Studies on Myostatin Expression in Silver Sea Bream *Sparus sarba*

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Abstract of thesis entitled:

Studies on myostatin expression in silver sea bream *Sparus sarba*

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In the first part of the present study, myostatin-1 and myostatin-2 cDNAs have been isolated from silver sea bream (*Sparus sarba*). The full length myostatin-1 cDNA comprises a 1140 bp open reading frame encoding 379 amino acids whereas the partial myostatin-2 cDNA has 363 bp which encodes for 121 amino acids. Myostatin-1 was expressed in many different organs in silver sea bream; whereas myostatin-2 was mainly expressed in muscle and brain. Expression levels of both myostatin-1 and myostatin-2 in tissues of sea bream in different seasons were also evaluated through real-time PCR. Myostatin was expressed at a higher level in summer compared to that in winter in silver sea bream. The different expression levels of myostatin through different seasons suggest that myostatin may have functions in growth which was affected by environmental factors.

In the second part of the study, silver sea bream were injected with growth hormone (GH), 11- ketotestosterone (11KT) or cortisol. Myostatin expression levels

have been measured by using RT-Real-time PCR. Growth hormone resulted in a decreased myostatin-1 expression in white muscle and an increased myostatin expression in red muscle. 11KT decreased the myostatin-1 mRNA amount in red muscle. Cortisol injection resulted in an increased myostatin-1 expression in red muscle which is opposite to the expected. These results suggest that myostatin is involved in the complex hormone regulation in growth progress.

In the third part, myostatin-1 expression levels were examined in silver sea bream exposed to different salinities. Two sets of experiments were carried out: the first experiment involved the long term adaptation of fish to 33ppt, 12ppt and 6ppt respectively and the second experiment involved abrupt transfer of fish from 33ppt to 6ppt. Expression levels of myostatin-1 in white muscle, red muscle, gill and kidney were recorded. The expression of myostatin-1 in all these tissues was unchanged over the salinity range tested in both the long term adaptation experiment and the abrupt transfer experiment for 72h. However, there were significant increases of myostatin-1 expression in red muscle and gill and a decrease of myostatin-1 in kidney in silver sea bream 24h after abrupt transfer from 33ppt to 6ppt. These results showed that myostatin-1 may be involved in short term adaptation for salinity change.

摘要

本研究第一部分完成了黃錫鯛（*Sparus sarba*）肌肉抑制素-1 的完整基因序列和肌肉抑制素-2 的片段基因序列的克隆。肌肉抑制素-1 完整基因的開放閱讀框總長 1140 個城基對，編碼 379 個氨基酸。肌肉抑制素-2 的克隆片段含有 363 個城基對，編碼 121 個氨基酸。肌肉抑制素-1 在黃錫鯛多種器官中均有表達，而肌肉抑制素-2 則主要表達於骨骼肌和腦組織。在不同季節中，肌肉抑制素-1 和-2 於不同器官的基因表達量均有明顯變化。其於夏季的基因表達量明顯高於冬季。肌肉抑制素表達的季節性差異指出環境因素可影響其在魚類生長中的作用。

在本研究第二部分中，生長激素、睾丸硬甾酮以及皮質醇以腹腔注射的方法被分別注射于黃錫鯛中。 此後對其肌肉抑制素的表達情況進行研究。結果發現生長激素注射抑制了肌肉抑制素-1 於白肌中的表達，但同時促進了其在紅肌中的表達。睾丸硬甾酮注射令到紅肌肌肉抑制素-1 的表達被抑制。皮質醇則促進了肌肉抑制素-1 於紅肌中的表達。以上結果反映出肌肉抑制素被多種和生長密切相關的激素所調節。

在第三部分中，由於肌肉抑制素-1 在黃錫鯛鰓及腎臟中均有表達，此部分探索了肌肉抑制素於與滲透壓調節相關器官中的功能的可能性。第一組實驗將黃錫鯛長期馴養於正常海水鹽度（千分之三十三）、等滲鹽度（千分之十二）和低滲鹽度（千分之六）中四個星期。第二組實驗將馴養於海水鹽度中的黃錫鯛直接

轉移至低滲鹽度（千分之六）環境中 24 小時及 72 小時，並與對照組轉移即將海水中馴養之黃錫鯛直接轉移至海水中進行比較。結果表明，在長期馴養試驗和直接轉移 72 小時試驗中，肌肉抑制素-1 於白肌、紅肌、腮和腎臟中的表達沒有因環境鹽度因素而引起變化。在直接轉移后 24 小時試驗中，肌肉抑制素-1 在紅肌及腮中的表達量則有明顯增加，同時，其於腎臟中的表達則明顯降低。以上結果表明，肌肉抑制素-1 有可能在黃錫鯛對不同鹽度進行快速適應中起到一定作用。

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Chapter 1:

General Introduction

Silver sea bream (*Sparus sarba*) used in this study is a kind of excellent food fish with high commercial value. The species is characterized by having an extensive period of postlarval muscle growth (Rowlerson and Veggetti 2001). Compared to traditional fish models such as zebrafish which show little postlarval skeletal muscle growth and reach only small adult sizes, species like sea bream is a better model organism for studying muscle development and myostatin gene in teleost fish (Maccatrozzo et al., 2001b).

Myostatin (MSTN) is a member of the transforming growth factor-ß (TGF-ß) superfamily discovered about 10 years ago (McPherron et al., 1997). The TGF-β superfamily encodes secreted factors that are important for regulating embryonic development and tissue homeostasis in adult, for example, the well known activin and bone morphogenetic proteins (BMPs) (Crampton and Luckhart, 2001). Myostatin regulates muscle growth negatively by inhibiting the proliferation and differentiation of myoblast through a TGF-β signaling pathway (Rios et al., 2002; Thomas et al., 2000), resulting in myostatin deficient animals such as myostatin knockout mice displaying double muscling phenotype (McPherron et al., 1997). In mammals, such as mice, the expression of myostatin is almost exclusively found in skeletal muscle among the different tissues (McPherron et al., 1997). In fish, a second myostatin isoform was also found in 2001 by Robert and Goetz in brook trout (Roberts and

Goetz, 2001) and in some other fish species (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001). Myostatin-1 mRNA was expressed in many different tissues such as gill and kidney, but myostatin-2 mRNA was mainly expressed in brain and muscle (Helterline et al., 2007; Rescan et al., 2001). Several studies have shown that myostatin can be regulated by many hormones such as growth hormone, testosterone, 11-ketotestosterone and cortisol in both mammalian and teleostean species (Biga et al., 2004; Liu et al., 2003; Mendler et al., 2007; Roberts et al., 2004; Rodgers et al., 2003). However, no studies on myostatin have been conducted using silver sea bream and the results on other teleost species were far from conclusive. As such, elucidation of the hormonal regulation of myostatin expression is the main focus of the present study.

In Chapter 2, the basic background information about myostatin and the literature on previous studies on myostatin in both mammalian and teleostean species will be reviewed in detail.

Several studies have shown that the structure of myostatin shares many common characteristics with all members of the family: a hydrophobic core of amino acids at the N-terminal that functions as a secretory signal, a proteolytic processing site (RXXR), a cysteine knot containing nine cysteine residues which is essential for TGF-β activity, and a bioactive C-terminal domain. Proteolytic processing of

myostatin results in a N-terminal Latency Associated peptide (propeptide) and the C-terminal mature myostatin peptide (Thomas et al., 2000). Myostatins of many different species exhibit highly conserved C-terminal bioactive region, indicating that myostatin may have similar function throughout evolutionary diverse animals. In Chapter 3, the first part of this study, characteristics of both myostatin-1 and myostatin-2 in silver sea bream will be examined. The mRNA expressions of these two myostatins in silver sea bream in different seasons were quantified.

In Chapter 4, the effects of growth hormone, 11-ketotestoetrone and cortisol on myostatin mRNA expression in silver sea bream were studied. Myostatin can be regulated by many hormones. In a murine C2C12 cell line, growth hormone treatment resulted in inhibition of myostatin mRNA (Liu et al., 2003). In another study on rainbow trout, growth hormone injection resulted in differential regulation of myostatin-1 and myostatin-2 as growth hormone induced myostatin-1 but reduced myostatin-2 (Biga et al., 2004). Testosterone has been shown to inhibit myostatin expression in rat (Mendler et al., 2007). Glucocorticoids such as dexamethasone and cortisol also have effects on myostatin. However, results of different studies are in conflict. In rat, myostatin mRNA was increased significantly after a single injection of dexamethasone (Lang et al., 2001). However, some studies on fish species showed a opposite result: In tilapia larvae, myostatin mRNA was reduced following cortisol

treatment (Rodgers et al., 2003).

In the present study, silver sea bream were treated with growth hormone (GH), 11-ketotestosterone (11KT) or cortisol. Expression levels of myostatin-1 in white muscle and myostatin-2 in both white and red muscle from hormone treated fish were compared to those in controls to evaluate the effect of hormones on myostatin expression in silver sea bream.

Studies on fish myostatin showed that myostatin-1 was expressed in gill and kidney in many teleost species such as sea bream, zebrafish and salmon (Ostbye et al., 2001; Radaelli et al., 2003). Gill and kidney are important organs responsible for osmoregulation in fish, suggesting that myostatin may be involved in the osmoregulatory processes. The euryhaline fish silver sea bream is an excellent model for studying osmoregulation by virtue of its wide tolerance of a wide range of salinities (0-70ppt) (Deane and Woo, 2004, 2006; Wong et al., 2006). Thus in Chapter 5, the possible osmoregulatory role of myostatin in silver sea bream will be examined by observing myostatin-1 expression levels in gill and kidney in silver sea bream following exposure to different salinities.

The overall objective of the present thesis is to complete some basic studies on myostatin gene expression; to investigate the effects of different hormones on muscle myostatin mRNA expression; and to evaluate the effects of different salinity changes

on myostatin-1 mRNA expression in the main organs of osmoregulation in order to deduce if there is any unreported function of myostatin on osmoregulation in silver sea bream. These investigations may possibly lead to a better understanding about the physiology of myostatin in teleost species.

Chapter 2

Literature Review

2.1 An introduction to myostatin

2.1.1 A general introduction

The myostatin gene belongs to the transforming growth factor-β (TGF- β) superfamily which encodes secreted factors that are important for regulating embryonic development and tissue homeostasis in adult, for example, the well known activin and bone morphogenetic proteins (BMPs). Myostatin inhibits myoblasts proliferation and differentiation which results in inhibition of muscle growth (Rios et al., 2002; Thomas et al., 2000). Mice with a targeted disruption of the myostatin gene display a marked increase in muscle mass, up to three times normal size, as a result of a combination of muscle fiber hyperplasia and hypertrophy (McPherron et al., 1997). Hyperplasia means the proliferation of cells, and hypertrophy which is different from hyperplasia is the increase of size of cell. The obvious increase in muscle mass is also observed in cattle Belgian Blue which has a natural mutation on the myostatin gene (McPherron and Lee, 1997). Myostatin is expressed initially in the myotomal compartment of developing somites and continues to be expressed in muscle throughout development and in adult animals (McPherron et al., 1997). In mammals, such as mice, there was only one type of myostatin and its expression is almost exclusively confined to the skeletal muscle among the different tissues (McPherron et al., 1997). However, in fish, a second myostatin isoform was also found in 2001 by

Robert and Goetz in brook trout (Roberts and Goetz, 2001) and in some other fish species (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001). They found that the fragment obtained by PCR from ovary was different from that in muscle and brain. The ovary myostatin (ov MSTN or MSTN1) was expressed in many different tissues in include eye, gill, kidney, intestine, ovary and brain (Ostbye et al., 2001), and the brain and muscle myostatin (b/m MSTN or MSTN2) was mainly expressed in brain and muscle (Helterline et al., 2007; Rescan et al., 2001).

2.1.2 Myostatin identification

Myostatin was originally identified in a screen for novel mammalian members of the transforming growth factor-ß (TGF-ß) superfamily of growth and differentiation factors (McPherron et al., 1997). These authors carried out PCR on mouse genomic DNA by using degenerate primers which have been designed according to the conserved regions of known TGF-beta family members.

A second myostatin was first found in 2001 by Robert and Goetz in brook trout (Roberts and Goetz, 2001). They found that the fragment obtained by PCR from the ovary was different from that in muscle and brain. The ovary myostatin (ov MSTN or MSTN1) was expressed in many different tissues, but the brain and muscle myostatin

(b/m MSTN or MSTN2) was mainly expressed in brain and muscle. Subsequently, it is known that there are two myostatin isoforms in many fish species, such as gilthead sea bream, Atlantic salmon and white bass (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001).

2.1.3 Structural studies of myostatin

As a member of the TGF-beta family, the structure of myostatin shares many common characteristics with all members of the family: a hydrophobic core of amino acids at the N-terminal that functions as a secretory signal, a proteolytic processing site (RXXR), a cysteine knot contains nine cysteine residues which are essential for TGF-β activity and a bioactive C-terminal domain (Derynck and Miyazono, 2008). Proteolytic processing of myostatin results in a N-terminal latency associated peptide (propeptide) and the C-terminal mature myostatin peptide (Thomas et al., 2000). Myostatin of many different species possess a highly conserved C-terminal bioactive region, indicating that myostatin may have a similar function throughout evolution.

2.1.4 Phenotype of myostatin-null animals or transgenic animals

A. Mammals

Myostatin expression in mammals is limited primarily to skeletal muscle, which

in mammals appears to be the principal target tissue. The muscle mass of myostatin-null cattle, dog, mice, and humans is much higher than normal animals and produces "double muscling" phenotype. Enhanced muscle growth in these animals may be due to increases in both hyperplastic growth and hypertrophic growth (McPherron et al., 1997). Studies on myostatin null mice showed that total muscle cell number was 86% higher in mutant animals compared to wild-type mice. Total DNA of the muscle mass of myostatin null mice is also increased. These results indicate a hyperplastic growth of muscle when the myostatin gene is silenced. Hypertrophic growth is manifest as increased fiber diameter (McPherron et al., 1997). In another study using myostatin overexpressed mice, results showed that myostatin can induce muscle atrophy (Reisz-Porszasz et al., 2003). Myostatin null phenotype has also been found in some mutant cattle breeds such as Piedmontese, Belgian Blue, and Marchigiana (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997).

B. Fish

In fish species, myostatin is expressed in many different tissues, suggesting that myostatin may have function not only in skeletal muscle. Results from different studies on even the same fish species were very different so that there may be many different complex functions of myostatin in fish. Dramatic increase in body weight of zebrafish was obtained with the use of RNAi methods. The body weight of zebrafish

microinjected with 5.5×10^6 molecules of dsRNA for 75days was increased by 45% when compared with the control (Acosta et al., 2005). A double-muscled effect in transgenic zebrafish was also observed in another study using RNAi method as well (Lee et al., 2009). In that study, a hereditarily stable myostatin gene knockdown zebrafish strain with the double-muscle phenotype was established by microinjection of an antisense RNA-expressing vector. The resulting homozygous transgenic zebrafish were 45% heavier than control fish. The myogenic genes like myogenin and MyoD were also evaluated in transgenic fish and both exhibited dramatic upregulation (Lee et al., 2009). However, these results were very different from that of another study in which a transgenic zebrafish line overexpressing the myostatin prodomain in skeletal muscles was generated. The transgenic zebrafish have no significant change in muscle fiber size, and only exhibit 10% more myofibers than the non-transgenic fish. In addition, there are also no significant difference in myogenic gene expression and differentiation of muscle cells (Xu et al., 2003). Elucidation of the reasons for such discrepant results in different studies will await further studies.

2.2 Regulation of myostatin

2.2.1 Biosynthesis of myostatin

Myostatin is synthesized primarily in muscle cell and then circulates into the

blood stream. Myostatin is synthesized as a precursor protein first, which is similar to other members of the TGF-beta family. The bioactive form of myostatin is generated by two proteolytic processing events on the precursor protein. The first processing event cuts a 24-amino acid signal peptide which is necessary for targeting the protein to the secretory pathway (McFarlane et al., 2005). The second cleavage occurs at the RXXR site and generates a N-terminal propeptide and a bioactive C-terminal part which possesses receptor binding activity (McFarlane et al., 2005). The second cleavage may be carried out by paired dibasic amino acid-cleaving enzyme (PACE or furin). Furin is a member of subtilisin-like serine proteinases family. In Lee and McPherron's study in 2001, they used a soluble form of furin to cut myostatin precursor protein at the proteolytic site RXXR in CHO cell (Lee and McPherron, 2001). The propeptide plays an important role in the proper folding of the C-terminal domain into a cysteine knot as in other TGF- beta family members. Two C-terminal fragments which folded into a cysteine knot forms a disulfide-linked dimer (McFarlane et al., 2005).

2.2.2 Regulation of myostatin expression

Myostatin can be regulated and affected by many factors, for example, hormones, transcriptional factors and other proteins. The highly conserved regulatory region of

the myostatin gene has several hormone-response site such as growth hormone response element, glucocorticoid response element, thyroid hormone response element and androgen response element (Du et al., 2005; Ma et al., 2001; Ma et al., 2003; Roberts and Goetz, 2003). Transcription factor-binding motifs was also present such as MyoD binding site (Salerno et al., 2004; Spiller et al., 2002). Some studies showed that the myostatin gene is a downstream target gene of basic Helix-Loop-Helix transcription factor MyoD. There is a significant reduction in the myostatin promoter activity in cells that lack MyoD expression (Spiller et al., 2002). These response elements for different factors indicated that myostatin can be regulated at the transcription level.

Myostatin is known to be regulated by various hormones. For example, testosterone has been shown to inhibit myostatin expression (Mendler et al., 2007). And as described, a glucocorticoid response element was identified on the myostatin promoter region. However, results from different studies are in conflict. In rat, myostatin mRNA was increased 2.7-fold after a single injection of the synthetic glucocorticoid, dexamethasone. Pretreatment with the glucocorticoid receptor antagonist RU486 completely prevented the dexamethasone-induced increase in myostatin, confirming the effect of the glucocorticoid (Lang et al., 2001). However, some studies on fish species showed an opposite result: In tilapia larvae, myostatin

mRNA was reduced by 66 and 75% under cortisol treatment at 3h and 6h respectively (Rodgers et al., 2003). Results similar to those in tilapia were found in channel catfish in which a decrease in myostatin expression was recorded at 12 hours post dexamethasone (Dex) injection (Weber et al., 2005). Furthermore, fasting and stress which can lead to an increase in cortisol also resulted in decreased myostatin expression in fish (Rodgers et al., 2003; Vianello et al., 2003). Growth hormone response element was also identified upstream from the transcription start site in the myostatin gene of trout and mammal (Roberts and Goetz, 2001; Taylor et al., 2001). In mouse C2C12 cell line, treatment of growth hormone resulted in inhibition of myostatin mRNA (Liu et al., 2003). Recently, there was a report of differential myostatin expression following growth hormone injection between two closely-related fish species, giant danio and zebrafish. In this study, myostatin was down-regulated in growth hormone-treated giant danio but up-regulated in zebrafish administered with growth hormone (Biga and Meyer, 2009). In a study using transgenic salmon overexpressing growth hormone as model, growth hormone had no effect on myostatin-1, but resulted in a decrease in white muscle myostatin-2 expression and an increase in red muscle myostatin-2 expression. At the protein level, myostatin protein was decreased in both red and white muscle (Roberts et al., 2004). Growth hormone injection in rainbow trout resulted in differential regulation of

myostatin-1 and myostatin-2: growth hormone induced myostatin-1 but reduced myostatin-2 (Biga et al., 2004).

2.2.3 Regulation of myostatin protein

In addition to regulation at the transcription level, myostatin also can be regulated at the protein level. Myostatin is found in serum and muscle tissue in several forms, bound to different protein partners. These proteins bind to myostatin protein, modulate its activation, secretion or receptor binding (Table 2.1).

Follistatin

Follistatin is an autocrine glycoprotein and can inhibit the activity of several members of the TGF beta family including the mature myostatin. Myostatin can bind to follistatin in skeletal muscle as mature myostatin form. As a consequence, this binding to follistatin inhibits binding of myostatin to its receptor (Lee and McPherron, 2001). Muscle mass of transgenic mice overexpressing follistatin obtained by pronuclear injection of follistatin construct was increased by 194–327% relative to control animals, the larger muscle mass resulted from a combination of hyperplasia (66% increase in fiber number) and hypertrophy (28% increase in fiber diameter) (Lee and McPherron, 2001).

FLRG

FLRG (follistatin-related gene) can directly bind to mature myostatin with high affinity and inhibit myostatin activity in a concentration-dependent manner as assessed by reporter gene assays. (Hill et al., 2002)

GASP-1

GASP-1 (Growth and differentiation factor-associated serum protein-1) was discovered as a protein associated with endogenous myostatin in mice and human serum. GASP-1 can directly bind to both mature myostatin and propeptide. Structural study showed that GASP-1 contains multiple protease-inhibitory domains. So GASP-1 is likely to inhibit the activity of proteases and as a consequence inhibit myostatin activity (Hill et al., 2003).

Propeptide

Propeptide is also very important in myostatin regulation. Using purified recombinant myostatin and propeptide protein as standards in Western blots to estimate the amount of these proteins purified from serum, Hill found that the majority (>70%) of circulating myostatin is bound to propeptide (Hill et al., 2002). Experiment carried out in CHO cells also showed that the bioactive C-terminal dimer forms a noncovalent complex with the propeptide. The propeptide can maintain the C terminal bioactive myostatin in an inactive, latent state (McFarlane et al., 2005). The

ability of the propeptide to block the activity of the purified myostatin C-terminal has been documented in vitro and in vivo (Lee and McPherron, 2001; Thies et al., 2001). In Lee and McPherron's study (Lee and McPherron, 2001), purified propeptide can block the activity of the purified myostatin C-terminal dimer in vitro in Act RIIB receptor binding assay. In *in vivo* test, transgenic mice overexpressing myostatin propeptide obtained by pronuclear injection of propeptide construct showed increases in both muscle fiber number and size (Lee and McPherron, 2001). Consequently, one regulatory mechanism to activate myostatin C-terminal dimer is proteolytic cleavage of the propeptide. Injection of a mutant form of the propeptide (aspartate 76 was mutated to alanine) made resistant to proteolysis by the BMP-1/tolloid proteinases in vitro into mice for four weeks resulted in increases in muscle growth of 25% (Wolfman et al., 2003).

hSGT

Human small glutamine-rich tetratricopeptide repeat-containing protein (hSGT) can bind to the myostatin N-terminal signal peptide region and inhibits its secretion and activation (Wang et al., 2003).

Decorin

A proteoglycan found in the extracellular matrix, decorin, plays an important role in cell growth through the modulation of growth factor activities (Patel et al., 1998; Yamaguchi et al., 1990). It also binds to TGF-β via its core protein and modulates its

activity. Some studies showed that decorin interacts with myostatin and suppresses myostatin-mediated inhibitory action to muscle cell growth by keeping myostatin away from its receptor on muscle cells (Kishioka et al., 2004; Kishioka et al., 2008).

T-cap

Titin-cap (T-cap) is a sarcomeric protein that binds to the N-terminal domain of Titin and is a substrate of the titin kinase (Nicholas et al., 2002). Several assays including mammalian two-hybrid studies, in vitro binding assays and yeast two-hybrid system suggested the specific interaction between processed mature myostatin and full-length T-cap (Nicholas et al., 2002). The rate of cell proliferation was significantly increased in T-cap overexpressing C2C12 myoblasts. Western analyses showed that production and processing of myostatin were not affected in T-cap overexpressing myoblast, however, an increase in mature myostatin indicated that T-cap may block myostatin secretion. These results suggest that T-cap may control myostatin secretion in myoblast but does not affect the proteolytic processing step of precursor myostatin (Nicholas et al., 2002).

Table 2.1

Myostatin binding proteins

Binding molecule	***Binding form***	***Consequence of binding***
Myostatin propeptide	Mature myostatin	Inhibits myostatin receptor binding
GASP1	Mature myostatin & propeptide	Inhibits myostatin activation
FLRG	Mature myostatin	Inhibits myostatin activation
hSGT	Myostatin N-terminal signal peptide region	Inhibits myostatin secretion and activation
Titin cap	Mature myostatin	Inhibits myostatin secretion
Follistatin	Mature myostatin	Inhibits myostatin receptor binding

2.3 Myostatin effect

2.3.1 The myostatin signaling pathway

As a member of the TGF-beta family, the signaling pathway of myostatin is

similar to most other TGF-beta ligands, which signal through high affinity transmembrane receptors, the type I and type II serine/threonine kinase receptors. In the absence of ligand, type II and type I receptors homodimerize at the cell surface. After ligand binding to type II receptor occurs, the type I receptor was phosphorylated by the ligand-bound type II receptor. This leads to type I serine/threonine kinase receptor activation which in turn phosphorylates the Smad protein (Joulia-Ekaza and Cabello, 2006) (Fig 1).

