Osmotic induction of stress proteins in nemerteans

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Abstract
Nemerteans can experience fluctuating salinity during exposure to low tides in intertidal mudflats. This study attempted to determine if stress proteins (SPs) can be induced in nemerteans exposed to fluctuating salinities under laboratory conditions. Paranemertes peregrina Coe 1901 were collected from intertidal mudflats. The ribbon worms were then acclimated at 34% for 3–4 days prior to the salinity stress experiments. For the hyperosmotic experiment, 39 and 44% were selected. Salinities of 24 and 29% were used for the hyposmotic experiment. Both experiments used 34% as the control. Three worms were sampled at 0, 2, 4, 6, 12, 18 and 24 h after incubation at each of the salinities. Weights were recorded before and after the sampling periods to determine water efflux and influx under hyperosmotic and hyposmotic conditions, respectively. SPs detected in the nemertean proteins were separated by SDS–PAGE electrophoresis and identified using Western immunoblots with specific antibodies. SP levels were then quantified by densitometry. Worms from the 39 and 44% treatments showed significant weight loss compared with control worms. Worms from the 24 and 29% treatments had significant weight gain compared with control worms. SP70 and SP90 were identified. Two-way analysis of variance showed varying significant effects of salinity, time and the interaction between the two parameters on the induction of SP70 and SP90 in both the hyperosmotic and hyposmotic experiments. Detection of SPs demonstrates their importance in the physiological response of nemerteans to fluctuating salinities.

Keywords: Stress proteins, heat shock proteins, salinity, nemerteans, Paranemertes peregrina

Introduction
In intertidal mudflats, the osmoconforming ribbon worm, Paranemertes peregrina Coe 1901 can experience fluctuating salinities during low tidal exposure. Hyposmotic conditions can occur during heavy rainfall from storms or from spring, creek or river runoff. Conversely, hyperosmotic conditions can occur when the ribbon worms are stranded in very shallow pools or puddles that undergo rapid evaporation during daylight.
The role of the cephalic gland, the cerebral organ and the cerebral ganglia in nemerteans during responses to osmotic variation has been reviewed by Ferraris (1985a). Water and solute regulation in relation to these structures in several nemertean species has been extensively studied (Amerongen and Chia 1983; Ferraris 1985b; Ferraris and Norenburg 1988; Ferraris and Schmidt-Nielsen 1982). Amerongen and Chia (1983) reported that *Paranemertes peregrina* without cerebral organs gained more water under hyposmotic conditions compared with worms that possessed cerebral organs. Under hyposmotic conditions *P. peregrina* may be able to regulate its volume through elimination of extracellular water, Na$^+$ and Cl$^-$, decreasing permeability of body and gut epithelia to inorganic ions, and reducing intracellular volume through regulation of organic solutes (Ferraris 1985b). *Procephalothrix spiralis* Coe 1930 was found to regulate water content through Na$^+$ and Cl$^-$ efflux under hyposmotic conditions (Ferraris and Schmidt-Nielsen 1982; Ferraris and Norenburg 1988).

The function of intracellular proteins can be significantly affected by changes in Na$^+$ and K$^+$ concentrations, which affect catalytic rates of enzymes and their substrate interactions, as well as cellular membrane transport (Levinton 2001). Yancey (2001) reports that salt ions can perturb macromolecules, such as proteins. Also changes in concentrations of water molecules can possibly affect hydration of proteins when their surfaces interact with the surrounding water molecules (Timasheff 2002). Since both water and proteins are dipolar, ions are hypothesized to affect the hydrogen-bonding properties of water (Collins and Washabaugh 1985) and to either salt-out non-polar groups or salt-in peptide groups (Baldwin 1996). Furthermore, Diamant et al. (2001) discussed how increasing concentrations of solutes, ions and water molecules in a confined area (i.e. cell) can result in molecular crowding, which can induce either more protein–protein interactions or between unstable folding intermediates. These interactions can lead to either protein aggregation or more favorable binding of unstable folding intermediates to molecular chaperones during stabilization of the native proteins (Diamant et al. 2001).

Since inorganic intracellular solute concentrations are closely regulated, marine organisms generally rely on organic solutes, such as free amino acids, to help adapt to fluctuating osmotic conditions (Schmidt-Nielsen 1999). Pierce (1982) reported that invertebrates under hyposmotic stress responded through efflux of inorganic ions (K$^+$ and Cl$^-$), organic osmolytes and certain free amino acids. Organic osmolytes, such as amino acids, polyols, sugars, methylamines and urea, are utilized by marine invertebrates in response to hyperosmotic stress (Yancey et al. 1982; Yancey 2001). The role of these osmolytes, known as chemical chaperones, has been attributed to maintaining protein stability and its folding (see reviews of Bolen and Baskakov 2001; Roberts 2005).

