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Identification and partial characterisation of the HSP70 gene of the South African abalone *Haliotis midae*.

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## Abstract

Identifying genes which are up-regulated in response to stress has become important in understanding invertebrate immunity. The HSP70 gene from *Haliotis midae* was successfully cloned and sequenced, and was found to have highest similarity (99%) with *Haliotis diversicolor*. Protein and RNA were isolated from haemocytes extracted from *H. midae* exposed to heat stress at  $21 \pm 1^\circ\text{C}$  at 15 minute time intervals up to one hour, and then every 30 minutes up to three hours. Real-time PCR amplification revealed that HSP70 gene expression increased after 60 minutes of heat shock exposure, but then decreased to the same levels as time point 0 after 150 minutes exposure. Western hybridisation analysis was performed and the results revealed increased expression at all time points in relation to time point zero. The findings of this study reveal that *H. midae* does indeed possess the HSP70 gene and that its mRNA expression increases after 60 minutes of heat stress. HSP70 protein expression is immediate and remains at higher levels than the control time point zero for the entire time course. This study is an important step in finding a viable biomarker in *Haliotis midae*, which could aid in early detection and eradication of stress caused by elevated temperatures in an aquaculture environment.

## 1. Introduction

### 1.1. Abalone and Aquaculture

Abalone are marine gastropods that belong to the family *Haliotidae*. Gastropods include the snails and slugs, and are characterised by their muscular “feet” upon which they move. The *Haliotis* genus is identified by their flat, ear-shaped shell, with respiratory holes found dorsally. Six species of *Haliotis* are found in the coastal waters of southern Africa (Sales and Britz, 2001). In South Africa, *Haliotis midae* is farmed commercially. Wild *H. midae* prefer the cold temperatures of the coast of southern Africa (between 12-13 °C), although they can be found in water up to 20 °C, despite evidence that elevated water temperatures can restrict growth rate and reproduction (Sales and Britz, 2001).

Abalone have been utilised as a source of food for generations. This valuable marine resource is worth between US\$34 - US\$36 per kg (Macey and Coyne, 2005). The major market for abalone is concentrated in the Far East, although Mexico, USA and Europe also consume abalone (Robertson-Anderson, 2003; Oakes and Ponte, 1996). Its high value has resulted in poaching becoming an ever-increasing problem. This has resulted in increasing interest in aquaculture.

Aquaculture can be defined as the farming of aquatic plants and animals (Middlen and Redding, 1998; De Silva and Anderson, 1994). With pressures placed on the environment by humans, it seems only logical that such practices be favoured. The high stocking densities maintained on aquaculture facilities, along with fluctuations in environmental conditions, including temperature,

bacterial infection, fungal infection and red tide phenomena, cause unnecessary stress on cultured organisms.

## 1.2. Stress and immunity in abalone and gastropods

Stress and disease are major influences on the biological processes of an organism's metabolism, as they disrupt the homeostasis of the body and lead to a decrease in growth, which is of economic importance to farmers (Lacoste *et al.*, 2002). When a stress causes a shift in the equilibrium between host, pathogen and environment, disease occurs (Bachere, 2003). Invertebrate response to stressors such as oxygen limitation, altered salinity and elevated temperature has been linked to the onset of disease due to decreased immune capacity (Malham *et al.*, 2003, Lacoste *et al.*, 2002).

Invertebrates exhibit innate immunity, which involves humeral responses such as anti-microbial peptide production, melanisation and the production of reactive oxygen species (Marmaras and Lampropoulou, 2009), as well as cellular responses of circulating haemocytes (Tincu and Taylor, 2004). Haemocytes are derived from undifferentiated mesoderm cells (Marmaras and Lampropoulou, 2009), which differentiate into specific types such as prohaemocytes, granulocytes, plasmacytes, spherulocytes and oenocytoids (Martin *et al.*, 2007). Haemocytes have the capacity to perform a wide variety of functions, including metabolite transport, wound repair and more notably, phagocytosis. Phagocytosis is the means by which pathogens are removed from the invertebrate body, and as such, a decrease in immune capacity would allow for opportunistic infection.

Currently, knowledge of the immune system of *H. midae* is limited. A better understanding of the abalone immune system and the genes involved in immunity is required to facilitate the development of proper tools for the accurate identification of stress within *H. midae*.

Common physiological *in vitro* tests used to indicate stress in abalone include the haemocyte count, phenoloxidase measurement, superoxide production, phagocytosis assay and bacterial clearing assays (Hooper *et al.*, 2007). These *in vitro* tests however, have not been specifically designed for abalone and may show varying results. The ability to predict the vulnerability of a species to stress is best achieved at the molecular level. Genes that are differentially regulated during stress or disease could be used in a laboratory-based test. Candidate genes for such tests include those that have been shown in literature to be differentially regulated under stress (Feder and Hofmann, 1999). Hence there is now interest in developing molecular biomarkers for environmental stress, with large amounts of research concentrated on the response to heat stress or elevated temperature (Clark and Peck, 2009).

A biomarker can be defined as “a measurement of a molecular or chemical substance or event in a biological system” (Wilson and Suk, 2002). Biomarkers can be used to detect changes in an organism’s physiological state (Centre for Environmental Health Sciences at Dartmouth, 2001), thereby providing an early indication of stress and the possibility of eliminating that stress. Identification of viable biomarkers can also help aquaculture facilities improve conditions associated with mass mortalities.

At increased temperatures, marine invertebrates develop internal hypoxia, even in fully oxygenated waters, a reduction in metabolic rate and aerobic capacity (Portner *et al.*, 2005). However, organisms respond to stress in many different ways, with the only known universal response occurring at the molecular level (Gross, 2004). The most commonly studied molecular response is that of the heat shock proteins (HSPs). The most studied of these HSPs are the 70 kDa heat shock proteins (HSP70) (Clark and Peck, 2009). HSP70 may be used as a biomarker, since its upregulation has been reported by Plakidou-Dymock and McGivan (1994) in response to heat shock, amino acid deprivation and viral infection.

### 1.3. HSP70 function and regulation

The term stress proteins, recognises that many of these proteins are induced by a variety of stimuli (Tavaria *et al.*, 1996). Stress proteins associate with denatured or partially unfolded proteins, protecting them from further denaturation. This ability to bind to other proteins appears to be a common feature of stress proteins and has led to their inclusion in the molecular chaperone superfamily.

HSPs are highly conserved throughout species and are synthesised in almost all organisms studied to date in response to thermal stress. HSPs are classified into families whose members are grouped according to their molecular weights. They may have different locations within the cells as well as different levels of expression (De Maio 1999).

One of the most prominent and best characterised families of stress proteins is the HSP70 family. This family consists of proteins ranging in size from 66 kDa to 78 kDa (Tavaria *et al.*, 1996). HSP70 proteins have been shown to function in the process of protein folding by stabilizing the nascent polypeptide chain preventing incorrect folding. Apart from its peptide chaperone capacity, HSP70 is widely believed to participate in immunity and adaptation of an organism to stress (Martynova *et al.*, 2007).

Sriram *et al.* (1997) reported that human HSP70 chaperone is a 640 amino acid protein consisting of two major domains. A 44 kDa, 388 amino acid N-terminal domain, responsible for binding and hydrolysing ATP, and a C-terminal domain responsible for binding peptides and folding non-native polypeptides. The C-terminal domain can be divided into two functionally relevant sub-domains, an 18 kDa peptide-binding domain and a 10 kDa C-terminal domain that contains the Glu-Glu-Val-Asp (EEVD) regulatory motif identified by Freeman *et al.* (1995). Communication between these domains appears to be an important component of HSP70 chaperone activity (Buchberger *et al.*, 1995, Freeman *et al.*, 1995).

The HSP70 open reading frame of *Haliotis discus hannai* is 1968 bp in length, and encodes a 655 amino acid protein (Cheng *et al.*, 2007). The HSP70 is composed of a 44 kDa N-terminal domain responsible for binding ATP, a substrate peptide binding domain of 18 kDa, and a C-terminal domain of 10 kDa.

HSP70 family molecular chaperones require specific monovalent and divalent metal ions for ATP binding and hydrolysis (Sriram *et al.*, 1997). ATP hydrolysis and phosphate release is coupled with

HSP70 molecular chaperone function. The ADP-bound state shows higher affinity for polypeptides than its ATP-bound state. Sriram *et al.* (1997) also showed that ATP binding and hydrolysis by HSP70 affects the relative conformation of its domains and its interactions with other protein cofactors, thus affecting the chaperone function of HSP70.

HSP70 holds unfolded substrates in an intermediately folded state to prevent irreversible aggregation and catalyses the refolding of unfolded substrates in an energy and co-chaperone-dependent reaction (Cheng *et al.*, 2007). The N-terminal ATPase domain interacts with co-chaperones and the C-terminal substrate-binding domain with substrates (Cheng *et al.*, 2007). ATP hydrolysis-induced conformational changes in the ATPase domain (44 kDa) of HSP70 regulates the binding. This binding of substrate is stimulated by the co-chaperone HSP40. Release of substrates requires the binding of ATP to HSP70, after which the substrates either enter a new cycle of binding and release, or fold into their native conformation. Co-chaperone interactions can influence the HSP70-substrate binding and release cycle by stimulating, inhibiting or altering the trafficking of HSP70-interacting substrates (Cheng *et al.*, 2007). Kohler and Eckwert (1997) showed that the kinetic reaction of the HSP70 activation at increasing exposure to stressors described an optimum curve: both low and very high stress loads resulted in a low HSP70 level. This is because high stress intensities over-charge the protective HSP70 system. The major inducing factor for HSP70 upregulation is the presence of damaged cellular protein (Ananthan *et al.*, 1986).

The regulation of expression of HSP70 genes occur mainly at the transcriptional level. Cheng *et al.* (2007) and Piano *et al.* (2005) examined the HSP70 responses in *Haliotis discus hannai* muscle and gill to both thermal stress and *Vibrio anguillarum* infection. Both tissues showed a marked increase

in HSP70 expression upon recovery after heat shock. HSP70 mRNA expression increased two hours into recovery reaching a maximum level at 12 and 24 hrs (Cheng *et al.*, 2007).

#### 1.4. Aims of this study

There is currently no information pertaining to the regulation of the HSP70 gene in *Haliotis midae*.

The first aim of this study was to identify the HSP70 gene in *Haliotis midae*. Degenerate and gene specific primers were used to amplify the HSP70 gene from both genomic and complementary DNA (cDNA) from *H. midae* haemocytes.

The second aim of this study was to investigate the molecular response of HSP70 in *H. midae* exposed to elevated temperature and to determine the potential of HSP70 as a suitable biomarker. Quantitative real time PCR amplification and western hybridisation analysis were used to determine the transcriptional and translational regulation, respectively, of HSP70 in *H. midae* haemocytes in response to heat stress. For this study, *H. midae* were heat-shocked at  $21 \pm 1$  °C. Haemocytes isolated from *H. midae* were assayed for their response to heat shock over time with a single recovery point included.

This study attempts to comprehensively test how HSP70 levels vary over time in response to a sudden elevation in temperature. In this work we report the partial cDNA nucleotide sequence of the *H. midae* HSP70 gene and examine the effects of thermal stress on the relative HSP70 mRNA and protein expression patterns.

## 2. Materials and Methods

### 2.1. Animals

*Haliotis midae* used in this study were kindly donated by Global Ocean (Kleinmond, South Africa) and kept at the Marine and Coastal Management Research Aquarium (Cape Town, South Africa). Animals were maintained in polyethylene tanks containing 100 litres of aerated and continuously flowing (330l/h) 10 µm filtered sea water at 15 °C. Animals were fed a diet of kelp, *Ecklonia maxima*.