Three kinds of Smad proteins are involved in TGF-beta ligand family signaling pathway: receptor regulated Smad (R-Smads), common mediator Smad (Co-Smads) and inhibitory Smad (I-Smads). R-Smads including Smad 1, 2, 3, 5 and 8 are Smad proteins activated by type I receptors through phosphorylation (Zhu et al., 2004). After phosphorylation or activation of R-Smads, Co-Smads bind to the activated R-Smads and the complex can accumulate in the cell nucleus where they regulate transcription of specific target genes. I-Smads inhibit the TGF-beta pathway by two mechanisms. Firstly, I-Smads can compete with R-Smads for binding to activated receptors. Secondly (Hata et al., 1998), I-Smads compete with co-Smads for binding of activated R-Smads. Expression of I-Smads can be stimulated by TGF-beta ligand. So I-Smads can regulate the TGF-beta pathway through negative feedback (Imamura et al., 1997).

There are several kinds of type II receptors which respond to different ligands, such as ActRIIA (activin type II receptor), ActRIIB and BMPRII (bone morphogenetic protein type II receptor). In Lee and McPherron's cross-linking experiments in 2001, COS-7 cells transfected with expression constructs for the indicated receptors were incubated with ^{125}I-myostatin and crosslinking agent and analyzed by SDS/PAGE. Results showed that both ActRIIA and ActRIIB can bind to myostatin. The ActRIIB receptor exhibits a much higher level of binding than ActRIIA (Lee and McPherron, 2001). Injection of recombinant extracellular domain of goldfish ActRIIB also led to an increase in muscle mass and protein content in goldfish (Carpio et al., 2009).

Studies have established that R-Smads Smad2 and Smad3 and co-Smad Smad4 are involved in myostatin signaling. I-Smad Smad7 can inhibit myostatin signal. In addition, myostatin expression of Smad7 is induced by myostatin, leading to a negative regulatory feedback loop mechanism (Zhu et al., 2004).

The myostatin signaling pathway is shown in Figure 2.1 which was excerpted from Joulia-Ekaza and Cabello's paper in 2007 (Joulia-Ekaza and Cabello, 2007).

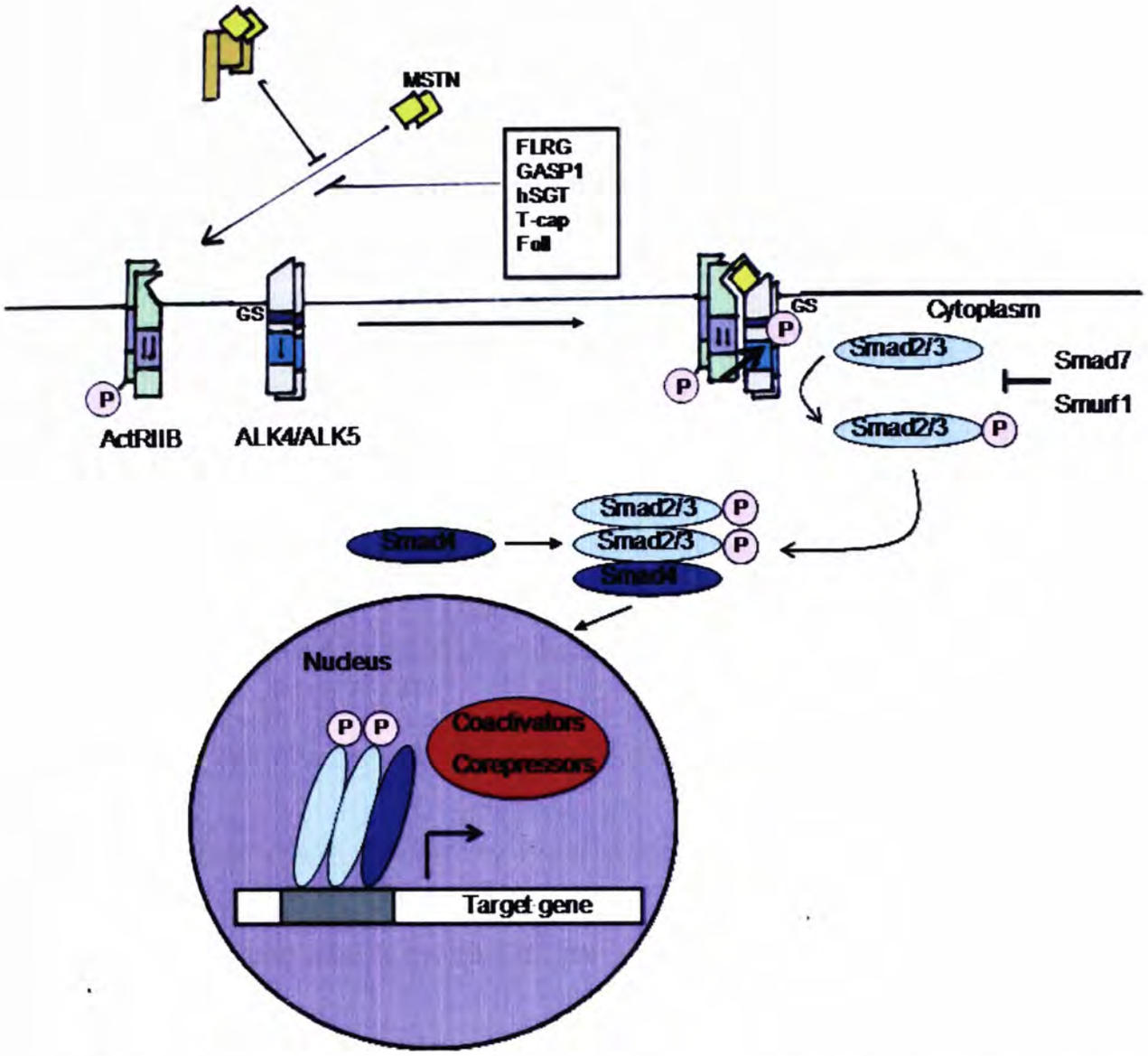


Fig. 2.1. Myostatin signaling pathway. Redrawn based on Figure 1 from *The myostatin gene: physiology and pharmacology relevance* (Joulia-Ekaza and Cabello, 2007).

2.3.2 Cellular response for myostatin signaling

It is known that myostatin inhibits skeletal muscle growth by inhibiting the proliferation and differentiation of myoblasts (Rios et al., 2002; Thomas et al., 2000). The whole process is shown in Figure 2.2.

The growth of myoblast is inhibited by incubation with myostatin-plus medium.

Treatment with myostatin also results in increased G1 phase cell number and decreased S phase cell number, which indicates that myostatin can lead to cell withdrawal from the cell cycle and consequently leading to arrest in G1 phase (Thomas et al., 2000). So the process of inhibiting cell proliferation by myostatin may occur through the regulation of myoblast cell cycle by interrupting the transition of G1 phase to S phase. G1-S phase transition required S phase specific transcription factor which is inhibited by Rb (retinoblastoma) protein binding. Cyclin-Cdk complex can phosphorylate Rb protein and the phosphorylated Rb can no longer bind to transcription factor and permitting transcription of S phase genes. Cyclin-Cdk inhibitors (CKIs) can inhibit cyclin-Cdk and as a consequence inhibit the G1-S phase transition(Becker et al., 2000). A CKI p21 seems to be promoted by myostatin. p21 protein was induced and Cdk2 activity was decreased in myostatin-treated myoblast as measured by Western blot. Myostatin treatment also causes a decrease in the hyperphosphorylated form of Rb and a corresponding increase in the hypophosphorylated form Rb protein. The increased hypophosphorylated form Rb may be caused by the inhibitory effect of myostatin-promoted p21 on Cyclin-Cdk (Thomas et al., 2000).

Myotube formation was inhibited in C2C12 cells overexpressing myostatin, indicating that myostatin can inhibit muscle cell differentiation. This inhibitory action

of myostatin may be mediated by the down-regulation of the transcription factor required for myoblast differentiation, MyoD and myogenin (Rios et al., 2002). Expression of MyoD, myogenin and Mrf 4 were found to be increased in myostatin gene knockdown zebrafish (Lee et al., 2009). Some studies showed that myostatin can down-regulate MyoD expression by increasing Smad 3 •MyoD association. Myostatin signaling specifically induced Smad 3 phosphorylation and increased Smad 3-MyoD association, suggesting that Smad 3 may mediate the myostatin signal by interfering with MyoD activity and expression (Langley et al., 2002).

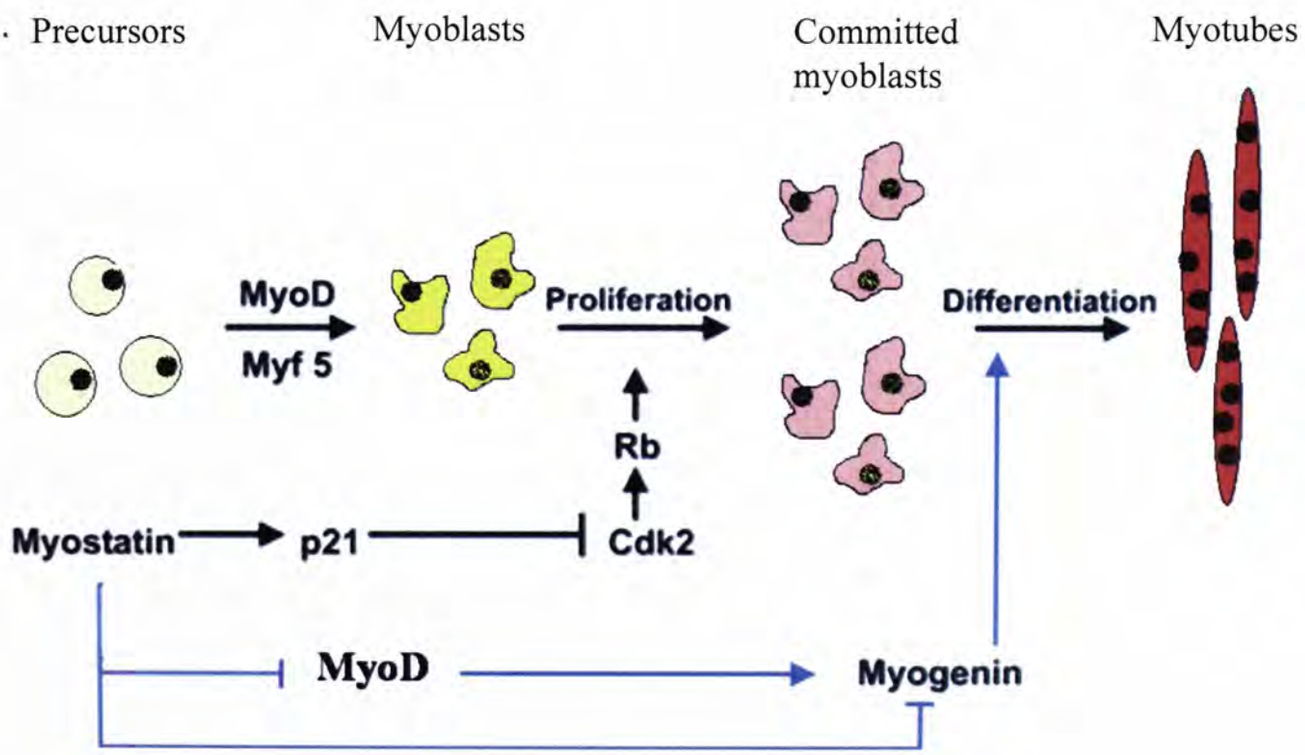


Fig. 2.2. Myostatin inhibits myoblast proliferation and differentiation

2.4 Functions in tissues other than muscle

Myostatin is also expressed minimally in some other tissues in mammals such as adipose tissue and heart (Rodgers and Garikipati, 2008). Myostatin can inhibit the differentiation of 3T3-L1 preadipose cells from human *in vitro* (Kim et al., 2000; Kim et al., 2001). In contrast, myostatin is shown to promote adipogenesis *in vitro* in Artaza's study (Artaza et al., 2006). Myostatin can inhibit the growth of cardiomyoblast and can also suppress cardiomyocyte hypertrophic growth responses (Morissette et al., 2006; Shyu et al., 2005). Myostatin may play similar function in heart with what it does in muscle (Rodgers and Garikipati, 2008).

Many studies have shown that myostatin is expressed in different brain regions of different vertebrates (Radaelli et al., 2003; Rodgers and Garikipati, 2008), although its function in the brain is not clear. As a growth differentiation factor GDF-11 shares a very similar bioactive domain with myostatin, suggesting that myostatin may be similar to GDF-11 in playing a role in neurogenesis. GDF-11 regulates neurogenesis negatively, and its inhibitory effect on neurogenesis through inducing cell cycle arrest in neuroprogenitors cells was proved in studies in olfactory epithelium (Wu et al., 2003). Both myostatin 1 and myostatin 2 were expressed in fish brain with high levels, suggesting that myostatin may play a similar function with GDF-11 in brain (Rodgers and Garikipati, 2008).

2.5 Myostatin in fishes

2.5.1 Introduction to silver sea bream (*Sparus sarba*)

The scientific name of Silver sea bream is *Sparus sarba*, its synonym name is *Rhabdosargus sarba*. This species belong to order Perciformes, family Sparidae. They have deep, ovate and compressed body. They have round head and obtuse snout. The caudal fin is forked. They are called silver sea bream or gold-line sea bream as their bodies are silver in color with yellow longitudinal stripes on both sides of the body. The maturity size is about 26 cm TL and the maximum size is 80 cm TL (WWF). Silver sea bream is a kind of euryhaline fish which are able to adapt a wide range of salinities. The fish is a kind of excellent food fish with high commercial value. FAO reported that world supply of sea bream has increased over time and silver sea bream represents a large percentage (about 30%) (FAO). Silver sea bream show extensive postlarval muscle growth and can reach large adult size (to at least 26 cm TL). So it is a suitable model for studying muscle development of teleost fish.

2.5.2 Studies carried on in fishes

In fish species, there are two myostatin genes. The second myostatin was first found in 2001 by Robert and Goetz in brook trout (Roberts and Goetz, 2001). After that, the two myostatin genes were identified in many other teleosts such as gilthead

sea bream, Atlantic salmon and white bass (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001).The expression pattern of myostatin in fish species was very different from that in mammals. Myostatin-1 was shown to have a far more diverse expression pattern than that in mammal (Johansen and Overturf, 2005; Maccatrozzo et al., 2001b; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2003). The expression pattern suggests that myostatin may have function not only in skeletal muscle. Myostatin-2 was mainly expressed in muscle and brain (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001).

Fish meat is one of the most important foods in the world. The demand is very high and it is a healthy food because of its high content of Omega 3 fatty acid. Omega 3 fatty acids are good for the heart and it has been suggested to reduce risk for cardiovascular diseases. So a study about fish muscle growth is important and useful to the production of food fish. Manipulating myostatin gene expression seems to be a possible way to increase the fish muscle mass as myostatin is suggested to be a negative regulator for muscle growth in mammals (McPherron et al., 1997). People try to study the function of myostatin in fish by overexpressing or silencing the gene. Dramatic increase in body weight of zebrafish was obtained by RNAi methods. The body weight of zebrafish microinjected with 5.5×10^6 molecules of dsRNA for 75days

was increased by 45% when compared with the control (Acosta et al., 2005). However, the results were very different from that of another study in which a transgenic zebrafish line overexpressing the myostatin prodomain in skeletal muscles was generated. The transgenic zebrafish have no significant change in muscle fiber size, and only exhibit 10% more myofibers than the non-transgenic fish (Xu et al., 2003).

The regulation of myostatin at the RNA or protein levels was also very important and was studied by many groups. In mammals, there are many factors which have been shown to regulate myostatin at the protein level (Table 2.1). In addition, several hormones response elements were found in the 5' regulatory region on the myostatin gene. Hormones response elements were also found in several fish species. Interactions of myostatin with some important growth related hormones have been studied, for example, growth hormone and cortisol. In a study using transgenic salmon overexpressing growth hormone as model, growth hormone had no effect on myostatin-1, but resulted in a decrease in white muscle myostatin-2 expression and an increase in red muscle myostatin-2 expression. At the protein level, myostatin protein was decreased in both red and white muscle (Roberts et al., 2004). Growth hormone injection in rainbow trout results in differential regulation of myostatin-1 and myostatin-2: growth hormone induced myostatin-1 but reduced myostatin-2 (Biga et

al., 2004). In tilapia larvae, myostatin mRNA was reduced by 66 and 75% following cortisol treatment at 3h and 6h respectively (Rodgers et al., 2003). Although these results were opposite to the findings in mammals, they were consistent with those in channel catfish in which a decrease in myostatin expression was recorded at 12 hours post dexamethasone (Dex) injection (Weber et al., 2005). Results of studies on hormones effect on myostatin in fish were very different from those in mammals or even in different fish species. It indicates a possible different function of myostatin in teleosts and more studies are required to elucidate these possible novel functions.

2.5.3 Possible novel functions of myostatin in fishes

Myostatin was expressed in gill and kidney in many teleost species such as sea bream, zebrafish and salmon (Ostbye et al., 2001; Radaelli et al., 2003). Gill and kidney are important organs responsible for osmoregulation in fish. So myostatin may play roles in the osmoregulation processes. In gills, myostatin was localized in either the chloride or mucus cell at the primary lamellae in Ostbye's study (Ostbye et al., 2001). Considering the function of myostatin in myoblast and the role of TGF-beta family members in the cell, myostatin may play a role in chloride cell differentiation in fish gills, but this aspect of chloride cell function has never been fully investigated.

Chapter 3

Characterization of myostatin gene in the silver sea bream (*Sparus sarba*)

3.1 Abstract

Myostatin-1 and myostatin-2 cDNAs have been isolated from silver sea bream (*Sparus sarba*). The full length myostatin-1 cDNA contains 1140 bp open reading frame encoding 379 amino acids whereas the partial myostatin-2 cDNA has 363 bp which encodes for 121 amino acids. The amino acid sequence of silver sea bream myostatin-1 is highly conserved with those of other fish and mammalian species especially for the bioactive C-terminal region. Phylogenetic analysis of myostatin in different animals suggests close evolutionary relationships. Myostatin is a member of TGF-beta family and the sequence isolated in this study possesses all the characteristics of that family. Reverse transcriptase polymerase chain reaction (RT-PCR) on total RNA extracted from different sea bream tissues revealed myostatin-1 and myostatin-2 expression in skeletal muscle and non-muscle tissues such as brain, gill and kidney. The expression pattern of myostatin in fish differs from that in mammals, suggesting possible additional functions of myostatin in teleost species. Expression levels of both myostatin-1 and myostatin-2 in tissues of sea bream in different seasons were also evaluated through real-time PCR. Myostatin was expressed at a higher level in summer compared to that in winter in silver sea bream. The different expression levels of myostatin through different seasons suggest that myostatin may have functions in growth which are affected by environmental factors.

3.2 Introduction

Myostatin (MSTN) is a member of the recently discovered family of proteins, the transforming growth factor-ß (TGF-ß) superfamily (McPherron et al., 1997). The TGF-β superfamily encodes secreted factors that are important for regulating embryonic development and tissue homeostasis in adult, for example, the well known activin and bone morphogenetic proteins (BMPs) (Crampton and Luckhart, 2001). Myostatin regulates muscle growth negatively by inhibiting the proliferation and differentiation of myoblast through a TGF-β signaling pathway (Rios et al., 2002; Thomas et al., 2000), resulting in myostatin deficient animals such as myostatin knockout mice displaying double muscling phenotype (McPherron et al., 1997).

In mammals, such as mice, the expression of myostatin is almost exclusively found in skeletal muscle among the different tissues (McPherron et al., 1997). In fish, a second myostatin isoform was also found in 2001 by Robert and Goetz in brook trout (Roberts and Goetz, 2001) and in some other fish species (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001). These authors found that the fragment obtained by PCR from ovary was different from that in muscle and brain. The ovary myostatin (ov MSTN or MSTN1) was expressed in many different tissues, and the brain and muscle myostatin (b/m MSTN or MSTN2) was mainly expressed in brain and muscle (Helterline et al., 2007; Rescan et al.,

2001).

As a member of the TGF-beta family, the structure of myostatin shares many common characteristics with all members of the family: a hydrophobic core of amino acids at the N-terminal that functions as a secretory signal, a proteolytic processing site (RXXR), a cysteine knot containing nine cysteine residues which is essential for TGF-β activity, and a bioactive C-terminal domain. Proteolytic processing of myostatin results in a N-terminal Latency Associated peptide (propeptide) and the C-terminal mature myostatin peptide (Thomas et al., 2000). Myostatins of many different species exhibit highly conserved C-terminal bioactive region, indicating that myostatin may have similar function throughout evolutionary diverse animals.

Silver sea bream (*Sparus sarba*) used in this study is a kind of excellent food fish with high commercial value. It has an extensive period of postlarval muscle growth (Rowlerson and Veggetti 2001). Compared to traditional fish models such as zebrafish which show little postlarval skeletal muscle growth and reach only small adult sizes, species like sea bream is a better model organism for studying muscle development in teleost fish (Maccatrozzo et al., 2001b).

3.3 Materials and methods

3.3.1. Experimental fish

Silver sea bream (*Sparus sarba*) were obtained from a local fish farm and kept in recirculating and aerated seawater [33 parts per thousand (ppt)] in the Simon FS Li Marine Science Laboratory, the Chinese University of Hong Kong. Fish were fed daily with a formulated diet (Woo and Kelly, 1995). Tissue samples collected from fish were stored in TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) in -80°C until extracted for total RNA. The fish used for the analysis of seasonal expression pattern of myostatin were obtained in June 2008 and allowed to grow in tanks and sampled in August, October, February and April respectively. The weights of the fish sampled in August, October, February, and April were 52.01±2.25, 69.63±1.20, 86.89±2.67 and 109.48±2.35 respectively.

3.3.2 Total RNA extraction and cDNA cloning of myostatin-1 and myostatin-2 in silver sea bream

Total RNA was extracted from muscle of silver sea bream by using TRI Reagent® (Molecular Research Center, Inc.) according to the manufacturer's protocol. The concentration of total RNA was determined by measurement of absorbance at 260 nm (conversion factor: 1OD = 40ng RNA/ml). The purity of RNA was checked by

ratio of absorbance at 260nm over which at 280nm. RNA integrity (intensity of 18s and 28s ribosomal RNA on gel) was assessed by gel electrophoresis of total RNA.

Before performing reverse transcription, the extracted total RNA which was re-suspended in DEPC water was treated with deoxyribonuclease I (amplification grade, Invitrogen, Carlsbad, CA) to eliminate possible genomic DNA contamination. The total RNA was then transcribed reversely to first-strand cDNA by M-MLV Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's instruction using oligo$(dT)_{15}$ primer.

Two gene specific primers (5'-TTCTCGACCAGTACGACGTG-3' and 5'-TAATCCAGTCCCAGCCAAAG-3') were designed based on gilthead sea bream myostatin-1 cDNA sequence (GenBank Accession no.: AF258448) and the other two gene specific primers (5'-CCCAGGACGAGTTGACCTCA-3' and 5'-CGGACTCTGATTCGGGT GTT-3') were designed based on gilthead sea bream myostatin-2 cDNA sequence (GenBank Accession no.: AY046314). These primers were used to isolate a partial region of silver sea bream myostatin-1 and myostatin-2 cDNA within the coding region respectively. The reaction mixture for the polymerase chain reaction consisted of 0.8 µl first strand cDNA, 1x PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTP, 0.2 µM of each gene-specific primer and 0.4 U units of Taq DNA polymerase (total volume 20µl). The PCR amplification was carried out for 35 cycles

after the initial denaturing step at 94°C for 5min, and each cycle consisted of 30 sec denaturing at 94°C, 60 sec at 60°C for annealing, and 30 sec at 72°C for extension. The 35 cycles were followed by 10 min at 75°C for final extension. The PCR products were ligated to pCR®-TOPO® vector by using TOPO TA cloning® Kit (Invitrogen, Carlsbad, CA). NucleoSpin® Plasmid DNA Purification Kit (Macherey-Nagel, Brockville, Ontario) was used to extract plasmids containing target DNA from bacteria cells. The plasmids were subsequently sequenced.

The 5' and 3' ends of silver sea bream myostatin-1 cDNA were obtained by Rapid Amplification of cDNA Ends (RACE) (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Based on the nucleotide sequence of the partial cDNA of silver sea bream, the antisense primer 5'-TCGAATCGAAGGCGTTAATC-3' was used in reverse transcription for 5'RACE and antisense primer 5'-TTCAGGGAGCGGATGTGTAT-3' and 5'-ACGACGGACTCAGGTTCAGT-3' and 5'-ACATCCCTGTTGTCGTCTCC-3' were used for PCR in 5'RACE together with Abridged Anchor Primer (AAP) and Abridged Universal Amplification Primer (AUAP). In 3' RACE, Adaptor Primer (AP) was used for first strand cDNA synthesis in reverse transcription. Two gene specific sense primers 5'-GTGGTTATGGAGGAGGACGA-3' and 5'-AACCGTTCATGGAGGTGAAGA-

3' were used for PCR together with AUAP. The PCR products of 5' and 3' RACE were subcloned as described above.

3.3.3 Multiple sequence alignment and unrooted trees

The amino acid sequences of myostatin-1 and myostatin-2 of silver sea bream were aligned with those of other fish species using the multiple sequence alignment program T-Coffee (European Bioinformatics Institute, website: http://www.ebi.ac.uk/t-coffee). Unrooted trees were constructed using CLUSTALW (Kyoto University Bioinformatics Center, website: http://align.genome.jp).

