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Heat-shock protein adaptation in abyssal and hadal amphipods

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Abstract

Heat-shock proteins (HSPs) are a prominent family of cellular chaperones that are involved in the folding, assembly and degradation of cellular proteins, cell-cycling and signal transduction. HSPs are highly conserved across taxa and form a key component of the stress response with signatures of molecular adaptation in some species exposed to extreme environmental stressors such as dehydration, heavy metal pollutants and arctic temperatures. Here we characterise two key heat-shock protein genes (hsp70 and hsp90) in deep-sea Lysianassoidea amphipods, with a focus on copy number variation and signatures of selection on the DNA sequences. Four phylogenetically distinct isoforms were resolved for both hsp70 and hsp90, with one isoform in each gene being exclusive to the hadal genus *Hirondellea*. Signatures of purifying selection were shown across hsp70 and hsp90 from dN:dS ratios. The GC content of each gene was lower, and the number of codons used was higher, than in shallow water amphipods suggesting a relaxation in codon usage bias. Such observations suggest that increased hydrostatic pressure is an important environmental stress that shapes the adaptation of heat-shock protein genes in deep-sea amphipods.

Keywords: Heat-shock proteins; Lysianassoidea amphipods; deep sea; adaptation; evolution

1. Introduction

Understanding how natural populations adapt to fluctuating and extreme environmental conditions is an important and enduring theme in evolutionary biology. Elucidating the genetic changes that underpin adaptation to environmental pressures helps to inform the underlying evolutionary drivers that determine patterns of biodiversity, define a species' ability to colonise new ecological niches and ultimately establish the limits of life (Lavergne et al., 2010; Hoffman and Sgrò, 2011). It is also important to understand how the processes driving these patterns are influenced by different environmental conditions in a diverse range of biomes (Poiani et al., 2011). Utilising 'extreme' environments to examine such phenomena provides a powerful opportunity to investigate the patterns of adaptation to a suite of exaggerated selection pressures. These extreme environmental pressures can result in the manifestation of a wide-range of behavioural, physiological or biochemical phenotypes (Stillman and Somero, 1996) where these adaptations can be environment-, taxa- or species-specific (Castoe et al., 2013; Stein and Moser, 2014). A classic example of this are the Cichlid fishes of a hypersaline soda lake that showed adaptation in a suite of highly expressed genes responsible for osmoregulation, energy metabolism, ion transport and chemical detoxification as a response to elevated levels of pH, salinity, temperature and fluctuating levels of O₂ (Kavembe et al., 2015).

One recognised "universal" response to environmental pressures is the production of stress proteins (Kültz, 2005). There is a large complement of stress proteins involved in the stress response but the majority studied are chaperone proteins, which play an essential role in cell function and maintenance (Feder and Hofmann, 1999). The most prominent chaperone proteins belong to the heat-shock protein (HSP) family that are involved in the folding, assembly and degradation of cellular proteins (Beckham et al., 1990), cell-cycling (Nakai and Ishikawa, 2001) and signal transduction (Voellmy, 1994). Heat-shock proteins can be expressed constitutively, or as a response to cellular stress. They are highly conserved proteins, which have been found across most known taxa and many have been shown to have multiple isoforms (Schlesinger, 1990). Two of the most important HSPs are the 70 kDa and 90 kDa proteins, which are known as hsp70 and hsp90, respectively. Hsp70 provides a broad spectrum of essential housekeeping cellular functions involving folding and signal transduction pathway and protein quality control. It has been achieved through the diversification and specialisation of hsp70 chaperones (Mayer and Bukau, 2005). Hsp90 prevents protein aggregation and promotes protein refolding *in vitro* and is an important component of many multiprotein complexes *in vivo* (Prodromou et al., 1997). Hsp90 has also been suggested as a molecular mechanism for uncovering morphological variants that can drive evolutionary change in response to environmental cues (Rutherford and Lindquist, 1998).

Both hsp70 and hsp90 have shown signatures of selection to environmental conditions across a variety of taxa, despite them both being highly conserved proteins. These include biased rates of synonymous to non-synonymous nucleotide substitutions consistent with directional selection (Yang and Bielawski, 2000), an increase in GC content to increase thermal stability (Marmur and Doty, 1962) and a propensity towards codon usage bias that increases translational efficiency (Sharp and Li, 1987). Further adaptation in HSPs have been shown in an increase in gene copy number to facilitate greater levels of expression (Zhou et al., 2011) and hypermethylation of gene sequences to induce expression as a response to environmental stress (Gavery and Roberts, 2010). It is well documented that HSPs have these signatures of adaptation as a response to extreme environmental stressors such as dehydration (Benoit et al., 2010), osmotic stress (Sun et al., 2001), heavy metal pollutants (Lewis et al., 1999) and extreme temperatures (Trent et al., 1990; Clark et al., 2009).

