of the muscle cross-sectional area, to be representative of the differences that might occur at different parts of the muscle (Johnston et al. 1999). The number of fibres in each field was also counted. Total cross-sectional muscle area was also computed after tracing the physical limits of interest of the section on the monitor. Total number of fibres in 83 and 100 dph juveniles was estimated by extrapolation of the mean number of fibres per field relatively to the total muscle cross-sectional area. The outlines of muscle fibres were traced using a 400 x magnification. The fibre diameter (µm) was estimated indirectly, as the diameter of a circle having the same area of a fibre in a perfect cross-section. Maximum fibre diameter (D_{max}) in 100 dph juveniles was estimated by averaging the 20 largest fibre diameters of each fish. This study was performed using an Olympus BX51 microscope (Olympus Europa GmbH, Hamburg, Germany) with the Cell[^]B Basic imaging software.

The relative contribution of hypertrophy and hyperplasia towards the increase in muscle cross-sectional area was estimated as reported elsewhere (Valente et al. 1999), as follows: ΔA (µm²) = $N_m \Delta \bar{a}$ (µm²) + \bar{a}_m (µm²) ΔN , where Δ is calculated between two consecutive sampling times (t and $t+1$) and N_m and \bar{a}_m refer to the mean total number of fast fibres and fibre area at t , respectively.

4.2.5. Statistical analysis

Unless otherwise stated, all statistical analyses were performed using the STATISTICA 8.0 software package (StatSoft, Inc., Tulsa, USA). Data are presented as Mean \pm SD and in all cases, significance levels were set at $P < 0.05$. The effects of the temperature on N and A were evaluated using a covariance analysis (ANCOVA), in which temperature was set as the independent variable and the total length as a covariate ($N = 6$). When data did not meet the normality and/or equal variance requirements, a Kruskal–Wallis one-way ANOVA on ranks with a median test was performed instead. The effect of temperature on fibre size was evaluated using all fibres and through a Kruskal–Wallis one-way ANOVA on ranks with a median test.

To compare the distribution of muscle fibre size, a nonparametric method was used to fit smoothed probability density functions (PDFs) using the statistical program for the analysis of muscle fibre populations *FibreA* (Johnston et al. 1999). Bootstrapping was used to distinguish random variation in diameter distribution from treatment differences. The Kolmogorov–Smirnov test was used to test the null hypothesis that PDFs of muscle fibre diameter in the three treatments were identical.

Evaluation of expression stability for the three reference genes was performed using the statistical application *geNorm* (<http://medgen.ugent.be/>) as previously reported (Fernandes et al. 2008). Differences in growth rate and relative expression of target genes with temperature were examined by a one-way ANOVA, using the Tukey HSD *post-hoc* test. When data did not meet the normality and/or equal variance requirements, a Kruskal–Wallis one-way ANOVA on ranks with a median test was performed instead. A Pearson's product–moment correlation (Zar 1996) was used to compare normalised expression data in muscle of 83 dph and 100 dph fish *versus* RGR values and muscle cellularity using the mean value of each triplicate tank ($N = 3$).

4.3. Results

4.3.1. Development and growth

Rearing temperature during larvae pelagic phase greatly influenced Senegalese sole larval growth and development. A temperature of 15 °C clearly delayed larvae development, as observed by the higher number of days needed to reach pre-metamorphic, metamorphic and post-metamorphic stages amongst the different temperatures (Figure 1). Furthermore, during pelagic phase, dry weight (DW), total length

(L_T), RGR (% day⁻¹) and condition factor (K) were also deeply affected by rearing temperature (Figure 2A,B; Table 2). DW at pre-metamorphosis differed significantly among all groups ($P < 0.001$) being almost 3-fold higher at 21 °C than at 15 °C (0.16 ± 0.05 , 0.10 ± 0.03 and 0.06 ± 0.02 mg at 21 °C, 18 °C and 15 °C, respectively). During metamorphosis, DW was also significantly higher at 21 °C relatively to 15 °C and 18 °C groups (0.73 ± 0.22 , 0.50 ± 0.16 and 0.27 ± 0.15 mg at 21 °C, 18 °C and 15 °C, respectively) ($P < 0.001$). In post-metamorphic larvae DW at 21 °C and 18 °C were similar and significantly higher than that at 15 °C ($P < 0.05$) (Figure 2A).

At 83 dph, body weight was significantly higher in post-larvae from 21 °C than in the 18 °C or 15 °C ones ($P < 0.001$) (Figure 2A). However, after the post-metamorphic stage, when all larvae were transferred to the same temperature, that is 20 °C, there was an inversion in the RGR trend, which became evident between 83 dph and 100 dph (Table 2). The larvae initially reared at 15 °C seemed to activate a process of compensatory growth after transfer to 20 °C (RGR % day⁻¹ between 83 dph – 100 dph: 3.02 ± 0.62 , 3.96 ± 1.75 and 7.46 ± 0.04 at 21 °C, 18 °C and 15 °C respectively) ($P < 0.05$) that was reflected on increased DW, since at 100 dph juveniles from 15 °C presented similar weights to those at 18 °C, but significantly lower than the 21 °C ones (100.34 ± 26.46 , 100.89 ± 15.41 and 134.82 ± 13.59 mg at 15 °C, 18 °C and 21 °C, respectively) ($P < 0.05$) (Figure 2A).

Total length (L_T) of larvae during MO, pre-metamorphosis and metamorphosis was significantly affected by temperature. During these stages larvae reared at 21 °C were always significantly longer than larvae from 15 °C ($P < 0.001$) (Figure 2B). Condition factor K was significantly higher in pre-metamorphic larvae from 18 °C and 21 °C than in the 15 °C group ($P < 0.05$) and significantly higher during metamorphosis in larvae from 21 °C relatively to the 18 °C and 15 °C groups ($P < 0.05$) (Table 2). However, no significant differences were observed in L_T and K of post-metamorphic larvae, which were still sampled at different days amongst temperatures (Figure 2B and Table 2). By 83 dph, L_T was higher in post-larvae initially reared at 21 °C than at 18 °C or 15 °C ($P < 0.05$ and $P < 0.001$, respectively) but by 100 dph no significant differences in total length persisted among treatments (Figure 2B). Nevertheless, condition factor was still significantly higher in 100 dph post-larvae from 21 °C and 18 °C relatively to the 15 °C ones ($P < 0.001$ and $P < 0.05$, respectively), because at this point fish weight was still higher in the 21 °C group (Figure 2A).

Survival rates of newly-settled larvae were greatly affected by rearing temperature, since larvae from 21 °C (27.70 ± 6.68 %) had an almost 3-fold higher survival rate relatively to the 15 °C ones (10.17 ± 9.10 %) ($P < 0.05$). Newly-settled larvae from 18 °C showed an intermediary survival rate of 25.08 ± 1.84 % (Table 2). However, by 100 dph

the survival rates of juveniles did not differ significantly amongst treatments (47.99 ± 0.9 , 60.00 ± 5.01 and 39.21 ± 14.97 % at 15, 18 and 21 °C, respectively), fact mainly due to the high deviation of the 21 °C group, which was also the group with the lowest survival (Table 2).

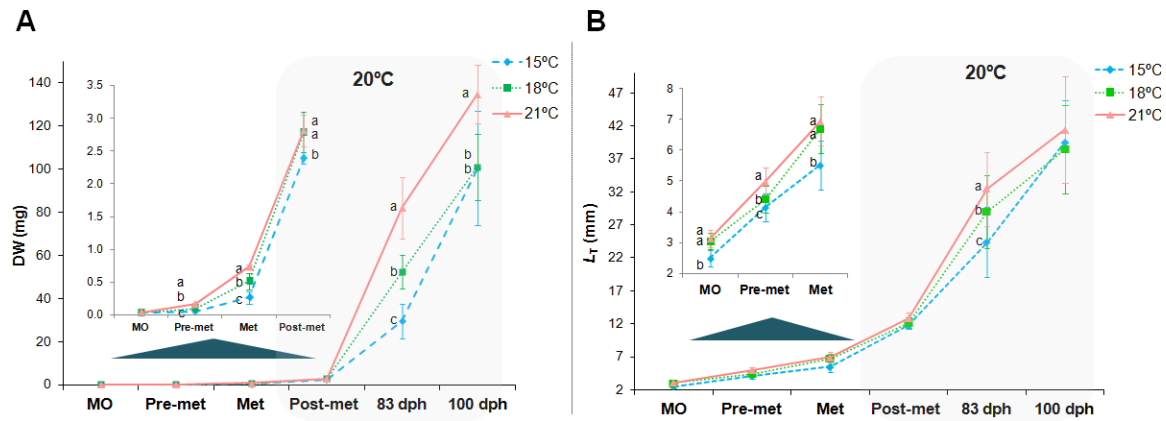


Figure 2. Growth of Senegalese sole larvae and post-larvae reared at 15, 18 and 21 °C during pelagic phase and transferred to ~ 20°C after settlement. A) Dry weight (DW, mg) during development ($N = 20$). B) Total length (L_T , mm) during development ($N = 20$). MO (mouth-opening), Pre-Met (pre-metamorphosis), Met (metamorphosis), Post-Met (post-metamorphosis), 83 dph (83 days post hatching) and 100 dph (100 days post hatching). Error bars indicate the standard deviation of the mean for each treatment and different letters indicate significant differences between temperatures of at least $P < 0.05$.

Table 2.

Relative growth rate, condition factor and survival of larvae and early juveniles of Senegalese sole reared at 15 °C, 18 °C or 21 °C during the pelagic phase and transferred to 20 °C until 100 dph.

Pelagic phase	15 °C	18 °C	21 °C
¹ RGR MO – Pre-Met	4.20 ± 3.75 ^b	15.58 ± 2.36 ^a	25.46 ± 3.64 ^a
² K Pre-met	0.08 ± 0.02 ^b	0.12 ± 0.01 ^a	0.13 ± 0.0 ^a
¹ RGR Pre-met – Met	15.29 ± 1.01 ^c	31.25 ± 6.32 ^b	46.44 ± 5.17 ^a
² K Met	0.15 ± 0.02 ^b	0.17 ± 0.01 ^b	0.22 ± 0.02 ^a
Survival newly-settled larvae (%)	10.17 ± 9.10 ^b	25.08 ± 1.84 ^{a,b}	27.70 ± 6.68 ^a
Benthic phase	20 °C	20 °C	20 °C
¹ RGR Met – Post-met	7.48 ± 0.57	7.35 ± 0.66	6.03 ± 0.19
² K Post-met	0.15 ± 0.00	0.15 ± 0.01	0.13 ± 0.01
¹ RGR Post-met – 83 dph	8.09 ± 0.79	6.88 ± 0.60	7.26 ± 0.60
² K 83 dph	1.20 ± 0.27 ^b	1.22 ± 0.21 ^b	1.38 ± 0.33 ^a
¹ RGR 83 dph – 100 dph	7.46 ± 0.04 ^a	3.96 ± 1.75 ^{a,b}	3.02 ± 0.62 ^b
² K 100 dph	0.92 ± 0.18 ^b	1.01 ± 0.18 ^a	1.07 ± 0.21 ^a
Survival 100 dph (%)	47.99 ± 0.88	60.00 ± 5.01	39.21 ± 14.97

¹ Relative growth rate (RGR, % day⁻¹) was determined as: $(e^g - 1) \times 100$ with $g = [(\ln_{\text{final weight}} - \ln_{\text{initial weight}})/\text{days}]$, in DW (mg).

² Condition factor K was determined as: $(\text{fish DW}/L_T^3) \times 100$, where L_T indicates total length.

(^{a,b,c}) Different superscript letters indicate statistical differences of at least $P < 0.05$ between different treatments at the same stage/age.

4.3.2. Rearing temperature effect on fast skeletal muscle growth

Temperature had a great impact on Senegalese sole fast skeletal muscle growth, not only when larvae were exposed to different temperatures (up to metamorphosis completeness, that is the acquirement of a benthic lifestyle), but also after transfer to a common temperature (from post-metamorphosis up to 100 dph) (Figure 3A-J) showing a prolonged effect.

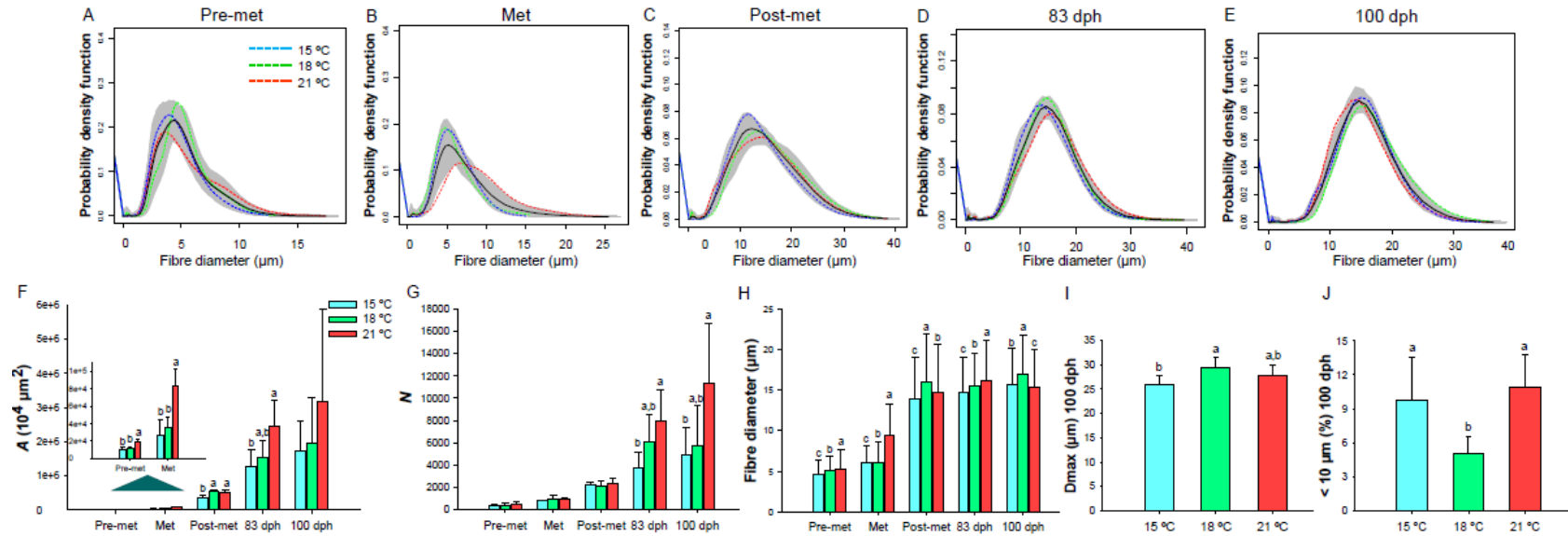


Figure 3. Muscle cellularity of Senegalese sole reared at 15 °C (blue), 18 °C (green) and 21 °C (red) during pelagic phase and transferred to 20 °C after settlement. Probability density functions (PDFs) distributions of fast muscle fibre at A) pre-met, B) met, C) post-met, D) 83 dph and E) 100 dph. The dashed lines show the average PDF for each group and the solid line central to the shaded area is the average PDF for combined groups (15 °C, 18 °C and 21 °C). The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter. F) total cross-sectional muscle area (A) (μm^2), G) total number of fibres (N) and H) average fibre diameter (μm) of fast muscle in Senegalese sole at pre-met, met, post-met, 83 dph and 100 dph ($N = 6$). I) maximum fibre diameter (D_{max}) (μm) in 100 dph early juveniles ($N = 6$). J) percentage of fibres smaller than 10 μm in 100 dph early juveniles ($N = 6$). Pre-met (pre-metamorphosis), Met (metamorphosis), Post-met (post-metamorphosis), 83 dph (83 days post hatching) and 100 dph (100 days post hatching). Error bars indicate the standard deviation of the mean for each treatment and different letters indicate significant differences between temperatures in the same stage ($P < 0.05$).

Pre-metamorphosis and metamorphosis

During pre-metamorphosis and metamorphosis stages, probability density functions (PDFs) showed a progression towards the right-hand tail of the distribution, indicating an enlargement of fibre size throughout development (Figure 3A,B). Muscle cross-sectional area (A) of larvae raised at the lowest temperature was significantly smaller than those raised at 21 °C ($P < 0.05$) (Figure 3F). The diameter of fast muscle fibres was significantly reduced at 15 °C compared to 18 °C and 21 °C at those developmental stages ($P < 0.001$) (Figure 3H). However, no significant differences were observed in number of fibres (N) amongst temperatures (Figure 3G).

Post-metamorphosis until 100 dph

Between metamorphosis and post-metamorphosis there was a great enlargement of fibre size, reflected on the shift of PDFs distributions towards the right-hand of the graphic (Figure 3C). Fibre diameter in post-metamorphic larvae was significantly higher at 18 °C ($16.0 \pm 5.9 \mu\text{m}$) relatively to the 21 °C ($14.7 \pm 5.9 \mu\text{m}$) and 15 °C treatments ($13.8 \pm 5.2 \mu\text{m}$) ($P < 0.001$) (Figure 3H). A was 1.5 and 1.6-fold larger at 21 °C and 18 °C than at 15 °C ($P < 0.05$) (Figure 3F). However, no significant differences were found in the N of post-metamorphic larvae (Figure 3G). The percentage of fibres smaller than 10 μm was not significantly different amongst temperatures at this stage, despite the lowest value of the 18 °C group (21.87 ± 10.39 , 15.34 ± 5.58 and 21.42 ± 8.51 % at 15 °C, 18 °C and 21 °C, respectively).

By 83 dph, PDF distribution showed that the 21 °C group skewed to the right-hand tail of the distribution, although not significantly (Figure 3D). Mean fibre diameter (Figure 3H) was significantly larger in the 21 °C group ($16.2 \pm 4.1 \mu\text{m}$) compared with the 18 °C or 15 °C treatments (15.5 ± 4.1 and $14.7 \pm 4.3 \mu\text{m}$, respectively) ($P < 0.001$). A was 1.9 and 1.6-fold larger in the 21 °C group relatively to the 15 °C or 18 °C ones, respectively ($P < 0.05$) (Figure 3F), and N was significantly higher at 21 °C than at 15 °C ($P < 0.05$) (Figure 3G).

In 100 dph early juveniles there was an inversion in the mean fibre diameters between groups (Figure 3E), as mean diameter of fast fibres from the 18 °C group ($17.1 \pm 4.8 \mu\text{m}$) was significantly larger than the 15 °C ($15.7 \pm 4.4 \mu\text{m}$) and 21 °C ones ($15.3 \pm 4.6 \mu\text{m}$) ($P < 0.001$) (Figure 3H). At this stage, the maximum fibre diameter D_{max} was higher at 18 °C compared to the 15 °C group ($P < 0.05$), however not significantly different from the 21 °C ones (Figure 3I), and interestingly, the percentage of fibres with diameter smaller than 10 μm was significantly higher at 21 °C (10.9 ± 2.9 %) and 15 °C (9.8 ± 3.8 %).

%) relatively to the 18 °C treatment ($5.1 \pm 1.5 \pm 4.7$ %) ($P < 0.05$) (Figure 3J). *N* was significantly higher at 21 °C than at 15 °C ($P < 0.05$) (Figure 3G) and despite the increasing values of *A* towards the 21 °C treatment, there were no significant differences amongst temperatures (Figure 3F). Between 83 dph and 100 dph, the relative contributions of hyperplasia/hypertrophy towards fast muscle growth differed between the 18 °C and 21 °C treatments, since the former grew mainly by hypertrophy (74.2 ± 41.0 %) and the latter grew by fibre hyperplasia (100 ± 0.0 %) ($P < 0.05$).

4.3.3. Thermal plasticity of growth-related genes

Pre-metamorphosis and metamorphosis

The effect of rearing temperature on myogenic regulator factors (*MRFs*) transcript levels was more prominent during pre-metamorphic and metamorphic stages, when larvae were still exposed to different temperatures (Figure 4A,B). *Myf5*, *myod2*, *mrf4* and *myog* were all expressed at higher levels at 21 °C than at 15 °C at pre-metamorphosis ($P < 0.05$) (Figure 4A). A similar trend was observed during metamorphosis in *myod2* and *myog* ($P < 0.05$) but *mrf4* was more expressed at 18 °C than at 15 °C at this stage ($P < 0.05$) (Figure 4B). *Myod1* transcript levels did not show any significant differences between temperatures (Figure 4A,B). *Myhc2* expression during pre-metamorphosis was 1.9 and 1.6-fold higher at 21 °C compared to 15 °C ($P < 0.001$) or 18 °C ($P < 0.05$), respectively, and 3-fold higher at 21 °C than at 15 °C during metamorphosis ($P < 0.001$). *MyHC* expression profile during pre-metamorphosis and metamorphosis followed a similar trend to *myhc2*, with the highest mRNA levels observed at 21 °C ($P < 0.05$) (Figure 4A,B).

Mstn1 expression was significantly higher at 21 °C than at 15 °C during pre-metamorphosis ($P < 0.05$) but that did not persist at met and post-met stages. *Fst* mRNA levels were not significantly different amongst temperatures (Figure 4A). Temperature influenced *fgf6* expression during metamorphosis, with higher expression levels at 21 °C than at 15 °C ($P < 0.05$) (Figure 4B). A similar trend was observed in *hsp70* during metamorphosis ($P < 0.05$).

During metamorphosis, *igf-I* expression was 1.7-fold higher in larvae from the 21 °C group relatively to the 15 °C ones ($P < 0.05$) (Figure 4B). A significant difference in *igf1r* transcript levels was also observed between temperatures at this stage, with larvae from 15 °C showing a higher *igf1r* expression than those from 18 °C ($P < 0.05$). *Igf-II* was very stable across temperatures and no significant differences could be observed in its expression among treatments (Figure 4A,B).

Post-metamorphosis until 100 dph

In post-metamorphic larvae, *mylc2* showed higher expression at 15 °C and 21 °C compared to the 18 °C group ($P < 0.05$) and *myHC* mRNA levels were also higher at 15 °C relatively to the 18 °C treatment ($P < 0.05$) (Figure 4C).