3.3.4 Detection of myostatin-1 and myostatin-2 mRNA in different tissues

Based on the cDNA sequence of the previously cloned silver sea bream myostatin-1 and myostatin-2, gene specific primers were designed (5'-GGAGACGACAACAGGGATGT-3' and 5'-TTCAGGGAGCGGATGTGTAT-3' for MSTN1; 5'- CCCAGGACGAGTTGACCTCA-3' and 5'-CGGACTCTGATTCGGGTGTT-3' for MSTN2). By using these gene-specific primers, expression of myostatin-1 and myostatin-2 mRNA in different tissues (white muscle, red muscle, gill, kidney, intestine, heart, spleen, whole brain and several brain regions: olfactory bulb, telencephalon, hypothalamus, optic tectum, cerebellum,

medulla oblongata, spinal cord and pituitary) were examined by RT-PCR as previously described. The PCR products were checked using electrophoresis on agarose gel with ethidium bromide staining under UV illumination and documented using BIO-RAD gel doc.

3.3.5. Real-time PCR for quantification of myostatin-1 and -2 mRNA expression

Real-time quantitative RT-PCR was used to measure the mRNA expression of myostatin-1, myostatin-2 and a housekeeping gene 18s rRNA which acts as internal control for normalization. The standards for myostatin-1, myostatin-2 and 18s rRNA were prepared by RT-PCR amplification with the gene-specific primers (5'-GGAGACGACAACAGGGATGT-3' and 5'-TTCAGGGAGCGGATGTGTAT-3' for MSTN1; 5'- CCCAGGACGAGTTGACCTCA-3' and 5'-CGGACTCTGATTCGGGTGTT-3' for MSTN2; 5'-CTTGGATGTGGTAGCCGTTT-3' and 5'-GGATGCGTGCATTTATCAGA-3' for 18s rRNA, designed according to the partial sequence of silver sea bream 18s rRNA (GenBank Accession no.: EF494673). Real time PCR was carried out using iCycler iQ ™ Real-Time PCR Detection System (BioRad, Hercules, CA). The real-time PCR reactions were carried out in a total volume of 30µl containing 10µl diluted RT reaction mix, 15µl iQ ™ SYBR® Green Supermix (BioRad, Hercules, CA) and 0.2µM

of each primer pair as described above. The reaction consisted of an initial cycle of denaturation at 95°C for 4 minutes, 40 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 40 seconds and signal detection at 84°C for 7 second. A melting curve analysis consisting of 176 cycles of 7 seconds with temperature increase of 0.2°C / cycle was carried out at the end of the reaction to ensure the specificity of the reaction.

3.3.6 Data processing and statistical analysis

The mRNA expression levels of myostatin-1 and myostatin-2 were normalized by 18s rRNA expression for each sample. Data were presented as mean ± SEM. Statistical analyses were performed using statistical program SIGMASTAT 2.0 and the differences were considered to be significant at p-value < 0.05 by one-way analysis of variance (ANOVA) and Newman-Keuls Multiple Comparison Test.

3.4 Results

3.4.1 Cloning of myostatin-1 and myostatin-2 cDNA

In the first round PCR, cDNA fragments of 584 bp and 363 bp were amplified using the gene-specific primers designed according to the sequence of gilthead sea bream myostatin-1 and myostatin-2 cDNA respectively.

The full length myostatin-1 sequence was obtained by RACE (Rapid Amplification of cDNA Ends). 5' and 3' RACE yielded products of 412 bp in the 5' region and 611 bp in the 3' region. Excluding the poly(A) tail, the full-length silver sea bream myostatin cDNA consists of 1631 nucleotides. This sequence is composed of a 129 bp 5' untranslated region, a 1140 bp open reading frame encoding 379 amino acid, and a 362 bp 3' untranslated region. As with all other members of TGF-beta family, silver sea bream myostatin gene possesses a proteolytic processing site RVRR (RXXR) and nine cysteine residues in the carboxy-terminal portion of the coding region. The proteolytic processing results in both a N-terminal propeptide (latency associated peptide, LAP) and a C-terminal mature myostatin peptide. Eight of the nine cysteine residues form disulfide bonds to create a cysteine knot and the ninth one forms a bond with the ninth cysteine of another myostatin molecule to form the dimer (Fig. 3.1 & Fig. 3.2).

Alignment of the deduced amino acid sequence of silver sea bream myostatin-1 and myostatin-2 with the amino acid sequences of human (GenBank Accession no.:NM 005259), mouse (NM 010834), and several teleost species including zebrafish (MSTN1: AY 258034; MSTN2: AY 687474), fugu (MSTN1: AY 445322; MSTN2: AY 445321), rainbow trout (MSTN1a: AF 273035; MSTN1b: AF 273036; MSTN2a: DQ 417326) and gilthead sea bream (MSTN1: AF258448; MSTN2: AY 046314)

produced by T-Coffee is shown in Fig 3.3. Results show that the bioactive C-terminal domains are highly conserved among various groups. Relationship among different animals was built up as unrooted trees calculated by CLUSTALW (Fig. 3.4).

3.4.2. Myostatin tissue distribution and seasonal pattern

Silver sea bream myostatin-1 expression was detected mainly in white muscle, red muscle and brain. Low level of its expression was also detected in some other tissues such as gill and kidney (Fig 3.5). Myostatin-2 expression was only detected in red muscle, brain and a very low level in white muscle (Fig 3.5). Figure 3.6 shows the result of MSTN expression level measured by real-time PCR in three different tissues: white muscle, red muscle and whole brain. Expressions of myostatin-1 in all these three tissues were higher than that of myostatin-2. Myostatin-1 expression was highest in red muscle which is 3.6 fold of white muscle. Myostatin-2 was mainly expressed in red muscle and whole brain which were respectively 107-fold and 155-fold higher than that in white muscle.

Myostatin-1 expression in white muscle was highest in summer (August) (Fig. 3.7A), while peak level of myostatin-1 expression in red muscle occurred in August and October (Fig. 3.7B). For both white and red muscle, myostatin-1 had lowest

expression in the spawning season (February). For myostatin-2 mRNA abundance, the highest expression was recorded in October and the lowest value was also found in February just like myostatin-1 (Fig. 3.7C).

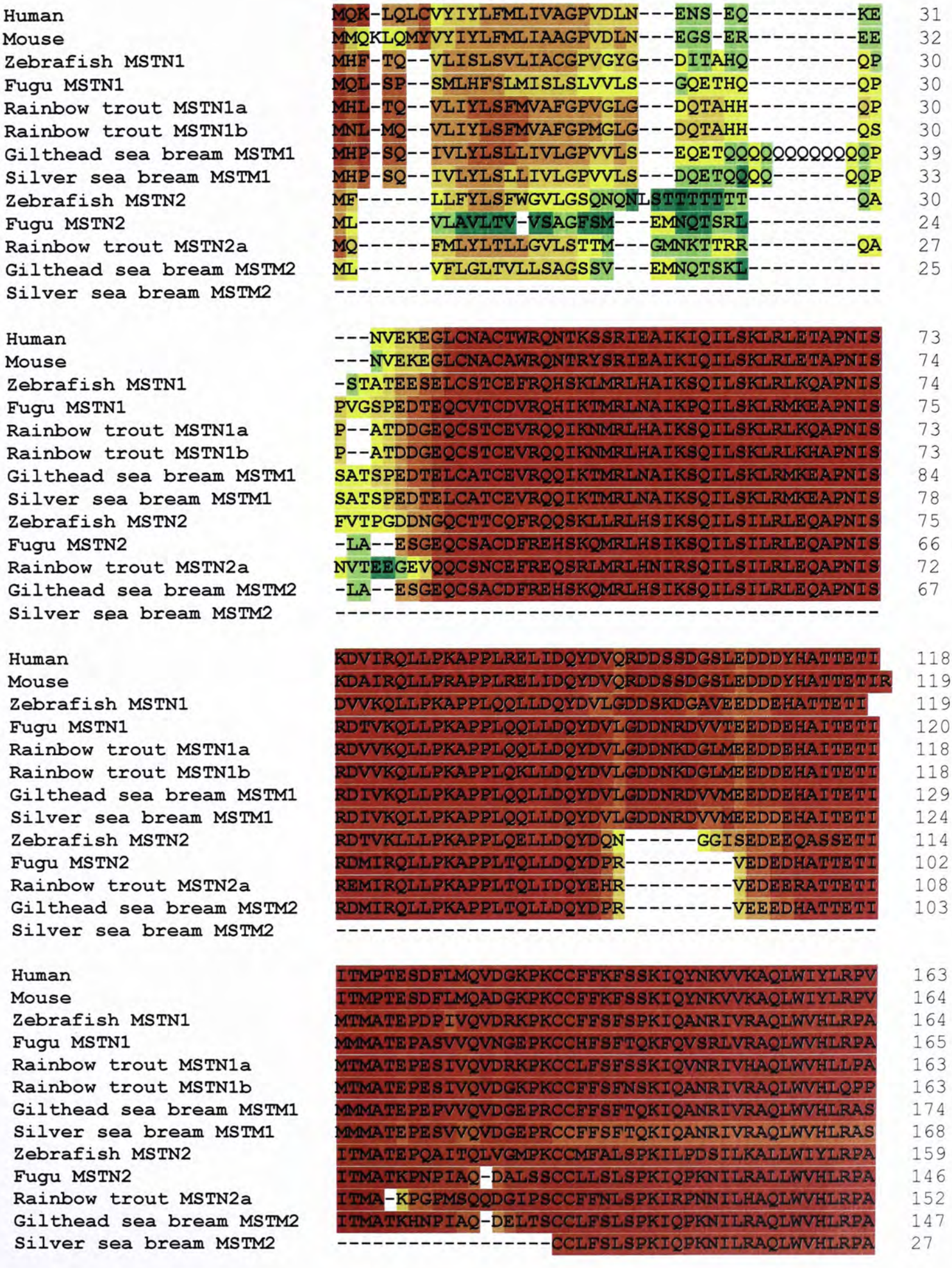
```
   1  GGGGGGGGGGGGGGGAGGCGCCTGTCAGTGTGGGACTTTAATCCAAACCCAGTCCAGCCG   60
  61  CGCGTCACGTCCAGCGCAACCCAACGGATCTTTTTCTCCTTAAAACCAAACTTTCACACC   120

   1                M  H  P  S  Q  I  V  L  Y  L  S  L  L  I  V  L  G   17
 121  TTAGAGAGAATGCATCCGTCTCAGATTGTGCTCTATCTTAGCTTGCTGATTGTTTTGGGT   180

  18   P  V  V  L  S  D  Q  E  T  Q  Q  Q  Q  Q  Q  P  S  A  T  S   37
 181  CCAGTAGTTTTGAGCGACCAAGAGACGCAGCAGCAGCAGCAGCCCTCCGCCACCAGC   240

  38   P  E  D  T  E  L  C  A  T  C  E  V  R  Q  Q  I  K  T  M  R   57
 241  CCAGAAGACACGGAGCTGTGTGCAACCTGCGAGGTCCGGCAGCAGATCAAAACCATGCGA   300

  58   L  N  A  I  K  S  Q  I  L  S  K  L  R  M  K  E  A  P  N  I   77
 301  TTAAACGCCATAAAGTCTCAGATTCTGAGCAAACTGCGAATGAAAGAGGCTCCAAATATC   360

  78   S  R  D  I  V  K  Q  L  L  P  K  A  P  P  L  Q  Q  L  L  D   97
 361  AGCCGGGACATCGTGAAGCAGCTCCTGCCCAAAGCGCCGCCGCTGCAGCAGCTTCTCGAC   420

  98   Q  Y  D  V  L  G  D  D  N  R  D  V  V  M  E  E  D  D  E  H  117
 421  CAGTACGACGTGCTGGGAGACGACAACAGGGATGTGGTTATGGAGGAGGACGATGAGCAC   480

 118   A  I  T  E  T  I  M  M  M  A  T  E  P  E  S  V  V  Q  V  D  137
 481  GCCATCACGGAGACGATTATGATGATGGCCACTGAACCTGAGTCCGTCGTCCAGGTGGAT   540

 138   G  E  P  R  C  C  F  F  S  F  T  Q  K  I  Q  A  N  R  I  V  157
 541  GGGGAGCCGAGGTGCTGCTTTTTCTCCTTCACTCAAAAGATTCAAGCCAATCGAATCGTA   600

 158   R  A  Q  L  W  V  H  L  R  A  S  D  E  A  T  T  V  F  L  Q  177
 601  CGAGCGCAGCTCTGGGTGCATCTGCGCGCGTCGGACGAGGCGACCACGGTGTTCCTGCAG   660

 178   I  S  R  L  M  P  V  T  D  G  N  G  H  I  H  I  R  S  L  K  197
 661  ATCTCCCGCCTGATGCCGGTCACGGACGGGAACGGGCACATACACATCCGCTCCCTGAAG   720

 198   I  D  V  N  A  G  V  G  S  W  Q  S  I  D  V  K  Q  V  L  S  217
 721  ATCGACGTGAACGCCGGGGTCGGCTCTTGGCAAAGTATAGACGTCAAACAAGTGTTGAGC   780

 218   V  W  L  R  Q  P  E  T  N  W  G  I  Q  I  N  A  F  D  S  R  237
 781  GTGTGGCTGCGGCAGCCGGAGACCAACTGGGGCATCCAGATTAACGCCTTCGATTCGAGG   840

 238   G  N  D  L  A  V  T  S  A  E  P  G  E  D  G  L  Q  P  F  M  257
 841  GGGAATGACTTGGCCGTGACCTCCGCGGAGCCTGGAGAGGACGGACTGCAACCGTTCATG   900

 258   E  V  K  I  S  E  G  P  K  R  V  R  R  D  S  G  L  D  C  D  277
 901  GAGGTGAAGATCTCAGAAGGGCCCAAGCGTGTCCGGAGAGACTCGGGCCTGGACTGTGAC   960

 278   E  N  S  P  E  S  R  C  C  R  Y  P  L  T  V  D  F  E  D  F  297
 961  GAGAACTCTCCAGAGTCCCGGTGCTGCCGCTACCCGCTCACGGTGGACTTCGAAGACTTT  1020

 298   G  W  D  W  I  I  A  P  K  R  Y  K  A  N  Y  C  S  G  E  C  317
1021  GGTTGGGACTGGATTATTGCCCCAAAGCGCTACAAGGCCAACTATTGCTCCGGGGAGTGT  1080

 318   E  Y  M  H  L  Q  K  Y  P  H  T  H  L  V  N  K  A  Y  P  R  337
1081  GAGTACATGCACTTGCAGAAGTACCCGCACACCCACCTGGTGAACAAAGCCTACCCCAGA  1140

 338   G  S  A  G  P  C  C  T  P  T  K  M  S  P  I  N  M  L  Y  F  357
1141  GGGTCCGCGGGCCCCTGCTGTACCCCCACCAAGATGTCGCCCATCAACATGCTCTACTTT  1200
```

```
 358    N  R  K  E  Q  I  I  Y  G  K  I  P  S  M  V  V  D  R  C  G    377
1201    AACCGAAAGGAGCAGATCATCTACGGCAAGATCCCCTCCATGGTGGTAGACCGTTGTGGA  1260

 378    C  S  *                                                        379
1261    TGCTCTTGAGTCGGGAGGGAGATCTCGGCGAGGTTCGGACGGCTCGGTCCGGGCCTCCAG  1320

1321    TTTTCAGACTCTTTGACACAACCGATCCACCAGTTCCAGTGCTTCTCCTGCAGCAACACA  1380
1381    GTGCAATAGAAACCAGAGTAGAGGCCACAATCAAGCCCGACCTTCCCGCAGGGCAGCGCT  1440
1441    GCTTTCACATCCGGCACAGCTCTCACCTTTTTCTTTCCTCCTGTGAAATCTTAGTTTGAC  1500
1501    GTCAGATGGATGCAGGAACACACACACACACACACACACACACACACACACACTTCAGTC  1560
1561    GACGCAGATTCAAGCAGCTCATTTGTTGGTTTCATGATCACTTTTCTCAATAAAAGTAAT  1620
1621    TTATCATTTCC                                                    1631
```

Figure 3.1 Full-length nucleotide sequence and deduced amino acid sequence of silver sea bream myostatin-1 cDNA, including the 1140 bp complete open reading frame (ORF) encoding for 379 amino acid with additional 412 bp 5' and 611 bp 3' untranslated regions (UTR). Asterisk indicates a stop codon. The nine cysteine residues and proteolytic processing site are indicated with gray shading.

```
  1   C  C  L  F  S  L  S  P  K  I  Q  P  K  N  I  L  R  A  Q  L   20
  1  TGTTGTCTCTTCAGTCTCAGTCCGAAGATCCAGCCCAAAAACATCCTGCGCGCTCAGCTG  60

 21   W  V  H  L  R  P  A  D  I  V  T  T  V  F  L  Q  I  S  R  L   40
 61  TGGGTCCACCTGCGGCCGGCCGACATAGTCACCACCGTATTCCTGCAGATCTCCCGCCTC  120

 41   K  P  G  K  E  G  N  N  T  R  I  R  V  R  S  L  K  I  D  T   60
121  AAACCTGGAAAGGAGGGAAACAACACCCGAATCAGAGTCCGGTCCCTGAAGATTGACACC  180

 61   D  A  G  A  G  S  W  Q  S  I  D  I  K  S  L  L  Q  A  W  L   80
181  GACGCCGGCGCCGGCTCCTGGCAAAGCATCGACATCAAGTCTCTGCTGCAGGCTTGGCTG  240

 81   R  Q  P  E  T  N  Y  G  I  E  I  N  A  Y  D  S  K  G  E  D  100
241  CGCCAACCGGAGACCAACTACGGCATTGAGATCAACGCCTACGACTCCAAAGGAGAAGAT  300

101   L  A  V  T  S  A  E  P  G  E  E  G  L  Q  P  F  I  E  V  K  120
301  CTGGCTGTCACCTCAGCAGAGCCAGGAGAGGAAGGACTGCAACCGTTCATCGAAGTGAAG  360

121   I
361  ATC
```

