Regulation of growth - understanding the myostatin functioning in two important aquaculture species, barramundi (*Lates calcarifer*) and black tiger shrimp (*Penaeus monodon*)

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The completion of this work represents an important milestone into my life. With time I have come to realize that learning how to make science is what I believe being only one of my accomplishments. I have in fact evolved into a completely different person, one that I quite like today, and this growth has only been possible thank to the day to day interaction with a number of key-people that have surrounded me along this journey and whose contribute has been quintessential.

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Abstract

Growth has traditionally been the most important trait amongst the many targeted to boost the productivity and profitability of aquaculture farms. Several genetic approaches have been investigated and employed in recent years to enhance the growth rate of aquatic species. Of these, the use of a single gene approach has recently gained popularity amongst aquaculture scientists looking at the improvement of productivity. The adoption of a single gene approach has spiked the characterization and study of a number of candidate genes of major effect on growth and one such gene is that of myostatin (*Mstn*). MSTN is a protein that inhibits muscle growth and that in beef cattle is responsible for inducing "double muscling", a phenotype with increased muscle mass. A number of studies have shown that genes similar to that of the cattle *Mstn* are present in the whole animal kingdom and in many species have a significant association with growth functions. Because of its central role in the regulation of growth, *Mstn* is therefore a very interesting candidate gene for the improvement of aquaculture productivity.

Detailed evolutionary analyses have revealed that *Mstn* comprises with its closest relative growth and differentiation factor-11 (*Gdf-11*) a small gene family that evolved from multiple events of gene or genome duplication. The structure of the *Mstn/Gdf11* family resembles the "one-to-four" model seen for many genes isolated from species encompassing the diversity of the animal kingdom. In the one-to-four gene model invertebrates possess a single gene homologue (i.e. *Mstn/Gdf11*), higher vertebrates like mammals possess two paralog genes (i.e. *Mstn* and *Gdf11*) and at least four paralogs are present in teleost fish (i.e. *Mstn-1* and *Mstn-2*). Gene duplication events may have engendered the origin of new functions compared to those initially exerted by the *Mstn/Gdf11* ancestor. While some of these new physiological roles arisen following duplication are well studied and understood in mammalian model

species, the history of functional evolution of the *Mstn/Gdf11* family in species of high relevance for aquaculture production is still largely unclear. This thesis addresses key issues important for providing significant advances in the understanding of *Mstn*-like genes in aquaculture species using barramundi (*Lates calcarifer*) and the black tiger prawn (*Penaeus monodon*) as model species.

As at least two *Mstn* paralogs (*Mstn-1* and *Mstn-2*) are present in fish like *L. calcarifer*, it is important for the purpose of growth improvement to understand whether subfunctionalization occurred following the event of genome duplication. In this thesis the *Mstn-2* gene paralog, including its upstream region, was isolated and characterized from the *L. calcarifer*. Through a detailed analysis of gene expression this thesis provides preliminary evidence of differentiation of MSTN paralogs. Firstly, differential regulation as well as specific tissue-responses in the muscle, liver, gill and brain of *L. calcarifer* was observed after nutritional deprivation. In particular, the *LcMstn-1* expression increased in liver (~4 fold) and muscle (~3 fold) and diminished in brain (~0.5 fold) and gill (~0.5 fold), while that of *LcMstn-2* remained stable in brain and muscle and was up regulated in gill (~2.5 fold) and liver (~2 fold). In addition, it also suggested that *Mstn* genes in fish may regulate different growth and developmental processes by revealing an independent regulation of each paralog throughout the embryonic development with the *Mstn-2* generally more abundant than the *Mstn-1* and a diametrically opposite correlation of their expression with muscle hypertrophy in juvenile fish. Analyses of relationship between *Mstn* transcript abundance and muscle hypertrophy showed in fact that the expression of *LcMstn-1* was only marginally associated with fiber size ($r = 0.384$, $p = 0.064$) while that of *LcMstn-2* showed a highly significant negative correlation ($r = -0.691$, $p < 0.0001$). Differential regulation of *Mstn* paralogs was supported by *in silico* analyses of regulatory motifs that revealed, at least in the immediate region upstream the genes, a differentiation between *Mstn-1* and *Mstn-2*. The *Mstn-1* in particular showed a significantly higher conservation of regulatory sites among teleost species compared to its paralog indicating that this gene might have a highly conserved function in the taxon.

Conversely, invertebrates possess a single ortholog of the MSTN/GDF11 family. In this thesis the *Mstn/Gdf11* gene ortholog was identified and characterized in the Penaeid shrimp, *Penaeus monodon*. The overall protein sequence and specific functional sites were highly conserved with other members of the MSTN/GDF11 family. Gene transcripts of *pmMstn/Gdf11*, assessed by real-time PCR, were detected in a variety of tissue types and were actively regulated in muscle across the moult cycle. To assess phenotypic function in shrimp, the *pmMstn/Gdf11* gene expression was down-regulated by tail-muscle injection of sequence-specific double-stranded RNA. Shrimp with reduced levels of *pmMstn/Gdf11* transcripts displayed a dramatic slowing in growth rate compared with that of control groups. Findings from this study place the MSTN/GDF11 gene at the centre of growth regulation in shrimp suggesting that this gene has an opposite role in invertebrates compared to higher vertebrates. In the former, levels of gene expression may positively regulate growth.

The outcomes of this thesis have provided significant advances in the understanding of *Mstn*-like genes in aquaculture species such as barramundi (*L. calcarifer*) and the black tiger prawn (*P. monodon*), towards the development of MSTN-based technologies for the enhancement of growth. In particular, this research emphasized that significant differences exist between *Mstn-1* and *Mstn-2* in fish whereby an efficient enhancement of growth may arise from specific targeting of one paralog only. It also revealed for the first time that the invertebrate *Mstn/Gdf11* does not inhibit growth. This last finding in particular will cause an inversion of tendency, whereby research aiming to improve growth of invertebrates like crustaceans should investigate strategies to enhance the activity of MSTN/GDF11 and not reduce it, such as the case of vertebrate MSTN.

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1 General Introduction

Aquaculture is the fastest growing food production sector growing annually at the rate of 6.9% (FAO, 2009). Success of the aquaculture industry along with the decline of world fisheries production has dramatically increased the significance of farmed seafood for human supply to the point where in 2008 50% of the seafood consumed was aquaculture derived. Among species farmed, marine shrimps and finfish accounted for those with the highest commercial value (FAO, 2009).

In developed economies and for established cultured species, a major constraint on the steady growth of aquaculture has been an inability of farms to minimize production costs and supply products at a highly competitive market price. In the development of aquaculture for various species R&D resources initially have been targeted towards optimum feed formulation and/or refined culturing procedures. Whilst R&D in these areas has led to closure of life-cycles and establishment of viable culture practices many industries have not continued to improve per unit productivity through these approaches and, particularly for those species where an optimum of culturing practices has been reached, significant increases in productivity will now only be achieved through the adoption of genetic technologies.

Depending on the species under culture, there are many traits that have been targeted to increase the productivity and ultimately profitability of aquaculture farms. Of these, growth has traditionally been the most influential. Through increased growth rate in fact an animal's harvest size is reached in a shorter period of time thus speeding up the production cycle and maximizing both fixed and variable input costs associated with a crop. As has been experienced for terrestrial livestock industries like beef cattle, swine and poultry, the application of various genetic technologies aiming to enhance growth has dramatically

improved the performance of farmed stocks and has been one of the single factors leading to rapid industry expansion and profitability. Improving growth and reducing the time to harvesting is an even more critical factor for aquaculture production, where costs and risk of disease outbreaks are generally higher than in terrestrial livestock systems.

Several genetic approaches have been investigated and employed in recent years to enhance the growth rate of aquatic species. These include *selective breeding programs*, where fast-growing individuals are selected and mated to produce improved progeny (Circa et al., 1995; Gjoen and Bentsen, 1997), *chromosome manipulation* used to produce, for example, enhanced stock with an extra set of chromosome (i.e. triploids) (Liu et al., 2004), use of stocks of *single, fast-growing sex* (i.e. all-male tilapia) (Mair et al., 1997) and finally *genetic modification* of growth related genes (i.e. transgenic salmon over expressing the growth hormone) (Du et al., 1992). While in certain species these techniques have provided extremely positive results with respect to production improvement, their extensive application is still very restricted by a number of limitations. Above all, many aquaculture species still cannot be bred efficiently in captivity and this is an obvious impediment for most of the above-mentioned approaches. As new aquaculture species are domesticated this problem is likely to persist. It is therefore evident that a strategy for growth improvement, more independent from the reproductive cycle would be beneficial to the future maturity of many industries.

One promising approach to improve species where reproductive control limits the application of more traditional genetic methods uses molecular technologies like RNA interference, protein-protein interaction, etc to interfere with the normal functioning of gene/protein pathways controlling growth. Interesting prospects in the development of these technologies have come from the field of biomedical research that is recently directing a large amount of resources towards what is known as “gene therapy”. This branch of medicine investigates

viable ways to deliver nucleic acids into somatic cells in order to interfere with mutated gene pathways in a highly specific manner. Although still in its infancy, interesting progress has been achieved and in the foreseeable future this technology is expected to revolutionize the entire field of medicine (Cavazzana-Calvo et al., 2004). Animal production, including the aquaculture industry, will greatly benefit from the developments and advances in biomedical fields and may adapt those technologies to target growth genes in order to improve production. In this regard, it is essential to characterize and understand genes of major effect on growth. One such gene with potential for enhanced growth improvement is that of myostatin (*Mstn*).

1.1 Myostatin in animal production - what is the fuss?

Myostatin (MSTN) is a protein that inhibits muscle growth. The excitement around the MSTN is associated with two major factors. First and foremost, MSTN has a major effect on muscle growth thereby presenting a vast potential for production improvement. "Double muscling" in cattle, for example, is a phenotype exhibiting significantly higher muscle mass that has appealed to cattle farmers and that in some breeds such as the Belgian Blue has been exploited for at least half a century in order to improve meat production. The factor responsible for this appealing trait is a single genetic mutation occurring at the gene encoding for MSTN resulting in an inactive form of this protein (Kambadur et al., 1997; McPherron et al., 1997). No less important, however, is the fact that MSTN acts as a negative regulator of muscle growth, with muscle growth through increased cell division and/or hypertrophy evident when lower levels of the protein are present, or when the protein is inactive (McPherron et al, 1997; Thomas et al, 2000). The possibility of increasing growth through reducing levels of the MSTN protein lends itself to several biotechnological approaches and as a consequence reducing or inhibiting the activity of this gene/protein has been the primary focus of research efforts to date.

Researchers have explored MSTN-based approaches in other farmed species in an effort to reproduce a similar phenotype as that naturally found in cattle by reducing or nullifying the bioactivity of the protein. Two strategies resulting in reduced bioactive MSTN levels have been primarily investigated. The first aimed to identify single mutations associated with increased growth of skeletal muscle in order to provide a molecular background to assist breeding programs. This strategy has proven highly successful in cattle where individual null-mutations were identified that result in an inactive form of MSTN, thus increasing muscle mass by up to 25% (Grobet et al., 1998; Kambadur et al., 1997). In mice it was shown that a similar, but induced, null *Mstn* mutation could result in a 200-300% increase in muscle mass, although this greater improvement might reflect the fact that livestock animals have already been heavily selected for high growth rate in recent years (McPherron et al., 1997). The hypermuscular compact phenotype created during a selection experiment in Germany also showed to possess a 12 base pairs deletion in the *Mstn* gene (Varga et al., 2003). In other farmed animals no cases of natural null-mutation have been described. However, *Mstn* polymorphisms in non-coding regions significantly associated with growth were identified in sheep (1.5% yield increment), pig (up to 35% heavier weight) and chicken (Hickford et al., 2010; Ye et al., 2007; Zhang et al., 2009). The second avenue of research investigated strategies that directly interfere with MSTN bioactivity or circulating levels. These strategies included for example the use of RNA interference (RNAi) to down-regulate the *Mstn* gene (Jain et al., 2010), as well as the application of MSTN antagonists like follistatin or the MSTN pro-peptide to neutralize MSTN protein bioactivity (Lee and McPherron, 2001). In livestock, this last approach is still in its infancy and its potential to improve production has not been reported in the literature.

1.2 Molecular and evolutionary background

Myostatin (MSTN) is also known as growth and differentiation factor (GDF)-8 and constitutes with its closest relative GDF11 a family of proteins (MSTN/GDF11 family) belonging to the transforming growth factor-β (TGFβ) superfamily. The TGFβ include proteins that share structural features and signaling mechanisms and that in metazoans regulate a number of important biological processes including cell growth, proliferation and differentiation (reviewed in Herpin et al., 2004). Understanding the molecular basis that underlies the functioning of MSTN, including its structural features and mechanisms of maturation, is pivotal in order to comprehend the applications developed to interfere with this protein's activity and ultimately improve muscle growth.

Similarly to other TGFβ proteins, MSTN is secreted by the cell in an immature form (pre-pro-peptide) encompassing three domains including a hydrophobic signal sequence, a pro-peptide and a mature region (Fig. 1) (mechanisms of action of MSTN are reviewed in Rodgers and Garikipati, 2008). The immature MSTN undergoes a number of post-translational modifications that involve first the removal of the signal sequence and subsequently the proteolysis of the pro-peptide from the mature MSTN sub-unit. Two mature MSTN sub-units ultimately dimerize by forming interchain disulphide-bonds through conserved cysteine residues generating the bioactive dimer (Daopin et al., 1992). The bioactive MSTN can be found in a latent form where it binds to the previously cleaved pro-peptide (a complex a.k.a. latency-associated protein or LAP) or is bioneutralized by other antagonists like the protein follistatin (Fig. 1) (Lee and McPherron, 2001). When not in a latency complex, MSTN can signal with the activin type-2 receptor on the cell surface that in turns recruits and trans-phosphorylates the activin type-1 receptor, activates the intracellular SMAD protein cascade and ultimately regulates the transcription of target genes in the nucleus of cells (Massague and Wotton, 2000). One important growth pathway that is a downstream target of MSTN is that of the Akt/mammalian target of rapamycin (a.k.a. mTOR) (Amirouche et al., 2009).

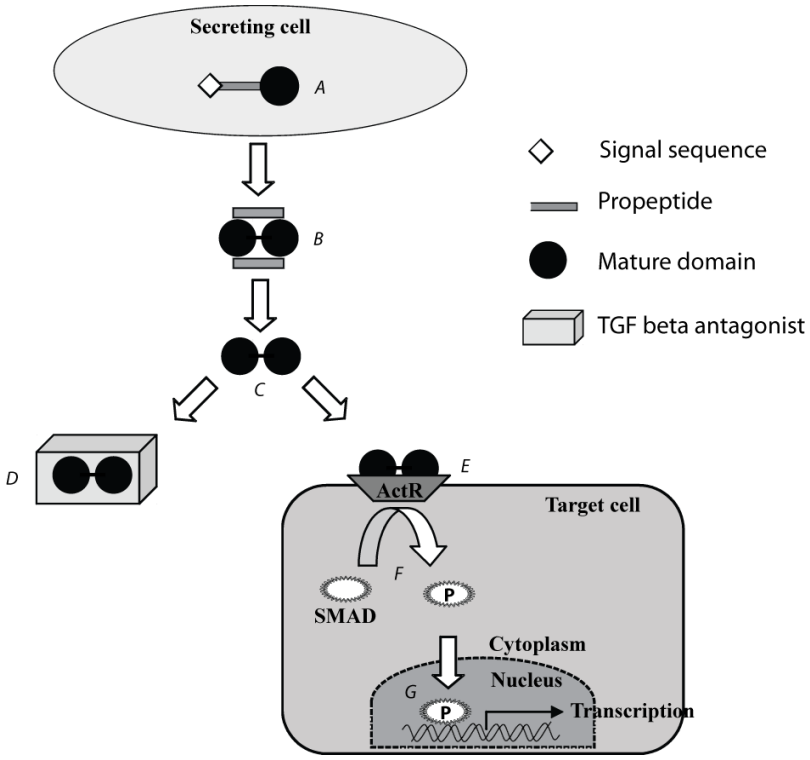

Figure 1.1. Secretion and cell signaling pathway of MSTN. MSTN is secreted by the cell in an immature form (A). After post-translational modifications, it can be found in a latent form where binds to its pro-peptide (B) or to antagonists like follistatin (D). When in its active form (C), MSTN signals with Activin receptors type-1 and -2 (E) that in turn phosphorilate intracellular SMAD proteins (F) and induce transcription in the nucleus (G).

Since its discovery (McPherron et al., 1997), a large number of MSTN (and/or GDF-11) sequences have been reported in the literature, primarily from vertebrates including mammals, aves and fish, but also from a few invertebrates. The abundance of sequence submissions has facilitated the reconstruction of a detailed phylogenetic history providing a deeper understanding of *Mstn/Gdf11* gene family evolution (Fig. 2) (Saina and Technau, 2009; Xing et al., 2007). Recent phylogenetic analyses show that the *Mstn/Gdf11* gene family resembles the "one-to-four" model seen for many genes isolated from species encompassing the diversity of the animal kingdom. In the one-to-four' gene model invertebrates possess a single gene homologue, higher vertebrates like mammals possess two paralog genes and at least four

paralogs are present in teleost fish (Ohno, 1999). The one-to-four is a very common gene family structure in the animal kingdom and reflects specific events of duplication that occurred during the evolution of gnatostomes (Dehal and Boore, 2005). To date, it appears in fact that invertebrates only possess a single gene of *Mstn/Gdf11* (Saina and Technau, 2009). A whole-genome duplication event and subsequent gene divergence occurred early during the evolution of vertebrates and separated *Mstn* and *Gdf11* sub-families (Rodgers et al., 2007). Indeed, these genes have been described from both mammals and fish (Biga et al., 2005; Funkenstein and Olekh, 2010; Nakashima et al., 1999). A second round of genome duplication occurred at the origin of ray-finned fish and generated two *Mstn* paralogs named *Mstn1* and *Mstn2* (Rodgers et al., 2007). Although only one copy of *Gdf11* has been isolated in teleosts thus far, it stands to reason that two paralogs of this gene might also exist as a result of the teleost genome duplication. Noteworthy, within the teleost taxon a further duplication occurred in salmonids, following tetraploidization (Phillips and Rab, 2001). Thus, as shown in *Oncorhynchus mykiss* all salmonid species should possess four *Mstn* paralogs (*Mstn*1a, *Mstn*1b, *Mstn*2a, *Mstn*2b) (Garikipati et al., 2007; Garikipati et al., 2006).

Gene duplication may have engendered the origin of new functions compared to those initially exerted by the *Mstn/Gdf11* ancestor. While some of these new physiological roles arisen following duplication are well studied and understood in mammalian model species (i.e. functions of MSTN and GDF11 in mice), the history of functional evolution of the *Mstn/Gdf11* family is still largely unknown. It is interesting that species of high relevance for aquaculture production are those that compared with mammals either did not undergo genome duplication (i.e. crustacean and mollusk) or were subject to one or two additional rounds of duplication (i.e. teleost fish). This poses a significant limitation on transferring knowledge acquired from mammalian systems where these proteins are studied in detail, and encourage specific experiments questioning the physiological functions of the MSTN/GDF11 family in aquaculture species.

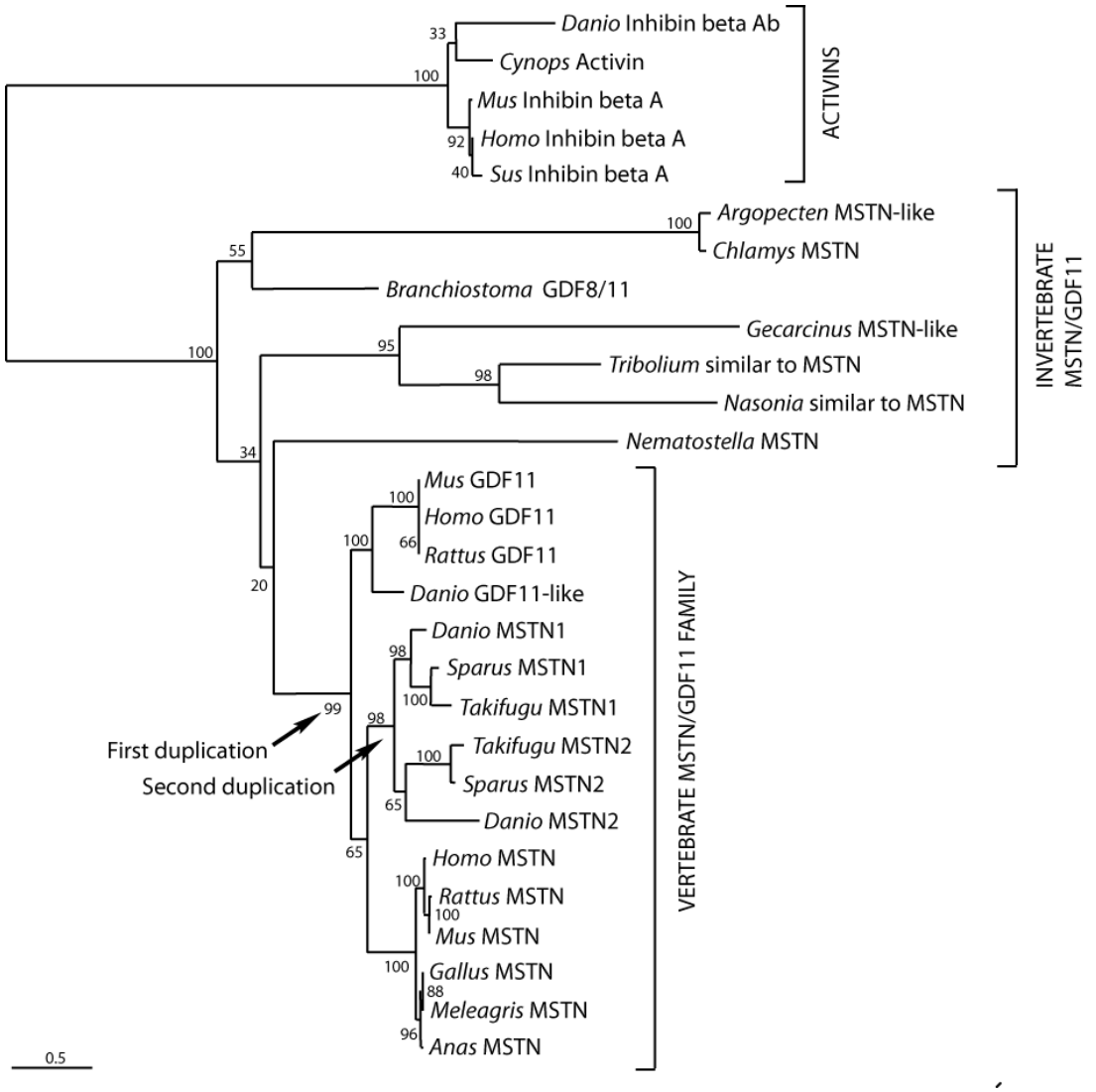

Figure 1.2. Phylogenetic history of MSTN/GDF11 family (amino acid). Figure adapted from Saina and Technau (2009). Putative evolutionary location of duplication events are marked with an arrow.

1.3 An overview of the physiological functions of the MSTN/GDF11 family

The MSTN and GDF11 sub-families arose from a common ancestor after the first duplication event, early during the history of vertebrates. Their mature domains share approximately 90% identity (Funkenstein and Olekh, 2010; McPherron et al., 2009). As revealed by functional studies in model mammalian systems, despite their similarity, MSTN and GDF11 regulate very different biological processes. In mice, MSTN is detected primarily in muscle and its principal and most investigated function in this tissue is to inhibit cell proliferation (by

preventing cell cycle progression through the G_1 and G_2 stages) and protein synthesis (reviewed in Rodgers and Garikipati, 2008). Thus, mice with reduced MSTN levels exhibit an increase of muscle mass due to increased number of muscle fibers (hyperplasia) and increased fiber size (hypertrophy) and resulting in a 2 to 3 fold heavier muscle phenotype (McPherron et al., 1997). Roles of MSTN are not limited to skeletal muscle, but extend to cardiac muscle where it also inhibits fiber growth and proliferation (Heineke et al., 2010). Expression of MSTN in the heart has been confirmed in a number of mammals including mice, sheep and chicken (Sharma et al., 1999; Sundaresan et al., 2008). On the contrary, GDF-11 is expressed in different tissue types from those where its close relative MSTN is found. These include but are not limited to brain and dental pulp (Nakashima et al., 1999). GDF11 has been associated with a number of functions; above all regulation of neurogenesis, but it is also involved in the development of kidney, pancreas and retina tissue (Dichmann et al., 2006; Esquela and Lee, 2003; Kim et al., 2005; Wu et al., 2003). Most important, however, is the notion that despite functioning in very diverse tissue types both the MSTN and GDF11 appear to have a negative effect on their targets, a feature thereby shared by the whole MSTN/GDF11 family. Evidence also exists suggesting that for certain biological processes (i.e. skeletal patterning) MSTN and GDF11 have redundant functions, an inheritance from their common ancestor before duplication (McPherron et al., 2009). In contrast with mammals where MSTN and GDF11 are expressed in a limited number of tissues, the expression of these genes in teleost fish is rather ubiquitous and in these species they may affect a number of tissue types as diverse as their transcription profiles (Biga et al., 2005; De Santis et al., 2008; Helterline et al., 2007; Rescan et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001).

The use of MSTN-based applications for the improvement of growth in aquaculture species is strictly dependant upon the correct understanding of this gene's functionality. However, studies attempting to elucidate the physiological significance of MSTN in teleosts have been far from conclusive. Two questions still remain unanswered. First and foremost, since teleost *Mstn* genes are expressed in a number of tissue types many authors have speculated that,

unlike mammals, their physiological role might extend to non-muscle tissue. The second question relates to the subfunctionalization, if any, of MSTN-1 and MSTN-2 that may have occurred after the second duplication event (in teleosts only) (Fig 2). In fish muscle where the roles of MSTNs have been investigated in more detail, these proteins play an important role in regulating growth. In fact, a significant change in muscle growth and/or structure was achieved by reducing the circulating levels of MSTNs (Acosta et al., 2005; Lee et al., 2009), overexpressing the MSTN-1 prodomain (Xu et al., 2003), immersing fish in a solution containing soluble MSTN-1 prodomain (Lee et al., 2010), or by targeted *Mstn* gene mutagenesis (Sawatari et al., 2010). While overall promising outcomes have been achieved, previous results have been inconsistent regarding the overall increase of muscle mass and understanding the reasons beneath this may maximize the design of strategies aiming to improve production of aquaculture species.

A main reason of confusion might be identified in the poor understanding of functional differentiation between *Mstn-1* and *Mstn-2* paralogs, along with the inability or the unawareness of certain studies to discern among them (as suggested by Helterline et al., 2007; Kerr et al., 2005). Initial reports referred in fact simply to "the MSTN" in fish, without considering the presence of two genes (Acosta et al., 2005; Xu et al., 2003). Subsequent studies have shown that due to high similarity between paralogs, even when only one *Mstn* is targeted the expression of both *Mstn* genes will be affected (Lee et al., 2009). This implies that a number of previous studies (particularly those using approaches similar to that of Lee et al, 2009 – i.e. morpholinos or RNAi) most likely assessed growth functions of the teleost *Mstn* gene sub-family as a whole, rather than of individual *Mstn* paralogs. While one may argue that proteins such as MSTN-1 and MSTN-2 showing approximately 90% or more similarity in their bioactive domain might have very similar if not identical effects on an organism's physiology, the possibility that they might have evolved diverse functions should not be disregarded. In mammals, very different biological processes are in fact regulated by

MSTN and GDF11, two paralogs that share approximately 90% of their bioactive domain (McPherron et al., 2009). In support of this hypothesis, very recent reports indicated that by affecting the functionality of the MSTN-1 only, growth is not affected although muscle hyperplasia is significantly stimulated (Sawatari et al., 2010). Conversely, other studies reported that by over-expressing the *Mstn-2* gene muscle attachment failure was observed, eventually leading to muscle loss (Amali et al., 2008). These results represent strong evidence that, at least with regard to muscle growth, *Mstn-1* and *Mstn-2* may regulate different processes with regards to muscle growth and it will be therefore beneficial to determine if one paralog in particular is responsible for stimulating growth.

While the understanding of MSTN functioning in teleosts compared with mammals is complicated by an additional round of duplication hence introducing the likelihood of subfunctionalization, invertebrates only possess a single member of the *Mstn/Gdf11* family (Fig. 2) that conceivably resembles the archetypal ancestor. The invertebrate Mstn/Gdf11 may therefore maintain to some extent a combined functional role of the vertebrate MSTN and GDF11. Thus far, little evidence has been presented supporting this hypothesis of a combined role. In decapod crustaceans the MSTN/GDF11 is significantly regulated in muscle during molt-associated atrophy indicating that like the vertebrate MSTN this protein might be involved in the regulation of growth (Covi et al., 2010). In addition, loss-of-function mutation at the gene coding for the receptor of MSTN/GDF11 in *Drosophila melanogaster* was associated with a delay of synaptic development, hence resembling neural functions of the mammalian GDF11 (Aberle et al., 2002; Lee-Hoeflich et al., 2005). While supporting an interesting evolutionary hypothesis and encouraging further studies in this direction, these results do not suffice to clearly define the physiological role of the invertebrate MSTN/GDF11.

1.4 *Lates calcarifer* and *Penaeus monodon* as model commercial species

This research used the barramundi (*Lates calcarifer*) and the black tiger shrimp (*Penaeus monodon*) as model species to advance our understanding of the biological relevance of the MSTN/GDF11 family in fish and crustaceans. Both species are widely cultured in tropical and sub-tropical regions of Australia and particularly in Queensland represent very important aquaculture industries. The production volume of these two species combined exceeds 90% of the total Queensland production according to the most recent survey (Lobegeiger and Wingfield, 2009). Although marine shrimp still represent the prominent industry, barramundi aquaculture is the fastest growing and its production value has grown at an average rate of 35% a year since 2003 defining alone the trend of the whole Australian industry. Besides their commercial importance, barramundi and black tiger shrimp were chosen as model species for this thesis because of the ease of obtaining larvae and juveniles in the location where experiments were conducted. Also, both these species have exceptionally fast growth rates allowing growth differences and their association with *Mstn*-like genes to be identified in short periods of time.

