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# Hormonal Regulation of Myostatin Expression in Silver Sea Bream Muscle

Chaoxiong Zhang and Norman Y. S. Woo+

School of Life Sciences, The Chinese University of Hong Kong, Hong Kong SAR, China

**Abstract.** Two myostatin genes (MSTN1 and MSTN2) were isolated and their mRNA expression profiles studied in the silver sea bream (Sparus sarba). Full length MSTN1 cDNA contains 1140 bp encoding 379 amino acids whereas the partial MSTN2 cDNA has 363 bp encoding for 121 amino acids. MSTN1 mRNA was expressed in a variety of tissues whereas MSTN2 mRNA was expressed only in red muscle and brain tissues. Sea bream were administered either growth hormone (GH), 11-ketotestosterone (11KT) or cortisol, and using quantitative real time PCR analysis it was found that GH caused significant decrease of MSTN1 transcript in white muscle but elevated the abundance of this transcript in red muscle. Injection of 11KT and cortisol resulted in decreased MSTN1 mRNA in red muscle whereas the abundance of MSTN2 mRNA remained relatively unchanged following hormone administration. The findings from this study indicate that GH, 11KT and cortisol modulate MSTN1 mRNA expression in silver sea bream.

**Keywords**: teleost; myostatin; gene expression; muscle

## 1. Introduction

Myostatin (MSTN) is a member of the transforming growth factor-ß (TGF-ß) superfamily and regulates muscle growth negatively by inhibiting the proliferation and differentiation of myoblasts through a TGF-β signaling pathway [20], consequently, MSTN-deficient animals such as MSTN knockout mice will display a double muscling phenotype [9]. In mammals, expression of MSTN is almost exclusively restricted to skeletal muscle [9], however, this is not the case for fishes. Fish generally possess two MSTN isoforms, MSTN1 and MSTN2, the latter was first found in brook trout [21] and later found in other fish species [2, 16, 20, 29]. MSTN1 is expressed in many different tissues but MSTN2 is mainly expressed in brain and muscle [7, 23]. Such a differential expression pattern of MSTN isoforms suggests diverse functions for MSTNs in fish.

MSTN is regulated by anabolic and catabolic hormones. In mammalian muscle, the anabolic effect of growth hormone (GH) acts via inhibition of MSTN mRNA expression [10] and a similar mechanism may operate in fish since a GH-response element has been identified upstream from the transcription start site in the MSTN gene of fish [21]. However, the regulatory pattern is not as well-defined as that in mammals since fish generally has two MSTNs and a double set of musculature (white and red muscle). Transgenic salmon overexpressing GH exhibited a decrease in white muscle MSTN2 mRNA expression, an increase in red muscle MSTN2 mRNA expression, but a decline in MSTN protein in both red and white muscle [22]. In rainbow trout, GH injection induced MSTN1 but reduced MSTN2 mRNA abundance [1]. The male sex hormone, testosterone, is generally anabolic in mammals and acts via inhibiting MSTN expression [16] but it is not certain if the anabolic action of the fish specific androgen, 11-ketotestosterone (11KT) [3] is in any way related to modulation of MSTN expression. Glucocorticoids such as cortisol are generally catabolic in mammals [26] and fish [28]. A glucocorticoid response element (GRE) identified on the MSTN promoter region indicates a possible regulatory effect of glucocorticoids on MSTN [7]. However, a direct relationship

+ Corresponding author. Tel: +852-39436148; Fax: +852-26035646
*Email address*: normanwoo@cuhk.edu.hk

between glucocorticoid and MSTN expression in controlling muscle mass has not been clearly established, and available results are often contradictory. MSTN mRNA was markedly increased (+270%) after a single injection of dexamethasone in the rat [7] but was drastically lowered (-75%) in tilapia larvae following cortisol treatment [25]. Given that the relationship between MSTN and hormones have not been clearly defined in fish, the present study was carried out to delineate the effects of exogenous GH, 11KT and cortisol on mRNA expression levels of MSTN1 and MSTN2 in the white and red musculature of silver sea bream.

