

Research Highlights

- We identified 8 heat shock proteins (3 small Hsps, 4 Hsp70's, Hsp90).
- Hsp70a (cytosolic clade) and Hsp21, and Hsp22 were induced by handling stress.
- Hsp70a expression was elevated in shallow, active animals.
- Hsp22 expression was elevated in deep, diapausing animals.

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

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

Marine food webs depend on herbivorous zooplankton such as copepods to process and repack 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; Durbin et al., 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*, including a preparatory phase that precedes unfavorable environmental conditions (Dahms, 1995; Hirche, 1996), a dormancy phase characterized by an endogenous 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 facultative diapause; however, we recognize that not all individuals or populations of *C. finmarchicus* appear to enter a diapause state at the same time

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4 or of similar intensity (Hirche, 1996), which is dissimilar from the true diapause observed in
5 other well-characterized organisms (e.g. *Artemia* cysts, overwintering insects).
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9 Diapause in *C. finmarchicus* is identified by a combination of classic behavioral,
10 morphological, and biochemical characteristics: diapausing C5 copepodids accumulate at depths
11 below 200 to 300 m in oceanic waters (Heath et al., 2004; Miller et al., 1991; Sameoto and
12 Herman, 1990), have empty guts with thin epithelia (Bonnet et al., 2007; Hirche, 1983), and have
13 large oil sacs (Miller et al. 2000). Indicative of arrested development, diapausing copepods have
14 reduced transcriptional activity (i.e. low RNA:DNA ratios) (Wagner et al., 1998), low
15 ecdysteroid levels (Johnson, 2004), and delayed molt progression (i.e. cessation of tooth
16 formation) (Miller et al., 1991). Molecular markers of diapause include low expression of genes
17 related to lipid synthesis, transport, and storage (*ELOV*, *FABP*, *RDH*) and high expression of
18 *ferritin* and *ecdysteroid receptor (EcR)* in diapausing copepods (Tarrant et al., 2008). Further
19 exploration of *C. finmarchicus* diapause using molecular techniques is required to understand the
20 mechanisms regulating the physiological changes that characterize the diapause response.
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32 Heat shock proteins (*Hsps*) are a highly conserved superfamily of molecular chaperones
33 that facilitate proper protein folding and localization while preventing protein aggregation (Feder
34 and Hofmann, 1999; Hartl and Hayer-Hartl, 2002). Furthermore, previous research has shown
35 that *Hsps* play a major role in diapause regulation in a wide range of organisms (Denlinger et al.,
36 2001; MacRae, 2010; Qiu, 2008; Yuan et al., 1996). Induction of *Hsps* by protein denaturing
37 stressors (e.g., heat, toxins) appears to be an ancient and universal response within all examined
38 taxa ranging from bacteria to plants, flies, and human beings (Hansen et al., 2008; Lindquist,
39 1986; Sorensen et al., 2003). In addition to their well-known roles in stress tolerance, *Hsps* are
40 integral to normal cell growth and development. Through protein-protein interactions, *Hsps* help
41 to regulate fundamental cellular processes such as protein turnover, mitochondrial and
42 endoplasmic reticulum trafficking, cell cycle progression, and steroid signaling (Beato and Klug,
43 2000; Helmbrecht et al., 2000; Pratt, 1997; Taipale et al., 2010). During diapause, *Hsps* are
44 thought to contribute to cell cycle arrest and increased stress (e.g. cold) resistance (Denlinger et
45 al., 2001; MacRae, 2010; Rinehart et al., 2007).
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58 Unlike the classic stress response which is characterized by transient and universal
59 upregulation of a wide range of *Hsps*, *Hsp* expression patterns observed during dormancy may
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4 be prolonged and highly variable among species and *Hsp* types (Denlinger et al., 2001).
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6 Comparison of *Hsp70* regulation during the diapause periods of several insect species
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8 demonstrates how the participation of a given class of *Hsp* in the diapause response can differ
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10 significantly; for example, *Hsp70* is highly expressed during the pupal diapause of the flesh fly
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12 *Sarcophaga crassipalpis* (Rinehart and Denlinger, 2000), but expression is low during the adult
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14 diapause of the Colorado potato beetle *Leptinotarsa decemlineata* (Yocum, 2001) and the larval
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16 diapause of the bamboo borer *Omphisa fuscidentalis* (Tungjitwitayakul et al., 2008). In addition,
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18 different classes of *Hsps* can play distinct roles in dormancy *within* a species, as indicated by
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20 discordant expression patterns: in the pupal diapause of *S. crassipalpis*, *Hsp90* is downregulated,
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22 while *Hsp70* and several small *Hsps* are upregulated (Rinehart and Denlinger, 2000; Rinehart et
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24 al., 2007; Rinehart et al., 2000). However in some species, such as in the fruit fly *Drosophila*
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26 *triantararia*, *Hsps* do not appear to participate in diapause at all (Goto and Kimura, 2004; Goto et
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28 al., 1998).

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30 In light of the involvement (albeit varied) of *Hsps* in the diapause response of many
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32 organisms, we hypothesized that *Hsps* play a role in regulating *C. finmarchicus* diapause. In
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34 addition to their role in the stress response, *Hsp70* and *Hsp90* expression varies during the
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36 crustacean molt cycle (Cesar and Yang, 2007; Spees et al., 2003) and could therefore help
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38 regulate the developmental delay associated with diapause in *C. finmarchicus*. Production of
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40 *Hsp70* and *Hsp90* transcripts in *C. finmarchicus* has been previously examined in response to
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42 stressors such as increased temperature and exposure to toxins (Hansen et al., 2007; Hansen et
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44 al., 2008; Voznesensky et al., 2004), but not as a factor regulating diapause. Small *Hsps* have
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46 been shown to play an important role in stress tolerance during diapause in the crustacean
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48 *Artemia franciscana* (Clegg et al., 1999; Qiu, 2008; Qiu and MacRae, 2008), but have yet to be
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50 investigated in calanoid copepods. In this study, we examined the expression patterns of several
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52 large and small *Hsps* (*Hsp90*, 4 forms of *Hsp70*, *Hsp21*, *Hsp22*, *p26*) in individual diapausing
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54 and active *C. finmarchicus* C5 copepodids by quantitative real-time PCR (qRT-PCR). We also
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56 characterized expression of these genes during exposure to handling stress to identify inducible
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58 forms of *Hsps* and to confirm that diapause-associated patterns of *Hsp* expression were not a by-
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60 product of incidental stressors. We further examined the phylogenetic relationships among the
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62 large *Hsps* to facilitate comparisons with inducible or diapause-associated *Hsps* in other taxa.
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64 This study represents the first characterization of *Hsp* expression in association with diapause in
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4 a calanoid copepod species and expands the current understanding of the molecular regulation of
5 diapause.
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10 11 **2. Materials and Methods**

12 13 *2.1 Identification and cloning of C. finmarchicus Hsp genes:*

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17 Material for initial cloning of *Hsps* was obtained from bulk samples of *C. finmarchicus*
18 C5 copepodids that were collected in 2005, preserved in RNAlater (Ambion) at -80°C, and
19 described previously (Tarrant et al., 2008). We searched the NCBI Expressed Sequence Tag
20 (EST) database for *C. finmarchicus Hsp* sequences using the tblastn algorithm with selected
21 crustacean *Hsp21*, *Hsp22*, *p26*, *Hsp70* and *Hsp90* sequences (Table 1). Specific primers were
22 designed and commercially synthesized (Eurofins MWG Operon) to amplify partial *Hsp*
23 sequences for cloning. *Hsps* were amplified from pooled *C. finmarchicus* cDNA using 0.25 µl
24 Amplitaq gold polymerase per 50 µl reaction. The PCR conditions were as follows: 94°C/10
25 min; 40 cycles of 94°C/15 sec, 60-67°C/30 sec, 68°C/7 min; 68°C/7 min; hold at 4°C. Products
26 were visualized on 1% agarose gels, excised, and purified using the MinElute gel extraction kit
27 (Qiagen). PCR products were cloned into pGEM-T Easy (Promega) and sequenced.
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41 *2.2 Phylogenetic analysis*