In addition to chemical chaperones, marine invertebrates have been reported to produce molecular chaperones/stress proteins (SPs), more commonly called heat shock proteins (HSPs) (see reviews by Feder and Hofmann 1999; Sorenson et al. 2003). During environmental challenges, SPs are induced to maintain physiological integrity of cellular proteins (Feder 1999). SPs serve as molecular chaperones and they help to properly fold and compartmentalize proteins during their biosynthesis and to prevent renaturation and aggregation of damaged proteins (Hartl 1996; Rassow et al. 1997).

Much research on the induction of SPs in various intertidal organisms has focused on temperature stress (Margulis et al. 1989; Sanders et al. 1991, 1992; Hofmann and Somero 1995, 1996; Roberts et al. 1997; Clegg et al. 1998; Tomanek and Somero 1999; Spees et al. 2002b; Hamdoun et al. 2003; Tomanek and Sanford 2003). Intertidal species have also been reported to induce SPs in relation to pollutant exposure (Steinert and Pickwell 1988;
Werner and Nagel 1997), food availability (Dahlhoff et al. 2001) and aggressive encounters (Rossi and Snyder 2001).

The coordination between chemical and molecular chaperones appears to be important in maintaining the integrity and stability of intracellular proteins (Diamant et al. 2001). In *Escherichia coli* cells preadapted to high salinity, Diamant et al. (2001) found that increased glycine betaine prevented protein aggregation. Under hyperosmotic stress, low concentrations of osmolytes, including proline, glycerol and especially glycine betaine, activated molecular chaperones (SPs) by assisting in folding-in chaperone-bound polypeptides, as well as stabilizing native protein end products (Diamant et al. 2001).

Very few studies have been reported on the induction of SPs in invertebrates during osmotic stress (Gonzalez and Bradley 1994; Werner and Hinton 2000; Drew et al. 2001; Spees et al. 2002b; Lyons et al. 2003). All of these previous studies involved hard-shelled bivalves and crustaceans with exoskeletons to minimize osmotic and solute fluxes during osmoregulation.

Firstly, to address the lack of data on soft-bodied invertebrates, this study was initiated to investigate whether SPs are induced during osmotic stress in the intertidal nemertean, *Paranemertes peregrina*. Secondly, this study attempted to determine whether SP concentrations vary in relation to salinity and exposure time. Since our previous work has shown the presence of HSP70 and HSP90 in *P. peregrina* during exposures to sublethal temperatures (Okazaki et al. 2001), we hypothesize that this nemertean species would similarly utilize these two proteins during its osmotic stress response. Studies on the osmotic stress response in this important predator may lead to the further understanding of how soft-bodied helminthes adapt to salinity changes in the intertidal.

**Materials and methods**

**Collection**

*Paranemertes peregrina* were collected from the intertidal mudflats of Bodega Harbor, Bodega Bay, CA during low tides in June 2002. The worms were acclimated to 11°C in flow-through seawater at the University of California Bodega Marine Laboratory for 3–4 days.

**Osmotic stress experiments**

Two separate osmotic stress experiments were conducted. For the hyperosmotic experiment, the following salinities were selected: 34 (control), 39 and 44%. Dissolved sea salts (Sigma Chemicals, St. Louis, MO, USA) were added to filtered seawater (34%) to yield 39 and 44%. For the hyposmotic experiment, filtered sea water (34%) was diluted with distilled de-ionized water to yield 29 and 24%. All salinities were verified with a Wescor vapor pressure osmometer.

Individual worms were weighed and then separately placed into aerated 125-ml Nalgene bottles with their respective salinities. All bottles were placed into seawater troughs at 11°C. The worms were immersed in the salinities for the following time exposures: 0, 2, 4, 6, 12, 18 and 24 h. Three worms were used for each salinity and time exposure.

Weights were recorded after salinity exposure to determine the volume of water loss or gain. After placement on filter paper to remove excess moisture and mucus, the worms were weighed to the nearest 0.1 mg on a Mettler Toledo AB54 microbalance. Each worm
was allowed to relax and then was cut transversely in half. The posterior portion was immediately frozen in liquid N$_2$ for SP analysis. The anterior portion was placed into RNALater (Ambion, Austin, TX, USA) for future mRNA studies.