Heat-shock protein adaptation has also been shown as a response to high hydrostatic pressure but only for *in vitro* isolated cells (Takahashi et al., 1997; Salvador-Silva et al., 2001) and bacteria (Aertsen et al., 2004). The deep sea is a prime habitat for examining the *in situ* evolution of HSPs in response to hydrostatic pressure as the increasing depth provides a gradient of hydrostatic pressure in a mostly otherwise stable environment where range expansion in the deep sea has been predicted to mirror a tolerance of hydrostatic pressure (Young et al., 1997; Thatje et al., 2005). A limited number of studies have focused on deep-sea taxa but these have all centred on classical thermotolerance responses to the extreme and fluctuating temperature of hydrothermal vents, which are capable of producing temperatures exceeding 350°C (Ravaux et al., 2003; Shillito et al., 2006; Cottin et al., 2008). These studies have highlighted that HSP responses are essential to survival in such a habitat (Ravaux et al., 2009) but the assertion that HSP responses are a result of strictly temperature adaptation is confounded by the effects of non-thermal stresses associated with hydrothermal vents such as pH, redox state and hydrostatic pressure (Holden and Baross, 1990).

Here we test for classical signatures of selection operating on two key heat-shock protein genes (hsp70 and hsp90) in Lysianassoidea amphipods. This group provides an excellent model system for examining the effects of hydrostatic pressure as they are ubiquitous to bathyal (200 – 2000 m), abyssal (2000 – 6000 m) and hadal (6000 - ~ 10,000 m) depths and are one of the few eukaryote species that have evolved to withstand the extreme hydrostatic pressures of the deep sea (Jamieson et al., 2010). We examine how variation in gene copy number, relative rates of synonymous and non-synonymous substitution, GC content and codon usage bias varies among species, across different deep-sea trenches, and between deep ocean and shallow water species.

2. Materials and Methods

2.1. Sample collection

A total of 12 species from seven families within the Lysianassoidea and one species from the Lanceolioidea were collected over the course of six sampling cruises to seven trenches; Kermadec and Tonga trenches (2007; Cruise SO197), the Japan Trench (2007; Cruise KH0703), the Mariana Trench (2007; KR0716), the Izu-Bonin Trench (2009; Cruise KT0902), the Peru-Chile Trench (2010; Cruise SO209) and the New Hebrides Trench (2013; Cruise KAH1310) (Table 1). In all cases an autonomous full ocean depth rated lander vehicle, which incorporated small baited funnel traps for sample collection, was deployed to the seafloor for up to eight hours (for details see Table 1 and Ritchie et al., (2015)). Upon recovery of the lander vehicles, samples were transferred immediately to 99% ethanol prior to morphological identification in a shore-based laboratory (National Institute for Water and Atmospheric Research, New Zealand, or latterly the Australian Museum).

Total genomic DNA was subsequently extracted from either the sixth pereopod or the whole body of individual specimens from all 13 species using a standard phenol-chloroform approach.

2.2. Characterisation of *hsp70* and *hsp90* genes

Degenerate PCR primers for *hsp70* and *hsp90* were designed from multiple alignments of homologous sequences using PRIFI (Fredslund et al., 2005) where the maximum number of ambiguous positions was set to four bases and the critical ambiguous position distance from the 3' end was set to five bases. For *hsp70* the multiple alignment consisted of crustacean sequences; *Penaeus monodon* (AF474375), *Artemia franciscana* (AF427596), *Eulimnogammarus cyaneus* (JN704343), *Eulimnogammarus varrucosus* (JN704341), *Eulimnogammarus vittatus* (JN704342), *Gammarus lacustris* (JN704340) and *Marsupeneas japonicus* (EF091692). For *hsp90* the multiple alignment consisted of crustacean sequences; *Penaeus monodon* (EF015590), *Fenneropenaeus chinensis* (EF032650), *Marsupeneas japonicus* (AB520827), *Litopenaeus vannamei* (HQ008268), *Metapeneas ensis* (EF470247) and *Scylla paramamosain* (JF265066).

Initial PCR amplification was conducted using the degenerate *hsp70* non-specific and *hsp90* non-specific primers (Supplemental Table 1) where amplification reaction mixes contained 0.2 mM each dNTPs, 2.5 mM MgCl₂, 0.5 μM each primer, 0.5 U of BioTaq DNA polymerase (Bioline), 5-20 ng DNA template in 1^x NH₄ buffer (Bioline: 16 mM (NH₄)_xSO₄, 67 mM Tris-HCl) in a total reaction volume of 20 μL. PCR amplification was performed using a G-storm thermal cycler (G-storm Ltd, Surrey, UK) with the following touch-down conditions:

initial denaturation at 92°C for 2 min, followed by; 20 cycles of denaturation at 92°C for 30s, annealing at 65°C – 55°C (-0.5°C/cycle) for 30s, extension at 72°C for 30s; 20 cycles of denaturation at 92°C for 30 s, annealing at 55°C for 30s, extension at 72°C for 30s before a final elongation step at 72°C for 5 min.

Sequencing using the degenerate primers identified multiple isoforms for both hsp70 and hsp90. Isoform-specific primers were designed for each isoform using the obtained amphipod sequence data. Due to the poor quality and high degradation of DNA in deep-sea animals (Dixon et al., 2004), it is difficult to determine whether the amplification failure of an isoform is a consequence of its absence in a given species or PCR failure as a result of DNA degradation preventing sequencing of a large fragment. Therefore, isoform-specific primers were designed to amplify the full length of the fragment being investigated but isoform-specific primers were also designed to amplify a small part of the fragment to rule out false negatives (Supplemental Table 1). All isoform-specific primers were PCR amplified using the same reaction mixes and PCR conditions as stated above.