### 2.2. Haemocyte Collection

Haemolymph (1 ml) was collected from the pedal sinus of abalone (60 mm ± 5 mm) using sterile syringes and 26G X ½ inch needles. At each time point, an equal volume of haemolymph from five animals was pooled and aliquoted into 1 ml fractions. Haemocytes were collected by centrifugation at 4 500 x g for one minute. The supernatant (haemolymph) was discarded. Haemocytes were immediately frozen in liquid nitrogen. Haemocytes were stored in liquid nitrogen for 30 minutes before being placed at – 80 °C, where they were stored for a period of no longer than six months.

### 2.3. Genomic DNA isolation

Haemocytes were thawed on ice and washed in phosphate buffered saline (PBS) [8% (w/v) NaCl, 0.2% (w/v) KCl, 1.44% (w/v) Na<sub>2</sub>HPO<sub>4</sub>, 0.24% (w/v) KH<sub>2</sub>PO<sub>4</sub>] by centrifugation at 4500 x g for one minute. Haemocytes were re-suspended in lysis buffer (10mM Tris, 1mM EDTA, 2% (w/v) sodium

dodecyl sulphate (SDS), 0.1 mg/ml Proteinase K, pH 7.6) and incubated for one hour at 37 °C. One hundred microlitres of 5 M NaCl and 80 µl CTAB/NaCl was added and the solution incubated at 65°C for 10 minutes. An equal volume of chloroform:isoamyl (24:1) was added and the samples centrifuged for 5 minutes at 5000 X g. The aqueous phase was removed and aliquoted into fresh microfuge tubes where 0.6 volume of isopropanol was added and the resultant solution mixed by inversion. The sample was centrifuged for 15 minutes at 5000 X g. The supernatant was discarded and the genomic DNA washed with 70% ethanol and centrifuged for 5 minutes at 5000 X g. The genomic DNA was air dried for 5-10 minutes to allow the excess ethanol to be evaporated and subsequently re-suspended in Tris-EDTA buffer (10 mM Tris, 1mM EDTA, pH 7.6) with 1 µl of 10 mg/ml (*w/v*) RNase A (Sigma). The DNA was quantified spectrophotometrically (NanoDrop® ND-1000 Spectrophotometer, NanoDrop 1000 software version 3.7.1) and visualized on a 0.8% 1X TAE agarose gel containing 0.4 µg/ml ethidium bromide. Genomic DNA samples were stored at 4 °C.

## 2.4. Cloning and Sequencing of HSP70 from *H. midae*

### 2.4.1. Primer Design

Degenerate primers were designed to amplify a portion of the HSP70 gene from *H. midae*. The *Haliotis diversicolor* HSP70 nucleotide sequence (nucleotide accession number, ACO36047.1) was used to find similar sequences using the BLAST algorithm (Altschul *et al.*, 1997) provided by the internet service of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST/>). Ten protein sequences (Table 2.1) were aligned using ClustalW Multiple Sequence Alignment version 1.4 (Thompson *et al.*, 1994). The alignment was exported into the CODEHOP algorithm (Rose *et al.*, 1998) and the degenerate primers HSPDF and

HSPDR (Table 2.2) were selected from a list generated by the CODEHOP algorithm. The expected product size is approximately 530 base pairs (bp). The sequence of this amplification product was used to design gene-specific primers HSPF and HSPR (Table 2.2). Primers HSPF and HSPR were designed to the 3' end of the HSP70 gene using Beacon Designer design software and FastPCR Professional (Table 2.2). The expected HSP70 product size is 82 bp.

Table 2.1: HSP70 protein sequences used to design degenerate primers for PCR amplification of the *H. midae* HSP70

<b>Name</b>	<b>Accession Number</b>
<i>Aplysia California</i>	<b>CAA78757.1</b>
<i>Biomphalaria glabrata</i>	<b>AAB99911.1</b>
<i>Chlamys farreri</i>	<b>ABE77386.1</b>
<i>Crassostrea gigas</i>	<b>BAD15286.1</b>
<i>Crassostrea ariakensis</i>	<b>AAO41703.1</b>
<i>Drosophila auraria</i>	<b>CAA04699.1</b>
<i>Haliotis discus hannai</i>	<b>ABC54952.1</b>
<i>Haliotis tuberculata</i>	<b>CAK95236.1</b>
<i>Mytilus galloprovincialis</i>	<b>AAW52766.1</b>
<i>Ostrea edulis</i>	<b>AAM46635.1</b>

Table 2.2: Oligonucleotide primer sequences used for amplification of HSP70 and real-time quantitative PCR (qPCR).

Primer name	Nucleotide sequence	Reference
<b>Degenerate primers</b>		
HSPDF	5'- GAGAACAAGATCACCATCACCa(C/T)ga(C/T)aa(A/G)gg -3'	This study.
HSPDR	5'- CCTGGTACAGCTTGGTGATGAT(A/G/C/T)GG(A/G/C/T)(A/G/T)T(A/G)CA-3'	This study.
<b>Gene-specific primers</b>		
HSPF	5'- TCGAGAGCGGAGAGCTTGA -3'	This study.
HSPR	5'- CAATCCTCGGGCTTGGTGT -3'	This study.
M13F	5'- CGCCAGGGTTTTCCCAGTCACGAC -3'	Yanisch-Perron <i>et al.</i> (1985)
M13R	5'- GAGCGGATAACAATTCACACAGG -3'	Yanisch-Perron <i>et al.</i> (1985)
L28F	5'- TGGACTCACCGTCAGCACG -3'	Arendze-Bailey (Unpublished data)
L28R	5'- TTCTGGTGACCAAGACAACGCC -3'	Arendze-Bailey (Unpublished data)

#### 2.4.2. Amplification of *H. Midae* HSP70

Initially gDNA (section 2.3) was amplified using an annealing temperature gradient from 50 °C to 60 °C and MgCl<sub>2</sub> concentrations between 1.5 mM and 3.0 mM to determine the optimal reaction conditions. Amplification reactions contained 0.5 U *Taq* polymerase, 0.2 mM dNTPs and 0.25 mM of each of HSPDF and HSPDR primers (Table 2.2). The cycling conditions were 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 50 °C – 60 °C for 30 seconds, 72 °C for 30 seconds, and finally, 72 °C for 10 minutes. Amplified products were analyzed on a 1.5% 1X TAE agarose gel containing 0.4 µg/ml ethidium bromide. Optimal amplification was at 54.5 °C and 3 mM MgCl<sub>2</sub> concentration. The 523 bp product was extracted from the gel using peQLab's E.Z.N.A Gel Extraction Kit (peQLab, Germany) according to the manufacturer's instructions.

#### 2.4.3. Cloning and Sequencing of HSP70

The purified 523 bp fragment was ligated into plasmid vector pTZ57R/T as described in the Fermentas InsTaclone PCR cloning kit #K1214 (Fermentas, USA). The reaction was incubated at 22 °C for 16 hours and transformed into *Escherichia coli* DH5 $\alpha$  competent cells prepared according to the method described by Inoue *et al.*, (1990). Transformants were inoculated onto Luria agar (LA) solid media (10% (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar) containing 100  $\mu$ g/ $\mu$ l ampicillin, 2% (w/v) X-gal and 100mM IPTG and incubated at 37 °C for 16 hours. Positive transformants were inoculated onto LA solid media containing 100  $\mu$ g/ml ampicillin and incubated at 37 °C for 16 hours. Transformants were then inoculated into Luria broth (LB) (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) containing 100  $\mu$ g/ml ampicillin and incubated at 37 °C for 16 hours on an orbital shaker.

Plasmid DNA was isolated as described by Sambrook *et al.*, (1989) and quantified spectrophotometrically (NanoDrop® ND-1000 Spectrophotometer, NanoDrop 1000 software version 3.7.1). The presence of insert was confirmed by digesting one microgram of plasmid DNA with *Hind*III (1U) and *Eco*RI (1U) in a 20  $\mu$ l reaction. Restriction enzyme digest products were visualised on a 1% TAE agarose gel containing 0.4  $\mu$ g/ml ethidium bromide.

Plasmid DNA was isolated from transformants containing the correct insert using peQLab's E.Z.N.A Cycle Pure Kit (peQLab, Germany) and sequenced by Macrogen Inc. (World Meridian Centre 60 – 24, Gasandong Geumchun-gu, Seoul Korea 153 – 021). The plasmid was sequenced using M13F and M13R primers (Table 2.2). Sequence data were analysed and edited using Chromas version

2.33 (Conor McCarthy, 1996) and DNAMAN version 4.13 (Lynnon BioSoft). Sequence homology searches were performed using the BLAST algorithm (Altschul *et al.*, 1997) provided by the internet service of the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>, 03-2009).

BioEdit Sequence Alignment Editor © (Version 7.0.5.2) was used to perform all multiple sequence alignments using the ClustalW (Thompson *et al.*, 1994) multiple sequence alignment algorithm. The BLASTX algorithm was used to determine the identity of the sequence obtained by aligning it with other protein sequences from the National Centre for Biotechnology Information (NCBI) online database ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)). The conserved domains and open reading frame were found using the conserved domain finder and the ORF-finder features on the NCBI website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Sequences available on the NCBI database (Table 2.3) were used to construct a neighbour joining evolutionary tree with bootstrapping (1000 replicates) using the MEGA software (MEGA version 4.0, Tamura *et al.*, 2007).

Table 2.3: Amino acid sequences used in the construction of a phylogenetic tree.

<b>Sequence name</b>	<b>Accession number</b>
<i>Alvinella pompejana</i>	CBM42048.1
<i>Argopecten irradians</i>	AAS17723.1
<i>Chlamys farreri</i>	AAO38780.1
<i>Crassostrea ariakensis</i>	AAO41703.1
<i>Crassostrea gigas</i>	BAD15286.1
<i>Dendrolimus tabulaeformis</i>	ABM90802.1
<i>Haliotis discus hannai</i>	ABC54952.1
<i>Haliotis tuberculata</i>	CAK95236.1
<i>Helicoverpa armigera</i>	ACL31668.1
<i>Hepialus pui</i>	ADA61012.1
<i>Locusta migratoria</i>	AAP57537.3
<i>Mizuhopecten yessoensis</i>	AAS17724.1
<i>Mythimna separata</i>	ABY55233.1
<i>Mytilus galloprovincialis</i>	AAW52766.1
<i>Perna indica</i>	ADC91992.1
<i>Perna viridis</i>	ABJ98722.1
<i>Phascolosoma esculenta</i>	ACB47483.1
<i>Pteria penguin</i>	ABJ97377.1
<i>Trichoplusia ni</i>	ABH09734.1

## 2.5. Heat Shock Treatment Experimental Design

*H. midae* were subjected to temperature stress exposure to determine whether the HSP70 mRNA transcript and protein were differentially expressed in response to elevated water temperature. A single 100 litre tank and 50 abalone of 60 mm  $\pm$  5mm in length were used for this experiment. Two 100 watt thermostatically controlled heaters were placed in the tank and the tank's temperature was adjusted to 22 °C  $\pm$ 1 °C. Haemolymph was extracted (section 2.2) from five individuals at ambient temperature (15 °C), t = 0. The 45 remaining abalone were placed in the 100 litre tank at 22 °C and haemolymph was extracted at 15, 30, 45, 60, 90, 120, 150 and 180 minutes. The heaters were removed at this point and 24 hours later, an additional haemolymph sample was collected.

## 2.6. RNA Isolation

Haemocytes were collected as described in section 2.2.