In fast muscle of 83 dph juveniles, expression of *mrf4* was 2.4 and 1.6-fold higher at 15 °C than at 18 °C or 21 °C, respectively ($P < 0.05$), and a higher *myHC* transcript levels was also found in the 15 °C compared with the 21 °C ones ($P < 0.05$) (Figure 4C). In contrast, *mstn1* was 3-fold higher in muscle of 83 dph fish initially reared at 18 °C and 21 °C compared to those reared at 15 °C ($P < 0.05$) (Figure 4D). Interestingly, at 83 dph, the expression of several genes in muscle was significantly correlated with RGR values between 83-100 dph. *MyHC* had a positive correlation with RGR ($R = 0.84$, $P < 0.05$), as well as *myog* ($R = 0.76$, $P < 0.05$) or *pax7* ($R = 0.85$, $P < 0.05$), and *mstn1* had a negative correlation with RGR ($R = -0.84$, $P < 0.05$). *Mrf4* was not significantly correlated with RGR but its correlation value was high ($R = 0.69$) (Table 3).

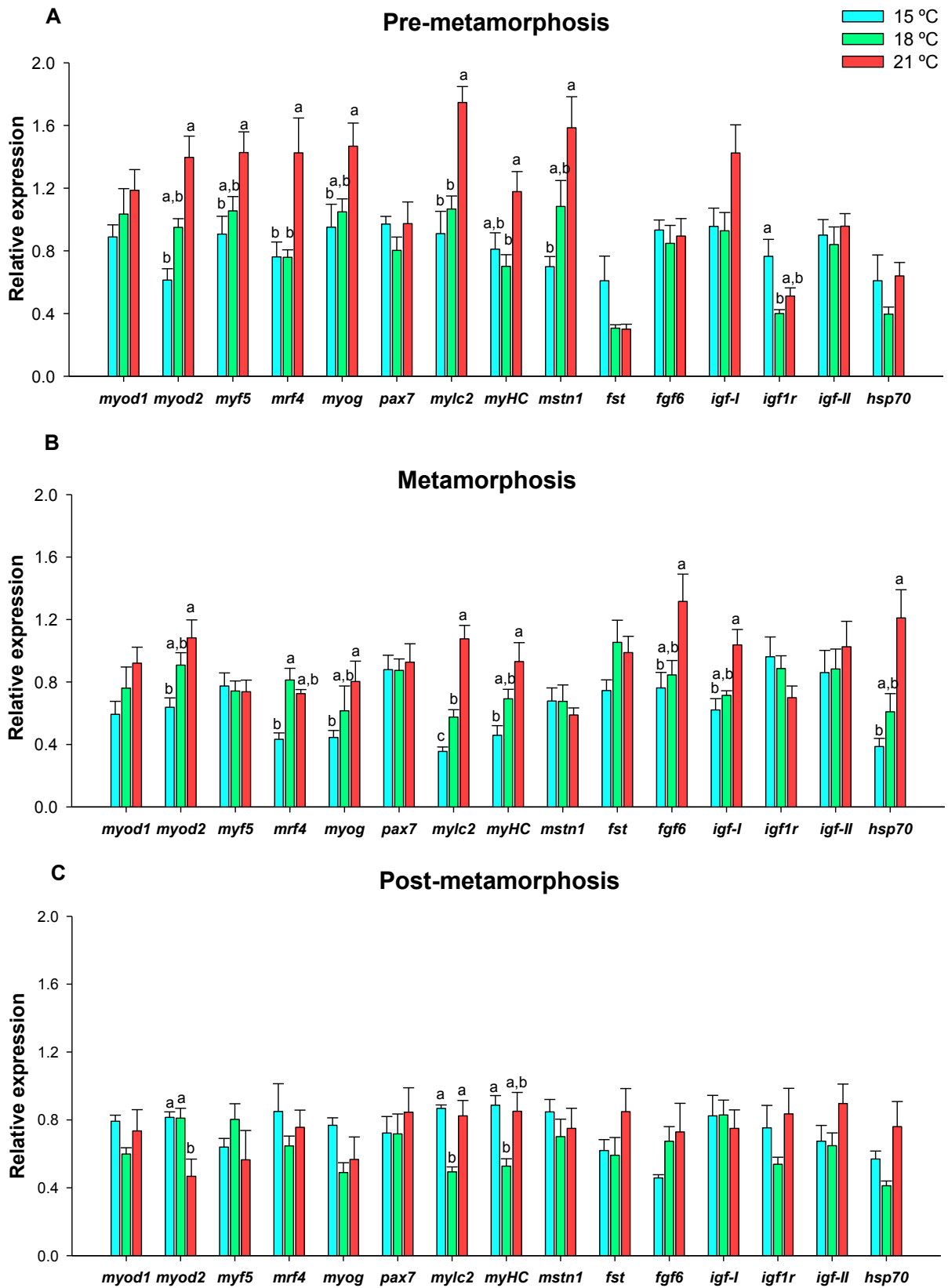
In fast muscle of 100 dph fish, expression of *myod1* was significantly lower at 18 °C than at 15 °C or 21 °C ($P < 0.05$), and *myod2* followed a similar trend (Figure 4E). *Igf1r* mRNA levels in muscle were higher at 18 °C than at 21 °C ($P < 0.05$) (Figure 4E). Furthermore, at this age *myod1* and *myf5* expression were negatively correlated with fibre diameter ($R = -0.77$, $P < 0.05$ and $R = -0.73$, $P < 0.05$, respectively). In contrast, *igf1r* transcript levels was positively correlated with fibre diameter ($R = 0.84$, $P < 0.05$) (Table 3).

Table 3.

Correlations between gene expression in muscle of 83 dph juveniles and RGR, and gene expression in muscle of 100 dph juveniles and fibre diameter.

Genes	Tissue	RGR 83-100 dph	fibre diameter 100 dph
<i>myod1</i>	muscle	NS	$P = 0.02$, $r = -0.77$
<i>myf5</i>	muscle	NS	$P = 0.03$, $r = -0.73$
<i>myog</i>	muscle	$P = 0.03$, $r = 0.76$	NS
<i>pax7</i>	muscle	$P = 0.008$, $r = 0.85$	NS
<i>myHC</i>	muscle	$P = 0.009$, $r = 0.84$	NS
<i>igf1r</i>	muscle	NS	$P = 0.005$, $r = 0.84$
<i>mstn1</i>	muscle	$P = 0.01$, $r = -0.84$	NS

NS: not significant.



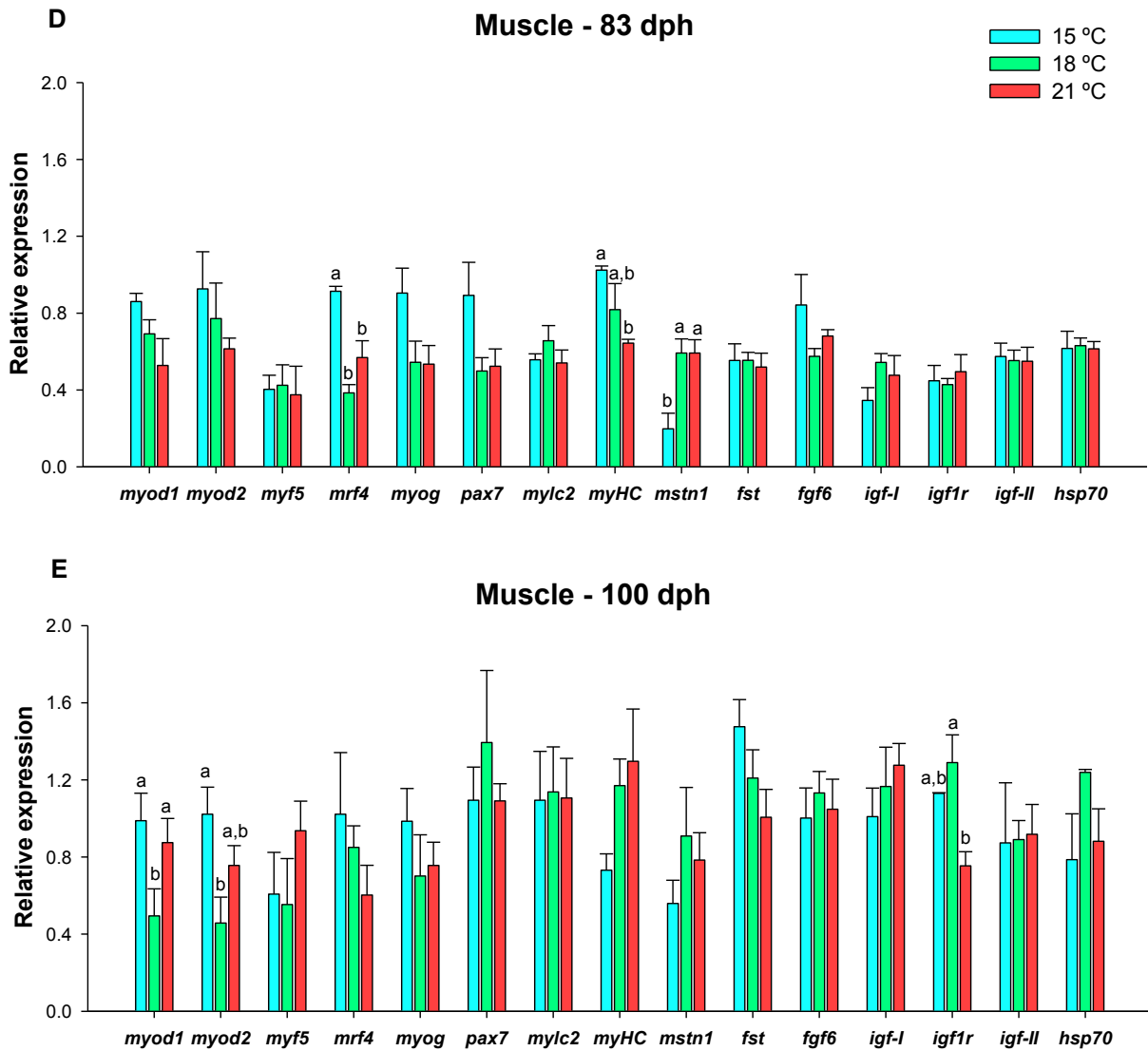


Figure 4. Relative expression of *myod1*, *myod2*, *myf5*, *mrf4*, *myog*, *pax7*, *mylc2*, *myHC*, *mstn1*, *fst*, *fgf6*, *igf-I*, *igf1r*, *igf-II* and *hsp70* in Senegalese sole larvae reared at 15 °C (blue bars), 18 °C (green bars) and 21 °C (red bars) during the pelagic phase. After this period, fish were transferred to 20 °C until 100 days post-hatch (dph) (see main text for details). Developmental stages were pre-metamorphosis stage 0 (A), metamorphosis stage 2 (B), post-metamorphic larvae (C), 83 dph (D) and 100 dph (E). At 83 dph and 100 dph only fast skeletal muscle was analysed. Transcript levels were determined by qPCR and normalised within each developmental stage using *eef1a1*, *rps4* and *ubq* as endogenous reference genes. Error bars represent the standard error of the mean for each treatment ($N = 6$) and significant differences between temperatures are indicated by different letters ($P < 0.05$).

4.4. Discussion

The present work investigated how somatic growth, gene expression and muscle cellularity of Senegalese sole larvae and early juveniles were affected by early thermal history. Three rearing temperatures (15, 18 or 21 °C) were used during Senegalese sole larval pelagic phase (short-term effect), before transfer to a common temperature (20 °C) until 100 dph (long-term effect). Data demonstrated that early temperatures greatly affected somatic growth of larvae up to the juvenile phase. Fish initially reared at the lowest temperature (15 °C) had the lowest growth before transfer, but exhibited compensatory growth and reached a similar body weight to those reared at 18 °C at 100 dph. Samplings at 83 dph and 100 dph were performed at a different number of days after weaning (depending on the temperature) and fish were hence fed with inert diets for a different number of days. The transition from live food to inert diets is critical in all marine fish species and can impact growth and skeletal muscle morphology. Engrola et al. (2007) reported that *Artemia* alone is not a suitable food to sustain maximum growth in sole after 40 dph (at 21 °C) and that better growth is obtained when post-larvae are fed with inert diet alone. The compensatory growth displayed by the post-larvae in the 15 °C group is probably an association of increased protein intake after change to the inert diet with a higher feed intake promoted by increased water temperature (15 to 20 °C).

Rearing temperature had some direct effects on developmental rate and growth of pre-metamorphic and metamorphic larvae, which resulted in important differences in larvae size, RGR and condition factor amongst temperature groups (Figure 2, Table 2). Larvae reared at 15 °C showed delayed development and a clear reduction in size when compared to the other groups. Temperature is also known to be associated with fish survival (Pepin 1991). In our study, survival of newly-settled larvae was very low in the 15 °C group (Table 2), indicating that this temperature was less appropriate for Senegalese sole pelagic larval development. Interestingly, in a previous study, changes in embryonic temperature did not significantly affected survival of larvae (Campos et al. 2013c) and therefore it seems that the effects of water temperature on this parameter are much dependent on developmental stage.

In the previous study changes in embryonic temperature had an effect on size and muscle growth of Senegalese sole larvae up to 30 dph, with incubation temperatures of 18 °C and 21 °C producing larger larvae with more and larger fast fibres (Campos et al. 2013c). In the present work embryos were incubated at a similar temperature, but larvae submitted to high temperatures during the pelagic phase also resulted in increased fast muscle fibre size during pre-metamorphic and metamorphic stages (Figure 3). Increased fibre size might be related to a higher gene expression for most growth-related genes that

were analysed (Figure 4A,B). The majority of the *MRFs* as well as *myosins*, *fgf6* and *igf-I* were up-regulated at 18 °C and/or 21 °C relatively to 15 °C, in both of these developmental stages, whereas *pax7* expression was not affected. In fact, *myod* and *myog* are useful markers of myogenic cell recruitment for second-phase myogenesis (stratified hyperplasia) (Steinbacher et al. 2007), which occurs in Senegalese sole at these larval stages (Campos et al. 2013c). Methylation levels of the *myog* putative promoter in skeletal muscle of Senegalese sole larvae were found to be affected by rearing temperature at metamorphosis, with higher methylation levels at 15 °C than at 21 °C (Campos et al. 2013b), which is consistent with the lowest *myog* expression observed at that stage in the 15 °C group. In seabass and rainbow trout (*Oncorhynchus mykiss*) larvae there seems to exist an optimal temperature for higher transcript levels of *MRFs* and *myHC*, which have been positively related with growth (Wilkes et al. 2001). Interestingly, *mstn1* presented a higher expression at 21 °C than at 15 °C during pre-metamorphosis, a fact no longer observed in larvae undergoing metamorphosis (Figure 4A,B). In Senegalese sole, as in other teleosts, *mstn* does not seem to be only involved in skeletal muscle growth regulation, since high transcript levels have been also found in liver (Campos et al. 2010). Since during pre-metamorphosis and metamorphosis stages gene expression was analysed in whole larvae and not only in muscle tissue, it is plausible that the higher expression observed at 21 °C during pre-metamorphosis is associated with physiological mechanisms other than muscle growth repression.

Deposition of protein during fish growth is largely controlled by the Igf system (De-Santis and Jerry 2007). In the present study, *igf-I*, *igf-II* and *igf1r* showed different expression profiles across temperatures, which were also dependent on the developmental stage. The expression of *igf-I* increased with temperature during metamorphosis, whereas *igf1r* showed an inverse trend during pre-metamorphosis. No temperature effect was observed on *igf-II* transcript levels at any stage (Figure 4A,B). In a study with rainbow trout post-larvae fed *ad libitum*, a higher rearing temperature led to increased levels of *igf-I* mRNA in liver and also plasma Igf-I, which correlated with growth rate (Gabillard et al. 2003). Furthermore, *igf-II* levels did not seem to be affected by temperature, which is in line with our results. Interestingly, in adult rainbow trout it was suggested that increased abundance of Igf-I receptors during periods of food restriction increased the sensitivity of the muscle to circulating Igfs (Chauvigne et al., 2003). In this way, and considering that 15 °C had a striking negative impact on Senegalese sole growth during the pelagic phase, a putative lower nutritional status may have contributed to a higher *igf1r* expression at 15 °C during pre-metamorphosis since temperature is known to highly influence food consumption of fish (Imsland et al. 2001; Handeland et al. 2008). Interestingly, despite the low feed consumption of Atlantic halibut juveniles reared at a low

temperature and under a continuous light regime, these fish presented a higher growth rate and also a higher muscle fibre hypertrophy compared to higher temperature groups (Lohne et al. 2012). Moreover, suppression of feed intake associated with cold water temperature or with feed restriction at a warm temperature, both reduced growth in channel catfish (*Ictalurus punctatus*); however, the decreased growth rate of the fasted fish was independent of changes in the expression of *mstn*, *myHC* or PCNA (Weber and Bosworth 2005). These studies stress how rearing temperature effects might be complex at gene regulation and muscle cellularity levels. It is plausible that food ingestion indirectly contributed to differences in muscle cellularity and gene expression via rearing temperature in Senegalese sole pelagic larvae, given the lowest muscle growth and gene expression of the 15 °C group (although we did not measure *Artemia* consumption in the rearing tanks due to its great difficulty).

The higher expression of *hsp70* during metamorphosis at 21 °C (Figure 4B) seems to indicate a natural response to warmer temperatures; however, nothing indicates that this was an extreme response, considering the range of temperatures that Senegalese sole can be exposed to in natural environments. Furthermore, there is a high variability in HSPs responses in diverse species and therefore, the use of HSPs as stress indicators in fish should be cautious (Iwama et al. 2004).

After metamorphosis, the larvae were transferred to a common temperature (20 °C) and a different trend in growth was observed, particularly in the group previously reared at 15 °C, since its relative growth rate became comparable to that of other groups (Table 2). Consistent with these results, Campos et al., (2013a) found that post-larvae initially reared at 15 °C during the pelagic phase and then transferred to 20 °C presented a higher feed intake and protein digestibility than the ones initially reared at either 18 °C or 21 °C, further supporting the compensatory growth of this group. Furthermore, expression levels of important myogenic genes such as *myod2*, *mylc2* or *myHC* in post-larvae were also among the highest at 15 °C (Figure 4C). The existence of two *myod* paralogues (*myod1* and *myod2*) may implicate some degree of subfunctionalisation and therefore some differential gene expression, which may become more evident at specific stages.

A mechanism of compensatory growth could be suggested by 83 dph. At this age, gene expression in fast muscle was consistent with the highest growth rate presented by the 15 °C group during this period that resulted in increased skeletal muscle area compared to the other two groups (Figure 3F, Figure 4D, Table 2). Significantly higher mRNA levels of *mrf4* and *myHC* in the 15 °C fish indicates that at this point there was a greater effort in building muscle tissue relatively to that observed in the 21 °C group. Genes such as *myosins* or those belonging to the *MRF* group are implicated in muscle fibre differentiation, protein accretion and growth, and in diverse teleost species such as

Atlantic salmon (Hevrøy et al. 2006), pacu (de Almeida et al. 2010) or Senegalese sole (Campos et al. 2010), some have been positively correlated with muscle or somatic growth. In the present study, significant positive correlations were also found between *myHC*, *pax7* and *myog* expression at 83 dph in muscle and RGR values (Table 2), which are indicative of the importance of these genes for growth. Moreover, at 83 dph *mstn1* presented a higher expression in fast muscle of 18 and 21 °C fish relatively to the 15 °C ones and was negatively correlated with RGR (Table 2), supporting the idea that *mstn1* is a negative regulator of muscle growth in Senegalese sole.

In 100 dph juveniles, the 15 °C group reached the same weight as the 18 °C and the same length and muscle area as the 18 °C and 21 °C groups (Figure 2A,B). Therefore, it seems that a mechanism of compensatory growth was in fact activated in the 15 °C group after transfer to 20 °C, confirmed by the highest RGR value between 83 dph and 100 dph (Table 2). Interestingly, the 18 °C treatment did not seem to compensate weight relatively to the 21 °C group. In fact, in a parallel study it was found that by 121 dph, sole juveniles initially reared at 15 °C fully compensated body weight relatively to the 21 °C ones, whereas the 18 °C group remained significantly smaller than both 15 °C or 21 °C (Campos et al. 2013a), which corroborates the present findings. During the benthic phase and up to 100 dph, survival of fish, which is an important factor considering production, was not significantly affected by early thermal history and was comparable to what has been found in other studies with Senegalese sole post-larvae/young juveniles (Engrola et al. 2005). However, we must bear in mind the highest mortality registered at the lowest temperature (15 °C) during the pelagic phase. Regarding the muscle phenotype, it was found that by 100 dph the 18 °C group presented the largest mean fibre size and Dmax (Figure 3H and I) but the lowest % of small-sized fibres; hence, fibre hypertrophy was the most important mechanism of muscle growth in this treatment between 83 dph and 100 dph. On the contrary, at this age, the 21 °C treatment induced the lowest mean fibre diameter but the highest number of fibres, and together with 15 °C, the highest percentage of small fibres (Figure 3J), suggesting that fibre hyperplasia was a major mechanism of growth in these two groups. Such mechanisms of hyperplasia and hypertrophy are consistent with the observed expression of some genes in sole fast muscle (Figure 4E). *Myod1*, higher expression in muscle of 15 °C and 21 °C 100 dph juveniles, and *myod2*, with higher transcript levels at 15 °C compared with 18 °C, might be related with hyperplastic growth. This is further supported by the significant negative correlation found between *myod1* expression and fibre diameter (Table 2). Such implication of *myod* in hyperplastic growth has also been observed in other teleost such as the rainbow trout (Johansen and Overturf 2005) or the pacu (de Almeida et al. 2008). Interestingly, *igf1r*, which was found positively correlated with fibre diameter and showed higher transcript levels in muscle of the 18 °C

juveniles, might be related with increased fibre hypertrophy. The Igf-I effects on myoblast proliferation and differentiation (Coolican et al. 1997) and on myotube hypertrophy (Rommel et al. 2001) are mediated through Igf1R. This interaction promotes the activation of two major intracellular signaling pathways, the mitogen-activated protein kinases (MAPKs), and the mTOR (mammalian target of rapamycin) through phosphatidylinositol 3 kinase (PI3K)/Akt (Nave et al. 1999). MAPK pathway is essential for muscle cell proliferation (Jones et al. 2001), whereas Akt/mTOR activation stimulates protein synthesis and muscle hypertrophy (Bodine et al. 2001; Rommel et al. 2001). Whether these pathways were differentially activated in sole muscle at 100 dph is currently unknown; however, it is plausible that there was a higher activation of the latest in the 18 °C group. In fact, it was found that *igf1r* over-expression in mammal skeletal muscle results in myotube hypertrophy (Quinn et al. 2007).