## 2. Materials and methods

Eight groups of silver sea bream (*Sparus sarba*) were used for experiments. Fish groups 1, 2, and 3 were respectively given daily intraperitoneal injections of 0.8% NaCl, 10 ng/g and 1 µg/g recombinant sea bream (*Acanthopagrus butcheri*) GH for four days. Groups 4, 5, and 6 were given single intraperitoneal injections of dimethyl sulfoxide (vehicle), 50 ng/g or 2.5 µg/g 11-KT respectively. Groups 7 and 8 were given either single intraperitoneal injection of 0.8% NaCl or 50 µg/g cortisol. Twenty four hours after injections, fish were killed and white and red muscle samples were collected and stored in TRI Reagent® at -80°C. Muscle total RNA was extracted using TRI Reagent®, treated with deoxyribonuclease I and reverse transcribed to first-strand cDNA by M-MLV Reverse Transcriptase using oligo$(dT)_{15}$ primer. Gene specific primers for the isolation of partial regions of silver sea bream MSTN1 (5'-TTCTCGACCAGTACGACGTG-3' and 5'-TAATCCAGTCCCAGCCAAAG-3') and MSTN2 (5'-CCCAGGACGAGTTGACCTCA-3' and 5'-CGGACTCTGATTCGGGTGTT-3') cDNA were designed based on gilthead sea bream MSTN1 (GenBank AF258448) and MSTN2 cDNA sequence (GenBank AY046314) respectively. The PCR mixture consisted of first strand cDNA, PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each gene-specific primer and Taq DNA polymerase. After denaturation (94°C, 5 min), 35 cycles of PCR amplification was carried out and each cycle consisted denaturing (94°C, 30 sec), annealing (55°C, 60 sec) and extension (72°C, 30 sec) followed by final extension (72°C, 10 min). PCR products were ligated to pCR®-TOPO® vector, mixed with competent cell (DH5α), placed on ice for 15 min, transferred to 42 ºC for 30 sec, and then transferred into ice (5 min), after which S.O.C. Medium was added and the mixture shaken at 37 ºC for 1 h. About 50 -100 µl of the transformed bacteria were spread on a pre-warmed LB plate containing 50 µg/ml ampicillin and incubated overnight at 37 ºC. PCR was used to screen for positive transformations and plasmids containing target DNA were extracted using NucleoSpin® Plasmid DNA Purification Kit and sequenced.

The 5' and 3' ends of putative sea bream MSTN1 cDNA were obtained using Rapid Amplification of cDNA Ends. Based on nucleotide sequence of the partial cDNA of putative sea bream MSTN1, the antisense primer 5'-TCGAATCGAAGGCGTTAATC-3' was synthesized and used in reverse transcription for 5'RACE and the antisense primers 5'-TTCAGGGAGCGGATGTGTAT-3', 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. 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. Based on the cDNA sequence of silver sea bream MSTN1 and MSTN2, gene specific primers were designed (5'-GGAGACGACAACAGGGATGT-3' and 5'-TTCAGGGAGCGGATGTGTAT-3' for MSTN1; 5'-CCCAGGACGAGTTGACCTCA-3' and 5'-CGGACTCTGATTCGGGTGTT-3' for MSTN2) and used to examine the expression of MSTN1 and MSTN2 mRNA in various sea bream tissues using RT-PCR. The PCR products were checked using electrophoresis on agarose gel with ethidium bromide staining under UV.