**Sample preparation**

For detection of SPs, the worm samples were homogenized in hypotonic lysing buffer (40 mM HEPES, 5 mM MgSO$_4$, 10 mM KCl, and 20 mM NaCl, pH 7.5 with a protease inhibitor (Boehringer–Mannheim, Mannheim, Germany). After centrifugation at 15 339 g in an Eppendorf 5810R microcentrifuge, the supernatants were collected. The pellets were then washed with lysing buffer and centrifuged again to ensure complete extraction of the protein. Aliquots of the supernatants were analyzed for total proteins using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) with a Molecular Devices 9600 microplate reader.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)**

Aliquots of the supernatant were combined with equal volumes of 2 × SDS sample buffer (Laemmli 1970) and heated to 100°C for 5 min. Aliquots of the supernatants with the SDS buffer were loaded, based on equal total protein concentrations (either 10 or 15 µg), onto 10-well, 13% sodium dodecyl sulfate polyacrylamide gels (8 × 10 cm) to separate the proteins. Positive controls, 50 ng of HSP70 and 150 ng of HSP90 (StressGen, Victoria, BC, Canada) were also run simultaneously with the experimental and control samples. Gels were then electroblotted onto 0.45-µm Immobilon PVDF membranes (Millipore, Bedford, MA, USA).

**Immunoblot analysis**

The blotted proteins were placed in a TBS–T buffer (Tris-buffered saline and 0.001% Tween 20, pH 7.4 plus 5% w/v nonfat dry milk) to block non-specific binding sites. After several rinses with TBS–T, the blotted proteins were then incubated with anti-mouse SP70 (1:1000) and SP90 (1:500) antibodies (abs) (StressGen). After blocking overnight with 5% nonfat dry milk in TBS–T buffer (150 mM NaCl, 10 mM Tris, 1 ml l$^{-1}$ Tween 20, pH 7.4), and washing in TBS–T, the blotted proteins were incubated in mouse HSP70 antiserum for 1 h (1:1000; StressGen SPA-822), washed and incubated in mouse HSP90 antiserum for 2 h (1:500; StressGen SPA-830). After washing, the secondary ab was added for 1 h. The secondary ab was HRP-conjugated goat anti-mouse IgG (1:1000; Sigma A-4416).

After several washes with TBS–T solution, the blotted proteins and their respective abs were exposed to a secondary ab (1:1000) consisting of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma Chemicals). The membranes were then subjected to a chemi-luminescent reaction (CLR). This process allowed a catalytic reaction to occur between HRP of the secondary ab with 4 µl of 30% H$_2$O$_2$ contained in coumaric acid, luminol and dimethyl sulfoxide in a CLR buffer, pH 8.5. The reaction was captured on single emulsion X-ray films (Kodak BioMax MR). The protein bands on the films were quantified by normalizing with the positive standards and analyzed by densitometry using NIH Imaging software.
Statistical analysis

A two-way analysis of variance (ANOVA) (Sokal and Rohlf 1981) was used to determine significant differences \((P<0.05)\) among the proportional weight changes and SP concentrations in relation to either salinity, time or their interactions. The percentage weight changes were first arcsine-root transformed before performing the statistical analysis.

Results

Weight changes

All nemerteans survived the hyperosmotic and hyposmotic exposures. The hyper-exposed ribbon worms showed a greater weight loss 10–14 and 16–23\% for exposures of 39 and 44\%, respectively, when compared to the controls (2–7\%) (Figure 1). Salinity, exposure time and their interactions had highly significant effects \((P<0.001)\) on weight loss.

The hypo-exposed ribbon worms displayed a greater weight gain of 3–7 and 15–20\% for exposures to 29 and 24\%, respectively, when compared to the controls (2–5\%) (Figure 2). Highly significant effects on weight gain were observed for salinity \((P<0.001)\), exposure time \((P=0.0024)\) and for the interaction between salinity and time \((P=0.0003)\).