Figure 3.2 Partial nucleotide and deduced amino acid sequence of silver sea bream myostatin-2 cDNA.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Fugu MSTN1 MQL-SP--SMLHFSLMISLSLVVLS---GQETHQ---------QP 30
Rainbow trout MSTN1a MHL-TQ--VLIYLSFMVAFGPVGLG---DQTAHH---------QP 30
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Silver sea bream MSTM1 MHP-SQ--IVLYLSLLIVLGPVVLS---DQETQQQQ------QQP 33
Zebrafish MSTN2 MF------LLFYLSFWGVLGSQNQNLSTTTTTTT---------QA 30
Fugu MSTN2 ML------VLAVLTV-VSAGFSM---EMNQTSRL---------- 24
Rainbow trout MSTN2a MQ------FMLYLTLLGVLSTTM---GMNKTTRR---------QA 27
Gilthead sea bream MSTM2 ML------VFLGLTVLLSAGSSV---EMNQTSKL---------- 25
Silver sea bream MSTM2 ----------
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Mouse ---NVEKEGLCNACAWRQNTRYSRIEAIKIQILSKLRLETAPNIS 74
Zebrafish MSTN1 -STATEESELCSTCEFRQHSKLMRLHAIKSQILSKLRLKQAPNIS 74
Fugu MSTN1 PVGSPEDTEQCVTCDVRQHIKTMRLNAIKPQILSKLRMKEAPNIS 75
Rainbow trout MSTN1a P--ATDDGEQCSTCEVRQQIKNMRLHAIKSQILSKLRLKQAPNIS 73
Rainbow trout MSTN1b P--ATDDGEQCSTCEVRQQIKNMRLHAIKSQILSKLRLKHAPNIS 73
Gilthead sea bream MSTM1 SATSPEDTELCATCEVRQQIKTMRLNAIKSQILSKLRMKEAPNIS 84
Silver sea bream MSTM1 SATSPEDTELCATCEVRQQIKTMRLNAIKSQILSKLRMKEAPNIS 78
Zebrafish MSTN2 FVTPGDDNGQCTTCQFRQQSKLLRLHSIKSQILSILRLEQAPNIS 75
Fugu MSTN2 -LA--ESGEQCSACDFREHSKQMRLHSIKSQILSILRLEQAPNIS 66
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Silver sea bream MSTM2 ----------
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Mouse KDAIRQLLPRAPPLRELIDQYDVQRDDSSDGSLEDDDYHATTETIR 119
Zebrafish MSTN1 DVVKQLLPKAPPLQQLLDQYDVLGDDSKDGAVEEDDEHATTETI 119
Fugu MSTN1 RDTVKQLLPKAPPLQQLLDQYDVLGDDNRDVVTEEDDEHAITETI 120
Rainbow trout MSTN1a RDVVKQLLPKAPPLQQLLDQYDVLGDDNKDGLMEEDDEHAITETI 118
Rainbow trout MSTN1b RDVVKQLLPKAPPLQKLLDQYDVLGDDNKDGLMEEDDEHAITETI 118
Gilthead sea bream MSTM1 RDIVKQLLPKAPPLQQLLDQYDVLGDDNRDVVMEEDDEHAITETI 129
Silver sea bream MSTM1 RDIVKQLLPKAPPLQQLLDQYDVLGDDNRDVVMEEDDEHAITETI 124
Zebrafish MSTN2 RDTVKLLLPKAPPLQELLDQYDQN------GGISEDEEQASSETI 114
Fugu MSTN2 RDMIRQLLPKAPPLTQLLDQYDPR---------VEDEDHATTETI 102
Rainbow trout MSTN2a REMIRQLLPKAPPLTQLIDQYEHR---------VEDEERATTETI 108
Gilthead sea bream MSTM2 RDMIRQLLPKAPPLTQLLDQYDPR---------VEEEDHATTETI 103
Silver sea bream MSTM2 ----------
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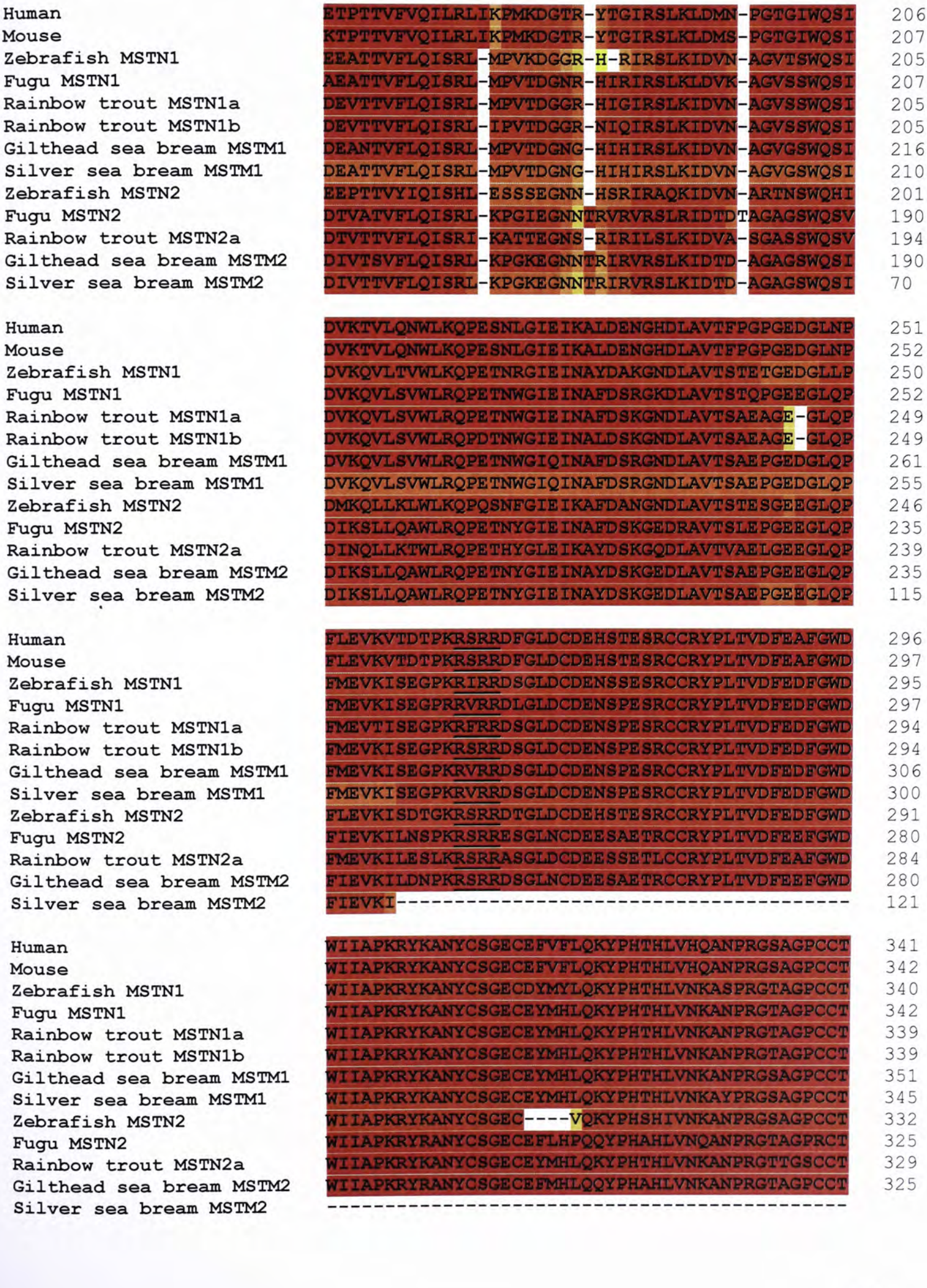
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Zebrafish MSTN1 EEATTVFLQISRL-MPVKDGGR-H-RIRSLKIDVN-AGVTSWQSI 205
Fugu MSTN1 AEATTVFLQISRL-MPVTDGNR-HIRIRSLKLDVK-AGVSSWQSI 207
Rainbow trout MSTN1a DEVTTVFLQISRL-MPVTDGGR-HIGIRSLKIDVN-AGVSSWQSI 205
Rainbow trout MSTN1b DEVTTVFLQISRL-IPVTDGGR-NIQIRSLKIDVN-AGVSSWQSI 205
Gilthead sea bream MSTM1 DEANTVFLQISRL-MPVTDGNG-HIHIRSLKIDVN-AGVGSWQSI 216
Silver sea bream MSTM1 DEATTVFLQISRL-MPVTDGNG-HIHIRSLKIDVN-AGVGSWQSI 210
Zebrafish MSTN2 EEPTTVYIQISHL-ESSSEGNN-HSRIRAQKIDVN-ARTNSWQHI 201
Fugu MSTN2 DTVATVFLQISRL-KPGIEGNNTRVRVRSLRIDTDTAGAGSWQSV 190
Rainbow trout MSTN2a DTVTTVFLQISRI-KATTEGNS-RIRILSLKIDVA-SGASSWQSV 194
Gilthead sea bream MSTM2 DIVTSVFLQISRL-KPGKEGNNTRIRVRSLKIDTD-AGAGSWQSI 190
Silver sea bream MSTM2 DIVTTVFLQISRL-KPGKEGNNTRIRVRSLKIDTD-AGAGSWQSI 70
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Mouse DVKTVLQNWLKQPESNLGIEIKALDENGHDLAVTFPGPGEDGLNP 252
Zebrafish MSTN1 DVKQVLTVWLKQPETNRGIEINAYDAKGNDLAVTSTETGEDGLLP 250
Fugu MSTN1 DVKQVLSVWLRQPETNWGIEINAFDSRGKDLAVTSTQPGEEGLQP 252
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Gilthead sea bream MSTM1 DVKQVLSVWLRQPETNWGIQINAFDSRGNDLAVTSAEPGEDGLQP 261
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Fugu MSTN2 DIKSLLQAWLRQPETNYGIEINAFDSKGEDRAVTSLEPGEEGLQP 235
Rainbow trout MSTN2a DINQLLKTWLRQPETHYGLEIKAYDSKGQDLAVTVAELGEEGLQP 239
Gilthead sea bream MSTM2 DIKSLLQAWLRQPETNYGIEINAYDSKGEDLAVTSAEPGEEGLQP 235
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Human FLEVKVTDTPKRSRRDFGLDCDEHSTESRCCRYPLTVDFEAFGWD 296
Mouse FLEVKVTDTPKRSRRDFGLDCDEHSTESRCCRYPLTVDFEAFGWD 297
Zebrafish MSTN1 FMEVKISEGPKRIRRDSGLDCDENSSESRCCRYPLTVDFEDFGWD 295
Fugu MSTN1 FMEVKISEGPRRVRRDLGLDCDENSPESRCCRYPLTVDFEDFGWD 297
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Rainbow trout MSTN1b FMEVKISEGPKRSRRDSGLDCDENSPESRCCRYPLTVDFEDFGWD 294
Gilthead sea bream MSTM1 FMEVKISEGPKRVRRDSGLDCDENSPESRCCRYPLTVDFEDFGWD 306
Silver sea bream MSTM1 FMEVKISEGPKRVRRDSGLDCDENSPESRCCRYPLTVDFEDFGWD 300
Zebrafish MSTN2 FLEVKISDTGKRSRRDTGLDCDEHSTESRCCRYPLTVDFEDFGWD 291
Fugu MSTN2 FIEVKILNSPKRSRRESGLNCDEESAETRCCRYPLTVDFEEFGWD 280
Rainbow trout MSTN2a FMEVKILESLKRSRRASGLDCDEESSETLCCRYPLTVDFEAFGWD 284
Gilthead sea bream MSTM2 FIEVKILDNPKRSRRDSGLNCDEESAETRCCRYPLTVDFEEFGWD 280
Silver sea bream MSTM2 FIEVKI---------- 121
Human WIIAPKRYKANYCSGECEFVFLQKYPHTHLVHQANPRGSAGPCCT 341
Mouse WIIAPKRYKANYCSGECEFVFLQKYPHTHLVHQANPRGSAGPCCT 342
Zebrafish MSTN1 WIIAPKRYKANYCSGECDYMYLQKYPHTHLVNKASPRGTAGPCCT 340
Fugu MSTN1 WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKANPRGTAGPCCT 342
Rainbow trout MSTN1a WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKANPRGTAGPCCT 339
Rainbow trout MSTN1b WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKANPRGTAGPCCT 339
Gilthead sea bream MSTM1 WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKANPRGSAGPCCT 351
Silver sea bream MSTM1 WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKAYPRGSAGPCCT 345
Zebrafish MSTN2 WIIAPKRYKANYCSGEC----VQKYPHSHIVNKANPRGSAGPCCT 332
Fugu MSTN2 WIIAPKRYRANYCSGECEFLHPQQYPHAHLVNQANPRGTAGPRCT 325
Rainbow trout MSTN2a WIIAPKRYKANYCSGECEYMHLQKYPHTHLVNKANPRGTTGSCCT 329
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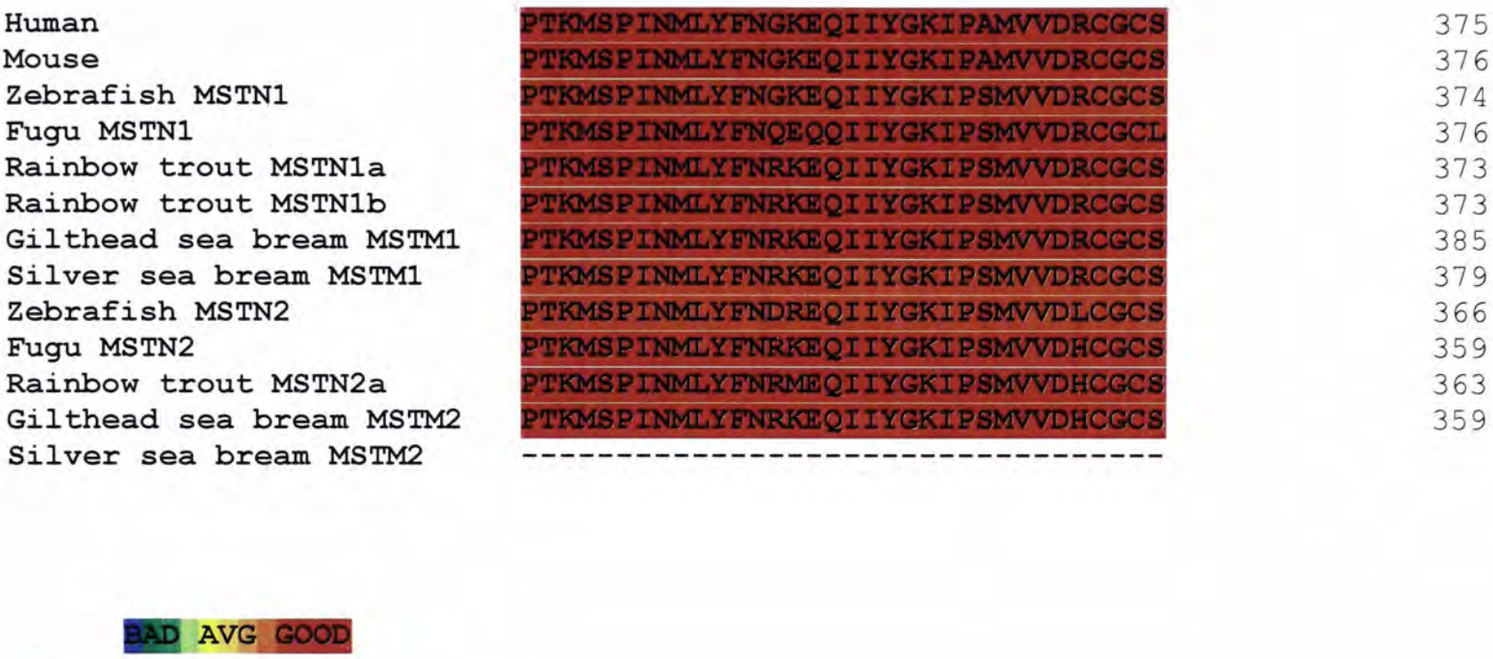
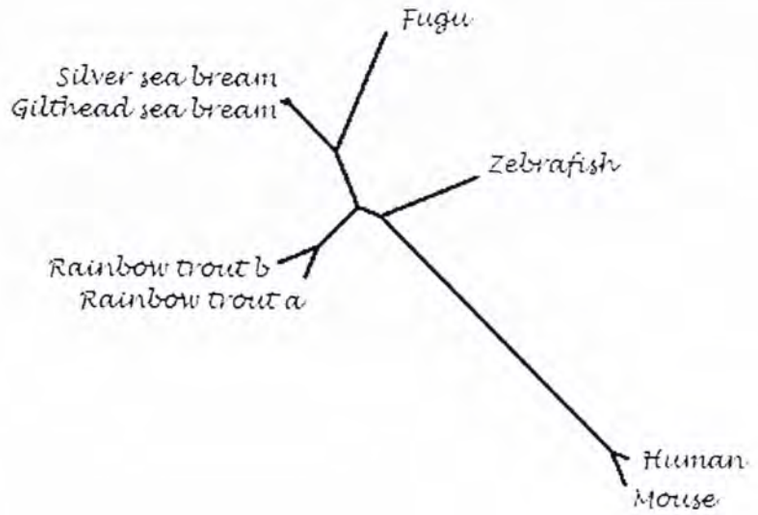
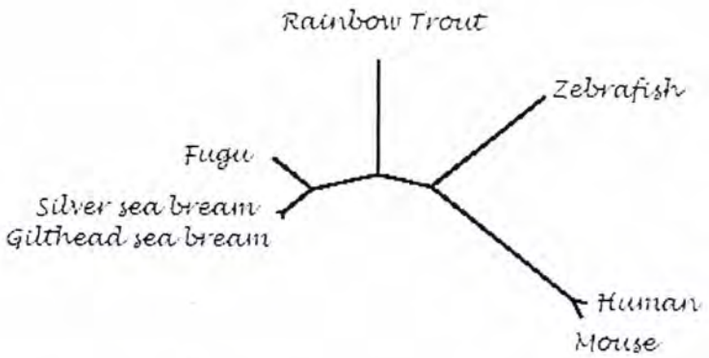


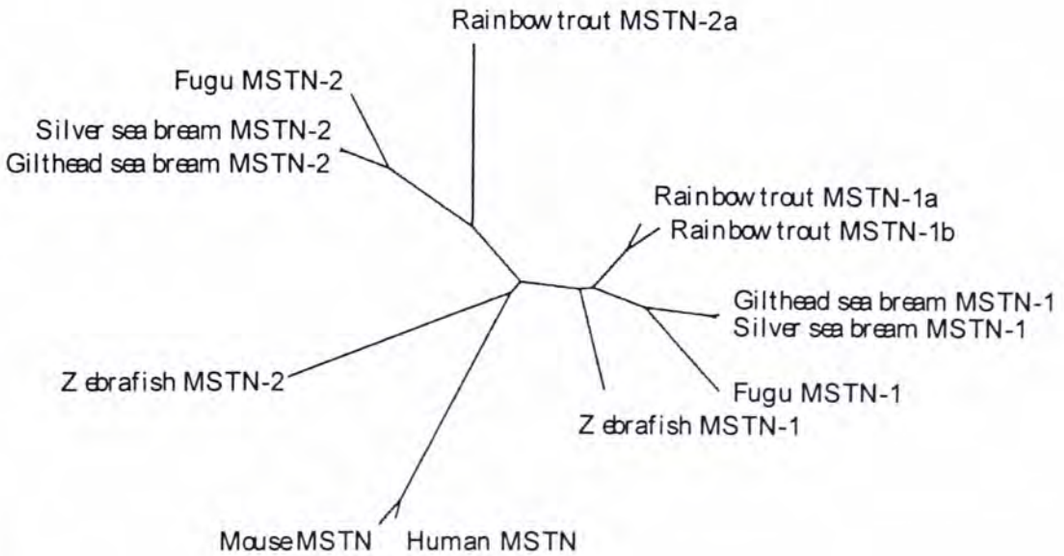
Figure 3.3 Multiple alignment of myostatin-1 and myostatin-2 amino acid sequence using T-Coffee. Deduced amino acid sequences of silver sea bream myostatin-1 and partial myostatin-2 were aligned with the amino acid sequences of myostatin of human, mouse, zebrafish, fugu, rainbow trout and gilthead sea bream using the multiple sequence alignment program T-Coffee. The legend below the alignment shows the different colors used to represent the degrees of amino acid homology among the vertebrate being considered. The proteolytic processing sites are underlined.



MSTN1



MSTN2



MSTN1 & 2

Figure 3.4 Unrooted neighbor-joining trees of myostatin--1 and myostatin--2 amino acid sequences in different vertebrates. Unrooted trees were constructed from the multiple sequence alignment CLUSTALW.

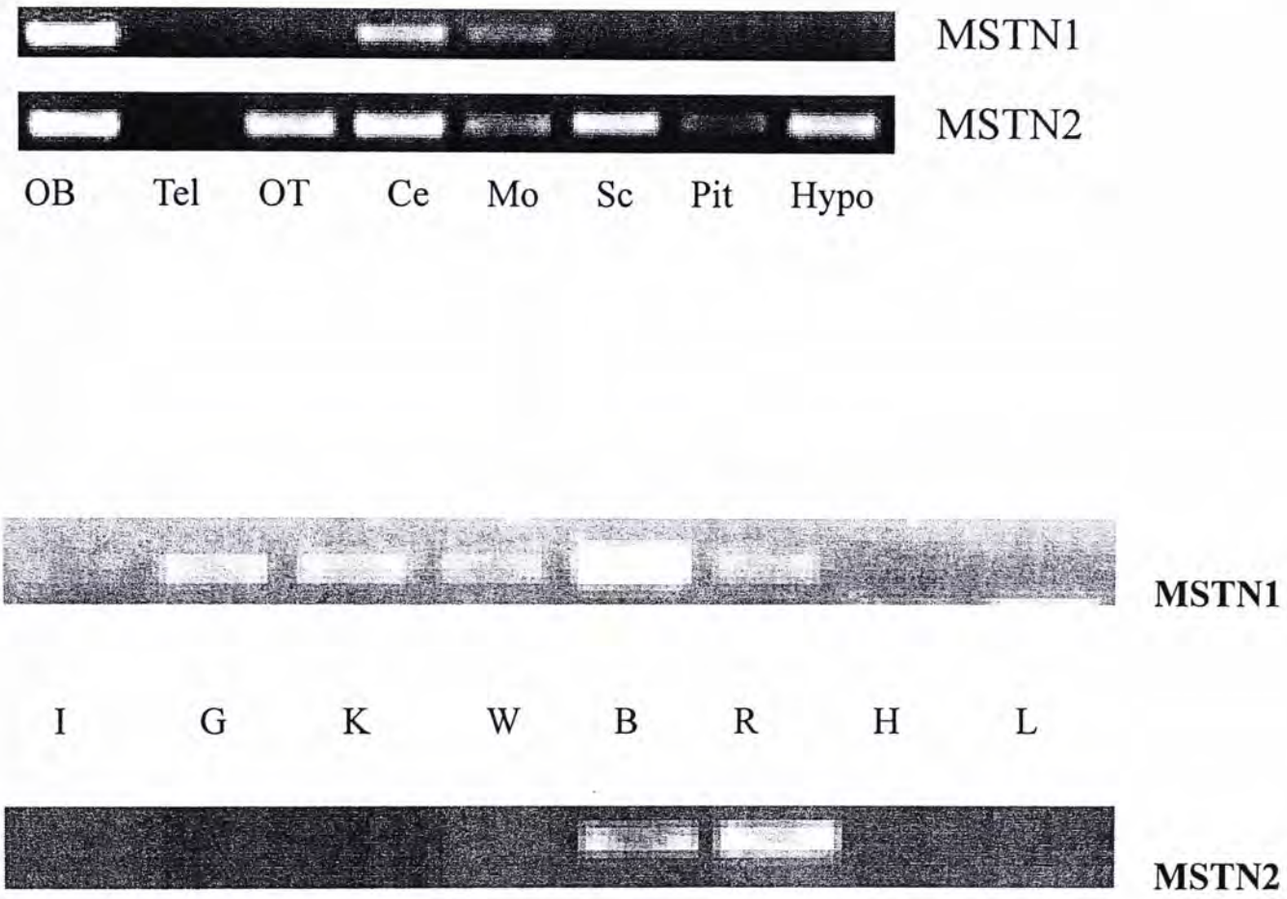


Figure 3.5 Tissue distribution of myostatin-1 (MSTN1) and myostatin-2 (MSTN2) mRNA expression. OB : olfactory bulb ; Tel : telencephalon ; OT : optic tectum ; Ce : cerebellum ; Mo : medulla oblongata ; Sc : spinal cord; Pit: pituitary; Hypo: hypothalamus. I: intestine; G: gill; K: kidney; W: white muscle; B: whole brain; R: red muscle; H: heart; L: liver.

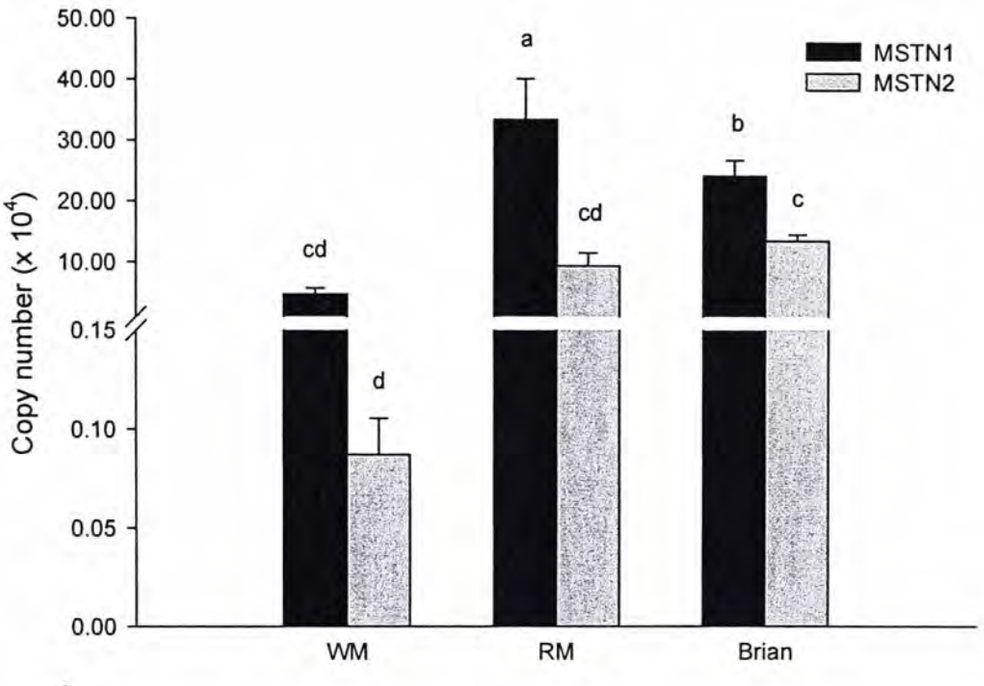
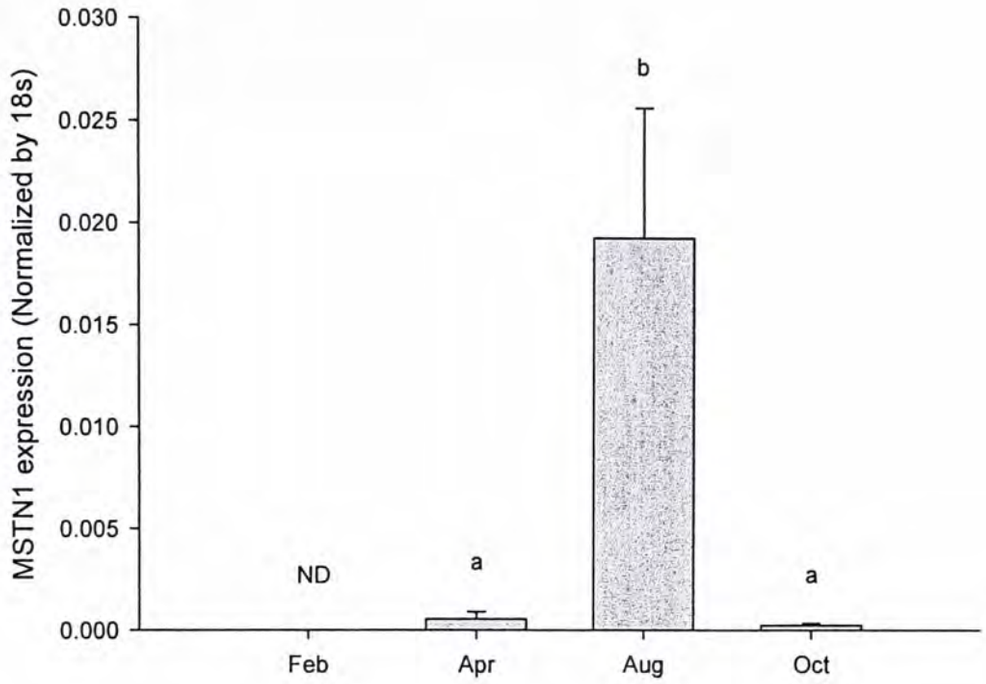
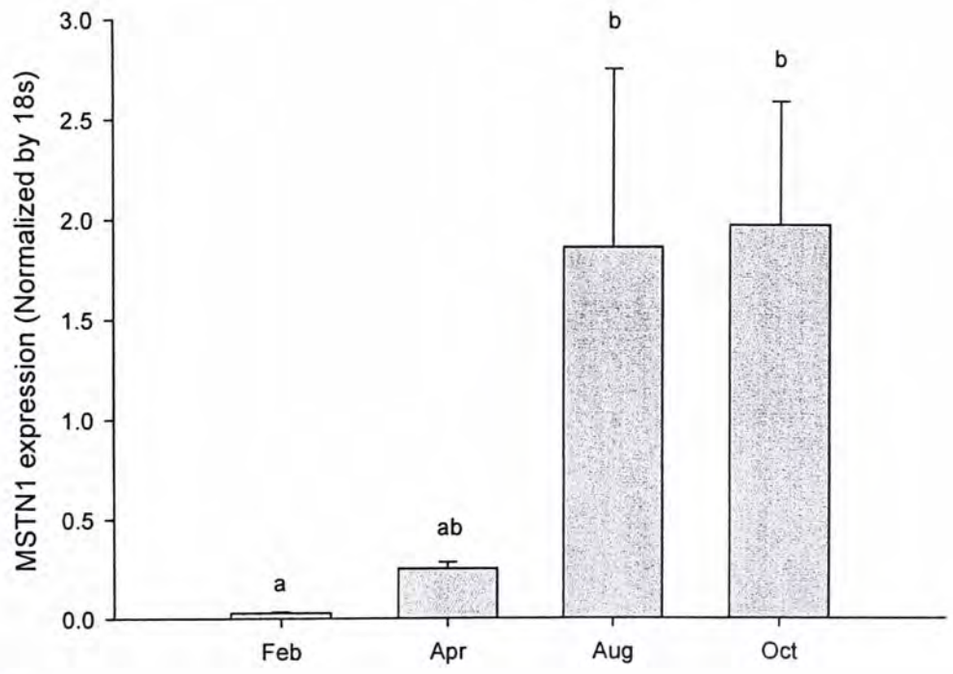


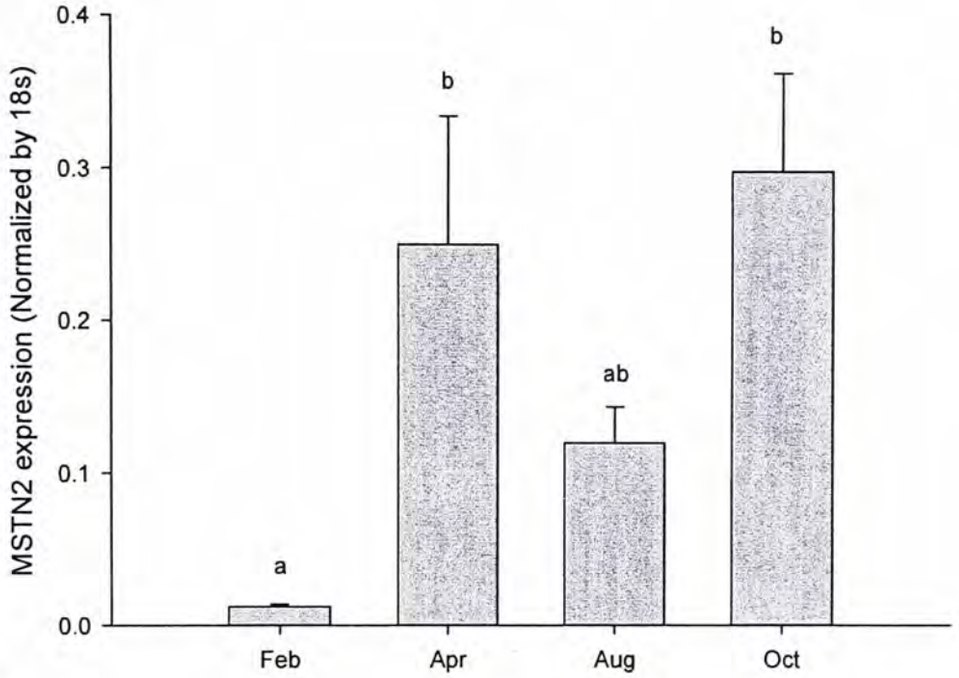
Figure 3.6 Expression of myostatin-1 (MSTN1) and myostatin- 2 (MSTN2) in white muscle, red muscle and whole brain. WM: white muscle; RM: red muscle; Brain: whole brain. Data are presented as means ± SEM (n = 6-7 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.


