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ORIGINAL PAPER

Stress levels over time in the introduced ascidian *Styela plicata*: the effects of temperature and salinity variations on hsp70 gene expression

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Abstract Species distribution, abundance, and long-term survival are determined by biotic and abiotic regimes. However, little is known about the importance of these factors in species range expansion. *Styela plicata* is a solitary ascidian introduced all over the world by ship fouling, including salt marsh habitats, where introduced populations must tolerate high seasonal variations in temperature and salinity. To determine the seasonal stress levels in a salt marsh population of *S. plicata*, we quantified heat shock protein (hsp70) gene expression using quantitative real-time PCR throughout a 2-year cycle. Results showed that hsp70 expression varied over time, with higher stress levels recorded in summer and winter. Periodic conditions of high temperatures, particularly when coupled with low salinities, increased hsp70 gene expression. Mortality events observed every year around June were concurrent with sharp increases in temperature (>6°C), indicating that drastic changes in abiotic factors may overwhelm the observed stress response mechanisms. Determining the ability of introduced species to cope with stress, and the thresholds above which these mechanisms fail, is fundamental to predict the potential expansion range of introduced species and design efficient containment plans.

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Introduction

Stress response mechanisms allow marine organisms to cope with unexpected or sharp fluctuations in one or several biotic or abiotic factors (Aruda et al. 2011; Clark and Peck 2009; Cottin et al. 2010; Huang et al. 2011; Lockwood et al. 2010). Depending on the extent and duration of the stress, organisms can recover, survive for a time with an impaired fitness, or die. The persistence of stress factors can shape an organism's distribution, excluding it from some locations (e.g., Osovitz and Hofmann 2005). Physical parameters such as temperature and salinity can vary over time, especially in particular habitats such as marginal marine and anthropogenic environments (estuaries, bays, and harbors). At a broader scale, climate change will yield a global increase of seawater temperature, and current studies suggest that most marine organisms do not posses the necessary mechanisms to deal with this stress and will be replaced by species better adapted to warm environments (e.g., Helmuth et al. 2005; Somero 2010). Biological factors such as space competition, epibiosis, disease, and predation may also stress an organism. The impact of these biological factors on a given population is often limited, as only a few individuals within a community are generally involved in a particular interaction. On the other hand, the arrival and establishment of a non-indigenous species may alter the biological interactions of a whole community, yielding a significant disruption of well-established networks (e.g., Harris and Tyrrell 2001; Strayer et al. 2006).

From the point of view of an introduced species, successful colonization of a new environment also depends on the

occurrence of adequate physical and biological conditions, both for adults and larvae (Stachowicz et al. 2002; Verween et al. 2007; Blackburn and Duncan 2001; Fowler et al. 2011; Zerebecki and Sorte 2011). Thus, widely introduced species should be opportunistic and able to colonize new habitat rapidly, often exploiting temporal windows of tolerable conditions (McKinney 2002). Among fluctuating environments, salt marsh communities provide an ideal setting to assess the natural ability of a species to cope with strong changes in salinity and seawater temperature (Weinstein 1996; Gascon et al. 2005). Only those organisms adapted to wide environmental fluctuations can survive in the long term, successfully colonizing these habitats [e.g., the polychaete *Nereis diversicolor* (Paramor and Hughes 2004; Aberson et al. 2011) and the limpet *Crepidula fornicata* (Blanchard 1997; Bishop 2005)]. In order to cope with sharp abiotic changes that can yield suboptimal and stressful conditions, successfully introduced species should be equipped with efficient physiological mechanisms to respond to stress (Thomsen and McGlathery 2007; Piola and Johnston 2008; Dafforn et al. 2009).

Heat shock protein response is the first mechanism deployed by eukaryotes to deal with an accumulation of non-native proteins in stressed cells through increased expression of heat shock proteins (hsps; Voellmy and Boellmann 2007). Hsps are involved in proper folding or unfolding of proteins and participate in the removal of non-native or aggregated proteins from the cell (Gething and Sambrook 1992; Parcell and Lindquist 1993; Feder and Hofmann 1999). To date, it is unclear whether changes in hsp expression can be directly correlated with protein abundance (Vogel et al. 2010), although recent studies suggested that for most common heat shock proteins, an immediate induction of expression is followed by a subsequent increase of the corresponding protein abundances (Maier et al. 2011). Thus, increased transcription of stress-related genes can be considered an early indicator of stress, which is of utmost importance when dealing with invasive species.