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43 The phylogenetic relationships among the *C. finmarchicus Hsp70* and *Hsp90* partial
44 sequences and *Hsps* from a broad selection of taxa were determined using maximum likelihood
45 analyses. We retrieved representative *Hsp70* sequences from yeast, bacteria, red algae, human,
46 insects, and crustaceans (Table B.1), including two *Hsp70* sequences that had previously been
47 reported from *C. finmarchicus* (Hansen et al., 2008; Voznesensky et al., 2004). The selected
48 *Hsp70* sequences encompass all four monophyletic groups of eukaryotic *Hsp70*s as defined by
49 intracellular localization (i.e., cytosol, endoplasmic reticulum, mitochondria, and chloroplast)
50 (Boorstein et al., 1994; Daugaard et al., 2007; Rhee et al., 2009). Similarly, for phylogenetic
51 analysis of *Hsp90* we retrieved sequences from yeast, plants, human, nematodes, insects, and
52 crustaceans that are representative of the four eukaryotic *Hsp90* subfamilies: cytosolic (*Hsp90A*),
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4 endoplasmic reticulum (*Hsp90B*), mitochondrial (*TRAP*), and chloroplast (*Hsp90C*) (Chen et al.,
5 2006) (Table B.2). This dataset also included one *Hsp90* EST previously examined in *C.*
6 *finmarchicus* (Hansen et al., 2007). Using the multiple sequence alignment program MUSCLE
7 (Edgar, 2004), we aligned the *Hsp* sequences and then trimmed the variable 5' and 3' regions for
8 a total sequence length of ~ 650 amino acids for each *Hsp* family. We first conducted analyses
9 of the *Hsp70* and *Hsp90* sequences in order to classify the *Hsp* subfamily types represented by
10 our cloned sequences. Maximum likelihood analyses were run using RaxML (v7.0.4, Stamatakis,
11 2006) under a RTREV+G model of protein evolution (selected by AIC with ProtTestv1.4,
12 Abascal et al., 2005). In a second analysis we retrieved additional insect *Hsp70* and *Hsp90*
13 sequences that had been studied for their role in diapause (reviewed by MacRae, 2010). We
14 aligned these sequences with representative *Hsps* from each subfamily and conducted maximum
15 likelihood analyses as described above. Support for nodes was assessed as a proportion of 1000
16 bootstrap replicates and the most likely trees were constructed and visualized in FigTree v1.3.1
17 (<http://tree.bio.ed.ac.uk/software/figtree/>) with bootstrap values > 50% reported.
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33 2.3 Handling stress effects on gene expression 34 35

36 To test whether handling stress affects *Hsp* expression in *C. finmarchicus*, shallow C5
37 copepodids were collected in the southwestern Gulf of Maine at a station 25.5 km east/northeast
38 of Chatham, Massachusetts, USA (41° 46' N/ 69° 38' W) on 18 May 2010 during a cruise
39 aboard the NOAA Ship *Delaware II*. Zooplankton were collected between 13:10 and 13:30 local
40 time from 0-20 m using a 70-cm diameter ring outfitted with a 150 µm conical mesh net. Once
41 the net was recovered, the contents of the cod end were poured onto a 150-µm mesh sieve and 10
42 ml of copepods were added to each of three, covered, black, 7.6-liter ice-chilled containers of
43 ambient filtered seawater stored in several closed ice chests. To mimic the stress that may be
44 experienced during extended waiting times associated with the processing of collected copepod
45 samples, each 7.6-liter ice-chilled container was left for a specific time (t = 0, 2, 3 hours) before
46 its contents were gently sieved and transferred to an ice-chilled Petri dish. From the Petri dish,
47 live *C. finmarchicus* C5 copepodids were individually captured using a wide-bore glass Pasteur
48 pipette, mounted on a depression slide, photographed on a stereomicroscope equipped with a
49 digital camera (see Section 2.4), preserved in microcentrifuge tubes with 500 µl RNAlater
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4 (Ambion), and stored at -20°C until analysis. For this study, we pooled three C5 copepodids per
5 tube to increase sample RNA yield for 10 (t = 0 h and 2 h) and 9 (t = 3 h) total samples. For each
6 of the three time points, we used qRT-PCR (see Section 2.6) to measure expression of six
7 selected *Hsp* transcripts (i.e. *Hsp70A*, *Hsp70B*, *Hsp70D*, *Hsp90*, *Hsp21*, and *Hsp22*; in a pilot
8 study *Hsp70C* was not reliably amplified and *p26* did not appear to be induced) and three
9 previously identified molecular markers of diapause (*ELOV*, *RDH*, *ferritin*) (Tarrant et al., 2008).
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19 *2.4 Sampling of deep and shallow C. finmarchicus*

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21 To assess the relationship between diapause and *Hsp* expression, *C. finmarchicus* C5
22 copepodids were collected in the southwestern Gulf of Maine during a cruise in 2006 aboard the
23 NOAA Ship *Albatross IV* using a 1 m² Multiple Opening-Closing Net and Environmental
24 Sensing System (MOCNESS) (Wiebe et al., 1976) outfitted with 333 µm mesh nets.
25 Zooplankton were collected in two depth strata: 169 to 208 m and 0 to 50 m (hereafter referred to
26 as the deep and shallow samples, respectively). On 20 May 2006 the deep sample was collected
27 at a station in Franklin Basin just to the north of Georges Bank (41° 54' N/68° 16' W) between
28 11:15 and 11:50 local time. The shallow sample was collected at a station 83 km to the west of
29 the Franklin Basin station in southern Wilkinson Basin (41° 53' N/69° 17' W) between 17:08
30 and 17:21 local time on the same day. Water depths at the stations where the deep and shallow
31 samples were collected were 220 m and 198 m, respectively. Upon recovery of the MOCNESS,
32 the contents of the cod end were immediately poured into a transparent, ice-chilled 1.5-liter
33 container and stored in a closed ice chest. Live *C. finmarchicus* were periodically transferred
34 from this container to an ice-chilled Petri dish with a 44-ml Pasteur pipette. From the Petri dish
35 they were individually captured using a wide-bore glass Pasteur pipette, mounted on a depression
36 slide, photographed, individually preserved in microcentrifuge tubes with 500 µl RNAlater
37 (Ambion), and frozen (-20°C). Observations of gut contents or fecal pellet production were noted
38 while viewing the live animals. Photographs of single animals were taken with a Canon EOS-
39 20D digital camera mounted on a Zeiss Stemi 2000C stereomicroscope, and all measurements
40 were calibrated with digital photographs of a stage micrometer taken just prior to sampling. The
41 length, width, oil sac volume, and fractional fullness (after Miller et al., 2000) of each copepodid
42 were estimated from these photographs as described previously (Tarrant et al., 2008).
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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 x g/ 30s) Phase Lock Gel Heavy 2 ml tubes (5 PRIME), mixed with 200 µl of chloroform and centrifuged at 14,000 x g for 5 min at 4°C. The upper aqueous phase was mixed (1:1) with isopropanol, 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* *Hsps* 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 hours in an ice-chilled bucket) and used to prepare cDNA (300 ng RNA per 20 µl reaction). Expression of *ferritin*, *RDH*, *16S* and selected *Hsps* 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 *Hsps*, *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.

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4 All qRT-PCR reactions were run in an iCycler iQ Real-Time PCR detection system (Bio-
5 Rad). The iQ SYBR Green PCR mixture consisted of 11 μ l molecular biology grade distilled
6 water, 12.5 μ l iQ SYBR Green Supermix, 0.25 μ l 5'-primer (10 μ M), 0.25 μ l 3' primer (10 μ M),
7 and 1 μ l cDNA. The PCR conditions were: 95°C/30 min; 40 cycles of 95°C/15 s, 64-66°C/45 s.
8 The SsoFast EvaGreen PCR mixture consisted of 9 μ l molecular biology grade distilled water
9 12.5 EvaFast, 1.25 μ l 5'-primer (10 μ M), 1.25 μ l 3' primer (10 μ M), and 1 μ l cDNA. The PCR
10 conditions were: 95°C/30 min; 40 cycles of 95°C/5 s, 62-64°C/45 s. After amplification by either
11 procedure, PCR products from each reaction were subjected to melt-curve analysis to ensure that
12 only a single product was amplified. Selected products were also visualized on 15% TBE gels
13 and consistently yielded single bands.
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26 2.7 Normalization and analysis of qRT-PCR expression data