1.5 Thesis aims and structure

This thesis aimed to address key questions necessary to advance the understanding of *Mstn*-like genes in two important aquaculture species and consequently to provide a foundation for future studies investigating strategies for production improvement. The structure and aims of these thesis chapters are as follows.

A major focus of this thesis was the understanding of the regulation of *Mstn* genes abundance using real-time PCR. *Chapter 2* investigated the reliability of three commonly employed methods of normalization for the assessment of gene expression data using barramundi as a

model. Robustness and reliability of the ΔC_q approach (normalizing to cDNA input), the $\Delta\Delta C_q$ method (normalizing to a single reference gene) and normalizing to an averaging of multiple reference genes selected using geNorm software were evaluated. *Chapter 2* reports on the variable and potentially misleading outcomes that may be generated from incautious use of these normalization approaches.

Chapter 3 & 4 aimed to provide evidence of *Mstn-1* and *Mstn-2* differentiation. *Chapter 3*, in particular, addressed the question of whether *Mstn-1* and *Mstn-2* are biologically redundant or may have evolved different tissue-functions in *L. calcarifer*. Due to similarity between genes it is extremely challenging to approach direct functional studies of individual paralogs (Lee et al., 2009), let alone in species with long generation time and very small-sized eggs such as *L. calcarifer*. This chapter has approached the issue of biological redundancy in an indirect manner. In particular, since genes with redundant physiological roles should be at least similarly regulated in this chapter a transcriptional response was induced by subjecting barramundi juveniles to a four week fasting trial and subsequent measurement of the tissue-specific abundance of *Mstn1* and *Mstn2*. In order to further elucidate the evolution of these genes the conservation of *cis*-regulatory elements in the *L. calcarifer* proximal region of *Mstn* genes and in that of other fish species was also analysed.

C*hapter 4* also comparatively assessed the relative abundance of *Mstn-1* and *Mstn-2* in barramundi. Differently from *Chapter 3* where differences in growth were induced by nutritional alteration, *Chapter 4* focused on the transcript abundance in fish exhibiting natural differences in growth rate. Firstly, the expression profiles of *Mstn-1* and *Mstn-2* were determined during embryonic and larval development to provide a representative model for other teleosts with a pattern of growth similar to that of barramundi. More importantly, the abundance of *Mstn-1* and *Mstn-2* was measured in juveniles exhibiting different growth rate and associated with size of muscle fibers (hypertrophy). This chapter provides further

evidence that, despite their similarity and at least in *L. calcarifer*, *Mstn-1* and *Mstn-2* may have a diverse physiological relevance.

Chapter 5 tested the hypothesis of whether the *Mstn/Gdf11* maintains the role of negative regulator of muscle growth in crustaceans and may therefore be directly employed for growth improvement by down-regulating its expression. The complete coding sequence of *Mstn/Gdf11* was isolated from the black tiger shrimp and the differential expression pattern was assessed across various tissue types as well as during the molting cycle. *Mstn/Gdf11* expression was down-regulated using RNAi and the phenotypic effects on growth were measured. *Chapter 5* revealed that in invertebrates like crustaceans MSTN/GDF11 might have an inverted role whereby reduced levels of this gene caused a dramatic slowing of growth rate.

Each data chapter (2-5) of this thesis is presented with a stand-alone introduction, materials & methods, results and discussion section. At the time of the submission of this thesis all data chapters, with the exception of *Chapter 4*, were accepted for publication in peer-reviewed international journals and are presented as published herein with minor modifications.

2 An appraisal of normalization approaches and internal reference genes for quantification of gene expression

2.1 Introduction

Real-time quantitative reverse transcription PCR (RT-qPCR) is a sensitive and target-specific technique that has been widely employed for the quantification of messenger RNA (mRNA) transcripts. Producing a valid and reliable RT-qPCR assay is not an easy task and involves a number of strict requirements, including a procedure to generate a reliable comparative baseline for robust cross-sample comparisons as recently outlined in the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009). The most commonly used methods to produce such a comparative baseline are *i)* ΔC_q which standardizes against the input amount of nucleic acids, usually total RNA, (*ii)* $\Delta\Delta C_q$ which normalizes target gene transcript abundance against a single internal reference gene and requires a strong correlation between reference gene transcript abundance and total amount of RNA and *iii)* geometric averaging of the abundance of multiple reference gene transcripts chosen using geNorm (or similar) software (for example see Vandesompele et al., 2002).

The choice of a normalization approach depends on a number of factors that must be carefully evaluated. Normalizing target gene transcript abundance against the input of nucleic acids is simpler and less costly than approaches using internal reference genes, but if total input RNA is used as the normalizer this technique may not entirely remove the variation introduced during laboratory procedures (i.e. differences in reverse transcriptase (RT) and/or PCR efficiency between samples). To combat this the use of internal references (single or multiple) is considered more desirable by authoritative authors in the biomedical field but this usually requires a careful evaluation of the expression of several candidate reference genes under the

experimental conditions to be assessed (reviewed in Bustin et al., 2009). Omitting to validate the appropriateness of the selected reference gene(s) can lead to inaccurate and misleading results when the reference gene used does not correlate with total amount of RNA but is itself up or down regulated under the selected experimental conditions (Filby and Tyler, 2007). Presently, the majority of investigators favor the use of internal references as the normalization approach but as each additional gene analyzed increases the costs substantially most biological studies can afford to use and/or evaluate only a single reference gene. Fifty per cent of recent RT-qPCR studies in fish used *β-actin* as the only internal reference gene, while genes coding for ribosomal subunits (mainly *18S*) and *elongation factor-1 α* (*ef1-α*) were adopted in a further 30% and 10% of cases respectively (Jorgensen et al., 2006). Most of these studies, however, do not provide evidence of proper validation (Gutierrez et al., 2008) and the use of *β-actin* as a suitable internal control is now widely disputed (for example see Ruan and Lai, 2007; Selvey et al., 2001). Traditionally, reference genes used in lower vertebrates such as fish were chosen based largely on stable expression patterns in mammalian models. Only very recently has the appropriateness of several candidate reference genes been evaluated in fish under multiple conditions including estrogen exposure (Filby and Tyler, 2007) and during embryological development (Infante et al., 2008; Zhong et al., 2008b) as well as during hormonal treatment and feed restriction (Small et al., 2008).

Feed restriction is an approach widely adopted to elucidate the biochemical pathways regulating muscle growth in fish. Compensatory growth, a period of accelerated growth rate that generally follows nutritional restriction, has attracted considerable attention as a means of improving growth rates in aquaculture production systems (Hayward et al., 1997) but is also of considerable interest to other fields of fish biology and fisheries management. Feed distribution in the wild is often patchy, particularly during planktonic dispersal phases, and may be seasonably variable leading to repeated periods of feed restriction followed by

accelerated growth (Ali et al., 2003). The most accredited hypotheses attempting to explain compensatory growth in finfish involve behavioral changes such as increased feed intake (Hayward et al., 1997; Nikki et al., 2004) and/or physiological responses including rapid recovery of hormone production upon re-feeding (Gaylord et al., 2001). With the establishment of RT-qPCR the number of works investigating gene expression in an attempt to determine the underlying molecular basis of compensatory growth in finfish has notably increased (Chauvigne et al., 2003; Johansen and Overturf, 2006; Terova et al., 2006; Weber and Bosworth, 2005).

Paradoxically, studies assessing and evaluating gene expression responses associated with nutritional deprivation in several fish species have to date reported dissimilar expression profiles for the same growth related genes. For example, a key target of many compensatory growth studies in fish is the *Mstn-1* gene. As an important regulator of growth, the characterization of the *Mstn-1* gene expression profile may help pinpoint the time point when muscle growth is turned on and off in growth studies. The expression profile of the *Mstn-1* gene during nutritional restriction has in fact been routinely monitored in muscle using RT-qPCR, but with varying outcomes in different fish species (Johansen and Overturf, 2006; Rodgers et al., 2003; Terova et al., 2006). After four weeks of fasting and generally significant muscle loss the *Mstn-1* gene is apparently stably expressed in adult tilapia muscle (*Oreochromis mossambicus*) when normalized to *18S* reference gene expression (Rodgers et al., 2003), but appears down regulated in rainbow trout (*Onchorynchus mykiss*) when data is normalized to *β-actin* (Johansen and Overturf, 2006) and up regulated when normalized to *β-actin* in European sea bass (*Dicentrarchus labrax*) (Terova et al., 2006). As *Mstn-1* is indisputably a negative muscle growth regulator a significant increase of *Mstn-1* expression would be expected upon fasting and loss of muscle mass. Physiological differences between these three fish species, together with experimental differences, may in part explain the different responses observed at the mRNA level but the possibility of these results being affected by the use of inappropriate reference genes or inadequate normalization approaches

should not be disregarded (Gutierrez et al., 2008). The lack of validation information to support the use of the *18S* and *β-actin* genes as references in the aforementioned studies substantially impedes the ability to critically evaluate and compare the results presented in each case.

Herein, the reliability of the three most commonly employed methods of RT-qPCR normalization for the assessment of gene expression data were assessed utilizing as a case study a four week nutritional fasting experiment in barramundi (*Lates calcarifer*). This chapter evaluated robustness and reliability of measuring the relative expression of the key muscle growth regulator, *Mstn-1*, in response to fasting in barramundi using the ΔC_q approach (normalizing to cDNA input), the $\Delta\Delta C_q$ method (normalizing to a single reference gene) and normalizing to an averaging of multiple reference genes selected using geNorm software. The variable and potentially misleading outcomes that may be generated with these commonly used normalization approaches is reported. In addition, the suitability of a panel of seven candidate reference genes was evaluated using the ΔC_q method, and by assessing gene stability with geNorm software. The candidate reference genes evaluated were chosen on the basis of previously demonstrated stability during fasting in fish (*gapdh, cat-D, ef1-α, 18S*) (Hagen et al., 2009; Rodgers et al., 2003; Small et al., 2008) or for their known involvement in fundamental physiological processes (*α-tub, rpl8, ubq)*.

2.2 Materials and methods

2.2.1 Animals, experimental rearing condition and sample collection

Barramundi (*Lates calcarifer*) juveniles from a single spawning event were obtained from a commercial hatchery and graded to a similar size (~ 7g). Fish were stocked individually into large-mesh plastic net cages to prevent cannibalism and minimize adverse social interactions.

Individual cages were joined together in a single floating unit within a 5000L freshwater circular tank. Experimental treatments were repeated in duplicate (2 x 5000L tanks). To eliminate growth variation that may have been associated with fish cage position the entire floating unit within each tank was rotated ~ 90° every three days. Each tank contained both fasting treatments (n = 25) and control individuals (n = 25), each treatment being randomly allocated to one half of the tank. Water quality was regularly monitored and no differences were recorded between tanks during the entire experimental period.

Before fasting, fish were acclimatized to the cage system, during which time they were fed once a day to satiation with 3mm formulated commercial pellets (Skretting, Australia). As the time taken for each fish to accept food was not consistent among individuals the experimental batch was only considered fully acclimatized when every fish had been actively feeding for at least three weeks. At the end of the acclimatization period, eight fish were randomly sampled from each tank (T_0, n = 8 per treatment) to provide starting levels of gene expression. Half the remaining fish were then fasted, while an equal number of control samples were fed to satiation once a day. Fish were then periodically sampled over a 30 day period at T_1 (4 days of fasting), T_2 (10 days of fasting), T_3 (20 days of fasting) and T_4 (30 days of fasting). Each sampling time comprised 4 fasted and 4 control fish from each of the two replicate tanks (n = 8 per treatment). Fish were humanely euthanized using clove oil and white muscle tissue was immediately dissected from the region adjacent the caudal fin, snap frozen in liquid nitrogen and preserved at -80°C until further processing.

2.2.2 RNA preparation (extraction and quality control), cDNA synthesis and quantification

Total RNA was extracted by homogenizing samples using a PRO200 homogenizer (PRO scientific, Oxford, CT) in Ultraspec RNA (Biotecx, Houston, TX) and precipitated by adding 0.5 volumes of isopropyl alcohol and 0.5 volumes of RNA precipitation solution (1.2 M sodium chloride, 0.8M disodium citrate); the addition of the RNA precipitation solution has

been found to substantially improve RNA purity from fish tissues in our laboratory (Sambrook et al., 1989). Quality of RNA was verified on agarose gels by visual inspection of 18S and 28S ribosomal RNA bands and lack of visible genomic DNA contamination as well by $OD_{260/280}$ and $OD_{260/230}$ absorbance ratios (range: 1.95-2.08) measured on a nanodrop spectrophotometer (Nanodrop technology, Wilmington, DE). RNA samples were quantified by absorbance at 260nm on the nanodrop spectrophotometer, aliquoted and then diluted to a final concentration of 200ng/µl. To eliminate any residual traces of DNA contamination a total of 10µg of total RNA for each sample was treated with a Turbo DNA-free kit (Ambion, Austin, TX), including an ammonium acetate precipitation. First strand complementary DNA (cDNA) was synthesized from approximately 2-3µg of DNAse treated RNA using Superscript III first-strand synthesis supermix (Invitrogen, Carlsbad, CA) with 25µM oligo$(dT)_{20}$ and 25µM random hexamers (Resuehr and Spiess, 2003). For verification of complete DNA removal, an aliquot of each sample's DNase treated RNA was diluted to the same concentration as the RNA used in the cDNA syntheses, this was later PCR amplified using *α-tub* specific primers as a no-amplification control (NAC) ($C_{q\ (NAC\ control)} - C_{q\ (cDNA\ synthesis)} > 10$). The RNA strand was digested from all cDNA synthesis reactions using 1µl of RNAse cocktail (Ambion) in a 20µl cDNA reaction and incubated at 37°C for 30 min followed by deactivation at 70°C for 10 min. To remove any potential PCR inhibitors and facilitate quantification, single strand cDNA was purified using Nucway spin columns (Ambion). The cDNA was then quantified in triplicate using a Quant-it Oligreen ssDNA kit (Invitrogen) and measuring fluorescence with a Chromo 4 detector (Biorad, Hercules, CA) attached to an MJ research DNA engine running Opticon Monitor 3.0 software (Biorad). This cDNA quantification step was undertaken to allow the amount of input cDNA to be standardized to each quantitative PCR assay removing any bias that might otherwise be introduced due to variable RT efficiencies between samples. An aliquot of each cDNA

sample was diluted to a final standardized concentration of 2ng/μl prior to RT-qPCR analyses.

Table 2.1. Sequence discovery of candidate reference genes under investigation. Primer sequences and annealing temperature used for cDNA sequencing in *L. calcarifer*. GenBank accession numbers of all sequences used for alignment and subsequent gene discovery primer design are presented.

Candidate Reference Gene	Primer pair (gene sequence discovery)[a]	Aligned sequence accession numbers	Anneal Temp (°C)
Elongation factor-1α (ef1-α)	JCU_EFIa(v)_F1: GAYCCACATYAACATCGTG JCU_EF1a(v)_R1: GGTGGTTCAGGATGATGAC	AB056104, AF485331, AY643400, AF184170, NM_131263, AB075952, NM_001104662, NM_001037873, DQ402371, AY190693, AB032900	52
Ubiquitin (ubq)	JCU_UBQ(f)_F1: GATTTTCGTGAAGACGTTG JCU UBQ(f)_R1: GCATATCATCTTGTCGCAGT	AY909446, EB038831, AY190746, AB291588	52
Glyceraldehyde-3-phosphate dehydrogenase (gapdh)	JCU_GAPD(f)_F1: GGTBTACATGTTCAAGTATG JCU_GAPD(f)_R1: CATCAAAGATGGAGGAGTG	AB075021, AB300322, AY863148, EU828449, AJ937522, DQ641630, AJ937522, NM_001124209	52
Ribosomal protein L8 (rpL8)	JCU_rpL8(f)_F1: AAAGGTGCYGCTAAACTC JCU_rpL8 (f)_R1: CCTGGACGGTCTTTGTTC	BC065432, EF584753, BT028123	52
18S ribosomal RNA (18S)	JCU_18S(v)_F1: CAATACAGGACTCTTTCGAG JCU_18S(v)_R1: CACTAAACCATCCAATCGGTAG	X98842, X9884, X98844, X98840, X98846, X98843, X98838, NR_003286	55

[a] primer names indicate the taxonomic specificity of generic primers used for sequence discovery, (f) indicates fish sequences only were used for the alignment while (v) indicates other vertebrate sequences were also included in the alignment.

2.2.3 Real time PCR: primer design

No barramundi specific gene sequence information was initially available for five of the seven candidate reference genes, namely *ef1-α*, *ubq*, *gapdh*, *rpl8* and *cat-D*. To enable species specific PCR primers to be developed degenerate or universal primer pairs for each gene were designed from cross-species comparative alignments of fish and/or other vertebrate sequences available from GenBank (Table 2.1). These primers were designed using PerlPrimer v1.1.17 (Marshall, 2004). Barramundi specific sequences were retrieved by PCR amplification of barramundi muscle cDNA in 30 μl reactions containing 1x NH_4-based reaction buffer (Bioline, Taunton, MA), 2.5 mM $MgCl_2$, 200 μM dNTP mix, 0.2 μM of each primer, 1 unit of Biotaq DNA polymerase (Bioline) and approximately 4ng of muscle cDNA. Amplification

cycles, performed using a MJ research thermal cycler, consisted of an initial denaturation step of 2 min at 94°C followed by 32 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at primer specific temperature (Table 2.1) and elongation at 72°C for 45 sec, followed by a final elongation step of 10 min at 72°C. PCR fragments were visualized on a 1.5% agarose gel, cloned into a pGEM-T Easy Vector System (Promega, Madison, WI) and sequenced in both direction using the M13 universal sequencing primers USP-17MER and RSP-25MER (Geneworks, Australia). Sequencing was performed at a commercial facility (AGRF, Brisbane, Australia). The resulting sequences were blasted against those available from the public domain using BLASTN (Altschul et al., 1990) and the barramundi sequences obtained from muscle cDNA were deposited in GenBank (Table 2.2). These barramundi specific sequences were used to design primer pairs for RT-qPCR assays (Table 2.2) using PerlPrimer v1.1.17 (Marshall, 2004). For the remaining two candidate reference genes, *18S* and *a-tub*, the RT-qPCR primers utilized were those previously developed elsewhere (Kumar et al., 2000; Xu et al., 2006). PCR primers for the analysis of the target gene *Mstn1* (Table 2.3) were also developed using PerlPrimer v1.1.17 based on our previous characterization of genomic and mRNA sequences for this species (De Santis et al., 2008).

Table 2.2. RT-qPCR primers used for assessing mRNA expression changes in fasting barramundi muscle. Size of the amplified fragments in barramundi and specific annealing temperatures for each primer pair are presented.

	Gene	Symbol	*L. calcarifer* accession no	Primer pair (RT-qPCR)a	Annealing T (°C)	Amplicon size (bp)
Candidate reference genes	Elongation factor-1α	ef1-α	GQ507427	Lc_ef1α_F: AAATTGGCGGTATTGGAAC Lc_ef1α_R: GGGAGCAAAGGTGACGAC	58	83
	Ubiquitin	Ubq	GQ507428	Lc_ubq_F: ACGCACACTGTCTGACTAC Lc_ubq_R: TGTCGCAGTTGTATTTCTGG	60	119
	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	GQ507430	Lc_gapdh_F: TACGACGACATCAAGAAGG Lc_gapdh_R: CTGGTGCTCTGTGTATCC	56	78
	Ribosomial protein L8	rpL8	GQ507429	Lc_rpl8_F: AACCAAGAAGTCCAGAGTC Lc_rpl8_R: TTGTCAATACGACCACCAC	57	105
	18S ribosomial RNA	18S	GQ507431	18_F: TGGTTAATTCCGATAACGAACGA 18S_R: CGCCACTTGTCCCTCTAAGAA	58	94
	α-Tubulin	α-tub	EU136175	Lca_tub_F: GGCACTACACAATCGGCAAAGAGA Lca_tub_R: TCAGCAGGGAGGTAAAGCCAGAGC	60	144

	Cathepsin D	cat-D	EU143237	Lc_catD_F: AGAAGTTCCGTTCCATCAG Lc_catD_R: GGCGTCAAGGTAGTTCTTC	57	149
GOI	Myostatin-1	Mstn1	EF672685	Lc_mstn_F: ATGTAGTTATGGAGGAGGATG Lc_mstn_R: CTTGGACGATGGACTCAG	58	84

[a] primers for *α-tub* are taken from Xu et al., 2006 and those for *18S* are taken from Kumar et al., 2000.

Table 2.3. Quality control of RT-qPCR amplifications of fed (control) versus fasted barramundi after 4 weeks of experimental treatment.

		Control			Fasting		
Gene	E-1 (%)	Mean C_q	SD	CV (%)	Mean C_q	SD	CV (%)
Ef1-α	96.7	13.26	0.36	2.7	11.52	0.46	4.0
18S	102.9	14.33	0.27	1.2	13.87	0.17	1.2
RpL8	95.7	16.83	0.42	2.5	14.95	0.42	2.8
Gapdh	91.5	16.87	0.29	1.7	18.04	0.41	2.3
Ubq	95.6	19.16	0.08	0.4	17.41	0.07	0.4
α-tub	100.6	22.33	0.10	0.4	22.66	0.12	0.5
Cat-D	93.3	26.04	0.14	0.5	23.24	0.10	0.4

PCR efficiencies (E) are expressed as % and calculated according to the equation [E = $10^{(-1/slope)}$-1]*100. Mean C_qs (quantification cycles) are presented as a level of overall gene abundance. SD is the mean standard deviation of the RT-qPCR triplicates, while CV is the mean coefficient of variation of the RT-qPCR triplicates. Both SD and CV represent a value of intra-assay variability.

2.2.4 RT-qPCR optimization, assay validation and quality control

RT-qPCR reactions were carried out in a final volume of 12µl, using 1x Sybr GreenER qPCR Supermix Universal (Invitrogen), 2.5µM rox reference dye and 0.2µM of each primer and 2ng of cDNA template. Reactions were run in triplicate. PCR amplification cycles were performed on a MJ research DNA engine fitted with a Chromo 4 detector running Opticon Monitor 3.0 (Biorad). Reaction conditions were 2 min at 50°C, 10 min at 95°C followed by 40 cycles of denaturation at 95°C for 15 sec and joined annealing/extension for 30 sec at primer specific temperatures (Table 2.2). A melting curve analysis was performed after every amplification program to verify specificity of the target and absence of primer dimers and a no-template control (NTC) was included with each assay to verify that PCR master mixes were free of contamination ($NTC_{(18S)}$=39.45; $NTC_{(remaining\ genes)} > 40$).

To ensure PCR conditions were optimal a $\log_{10}$ dilution series was produced starting from undiluted cDNA pooled together from three randomly selected treatment and three randomly

selected control samples (T_4). The dilution series was used to generate a standard curve by plotting the quantification cycle (C_q) for each dilution point against the starting quantity of cDNA. Standard curves were used to estimate efficiency (E) and reproducibility of the assay and were run in triplicate on each PCR run (a single assay). E was determined by the equation [$E = 10^{(-1/slope)}$] (Rasmussen, 2001) and ranged from 91.5 % to 102.9 % (Table 2.3). Reproducibility was represented by the R^2 value of the standard curve and was always greater than 0.99. Repeatability (intra-assay variability) was also measured as standard deviation (SD) for the C_q variance (Table 2.3, SD < 0.46, mean SD = 0.24) as indicated in the MIQE guidelines (Bustin et al., 2009). However, to allow comparison with previous studies, the coefficient of variation (CV) (%) of C_q values was also calculated according to Murray & Laurence (1993) (Table 2.3, CV < 0.04, mean CV = 0.015). To avoid confounding technical variation between runs (inter-assay variability), with biological variation between treatments, all treatment and control samples from the same time point were analyzed on the same plate for each gene and standard curves for determining PCR efficiency were included on every plate. Since physiological changes in fasting fish were thought more likely to be evident in the long term, initial investigations of candidate reference gene stability were conducted on fish from the final sampling time T_4 (four weeks fasted) and consisted of 8 fasted and 8 control samples chosen at random from across the two replicate tanks. Following the analyses of sampling time T_4, only candidate reference genes whose expression did not vary between control and fasted fish at T_4 were selected and their expression was analyzed for the intervening sample times (T_0, T_1, T_2, T_3).

2.2.5 Comparison of normalization methods

Three widely used methods of normalization were compared in the present study, including *i)* standardizing the input amount of nucleic acids (ΔC_q method) *ii)* normalizing target gene transcript abundance against a single internal reference gene ($\Delta\Delta C_q$ method) and *iii)*

geometric averaging the abundance of multiple reference genes using geNorm. The *Mstn-1* gene was used as the target gene of interest (GOI) for comparison of these three different methods of normalization based on fish sampled only at T_4 (four weeks). Relative *Mstn-1* expression at this time point was normalized against amount of input nucleic acid (ΔC_q method), in this case quantified cDNA, and was calculated according to the equation [Ratio $_{(test/calibrator)} = E^{\,Cq\ (calibrator)\ -\ Cq\ (test)}$] with individual fish as the test and the average of all eight control fish from the same time point as the calibrator. Normalization against a single internal reference was obtained using the $\Delta\Delta C_q$ method according to the equation [Ratio $_{(test/calibrator)} = (E_{\ target})^{\,\Delta Cq\ target}/(E_{\ normalizer})^{\,\Delta Cq\ normalizer}$] (Pfaffl, 2001). The most stable reference gene (*α-tub*) selected using the ΔC_q method was used as a normalizer (see results). Finally, geometric averaging of multiple reference genes was also performed using geNorm software (Vandesompele et al., 2002). Briefly, the pair wise variation *V* between two sequential normalization factors was calculated and used to determine the minimum number of genes required for a reliable normalization as indicated by the geNorm authors (Vandesompele et al., 2002). This analysis indicated that only two genes were required ($V_{2/3}$ = 0.071). Subsequently, expression stability *M* for each candidate reference gene and appropriate normalization factors were calculated as described in Vandesompele et al. (2002). The final relative expression of *Mstn-1* was obtained by dividing relative expression levels obtained with the ΔC_q method by these geNorm normalization factors.

2.2.6 Statistical analysis

Gene expression levels of control and treatment samples for each gene were statistically compared using separate one-way ANOVAs. When examining the expression of candidate reference genes at different time points each time point was considered separately. Differences of $P < 0.05$ were considered significant. Assumptions for homogeneity of variance were tested using a Levene's test and as all data conformed no transformations were

necessary. All statistical analyses were carried out using SPSS statistical software (SPSS Inc., 2006).

2.3 Results

2.3.1 Evaluation of candidate reference gene stability using ΔC_q method

Relative expression levels of the seven candidate reference genes assessed were obtained by normalizing against cDNA input using the ΔC_q method. The expression level of six out of seven candidate reference genes was significantly different between control (fed) and fasted barramundi after four weeks of nutritional deprivation ($P < 0.01$). Although involved in opposite cellular pathways the transcript levels of *18S, ef1-α* and *rpl8* (protein synthesis) as well as *cat-D* and *ubq* (protein degradation) were all significantly higher, while *gapdh* (involved in glycolysis) was significantly lower, in fasted compared to control fish after 4 weeks (Fig. 2.1).

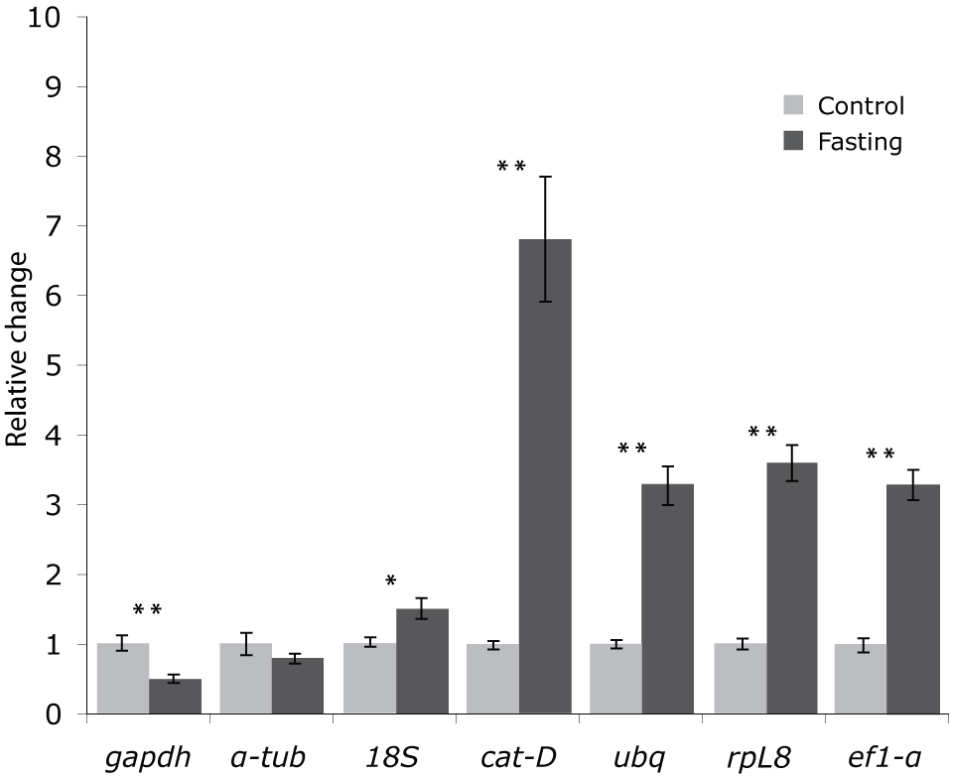

Figure 2.1. Mean (± SEM) expression levels of seven candidate reference genes normalized by ΔC_q (cDNA input) after four weeks (T_4) of fasting in barramundi muscle. Expression is presented as a relative transcript abundance in fasted (n=8) compared with control (n = 8) fish. Gene abbreviations as presented in table 2.2. Genes marked with * ($p < 0.05$) and ** ($p < 0.01$) are statistically different (ANOVA, treatment vs control).