RNA was isolated according to Chomczynski and Sacchi (1987) with some modifications. Briefly, 500  $\mu$ l of Solution D (4M guanidium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarkosyl, 0.7% (w/v) 2-mercaptoethanol) was added to concentrated haemocytes. The sample was mixed and maintained on ice for the remainder of the procedure. Five hundred microlitres of phenol (pH 4) was added to the solution and mixed by inversion after which 150  $\mu$ l of chloroform:isoamyl (49:1) was added. The solution was incubated on ice for 10 minutes and subsequently centrifuged for 30 minutes at 9 000 x g at 4 °C. The aqueous phase was removed and dispensed into fresh 1.5ml eppendorf tubes. One volume of isopropanol was added to each sample, mixed by inversion and

incubated for approximately 16 hours at  $-70^{\circ}\text{C}$ . The samples were centrifuged for 10 minutes at  $9\ 000\ \text{X}\ g$  at  $4^{\circ}\text{C}$  and the supernatant removed. RNA was re-suspended in  $150\ \mu\text{l}$  Solution D and 1 volume of isopropanol was added. RNA was allowed to precipitate for 1 hour at  $-20^{\circ}\text{C}$ . The RNA was concentrated by centrifugation for 10 minutes at  $9\ 000\ \text{X}\ g$  at  $4^{\circ}\text{C}$ , and washed with 96% ethanol followed by a 70% ethanol wash. The RNA was air dried for 5 minutes to remove excess ethanol and re-suspended in diethylpyrocarbonate (DEPC) water. RNA was quantified spectrophotometrically (NanoDrop ND-1000 Spectrophotometer, NanoDrop 1000 software version 3.7.1) by measuring the absorbance at 260 nm. A DNase treatment was included to remove contaminating genomic DNA. One unit of RNase free DNase I (Fermentas) was added per microgram of RNA in a  $50\ \mu\text{l}$  reaction and incubated at  $37^{\circ}\text{C}$  for 1 hour according to the manufacturer's instructions. Fifty microlitres of phenol (pH 4):chloroform:isoamyl (25:24:1) was added to the samples and mixed by inversion. The samples were centrifuged for 10 minutes at  $9\ 000\ \text{X}\ g$  at  $4^{\circ}\text{C}$ . The aqueous phase was recovered and mixed with  $5\ \mu\text{l}$  of 3 M Na-acetate (pH 5.2) and  $150\ \mu\text{l}$  of 100% ethanol. The samples were centrifuged for 10 minutes at  $9\ 000\ \text{X}\ g$  at  $4^{\circ}\text{C}$  to concentrate the RNA. RNA was air dried for 5 minutes and re-suspended in  $25\ \mu\text{l}$  DEPC treated water. RNA was quantified spectrophotometrically as described above.

RNA integrity was determined by visualisation on a formaldehyde 1.2 % (w/v) agarose gel (Sambrook *et al.*, 1989). RNA loading buffer (4.5% (w/v) MOPS, 5.3% (w/v) formaldehyde, 60% (w/v) formamide, 0.7% (w/v) bromophenol blue, 0.7% (w/v) xylene cyanol) was added to one microgram of sample (1:1) and heated at  $65^{\circ}\text{C}$  for 5 minutes, cooled on ice and electrophoresed at 80 volts in 1X MOPS.

## 2.7. RNA Retrotranscription

Two micrograms of total RNA was retrotranscribed to cDNA using the Promega ImpromII® Reverse Transcriptase kit (Promega, USA) according to the manufacturer's instructions. Conversion reactions were performed in a total volume of 40 µl. cDNA was primed by adding a combination of random hexamers (Promega) and Oligo dTs (Promega) to the cDNA synthesis reaction at a ratio of 1:1. The cDNA reactions were incubated at 42°C for 16 hours. The reactions were heat inactivated for 15 minutes at 75°C and subsequently aliquoted into 10 µl volumes. Ninety microlitres of sterile water was added and samples stored at - 80 °C for no longer than three months. One microlitre of diluted cDNA was used as template in PCR amplification reactions.

## 2.8. Semi-quantitative RT-PCR

One microlitre of cDNA was amplified using either Ribosomal protein L28 primers (L28F and L28R) or gene-specific primers HSPF and HSPR (Table 2.2) prior to real-time PCR amplification to confirm the integrity of the template. The PCR amplification reaction contained 3 mM MgCl<sub>2</sub>, 0.5 U *Taq* polymerase, 0.2 mM dNTPs and 0.3 µM of each of the primers. The following cycling conditions were used for both sets of primers: 95°C for 10 minutes, followed by 30 cycles of 95°C for 10 seconds, 60°C for 15 seconds and 72° for 20 seconds, with a final extension step at 72°C for 5 minutes. The PCR amplification products were electrophoresed on a 2 % TAE-agarose gel for 45 minutes at 80 V in order to visualize the products.

The RNA isolated at each time point of the heat shock treatment (section 2.5) was used as template in a PCR amplification reaction to confirm the absence of contaminating DNA in each sample. The reaction contained 0.3  $\mu\text{M}$  of each gene specific primer (HSPF and HSPR, Table 2.2), 3 mM  $\text{MgCl}_2$ , 0.5 U *Taq* polymerase and 0.2 mM dNTPs. The same cycling conditions were used as described above and the amplification products were visualised by electrophoresis as described previously.

## 2.9. Real-time PCR

An Oligo dT Sybr green Sensi mix kit (Quantace) was used for real time quantitative PCR (qPCR) analysis to investigate transcriptional regulation of the HSP70 gene in response to heat stress. Primer pairs for both the gene of interest (HSF and HSR, Table 2.2) and the reference gene (L28F and L28R, Table 2.2) were optimised for use in amplification reactions containing 3 mM  $\text{MgCl}_2$ . The reactions were performed according to the manufacturer's instructions in 12.5  $\mu\text{l}$  reaction volumes in 0.1 ml Strip tubes (Qiagen). Primers were used at a final concentration of 16  $\mu\text{M}$ . An amount of cDNA corresponding to 400 ng of converted RNA was used as template in each real-time PCR reaction. The reactions were analysed in the Rotor-Gene™ 6000 system (Corbett Research) using Rotor-Gene™ Series Software version 1.7. Equal amounts of starting template were used for both control and experimental samples for each gene. The cycling conditions for Ribosomal protein L28 primers were as follows: 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 60°C for 15 seconds and 72° for 20 seconds, with a final extension step at 72°C for 5 minutes. The cycling conditions for HSP70 were as follows: 95°C for 10 minutes, followed by 35 cycles of 95°C for 10 seconds, 65°C for 15 seconds and 72° for 30 seconds, with a final extension step at 72°C for 5

minutes. A no template control was also included for each primer set to confirm the absence of contaminating DNA.

Amplification of single PCR products using each primer pair was confirmed by comparing melt curves. A standard curve was generated for each pair of primers using a ten-fold dilution series of the pooled cDNA. The comparative threshold cycle (Ct) method ( $\Delta\Delta$  Ct) developed by PE Applied Biosystems (Perkin Elmer) was used to calculate the relative fold-changes of HSP70 transcripts between experimental and control samples. The first sampling point (time = 0) was used as the reference sample. The relative expression level values of HSP70, normalized to the reference gene, are presented as  $2^{-\Delta\Delta Ct}$  for both experimental and control groups ( $\Delta Ct = Ct$  of HSP70 minus  $Ct$  of reference gene,  $\Delta\Delta Ct = \Delta Ct$  of specific time point minus  $\Delta Ct$  of time = 0).

#### 2.10. Protein isolation for Western Hybridisation Analysis

Haemocytes (section 2.2) from heat stressed *H. midae* were allowed to thaw on ice for 30 minutes and then washed in anti-coagulation buffer (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6). Haemocytes were re-suspended in 200  $\mu$ l lysis buffer (200 mM NaCl, 1mM EDTA, 0.1 % Triton X100, 1mM phenylmethylsulphonyl, 1mM DTT, 20 mM Tris-Cl pH 7.5) and mixed for ten minutes with 30 second pulses on a vortex mixer. Samples were centrifuged at 9 000 x g for 30 minutes at 4 °C and the supernatant was transferred to a fresh tube. Protein was precipitated at -20 °C for 16 hours using 0.1 M ammonium acetate in methanol. Protein was concentrated by centrifugation at 14 000 rpm for 10 minutes at 4 °C. The pellet was washed twice with 80 % acetone, air-dried and re-suspended in urea lysis buffer (ULB) [8 M Urea, 5

mM dithiothreitol, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5)] for 30 minutes before being stored at -70 °C.

Protein was quantified using the Bradford protein assay (Bradford, 1976) with the following modifications: 900 µl of protein assay dye (Bio-Rad) (1X) was added to 80 µl of 0.1 M HCl and 20 µl of diluted protein. The colour reaction was allowed to develop for 5 minutes at room temperature, after which, colour intensity was measured spectrophotometrically at 595 nm. A standard curve was constructed using bovine serum albumin at concentrations between 1 µg/µl and 0.1 µg/µl in ULB.

#### 2.11. Poly-Acrylamide Gel Electrophoresis (PAGE) and Western Hybridisation

Protein sample application buffer (1M Tris-HCl, 50% (v/v) glycerol, 2% (w/v) SDS, 5 mg bromophenol blue, 1% (v/v) 2-mercaptoethanol, pH 6.8) was added to protein samples (1:5, v/v) and incubated at 95 °C for 5 minutes. Twenty-five micrograms of each protein sample was resolved on a SDS-PAGE gel (4.5% stacking gel at pH 6.8 and 8% separating gel at pH 8.8) at 120 V. A pre-stained protein molecular mass marker (Fermentas, USA) was used to determine the size of the proteins. Samples were separated on duplicate gels and one of these stained with Coomassie Brilliant Blue at 37 °C for one hour. Protein was visualised by incubating the gel in de-stain (25% (v/v) methanol, 10% (v/v) acetic acid) for 16 hrs at 22 °C. Protein separated on the second gel was electro-transferred to a nitrocellulose membrane (Whatman®, Protran® 0.2 µm pore size) using the Bio-Rad Trans-Blot cell (Bio-Rad) at 100 V for 1 hr at 4 °C. The membrane was incubated in blocking solution (5% skim milk powder, 50 mM Tris, 150 mM NaCl, pH 7.4) for 1 hr at 4 °C. The membrane was incubated with 1:5000 anti-HSP70 antibody (Stressgen, Cat # SPA-810) in blocking

solution (5% skim milk powder, 50 mM Tris, 150 mM NaCl, pH 7.4) for 16 hours at 4 °C and then washed with TBST buffer (50 mM Tris, 150 mM NaCl, 1% Tween 20, pH 7.4) for 90 minutes. The membrane was then incubated in 1:10 000 goat-anti-rabbit peroxidase labelled secondary antibody (Kirkegaard and Perry Laboratories, USA) in blocking solution (5% skim milk powder, 50 mM Tris, 150 mM NaCl, pH 7.4) for three hours at 4 °C. The membrane was washed with TBST solution for 90 minutes, with a final wash in 1 X TBS solution. Proteins were visualised chromogenically by incubating the membrane in TBM membrane peroxidase substrate according to the manufacturer's instructions (Kirkegaard and Perry Laboratories, USA) as well as chemiluminescently using the Bio-Rad Immun-Star WesternC chemiluminescent detection kit (Bio-Rad) according to manufacturer's instructions.

Analysis of the western blot data was performed using Quantity One software (Bio-Rad). A volume analysis report was generated and the density (Intensity/mm<sup>2</sup>) of the protein bands determined.

## 2.12. Statistical Analysis

Microsoft Excel (Microsoft Office 2007, Microsoft Corporation) was used to determine the descriptive statistics (mean, standard deviation and standard error) for all data acquired. All statistical analysis was performed using SigmaStat version 3.1 (Systat Software™ Inc. GmbH, 2004). An ANOVA on ranks was performed on transformed (log<sub>10</sub>) qPCR data from the heat stress trial, with the addition of an Ad hoc Tukey test. Differences were considered significant at  $P < 0.05$ .

### 3. Results

#### 3.1 Cloning and sequencing of the *H. midae* HSP70 Gene

In order to assess the feasibility of HSP70 as a biomarker, it was necessary to first identify the gene in *H. midae*. A 523 bp fragment of *H. midae* HSP70 was amplified from genomic DNA (Appendix A1) using HSPDF and HSPDR (Table 2.2) and cloned into the cloning vector pTZ57R/T to create pTZ57R/T70. Restriction enzyme digestion of recombinant plasmid DNA isolated from *E. coli* transformants with *Hind*III and *Eco*RI (Figure 1) and PCR amplification with M13 primers (M13F and M13R, Table 2.2) confirmed the presence of a 523 bp insert (Appendix A2).