27 For both the handling experiment and the diapause study, *16S* ribosomal RNA was
28 used as a housekeeping gene (Tarrant et al., 2008). Expression was normalized using the Pfaffl
29 method (Pfaffl, 2001), a relative quantification approach that allowed for consistent comparison
30 of gene expression without requiring plasmid standards ('Expression' = $E_{\text{target}}^{\Delta C_t \text{ target}(\text{sample-}$
31 $\text{calibrator})} / E_{16S}^{\Delta C_t 16S(\text{sample-calibrator})}$). For each of the *Hsps* the amplification efficiency was calculated
32 from a standard curve generated by amplification of serially diluted plasmid standards. The
33 amplification efficiencies for *ELOV*, *RDH*, and *ferritin* were previously calculated from a
34 relative standard curve of serially diluted cDNA (Tarrant et al., 2008). To calculate the
35 'Expression' of each *Hsp*, the mean threshold cycle (C_t) of the 0-hour treatment samples
36 (handling experiment) or of the shallow samples (diapause study) was used as the 'calibrator'
37 (also known as the reference sample). The C_t values for duplicate wells were averaged and
38 relative expression values were base-10 log-transformed for statistical analysis.
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52 2.8 Statistical analysis

53 Two-sample, two-tailed t-tests for the morphometric parameters, RNA:DNA ratios, and
54 expression of *ELOV*, *RDH*, and *ferritin* were performed to confirm that deep and shallow
55 samples represented diapausing and active copepods, respectively. *16S* and *Hsp* expression in
56 deep and shallow samples were also evaluated with two-sample, two-tailed t-tests. One-way
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4 ANOVAs were used to compare qRT-PCR expression between handling stress treatments (0, 2,
5 and 3 hour). Planned posthoc comparisons (Dunnett test) in genes with significant ANOVA
6 results compared the 0-hour treatment mean (control) with the 2- and 3- hour treatment sample
7 means.
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11 12 13 14 **3. Results**

15 16 17 *3.1 Identification of C. finmarchicus Hsp genes and phylogenetic analyses*

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20 Through a search of the *C. finmarchicus* ESTs at NCBI, we identified eight different
21 putative *Hsp* sequences (*Hsp90*; 4 forms of *Hsp70* designated *Hsp70A*, *Hsp70B*, *Hsp70C* and
22 *Hsp70D*; *Hsp21*; *Hsp22*; and *p26*; Table 1). The *Hsp70* forms and *Hsp90* were highly conserved,
23 with Expect values (E-values) less than 10^{-40} and about 50% identity with *Hsp* proteins
24 previously annotated from *Artemia*. In comparison, the small *Hsps* (*Hsp21*, *Hsp22*, *p26*) were
25 more variable, with larger E-values and about 30% identity with amino acid sequences from
26 *Artemia*. The partial *Hsp* sequences we amplified ranged in size from 361 to 637 bp (Table 1).
27 Alignments of our *C. finmarchicus* clones with several crustacean and insect *Hsp* sequences
28 demonstrated that the cloned *Hsp70A* and *Hsp90* sequences encode the 3' ends of the predicted
29 proteins, while *Hsp70* (B, C, and D), *Hsp21*, *Hsp22*, and *p26* all encode the 5' ends (Fig. A.1-5,
30 respectively).
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41 Phylogenetic analyses based on maximum likelihood criteria confirm that the *C.*
42 *finmarchicus* sequences are members of the *Hsp70* and *Hsp90* families (Fig. 1 and 2,
43 respectively). In the *Hsp70* analysis we considered the sequences of bacterial origin (i.e., the
44 monophyletic group of bacteria, plastid, and mitochondrial sequences) as the outgroup.
45 Similarly, bacterial *Hsp90* homologs (high-temperature protein G, HTPG; Chen et al., 2006)
46 were used to root the *Hsp90* tree. All three monophyletic groups of the *Hsp70* family that have
47 been described in animals are represented by our four partial *Hsp70* sequences: one form is
48 closely related to cytosolic forms of *Hsp70* (i.e., *Hsp70A*), two to mitochondrial forms (*Hsp70B*,
49 and *Hsp70C*), and one to endoplasmic reticulum-associated forms (*Hsp70D*). The *Hsp70*
50 sequences that were identified in our study are distinct from those previously examined in *C.*
51 *finmarchicus* (Hansen et al., 2007; Voznesensky et al., 2004) (Fig. 1). The *Hsp70* described by
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4 Voznesensky (2004) falls into a clade with *Hsp70A* from our study and an *Hsp70* from the
5 intertidal copepod *Tigriopus japonicus* (Rhee et al., 2009) although bootstrap support for this
6 clade is weak (< 50%). The two *C. finmarchicus* sequences in this clade share only 58% identity,
7 suggesting that they represent distinct genes. The *C. finmarchicus Hsp70* described by Hansen et
8 al. (2008) falls into a clade distinct from the subfamilies associated with the cytosol, endoplasmic
9 reticulum, and bacterial origin. Sequences within this clade include divergent human and
10 *Drosophila Hsp70*-like genes (e.g., human *Hsp70_14* and *Hsp70_4*) that have been demonstrated
11 in human to be functionally distinct from other forms of *Hsp70* (Kaneko et al., 1997; Wan et al.,
12 2004). The *Hsp90* that we have identified in *C. finmarchicus* is identical to an EST sequence
13 previously reported by Hansen et al. (2007) and it falls into a well-supported clade (100%
14 bootstrap support) with cytosolic (*Hsp90A*) sequences from other organisms including
15 crustaceans, insects, and human (Fig. 2).
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30 *3.2 Handling experiments*

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32 In a pilot study (data not shown), *p26* was not as strongly induced as other small *Hsps*,
33 and our assay did not reliably amplify *Hsp70C* (melt curves indicated non-specific products);
34 therefore, expression of *p26* and *Hsp70C* were not measured in the handling experiment. Of the
35 six examined *Hsps* (*Hsp90*, *Hsp70A*, *Hsp70B*, *Hsp70D*, *Hsp21* and *Hsp22*), expression of three
36 of these was significantly induced in shallow animals by a handling stress of increased waiting
37 time before sampling: *Hsp70A* (one-way ANOVA; $F = 4.38$, $p = 0.023$), *Hsp21* ($F = 4.99$, $p =$
38 0.015) and *Hsp22* ($F = 4.22$, $p = 0.027$). Expression of *Hsp70A*, *Hsp21*, and *Hsp22* was
39 significantly higher in the 3-hour treatment relative to the control treatment at hour 0 (Dunnett's
40 test: $D = 2.34, 2.34, 2.35$, respectively; Fig. 3). The median expression in the 3-hour treatment
41 for *Hsp70A*, *Hsp21*, and *Hsp22* was 2.95, 2.11, and 1.82 times higher than in the 0-hour
42 treatment, respectively. Expression of these three *Hsp* transcripts was not significantly different
43 between the 2-hour and 0-hour treatments. Genes previously identified as molecular markers of
44 diapause (*ELOV*, *ferritin*, *RDH*) (Tarrant et al., 2008) showed no significant change in
45 expression with handling stress (one-way ANOVA; $F = 2.087$, $p = 0.15$ for *ELOV*, $F = 0.21$, $p =$
46 0.81 for *ferritin*, $F = 3.13$, $p = 0.061$ for *RDH*; there was suggestive, but inconclusive, evidence
47 that expression of *RDH* was lower during later sampling periods when compared to hour zero).
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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 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 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 *Hsps* 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 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 *Calanus finmarchicus*. The cloned *C. finmarchicus* *Hsps* 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 *Hsps*. These findings are consistent with observations that small *Hsps* 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; Dugaard 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