Where PCR amplification resulted in multiple banding or insufficient quantity of product bands were picked for re-PCR. PCR products were run on an agarose gel to differentiate the multiple bands before the target bands were picked with a microfilter tip which was then placed into a 0.2µL PCR tube containing an amplification reaction mix of 0.5 µM each primer and 1 U of MyFi DNA polymerase (Bioline) in 5^x buffer (Bioline: 1mM dNTPs, 3mM MgCl₂ and enhancers). Re-PCR amplification was performed using a G-storm thermal cycler with the following conditions: initial denaturation at 92°C for 2 min, followed by; 25 cycles of denaturation at 95°C for 15s, annealing at 65°C for 15s, extension at 72°C for 20s before a final elongation step at 72°C for 2 min.

PCR products were enzymatically purified using ExoSAP-IT and quantified with lambda DNA size standards on a 1% TBE agarose gel. Sequencing was undertaken with an ABI 3730xl automated DNA sequencer (MWG Eurofins Ebersberg, Germany) using the same PCR primers as used in the original PCR.

2.3. Phylogenetic reconstruction

Electropherograms were viewed in MEGA v.6.0.5 (Tamura et al., 2013) and nucleotide alignments were constructed on the webPRANK server (Löytynoja and Goldman, 2010) and confirmed by eye. Sequence identity was also confirmed using NCBI BLASTn (Altschul et al., 1990) before nucleotide sequences were translated to amino acid sequences to check for the presence of stop codons.

The optimal evolutionary models for both nucleotide and amino acid sequences, for both hsp70 and hsp90, were identified by JModelTest 2.1.6 (Darriba et al., 2012) using both

the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). Both AIC and BIC identified the same best-fit models for hsp70 and hsp90 (nucleotide: TN93 + G + I and amino acid: Blosum92 + G + I).

Phylogenetic topologies for both hsp70 and hsp90 were inferred by both maximum-likelihood and Bayesian approaches using PhyML v.3.1 (Guindon et al., 2010) and *BEAST (Drummond et al., 2012), respectively. Maximum-likelihood analyses were conducted with a neighbour-joining starting tree and using nearest neighbour interchange branch swapping using the model of sequence evolution estimated by JModelTest2 but with the parameters estimated by PhyML. The stability of nodes was assessed from bootstrap support based upon 10,000 iterations. Bayesian analyses were run for 10,000,000 generations, sampling every 500,000 trees and using the model of sequences evolution estimated by jModelTest2 but with the parameters estimated by *BEAST. The first 100,000 trees were discarded as burn-in where the partition frequencies among the remaining trees give the posterior probabilities to provide an estimate of clade credibility. The likelihood of observed versus phylogenetically constrained alternative topologies were tested using Shimodaira-Hasegawa tests within CONSEL.

2.4. Nucleotide composition analysis

GC content was calculated for the entire coding sequence of hsp70 in: 1) four deep-sea amphipod hsp70 isoforms (see Table 1 for details); 2) the average deep-sea amphipod hsp70 sequence; 3) the average Antarctic euphausiid hsp70 (*Euphausia superba*; *E. crystallorophias*); 4) a shallow water amphipod hsp70 (*Eulimnogammarus verrucosus*; *E. vittatus*; *E. cyaneus*; *Gammarus lacustris*); 5) a non-amphipod crustacean hsp70 (*Marsupenaeus japonicus*; *Litopenaeus vannamei*; *Penaeus monodon*) using CodonW (<http://www.codonw.sourceforge.net>). GC content was also calculated for the entire coding sequence of hsp90 in: 1) four deep-sea amphipod hsp90 isoforms (see Table 1 for details), 2) the average deep-sea amphipod hsp90 sequence and 3) a non-amphipod crustacean hsp90 (*Marsupenaeus japonicas*; *Litopenaeus vannamei*; *Penaeus monodon*). GC content was compared between groups using t-tests. The number of GC bases in first and second, and third base codon positions were also calculated using the method in Xiang et al., (2015). The percentage of non-synonymous first and second GC codon positions were also calculated for both hsp70 and hsp90 using the phylogenetically closest non-deep-sea amphipod sequence as the baseline changes were measured against.

2.5. Tests for selection

The signatures of selection pressures acting on hsp70 and hsp90 can be identified from the dN:dS ratios calculated using DNAsp v.5 (Librado and Rozas, 2009). A dN:dS = 1 suggests there is no evidence of selection, dN:dS > 1 indicates positive selection and dN:dS < 1 indicates purifying selection. Variances of dN (Var(dN)) and dS (Var(dS)) were also estimated to perform Z-tests of selection where $Z = (dN - dS) / \sqrt{\text{Var}(dS) + \text{Var}(dN)}$ in MEGA v.6.0.5 (Tamura et al., 2013). Z-tests were conducted across all groups of interest to test the null hypothesis of no selection (H0: dN = dS), an alternative hypothesis of positive selection (H1: dN > dS) and an alternative hypothesis of purifying selection (H2: dN < dS).

Maximum-likelihood analysis of sequence evolution of the multiple isoforms of hsp70 and hsp90 was conducted using codeML within PAML v.4.9 (Yang, 1997). Each isoform was tested using two models; a One Ratio model (MO) which assumes the same ω ratio across all branches, and a Free Ratio model (FR) which allows for different ω ratios across branches. Subsequently the two models were compared using a likelihood ratio test (LRT) to determine which model represented a better fit for each isoform.