The development of new genetic tools such as gene expression quantification has allowed for the detection of minute changes in the stress response of marine organisms and provided insight into their tolerance thresholds and role in resilience (Hofmann and Place 2007). To date, most of the studies ascertaining stress levels through quantification of gene expression in marine organisms have targeted the heat shock protein 70 (hsp70) and have focused on thermal resilience (e.g., Osovitz and Hofmann 2005; López-Legentil et al. 2008; Henkel and Hofmann 2008; Feidantsis et al. 2009; Rodriguez-Lanetty et al. 2009).

Ascidians, or sea squirts, are conspicuous components of epibenthic marine communities all over the globe (e.g., Glasby 2001; Voultsiadou et al. 2007) and are among the most important marine invaders worldwide (Lambert 2002, 2007). Most of these species are known to rely on anthropogenic transport for long-distance dispersal and new habitat colonization (e.g., López-Legentil et al. 2006; Rius et al. 2008; Barros et al. 2009; Pineda et al. 2011). Little is known about stress tolerance in ascidians and the genes involved in stress response and regulation. In fact, stress-related genes have only been described to a significant extent for one species, the phlebobranch ascidian *Ciona intestinalis*, for which the complete genome has been sequenced (Dehal et al. 2002, Fujikawa et al. 2010).

Styela plicata (Lesueur, 1823) is a solitary ascidian commonly found inhabiting harbors of warm and temperate oceans, usually at high densities. In spite of its broad geographical distribution, the native range of this species is not yet elucidated (Lambert 2001; Pineda et al. 2011). The introduction success of *S. plicata* to new regions has been attributed to its high tolerance of polluted waters (Naranjo et al. 1996) and changes in temperature and salinity (Sims 1984; Thiyagarajan and Qian 2003). A prompt response to stressors during both larval and adult stages are critical for the long-term establishment of ascidians in a new habitat (e.g., Dybern 1967; Vázquez and Young 1996, 2000).

In the USA, the Atlantic Intracoastal Waterway extends along most of the Eastern Seaboard, from Norfolk, VI to Miami, FL. The waterway was built to provide a navigation channel for trade and transport and is periodically dredged to allow passage of deep-draught ships. Along its length, natural areas (rivers, bays, and sounds) alternate with artificial stretches and numerous inlets that communicate the waterway with the Atlantic Ocean. In the Wilmington stretch (North Carolina), the waterway is surrounded by *Spartina alterniflora* salt marsh habitat and separated from the Atlantic by the Masonboro Island (Mallin et al. 2000). The Masonboro Sound is characterized by strong salinity and temperature oscillations (Sutherland 1974), and fast terrestrial development, which has exposed the benthic communities living in the Sound to increased sediment runoff, nutrient, and organic inputs (Mallin et al. 1999).

The goal of this study was to advance our understanding of the factors shaping the distribution of the introduced ascidian *S. plicata* by monitoring stress responses in a salt marsh population exposed to wide temperature and salinity fluctuations. To achieve this goal, we measured temperature and salinity changes over a 2-year period and quantified hsp70 gene expression using quantitative real-time PCR (QRT-PCR). We hypothesized that *S. plicata* will feature a high plasticity in the production of stress proteins and will respond to sharp fluctuations in temperature and salinity by increased transcription of these proteins.