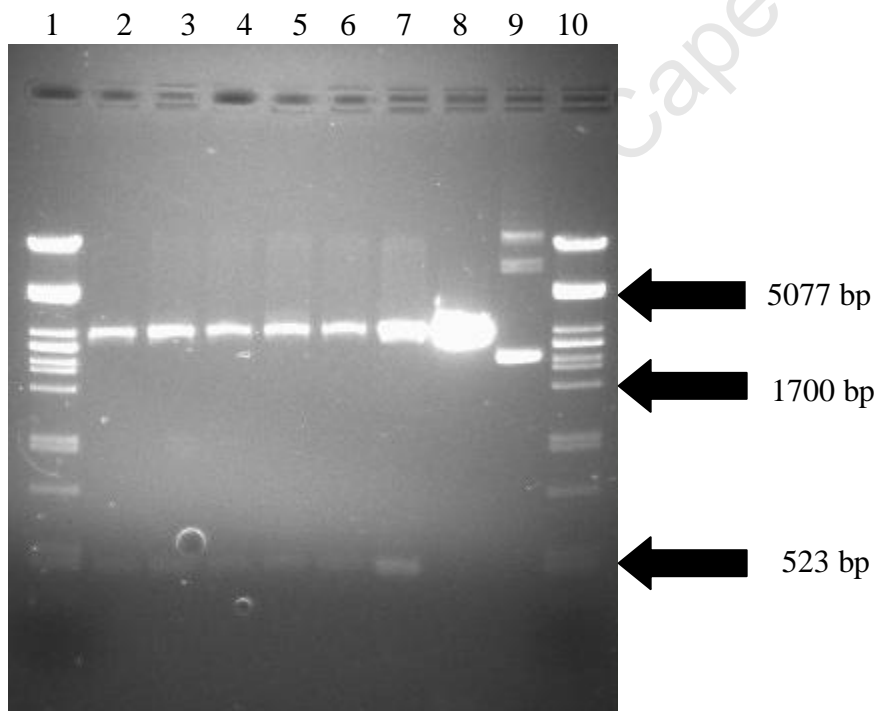


Figure 1: Restriction enzyme digest products of pTZ57R/T70 using *Eco*RI and *Hind*III confirming the presence of a 523 bp insert indicated by the arrow. Lanes 1 & 10:  $\lambda$ *Pst*I molecular marker; lanes 2 – 8 pDNA from positive transformants digested with *Eco*RI and *Hind*III; lane 9: Undigested pTZ57R/T.

The nucleotide sequence of the cloned 523 bp fragment was found to be similar to other HSP70 sequences following a BLASTX search of the GenBank database (Table 3.1). The sequence (Figure 2), designated 1a-pHSP, showed maximum similarity (99%) to the HSP70 gene of *Haliotis diversicolor* (nucleotide accession number ACO36047.1) using the BLASTX algorithm.

Table 3.1: Top ten alignments to *H. midae* HSP70 amino acid sequence following BLASTX search.

Accession number	Protein name	Species name	E value
ACO36047.1	heat shock cognate protein 70	<i>H. diversicolor</i>	3e <sup>-53</sup>
CAK95236.1	71 kDa heat shock protein	<i>H. tuberculata</i>	3e <sup>-53</sup>
ABC54952.1	heat shock protein 70	<i>H. discus hannai</i>	3e <sup>-53</sup>
ABU63809.1	heat shock protein 70 form 2	<i>P. grasslei</i>	6e <sup>-46</sup>
CBM42048.1	heat shock protein-70 kDa	<i>A. pompejana</i>	2e <sup>-45</sup>
ACB47483.1	heat shock protein 70	<i>P. esculenta</i>	5e <sup>-45</sup>
AAW52766.1	HSP70	<i>M. galloprovincialis</i>	6e <sup>-45</sup>
CAH04109.1	heat shock cognate 71	<i>M. galloprovincialis</i>	6e <sup>-45</sup>
XP002733703.1	PREDICTED: heat shock cognate 71 kDa protein-like	<i>S. kowalevskii</i>	8e <sup>-45</sup>
AAO38780.1	heat shock protein 70	<i>C. farreri</i>	8e <sup>-45</sup>

A phylogenetic tree was constructed to determine the evolutionary relationship between the *H. midae* HSP70 amino acid sequence and the HSP70 amino acid sequences from other organisms (Figure 3). Phylogenetic analysis of the HSP70 amino acid sequences identified three groups of

invertebrate HSP70 proteins. Arthropoda HSP70 clustered with HSP70 proteins from bivalves (mussels and oysters), while another group of bivalve HSP70 proteins (clams/scallops) clustered with the HSP70 proteins of the gastropods and annelids. The *H. midae* HSP70 sequence clustered in the phylum Gastropoda. The phylogenetic tree revealed that *H. midae* HSP70 was most strongly related (bootstrap value of 98) to both the *H. tuberculata* and *H. discus hannai* HSP70 proteins. The *H. midae* HSP70 DNA sequence was used to design specific primers for use in real-time PCR (Figure 2).

TGAGAACAAGATCACCATCACCAATGATAAGGGTGTGTGCCAAAATCAAGCCTCATTG  
GGTATTTTTGACGTATCAAATCTAGTTTCGGTTTGATCTCTTTACATTAACATTTAAGC  
TTTATGAACTGTTTCATGTAATGTTTTCTAGATAGCTTGGCAGGTTAATATTTTTAAAGTG  
GACTTATGAAAATTTGAATTTTTCTTCAGGTCGCTTGTCGAAGGAGGAGATTGAGCGTA  
TGGTTAATGAGGCTGAGAACTACAAGGCGGAGGATGAGAAGCAGAAAGATCGTATCCA  
GGCCAAGAATGGTCTGGAAAGCTATGCCTTCAACATGAAGTCAACTGTAGAGGATGAA  
AACTGAAGGACAAGATCAGTGAAGATGACAAGAAGACCATCACTGACAAGTGTAACG  
ATGTCATTAGCTGGTTAGACTCTAACCAACTGGCTGAGAAGGATGAGTTTGAACACAAG  
CAGAAGGAGTTGGAGGGTGTGTGCACCCCGATCATCACCAAGCTGTACCAGA

Figure 2: Nucleotide sequence of a fragment of the *H. midae* HSP70 gene. Sequence specific primers HSPF and HSPR were designed to underlined sequences.

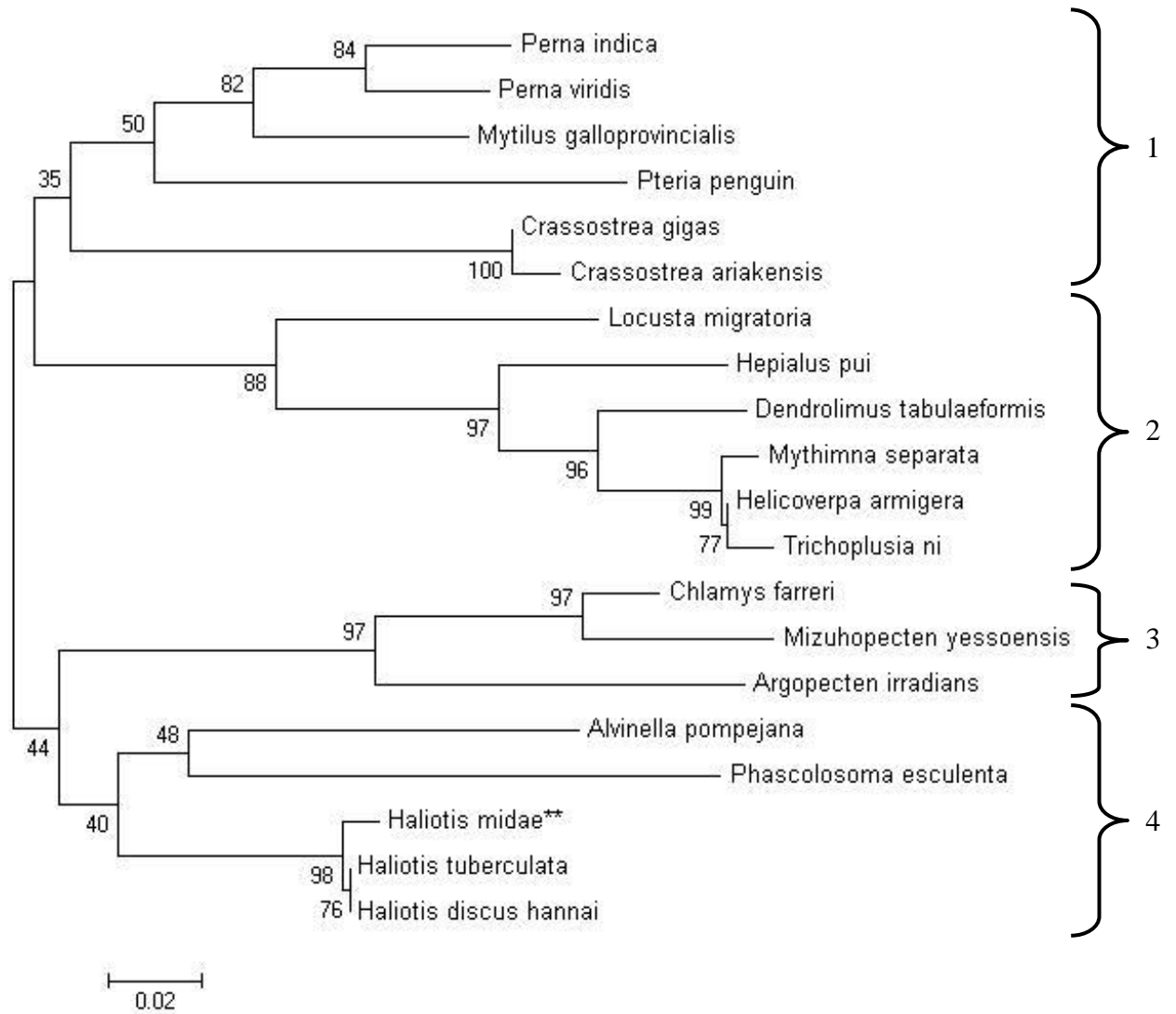


Figure 3: A neighbour joining phylogenetic tree (unrooted) displaying evolutionary relationships between the *H. midae* HSP70 and HSP70 sequences from invertebrate species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches of the tree. Groups 1, 2, 3 and 4 are bivalves (mussels and oysters), arthropods, bivalves (scallops and clams) and gastropods/annelids respectively.

## 3.2. Differential Expression of *H. midae* HSP70 in haemocytes

### 3.2.1. Quantitative Real-time PCR Amplification

The relative levels of mRNA expression of HSP70 in haemocytes sampled from *H. midae* exposed to heat stress was determined using qPCR. The quality of the total RNA isolated for conversion to cDNA was confirmed by visualisation on a 1.2% formaldehyde gel (Appendix B1). High molecular weight contaminants (gDNA and protein) would have been visible in the wells of the gel. Appendix B1 shows no contaminating products in lanes 1-10. The presence of contaminating genomic DNA was further evaluated by amplifying RNA with L28 ribosomal protein primers (Appendix B2). The efficiency of both the gene-specific (HSPF and HSPR) and L28 ribosomal protein primers (L28F and L28R) was evaluated by generation of standard curves (Appendix B3). Melt curves (Appendix B3) for both HSP70 and Ribosomal protein L28 amplifications indicated that a single product was amplified for both primer sets. Standard curves were generated for both the reference and HSP70 genes in order to determine Ct values for normalization and calculation of relative gene expression.