In particular, *cat-D*, *ef1-α*, *ubq* and *rpl8* transcript abundances were 3-6 fold higher in unfed fish ($P < 0.01$), whereas *18S* (~ 0.5 fold higher, $P < 0.05$) and *gapdh* (~ 0.5 fold lower, $P < 0.01$) were only slightly different between control and fasted fish. The only one gene whose expression appeared unchanged after four weeks of nutritional fasting was that of *α-tub*. The transcript levels of *α-tub* were not significantly different in fed versus fasted fish after 4 weeks of fasting ($P > 0.05$) (Fig. 2.1).

2.3.2 Temporal stability of α-tub expression (ΔC_q method)

As *α-tub* showed the greatest uniformity between control and fasted fish after four weeks of nutritional deprivation, the temporal stability of this gene over the full time course of the experiment was examined using ΔC_q analysis to see if this gene was suitable as an internal reference at all time points. At the beginning of the experimental period (T_0) there was no significant difference in *α-tub* transcript abundance between treatment groups (Fig. 2.2). Interestingly, however, *α-tub* exhibited a variable pattern of expression at the intermediate sampling times (Fig. 2.2). In particular, *α-tub* transcript levels were significantly lower in fasted compared to control fish after just 4 days of fasting (~ 0.4 fold lower, $P < 0.01$) and after 10 days (~ 0.5 fold lower, $P < 0.05$; Fig. 2.2). By day 20 (T_3) the expression of *α-tub* in unfed fish stabilized to levels comparable with that of controls as was the case after 4 weeks of fasting ($P > 0.05$, Fig. 2.2).

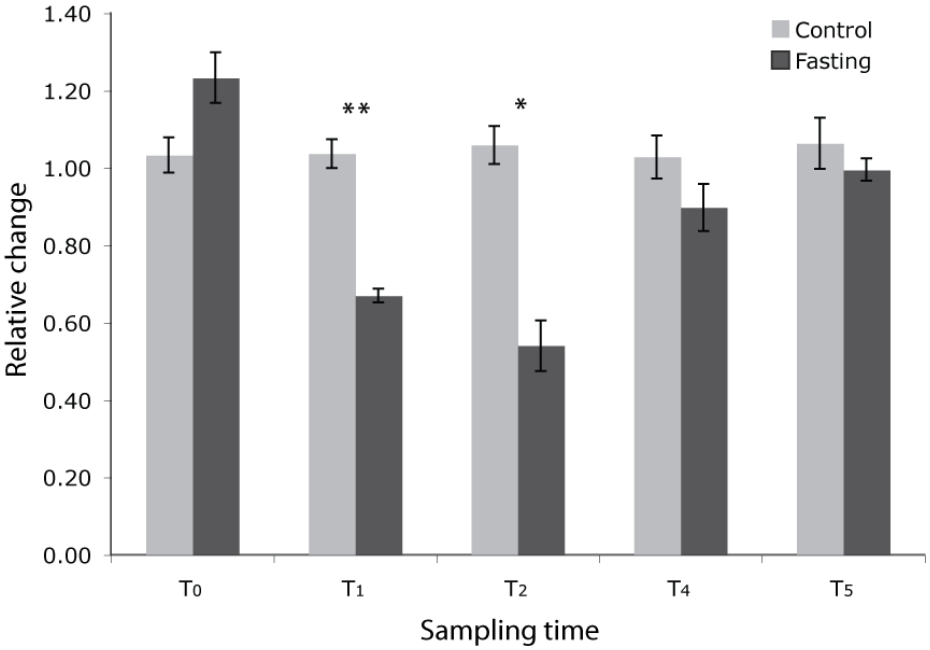

Figure 2.2. Mean (± SEM) expression levels of the candidate reference gene *α-tub* normalized by ΔC_q (cDNA input) during four weeks of fasting in barramundi muscle. Expression is presented as a relative measure of transcript abundance in fasted (n = 8) compared with control fish (n = 8). Sampling times include T_0 (experiment start), T_1 (4 days of fasting), T_2 (10 days of fasting), T_3 (20 days of fasting) and T_4 (30 days of fasting). Sampling times marked with * ($p < 0.05$) and ** ($p < 0.01$) are statistically different (ANOVA, treatment vs control). Note: sampling times are independent from one another; the trend does not represent *α-tub* changes over time but a change of *α-tub* in fasted barramundi compared with control.

2.3.3 Comparison of normalization methods: Mstn-1 case study

All comparisons of normalization methods were performed on fish from sampling time T_4 (4 weeks of fasting) and were based on *Mstn-1* as the target gene of interest. Both normalization against cDNA input using the ΔC_q approach and normalization against *α-tub* using the $\Delta\Delta C_q$ method produced identical results (Fig. 2.3) suggesting that *Mstn-1* transcriptional levels were approximately three fold higher in fasted compared to control fish as is expected for a negative muscle growth regulator. The variation of the distribution around the mean was similar for both methods (Fig. 2.3) indicating that both ΔC_q and $\Delta\Delta C_q$ approaches were equally effective to remove variability introduced during laboratory procedures.

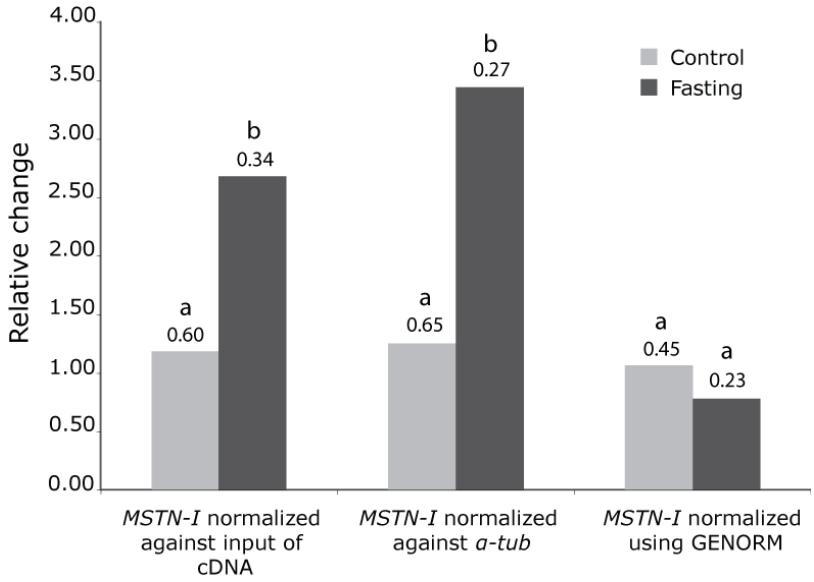

Figure 2.3. Mean expression levels of *mstn-I* after four weeks of fasting in barramundi muscle normalized with three different methods: input cDNA, single experimentally validated reference gene (*a-tub*), geometric averaging of multiple reference genes (*18S and rpL8*) using geNorm. Expression is presented as a relative measure of fasting compared with control fish. Expression levels marked with the different letter are statistically different ($p < 0.01$). Same letter indicates $p > 0.05$. Numbers represent between-sample variation (coefficient of variation).

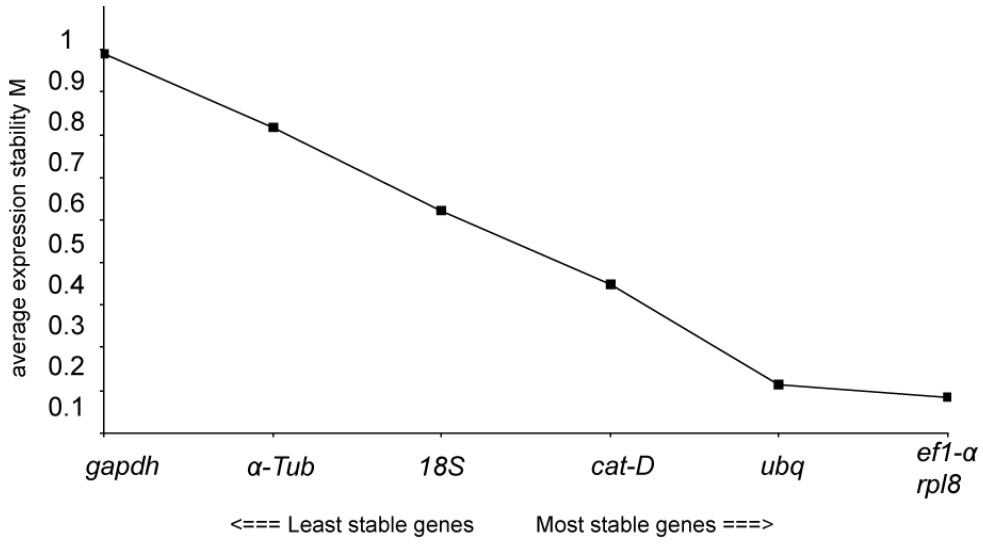

Figure 2.4. Average expression stability (M) of the seven candidate reference genes calculated by the program geNorm. Low M values correspond to high expression

2.4 Discussion

Unveiling expression profiles of growth genes following nutritional deprivation in fish muscle is an essential step towards the understanding of molecular mechanisms regulating muscle

wasting and energy redistribution. To date a stable internal reference and/or a valid normalization approach has not been accurately validated for *L. calcarifer*. In the present case study, seven candidate reference genes were evaluated and three widely used methods of RT-qPCR normalization were compared, including *i)* standardizing the input amount of cDNA *ii)* normalizing target gene transcript abundance against a single internal reference gene and *iii)* geometric averaging of the abundance of two reference gene transcripts chosen using geNorm software (Vandesompele et al., 2002). In the case study of fasting barramundi it is shown that quantifying and standardizing the input level of cDNA and using the ΔC_q method produces the most robust and biologically meaningful assessment of gene expression for a key gene involved in compensatory fish growth (*Mstn-1*). It is also provided experimental evidence that inappropriateness of the normalization method adopted can lead to inaccurate and misleading interpretation of gene expression data, highlighting the importance of always verifying the reliability of the normalization approach employed.

When analyzing gene expression data, stability of candidate reference genes and an appropriate method of normalization must be carefully evaluated. The recently developed MIQE guidelines (Bustin et al., 2009) suggest employing the geometric average of multiple reference genes and assessing gene stability with the support of validated mathematical models such as geNorm (Vandesompele et al., 2002). GeNorm is a user friendly Microsoft Excel application that evaluates the expression stability of internal control genes based on the principle that the expression ratio of two stable reference genes should be the same in all samples (Vandesompele et al., 2002). In this study, the geNorm expression stability analysis indicates that e*f1-α* and *rpL8* had the highest stability value. However, the higher stability of e*f1-α* and *rpL8* was in contrast with the obvious experimental variability of these genes that was observed using the ΔC_q approach. From ΔC_q it was evident that the most uniformly expressed gene is in fact that of *α-tub* (which was ranked by geNorm as the second least

stable) while *ef1-α* and *rpL8* are amongst the most highly affected by the nutritional deprivation imposed during the experiment. The failure of geNorm to identify *α-tub* as the most stable gene indicates an important limitation of this widely used mathematical modeling software. In fact, because the geNorm model estimates are based solely on expression ratios of two genes in different samples, the resulting outcomes are not an indication of stability per se, but are instead an estimate of expression similarities. The ΔC_q analysis showed that e*f1-α, ubq* and *rpL8,* ranked as the most stable genes by geNorm, were simply the genes with the most similar mean expression patterns. Similarly, geNorm also failed to identify the most developmentally stable reference genes during an embryological study of *Paralichthys olivaceus* (Zhong et al., 2008b). A correct prediction of the geNorm model is based on assumptions that at least two stable genes are present and that the transcriptional levels of two most stable genes are more similar to one another than to the expression of any other paired genes. The present appraisal in *L. calcarifer* suggests that the model fails to accurately evaluate gene stability when only one stable gene is present within the candidate panel, as was the case with *α-tub* in this examination of the effects of nutritional deprivation on seven candidate reference genes.

Studies employing RT-qPCR to assess gene expression in fish are increasingly using the geNorm application (for example Small et al., 2008, Hagen et al., 2009). This approach is regarded as the best way for identification of the optimal number and choice of reference genes in biomedical studies (Bustin et al., 2009). Herein, experimental evidence is provided showing that averaging expression of two or more reference genes using geNorm in biological studies can generate misleading outcomes when only one stable reference gene is present within the relatively small but typical panel of candidate references genes examined. The present chapter examined the relative expression of the target gene of interest, *Mstn-1*, normalized to *i)* cDNA input via the ΔC_q method, *ii)* one single reference gene (*α-tub*) ($\Delta\Delta C_q$ method) and *iii)* geometric averaging of e*f1-α* and *rpL8* using geNorm. Both the ΔC_q and

$\Delta\Delta C_q$ methods showed a very similar and biological relevant pattern of *Mstn-1* expression, indicating an approximately three fold relative increase of *Mstn-1* expression in four week fasted barramundi, with low variation around the mean. Alternatively, the spurious interpretation of gene stability performed by geNorm triggered the cascade of analyses that generated a false comparative baseline built on genes (e*f1-α* and *rpL8*) that were up regulated rather than stable under the experimental conditions employed here. When *Mstn-1* expression was normalized using the correction factors proposed by geNorm the outcome indicated that no significant differences between *Mstn-1* transcriptional levels existed between fasted and fed fish. This is contrary to biological expectation as expression levels of *Mstn-1,* a known negative muscle growth regulator gene, have been shown to increase under conditions not favoring muscle growth in numerous other experimental models (Amali et al., 2004; Lee, 2004; Lee and McPherron, 2001; Ma et al., 2003). When the barramundi *Mstn-1* dataset was corrected using geNorm-generated correction factors a significant reduction of technical variation was observed, indicating that averaging two or more reference genes is at least an effective way of reducing introduced technical variation. However, the present results support those of Zhong et al. (2008b) and strongly suggest that geNorm should not be relied upon alone to select suitable reference genes. Candidate reference genes should also be evaluated by the ΔC_q method including cDNA quantification within the RT-qPCR protocol and careful standardization of input cDNA amounts for qPCR. A reliable cDNA quantification step, in which RNA strands are digested from the cDNA preparation and digested RNA fragments removed, is vital in order to properly validate the gene stability pattern proposed by geNorm.

Using the ΔC_q method I have demonstrated that after four weeks of nutritional restriction in barramundi the most stable candidate reference gene was that of *α-tub.* Surprisingly, although transcriptional levels of *α-tub* remained unchanged compared with that of control fish after 30 days of fasting, a clear pattern of varying expression was observed after four and ten days of

fasting. Ideally, reference gene(s) should not exhibit changes of expression levels under the assessed experimental conditions. Evaluating a reference gene's stability at only one sampling point may lead to the false selection of a variable and therefore unsuitable reference gene, because initial investigations missed the "window" of variable gene transcription. These results suggest that for a robust appraisal of a candidate reference gene's stability investigators should provide evidence of the stability of the chosen reference gene for each time point to be examined. Such validation data should be presented to reviewers as a minimum requirement to prove the robustness of RT-qPCR assays and ensure only valid and biologically meaningful gene expression data are published.

Recently, the stability of several candidate reference genes was investigated in channel catfish (*Ictalurus punctatus*) under a variety of experimental conditions including fasting (Small et al., 2008). Four of the seven genes under investigation in channel catfish were also evaluated in the present study. In contrast with findings in barramundi, Small *et al.* (2008) found that *α-tub* was up regulated after four weeks of fasting when compared with control fish but *ef1-α, 18S* and *gapdh* were stably expressed. In channel catfish five of the seven candidate reference genes evaluated were in fact stably expressed in muscle tissue although body weight was dramatically affected by the end of the fasting trial (Small et al., 2008). These results suggest that in barramundi *α-tub* is down regulated after four and ten days of fasting but gene transcript abundance returns to levels comparable with that of control fish by day 20 and is not significantly higher than controls after four weeks. In barramundi, *ef1-α,* and *18S* were up regulated in fasted fish relative to 4 week controls and *gapdh* appeared down regulated at this time point. The fact that after four weeks of nutritional restriction in barramundi the transcript levels of six out of seven candidate reference genes under investigation were significantly altered suggests that muscle tissue in this species undergoes a substantial rearrangement of gene expression in response to fasting.

As observed for barramundi and channel catfish in the specific case of fasting, fish species can respond very differently to stress as a result of adaptation to varying habitats. The extremely variable biological response to stress often corresponds to different underlying gene expression profiles, such as those observed in barramundi and channel catfish. The use of internal reference gene(s), as advocated in the MIQE guidelines for normalization of RT-qPCR derived gene expression data, remains the best way to control for technically induced variation such as variable reverse transcription yield and PCR efficiency differences between samples (Bustin et al., 2009). However, since there is large biological variability among fish species, appropriate validation of gene stability can not rely solely on previous studies but must be assessed for every new experimental model. For non-model organisms this may require substantial investment in gene discovery and sequencing of candidate reference genes before an even larger investment is required to evaluate the expression patterns of up to seven or more candidate reference genes to identify at least one and preferably two or three genes that are truly stable under the experimental conditions employed. In addition, as shown in the present study of barramundi muscle, many of the candidate genes investigated may undergo a stage of regulatory change reducing the likelihood of identifying stable reference genes that can be widely employed across biological time series experiments.

Alternatively, the ΔC_q approach with adequate biological replication, and careful control of input cDNA levels, can be successfully employed to produce biologically meaningful results. The ΔC_q approach as described in the present study and that of Filby and Tyler (2007), utilizes accurate cDNA quantification and post reverse-transcriptase purification techniques and therefore removes completely the technical variation associated with differences in reverse transcription yield. The post reverse transcription clean-up minimizes the carry over of possible PCR inhibitors and results in routinely high PCR efficiencies (91.5% to 102.9% in the present chapter) and acceptably low intra-assay variances (CV 0.4 to 4.0% in this chapter)

as well as removes digested RNA assuring a reliable quantification of the cDNA templates. It must be remembered, however, that normalizing to the input cDNA amount does not remove variation associated with loading errors made at the cDNA dilution stage and during template addition to PCR reactions. Before considering reference gene based approaches it should also be pointed out that additional pipetting errors may also be introduced at the PCR template addition stage if target and reference genes are assayed separately rather than multiplexed in the same reaction. The complications of optimizing multiplexed reactions and the added costs of requiring appropriately labeled primers or probes for multiplexing may substantially outweigh the costs of purifying cDNA and undertaking accurate cDNA quantification.

In conclusion, the results of this chapter suggest that using an appropriate number of biological replicates, the ΔC_q approach to RT-qPCR normalization described here ensures a meaningful and biologically significant appraisal of gene expression, and furthermore eliminates the risks of false outcomes associated with inappropriate use of the relatively limited number of reference gene(s) usually available as candidates in non-model organisms including barramundi and other finfish.

3 Differential tissue-regulation of myostatin genes in the teleost fish *Lates calcarifer* in response to fasting. Evidence for functional differentiation

3.1 Introduction

Gene or genome duplication is a fundamental evolutionary mechanism leading to increases in genetic variability and phenotypic complexity of organisms (Donoghue and Purnell, 2005). Duplication events are thought to generate daughter proteins with transitory functional redundancy whereby two or more paralogs generally perform identical physiological roles (Dehal and Boore, 2005). After a transitional period of redundancy, each paralog protein can specialize into new functions from those primarily performed by the ancestral gene, at first by changes of regulatory mechanisms, and finally by modification in peptide sequence and structure (Hughes, 1994). Gene duplication is believed to be the root of the diversity and complexity observed between vertebrates, which have undergone at least two rounds of whole genome duplication, one at the origin of gnatostomes and the second early during the evolution of ray-finned fish (Christoffels et al., 2004; Dehal and Boore, 2005; Donoghue and Purnell, 2005). As a result, the one-to-four rule applies whereby many genes tend to be present in single-copy in invertebrates, with higher vertebrates possessing two and teleosts at least four copies of the same gene (Ohno, 1999). The *Mstn/Gdf11* gene family structure appears to reflect the multiple rounds of duplication that occurred during the evolution of vertebrates with teleost fish, in particular, possessing at least two *Mstn* paralogs (*Mstn-1* and *Mstn-2*) (Rodgers et al., 2007). Evidence suggests that in some aspects the role of *Mstn* in fish may not be as confined to muscle tissue and therefore not as specialized as its role appears to be in mammals. Either copy of teleost *Mstn* genes are in fact differentially expressed in a variety of tissue types underlying an array of possible functions as diverse as their

transcription profiles (De Santis et al., 2008; Garikipati et al., 2007; Helterline et al., 2007; Ostbye et al., 2001).

Although it is expected that both *Mstn* paralogs in teleost fish may have retained the role of a negative regulator observed in mammals, it has been problematic to distinguish whether functional specialization, if any, exists between the two genes. Direct physiological assessment of MSTN in fish has been conducted primarily through RNA interference (Acosta et al., 2005; Lee et al., 2009) that was proven incapable of selectively silencing only one paralog due to high similarity between the two gene sequences (Lee et al., 2009). Besides, previous authors also suggested that a number of studies trying to infer functions indirectly (i.e. gene or protein expression studies) were either conducted before the discovery of both teleost *Mstn* or did not consider/report the effects of primer/probe cross-hybridization (Helterline et al., 2007; Kerr et al., 2005). It stands to reason that a number of previous results may have biased the ability of establishing functional or regulatory differences that have evolved after duplication of *Mstn-1* and *Mstn-2*.

While the amino acid sequence of the MSTN-1 bioactive domain shares approximately 90% identity with that of the MSTN-2 [i.e. 89.9% similarity in the teleost *Sparus aurata*; (Funkenstein and Olekh, 2010)], precursors of these proteins are differentially expressed depending upon tissue types and it is conceivable that despite the high similarity they might have different functions, an occurrence also observed between mammalian MSTN and GDF11 (McPherron et al., 2009). Since genes with identical functions are expected to exhibit similar responses to an exogenous stress, the present study aimed to elucidate the specific transcriptional response of *Mstn-1* and *Mstn-2* comparatively analyzed in various tissues of the Asian sea bass (*Lates calcarifer*) when fish were subjected to fasting. In order to understand in further details the evolution of *Mstn-1* and *Mstn-2* after duplication the composition of *cis*-regulatory elements in *L. calcarifer* as well as assessed their conservation using representative species of teleosts was determined.

3.2 Material and methods

3.2.1 Sample collection, RNA preparation and cDNA synthesis

The barramundi (*L. calcarifer*) used in this chapter were the same as those used in *chapter 2*, where experimental design and rearing conditions are described in detail. In brief, fish were stocked individually into large-mesh plastic net cages to prevent cannibalism (likely to increase during fasting) and minimize adverse social interactions. The experiment was run in duplicate tanks, with each tank containing both treatment (non-fed) and control individuals (fed). Both treatment and control fish were sampled at the beginning of the experimental trial and after 30 days of fasting (n=8 per treatment). White muscle, liver, brain and gill tissues were immediately dissected, snap frozen in liquid nitrogen and preserved at -80°C until further processing. For consistency white muscle tissue was always dissected from the region adjacent the caudal fin. Fin tissues were also collected and stored in 70% ethanol for extraction of genomic DNA (gDNA).

Genomic DNA was extracted from fin clips using a DNA blood and tissue kit (Qiagen, Valencia, CA). The protocol for total RNA extraction, DNAse treatment, cDNA synthesis and quantification protocols were identical to those described in *chapter 2*. Briefly, RNA was extracted by homogenizing samples in Ultraspec RNA (Biotecx). Quality of RNA was verified on agarose gels by visual inspection of 18S and 28S ribosomal RNA bands and lack of visible gDNA contamination, as well as by $OD_{260/280}$ (range: 1.98-2.13; average 2.02) and $OD_{260/230}$ (range: 1.70-2.23; average: 1.98) absorbance ratios measured on a Nanodrop spectrophotometer (Nanodrop technology). A Turbo DNA-free kit (Ambion) was used for DNA removal. For verification of complete DNA removal, RNA only was later PCR

amplified using *LcMstn-1* and *LcMstn-2* specific primers (intron-spanning; Table 3.1) as a no-amplification control (NAC) ($C_{q\ (NAC\ control)} - C_{q\ (cDNA\ synthesis)} > 10$).

Table 3.1. Name, sequence, annealing temperature (T) of primers used in the present study to *i*) isolate the full sequence of the *LcMstn-2* gene including its 5'-upstream region and *ii*) to quantify the transcript abundance through Real-time PCR

Primer name	Primer sequence (5'-3')	Annealing T	Application
LcMSTN2_jcu_F	AACATCAGCCGGGACATGATC	55°C	Gene discovery
LcMSTN2_jcu_R	TGGGAGCGATGATCCAGTC	55°C	Gene discovery
LcMSTN2_5'RACE_R	GCTGGATCTTTGGACTGAGGCTGAA	72°C	RACE
LcMSTN2_3'RACE_F	GACTGCAACCGTTCATCGAAGTGAA	70°C	RACE
LcMSTN2_3'RACE_nest_F	CCTCAACTGCGACGAGGAGTCG	70°C	RACE
LcMSTN2_IPCR_F	GACGAACCAGACCTCCAAG	55°C	Inverse PCR
LcMSTN2_IPCR_R	GTGAGACAGAACAAGAGG	55°C	Inverse PCR
LcMSTN2IPCR_conf_F	AACTAACTGAACTGAAGGCA	57°C	Inverse PCR confirmation
LcMSTN2IPCR_conf_R	ACGGAAGTCAAGTCAATCAA	57°C	Inverse PCR confirmation
LcMSTN2_qPCR_F	ACGACAGAGACCATCATCAC	60°C	Real-Time PCR
LcMSTN2_qPCR_R	TGAACAGACAACACAAGGAC	60°C	Real-Time PCR

3.2.2 Nucleotide sequence isolation

No previous sequence information was available on the *L. calcarifer Mstn-2* gene (*LcMstn-2*). At first, a fragment of the *LcMstn-2* gene was isolated by the design of primers (LcMSTN2_jcu_F and LcMSTN2_jcu_R, Table 3.1) based on conserved regions between *Sparus aurata*, *Danio rerio*, *Takifugu rubripes*, *Larimichthys crocea*, (Accession numbers AY046314, AY687474, AY445321, EU571244 respectively). Primers for the amplification of the *LcMstn-2* gene were preferentially designed in regions of low identity with that of *LcMstn-1* (EF672685). A mix of muscle cDNA (4ng) from each individual fish and gDNA (4ng) were separately amplified using the aforementioned primer pair with the following conditions: 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 1 unit of Taq polymerase (Bioline) and a standard 35 cycles PCR amplification program (30 s at 94ºC, 30 s at 55ºC and 60 s at 72ºC). For cDNA, a single PCR amplification product of ~663 bp was

visualized on a 1.5% agarose gel stained with GelGreen (Biotium, Hayward, CA), while amplification of gDNA resulted in a ~1300 bp fragment. PCR fragments were cloned into a pGEM-T Easy Vector System (Promega) and sequenced at a commercial sequencing facility (Macrogen Inc., Korea) in both directions using M13 universal sequencing primers. The specificity of the isolated sequences (gDNA, cDNA and translated amino acids) was verified by performing a BLAST search against the entire nucleotide and protein collections available on the public domain (Benson et al., 2007). Both nucleotide and translated protein sequences were also visually inspected by direct alignment with other vertebrate *Mstn* genes. The full-length 5' and 3' ends were isolated using GeneRacer Advanced RACE kit (Invitrogen) and gene-specific primers (Table 3.1) designed on the partial *LcMstn-2* sequence. In addition to the complete mRNA sequence, the 5'-flanking sequence was isolated by inverse PCR (Ochman et al., 1988). Briefly, gDNA was digested using the *HaeIII* restriction enzyme (New England Biolabs, Ipswich, MA), intramolecularly ligated using T4 DNA ligase (New England Biolabs) and finally amplified using primers oriented in the opposite direction of the usual orientation (LcMSTN2_IPCR_F and LcMSTN2_IPCR_R; Table A.1). The identity of 5'-flanking region was confirmed by standard PCR (MSTN2IPCR_conf_F and MSTN2IPCR_conf_R; Table 3.1).

3.2.3 Real-time PCR: assay design and data analysis

The newly isolated *LcMstn-2* gene sequence was used to design an intron-spanning primer pair (Table 3.1) for RT-qPCR using PerlPrimer (Marshall, 2004). An assay for the quantification of *LcMstn-1* transcripts was designed and validated in *chapter 2* where reaction conditions are also reported. Primer specificity and cross-hybridization were verified as suggested by Helterline et al. (2007). Briefly, partial fragments of the *LcMstn-1* and *LcMstn-2* genes containing the entire RT-qPCR amplicon were amplified using Myo-Up1/Myo-L5 (De-Santis et al., 2008) and LcMstn2_jcu_F/LcMstn2_jcu_R (Table 3.1) respectively. PCR

fragments were cloned for sequence verification. RT-qPCR primers were validated using a mismatch assay by cross-pairing plasmids (1 ng) containing *LcMstn-1* or *LcMstn-2* with each gene's specific primer pair. Reactions were run in duplicate, in the same plate and along a temperature gradient (56.4-67°C). The primer specificity assay revealed that at 60°C each primer pair had low affinity for the non-specific *LcMstn* gene (Table 3.2). Routinely, real-time PCR amplicon specificity was confirmed by cloning and sequencing at least once, as well as by melt-curve analyses after each run. Optimal PCR conditions were verified by calculating reaction efficiencies (Rasmussen, 2001). Efficiencies ranged from 98.2% (*LcMstn-2*) to 100.1% (*LcMstn-1*) (Table 3.3). The R^2 value of the standard curve was always greater than 0.99. Low intra-assay variability, measured as standard deviation (SD) for the C_q variance (SD < 0.77, mean SD = 0.31; Table 3.3) and also as coefficient of variation (CV) (%) of C_q values according to Murray and Laurence (1993) (CV < 2.38, mean CV = 1.62; Table 3.3), indicates high experimental reproducibility. For each gene, all treatment and control samples were analyzed on the same plate.

Table 3.2. Primer specificity assays (raw C_q values ± SEM) for the primer pairs LcMSTN1_qPCR_F and LcMSTN1_qPCR_R (1) and LcMSTN2_qPCR_F and LcMSTN2_qPCR_R (2). The primer specificity assay revealed that at 60°C each primer pair had low affinity for the non-specific *Mstn* paralog.