Materials and methods

Hsp70 gene characterization and amplification

The first objective of this study was to localize, isolate, and sequence the hsp70 gene for the ascidian *Styela plicata*. To achieve this goal, two individuals of *S. plicata* from each of the following Spanish populations: Blanes (41°40′29″ N, 2°47′56″ E), Vilanova i la Geltrú (41°12′53″ N, 1°44′10″ E), San Fernando (36°28′51″ N, 6°10′52″ W), and Wilmington, NC in the USA (34°8′24″ N, 77°51′44″ W), were collected in 2008 and kept in absolute ethanol until processed. Samples were collected from different countries to increase our probability of finding different alleles and locating conserved regions in *S. plicata*'s hsp70 gene. DNA extractions were obtained using the Puregene and the QIAamp DNA Mini Kit kits (Qiagen). For amplification of the target gene (hsp70), a nested PCR was performed using the primers described in Borchiellini et al. (1998) for sponges in the first PCR, and after obtaining some preliminary sequences, the newly designed primer set SPNC-INT A: 5′-TCC GGA AGA AAT CAG CTC AAT GGT-3′ and SPNC-INT B: 5′-ATG CAA CAG CTT CGT CTG GAT TGA-3′ for the second. For the first PCR, conditions were as follows: A single soak at 95°C for 5 min, 35 amplification cycles (denaturation at 95°C for 1 min; annealing at 45°C for 1 min; and extension at 68°C for 3 min), and a final extension at 72°C for 10 min. Conditions for the second PCR consisted of a single soak at 95°C for 5 min, 35 amplification cycles (denaturation at 95°C for 1 min; annealing at 50°C for 1 min; and extension at 68°C for 2 min), and a final extension at 72°C for 10 min. Amplification for the San Fernando and Wilmington samples was carried out in a Peltier PTC-200, and for the Blanes and Vilanova i la Geltrú samples, in an Eppendorf Mastercycler machine. To obtain purified amplification products, amplification bands were cut from a low melting point agarose gel (1%) following the PerfectPrep Gel Cleanup kit procedure (Eppendorf). The purified DNA was cloned in *Escherichia coli* using the TOPO® TA Cloning® Kit and One Shot® TOP10 competent cells, according to manufacturer's instructions (Invitrogen). Sixteen positive colonies from each population were sequenced using the BigDye TM terminator v. 3.1 and the plasmid primers T7 and M13R. Sequences were obtained on an ABI Prism 3100 automated sequencer located at the Center for Marine Science (UNC Wilmington) or at the Scientific and Technical Services of the University of Barcelona (Genomics Unit).

Hsp70 phylogeny

The phylogenetic relationships of the 22 hsp70 gene sequences obtained in this study were determined by comparison with previously reported hsp70 family sequences in GenBank derived from marine invertebrates (n=20; representing 15 species from 4 phyla) and two outgroup sequences from fungi. Only four sequences from ascidians were found, two for the phlebobranch *C. intestinalis* (Fujikawa et al. 2010) and two for the phlebobranch *Ecteinascidia turbinata* (López-Legentil and Turon 2007). Nucleotide sequences presented numerous deletions and mutations and could not be unambiguously aligned using standard alignment algorithms. Thus, we translated all nucleotide sequences to amino acid sequences and aligned them using the ClustalW Multiple Alignment tool in Bioedit® v.7.0.5.3 (Hall 1999). This final alignment was used to build a consensus neighbor-joining tree using MEGA v.5.0 (Tamura et al. 2011). Confidence in the nodes was assessed by 10,000 bootstrap replicates (Felsenstein 1985).

Hsp70 temporal variation samples and environmental data

Six to seven adults of *S. plicata* were collected monthly from April 2007 to July 2009 (28 months) from the Center for Marine Science docks. The docks are located in a salt marsh area within the Intracoastal Waterway (UNC Wilmington; 34°8′24″ N, 77°51′44″ W). Seawater temperature and salinity were measured with a digital thermometer and a refractometer, respectively. Samples were handpicked, immediately placed in a bucket with ambient seawater, and transported to the lab (less than 100 m away). Once in the lab, ascidians were carefully dissected to avoid puncturing their stomach and digestive tract, and branchial tissue was immediately frozen and stored at −80°C.

RNA extraction and cDNA synthesis

From each individual, 100 mg of tissue from the branchial sac was carefully sampled and homogenized in TRIzol® reagent (Invitrogen). The Micro-to-Midi RNA purification kit (Invitrogen) was subsequently used to purify RNA, according to manufacturer's instructions. RNA was re-suspended in 100 μL nuclease-free water. In order to eliminate any remaining DNA from the RNA extractions, all samples were DNAse-treated using DNAse Amplification Grade I (Invitrogen). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the SuperScript Reverse Transcriptase II kit (Invitrogen) following manufacturer's instructions. Reactions to create cDNA were carried out with the specific primer for hsp70 SPNC-INT B described above and a newly designed primer for 18S rRNA gene 5′-AAG ACT TTG GTT TCC CGG AAG CTG-3′, based on 14 sequences of *Styela* spp. available in GenBank (FM897318 to FM897325, L12442 to L12444, AH001758, AY903923, and M97577). To our knowledge, no previous study aiming to quantify gene expression in ascidians exists, and therefore, few sequences for potential reference genes are available. On the other hand, previous studies have demonstrated that 18S rRNA transcript abundance is stable under

differing conditions (Marino et al. 2003; Kim et al. 2003; Li et al. 2011), and this gene is commonly used in ascidians to perform phylogenetic analysis (e.g., Zeng et al. 2006; Pérez-Portela et al. 2009). Thus, based on current information and available data, we decided to use a fragment of the 18S rRNA gene as an internal reference gene for this study.

