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Heat shock protein expression during stress and diapause in the marine copepod *Calanus finmarchicus*

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ABSTRACT

Calanoid copepods, such as *Calanus finmarchicus*, are a key component of marine food webs. *C. finmarchicus* undergo a facultative diapause during juvenile development, which profoundly affects their seasonal distribution and availability to their predators. The current ignorance of how copepod diapause is regulated limits understanding of copepod population dynamics, distribution, and ecosystem interactions. Heat shock proteins (*Hsps*) are a superfamily of molecular chaperones characteristically upregulated in response to stress conditions and frequently associated with diapause in other taxa. In this study, 8 heat shock proteins were identified in *C. finmarchicus* C5 copepodids (*Hsp21*, *Hsp22*, *p26*, *Hsp90*, and 4 forms of *Hsp70*), and expression of these transcripts was characterized in response to handling stress and in association with diapause. *Hsp21*, *Hsp22*, and *Hsp70A* (cytosolic subfamily) were induced by handling stress. Expression of *Hsp70A* was also elevated in shallow active copepodids relative to deep diapausing copepodids, which may reflect induction of this gene by varied stressors in active animals. In contrast, expression of *Hsp22* was elevated in deep diapausing animals; *Hsp22* may play a role both in short-term stress responses and in protecting proteins from degradation during diapause. Expression of most of the *Hsps* examined did not vary in response to diapause, perhaps because the diapause of *C. finmarchicus* is not associated with the extreme environmental conditions (e.g., freezing and desiccation) experienced by many other taxa, such as overwintering insects or *Artemia* cysts.

1. Introduction

Marine food webs depend on herbivorous zooplankton such as copepods to process and repackage energy harnessed by photosynthetic primary producers. Representing often more than half of the zooplankton biomass in the temperate North Atlantic (Planque and Batten, 2000; Williams et al., 1994), the calanoid copepod *Calanus finmarchicus* provides an essential route of energy transfer to higher trophic levels either via direct predation or trophic links. The ecological success of *C. finmarchicus* is facilitated by its ability to avoid adverse seasonal conditions and high predation risk (Kaartvedt, 1996) by vertically migrating to depth and entering a facultative diapause during the last juvenile stages (typically stage C5) (Hirche, 1996). In the Gulf of Maine, a portion of the *C. finmarchicus* population enters into this diapause period during the warm spring and summer months and exits during mid to late winter to molt into adults; however, some C5 juveniles skip diapause and proceed to molt into adults, reproduce, and spawn another generation (Durbin et al., 2000, 1997). The appropriate translation of environmental cues into the physiological changes required for diapause in *C. finmarchicus* suggests the involvement of complex but flexible internal regulatory processes. Despite the ecological implications of seasonal dormancy, shockingly little is understood about the factors that regulate the adaptive diapause response in calanoid copepods.

True diapause, an endogeneously regulated process during which organisms undergo progressive physiological changes over several successive phases, is distinct from quiescence, an immediate response to changes in limiting environmental factors that is not restricted to a specific ontogenetic stage (Kostál, 2006). Diapause is characterized by a persistent reduction of metabolism, increased stress resistance, and an arrest of development at a specific life stage (Kostál, 2006). Many aspects of true diapause are observed in the physiology of dormant *C.* finmarchicus; this includes a preparatory phase that precedes unfavorable environmental conditions (Dahms, 1995; Hirche, 1996), a dormancy phase characterized by an endogeneous arrest in development, reduction of metabolism and respiration (Hirche, 1983; Ingvarsdóttir et al., 1999), and gene expression patterns consistent with increased stress resistance (Tarrant et al., 2008), and a distinct post-dormancy phase when development resumes (Hirche, 1996). Therefore, we refer here to the *C. finmarchicus* dormancy as a

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facultative diapause; however, we recognize that not all individuals or populations of *C. finmarchicus* appear to enter a diapause state at the same time or of similar intensity (Hirche, 1996), which is dissimilar from the true diapause observed in other well-characterized organisms (e.g., *Artemia* cysts and overwintering insects).

Diapause in *C. finmarchicus* is identified by a combination of classic behavioral, morphological, and biochemical characteristics: diapausing C5 copepodids accumulate at depths below 200–300 m in oceanic waters (Heath et al., 2004; Miller et al., 1991; Sameoto and Herman, 1990), have empty guts with thin epithelia (Bonnet et al., 2007; Hirche, 1983), and have large oil sacs (Miller et al., 2000). Indicative of arrested development, diapausing copepods have reduced transcriptional activity (i.e., low RNA:DNA ratios) (Wagner et al., 1998), low ecdysteroid levels (Johnson, 2004), and delayed molt progression (i.e., cessation of tooth formation) (Miller et al., 1991). Molecular markers of diapause include low expression of genes related to lipid synthesis, transport, and storage (*ELOV*, *FABP*, *RDH*) and high expression of *ferritin* and *ecdysteroid receptor* (*EcR*) in diapausing copepods (Tarrant et al., 2008). Further exploration of *C. finmarchicus* diapause using molecular techniques is required to understand the mechanisms regulating the physiological changes that characterize the diapause response.

Heat shock proteins (*Hsps*) are a highly conserved superfamily of molecular chaperones that facilitate proper protein folding and localization while preventing protein aggregation (Feder and Hofmann, 1999; Hartl and Hayer-Hartl, 2002). Furthermore, previous research has shown that *Hsps* play a major role in diapause regulation in a wide range of organisms (Denlinger et al., 2001; MacRae, 2010; Qiu and MacRae, 2008a,b; Yuan et al., 1996). Induction of *Hsps* by protein denaturating stressors (e.g., heat and toxins) appears to be an ancient and universal response within all examined taxa ranging from bacteria to plants, flies, and human beings (Hansen et al., 2008; Lindquist, 1986; Sorensen et al., 2003). In addition to their well-known roles in stress tolerance, *Hsps* are integral to normal cell growth and development. Through protein–protein interactions, *Hsps* help to regulate fundamental cellular processes such as protein turnover, mitochondrial and endoplasmic reticulum trafficking, cell cycle progression, and steroid signaling (Beato and Klug, 2000; Helmbrecht et al., 2000; Pratt, 1997; Taipale et al., 2010). During diapause, *Hsps* are thought to contribute to cell cycle arrest and increased stress (e.g., cold) resistance (Denlinger et al., 2001; MacRae, 2010; Rinehart et al., 2007).

Unlike the classic stress response which is characterized by transient and universal upregulation of a wide range of *Hsps*, *Hsp* expression patterns observed during dormancy may be prolonged and highly variable among species and *Hsp* types (Denlinger et al., 2001). Comparison of *Hsp70* regulation during the diapause periods of several insect species demonstrates how the participation of a given class of *Hsp* in the diapause response can differ significantly; for example, *Hsp70* is highly expressed during the pupal diapause of the flesh fly *Sarcophaga crassipalpis* (Rinehart and Denlinger, 2000), but expression is low during the adult diapause of the Colorado potato beetle *Leptinotarsa decemlineata* (Yocum, 2001) and the larval diapause of the bamboo borer *Omphisa fuscidentalis* (Tungjitwitayakul et al., 2008). In addition, different classes of *Hsps* can play distinct roles in dormancy *within* a species, as indicated by discordant expression patterns: in the pupal diapause of *S. crassipalpis, Hsp90* is downregulated, while *Hsp70* and several small *Hsps* are upregulated (Rinehart and Denlinger, 2000; Rinehart et al., 2007, 2000). However in some species, such as in the fruit fly *Drosophila triauraria*, *Hsps* do not appear to participate in diapause at all (Goto and Kimura, 2004; Goto et al., 1998).