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4 benefit from measuring both mRNA and protein expression, as these two can indicate different
5 regulatory processes and timescales of response.
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9 Exposure to a handling stress of increased waiting time before sampling induced two
10 small *Hsps* (*Hsp21* and *Hsp22*), as well as *Hsp70A*. Induction of small *Hsps* in *C. finmarchicus*
11 was not surprising, as they play a direct role in stress tolerance in arthropods (Clegg et al., 1999;
12 MacRae, 2010; Qiu and MacRae, 2008). The cytosolic *Hsp70* subfamily includes a mixture of
13 constitutively expressed (cognate) and inducible forms (Daugaard et al., 2007), with the
14 inducible genes varying among taxa (e.g., in mammals the cognate and inducible forms of
15 cytosolic *Hsp70* resulted from lineage-specific duplication and subsequent diversification).
16 Induction of *Hsp70* has been reported following thermal stress in *C. finmarchicus* (Voznesensky
17 et al., 2004) and following thermal stress or metal exposure in the intertidal copepod *Tigriopus*
18 *japonicus* (Rhee et al., 2009). Our phylogenetic analysis showed that the sequences from
19 Voznesensky et al. (2004) and Rhee et al. (2009) fall within the cytosolic *Hsp70* subfamily and
20 form a clade with *C. finmarchicus Hsp70A*, which was induced by handling in our study (Fig. 1).
21 In contrast, *Hsp70B*, *Hsp70C*, and *Hsp70D* clustered with constitutively expressed mitochondrial
22 and endoplasmic reticulum forms, so we expected that they would not be induced by stress. Our
23 results confirm this, as none of these transcripts were significantly induced by handling stress.
24 Our phylogenetic analysis also clustered the *C. finmarchicus Hsp70* sequence previously
25 identified by Hansen et al. (2008) with human *Hsp70*-like proteins (*Hsp70_14*, *Hsp70_4*) that are
26 known to share low sequence and functional similarity with cytosolic *Hsp70* forms (Kaneko et
27 al., 1997; Wan et al., 2004). Therefore, previously reported differences in *C. finmarchicus Hsp70*
28 inducibility by stressors (Hansen et al., 2008; Voznesensky et al., 2004) may be attributed, at
29 least in part, to differences in the ‘*Hsp70*’ transcripts examined. Cytosolic forms of *Hsp90* are
30 also sometimes induced in response to stress (Chen et al., 2006; Taipale et al., 2010); however,
31 this is not observed in all species. Cytosolic *Hsp90* was not significantly induced by thermal
32 stress in *T. japonicus* (Rhee et al., 2009) or by naphthalene exposure in *C. finmarchicus* (Hansen
33 et al., 2008), although Hansen et al. (2007) suggested that *Hsp90* may be induced by combined
34 chemical and thermal stress in *C. finmarchicus*. *Hsp90* was not induced by handling in our study.
35 Unlike the small *Hsps*, *Hsp90* does not play a direct protective role in stabilizing denatured
36 proteins (reviewed by MacRae, 2010) but acts as a molecular chaperone to enable the proper
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4 folding of a wide range of cellular proteins (Taipale et al., 2010), which may help explain our
5 findings.
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9 Two of the genes induced by handling stress in shallow samples, *Hsp70A* and *Hsp22*,
10 were also differentially expressed between active and diapausing copepods. Diapause was
11 classified using both traditional and molecular markers (Table 3), and associations between these
12 two markers were identical to those observed by Tarrant et al. (2008). Expression of *Hsp70A* was
13 both elevated in active animals and induced upon handling in shallow samples. We speculate that
14 the observed differential expression of this gene reflects greater exposure of copepods to
15 stressors while active in the upper ocean, such as temperature gradients encountered during diel
16 vertical migrations, visual predators, solar radiation (including ultraviolet radiation; Wold and
17 Norrin, 2004), turbulence, and starvation. In contrast, diapausing copepods likely experience a
18 more stable environment at depth with far fewer stressors. Like *Hsp70A*, *Hsp22* was induced
19 upon handling in shallow samples; however, *Hsp22* expression was elevated in diapausing
20 animals. These observations could be explained by differential induction of *Hsp22* during the
21 process of collecting active and diapausing samples (i.e., if *Hsp22* is more inducible in
22 diapausing copepods); however, we consider this unlikely since diapausing copepods are
23 expected to be in a state of torpor with reduced responsiveness to external stimuli (Hirche, 1983).
24 Instead, we speculate that *Hsp22* plays a role in both stress response and diapause in *C.*
25 *finmarchicus*. Qiu and MacRae (2008) found that in *Artemia*, *Hsp22* is similarly induced both in
26 response to thermal stress and in preparation for diapause.
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43 The role of *Hsp70* and *Hsp90* in mediating diapause varies greatly among insect taxa. For
44 example, while changes in *Hsp70* expression are not a component of the larval diapause of the
45 blowfly (Tachibana et al., 2005), the pupal diapause of the corn earworm (Zhang and Denlinger,
46 2009) or the adult diapause of a fruit fly (Goto et al., 1998), *Hsp70* is highly induced in the pupal
47 diapause of the solitary bee (Yocum et al., 2005) and the onion maggot (Chen et al., 2006).
48 Conversely, *Hsp70* is down-regulated in the larval diapause of the corn stalk borer (Gkouvitsas
49 et al., 2009) and the bamboo borer (Tungjitwitayakul et al., 2008). *Hsp90* demonstrates similar
50 discrepancies in diapause-related expression patterns, including down-regulation in the pupal
51 diapause of the flesh fly (Rinehart and Denlinger, 2000), up-regulation at the termination of the
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4 larval diapause of the blow fly (Tachibana et al., 2005), and constant expression in the pupal
5 diapause of the solitary bee (Yocum et al., 2005).
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9 The variation in diapause-associated expression patterns of *Hsp70* and *Hsp90* among taxa
10 cannot be attributed to differences in the developmental stage at which diapause occurs. In
11 addition, our phylogenetic analyses demonstrate that all of the genes described in the studies
12 cited in the preceding paragraph belong to the cytosolic subfamilies (Fig. B.1 and B.2); therefore,
13 variability in *Hsp70* and *Hsp90* expression patterns among taxa cannot be attributed to different
14 subfamily membership. However, within the *Hsp* subfamilies, more recent duplication events
15 have given rise to multiple isoforms of cytosolic *Hsp70* and *Hsp90* genes (Chen et al., 2006;
16 Daugaard et al., 2007; Taipale et al., 2010). In our analyses, non-inducible and inducible (both
17 positively and negatively by diapause) *Hsp70* and *Hsp90* isoforms were distributed throughout
18 the clusters of the cytosolic subfamily, which highlights the need for studies of the inducibility
19 and function of individual *Hsp* isoforms.
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30 In conclusion, this study represents the first characterization of *Hsp* expression in
31 association with diapause in a calanoid copepod and expands the current understanding of the
32 molecular regulation of diapause. We have identified a small *Hsp* (*Hsp22*) that is upregulated
33 during diapause. We have also identified three *Hsps* (*Hsp70A*, *Hsp21*, and *Hsp22*) that were
34 induced by handling stress. We now know that diapause in *C. finmarchicus* is marked by
35 changes in lipid metabolism (*FABP*, *ELOV*, *RDH*), endocrine signaling (*EcR*), and protection
36 from cellular stress and protein degradation (*Hsp22*, *ferritin*) (Tarrant et al., 2008, current study).
37 Enhanced stress tolerance is a particularly common feature of diapause, regardless of the
38 developmental stage during which diapause occurs and/or differences in the degree of metabolic
39 arrest (MacRae, 2010; Qiu, 2008; Rinehart et al., 2007; Sonoda et al., 2006). In the case of *C.*
40 *finmarchicus*, the environmental conditions experienced during diapause are relatively minor
41 (i.e., small variation in temperature, not subject to freezing or desiccation) compared to those
42 experienced by overwintering insects or *Artemia* cysts, so the induction of *ferritin* and *Hsp22*
43 may be sufficient for protection of proteins and other cellular components. Future work will be
44 needed to identify the full complement of genes and protein products associated with diapause,
45 and particularly the mechanisms that regulate preparation for and emergence from diapause.
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4 **Figure legends:**
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7 Fig. 1 : Maximum likelihood analysis of *Hsp70* from *Calanus finmarchicus* and representative
8 *Hsp70* sequences. Bootstrap percentages of 1000 replicates are indicated above branches when
9 they are greater than 50%. Sequences of bacterial origin (i.e., the monophyletic group of
10 bacteria, plastid, and mitochondrial sequences) were used as the outgroup. Sequences selected
11 for these analyses are principally from Rhee et al. (2009), Boorstein et al. (1994), and Daugaard
12 et al. (2007) with some additions (Table B.1 for full list of sequences and accession numbers).
13 ‘*C. finmarchicus 70 (V)*’ and ‘*C. finmarchicus 70 EST*’ represent *Hsp70* sequences previously
14 characterized by Voznesensky et al. (2004) and Hansen et al. (2008), respectively. Symbols
15 denote *Hsp70* forms that are non-inducible [●] and inducible [(+)] by stress as described in this
16 study as well as in studies listed in Table B.1. Distance bar at the bottom of the tree indicates
17 branch length scale, or the number of substitutions per amino acid site.
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31 Fig. 2: Maximum likelihood analysis of *Hsp90* from *Calanus finmarchicus* and representative
32 cytosolic (*Hsp90A*), endoplasmic reticulum (*Hsp90B*), chloroplast (*Hsp90C*) and mitochondrial
33 (TRAP) *Hsp90* genes. Bootstrap percentages of 1000 replicates are indicated above branches
34 when they are greater than 50%. Bacterial *Hsp90* homologs (i.e., high-temperature protein G,
35 HTPG) sequences were used to root the tree. Sequences are primarily from Chen et al. (2005;
36 2006) with some additions (see Table B.2 for full list of sequences and accession numbers).
37 Symbols denote copepod *Hsp90* forms that are non-inducible [●] and inducible [(+)] by stress as
38 described in this study and Rhee et al. (2009). Distance bar at the bottom of the tree indicates
39 branch length scale, or the number of substitutions per amino acid site.
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51 Fig. 3 (a-f). Sample means (\pm 95% CI) and jitter plots of base-10 log-transformed gene
52 expression of pooled (3 individuals/tube) shallow *Calanus finmarchicus* C5 copepodids exposed
53 to handling stress. Expression was calculated relative to mean expression at t = 0 hour and
54 normalized to *16S* rRNA expression. Expression of *Hsp70A*, *Hsp21*, and *Hsp22* was significantly
55 higher in the 3-hour treatment than in the 0-hour treatment (Dunnett test values represented by
56 ‘D’ = 2.34, 2.34, 2.35, respectively; $p < 0.05$).
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7 Fig. 4 (a-d). Sample means (\pm 95% CI) and jitter plots of base-10 log-transformed gene
8 expression of *ELOV*, *RDH*, *ferritin*, and *16S* in individual deep (\circ) and shallow (\blacktriangle) *Calanus*
9 *finmarchicus* C5 copepodids. Expression values are calculated relative to mean expression in
10 shallow samples and normalized to *16S* rRNA expression (for *ELOV*, *RDH*, and *ferritin*).
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18 Fig. 5 (a-g). Sample means (\pm 95% CI) and jitter plots of base-10 log-transformed *Hsp*
19 expression in individual deep (\circ) and shallow (\blacktriangle) *Calanus finmarchicus* C5 copepodids.
20 Expression was calculated relative to mean expression in shallow samples and normalized to *16S*
21 rRNA.
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(Legends for Supplementary Figures, Online Content)