Real-time quantitative RT-PCR was used to measure mRNA expression of MSTN1, MSTN2 and a housekeeping gene 18s rRNA (for internal normalization). The templates for the standard curves for MSTN1, MSTN2 and 18s rRNA were prepared by PCR amplification of cDNA fragments 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) [silver sea bream 18s rRNA, GenBank EF494673] designed according to the corresponding silver sea bream cDNA

sequences. The amplicon was purified and quantified, and copy number of DNA molecule was calculated as templates of standard curve. Defined copies of amplified DNA of each gene at 10-fold dilutions were subjected to Real-time PCR to construct the standard curve. Real-time PCR analyses were performed using iQ ™ SYBR® Green Supermix (BioRad, USA) on a iCycler iQ™ Real-Time PCR System. PCR reaction protocol consisted of 4 min at 95 ºC, 40 cycles of denaturing at 95 ºC for 30s, annealing at 60 ºC for 30s, extension at 72 ºC for 40s and signal detection at 84 ºC for 7s. Abundance of MSTN1 and MSTN2 gene transcripts were normalized to 18s rRNA transcript abundance for each sample. Data presented as means ± SEM were subjected to either a Student's t-test (for 2 groups) or a one-way ANOVA to test for significance followed by a Newman-Keuls Multiple Comparison Test (for 3 groups) to delineate significance among groups.

## 3. Results

Excluding the poly(A) tail, full-length silver sea bream MSTN1 cDNA consists of 1631 nucleotides which includes a 129 bp 5' untranslated region, a 1140 bp open reading frame encoding 379 amino acid, and a 362 bp 3' untranslated region (Fig. 1). Silver sea bream MSTN1 possesses a RXXR (RVRR) proteolytic processing site, nine cysteine residues corresponding to TGF-β activity and a bioactive C-terminal domain and microsatellite repeats (including a CAG repeat) at the N-terminal of the coding region to encode a polyglutamine stretch (6 residues) and a (AC) repeat at the 3'-UTR (Fig. 1). The silver sea bream MSTN1 gene sequence has been placed on GenBank (Accession FJ972540). Only a fragment of the silver sea bream MSTN2 was obtained and its sequence deposited with GenBank (Accession FJ972450). Comparing with the amino acid sequences of MSTNs from other species, silver sea bream MSTN1 shares 98% homology with MSTN1 of gilthead sea bream (GenBank AF258448), 85% with fugu (AY445322), 79% with zebrafish (AY258034), 83% with rainbow trout MSTN1a, 82% homology with rainbow trout MSTN1b and 62% homology with human (NM005259) and mouse (NM010834) MSTNs. The silver sea bream MSTN2 fragment shares 99% homology with the MSTN2 of gilthead sea bream (AY046314), 88% with fugu (AY445321), 59% with zebrafish (AY687474) and 72% with rainbow trout MSTN2a (DQ417326).

MSTN1 mRNA was detected in white and red muscle, several brain regions, gill, kidney, but MSTN1 mRNA in the intestine was only barely detectable (Fig. 2). MSTN2 mRNA was expressed in red muscle and brain but was barely detectable in white muscle (Fig. 2). MSTN2 mRNA was not detected in other tissues (Fig. 2). in red muscle MSTN2 mRNA following GH injection, these apparent changes were not statistically significant (Fig. 3c). Injection of 11KT (2.5 μg/g) into sea bream was followed by marked decrease in MSTN1 mRNA in red muscle (Fig. 3b), although there was no statistically significant change in white muscle MSTN1 mRNA (Fig. 3a) and red muscle MSTN2 mRNA expression (Fig. 3c) following 11KT injections. In sea bream injected with 50 μg/g cortisol, MSTN1 mRNA expression level in white muscle decreased significantly by 70% (Fig. 3a), while in the red muscle, both MSTN1 and MSTN2 mRNA expression were unchanged (Fig. 3b, c).