	Plasmid →	LcMstn-2		LcMstn-1	
	Primers →	1	2	1	2
Temperature	56.4°C	35.9±0.76	11.2±0.03	11.3±0.25	32.6±0.09
	57.1°C	35.6±1.11	10.7±0.24	10.7±0.26	32.9±0.13
	58.0°C	36.9±0.96	10.9±0.03	11.12±0.05	33.4±0.10
	59.4°C	36.4*	10.8±0.00	11.2±0.12	35.6±0.02
	61.2°C	37.4*	11.2±0.14	11.4±0.02	35.7±0.04
	63.2°C	NA	11.7±0.01	12.1±0.14	NA
	64.9°C	NA	12.4±0.01	14.8±0.74	39.3
	66.2°C	NA	14.4±0.13	22.2±1.80	NA
	67.0°C	NA	17.5±0.38	30.5*	NA

* indicate that only one of the duplicate C_q values was below 40

Raw C_q data was analyzed using the ΔC_q method whereby the relative expression of each gene was calculated according to the equation [Ratio $_{(test/calibrator)}$ = E $^{Cq\ (calibrator)\ -\ Cq\ (test)}$] with

individual fish as the test and the average of all eight control fish as the calibrator in a tissue-specific manner. *Chapter 2* showed that a careful quantification of nucleic acids provided a safe and more significant biological interpretation of the data when compared to that obtained from using reference genes for normalization. Therefore, in this chapter the same protocols of RNA extraction, cDNA synthesis and quantification as that of *chapter 2* were used to generate a reliable baseline input of genetic material. Normalized expression values of control and treatment samples for each gene were statistically compared using separate one-way ANOVAs. When examining the expression of target genes in different tissues, each tissue was considered separately. Differences of $P < 0.05$ were considered significant. Assumptions for homogeneity of variance were tested using a Levene's test and as all data conformed no transformations were necessary. All statistical analyses were carried out using SPSS statistical software (SPSS Inc., 2006).

Table 3.3. Raw values of *LcMstn-1* and *-2* gene expression.

			Control			Fasting		
Gene	Tissue	E-1 (%)	Mean C_q	SD	CV (%)	Mean C_q	SD	CV (%)
LcMstn1	Muscle	100.1	26.93	0.19	0.70	25.44	0.28	1.09
	Liver		32.86	0.77	2.37	31.09	0.63	2.00
	Brain		27.99	0.29	1.02	28.71	0.24	0.84
	Gill		26.20	0.62	2.36	27.36	0.56	2.06
LcMstn2	Muscle	98.2	31.60	0.68	2.38	31.39	0.56	1.78
	Liver		30.80	0.70	2.25	29.57	0.30	1.00
	Brain		24.34	0.45	1.84	23.81	0.19	0.80
	Gill		24.64	0.51	2.06	23.25	0.32	1.40

Amplification efficiency (E-1 %), mean quantification cycle (C_q), standard deviation (SD), coefficient of variation (CV) was measured in muscle, liver, brain and gill of control and fasting animals.

3.2.4 Teleost promoters analysis

DNA sequences of both *LcMstn-1* (EF672685) and *LcMstn-2* (GU590863) upstream sequences were searched for transcription factor binding sites (TFBS) using the online-based

Alibaba 2.1 search tool (Grabe, 2002), using default parameters and 80% minimum matrix conservation.

Conserved nucleotide stretches between teleost species were otherwise searched using the web-based tool MEME (Multiple Em for Motif Elicitation) (Bailey and Elkan, 1994). Intron-1, intron-2 and 5'-flanking region of piscine *Mstn-1* and *Mstn-2* genes were analyzed independently. False discovery rate was limited by using teleost species evolutionarily distant from each other including *D. rerio* (AY323521, DQ451548), *Oncorhyncus mykiss* (DQ136028, DQ138301), *L. calcarifer* (EF672685, GU590863), *Tetraodon nigroviridis* and *T. rubipres* (sequences for these species were searched on the Ensembl database using *LcMstn-1* and *LcMstn-2* gene as a query) and estimating significance by comparing the *E*-value of the identified motifs with the *E*-value originated by searching shuffled sequences. The lowest *E*-value of the shuffled run was selected as the threshold of significance and results above this value were discarded. Motifs resulting from the described search were queried against the TRANSFAC database using the web-based tool TOMTOM (Gupta et al., 2007; Matys et al., 2003). Only putative targets with *q*-value lower than 0.5 were accepted.

3.3 Results

3.3.1 Characterization of the L. calcarifer Mstn-2 gene

The full-length *LcMstn-2* gene (GU590863), including a portion of the upstream sequence, was cloned and characterized in this study. The full-length mRNA was 1238 bp long and contained putative start (ATG) and stop (TGA) codons that defined an open reading frame (ORF) of 1080 bp (Fig 3.1). Gene structure, defined by comparison between gDNA and cDNA, revealed the presence of two introns of 535 bp (intron 1) and 186 bp (intron 2) and three exons of 328 bp (exon 1), 371 bp (exon 2) and 381 bp (exon 3) (Fig 3.1). This gene structure was consistent with that of the *LcMstn-1* as well as of other members of the vertebrate *Mstn* gene sub-family. Both introns contained distinctive splicing signals at the 5'

(GT) and 3' end (AG) (Fig 3.1). The 3'UTR (133 bp) terminated with the typical polyadenylation signal and presented two putative recognition sites ATTAAA and TGTAN for the binding of CPSF (Cleavage and Polyadenylation Specificity Factor) and CFI (Cleavage Factor I) respectively. These sites were located 22 bp (ATTAAA) and 65 bp (TGTAN) from the polyA signal (Fig 3.1). The *LcMstn-2* nucleotide sequence, when BLAST-searched against the entire database, shared highest similarity with other teleost *Mstn-2* genes including those of *S. aurata* (92%), *L. crocea* (92%) and *Umbrina cirrosa* (90%) and comprised among the first 50 hits other teleost *Mstn-1* and *Mstn-2* genes.

The translated protein sequence accounted for 359aa (amino acids) and comprised a signal peptide (1-16aa) and a TGFβ domain (265-359aa) [predicted by online-tool ELM (Gould et al., 2010)]. The TGFβ domain is the bioactive region of the protein and it is cleaved from the precursor peptide at the R-X-R/K-R proteolytic cleavage site [RSRR (247-251aa) in LcMSTN-2 (Fig 3.2)]. The TGFβ domain also contained nine cysteines (Fig 3.2) conserved between most known vertebrate and invertebrate MSTN. The precursor LcMSTN-2 protein encompassed three putative generic motifs of N-glycosylation (N-X-S/T) at residues 19-24 (TNQTSK), 64-69 (PNISRD) and 166-171 (GNNTRV). Of these, the motif at position 64-69aa (Fig 3.2) was the most conserved between a number of vertebrate and invertebrate species (Fig 3.3) and, as previously shown for other TGFβ proteins (Brunner et al., 1992), most likely maintained a role in the protein functionality. The peptide sequence, particularly that corresponding to the bioactive domain, was extremely conserved between the two paralogs. The most noteworthy difference between LcMSTN-1 and LcMSTN-2 was a 9aa insertion/deletion in the propeptide region (Fig 3.2).

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AGATGTGTTT GTAGCTCCTG CTGTTGTCCA CCAGGTGGCG CCAACAGACC AGGAAACATT [  60]
AAACTAACTG AACTGAAGGC AGAAATAATA ATCAATGATT AAATCAAATA ATCACACAGC [ 120]
TAATAACTGA ACTATAGAAA TACATTTTAT GAAAATGTGA AATAAAAAGA TATTAAGATG [ 180]
CGAATACAAT TACAGTTGTT GTTCTTTACT GTTATGAATC AGAGAGAGAT AAAGTTGATA [ 240]
CCTGCAGGAT AAAAGTAGAA GCTGTGCTCT TTATTTTCTG CCTAAAAACT TTCCACGATA [ 300]
AAAACAAAAG TCCTGTTTGT CTTTGCTGTC TTGGTGTTGC TGAAAAAAGC TGAAAGCACC [ 360]
GTGAAAACAA GAGTTGACAG ACTCCACAGT GGATCCAGAT GAATTAACCA TACCCAACCT [ 420]
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GCTTAAGTGT CCTTGAGCAA GAGGCAATGA CTGCTCCCCC ATCTTCTTTT AACTAAAGTT [ 480]
TCTAAAGCGT TAAATCAGTG GTAAAATGTA GAGCATGTCC TTAAAGATGA GAAAATCTTA [ 540]
ACTCAAGCTT CATTTAAAGA TCATAAAATT GATCATAAAA TCTTGCTACC TCTTTAAATC [ 600]
                                                      TATA
TCCTCTCTCT GATTAAAAAA AGATCCTCCT TGCAGCCAAT CATAAACTCC TGAGGTCCGG [ 660]
                                        CAAT
GGACAGACTC ATATCAGCCA GAGTCGCTGC ACTGACCTCT TCTTCTCTGC AGG        [ 713]
                                          ---------->
                                                mRNA
M   L   L   L   F   C   L   T   L   F   F   S   A   A   F   S   M   E   T   N
ATG CTC CTC TTG TTC TGT CTC ACC CTC TTC TTC TCT GCG GCT TTT TCC ATG GAG ACG AAC [ 773]
Q   T   S   K   L   L   A   E   S   G   E   Q   C   S   A   C   D   F   R   E
CAG ACC TCC AAG CTG CTG GCG GAG AGC GGA GAG CAG TGC TCG GCC TGC GAC TTC CGG GAG [ 833]
H   S   K   Q   M   R   L   H   S   I   K   S   Q   I   L   S   I   L   R   L
CAC AGC AAG CAG ATG AGG CTC CAC AGC ATC AAG TCC CAG ATC CTC AGC ATC CTG CGG CTC [ 893]
E   Q   A   P   N   I   S   R   D   M   I   R   Q   L   L   P   K   A   P   P
GAG CAG GCG CCC AAC ATC AGC CGG GAC ATG ATC CGC CAG CTG CTC CCC AAA GCG CCT CCT [ 953]
L   T   Q   L   L   D   Q   Y   D   P   R   V   E   D   E   D   H   A   T   T
CTG ACG CAG CTC CTG GAC CAG TAC GAC CCG CGG GTG GAG GAC GAG GAC CAC GCC ACG ACA [1013]
E   T   I   I   T   M   A   T   K   H
GAG ACC ATC ATC ACT ATG GCC ACC AAG C                                        [1041]
GTAACTAAAC CAAACCATAC AATACTTTTC AACACTTTCC AAAGTATAAT GTTTATATCT [1101]
GTGGGAGTAG AAGAACAAAA CATCAGAGCA GATCCAATAA TTGAGTTAAT TACATGAACA [1161]
TTTCTGCTTG AAGTCACGTT CAAGTCTTAT TTAAAGCAAC AGTATGTAAC TATATCCCAG [1221]
CTTCAGTGTT ACTGTGACGG TTCACTGACT TAATAAACAG AGTTTATTCC ATCTCCCTCT [1281]
AACTGAGTGA AAGTTCCAGA GTAAAGTAAA AAAAACAAAA AAACAGTTCA TCTCCAGCCA [1341]
GAAAACTGAC AGAAATATCA AGAGTTTTAA ATGGAGTTTG GTGTGTTTAT TTGAGCCACA [1401]
GCACTTCCTG CTGCAGAAGC CGGGGATCAT GGGTAATATA CACCGTTCAG CTGTGTTTCA [1461]
GTGTTAAATA TATGATTTTT TTCTGGTGGT GGTGTTTGAG GAAAGGTCAG GAGGTCACCA [1521]
GATTAACCAG GTGAAAAATT TTATATGTGT GATGTATGTG AATGATTTCC TCCAG      [1576]
   N   P   V   A   Q   D   E   L   S   L   C   C   L   F   S   L   S   P   K   I
AT AAT CCC GTC GCC CAG GAC GAG TTG TCC TTG TGT TGT CTG TTC AGC CTC AGT CCA AAG A [1636]
   Q   P   K   N   I   L   S   A   Q   L   W   V   H   L   R   P   A   D   M   V
TC CAG CCC AAA AAC ATC CTG AGT GCT CAG CTG TGG GTC CAC CTG CGT CCG GCC GAC ATG G [1696]
   T   T   V   F   L   Q   I   T   H   L   K   P   G   K   E   G   N   N   T   R
TC ACC ACC GTC TTC CTG CAG ATC ACC CAC CTC AAA CCG GGC AAA GAG GGA AAC AAC ACC C [1756]
   V   R   V   R   S   L   K   I   D   T   D   A   G   A   G   S   W   Q   S   V
GG GTC CGA GTC CGC TCC CTG AAG ATC GAC ACC GAC GCC GGC GCC GGT TCC TGG CAA AGC G [1816]
   D   I   K   S   L   L   Q   A   W   L   R   Q   P   E   T   N   Y   G   I   E
TC GAC ATC AAG TCC CTG CTG CAG GCG TGG CTG CGT CAG CCG GAG ACC AAC TAT GGC ATC G [1876]
   I   N   A   Y   D   S   K   G   E   D   L   A   V   T   S   A   E   P   G   E
AG ATC AAC GCC TAC GAC TCC AAG GGC GAA GAC CTG GCT GTC ACG TCT GCG GAG CCC GGA G [1936]
   E   G   L
AG GAA GGA CTG                                                               [1947]
GTGAGCTCAC AACAAATCAA AATAGAATAG ACAGAGAAAT TAGCATTTTT CATAATAGTT [2007]
CTAATCGTTA ATTTCCATGA AAGTGATGAT ATTTCTGATG AACCACCTCA CTGATCTTTT [2067]
TTTATGTAGA GTCATCAGTT TCACCCCGGT CCAGAGTTTT GACCTCGTCT CTTTAATTTC [2127]
CCTCCTAG                                                         [2135]
Q   P   F   I   E   V   K   I   L   D   S   P   K   R   S   R   R   D   S   G
CAA CCG TTC ATC GAA GTG AAG ATC CTC GAC AGC CCC AAG AGA TCC CGC CGT GAC TCG GGC [2195]
L   N   C   D   E   E   S   A   E   T   R   C   C   R   Y   P   L   T   V   D
CTC AAC TGC GAC GAG GAG TCG GCG GAG ACG CGC TGC TGC CGC TAC CCG CTC ACC GTC GAC [2255]
F   E   E   F   G   W   D   W   I   I   A   P   K   R   Y   R   A   N   Y   C
TTC GAG GAG TTC GGC TGG GAC TGG ATC ATC GCG CCC AAA CGC TAC CGG GCC AAC TAC TGC [2315]
S   G   E   C   E   F   M   H   L   Q   Q   Y   P   H   A   H   L   V   N   K
TCA GGG GAG TGT GAG TTC ATG CAC CTG CAG CAG TAC CCG CAC GCG CAC CTG GTG AAC AAG [2375]
A   N   P   R   G   T   A   G   P   C   C   T   P   T   K   M   S   P   I   N
GCC AAC CCA CGG GGC ACG GCG GGG CCC TGC TGC ACG CCC ACC AAG ATG TCG CCC ATC AAC [2435]
M   L   Y   F   N   R   K   E   Q   I   I   Y   G   K   I   P   S   M   V   V
ATG CTC TAC TTC AAC CGC AAG GAG CAG ATC ATC TAC GGG AAG ATC CCG TCC ATG GTG GTC [2495]
D   H   C   G   C   S   *
GAC CAC TGC GGC TGC TCC TGA                                                  [2516]
GGAAACCCCT GCAGGAGTCA GGGTCGGTCC TGGGAGTAGG ATATGATGGA AACAGTAGAT [2576]
TCTGAAGGTT GTAACGGTGG TAAAATCACA TCAACGATGG CAGAAATGTG TGATTAAATG [2636]
          CFI                                                 CPSF
ATAGAAATGT GACAAAAAAAAAAAAAAAAAAAA                                [2670]
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Figure 3.1. (Previous page). Genomic sequence of the *LcMstn-2* gene. Non-coding regions are italicized. Coding regions are in normal capital letters and the translated amino acid sequence is reported in bolded single-letter code (* indicates the STOP codon). Putative TATA and CAAT boxes are underlined and marked as well as the mRNA starting nucleotide. Splicing sites (GT…AG) are underlined in the intron sequences. Sites for the binding of CPSF (Cleavage and Polyadenylation Specificity Factor) and CFI (Cleavage Factor I) are also underlined. Numbers at the end of the lines indicate nucleotide position.

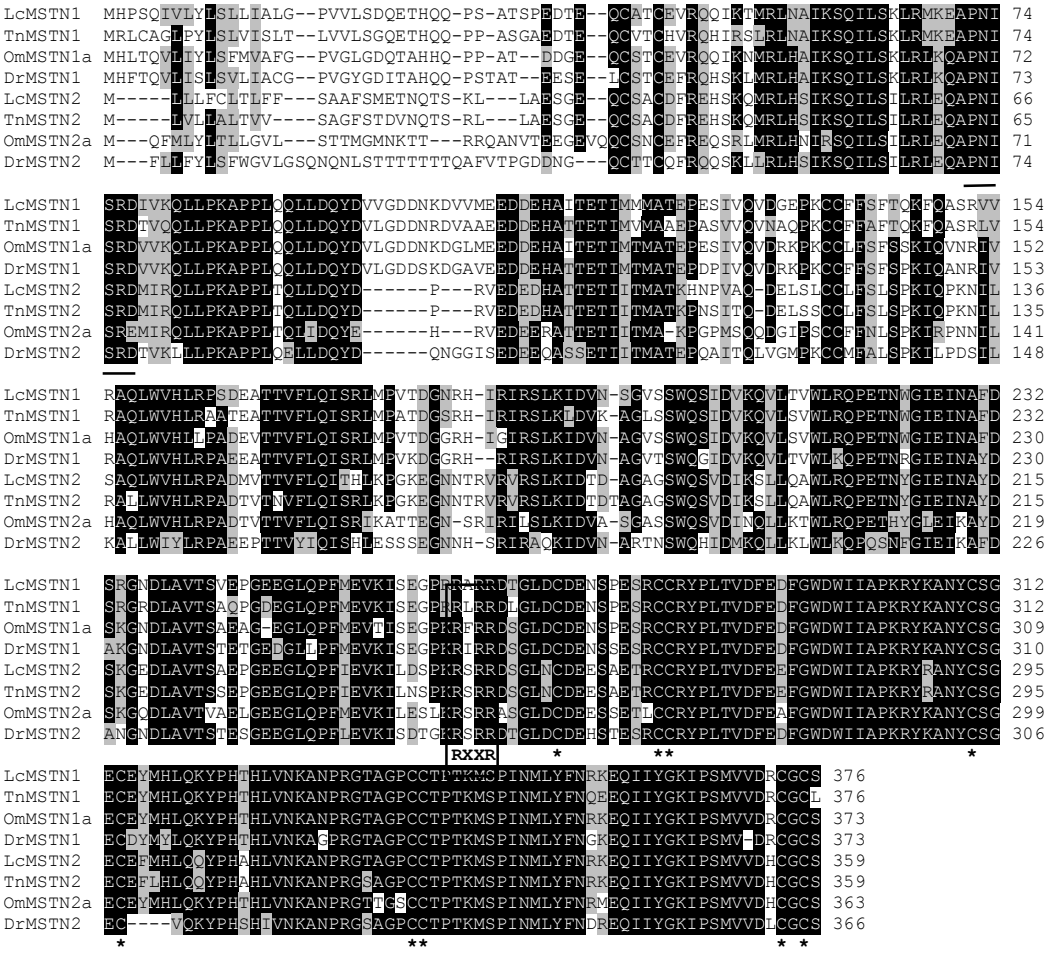


Figure 3.2. Complete protein alignment of piscine *Mstn-1* and *-2* genes. *Lates calcarifer* (LcMSTN1 and LcMSTN1), *Tetraodon nigroviridis* (TnMSTN1 and TnMSTN2), *Oncorhynchus mykiss* (OmMSTN1a and OmMSTN2a) and *Danio rerio* (DrMSTN1 and DrMSTN2) are reported. Underlined is the conserved glycosidation site (PNISRD), boxed is the proteolytic site and asterisks denote the conserved cysteines.

HsMSTN	APNISKD
DrMSTN1	.....R.
DrMSTN2	.....R.
HsGDF11	.....RE
DrGDF11	.....RE
TcMSTN	...VTGR
AiMSTN	M..TTAK
DmMGLN	L...T.P
NvMSTN	...LTDP

Figure 3.3. Snapshot of the glycosidation site across vertebrate and invertebrate species including *Homo sapiens* MSTN and GDF11 [HsMSTN (BC074757) and HsGDF11(NM_005811)], *Danio rerio* MSTN1, MSTN2 and GDF11 [DrMSTN1 (AY323521), DrMSTN2 (AY687474) and DrGDF11 (NM212975)], *Tribolium castaneum* predicted MSTN (TcMSTN; Q26974), *Agropecten irradians* MSTN-like (AiMSTN; AY553362), *Drosophila melanogaster* myoglanin (DmMGLN; AAD24472) and *Nematostella vectensis* MSTN/GDF11 (NvMSTN; XM_001641548). Residues identical to those of HsMSTN are indicated with a dot. It is evident the complete conservation of the core site N-X-S/T.

3.3.2 Comparative tissue-specific regulation of Mstn genes after fasting

Fasting is a known stressor that influences the expression levels of *Mstn* genes in teleost fish (Rodgers et al., 2003; Terova et al., 2006). *L. calcarifer* juveniles were deprived with food for 30 days to induce changes of *Mstn* expression that were measured in muscle, gill, liver and brain of fasted fish and compared with those of control fed animals. Transcriptional levels of both *LcMstn* genes of treatment and control animals measured at the start of the trial (day 0) were not significantly different from that of control fish at day 30 showing that expression of *LcMstn* genes in the control group was unchanged throughout the experiment and that changes observed in the fasting treatment were in response to this stressor. Altogether, both *LcMstn-1* and *LcMstn -2* were detected in every tissue analyzed in this study. While *LcMstn-1* was detected at similar levels of expression in muscle, gill and brain, *LcMstn-2* transcripts were most abundant in brain and gill compared with the other tissues (Fig 3.4). With respect to each individual gene, the regulatory response of *LcMstn-1* and *LcMstn -2* varied greatly in a tissue-dependent manner (Fig 3.5). The *LcMstn-1* was always significantly affected by fasting and was up regulated in liver (~ 4 fold; $P < 0.05$) and muscle (~3 fold; $P < 0.01$) while expression decreased in brain (~0.5 fold; $P < 0.05$) and gills (~0.5 fold; $P < 0.01$). Alternatively, the *LcMstn-2* expression remained stable in brain and muscle, but increased dramatically in gill (~2.5 fold; $P < 0.01$) and liver (~2 fold; $P < 0.05$) (Fig 3.5). Noteworthy, while in fish like *O. mykiss Mstn-2* was found in both spliced and unspliced forms depending upon tissue types (Garikipati et al, 2007), melting curve analyses performed after every run of amplification indicated that in the analysed tissues of *L. calcarifer* only one size amplicon was present that corresponded to the spliced form of this gene. The most remarkable aspect of *Mstn* regulation in response to fasting in *L. calcarifer* was that all tissues except liver displayed a differential response of *LcMstn-1* and *LcMstn-2* (Fig 3.5), which suggested that

these genes are not biologically redundant in function, but that they are independently regulated in a tissue-specific manner.

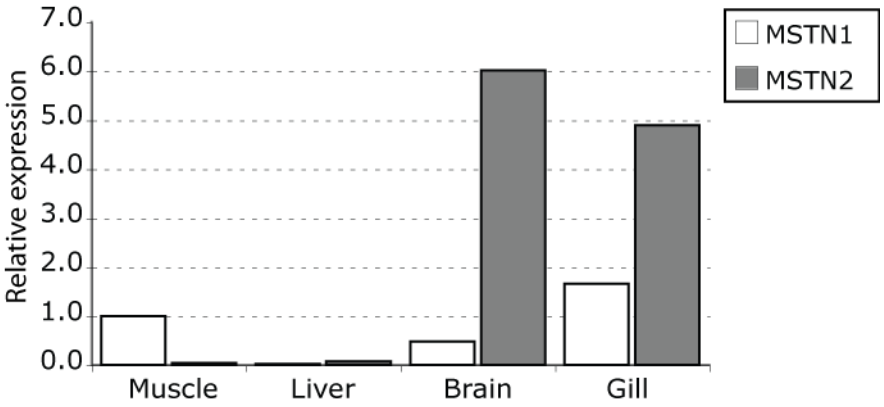


Figure 3.4. Relative tissue expression of *LcMstn1* and *LcMstn2* calculated on average raw C_q value of control fish.

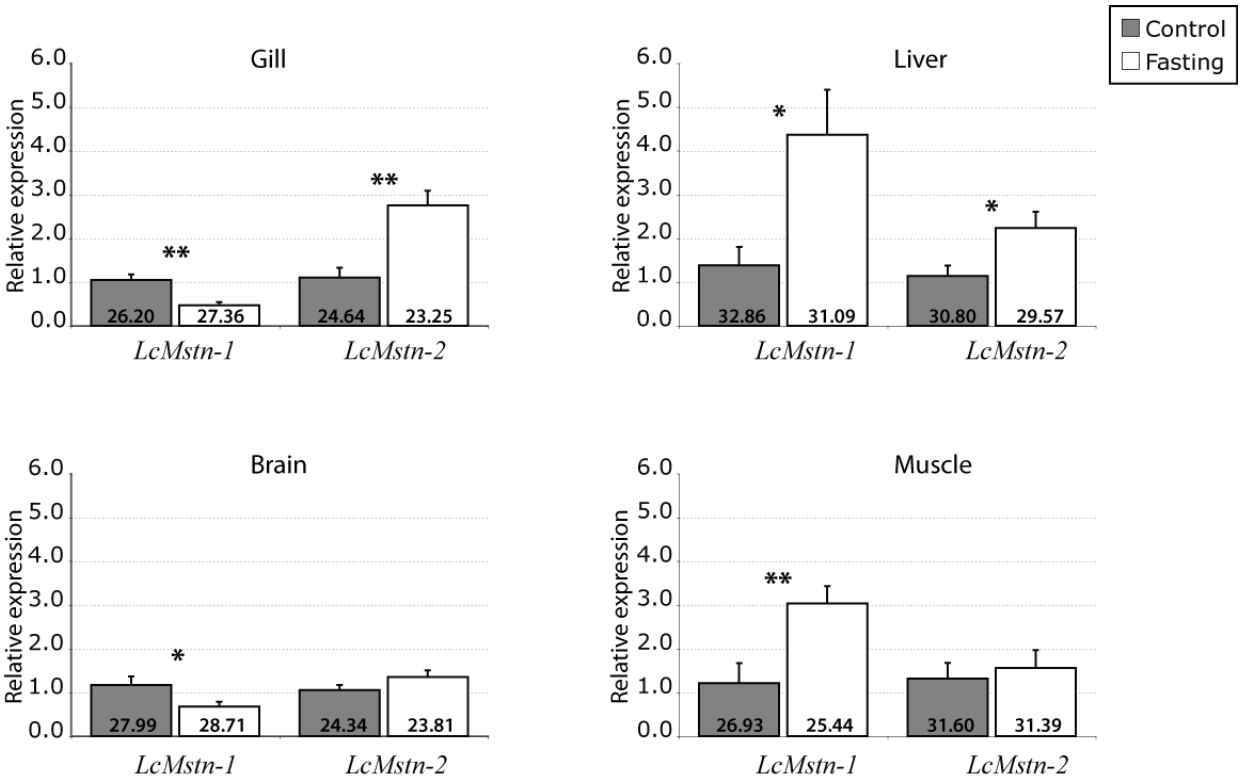


Figure 3.5. Tissue-specific expression of *LcMstn1* and *LcMstn2* in fasted versus control fish. Expression values of treatments are presented relatively to the corresponding control for each tissue.

Numbers inside the column represent the average raw C_q values for that group. Bars are representative of the SEM and asterisks denote statistical significance between fasted and control groups (* = P<0.05; ** = P<0.01)

3.3.3 Comparative characterization of Mstn gene's regulatory motifs

A 689 bp fragment from the *LcMstn-2* upstream region was isolated by inverse-PCR. The *LcMstn-2* 5'-flanking sequence aligned in the proximal region with that of *T. nigroviridis* and *T. rubripes,* but not *D. rerio* and *O. mykiss* (Fig 3.6). A first visual evaluation of sequence alignments indicated that the teleost *Mstn-1* proximal region presented a higher degree of conservation compared with that of *Mstn-2* genes (Fig 3.6 & 3.7). MEME analysis confirmed this trend revealing that the number of conserved sequence stretches was higher and more significant in the *Mstn-1* gene (Fig 3.8). A number of hits, although highly conserved, did not match a known target when queried against the TRANSFAC database using the TOMTOM application and for clarity were excluded from the graphic representation.