Hyperosmotic induction of stress proteins

In the hyperosmotic experiment, SP70 and SP90 were detected in both the experimental and control worms (Figure 3). SP70 concentrations varied significantly in relation to salinity \((P=0.038)\) and to exposure time \((P=0.015)\) (see Figure 4a). At 44\% with a 2–6-h

![Figure 1. Weight changes in Paranemertes peregrina during exposure to 34\% (control), and 39 and 44\% (hyperosmotic) conditions for 0, 2, 4, 6, 12, 18 and 24 h. Values (\%) are expressed as means ± standard error.](image-url)
exposure, elevated SP70 concentrations were somewhat higher (1.0–2.7 pg m$^{-2}$) compared to control (0.5–0.9 pg m$^{-2}$). The interaction between salinity and exposure time had no significant effect on SP70 concentrations.

SP90 concentrations fluctuated significantly ($P<0.0001$) in relation to exposure time only (Figure 4b). After a 2-h exposure to 39 and 44%, higher SP concentrations (5.5–8.0 pg m$^{-2}$) were observed compared with control (3.0 pg m$^{-2}$). Neither salinity nor the interaction between salinity and exposure time had significant effects on SP90 concentrations.

**Hyposmotic induction of stress proteins**

In the hyposmotic experiment, SP70 and SP90 were detected in both the experimental and control worms (Figure 5). Exposure duration had a highly significant effect ($P=0.002$) on
SP70 concentrations (Figure 6a). SP70 concentrations in the experimental worms appeared to be generally lower from 2- to 18-h exposures compared with 0- and 24-h. At 29%, SP70 increased to a high concentration (2.8 pg µg⁻¹) after 24 h. Neither salinity nor the interaction between salinity and exposure time had significant effects on the SP70 concentrations.

Hyposmotic conditions profoundly affected SP90 concentrations (Figure 6b). SP90 was highly induced by salinity (P=0.0009), exposure time (P<0.0001), and by the interaction...
between salinity and exposure time \((P<0.0001)\). SP90 concentrations \((10\text{ and }8\text{ pg}\mu\text{g}^{-1})\) increased in worms from 29 and 24\%\text{\textsubscript{s}}, respectively, after a 6-h exposure compared with controls \((3.5\text{ pg}\mu\text{g}^{-1})\). Similar to SP70 levels after 24 h, worms from the 29\%\text{\textsubscript{s}} exposure group had high SP90 concentrations \((23\text{ pg}\mu\text{g}^{-1})\). However, after 24 h, low SP90 concentrations \((2.0\text{ pg}\mu\text{g}^{-1})\) were observed in the worms from the 24\%\text{\textsubscript{s}} exposure group. Low SP90 concentrations were generally observed at 2, 4, 12 and 18 h for both the experimental and control worms.

**Discussion**

**Osmotic flux**

This study has shown that *Paranemertes peregrina* from intertidal mudflats is able to osmoregulate under both hyperosmotic and hyposmotic stress. Ferraris (1985) observed that *P. peregrina* behaved as a hyposmotic osmoconformer at 100 and 70\% salinities during the first 6 h of exposure. Since nemerteans lack any hard or calcified epidermal structures, weights are expected to fluctuate accordingly to changing salinity conditions. In both hyperosmotic and hyposmotic conditions, the worms showed dramatic weight changes, but survived water efflux and influx. No deaths were observed either in the highest hyperosmotic or lowest hyposmotic salinity, 44 or 24\%\text{\textsubscript{s}}, after 24 h. All of the worms from
both the hyper- and hyposmotic treatments were very lethargic. The nemerteans from the hyperosmotic treatments were shriveled while the hyposmotic-stressed worms were bloated and generally produced copious amounts of mucus. Ferraris (1979) also reported copious mucus production by the cephalic glands in *Lineus socialis* Leidy, 1855 and *Amphiporus lactifloreus* Johnston, 1828 when exposed to hyposmotic conditions. In contrast, Spees et al. (2002a) observed that *Homarus americanus* Milne Edwards, 1837 with its hard exoskeleton was an inefficient osmoregulator. They found that hemolymph osmolarity fluctuated significantly within 30 min in both hyperosmotic and hyposmotic conditions.