Our findings show that control of water temperature is essential for optimal larval and young juvenile production of Senegalese sole. A low temperature (15 °C) during pelagic phase directly increases larval mortality and significantly delays development, but a good compensatory growth was observed after transfer to a more suitable temperature (20 °C). It also seems that even if larvae from the 18 °C group initially presented a better somatic growth and survival than the 15 °C one, by 100 dph they did not compensate growth relatively to the 21 °C group favoring hypertrophy of existing fibres rather than hyperplasia. A positive relation between somatic growth and muscle sustained hyperplasia has been proposed in several fish species (Valente et al. 1999; Zimmerman and Lowery 1999; Alami-Durante et al. 2007). Furthermore, previous studies showed that changes in muscle phenotype with temperature are not always linear in fish. For example, in Atlantic salmon an incubation temperature of 5 °C until the “eyed stage” produced a higher final fibre number and fibres with smaller diameter in adults than 2 °C, 8 °C or 10 °C, despite the highest weight of the 8 °C fish (Macqueen et al. 2008). Water temperature during the larval period was found to be critical for later muscle development in seabass (López-Albors et al. 2008) but in Senegalese sole it remains to be ascertained whether phenotype differences registered during larval stages will persist at harvest.

In conclusion, our study clearly shows that temperature differences during the larval pelagic phase alone greatly influence the growth trajectory of Senegalese sole juveniles: i) a temperature of 15 °C initially induced a lower growth but a good compensatory growth was observed afterwards and was accompanied by an up-regulation of *mrf4* and *myHC* in fast muscle and down-regulation of *mstn1* in the 83 dph juveniles; ii) the 18 °C group showed an intermediate growth capacity favoring fibre hypertrophy, and iii) the 21 °C treatment clearly favoured growth of larvae concomitantly with a high gene expression, and promoted a good growth of juveniles. It is plausible that rearing temperature

influenced mechanisms of gene regulation such as DNA methylation. Further studies are required to determine if these patterns of growth and muscle phenotype are reflected at the commercial size.

4.5. Acknowledgments

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Chapter5

Temperature affects methylation of the *myogenin* putative promoter, its expression and muscle cellularity in Senegalese sole larvae

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RESEARCH PAPER

Temperature affects methylation of the *myogenin* putative promoter, its expression and muscle cellularity in Senegalese sole larvae

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Keywords: *Solea senegalensis*, thermal plasticity, *myogenin*, methylation, epigenetic regulation, myogenesis

Temperature affects methylation of the *myogenin* putative promoter, its expression and muscle cellularity in Senegalese sole larvae

Abstract

Myogenin (*myog*) encodes a highly conserved myogenic regulatory factor that is involved in terminal muscle differentiation. It has been shown in mammals that methylation of cytosines within the *myog* promoter plays a major role in regulating its transcription. In the present study, the Senegalese sole (*Solea senegalensis*) *myog* putative proximal promoter was identified and found to be highly conserved among teleosts. Therefore, it is plausible that it plays a similar role in controlling *myog* expression. Cytosine methylation of the *myog* promoter in skeletal muscle of Senegalese sole larvae undergoing metamorphosis was influenced by rearing temperature. A lower temperature (15 °C) significantly increased *myog* promoter methylation in skeletal muscle, particularly at specific CpG sites, relatively to higher rearing temperatures (18 and 21°C). *Myog* transcription was downregulated at 15°C, whereas expression of *dnmt1* and *dnmt3b* was upregulated, consistently with the higher *myog* methylation observed at this temperature. Rearing temperature also affected growth and fast muscle cellularity, producing larger fibers at 21°C. Taken together, our data provide the first evidence of an epigenetic mechanism that may be underlying the temperature-induced phenotypic plasticity of muscle growth in teleosts.

Keywords: *Solea senegalensis*, thermal plasticity, *myogenin*, methylation, epigenetic regulation, myogenesis

5.1. Introduction

The development of vertebrates is largely regulated by epigenetic events like DNA methylation of specific cytosine residues in the genome, which is stably inherited through cell division.(Goll and Bestor 2005) DNA cytosine methylation is carried out by a group of DNA (cytosine-5)-methyltransferases, known as Dnmts. Dnmt1 is involved in maintaining existing methylation patterns and has a direct role in histone methylation (Rai et al. 2006), whereas Dnmt3a and 3b are two functionally related proteins that are essential for *de*

novo methylation (Chen et al. 2003; Goll and Bestor 2005; Li et al. 2007).

Myogenin (*myog*) belongs to a family of four *myogenic regulatory factors* (*MRFs*), which are critical regulators of myogenesis and highly conserved amongst vertebrates. It encodes a transcription factor of the basic-helix-loop-helix (bHLH) protein family and plays an essential role in the specification and differentiation of myoblasts (Bergstrom and Tapscott 2001). In zebrafish embryos, muscle-specific expression of *myog* was shown to be controlled by MEF2 and MEF3 binding sites in the promoter and by two non-canonical Enhancer Boxes (E-box), which are the *MRF* protein binding sites conferring muscle-specificity (Du et al. 2003). Also in striped bass (*Morone saxatilis*), a 0.6 kb sequence of the *myog* promoter containing regulatory elements was enough to drive muscle-specific *myog* expression (Tan et al. 2002). In the mouse embryo *myog* promoter, the Myocyte Enhancer Factor-2 (MEF2) (which is bound by elements of the MEF2 family of transcription factors) and MEF3 binding sites were shown to be critical for the correct temporal and spatial expression of *myog* (Cheng et al. 1993; Spitz et al. 1998). Moreover, the E-box (E1) present between the TATA box and the transcription start site (TSS) was shown to be the binding site for myogenic bHLH protein complexes, and mutation of this E-box was shown to block *myog* expression in the myotome during development (Yee and Rigby 1993). The *myog* promoter has a relatively low density of CpG residues (Fuso et al. 2010) but methylation of cytosine nucleotides within this region plays a role in the negative regulation of transcription (Fuso et al. 2010; Palacios et al. 2010). During mouse early development, *myog* promoter was shown to be initially methylated but becomes demethylated as development proceeds (Palacios et al. 2010). Furthermore, in murine mesenchymal progenitor cell cultures treated with a DNA methylation inhibitor, *myog* was up-regulated at the myoblast stage and myogenesis was promoted (Hupkes et al. 2011).

Myog expression is known to vary with temperature in some teleost species. For example, in tiger pufferfish (*Takifugu rubripes*) embryos, the peak *myog* expression occurred later with respect to developmental stage at a higher incubation temperature (Fernandes et al. 2006). In early larvae of rainbow trout (*Oncorhynchus mykiss*) and seabass (*Dicentrarchus labrax*), *myog* expression increased towards the temperature to which these species are naturally exposed in the wild (Wilkes et al. 2001). Nevertheless, in spite of the vital importance of *myog* in muscle development and growth, virtually nothing is known about the molecular mechanisms underlying the epigenetic regulation of *myog* expression by temperature. In natural conditions, the marine flatfish Senegalese sole (*Solea senegalensis*) faces temperature fluctuations between 13 and 28 °C (Vinagre et al. 2006), and large thermal variation have also been observed in aquaculture conditions (Imsland et al. 2003). Incubation temperature has been reported to influence muscle cellularity up to 30 days post-hatching (dph), as for example larvae initially

incubated at 18 or 21 °C had 11 and 9 % more muscle fibres than those incubated at 15 °C, respectively (Campos et al. 2013). An increase in muscle growth was observed particularly during and after metamorphosis. Rearing temperature was also found to highly influence protein metabolism in Senegalese sole larvae and post-larvae (Campos et al. 2013). In the present study, we hypothesized that rearing temperature post-hatching could influence the muscle phenotype of Senegalese sole larvae and the methylation status of the *myog* promoter in skeletal muscle.

5.2. Material and methods

5.2.1. Fish husbandry and sampling

The Senegalese sole incubation experiment took place at the LEOA facility, University of Algarve, Portugal. Embryos were incubated in a 200L cylindro-conical tank at 20.2 ± 0.5 °C. Newly hatched larvae were then transferred to 9 fiberglass conical tanks (100 L) tanks in a closed recirculation system with an initial density of 60 larvae L⁻¹. Water temperature, salinity, O₂, pH and nitrogenous compounds were monitored regularly and larvae were exposed to an artificial photoperiod of 12h light: 12h dark. They were reared at three temperatures: 15.2 ± 0.5 , 18.3 ± 0.6 or 21.1 ± 0.4 °C, in triplicate groups until complete metamorphosis. At mouth opening, larvae were fed rotifers (*Brachionus* sp.) enriched with DHA Selco® (Inve, Belgium) for 6 hours prior to harvesting. *Artemia* AF Strain nauplii (na) (Inve) were introduced between 5 dph and 8 dph and metanauplii between 9 and 16 dph, according to rearing temperature. *Artemia* enrichment was done at 250,000 nauplii/metanauplii L⁻¹, with 0.4 g L⁻¹ supplementation of Easy DHA Selco® (Inve) and Micronised Fishmeal® (Ewos, UK) added in two doses of a 1 : 1 mixture (weight basis).

Metamorphic larvae (Met) at stage 2, according to the eye-translocation stage (Fernandez-Diaz et al. 2001), were sampled at 12 dph at 21 °C, 15 dph at 18 °C and 23 dph at 15 °C. Larvae were killed by over-anaesthesia with MS-222 (400 mg·L⁻¹). Samples for nucleic acid extraction were then snap-frozen in liquid nitrogen and stored at -80 °C. Dry weight (DW, mg) was measured on -80 °C freeze-dried sole larvae ($n = 3$ in pools of 25 larvae, ± 0.001 mg) and total length (L_T , mm) was measured on 20-30 larvae per tank.

Animal handling protocols were conducted according to the directive of November 24th, 1986 (86/609/EEC) from the European Economic Community concerning animal experimentation guidelines.

5.2.2. RNA and genomic DNA extraction

Total RNA was extracted from 6 pools of 15 larvae per temperature group (2 pools per tank) using Qiazol (Qiagen, Germany). Assessment of RNA quality was performed by agarose gel electrophoresis and its quantity determined with a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). Absorbance ratios (260/280 nm) were greater than 1.9, indicating high purity RNA. Genomic DNA was isolated from larvae skeletal muscle (6 larvae per temperature) using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. Quality and quantity of genomic DNA were determined as indicated above for RNA. Genomic DNA was also extracted from Senegalese sole gut ($n = 6$), to serve as a control of methylation levels (see below).

5.2.3. Cloning and sequencing the putative 5'UTR and proximal promoter of the *myog* gene

Senegalese sole *myog* putative 5'UTR and promoter were isolated by PCR as two overlapping DNA fragments from sole DNA libraries using the Genome Walker™ Universal Kit (Clontech, CA, USA). Briefly, Senegalese sole genomic DNA was completely digested with restriction enzymes (*DraI*, *EcoRV*, *PvuII* and *StuI*) to produce blunt-ended DNA fragments. The digested DNA was then ligated with a DNA adapter (Clontech) and the resulting DNA fragments were used as templates for two rounds of primary and nested PCR amplifications using two adapter-specific primers together with two *myog* specific primers (GSPs) in the first exon (GenBank accession number EU934044). The remaining sequence of the first exon, the 5' flanking region and part of the promoter was isolated using the GSP1 5'-CGCCTCCAGACAGACTCGCACACAAG and GSP2 5'-CTGGTCAGGGAAGAAATAGGGGTTGGTC; the remaining part of the promoter was isolated using the GSP1 5'-GGACGGATAATCTCGGATCAAATATGCC, GSP2 5'-TCTTGTCTCCCTCCATGACACCATACAT. Cloning and sequencing were essentially performed as previously reported (Campos et al. 2010).

5.2.4. DNA bisulfite treatment and sequencing

Bisulfite modification of muscle genomic DNA extracted from stage 2 larvae was performed using the EpiTect Bisulfite Kit (Qiagen), according to the manufacturers' instructions. Bisulfite treated DNA from each temperature group (15, 18 and 21 °C) were pooled, and then amplified by PCR (forward: 5'-ATGTATGGTGTATGGAGGGAGA;

reverse: 5'-ACTAAACAACGCTATAATCTAAATTA), to obtain a 523 bp fragment of the putative 5'UTR and promoter of the Senegalese sole *myog* gene. PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen) and cloned onto a pCR4-TOPO® plasmid vector (Invitrogen), as reported (Campos et al. 2010). Twenty one individual colonies per temperature were picked and lysed in H₂O at 99 °C for 5 min. Following centrifugation at 16 x *g* for 3 min, 1 µl of the supernatant was used for PCR amplification with M13 primers and an annealing temperature of 55 °C. PCR products were visualised on agarose gel, purified with the ExoSAP-IT® PCR Product Cleanup (Affymetrix, CA, USA) and sequenced as described elsewhere (Campos et al. 2010).

The efficiency of the bisulfite conversion step and methylation levels at each CpG site were evaluated with the Bisulfite Sequencing data Presentation and Compilation software (<http://biochem.jacobsuniversity.de/BDPC/>) and the BiQ Analyzer (<http://biq-analyzer.bioinf.mpi-inf.mpg.de/>). The mean percentage of converted cytosines was 96.2%, 96.4% and 96.6% for the 15 °C, 18 °C and 21 °C group, respectively. The total number of methylated cytosines (CpG sites + non-CpG sites) was calculated as the average of total number of methylated cytosines across the 21 clones, for each temperature.

5.2.5. *Dnmt* cloning and sequencing

BLAST similarity searches against the nr database (<http://blast.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org/>) were performed to identify orthologues of *dnmt1*, *dnmt3a* and *dnmt3b* in other teleost species. Degenerate primers were designed against the most conserved regions of the sequences. Cloning and sequencing were essentially performed as previously reported (Campos et al. 2010). DNA sequences were analysed with the CodonCode Aligner v.3.7.1 software (CodonCode Corporation, USA) and their identity determined by BLASTN similarity searches against the NCBI and Ensembl databases. Partial coding sequences of 259 bp, 750 bp and 469 bp were obtained for Senegalese sole *dnmt1*, *dnmt3a* and *dnmt3b*, respectively (See Table 2 for GenBank Accession numbers).

5.2.6. Quantitative real-time PCR (qPCR)

One microgram of total RNA from each pool of larvae during metamorphosis was used to synthesise cDNA with the QuantiTect reverse transcription kit (Qiagen), as reported elsewhere (Fernandes et al. 2008). Specific qPCR primers were designed for the sole *myog* and *dnmts* sequences. Whenever possible, primers were designed to span at

least one intron/exon border to avoid amplification of potential contaminating genomic DNA, and then analysed with Netprimer (<http://www.premierbiosoft.com/>), as previously described (Fernandes et al. 2008). *Elongation factor 1 α* (*eef1a1*), *ribosomal protein 4* (*rps4*) and *ubiquitin* (*ubq*) were used as endogenous reference genes to normalise target gene expression. Primer sequences, amplicon sizes and qPCR amplification efficiencies are shown in Table 2. Quantification of gene expression was performed by qPCR with SYBR Green chemistry (Roche) on a LightCycler® 480 (Roche), as detailed elsewhere (Fernandes et al. 2008; Campos et al. 2012).

5.2.7. Muscle morphometry

Two fish at stage 2 metamorphosis were collected per tank and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) in phosphate buffered saline (PBS tablets, Sigma-Aldrich, St. Louis, Missouri, USA) for 6-12 h, and washed in PBS. Samples were then dehydrated in a graded ethanol (AGA, Prior Velho, Portugal) series, cleared in xylol (Prolabo, VWR International LLC, Radnor, PA, USA) and finally included in paraffin Histosec® (Merck, Whitehouse Station, NJ, USA). Larvae were sectioned (10 μ m) transversely to the body axis just posterior to the anus. Sections were then mounted on slides coated with aminopropyltriethoxysilane (APES) (Sigma-Aldrich, St. Louis, Missouri, USA), to improve adhesion and then stained with haematoxylin–eosin (Merck, Whitehouse Station, NJ, USA). Total cross-sectional muscle area [A (mm^2) (muscle)], the total number of fibre [N (fibres)] and the fibre cross-sectional area [\bar{a} (μm^2) (muscle fibre)] were measured. The total cross-sectional muscle area [A (fast muscle)] was computed after tracing the physical limits of interest of the section on the monitor, at a 200 x magnification. The outlines of muscle fibre were traced using a 400 x magnification. The fibre diameter (μm) was estimated indirectly, as the diameter of a circle having the same area of a fibre in a perfect cross-section. This study was performed using an Olympus BX51 microscope (Olympus Europa GmbH, Hamburg, Germany) with the Cell^B Basic imaging software.

5.2.8. Statistical analysis

The effects of the temperature on fast muscle fibres were evaluated using a covariance analysis (ANCOVA), in which temperature was set as the independent variable while the total length was set as a covariate.

Evaluation of expression stability for the three reference genes was performed using the statistical application *geNorm* (<http://medgen.ugent.be/>), as previously reported

(Fernandes et al. 2008). Expression of target genes was evaluated by the relative quantification method (Fernandes et al. 2008). Differences in the expression of target genes with temperature were examined by a one-way ANOVA with Holm-Sidak *post-hoc* tests using the SigmaPlot 11.0 statistical package (Systat software, London, UK). When the data did not meet the normality or equal variance requirements, a Kruskal-Wallis one-way ANOVA on ranks with a median test was performed instead. Significance levels were set at $P < 0.05$.

To check for differences in methylation levels at specific CpG positions of the *myog* putative promoter, an analysis of molecular variance (AMOVA) was performed using the Arlequin 3.5.1.2 software (<http://cmpg.unibe.ch/software/arlequin3/>). Each CpG site was trimmed from the original sequence (7-26 nucleotides in length), with two possible variants for each cytosine nucleotide: C if methylated and T if unmethylated. Then, all sequences from the same temperature were considered as one group, with size equivalent to the number of sequences analysed for each temperature ($n = 21$). Significance level was set at $P < 0.05$.

5.3. Results

5.3.1. Growth

Rearing temperature significantly affected dry weight (DW) and total length (L_T) of Senegalese sole larvae at stage 2 metamorphosis (Table 1). DW was 0.7 ± 0.2 , 0.5 ± 0.2 and 0.3 ± 0.2 mg at 21 °C, 18 °C and 15 °C, respectively ($P < 0.001$).

Table 1. Age, dry weight and total length of Senegalese sole larvae reared at 15, 18 or 21 °C.

Metamorphosis	21 °C	18 °C	15 °C
Days post hatching	12	15	23
DW (mg) (Mean \pm SD)	0.7 ± 0.2^a	0.5 ± 0.2^b	0.3 ± 0.2^c
L_T (mm) (Mean \pm SD)	6.9 ± 0.8^a	6.7 ± 0.8^a	5.5 ± 0.8^b

Different superscript letters indicate statistically significant differences ($P < 0.05$) between different treatments.

5.3.2. Characterization of the putative promoter and 5' UTR of the *myog* gene in Senegalese sole

A sequence of 523 bp comprising 187 bp of the putative *myog* 5' untranslated region (UTR) and 336 bp of its proximal promoter has been identified in Senegalese sole (Genbank accession number KC404969). Binding sites for MEF2, MEF3, TATA, Sox6 and TAF (putative TATA box binding protein associated factor) were present in this sequence and two putative non-canonical, muscle-specific E-box binding sites (5'-CAGTTG-3') were found separated by 8 bp (Figure 1). The most proximal elements to the translation initiation codon (ATG) are the two E-boxes in the 5' UTR. Putative binding sequences for MEF3 and MEF2 were located at positions -92 and at -70 from the transcription starting site (TSS), respectively, and three putative TATA box binding protein associated factors (TAF) were found at positions -213, -73 and +44 bp from the TSS.

The organisation of putatives E-boxes, MEF2 and MEF3-B binding sites in the Senegalese sole *myog* promoter was conserved amongst different fish species (Figure 1). Identity at the nucleotide level across Acanthopterygii was 100 %, 92 % and 100 % for the E-boxes, MEF3 and MEF2), respectively.

5.3.3. In muscle of Senegalese sole larvae, DNA methylation of the *myog* promoter is affected by temperature

To avoid any possible bias of the sequencing, the CpG site located in the reverse primer was not included in the analysis. Therefore, 21 CpG nucleotides up to 523 bp upstream of the ATG start codon were analysed for global methylation patterns and total number of methylated cytosines (Figure 1). A rearing temperature of 15 °C significantly increased overall methylation levels of the *myog* putative promoter (21.8 ± 4.9) in muscle of larvae undergoing metamorphosis relatively to the 21°C (17.8 ± 6.0) ($P < 0.05$) (Figure 2A). The methylation patterns proportions, expressed as percentage of the 144 bisulfite patterns in all temperature groups (CG, CHG and CHH, where H = A, C or T), were similar amongst temperatures: around 15% for CGN (CpG methylation), 20% for CHG (non-CpG methylation) and 65% for CHH (non-CpG methylation).

At several CpG sites, the cytosines were differentially methylated between temperature groups (Figure 2B). Significant differences were observed at position -203 (located 4 bp downstream of a TAF binding site) ($F_{st} = 0.122$, $P = 0.015$), position -165 ($F_{st} = 0.221$, $P = 0.000$) and position -72 from the TSS (located in a TAF binding site and separated by 1 bp from a MEF2 site) ($F_{st} = 0.148$, $P = 0.013$). CpG sites were also identified in the MEF3 (-88 bp) and in the TAF binding sites (-209 bp), even if no

significant differences were observed between temperatures. In fact, the CpG located in this latter TAF motif was one of the less methylated across all temperatures. Analysis of *myog* methylation in a non-muscle tissue (gut, $n = 6$) revealed that all CpG sites located in critical binding sites for muscle *myog* expression, such as MEF2 or MEF3, were 100% methylated at all temperatures (Supplementary Figure 1).