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31 Fig. A.1: Alignment of translated *Hsp70* sequences from *Calanus finmarchicus* (this study),
32 *Bombyx mori* (BAA32395), *Drosophila auraria* (CAA04699), *Artemia franciscana*
33 (AAL27404), *Mytilus galloprovincialis* (M.gall; AAW52766), and *Manduca sexta* (AAF09496).
34 The *Hsp70A* sequence identified in this study is 210 amino acids and aligns at the 3' end of the
35 approximately 640 amino acid long *Hsp70* protein while the *Hsp70B*, *Hsp70C* and *Hsp70D*
36 clones range in size from 150-200 bp and align at the 5' end.
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46 Fig. A.2: Alignment of translated *Hsp90* sequences from *Caenorhabditis elegans*
47 (NP_506626.1), *Calanus finmarchicus* (this study), *Metapenaeus ensis* (ABR66910.1), *Penaeus*
48 *monodon* (ABM54577.1), *Drosophila melanogaster* (D.mel; NP_523899.1), and *Apis mellifera*
49 (NP_001153536.1). The partial *Hsp90* sequence identified in this study represents 119 amino
50 acids and aligns at the 3' end of *Hsp90A* (~720 amino acids).
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58 Fig. A.3: Alignment of translated *Hsp21* sequences from *Calanus finmarchicus* (this study),
59 *Artemia franciscana* (ABD19712), and *Bombyx mori* (BAD74197.1). The *Hsp21*-like sequence
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4 identified in this study represents 181 amino acids aligned towards the middle of an
5 approximately 200 amino acid long Hsp21 protein.
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11 Fig. A.4: Alignment of translated *Hsp22* sequences from *Calanus finmarchicus* (this study),
12 *Artemia franciscana* (ABD19713), and *Culex quinquefasciatus* (XP_001847194). The *Hsp22*-
13 like sequence identified in this study represents 168 amino acids aligned towards the 5' end of an
14 approximately 200 amino acid long Hsp22 protein.
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22 Fig. A.5: Alignment of translated *p26* sequences from *Calanus finmarchicus* (this study),
23 *Artemia sinica* (ABC41137.1), and *Artemia franciscana* (ABC41138). The *p26*-like sequence
24 identified in this study represents 180 amino acids aligned towards the 5' end of an
25 approximately 200 amino acid long p26 protein.
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33 Fig. B.1: Maximum likelihood analysis of *Hsp70* from *Calanus finmarchicus*, several diapausing
34 insect species, and representative cytosolic, endoplasmic reticulum, mitochondrial, plastid, and
35 bacterial *Hsp70* members. Bootstrap percentages of 1000 replicates are indicated above branches
36 when they are greater than 50%. Sequences of bacterial origin were used as the outgroup.
37 Sequences selected for these analyses are principally from Rhee et al. (2009), Boorstein et al.
38 (1994), Daugaard et al. (2007), and MacRae (2010) with some additions (see Table B.1 for full
39 list of sequences and accession numbers). Symbols and colors denote *Hsp70* forms that are non-
40 inducible [●; blue], inducible [(+); red], down-regulated [(-); green], or show variable expression
41 in response to diapause [~; pink] according to findings in our study and those reviewed by
42 MacRae (2010). Distance bar at the bottom of the tree indicates branch length scale, or the
43 number of substitutions per amino acid site.
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57 Fig. B.2: Maximum likelihood analysis of *Hsp90* from *Calanus finmarchicus*, several diapausing
58 insect species, and representative cytosolic (*Hsp90A*), endoplasmic reticulum (*Hsp90B*),
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4 chloroplast (*Hsp90C*), and mitochondrial (TRAP) *Hsp90* members. Bootstrap percentages of
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6 1000 replicates are indicated above branches when they are greater than 50%. Bacterial *Hsp90*
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8 homologs (i.e., high-temperature protein G, HTPG) were used to root the *Hsp90* tree. Sequences
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10 selected for these analyses are primarily from Chen et al. (2005; 2006) with some additions (see
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12 Table B.2 for full list of sequences and accession numbers). Symbols and colors denote *Hsp90*
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14 forms that are non-inducible [●; blue], inducible [(+); red], down-regulated [(-) ; green], or show
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16 variable expression in response to diapause [~ ; pink] according to findings in our study and
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18 those reviewed by MacRae (2010). Distance bar at the bottom of the tree indicates branch
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20 lengths or the number of substitutions per amino acid site.
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Figure 1