In light of the involvement (albeit varied) of *Hsps* in the diapause response of many organisms, we hypothesized that *Hsps* play a role in regulating *C. finmarchicus* diapause. In addition to their role in the stress response, *Hsp70* and *Hsp90* expression varies during the crustacean molt cycle (Cesar and Yang, 2007; Spees et al., 2003) and could therefore help regulate the developmental delay associated with diapause in *C. finmarchicus*. Production of *Hsp70* and *Hsp90* transcripts in *C. finmarchicus* has been previously examined in response to stressors such as increased temperature and exposure to toxins (Hansen et al., 2007, 2008; Voznesensky et al., 2004), but not as a factor regulating diapause. Small *Hsps* have been shown to play an important role in stress tolerance during diapause in the crustacean *Artemia franciscana* (Clegg et al., 1999; Qiu and MacRae, 2008a,b), but have yet to be investigated in calanoid copepods. In this study, we examined the expression patterns of several large and small *Hsps* (*Hsp90*, 4 forms of *Hsp70*, *Hsp21*, *Hsp22*, *p26*) in individual diapausing and active *C. finmarchicus* C5 copepodids by quantitative real-time PCR (qRT-PCR). We also characterized expression of these genes following exposure to handling stress to identify inducible forms of *Hsps* and to confirm that diapause-associated patterns of *Hsp* expression were not a by-product of incidental stressors. We further examined the phylogenetic relationships among the large *Hsps* to facilitate comparisons with inducible or diapause-associated *Hsps* in other taxa. This study represents the first characterization of *Hsp* expression in association with diapause in a calanoid copepod species and expands the current understanding of the molecular regulation of diapause.

2. Materials and methods

2.1. Identification and cloning of C. finmarchicus Hsp genes

Material for initial cloning of *Hsps* was obtained from bulk samples of *C. finmarchicus* C5 copepodids that were collected in 2005, preserved in RNAlater (Ambion) at −80 °C, and described previously (Tarrant et al., 2008). We searched the NCBI Expressed Sequence Tag (EST) database for *C. finmarchicus Hsp* sequences using the tblastn algorithm with selected crustacean *Hsp21*, *Hsp22*, *p26*, *Hsp70* and *Hsp90* sequences (Table 1). Specific primers were designed and commercially synthesized (Eurofins MWG Operon) to amplify partial *Hsp* sequences for cloning. *Hsps* were

Table 1
Annotation of cloned *Calanus finmarchicus Hsps*. Accession numbers are given for the selected crustacean *Hsps* used to search the EST database ("BLAST input") and the subsequent *C. finmarchicus* EST hits. Portions of each EST sequence were cloned in the present study. The percent identity was calculated as the percentage of identical amino acids in the input sequence relative to the *C. finmarchicus* EST.

Gene name	BLAST input	*Calanus* EST hit	EST length (bp)	[a]*E*-value	Input/EST identity (% identity)	Cloned sequence length (bp)
Hsp90	*Metapenaeus ensis* (ABR66910)	ES414827	413	7.00E−41	104/128 (81)	361
Hsp70A	*Artemia franciscana* (AAL27404)	EL965576	667	1.00E−71	152/221 (53)	630
Hsp70B	*Artemia franciscana* (AAL27404)	EH666605	650	6.00E−57	110/205 (53)	586
Hsp70C	*Artemia franciscana* (AAL27404)	ES237720	665	1.00E−55	110/211 (52)	637
Hsp70D	*Artemia franciscana* (AAL27404)	FG342764	496	5.00E−52	102/164 (62)	463
Hsp21	*Artemia franciscana* (ABD19712)	EH667182	700	4.00E−07	30/102 (29)	536
Hsp22	*Artemia franciscana* (ABD19713)	FK041659	665	1.00E−09	30/87 (34)	502
p26	*Artemia franciscana* (ABC41138)	EH666286	634	3.00E−13	34/97 (35)	551

[a] *E*-values based on BLAST search on 5 August 2010.

amplified from pooled *C. finmarchicus* cDNA using 0.25 μl Amplitaq gold polymerase per 50 μl reaction. The PCR conditions were as follows: 94 °C/10 min; 40 cycles of 94 °C/15 s, 60–67 °C/30 s, 68 °C/7 min; hold at 4 °C. Products were visualized on 1% agarose gels, excised, and purified using the MinElute gel extraction kit (Qiagen). PCR products were cloned into pGEM-T Easy (Promega) and sequenced.

2.2. Phylogenetic analysis

The phylogenetic relationships among the *C. finmarchicus Hsp70* and *Hsp90* partial sequences and *Hsp*s from a broad selection of taxa were determined using maximum likelihood analyses. We retrieved representative *Hsp70* sequences from yeast, bacteria, red algae, human, insects, and crustaceans (Table B.1), including two *Hsp70* sequences that had previously been reported from *C. finmarchicus* (Hansen et al., 2008; Voznesensky et al., 2004). The selected *Hsp70* sequences encompass all four monophyletic groups of eukaryotic *Hsp*70s as defined by intracellular localization (i.e., cytosol, endoplasmic reticulum, mitochondria, and chloroplast) (Boorstein et al., 1994; Daugaard et al., 2007; Rhee et al., 2009). Similarly, for phylogenetic analysis of *Hsp90* we retrieved sequences from yeast, plants, human, nematodes, insects, and crustaceans that are representative of the four eukaryotic *Hsp90* subfamilies: cytosolic (*Hsp90A*), endoplasmic reticulum (*Hsp90B*), mitochondrial (*TRAP*), and chloroplast (*Hsp90C*) (Chen et al., 2006) (Table B.2). This dataset also included one *Hsp90* EST previously examined in *C. finmarchicus* (Hansen et al., 2007). Using the multiple sequence alignment program MUSCLE (Edgar, 2004), we aligned the *Hsp* sequences and then trimmed the variable 5′ and 3′ regions for a total sequence length of ~650 amino acids for each *Hsp* family. We first conducted analyses of the *Hsp70* and *Hsp90* sequences in order to classify the *Hsp* subfamily types represented by our cloned sequences. Maximum likelihood analyses were run using RaxML (v7.0.4, Stamatakis, 2006) under a RTREV+G model of protein evolution (selected by AIC with ProtTestv1.4, Abascal et al., 2005). In a second analysis we retrieved additional insect *Hsp70* and *Hsp90* sequences that had been studied for their role in diapause (reviewed by MacRae, 2010). We aligned these sequences with representative *Hsp*s from each subfamily and conducted maximum likelihood analyses as described above. Support for nodes was assessed as a proportion of 1000 bootstrap replicates and the most likely trees were constructed and visualized in FigTree v1.3.1 (http://www.tree.bio.ed.ac.uk/software/figtree/) with bootstrap values >50% reported.

2.3. Handling stress effects on gene expression

To test whether handling stress affects *Hsp* expression in *C. finmarchicus*, shallow C5 copepodids were collected in the southwestern Gulf of Maine at a station 25.5 km east/northeast of Chatham, Massachusetts, USA (41° 46′ N/69° 38′ W) on 18 May 2010 during a cruise aboard the NOAA Ship *Delaware II*. Zooplankton were collected between 13:10 and 13:30 local time from 0 to 20 m using a 70-cm diameter ring outfitted with a 150 μm conical mesh net. Once the net was recovered, the contents of the cod end were poured onto a 150-μm mesh sieve and 10 ml of copepods were added to each of three, covered, black, 7.6-l ice-chilled containers of ambient filtered seawater stored in several closed ice chests. To mimic the stress that may be experienced during extended waiting times associated with the processing of collected copepod samples, each 7.6-l ice-chilled container was left for a specific time (t = 0, 2, 3 h) before its contents were gently sieved and transferred to an ice-chilled Petri dish. From the Petri dish, live *C. finmarchicus* C5 copepodids were individually captured using a wide-bore glass Pasteur pipette, mounted on a depression slide, photographed on a stereomicroscope equipped with a digital camera (see Section 2.4), preserved in microcentrifuge tubes with 500 μl RNAlater (Ambion), and stored at −20 °C until analysis. For this study, we pooled three C5 copepodids per tube to increase sample RNA yield for 10 (t = 0 h and 2 h) and 9 (t = 3 h) total samples per treatment. For each of the three time points, we used qRT-PCR (see Section 2.6) to measure expression of six selected *Hsp* transcripts (i.e., *Hsp70A*, *Hsp70B*, *Hsp70D*, *Hsp90*, *Hsp21*, and *Hsp22*; in a pilot study *Hsp70C* was not reliably amplified and *p26* did not appear to be induced) and three previously identified molecular markers of diapause (*ELOV*, *RDH*, *ferritin*) (Tarrant et al., 2008).