2.6. Codon usage

The analyses of codon usage bias were conducted using the programme CodonW. The effective number of codons (N_c) was calculated for both hsp70 and hsp90 from codon usage data and visualised using the ggplot2 package (Wickham, 2011) in R v.3.2.4. N_c can range from 20 to 61 where 20 exemplifies extreme codon bias where a single codon is used to code for each amino acid, and 61 represents the equal use of every codon for each amino acid.

The relative synonymous codon usage (RSCU) was also calculated for each of the two genes from codon usage data. RSCU values are the observed frequency of a given codon divided by the expected frequency when there is no codon usage bias. Values greater than one indicate that a codon is experiencing usage more frequently than expected under no selection bias and values less than one indicate a codon is used less frequently than expected. A heat map of RSCU values for each gene was drawn using CIMMiner (<http://discover.nci.nih.gov/cimminer>) by implementing a Euclidean distance method and an average linkage cluster algorithm. Cell colours indicate RSCU values such that blue is <1, black is ~1 and red is >1.

3. Results

3.1. Characterisation of hsp70 and hsp90 genes

Sequencing identified four isoforms within both the hsp70 and hsp90 genes. Hsp70 was characterised in eight species and each species had between one and four isoforms. In total, 945 bp were sequenced in the four hsp70 isoforms which covers the majority of exon 2 through to the majority of exon 5. Within exon 5, 201 bp cover part of the peptide binding domain. Hsp90 was characterised in 12 species and each species had between one and three isoforms. In total, 627 bp were sequenced in the four hsp90 isoforms which covers the majority of exon 7 through to approximately half of exon 10. From exon 7 through to exon 9 and from exon 9 to exon 10 294 bp and 273 bp, respectively, cover part of the ribosomal protein S5 domain.

Sequence analysis revealed no introns were present in any isoforms in both hsp70 and hsp90 genes. A lack of intronic sequences have been previously shown in the Crustacea for hsp70 (e.g. Liu et al., 2004) and in the Lepidoptera for hsp90 (e.g. Sonoda et al., 2006). Isoform sequences have been annotated and deposited into GenBank (Table 1).

3.2. Phylogenetic analysis

The phylogenetic relationships for the four hsp70 deep-sea amphipod isoforms are given in Figure 1 using both nucleotide and amino acid sequences. The four isoforms each formed distinct clades and species were not monophyletic. This was consistent across both the nucleotide and amino acid phylogenies and the relative relationship between the isoform clades did not vary considerably between phylogenies. In both phylogenies hsp70-4 is separated from hsp70-1 to hsp70-3. *Hirondellea dubia* is the only species that has been shown to contain all four isoforms and the isoform hsp70-4 is exclusive to the *Hirondellea* genus.

The phylogenetic relationships for the four hsp90 deep-sea amphipod isoforms are given in Fig. 2 using both nucleotide and amino acid sequences. The four isoforms again formed distinct clades with species not forming monophyletic groupings. The phylogenetic relationships varied between the nucleotide and amino acid phylogenies. No species have been shown to contain all four hsp90 isoforms but *Hirondellea gigas* has three (hsp90-1, 2 and 3) and the hsp90-2 isoform is exclusive to *Hirondellea*.

Additional phylogenies were created for both the hsp70 and hsp90 isoforms to show their relation to shallow water amphipod and wider crustacean sequences that were available on Genbank (hsp70, Supplemental Fig. 3; hsp90, Supplemental Fig. 4; Accession numbers provided in figure legend). The likelihood of our observed topology where the deep-sea and shallow water isoforms were reciprocally monophyletic was significantly

greater than any constrained topology where they were para- or polyphyletic (Shimodaira-Hasegawa Test hsp70, $p < 0.05$; hsp90, $p < 0.05$).

3.3. Nucleotide composition analysis

An amino acid alignment was constructed for hsp70 using the four deep-sea amphipod isoforms, five Antarctic euphausiid isoforms, shallow water amphipods and non-amphipod crustaceans (Supplemental Fig. 1; Accession numbers provided in figure legend). The amino acid alignment of the hsp70 deep-sea amphipod isoforms shows none of the isoforms have any insertions or deletions of whole amino acids. In total, 26 amino acids (8.25%) differed across the four hsp70 isoforms.

An amino acid alignment was also constructed for hsp90 using the four deep-sea amphipod isoforms and non-amphipod crustaceans (Supplemental Fig. 2; Accession numbers provided in figure legend). The amino acid alignment of the hsp90 deep-sea amphipod isoforms shows none of the isoforms have any insertions or deletions of whole amino acids. In total, 23 amino acids (11.06%) differed across the four hsp90 isoforms.

3.4. Tests for selection

Across the four hsp70 isoforms and dN:dS ratios were less than 1, which is consistent with the effects of purifying selection (Table 2). The presence of purifying selection was also tested using the Z-test statistic, which allowed us to reject the null hypothesis of neutral evolution and accept an alternative hypothesis of purifying selection (Table 2). This was also shown for the four hsp90 isoforms (Table 2).

For all isoforms, across both hsp70 and hsp90, the Free-Ratio model (FR) provided a better fit for our data (Table 2). However, this was not shown to be significant in any case meaning we cannot explicitly reject the null One Ratio model (MO) for any of the hsp70 or hsp90 isoforms. As the FR model is not significantly favoured we cannot conclude that there is variable selective pressure acting on the isoforms.