QRT-PCR primer design

The QRT-PCR primer set 5′-GYG GAA CAT TGG AAC CAG-3′ (forward) and 5′-CAG CTT CGT CTG GAT TGA TTG-3′ (reverse) was designed against a 135-base pair region of the hsp70 gene. The primers 5′-GGA AGA CGA ACT ACT GCG AAA GCA-3′ (forward) and 5′-AAG ACT TTG GTT TCC CGG AAG CTG-3′ (reverse) were designed against a 130-base pair region of the 18S RNA gene of *S. plicata*. All primers for QRT-PCR were designed using the Primer Express software (Applied Biosystems).

QRT-PCR of hsp70 transcripts

To quantify mRNA abundance of the hsp70 gene, we used a 7700 Applied Biosystems quantitative real-time PCR and the standard curve method. Standards for the 18S rRNA gene (reference gene) and the hsp70 gene (target gene) were obtained by cloning (TOPO TA Cloning® Kit, Invitrogen). Positive colonies were analyzed by PCR using specific primers targeting the plasmid. Colonies containing the correct insert were grown overnight in an LB liquid media containing kanamycin. Plasmid extraction was performed using the PerfectPrep plasmid mini kit (Eppendorf) and sequenced to verify again that the correct fragment of 18S rRNA or hsp70 gene was present. QRT-PCR reactions were performed with 2 μL of hsp70 cDNA or 1 μL of 18S cDNA (previously diluted to 1:100 *v*/*v*), in 10 μL SYBR GreenER SuperMix (Invitrogen) and nuclease-free water to a final volume of 20 μL. The PCR conditions were as follows: a single soak at 50°C for 2 min and 95°C for 10 min and was followed by 40 amplification cycles (95°C for 15 s, 58°C for 15 s, and 68°C for 45 s); finally, the dissociation step consisted on an extra cycle of 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. Each 96-well plate contained samples in triplicates, as well as 7-fold serial dilution of the corresponding standard and negative controls in duplicates, for both the target and reference genes. Melt curve analysis was performed following each PCR to confirm that a single product was amplified. Relative abundances were calculated for each triplicate according to a reference standard curve. These triplicate QRT-PCR values were averaged to obtain a single value per sample and gene (target and reference). To obtain the ratio of the target gene corrected for the reference gene, we divided the averaged value of the target gene by the one of the reference gene. This ratio value was used to obtain monthly averages and for statistical inference (see below).

Data analysis

A non-parametric Kruskal–Wallis one-way analysis of variance was performed to assess whether there were significant differences in hsp70 gene expression among months. Post hoc comparisons were made using the Dunn's method. Likewise, a two-way ANOVA was performed to test for significant effects and potential interaction of temperature and salinity on hsp70 gene expression, according to preestablished groups for temperature (<20°C, 20–25°C, and >25°C) and salinity (<28‰, 28–32‰, and >32‰). Data were rank-transformed (Conover and Iman 1981) prior to this analysis to meet the assumptions of normality and homoscedasticity. In the presence of a significant interaction (see "Results" section), comparisons using the Student–Newman–Keuls (SNK) test were made for levels of one factor at each level of the other factor using the common error mean square (Quinn and Keough 2002).

In addition, hsp70 gene expression over time was related to temperature and salinity variations using monthly means and cross-correlation analyses (using the Pearson coefficient). In these analyses, the values of one variable were correlated with the values of the other at different time lags (months). All analyses were performed using the software SYSTAT v. 12 (©SYSTAT Software, Inc. 2007) and SigmaStat v. 3.11 (©SYSTAT Software, Inc. 2004).

Results

A total of 50 sequences of 761 base pairs were obtained for the hsp70 gene of *S. plicata* (GenBank accession nos. JN593023 to JN593072). Further analyses revealed 30 unique sequences with an overall nucleotide diversity of 0.07167±0.00213. Translation of these sequences yielded 22 unique amino acid sequences and a total amino acid variability of 0.035 substitutions per site.