## 4. Discussion

Excluding the poly(A) tail, full-length silver sea bream MSTN1 cDNA comprises 1631 nucleotides which includes a 129 bp 5' untranslated region, a 1140 bp open reading frame encoding 379 amino acid, and a 362 bp 3' untranslated region. Similar to other TGF-β family members [9], silver sea bream MSTN1 possessed a RXXR (RVRR) proteolytic processing site, nine cysteine residues corresponding to TGF-β activity and a bioactive C-terminal domain. Proteolytic processing results in the formation of a N-terminal propeptide (latency associated peptide) and a C-terminal mature MSTN peptide [9]. Eight of the nine cysteine residues form disulfide bonds to create a cysteine knot and the ninth cysteine forms a bond with the ninth cysteine of another MSTN molecule to form a dimer [9]. Silver sea bream MSTN1 gene contains microsatellite repeats including (CAG) repeat at N-terminal of the coding region to encode a polyglutamine stretch (6 residues) and a (AC) repeat at the 3'-UTR. Multiple alignment of silver sea bream MSTN sequence with those from other animals revealed the existence of a similar polyglutamine stretch in gilthead sea bream MSTN (12 residues), but no such polyglutamine stretch could be found for other vertebrate MSTNs. It is possible that expansions of the polyglutamine tract would lead to a gene either losing its

function or gaining new functions, as with the case for several human disease genes such as the Huntington's gene, in which expansion of the polyglutamine tract has resulted in neutrophil aggregates [9, 21].

Sea bream injected with GH displayed significant decrease (-80%) in white muscle MSTN1 mRNA (Fig. 3a) but elevated MSTN1 mRNA in red muscle (+300%) (Fig. 3b). Although there was a trend of elevation Expression patterns of MSTN isoforms in fish are different from that of mammals and different from each other. MSTN1 is expressed in many tissues in fish [13, 17, 24], whereas MSTN2 expression is primarily confined to the brain [12]. In silver sea bream, MSTN1 mRNA was detected in white and red muscle, gill, kidney, several brain regions, and a very low expression level in the intestine whereas MSTN2 mRNA was abundantly expressed in red muscle and brain but was barely detectable in white muscle. This expression pattern is consistent with results reported for brook trout [21] and gilthead sea bream [19]. In mammals, MSTN is expressed mainly in skeletal muscle [9]. In silver sea bream, MSTN2 mRNA were expressed at higher levels in muscle than in white muscle, consistent with the findings in gilthead sea bream and sole [19]. This differential MSTN expression pattern in red and white muscle may indicate a role of MSTN in regulating the growth of different muscle fibers in fish. Red muscle generally grows slower than white muscle [19] and being a negative regulator of muscle growth, MSTN may act to inhibit red muscle bulk, which in less active fish like the sea bream (Sparidae), red muscle only comprises about 10-15% of the total musculature [15]. With such a MSTN distribution pattern, it appears that the function of MSTN may not be restricted to regulation of muscle growth. In sea bream and other fish [19], both MSTN1 and MSTN2 mRNA were abundantly expressed in brain, suggesting some neural function for MSTN. MSTN may have a function similar to a growth differentiation factor such as GDF-11, as both molecules share a highly similar bioactive domain [23]. MSTN mRNA is expressed in gill and kidney of silver sea bream, zebrafish and salmon [20, 22], suggesting that MSTN may play certain roles in the physiology of these organs but the precise role of MSTN in gill and kidney is unknown.

The two sets of musculature in sea bream exhibit opposite responses to GH injection: MSTN1 mRNA was decreased in white but elevated in red muscle. MSTN2 mRNA abundance in red muscle did not change following GH injection. In mammals, GH mostly exerts its muscle anabolic effect by inhibiting MSTN expression [10]. In fish, a suppressive relationship of GH on muscle MSTN mRNA expression has not been firmly established although the identification of GH cell specific elements upstream of the transcriptional start site of both MSTN1 and MSTN2 [21] does lend support to the association. Transgenic salmon overexpressing GH had no change in MSTN1 mRNA in white and red muscle, but had lowered white muscle MSTN2 mRNA and elevated red muscle MSTN2 mRNA levels [22]. However, MSTN protein was decreased in both red and white muscle [22]. In white muscle of rainbow trout, GH had a differential effect on MSTN mRNA expression; it induced MSTN1 mRNA but reduced MSTN2 mRNA [1]. It appears that white and red muscles appear to respond differentially to GH.