3.4. Codon usage

Across the four hsp70 isoforms GC content varied from 43.72 % in hsp70-4 to 48.26% in hsp70-2 with an average GC content of 45.26% (Table 3). The average GC content of

deep-sea amphipods is significantly higher than the average GC content of Antarctic euphausiids (41.59%; $p < 0.001$), but significantly lower than shallow water amphipods (51.79%; $p < 0.001$) and non-amphipod crustaceans (54.20%; $p < 0.001$). GC content in the deep-sea isoforms has been increased on average by 3.67% from Antarctic euphausiids, and reduced on average by 6.53% from shallow water amphipods and 8.94% from non-amphipod crustaceans. The percentage of GC content in CP1 and CP2 that resulted in amino acid changes ranged from 0.35% in hsp70-2 and hsp70-3, to 5.71% in hsp70-4 with an average of 1.96% across the four isoforms.

Across the four hsp90 isoforms GC content varied from 40.46% in hsp90-1 to 49.95% in hsp90-3 with an average GC content of 47.02% (Table 3). The average GC content of deep-sea amphipods is statistically lower than the average GC content of non-amphipod crustaceans (50.99%; $p = 0.05$). GC content in the deep-sea isoforms has been reduced on average by 3.97% from the average GC content of non-amphipod crustaceans. The percentage of GC content in CP1 and CP2 that resulted in amino acid changes ranged from 1.32% in hsp90-2 to 2.01% in hsp90-1 with an average of 1.79% across the four isoforms.

Codon usage bias for the four hsp70 isoforms varied from 45 codons in hsp70-3 to 51 codons in hsp70-4 compared to the range of 40 to 51 codons in Antarctic euphausiids isoforms, 43 codons in shallow amphipods and 40 codons in non-amphipod crustaceans. Deep-sea amphipods use 4 codons (6.56%) more on average than Antarctic euphausiids, 6 codons (9.84%) more than shallow amphipods and 9 codons (14.75%) more than non-amphipod crustaceans indicating a relaxation in codon usage bias in hsp70. The increased use of codons is also reflected in patterns of codon usage bias from RSCU values, which showed no large selection sweeps towards biased use of codons (Supplemental Figure 5).

Codon usage bias for the four hsp90 isoforms varied from 41 in codons in hsp90-3 to 52 codons in hsp90-1 compared to the 32 codons used in non-amphipod crustaceans. Deep-sea amphipods use 14 codons (23.0%) more on average than non-amphipod crustaceans indicating a relaxation in codon usage bias in hsp90. Again this increased use of codons is reflected in patterns of codon usage bias from RSCU values, which showed no large selection sweeps towards biased use of codons (Supplemental Figure 6).

4. Discussion

Heat-shock proteins play an important role in cell maintenance by performing essential chaperone functions in response to environmental stimuli. Responses of HSPs have been shown across a wide variety of extreme environmental conditions such as high altitudes (Zhong et al., 2000), extreme low (Clark and Peck, 2009) and high temperatures (Trent et al., 1990), solar radiation (Keyse and Tyrrell, 1987) and halophilic environments (Shukla, 2006). Heat-shock adaptation has also been postulated as crucial to colonising the

deep sea (Brown and Thatje, 2014) and this has been shown in the deep sea, with hydrothermal vent shrimp *Rimicaris exoculata* and annelid *Paralvinella grasslei* being able to survive in temperatures exceeding 30°C due, in part, to their heat-shock protein response (Ravaux et al., 2003; Shillito et al., 2006).

The salient finding of this study is the identification of signatures of HSP adaptation in Lysianassoidea amphipods from abyssal and hadal depths. Most strikingly, this current study unveiled four distinct isoforms for both the hsp70 and hsp90 genes. Duplication of genes can be an important mechanism to allow for an increase in gene expression, development of more efficient copies of the genes and it is an important source for novel gene applications (Kondrashov, 2012; Magadum et al., 2013). The multiple copies discovered here for both HSP genes are a signature of selection and may be crucial to the evolutionary potential of deep-sea amphipods. However, it should also be noted that all four isoforms in each of the genes are not present in all species and it is unknown if the distribution of isoforms between species is due to different ecological adaptations.

Notwithstanding, each of the isoforms showed an increase in synonymous substitutions per synonymous site (dS) compared to non-synonymous substitutions per non-synonymous site (dN), which is consistent with the effects of purifying selection (Kimura, 1977). The dN:dS ratio infers the direction and magnitude of natural selection on protein coding genes. A higher number of non-synonymous (dN) changes is consistent with the effects of positive selection whereas an excess in synonymous (dS) substitutions indicates purifying selection is acting to maintain a stable optimum (Yang and Bielawski, 2000). In this case we detected a strong signature of purifying selection, consistent with a scenario that different hsp70 and hsp90 isoforms are optimally adapted to the prevailing environmental conditions. Indeed, purifying selection acting upon HSPs is common across the different HSP families and occurs in wide variety of taxa including hsp90 in fish (Wei et al., 2013), small HSPs in plants (Bondino et al., 2012), hsp70 in mice (McCallister et al., 2015) and GroEL in *E. coli* (Fares et al., 2002).

