# Journal of
# Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

Journal of Fisheries and Aquatic Science 3 (5): 298-301, 2008
ISSN 1816-4927


# Heat Shock Protein70 mRNA Expression as a Biomarker for Stress Evaluation in Blue Green Damselfish *Chromis viridis*

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**Abstract:** cDNA partial sequence of heat shock protein 70 (HSP70) were isolated from blue green damselfish *Chromis viridis*, with highly homologous to other teleost HSP70 genes. To evaluate the stress response of HSP70 gene in damselfish, RT-PCR was conducted after 90 days of daily exposure to excess level of water stream. Increased expression of HSP70 mRNA was found in the brains and the gills of experimental fish compared to control fish. This result suggests that HSP70 mRNA may provide a useful biomarker for the evaluation of stress levels in small ornamental fish with difficulty of blood sampling.

**Key words:** HSP70, mRNA, stress, ornamental fish, damselfish

## INTRODUCTION

The trade of live marine ornamental fish is a global industry. Unlike freshwater aquaria species, where 90% of fish species are currently farmed, the great majority of marine aquaria are stocked from wild caught species (Wabnitz *et al*., 2003). For possible over-harvesting of some species, the aquarium industry has attracted some controversy, particularly regarding its sustainability. Moreover, fish that are caught in the wild often suffer from poor handling resulting in poor health (Bunting *et al*., 2003).

Therefore, physiological research on marine ornamental fish as well as mariculture species is needed in order to conduct proper husbandry under suitable condition. Among all, stress evaluation is essential, but as for the small ornamental species, such as damselfish (Pomacentridae) has a difficulty in blood sampling to assess the blood cortisol level, which is generally used for the stress evaluation.

In this study, the development of new method for stress evaluation as an alternative to blood cortisol measurement was conducted using heat shock protein (HSP) 70 mRNA transcription level in the blue-green damselfish (*Chromis viridis* Cuvier), one of the most traded fish worldwide (Wabnitz *et al*., 2003). HSP70 is known to be synthesized in response to various environmental stresses (Elyse Ireland *et al*., 2004) and expected to a new useful biomarker for the stress evaluation as well (Gornati *et al*., 2004; Hoekstra *et al*., 1998). And for the stress exposure, the daily exposure to the excess level of water stream formed by the generation of micro bubbles, of which diameter approximately 1 mm, was used for the chronic stress.

## MATERIALS AND METHODS

*C. viridis* was obtained from the commercial pet shop (Negishi Sango-en, Fukushima, Japan). The liver was extracted and soaked in 500 μL RNA stabilization solution RNAlater (Applied Biosystems,

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California, USA) in a 1.5 mL microfuge tube. Total RNA was purified by Rneasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of the RNA was performed by ThermoScript RT-PCR System (Invitrogen, California, USA). HSP70 cDNA was amplified by PCR with Takara Ex Taq Reacition Kit (Takara Bio, Shiga, Japan). Total PCR reaction volume of 30 μL was composed of 3.0 μL 10X Ex Taq Buffer, 3.0 μL dNTP mixture, 2.1 pmol of each primer, 0.8 units Ex Taq and 1.0 μL DNA solution containing 0.15 μg cDNA. Degenerate oligonucleotide primers used in PCR (Chr-HSP70-F; 5'-CARGAYTTYTTYAAYGGAAARGA -3', Chr-HSP70-R; 5'-CCCCCAGCACTYTGRTANAGKTT-3') were based on previous reports (Deane *et al*., 2000). The profile of PCR conditions was as follows: initial denaturation at 95°C for 5 min 35 cycles of denaturation at 95°C for 30 sec, annealing at 48°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products of HSP genes were ligated into the pCR 2.1-TOPO vector by the use of TOPO TA Cloning (Invitrogen). The sequences of HSP70 gene was confirmed by DNA sequencing with ABI PRISM™ 3730xl DNA Analyzer (Applied Biosystems). The HSP70 gene sequence was compared with all other known gene sequences through a BLAST search. Similar DNA sequences were downloaded from the DNA Data Bank of Japan (DDBJ) and aligned with our sequences.