2.4. Sampling of deep and shallow C. finmarchicus

To assess the relationship between diapause and *Hsp* expression, *C. finmarchicus* C5 copepodids were collected in the southwestern Gulf of Maine during a cruise in 2006 aboard the NOAA Ship *Albatross IV* using a 1-m^2 Multiple Opening-Closing Net and Environmental Sensing System (MOCNESS) (Wiebe et al., 1976) outfitted with 333 μm mesh nets. Zooplankton were collected in two depth strata: 169–208 m and 0–50 m (hereafter referred to as the deep and shallow samples, respectively). On 20 May 2006 the deep sample was collected at a station in Franklin Basin just to the north of Georges Bank (41° 54′ N/68° 16′ W) between 11:15 and 11:50 local time. The shallow sample was collected at a station 83 km to the west of the Franklin Basin station in southern Wilkinson Basin (41° 53′ N/69° 17′ W) between 17:08 and 17:21 local time on the same day. Water depths at the stations where the deep and shallow samples were collected were 220 m and 198 m, respectively. Upon recovery of the MOCNESS, the contents of the cod end were immediately poured into a transparent, ice-chilled 1.5-l container and stored in a closed ice chest. Live *C. finmarchicus* were periodically transferred from this container to an ice-chilled Petri dish with a 44-ml Pasteur pipette. From the Petri dish they were individually captured using a wide-bore glass Pasteur pipette, mounted on a depression slide, photographed, individually preserved in microcentrifuge tubes with 500 μl RNAlater (Ambion), and frozen (−20 °C). Observations of gut contents or fecal pellet production were noted while viewing the live animals. Photographs of single animals were taken with a Canon EOS-20D digital camera mounted on a Zeiss Stemi 2000C stereomicroscope, and all measurements were calibrated with digital photographs of a stage micrometer taken just prior to sampling. The length, width, oil sac volume, and fractional fullness (after Miller et al., 2000) of each copepodid were estimated from these photographs as described previously (Tarrant et al., 2008).

2.5. RNA extraction

Total RNA was extracted from preserved individual or pooled C5 copepodids using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad) with slight modification. C5 copepodids were homogenized in 1 ml PureZOL using a teflon homogenizer. The homogenate was added to pre-spun (16,000 × g/30 s) Phase Lock Gel Heavy 2 ml tubes (5 PRIME), mixed with 200 μl of chloroform and centrifuged at 14,000 × g for 5 min at 4 °C. The upper aqueous phase was mixed (1:1) with 70% ethanol, added to the extraction columns, and processed according to the manufacturer's protocol, including on-column DNase digestion. RNA yield and purity were quantified using a Nanodrop ND-1000 spectrophotometer. RNA quality was visualized for selected individual samples on a denaturing agarose gel.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

ELOV, *ferritin*, *RDH*, and *16S* qRT-PCR primers and assay conditions have been described previously (Tarrant et al., 2008). Assays were developed to measure expression of *C. finmarchicus Hsp*s by qRT-PCR. Oligonucleotide primers were designed against the cloned *Hsp* sequences to target 75–150 bp amplicons (Table 2). For the handling stress assays, total RNA was extracted from pooled (3 individuals/tube) shallow C5 copepodids collected at three time treatments (0, 2 and 3 h in an ice-chilled bucket) and used to prepare cDNA (300 ng RNA per 20 μl reaction). Expression of *ferritin*, *RDH*, *16S* and selected *Hsp*s was measured by qRT-PCR using SsoFast EvaGreen Supermix (Bio-Rad) from 10 (t = 0 h and t = 2 h) or 9 (t = 3 h) pooled samples. To compare expression between deep and shallow animals, total RNA was extracted from individual deep and shallow C5 copepodids and used to prepare cDNA (200 ng RNA per 20 μl reaction). Expression of the eight *Hsp*s, *ELOV*, *ferritin*, *RDH*, and *16S* (housekeeping gene) was quantified via qRT-PCR from 21 individual deep and 21 individual shallow C5 copepodids. Expression of *Hsp21*, *Hsp22*, and *RDH* was measured using SsoFast EvaGreen Supermix, and the other genes were measured using iQ SYBR Green Supermix (Bio-Rad). In all *Hsp* assays, plasmid standards were run in duplicate on the same plate. For each gene, all samples were run in duplicate wells on a single plate.

All qRT-PCR reactions were run in an iCycler iQ real-time PCR detection system (Bio-Rad). The iQ SYBR Green PCR mixture consisted of 11 μl molecular biology grade distilled water, 12.5 μl iQ SYBR Green Supermix, 0.25 μl 5′ primer (10 μM), 0.25 μl 3′ primer (10 μM), and 1 μl cDNA. The PCR conditions were: 95 °C/30 min; 40 cycles of 95 °C/15 s, 64–66 °C/45 s. The SsoFast EvaGreen PCR mixture consisted of 9 μl molecular biology grade distilled water 12.5 EvaFast, 1.25 μl 5′ primer (10 μM), 1.25 μl 3′ primer (10 μM), and 1 μl cDNA. The PCR conditions were: 95 °C/30 min; 40 cycles of 95 °C/5 s, 62–64 °C/45 s. After amplification by either procedure, PCR products from each reaction were subjected to melt-curve analysis to ensure that only a single product was amplified. Selected products were also visualized on 15% TBE gels and consistently yielded single bands.

Table 2
Oligonucleotide primer sequences and annealing temperatures (T_m) used in qPCR assays. IQMix and EvaGreen are distinct qPCR reagents (see text for additional details).

Gene	Primer sequence	Tm iQMix	Tm EvaGreen
Hsp90	F: 5′-TCATCCGGATTCAGCTTGGAG-3′	64	60
	R: 5′-GGTGGCATGTCGCTGTCATC-3′	64	60
Hsp70A	F: 5′-CGAAACAGCAGGAGGAGTGATG-3′	64	60
	R: 5′-TGACAGCAGGTTGGTTGTCTTG-3′	64	60
Hsp70B	F: 5′-TGGAGGGAAAGGCAGCTAAAG-3′	66	60
	R: 5′-CATCGCTGGAACTAACCCAAGC-3′	66	60
Hsp70D	F: 5′-GGGTGGAGGTGATCCCTAATG-3′	66	60
	R: 5′-TGCACCACTTCATCAGTCCAC-3′	66	60
Hsp22	F: 5′-GGCTACAAGCCAAGTGAGCTG-3′	–	64
	R: 5′-GAGACCATGGTGTGGCCTTC-3′	–	64
Hsp21	F: 5′-TGCAAACACAGCAACAAGCTG-3′	–	62
	R: 5′-GCCTCGGAAAGAGCATTCTTC-3′	–	62
p26	F: 5′-CTTGCCAAGCATGAGACCAAG-3′	64	60
	R: 5′-GGATTGACCCCAGATGGTAATG-3′	64	60
ELOV	F: 5′-GTCTGGTGGTGTTTCTTCTCC-3′	64	60
	R: 5′-CACATGCAGAGAGGTAAGTTGG-3′	64	60
RDH	F: 5′-CTAGCCAGGTTGCTGATGAAG-3′	–	64
	R: 5′-TCTTGGAGATGGTGAGGTCTG-3′	–	64
ferritin	F: 5′-AATATCAGACCAAGCGTGGAG-3′	64	60
	R: 5′-AGCTTCCATTGCCTGAATAGG-3′	64	60
16S	F: 5′-AAGCTCCTCTAGGGATAACAGC-3′	64	62
	R: 5′-CGTCTCTTCTAAGCTCCTGCAC-3′	64	62