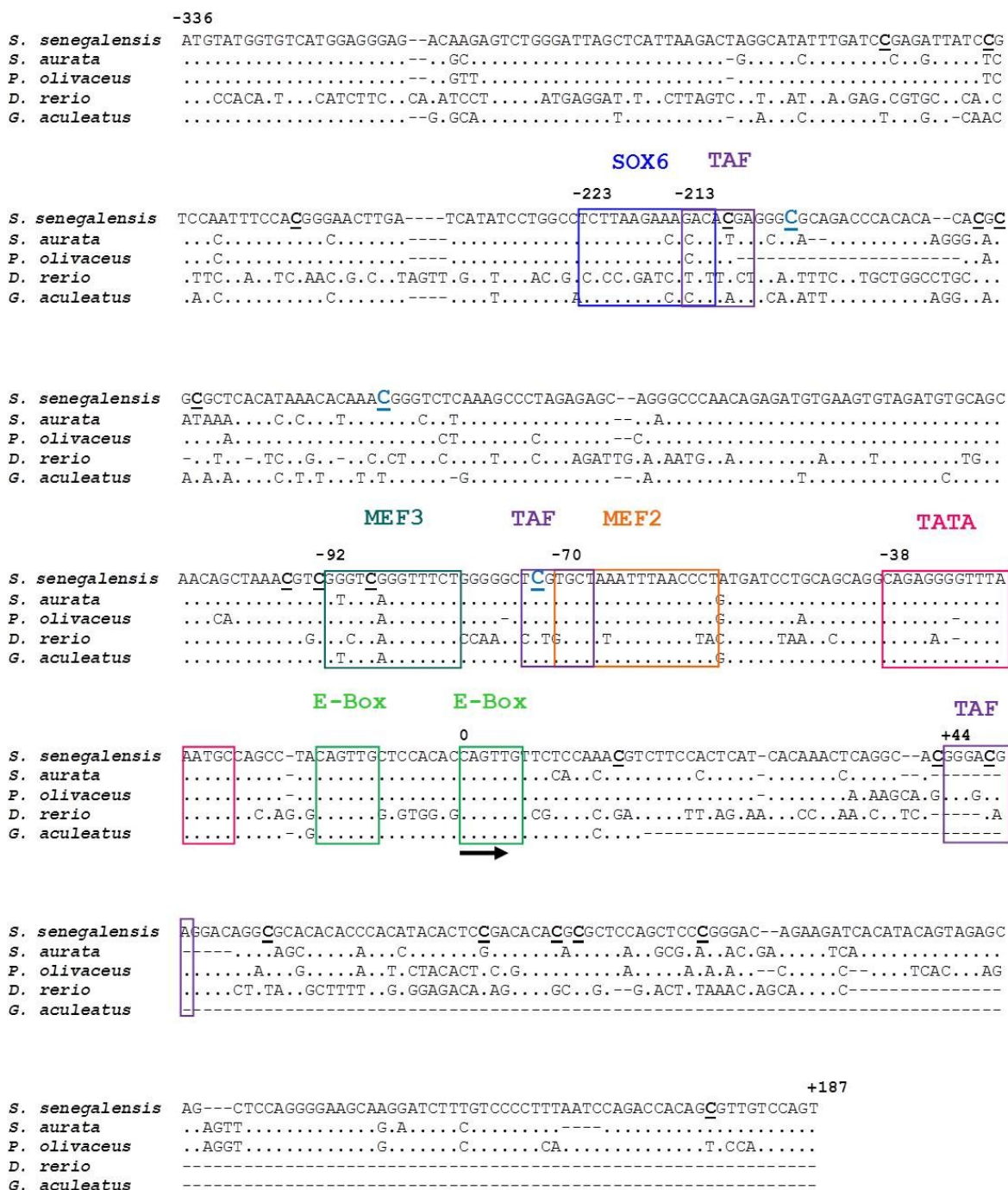


Figure 1. Comparison of the 5'-flanking UTR and putative promoter sequence of *myog* genes from Senegalese sole and other fish sequences. SOX6, TAF, MEF3, MEF2, TATA binding sites and the two proximal E-boxes are indicated. The sequence and localization the E-boxes, MEF2, MEF3 and TATA are particularly conserved among *myog* promoters from all five fish species. The predicted transcription starting site is indicated (arrow). The location of the 22 CpG sites is indicated by bold, underlined Cs. GenBank accession numbers for the sequences are: KC404969 (*Solea senegalensis*), EF462192 (*Sparus aurata*), EF144128 (*Paralichthys olivaceus*) and AY124482 (*Danio rerio*). The Ensembl accession number for *Gasterosteus aculeatus* is ENSGACG00000000349.

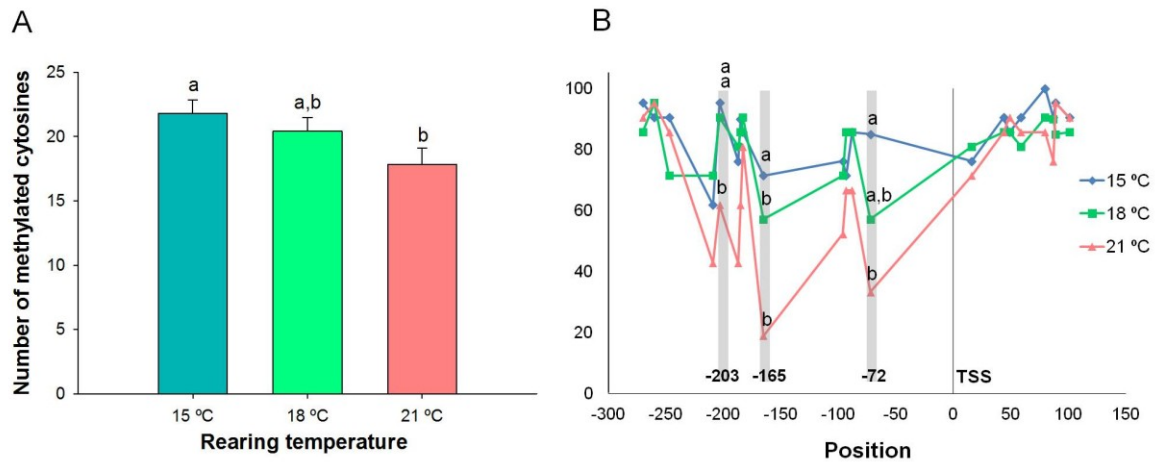


Figure 2. (A) Total number of methylated cytosines in the Senegalese sole myog proximal promoter in muscle of larvae reared at 15, 18 and 21 °C. Error bars indicate the standard error of the means for each treatment (N = 21). Different letters (and no common letters) indicate statistically significant differences between temperatures ($P < 0.05$) as determined by ANOVA. (B) Percentage of cytosine methylation at 21 CpG sites in Senegalese sole myog proximal promoter in muscle of larvae reared at different temperatures (15 °C, 18 °C and 21 °C). Different letters indicate significant differences between temperatures at each CpG position ($P < 0.05$), as determined by ANOVA. TSS represents the transcription starting site.

5.3.4. Expression of myog and dnmts homologues in Senegalese sole larvae changes with temperature

During metamorphosis, *myog* expression in Senegalese sole larvae was affected by rearing temperature (Figure 3) and 1.8-fold higher at 21 °C than at 15 °C ($P < 0.05$). In contrast, *dnmt1* expression was higher in the 15 °C group than in the 21 °C one ($P < 0.05$) and *dnmt3b* transcript levels were higher at 15 °C than at either 18 or 21 °C ($P < 0.05$). The *dnmt3a* paralogue had a uniform expression profile across temperatures (Figure 3).

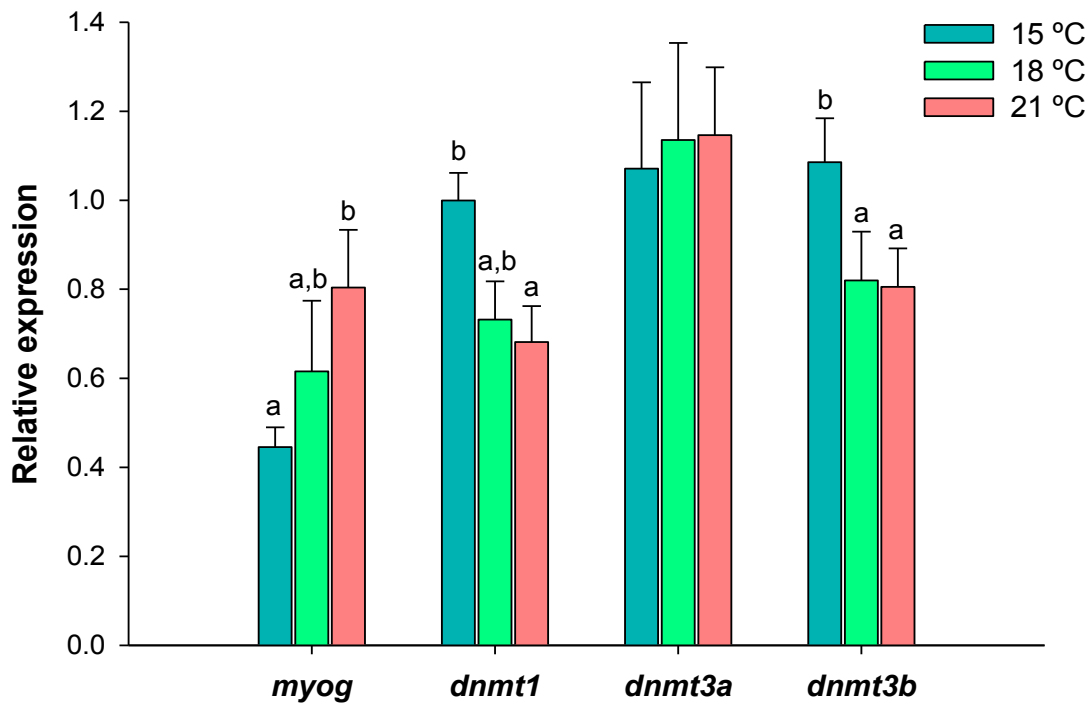


Figure 3. Relative expression of *myog*, *dnmt1*, *dnmt3a* and *dnmt3b* in Senegalese sole larvae reared at 15 °C (blue bars), 18 °C (green bars) and 21 °C (red bars) until metamorphosis stage 2 (see main text for details). Transcript levels were determined by qPCR and normalised within each developmental stage, using *eef1a1*, *rps4* and *ubq* as endogenous reference genes. Error bars indicate the standard error of the means for each treatment ($N = 6$). For each gene, significant differences between temperatures are indicated by different letters (without letters in common, $P < 0.05$).

5.3.5. Phenotypic plasticity of muscle growth in Senegalese sole larvae

Rearing temperature significantly influenced Senegalese sole fast skeletal muscle growth (Figure 4). Larvae reared at 21 °C showed a 1.6-fold increase in fibre diameter ($P < 0.001$) (Figure 4A) (6.0 ± 2.9 , 6.1 ± 2.6 and $9.5 \pm 3.8 \mu\text{m}$ at 15 °C, 18 °C and 21 °C, respectively) and a 3.1-fold increase in total-cross sectional area (A) ($P < 0.05$) (Figure 4B), relatively to fish from the 15 °C group. The mean number of fast fibres (N) was not significantly different between temperatures, despite the moderately higher number of fibres of the 21 °C treatment.

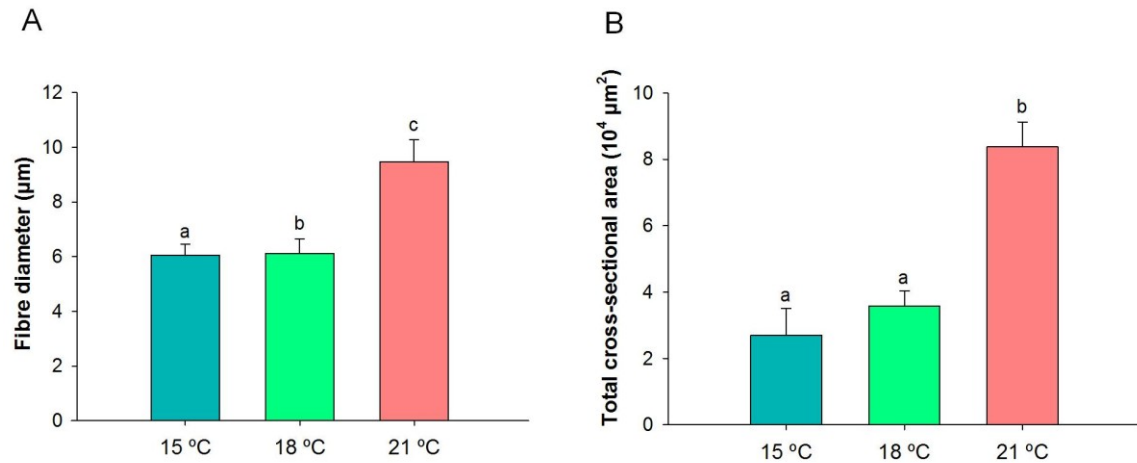


Figure 4. (A) Fiber diameter (μm) and (B) total cross-sectional muscle area A (μm²) of fast muscle in Senegalese sole larvae reared at 15 °C (blue bars), 18 °C (green bars) and 21 °C (red bars). Error bars indicate the standard error of the means for each treatment ($N = 6$) and different letters indicate significant differences between temperatures ($P < 0.05$).

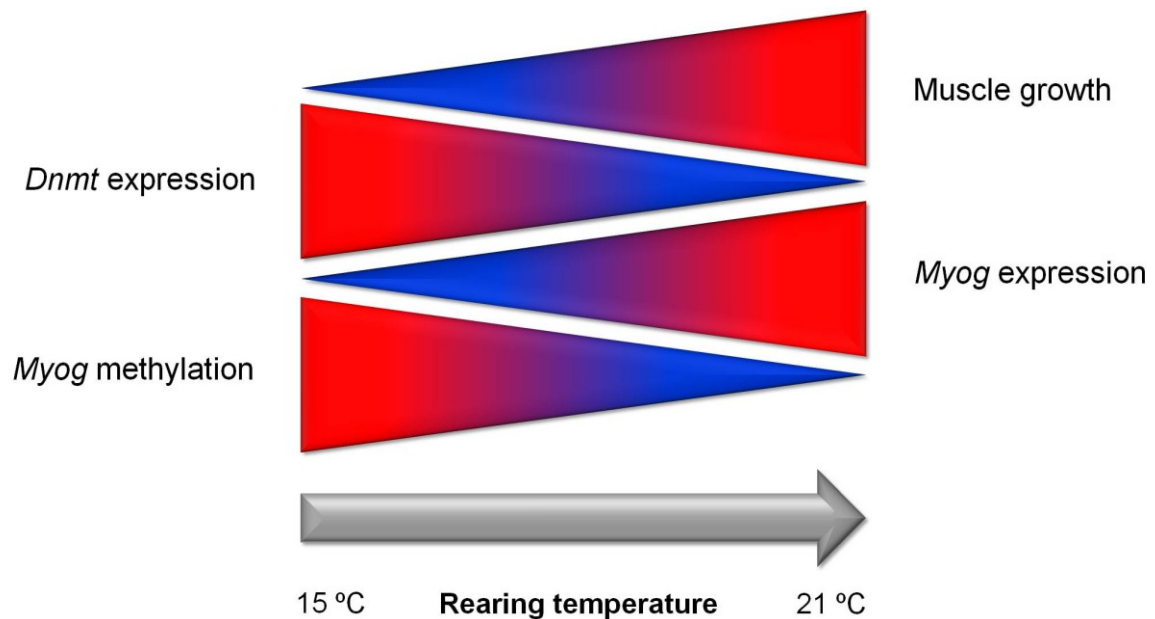


Figure 5. Simplified model of temperature-induced phenotypic plasticity of muscle growth in Senegalese sole. A higher rearing temperature (21 °C vs. 15 °C) lead to lower methylation levels of the *myog* proximal promoter in fast muscle, which correlated with a decrease in *dnmt1* and *dnmt3b* transcripts and an increase in *myog* expression and muscle growth. Blue and red indicate lower and higher levels of methylation, gene expression and muscle growth, respectively.

5.4. Discussion

5.4.1. *The putative proximal promoter of Senegalese sole myog is highly conserved and its methylation levels in skeletal muscle increase with lower rearing temperatures*

A higher methylation level of the *myog* promoter in skeletal muscle of Senegalese sole larvae was observed at 15 °C both in the global methylation of the promoter and specifically in CpG sites (Figures 2A and B). Non-CpG methylation has been shown to play an important role in mammals. For example, non-CpG hypermethylation of the *peroxisome proliferator-activated receptor-γ coactivator* promoter in skeletal muscle was found to decrease the mitochondrial density in diabetics type 2, contributing to impaired fat oxidation and excess lipid storage (Barres et al. 2009). In the Senegalese sole *myog* promoter, several CpC sites were also found dispersed throughout the sequence, raising the hypothesis that their differential methylation may influence *myog* transcription, alongside with methylation at specific CpG sites. It has been suggested that CpG and non-CpG methylation are in a dynamic equilibrium controlling *myog* transcription, and that active non-CpG demethylation during muscle differentiation occurs faster than CpG demethylation (Fuso et al. 2010).

The Senegalese sole *myog* promoter putative regulatory sequences were conserved relatively to other vertebrate species and it is likely that they play a similar role in controlling transcriptional activity. This region of the *myog* promoter contains several conserved DNA binding elements, such as the E-boxes, MEF2 and MEF3 binding sites and the TATA box. In the present study, three CpG sites were significantly hypermethylated at 15 °C compared to 21 °C and most of the others CpGs also had higher methylation levels at 15 °C, albeit not statistically significant (Figure 2B). One of the 15 °C-hypermethylated CpGs was located in a TAF (putative TATA box binding protein associated factor) binding site, which is involved in establishing the transcription initiation factor TFIID multimeric protein complex and plays a central role in mediating promoter responses to activation and repression (Verrijzer and Tjian 1996). Another 15 °C-hypermethylated CpG was also located 4 bp downstream of a putative TAF motif (-209 bp), which is relevant for the regulation of *myog* transcription. The putative TATA binding site was found to be highly conserved across species.

The MEF2 site, which is bound by elements of the MEF2 family of transcription factors (such as MEF2A, MEF2C or MEF2D), seems to be required for the activation of *myog* in some developmental contexts (Faralli and Dilworth 2012). In seabream (*Sparus aurata*) and zebrafish, deletion of the MEF2 binding site significantly reduced muscle-

specific expression of *myog* (Du et al. 2003; Codina et al. 2008). Given the known importance of the MEF2 regulatory motif, the status of cytosine methylation at this site is likely to interfere with the transcriptional activity of the *myog* promoter in Senegalese sole. The closest CpG site to the E-boxes significantly affected by temperature was the one located in a TAF and very close to the MEF2 motifs. It is plausible that the methylation status of regions flanking E-boxes in the of Senegalese sole *myog* promoter affect the binding of bHLH complexes, since it has been reported that flanking sequences, methylation status and interaction with adjacent regulatory elements contribute to selection of particular protein complexes by the E-boxes.(Luscher and Larsson 1999; Salero et al. 2003).

5.4.2. *Myog and dnmt expression are affected by temperature in Senegalese sole larvae*

In teleosts, *myog* transcripts are present during early development from somitogenesis onwards (Fernandes et al. 2006) and in post-embryonic stages its transcription is related to muscle growth by hyperplasia and hypertrophy (Johnston 2006). For example, in brown trout (*Salmo trutta*) larvae, *myog* expression persisted in zones of intense muscle hyperplasia at the dorsal and ventral apices of the myotome and next to the horizontal septum (Steinbacher et al. 2007).

In a previous study with Senegalese sole juveniles, *myog* expression in fast skeletal muscle was influenced by diet and positively correlated with growth parameters such as daily growth index or protein gain, which is indicative of *myog* importance in growth and protein accretion processes (Campos et al. 2010). We have found that expression of *myog* in Senegalese sole larvae undergoing metamorphosis was lower at 15 °C compared to 21 °C. This is consistent with the cytosine methylation pattern of the *myog* proximal promoter observed in muscle at different rearing temperatures, supporting the hypothesis that methylation of CpG sites and/or non-CpG methylation may influence *myog* expression in Senegalese sole larvae (Figure 5).

Expression of *dnmt1* and *dnmt3b* was significantly higher at 15 °C during metamorphosis, whereas lower mRNA levels were observed in larvae from the 18 and 21 °C groups. This corroborates the methylation results discussed above, since Dnmts are directly involved in DNA-methylation of cytosines. In mammals, DNA methylation patterns are somatically heritable through the action of Dnmt1, which is the maintenance methyltransferase (Reik 2007). The Senegalese sole Dnmt1 putative partial protein was shown to be highly conserved (94% and 83% identity with zebrafish and human counterparts, respectively) and it is likely that it plays similar functions across different

vertebrate taxa. Few studies on *dnmt1* expression have been performed in fish but Rai et al. (2006) reported that zebrafish *dnmt1* morphants exhibited a dramatic reduction of genomic cytosine methylation. Interestingly, expression patterns of *dnmt3a* and *dnmt3b* paralogues during zebrafish embryonic development were strikingly different. Moreover, temperature clearly influenced expression of these two genes in a different manner, suggesting that *dnmt3* paralogues are diverging and that *dnmt3a* and *dnmt3b* may play different roles in thermal epigenetic regulation of gene expression during early development in zebrafish (Campos et al. 2012). Given the higher similarity of zebrafish *dnmt3a* across vertebrate taxa and low non-synonymous/synonymous substitution ratios in the sequence, *dnmt3a* may have a more conserved function in vertebrate physiology than *dnmt3b* (Campos et al. 2012). It is not known if a specific Dnmt targets specific cytosines according to its location in a CpG or non-CpG context in the *myog* promoter. Nevertheless, such possibility should not be discarded given the distinct expression of *dnmt3* genes in Senegalese sole larvae among temperatures.