The expression levels of HSP70 mRNA showed an increasing trend after *H. midae* had been exposed to elevated water temperatures for 45 minutes until 150 minutes (7.80 fold) (Figure 4). After 180 minutes (2.45 fold) of exposure to elevated water temperature, the HSP70 mRNA decreased to that of basal levels (T=0). HSP70 expression in *H. midae* haemocytes sampled after 24 hours of recovery from heat stress was at 0.27 fold below basal levels. However, a statistically significant ( $P < 0.05$ ) increase in HSP70 expression levels was only observed after 90 minutes following the onset of heat stress. This was maintained up to 150 minutes of exposure to elevated water

temperature, after which there was a significant decrease in HSP70 mRNA (180 minutes of heat stress) to that of basal levels of expression.

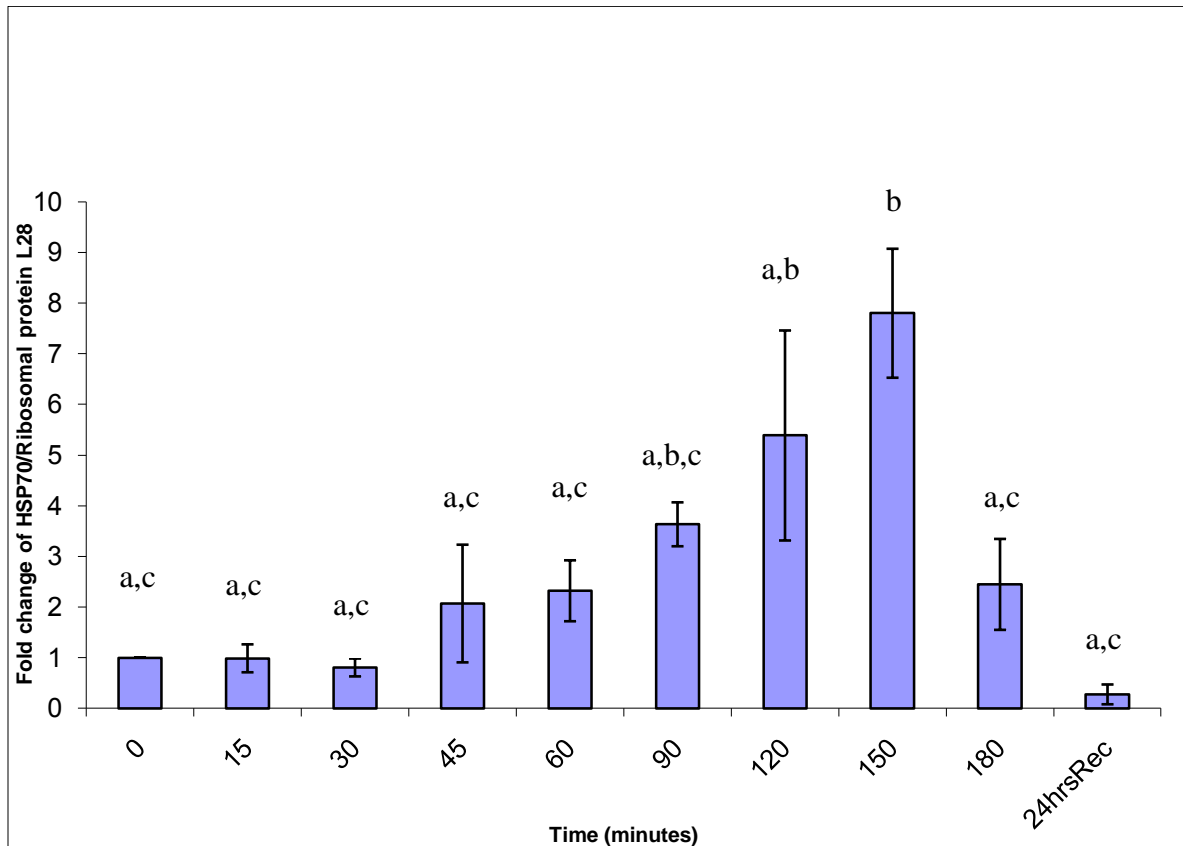
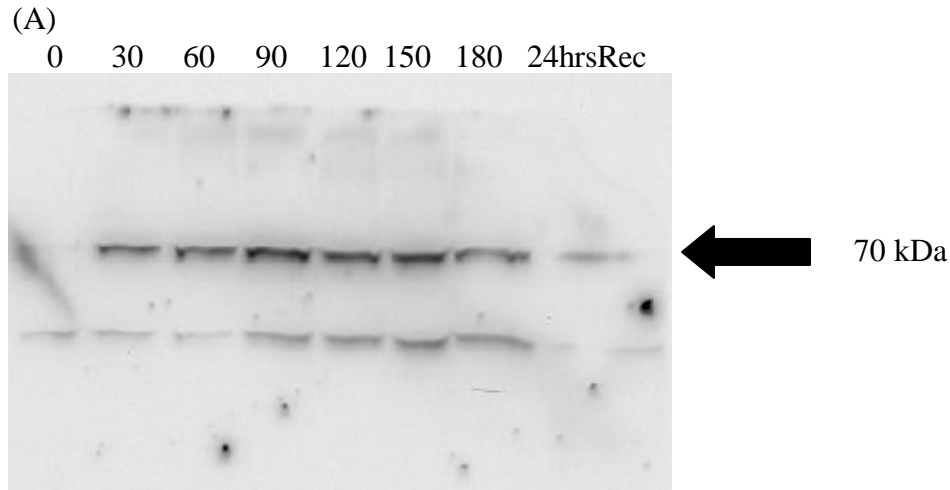


Figure 4: The level of expression of HSP70 in *H. midae* haemocytes, normalized to Ribosomal protein L28 at each time-point after heat shock, as determined by qPCR analysis. The data is presented as the mean  $\pm$  SEM of five pooled individuals, from three independent heat shock treatments. Significant differences ( $P < 0.05$ ) are represented by different letters. Comparative threshold cycle (Ct) method was used for calculating relative fold change with T = 0 as calibrator.

### 3.2.2. Western Hybridisation Analysis of *H. midae* HSP70

Differential expression of HSP70 protein in haemocytes sampled from *H. midae* during heat shock was determined using western blot analysis. Coomassie staining of a duplicate SDS-PAGE gel confirmed equal loading of the protein samples in each lane (Appendix B4).

Hybridisation with anti-HSP70 antibody (Stressgen) produced a single band with the expected size of 70 kDa in all haemocyte samples (Figure 5A). The density of the HSP70 bands detected in the sampled haemocytes was determined and plotted against time (Figure 5B). Expression of the *H. midae* HSP70 protein increased rapidly following the onset of heat shock. Although ANOVA analysis showed that there was a significant difference in fold-change in HSP70 expression over the time course of the experiment ( $P < 0.05$ ), the post-hoc Tukey HSD test showed no significant difference in HSP70 levels between the time points. Expression levels increased after 30 minutes of exposure to heat stress. This increased level of expression was maintained for a further 150 minutes (180 minutes of exposure to elevated water temperature) at which point the water was allowed to cool down to ambient temperature. At 24 hours after recovery from heat stress, HSP70 protein expression had returned to basal levels ( $t=0$  min). However, a statistically significant ( $P < 0.05$ ) increase in HSP70 protein levels was only observed at 90 minutes of exposure to heat stress. It is possible that additional biological repeats are required to increase the power of the statistical analysis.



(B)

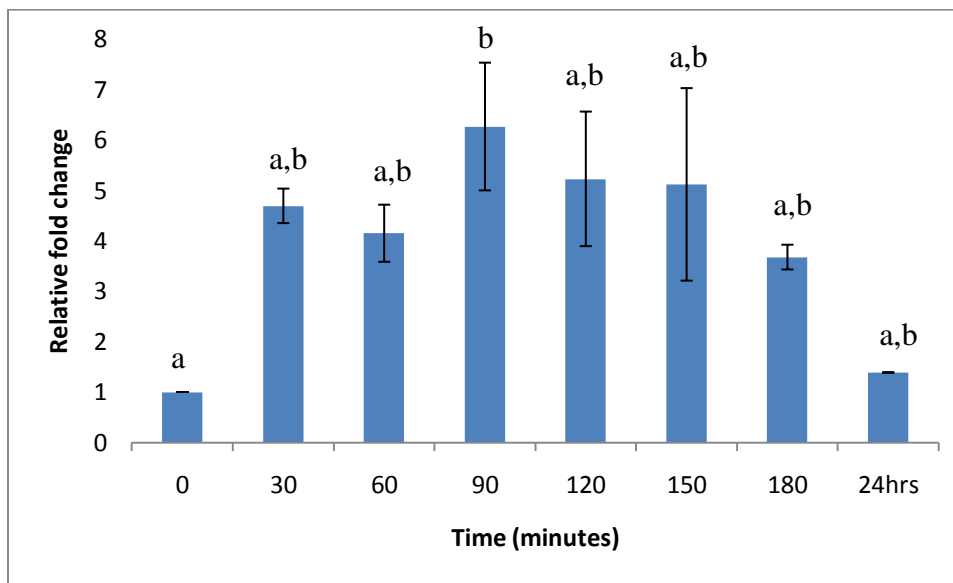


Figure 5: Western hybridisation analysis of haemocyte protein using anti-HSP70 antibody.

(A) Western hybridisation image of the heat shocked haemocyte proteins over the time course of the heat stress experiment; (B) mean fold-change compared to  $t = 0$  of HSP70 protein over the time-course of the heat shock experiment. The data is presented as the mean  $\pm$  SEM of five pooled individuals from three heat shock experiments.

#### 4. Discussion

Currently, knowledge of the immune system of *H. midae* is limited. A better understanding of the abalone immune system and the genes involved in immunity is required to facilitate the development of proper tools for the accurate identification of stress within *H. midae*.

Heat-inducible forms of HSP70 play a central role in stress tolerance by promotion of growth at moderately high temperatures and protecting organisms from death at extreme temperatures (Ravi *et al.*, 2004). Cheng *et al.* (2007) investigated the response of HSP70 mRNA in *H. discus hannai* to heat shock and showed that HSP70 had a defining stress-associated role. Heat shock treatment of cells is known to cause denaturation and aggregation of proteins, disrupt the integrity of cell organelles and inhibit transcription and mRNA translation (Welch, 1992). HSP70 binds heat-unfolded proteins to prevent their aggregation and catalyse their refolding to their native state (Welch, 1992).

There is currently no information pertaining to the regulation of the HSP70 gene in *Haliotis midae*. Several recent studies have greatly advanced our knowledge of the effect of stress on the immune response of abalone, however few studies have contributed to our knowledge of the immune response in *H. midae*. This study has given more insight into the molecular responses of *H. midae* to thermal stress.

The synthesis of HSPs is induced when environmental variation disrupts an organism's physiological system to the extent that its proteins denature. HSP70 is the major stress inducible HSP and is induced by many stressors including heat shock, parasitism, heavy metals and overcrowding (Cheng *et al.*, 2007). HSP70 is a molecular chaperone that plays an important role in promoting the proper folding or refolding of proteins. HSP70 also prevents the potentially damaging interactions of protein aggregations and aids in the disassembling of already formed protein aggregates (Cheng *et al.*, 2007). Stress-inducible HSP70 is one of the well recognised members of HSP family of proteins whose level is enhanced in response to heat (Hofmann and Somero, 1996).

The CODEHOP algorithm was used to generate multiple degenerate primer options. Degenerate primer pairs were selected from the 3' end of the HSP70 sequence alignments. A 523 bp fragment of *H. midae* HSP70 was amplified from genomic DNA using HSPDF and HSPDR and cloned into *E. coli*. Sequence analysis showed that the 523 bp sequence was similar to other HSP70 sequences (Table 3.1). The sequence was designated 1a-pHSP and showed maximum similarity (99%) to the HSP70 gene of *Haliotis diversicolor* (nucleotide accession number ACO36047.1), using the BLASTX algorithm. The deduced amino acid sequence of 1a-pHSP revealed a peptide of 105 amino acids. Alignment of 1a-pHSP with the full length amino acid sequence of HSP70 from *Haliotis discus hannai* revealed the relative location of the isolated fragment. The amino acid fragment 1a-pHSP comprised portions of both the substrate peptide binding domain and the C-terminal domain of *H. discus hannai* isolated by Cheng *et al.* (2007). The results showed that the genes encoding HSP70 are highly conserved between species. The phylogenetic tree analysis showed highest evolutionary homology to two other *Haliotis* species confirming that the isolated fragment was indeed a portion of the HSP70 gene in *Haliotis midae*.