It has previously been suggested that increases in GC content and codon usage bias are indicative of selection pressures acting to maintain DNA stability and translational efficiency, and that this is often more pronounced in highly expressed genes such as heat-shock protein genes (Grosjean and Fiers, 1982). This has not been shown here. In each isoform of both genes the GC content is lower than in the non-amphipod crustaceans. Previous studies have suggested that a GC rich template may not be necessary for the efficient expression of HSP genes (Kudla et al., 2004). It has also been suggested that in lower organisms the GC content of hsp70 homologs are usually similar to the average GC content of their genomes (Kudla et al., 2004). This is likely true for amphipods as the deep-sea amphipod hsp70 isoforms had an average GC content of 45.26% and the total transcriptome GC content for the shallow water amphipod *Parhyale hawaiiensis* is ~45% (Whittle and Extavour, 2015). Often an increase in GC content is linked with codon usage bias as the majority of bias realised is a selection towards GC third codon positions (GC3)

however, this may not always be true for Arthropods as bias has been shown for both GC3 (Duret and Mouchiroud, 1999) and for AT third codon positions (AT3) (Behura and Severson, 2012). Regardless, no signatures of codon bias were seen for either GC3 or AT3.

The novel finding of this study is the presence of four isoforms in both the hsp70 and hsp90 genes in deep-sea amphipods. Copy number variation in hsp70 has been used to infer the effects of selection in several taxa, including five isoforms in Antarctic euphausiids (Casella et al., 2015), two genes in the hydrothermal vent shrimp *Rimicaris exoculata* (Ravaux et al., 2007), and between two and five copies in different species of *Drosophila* (Bettencourt et al., 2008). The evolutionary divergence between the deep-sea amphipod isoforms is not as pronounced as the Antarctic euphausiids although the majority of their divergence is driven by a mitochondrial isoform (hsp70-D). The most distinctive deep-sea isoform is hsp70-4 as it is the most evolutionary removed from the rest and it is exclusive to species of *Hirondellea* which is the deepest living genus of amphipods.

Hsp90 is known to have two major cytoplasmic isoforms that originated from a duplication event ~500MYA (Gupta, 1995) but it was initially believed that invertebrates only had a single cytoplasmic isoform. A small number of studies have since discovered two hsp90 isoforms in some invertebrate species including the marine crab *Portunus trituberculatus* (Cui et al., 2010) but there have been no studies that have identified multiple isoforms comparable to those that we have resolved in the deep-sea amphipods where *Hirondellea gigas* has three hsp90 isoforms in total. The most distinctive isoform is hsp90-2 which is exclusive to species of *Hirondellea* in the same way as hsp70-4 suggesting these isoforms may be hadal specific.

Within this study the inference of the number of isoforms is based upon a limited amount of sequencing within each allele, and represents a minimum number of variants. Clearly, an improved analysis would incorporate full length sequences of the genes to ensure that no extra variation could be resolved and that the full complement of isoforms has been characterised within each species. In the hsp70 gene it would be interesting to sequence the entirety of the peptide binding regions as well as portions of the gene that contain motifs implicated in ATP/ATG interactions and glycosylation sites as variability in these regions suggests differentiation of isoform function (Casella et al., 2015). Hsp90 isoforms also contain conserved regions called 'signature sequences' which are three N-terminal domains and two middle region domains that may also suggest functional differentiation (Sreedhar et al., 2004). Differentiation of gene expression has been shown between the hsp70 isoforms of two Antarctic euphausiids species (Casella et al., 2015), which suggests they have adapted different response patterns due to their different habitats.

Overall, this study has highlighted a signature of selection in deep-sea amphipod heat-shock protein genes, which forms part of the stress response to hydrostatic pressure. This signature of purifying selection indicates that the heat-shock protein isoforms are well

adapted in Lysianassoidea amphipods to deal with hadal selection pressures although this does not discount the involvement of other genes in the stress response. Our understanding of the subtle interplay between genes will be greatly enhanced with the emerging comparative transcriptomic approaches to evolutionary analyses.