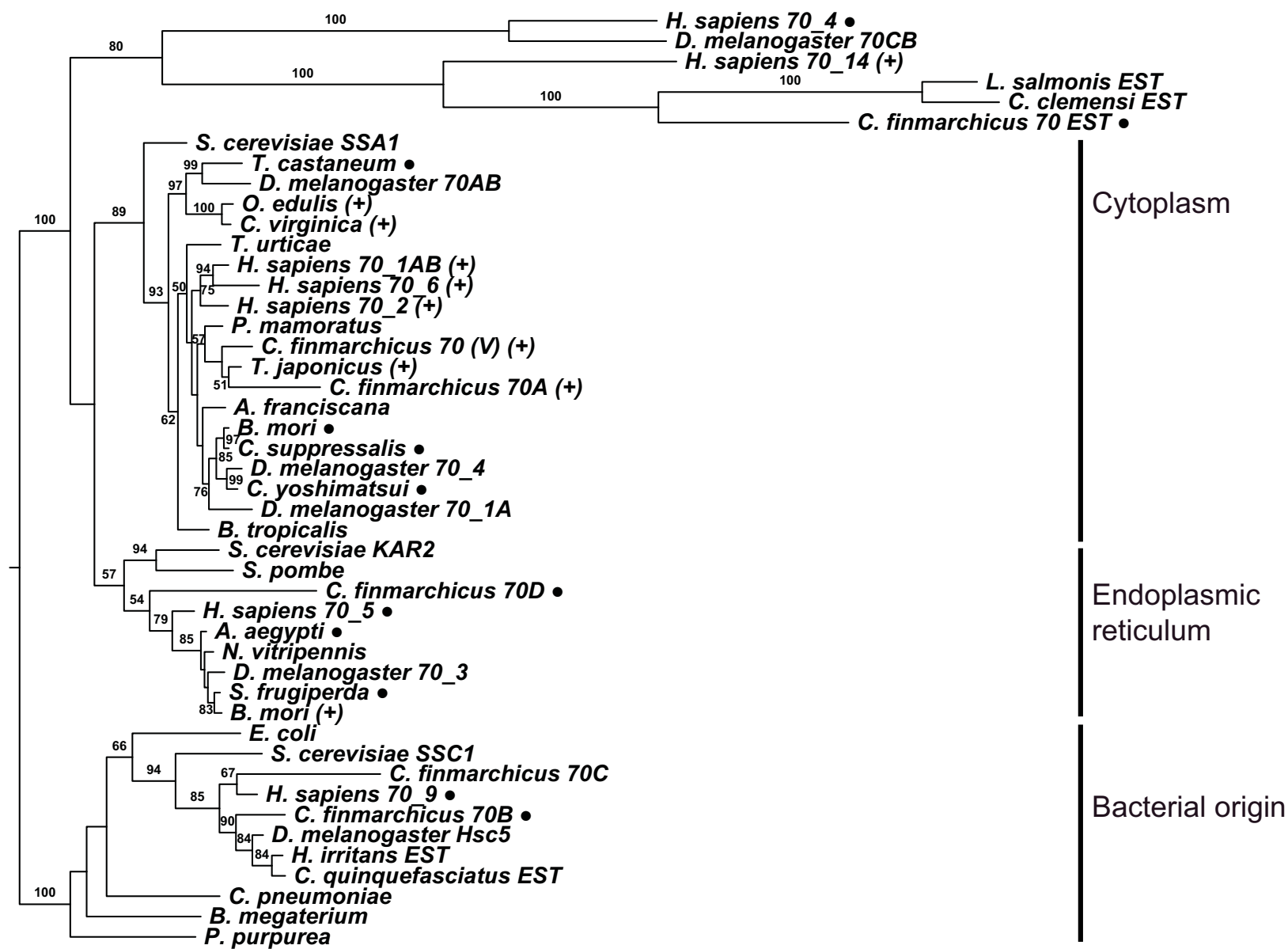


Figure 2

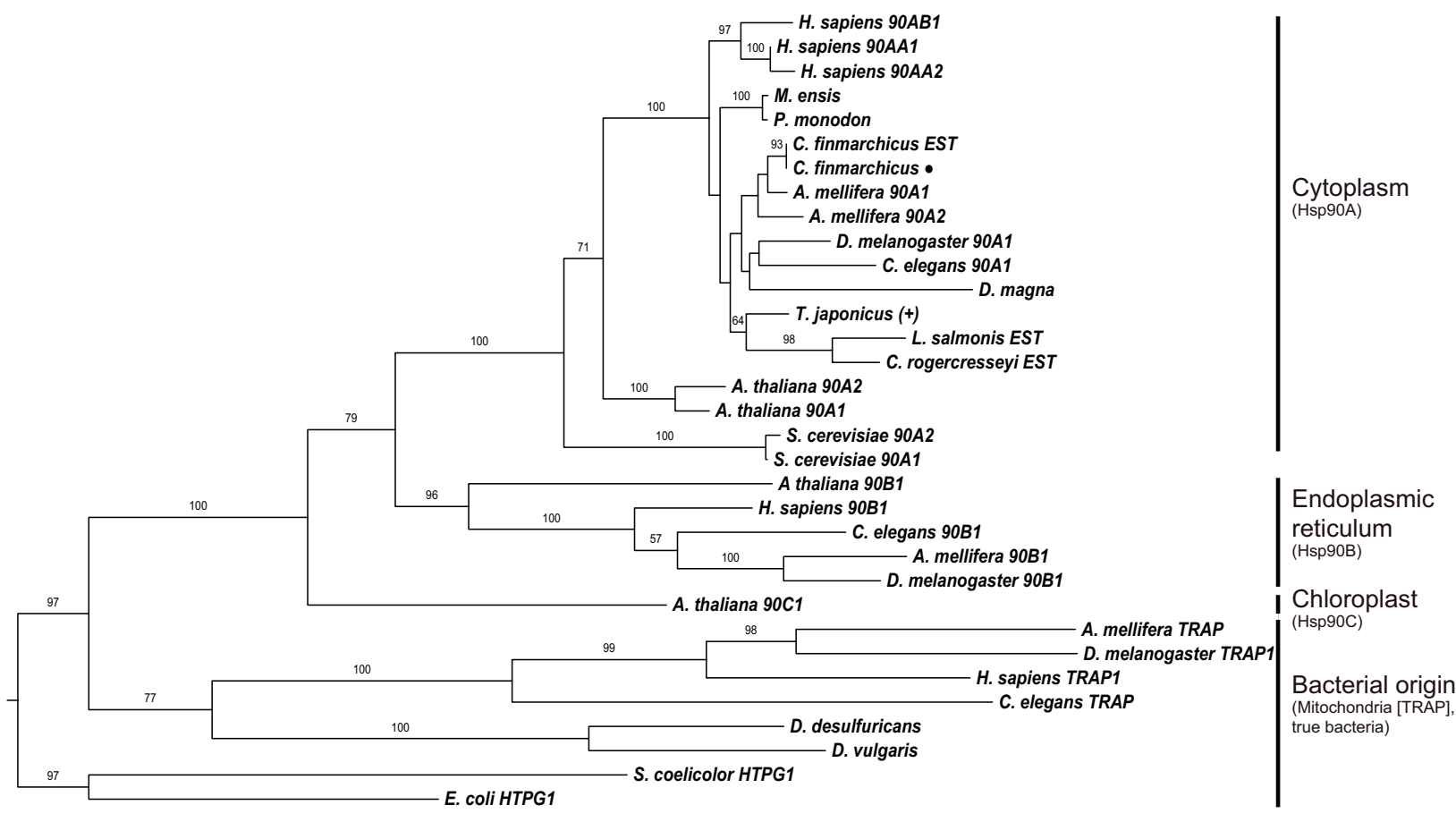


Figure 3

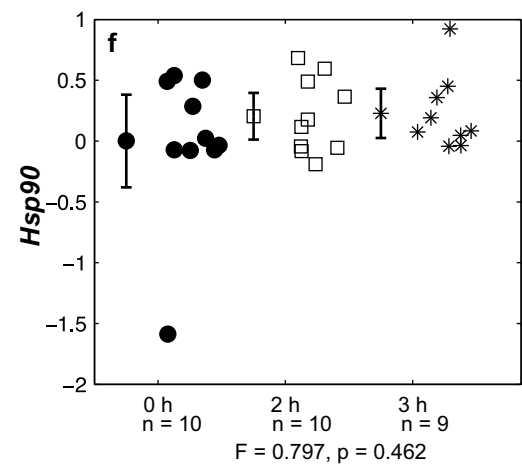
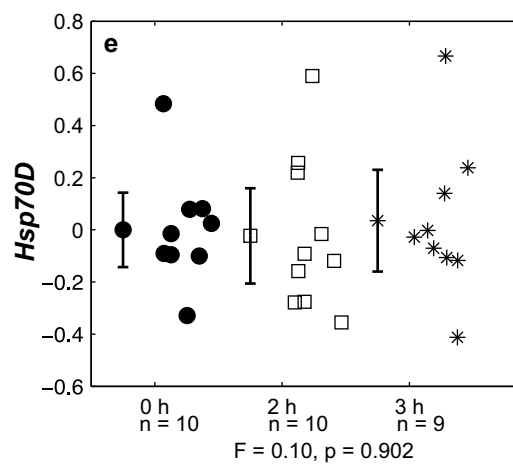
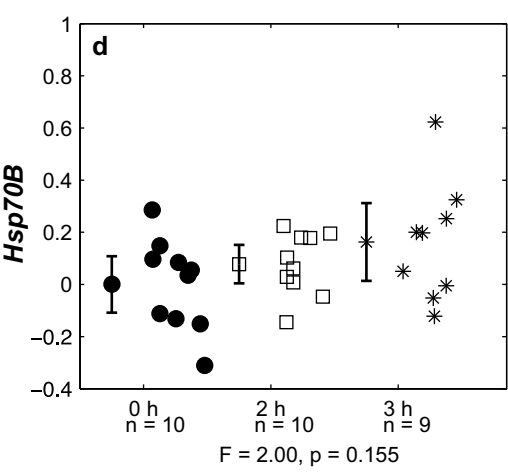
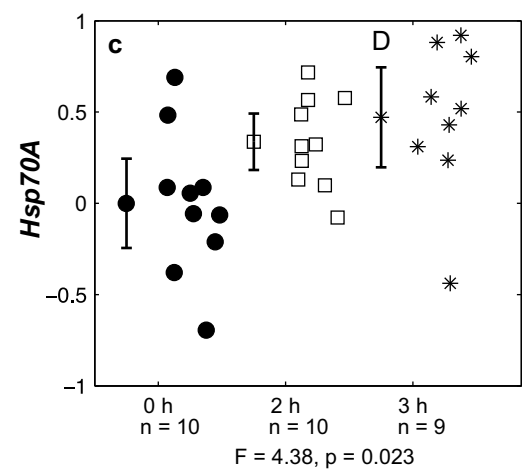
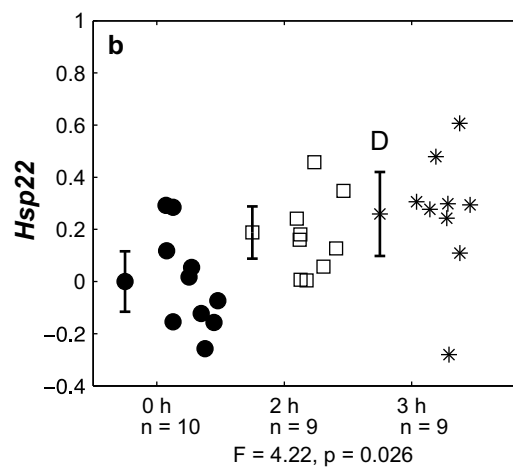
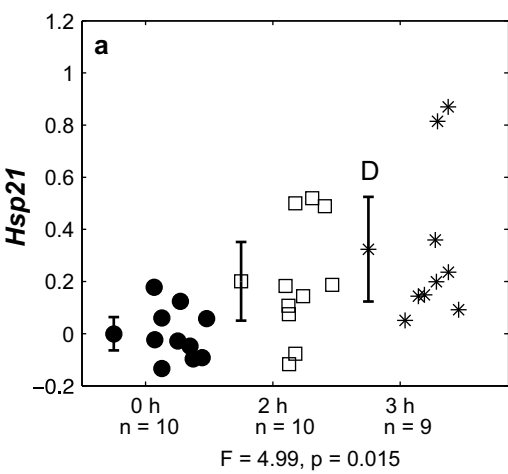