MSTN1 expression (Normalized by 18s)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
ND
a
b
a
Feb
Apr
Aug
Oct

(A)


MSTN1 expression (Normalized by 18s)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
Feb
Apr
Aug
Oct

(B)



(C)

Figure 3.7 Seasonal variation of myostatin-1 expression in white muscle (A), red muscle (B) and myostatin-2 expression of red muscle (C) in silver sea bream. Data are presented as means ± SEM (n = 5-6 for each group). ND: not detectable. Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.

3.5 Discussion

In the present study, silver sea bream myostatin-1 (full length) and myostatin-2 (partial) cDNA were isolated and sequenced. The amino acid sequences of myostatin-1 and myostatin-2 were deduced according to the cDNA sequence (Fig. 3.1 & Fig. 3.2). For myostatin-1, the RT-PCR and RACE approaches generate 1631 nucleotides and contained a 1140 bp open reading frame encoding 379 amino acid (Fig. 3.1). For myostatin-2, only a partial sequence of 121 amino acids was obtained because reactions involving RACE had failed.

Myostatin is a member of the TGF-beta family. Similar to all other members in that family, the silver sea bream myostatin-1 possesses a RXXR (RVRR) proteolytic processing site, nine cysteine residues which correspond to TGF-beta activity and a bioactive C-terminal domain (McPherron et al., 1997) (Fig. 3.1). Silver sea bream myostatin-1 gene contains some microsatellite repeats. A (CAG) repeat was found in the N-terminal of the coding region to encode a polyglutamine stretch (6 residues). In 3'-UTR, there was a (AC) repeat. According to the result of multiple alignment of silver sea bream myostatin sequence with those of other animals, a noticeable site is the polyglutamine stretch in the myostatin of gilthead sea bream (12 residues) and silver sea bream (6 residues). There is no such site in the myostatins of higher vertebrates. It is possible that expansions of the polyglutamine tract would cause a

gene losing its function or gaining new functions, such as the case which has been reported for several human genetic disease genes such as the Huntington gene involved in Huntington's disease, in which expansion of the polyglutamine tract causes neutrophil aggregates (Kocabas et al., 2002; Paulson et al., 2000).

Multiple alignment of sea bream myostatin with those of other teleost and mammalian species showed that silver sea bream myostatin amino acid sequence was highly conserved throughout evolution, especially in C-terminal bioactive region (Fig. 3.3.). The high level of evolutionary constraints suggests the importance of the function of this gene.

In mammalian species, myostatin is expressed mainly in skeletal muscle (McPherron et al., 1997), with expression in adipose tissue (McPherron et al., 1997), cardiac muscle (Sharma et al., 1999) and mammary gland (Ji et al., 1998) occurring at a lower level. In teleost species, there are two isoforms myostatin-1 and myostatin-2. The expression patterns of these two isoforms were different from that of mammalian species, and were also different from each other. Myostatin-1 was expressed in a variety of tissues in many fish species such as gilthead sea bream (Maccatrozzo et al., 2001b), Atlantic salmon (Ostbye et al., 2001) and tilapia (Rodgers et al., 2001). Different from myostatin-1, the expression of myostatin-2 was primarily expressed in brain (Maccatrozzo et al., 2001a). In silver sea bream, myostatin-1 mRNA was

detected in white muscle, red muscle, several regions of the brain, gill, kidney and very low expression level was detected in heart and intestine (Fig. 3.5); myostatin-2 was expressed only in brain and red muscle and a very low level in white muscle, a finding which is consistent with results found in brook trout (Roberts and Goetz, 2001) and gilthead sea bream (Radaelli et al., 2003).

Real-time quantitative RT-PCR showed that the expression level of silver sea bream myostatin-1 was higher than myostatin-2 in white muscle, red muscle and brain. myostatin-1 expression was highest in red muscle which was 3.6 fold of white muscle. myostatin-2 was mainly expressed in red muscle and whole brain which were respectively 107-fold and 155-fold higher than that in white muscle (Fig. 3.6). Our results are consistent with the findings in gilthead sea bream and sole (Radaelli et al., 2003). However, in rat and little tunny, myostatin expression is higher in white muscle (Matsakas et al., 2006; Roberts and Goetz, 2003). The high expression level of myostatin in red muscle and low expression level in white muscle suggest that myostatin may play a regulatory role in growth of different types of muscle fiber. In the sea bream, red muscle grows slowly compared to white muscle (Radaelli et al., 2003). As a potent muscle negative regulator, myostatin may inhibit red muscle growth to adjust the growth rate of this muscle type. In fact, physiologically, white muscle and red muscle are very different in fish. White muscle or fast twitch muscle

is anaerobic and glycolytic and is mainly used for rapid burst activities. In general, white muscle fibers make up the bulk of fish, about 90%. The white muscle is white in color because of the presence of little amounts of myoglobin and thus the muscle is mainly anaerobic (Altringham and Ellerby, 1999). The oxidative slow twitch red muscle forms a relative small proportion of the total muscle mass and is usually located around the lateral line. Red muscle is used for slow steady swimming and its proportion is related to the ecology of the fish: constantly swimming pelagic species have more slow muscle than benthic species, and red muscle is entirely absent in some sluggish benthic species (Sanger and Stoiber, 2001; (Altringham and Ellerby, 1999).

The different patterns of myostatin- expression in fish and mammal suggest that the function of myostatin in fish may not be restricted to regulation of muscle growth. Many other studies also showed that myostatin is expressed in different brain regions (Radaelli et al., 2003; Rodgers and Garikipati, 2008). Some studies suggest that myostatin may have a similar function to a growth differentiation factor GDF-11 which plays a role in neurogenesis as GDF-11 shares a very similar bioactive domain with myostatin. GDF-11 regulates neurogenesis negatively, and its inhibitory effect on neurogenesis through inducing cell cycle arrest in neuroprogenitors was proved in studies in olfactory epithelium (Wu et al., 2003). Both myostatin 1 and myostatin 2

were expressed in silver sea bream brain at high levels (Fig. 3.6) and therefore myostatin may play a similar function with GDF-11 in brain of fish species (Rodgers and Garikipati, 2008). Myostatin was expressed in gill and kidney in many teleost species such as gilthead sea bream, zebrafish, salmon (Ostbye et al., 2001; Radaelli et al., 2003) and silver sea bream (Fig. 3.5). Gill and kidney are important organs responsible for osmoregulation in fish, suggesting that myostatin may also participate in modulating fish osmoregulation process. In gills, myostatin was found in either chloride or mucus cell in Ostbye's study (Ostbye et al., 2001). Considering the function of myostatin in myoblasts and the role of TGF-beta family members in the cell, it is possible that myostatin may play a role in chloride cell differentiation.

In the present study, the expression level of myostatin in sea bream was shown to be different in different seasons. Real-time PCR measured that myostatin was expressed at a higher level in summer compared to that in winter in both white and red muscle in silver sea bream. For silver sea bream, winter (February) is the spawning season during which growth is generally suppressed (Tveiten et al., 1998). Suppression of growth in the spawning season is expected to coincide with elevation of myostatin expression, and indeed some studies point towards this direction. For example in both brook trout and rainbow trout, although there was no change in myostatin mRNA expression (Johansen and Overturf, 2005; Roberts and Goetz, 2003),

muscle myostatin protein level was significantly elevated during spawning in brook trout (Roberts and Goetz, 2003). However, other studies recorded a decrease of myostatin mRNA expression in the spawning season, for example, expression of myostatin-2 in both white and red muscle of rainbow trout was dramatically decreased at the time of spawning in both males and females (Rescan et al., 2001). Furthermore, some studies showed that MyoD2 expression was dramatically increased in spawning female rainbow trout and muscle hypertrophy occurred in spawning fish (Johansen and Overturf, 2005). Since myostatin can downregulate MyoD expression (Langley et al., 2002; Rios et al., 2002) and myostatin is a downstream target of MyoD (Spiller et al., 2002), a possible explanation of decreased myostatin expression is that although the growth rate decreased during spawning, myostatin and MyoD can keep muscle growth and avoid muscle atrophy.

Chapter 4

Effects of growth hormone, 11-ketotestosterone and cortisol on myostatin mRNA expression in silver sea bream (*Sparus sarba*)

4.1 Abstract

Myostatin (MSTN), which was first cloned in 1997, is an important negative regulator of skeletal muscle growth and belongs to the TGF-β superfamily. Targeted disruption of myostatin gene in mice results in a marked increase in muscle mass, contributed by a combination of hypertrophy and hyperplasia. Growth hormone-responsive element and glucocorticoid-responsive element were recently identified upstream of the myostatin gene promoter region in some teleost species such as salmon. It indicates that growth hormone and cortisol may be possible regulators for myostatin. In order to study the relationship between these hormones and myostatin, *in vivo* and *in vitro* tests were carried out. Silver sea bream were injected with growth hormone (GH), 11- ketotestosterone (11KT) or cortisol. Myostatin expression levels have been measured by using RT-Real-time PCR. Growth hormone resulted in a decreased myostatin-1 expression in white muscle and an increased myostatin expression in red muscle. 11KT decreased the myostatin-1 mRNA amount in red muscle. Cortisol injection resulted in an increased myostatin-1 expression in red muscle which is opposite to the expected. This anomalous result may be due to the stimulatory effect of cortisol on growth hormone secretion. Therefore an experiment was carried out to investigate the effect of cortisol on growth hormone secretion in silver sea bream pituitary cell culture which confirms the

stimulatory effect of cortisol.

4.2 Introduction

Myostatin (MSTN) is a member of transforming growth factor-ß (TGF-ß) superfamily discovered about 10 years ago (McPherron et al., 1997). Myostatin regulates muscle growth negatively by inhibiting the proliferation and differentiation of myoblast through a TGF- β signaling pathway (Rios et al., 2002; Thomas et al., 2000). Myostatin knockout mice display double muscling phenotype (McPherron et al., 1997). In most fish species, there are two myostatin genes (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001) with different expression patterns. Myostatin-1 was expressed in many different tissues, but myostatin-2 was mainly expressed in brain and muscle (Helterline et al., 2007; Rescan et al., 2001).

Myostatin can be regulated by many hormones. A growth hormone response element was also identified upstream from the transcription start site in the myostatin gene in trout and mammal (Roberts and Goetz, 2001; Taylor et al., 2001). In mouse C2C12 cell line, treatment of growth hormone resulted in inhibition of myostatin mRNA (Liu et al., 2003). In a study using transgenic salmon overexpressing growth hormone as a model, growth hormone was shown to exert no effect on myostatin-1

expression, but resulted in a decrease in white muscle myostatin-2 expression and an increase in red muscle myostatin-2 expression. In the same study, myostatin protein was decreased in both red and white muscle (Roberts et al., 2004). In another study on rainbow trout, growth hormone injection resulted in differential regulation of myostatin-1 and myostatin-2 as growth hormone induced myostatin-1 but reduced myostatin-2 (Biga et al., 2004).

11-ketotestosterone is a kind of fish specific testosterone which has profound anabolic effect (Gazola and Borella, 1997). Anabolic steroids such as testosterone has been shown to inhibit myostatin expression (Mendler et al., 2007).

A glucocorticoid response element was also identified on the myostatin promoter region (Funkenstein et al., 2009; Ma et al., 2001). However, results of different studies are in conflict. In rat, myostatin mRNA was increased 2.7-fold after a single injection of dexamethasone. Pretreatment with RU486 completely prevented the dexamethasone-induced increase in myostatin, confirming the effect of the glucocorticoid (Lang et al., 2001). However, some studies on fish species showed a opposite result: In tilapia larvae, myostatin mRNA was reduced by 66 and 75% under cortisol treatment at 3h and 6h respectively (Rodgers et al., 2003).

In the present study, silver sea bream were treated with growth hormone (GH), 11-ketotestosterone (11KT) or cortisol. Expression levels of myostatin-1 in white

muscle and myostatin-2 in both white and red muscle from hormone treated fish were compared to those in controls to evaluate the effect of hormones on myostatin expression in silver sea bream.

Silver sea bream used in this study is a kind of excellent food fish with high commercial value. It has extensive postlarval muscle growth and can reach large adult size (Rowlerson and Veggetti 2001). So it is an excellent model for studying muscle development of teleost fish (Maccatrozzo et al., 2001b).

4.3 Materials and methods

4.3.1 Experimental fish

Silver sea bream (*Sparus sarba*) weighing from 40 to 60 g were obtained from a local fish farm and kept in recirculating and aerated seawater [33 parts per thousand (ppt)] in the Simon FS Li Marine Science Laboratory, the Chinese University of Hong Kong. Fish were fed daily with a formulated diet (Woo and Kelly, 1995). All the hormone injection experiments were carried out in the summer when water temperatures reached 26-28 °C.

4.3.2 Growth hormone injection

Fish were randomly divided into three groups and maintained separately in

aerated seawater tanks for one month before growth hormone or saline injection. Three groups of fish received either daily intraperitoneal injection of saline (0.8% NaCl), 10ng/g BW recombinant bream growth hormone (rbGH) (Hospira, Lake Forest, IL, USA), or 1μg/g BW rbGH for four days. Injections were given between 10-11am, and the last injection was administered 24h prior to sacrifice. Fish were fed daily with a formulated diet (Woo and Kelly, 1995). After the injection protocol was complete, fish were removed from water and killed by spinal transection. White and red muscle samples collected from fish were stored in TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) in -80°C until extracted for total RNA. RT - Realtime PCR were performed to evaluate the myostatin expression by using methods described in Chapter 3.

4.3.3 11-ketotestosterone and cortisol injection

For 11-ketotestosterone injection experiment, fish were randomly divided into three groups and maintained separately in aerated seawater tanks for one month before 11-ketotestosterone or saline injection. Three groups of fish received either single intraperitoneal injection of DMSO, 50 ng/g BW 11-ketotestosterone (Cosmo Bio, Tokyo, Japan), or 2.5 μg/g BW 11-ketotestosterone.

For cortisol injection experiment, fish were randomly divided into two groups

and maintained separately in aerated seawater tanks for one month before cortisol injection. Two groups of fish weighing from 40 to 60 g were received either single intraperitoneal injection of saline (0.8% NaCl), or 50 μg/g BW cortisol (Solu-Cortef, Pharmacia & Upjohn, Belgium).

Injections were given between 10-11am. Fish were fed daily with a formulated diet (Woo and Kelly, 1995). Fish were removed from water and killed by spinal transaction on the third day. Tissue samples including white muscle and red muscle collected from fish were stored in TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) in -80°C until extracted for total RNA. RT - Realtime PCR were performed to evaluate the myostatin expression by using methods described in chapter 3.

4.3.4 Muscle tissue culture and hormone exposure

According to Negatu's method (Negatu and Meier, 1995), both white and red muscle were collected and chopped into 1mm thick pieces using McIlwain tissue chopper (Brinkmann, Westbury, NY). Each piece had a surface of nearly 4 by 5 mm for white muscle and 2 by 4 mm for red muscle. The muscle explants were washed with DMEM (Invitrogen, Carlsbad, CA) with 10^5 U/L penicillin, 10^5 U/L streptomycin and 6 g/L HEPES. Muscle explants were divided to four groups: control

DMEM, DMEM with 100 ng/ml sea bream growth hormone, DMEM with 100 ng/ml 11-ketotestosterone and DMEM with 1000 ng/ml cortisol. These doses were chosen according to previous studies of our laboratory (Leung et al., 2008) and my preliminary results where significant metabolic effects of these hormones have been observed. The four groups of tissue slices were incubated at 28℃ for 24 h before collection. Collected sample were stored in TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) in -80°C until extracted for total RNA. RT - Realtime PCR were performed to evaluate to myostatin expression by using methods described in Chapter 3.

4.3.5 Primary pituitary cell culture and cortisol exposure

To check the possible effect of cortisol on growth hormone secretion, primary pituitary cell culture was performed based on the protocol developed by Kwong et al (Kwong et al., 2009). Pituitaries of silver sea bream were removed and placed in Leibovitz's L15 medium. Pituitaries were washed and diced into 0.5 mm x 0.5mm fragments using a McILWAIN tissue chopper (Brinkmann, Westbury, NY). Pituitary fragments were washed and then incubated with 0.25% trypsin (Sigma, St Louis, MO) at 28℃ with gentle shaking for 45 minutes. After digestion, cells were incubated in L15 medium containing 10% fetal bovine serum (FBS) for 5 minutes and then in L15

medium containing DNase II (10 μg/ml) for another 5 minutes. Mechanical dispersion was performed and then the cells were filtered by cell strainer (40 μm, BD Biosciences, Bedford, MA). The dispersed pituitary cells were centrifuged at 300 x g for 10 minutes and resuspended in L15 medium and then plated in BD Falcon® 24-well plate (Becton Dickinson, Franklin Lakes, NJ) at a density of 0.4 million cells per well. Total cell count was estimated using a hemocytometer. Cell viability was above 90% as checked by using trypan blue exclusion. The primary pituitary cells were allowed to recover overnight at 28℃ in a humidified atmosphere. After overnight incubation, the pituitary cells were incubated in fresh L15 medium for 30 minutes first and then the medium was replaced by 1μM (about 360ng / ml) cortisol containing L15 medium. After 2 hours of cortisol exposure, the medium was collected for measurement of growth hormone using enzyme-linked immunosorbent assay (ELISA).

4.3.6 Measurement of growth hormone secretion by ELISA

100 μl medium samples and standards were coated in Costar® 9018 EIA/RIA 96-well plate (Corning, Corning, NY) at 4℃ for overnight. After washing with PBS, the plate was incubated in blocking buffer containing 5% (w/v) skimmed-milk in PBS-T for 2 hours at 37℃. Then the wells were washed with PBS and incubated in

1:500 diluted anti-sea bream growth hormone antibody prepared previously by Deane and Woo (2006) in PBS-T for 1 hour at room temperature. After washing with PBS, anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham) was added to each well at a dilution of 1:1000 and incubated for 1 hour at room temperature. After the 1 hour incubation, the plate was washed with PBS and added with 3, 3', 5, 5' – tetramethylbenzidine (TMB) (Sigma) in 0.05M phosphate-citrate buffer with 0.03% sodium perborate (pH 5) (Sigma) as a substrate. After 10 minutes of incubation, 2M H2SO4 was added to each well to stop color development and absorbance was read in a microplate spectrophotometer (SPECTRAmax^{TM}250, Molecular Devices, Sunnyvale, CA) at wavelength of 450nm.

4.3.7 Data processing and statistical analysis

The mRNA expression levels of myostatin-1 and myostatin-2 were normalized against 18s rRNA expression and expressed relative to the expression level of the control injection group, which was assigned a value of 100%. Data were presented as mean ± SEM. Statistical analyses were performed by statistical program SIGMASTAT 2.0 and the differences were considered to be significant at p-value < 0.05 by one-way Analysis of Variance (ANOVA) and Newman-Keuls Multiple Comparison Test for more than two groups and by T-test for two groups.

4.4 Results

4.4.1 Growth hormone injection

In silver sea bream injected intraperitoneally with growth hormone at doses of 10 ng/g BW and 1μg/g BW, myostatin-1 mRNA abundance in white muscle decreased dramatically by 83% and 88% respectively (Fig. 4.1 A). Different from white muscle, myostatin-1 expression in red muscle was increased significantly by 3.4-fold following injection of 10 ng/g BW growth hormone (Fig. 4.1 B). There was no significant change in red muscle myostatin-2 expression following growth hormone injection (Fig. 4.1C).

4.4.2 11-ketotestosterone injection

In silver sea bream injected intraperitoneally with 11-ketotestosterone at doses of 50 ng/g BW and 2.5 μg/g BW, myostatin-1 mRNA expression level was decreased significantly in red muscle at the dose 2.5μg/g BW (Fig. 4.2 B). There were no significant differences in white muscle myostatin-1 expression and in red muscle myostatin-2 expression (Fig. 4.2 A & C).

4.4.3 Cortisol injection

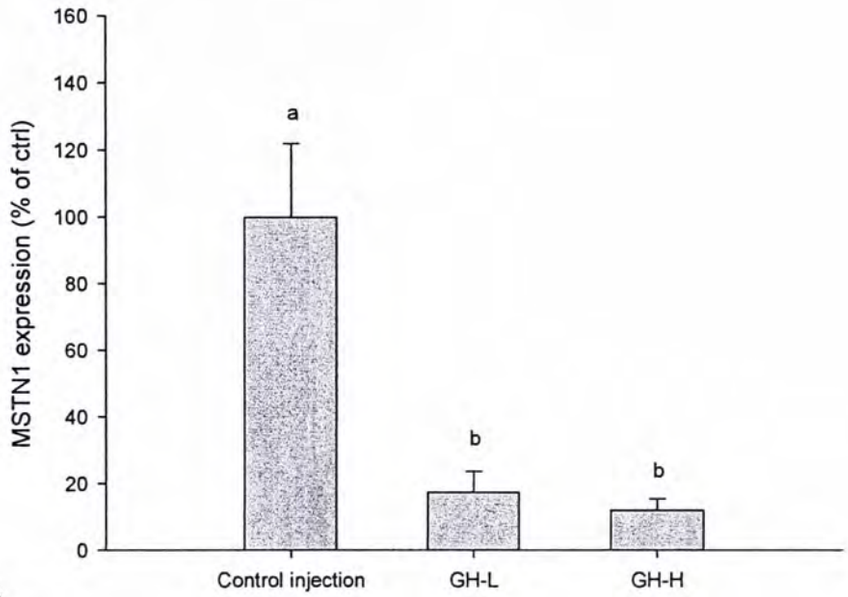
In silver sea bream injected intraperitoneally with 50 μg/g BW cortisol, myostatin-1 mRNA expression level in white muscle decreased significantly by 70% (Fig. 4.3 A) while in the red muscle, both myostatin-1 and myostatin-2 expression were unchanged following cortisol treatment (Fig. 4.3 B &C).

4.4.4 *In vitro* hormone treatment –growth hormone, 11-ketotestosterone and cortisol

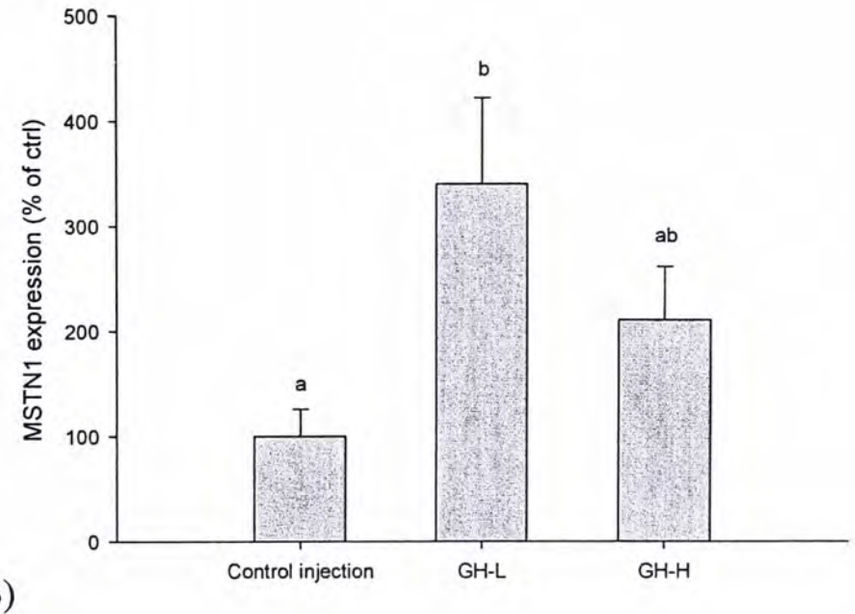
In red muscle explants culture exposed to growth hormone (100 ng/ml), myostatin-1 expression was stimulated 1.81-fold while for myostatin-2, it remained unchanged on exposure to growth hormone (Fig. 4.4 A & B). Both cortisol and 11-ketotestosterone had no significant effect on myostatin-1 and myostatin-2 mRNA expression level in red muscle explants culture (Fig. 4.4 A & B).