5.4.3. Muscle cellularity and growth are affected by temperature in Senegalese sole larvae

The ultimate size of a fish is shaped by the balance between recruitment and enlargement of muscle fibres and water temperature is one of the main constraints affecting muscle phenotype but the molecular basis of such phenotypic plasticity is still poorly understood (Johnston 2006). However, temperature impact on muscle growth depends largely on the species and on the temperatures to which they are exposed in their natural environment. For example, raising seabass larvae at 15 °C induces a higher muscle growth than higher or lower temperatures, but in rainbow trout, optimal growth is observed at 4 °C compared to 8 °C (Wilkes et al. 2001).

Senegalese sole is a species of relatively temperate waters and natural spawning of broodstock in captivity has been observed between 16 °C and 22 °C (for a review see Imsland et al. 2003). In the present study, fast muscle growth and cellularity in this species were largely affected by rearing temperature. To the best of our knowledge, this is the first study indicating that an epigenetic mechanism such as *myog* methylation in muscle of Senegalese sole larvae reared at different temperatures can affect gene expression and muscle growth (Figure 5). It is plausible that developmental time difference, an indirect effect of temperature, also affected DNA methylation and *myog* expression. It remains to be seen if the epigenetic events herein reported are conserved in other temperate-water fish species, with its implications for the aquaculture industry.

5.5. Acknowledgements

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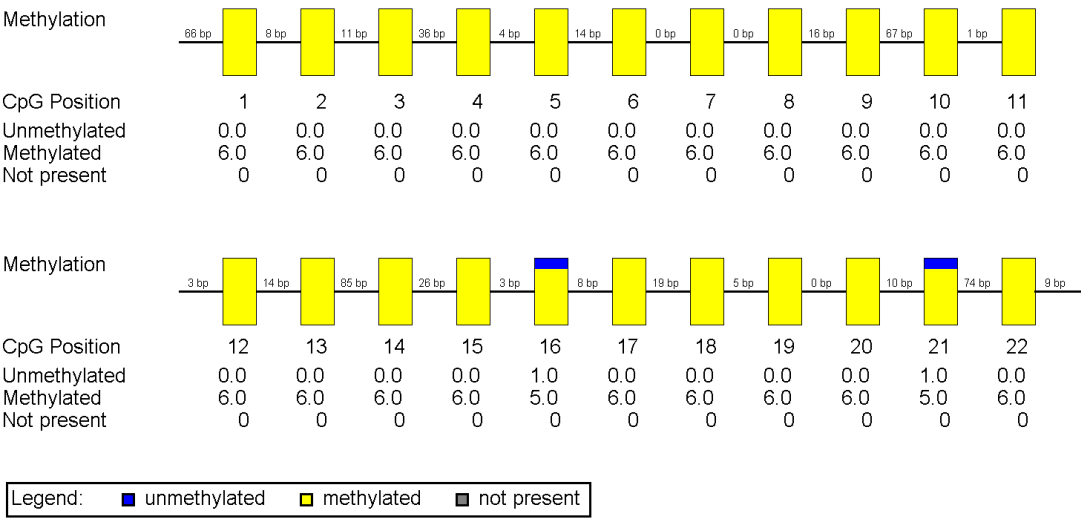
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Supplementary Figure 1.

Chapter 6

Rearing temperature affects Senegalese sole (*Solea senegalensis*) larvae protein metabolic capacity

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Rearing temperature affects Senegalese sole (*Solea senegalensis*) larvae protein metabolic capacity

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Rearing temperature affects Senegalese sole (*Solea senegalensis*) larvae protein metabolic capacity

Abstract

The present work examined the short- and long-term effects of three rearing temperatures on protein metabolism and growth trajectories of Senegalese sole larvae using ^{14}C -labelled *Artemia* protein as feed. A first feeding trial was performed on larvae reared at 15, 18 and 21 °C (at 26, 17 and 14 days post-hatching (dph), respectively) and a second trial conducted on post-larvae after transfer to the same rearing temperature (~ 20 °C) (49, 35 and 27 dph, in larvae initially reared at 15, 18 and 21 °C, respectively). Temperature greatly influenced larvae relative growth rate (RGR) and survival, since growth at 15 °C was severely depressed. Protein digestibility and retention was highest at 18 °C during the first trial (85.35 ± 1.16 and $86.34 \pm 2.33\%$, respectively). However, during the second trial, post-larvae from 15 °C had the highest feed intake and protein digestibility (3.58 ± 1.54 and $75.50 \pm 1.35\%$, respectively), although retention was similar between treatments. Furthermore, after transfer to 20 °C larvae from 15 °C experienced compensatory growth, which was observed until 121 dph, and confirmed by RGR values, which were significantly higher at 15 °C than at 21 °C or 18 °C. Results from the present study show that *Solea senegalensis* larval development, survival and protein digestion and retention are highly affected by thermal history.

Keywords: rearing temperature, *Artemia* intake, protein digestibility, retention efficiency, compensatory growth, *Solea senegalensis*

6.1. Introduction

Behavioural and physiological mechanisms in fish are highly modulated by thermal conditions, since their body temperature is very close to that of the surrounding water (reviewed by Angilletta et al. 2002). Temperature acclimation can result in changes in enzyme copy number and tissue activity (Somero 2004). In Senegalese sole (*Solea senegalensis*) juveniles, different temperatures lead to changes in plasma metabolites and increasing temperature raises metabolic activity (Costas et al. 2011). It has also been

shown that this species adjusts its osmoregulatory system to compensate the effects of temperature on electrolyte transport capacity and that thyroid hormones are implicated in temperature acclimation (Arjona et al. 2010). However, fish larvae are generally more sensitive to water temperature than juveniles or adults since their development is not complete (Johnston and Hall 2004) and have a much higher growth rate (Conceição et al. 1998).

Growth in fish is primarily due to protein deposition, and most marine teleost larvae have a high requirement of amino acids for protein deposition and growth, and also as major source of energy (reviewed by Rønnestad et al. 2003). Amino acid utilisation by fish larvae depends on their profile in the diet, on their digestibility and rate of absorption, on the profile of synthesised proteins and on their individual use for energy (Rønnestad and Conceição 2005). As larval ontogenic development takes course, their requirements of amino acids can change and some can become more efficiently absorbed than others (Finn and Fyhn 2010; Conceição et al. 2011). Senegalese sole larvae were shown to have a high *Artemia* protein digestibility between 12 and 35 dph (days post-hatching) as determined by ¹⁴C-labelling tracer studies (Morais et al. 2004). This indicates a high capacity for digesting live prey from a young age, which fuels their tremendous growth rate during that period (Conceição et al. 2007). Moreover, *Artemia* protein digestibility in this species is highly sensitive to nutritional history and physiological condition (Engrola et al. 2009; Engrola et al. 2010).

Variations in rearing temperature are particularly important in early stages of development of farmed species, as they can irreversibly affect fish potential to grow (Johnston and Hall 2004). In nature, Senegalese sole inhabits coastal and estuarine areas where temperatures can range between 13 and 28 °C (Vinagre et al. 2006), and large thermal variations have also been observed in aquaculture conditions (Imsland et al. 2003). Several studies have examined the effects of temperature on metabolism and swimming activity of fish larvae (von Herbing 2002; Lankin et al. 2008); however, the effects of temperature on protein digestibility and retention have been overlooked. Nevertheless, it was found that for example higher rearing temperatures increased the absorption and depletion rates of amino acids and also led to a higher retention efficiency of yolk nutrients in larvae of African catfish (*Clarias gariepinus*) (Conceição et al. 1998). Therefore, in the present study we examined the short and long term effects of different rearing temperatures during larval pelagic phase (from hatching until settlement on the bottom) on protein and amino acid absorption and retention in Senegalese sole larvae and post-larvae using an *Artemia* radiolabelling method.

6.2. Material and methods

6.2.1. Fish rearing and sampling

Senegalese sole eggs were incubated in a 200L cylindro-conical tank at a temperature $20.2 \pm 0.5^\circ\text{C}$. Newly hatched larvae were transferred to 9 fibreglass conical tanks (100L) tanks in a closed recirculation system with an initial density of 60 larvae L^{-1} . Three rearing temperatures were used (15.2 ± 0.5 , 18.3 ± 0.6 or $21.1 \pm 0.4^\circ\text{C}$) in triplicate groups. At mouth opening, larvae were fed rotifers (*Brachionus* sp.) enriched with DHA Selco® (Inve, Dendermonde, Belgium). *Artemia* was introduced according to developmental stage, which corresponded to different days in the three rearing temperatures. *Artemia* AF nauplii (na) (Inve) were introduced between 5 dph and 8 dph and metanauplii between 9 and 16 dph. *Artemia* enrichment was done at 250 000 nauplii/metanauplii L^{-1} , with 0.4 g L^{-1} supplementation of Easy DHA Selco® (Inve) and Micronised Fishmeal® (Ewos, Bathgate, UK) added in two doses of a 1 : 1 mixture (weight basis). Metanauplii supply was gradually changed from live to frozen *Artemia* from during settling at the bottom of the tank. After settling, larvae were transferred to flat-bottom plastic tanks (10 L volume, 600 larvae per tank), and maintained all at the same temperature ($20.4 \pm 0.5^\circ\text{C}$). Estimations of survival in newly-settled larvae were performed for all treatments. Weaning was initiated at 53 dph in post-larvae from 21°C , 57 dph in post-larvae from 18°C and 69 dph in the 15°C ones. Post-larvae were initially hand fed, twice a day (10.00 h and 17.00 h) with the diet AgloNorse no.1 (EWOS, Scotland) (0.2–0.6 mm) and co-fed with frozen *Artemia* metanauplii during one week. *Artemia* supply was gradually decreased to zero, and inert diet was then supplied by automatic feeders for 24 h per day. Juveniles were kept until 121 dph, where growth was measured. Survival rates of 121 dph juveniles (relatively to the number of benthic post-larvae placed in the flat-bottom tanks) were estimated for all treatments.

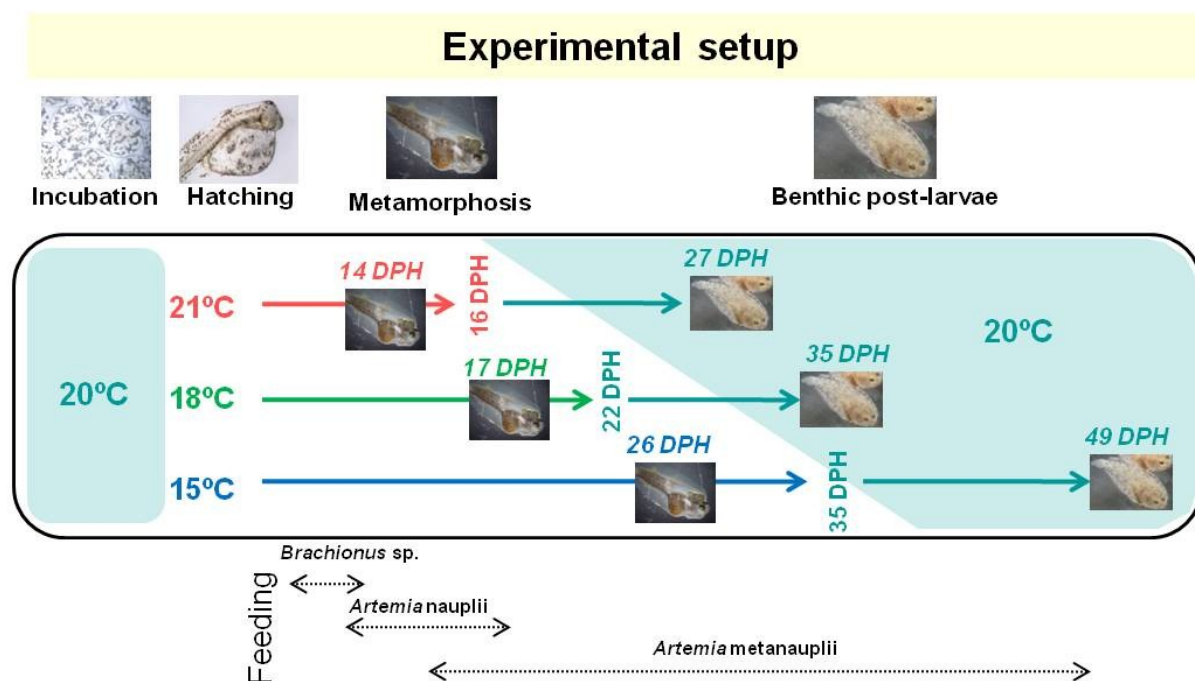


Figure 1. Experimental set-up. Senegalese sole larvae were reared at 21 (red), 18 (green) and 15 °C (blue) until acquiring a benthic lifestyle which occurred at 16 days post-hatching (dph) in larvae from 21 °C, 22 dph in larvae from 18 °C and 35 dph in larvae from 15 °C. Newly settled larvae were transferred all to the same rearing temperature (~ 20 °C, shaded area) at 16, 22 and 35 dph for 21, 18 and 15 °C, respectively. Samples for *Artemia* radiolabelling trials were taken at two developmental stages: metamorphosis and benthic post-larvae. Samples for the first trial were taken at 14, 17 and 26 dph (for 21, 18 and 15 °C, respectively) (italic), and for the second trial at 27, 35 and 49 dph (for larvae initially reared at 21, 18 and 15 °C, respectively) (italic). (Color figure online)

6.2.2. *Artemia* radiolabelling trials

Artemia radiolabelling trials were conducted at two developmental stages, corresponding to different ages according to larval rearing temperature (Figure 1). Metamorphosis (stage 3 according to Fernández-Díaz et al. (2001)), occurred at 14 dph at 21 °C, 17 dph at 18 °C and at 26 dph at 15 °C. The second *Artemia* labelling was conducted on benthic post-larvae that had been maintained at the same temperature since settlement (~20 °C): larvae initially reared at 21 °C: 27 dph (12 days after transfer); larvae initially reared at 18 °C: 35 dph (13 days after transfer); larvae initially reared at 15 °C: 49 dph (14 days after transfer) (Figure 1).

Before the radiolabelling trials, 10 larvae from each temperature at metamorphosis stage and 50 post-larvae from each temperature at benthic stage were acclimatised to room temperature (20 °C) and fasted for 16 h. *Artemia* was radiolabelled according to the

method developed by Morais et al. (2004). *Artemia* nauplii were enriched with a [$U-^{14}C$] uniformly labelled protein hydrolysate (3.7 MBq ml; American Radiolabeled Chemicals, Inc., Saint Louis, U.S.A.) during a 16 h period, at a density of 200 *Artemia* mL⁻¹ in a sealed incubation system at 28°C and with a dose of 3.3 µL of the [$U-^{14}C$] protein hydrolysate per mL of seawater. The incubation system was connected to a KOH trap (1 L, 0.5 M) to capture the radiolabelled $^{14}CO_2$, avoiding its release into the atmosphere. At the end of each enrichment period, *Artemia* were thoroughly washed and triplicate samples of *Artemia* and *Artemia* incubation water (3 mL) were taken to measure the incorporated radiolabel using a 6 % trichloroacetic acid (Sigma-Aldrich, St. Louis, Missouri, USA) (TCA) extraction method, to be able to correct for the ^{14}C present in the incubation seawater.

At each trial, dry weight (DW) was measured on -80 °C freeze-dried sole larvae ($n = 3$ in pools of 20 larvae, ± 0.001 mg). In the second radiolabelling trial a photo was also taken from each post-larva (50 per temperature), and from the calibration eyepiece (15 mm). The total length (mm) (L_T) of larvae was determined using UTHSCSA Image Tool 3.00 software (University of Texas Health Science Center, Texas, USA).

First radiolabelling trial

After the acclimation period, 10 larvae per temperature at the metamorphosis stage were allowed to feed for a 30 min period in a common tank and then carefully transferred one by one with an inverted Pasteur pipette through two tanks with clean seawater (to eliminate any ^{14}C amino acids that could be present in the surface of the larvae), and subsequently transferred to an incubation vial. This feeding period of 30 min is shorter than sole larvae gut transit time (Morais et al. 2004), and sole ceased feeding activity in all trials groups well before the 30 min exposure. Individual larva were kept in a sealed chamber containing 7 ml of seawater (considered to retain all labelled ^{14}C from larvae evacuation), which was connected by a capillary to a $^{14}CO_2$ trap (5 ml KOH, 0.5 M). After 24 h, larvae were removed from the vials, and rinsed in clean seawater. No mortalities or perceptible changing of behaviour were observed during this period. Following larval sampling, the incubation vial was resealed and 1 ml of hydrochloric acid (0.1 M HCl) was added in a series of gradual steps, resulting in a progressive decrease of pH that causes the rapid diffusion of any remaining CO_2 from the water into the metabolic trap (Ronnestad et al. 2001). Larvae were placed in separate 6 ml scintillation vials (Sarstedt) and were submitted to a 24 h, 6 % TCA solution for free amino acid (FAA) extraction (0.5 ml, with periodical shakings, at 4 °C). After removal of the TCA soluble fraction into a clean 6 ml vial, the TCA precipitate was solubilised with 500 µL Solvable (PerkinElmer, U.S.A.) at 50 °C during 24h.

Second radiolabelling trial

In the second trial, 50 post-larvae at the benthic stage with distinct temperature histories (15, 18 and 21 °C) were sampled 12 - 14 days after transfer to 20 °C. After the acclimation period, post-larvae were fed over a period of 30 min, and transferred into the incubation chambers. Samples were taken in a time-course at 2, 4, 6, 8 and 24 hours after feeding (HAF) (meaning $n = 10$ at each sampling point during the time-course). One dead larva was observed in the 21 °C group and one in the 18 °C group. No perceptible changing of behaviour was observed during this period. HCl (0.1 M) was added to the incubation vial in a series of gradual steps, as above. Samples were then submitted to a 24 h, 6 % TCA solution for FAA extraction. After removal of the TCA soluble fraction into a clean 6 ml vial, the TCA precipitate was solubilised with 500 µL Solvable at 50 °C during 24h.

In both trials, the TCA soluble and precipitate fraction were prepared for radioactivity counting by adding 5ml of scintillation cocktail (Ultima Gold XR, PerkinElmer, U.S.A.), and 15 ml were also added to the incubation water and KOH-CO₂ trap. The samples were counted on a Tri-Carb 2910TR liquid scintillation analyser (PerkinElmer, U.S.A.) and the results are presented as a percentage of the counts (DPM: disintegrations per minute) found in each compartment in relation to the total counts.

Artemia intake (AI, % BDW – body dry weight) and protein utilisation was determined in both radiolabelling experiments. *Artemia* intake after a single meal of radiolabelled *Artemia* was determined according to Conceição et al. (1998):

$$AI = [(R_{total} / SR_{Artemia}) / DW_{fish}] \times 100$$

where R_{total} is the sum of the radioactivity in the incubation seawater, in the metabolic trap and in the larvae (DPM), $SR_{Artemia}$ is the specific radioactivity per *Artemia* samples (DPM mg⁻¹, *Artemia* DW considered as 0.002 mg) and DW_{fish} is the fish dry weight (mg). Sole that did not ingest any prey were eliminated from the analysis.

Protein utilisation was determined based on protein digestibility (D, %), protein retention efficiency (R, %), catabolism fraction (C, %), and evacuation (E, %). These estimates were determined as:

$$D = [(R_{body} + R_{CO_2 \text{ trap}}) / (R_{body} + R_{CO_2 \text{ trap}} + R_{water})] \times 100;$$

$$R = [R_{body} / (R_{body} + R_{CO_2 \text{ trap}})] \times 100;$$

$$C = [R_{CO_2 \text{ trap}} / (R_{body} + R_{metabolic \text{ trap}})] \times 100;$$

$$E = [(R_{\text{water}})/(R_{\text{body}} + R_{\text{CO}_2 \text{ trap}} + R_{\text{water}})] \times 100;$$

where R_{body} is the total radioactivity in fish body (DPM), $R_{\text{CO}_2 \text{ trap}}$ is the total radioactivity per CO_2 trap (DPM), and R_{water} is the total radioactivity in the incubation seawater (DPM).

6.2.3. Data analysis

Growth was expressed as relative growth rate (RGR, % day⁻¹) between mouth-opening and metamorphosis (stage 3), metamorphosis (stage 3) and post-metamorphosis, and post-metamorphosis and 121 dph. RGR was determined as: $(e^g - 1) \times 100$ with $g = [(\ln_{\text{final weight}} - \ln_{\text{initial weight}})/\text{time}]$ in DW (mg) (Ricker 1958).

Absorbed (trap, gut, gut FAA, body and body FAA) and evacuated (water) portions were expressed as DPM percentage of total tracer fed to a given larvae. Catabolism (trap) and retention (FAA and body) were expressed as a percentage of total absorption (trap, FAA and body). Metabolic budgets were calculated after subtraction of the blanks of each fraction.

Data were tested using a one-way ANOVA following Tukey HSD post-hoc tests. Significance levels were set at $P < 0.05$. When the data did not meet the normality and/or equal variance requirements, a Kruskal–Wallis one-way ANOVA by ranks and Median test was performed instead. All results expressed as percentage were previously arcsine transformed. Data are presented as Mean \pm SD. Statistical analyses were performed using the STATISTICA 8.0 software package (StatSoft, Inc., Tulsa, USA).