In mammals, the anabolic effect of androgens on muscle growth [14] is mediated via inhibition of MSTN expression [16] and 11KT is an anabolic fish androgen [3]. In sea bream, 11KT injection decreased MSTN1 mRNA in red muscle. The present report is the first to demonstrate the mechanism of the anabolic effect of 11KT on fish muscle via its downregulatory effect on muscle MSTN expression. In sea bream, cortisol injection led to marked decline in MSTN1 mRNA expression in white muscle, but in red muscle, both MSTN1 and MSTN2 mRNA expression were unaffected. Cortisol is a catabolic hormone and exerts negative effect on growth in mammals [26] and fish [28]. A glucocorticoid response element has been identified on the MSTN promoter region [11], substantiating the interaction between glucocorticoid and MSTN. However, results of different studies are in conflict. In rat, MSTN mRNA was increased after injection of dexamethasone [7] and MSTN gene deletion prevented glucocorticoid-induced muscle atrophy [4]. However, the present and other studies on fish showed the contrary: muscle MSTN mRNA was reduced in tilapia larvae immersed in water containing cortisol [25], in channel catfish following dexamethasone injection [30], and in silver sea bream following cortisol administration (this study). Hypercortisolemia following fasting also lowered MSTN mRNA level in fish [30]. At present, it is unknown whether glucocorticoids regulate MSTN expression directly or indirectly in vivo, as in channel catfish, dexamethasone injection led to decreased MSTN but had no effect on MSTN mRNA in primary muscle cell culture [30]. It is possible that

dexamethasone or cortisol injection indirectly regulates MSTN expression through alteration of other factors such as GH as cortisol has been reported to stimulate GH secretion in fish [27].

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   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  CCAGTAGTTTTGAGCGACCAAGAGACGCAGCAGCAGCAGCAGCAGCCCTCCGCCACCAGC  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  TTAAACCCCATAAAGTCTCAGATTCTGAGCAAACTCCGAATGAAAGAGGCTCCAAATATC  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  GCCATCACGGAGACGATTATGATGATGGCCACTGAACCTGAGTCCGTCGTCCAGTGGAT  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  GAGTACATGCACTTGCAGAAGTACCCGCACACCCACCTGGTGAACAAAGCCTACCCAGA 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  GTGCAATAGAAACCAGAGTAGAGGCCACAATCAAGCCCGACTTCCCGCAGGGCAGCGCT 1440
1441  GCTTTCACATCCGGCACAGCTCTCACCTTTTTCTTTCCTCCTGTGAAATCTTAGTTTGAC 1500
1501  GTCAGATGGATGCAGGAACACACACACACACACACACACACACACACACACTTCAGTC 1560
1561  GACGCAGATTCAAGCAGCTCATTTGTTGGTTTCATGATCACTTTTCTCAATAAAAGTAAT 1620
1621  TTATCATTTCC                                                 1631
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Fig. 1: Full-length nucleotide sequence and deduced amino acid sequence of silver sea bream MSTN1 cDNA. The sequence consists of a 1140 bp open reading frame (ORF) encoding for 379 amino acids with additional 412 bp comprising the 5'- and 611 bp the 3'-untranslated regions. An asterisk indicates a stop codon. The nine cysteine residues and proteolytic processing site are indicated in gray shading.

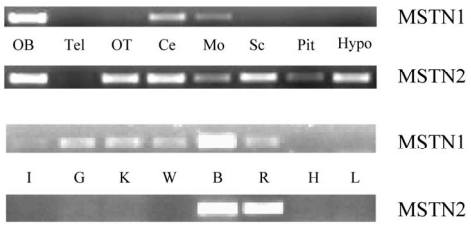


Fig. 2: Distribution of MSTN1 and MSTN2 mRNA expression in tissues of silver sea bream. OB : olfactory bulb ; Tel : telencephalon ; OT : optic tectum ; Ce : cerebellum ; Mo : medulla oblongata ; Sc : spinal cord; Pit: pituitary; Hypo: hypothalamus; I: intestine; G: gill; K: kidney; W: white muscle; B: whole brain; R: red muscle; H: heart; L: liver.