The amino acid sequences obtained here for *S. plicata* were distributed in two clades (Fig. 1), with a between groups mean distance of 0.057 substitutions per site. Both clades were further grouped with one hsp70 sequence described for the ascidian *Ciona intestinalis* and were part of the largest clade retrieved in the analysis (Fig. 1). This large clade also included sequences from Cnidaria and Porifera, which formed two moderately supported clades (bootstrap values >60), and the Arthropoda, which appeared as a polyphyletic group (Fig. 1). Other ascidian sequences for *C. intestinalis* and *Ecteinascidia turbinata* formed a well-supported

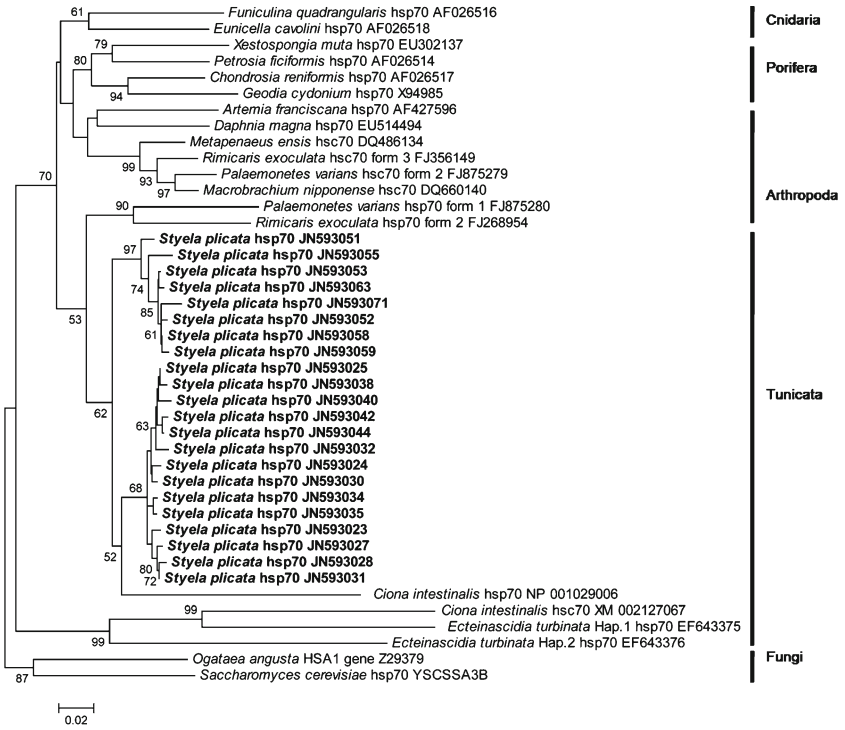

Fig. 1 Phylogeny of partial hsp70 amino acid sequences from marine organisms highlighting the phylogenetic position of the 22 unique sequences obtained in this study for the ascidian *Styela plicata* (*bold letter*). Two fungi sequences were used as outgroup taxa. *Labels* on terminal nodes of reference sequences indicate the species, gene, and GenBank accession numbers. Tree topology was obtained from neighbor-joining analysis and bootstrap values above 50% confidence level are shown above the nodes. *Scale bar* represents 0.02 substitutions per site

clade (bootstrap support=99), but its position within the tree could not be resolved.

The temperature showed a clear seasonal trend, with peaks above 30°C in summer and reaching down to less than 9°C in winter 2008, while in winter 2009, the values were ca. 5°C higher (Fig. 2). The salinity values ranged between 26‰ and 38.5‰ and showed a less clear trend, with generally higher values in autumn–winter and lower values in spring–summer. However, abrupt fluctuations from 1 month to the next were also observed (e.g., December 2008; Fig. 2).

There were wide fluctuations in hsp70 ratio values during the study period (Fig. 2). These values ranged between 0.00011 (±SE 0.00042) in June 2008 and 0.00178 (±SE 0.00069) in August 2007, with an overall mean of 0.00048 (±SE 0.00008). Inter-individual variability was also observed within months (as revealed by wide error bars in Fig. 2). The monthly coefficient of variation (ratio between standard deviation and mean) of hsp70 values was of 0.71. In contrast, the intra-individual replicates had a coefficient of variation of 0.15.