In fish and abalone, heat-inducible HSP70 is expressed at low levels in unstressed cells and is up-regulated in response to stress (Farcy *et al.*, 2007). To our knowledge, this is the first investigation into how *H. midae* HSP 70 gene expression changes over a time course during a heat shock event. It is clear from this study that HSP70 expression is affected by a temperature change at both the mRNA and protein level. mRNA transcripts of the *H. midae* HSP70 gene in haemocytes increased 45 minutes post heat shock. mRNA expression increased steadily for a further 105 minutes after the initial 45 minutes, followed by a sudden decrease at time point 180 minutes. Western hybridisation analysis of the HSP 70 protein showed a marked increase in HSP70 levels 30 minutes after heat shock. HSP70 protein further increased up to 90 minutes post heat shock and then steadily decreased over the next 90 minutes. At 24 hours into recovery, both the mRNA and protein expression levels had decreased to basal levels. Such a rapid response in both mRNA and protein is expected in haemocytes, since haemocytes are the most important feature of any invertebrates' innate immune system. Invertebrates rely heavily on haemocytes as their primary defence mechanism against pathogens and environmental stressors. Farcy *et al.* (2007) reported an increase in HSP70 expression over a one hour heat shock event in cultured haemocytes isolated from *H. tuberculata*. The data showed an increase in HSP70 mRNA at 30 minutes heat exposure and a sharp rise in expression at 60 minutes of heat exposure. No recovery time point was included to assess whether or not HSP70 mRNA levels returned to basal levels of expression. There are currently very few studies which evaluate mRNA and protein expression levels of HSP70 in haemocytes over a heat shock time course experiment.

It is however not conceivable that the HSP70 mRNA would up-regulate after protein expression, as mRNA is required to synthesise protein. The HSP70 multigene family includes both constitutive and inducible forms (Fabbri *et al.*, 2008). The increase in HSP70 mRNA expression at 45 minutes post heat shock (Figure 4) compared to the abrupt increase of HSP70 protein 30 minutes post heat shock (Figure 5) could be explained by the findings of Fabbri *et al.* (2008). It is possible that the HSP70 mRNA expression analysis corresponds to the constitutive form, and the expression analysis of HSP70 protein is indeed the inducible form of HSP70. Prolonged exposure of organisms to stress factors could cause over expression of constitutive forms of HSP70 (Fabbri *et al.*, 2008), which could explain the eventual increase in expression at 90 minutes post heat shock of HSP70 mRNA in *H. midae*.

HSP expression varies among species that differ in thermal tolerance (Feder and Hofmann, 1999), and the range over which HSPs are induced correlates with normally encountered temperatures (Sanders, 1993). Thus, differences in HSP synthesis are a function of an organism's thermal habitat, including intertidal versus sub-tidal location, shallow as opposed to deep water, and the extent of seasonal fluctuations in temperate and tropical environments (Choresh *et al.*, 2001; Norris *et al.*, 1995).

Heat shock treatment of cells is known to cause denaturation and aggregation of proteins, disrupt the integrity of essential organelles and inhibit vital processes such as transcription and mRNA translation. HSP70 binds nascent as well as unfolded proteins to prevent them from aggregating and to catalyse their refolding to the native state (Welch, 1992). Temperatures of 3 to 15 °C above normal body temperature generally result in time-dependant expression of HSPs (Lindquist, 1986).

This was demonstrated by Ireland et al. (2004) in *Fucus serratus* and *Lemna minor*, where HSP70 expression in *L. minor* increased to maximum levels after 4 hours of exposure and then declined to control levels. The data supports the hypothesis that the production of HSP70 mRNA is energetically expensive to the cells, hence the abrupt termination of production. It is likely that transcription of other HSPs follows this abrupt halt in HSP70 production.

Cheng *et al.* (2007) showed that HSP70 mRNA expression in muscle and gill tissues from *H. discus hannai* increased after a heat shock event and returned to basal levels after 96 hours of recovery. Our study focused on the expression of HSP70 in haemocytes within the first three hours of heat stress. Inclusion of a single recovery time point at 24 hours after the heat shock event confirmed that HSP70 mRNA and protein expression had returned to basal levels and that HSP70 was no longer induced. HSP70 expression in different tissues may vary. Since haemocytes are an important component of the primary defence mechanism against pathogens and stressors, this may be a reason for the abrupt increase in expression levels of HSP70 observed in our study.

This study has shown that expression of an *H. midae* HSP70 is regulated in haemocytes upon exposure to an elevated temperature of  $21 \pm 1$  °C. This indicates that this gene may be involved in the immune response of *H. midae* with respect to a heat shock, and thus, is a candidate as a biomarker of stress. Further studies could include the investigation of HSP70 expression in response to stressors such as pathogen infection (*Vibrio anguillarum*), handling and increased environmental toxins, as well as, identifying and characterising HSP70 in different tissues of *H. midae*. Such studies would aid aquaculture facilities in their understanding and control of the stress responses in *H. midae* and further our understanding of HSP70's role in the immune response. Cheng *et al.*

(2007) showed that challenging *H. discus hannai* with heat shock or the pathogenic bacterium *V. anguillarum* resulted in a dramatic increase in HSP70 mRNA levels in muscle and gills.

Selvakumar and Geraldine (2005) showed that induction of Hsp70 protein was observed only in gill and heart tissues of *Macrobrachium malcolmsonii*, and not in hepatopancreas or skeletal muscle. This is evidence of a tissue-specific variation in the stress response in this prawn. This tissue-specific variation of HSP70 expression may also occur in *H. midae*. Further studies on HSP70 mRNA and protein expression in multiple tissues of *H. midae* would improve our understanding of HSP70 expression and its use as a biomarker in *H. midae*.

Singer *et al.* (2005) investigated the induction of HSP70 in *Dreissena polymorpha* exposed to  $\text{Pt}^{4+}$  and found that HSP70 was induced between 43 – 58  $\mu\text{g Pt}^{4+}/\text{g}$  dry weight soft tissue. Investigating the *H. midae* HSP70 response to heavy metal exposure is necessary to comparatively assess the risk of elements extruded into the coastal waters of South Africa and the effect such activities have on aquaculture.

Wang *et al.* (2008) reported the expression of HSP70 in haemocytes of *Pinctada fucata* upon infection with *V. alginolyticus*. HSP70 mRNA increased to maximum levels after 4 hours of infection and returned to basal levels 32 hours post-infection. A similar study performed on *A. irradians* by Song *et al.* (2006) showed an increase in HSP70 mRNA, which reached a maximum level of expression at 8 hours and lasted to 16 hours post-infection with *V. anguillarum*.

A single fragment of the HSP70 gene in *H. midae* was amplified by PCR over the course of this study. Future work may include the use of 5' RACE to amplify the 5' region of the HSP70 gene from *H. midae*. Obtaining additional sequence information for the *H. midae* HSP70 would allow for further bioinformatic and phylogenetic studies.

The development of haemocyte monolayer culture will allow for a less invasive method of studying haemocyte response to stress, both physiologically and at the molecular level. Studies performed by Farcy *et al.* (2007) showed that these *in vivo* studies are indeed feasible. Farcy *et al.* (2007) showed that HSP70 mRNA expression is inducible when haemocyte monolayers were exposed to heat stress.

This study provides the first step to gaining a more in-depth understanding of the *H. midae* immune response. Our current knowledge regarding the abalone immune system is limited and largely reliant on data obtained from bivalves that have been more extensively studied (Hooper *et al.*, 2007). Therefore, further insight into the abalone immune system is necessary if genetic tools are to be developed for detection of pathogenic stress within farmed abalone.

In summary, we have cloned a portion of the HSP70 gene from *Haliotis midae*. The upregulated mRNA and protein expression of HSP70 in the abalone following heat shock indicates that the HSP70 gene is inducible making it an ideal candidate for further investigation into its use as a biomarker.

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6. Appendices

Appendix A

Identification of HSP70 in *Haliotis midae*.

A1 – Amplification of the *H. midae* HSP70 from genomic DNA

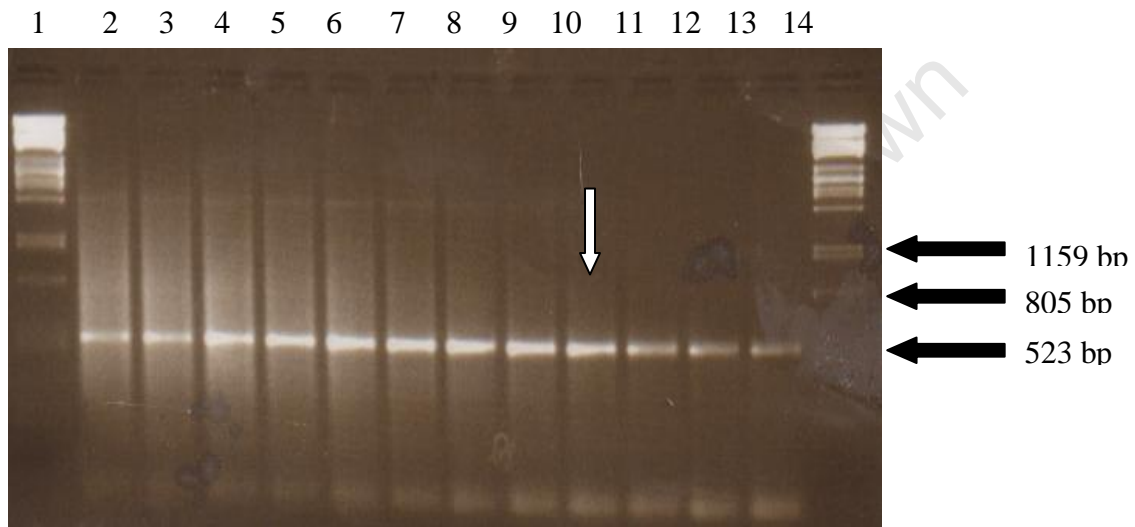


Figure A1: Amplification of the putative HSP70 gene from *H. midae* genomic DNA using the degenerate primers HSPDF and HSPDR. Lanes 1 and 14:  $\lambda$  PstI DNA marker. Lanes 2 – 13: amplified product with increasing annealing temperatures. The 500 bp fragment (lane 10) was purified, cloned and sequenced.