Figure 4

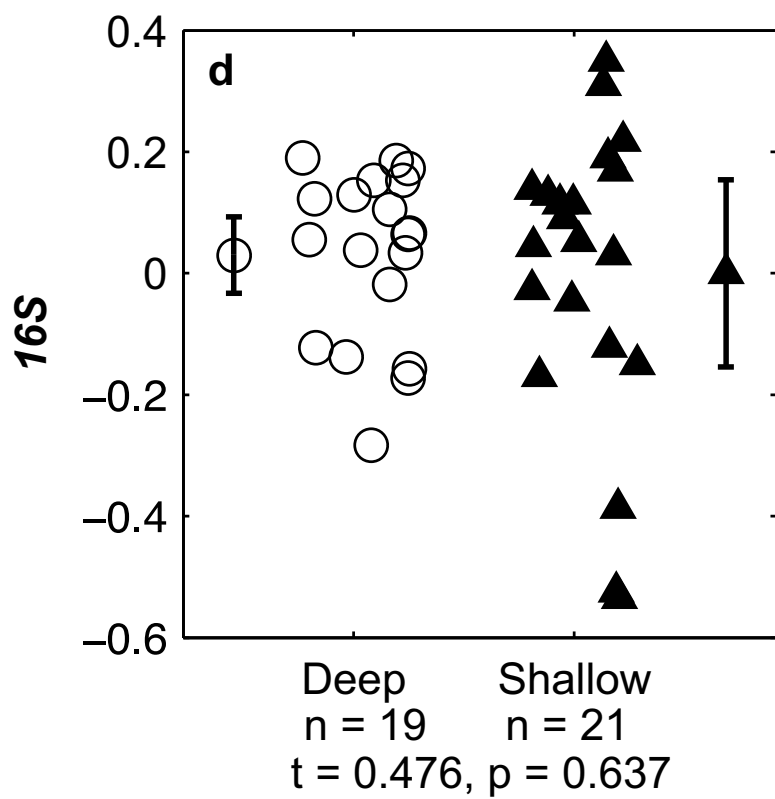
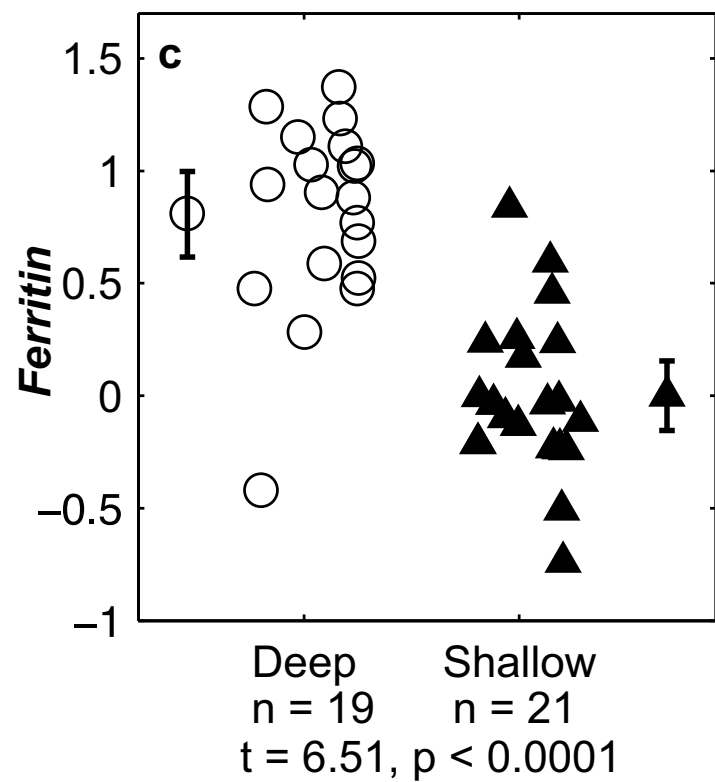
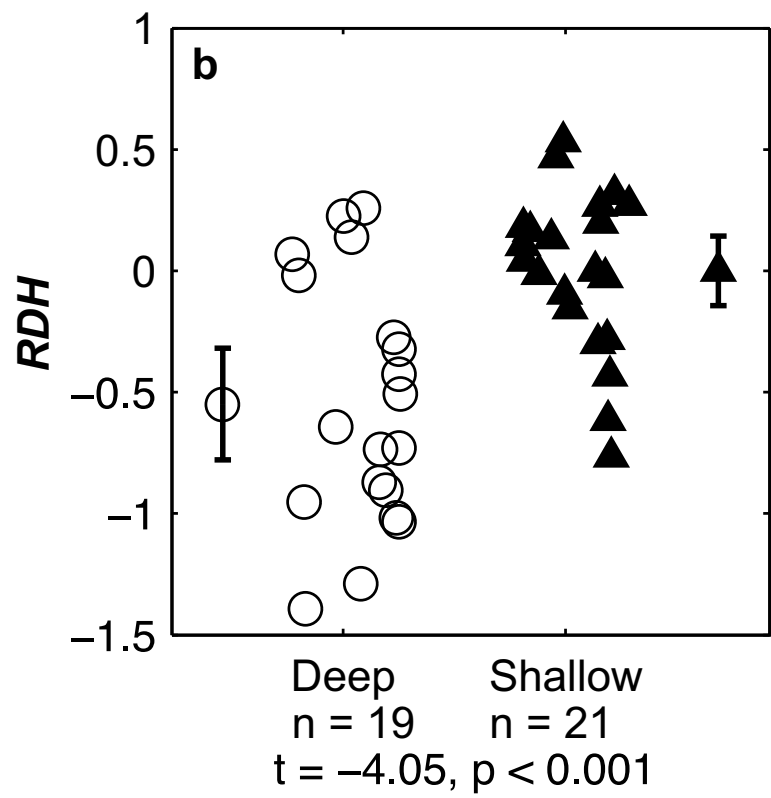
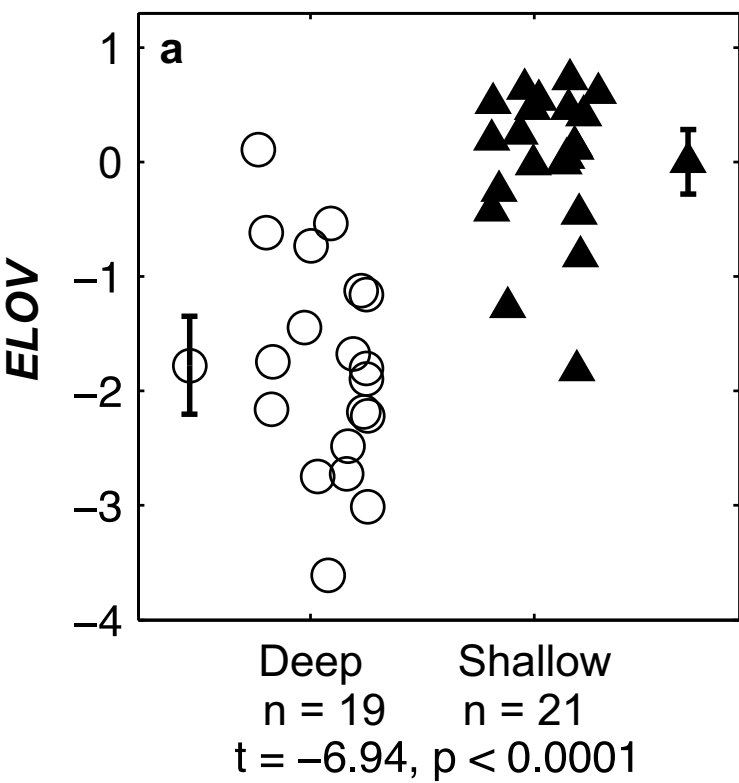