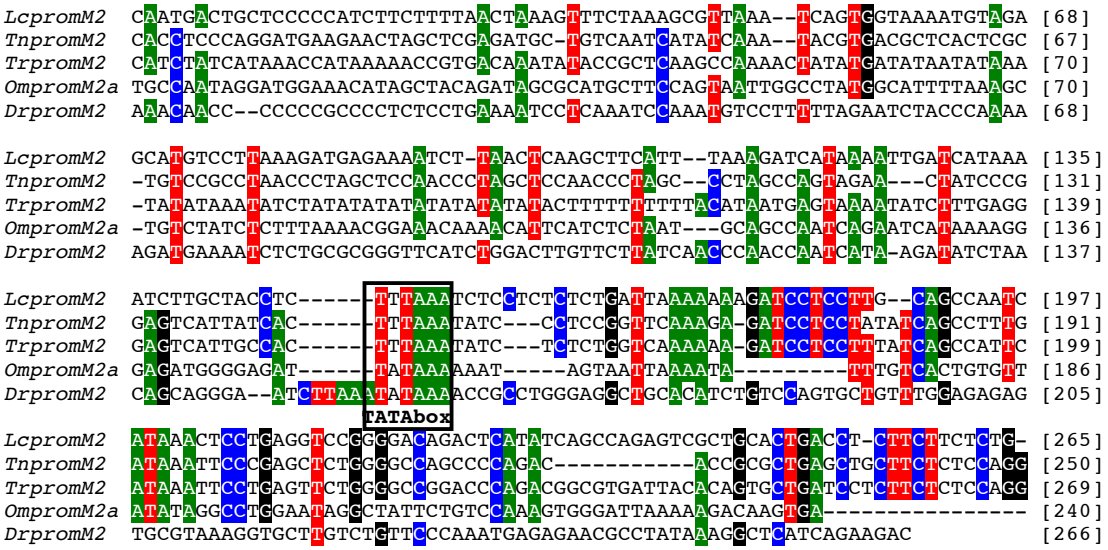
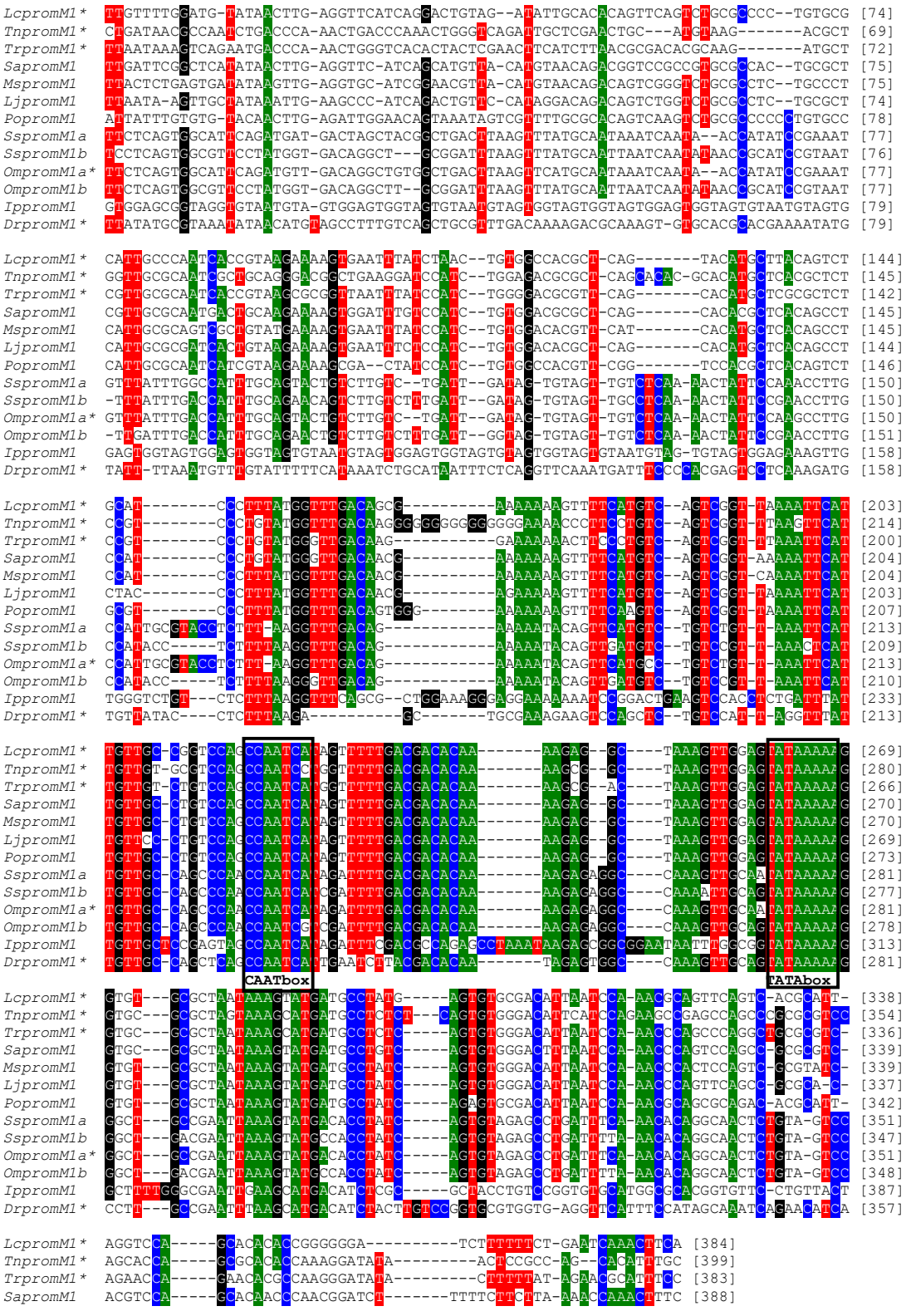


Figure 3.6. Nucleotide alignment of the *Mstn-2* proximal promoter (promM2) of *Lates calcarifer* (*Lc*), *Tetraodon nigroviridis* (*Tn*), *Takifugu rubripes* (*Tr*), *Oncorhynchus mykiss* (*Om*) and *Danio rerio* (*Dr*). Boxed is the putative TATA box. Numbers at the end of the lines indicate nucleotide position.

First and foremost, canonical TATA motif and a CAAT box (Fig 3.7 and Fig 3.8) were found in teleost *Mstn-1* genes at their typical locations that were identical to those of other vertebrates (Grade et al., 2009; Mantovani, 1999; Smale and Kadonaga, 2003). The *Mstn-2,*

on the contrary, presented only a modified TATA and a CAAT box not fully conserved across species (Fig 3.6 and Fig 3.8).



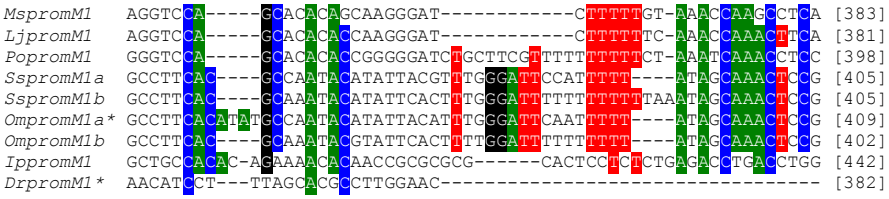


Figure 3.7. Nucleotide alignment of the *Mstn-1* proximal promoter (promM2) of *Lates calcarifer* (Lc), *Tetraodon nigroviridis* (Tn), *Takifugu rubripes* (Tr), *Oncorhynchus mykiss* (Om), *Danio rerio* (Dr), *Sparus aurata* (Sa; EU881511), *Micropterus salmoides* (Ms; EF071854), *Lateolabrax japonicus* (Lj; AY965685), *Paralichthys olivaceus* (Po; DQ997779), *Salmo salar* (Ss1a EF392862 and Ss1b AJ316006), *Ictalurus punctatus* (Ip; AF396747). Boxed are the putative TATA and CAAT boxes. Numbers at the end of the lines indicate nucleotide position.

The modified TATA box did not produce significant hits when searched using TOMTOM, but it was previously shown to efficiently promote transcription (Yaneva et al., 2006). Teleost *Mstn-1* genes also contained three additional sites with high binding affinity for YY1 (Do Kim and Kim, 2009), a factor that can operate as an activator or repressor depending upon the interaction with other proteins (Shi et al., 1991), the Sox family that is very important during the development of vertebrates (Sohn et al., 2006; Wegner, 1999) and MEIS1, a mediator between MyoD and its target (Chang et al., 1997; Grade et al., 2009; Heidt et al., 2007). The analysis of *LcMstn* upstream regions alone using Alibaba 2.1 confirmed the presence of a TATA and two CAAT boxes in the *LcMstn-1* gene and a CAAT box in the *LcMstn-2* gene (Table 3.4 and 3.5). Most notably, the *LcMstn-1* gene presented binding sites for estrogen and glucocorticoid receptors (ER & GR) that were absent in the upstream sequence stretch of *LcMstn-2* isolated herein (Table 3.4 and 3.5). Both *LcMstn* genes contained putative canonical E-boxes consensus motifs (CANNTG) (Fig 3.8), known to bind myogenic regulatory factors through interaction with the basic-helix-loop-helix structural motif.

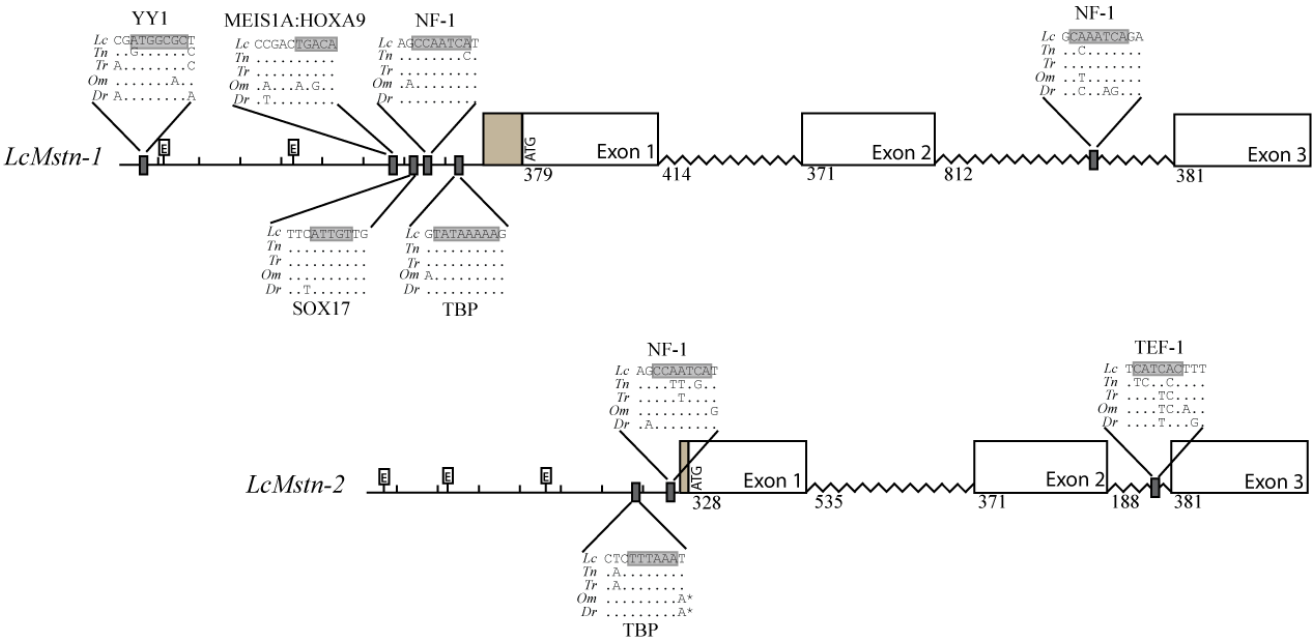


Figure 3.8. Gene structure and transcription factor binding sites (MEME analysis) of *LcMstn-1* and *LcMstn-2*. Numbers indicates the base pair-length of the corresponding gene regions (Exon or intron). The size of exon 1 does not include the untranslated region. Small grey boxes indicates the position of the identified motifs relative to the ATG starting codon in *L. calcarifer*. For each motif, an alignment of *L. calcarifer* (*Lc*), *T. nigroviridis* (*Tn*), *T. rubripes* (*Tr*), *O. mykiss* (*Om*) and *D. rerio* (*Dr*) is presented with dots representing nucleotides conserved with the sequence of *L. calcarifer*. For each alignment the conserved core of the motif is grey-shaded. Most significant transcription factors targeting the binding sites are indicated above each alignment. E-boxes (CANNTG) are also reported. *Mstn-2* most similar TATA motifs identified in *Dr* and *Om* by MEME analysis (marked with *) were in a different position from those found in the remaining species. However, in *Dr* and *Om* a putative TATA box was present at the position indicated on the *Lc* sequence and it is showed in Fig. 3.6.

Table 3.4. Transcription Factor Binding Sites identified in the LcMstn-1 upstream region (EF672685) using the online-tool Alibaba 2.1.

Binding Affinity	Position[a]	Motif
Zinc-coordinating DNA-binding domains		
Estrogen Receptor (ER)	-627/-618	tcagactgac
Glucocorticoid Receptor (GR)	-402/-393	atctgttgtt
Specificity Protein-1 (Sp1)	-453/-444	gggggtgtgg
	-445/-436	ggagggaagg
	-339/-330	tctgcgcccc
GATA-1	-518/-509	cttatcatgt
	-359/-350	agatattgca
p40x	-736/-727	cttttccgtt
Helix-turn-helix		
Octamer binding factor-1 (Oct-1)	-722/-713	agatcatgca
	-601/-592	attcttattt
Antennapedia (Antp)	-604/-595	attattctta
Basic domains		
CAAT Enhancer Binding Protein alpha (C/EBPalpha)	-476/-467	ttttgaaatg
	-423/-414	cattgcccaa
	-323/-314	cattgcccaa
cAmp Response Element-Binding protein (CREB)	-409/-400	tgatatcatc
Others		
Nuclear Factor-kappaB (NF-kB)	-765/-756	agggattttc
CAAT Transcription Factor (CTF)	-317/-308	ccaatcaccg
	-184/-175	ccagccaatc
TATA-box-Binding Protein (TBP)	-141/-132	ggagtataaa

[a] Position [-1] was assigned to the first nucleotide 5' to the ATG first codon

Table 3.5. Transcription Factor Binding Sites identified in the *LcMstn-2* upstream region (GU590863) using the online-tool Alibaba 2.1.

Binding Affinity	Position[a]	Motif
Zinc-coordinating DNA-binding domains		
Retinoid X Receptor beta (RXR-beta)	-23/-14	actgacctct
Specificity Protein-1 (Sp1)	-682/-673	caggtggcgc
	-263/-254	ctgctccccc
GATA-3	-490/-481	gagagataaa
Helix-turn-helix		
Octamer binding factor-1 (Oct-1)	-566/-557	tatgaaaatg
	-560/-551	aatgtgaaat
Hepatocyte Nuclear Factor-1 (HNF-1)	-628/-619	taataatcaa
Basic domains		
CAAT Enhancer Binding Protein alpha (C/EBPalpha)	-446/-437	tctttatttt
CAAT Enhancer Binding Protein (C/EBP)	-77/-68	caatcataaa
Others		
Serum Response Factor (SRF)	-200/-191	catgtcctta
CAAT Transcription Factor (CTF)	-84/-75	tgcagccaat

[a] Position [-1] was assigned to the first nucleotide 5' to the ATG first codon.

3.4 Discussion

Gene duplication is a central evolutionary mechanism driving the increase of genetic and phenotypic complexity of living organisms. In higher vertebrates, MSTN is a potent inhibitor of muscle growth that operates in a very specialized manner (McPherron et al., 1997). As a result of a genome duplication, teleost fish possess two MSTN paralogs that to some extent and at least in muscle play a function similar to that of mammalian counterparts (Amali et al., 2008; Amali et al., 2004; Lee et al., 2009), but are also thought to be involved in a broader spectrum of physiological activities. Although a number of studies have reported that teleost *Mstn-1* and *Mstn-2* displayed a differential tissue-expression, only in *D. rerio* and *O. mykiss* has the regulation of these genes been comparatively investigated in response to exogenous stresses like fasting and over-crowding (Helterline et al. 2007; Johansen and Overturf, 2006).

Gene expression is finely regulated in a tissue and/or stage-specific manner mediated by *trans*-regulatory elements like transcription factors (TF) that bind selectively to consensus DNA motifs (Latchman, 1997). When gene duplication occurs, the purifying selection acting on the two daughter genes becomes more relaxed causing one or both of their sequences, including the aforementioned DNA binding sites, to mutate and differentiate. In the specific case of *L. calcarifer*, this study revealed a high degree of differentiation between regulatory regions of *LcMstn-1* and *LcMstn-2*. The conservation of some *cis*-elements may be interpreted as an evidence of remaining biological redundancy that, as similarly showed for the mammalian paralogs MSTN and GDF11 (McPherron et al, 2009), is very likely to exist also among MSTN-1 and MSTN-2 in teleosts. More importantly, based on the higher sequence conservation observed between teleost *Mstn-1* proximal regions it can be hypothesized that this paralog have maintained a similar physiological role within the whole taxon after duplication. It is conceivable that this/these functions are not limited to muscle tissue but they may extends to several cell types. In fact, not only *Mstn-1* transcripts are ubiquitously expressed, but also *cis*-elements (conserved between teleost species) that targets ubiquitously expressed transcription factors such as YY1 and Sox proteins were herein identified. Finally, as previous authors indicated that a number of *cis*-regulatory elements of the *Mstn-1* were also conserved in higher vertebrates (Grade et al., 2009), it stands to reason that the cellular functions of MSTN-1 most likely resemble those of the common ancestor before duplication. Conversely, while the *Mstn-1* might have maintained functions common with the common ancestor, the lack of an evolutionary conserved region upstream the *Mstn-2* suggests that more relaxed selective pressure might have induced this gene to separate from its paralog. Since duplication occurred early during the teleost lineage, it is conceivable that the *Mstn-2* might have evolved in a taxon-specific manner.

The differentiation of the proximal promoters of *Mstn-1* and *Mstn-2* genes is likely to be a major factor in determining the differential response of these gene paralogs to stimuli like fasting and should be further investigated. In the muscle of *L. calcarifer* in fact, the *Mstn-2*

paralog remained stable after 30 days of fasting as opposed to levels of *Mstn-1* that were found to increase. Since nutritional restriction reduces growth and encourage protein breakdown in muscle, increased levels of *Mstn-1* agree with its functional role established in several other vertebrate species of inhibitor of growth through myocyte proliferation (reviewed in Rodgers and Garikipati, 2008). A similar result as that described in this study was observed in *O. mykiss* where differential muscle transcriptional response of *Mstn* paralogs to 30 days of fasting was also reported. In *O. mykiss* however stable *Mstn-2* was opposed to decreased levels of *Mstn-1* (Johansen and Overturf, 2006). A similar pattern to that observed in muscle of *O. mykiss* was found in the brain of *L. calcarifer*, where the *Mstn-2* also remained unaffected by nutritional deprivation as opposed to diminished levels of *Mstn-1*. Since brain regulation and functions of *Mstn* genes in fish after fasting have never been investigated their physiological significance in this tissue is still a matter of speculation. At least with respect to muscle however, these findings as well as those of *O. mykiss* suggest that while *Mstn-1* is responsive to stimuli like fasting and this response can be different upon species, the *Mstn-2* gene has probably lost those *cis*-regulatory elements, and perhaps functions, responsible for its regulation in this tissue after fasting.

The loss of one candidate site that might explain the differential expression of *Mstn* paralogs in *L. calcarifer* is that of the glucocorticoid responsive (GR) element. Although the analyses of binding sites in this study were limited to short stretches of upstream sequences and results should be approached cautiously, a GR element was only identified in *LcMstn-1* but not *LcMstn-2*. It was shown that glucocorticoids hormones are responsible for the activation of the *Mstn* expression through binding to GR elements *in vitro* (Ma et al., 2001). Interestingly, in other vertebrates nutritional restriction was found to increase the plasma circulating levels of glucocorticoids, while decreasing the brain activity of these hormones (Barcellos et al., 2010; Lee et al., 2000). This work did not investigate circulating levels of glucocorticoids in *L. calcarifer*, however based on previous results and preliminary evidence of this chapter, it

can be hypothesized that these hormones may in turn increase muscle expression while decreasing brain regulation of the *Mstn-1* but do not affect the expression of *Mstn-2* due to lack of GR element in its proximal promoter.

In contrast with muscle and brain where only the *LcMstn-1* expression was affected by fasting, both gene paralogs were regulated in liver and gill. Yet, functions of MSTN in non-muscle tissues are largely unknown. It seems reasonable however that in fish liver, as in muscle, MSTN might participate in the cascade or pathway controlling cell apoptosis and/or proliferation. The liver is an important energy storage that undergoes a dramatic size reduction during fasting as a result of increased apoptosis, mobilization of glycogen reserves and inhibition of cell proliferation, functions known to be mediated by TGFβ proteins (Graslkraupp et al., 1994; Oberhammer et al., 1992; Rios et al., 2007; Tessitore and Bollito, 2006). Indeed, increased hepatic levels of both *LcMstn* transcripts support the hypothesis that both these genes may induce atrophy in this tissue. Noteworthy, the evidence that both *LcMstn* paralogs increased in liver after fasting as opposed to the muscle where only *LcMstn-1* was up regulated may indicate that different *trans*-regulatory factors activate regulate growth in these tissues. This is not surprising since the metabolic status of liver and muscle is affected at different time by nutritional restriction (Rios et al., 2006).

For the first time in fish, this study also reported a diametrically opposite regulation of *LcMstn-1* and *LcMstn -2* in gill where the former was significantly down regulated and the latter increased dramatically after fasting. Beside gas exchange, the gill's major function is to maintain the plasma osmotic homeostasis by regulating the ion exchange between the internal and the external environment. In *Oreochromis mossambicus* fasting caused a drop of osmoregulatory capacity that could not be explained by changes of Na^+/K^+-ATPase enzyme activity, growth hormone/cortisol release or amino acids/glucose mobilization (Vijayan et al., 1996). Thus, it is possible that osmoregulation is controlled by other factors involving,

directly or indirectly, TGFβ protein like MSTN and should be target of future investigations (Fiol and Kultz, 2005; Harms et al., 2000; Shibanuma et al., 1992).

In summary, this chapter presented evidence suggesting that after genome duplication teleost *Mstn-1* and *Mstn-2* underwent a substantial differentiation of non-coding regions ultimately resulting in the gain/loss of *cis*-elements. As shown in *L. calcarifer, D. rerio* and *O. mykiss*, the rearrangement of *cis*-regulatory regions of *Mstn* paralogs translated into differential responses of these genes in a tissue and stage-specific manner (Helterline, et al., 2007; Johansen, Overturf, 2006). These preliminary results strongly encourage future studies to investigate the activity of the putative *cis*-elements identified in fish to further discover function diversification of MSTN paralogs in teleost fish and explain the underlying regulatory mechanisms that control these functions. Many authors have approached *Mstn* as a possible target for the enhancement of fish production, but growth manipulation through this gene without unwanted side-effects will depend on a deeper understanding of its evolution.

4 Abundance of myostatin genes and their correlation with muscle hypertrophy during the development of *Lates calcarifer*

4.1 Introduction

MSTN is a key protein that regulates vertebrate muscle growth and development (see Rodgers and Garikipati, 2008 for extensive review of the topic). In mammalian cells, where its function was first proposed, MSTN inhibits muscle growth and affects both fiber hyperplasia and hypertrophy by preventing the normal progression of cell cycle in myoblasts and it has been shown to control myogenesis from early into embryonic development (*section V.B* of Rodgers and Garikipati, 2008). Unlike mammals where only one *Mstn* gene is present, teleosts possess at least two *Mstn* paralogs (*Mstn-1* and *Mstn-2*) that are independently regulated in a range of tissue types where they possibly have a diverse array of functions. Based solely on tissue expression patterns, many studies speculated that MSTN-1 may be the protein responsible for inhibition of muscle growth, while MSTN-2 was thought to be primarily associated with neural functions as its expression is most abundant in the brain (see *Chapter 3*; Rodgers and Garikipati, 2008). Nevertheless, recent works have refuted this hypothesis and provided evidence that modification of the muscle structure also occurred in *Danio rerio* over-expressing the *Mstn-2* gene hence indicating that MSTN-2 is at least capable of regulating myogenesis (Amali et al., 2008).

In the muscle of teleosts, the hypothesis that MSTN-1 and MSTN-2 are involved in growth regulation is supported by several studies. However, whether these proteins have redundant functions or they regulate different physiological muscle processes remains unclear. Rodgers et al. (2007) and others have suggested that failure to understand individual roles of MSTN-1 and MSTN-2 in regulating muscle growth may partially originate from previous confusing comparison between paralogs, thus they have increasingly encouraged a more attentive

comparative analysis to unravel the functional evolution of these closely related proteins (Helterline et al., 2007; Kerr et al., 2005; Rodgers et al., 2007). Whichever the reasons, assessments of MSTN functions in regulating fish muscle growth have been far from conclusive. For example, one approach that has been adopted is that of RNA interference (RNAi). By injecting double-stranded RNA designed on the tilapia *Mstn-1* into *D. rerio* embryos, Acosta et al. (2005) induced a "giant phenotype" with increased hyperplasia and hypertrophy, a phenotype similar to that observed in mice with disrupted MSTN (McPherron et al., 1997). A similar RNAi experiment, later conducted using *D. rerio*-specific *Mstn-2* double-stranded RNA, also measured increased muscle growth through at least hypertrophy, although growth through hyperplasia was not examined (Lee et al., 2009). Interestingly, Lee et al. (2009) reported that co-suppression of both *Mstn-1* and *Mstn-2* occurred even when only *Mstn-2* was targeted in a highly specific manner. This suggests that RNAi suppressed both genes in these experiments due to high similarity among paralogs, hence limiting the ability of attributing specific roles to one paralog or the other. A more selective approach that employed targeted mutagenesis revealed that decreasing the abundance of the *Mstn-1* only did not affect overall growth in medaka (*Oryzias latipes*) while inducing muscle hyperplasia (Sawatari et al., 2010). An identical phenotype to that observed in medaka was obtained by overexpressing the *Mstn-1* prodomain in zebrafish (Xu et al., 2003). Finally, growth improvement in teleosts was also achieved by blocking the activity of MSTN-1/MSTN2 increasing circulating levels of follistatin, MSTN prodomain and activin type 2 receptor (Carpio et al., 2009; Lee et al., 2010; Medeiros et al., 2009). Summarizing functional evidence arising from the studies abovementioned, one might only speculate that in teleosts *Mstn-1* appears to at least inhibit muscle hyperplasia but not hypertrophy, and that growth through hypertrophy is only attained when also the *Mstn-2* is down regulated. It is evident that more studies are needed to elucidate the specific roles of *Mstn-1* and *Mstn-2* in fish muscle.

Teleost *Mstn* genes, and particularly the *Mstn-1*, are dynamically regulated from early stages of embryonic development where they may control the commitment of germ cells to muscle lineages, or regulate the proliferation and growth of myoblasts (Garikipati et al., 2007; Garikipati et al., 2006; Helterline et al., 2007; Maccatrozzo et al., 2001b; Roberts et al., 2004; Xu et al., 2003; Zhong et al., 2008a). While several authors have reported detection of *Mstn-1* transcripts during early development, only in *D. rerio* has the distinct regulation of *Mstn-1* and *Mstn-2* genes been comparatively analyzed in this life stage (Helterline et al., 2007). During the development of *D. rerio, Mstn* paralogs exhibited a unique relative expression profile confirming that the two genes are at least switched on/off by different *trans*-regulatory factors (confirming the conjectures reported in *Chapter 3*) and suggesting that they may ultimately have evolved different physiological roles. Since *D. rerio* is the only fish species where the *Mstn-2* early developmental profile has been characterized in comparison with that of the *Mstn-1*, it is premature to conclude that this expression trend is representative of the entire teleost taxa. The lack of studies comparatively investigating the expression of *Mstn* paralogs in teleosts has even more significance in view of the fact that mechanisms of muscle growth have been shown to differ in fish with determinant (i.e. *D. rerio*) and indefinite (most commercial species) growth as early as embryonic development (Johnston, 2006). Chances exist that both *Mstn* paralogs might be involved in the development of fish muscle and that their expression profiles might differ in fish exhibiting different mechanisms of muscle growth. Thereby, elucidating the regulation of *Mstn* paralogs during the development of other fishes with determinant and indefinite growth may help clarify our understanding of their function and evolution in fish.

The Asian sea bass (*L. calcarifer*) is a commercially important aquaculture species renowned for its very fast indefinite growth and large overall adult body size (up to 137 cm and 45 kg). Cohorts of farmed sea bass exhibit large differences in growth rate that are evident even from very early life stages of development to the extent that standard culturing procedures involve continuous monitoring and size grading to avoid cannibalism. The present investigation

comparatively assessed the relative abundance of *Mstn-1* and *Mstn-2* in *L. calcarifer*. Firstly, the expression profiles of *Mstn-1* and *Mstn-2* during embryonic and larval development were determined in order to provide a representative model for other teleosts possessing an indefinite pattern of growth. More importantly, the expression of *Mstn-1* and *Mstn-2* in juveniles exhibiting different growth rate and associated the transcript abundance of these genes with size of muscle fibers were analyzed.

4.2 Materials and Methods

4.2.1 Sample collection, RNA preparation and cDNA synthesis

Lates calcarifer used in this study were obtained from a commercial hatchery in North Queensland (Australia). Broodstock spawning was performed according to commercial procedures at 28°C and fertilized eggs were collected immediately after first becoming visible in the water column using mesh nets. Further sampling of developing embryos and larvae included (in bracket is the time elapsed after eggs were first visible in the water column): 8-cell stage (CS) (1 hr), 64-CS (3 hrs), blastula (5 hrs), gastrula (6.5 hrs), neurola (8.5 hrs), fully formed embryo (12 hrs), hatching (17 hrs), 3, 9, 13, 20, 30 hrs and 4, 15 and 21 days after hatching. Larvae were euthanised on ice. Upon collection, stages of embryonic and larval development were visually confirmed under a light microscope (Tattanon and Tiensongrusmee, 1984; Tiensongrusmee et al., 1989). At 30 hours after hatching, larvae were transferred to the James Cook University aquarium facilities and raised according to commercial farming procedures until completion of the experiment (Schipp et al., 2007). All samples were stored in RNA later (Ambion) until further processing. Where the size of individuals did not suffice for RNA extraction (up to 30 hrs after hatching), pools of approximately 50 embryos/larvae were processed as a single biological replicate. At 21 days after hatching fish were graded into four size-classes based on their breadth using a slatted

grill with known gap size: *i)* small (< 1.5 mm), *ii)* medium (1.5 mm < 1.8 mm), *iii)* large (1.8 mm < 2 mm) and *iv)* extra-large (> 2 mm). Cross-section of muscle samples were obtained from the caudal region of fish, preserved in 10% formalin solution and later processed for histological analyses. RNA was extracted from the remaining part of the fish body. While it is known that that *Mstn-1* and *Mstn-2* are expressed in a number of mature and developing tissues (Helterline et al., 2007; Patruno et al., 2008), size of animals impeded to extract RNA from muscle or muscle-enriched tissue since a significant portion of this was used for histological analyses.