2.7. Normalization and analysis of qRT-PCR expression data

For both the handling experiment and the diapause study, *16S* ribosomal RNA was used as a housekeeping gene (Tarrant et al., 2008). Expression was normalized using the Pfaffl method (Pfaffl, 2001), a relative quantification approach that allowed for consistent comparison of gene expression without requiring plasmid standards ('Expression' = $E_{\text{target}}^{\Delta C_t\ \text{target(sample-calibrator)}} / E_{16S}^{\Delta C_t\ 16S\text{(sample-calibrator)}}$). For each of the *Hsp*s the amplification efficiency was calculated from a standard curve generated by amplification of serially diluted plasmid standards. The amplification efficiencies for *ELOV*, *RDH*, and *ferritin* were previously calculated from a relative standard curve of serially diluted cDNA (Tarrant et al., 2008). To calculate the 'Expression' of each *Hsp*, the mean threshold cycle (C_t) of the 0-h treatment samples (handling experiment) or of the shallow samples (diapause study) was used as the 'calibrator' (also known as the reference sample). The C_t values for duplicate wells were averaged and relative expression values were base-10 log-transformed for statistical analysis.

2.8. Statistical analysis

Two-sample, two-tailed *t*-tests for the morphometric parameters, RNA:DNA ratios, and expression of *ELOV*, *RDH*, and *ferritin* were performed to confirm that deep and shallow samples represented diapausing and active copepods, respectively. *16S* and *Hsp* expression in deep and shallow samples were also evaluated with two-sample, two-tailed *t*-tests. One-way ANOVAs were used to compare qRT-PCR expression between handling stress treatments (0, 2, and 3 h). Planned post hoc comparisons (Dunnett test) in genes with significant ANOVA results compared the 0-h treatment mean (control) with the 2- and 3-h treatment sample means.

3. Results

3.1. Identification of C. finmarchicus Hsp genes and phylogenetic analyses

Through a search of the *C. finmarchicus* ESTs at NCBI, we identified eight different putative *Hsp* sequences (*Hsp90*; 4 forms of *Hsp70* designated *Hsp70A*, *Hsp70B*, *Hsp70C* and *Hsp70D*; *Hsp21*; *Hsp22*; and *p26*; Table 1). The *Hsp70* forms and *Hsp90* were highly conserved, with expect values (*E*-values) less than 10^{-40} and about 50% identity with *Hsp* proteins previously annotated from *Artemia*. In comparison, the small *Hsp*s (*Hsp21*, *Hsp22*, *p26*) were more variable, with larger *E*-values and about 30% identity with amino acid sequences from *Artemia*. The partial *Hsp* sequences we amplified ranged in size from 361 to 637 bp (Table 1). Alignments of our *C. finmarchicus* clones with several crustacean and insect *Hsp* sequences demonstrated that the cloned *Hsp70A* and *Hsp90* sequences encode the 3′ ends of the predicted proteins, while *Hsp70* (B, C, and D), *Hsp21*, *Hsp22*, and *p26* all encode the 5′ ends (Figs. A.1–A.5, respectively).

Phylogenetic analyses based on maximum likelihood criteria confirm that the *C. finmarchicus* sequences are members of the *Hsp70* and *Hsp90* families (Figs. 1 and 2, respectively). In the *Hsp70* analysis we considered the sequences of bacterial origin (i.e., the monophyletic group of bacteria, plastid, and mitochondrial sequences) as the outgroup. Similarly, bacterial *Hsp90* homologs (high-temperature protein G, HTPG; Chen et al., 2006) were used to root the *Hsp90* tree. All three monophyletic groups of the *Hsp70* family that have been described in animals are represented by our four partial *Hsp70* sequences: one form is closely related to cytosolic forms of *Hsp70* (i.e., *Hsp70A*), two to mitochondrial forms (*Hsp70B* and *Hsp70C*), and one to endoplasmic reticulum-

associated forms (*Hsp70D*). The *Hsp70* sequences that were identified in our study are distinct from those previously examined in *C. finmarchicus* (Hansen et al., 2007; Voznesensky et al., 2004) (Fig. 1). The *Hsp70* described by Voznesensky et al. (2004) falls into a clade with *Hsp70A* from our study and an *Hsp70* from the intertidal copepod *Tigriopus japonicus* (Rhee et al., 2009) although bootstrap support for this clade is weak (<50%). The two *C. finmarchicus* sequences in this clade share only 58% identity, suggesting that they represent distinct genes. The *C. finmarchicus Hsp70* described by Hansen et al. (2008) falls into a clade distinct from the subfamilies associated with the cytosol, endoplasmic reticulum, and bacterial origin. Sequences within this clade include divergent human and *Drosophila Hsp70*-like genes (e.g., human *Hsp70_14* and *Hsp70_4*) that have been demonstrated in human to be functionally distinct from other forms of *Hsp70* (Kaneko et al., 1997; Wan et al., 2004). The *Hsp90* that we have identified in *C. finmarchicus* is identical to an EST sequence previously reported by Hansen et al. (2007) and it falls into a well-supported clade (100% bootstrap support) with cytosolic (*Hsp90A*) sequences from other organisms including crustaceans, insects, and human (Fig. 2).

3.2. Handling experiments

In a pilot study (data not shown), *p26* was not as strongly induced as other small *Hsp*s, and our assay did not reliably amplify *Hsp70C* (melt curves indicated non-specific products); therefore, expression of *p26* and *Hsp70C* were not measured in the handling experiment. Of the six examined *Hsps* (*Hsp90*, *Hsp70A*, *Hsp70B*, *Hsp70D*, *Hsp21* and *Hsp22*), expression of three of these was significantly induced in shallow animals by a handling stress of increased waiting time before sampling: *Hsp70A* (one-way ANOVA; $F = 4.38$, $p = 0.023$), *Hsp21* ($F = 4.99$, $p = 0.015$) and *Hsp22* ($F = 4.22$, $p = 0.027$). Expression of *Hsp70A*, *Hsp21*, and *Hsp22* was significantly higher in the 3-h treatment relative to the control treatment at hour 0 (Dunnett's test: $D = 2.34, 2.34, 2.35$, respectively; Fig. 3). The median expression in the 3-h treatment for *Hsp70A*, *Hsp21*, and *Hsp22* was 2.95, 2.11, and 1.82 times higher than in the 0-h treatment, respectively. Expression of these three *Hsp* transcripts was not significantly different between the 2-h and 0-h treatments. Genes previously identified as molecular markers of diapause (*ELOV*, *ferritin, RDH*) (Tarrant et al., 2008) showed no significant change in expression with handling stress (one-way ANOVA; $F = 2.087$, $p = 0.15$ for *ELOV*, $F = 0.21$, $p = 0.81$ for *ferritin*, $F = 3.13$, $p = 0.061$ for *RDH*; there was suggestive, but inconclusive, evidence that expression of *RDH* was lower during later sampling periods when compared to hour zero).