4.4.5 Pituitary cell growth hormone secretion under cortisol treatment

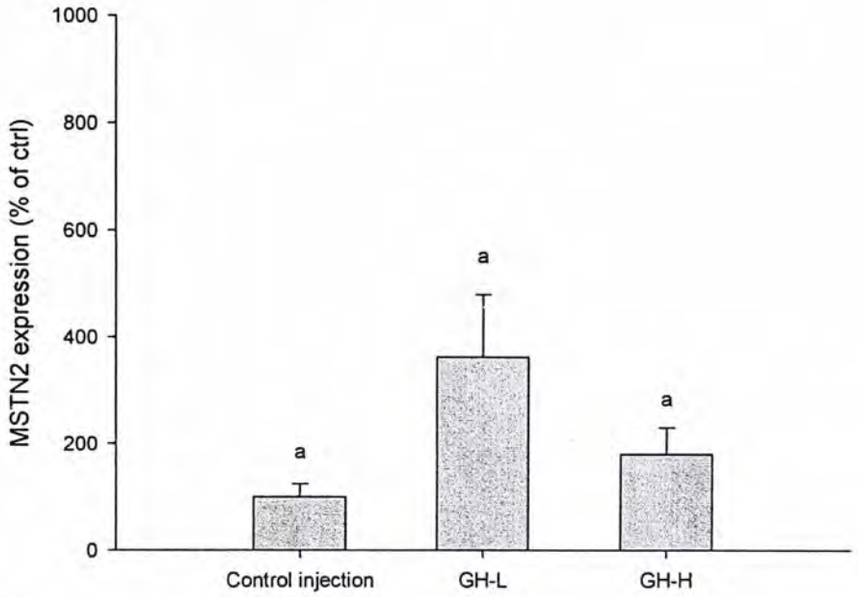
Growth hormone secretion was measured 2 h after pituitary cell incubation with 1μM cortisol. Pituitary cell growth hormone secretion was stimulated by cortisol significantly (Fig. 4.5).


MSTN1 expression (% of ctrl)
160
140
120
100
80
60
40
20
0
a
b
b
Control injection
GH-L
GH-H

(A)

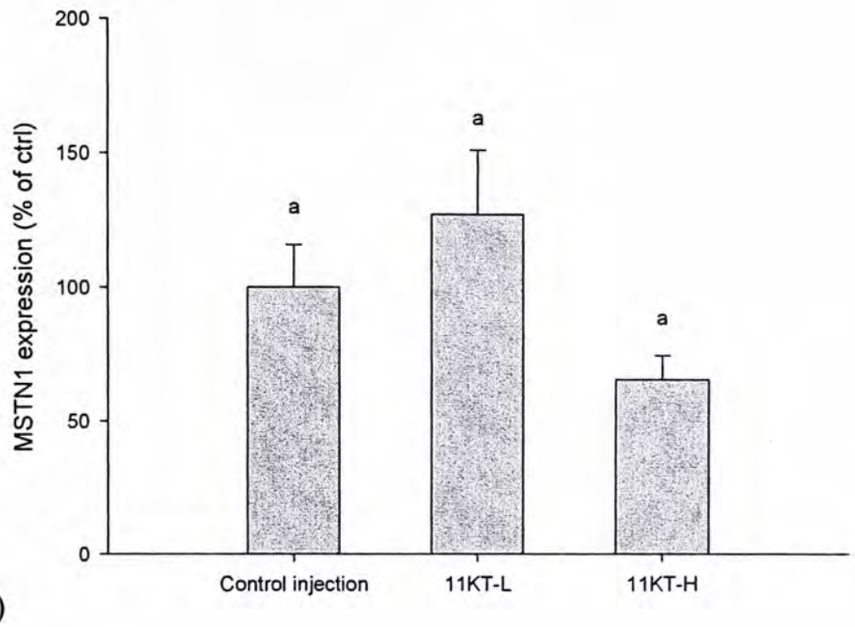

MSTN1 expression (% of ctrl)
500
400
300
200
100
0
a
b
ab
Control injection
GH-L
GH-H

(B)

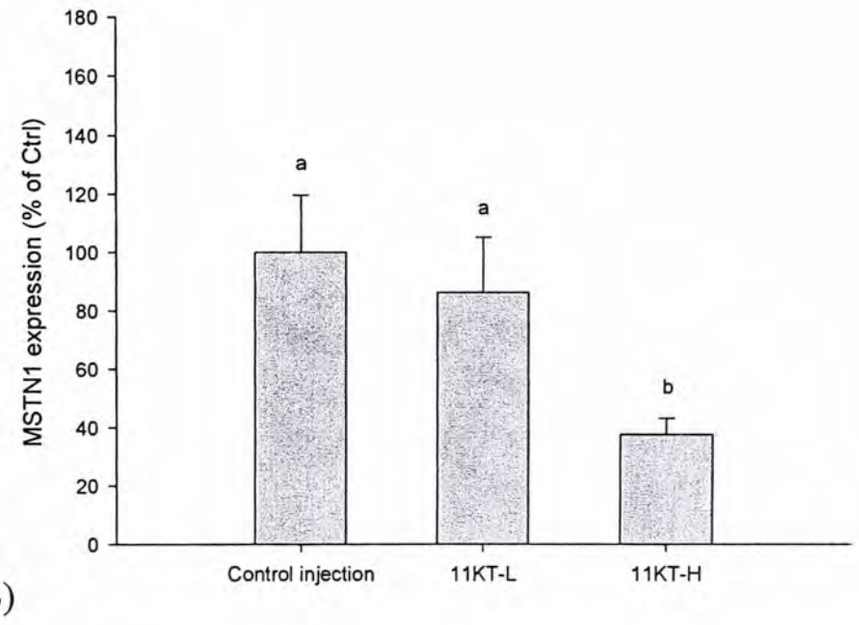


(C)

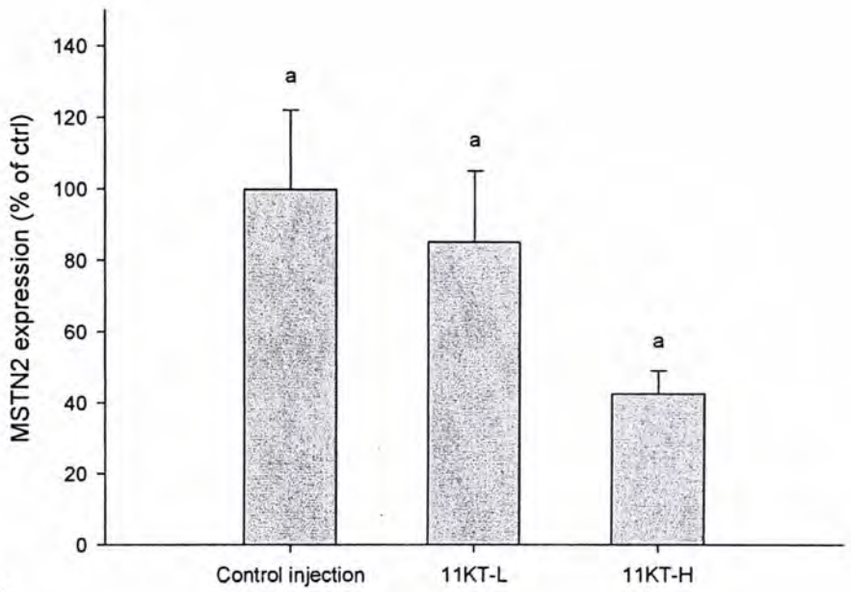
Fig. 4.1. Effect of intraperitoneal growth hormone injection on myostatin-1 (MSTN1) mRNA expression in white muscle (A), red muscle (B) and myostatin-2 (MSTN2) expression in red muscle (C). GH-L indicates (10 ng/g BW); GH-H indicates (1μg/g BW). Data are presented as means ± SEM (n = 5-7 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.


MSTN1 expression (% of ctrl)
200
150
100
50
0
a
a
a
Control injection
11KT-L
11KT-H

(A)

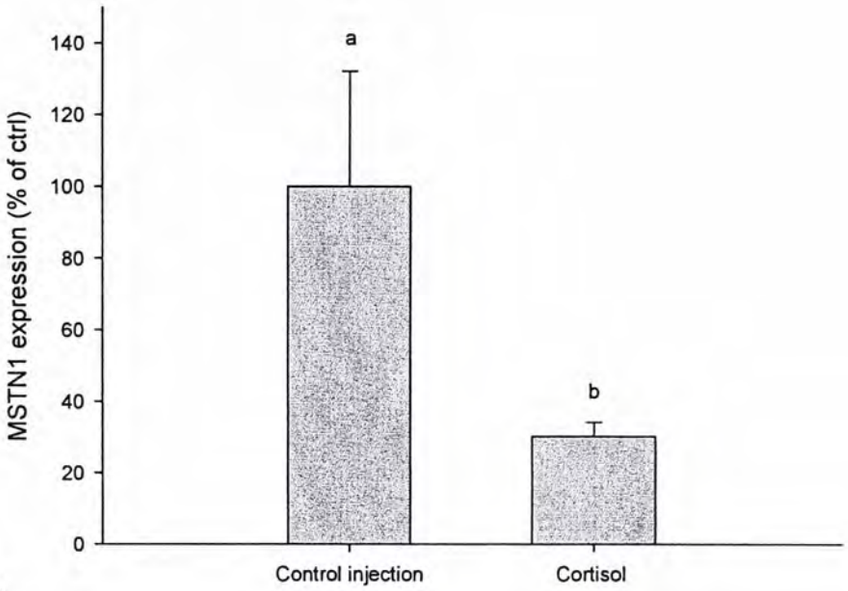

MSTN1 expression (% of Ctrl)
180
160
140
120
100
80
60
40
20
0
a
a
b
Control injection
11KT-L
11KT-H

(B)

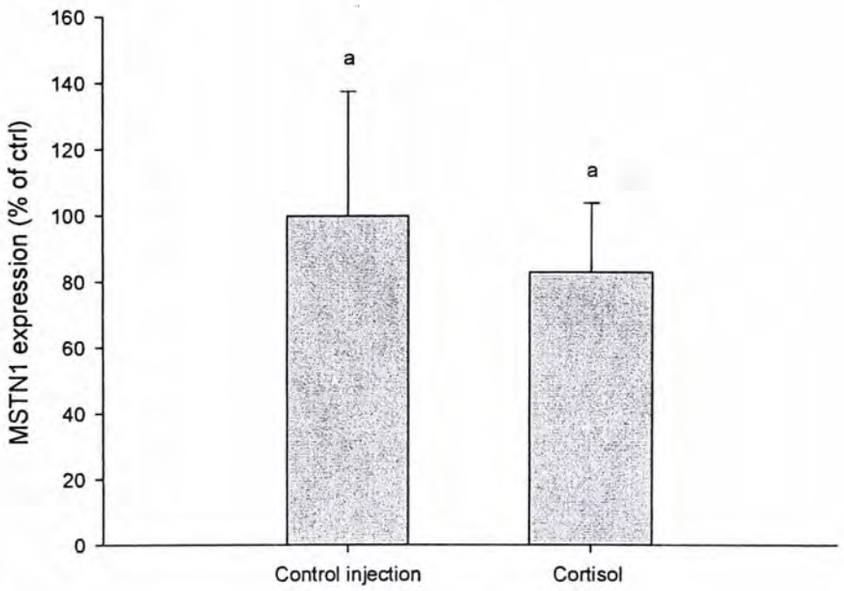


(C)

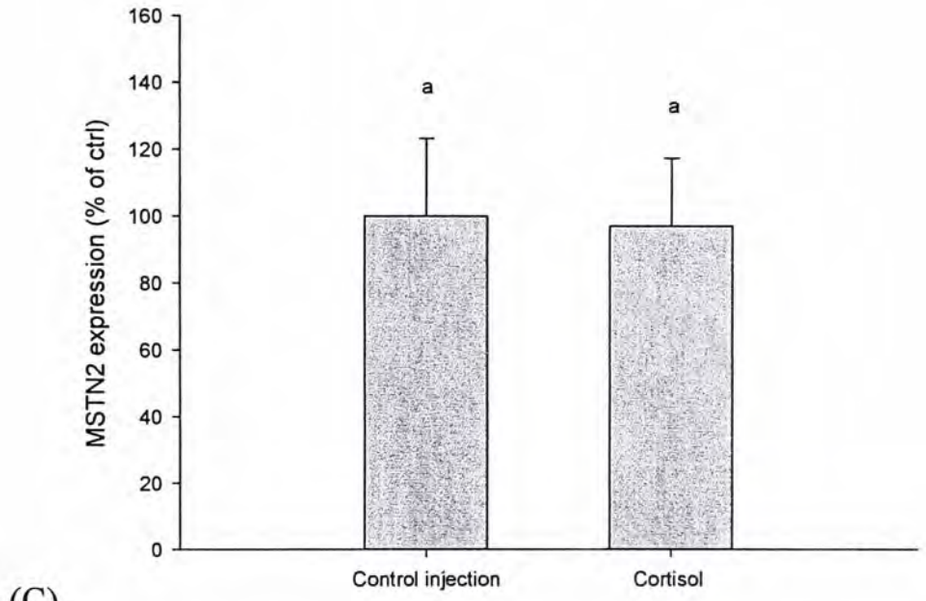
Fig. 4.2. Effect of intraperitoneal 11-ketotestosterone injection on myostatin-1 (MSTN1) mRNA expression in white muscle (A), red muscle (B) and myostatin-2 (MSTN2) expression in red muscle (C). 11KT5 indicates 50 ng/g BW. 11KT250 indicates 2.5 μg/g BW. Data are presented as means ± SEM (n = 5-7 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.


MSTN1 expression (% of ctrl)
140
120
100
80
60
40
20
0
a
b
Control injection
Cortisol

(A)

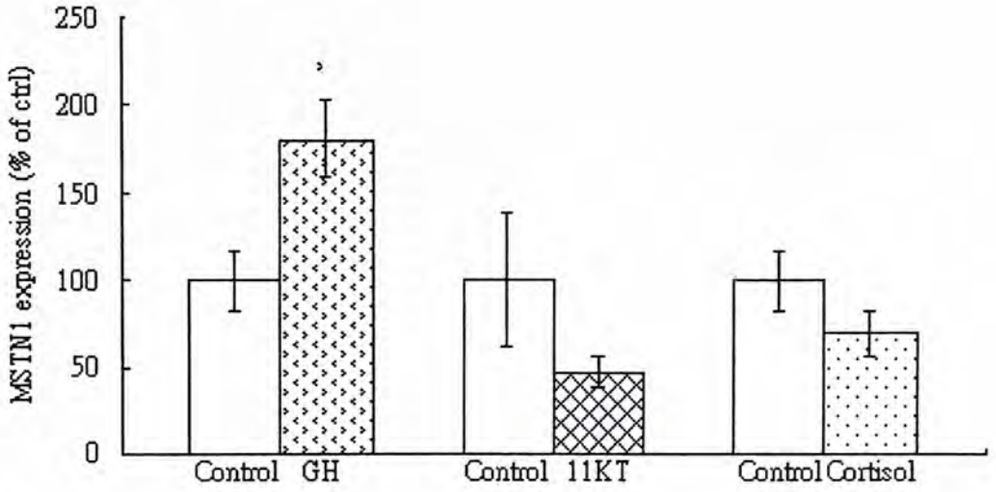

MSTN1 expression (% of ctrl)
160
140
120
100
80
60
40
20
0
a
a
Control injection
Cortisol

(B)

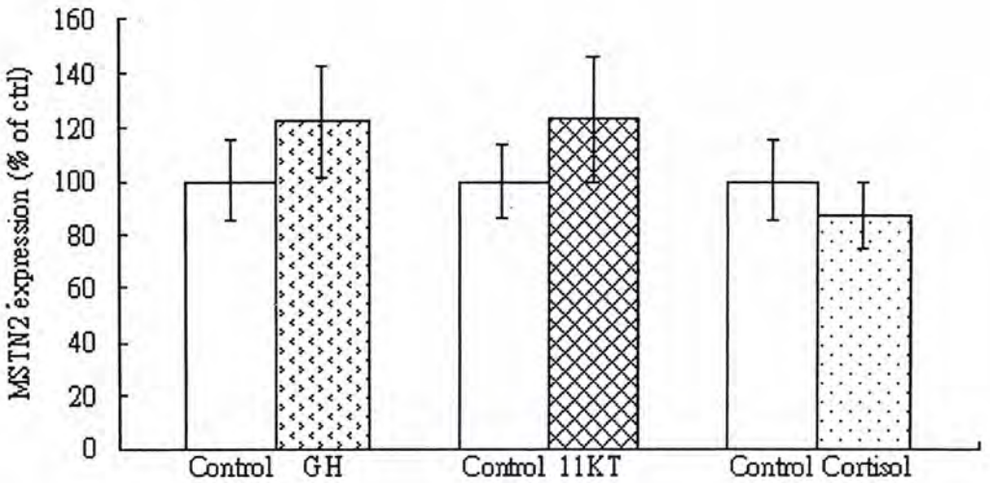


(C)

Fig. 4.3. Effect of intraperitoneal injection of cortisol (50 μg/g BW) on myostatin-1 (MSTN1) mRNA expression in white muscle (A), red muscle (B) and myostatin-2 (MSTN2) expression in red muscle (C). Data are presented as means ± SEM (n = 5-6 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.



(A)



(B)

Fig. 4.4. Effect of 24 h *in vitro* exposure to 100ng/ml growth hormone (GH), 100ng/ml 11 ketotestosterone (11KT) and 1000ng/ml cortisol on myostatin-1 (MSTN1) (A) and myostatin-2 (MSTN2) (B) mRNA expression of red muscle explant culture. Data are presented as means ± SEM (n = 6 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by asterisk.

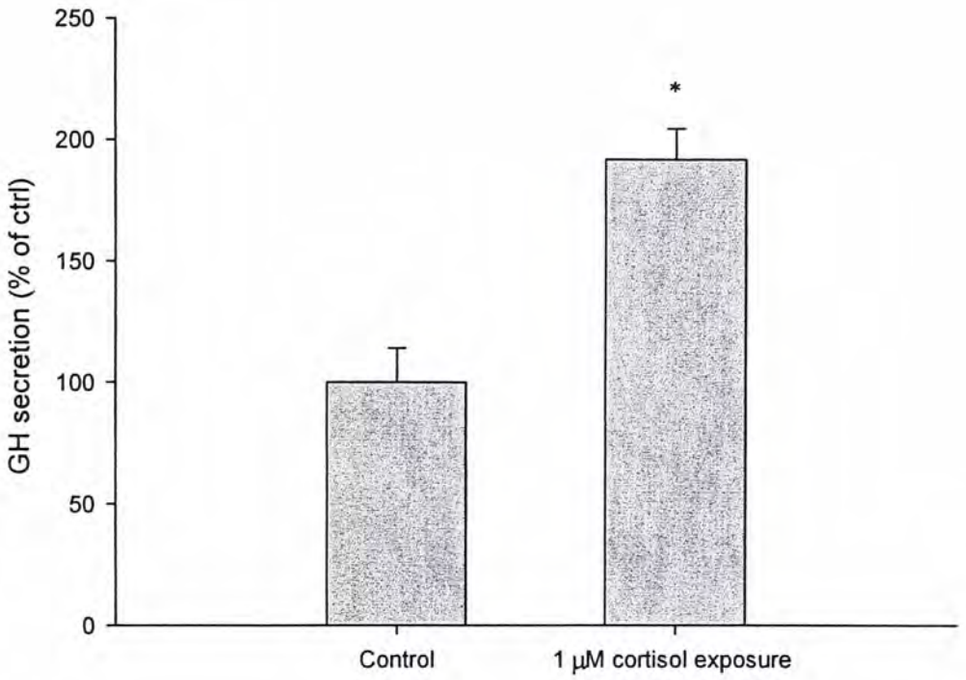


Fig. 4.5. Growth hormone secretion from sea bream pituitary cell culture upon *in vitro* cortisol exposure for 2 h. Growth hormone secretion was measured by ELISA. Data are presented as means ± SEM (n = 5 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by asterisk.

4.5 Discussion

We have investigated the effects of growth hormone, 11-ketotestosterone and cortisol on the expression of myostatin transcripts in the muscle of silver sea bream. All these three hormones play important roles in muscle growth. Growth hormone and testosterone are suggested to be anabolic. Growth hormone can stimulate muscle mass directly or indirectly through IGF-I (Aroniadou-Anderjaska et al., 1996; Gautsch et al., 1999; Ullman and Oldfors, 1989). Testosterone also has anabolic effect on skeletal muscle growth (Giannoulis et al., 2008; Mauras, 2006). Cortisol is a catabolic stress hormone and exerts negative effect on growth (Sherwood et al., 2005).

Growth hormone cell specific elements have been identified in the upstream of the transcriptional start site of both ov MSTN (MSTN1) and b/m MSTN (MSTN2) in brook trout and the element was also found in channel catfish (Roberts and Goetz, 2003). In addition, growth hormone receptor (GHR) expression showed a higher level in double muscled bovine when compared to the normal animals (Listrat et al., 2005). So these results suggest an interaction between growth hormone and myostatin. Although there were some studies which showed the lack of that relationship in human and porcine (Brill et al., 2002; Ji et al., 1998), other experiments carried out in hypopituitary growth hormone-deficient adults showed that growth hormone treatment significantly inhibited myostatin mRNA expression by 70%, and the

inhibitory effect was sustained during the period of growth hormone treatment (Liu et al., 2003). In the same study, the effect of growth hormone on myostatin was also tested *in vitro* in C2C12 cell line to evaluate the potent direct effect of growth hormone on myostatin regulation. Myostatin expression level was decreased 24 hours after growth hormone incubation with doses ranging from 50 ng/ml to 1000 ng/ml (Liu et al., 2003). In addition, a dose of 25 ng/ml growth hormone already resulted in inhibition of myostatin at the protein level (Liu et al., 2003).

There were also a few experiments studying growth hormone effect on myostatin expression in teleosts. In a study using transgenic salmon overexpressing growth hormone as model, growth hormone had no effect on myostatin-1, but resulted in a decrease in white muscle myostatin-2 expression and an increase in red muscle myostatin-2 expression. However, at protein level, mature myostatin protein was decreased in both red and white muscle (Roberts et al., 2004). Growth hormone injection in rainbow trout resulted in differential regulation of myostatin-1 and myostatin-2: growth hormone induced myostatin-1 but reduced myostatin-2 (Biga et al., 2004). Growth hormone injection regulate myostatin levels differentially between two species in another study in which myostatin was down-regulated in GH-treated giant danio and up-regulated in GH-treated zebrafish (Biga et al., 2004).

These results suggest the interaction of growth hormone and myostatin is

complex.

In the present study, in silver sea bream injected intraperitoneally with growth hormone, myostatin-1 mRNA abundance in white muscle decreased dramatically. This result is consistent with the expected in which growth hormone inhibits myostatin. However, different from white muscle, myostatin-1 expression in red muscle was increased significantly at the 10 ng/g BW dose by 3.4-fold. There was no significant change in red muscle myostatin-2 expression. The direct effect of growth hormone on myostatin in red muscle was also confirmed in red muscle explant culture in which a significant increase of myostatin-1 expression was found when expose red muscle to 100ng/ml growth hormone. Thus the responses of white muscle and red muscle to growth hormone were very different. Growth hormone receptor mRNA expression level was higher in fast white muscle than that in slow red muscle (Casse et al., 2003). In addition, our results showed that myostatin was differentially expressed in white muscle and red muscle of sea bream. There were some studies which showed that growth hormone could alter the ratio of white muscle to red muscle within the fish musculature, for example, growth hormone has been shown to induce white muscle proportion and inhibit red muscle proportion (Hennessey et al., 2001; Lange et al., 2002). However, there were also some other studies which indicated that growth hormone had no effect on muscle fiber composition (Cuneo et

al., 1992; Woodhouse et al., 1999). A possible explanation for the increased myostatin-1 mRNA expression in red muscle and the decreased myostatin-1 mRNA in white muscle is that growth hormone can regulate the ratio of white muscle to red muscle through the inhibitory action of myostatin on muscle growth.

11 ketotestosterone (11KT) is a kind of fish specific testosterone and testosterone is known to exert profound anabolic effects on animals. 11KT is therefore expected to inhibit myostatin mRNA expression in fish muscle as testosterone has been shown to inhibit myostatin expression in mammalian muscle (Mendler et al., 2007). Our results are also consistent with the expectation that 11KT injection caused a decreased myostatin-1 mRNA level in red muscle. Although there is no significant difference for myostatin-1 in white muscle, myostatin-2 in red muscle and myostatin-1 in red muscle explant culture, a trend towards downregulation of myostatin by 11KT treatment is evident.

A glucocorticoid response element was identified on the myostatin promoter region (Ma et al., 2001), indicating the presence of an interaction between glucocorticoid and myostatin. However, results of different studies are in conflict. In rat, myostatin mRNA was increased after injection of dexamethasone (Dex) in several studies (Lang et al., 2001; Ma et al., 2003). In addition, myostatin gene deletion can prevent glucocorticoid-induced muscle atrophy (Gilson et al., 2007). However, some

studies on fish species showed an opposite result: in cortisol-immersed tilapia larvae, myostatin mRNA was reduced at 3h and 6h respectively (Rodgers et al., 2003). Mammalian and tilapia myostatin mRNA appear to be differentially regulated by these stress hormones (Rodgers et al., 2003). Results similar to those in tilapia were found in channel catfish in which a decrease in myostatin expression was recorded at 12 hours post dexamethasone injection (Weber et al., 2005). Our results also showed a significant decrease in white muscle myostatin-1 following cortisol treatment, consistent with the studies carried out on tilapia and channel catfish. Furthermore, fasting and stress which can lead to an increase in cortisol also resulted in a decreased myostatin expression in fish (Rodgers et al., 2003; Vianello et al., 2003). At present, it is unknown whether glucocorticoid regulates the in vivo expression of myostatin in a direct or indirect fashion. Although dexamethasone injection led to decreased myostatin expression in channel catfish, it had no effect on the abundance of myostatin mRNA in channel catfish primary muscle cell culture (Weber et al., 2005). It is possible that dexamethasone or cortisol injection indirectly regulates the expression of myostatin through the alteration of other factors such as growth hormone. Cortisol is suggested to stimulate growth hormone secretion in tilapia (Uchida et al., 2004). The result of the present study performed on silver sea bream pituitary cell culture also showed that cortisol enhanced growth hormone secretion.