Overall, hsp70 expression varied widely over time, with higher stress levels recorded in summer and winter. The ANOVA (Kruskal–Wallis) showed significant differences between months (H=83.42, df=26, P<0.001). Hsp70 transcript levels were significantly higher in August 2007 and

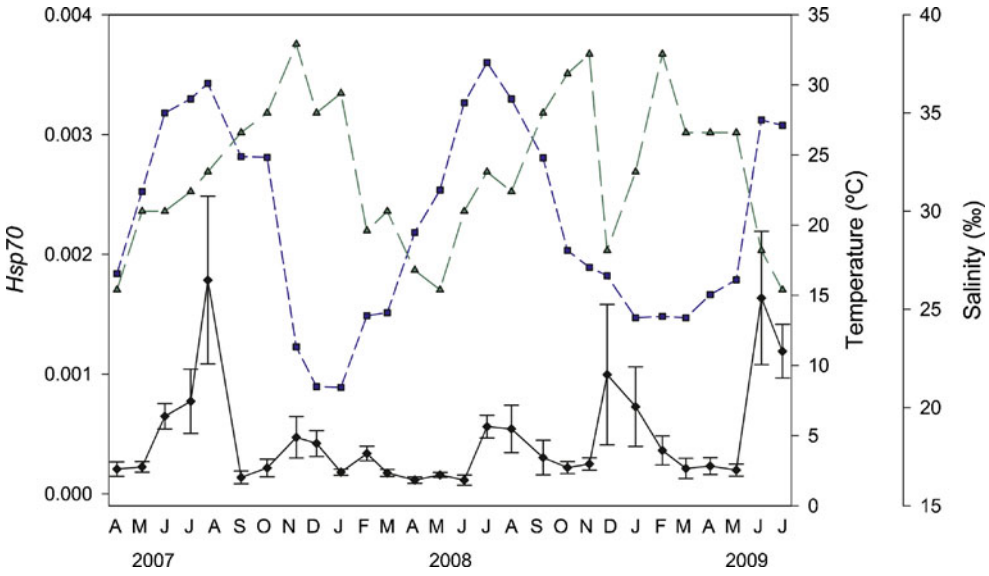

Fig. 2 Hsp70 gene expression from April 2007 to July 2009 (*black diamonds and continuous line*). Temperature and salinity values are superimposed (*squares and short dashes* for temperature; *triangles and long dashes* for salinity). *Vertical bars* denote standard errors

June–July 2009 than during the other months (Dunn test, $P<0.05$). The peak recorded in August 2007 corresponded to a sharp increase in temperature, while the increase in hsp70 gene expression observed in June–July 2009 corresponded to the conjunction of an increase in seawater temperature and a decrease of salinity values. Another increase in hsp70 transcript levels (albeit not significant due to large variance) was observed in December 2008, concomitant with a sharp drop in salinity values.

Cross-correlation analyses between hsp70 gene expression and temperature or salinity (Fig. 3) showed that the strongest correlation occurred at time lags of 0 (i.e., within readings from the same month), being positive in the case of temperature and negative in the case of salinity. A correlation at time lag 0 indicates that the effect of these variables, if any, is immediate and is not due to values in the preceding time periods. It should be noted, however, that the correlation was significant only for hsp70 and temperature at time lag 0 (Fig. 3a).

Examining hsp70 expression levels according to different temperature and salinity groupings revealed that high temperatures appeared to exacerbate the effects of salinity, especially in the low-salinity group (Fig. 4). Accordingly, a two-way ANOVA revealed a significant interaction between temperature and salinity (Table 1). Comparisons of salinity effects at each

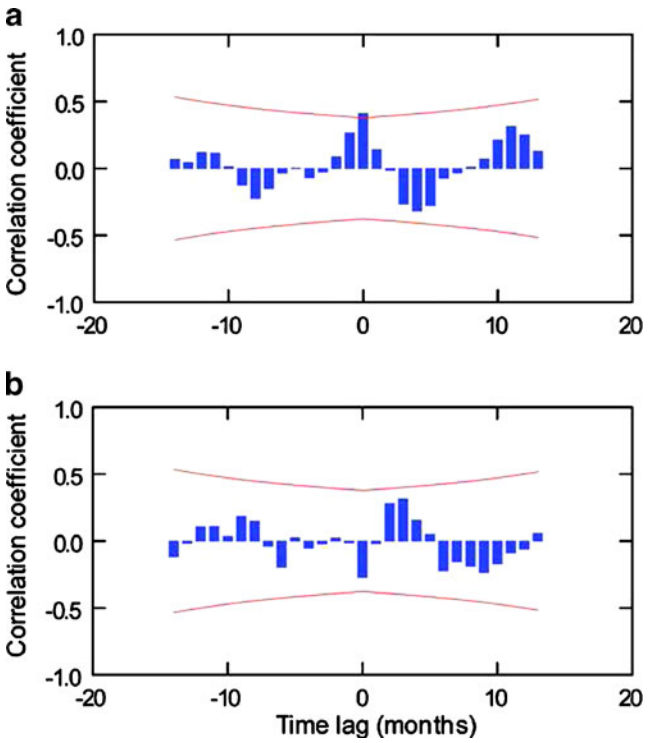

Fig. 3 Cross-correlation analyses between hsp70 gene expression and **a** temperature and **b** salinity. *Curved lines* bound the 95% confidence interval of the correlation coefficient in case of no association. Time lag is in months. Correlation at time lag 0 is the usual Pearson correlation