3.3. Characterization of diapause-associated expression

3.3.1. Outliers

Scatterplots of all morphological and gene-expression data were used to identify outliers (not shown). Two individual shallow *C. finmarchicus* C5 copepodids collected in 2006 for the diapause study had degraded oil sacs when photographed (thereby

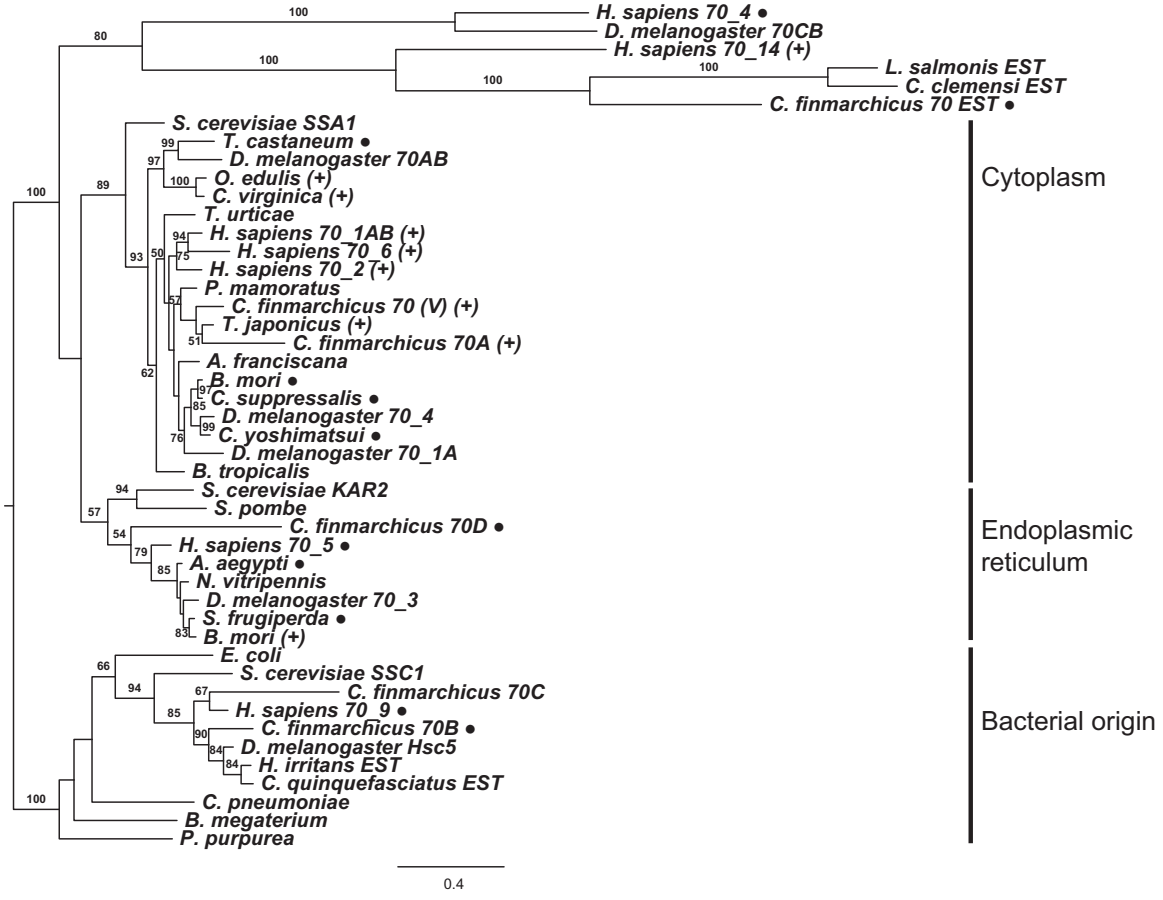

Fig. 1. Maximum likelihood analysis of *Hsp70* from *Calanus finmarchicus* and representative *Hsp70* sequences. Bootstrap percentages of 1000 replicates are indicated above branches when they are greater than 50%. Sequences of bacterial origin (i.e., the monophyletic group of bacteria, plastid, and mitochondrial sequences) were used as the outgroup. Sequences selected for these analyses are principally from Rhee et al. (2009), Boorstein et al. (1994) and Daugaard et al. (2007) with some additions (Table B.1 for full list of sequences and accession numbers). '*C. finmarchicus 70 (V)*' and '*C. finmarchicus 70 EST*' represent *Hsp70* sequences previously characterized by Voznesensky et al. (2004) and Hansen et al. (2008), respectively. Symbols denote *Hsp70* forms that are non-inducible [•] and inducible [(+)] by stress as described in this study as well as in studies listed in Table B.1. Distance bar at the bottom of the tree indicates branch length scale, or the number of substitutions per amino acid site.

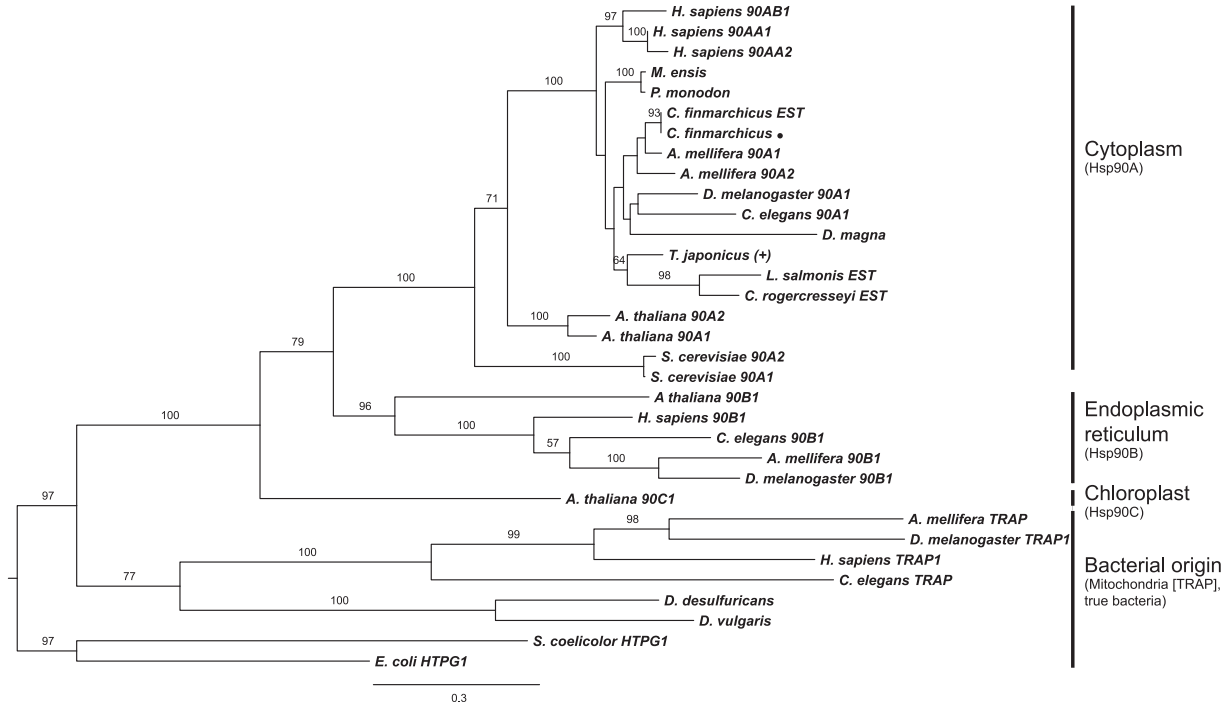

Fig. 2. Maximum likelihood analysis of *Hsp90* from *Calanus finmarchicus* and representative cytosolic (*Hsp90A*), endoplasmic reticulum (*Hsp90B*), chloroplast (*Hsp90C*) and mitochondrial (TRAP) *Hsp90* genes. Bootstrap percentages of 1000 replicates are indicated above branches when they are greater than 50%. Bacterial *Hsp*90 homologs (i.e., high-temperature protein G, HTPG) sequences were used to root the tree. Sequences are primarily from Chen et al. (2005a, 2006) with some additions (see Table B.2 for full list of sequences and accession numbers). Symbols denote copepod *Hsp90* forms that are non-inducible [•] and inducible [(+)] by stress as described in this study and Rhee et al. (2009). Distance bar at the bottom of the tree indicates branch length scale, or the number of substitutions per amino acid site.