A2 – PCR amplification of pTZ57R/T70 using M13 primers

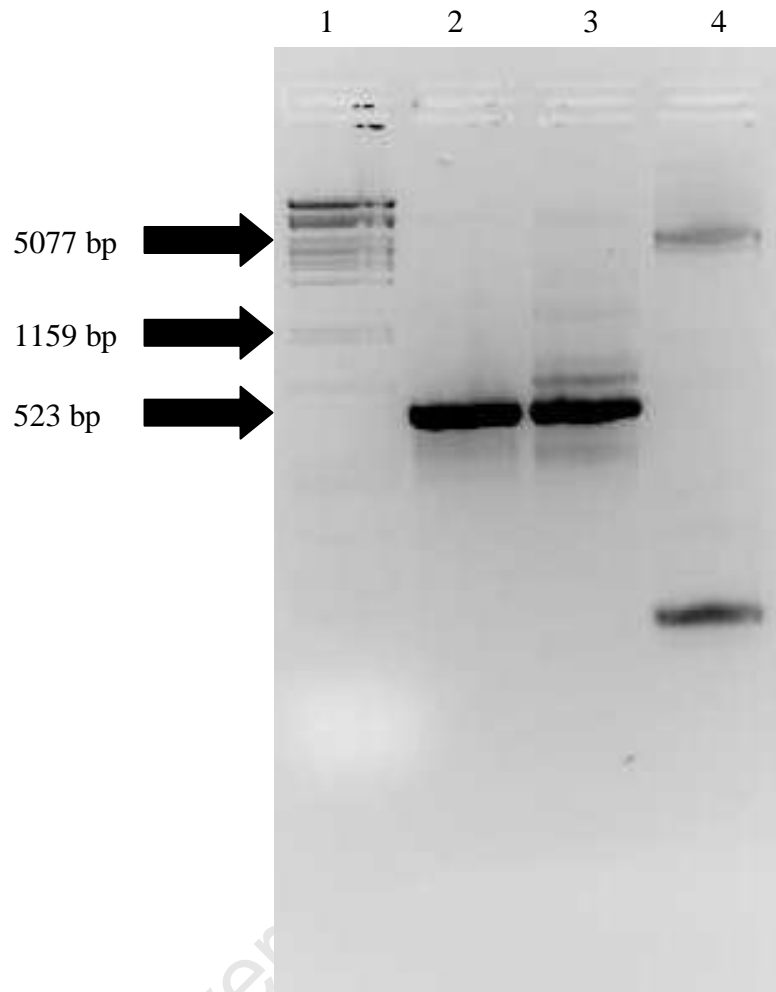


Figure A2: Amplification of cloned vector pTZ57R/T70. Lane 1:  $\lambda$  PstI DNA marker. Lanes 2-3: amplified pTZ57R/T70 displaying 523 bp products. Lane 4: amplified re-ligated vector pDNA.

A3 –  $\lambda$  – phage DNA digested with PstI

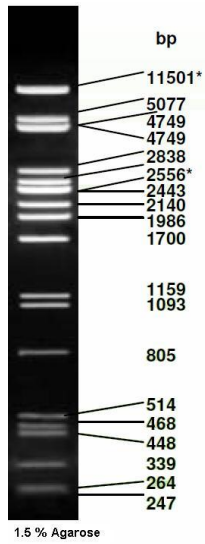


Figure A3: ‘Schematic’ of digested  $\lambda$  DNA used as reference to size electrophoresed DNA.

([http://www.taq-dna.com/phage-lambda-dna-psti-digest-ready-to-use-\\_144.html](http://www.taq-dna.com/phage-lambda-dna-psti-digest-ready-to-use-_144.html))

## Appendix B

### Differential expression of the *H. midae* HSP70 gene

#### Experimental controls

#### B1 – RNA isolation from haemocytes

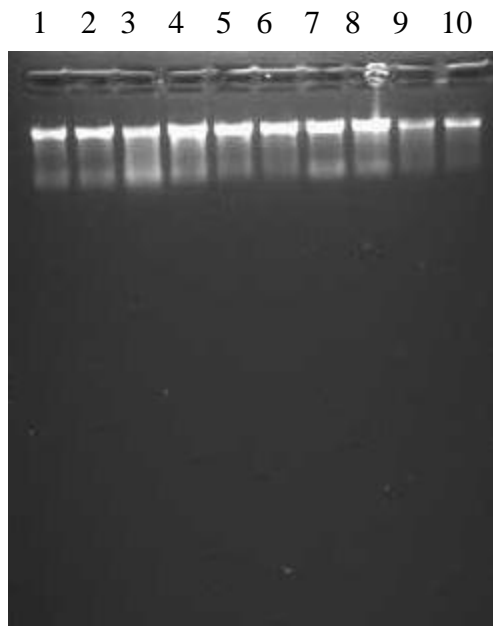


Figure B1: Total RNA isolated from haemocytes sampled from heat shocked *H. midae* electrophoresed on a 1.2 % (w/v) formaldehyde agarose gel. RNA isolated from haemocytes sampled at T=0 (lane 1), 15 minutes (lane 2), 30 minutes (lane 3), 45 minutes (lane 4), 60 minutes (lane 5), 90 minutes (lane 6), 120 minutes (lane 7), 150 minutes (lane 8), 180 minutes (lane 9) of exposure to heat stress and 24 hours recovery (lane 10).

B2 – Control PCR amplification of RNA

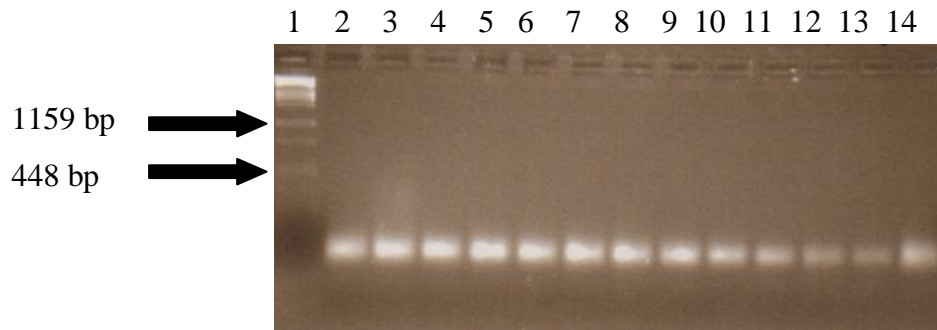


Figure B2: PCR products amplified using L28 ribosomal protein primers (L28F and L28R). Lane 1:  $\lambda$  PstI marker. Lanes 2 – 11: 1  $\mu$ l RNA from time points 0 min – 24 hrs post heat shock. Lanes 12 and 13 show PCR product obtained from 1  $\mu$ l of pooled RNA. Lane 14 is a negative (no template) control. The DNA product at 100bp found in all the lanes are primer dimers of L28.

B3 – Controls and Data obtained from qPCR analysis of *H. midae* HSP70 expression

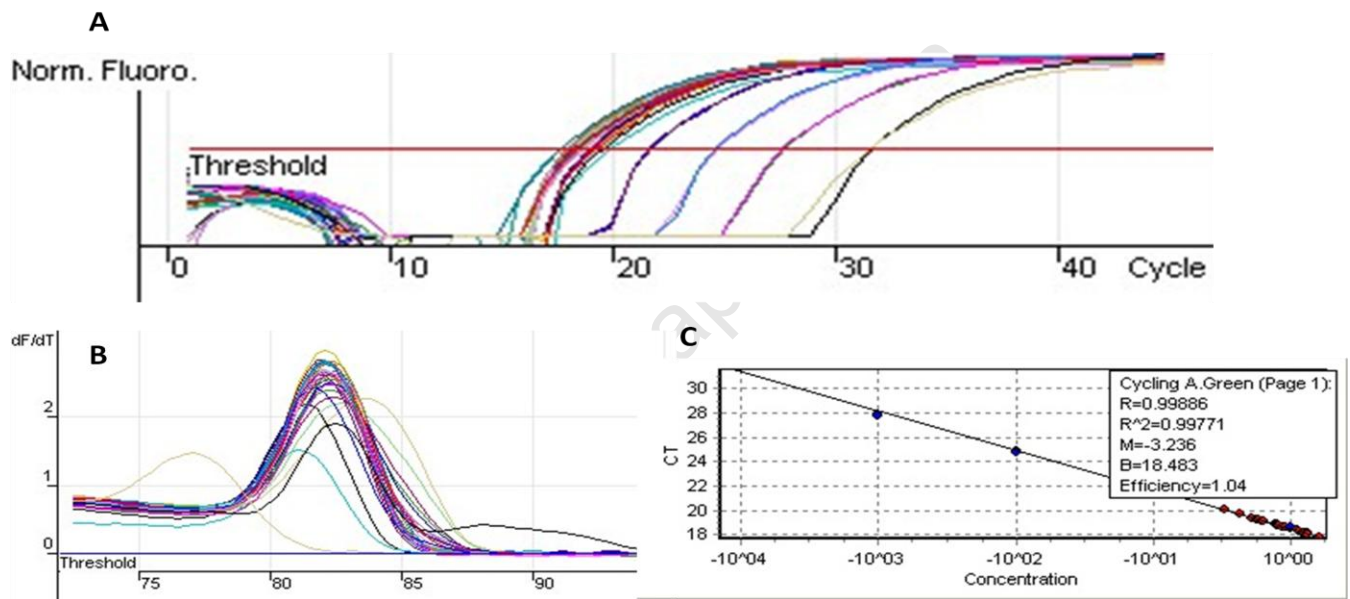


Figure B3.1.1: qPCR profile of the reference gene ribosomal factor L28 amplified from biological repeat 1. (A) run profile of the gene, (B) melt curve, (C) standard curve for the gene.

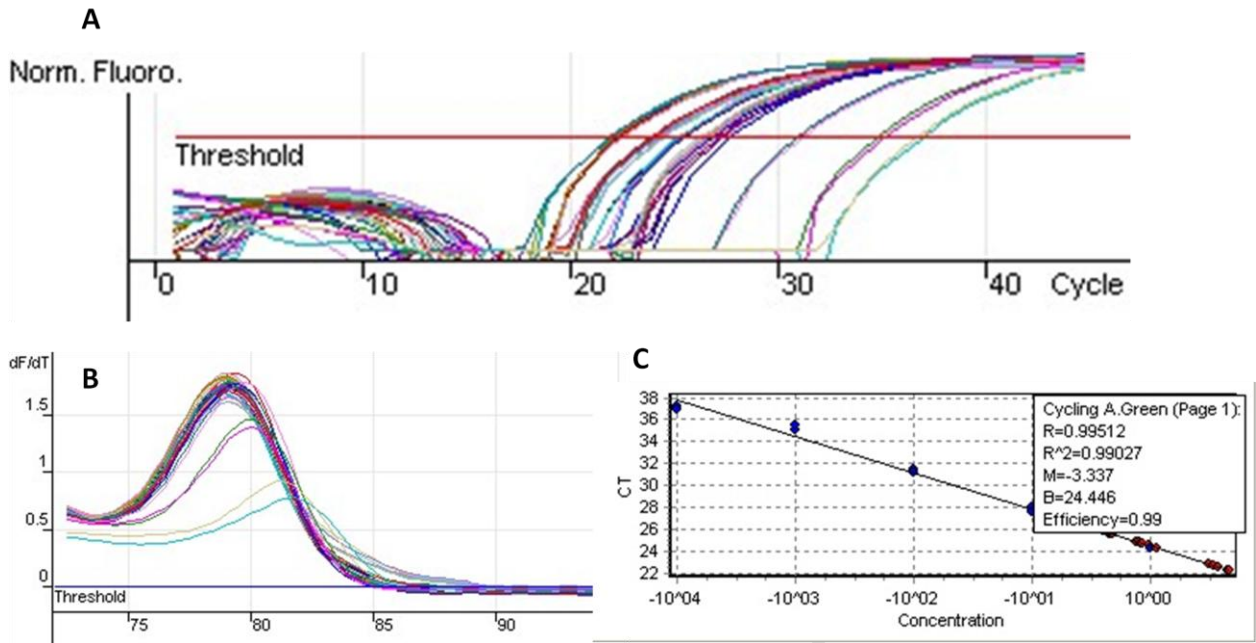


Figure B3.1.2: qPCR profile of the gene of interest (HSP70) amplified from biological repeat 1. (A) run profile, (B) melt curve and (C) standard curve for the gene.