Protocols for total RNA extraction, DNAse treatment, cDNA synthesis and quantification were as those described in *chapter 2*. Briefly, RNA was extracted by homogenizing samples in Ultraspec RNA (Biotecx). Quality of RNA was verified on agarose gels by visual inspection of 18S and 28S ribosomal RNA bands and lack of visible genomic DNA contamination as well as by $OD_{260/280}$ (range: 2.00-2.11; average 2.06) and $OD_{260/230}$ absorbance ratios (range: 1.65-2.14; average: 1.89) measured on a Nanodrop spectrophotometer (Nanodrop technology). A Turbo DNA-free kit (Ambion) was used for DNA removal. For verification of complete DNA removal, an aliquot of each sample's DNase treated RNA was diluted to the same concentration as that used in the cDNA syntheses, this was later PCR amplified using *LcMstn* gene specific primers as a no-amplification control (NAC) ($C_{q\ (NAC\ control)} - C_{q\ (cDNA\ synthesis)} > 10$). First strand complementary DNA (cDNA) was synthesized from 3 μg of DNAse treated RNA using Superscript III first-strand synthesis supermix (Invitrogen) and purified using Nucway spin columns (Ambion). The RNA strand was digested using RNAse cocktail (Ambion) and cDNA then quantified in triplicate using a Quant-it Oligreen ssDNA kit (Invitrogen).

4.2.2 Real-time PCR quality control and data analysis

Intron-spanning primer pairs for real-time PCR amplification of *LcMstn-1* (accession number: EF672685) (LcMstn_F: ATGTAGTTATGGAGGAGGATG and LcMstn_R: CTTGGACGATGGACTCAG) and *LcMstn-2* (accession number: GU590863) (LcMstn2_qPCR_F: ACGACAGAGACCATCATCAC and LcMstn2_qPCR_R: TGAACAGACAACACAAGGAC) were previously designed and validated in *chapter 2 & 3*, where reaction conditions are also reported. Primer specificity and cross-hybridization are reported in *chapter 3*, using the approach suggested by Helterline et al. (2007). The efficiency (E) was calculated from amplification of pooled developmental stages and was 99.6% and 98.1% for *LcMstn-1* and *LcMstn-2* respectively. The R^2 value of the standard curves were greater than 0.99. Experimental reproducibility was estimated by intra-assay variability, measured as standard deviation (SD) for the quantification cycle (C_q) variance (SD < 0.77, mean SD = 0.44) and also as coefficient of variation (CV) (%) of C_q values (CV < 1.47, mean CV = 0.88).

4.2.3 Choice of normalization method

Using fasting in *L. calcarifer* as a case study, *Chapter 2* demonstrated that normalization approaches that employ reference genes to reduce the variability among samples introduced during laboratory procedures present otherwise a high risk of introducing a strong bias in data interpretation. Conversely, it is shown that an attentive quantification of nucleic acids, and in particular cDNA, can ensure a meaningful and more significant biological interpretation of gene expression data. Additionally, in view of the results previously presented in *P. olivaceus* (Fig 4.1) showing that most commonly employed reference genes including *Ef1-α*, are highly variable during fish early development (Zhong et al., 2008b), the present chapter analyzed the raw C_q data using the ΔC_q method whereby the relative expression value was calculated

according to the equation [$\text{Ratio}_{(\text{test/calibrator})} = E^{\text{Cq (calibrator) - Cq (test)}}$] (See *Chapter 2* for further details). This choice was carefully made upon the following considerations: *i*) Zhong et al (2008b) showed that every reference genes analyzed in their study but *18S* were highly regulated during early embryogenesis (Fig 4.1), *ii*) the use of 18S as a reference gene has been strongly criticized due to the imbalance between ribosomal and messenger RNA (Solanas et al., 2001), *iii*) the bias eventually introduced by the use of reference genes commonly trusted to be stable such as *Ef1-α* (up to 200 fold increase during early development of *P. olivaceus* – Fig 4.1) would be certainly greater than that introduced by laboratory procedures. The use of a reference gene might in fact provide a false sense of accomplishment due to a sensible reduction of variation (i.e. see Fig 2.3 of *Chapter 2*) while increasing the risk of changing, if not totally inverting, the real biological trend. The risk of using a normalization to the input of nucleic acids, alternatively, would be only limited, in the worse case scenario, to introduction of random variation resulting in higher SD or CV. The use of triplicate samples (or pool of samples) dramatically reduces the probability that one particular measurement is affected by the cDNA quality. These considerations add up to the important investment required to analyze a panel of reference genes.

4.2.4 Muscle histology

For each fish analyzed in this study, a transverse section of approximately 3 mm was obtained from the area corresponding to the anal fin, oriented to allow a cross sectional cut, and then fixed in a 10% formalin solution for 24 h at 4 °C. Infiltration and embedding with paraffin wax were conducted according to standard laboratory procedures (Woods and Ellis, 1994). Samples were sectioned at a thickness of 5 μM using a manual rotary microtome (Leitz, Germany), mounted on slides and stained according to hematoxylin-eosin standard staining protocols (Woods and Ellis, 1994). Sections were examined using a DP12 Microscope Digital Camera System (Olympus, Japan). Muscle fiber numbers were obtained by counting every fiber falling into an area of 0.078012 mm² using ImageJ 1.42 (National Institutes of Health,

USA). Number of fibers within the unit area was used to determine the average size of individual fibers. Each muscle sample was analyzed by examining three locations on the slide and the average area of muscle fibers over all replicates was used for further correlation analyses.

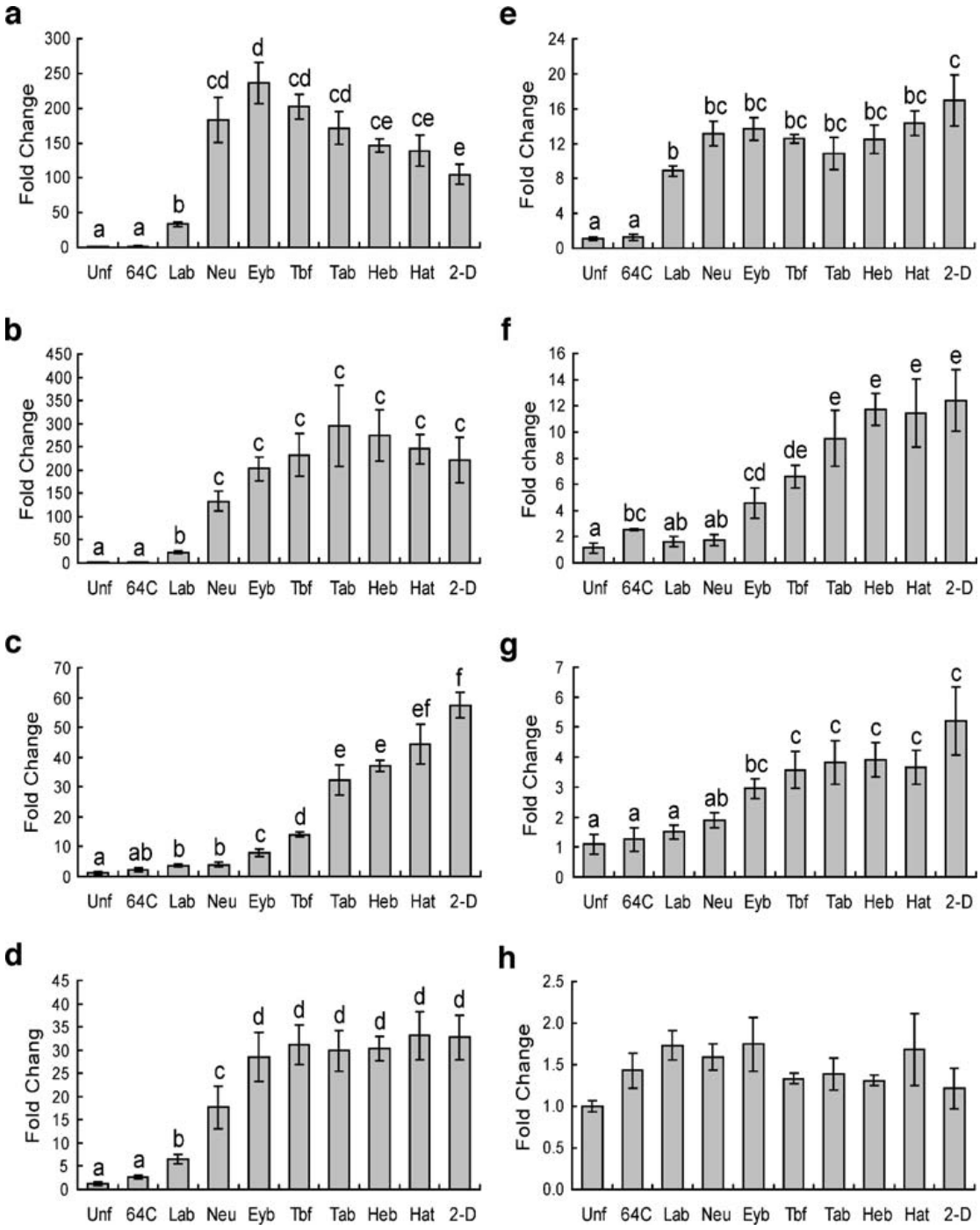


Figure 4.1. (Courtesy of Zhong et al, 2008b). Quantitative analyses of the expression profiles of eight reference genes at unfertilized egg (Unf), 64-cell (64C), late blastula (Lab), neurula (Neu), eye-bud (Eyb), tail-bud–forming (Tbf), tail-bud (Tab), heartbeating (Heb), hatching (Hat), and 2-day-old larva (2-D) stage. The expression of EF1-α (a), rpL17 (b), ACTB (c), α-Tub (d), B2M (e), GAPDH (f), and UbcE (g) were analyzed by $2^{-\Delta Ct}$ method. As for 18S rRNA gene, the amounts of the templates were determined from the standard curve of serial dilution plasmid containing 18S rRNA gene fragment and the expression variance is showed as ratio between the number of copies of 18S rRNA at a particular developmental stage and that in unfertilized eggs (h). Data are shown as means ± SEM (n=3).

4.2.5 Statistical analysis

All statistical analyses were performed using the SPSS software package (SPSS and inc., 2006). Assumptions for homogeneity of variance were tested using a Levene's test and data that did not conform was log or square root transformed. One-way analysis of variances (ANOVA) were used to compare expression values of *LcMstn-1* and *LcMstn-2* and muscle fiber size in graded groups (small, medium, large and extra large). *Post-hoc* multiple comparisons were made using Bonferroni tests. The relationship between muscle hypertrophy and *Mstn* abundance was investigated using the Pearson product-moment correlation (*r*). Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity. Only differences of $P < 0.05$ were considered significant.

4.3 Results

4.3.1 Expression of LcMstn-1 and LcMstn-2 genes during development

Expression levels of both *LcMstn-1* and *LcMstn-2* in fish as they progressed throughout development (until approximately metamorphosis) were assessed using real-time PCR. For better interpretation of the data, the expression profile of each *LcMstn* gene is reported relative to the initial abundance of each gene in fertilized eggs (Fig 4.2a & 4.2b); expression trends of *LcMstn-1* and *LcMstn-2* are also reported relatively to *LcMstn-1* abundance in fertilized eggs (Fig 4.2c). In fertilized eggs and throughout embryonic development until immediately beyond hatching *LcMstn-2* was found to be more abundant than *LcMstn-1* (~ 100 fold; Fig 4.2c). The detection of both gene transcripts in fertilized eggs and the subsequent

gradual decrease observed until blastulation (from 1.1 fold in fertilized egg to 0.6 fold in blastula for *LcMstn-1* and from 1.0 to 0.1 fold for the *LcMstn-2*; Fig 4.2a & 4.2b) were indicative of maternal *Mstn* origin.

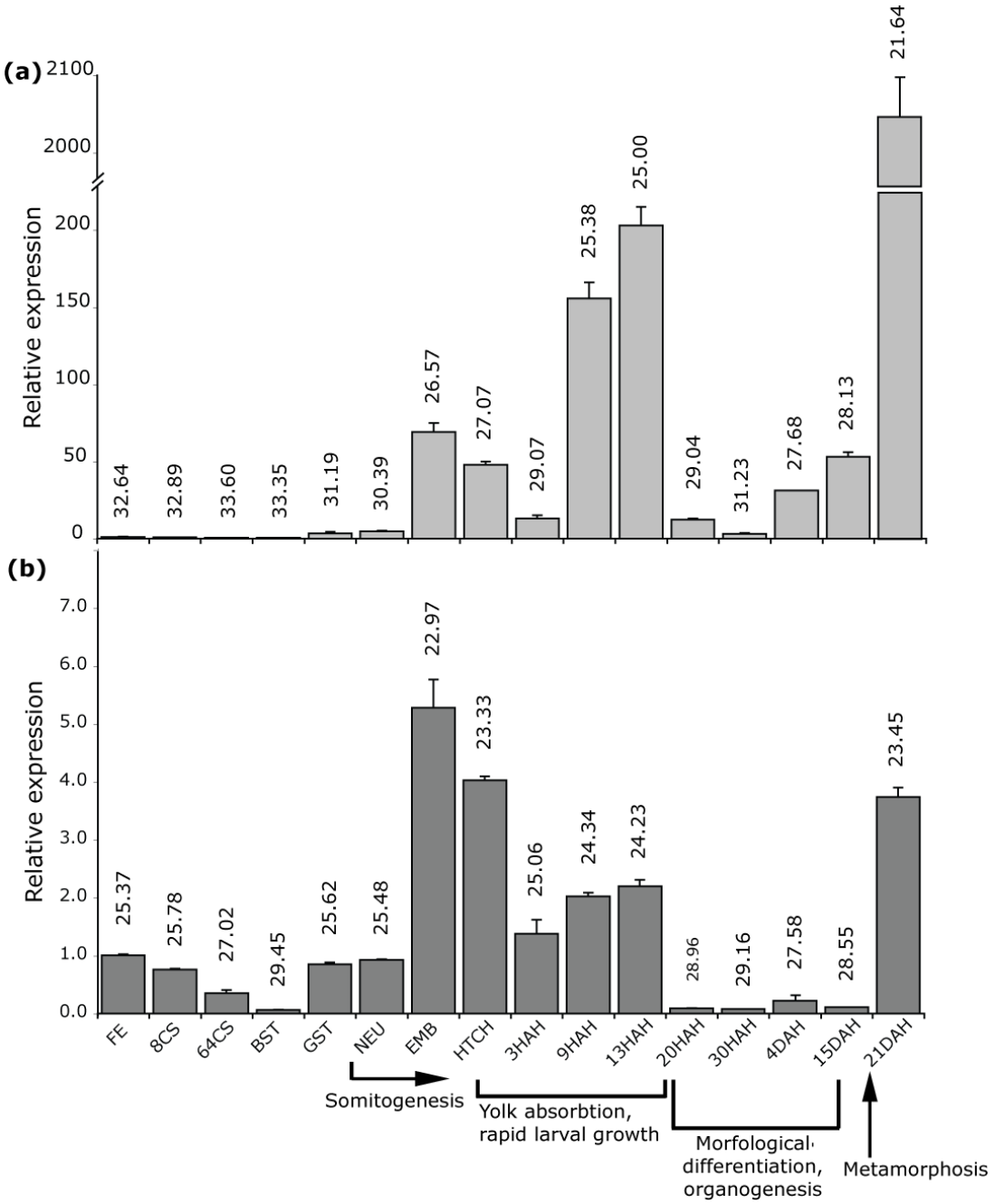


Figure 4.2. Developmental profiles of (a) *LcMstn-1* and (b) *LcMstn-2* expression measured at fertilized egg (FE), 8-cell stage (CS), 64-CS, blastula (BST), gastrula (GST), neurula (NEU), fully formed embryo (EMB), hatching (HTCH), 3, 9, 13, 20 and 30 hrs after hatching (HAH), 4, 15 and 21 days after hatching (DAH). For both genes, expression values are reported relative to those initially found in fertilized eggs. Numbers above each column indicate the average C_q value and is presented for

comparison among genes. Principal phases of *L. calcarifer* development are reported as described by Kohno et al. (Kohno et al., 1986). Bars are representative of SEM.

The expression of both *LcMstn-1* and *LcMstn-2* rose again at gastrula (to 3.4 and 0.9 fold respectively) and neurola stages (to 4.8 and 0.9 fold respectively), in correspondence with the onset of fish somitogenesis (Johnston et al., 2008) where expression was observed to peak in the fully formed embryos (69.0 and 5.3 fold respectively; Fig 4.2a & 4.2b). High transcriptional levels were detected for both *LcMstn-1* and *LcMstn-2* in the period around hatching, with the expression of *LcMstn-1* being prevalent after hatching (up to 202.6 fold; Fig 4.2a) and that of *LcMstn-2* being most abundant immediately before hatching (up to 5.3; Fig 4.2b). Noteworthy, a distinct drop in abundance of both gene transcripts were observed 3 hrs after hatching (from 47.9 to 13.1 fold for *LcMstn-1* and from 4.0 to 1.4 fold for *LcMstn-2;* Fig 4.2a & 4.2b) in correspondence with rapid yolk absorption by developing embryos and very fast growth rate, reported to occur during this developmental phase (Kohno et al., 1986). A second, more important reduction of the abundance of both genes was detected beyond 20 hrs after hatching (from 202.6 to 12.3 fold for *LcMstn-1* and from 2.2 to 0.1 fold for *LcMstn-2;* Fig 4.2a & 4.2b), when most morphological differentiation and organ maturation occurred (Kohno et al., 1986; Tiensongrusmee et al., 1989). After this time both *LcMstn* genes were present at similar levels of expression (Fig 4.3).

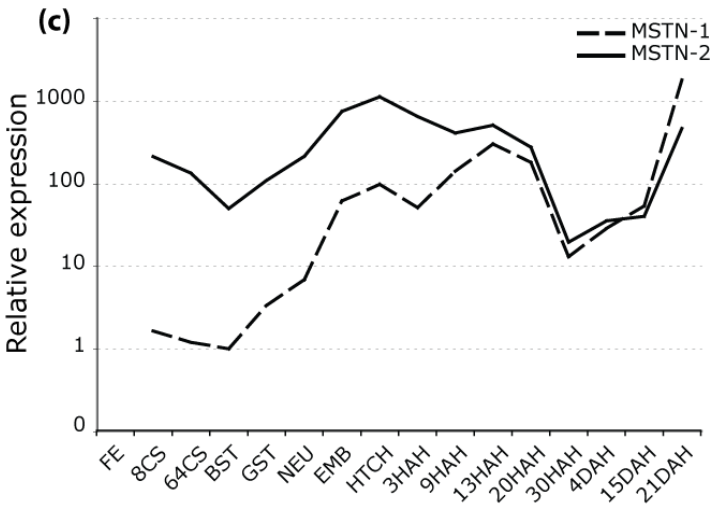


Figure 4.3. Relative expression of *LcMstn-1* and *LcMstn-2* relative to the abundance of *LcMstn-1* in FE. For clarity, data is presented on a logarithmic scale.

Between 20 hrs and 15 days after hatching the *LcMstn-2* expression remained at its minimal transcriptional levels while the *LcMstn-1* gene transcript abundance dropped at 30 hrs (from 12.3 to 3.2 fold) and gradually increased thereafter (31.2 fold and 53.0 fold at 4 and 15 days after hatching respectively; Fig 4.2a). The transcriptional activity of both *LcMstn* paralogs finally increased to 2104 (*LcMstn-1*) and to 3.8 (*LcMstn-2*) fold at day 21 after hatching, in proximity of the period where larvae underwent metamorphosis (Fig 4.2a & 4.2b).

4.3.2 Expression of LcMstn-1 and LcMstn-2 and correlation with muscle hypertrophy

By day 21 after hatching large differences in size were observed in the cultured batch of *L. calcarifer*. Fish of small, medium, large and extra-large grading classes weighed 0.018 g (± 0.0012 SEM), 0.038 g (± 0.0023 SEM), 0.075 g (± 0.0038 SEM) and 0.1771 g (± 0.006 SEM) respectively. Histological analyses revealed that size differences underlay a varied hypertrophy of muscle fibers (Fig 4.4). It was evident that muscle hypertrophy increased significantly in parallel with body size whereby small fish exhibited the lowest average fiber area (2004.3 μm^2) while extra large fish displayed the greatest fiber area (2980.0 μm^2; Fig 4.4, $P < 0.05$). The expression of both *LcMstn* genes presented an evident association with body size (Fig 4.5). Interestingly, while *LcMstn-1* expression rose in parallel with size of animals with large and extra large fish displaying more of this gene than small and medium grades ($P < 0.05$), that of *LcMstn-2* diminished ($P < 0.05$), hence exhibiting a diametrically opposite trend (Fig 4.5). Correlation analyses between *LcMstn* paralog expression and muscle fibers size at an individual level revealed that the abundance of the *LcMstn-2* gene was strongly correlated with fiber hypertrophy ($r = -0.691$, $p < 0.0001$), as opposed to that of

LcMstn-1 that showed only a marginal, non-significant association ($r = 0.384$, $p = 0.064$) (Fig 4.6).

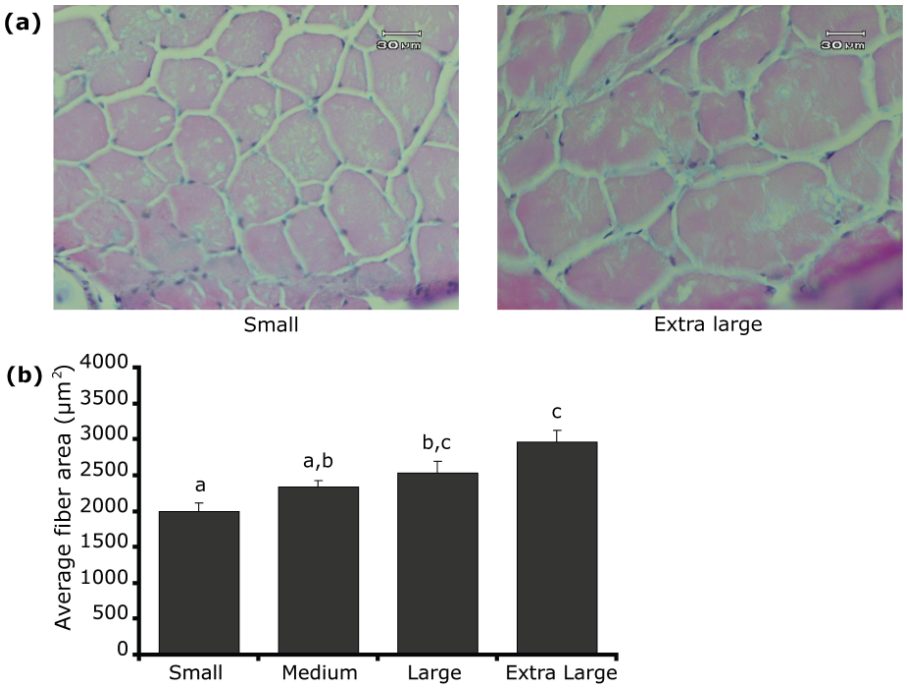


Figure 4.4. Histological analysis of muscle tissue from *L. calcarifer* juveniles of different sizes: (a) Digital images of muscle cross-section in small and extra large grades and (b) average fiber area measured in small, medium, large and extra large grades. Different letters denote statistical significant ($P < 0.05$).

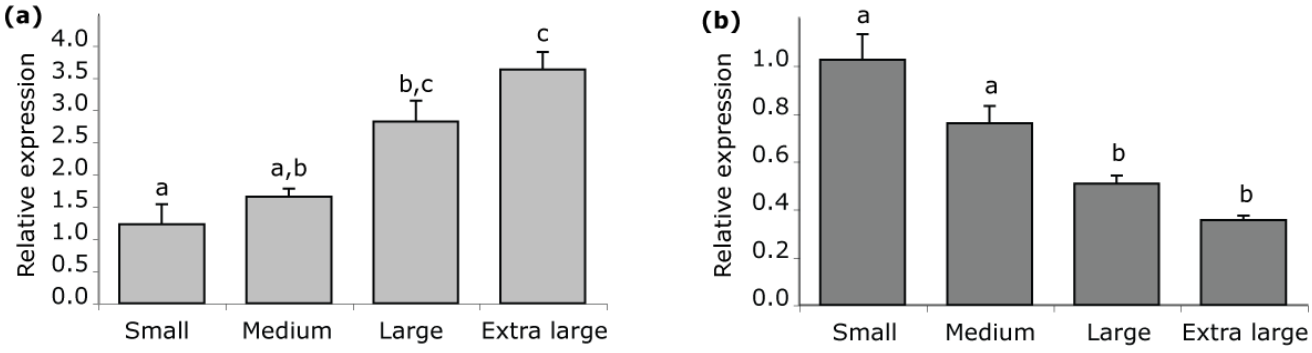


Figure 4.5. Relative expression of (a) *LcMstn-1* and (b) *LcMstn-2* measured in small, medium, large and extra large grades of *L. calcarifer*. Values are reported relatively to the small grade. Different letters denote statistical significant ($P < 0.05$).

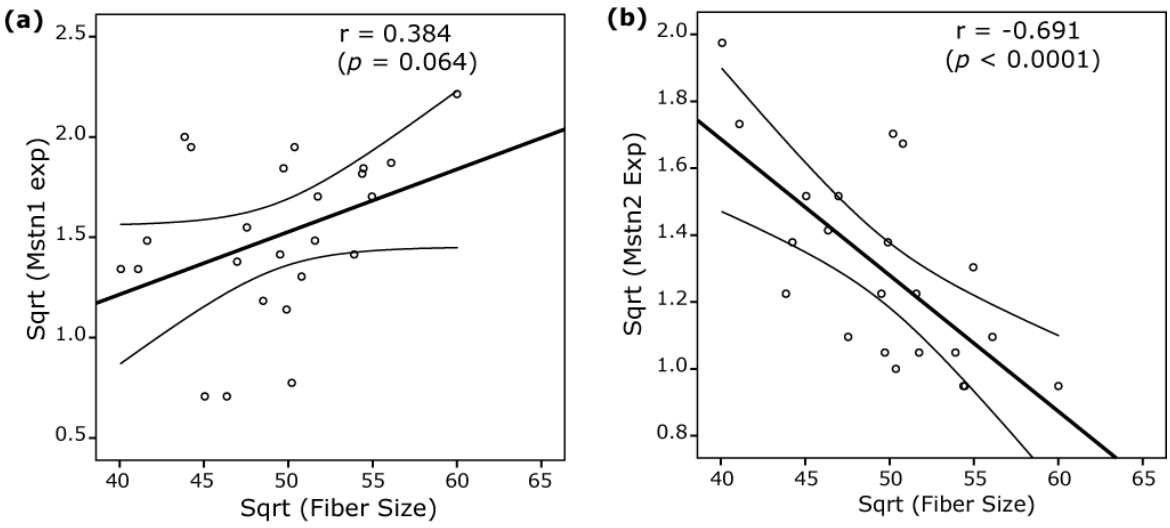


Figure 4.6. Scatter plots for *LcMstn-1* expression and fiber size (a) and *LcMstn-2* expression and fiber size (b). Best linear fit and mean confidence interval represent nature of the relationship. *r* indicates the value of Pearson product-moment correlation calculated on square root transformed data. Values were transformed to conform to the assumption of normality. Significance ($P < 0.05$) is reported in bracket.

4.4 Discussion

In vertebrates, MSTN is a key regulator of muscle growth and development (Rodgers and Garikipati, 2008). While in mammals only one MSTN has been reported, non-salmonid teleost fish possess two paralogs of this gene resulting from genome duplication. Specific roles of the MSTN paralog proteins in regulating fish muscle growth have not been clearly elucidated thus far. This chapter comparatively assessed the expression of *Mstn* genes in the teleost *L. calcarifer* in order to understand their differential regulation as well as their specific relevance to muscle growth and development.

Somitogenesis in fish starts during late gastrulation and progresses throughout embryonic and larval development (Johnston, 2006). Temporal expression profiles of *LcMstn* paralogs were consistent with the major phases of embryonic myogenesis suggesting that these genes may in fact possess a role in regulating teleost muscle development. In *L. calcarifer, LcMstn* paralogs exhibited similar transcription profiles that mostly differed in their relative abundance,

whereby the *LcMstn-2* was generally more abundant than its paralog. This finding is in sharp contrast with that of *D. rerio* where, with the exception of the very early development (up to 11.3 hrs post fertilization), the *Mstn-2* was generally less abundant than the *Mstn-1* (Helterline et al., 2007). It is important to point out that while two different normalization approaches were used to normalize RT-qPCR data in *D. rerio* (data normalized to *Ef1-α*) and *L. calcarifer* (data normalized to input cDNA), it is extremely improbable that the particular contrast observed between the *Mstn* expression level of these two species can be attributed to the methodology adopted. Different normalization approaches in fact, should in no way affect the relative expression of one paralog versus the other.

When analyzed individually however, the developmental expression profiles of *Mstn-1* and *Mstn-2* in *D. rerio* and L. *calcarifer* presented several similarities. The *Mstn-1* expression appears in fact to consistently first peak towards the end of somitogenesis and to generally increase throughout the embryonic and larval development with peaks up to thousands fold compared with starting levels in the egg. Similar results have also been reported in a number of other fish species suggesting that this expression trend should be common to most teleost fish (see for example Ko et al., 2007; Roberts et al., 2004; Zhong et al., 2008a). Conversely, the *Mstn-2* expression appears more stable compared with that of *Mstn-1*, with only minimal overall changes compared to initial stages of development both in *L. calcarifer* (up to 7 fold) and *D. rerio* (up to approximately 20 fold). Noteworthy, in *D. rerio* an isolated peak was reported at 4hrs post fertilization (blastula stage) for both *Mstn* genes, a finding that the authors interpreted as remaining maternal transcripts. This particular result was however in sharp contrast with most findings previously reported in the literature (these referring mostly to the *Mstn-1*), including those of the present chapter, and might simply represent an artifact of the reference gene used to normalize the data (*Ef1-α*). This eventuality is however difficult to assess *a posteriori* since evidence of *Ef1-α* gene stability was not reported in the study (Helterline et al., 2007).