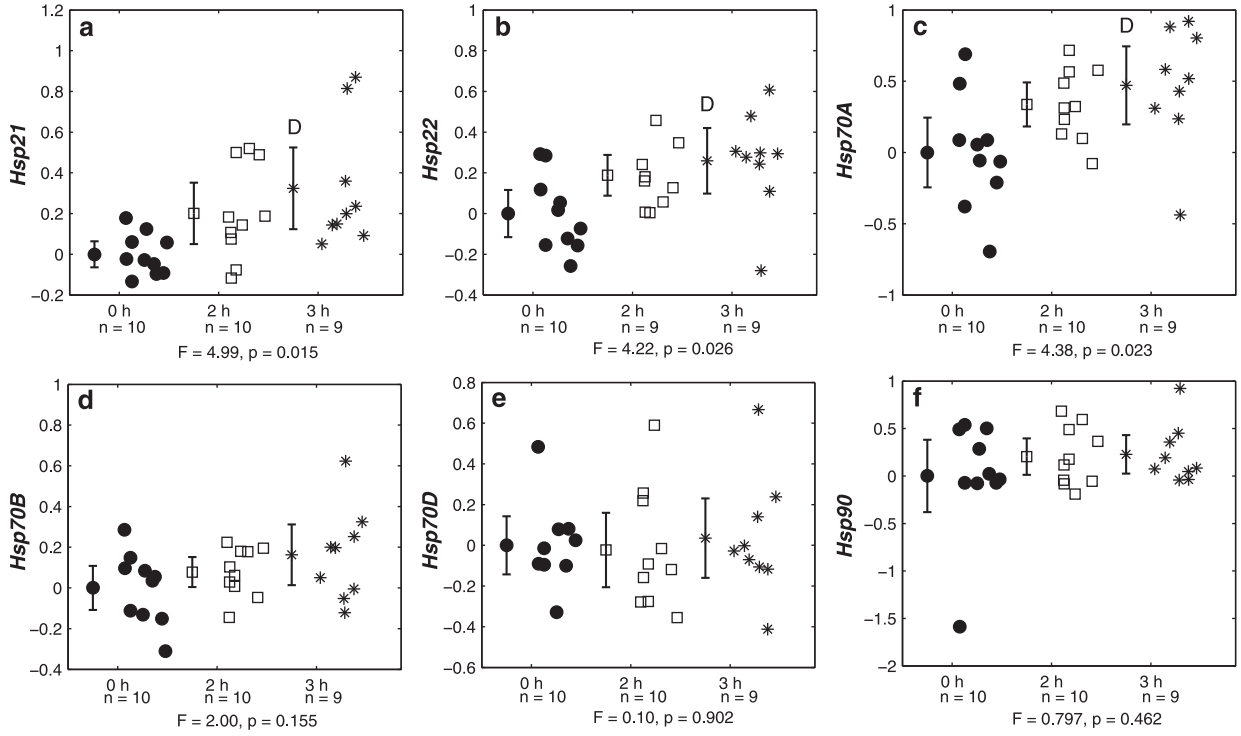

Fig. 3. (a–f) Sample means (±95% CI) and jitter plots of base-10 log-transformed gene expression of pooled (3 individuals/tube) shallow *Calanus finmarchicus* C5 copepodids exposed to handling stress. Expression was calculated relative to mean expression at $t = 0$ h and normalized to *16S* rRNA expression. Expression of *Hsp70A*, *Hsp21*, and *Hsp22* was significantly higher in the 3-h treatment than in the 0-h treatment (Dunnett test values represented by '*D*' = 2.34, 2.34, 2.35, respectively; $p < 0.05$).

eliminating the possibility of estimating oil sac volume and fractional fullness) and were therefore excluded from these morphometric analyses. Two individual deep *C. finmarchicus* C5 copepodids collected in 2006 were also excluded from further analysis based on very low expression of the housekeeping gene and every other gene examined, likely caused by mRNA degradation. Finally, a few individual wells produced poor melt curves during amplification of *p26* and *Hsp70D* so these wells and their replicates were eliminated from further analysis of *p26* and *Hsp70D*. Removal of all outliers due to low expression or poor melt

Table 3
Evidence for diapause in deep copepod samples. Two-sample, two-tailed *t*-tests were conducted using reported expectations as the alternative hypothesis. Sample sizes were as follows: $n = 21$ shallow, $n = 19$ deep. Gene expression reported as base-10 log-transformed differences relative to the average shallow sample expression. RNA:DNA ratios are also base-10 log transformed.

Indicator of diapause	Expectation	Deep	Shallow	*t*
ELOV expression	Deep < Shallow	-1.77 ± 0.43	0.00 ± 0.28	-6.94^{**}
RDH expression	Deep < Shallow	-0.55 ± 0.23	0.00 ± 0.14	-4.05^{*}
Ferritin expression	Deep > Shallow	0.81 ± 0.19	0.00 ± 0.15	6.51^{**}
Oil sac volume (mm^3)	Deep > Shallow	0.40 ± 0.053	0.14 ± 0.052	6.97^{**}
Oil sac fractional fullness (mm^3)	Deep > Shallow	0.78 ± 0.077	0.33 ± 0.098	7.43^{**}
RNA:DNA ratio	Deep < Shallow	0.30 ± 0.12	0.67 ± 0.13	-4.23^{*}
Empty gut (%)	Deep > Shallow	100%	9.5%	N/A

95% confidence intervals are provided for means.
* Indicates significance of the *t*-test $p = 0.0002$.
** Indicates significance of the *t*-test $p < 0.0001$.

curves yielded individual deep copepod samples of $n = 18$ (*p26*), $n = 16$ (*Hsp70D*), and $n = 19$ (all other genes), and $n = 21$ individual shallow copepod samples for all genes in the diapause study.

3.3.2. Hallmarks of diapause

For all examined physiological, biochemical, and molecular indicators of diapause, the shallow and deep samples followed patterns expected of active and diapausing copepods, respectively (Table 3). Morphometric analysis demonstrated that animals collected from deep water had significantly larger oil sac volumes and greater oil sac fractional fullness, as would be expected of diapausing copepods. A majority of the shallow animals had food in their guts while the guts of all examined individual deep copepodids were empty. Higher RNA to DNA ratios, reflective of increased transcriptional activity, were observed in shallow animals. Moreover, expression of genes related to lipid synthesis (*ELOV* and *RDH*) were significantly lower while *ferritin* expression was significantly higher in deep animals (Fig. 4a–c). As expected, expression of the housekeeping gene, *16S*, did not significantly differ between the shallow and deep samples (two-sample, two-tailed *t*-test; $t = 0.48$, $p = 0.93$; Fig. 4d).

3.3.3. Hsp expression in deep and shallow samples

Expression of the eight cloned *Hsp*s was quantified in deep and shallow copepod samples (Fig. 5); *Hsp70C* expression was below the sensitivity of our assay and was not analyzed further. Two of the *Hsps* (*Hsp70A* and *Hsp22*) exhibited significantly different expression between the deep and shallow samples (two-sample, two-tailed *t*-tests; *Hsp70A*: $t = -5.86$, $p < 0.0001$; *Hsp22*: $t = 3.15$, $p = 0.0032$). The median expression of *Hsp70A* was 7.52 times