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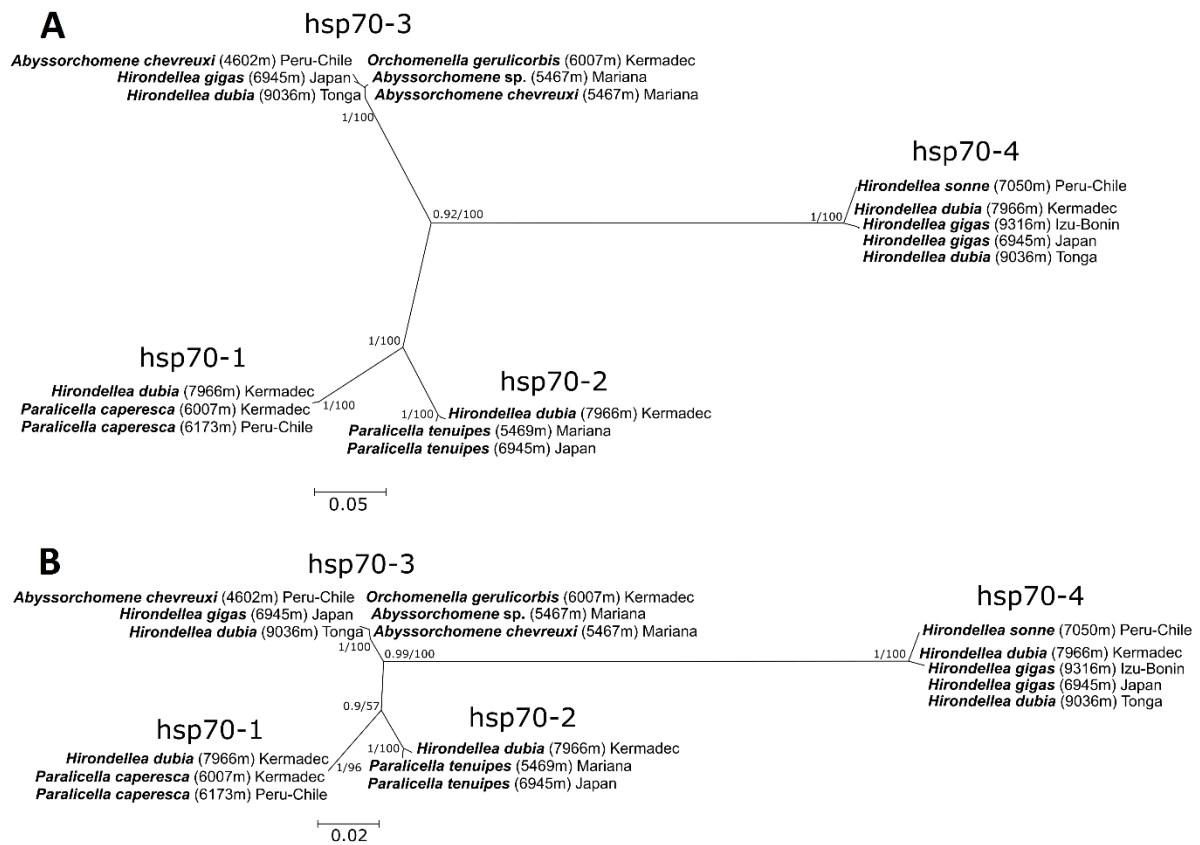


Figure 1. Unrooted maximum-likelihood tree showing the relationship between four deep-sea amphipod isoforms in the hsp70 gene for both A) nucleotide sequences and B) amino acid sequences. Bayesian posterior probabilities and maximum-likelihood bootstrap support are shown on major branch nodes.

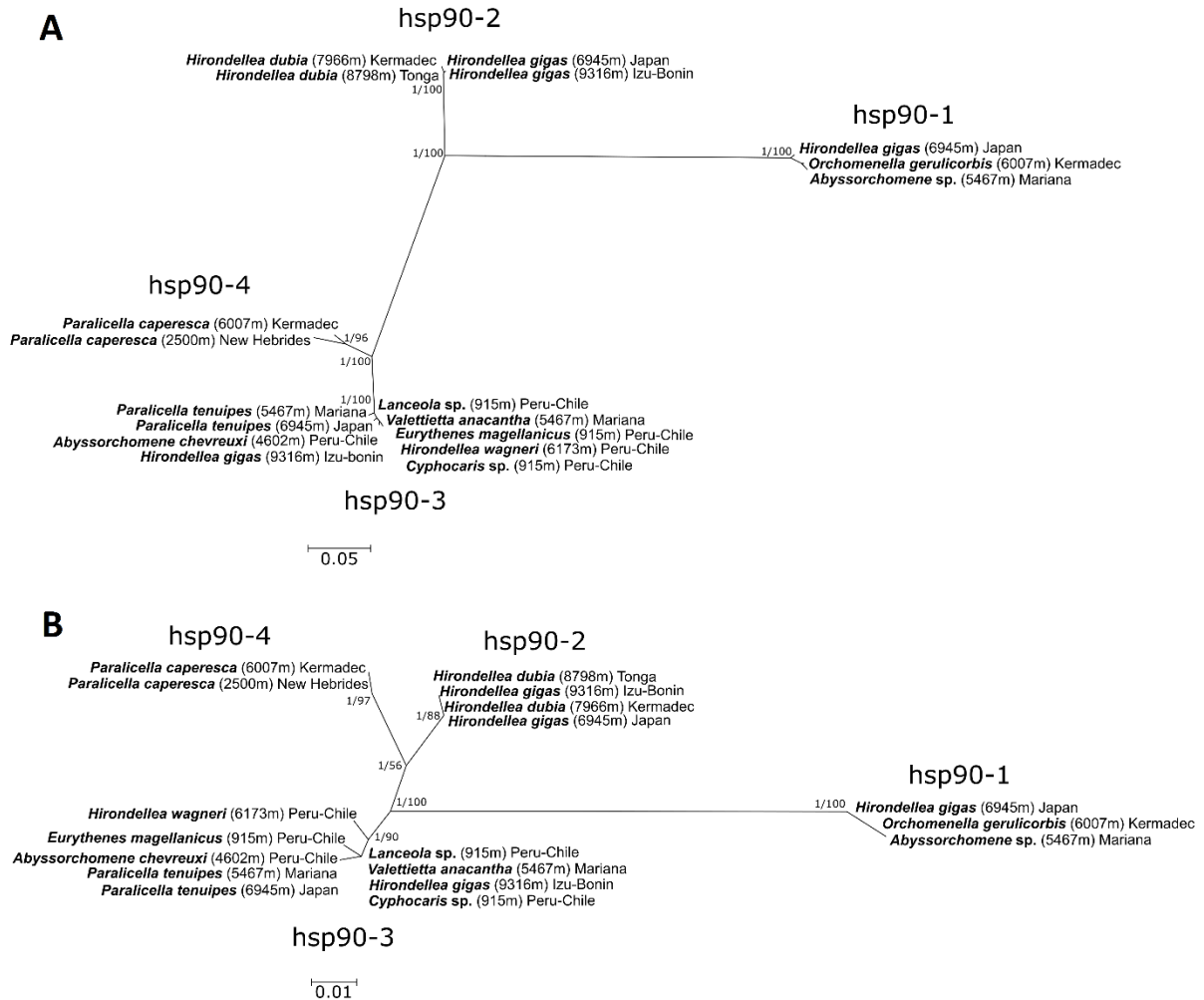


Figure 2. Unrooted maximum-likelihood tree showing the relationship between four deep-sea amphipod isoforms in the hsp90 gene for both A) nucleotide sequences and B) amino acid sequences. Bayesian posterior probabilities and maximum-likelihood bootstrap support are shown on major branch nodes.