Therefore, a possible mechanism for cortisol regulation on myostatin is that cortisol regulates myostatin expression through the action of growth hormone.

This study has involved treating silver sea bream with growth hormone, 11-keto testosterone and cortisol. Growth hormone was shown to inhibit myostatin-1 in white muscle but induce it in red muscle. 11KT decreased the expression level of myostatin-1 in red muscle. Cortisol treatment resulted in a decreased mRNA amount of myostatin-1 in white muscle. These results demonstrated that myostatin-1 has complex interactions with the hormones involved with growth regulation and further attest that hormones may regulate skeletal muscle growth through the action of myostatin. However, hormones have no effect on myostatin-2 expression, and that myostatin-2 may perform some unknown functions in silver sea bream.

Chapter 5

A possible new function of myostatin-1: Effect of extracellular osmolarity on myostatin in osmoregulatory organs in silver sea bream (*Sparus sarba*)

5.1 Abstract

Myostatin is a member of the transforming growth factor (TGF) -beta family. Members from this family play critical multiple roles in cell proliferation and differentiation. In gilthead sea bream, zebrafish, salmon and our model fish silver sea bream as well, myostatin-1 was expressed in osmoregulation related organs gill and kidney (Ostbye et al., 2001; Radaelli et al., 2003). In gills, myostatin-1 was found in either chloride or mucus cell in Ostbye's study (Ostbye et al., 2001). Considering the function of myostatin in myoblasts proliferation and differentiation and the role of TGF-beta family member in cellular differentiation, myostatin may play a role in chloride cell differentiation. In kidney, exposure to different salinities led to rapid changes in renal morphometrics in silver sea bream (Wong and Woo, 2006) and myostatin-1 may be involved in these changes. In the present study, two sets of experiments were carried out: the first experiment involved the long term adaptation of fish to 33ppt, 12ppt and 6ppt respectively and the second experiment involved abrupt transfer of fish from 33ppt to 6ppt. Expression levels of myostatin-1 in white muscle, red muscle, gill and kidney were recorded and compared among fish groups adapted to different salinity conditions. The expression of myostatin-1 in all these tissues was unchanged over the salinity range tested in long term adaptation experiment and abrupt transfer experiment for 72h. However, there were significant

increases of myostatin-1 expression in red muscle and gill and a decrease of myostatin-1 in kidney in silver sea bream 24h after abrupt transfer from 33ppt to 6ppt. These results showed that myostatin-1 may be involved in short term adaptation for salinity change.

5.2 Introduction

In fish species, there are two myostatin genes. The two myostatin genes were identified in many other teleosts such as gilthead sea bream, Atlantic salmon and white bass (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001).The expression pattern of myostatin in fish species was very different from that in mammals. Myostatin-2 was mainly expressed in muscle and brain. However, myostatin-1 was expressed in many different tissues, suggesting that in fish, the function of myostatin-1 may not be confined to the skeletal muscle alone (Maccatrozzo et al., 2001a; Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001).

Myotatin-1 was expressed in gill and kidney in many teleost species such as sea bream, zebrafish and salmon (Ostbye et al., 2001; Radaelli et al., 2003). Gill and kidney are important organs responsible for osmoregulation in fish, suggesting that myostatin may play roles in the osmoregulatory processes.

In gills, myostatin-1 was found in either chloride or mucus cell at primary lamellae in Ostbye's study (Ostbye et al., 2001). Chloride cells were especially important in the process osmoregulation (Hwang and Lee, 2007). They are major site of ion secretion and absorption (Kaneko et al., 2002). The morphology of chloride cells in seawater adapted fish and fresh water adapted fish are different. Many studies showed that chloride cells can alternate their morphology and ion-transporting functions when exposed to abrupt environmental osmotic changes (Kaneko et al., 2002; Katoh and Kaneko, 2003). Considering the inhibitory function of myostatin in myoblasts proliferation and differentiation and the role of TGF-beta family members in cellular differentiation, it is possible that myostatin may play a role in chloride cell differentiation in fish gills. In mouse, GDF-11 which shares a very similar bioactive domain with myostatin, was found to be expressed in the mandibular and hyoid arches. These two bones represent the first and second visceral arches which are mesenchymal structures in the region of the embryonic pharynx. Gill arches of fish constitute the 3rd±7th visceral arches (Ostbye et al., 2001). In view of the homology of the visceral arches between fish and mammals, and the similar localization of GDF-11 and myostatin in homologous organs, it is reasonable to postulate that myostatin-1 may have similar function with GDF-11 in the gill by affecting branchial cell differentiation.

Regarding the kidney, previous work of our laboratory has shown that there are rapid changes in renal morphometrics in silver sea bream on exposure to different salinities (Wong and Woo, 2006). Abrupt transfer of fish from 33ppt to 6ppt led to a dramatic rapid proliferation of collecting tubules after 24 hours. Myostatin which function in myoblast proliferation and differentiation may be involved in that process.

In the present study, fish were exposed to different salinity conditions to investigate whether myostatin-1 plays a role in osmoregulation in silver sea bream. Two sets of experiments were carried out: the first experiment involved the long term adaptation of fish to 33ppt, 12ppt and 6ppt respectively and the second experiment involved abrupt transfer of fish from 33ppt to 6ppt. Expression levels of myostatin-1 in white muscle, red muscle, gill and kidney were recorded and compared among fish groups adapted to different salinity conditions. Since both gill and kidney are blood rich organs, myostatin expression in whole blood has been tested first to eliminate the possible contamination by blood cell.

Silver sea bream used in this study is an excellent model for studying osmoregulation. Silver sea bream is a kind of euryhaline fish which can tolerate a wide range of salinities (0-70ppt) (Wong et al., 2006).

5.3 Materials and methods

5.3.1. Experimental fish

Silver sea bream (*Sparus sarba*) were obtained from a local fish farm and kept in recirculating and aerated seawater [33 parts per thousand (ppt)] in the Simon FS Li Marine Science Laboratory, the Chinese University of Hong Kong.

5.3.2 Long term salinity adaptation

Fish were randomly divided into three groups and maintained separately in aerated seawater tanks for one and half month before adaptation to three different salinities (6ppt, 12ppt and 33ppt). The low salinities (6ppt and 12ppt groups) were achieved by diluting seawater with dechlorinated tap water daily for two weeks until the final salinities were reached. Fish were fed daily with a formulated diet (Woo and Kelly, 1995) and cultured for one month before experiments.

5.3.3 Abrupt transfer from seawater to brackish water (6 ppt)

Hyposmotic brackish water (6ppt) was obtained by diluting seawater (33 ppt) with fully aerated tap water. Fish were then abruptly transferred from seawater holding tanks (33ppt) to experimental tanks containing brackish water at 6 ppt. Fish were sampled 24 and 72 hours after abrupt transfer. Control transfer groups were transferred from the original 33ppt seawater tank to another 33ppt seawater tank and

sampled at the same time intervals as the fish transferred to brackish water.

5.3.4 Data processing and statistical analysis

The mRNA expression of myostatin-1 were normalized against 18s rRNA expression and expressed relative to control group which was assigned a value of 100%. For the specific control groups, see figure legends for details. Data were presented as mean ± SEM. Statistical analyses were performed using statistical program SIGMASTAT 2.0 and the differences were considered to be significant at p-value < 0.05 by one-way Analysis of Variance (ANOVA) and Newman-Keuls Multiple Comparison Test.

5.4 Results

5.4.1 Long term adaptation to different salinities

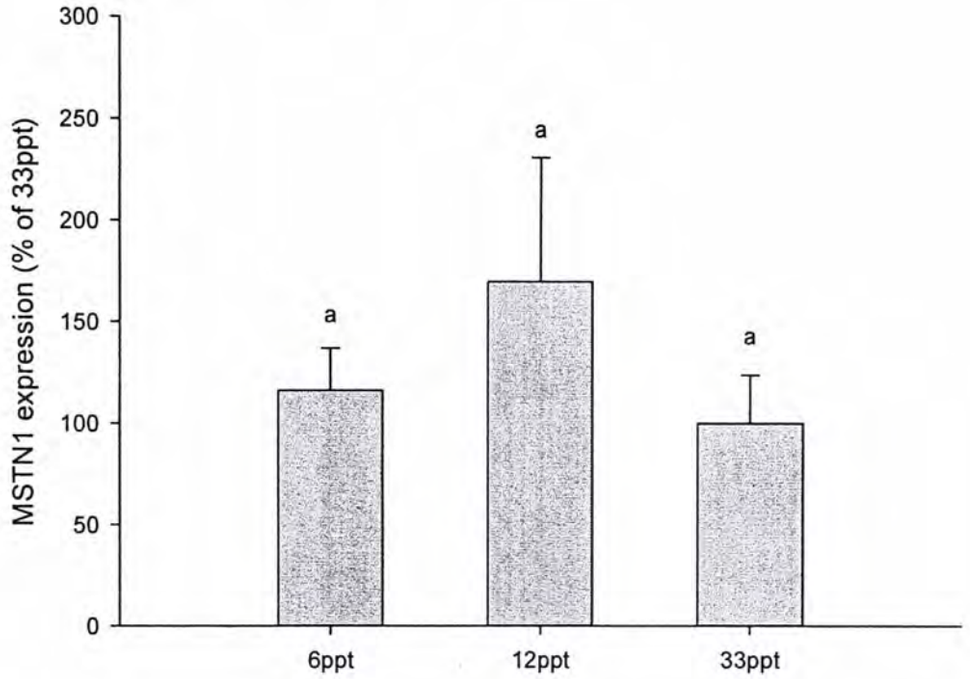
By using real-time PCR, the expression of myostatin-1 was studied in white muscle, red muscle, gill and kidney of silver sea bream adapted to different salinities for one month. The expression of myostatin-1 in all these tissues was unchanged over the salinity range tested. (Fig. 5.1)

5.4.2. Abrupt transfer from 33ppt to 6ppt – 24 h

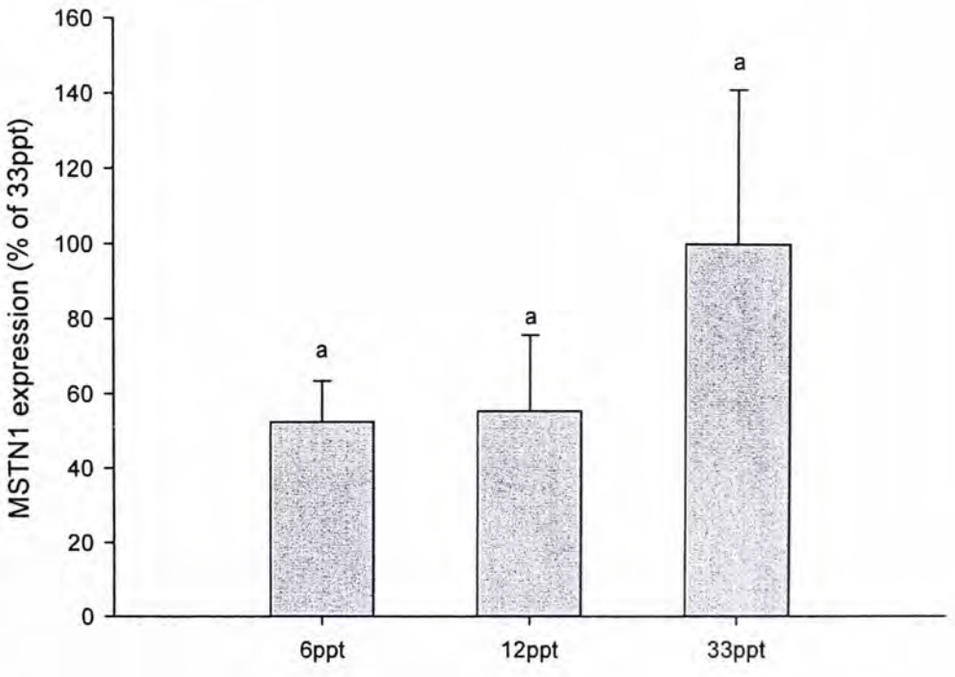
Silver sea bream were transferred from 33ppt to 6ppt, myostatin-1 expression of white muscle, red muscle, gill and kidney was measured using real-time PCR after 24 hours. Expression levels of a fish group that remained at 33ppt (no transfer) were also measured so as to evaluate effect of the transfer process *per se* as compared to the control transfer (33ppt-33ppt). There is no significant change in myostatin-1 expression in all tissues in fish subjected to control transfer except that the kidney exhibited a decrease after control transfer (Fig. 5.2D). Myostatin-1 expression in both red muscle and gill increased significantly 24h after transfer by 1.97 and 2.65-fold respectively (Fig. 5.2B & Fig. 5.2C) while in the kidney, abrupt transfer showed significant decrease in myostatin-1 mRNA level by 80%. Expression of myostatin-1 in white muscle and myostatin-2 in red muscle remained unchanged after abrupt transfer.

5.4.3 Abrupt transfer from 33ppt to 6ppt – 72 h

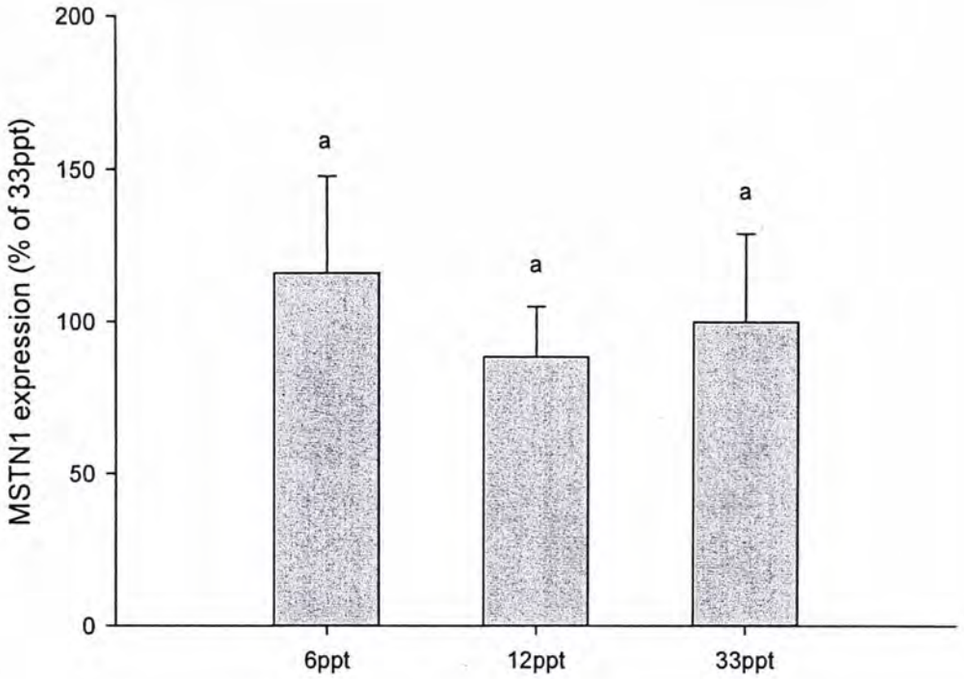
Silver sea bream were transferred from 33ppt to 6ppt, expression levels of myostatin-1 of white muscle, red muscle, gill and kidney were measured using real-time PCR after 72 hours. A group remained in 33ppt was set up to evaluate the effect of the transfer process *per se*. There was no significant change in myostatin-1 expression in all tissues in fish subjected to control (33ppt – 33ppt) transfer.


MSTN1 expression (% of 33ppt)
300
250
200
150
100
50
0
a
a
a
6ppt
12ppt
33ppt

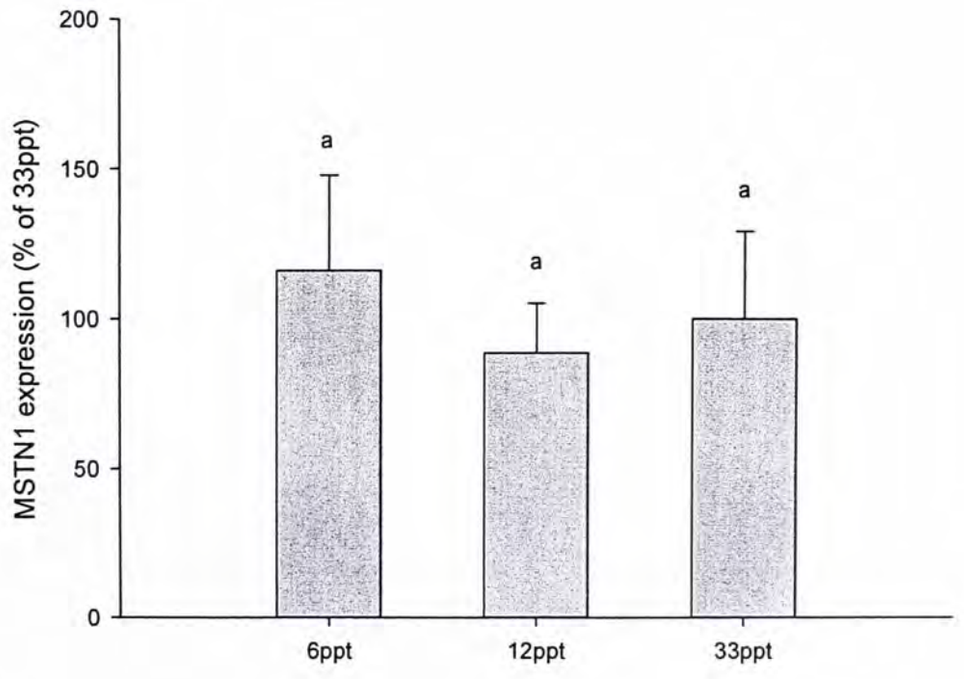
(A)


MSTN1 expression (% of 33ppt)
160
140
120
100
80
60
40
20
0
a
a
a
6ppt
12ppt
33ppt

(B)

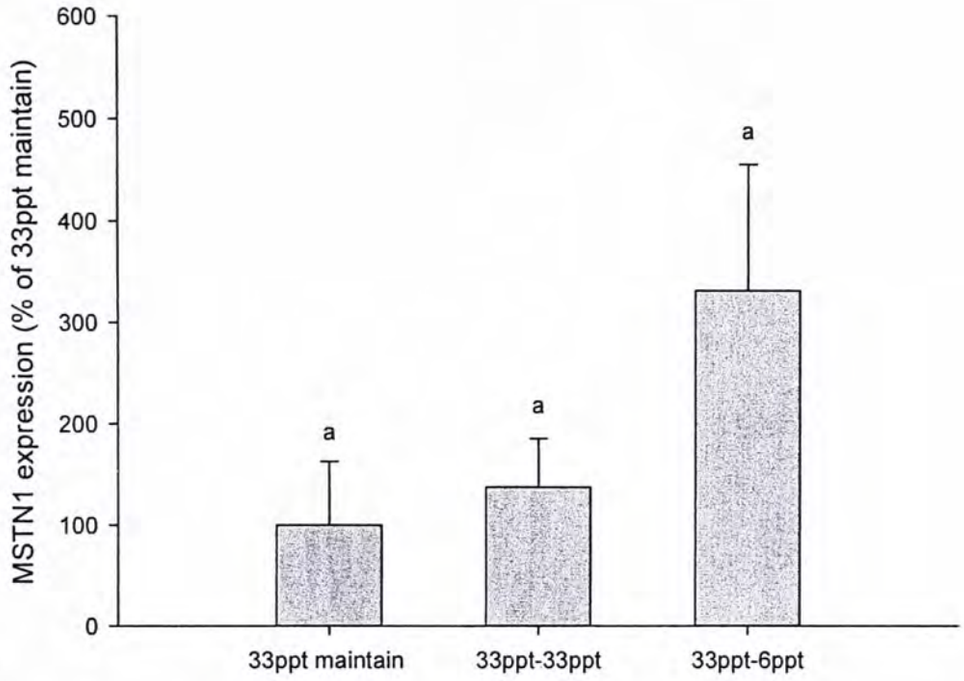

200
150
100
50
0
MSTN1 expression (% of 33ppt)
a
a
a
6ppt
12ppt
33ppt

(C)

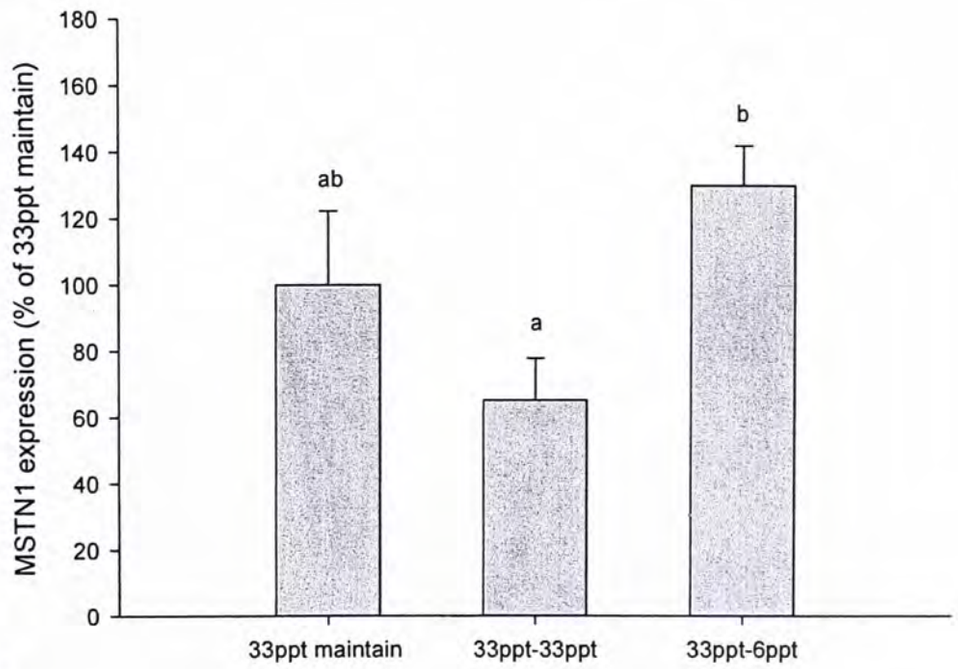

200
150
100
50
0
MSTN1 expression (% of 33ppt)
a
a
a
6ppt
12ppt
33ppt

(D)

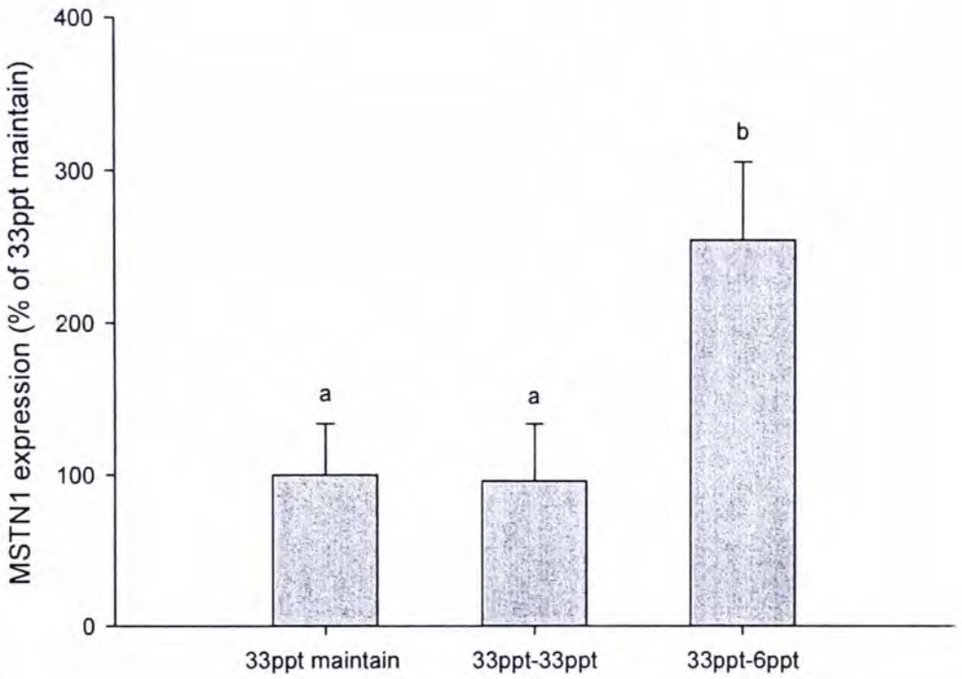
Figure 5.1 Myostatin-1 (MSTN1) expression of white muscle (A), red muscle (B), gill (C) and kidney (D) in silver sea bream adapted to salinities of 6 parts per thousand (ppt; hypoosmotic), 12 ppt (isoosmotic), 33 ppt (seawater, hyperosmotic) for 4 weeks. Control group: 33ppt. Data are presented as means ± SEM (n = 8-9 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.