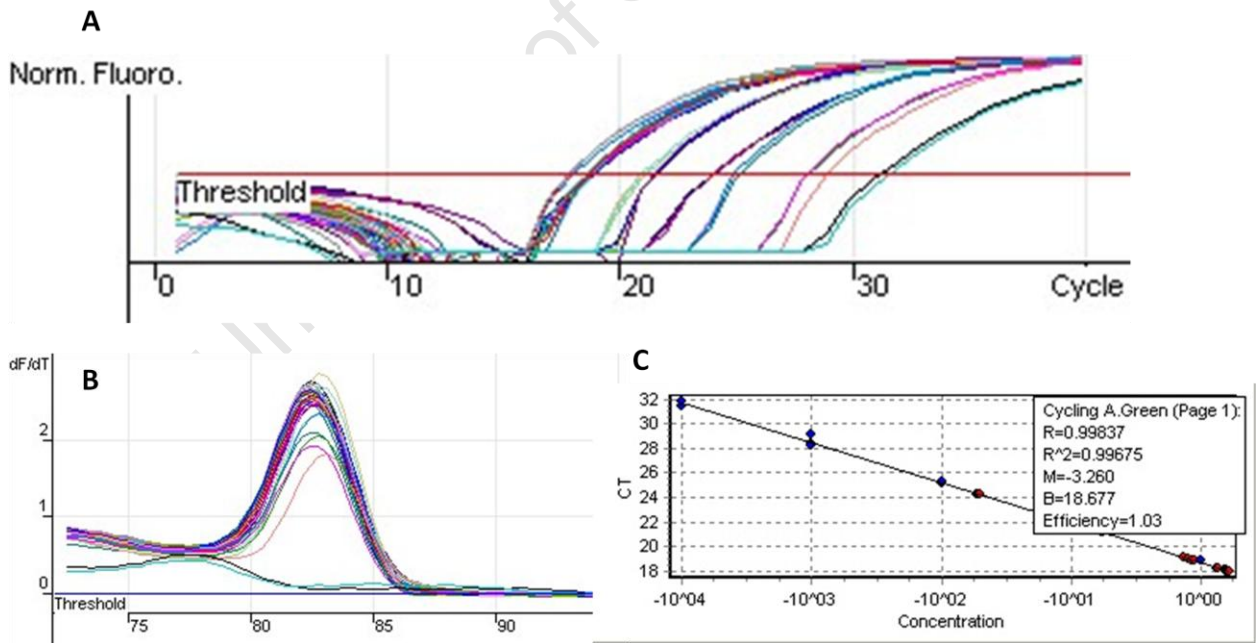


Figure B3.2.1: qPCR profile of the reference gene ribosomal factor L28 amplified from biological repeat 2. (A) run profile, (B) melt curve, (C) standard curve for the gene.

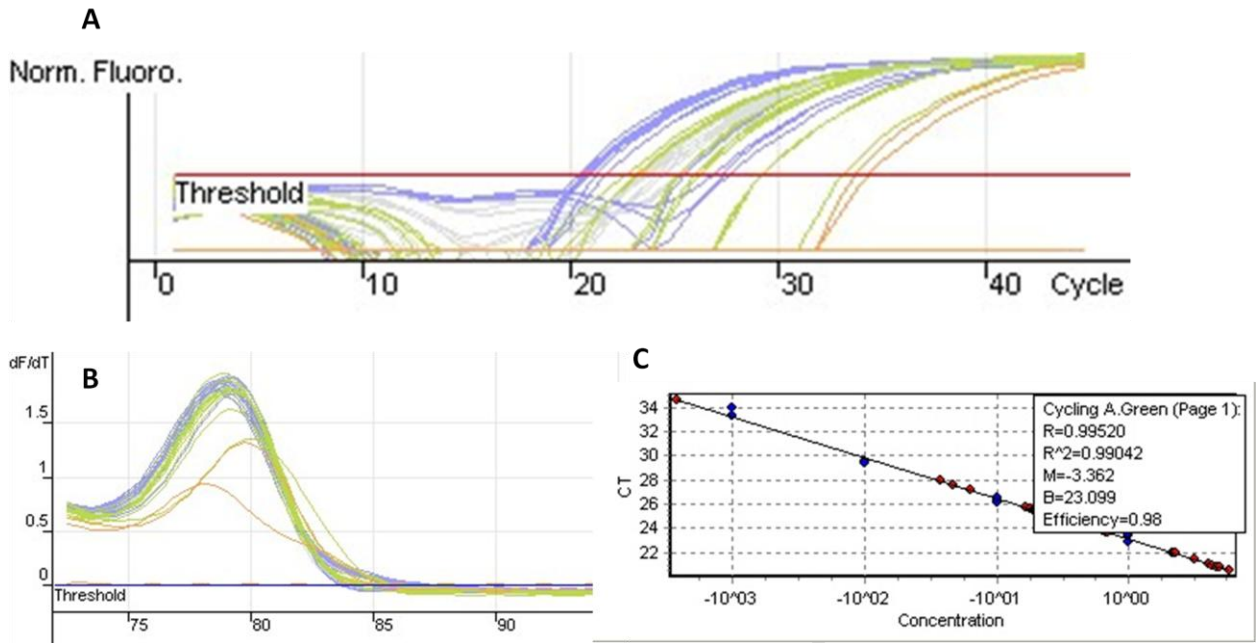


Figure B3.2.2: qPCR profile of the gene of interest (HSP70) amplified from biological repeat 2. (A) run profile (B) melt curve and (C) standard curve for the gene.

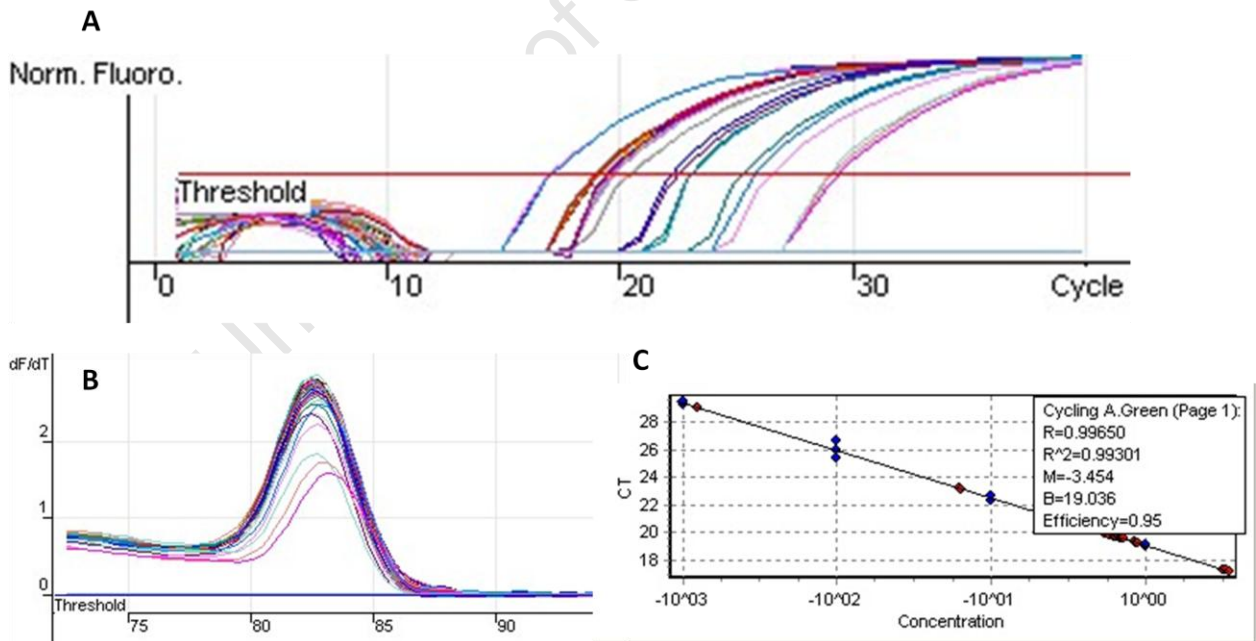


Figure B3.3.1: qPCR profile of the reference gene ribosomal factor L28 amplified from biological repeat 3. (A) run profile, (B) melt curve, (C) standard curve for the gene.

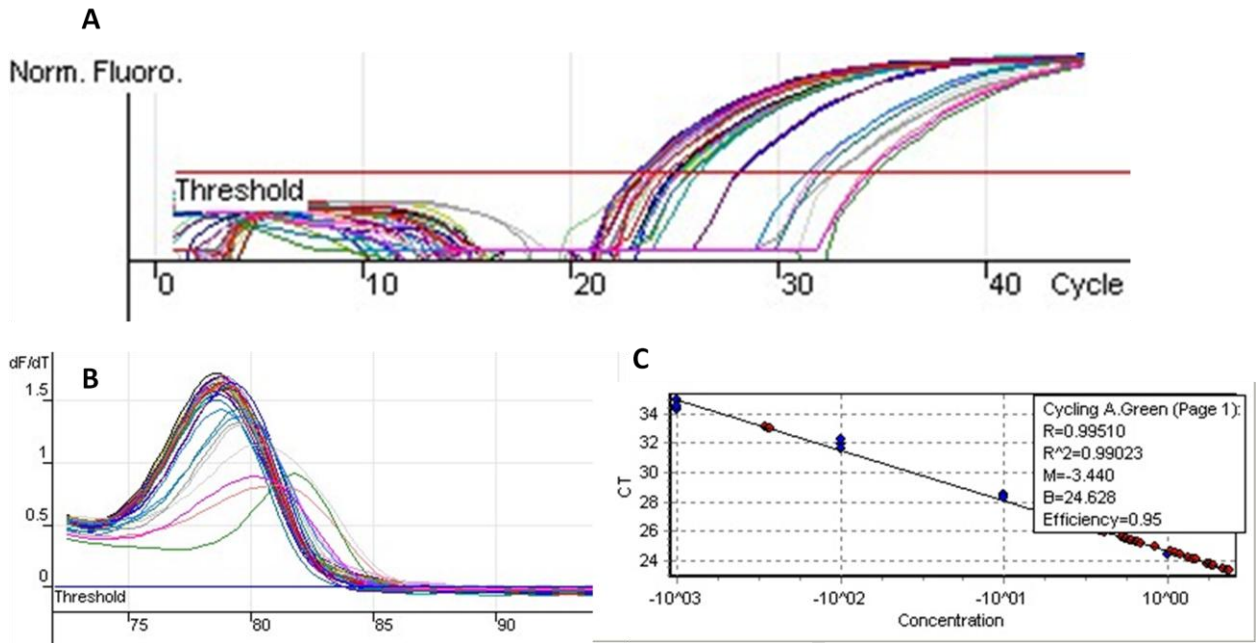


Figure B3.3.2: qPCR profile of the gene of interest HSP70 amplified from biological repeat 3.

(A) run profile, (B) melt curve and (C) standard curve for the gene.

B4 – Protein isolated from *H. midae* haemocytes

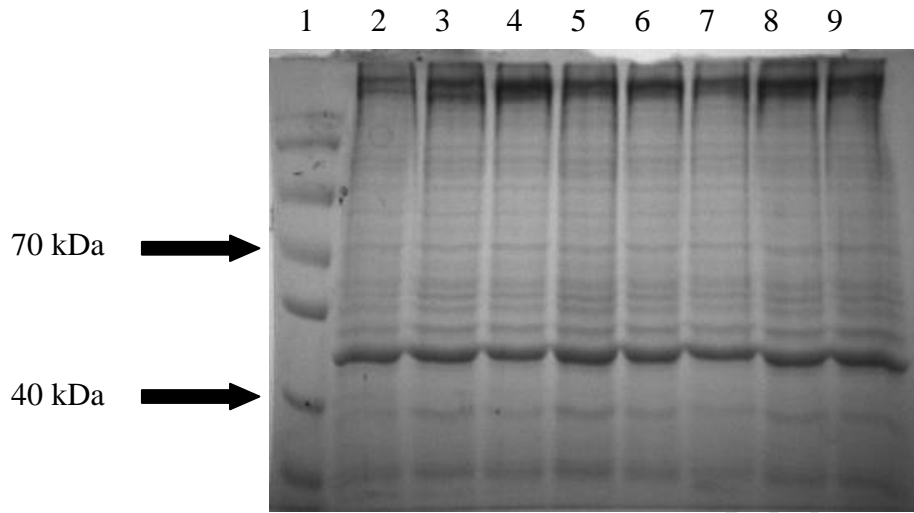


Figure B4: Protein isolated from haemocytes from heat stressed *H. midae*. Lanes 2 – 9 were loaded with 15µg total protein. Lane 1: Molecular weight marker (Bio-Rad). Lanes 2 – 8: Heat stress 0 – 180 minutes. Lane 9: 24 hour recovery following heat stress.