6.3. Results

6.3.1. Growth

Rearing temperature had a significant impact on larvae DW by the time of the first radiolabelling trial ($P < 0.001$), where larvae were still at different temperatures (15, 18 and 21 °C) (Table 1). This effect was not significant by the time of the second sampling, where larvae were already maintained at the same temperature (~20 °C) (Table 1). There were great differences in RGR (% day⁻¹) between temperatures at metamorphosis (Table 1), since RGR at 21 °C was significantly higher than at 18 °C ($P < 0.05$) and 15 °C ($P < 0.001$). Between metamorphosis and post-larvae, there were no significant differences in RGR between temperatures (Table 1). However, between post-larvae and juveniles at 121 dph, RGR was significantly higher in fish initially reared at 15 °C compared to 18 °C

and 21 °C ($P < 0.05$) (Table 1). Rearing temperature also affected the survival of newly-settled larvae, since larvae from 21 °C ($27.7 \pm 6.7\%$) had a higher survival rate of almost 3-fold relatively to the 15 °C ones ($10.2 \pm 9.1\%$) ($P < 0.05$). Larvae from 18 °C had a survival rate of $25.1 \pm 1.8\%$ (Table 1). However, survival of juveniles at 121 dph was not significantly different between temperatures (Table 1).

Table 1.

Growth in larvae and post-larvae of Senegalese sole reared at 15, 18 and 21 °C at the two *Artemia* radiolabelling feeding trials and at 121 days post-hatching.

First feeding trial – Sampled at same developmental stage ¹	21 °C	18 °C	15 °C
Developmental stage ¹	Metamorphosis (3)	Metamorphosis (3)	Metamorphosis (3)
Days post-hatching	14	17	26
DW (mg) (Mean ± SD)	0.9 ± 0.3 ^a	0.6 ± 0.2 ^b	0.4 ± 0.2 ^c
RGR ³ MO-Met (%/day) (Mean ± SD)	28.9 ± 0.8 ^a	20.9 ± 2.6 ^b	10.1 ± 1.8 ^c
Survival ² MO-Met (%) (Mean ± SD)	27.7 ± 6.7 ^a	25.1 ± 1.8 ^{a,b}	10.2 ± 9.1 ^b
Second feeding trial - Sampled at same developmental stage ¹	20 °C		
Developmental stage ¹	Post-metamorphic	Post-metamorphic	Post-metamorphic
Days post-hatching	27	35	49
Days after transfer to 20°C	12	13	14
DW (mg) (Mean ± SD)	2.1 ± 0.7	2.1 ± 0.6	2.0 ± 1.0
<i>L_T</i> (mm) (Mean ± SD)	11.9 ± 1.3 ^a	11.1 ± 1.2 ^b	12.0 ± 1.8 ^a
RGR ³ Met-Post-met (%/day) (Mean ± SD)	7.2 ± 0.0	7.1 ± 0.8	7.0 ± 0.4
DW 121 dph (mg) (Mean ± SD)	237.9 ± 151.4 ^a	187.7 ± 142.1 ^b	228.9 ± 155.4 ^a
RGR ³ Post-met-121dph (%/day) (Mean ± SD)	5.2 ± 0.2 ^a	5.4 ± 0.2 ^a	6.8 ± 0.4 ^b
Survival ² 121 dph (%) (Mean ± SD)	24.1 ± 5.8	33.0 ± 4.2	31.8 ± 9.3

¹ Developmental stages according to Fernandez-Diaz et al. (2001)

² Survival of newly settled larvae and by 121 dph

³ Relative growth rate (RGR, % day⁻¹) between mouth opening (MO) and post-metamorphosis, between metamorphosis and post-metamorphosis, and between post-metamorphosis and 121 dph juveniles was determined as: (eg-1) $9 \log_{10} \frac{g}{g_0}$ with $g = \frac{[\ln(\text{final weight}) - \ln(\text{initial weight})]}{\text{time}}$ in DW (mg)

Different superscript letters indicate statistical differences ($P < 0.05$) between different treatments

6.3.2. *Artemia* labelling at metamorphosis climax

***Artemia* intake**

Rearing temperature had a significant effect on sole larvae *Artemia* intake (% BDW) (Figure 2), when larvae from different rearing temperatures were assayed for feeding intake at the same temperature. Larvae from 15 °C showed a higher *Artemia* intake ($3.46 \pm 1.57\%$) than larvae from 18 °C ($1.71 \pm 0.45\%$) ($P < 0.05$) (Figure 2). Intake was not significantly different between the 18 and 21 °C groups. However, larvae from 21 °C ate a significantly higher number of preys (13.34 ± 5.42) than larvae from 18 °C (5.25 ± 1.39) ($P < 0.05$), whereas larvae from 15 °C ate a mean of 7.47 ± 3.39 *Artemia*.

Protein metabolism

The protein digestibility (absorbed fraction) was significantly higher in larvae from 18 °C than in larvae from 21 and 15 °C rearing temperatures ($80.35 \pm 1.16\%$, $71.28 \pm 1.58\%$ and $65.79 \pm 1.85\%$, respectively) ($P < 0.001$ between all treatments) (Figure 3A). It should be noted that larvae from different rearing temperatures were assayed for protein metabolism at a same temperature. Protein retention and catabolism were also affected by rearing temperature. Retention efficiency was higher in larvae from 18 °C ($86.34 \pm 2.33\%$) than in larvae from 15 °C ($80.70 \pm 5.31\%$) ($P < 0.05$) (Fig 3B). At 21 °C larvae retained $84.82 \pm 3.88\%$ of the labelled protein and catabolised $15.18 \pm 3.88\%$ (Figure 3B). Catabolism was lower at 18 °C ($13.66 \pm 2.33\%$) comparatively to larvae reared at 15 °C ($19.30 \pm 5.31\%$) ($P < 0.05$) (Figure 3B). Excreted portion was significantly different between all temperatures ($P < 0.001$) with highest values in larvae from 15 °C ($34.21 \pm 1.85\%$) ($19.65 \pm 1.16\%$ and $28.72 \pm 1.58\%$ at 18 and 21 °C, respectively) (Figure 3A).

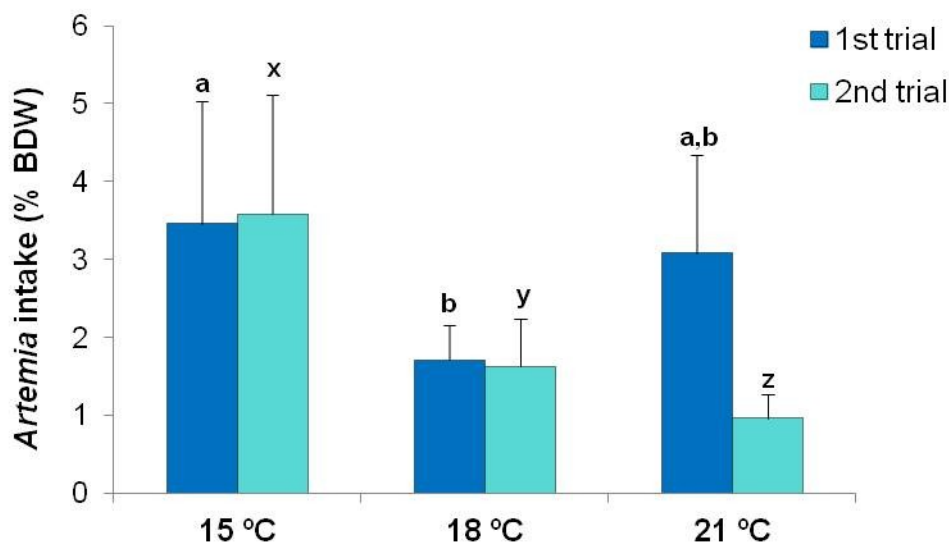


Figure 2. *Artemia* intake of Senegalese sole in the first trial (where larvae were at different rearing temperatures) and in the second experiment (where larvae from all groups were already at the same temperature for 12 or more days). Samples were taken at the same developmental stage in each experiment. Larvae from different rearing temperatures were assayed for *Artemia* intake at the same temperature (20 °C). Values are mean \pm SD of sole *Artemia* intake ($n = 10$ and 50 for each trial, respectively). Different letters indicate statistical differences between sole from different treatments in the first trial (a, b, c) ($P < 0.05$) and in the second trial (x, y, z) ($P < 0.001$).

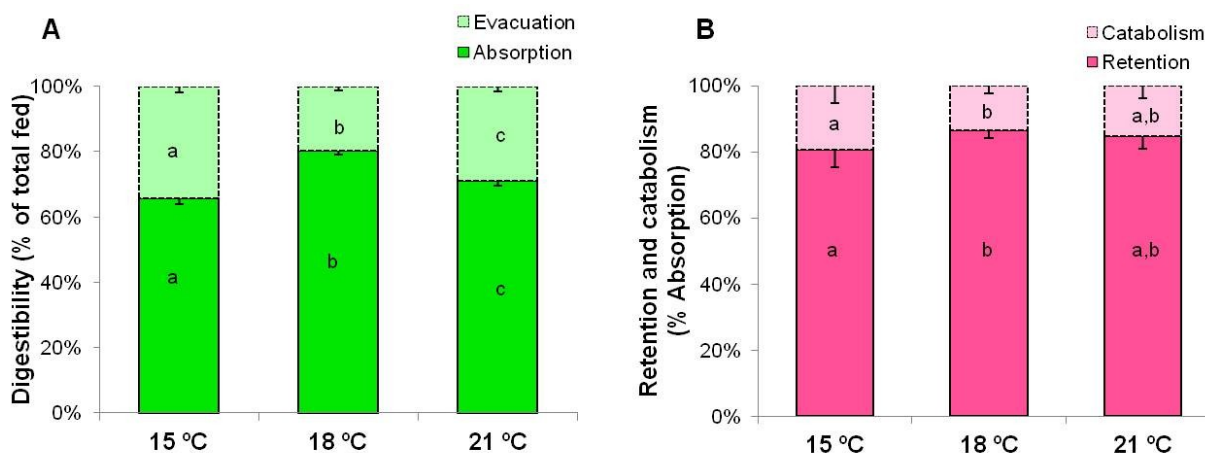


Figure 3. Protein digestibility (A) (% of radiolabel in the sole (absorption) and water (evacuation) in relation to the total quantity of radiolabelled feed) and retention and catabolism (B) (% of radiolabel in the sole in relation to absorption label) in sole larvae reared at 15 (26 dph), 18 (17 dph) and 21 °C (14 dph), 24 h after feeding a single meal of radiolabelled *Artemia* in trial 1. Larvae from different rearing temperatures were assayed for protein metabolism at the same temperature (20 °C). Values are mean \pm SD of sole retention and catabolism ($n = 10$). (a, b, c) Different letters indicate statistical differences ($P < 0.05$) between treatments.

6.3.3. Time-course experiment in sole post-larvae

Artemia intake

The number of *Artemia* ingested by Senegalese sole was significantly lower in larvae initially reared at 21 °C (10.21 ± 3.29) than in larvae from 15 °C rearing temperature (36.55 ± 15.69) ($P < 0.001$). Post-larvae from 18 °C ingested 17.23 ± 6.63 *Artemia* prey. *Artemia* intake (% BDW) showed a similar pattern, with values of $0.96 \pm 31\%$, $1.62 \pm 0.62\%$ and $3.58 \pm 1.54\%$ in larvae from 21, 18 and 15 °C temperatures, respectively ($P < 0.001$ for all treatments) (Figure 2).

Protein metabolism

Artemia digestion, absorption and metabolism patterns during the time-course showed comparable trends across temperatures, but also significant differences at specific time points between the different temperatures (Figure 4A-D). Generally, evacuation and catabolism increased over time until eventually reach a plateau and inversely, absorption and retention decreased over time.

Protein digestibility (absorbed portion) at 2 HAF was significantly higher at 15 °C than at 21 °C ($P < 0.05$), and the inverse occurred with evacuation (Figure 4A,C). Moreover, the total amount of radiolabel in the larvae was significantly higher in larvae from 15 °C and 18 °C compared to the 21 °C treatment ($P < 0.05$) (Figure 4B), with the correspondent inverse pattern in catabolism (Figure 4D).

Evacuated portion was significantly higher at 4 HAF than at 2 HAF, meaning that the digestive process was taking place ($P < 0.001$). Nevertheless, the amounts of radiolabel found in the larvae were significantly higher at 18 °C ($95.06 \pm 3.05\%$) than at 15 °C ($89.10 \pm 4.58\%$) ($P < 0.05$) or 21 °C ($86.22 \pm 4.42\%$) ($P < 0.001$) (Figure 4B). An inverse pattern was observed in the catabolised portion, since catabolism in the 18 °C treatment was lower than at 15 °C ($P < 0.05$) and 21 °C ($P < 0.001$) (Figure 4D).

Between 4 and 6 HAF, the catabolised and excreted fractions greatly increased in post-larvae from 18 °C ($P < 0.05$ and $P < 0.001$ for catabolism and evacuation, respectively) (Figure 4C, D). At 6 HAF, the protein digestibility was higher in larvae from 15 °C ($79.08 \pm 4.84\%$) than in the ones from 18 or 21 °C (62.16 ± 6.09 and $61.43 \pm 4.28\%$) ($P < 0.001$) (Figure 4A), and an inverse pattern was observed in the evacuated portion (Figure 4C). Nevertheless, there were no significant differences between temperatures in the amounts of radiolabel retained in the larvae (Figure 4B), as well as in the catabolised fraction (Figure 4D).

Between 6 and 8 HAF, there was a slight but non-significant increase in the catabolised and excreted fractions for each treatment (Figure 4C, D) and a concurrent decrease in the amounts of radiolabel in the larvae (Figure 4A, B). At 8 HAF, the absorbed portion was higher in post-larvae from the 15 °C treatment than in the 18 or 21 °C ones ($P < 0.001$) (Figure 4A), and the levels of excreted radiolabel were significantly higher in the 18 and 21 °C treatments compared with the 15 °C ($P < 0.001$) (Figure 4C). Nevertheless, there were no significant differences in the radiolabel retained in the larvae between temperatures at this point as well as in the catabolism (Figure 4B, D).

At 24 HAF, absorption was very similar to that at 8 HAF in all temperatures (Figure 4A) as well as the evacuated fraction (Figure 4C). Retention efficiency showed the lowest values of all time course and was significantly lower at 24 HAF than at 8 HAF for the 15 ($P < 0.05$) and 21 °C ($P < 0.001$) treatments (Figure 4B). In contrast, catabolism had the highest values of all time course and was significantly higher than at 8 HAF in the 15 °C treatment ($P < 0.05$) (Figure 4B). Protein digestibility at 24 HAF was significantly higher at 15 °C (75.50 ± 1.35) than in the 18 °C (59.56 ± 8.42) ($P < 0.05$) or 21 °C treatments (55.38 ± 1.75) ($P < 0.001$) (Figure 4A), and the excreted portion was higher in post-larvae from 21 to 18 °C than in 15 °C ones ($P < 0.05$) (Figure 4C). No differences were seen in the retention or catabolism values amongst temperatures (Figure 4B, D).

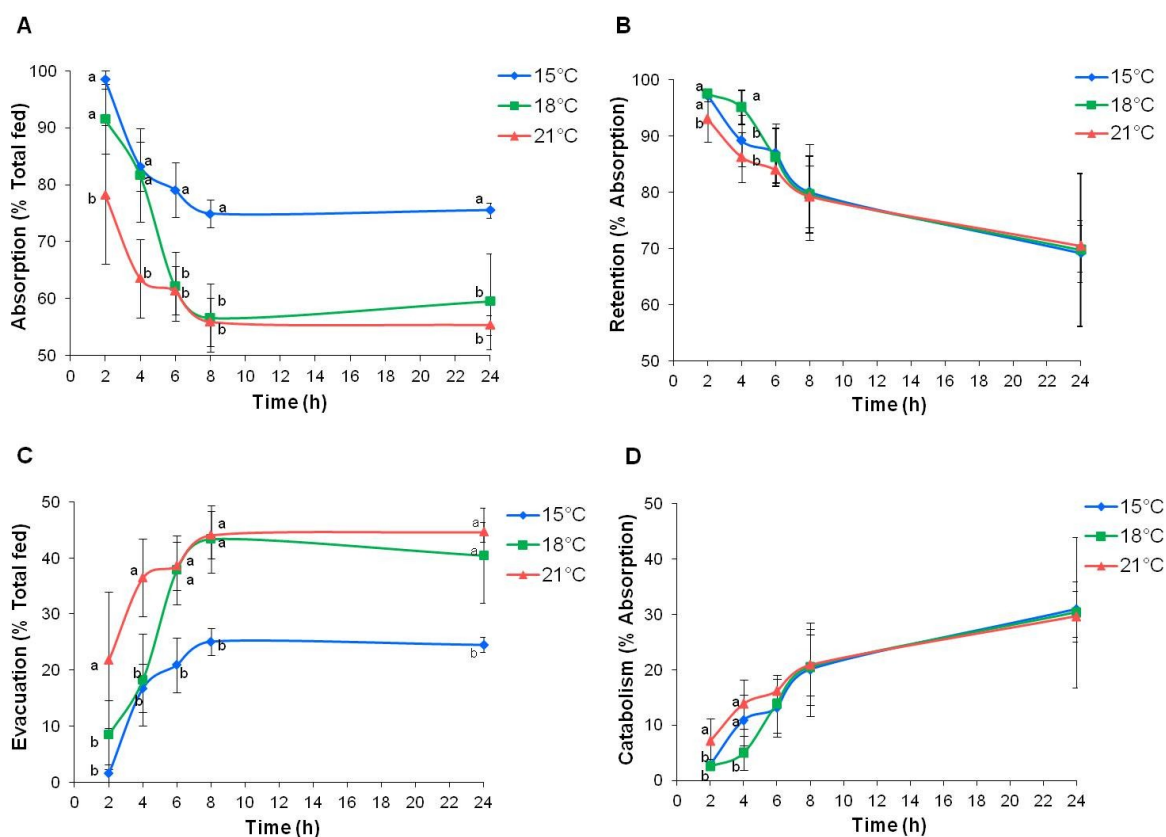


Figure 4. Percentage of protein digestibility A (% radiolabel in body + metabolic trap in relation to the total radiolabel fed), protein retention B (% of radiolabel in the metabolic trap in relation to digested label), evacuation C (% of radiolabel in the sole and water in relation to the total radiolabel fed) and catabolism D (% of radiolabel in the sole in relation to digested label), measured at 2, 4, 6, 8 and 24 HAF in sole post-larvae at the same developmental stage with 27, 35 and 49 dph (21, 18 and 15 °C, respectively), 12–14 days after transfer to the same rearing temperature (~ 20 °C). Values are mean \pm SD ($n = 10$). (a, b, c) Different letters indicate statistical differences ($P < 0.05$) between treatments.

6.3.4. Comparison of protein metabolism on 1st and 2nd trials

When comparing the two trials at 24 HAF, larvae from the 18 and 21 °C treatments showed a higher digestibility during metamorphosis ($P < 0.001$) (Figure 5A). An inverse pattern was observed in the larvae from the 15 °C group, since protein digestibility was higher in the older larvae ($P < 0.001$) (Figure 5A). Retention efficiencies in sole were higher during metamorphosis in all temperatures (Figure 5B) ($P < 0.001$ for 15 and 21 °C, and $P < 0.05$ for 18 °C).

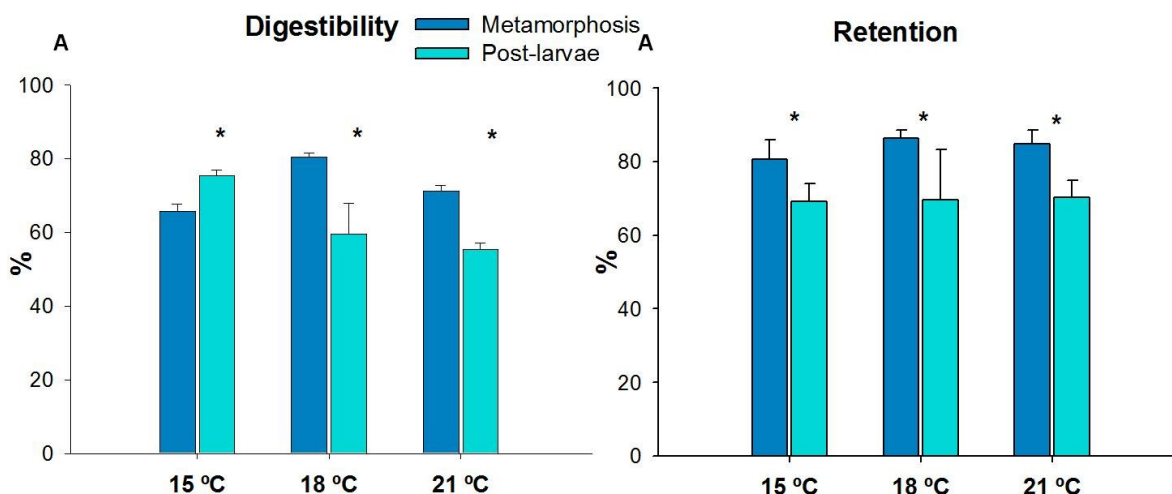


Figure 5. Protein digestibility (a) and retention (b) measured in sole larvae reared at 15 (26 dph), 18 (17 dph) and 21 °C (14 dph) during metamorphosis and after 24 HAF, and in post-larvae with 27, 35 and 49 dph (21, 18 and 15 °C, respectively) after transfer to the same rearing temperature (~ 20 °C) and after 24 HAF. Larvae from different rearing temperatures were assayed for protein metabolism at the same temperature (20 °C). Values are mean \pm SD ($n = 10$). Asterisk indicates statistical differences ($P < 0.05$) between trials.

6.4. Discussion

The present work examined the short and long term effects of three different rearing temperatures (15, 18 and 21 °C) during larval pelagic phase, on Senegalese sole larvae (metamorphosis climax) and post-larvae protein metabolism. At metamorphosis climax the objective was to study the effect of rearing temperature on the potential for feed intake, protein digestibility and catabolism and not to compare food intake and protein metabolism at the different temperatures (the results could be predicted once we are talking about a poikilotherm). The benthic post-larvae were evaluated at the same temperature as that of the rearing tanks (20 °C), and the long term effect of the initial rearing temperature was hence determined. It should be noted that larvae were transferred to the feeding trial temperature 16 hours prior to the trial itself to allow for physiological adaptation. Previous studies had shown that sole larvae tolerate and adapt quickly to temperature changes in the range 15 - 21 °C, a temperature fluctuation range which is normal in their natural habitat (Dinis 1986) e.g., shallow estuarine waters.