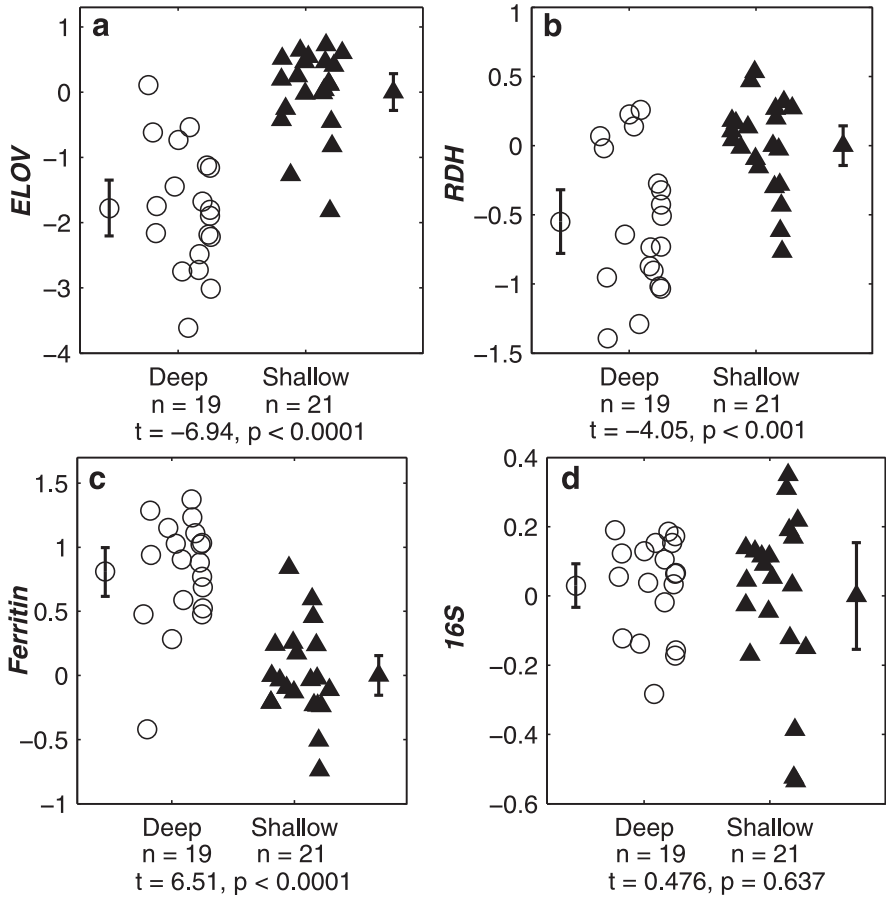

Fig. 4. (a–d) Sample means (±95% CI) and jitter plots of base-10 log-transformed gene expression of *ELOV*, *RDH*, *ferritin*, and *16S* in individual deep (○) and shallow (▲) *Calanus finmarchicus* C5 copepodids. Expression values are calculated relative to mean expression in shallow samples and normalized to *16S* rRNA expression (for *ELOV*, *RDH*, and *ferritin*).

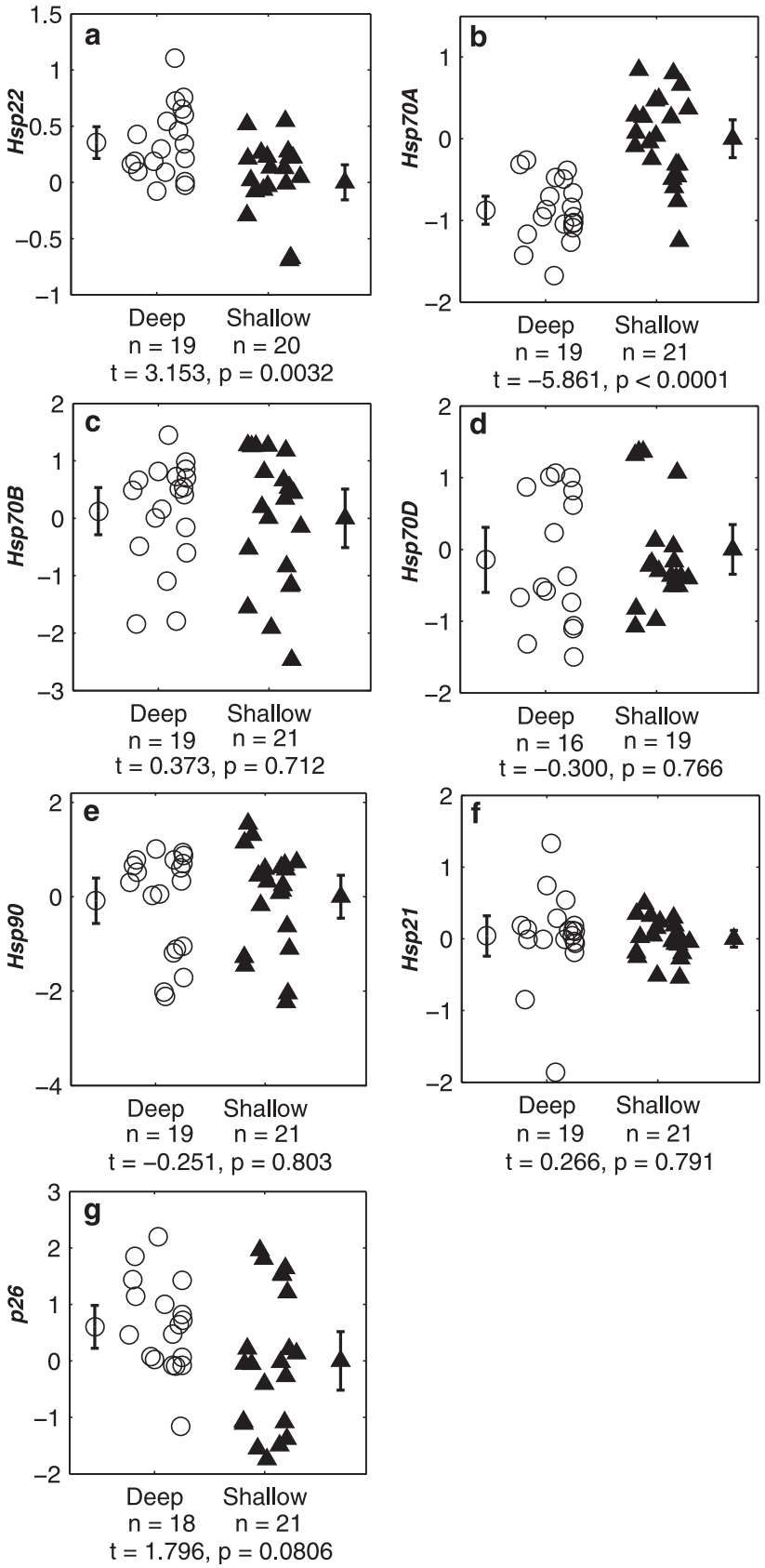

Fig. 5. (a–g) Sample means (±95% CI) and jitter plots of base-10 log-transformed *Hsp* expression in individual deep (○) and shallow (▲) *Calanus finmarchicus* C5 copepodids. Expression was calculated relative to mean expression in shallow samples and normalized to *16S* rRNA.

higher in the shallow samples than in the deep samples (95% CI: 3.74–15.09), while the median expression of *Hsp22* was 2.19 times higher in the deep samples than in the shallow samples (95% CI: 1.32–3.62). There was suggestive, but inconclusive, evidence that *p26* expression was also higher in the deep samples ($t = 1.80$, $p = 0.081$).

4. Discussion

In this study we isolated 8 *Hsp* transcripts (*Hsp90*, *Hsp70A*, *Hsp70B*, *Hsp70C*, *Hsp70D*, *Hsp21*, *Hsp22*, *p26*) and examined their expression in association with diapause and handling stress in the marine copepod *C. finmarchicus*. The cloned *C. finmarchicus Hsp*s showed high sequence similarity to corresponding genes from shrimp and *Artemia*, with the greatest conservation among *Hsp90* and *Hsp70* forms and relatively lower conservation among small *Hsp*s. These findings are consistent with observations that small *Hsp*s have diversified within many lineages and generally exhibit a lower degree of conservation (reviewed by Denlinger et al., 2001; Haslbeck et al., 2005). Phylogenetic analyses based on maximum likelihood trees indicate that the *C. finmarchicus Hsp70* and *Hsp90* transcripts represent cytosolic (*Hsp70A*, *Hsp90*), mitochondrial (*Hsp70B* and *Hsp70C*) and endoplasmic reticulum (*Hsp70D*) forms. Cytosolic forms of *Hsp70* and *Hsp90* are of particular interest for this study because these forms are typically induced in response to stress (Chen et al., 2006; Daugaard et al., 2007; Taipale et al., 2010). While *Hsp* mRNA expression has been shown in many studies to be an accurate indicator of stress responsiveness and/or preparation for diapause in a variety of species (e.g., Tungjitwitayakul et al., 2008; Yocum, 2001; Zhang and Denlinger, 2009), future studies may benefit from measuring both mRNA and protein expression, as these two can indicate different regulatory processes and time-scales of response.