**Stress proteins**

This study has demonstrated that SP70 and SP90 were induced in *Paranemertes peregrina* during exposure to hyper- and hyposmotic stress. Both stress proteins were also constitutively present in the control worms during the two experiments. Other invertebrates under osmotic stress have also demonstrated the presence of stress proteins. Only a 70-kDa protein (presumably SP70) was detected in the copepod *Eurytemora affinis* Poppe, 1880 from the hyperosmotic and control salinities, 20 and 10%, respectively (Gonzalez and Bradley 1994). However, this protein was not found in copepods from hyposmotic salinities (2 and 5%). The copepod did indeed show a 70-kDa protein in all salinities ranging from 2–20% when exposed to a high temperature of 30°C. HSP70 has also been reported for the Pacific clam *Potamocorbula amurensis* Schrenck, 1861 (Werner and Hinton 2000), the blacklip abalone *Haliotis rubra* Leach, 1814 (Drew et al. 2001) and the blue mussel *Mytilus edulis* Linnaeus, 1758 (Lyons et al. 2003). Spees et al. (2002b) reported finding both HSP70 and HSP90 in the lobster, *Homarus americanus* exposed to both hyper- and hyposmotic stress.

Both SP70 and SP90 changed in concentration in relation to salinity in both the hyperosmotic and hyposmotic experiments. However, in hyposmotic-stressed worms, only exposure time was found to be a significant factor for SP70 induction; whereas, salinity, time and their interactions significantly induced the production of SP90. Lobsters under hyposaline conditions have also been found to produce more SP70 and SP90 within 30–60 min – an indication of the effect of time on SP response to osmotic stress (Spees et al. 2002a; Chang 2005). Since cells under hyposaline conditions are perturbed by the low ion concentrations, which affect enzyme–ligand interactions rather than protein conformations (Somero and Yancey 1997), perhaps the increased induction of SPs are needed to adjust to this cellular activity.

In this study, higher SP70 concentrations in the hyposmotic-rather than hyperosmotic-stressed worms were observed. These results differ from the down regulation of HSP70 during response to low salinities by clams (Werner and Hinton 2000), abalones (Drew et al. 2001) and mussels (Lyons et al. 2003). Lyons et al. (2003) found 10-fold higher HSP70 concentrations in mussels from a high salinity habitat (36%) compared to low salinity habitats (6–21%). All three are molluscs that have hard shells to help buffer osmotic shock. They have perhaps evolved to utilize their SP70 differently than soft-bodied nemerteans, which require rapid physiological adjustment to osmotic changes. Invertebrates lacking shells or exoskeletons may need to use their SPs to adjust for the dilution or concentration of solutes, especially the production or movement of organic osmolytes to maintain their physiological integrity. Pierce and Warren (2001) reported the efflux of taurine in the bivalve *Noetia ponderosa* Say, 1822 during its response to hyposmotic stress. Lobsters under hyposmotic and hyperosmotic stress do show elevated hyperglycemic hormone
concentrations, which indicate increased glucose activity in response to osmotic stress (Chang et al. 1998; Chang 2005). Further comparative studies with soft-bodied and hard-shelled invertebrates during osmotic stress may elucidate interestingly different functional roles of SPs and their possible relationship in the production or movement of organic osmolytes.

In this study, the effects of salinity, exposure time and their interaction significantly affected the induction of SP90 especially in the hyposmotic-stressed worms. SP90 concentrations were observed to be higher compared to SP70, in both the hyposmotic- and the hyperosmotic-stressed worms. Okazaki et al. (2001) reported relatively low levels of SP90 in *Paranemertes peregrina* during its response to sublethal temperatures. These differences in the induction of SP90 between salinity and temperature stress could indicate its possible role in some membrane function, especially during ion and/or organic osmolyte fluxes. Ferraris (1985) postulated that *P. peregrina* under hyposmotic conditions may decrease permeability of its body and gut epithelia to inorganic ions. SP90 has previously been reported to be involved in signal transduction pathways for glucocorticoids (Hutchinson et al. 1994) and the induction of cytoplasmic dioxin receptors (Whitelaw et al. 1995).

This study has demonstrated the induction of SP70 and SP90 during the osmotic response of the nemertean to hyper- and hyposaline conditions. Both SPs are found ubiquitously in many different invertebrate taxa (Feder and Hofmann 1999; Okazaki et al. 2001). Like the nemertean from this study, Yancey (2001) found a decrease in HSP70 during hyperosmotic stress in vertebrate canine kidney cells. Thus, SP response to hyperosmotic stress appears to be highly conserved. Ferraris and Garcia-Perez (2001) reported that the accumulation of organic osmolytes in response to hyperosmotic stress is also highly conserved among different taxa ranging from bacteria and yeast to mammalian cells. Future work in our laboratory will analyze the remaining anterior portions of the worms to correlate transcriptional activity during the production of SP70/SP90 and accumulation of organic osmolytes. Further studies of nemertean SPs could be important in elucidating their ecological and evolutionary significance for the survival of marine organisms.

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