As also reported by previous authors, evidence exists to suggest that *Mstn* transcripts are produced in the ovary and subsequently inherited through the maternal line, a process still to be fully demonstrated but virtually common to all teleosts (Biga et al., 2005; Helterline et al., 2007; Zhong et al., 2008a). In *L. calcarifer* in fact, both *LcMstn-1* and *LcMstn-2* were detected in the fertilized egg and diminished to a minimum level of expression in the blastula. Interestingly, during blastulation of *D. rerio* both *Mstn-1* and *Mstn-2* were shown to be highly transcribed in comparison with other developmental stages and dropped to minimal level thereafter (Helterline et al., 2007). Assuming that the *Mstn-1* and *Mstn-2* expression levels shown during blastulation of *D. rerio* were not biased by the normalization approach, the transcriptional peaks observed at 4hrs post hatching may indicate a delayed degradation of maternal transcripts in this species compared with that observed in *L. calcarifer,* where the embryonic development occurs at a much faster rate. Whichever the case of maternal transcript degradation, most studies agree that a gradual rise of transcriptional activity of *Mstn* genes (in particular *Mstn-1*) is to be expected from gastrulation throughout the embryonic development (Helterline et al., 2007; Kerr et al., 2005; Roberts et al., 2004; Zhong et al., 2008a). This gradual rise could be interpreted as an indication that the level of these genes in fish may correlate with the onset of somitogenesis.

While being investigated during fish embryonic development, comparative assessments of *Mstn* transcriptional abundance after hatching have not been previously reported. These findings showed that large changes of *Mstn-1* and *Mstn-2* transcript abundance occurred in post-hatching phases. In *L. calcarifer*, the abundance of both *LcMstn* genes dramatically dropped immediately after hatching, most likely marking a phase of fast growth whereby larvae rapidly utilize most of the energy stored in the yolk (Kohno et al., 1986). It is also conceivable that while lower abundance of *LcMstn* paralogs during this phase correlated with enhanced growth rate, their subsequent increase depicted a slowing of growth due to the

lessening of yolk reserves. Yolk resorption is in fact known to terminate in *L. calcarifer* at about 15 hrs after hatching (Kohno et al., 1986) when the expression of both *LcMstn* was significantly high. A subsequent, longer period of reduced abundance of *LcMstn* commenced at 20 hr after hatching hence suggesting a restoration of faster growth rate. Although fast growth rate is known to resume approximately beyond 4 days after hatching, the earlier drop of expression might coincide with the phase of enhanced organ development and maturation that occurs in *L. calcarifer* during this time (Kohno et al., 1986). Previous authors have in fact provided evidence to support this hypothesis, indicating that MSTN-1 was detectable in muscle, heart, gut, kidney, skin, pancreas and liver of developing *Dicentrarchus labrax* larvae (Patruno et al., 2008). Being expressed in a range of developing tissue types, it is not to be excluded that fish MSTN paralogs, and in particular MSTN-1, may exert also important functions in controlling growth and differentiation of tissues other than muscle.

A central finding of this study was the diametrically opposite *LcMstn-1* and *LcMstn-2* expression trends observed among juvenile fish with different growth rates. These results supported those of *Chapter 3* where it is shown that *LcMstn* paralogs are differentially regulated possibly as a result of ongoing functional divergence. Most importantly, these studies demonstrated that muscle fiber size was significantly negatively correlated with *LcMstn-2* abundance, but not *LcMstn-1* that alternatively showed a marginal positive correlation. The most conservative hypothesis explaining these findings is that subfunctionalization occurred in the teleost *Mstn* gene sub-family. Cell cycle arrest is required for myosatellite cell differentiation and fusion and could be stimulated by *Mstn-1* (as suggested by higher levels of *LcMstn-1* in the larvae) whereas the *Mstn-2* might be in some way responsible of inhibiting the fusion of myocytes therefore low levels of this protein might result in muscle hypertrophy (as suggested by low levels of *LcMstn-2* in the larvae). Both these functions are in fact regulated by a single *Mstn* paralog in mammalian cells (Rodgers and Garikipati, 2008). The diametrically opposite expression patterns of *LcMstn-1* and *LcMstn-2* observed in this chapter could be indicative of a transition from predominantly

hyperplastic muscle growth mechanisms (i.e. stratified hyperplasia) to an hypertrophic type of growth (i.e. mosaic hyperplasia). This transition is documented to occur during fish metamorphosis (Johnston, 2006). These results should however be approached cautiously since transcript abundance was not representative of muscle tissue only. Further investigation will help clarifying the role of *Mstn-1* and *Mstn-2* in regulating mechanisms of muscle growth. In particular future studies should *i*) localize the tissue types where differences in expression are originated, *ii*) analyze a larger number of samples around metamorphosis (i.e. 15-25 days after hatching) and *iii*) assess both hypeplastic and hypertrophic growth and correlate them with the individual expression of *Mstn-1* and *Mstn-2* in the muscle or at least muscle enriched tissue.

In summary, results from the present study support the hypothesis that in fish functional divergence, possibly subfunctionalization of *Mstn-1* and *Mstn-2* is in place. This chapter showed that, consistently with other teleost species, these genes are dynamically regulated during the development of *L. calcarifer* in that they may control different physiological processes. This conjecture is supported by evidence showing that muscle hypertrophy, measured during the larval development, was highly correlated with the abundance of the *Mstn-2* gene but not with that of the *Mstn-1*.

5 An inverted role for the shrimp ortholog of vertebrate myostatin and GDF11

5.1 Introduction

Cell growth, proliferation and homeostasis are controlled by a number of structurally related proteins belonging to the transforming growth factor-β (TGFβ) superfamily. Myostatin (MSTN), formerly known as growth differentiation factor 8 (GDF-8), and GDF11 are closely related members of this superfamily that are thought to originate from one ancestral gene following gene duplication (Xing et al., 2007). In higher vertebrates, both MSTN and GDF11 are inhibitors of specific cellular functions, the former principally controlling growth of muscle (McPherron et al., 1997) and the latter regulating neurogenesis in the olfactory epithelium as well as the development of the axial skeleton (McPherron et al., 1999; Wu et al., 2003). Both these potent negative regulators and in particular MSTN have recently been under investigation to explain mechanisms underlying important human muscular diseases, as well as to select fast-growing breeding lines for the enhancement of livestock and aquaculture production (De Santis and Jerry, 2007; Lee, 2004).

Genes of the *Mstn/Gdf11* family have recently been isolated and characterized from a number of invertebrate species. Despite being reported under a diverse range of names such as myoglianin in *Drosophila melanogaster*, *Mstn*-like gene in *Argopecten irradians*, *Homarus americanus* and *Gecarcinus lateralis* , *Mstn/Gdf11* in *Nematostella vectensis* and *Gdf8/1*1 in *Branchiostoma belcheri*, invertebrates have only had one ortholog of vertebrate *Mstn* and *Gdf11* isolated to-date (Covi et al., 2008; Kim et al., 2004; Lo and Frasch, 1999; MacLea et al., 2010; Saina and Technau, 2009; Xing et al., 2007). For clarity, this chapter will refer to the abovementioned proteins as the invertebrate MSTN/GDF11 (iMSTN/GDF11).

While the role of both MSTN and GDF11 has been explored by functional studies in vertebrates (Lee et al., 2009; McPherron et al., 1997; McPherron et al., 1999; Wu et al., 2003), the physiological functioning of iMSTN/GDF11 is still not entirely understood. Previous findings reported that the *iMstn/Gdf11* gene showed a widespread tissue-expression suggesting that, unlike the mammalian orthologs, its physiological significance might not be restricted to specific tissues (Covi et al., 2008; Kim et al., 2004; MacLea et al., 2010; Saina and Technau, 2009).

In invertebrate muscle, however, *iMstn/Gdf11* is actively regulated and is thought to control functions such as growth. In decapod crustaceans for example, growth is a discontinuous, moult-dependent process that may require a drastic and reversible reduction of muscle size in order to undergo exuviation of body parts like claws from the old exoskeleton. In *G. lateralis* and *H. americanus*, this process of moult-induced rearrangement of the muscle structure is accompanied by a significant change in *Mstn/Gdf11* abundance (Covi et al., 2010; MacLea et al., 2010). In addition, the *Mstn/Gdf11* transcriptional changes strongly correlate with the level of circulating steroid hormones (i.e. ecdysteroids) that are known to regulate *Mstn* expression *in vitro* and *in vivo* in vertebrates (Ma et al., 2001; Ma et al., 2003). Evidence therefore suggests that similar to vertebrate MSTN, iMSTN/GDF11 is involved in the protein cascade that regulates muscle growth in invertebrate species.

In this chapter, the complete coding sequence of *Mstn/Gdf11* was isolated and characterized from the Penaeid shrimp *Penaeus monodon* (Fabricius, 1798), a commercially important decapod crustacean chosen as an experimental model due to its availability and high growth rate. This chapter reports on the *pmMstn/Gdf11* differential expression pattern across various tissue types as well as its relative abundance during the moult cycle. To infer the specific function of MSTN/GDF11 on the regulation of growth in crustacean, phenotypic changes induced by targeted down-regulation of the gene in *P. monodon* were assessed.

5.2 Materials and Methods

5.2.1 Sequence isolation and analysis

A partial fragment of the *pmMstn/Gdf11* gene sequence was isolated using a primer pair (pmMSTN_CSIRO_F and pmMSTN_CSIRO_R; Table 5.1) designed on a region conserved across crustaceans [*Litopaeneus vannamei* (Lve, ESTs FE172407 and FE046767), *G. lateralis* (Gla, EU432218), *Eriocheir sinensis* (Esi, EU650662)]. Mixed muscle cDNA was prepared as described below for differential tissue expression profiling and a single PCR amplification product of ~ 330 bp was obtained, cloned into a pGEM-T Easy (Promega, Sydney, NSW, Australia) and sequenced in both directions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Mulgrave, VIC, Australia). The full-length 3' end was isolated using GeneRacer advanced RACE kit (Invitrogen, Mulgrave, VIC, Australia) and gene-specific primers (RACE; Table 5.1). Attempts to amplify the full-length 5'end of the transcript using the RACE kit and gene specific nested primers (RACE; Table 5.1) yielded only a partial fragment (291 bp). Difficulties to RACE the 5'end of the *Mstn/Gdf11* gene have been previously reported for crustaceans (MacLea et al., 2010). The complete 5' ORF was isolated by PCR amplification of muscle cDNA using a 5' primer designed on a conserved region between *L. vannamei* and *G. lateralis Mstn/Gdf11* containing the ATG start codon and reverse primer designed on the known *pmMstn/Gdf11* sequence (5'ORF discovery; Table 5.1). To confirm the identity of the putative pmMSTN/GDF11 translated peptide sequence, evolutionary relationships were inferred between MSTN/GDF11 ortholog proteins (invertebrates and vertebrates), TGFβ1 and Inhibin that are the closest relatives of the MSTN/GDF11 sub-family (Herpin et al., 2004). An alignment was generated on the entire amino acid sequence using CLUSTALW (Thompson et al., 1994) and manually adjusted using MEGA 4.0 (Tamura et al., 2007). Evolutionary history was inferred using the Neighbour Joining (NJ) method using MEGA 4.0 with bootstrap support based on 1000

replicates and the JTT (+G) model. Finally, the full-length protein sequence was analyzed for putative functional sites using the web-based server ELM (Puntervoll et al., 2003).

Table 5.1. Primer sequences used in the present study and their annealing temperature (T), position on the sequence relative to the GenBank submission and application.

Primer name	Primer sequence (5'-3')	Annealing T	Position	Application
pmMSTN_CSIRO_F	ATGCGCCGTTGCTGGAGAT	55	209	Gene discovery
pmMSTN_CSIRO_R	TCGTAATACAGCATCTTCATCGG	55	532	Gene discovery
pmMSTN_Csiro_3'RACE_F	ACCAATGCACTACCAGCCAGATCGAGTC	70	278	RACE
pmMSTN_Csiro_5'RACE_R	CGACAGCAGCGGGACTCGATCTG	70	317	RACE
pmMSTN_Csiro_5'RACE_R_(nest)	GGTAGTGCATTGGTTTCGGCTGGAGTTC	68	291	RACE
pmMSTN_Csiro_5'ORF_F	CAGACAGACATGCAGTGG	52	0	5'ORF discovery
pmMSTN_Csiro_5'ORF_R	GGATTGTTCAGCCATTCTTG	52	1058	5'ORF discovery
pmMSTN_Csiro_qPCR_F	GAAACCAATGCACTACCAGC	60	275	Real-time PCR
pmMSTN_Csiro_qPCR_R	TGTGGGCGTATAGATAAGGG	60	427	Real-time PCR
pmMSTN_Csiro_dsRNA_F	GAATT**TAATACGACTCACTATAGG**GATCCCAGAGGATTACATCATAC	48*	4	dsRNA synthesis
pmMSTN_Csiro_dsRNA_R	GAATT**TAATACGACTCACTATAGG**GATCAGCTTCATATACCTTAGG	48*	387	dsRNA synthesis
LUC_Csiro_dsRNA_F	GAATT**TAATACGACTCACTATAGG**GATCGCGCCATTCTATCCTCTA	55*	34	dsRNA synthesis
LUC_Csiro_dsRNA_R	GAATT**TAATACGACTCACTATAGG**GATCTACATCGACTGAAATCCCT	55*	495	dsRNA synthesis
pmACT_Csiro_dsRNA_F	GAATT**TAATACGACTCACTATAGG**GATCATCATGTTCGAGACGTTC	50*	14	dsRNA synthesis
pmACT_Csiro_dsRNA_R	GAATT**TAATACGACTCACTATAGG**GATCCTCCTGCTTGCTGATCCA	50*	727	dsRNA synthesis

Boldface and underlines sections indicate the T7 promoter (as to differentiate from the gene-specific sequence).
An asterisk (*) denotes gene-specific annealing temperature only (see materials and methods for further details).

5.2.2 pmMstn/Gdf11 tissue expression and expression across the moult cycle

Expression profiles of *pmMstn/Gdf11* were analyzed in deep abdominal muscle, gill, hepatopancreas, eyestalk, heart and stomach. Each tissue was dissected from nine *P. monodon* and pooled into groups of three individuals per tissue type. Expression of *pmMstn/Gdf11* across the moult cycle was assessed in muscle tissue only from five *P. monodon* at each of the moult stages A, B, C, D_0, D_1, D_2, $D_{3/4}$ [as determined according to setal staging and epidermal withdrawal in uropods (Promwikom et al, 2004; Smith and Dall, 1985)]. All tissues were immediately stored in RNA later (Applied Biosystems).

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturers instructions, and precipitated by adding 0.5 volumes of isopropyl alcohol and 0.5 volumes of RNA precipitation solution for purity improvement (Sambrook et al., 1989). Total RNA was DNase digested with the Turbo DNA-free kit (Applied Biosystems) and quality and quantity were assessed by gel electrophoresis and on a Nanodrop spectrophotometer (Nanodrop technology). All RNA samples were diluted to 200 ng/μL using an epMotion 5070 (Eppendorf, North Ryde, NSW, Australia). Reverse transcription was performed on 1 ug total RNA using Superscript III (Invitrogen) with 25 μM oligo$(dT)_{20}$ and 25 μM random hexamers (Resuehr and Spiess, 2003). Expression of *pmMstn/Gdf11* in all samples was analyzed by real time PCR as described below.

Real-time PCR primers specific to *pmMstn/Gdf11* (Table 5.1) were designed with PerlPrimer v1.1.17 (Marshall, 2004). Real-time PCR amplification reactions were carried out using 1x SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μM of each primer and the equivalent of 7.5 ng reverse transcribed RNA. Amplification cycle conditions were 2 min at 50°C, 10 min at 95°C followed by 40 cycles of [15 s at 95°C and 40 s at 60ºC], and a dissociation melt curve analysis was performed. Reactions were set up using the epMotion 5070 (Eppendorf) and run in quadruplicates on a 7500 Real-time PCR system (Applied

Biosystems). Verification of gDNA contamination was carried out by PCR amplification of DNAse treated RNA samples using gene specific primers. Normalization was performed using the ΔC_q method as it was considered the least biased approach (*Chapter 2*; Zhong et al., 2008).

5.2.3 Down regulation of pmMstn/Gdf11

Sequence-specific dsRNAs were synthesized from verified gene specific PCR products using the MEGAscript RNAi kit (Applied Byosystems) according to the manufacturers' instructions. To synthesise dsRNA, primers were designed to amplify the targets *Luc* genes (accession number EU754723), *pmMstn/Gdf11* (HQ221765) and *β-actin* (Sellars et al., in Press) (Table 5.1). dsRNA synthesis primers contained a gene-specific portion and a modified T7 promoter extension (boldface and underlined in Table 5.1) reported to increase transcription efficiency (Milligan et al., 1987; Tang et al., 2005). Integrity and size of newly synthesized dsRNA were quantified by 1.5% agarose/EtBr gel electrophoresis.

P. monodon were collected from a commercial farm and maintained at CSIRO Marine and Atmospheric Research, Australia for one month prior to being stocked into the down-regulation experiment. On the day of stocking, shrimp were weighed, sexed and allocated randomly to tanks at a density of 4 shrimp per tank (2 males and 2 females). Shrimp (20 per treatment) were tail-muscle injected with the four experimental treatments: saline solution, *Luc*-dsRNA (5 μg), *pmMstn/Gdf11*-dsRNA (5 μg) and *βactin*-dsRNA (5 μg). Animals were weighed and re-injected with the assigned treatment every 10-11 days for the 45 days duration of the experiment. *pmMstn/Gdf11* transcript abundance was determined in pleopod (day 7) and tail muscle (day 45) of experimental treatments.

Tanks received 1.6 L^{-min}, 28 ± 1°C, 34 ± 2 ppt salinity seawater and had opaque white lids to reduce light intensity. Shrimp were fed a commercial feed (Lucky star) *ad libiutum* twice per day at 0900 and 1700 hours. The number of shrimp alive in each tank were counted and recorded at 0930 and 1750 hours every day for the 45 day experimental duration. Tanks were cleaned daily by siphoning and deaths removed.

Specific growth rates (SGR; %/d) [100 x [ln final weight- ln initial weight)/ growing period], average daily gain (ADG; g/d) [(final weight – initial weight)/growing period] and hepatosomatic index (HSI) [(Hepatopancreas weight/body weight)*100] were calculated at the end of the experiment.

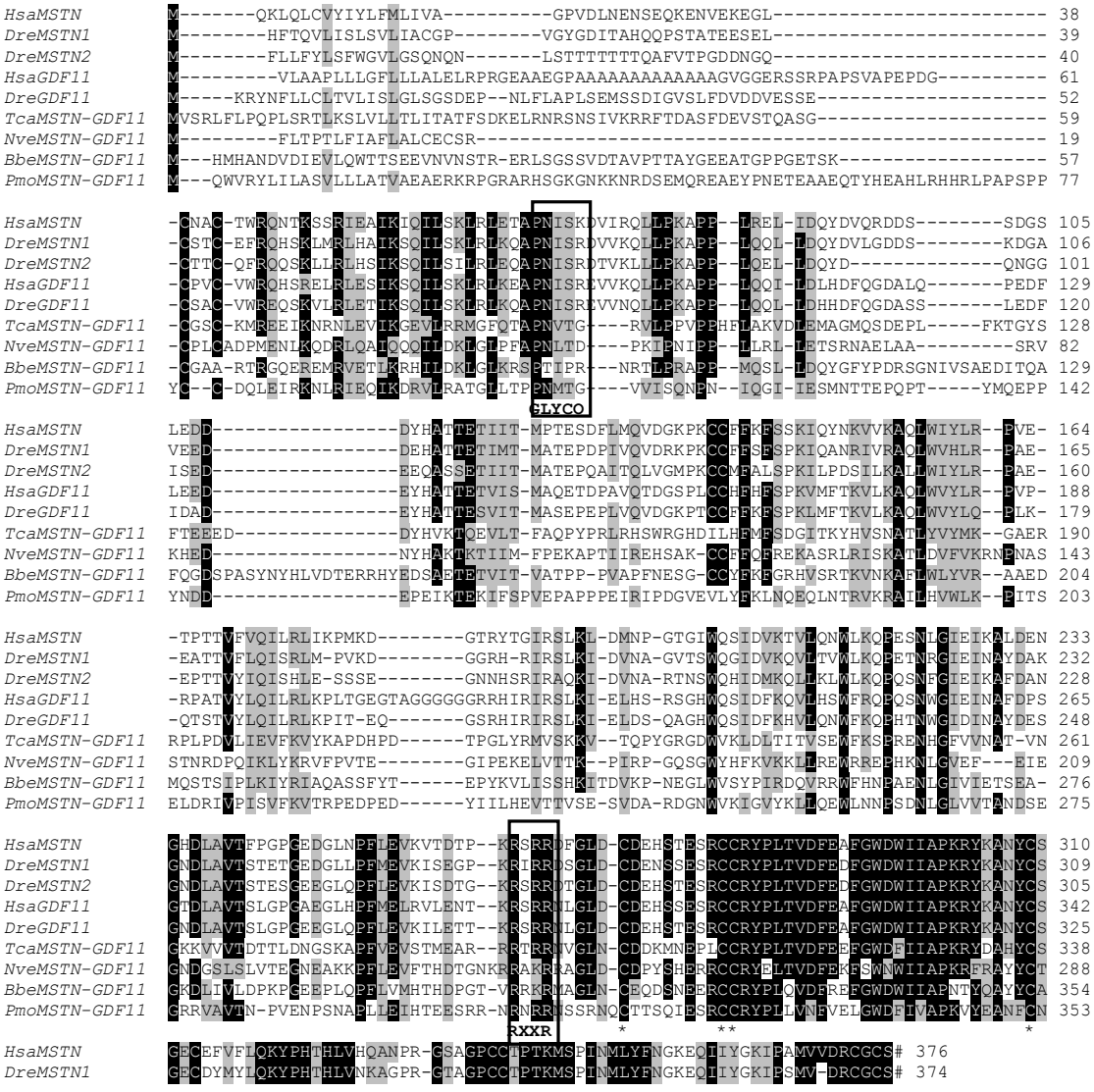
All statistical analyses were performed using the SPSS software (SPSS and inc., 2006). Assumptions for homogeneity of variance were tested using a Levene's test. Data that did not conform to the homogeneity of variance assumptions was log transformed. *PmMstn/Gdf11* expression (day 7 and day 45) and phenotypic (SGR, ADG, HSI) differences among treatments were tested using a two-way analysis of variances (ANOVA) using treatment and sex as the independent variables. *PmMstn/Gdf11* expression across the moult cycle was analyzed using a one-way ANOVA. *Post-hoc* multiple comparisons were made using Bonferroni tests. Differences of $P < 0.05$ were considered significant.

5.3 Results

5.3.1 Sequence characterization and analysis

The complete coding sequence of the *pmMstn/Gdf11* gene (accession number HQ221765) shared the highest nucleotide identity (>70%) with *G. lateralis*, *E. sinensis* and *H. americanus* orthologs. The *pmMstn/Gdf11* ORF was 1260 bp in length, with a 66 bp 3' untranslated region (UTR) terminated by a classical polyadenylation signal (Fig. 5.1). The translated protein was 419 aa (amino acids) in length (Fig. 5.1), shorter than that of other crustacean MSTN/GDF11 such as *G. lateralis* [497 aa (Covi et al., 2008)] and *E. seniensis* (468 aa).

Three domains could be recognized, including a signal sequence (position 1-21) possibly cleaved at a N-Arg dibasic convertase cleavage site matching the motif ERK (position 22-24) and a C-terminal domain shared by members of the TGF-β superfamily. The furin/subtilisin-like cleavage site RXXR (RNRR, position 302-305) represented the putative cleavage site for the mature C-terminal peptide. The mature domain contained nine conserved cysteines, shown to be essential for the formation of interchain disulphide bonds involved in the final step of maturation of the protein (dimerization) (Daopin et al., 1992) (Fig. 5.1). A general motif for N-glycosylation Asp-X-Ser/Thr (PNMTG, residues 108-113) was also conserved in other vertebrate and invertebrate MSTN/GDF11 (Fig. 5.1).



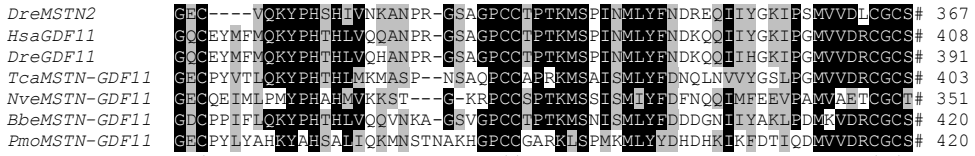


Figure 5.1. Alignment of the translated amino acid sequence of pmMSTN/GDF11 (GenBank accession number HQ221765) with other representative members of the MSTN/GDF11 gene family. The putative conserved glycosylation site (GLYCO) and the furin/subsitilin-like cleavage site (RXXR) are boxed. The conserved cysteines are marked with asterisks and the STOP codon with a dash. Species compared in this figure include *Homo sapiens* (Hsa, AAH74757, NP_005802), *Danio rerio* (Dre, AAP85526, AAT95431 and NP_998140), *Tribolium castaneum* (Tca, XP_966819), *Nematostella vectensis* (Nve, XP_001641598) and *Branchiostoma belcheri* (Bbe, EF634365).

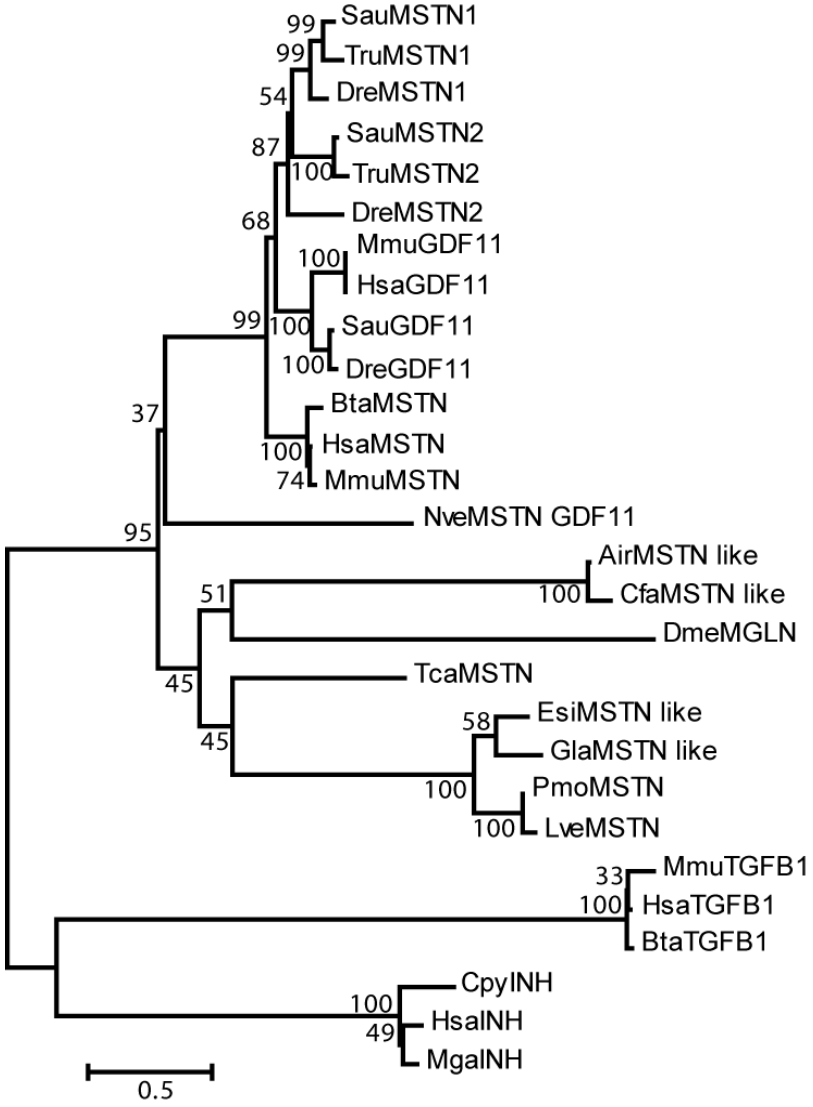


Figure 5.2 Evolutionary relationships among vertebrate and invertebrate MSTN/GDF11, TGFβ1 and INH proteins. Species used to reconstruct the MSTN/GDF11 evolutionary relationship included *Sparus aurata* (Sau, AF258448 and AY046314), *Takifugu rubipres* (Tru, Ensembl blast search), *Danio rerio* (Dre, AY323521, AY687474 and NM_212975), *Mus musculus* (Mmu, NM_011577, BC103678 and NM_010272), *Homo sapiens* (Hsa, NM_000660, BC074757, NM_005811 and NM_002192), *Bos taurus* (Bta, NM_001166068 and AF019620), *Argopecten irradians,* (Air, AY553362), *Tribolium castaneum* (Tca, XM_961726), *Penaeus monodon* (Pmo, current study) *Litopaeneus vannamei* (Lve, ESTs FE172407 and FE046767), *Gecarcinus lateralis* (Gla, EU432218), *Eriocheir sinensis* (Esi, EU650662), *Nematostella vectensis* (Nve, XM_001641548), *Chlamys farreri* (Cfa, EU563852), *Drosophila melanogaster*, (Dme, AAD24472), *Meleagris gallopavo* (Mga, AF336338) and *Cynops pyrrhogaster* (Cpy, D84516). The tree is drawn to scale, with branch lengths measured in the number of substitution per site. Values at the tree nodes indicate Bootstrap support values.

All crustacean MSTN/GDF11 orthologs, including pmMSTN/GDF11, formed a highly supported single clade (Fig. 5.2). Outside this crustacean clade were other invertebrate orthologs of the MSTN/GDF11 family, including those from insects, molluscs and cnidarians (Fig. 5.2). Invertebrate MSTN/GDF11 clustered with vertebrate members of the MSTN/GDF11 subfamily (bootstrap value 95%) and was clearly distinguished from the closest relatives to this subfamily, TGF-β1 and INH. The overall topology of the MSTN/GDF11 tree was consistent with the hypothesis that invertebrates contain a single MSTN/GDF11 gene, and that a duplication event separated the GDF11 and MSTN paralogs in vertebrates (Fig. 5.2).