Detection of HSP70 mRNA transcript was carried out using RT-PCR. Brains and gills of *C. viridis*, which was kept for 90 days in a tank (60×30×30 cm) equipped with micro bubbles generator for forming water stream, were used. The forming of the water stream was 1 h per day (10:00 am to 11:00 am daily) at flow rate of 1 L / min. HSP specific oligonucleotide primers used in RT-PCR (RT-HSP70-F; 5'- GTCTGAGAATGTGCAGGACTTGCT -3', RT-HSP70-R; 5'-ATGACCTCGTTGCACTTGTCCAAG -3') were designed by sequence data of *C. viridis* HSP70 gene reported in the present study. At the same time, amplification of β-actin was performed and the product was used as an internal standard of RT-PCR. β-actin detective primers (Actin-F; 5'-CAATGGATCCGGTATGTGC-3', Actin-R; 5'-CGTTGTAGAAGGTGTGATGCC-3') were based on previous report (Naito *et al*., 1998). RNA extraction, cDNA synthesis and PCR amplification methods were as previously described, though PCR amplifications were conducted at both 40 and 20 cycles in each sample for the semi-quantitative analysis. The results were compared to the control fish, which were kept in the same condition as experimental fish except the exposure to water stream.

## RESULTS AND DISCUSSION

A newly reported cDNA sequence was compared with the DDBJ database. *Chromis* HSP70 partial sequence (751 bp, Fig. 1) showed 92.7 % HSP70 sequence similarity to *Rhabdosargus sarba* Forsskål (accession No. AY436786) and 91.7% to *Dicentrarchus labrax* Linnaeus (AY423555), *Paralichthys olivace* Temminck et Schlegel (AB006814) and *Acanthopagrus schlegelii* Bleeker (accession No. AY762969) respectively. The cDNA sequence data reported in this study submitted to the DDBJ/EMBL/Gen-Bank database and was assigned the accession numbers AB360349.

RT-PCR showed that the constant transcription of HSP70 mRNA in brains and gills in both of experimental and control fish at 40 cycles of PCR amplification. However, at 20 cycles, higher transcription level of HSP70 mRNA was found in both tissues of experimental fish, especially in gills, compared to control fish with the slight individual variability (Fig. 2).

The present study reports the isolation and sequencing of cDNA partial clone corresponding to the *Chromis* HSP70 that have highly sequence homology with other teleost species. Though *Chromis* HSP70 mRNA was found in a certain level both in the brain and the gill under the normal condition, higher transcription was detected in stressed fish. HSP70 mRNA transcription level in the brain may reflect stress intensity by reason that HSP70 is shown to interact with the glucocorticosteroid receptor (GR) involving in correcting GR protein folding (Grad and Picard, 2007). Furthermore, HSP70 is

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GCTGAACAAGAGCATCAATCCAGATGAAGCTGTGGCCTATGGAGCTGCTGTCCAGGCTGCT
  L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A  A

ATCCTGTCTGGAGACAAGTCTGAGAATGTGCAGGACTTGCTGCTTCTGGATGTCACCCCT
 I  L  S  G  D  K  S  E  N  V  Q  D  L  L  L  L  D  V  T  P

CTGTCCCCTGGGTATTGAGACTGCTGGAGGTGTCATGACTGTCCTCATCAAACGTAACACC
 L  S  L  G  I  E  T  A  G  G  V  M  T  V  L  I  K  R  N  T

ACCATTCCTACCAAGCAGACCCAGACCTTCACTACCTATTCTGACAACCAGCCAGATGTG
 T  I  P  T  K  Q  T  Q  T  F  T  T  Y  S  D  N  Q  P  D  V

CTCATCCGGGTTTATGAGGGTGAACGTGCTATGACCAGGGACAACAACTTGCTGGGCAAG
 L  I  R  V  Y  E  G  E  R  A  M  T  R  D  N  N  L  L  G  K

TTTGAGCTGACAGGCATCCCTCCTGCTCCACGTGGTGTTCCCCAGATCGAAGTGACATTT
 F  E  L  T  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T  F