MSTN1 expression (% of 33ppt maintain)
600
500
400
300
200
100
0
a
a
a
33ppt maintain
33ppt-33ppt
33ppt-6ppt

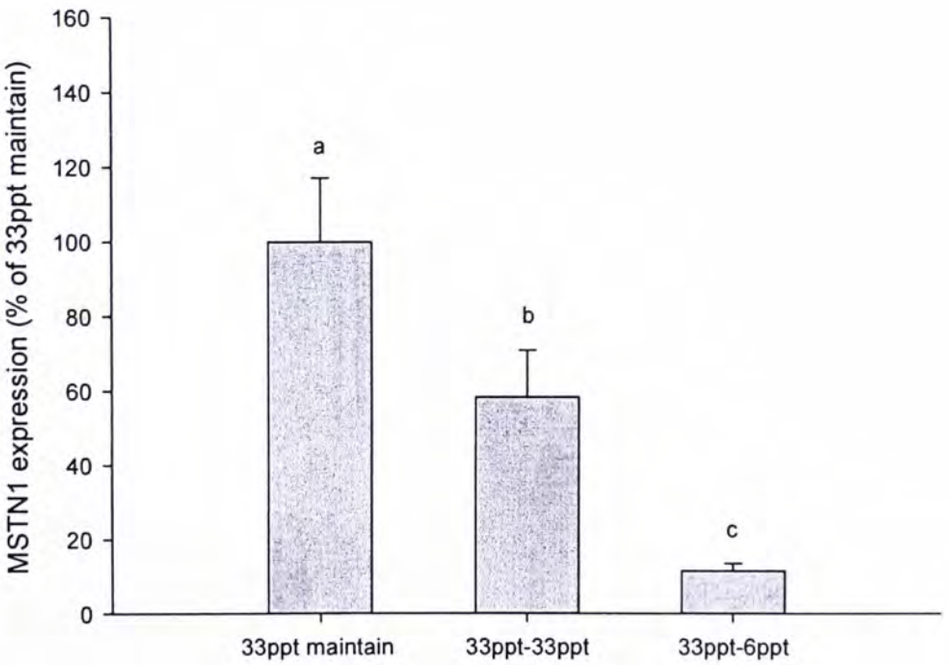
(A)


MSTN1 expression (% of 33ppt maintain)
180
160
140
120
100
80
60
40
20
0
ab
a
b
33ppt maintain
33ppt-33ppt
33ppt-6ppt

(B)

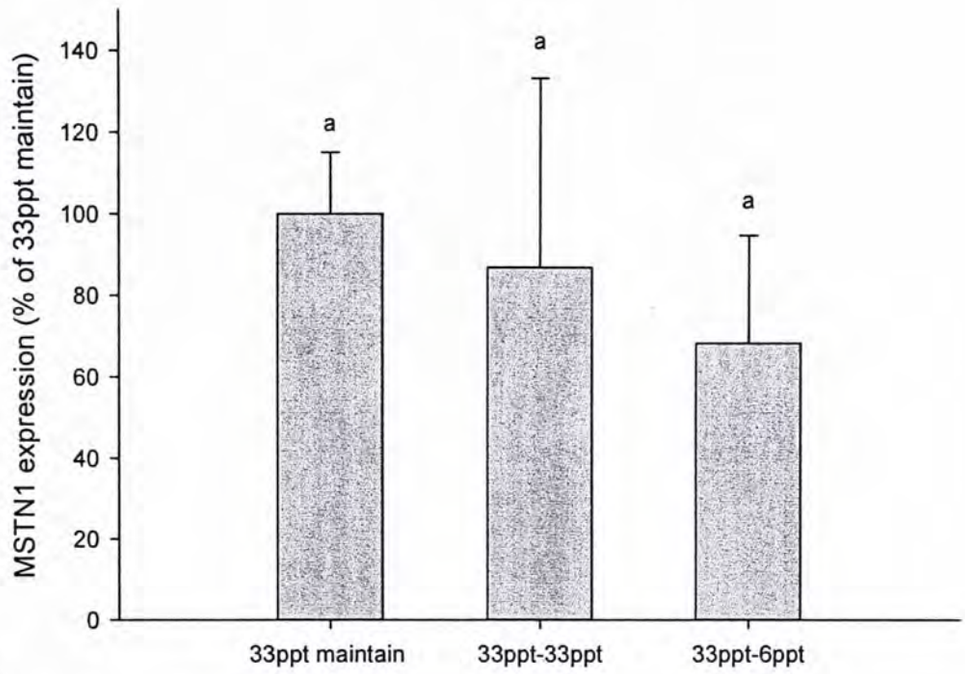

MSTN1 expression (% of 33ppt maintain)
400
300
200
100
0
a
a
b
33ppt maintain
33ppt-33ppt
33ppt-6ppt

(C)

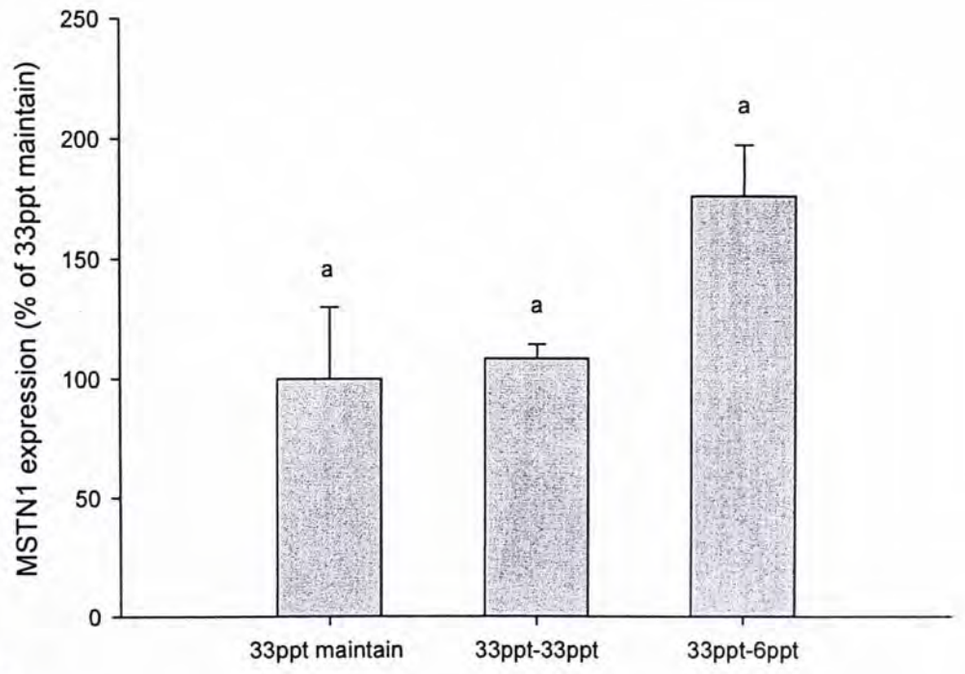

MSTN1 expression (% of 33ppt maintain)
160
140
120
100
80
60
40
20
0
a
b
c
33ppt maintain
33ppt-33ppt
33ppt-6ppt

(D)

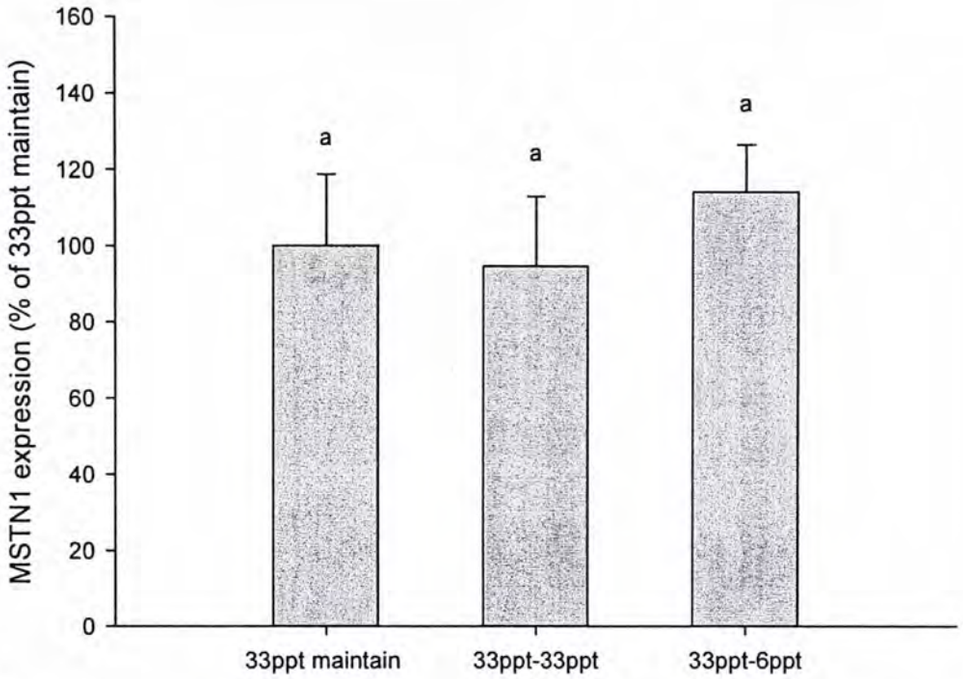
Figure 5.2 Myostatin-1 (MSTN1) expression of white muscle (A), red muscle (B), gill (C) and kidney (D) in silver sea bream abruptly transferred to hypoosmotic salinity (6ppt) after 24h. Control group: fish remained in 33ppt. Data are presented as means ± SEM (n = 5-6 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.


MSTN1 expression (% of 33ppt maintain)
140
120
100
80
60
40
20
0
a
a
a
33ppt maintain
33ppt-33ppt
33ppt-6ppt

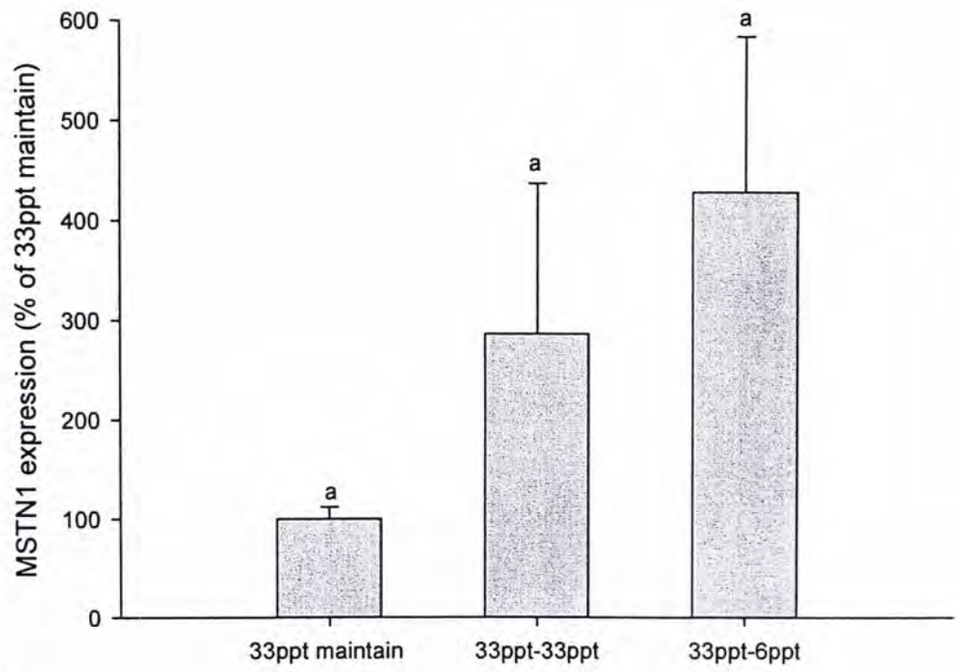
(A)


MSTN1 expression (% of 33ppt maintain)
250
200
150
100
50
0
a
a
a
33ppt maintain
33ppt-33ppt
33ppt-6ppt

(B)


MSTN1 expression (% of 33ppt maintain)
160
140
120
100
80
60
40
20
0
a
a
a
33ppt maintain
33ppt-33ppt
33ppt-6ppt

(C)


MSTN1 expression (% of 33ppt maintain)
600
500
400
300
200
100
0
a
a
a
33ppt maintain
33ppt-33ppt
33ppt-6ppt

(D)

Figure 5.3 Myostatin-1 (MSTN1) expression of white muscle (A), red muscle (B), gill (C) and kidney (D) in silver sea bream abruptly transferred to hypoosmotic salinity (6ppt) after 72h. Data are presented as means ± SEM (n = 5-6 for each group). Significant difference ($p < 0.05$) between groups is illustrated in each figure by different letters.

5.5 Discussion

We have investigated the changes in myostatin-1 expression level in seawater silver sea bream gill, kidney, as well as muscles after acclimation to brackish water (6 ppt) and upon abrupt transfer from seawater to brackish water. Myostatin was expressed in gill and kidney in many teleost species such as sea bream, zebrafish and salmon (Ostbye et al., 2001; Radaelli et al., 2003). In the fish model used in present study, silver sea bream, myostatin was also expressed in these two organs. Gill and kidney are key organs responsible for osmoregulation in fish.

Compare to silver sea bream adapted to seawater (33 ppt), there was no change in myostatin-1 mRNA expression in all tissues examined in fish adapted to both 12 ppt and 6 ppt. These data showed that long term adaptation of silver sea bream to isoosmotic and hypoosmotic salinities had no effect on myostatin expression and so the myostatin-1 gene may be not involved in osmoregulation.

Interestingly, in abrupt transfer experiments, myostatin-1 expression levels of red muscle and gill were increased significantly 24h after transfer to brackish water by 2-fold and 2.6-fold respectively when compared to those of the control transfer (33ppt-33ppt). However, for kidney, there was a significant decrease of myostatin-1 mRNA abundance 24h after abrupt transfer. For 72h after abrupt transfer from sea water to brackish water, there were no changes in myostatin-1 expression in all

tissues.

Changes in myostatin expression 24 hours after abrupt transfer in gill and kidney but no expression difference was observed after long term adaptation or 72 hours after abrupt transfer indicate that myostatin may be involved in the short term response of seawater to fresh water transfer.

Because of the very basic and experimental nature of our studies, and the lack of information on the role of myostatin in gill and kidney, it is difficult to draw any conclusions on any possible role for myostatin in fish osmoregulation. We can only suggest that myostatin may play a role in chloride cell differentiation in fish gills by considering the function of myostatin in myoblast, the role of TGF-beta family members in the cells and the likely occurrence of myostatin in gill chloride cell (Ostbye et al., 2001).

The morphology of chloride cells in seawater adapted fish and fresh water adapted fish are markedly different. Seawater-type chloride cells have a pit structure on the apical surface, whereas freshwater-type cells have microvilli on the flat or projecting apical membrane (Katoh and Kaneko, 2003; Kelly and Woo, 1999a, b; Wong et al., 2006) Many studies showed that chloride cells can alternate their morphology and ion-transporting functions when exposed to abrupt environmental osmotic changes (Hiroi et al., 1999; Kaneko et al., 2002; Sakamoto et al., 2000). In a

study carried out on killifish, seawater-type chloride cells were transformed into freshwater-type cells 12h after abrupt transfer to freshwater. During the transformation, an intermediate type with characteristics of both seawater and freshwater chloride cells was found and was most frequently observed at 3h after abrupt transfer (Katoh and Kaneko, 2003). For 3 days (72h) after freshwater transfer in the same study, 14.7% of seawater-type cells were replaced with newly differentiated freshwater-type chloride cells. This percentage is considerably higher than that in seawater maintained group (1.2%). These data showed that there were two mechanisms for chloride cell adaptive response. The first is the short term response of morphological and functional transformation of pre-existing chloride cells; the second is long-term adaptive response in which the part of pre-existing chloride cell degenerated and replaced by newly differentiated cells (Katoh and Kaneko, 2003). Our results of gill myostatin-1 expression at 24h and 72h after abrupt transfer may be related to these transformation, degeneration and differentiation of chloride cells.

For kidney, a significant decrease in myostatin-1 expression was observed 24h after abrupt transfer to 6 ppt. The previous work of our laboratory has shown that there are rapid changes in renal morphometrics in silver sea bream on exposure to different salinities (Wong and Woo, 2006). Abrupt transfer from 33ppt to 6ppt induced rapid changes in morphology of renal tubules. The abrupt transfer led to a dramatic

rapid proliferation of collecting tubules. Myostatin which function in myoblast proliferation and differentiation may be involved in that process but the mechanisms involved are yet unknown at this stage.

Lastly, it is worth mentioning that abrupt salinity transfer is known to cause stress to fish, and therefore there remains a possibility that the responses of myostatin expression to abrupt transfer as observed in the present study may actually be due to the induced stress, and such a possibility cannot be ignored.

Chapter 6

General Discussion and Conclusions

Myostatin is a negative regulator for muscle growth in mammals. One of the most important applications of studying myostatin is to find possible avenues for enhancing muscle growth. It is known that administration of myostatin antibody to animals generally does not result in changes in organ size and histology, or various serum parameters, suggesting that inhibition of myostatin in adults specifically increases skeletal muscle mass without side effect (Whittemore et al., 2003). In view of this, manipulation of endogenous myostatin levels may be feasible as one avenue for modulating muscle mass (muscle growth) which will find extreme usefulness in both agriculture and aquaculture. Manipulation of endogenous myostatin expression may have a greater commercial impact on fish culture than that of mammals as fish possess a larger ratio of muscle to non-muscle organs. So the study of myostatin in fish species is very important.

The present study has investigated the influence of growth-related hormones and salinity regimes on the regulation of myostatin expression in the silver sea bream. In the first part of this study, the characteristics of the expression profiles of both myostatin-1 and myostatin-2 in silver sea bream were examined. The mRNA expressions of these two myostatins in silver sea bream in different seasons and different organs were also quantified. Both myostatin-1and myostatin-2 were found to be expressed at lower levels in winter than that in summer. These finding suggested

that myostatin may be involved in the regulation of seasonal pattern for fish growth. In addition, the expression levels of both myostatin-1 and myostatin-2 were lower in white muscle than that in red muscle. These basic studies provide us with more knowledge about the expression pattern of the two forms of myostatins in silver sea bream.

In the second part of study, the effects of growth hormone, 11-ketotestosterone and cortisol on myostatin mRNA expression in silver sea bream were studied. Injection of growth hormone and 11-ketotestosterone resulted in a decreased myostatin-1 expression in white muscle and red muscle, respectively. Growth hormone and 11-ketotestosterone are well documented to be growth enhancing hormones. The present results suggest that the growth-promoting effect of growth hormone and 11-ketotestosterone may partially act via suppressing myostatin expression in muscle, and that myostatin in fish, may act in a similar way described for mammals by acting as a negative regulator of muscle growth. Different from that in white muscle, growth hormone injection induced myostatin-1 expression in red muscle, suggesting the existence of a unique regulatory mechanism of myostatin expression in red muscle. The distinctly different response to growth hormone treatment of myostatin in white muscle and red muscle suggest that myostatin may play a role in regulation of the ratio of white muscle to red muscle. Regarding the

effect of cortisol, some studies have shown a stimulatory effect of cortisol on myostatin expression (Lang et al., 2001; Ma et al., 2003), a finding which corroborates the generally observed negative effect of cortisol on muscle mass. However, in the present study, cortisol injection decreased the expression of myostatin-1 in white muscle. Studies on sea bream pituitary cell culture have shown that cortisol induced growth hormone secretion significantly in vitro. While other regulatory modes are possible, the reduction of myostatin expression by cortisol in the sea bream may likely to be mediated, at least partially, by a cortisol-stimulated growth hormone secretion. The three hormones we examined in the present study have different effects on myostatin gene expression. These three hormones are important growth related hormones. These results provide further evidence for the function of myostatin in growth regulation in fish.

Lastly, the expression levels of myostatin-1 were analyzed in silver sea bream exposed to different salinity conditions. The more diverse myostatin expression pattern in fish suggests that the function of myostatin may not be only restricted to the fish musculature. As a member of TGF-beta family, it may also function in cellular proliferation and differentiation in other organs. Our experiments involving exposure of fish to different salinities were designed to test the role of myostatin in gill and kidney indirectly. Although the difference in environmental salinities resulted in

marked changes in physiological parameters, most of these changes are closely related to osmoregulatory function. Long-term adaptation to different salinities and abrupt transfer into hyposmotic salinity for 72h had no effect on myostatin-1 expression. However, in samples collected 24h after abrupt transfer from 33ppt to 6ppt, myostatin-1 expression increased significantly in red muscle and gill but decreased dramatically in kidney. These findings reflected that myostatin-1 can be involved with short term salinity changes only. These results have been reported for the first time, and without further data, it is difficult to draw any conclusions on any possible role for myostatin in fish osmoregulation. We can only suggest that myostatin may be somehow involved in osmoregulatory processes especially during short-term salinity adaptation in silver sea bream.

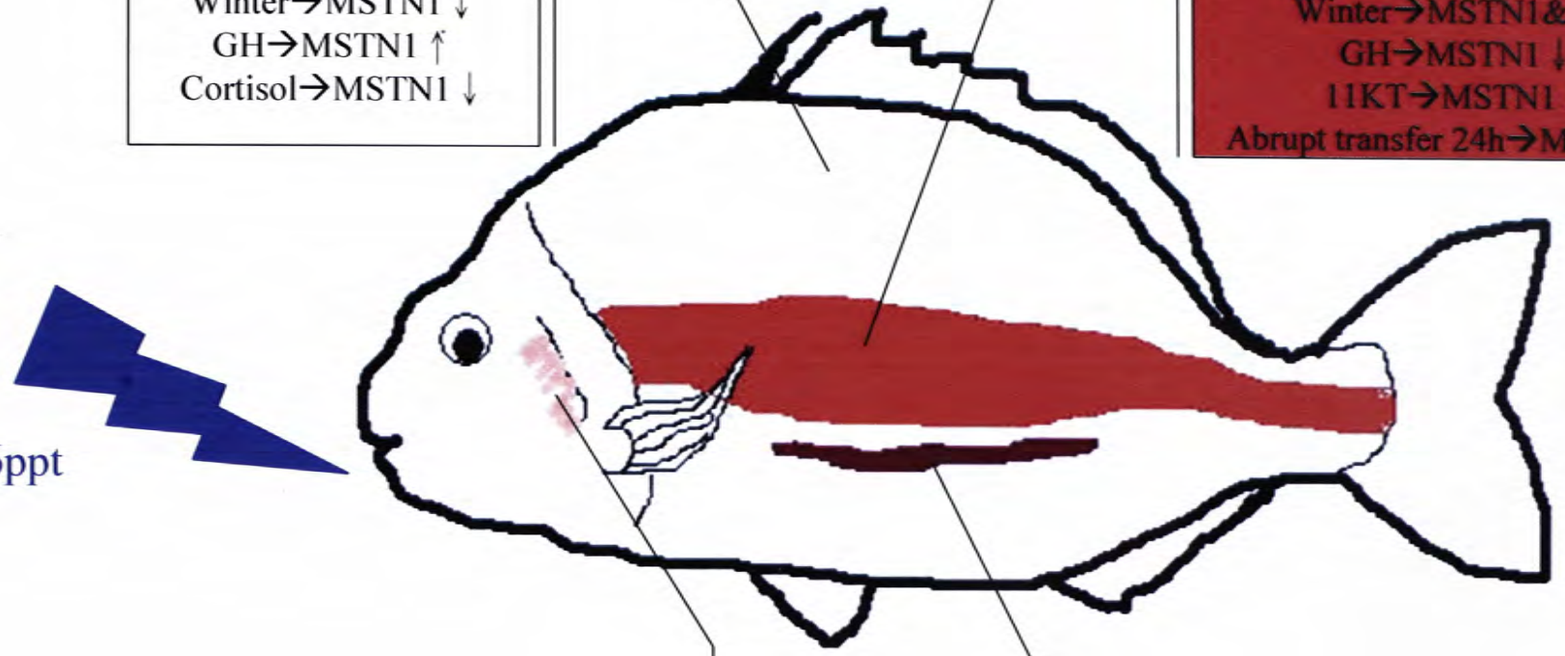
The present study has performed a basic research on several factors regulating myostatin expression in both red and white muscles and some non-muscle organs in silver sea bream. These findings enable us to know more about the function of myostatin in fish muscles and suggest a possible new function of myostatin in fish osmoregulatory physiology.

Hormone injection:

GH, 11KT, Cortisol

Salinities:

6, 12, 33ppt & 33→6ppt



WHITE MUSCLE

Winter→MSTN1 ↓
GH→MSTN1 ↑
Cortisol→MSTN1 ↓

RED MUSCLE

Winter→MSTN1&2 ↓
GH→MSTN1 ↓
11KT→MSTN1 ↓
Abrupt transfer 24h→MSTN1 ↑

GILL

Abrupt transfer 24h→MSTN1 ↑

KIDNEY

Abrupt transfer→MSTN1 ↓

Fig. 6.1: Summary of the main findings regarding the effects of hormones and salinity conditions on the expression pattern of myostatin-1 and myostatin-2 in different tissues and organs of silver sea bream (*Sparus sarba*).

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