5.3.2 pmMstn/Gdf11 tissue expression and expression across the moult cycle

The *pmMstn/Gdf11* gene was widely expressed across all tissues under investigation including muscle, hepatopancreas, eyestalk, heart, gill and stomach. The highest transcriptional activity was observed in heart (quantification cycle or C_q~13) where *pmMstn/Gdf11* mRNA copies were at least 100 fold higher than the remaining tissues (Fig. 5.3). Muscle, gill, eyestalk and stomach transcript abundance was altogether lower than that observed in heart and appeared similarly expressed in each of these tissues (Cq~17) (Fig. 5.3). *pmMstn/Gdf11* was detected at low levels in the hepatopancreas where the mRNA copy number was 100 fold lower than all other tissues (Cq~25) (Fig. 5.3).

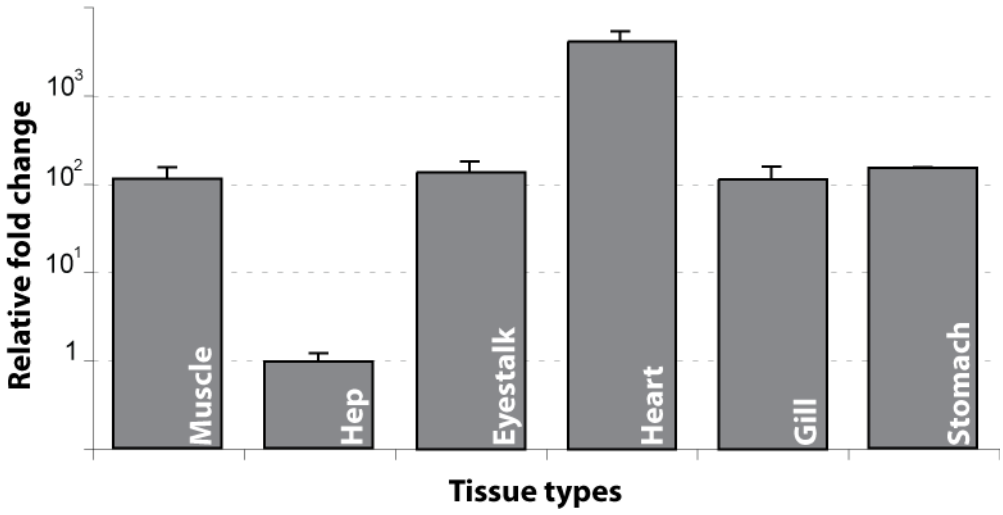


Figure 5.3. Relative tissue-specific expression of *pmMstn/Gdf11* gene. Values are reported relative to expression in the hepatopancreas (Hep). Bars indicated SEM (n = 3).

Muscle expression of *pmMstn/Gdf11* was regulated during the natural moult cycle (Fig 5.4). A sharp peak of expression was detected immediately after exuviation (A stage, 0-9 h post-ecdysis) and was followed by a 10 fold drop in the next phases (late post-moult/inter-moult stages, B-C, 9 to 48 h post-ecdysis; P <0.01) (Fig. 5.4). The pre-moult stages (D_0-D_4) displayed an overall higher *pmMstn/Gdf11* abundance than B-C stages (P <0.05) but did not statistically differ from the early post-moult (Fig. 5.4). During all stages of pre-moult the expression levels of *pmMstn/Gdf11* remained relatively stable until the following exuviation (Fig. 5.4).

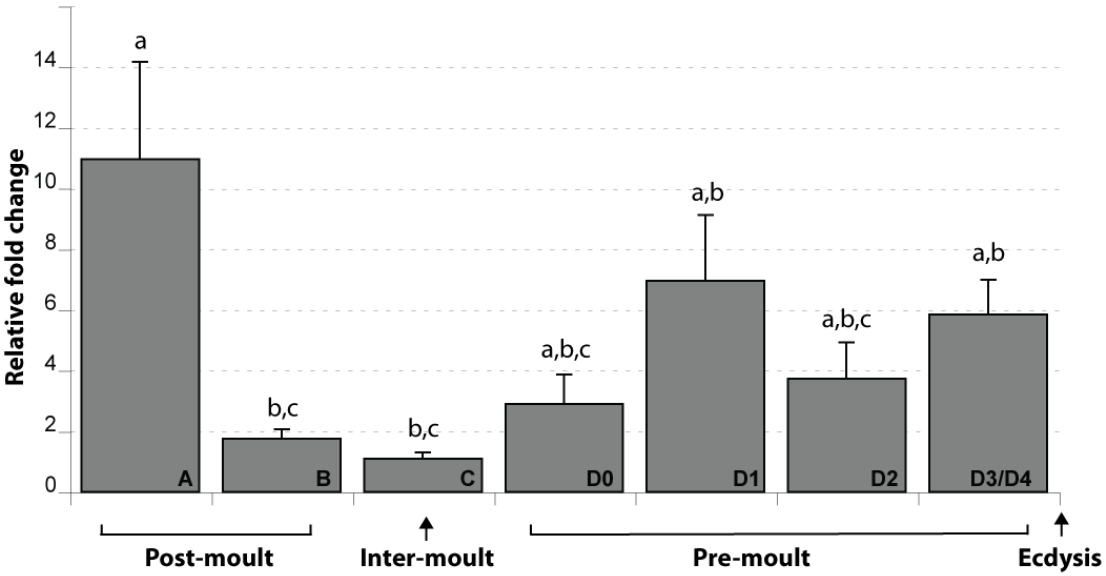


Figure 5.4. Relative *pmMstn/Gdf11* expression across the molt cycle. Values are reported relative to the C stage (inter-moult). Bars indicate SEM (n = 5). Columns denoted with same letter are not statistically significant (P <0.05).

5.3.3 Phenotypic response to pmMstn/Gdf11 down-regulation

The *pmMstn/Gdf11* gene was down-regulated by tail-muscle injection of sequence-specific dsRNAs (Fig. 5.5). Assessment of *pmMstn/Gdf11* expression among treatments revealed approximately 40% down-regulation of endogenous gene expression, which was evident at both days 7 (7 days post-injection) and day 45 (3 days post-injection) (Fig. 5.5).

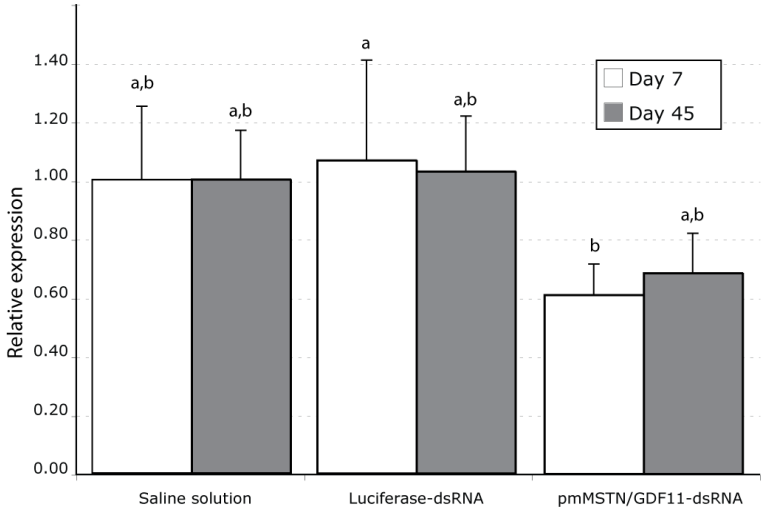


Figure 5.5. Relative expression of the *pmMstn/Gdf11* gene in animals injected with saline solution, *Luc*-dsRNA and *pmMstn/Gdf11*-dsRNA. Values are reported relative to control animals (Saline solution). White columns denote pleopod samples taken 7 days after the first injection, and grey columns denote muscle samples taken at day 45 (3 days after the last injection). At both 7 and 45 days a marginal reduction (~ 40%) of *pmMstn/Gdf11* mRNA abundance was observed in shrimp tail-muscle injected with *pmMstn/Gdf11*-dsRNA. Bars indicate SEM (n=20). Columns denoted with same letter are not statistically significant ($P < 0.05$).

As anticipated, positive control animals (n = 12) injected with *β-actin*-dsRNA died within 2-4 days post-injection, indicating endogenous gene silencing using tail-muscle injection of dsRNA was successful. Major growth differences were observed between the *pmMstn/Gdf11* treatment and controls, where shrimp injected with *pmMstn/Gdf11*-dsRNA showed a notably slower overall growth rate compared with *Luc*-dsRNA and Saline injected controls (Fig. 5.6 and Table 5.2). Initially evident as early as 11 days (Fig. 5.6) after the first injection, by day 45 shrimp that received *pmMstn/Gdf11*-dsRNA had only grown by 32%, compared with control animals that had more than doubled their initial size. Specific growth rates (SGR) were 0.67%/d in shrimp that received *pmMstn/Gdf11*-dsRNA, as opposed to 1.85%/d and 1.68%/d in *Luc* and saline controls respectively (P <0.01; Table 5.2). This translated to an average daily gain (ADG) of 0.06g/d, significantly slower than that of the *Luc* and saline controls at 0.23g/d and 0.18g/d respectively (P <0.01; Table 5.2). Interestingly, a marginal difference in ADG (P <0.01) and SGR was also observed between *Luc* and Saline controls (Table 5.2). No significant differences in hepatosomatic index (HSI) were observed, indicating a similar general health status of shrimp across all treatments and controls (Table 5.2), and there were no recorded deaths during the entire experimental period.

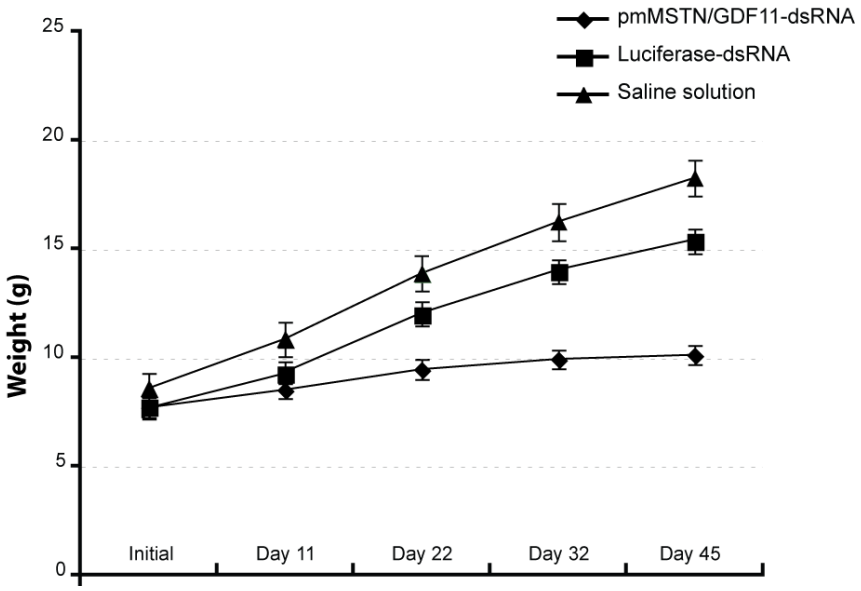


Figure 5.6. Weight measurements of experimental treatments (injection of saline solution, *Luc*-dsRNA and *pmMstn/Gdf11*-dsRNA) recorded every 10-11 days along the 45 day duration of the experiment. Bars indicate SEM (n = 20)

Table 5.2. Average (± SEM) daily gain (ADG), specific growth rate (SGR) and hepatosomatic index (HSI) of shrimp injected with saline solution, *Luc*-dsRNA and, *pmMSTN/GDF11*-dsRNA.

	Saline	*Luc*-dsRNA	pmMSTN/GDF11-dsRNA
ADG (g/day)	0.23 ± 0.011 [a]	0.18 ± 0.013 [b]	0.06 ± 0.005 [c]
SGR (%/day)	1.85 ± 0.12 [a]	1.68 ± 0.14 [a]	0.67 ± 0.07 [b]
HIS	3.81 ± 0.12 [nsd]	3.69 ± 0.14 [nsd]	3.58 ± 0.15 [nsd]

Within rows values with different superscripts are significantly different (P <0.05). nsd = no significant difference.

5.4 Discussion

Vertebrate MSTN is a negative regulator of growth whereby reducing *Mstn* expression induces increases in body weight (Acosta et al., 2005; Lee et al., 2009; McPherron et al., 1997). Loss-of-function mutations of the MSTN gene in vertebrates result in heavily muscled

phenotypes (McPherron and Lee, 1997), whilst increasing circulating levels of MSTN induces muscle atrophy (Ma et al., 2003; Shao et al., 2007; Wehling et al., 2000). Recently, invertebrate orthologs of genes similar to *Mstn* have been reported in a number of species including decapod crustaceans (Covi et al., 2010; Covi et al., 2008; MacLea et al., 2010), however, the function of these gene orthologs has not been specifically investigated.

In this chapter a previously unknown ortholog of *iMstn/Gdf11* named *pmMstn/Gdf11* was isolated from the shrimp, *Penaeus monodon*. The pmMSTN/GDF11 amino acid sequence contained all the principal functional sites common to all members of the MSTN/GDF11 family including the nine cysteines, a subtilisin proteolytic site and a glycosidation site. Conservation of functional sites and evidence of three-dimensional structure similarity between vertebrate and crustacean MSTN/GDF11 (MacLea et al 2010) suggests that this gene family may have maintained similar physiological functions throughout the animal kingdom.

Tail-muscle injection of a sequence specific dsRNA against the isolated pm*Mstn/Gdf11* resulted in a 68% reduction in *P. monodon* shrimp growth at the end of the experimental period. Interestingly, this response is opposite to that seen in higher vertebrates, suggesting that this gene is a positive growth regulator in this invertebrate species. In support of this theory, endogenous muscle expression of *Mstn/Gdf11* peaked immediately after moulting, a time where growth is not under the limitation of a hard exoskeleton and significant expansion of the animal occurs. During this time, potential induction of muscle growth factors are required to promote growth into the larger shell. This notion is supported by previous work in crustaceans, where *Mstn/Gdf11* abundance gradually decreased in the claws of *G. lateralis* during premoult stages (D_0-D_4) as the muscle underwent severe atrophy to allow the withdrawal of the claws from the old exoskeleton during ecdysis (Covi et al., 2010). This again provides a consistent positive relationship between *iMSTN/GDF11* expression and muscle size.

Vertebrate *MSTN* and *GDF11* are thought to have separated from a single gene, the archetypal *Mstn/Gdf11* gene (Xing et al., 2007; Xu et al., 2003). The function of this gene is presumed to resemble that of vertebrate *Mstn* (i.e. myogenesis) and *Gdf11* (i.e. neurogenesis). It stands to reason that a combined functional role of the *iMstn/Gdf11* gene may be reflected in a broader tissue gene expression profile, one that is less specialized than that of the vertebrate *Mstn* or *Gdf11* paralogs. Indeed, transcripts of the *pmMstn/Gdf11* gene were expressed in a wider variety of shrimp tissues including muscle, gills, heart, eyestalk and hepatopancreas, similar to the expression observed in other crustaceans like *G. lateralis* and *H. americanus* (Covi et al., 2008; MacLea et al., 2010).

Evidence also exists for a role for *iMstn/Gdf11* in neurogenesis in *D. melanogaster,* whereby mutant flies carrying a loss-of-function mutation at the gene coding for the MSTN/GDF11 receptor displayed a dramatic reduction of synaptic development at neuromuscular junctions (Aberle et al., 2002; Lee-Hoeflich et al., 2005). Interestingly, this functional role in insect synapses was also opposite to that observed in vertebrate systems, suggesting there may have been an inversion of the entire functional role for the ancestral *iMstn/Gdf11* gene. At this point, it is not possible to define where this inversion may have occurred, whether specifically within arthropods or elsewhere between arthropods and higher vertebrates. The precise functional role for *iMstn/Gdf11* in other crustacean tissues also requires further clarification, as this study specifically sought to investigate the role of *iMstn/Gdf11* in crustacean muscle and other phenotypes were not investigated.

In summary, here we characterize the *pmMstn/Gdf11* gene in the shrimp *Penaeus monodon*, including the tissue expression profile, regulation in muscle across the moult cycle and biological function. This chapter reconciles this gene and other invertebrate genes as an ortholog of the vertebrate MSTN/GDF11 family and provides clear evidence of a conserved growth regulation function of *iMstn/Gdf11* in muscle. It demonstrates a dramatic slowing of

the overall growth rate in response to down-regulation of the *iMstn/Gdf11* gene. This phenotypic effect in shrimp, in stark contrast to other vertebrate systems, appears to be positive rather than negative. As such, mechanisms that increase *pmMstn/Gdf11* gene expression may be valid targets to boost growth performance and improve shrimp production.

6 General Discussion

There is current interest in aquaculture in the potential of improving growth rates of finfish and crustaceans through the direct targeting of *Mstn*-like genes. Before manipulation or selection on *Mstn*-like genes occurs, however, it is critically important to have a strong understanding of the physiological functioning of these genes and possible genetic correlations related to changes in their expression with other phenotypic traits. In crustaceans, and invertebrates in general, our understanding of *Mstn*-like genes is only now beginning to accumulate with current knowledge on the *Mstn/Gdf11* gene being very preliminary. In fish, however, there has been increased research focus, but a clear understanding of *Mstn* (*Mstn-1* & *Mstn-2*) functioning has been limited by other factors such as the report of confounding results due to the existence of two paralogs (Helterline et al., 2007; Kerr et al., 2005), or the bias introduced through the improper use of normalization approaches adopted in studies investigating gene expression (*Chapter 2*; Zhong et al., 2008b). By paying particular attention to these limitations, the present research has advanced our understanding of *Mstn*-like genes functioning in fish and crustaceans highly relevant to aquaculture.

In *Chapter 3 & 4,* the comparative analyses of *L. calcarifer Mstn-1* and *Mstn-2* regulation provided support that these paralog genes are equally important for fish growth. However, differential expression and association with different growth phases suggest that they may have separate functions. In *Chapter 3* for example, it was shown that inducing a dramatic reduction of growth through fasting causes an increase of *Mstn-1* expression levels but not those of *Mstn-2*. Alternatively, in *Chapter 4* fish that were naturally growing at a faster pace displayed reduced expression levels of *Mstn-2*. This is of particular relevance to future aquaculture research aiming to target *Mstn* as a candidate gene to improve fish growth. The significantly higher number of published work targeting the *Mstn-1* compared with those

investigating the *Mstn-2* indicates that previous research has often disregarded the latter as an important growth gene. This tendency has probably been determined by former studies reporting expression of *Mstn-2* primarily in the brain and suggesting a role for this gene in neurogenesis rather than muscle growth (Maccatrozzo et al., 2001a). This earlier perceived role of *Mstn-2* appears, however, to be incorrect and it is now evident that the *Mstn-2* has an important role in controlling muscle growth.

Two *Mstn* paralog genes in fish appear therefore important for regulating growth. In particular, *Chapter 4* of this thesis suggests that *Mstn-2* may be at least responsible for regulation of muscle growth through hypertrophy since the abundance of this paralog is strongly correlated with this trait. This, in conjunction with studies reporting that the *Mstn-1* in fish muscle may only inhibit cell division (Sawatari et al., 2010; Xu et al., 2003), implies that two *Mstn* paralogs in fish may control functions that in mammals are regulated by a single *Mstn*. This hypothesis supports findings showing that fish phenotypes with increased body weight through muscle hypertrophy and hyperplasia have currently only been achieved by using RNAi (Acosta et al., 2005; Lee et al., 2009). RNAi in fact is shown to induce a co-suppression of both *Mstn* genes even when targeting one paralog only (Lee et al., 2009). Conversely, when a more specific approach is used (such as targeted mutagenesis of the *Mstn-1* gene or transgenic over-expression of the *Mstn-1* prodomain) fiber hyperplasia, but not hypertrophy, is affected with no significant changes in overall body weight (Sawatari et al., 2010; Xu et al., 2003). It is meaningful that to this date the best results in terms of growth improvement are achieved by using an approach with low specificity. Lee et al (2010) triggered an up to 42% growth improvement by immersing *O. mykiss* in a solution containing soluble *P. olivaceus* MSTN-1 prodomain. Similarly, Acosta et al (2005) increased growth of *D. rerio* by up to 45% using injection of *O. mossambicus Mstn-1* double-stranded RNA. It is conceivable that the lower specificity attained using a different species' *Mstn* construct may have increased the level of co-suppression hence producing better results in terms of fish growth. Whichever the case, these results stress the importance of understanding MSTN

function in fish in order to be able to employ MSTN-based technologies and efficiently improve muscle growth in fish.

This thesis also provided the first clear evidence that, at least in Penaeid shrimp, the invertebrate ortholog of the *Mstn* gene is a pivotal regulator of growth. However, despite growth being largely affected (up to 84% difference between treatment and control), down-regulation of the *Mstn/Gdf11* expression did not induce muscle enlargement as predicted from vertebrate knowledge of muscle functions of this gene. Conversely it caused a notable reduction of growth. These results support those from other invertebrates where the *Mstn/Gdf11* gene shows correlation with muscle size and is regarded as a prominent candidate gene for productivity improvement in cultured species (Covi et al., 2010; Covi et al., 2008; Wang et al., 2010). *Chapter 5* stresses the importance of unraveling mechanisms of function of *Mstn/Gdf11* in invertebrates like crustaceans prior to the development of approaches to modify the activity of this gene/protein for growth enhancement.

6.1 Myostatin in aquaculture – applications, achievements and prospects

As a consequence of the attractive outcomes of mammalian research, the prospect of improving muscle growth through a single gene approach is stimulating the research interest of a number of aquaculture scientists resulting in the characterization of many *Mstn*-like genes from several cultured species, primarily fish, but also crustaceans and mollusks (Covi et al., 2008; De Santis et al., 2008; Kim et al., 2004; Maccatrozzo et al., 2002; Rodgers et al., 2001). Following achievements and prospects of strategies for growth improvement that have used MSTN-based approaches are summarized.

6.1.1 Myostatin polymorphisms associated with production traits

While the discovery of MSTN spiked a fervent search for single nucleotide polymorphisms (SNP) associated with production traits in livestock this appeared not to be the tendency in aquaculture species. Extensive literature searches have only shown that while *Mstn*-like genes have been isolated and characterized in a large number of aquatic animals, SNPs were only reported in three fish species including *L. calcarifer*, *Ictalurus punctatus* and *Micropterus salmoides* (De Santis et al., 2008; Kocabas et al., 2002; Ling-yun et al., 2010) and the mollusk *Chlamys farreri*. Among these, a significant SNP association with growth traits was found in *M. salmoides,* where significant differences of up to 119% of body weight were reported (Ling-yun et al., 2010), and in *C. farreri* where SNPs in the *Mstn/Gdf11* gene were shown to be accountable for a body mass improvement of up to 60% (Wang et al., 2010). Whilst of great interest for assisted breeding programs, markers in the *Mstn* genes are, alas, still of limited use in aquaculture due to the inability to fully control the reproductive cycle of most aquatic species or to manage specific crosses between individuals.

6.1.2 Interference with gene expression

Strategies aiming to interfere with the expression of genes encoding for MSTN proteins have yielded very auspicious results, in particular with regard to fish species. These strategies made use mostly of RNAi or antisense technologies (i.e. morpholino) to reduce the level of *Mstn* gene expression. Growth improvement of up to 45% in as little as 75 days was achieved in *Danio rerio* by silencing the expression of *Mstn* genes through RNAi (Acosta et al., 2005; Lee et al., 2009). Experiments using morpholino injections did not report data on growth, however, a significant regulation of muscle-specific transcription factors such as MyoD and myogenin were observed suggesting that a similar improvement as that observed using RNAi might be achievable (Amali et al., 2004). Interestingly, increased rates of deformities and mortality were reported in fish with reduced amounts of circulating MSTNs, indicating that in

teleosts these proteins might have additional functions other than muscle growth (Lee et al., 2009).

The unwanted effects observed in fish with silenced *Mstn* genes shed light on the necessity to overcome a number of limitations in order to use technologies such as RNAi for growth improvement at a commercial scale. Firstly, a method to deliver the RNAi signal in a tissue specific manner is needed in order to reduce the impact of the treatment on other physiological processes controlled by *Mstn* genes. In addition, future studies should investigate methods to guarantee sufficient specificity that targets one *Mstn* paralog only (Lee et al., 2009). This is even more crucial since it is hypothesized that *Mstn-1* and *Mstn-2* might possess diverse functions in fish and there might be the requirement of targeting only one paralog to obtain faster growth and minor or nil counter-effects. Finally, a procedure to successfully deliver the RNAi signal into adult somatic cells of vertebrates must be developed (Mittal, 2004). Previous experimental fish models exhibiting RNAi-induced suppression of *Mstn* genes were produced by injecting the dsRNA-vector into one-cell stage embryos therefore avoiding the so called "mosaic effect" whereby gene silencing is not homogeneous across the whole animal (Amali et al., 2004; Lee et al., 2009; Xu et al., 2003). Since the area of research investigating delivery of RNAi signal into adult somatic cells of vertebrates is particularly active due to the vast potential of this technology in biomedical science, a major breakthrough in this direction is foreseeable. Improved delivery efficiency might allow, for example, the formulation of feed additives capable of inducing faster muscle growth through suppression of *Mstn* genes at any life stage.

Most of the considerations reported above that limit the use of technologies such as RNAi for growth improvement of fish species, seem not to be a reason of concern for invertebrate species relevant to aquaculture. First of all invertebrates only possess one gene of the *Mstn/Gdf11* family, hence reducing problems associated with the evolution of new genes or

genes functions. More importantly, inducing an RNAi response in invertebrates is easier than in vertebrate systems. A successful knock-down of endogenous genes can be achieved by injection, soaking, or feeding adult animals with dsRNA molecules (Tabara et al., 1998). While gene silencing through RNAi shows potential as a direct application for gene knockdown in invertebrates, *Chapter 5* indicates that this approach is not viable and that down regulation of the *Mstn/Gdf11* gene causes a slowing of growth rate. It is not yet fully understood if the *Mstn/Gdf11* has an inverted role compared with its vertebrate orthologs although preliminary evidence suggests so. If the hypothesis of an inverted role is confirmed, procedures for growth improvement in aquaculture invertebrates will have to look into strategies to up-regulate the *Mstn/Gdf11* gene expression or protein activity.

6.1.3 Interference with protein bioactivity

While silencing the expression of *Mstn*-like genes results in reduced levels of the translated protein, a number of peptides or compounds have been shown to directly affect the bioactivity of the MSTN dimer. *In vitro* experiments have shown that the peptides follistatin, ACTIIR and MSTN pro-domain (Lee and McPherron, 2001; Rebhan and Funkenstein, 2008), as well as sulphated polysaccarides (a.k.a. fucoidans) extracted from brown seaweeds (Ramazanov et al., 2003), are potent inhibitors on MSTN activity. Transgenic mice with higher levels of follistatin, ACTIIR or MSTN pro-domain for example exhibited a similar phenotype with increased muscle mass as that of mice with null-MSTN (Lee and McPherron, 2001).

Procedures increasing the amount of circulating follistatin or ACTIIR have also been tested on fish species and displayed excellent prospects for commercial applications. Recombinant ACTIIR and MSTN pro-domain delivered into the fish through immersion baths or injection engendered a growth improvement of up to 34% in 35 days in the goldfish *Carassius auratus* (ACTIIR) and up to 42% over an even shorter time frame (28 days) in the rainbow trout *O. mykiss* (MSTN-1 pro-peptide) (Carpio et al., 2009; Lee et al., 2010). The attractiveness of

these procedures resides in the fact that the recombinant protein can be transferred into the animals either during larval and adult stages (Carpio et al., 2009). In addition, for reasons still largely unknown soluble recombinant proteins can be uptaken through the gills into the organism, hence allowing several animals to be treated at the same time with minimal stress.

An attractive area for new research opportunities in aquaculture might involve the use of sulphated polysaccharides or fucoidans as MSTN inhibitors. Sulfated polysaccharides are extracted from seaweed which are intensely investigated as a way to treat wastewater of aquaculture production (Mata et al., 2010; Neori et al., 2004; Schuenhoff et al., 2006). While these compounds have been investigated as immunostimulants (Araujo et al., 2008; Lima et al., 2009; Rodrigues et al., 2009), they might also have a significant MSTN-mediated effect on growth. In juvenile *Marsupenaeus japonicus* it has been shown that supplementing the diet with sulphated polysaccharides induces a significant enhancement in growth that was observed to increase with increasing supplementation of this compound (Traifalgar et al., 2010). Whether the weight gain associated with sulphated polysaccharides supplementation observed at least in shrimp is mediated by a direct effect on the MSTN/GDF11 protein is not understood. This might however represent an interesting opportunity to produce a compound with growth-inducing properties while treating farm wastewater, hence capitalizing on a farm's by-product and maximizing profits.

6.2 Concluding remarks

The potential of MSTN for growth improvement in animal production has been largely investigated and it is now beyond any doubt that this protein is a major candidate for growth improvement of farmed animals. The outcomes of this thesis have provided significant advances in the understanding of *Mstn*-like genes in aquaculture species such as barramundi (*Lates calcarifer*) and the black tiger prawn (*Penaeus monodon*) towards the development of

MSTN-based technologies for the enhancement of growth. In particular, this research emphasized that significant differences exist between *Mstn-1* and *Mstn-2* in fish whereby an efficient enhancement of growth may arise from specific targeting of one paralog only. It also revealed for the first time that the invertebrate *Mstn/Gdf11* does not inhibit growth. This last finding in particular will cause an inversion of tendency, whereby research aiming to improve growth of invertebrates like crustaceans should investigate strategies to enhance the activity of MSTN/GDF11 and not reduce it, such as the case of vertebrate MSTN.

While most studies traditionally linked MSTN to livestock production improvement or biomedical applications, it is believed that in the field of aquaculture the largest potential to produce MSTN-based applications for growth improvement can be achieved. Differently from production of terrestrial livestock, the aquatic environment provides a unique medium that allows potentially to mass transferring nucleic acids or protein into the organism. This together with the fact that aquatic animals generally produce a large number of progeny per generation enables the treatment of large group of animals hence maximizing the costs and the benefits of using such approaches. Pivotal enquiries on the physiological roles of fish, crustacean and mollusk ortholog proteins of the mammalian MSTN are however still needed in order to define the best strategies to improve growth and reduce unwanted effects.

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