Similarly to what has been showed in other studies (Morais et al. 2004; Engrola et al. 2009), we found that Senegalese sole larvae and post-larvae have a great capacity to digest and absorb *Artemia* proteins and amino acids, thus confirming that feeding

radiolabelled *Artemia* to sole larvae is a suitable method to study protein metabolism in this species. The feed intakes observed in our study were generally lower than the intakes observed by other authors in Senegalese sole larvae (Mai et al. 2009; Engrola et al. 2010). However, that did not seem to influence the outcome of the tracer results nor the general fish growth, as DW in both experiments were on the expected range for this species at these developmental stages. *Artemia* labelling is a quite resilient method to study protein metabolism at different feed intakes providing good growth rates are obtained (Conceição et al. 2007).

Differences in water temperature during pelagic phase not only affected larvae growth, fact also reported in other marine fish species (Fonds 1979; Houde 1989; Green and Fisher 2004; Alami-Durante et al. 2007), but also protein metabolism in both the short term (metamorphosis) and the long term (benthic stage). There was a striking distinction regarding larvae size in the 15 °C treatment during the first trial since these larvae were particularly small, indicating that a lower temperature had a negative effect on growth and development, a fact also corroborated by the low RGR values. Survival rates of fish larvae have been largely correlated with water temperature (Houde 1974; Keller and Klein-MacPhee 2000; Dou et al. 2005), and in the present study the 15 °C clearly induced a higher mortality of the newly-settled larvae.

Different outcomes in larvae protein metabolism at 24 HAF in the two developmental stages must arise from their previous thermal histories and from the physiological characteristics of each developmental stage. In both trials the highest values of *Artemia* intake (% BDW) were observed in the 15 °C treatment. Nevertheless, given that the temperature in which the two trials were performed was around 20 °C, this may have influenced the feed intake of larvae, particularly in the 15 °C group. Considering that during the pelagic phase 15 °C had a striking negative impact on growth, these larvae were certainly eating less in the rearing tanks than the 18 or the 21 °C ones (although we did not measure the feeding intake in the rearing tanks), since temperature is known to highly influence the food consumption of fish (Imsland et al. 2001; Handeland et al. 2008). Therefore, the higher feed intake observed during the first trial in the 15 °C group might result from the change to a more favourable temperature (the *Artemia* feeding trial was conducted at 20 °C). However, in the first trial the number of *Artemia* prey ingested was quite in agreement with the higher DW at 21 °C, and in the second trial the higher *Artemia* ingestion of the 15 °C group was consistent with the suggested compensatory growth.

In the present study, protein digestibility during the first trial showed the highest values in larvae from the 18 °C group and retention efficiency in this treatment was also significantly higher than in the 15 °C one. Despite the fact that larvae from 15 °C showed the highest intake, one might suggest that their digestive capacity was compromised by a

slower maturation of the digestive system at a lower rearing temperature. In larvae of spotted wolffish (*Anarhichas minor*) or the lane snapper (*Lutjanus synagris*), a lower rearing temperature decreased the activity of several enzymes, including lactate dehydrogenase which is known to be involved in metabolic pathways that generate energy, and associated with the lower growth observed in this group (Savoie et al. 2008). Furthermore, in African catfish larvae it was found that retention efficiency of amino acids increased with temperature (Clarke et al. 1992; Conceição et al. 1998). Since during the first trial larvae from the 18 °C group presented the highest digestibility and retention values but size was lower than that of the 21 °C treatment, this indicates that digestive and metabolic capacities were good at 18 °C but feeding activity in the rearing tanks should be somewhat lower than at 21 °C. Moreover, the fact that the 18 °C group showed the lowest *Artemia* intake but the highest values of protein digestibility and retention seems to indicate that these larvae were in good condition.

During the second trial, where sole larvae were already at the same rearing temperature for 12-14 days, a similar trend of digestibility, retention, catabolism and evacuation could be observed among the different treatments during the 24 h time-course examined. However, the metabolic performance of the 15 °C group is noteworthy. These fish generally retained a higher percentage of radiolabel in relation to total fed during the time-course examined and by 24 HAF protein digestibility was also significantly higher in this group. On the other hand, retention efficiency at 24 HAF in relation to the digested label was very similar in all treatments, which indicates that even though the 15 °C group had the highest protein absorption and 21 °C the lowest, the subsequent retention did not coincide with these results. One can hypothesise that individuals from the 15 °C treatment were spending more energy (in absolute terms) than fish from the other temperatures in order to grow faster. The fact that during the second trial the DW and RGR was very similar between treatments indicates that post-larvae from the 15 °C group undergo compensatory growth after transfer to the 20 °C rearing temperature. This is also supported by the fact that during the second trial fish from 15 °C showed the highest feed intake. However, the difference in the number of days amongst temperature groups still observed at this second trial cannot be ignored. Nevertheless, the fact that by 121 dph juveniles initially reared at 15 °C showed a higher RGR and similar size to the 21 °C ones clearly indicates that these fish experienced compensatory growth after transfer to 20 °C. Furthermore, survival at 121 dph was not significantly affected by early thermal history, which indicates that the initial negative impact of the 15 °C was not verified any longer. Compensatory growth after sub-optimal rearing temperature conditions has been observed in juvenile teleost species like the brown flounder (*Paralichthys olivaceus*) (Huang et al. 2008) or the Atlantic cod (*Gadus morhua*) (Treberg et al. 2005).

Furthermore, in larvae of two strains of the European sea bass (*Dicentrarchus labrax*) reared at different temperatures, a higher growth was observed during exposure to higher temperatures, but fish exposed to lower temperatures exhibited compensatory growth once transferred to a higher temperature (Mylonas et al. 2005).

Differences in absorption and retention levels have been found in *S. senegalensis* larvae between 12 and 35 dph, as the younger showed a higher absorption and retention of *Artemia* protein, indicative of an effort to compensate their lower digestive capacity (Morais et al. 2004). However, Engrola et al. (2009) found that protein digestibility at 16 dph (metamorphosis climax at a rearing temperature of 19.9 °C) was lower than at younger or older ages, and related that with a reduction in larvae digestive capacity during metamorphosis climax. During metamorphosis, the digestive system of Senegalese sole larvae is not fully developed and intracellular digestion (pinocytosis) seems to be an important mechanism of food absorption (Ribeiro et al. 1999; Ribeiro et al. 1999). Similarly to what these studies described we also found that the retention efficiency at 24 HAF was always significantly higher in the larvae that were undergoing metamorphosis compared with the post-metamorphic larvae, which confirms that during metamorphosis climax larvae are more efficient in retaining dietary amino acids compared to later stages, even if they have different thermal histories. Nevertheless, the highest digestibility in the older larvae was found at the 15 °C treatment. Once again, this indicates that during the first trial digestive capacity was severely depressed in the 15 °C group, but after changing to the 20 °C a compensatory mechanism was activated. Moreover, the putative poorer developed digestive system at metamorphosis climax at 15 °C showed no long-term consequences and was probably quickly recovered once larvae were moved to 20 °C.

6.5. Conclusions

The present work shows that rearing temperature during Senegalese sole larval pelagic phase not only has an immediate effect on growth and protein metabolism but also has long-term consequences. Despite the inferior results regarding larvae growth, survival and protein metabolism initially obtained with a low temperature (15 °C), after transfer to a higher rearing temperature an inversion in the trend of these parameters amongst temperature groups was seen. Mechanisms of compensatory growth were activated after transfer to a higher temperature, as clearly observed in 121 dph juveniles that were initially reared at 15 °C.

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Chapter 7

General discussion, conclusions and perspectives

7.1. General discussion

The aim of the present Thesis was to provide knowledge on the epigenetic thermal regulation of muscle growth in Senegalese sole larvae by addressing molecular, physiological and morphological aspects of the thermal plasticity of muscle growth. The first part of this Thesis (**Chapters 2 and 3**) examined the effect of three different embryonic temperatures (15, 18 or 21 °C) and posterior transfer to a common temperature (21 °C), on Senegalese sole embryo and larval development, gene expression and muscle cellularity at equivalent ontogenic stages and until 30 days post-hatch (dph). In the second part of the dissertation (**Chapters 4, 5 and 6**), embryos were all incubated at 20 °C and newly-hatched larvae were transferred to three different rearing temperatures (15, 18 or 21 °C), where they were kept until metamorphosis completion and acquirement of a benthic lifestyle. Then, all larvae were transferred to a common temperature (20 °C) until 121 dph. The effects of the three different rearing temperatures on growth, gene expression and DNA methylation status, muscle cellularity and protein metabolism were assessed at equivalent developmental stages.

7.1.1 Embryonic temperature

Embryonic temperature affects larval muscle growth

The results obtained in **Chapter 2** of the present Thesis show that incubating Senegalese sole embryos at 18 °C or 21 °C and rearing larvae at 21 °C promote growth at 30 days post hatch (dph) relatively to incubating embryos at 15 °C and rearing larvae at 21 °C. Such temperatures fall within of the natural temperature range described for Senegalese sole (between 12 °C and 28 °C) (Vinagre et al. 2006). In fact, the use of 15 °C during embryonic development did not decrease hatching rate or increased mortality of larvae, and therefore its effects were exclusively observed at a growth level, supporting that 15 °C is not an extreme temperature for Senegalese sole, at least during embryonic development. By 30 dph it was observed an increase in weight of 25 % and 27 % of the 18 °C and 21 °C groups, respectively, relatively to the 15 °C one. In Atlantic salmon, incubation temperature alone was also shown to influence weight at two early developmental stages (Albokhadaim et al. 2007). However, it is most likely that if larvae from the 15 °C incubation group had also been reared at 15 °C until 30 dph the differences in size would be much more striking. In common carp, larvae incubated at 18 °C and reared at the same temperature until 26 dph had an almost 14-fold lower weight than larvae incubated and reared at 28 °C (Alami-Durante et al. 2000).

In **Chapter 2** an induced thermal-plasticity regarding the number and size of fast muscle fibres at equivalent ontogenic stages until 30 dph was also observed, which increased at higher incubation temperatures. At hatching there were differences in the size of fast fibres amongst temperature groups; however no differences were found in the number of fibres. Actually, it was only possible to observe significant differences in the number of fibres across temperatures at a later stage, during metamorphosis. Similarly, in newly-hatched larvae of seabass (Ayala et al. 2001; Ayala et al. 2003) and common carp (Alami-Durante et al. 2000) there were no effects of embryonic temperature in the number of fast fibres, and differences in number were only observed at later developmental stages. Also in turbot, embryo incubation at 12 °C instead of 16 °C produced larvae that at 1 dph had a lower mean cross-sectional area but the same number of fast fibres as 16 °C larvae (Gibson and Johnston 1995). In **Chapter 2**, the three different constant pre-hatch temperatures did not induce significant changes in the recruitment of new fibres during embryonic myogenesis. This demonstrates the great ability of Senegalese sole embryos to adapt to environmental temperature and to newly-hatched larvae maintain their programme of formation of fast fibres despite the temperature. Given that fibre mean diameter was larger at 18 °C and 21 °C than at 15 °C during hatching and mouth-opening, it seems that hypertrophy was the preferred thermal-induced muscle growth mechanism at these stages.

Little differences in fibre size and number were observed between hatching and mouth opening (**Chapter 2**). In the related species common sole (*Solea solea*), a temporary halt in new fibre production at hatching has been reported (Veggetti et al. 1999), which seems to agree with the findings in *S. senegalensis*. Hyperplasia was also a less significant mechanism of muscle growth during the yolk-sac stage of halibut larvae (Galloway et al. 1999). In **Chapter 2**, muscle hyperplastic growth began to be very evident during pre-metamorphic (8 to 9 dph) to metamorphosis climax (14 to 15 dph), initially by stratified hyperplasia, since a gradient in fast fibre size was observed across the apical regions of the myotome and the myoseptum to deeper areas of the myotome. In common sole, stratified hyperplasia was also observed at similar age (12 dph) (Veggetti et al. 1999). Mosaic hyperplasia, which involves the activation of myoblasts scattered throughout the myotome was only observed at a post-metamorphic stage (**Chapter 2**), and was concomitant with stratified hyperplasia until 30 dph.

The 18 °C during incubation increased the hyperplastic growth during pre-metamorphic to metamorphic stage. In sea bass, an increase of almost 3 °C during embryonic and pre-larval phases also promoted muscle growth of larvae (Lopez-Albors et al. 2003). By 30 dph, the 18 °C group presented a significantly higher number of fast fibres than the 15 °C one, and both 18 °C and 21 °C a higher mean fibre diameter relatively to

the 15 °C treatment (**Chapter 2**). However, at 30 dph there were no longer found differences in the number of fibres between the 15 and 21 °C groups and total cross-sectional area also did not differ amongst treatments. This seems to indicate a more balanced muscle growth between temperatures at post-metamorphic stages, which was clearly due to rearing all larvae at 21 °C. Likewise, differences in embryonic temperature in seabass were reflected in fast fibre number during larval stages, but such differences were not verified anymore in post-larval stages (Ayala et al. 2003). In this way, the results presented in **Chapter 2** indicate that even if a temperature of 21 °C during embryonic development promoted a faster development and a good somatic growth, by 30 dph differences in muscle cellularity parameters (except mean fibre diameter) relatively to the 15 °C group had disappeared. And given that the 18 °C treatment presented the higher number of fast fibres by 30 dph, this could have positive implications on muscle growth potential of this group. Interestingly, Dionísio et al. (2012) showed that Senegalese sole larvae initially incubated at a higher temperature (21 °C) showed increased number of skeletal deformities when compared to lower embryonic temperatures (15 and 18 °C). This also instigates the idea that 18 °C is indeed an optimal temperature to incubate *S. senegalensis* embryos.

The present results demonstrate the great ability of Senegalese sole to adapt to environmental temperature in early developmental stages. Yet, the long term effect of embryonic temperature on growth and muscle cellularity at commercial sized fish warrants further investigation.

Molecular basis of embryonic-induced myogenic thermal-plasticity and muscle growth in Senegalese sole

In **Chapter 2** it was investigated the expression profile and thermal plasticity of 16 genes involved in development and growth during Senegalese sole embryonic and larval development. Particular importance was given to genes related with muscle development and growth. The majority of sole *myogenic regulatory factors* (*MRFs*) (*myod1*, *myod2*, *myog* and *myf5*), which were previously shown to be highly conserved amongst teleost species (Campos et al. 2010), had a peak in expression during somitogenesis. The exception was *mrf4*, with higher transcript levels at a later stage of development. Since *mrf4* is mainly (but not only) involved in the muscle differentiation program (Buckingham 2001), it seems that in *S. senegalensis* the stages prior to and during metamorphosis are undergoing high muscle differentiation.

The two *myod* paralogues, *myod1* and *myod2*, have different expression profiles during Senegalese sole early development. *Myod2* was found throughout blastula stage to

30 dph, whereas *myod1* was firstly found during epiboly and at very low levels. Furthermore, the existence of high *myod2* transcripts during blastulation indicates maternal inheritance and much lower levels during epiboly are consistent with *myod2* degradation at the onset of zygotic expression. *Myod* gene duplication is common to other Acanthopterygian (Tan and Du 2002; Galloway et al. 2006), but is not found in zebrafish or carp, where it was probably lost during evolution (Macqueen and Johnston 2008). Macqueen et al. (2008) showed the presence of a differential evolutionary pressure on *myoD* paralogues. Acanthopterygian MyoD paralogues that arose during the teleost 3R whole genome duplication (Jaillon et al. 2004; Van de Peer 2004) have asymmetric evolutionary rates, which are higher for MyoD2 (Macqueen and Johnston 2008). The same authors reported that for example stickleback's (*Gasterosteus aculeatus* Linnaeus, 1758) MyoD2 and MyoD1 proteins have 40 and 8 unique substitutions, respectively, relatively to human MyoD, further indicating a faster evolution of MyoD2 relatively to MyoD1. Subfunctionalisation of duplicate genes can occur when each daughter gene adopts part of the functions of their parental gene (Hughes 1994) and the preservation of gene duplicates is thought to be maintained by the complementary loss of gene subfunctions or the acquisition of novel functions (Force et al. 1999). The temporal expression partitioning of *myod1* and *myod2* during early development of Senegalese sole is consistent with some type of gene subfunctionalisation. Also, 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.

In late embryonic rainbow trout, more than 6800 transcripts are significantly up-regulated in the superficial hyperplastic zones of the myotome compared to adult muscle, amongst them *pax7* and the *MRFs* (Rescan et al. 2013). It should be noted that in trout, stratified hyperplasia begins at a quite early stage of myotome development and the appearance of new muscle cells at the myotome extremes begins when the slow fibers have not yet finished their lateral migration (Steinbacher et al. 2007), whereas in Senegalese sole, as in other teleost, it seems that stratified hyperplasia becomes prominent later in development (**Chapter 2**). Nevertheless, a closer examination of stratified hyperplastic mechanism using specific cell markers such as proliferating cell nuclear antigen (PCNA) or phospho-histone H3 (H3P), which is a marker of mitotically dividing cells should be performed in sole to screen for myogenic cells that promote fast muscle stratified hyperplasia. In **Chapter 2**, *pax7* was found to have a peak in expression during hatching, probably associated with the mitotic division of active *pax7*-expressing

myoblasts cells, which might have contributed to the hyperplastic growth observed later in ontogeny. However, no thermal-induced effect was found on *pax7* expression during Senegalese sole embryogenesis except during blastulation (higher transcript levels at 18 °C). In hatchlings it was highest at 18 °C and lowest at 15 °C but without statistical significance. Such results are consistent with the fact that no significant differences in fast fibre number were found between 15 °C and 21 °C at 30 dph. The number of MPCs expressing *pax7* was not measured but the influence of temperature on *pax7* expression seems limited, at least in this range of temperatures and during the period where it was exerted.

Embryonic temperature promoted a transient differential gene expression at several stages of *S. senegalensis* development (**Chapter 2**). For instance, *myf5*, *myod2* and *fst* were highest at 21 °C during gastrulation and/or 20S stage. Importantly, despite its low levels, *myHC* and *mylc2* were highest at 18 or 21 °C in late embryos and/or hatchlings, indicating a more advanced state of muscle differentiation relatively to the 15 °C ones. It is also plausible that a lower embryonic temperature (which delayed embryo development) prolonged the expression of *MRFs* into developmental stages (for example in late embryos) where at higher temperatures it would already be reduced or at least similar to earlier stages of myogenesis. Temperature effect on *MRFs* and *myHC* transcript levels during early development determine muscle growth potential in rainbow trout and sea bass (Wilkes et al. 2001).

Furthermore, miRNAs SOLiD sequencing and qPCR analysis of miR-26a, miR-181a-5p and miR-206-3p revealed higher expression levels at 21 °C than at 15 °C during embryogenesis and/or at hatching (**Chapter 3**), which also indicates a higher activation of the myogenic process at a higher temperature. Since miR-181a-5p and miR-206-3p were predicted to target the 3' UTRs of *DDIT4* and *Sestrin1*, respectively, which are known mTOR inhibitors (Brugarolas et al. 2004; Budanov and Karin 2008; DeYoung et al. 2008), these results agree with a possible increased myogenic differentiation occurring at 21 °C. miR-181a-5p was also predicted to target *MAPK3* (member of MAPK/ERK pathway) and its upregulation at 21 °C at hatching might have led to a higher myogenic differentiation in this group. On the other hand, miR-181a-3p was predicted with confidence to target the 3' UTR of *Calpain 1*, which has been suggested to promote myoblast fusion (Dedieu et al. 2002; Honda et al. 2008). The fact that miR-181a-3p was upregulated at 15 °C during somitogenesis might indicate a repression of *Calpain 1* at this temperature. It should be noted that in **Chapters 2** and **3**, whole larvae and not only skeletal muscle were used to investigate mRNAs and miRNAs expression; hence, it is likely that expression levels of many mRNAs and miRNAs (except for example those of *MRFs* or miR-206, which are muscle specific) reflect a broader gene regulation and not just myogenesis. Furthermore,

miRNAs can have multiple mRNAs targets (Brennecke et al. 2005) and may regulate genes involved in the most diverse functions.

Long-term effects of embryonic temperature were found for *mrf4* expression, which was highest at 18 °C during mouth-opening and at 15 °C group during metamorphosis (**Chapter 2**). The latter seems to indicate an effort of this group towards an increasing muscle growth at this stage (and a similar trend is found in genes such as *myHC*, *igf-I*, *igf-II* or *igf1r*). Expression levels of several miRNAs also moderately increased towards the 15 °C group during metamorphosis (**Chapter 3**), which can be the result of an increased growth effort and tissue differentiation/proliferation in this group. In any case, the post-hatch transfer of all larvae to 21 °C seemed to have attenuated the differences on miRNAs levels between temperatures observed during embryogenesis. Senegalese sole larvae display a high growth rate and accumulate a huge amount of energetic compounds until metamorphosis onset (Yúfera et al. 1999); once it starts, growth rates decrease significantly (Fernández-Díaz et al. 2001). As mentioned in **Chapter 3**, the very high expression of several miRNAs at a pre-metamorphic stage can be associated with a high growth rate and preparation for the metamorphic process.

Similarly to our results, Funes et al. (2006) found very low levels of *igf-I* transcripts in Senegalese sole embryos but a great increase after hatching, whereas *igf-II* as well as *igf1r* levels were higher throughout development, indicating that these genes are developmentally regulated. Igf-I can induce proliferation and differentiation of myoblasts (Coolican et al. 1997) and myotube hypertrophy (Rommel et al. 2001) by activation of MAPK and mTOR pathways, which is mediated through the Igf1R. It is likely that *S. senegalensis* *igfs* had contributed to the observed growth thermal-plasticity. In seabream skeletal muscle, early temperature had effects on *igf-I* levels, but such effects only became visible when the nutritional input at a common temperature was altered, suggesting that muscle growth does not follow a rigid pattern (Garcia de la serrana et al. 2012). It is currently unknown if a similar response could be triggered in Senegalese sole subjected to feed constraints following thermal manipulation.