Exposure to a handling stress of increased waiting time before sampling induced two small *Hsp*s (*Hsp21* and *Hsp22*), as well as *Hsp70A*. Induction of small *Hsp*s in *C. finmarchicus* was not surprising, as they play a direct role in stress tolerance in arthropods (Clegg et al., 1999; MacRae, 2010; Qiu and MacRae, 2008a,b). The cytosolic *Hsp70* subfamily includes a mixture of constitutively expressed (cognate) and inducible forms (Daugaard et al., 2007), with the inducible genes varying among taxa (e.g., in mammals the cognate and inducible forms of cytosolic *Hsp70* resulted from lineage-specific duplication and subsequent diversification). Induction of *Hsp70* has been reported following thermal stress in *C. finmarchicus* (Voznesensky et al., 2004) and following thermal stress or metal exposure in the intertidal copepod *T. japonicus* (Rhee et al., 2009). Our phylogenetic analysis showed that the sequences from Voznesensky et al. (2004) and Rhee et al. (2009) fall within the cytosolic *Hsp70* subfamily and form a clade with *C. finmarchicus Hsp70A*, which was induced by handling in our study (Fig. 1). In contrast, *Hsp70B*, *Hsp70C*, and *Hsp70D* clustered with constitutively expressed mitochondrial and endoplasmic reticulum forms, so we expected that they would not be induced by stress. Our results confirm this, as none of these transcripts were significantly induced by handling stress. Our phylogenetic analysis also clustered the *C. finmarchicus Hsp70* sequence previously identified by Hansen et al. (2008) with human *Hsp70*-like proteins (*Hsp70_14* and *Hsp70_4*) that are known to share low sequence and functional similarity with cytosolic *Hsp70* forms (Kaneko et al., 1997; Wan et al., 2004). Therefore, previously reported differences in *C. finmarchicus Hsp70* inducibility by stressors (Hansen et al., 2008; Voznesensky et al., 2004) may be attributed, at least in part, to differences in the '*Hsp70*' transcripts examined. Cytosolic forms of *Hsp90* are also sometimes induced in response to stress (Chen et al., 2006; Taipale et al., 2010); however, this is not observed in all species. Cytosolic *Hsp90* was not significantly induced by thermal stress in *T. japonicus* (Rhee et al., 2009) or by naphthalene exposure in *C. finmarchicus* (Hansen et al., 2008), although Hansen et al. (2007) suggested that *Hsp90* may be induced by combined chemical and thermal stress in *C. finmarchicus*. *Hsp90* was not induced by handling in our study. Unlike the small *Hsp*s, *Hsp*90 does not play a direct protective role in stabilizing denatured proteins (reviewed by MacRae, 2010) but acts as a molecular chaperone to enable the proper folding of a wide range of cellular proteins (Taipale et al., 2010), which may help explain our findings.

Two of the genes induced by handling stress in shallow samples, *Hsp70A* and *Hsp22*, were also differentially expressed between active and diapausing copepods. Diapause was classified using both traditional and molecular markers (Table 3), and associations between these two markers were identical to those observed by Tarrant et al. (2008). Expression of *Hsp70A* was both elevated in active animals and induced upon handling in shallow samples. We speculate that the observed differential expression of this gene reflects greater exposure of copepods to stressors while active in the upper ocean, such as temperature gradients encountered during diel vertical migrations, visual predators, solar radiation (including ultraviolet radiation; Wold and Norrin, 2004), turbulence, and starvation. In contrast, diapausing copepods likely experience a more stable environment at depth with far fewer stressors. Like *Hsp70A*, *Hsp22* was induced upon handling in shallow samples; however, *Hsp22* expression was elevated in diapausing animals. These observations could be explained by differential induction of *Hsp22* during the process of collecting active and diapausing samples (i.e., if *Hsp22* is more inducible in diapausing copepods); however, we consider this unlikely since diapausing copepods are expected to be in a state of torpor with reduced responsiveness to external stimuli (Hirche, 1983). Instead, we speculate that *Hsp22* plays a role in both stress response and diapause in *C. finmarchicus*. Qiu and MacRae (2008b) found that in *Artemia, Hsp22* is similarly induced both in response to thermal stress and in preparation for diapause.

The role of *Hsp*70 and *Hsp*90 in mediating diapause varies greatly among insect taxa. For example, while changes in *Hsp*70 expression are not a component of the larval diapause of the blowfly (Tachibana et al., 2005), the pupal diapause of the corn earworm (Zhang and Denlinger, 2009) or the adult diapause of a fruit fly (Goto et al., 1998), *Hsp*70 is highly induced in the pupal diapause of the solitary bee (Yocum et al., 2005) and the onion maggot (Chen et al., 2005b). Conversely, *Hsp*70 is down-regulated in the larval diapause of the corn stalk borer (Gkouvitsas et al., 2009) and the bamboo borer (Tungjitwitayakul et al., 2008). *Hsp*90 demonstrates similar discrepancies in diapause-related expression patterns, including down-regulation in the pupal diapause of the flesh fly (Rinehart and Denlinger, 2000), up-regulation at the termination of the larval diapause of the blow fly (Tachibana et al., 2005), and constant expression in the pupal diapause of the solitary bee (Yocum et al., 2005).

The variation in diapause-associated expression patterns of *Hsp*70 and *Hsp*90 among taxa cannot be attributed to differences in the developmental stage at which diapause occurs. In addition, our phylogenetic analyses demonstrate that all of the genes described in the studies cited in the preceding paragraph belong to the cytosolic subfamilies (Figs. B.1 and B.2); therefore, variability in *Hsp*70 and *Hsp*90 expression patterns among taxa cannot be attributed to different subfamily membership. However, within the *Hsp* subfamilies, more recent duplication events have given rise to multiple isoforms of cytosolic *Hsp70* and *Hsp90* genes (Chen et al., 2006; Daugaard et al., 2007; Taipale et al., 2010). In our analyses, non-inducible and inducible (both positively and negatively by diapause) *Hsp70* and *Hsp90* isoforms were distributed throughout the clusters of the cytosolic subfamily, which highlights the need for studies of the inducibility and function of individual *Hsp* isoforms.

In conclusion, this study represents the first characterization of *Hsp* expression in association with diapause in a calanoid copepod and expands the current understanding of the molecular regulation of diapause. We have identified a small *Hsp* (*Hsp22*) that is upregulated during diapause. We have also identified three *Hsps* (*Hsp70A*, *Hsp21*, and *Hsp22*) that were induced by handling stress. We now know that diapause in *C. finmarchicus* is marked by changes in lipid metabolism (*FABP*, *ELOV*, *RDH*), endocrine signaling (*EcR*), and protection from cellular stress and protein degradation (*Hsp22, ferritin*) (Tarrant et al., 2008, current study). Enhanced stress tolerance is a particularly common feature of diapause, regardless of the developmental stage during which diapause occurs and/or differences in the degree of metabolic arrest (MacRae, 2010; Qiu and MacRae, 2008a,b; Rinehart et al., 2007; Sonoda et al., 2006). In the case of *C. finmarchicus,* the environmental conditions experienced during diapause are relatively minor (i.e., small variation in temperature, not subject to freezing or desiccation) compared to those experienced by overwintering insects or *Artemia* cysts, so the induction of *ferritin* and *Hsp*22 may be sufficient for protection of proteins and other cellular components. Future work will be needed to identify the full complement of genes and protein products associated with diapause, and particularly the mechanisms that regulate preparation for and emergence from diapause.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2011.03.007.

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