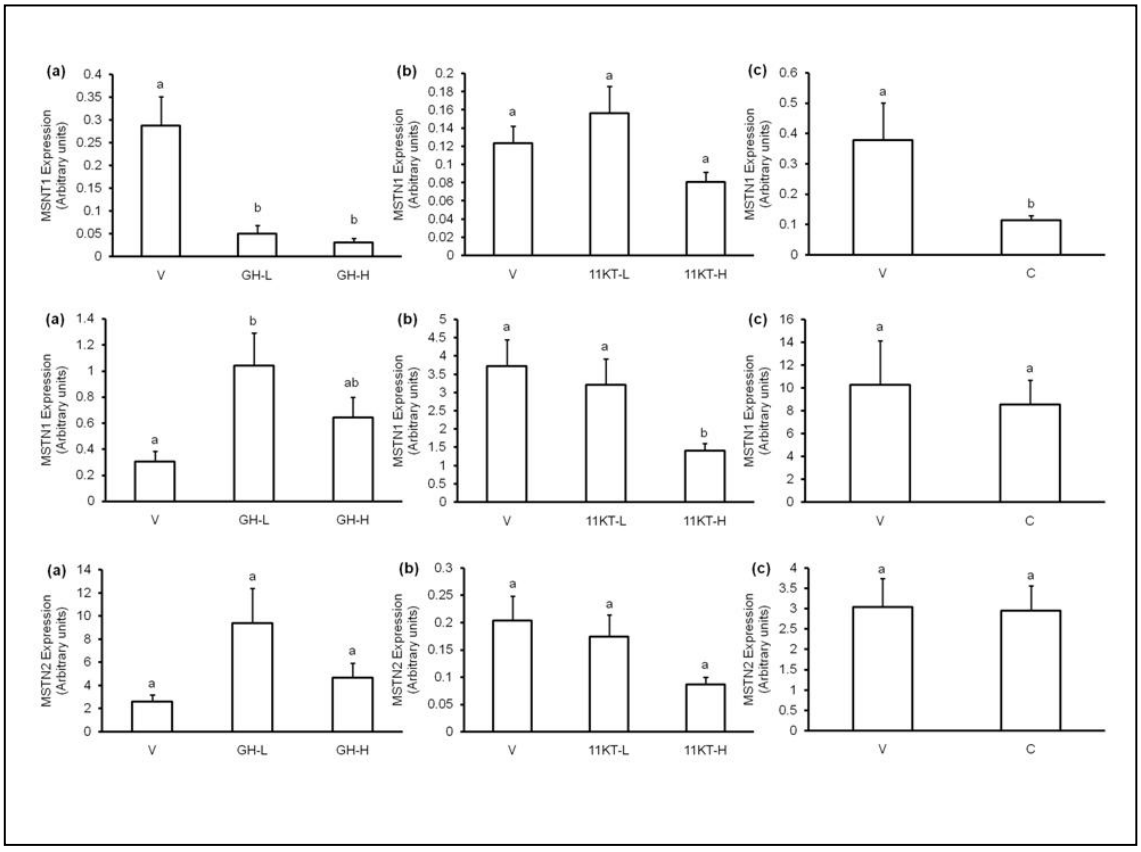


Fig. 3: Effect of intraperitoneal injection of growth hormone (GH), 11-ketotestosterone (11KT) and cortisol on MSTN1 mRNA expression in white muscle (a), red muscle (b) and MSTN2 expression in red muscle (c). V: vehicle; GH-L: 10 ng/g GH; GH-H: 1 µg/g GH; 11KT-L: 50 ng/g 11KT; 11KT-H: 2.5 µg/g 11KT; Cortisol: 50 µg/g cortisol. The amounts of transcript were normalized to corresponding 18s rRNA transcript abundance of each sample. Different alphabets indicate significant difference ($p$ <0.05) between groups.