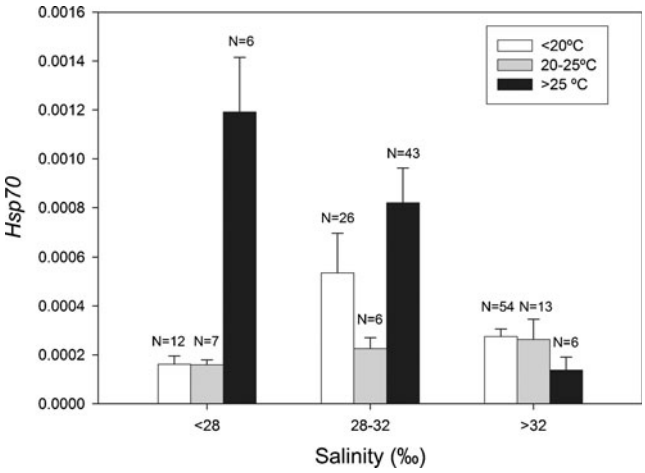

Fig. 4 Hsp70 gene expression over the 28 studied months grouped by temperature and salinity ranges. *Vertical bars* denote standard errors

temperature level (SNK tests) revealed that at seawater temperatures lower than 25°C, there was no clear effect of salinity on *hsp70* expression levels (Fig. 4, SNK tests all non-significant except for the comparison between low and intermediate salinities at <20°C). However, when seawater temperature reached values over 25°C, hsp70 gene expression increased with decreasing salinity values (Fig. 4), with hsp70 transcript levels significantly higher at <28‰ than at higher salinities (SNK test, $P=0.019$). Likewise, no significant effect of temperature was found at intermediate or higher salinities (SNK tests, all comparisons $P>0.05$). At low salinities (<28‰), hsp70 transcript levels were significantly higher at temperatures >25°C than for the other temperature groups (SNK test, $P<0.001$).

Discussion

Phylogenetic analysis showed a wide diversity in the hsp70-like proteins of marine invertebrates. Even the few ascidian sequences available in GenBank and included in this study

Table 1 Two-way ANOVA results to test for significant effects and potential interaction of temperature and salinity on *hsp70* gene expression

	SS	*df*	MS	*F* statistic	*P* value
Temperature	22,566.707	2	11,283.354	5.831	0.004
Salinity	20,789.934	2	10,394.967	5.372	0.005
Temperature × salinity	40,264.134	4	10,066.034	5.202	<0.001
Residual	317,327.181	164	1,934.922		
Total	431,462.000	172	2,508.500		

Salinity and temperature groups as in Fig. 4. Data were rank-transformed (see text)

SS sum of squares, *df* degrees of freedom, MS mean of square

were grouped in two distinct clades. Two distinct clades were also retrieved for our *S. plicata* sequences, both closely related to sequences described for the phlebobranch ascidian *C. intestinalis*. Our results, however, demonstrated that all hsp70 sequences recovered herein were closely related and probably belong to the same gene ortholog.

A seasonal trend in hsp70 gene expression was observed for the ascidian *S. plicata* in the studied salt marsh, indicating important changes in the physiological stress levels of this species over time. The observed variability in hsp70 expression levels among simultaneously sampled individuals (as reflected by the error bars in Fig. 2) was probably due to the presence of genetically distinct individuals in our sample set. Intraspecies variability in stress response has been reported in previous studies and is common in marine invertebrates (Agell et al. 2001; Osovitz and Hofmann 2005; Rossi et al. 2006; López-Legentil et al. 2008).