Table 1. Sample locations, depth and sequence accession numbers for all samples included in the analysis.

Species	Trench	Depth	Accession numbers							
			Heat-Shock Protein 70 (hsp70)				Heat-Shock Protein 90 (hsp90)			
			hsp70-1	hsp70-2	hsp70-3	hsp70-4	hsp90-1	hsp90-2	hsp90-3	hsp90-4
<i>Abyssorhynchomene</i> sp.	Mariana	546	-	-	KX500	-	KX500	-	-	-
		7m			367		378			
<i>Abyssorhynchomene chevreuxi</i>	Mariana	546	-	-	KX500	-	-	-	-	-
		7m	-	-	369	-	-	-	KX500	-
	Peru-Chile	460			KX500				392	
		2m			370					
<i>Cyphocaris</i> sp.	Peru-Chile	915	-	-	-	-	-	-	KX500	-
		m							384	
<i>Eurythenes magellanicus</i>	Peru-Chile	915	-	-	-	-	-	-	KX500	-
		m							388	
<i>Hirondella dubia</i>	Kermadec	796	KX500	KX500	-	KX500	-	KX500	-	-
		6m	361	365	KX500	374	-	382	-	-
	Tonga	879	-	-	366	-	-	KX500	-	-
		8m	-	-	-	KX500	-	383	-	-
		903				375				
		6m								
<i>Hirondella gigas</i>	Izu-Bonin	931	-	-	-	KX500	-	KX500	KX500	-
		6m	-	-	KX500	373	KX500	380	385-	-
	Japan	694			368	KX500	379	KX500		
		5m				372		381		
<i>Hirondella sonne</i>	Peru-Chile	705	-	-	-	KX500	-	-	-	-
		0m				376				
<i>Hirondella wagneri</i>	Peru-Chile	617	-	-	-	-	-	-	KX500	-
		3m							389	
<i>Lanceola</i> sp.	Peru-Chile	915	-	-	-	-	-	-	KX500	-
		m							386	
<i>Orchomenella gerulicorbis</i>	Kermadec	600	-	-	KX500	-	KX500	-	-	-
		7m			371		377			
<i>Paralicella caperesca</i>	Kermadec	600	KX500	-	-	-	-	-	-	KX500
		7m	362	-	-	-	-	-	-	393
	New Hebrides	250	-	-	-	-	-	-	-	KX500
		0m	KX500							394
		617	360							-
	Peru-Chile	3m								
<i>Paralicella tenuipes</i>	Japan	694	-	KX500	-	-	-	-	KX500	-
	Mariana	5m	-	363	-	-	-	-	390	-
		546		KX500					KX500	

		9m		364					391	
<i>Valettietta</i>	Maria	546	-	-	-	-	-	-	KX500	-
<i>anacantha</i>	na	7m							387	

Table 2. Analysis of directional selection in hsp70 and hsp90 genes in deep-sea amphipods and across other amphipod and crustacean groups using dN:dS, Z-test and codeML models. Likelihood ratio tests were also conducted between the two codeML models. Significance of the Z-test statistic and the LRTs are indicated by p<0.05* p<0.01.**

		dN	dS	dN:dS	Z	Model	Likelihood
hsp70	hsp70-1	0.10	0.86	0.11	18.05**	MO	-1359.83
						FR	-1359.60
	hsp70-2	0.10	0.81	0.12	17.71**	MO	-1318.66
						FR	-1318.66
	hsp70-3	0.10	0.85	0.12	18.05**	MO	-1453.45
						FR	-1451.79
	hsp70-4	0.14	0.84	0.17	16.62**	MO	-1456.87
						FR	-1456.41
	Antarctic A	0.09	0.83	0.12	17.99**		
	Antarctic B	0.10	0.87	0.12	19.11**		
	Antarctic C	0.12	0.88	0.14	18.00**		
	Antarctic D	0.36	0.78	0.46	8.98**		
	Antarctic E	0.09	0.77	0.12	16.97**		
	Shallow	0.11	0.83	0.13	19.30**		
hsp90	hsp90-1	0.17	0.77	0.22	11.86**	MO	-1227.86
						FR	-1224.12
	hsp90-2	0.16	0.78	0.21	11.52**	MO	-893.34
						FR	-892.74
	hsp90-3	0.14	0.63	0.22	8.34**	MO	-1288.27
						FR	-1274.42
	hsp90-4	0.15	0.68	0.22	11.00**	MO	-946.95
						FR	-946.95

dN = number of nonsynonymous mutations, dS = number of synonymous mutations, |Z| = Z-test, and models MO = one omega ratio model, FR = free omega ratio model.