GACATTGATGCCAATGGTATCATGAATGTCTCTGCTGTTGACAAGAGCACTGGAAAGGAA
 D  I  D  A  N  G  I  M  N  V  S  A  V  D  K  S  T  G  K  E

AACAAGATCACCATCACCAATGACAAGGGTCGCCTCAGCAAGGAGGACATTGAGCGCATG
 N  K  I  T  I  T  N  D  K  G  R  L  S  K  E  D  I  E  R  M

GTCCAGGAAGCTGAGAAGTACAAGGCTGAGGATGACGTTCAACGTGACAAGGTGTCCGCT
 V  Q  E  A  E  K  Y  K  A  E  D  D  V  Q  R  D  K  V  S  A

AAGAACGGCCTGGAGTCGTATGCTTTCAACATGAAGTCCACGGTGGAGGATGAGAAGCTT
 K  N  G  L  E  S  Y  A  F  N  M  K  S  T  V  E  D  E  K  L

GCCGGCAAAATCAGTGATGATGACAAGCAGAAGATCTTGGACAAGTGCAACGAGGTCATC
 A  G  K  I  S  D  D  D  K  Q  K  I  L  D  K  C  N  E  V  I

AGCTGGCTGGACAAGAACCAGACTGCAGAGAAGGATGAGTATGAGCATCAACAGAAGGAG
 S  W  L  D  K  N  Q  T  A  E  K  D  E  Y  E  H  Q  Q  K  E

CTGGAGAAAGTTTGCAACCCCATCATCACC
 L  E  K  V  C  N  P  I  I  T
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Fig. 1: *Chromis viridis* HSP70 cDNA partial sequence together with its deduced amino acid translation. GenBank DDBJ/EMBL/Gen-Bank database Accession No.AB360349

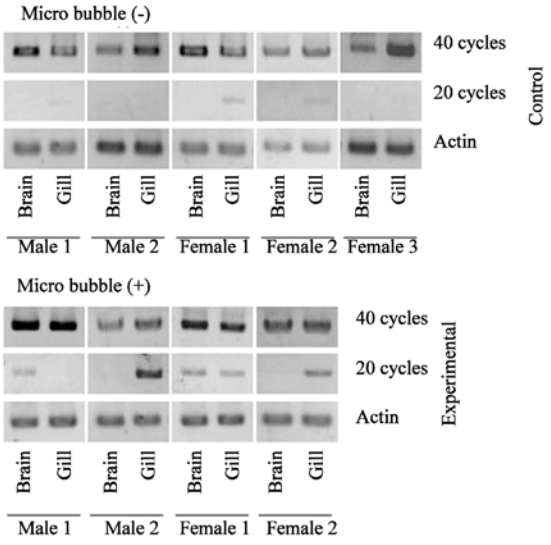


Fig. 2: Expression analysis in brains and gills of experimental (lower panel) and control (upper panel) fish. RT-PCR amplification products of HSP70 fragments at 20 and 40 cycles of PCR amplification and the constitutive control of β-actin fragment are shown

known to show high upregulation in gill by various environmental stresses; such as osmolality change (Deane and Woo, 2004) or exposure to metals (Hansen *et al.*, 2007). Micro bubbles that were used in the present study were reported to generate free radicals (Takahashi *et al*., 2007). High HSP70 mRNA transcription in the gill of experimental fish in this study suggested the mechanical damage from the

overexposure to micro bubbles. Actually, the thinning of epithelial cells and hemorrhagic focuses in gills were found in the experimental fish by the histological examination (data not shown).

The result in the present study suggested that the HSP70 mRNA transcription level in brains and gills represent easy and relatively inexpensive biomarkers to evaluate the welfare conditions of small ornamental fish with difficulty in the blood sampling.

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