High levels of hsp70 gene expression have been correlated with seawater temperature increases in many marine invertebrates (Osovitz and Hofmann 2005; López-Legentil et al. 2008; Pantile and Webster 2011). Accordingly, in this study, we found that significantly higher levels of hsp70 gene expression occurred during the summer months. Moreover, important mortality events occurred around June 2007, 2008, and 2009 when seawater temperatures reached values above 27°C. During these times, over 90% of the population of *S. plicata* disappeared or were dying, with an uncharacteristically soft and blackened tunic and the interior guts spilling out through the siphons or cuts in the tunic (authors' personal observation). Mortality or recovery of stressed animals is determined by the extent of damage to essential cellular structures (Downs et al. 2002). Minor damage can be repaired by an increase in hsp activity, while a prolonged exposure to stress leads to metabolic failure in a relatively short time (within a month in our case). Thus, our data suggested that extreme physiological stress resulting from a sharp increase in seawater temperature (>6°C between monthly readings) caused the massive mortality observed in *S. plicata*. Important episodic decreases in *S. plicata*'s populations were also reported in previous studies conducted in the same area (Sutherland 1974, 1978). However, those events were recorded in fall and were attributed to substrate inadequacy to support the large individuals resulting from summer growth.

Besides temperature, other factors are also known to significantly stress marine organisms, including sharp salinity decreases (e.g., Kültz 1996; Deane and Woo 2004; Yang et al. 2009), food constraints (e.g., Rossi et al. 2006), hypoxia (e.g., Ma and Haddad 1997), ocean acidification (e.g., O'Donnell et al. 2009), and the presence of pollutants (e.g., Müller et al. 1995; Agell et al. 2004; Azumi et al. 2004; Micovic et al. 2009; Su et al. 2010; Bozinovic and Oleksiak 2011). Several studies have also documented the physiological response of organisms under a combination of multiple potential stressors (O'Donnell et al. 2009; Lockwood et al. 2010; Monari et al. 2011). Thiyagarajan and Qian (2003) found that *S. plicata* recruitment success and post-larval growth in summer were impaired by high seawater temperatures (26–30°C) and low salinities (about 22–30‰). Similarly, in our study, we have found that the interaction between temperature and salinity on hsp70 gene expression was significant. In particular, at seawater temperatures over 25°C, hsp70 gene expression appeared to increase with decreasing salinity values. However, statistical significance was only recorded for the combination of high temperatures (>25°C) and low salinities (<28‰) recorded once in July 2009. Further experimentation in aquaria under tightly controlled environmental conditions is needed to pinpoint the effect of temperature and salinity fluctuations over several development stages of *S. plicata* and assess whether these factors are currently limiting the actual distribution of this species.

The biogeographic distribution of marine species is determined by each species tolerance to stress (Feder and Hofmann 1999), in which the heat shock response is a key factor. Thus, establishment of a new species is possible whenever the levels of environmental conditions fall within the tolerance range of the species. Likewise, if this range is wider for an introduced species than for directly competing native organisms, then the newcomer can become invasive (Stachowicz et al. 2002). For instance, Lockwood and Somero (2011) suggested that the success of the mussel *Mytilus galloprovincialis* over *Mytilus trossulus* in the west coast of the USA was due to the ability of *M. galloprovincialis* to deal with acute heat stress by producing more stress proteins. Although in this study we have not assessed the stress response of *S. plicata* to biotic factors such as competition with other species, the artificial substrates surveyed here were colonized in their nearly totality by *S. plicata*, and no conspicuous predators were observed. Thus, based on our results, it appears that *S. plicata*'s ability to thrive and colonize salt marsh habitats may depend on its ability to withstand severe abiotic changes.

In conclusion, *hsp70* gene expression in the introduced ascidian *S. plicata* varied over time and was significantly correlated to high seawater temperature. Low salinities also appeared to increase hsp70 gene expression, with highest levels of expression recorded at temperatures >25°C and salinities <28‰. The 15-fold variation in expression levels found here is consistent with the prediction that a certain degree of resilience to adverse environmental conditions has facilitated the worldwide distribution of this species. In addition, it is possible that this same ability to physiologically adjust to stressful conditions has allowed *S. plicata* to colonize fluctuating environments such as salt marshes. Even when severe changes in temperature or salinity overcome *S. plicata* tolerance thresholds (i.e., in

June), the species was able to completely refill the studied docks within a month (authors' personal observation), presumably by larvae originating from unknown reservoirs or from hulls of the many ships navigating the Atlantic Intracoastal Waterway. The fast growth rates recorded for *S. plicata* (Yamaguchi 1975; Sutherland 1978) should further allow this species to quickly repopulate any lost habitat. This study highlights the importance of understanding how introduced species respond to a combination of environmental factors in order to predict their invasive potential and prepare efficient containment plans.

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