Genes related to thermal stress and responsible for protein folding (*hsp90AA*, *hsp90AB* and *hsp70*) often respond differently to temperature. In seabream, *hsp90a* and *hsp70* are associated with muscle protein synthesis during refeeding and *hsp90a* also showed persistent effects induced to embryonic temperature during fish refeeding (Garcia de la serrana et al. 2012). During somitogenesis in Senegalese sole, *hsp70* had a general low expression, but higher at 15 °C than at 21 °C (**Chapter 2**); this response to cold-stress is currently unknown and needs to be further investigated. *Hsp90AA* transcript levels were very stable across temperatures in all larval stages. *Hsp90AA* is highly expressed in Senegalese sole skeletal muscle, whereas *hsp90AB* is not, and it was suggested a role

for *hsp90AA* in facilitating myosin folding and its assembly into organized myofibril filaments in sole skeletal muscle (Manchado et al. 2008). In the present work, embryonic temperature did not show long-term effects on *hsp90AA* mRNA levels of larvae.

By 30 dph, mRNA levels for most genes were very stable across temperature groups, except for *mrf4*, which was highest at 21 °C. These results indicate that, in this range of embryonic temperatures (15 °C - 21 °C), effects on gene expression in Senegalese sole larvae may be mostly transient.

7.1.2. Rearing temperature

Rearing temperature has a direct effect on muscle growth and gene regulation in Senegalese sole pelagic larvae

Senegalese sole larvae incubated at 20 °C and reared at 15 °C, 18 °C or 21 °C during pelagic phase showed striking differences regarding size and development. Larvae reared at 15 °C took more than twice the time to acquire a benthic lifestyle than larvae from 21 °C (35 dph and 16 dph, respectively) (**Chapters 4 and 6**). At metamorphic stage 2, larvae from 21 °C had a similar total length (6.94 ± 0.80 mm) to larvae from 18 °C (6.70 ± 0.79 mm); however both had a higher body weight than larvae from 15 °C (5.52 ± 0.79 mm). Metamorphosis in this species occurs when larvae attain a certain length rather than at a determined age (Fernández-Díaz et al. 2001). Flatfish metamorphosis is a particularly complex process, resulting in an asymmetrical craniofacial remodelling and lateralized behaviour (Schreiber 2006). It is energetically demanding, since energy requirements for physical remodeling (Fernández-Díaz et al. 2001) and hormone production required for metamorphosis (Manchado et al. 2008) increase, whereas growth rate decreases (Fernández-Díaz et al. 2001). Furthermore, Engrola et al. (2009) showed that protein digestibility at 16 dph (metamorphosis climax at a rearing temperature of 19.9 °C) was lower than at younger or older ages, and related that with a reduction in larvae digestive capacity during this phase. Also, prey capture in flatfish may be problematic since the transition from pelagic to benthic lifestyle means that new capture prey models must be learnt (Geffen et al. 2007), which may also contribute to a decrease in growth. In fact, feed intake of sole larvae was shown to decrease from pre-metamorphic to metamorphic stages (Engrola et al. 2009).

Weight of pre-metamorphic and metamorphic larvae was highest at 21 °C and lowest at 15 °C (**Chapters 4, 5 and 6**). This was reflected on condition factor, which was significantly lower at 15 °C and indicates nutritional deficiency (**Chapter 4**) and also in relative growth rate (RGR) values, which were remarkably low at 15 °C compared to 18

and 21 °C (**Chapters 4 and 6**). In flatfish, larval size at metamorphosis must be a major factor in determining the energy reserves and amount of time that an individual has in order to make a transition to a settled juvenile (Geffen et al. 2007). This is consistent with the fact that metamorphosis took 23 days to complete in larvae from 15 °C whereas at 21 °C it took only 8 days. Furthermore, 15 °C during the pelagic phase negatively affected survival of larvae, indicating that exogenous feeding larvae are more sensitive to low temperatures than embryos (**Chapter 2**).

Fast muscle bulk greatly increased in pelagic larvae. Between pre-metamorphosis and metamorphosis stage 2, 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 °C, respectively (**Chapter 4**). This indicates that within these developmental stages, a rearing temperature of 21 °C directly promoted general growth but also fibre hypertrophy relatively to lower temperatures. Such results are consistent with the gene expression patterns observed in **Chapter 4**.

In pre-metamorphic larvae, mRNA levels of all *MRFs* except those of *myod1* were highest at 21 °C, as well as *myosins* and *igf-I*, which supports the muscle growth results. However, since whole larvae were used in the qPCR analysis, it remains to be seen if *mstn1*, which was also up-regulated at 21 °C, played other functions than regulating muscle growth of pre-metamorphic larvae. Likewise, whether *mstn1* up-regulation during metamorphosis in the group incubated at 15 °C and reared at 21 °C (**Chapter 2**) was meaningful regarding the control of muscle growth is presently unknown, since in Senegalese sole (Campos et al. 2010), as in other teleost (Zhong et al. 2008; Funkenstein et al. 2009), *mstn1* might have additional functions other than just regulating muscle growth.

During metamorphosis, the majority of *MRFs* were also significantly up-regulated at 21 °C (**Chapter 4**). Furthermore, *igf-I*, *myosins* and *fgf6* showed higher transcript levels at 21 °C. 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 described in **Chapter 4** 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). Regardless of increased expression of *igf-I* at 21 °C in pre-metamorphic and metamorphic larvae, *igf-II* and *igf1r* transcript levels were remarkably different. Interestingly, the increased *mstn1* expression at 21 °C was no longer observed during metamorphosis. Moreover, *pax7* mRNA levels did not differ amongst temperatures, thus seeming that, in this regard, post-hatch stages are

less susceptible to suffer changes in the number of Pax7⁺ expressing cells. It would be convenient, however, to investigate how these cells change with rearing temperature at a histological level. Also, immunostaining for H3P and Myog could be helpful to better understand fast muscle thermal-plasticity dynamics.

Thermal-plasticity of epigenetic regulation in Senegalese sole

As previously mentioned on this Thesis, gene expression regulation is enabled by the alteration of chromatin states through epigenetic mechanisms such as DNA methylation and post-translational histone modifications. DNA methylation is carried out by Dnmt proteins, namely Dnmt1 and Dnmt3 (Goll and Bestor 2005) (Figure 1). DNA methylation plays a repressive role both in heterochromatin and in euchromatin (Herman and Baylin 2003) and cooperates with histone modifications to perform this repressive function (Bird and Wolffe 1999). Acetylation and methylation of histone 3 at lysine 9 (acetyl-H3K9 and methyl-H3K9, respectively) are two of the best studied modifications (Fuks 2005). Acetyl-H3K9 is known to be associated with active transcription whereas methyl-H3K9 with repressed transcription (Fuks 2005). 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 the terminal differentiation of particular tissues, such as skeletal muscle. In **Chapter 5**, *dnmt1* expression was up-regulated during metamorphosis in the 15 °C group. Senegalese sole Dnmt1 putative partial protein sequence is highly conserved amongst other vertebrates and it is likely to play similar functions in differentiation and development. Such higher expression at 15 °C is consistent with increased DNA methylation levels. Whether other mechanisms of gene repression such as histone methylation are activated at a lower temperature in Senegalese sole is currently unknown and should be investigated.

Zebrafish *dnmt3a* and *dnmt3b* paralogues may play different roles in thermal epigenetic regulation of gene expression during embryonic development (Campos et al. 2012). For instance, at several stages of development there was an up-regulation of *dnmt3b1* at the lowest incubation temperature (23 °C), whereas *dnmt3a1* and *dnmt3a2* were up-regulated at higher temperatures (27 °C or 31 °C). In **Chapter 5** it was observed a somewhat similar situation, since *dnmt3b* mRNA levels during *S. senegalensis* metamorphosis were highest at 15 °C but those of *dnmt3a* were very stable across temperatures. As suggested for zebrafish, this seems to indicate a subfunctionalisation of *dnmt3* genes in Senegalese sole.

The results of *dnmts* expression were consistent with the *myog* putative promoter methylation levels observed in Senegalese sole skeletal muscle during metamorphosis (**Chapter 5**). Overall cytosine methylation (including CpG and non-CpG sites) was highest at 15 °C. 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. As observed in zebrafish (Du et al. 2003) and seabream (Codina et al. 2008), the status of cytosine methylation in these regions is likely to interfere with the transcriptional activity of *myog* promoter. Results from sole *myog* methylation levels are consistent with the highest muscle growth at 21 °C in addition to the up-regulation of *myog* expression, highly suggesting that thermal-plasticity of an epigenetic mechanism can promote differential gene expression and modulate muscle growth in Senegalese sole.

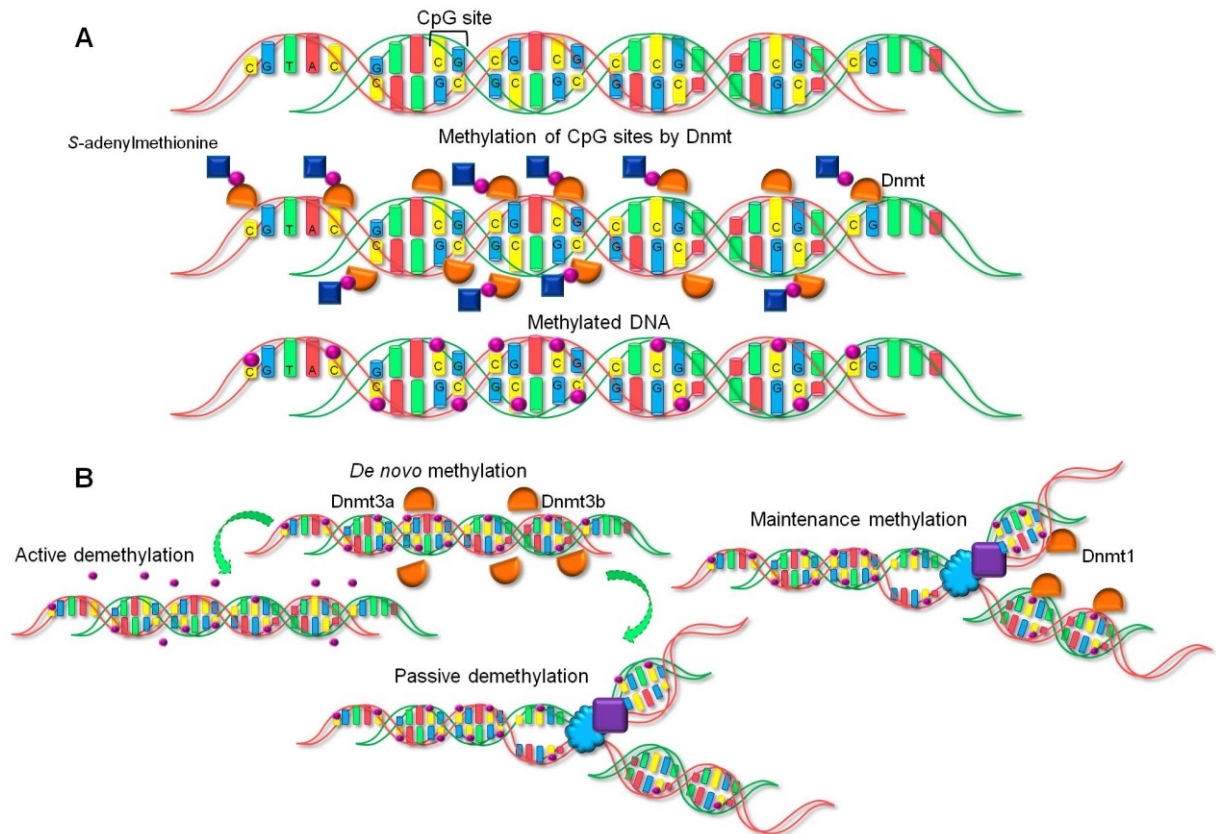


Figure1. Illustration of DNA methylation by Dnmts. A) Methylation of cytosine nucleotides at CpG sites within promoter regions generally leads to transcriptional inhibition and gene silencing. Covalent transfer of a methyl group (pink circles) from S-adenosylmethionine to the 5' position of a cytosine nucleotide is carried out by Dnmt enzymes (orange semi-circles), which prevents transcription. B) When DNA is replicated, the methyl group on the template strand is recognized and a new one is introduced on the daughter strand by Dnmt1. In the presence of Dnmt1, hemi-methylated DNA becomes fully methylated and DNA methylation patterns tend to be maintained. Dnmt3a and Dnmt3b are *de novo* methyltransferases that establish new methylation patterns. Passive demethylation takes place in the absence of methylation of newly synthesised DNA strands during replication. Active DNA demethylation occurs via direct removal of a methyl group independently of DNA replication.

Protein metabolism decreases at 15 °C in Senegalese sole metamorphic larvae

The tracer study described in **Chapter 6** showed that, despite the highest feed intake of the 15 °C larvae (probably due to the fact that the *Artemia* labelling feeding trial was conducted at 20 °C), the highest values of protein digestibility in metamorphic larvae were found in the 18 °C group and the lowest in the 15 °C one. This indicates a poorer digestive capacity of the 15 °C larvae, also predicted by their much reduced size and

developmental delay. It is plausible that the retention efficiency of amino acids had decreased at the lowest temperature, as 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 agree with the gene expression patterns and epigenetic regulation during the pelagic phase described in **Chapters 4 and 5**, since the expression of most growth-related genes was lowest at 15 °C and highest at 21 °C. The findings that a rearing temperature of 18 °C promoted the highest protein absorption but a lower size and RGR values relatively to 21 °C, point towards a good digestive capacity of the 18 °C group but perhaps a lower feeding activity in the rearing tanks comparatively to the 21 °C larvae. It would be pertinent to investigate digestive enzyme profile and the expression of genes related with the development of digestive system and digestion at different rearing temperatures.

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

Transfer of Senegalese sole larvae after completion of metamorphosis to a common rearing temperature (20 °C) brought a number of alterations at growth, protein metabolism and gene expression levels (**Chapters 4 and 6**). Particularly important is the fact that the 15 °C group initiated a process of compensatory growth after transfer to a more adequate temperature and overcame the initial growth limitations. The improvement of the digestive process of the 15 °C fish, shown by the *Artemia* labelling results described in **Chapter 6** probably had a major contribution to this. All post-larvae had a similar growth opportunity at 20 °C (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 (HAF), the 15 °C group had much higher protein digestibility (and also feed intake and number of *Artemia* prey ingested) than the 18 or 21 °C ones, despite the non-significant differences in protein retention. The latter fact was hypothesised as post-larvae from 15 °C were allocating more energy to somatic growth than post-larvae from the other temperatures. In fish, periods of low nutritional input induce changes in the storage reserves, particularly of lipids (reviewed by Ali et al. 2003). For example, in juvenile flounder that achieved full compensatory growth after thermal manipulation, the 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 changes had happened.

When comparing the protein digestibilities at the first and second feeding trials, the 15 °C group was the only one where digestibility is higher in post-larvae than in metamorphic larvae, giving one more indication that this temperature clearly held back the digestive process but in a transient way. It is currently unknown whether different amino acids are differentially retained at different rearing temperatures in Senegalese sole and that should be further examined.

As reported in **Chapter 4**, there was a great increase in fast muscle bulk between pelagic larvae and benthic stages such as post-larvae and early juveniles. For instance, between metamorphic larvae at stage 2 and early juveniles with 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 °C groups, respectively. It is remarkable however how the 18 and 15 °C fish presented a much higher increase than the 21 °C ones, which was mainly due to the fact that during pelagic phase the 21 °C group was already much larger than the other two groups. Such results agree with increasing RGR values towards the lowest temperatures, particularly the 15 °C 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 °C fish, even if at this age had not attained yet the size of 21 °C or 18 °C. Whether the more elevated levels of *mstn1* mRNA found in fish reared at 18 °C and 21 °C at 83 dph are associated with higher muscle protein catabolism remains to be determined; however, such results are consistent with the proposed compensatory growth of the 15 °C treatment.

At 100 dph, fast fibre hyperplasia seemed a major mechanism of muscle growth at 15 °C and 21 °C, whereas fibre hypertrophy appeared more relevant in the 18 °C group (**Chapter 4**). Furthermore, *myod1*, *myod2* and *igf1r* transcript levels in fast muscle agreed with the observed muscle thermal-plasticity. Given the stable *myog* expression levels, it is likely that the previously higher *myog* methylation levels at 15 °C during pelagic phase (**Chapter 5**) were mostly transient and related to rearing temperature. Interestingly, at this age it was found that the length of juveniles did no longer differ amongst temperatures. Moreover, juveniles from the 15 °C treatment had the same weight as the 18 °C ones. It could be hypothesized that the 18 °C fish would have a lower growth, since fibre hyperplasia is a mechanism that has been positively correlated with larger body sizes (Valente et al. 1999; Zimmerman and Lowery 1999; Alami-Durante et al. 2007). And in fact, by 121 dph it was found that the 15 °C group had attained the same weight as the 21 °C one and both were significantly higher than the 18 °C juveniles (**Chapter 6**). It is not well known why this intermediate temperature produced the smallest juveniles, since its initial performance was superior relatively to the 15 °C ones. But the fact is that initially

rearing larvae at 15 °C made them overcompensate the 18 °C ones. Perhaps the transition from 18 °C to 20 °C was not sufficient to induce a compensatory growth response or at least not as accentuated as at 15 °C and therefore these fish did not significantly change their growth curve. Nevertheless and considering the commercial production of Senegalese sole, one should keep in mind the initial lower survival of larvae from the 15 °C group.

As it was reported in **Chapters 2** and **4**, the relation between embryonic or rearing temperature and muscle cellularity is not always linear. For example, in juvenile seabream, temperature throughout embryo development until metamorphosis had a significant effect on fast muscle fibre production, since the low temperature produced juveniles with more 20 % of fibres than the high temperature, despite the initial lower size of the low temperature group (Garcia de la serrana et al. 2012). The comparison of results from **Chapters 2** and **4** shows that temperature has not the same outcome in Senegalese sole whether is applied during embryonic or larval periods, stressing its complex effects. For example, considering that 18 °C is a good embryonic temperature regarding somatic and muscle growth, its use during larval pelagic phase does not seem optimal, since on one hand it is below the growth observed at 21 °C and on the other hand compensatory growth at 15 °C after transfer to 20 °C surpassed it. Therefore, the impact of embryonic and rearing temperatures on Senegalese sole somatic growth and muscle cellularity should be further determined at commercial size.

7.2. Conclusions

The following conclusions can be highlighted from the results presented in this Thesis:

- Senegalese sole embryos and larvae can be reared in a temperature range from 15 °C to 21 °C; however, exogenous feeding pelagic larvae (**Chapter 4**) are more sensitive to the lowest temperature (15 °C) than embryos (**Chapter 2**), given that these larvae present a much lower growth and survival rates than those reared at 18 °C or 21 °C.
- An incubation temperature of 18 °C followed by transfer to 21 °C promoted larval muscle hyperplastic growth, which can have positive implications on muscle growth potential; however, at 30 dph the 15 and 21 °C groups did not differ significantly in fibre number or muscle area, highlighting how initial temperature effects may become attenuated or even reversed during development (**Chapter 2**).
- The developmental profile of 16 growth-related genes was described during Senegalese sole embryonic and larval development. *Myf5*, *fst*, *mrf4*, *mylc2* and *myHC* were amongst the genes most affected by embryonic temperature (**Chapter 2**), and thermal plasticity of miRNAs such as miR-17, miR-26a, miR-181a or miR-206 may have potential implications on thermal gene regulation (**Chapter 3**). Furthermore, computationally predicted mRNA targets for several miRNAs were related with the mTOR and MAPK pathways, which are directly involved in muscle growth (**Chapter 3**).
- Rearing pelagic larvae at 15 °C greatly decreased their growth and survival and delayed their development (**Chapters 4, 5 and 6**), decreased protein absorption and retention (**Chapter 6**) and increased DNA methylation levels of *myog* putative promoter in skeletal muscle (**Chapter 5**) comparatively to higher rearing temperatures (18 and 21 °C). Nevertheless, such negative effects were mostly shown to be transient once newly-settled larvae were transferred to 20 °C.
- Senegalese sole initially reared at 15 °C during the larval pelagic phase undergo a mechanism of compensatory growth and were equal in weight to the 21 °C group by 121 dph, being both groups larger than fish initially reared at 18 °C (**Chapter 6**). However, considering the commercial production of Senegalese sole, one should

keep in mind the initial lower survival of the 15 °C group. Muscle hyperplastic and hypertrophic growth mechanisms linked to their previous thermal histories are likely to be related to the growth results (**Chapter 4**).

7.3. Future perspectives

The overall aim of the present Thesis was to describe how changes in water temperature, applied at different stages of Senegalese sole early development (embryos and larvae), could affect growth performance and fast muscle cellularity of larvae and post-larvae and also to characterise changes in the underlying events at the molecular level, which could contribute to such thermal-induced phenotypic plasticity. The aims of this dissertation have been successfully fulfilled but several topics need a closer examination and should be considered in future studies.

Standard feeding conditions were used in the experiments described in the present Thesis. Nevertheless, over the past years there has been a great effort and large improvements in nutrition and feeding larvae and post-larvae of Senegalese sole. Whether Senegalese sole larvae and post-larvae arising from different embryonic temperatures are more or less susceptible to different feeding regimes is currently unknown, and it would be interesting to investigate the existence of interactions between these two variables and its effects on growth and muscle cellularity.

The existence of long-term effects of embryonic and rearing temperatures should be investigated in Senegalese sole up to commercial size. Furthermore, for a fine-tuning evaluation of thermal-plasticity on the different phases of myogenesis in embryos and larvae, studies involving *in situ* hybridisation and muscle immunolabelling for molecular markers such as Pax7, PCNA and H3P would be appropriate.

The contribution of the Akt/TOR and MAPK/ERK pathways to the thermal regulation of skeletal muscle growth should be examined. Both pathways are activated by Igf-I through Igf1R and it is likely that it would provide insights concerning the events controlling somatic growth in Senegalese sole. Moreover, it would be pertinent to experimentally validate the proposed miRNA targets involved in these pathways and their contribution to thermal regulation of growth.

7.4. References

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