Figure 5

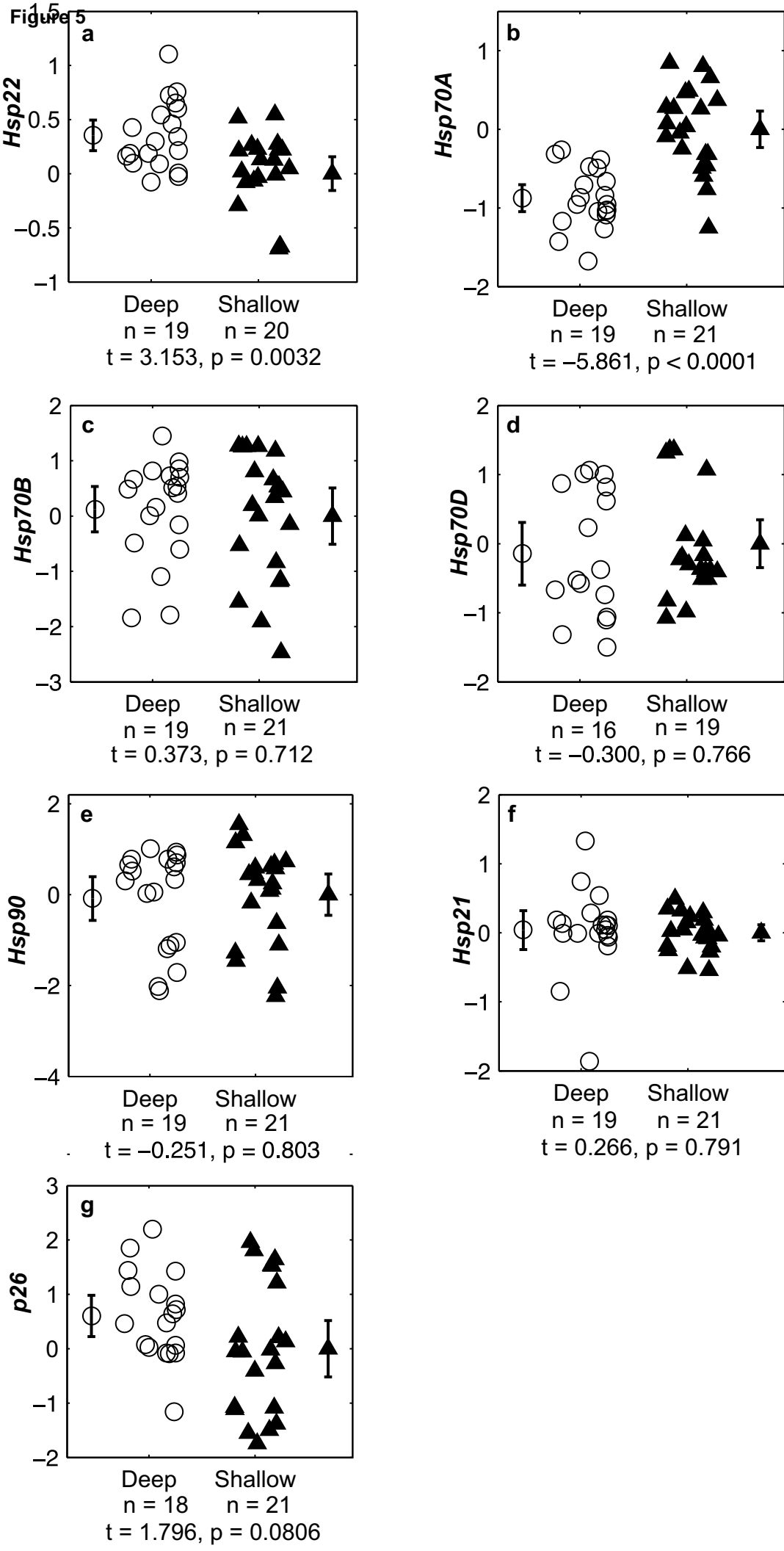


Table 1. Annotation of cloned *Calanus finmarchicus* Hsps. Accession numbers are given for the selected crustacean Hsps used to search the EST database (“BLAST input”) and the subsequent *C. finmarchicus* EST hits. Portions of each EST sequence were cloned in the present study. The percent identity was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

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

^aE-values based on BLAST search on 5 August 2010.

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

Gene	Primer Sequence	T_m IQMix	T_m EvaFast
<i>Hsp90</i>	F: 5'-TCATCCGGATTTCAGCTTGGAG-3'	64	60
	R: 5'-GGTGGCATGTCGCTGTCATC-3'	64	60
<i>Hsp70A</i>	F: 5'-CGAAACAGCAGGAGGAGTGATG-3'	64	60
	R: 5'-TGACAGCAGGTTGGTTGTCTTG-3'	64	60
<i>Hsp70B</i>	F: 5'-TGGAGGGAAAGGCAGCTAAAG-3'	66	60
	R: 5'-CATCGCTGGAACCTAACCCAAGC-3'	66	60
<i>Hsp70D</i>	F: 5'-GGGTGGAGGTGATCCCTAATG-3'	66	60
	R: 5'-TGCACCACTTCATCAGTCCAC-3'	66	60
<i>Hsp22</i>	F: 5'-GGCTACAAGCCAAGTGAGCTG-3'	—	64
	R: 5'-GAGACCATGGTGTGGCCTTC-3'	—	64
<i>Hsp21</i>	F: 5'-TGCAAACACAGCAACAAGCTG-3'	—	62
	R: 5'-GCCTCGGAAAGAGCATTCTTC-3'	—	62
<i>p26</i>	F: 5'-CTTGCCAAGCATGAGACCAAG-3'	64	60
	R: 5'-GGATTGACCCAGATGGTAATG-3'	64	60
<i>ELOV</i>	F: 5'-GTCTGGTGGTGTCTTCTCTCC-3'	64	60
	R: 5'-CACATGCAGAGAGGTAAGTTGG-3'	64	60
<i>RDH</i>	F: 5'-CTAGCCAGGTTGCTGATGAAG-3'	—	64
	R: 5'-TCTTGGAGATGGTGAGGTCTG-3'	—	64
<i>ferritin</i>	F: 5'-AATATCAGACCAAGCGTGGAG-3'	64	60
	R: 5'-AGCTTCCATTGCCTGAATAGG-3'	64	60
<i>16S</i>	F: 5'-AAGCTCCTCTAGGGATAACAGC-3'	64	62
	R: 5'-CGTCTCTTCTAAGCTCCTGCAC-3'	64	62

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. 95% confidence intervals are provided for means, and asterisks indicate significance of the t-test as follows: ** indicates $p < 0.0001$, * indicates $p = 0.0002$.

Indicator of diapause	Expectation	Deep	Shallow	t
<i>ELOV</i> expression	Deep < Shallow	-1.77 ± 0.43	0.00 ± 0.28	-6.94**
<i>RDH</i> expression	Deep < Shallow	-0.55 ± 0.23	0.00 ± 0.14	-4.05*
<i>Ferritin</i> 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