Expression of hsp70, hsp90 and hsf1 in the reef coral Acropora digitifera under prospective acidified conditions over the next several decades

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Summary
Ocean acidification is an ongoing threat for marine organisms due to the increasing atmospheric CO2 concentration. Seawater acidification has a serious impact on physiologic processes in marine organisms at all life stages. On the other hand, potential tolerance to external pH changes has been reported in coral larvae. Information about the possible mechanisms underlying such tolerance responses, however, is scarce. In the present study, we examined the effects of acidified seawater on the larvae of Acropora digitifera at the molecular level. We targeted two heat shock proteins, Hsp70 and Hsp90, and a heat shock transcription factor, Hsf1, because of their importance in stress responses and in early life developmental stages. Coral larvae were maintained under the ambient and elevated CO2 conditions that are expected to occur within next 100 years, and then we evaluated the expression of hsp70, hsp90 and hsf1 by quantitative real-time polymerase chain reaction (PCR). Expression levels of these molecules significantly differed among target genes, but they did not change significantly between CO2 conditions. These findings indicate that the expression of hsp70 is not changed due to external pH changes, and suggest that tolerance to acidified seawater in coral larvae may not be related to hsp expression.

Introduction
Ocean acidification is a result of anthropogenic climate change and is challenging marine ecosystems (Hoegh-Guldberg and Bruno, 2010). The surface layers of the ocean are acidifying due to the absorption of anthropogenic CO2 and have already absorbed approximately one-third of the human-produced CO2, resulting in a decrease in the ocean pH by 0.1 pH unit from the pre-industrial era (Doney et al., 2009). Atmospheric CO2 concentration, which is currently 380 ppm, is expected to rise to 1000 ppm by the end of the 21st century (Raven et al., 2005). Therefore, the ocean pH is expected to further decrease 0.3 to 0.5 pH units by 2100 (Caldeira and Wickett, 2005).

Seawater acidification has a serious impact on the physiologic processes in marine organisms throughout life, including fertility (e.g. Kurihara and Shirayama, 2004; Havenhand et al., 2008; Albright et al., 2010), calcification and/or growth rate (e.g. Fine and Tchernov, 2007; Kurihara et al., 2007; Dupont et al., 2008), metabolism (e.g. Reippschläger and Pörtner, 1996; Michaelidis et al., 2005), and metamorphosis/settlement (e.g. Albright et al., 2010; Nakamura et al., 2011). On the other hand, the survival of coral larvae are not obviously impacted by acidified seawater (Suwa et al., 2010; Nakamura et al., 2011), suggesting that coral larvae have some level of tolerance to external pH changes. The potential for metabolism suppression, a suggested survival strategy under stressful conditions (Guppy and Withers, 1999), has been reported for coral larvae (Nakamura et al., 2011). Information on the mechanisms underlying a tolerance response to acidified seawater, however, is scarce. Only a few studies have focused on the responses to environmental pH changes at the molecular level in marine invertebrates (Hauton et al., 2009; O’Donnell et al., 2009; Todgham and Hofmann, 2009; O’Donnell et al., 2010) and none of these studies have focused on corals, although profiling and quantifying gene expression will strongly enhance our understanding of the physiologic response of marine organisms in an acidifying ocean.

Heat shock proteins (Hsps) are involved in cellular defense and morphologic changes of organisms at developmental stages (Feder and Hofmann, 1999; Gunter and Degnan, 2007). Although not all Hsps are stress-inducible, they respond to various environmental stressors such as extreme temperatures, pH extremes, anoxia, and various toxic substances (Feder and Hofmann, 1999; Dahlhoff, 2004). Such hsp expression is mainly regulated by heat shock factors (HSFs) (Sorger, 1991; Pirkkala et al., 2001).

Here, we observed the transcription levels of two Hsps (hsp70 and hsp90) and an HSF (hsf1) when larvae of the scleractinian coral Acropora digitifera were exposed to acidified seawater for 1 to 7 days after fertilization. We also evaluated the expression
Materials and Methods

A scleractinian coral species, *Acropora digitifera*, was used in the present study. This coral species distributes commonly around Okinawa coral reefs and is also the most commonly used species in studies of the effects of acidified seawater on several early life stages in coral (Morita et al., 2010; Suwa et al., 2010; Nakamura et al., 2011). This species is also an important model species because their genome has recently been sequenced (Shinzato et al., 2011). This coral species distributes commonly around Okinawa coral reefs and is also the most important role in regulating metamorphosis and developmental plasticity in relation with environmental conditions (Bishop et al., 1998; Rutherford and Lindquist, 1998; Bishop et al., 2001; Queitsch et al., 2002; Baker, 2006). Therefore, evaluating the expression of *hsp70*, *hsp90*, and *hsf1* is a good starting point for investigating the relevancy of *hsp* expression in corals under acidified conditions with quantitative analyses.

Among the Hsp and HSF groups, we focused on the expression of *hsp70*, *hsp90*, and *hsf1* under acidified conditions to gain insight into the response to ocean acidification in coral larvae at the molecular level due to the importance of these genes in the stress response and in early life development. Hsp70 and Hsp90 are generally considered to contribute to stress responses (Freeman and Morimoto, 1996; Feder and Hofmann, 1999). In addition, their expression seems to be related to the activation of HSF1 (Morimoto, 1998; Zou et al., 1998). Hsp90s also have an important role in regulating metamorphosis and developmental plasticity in relation with environmental conditions (Bishop et al., 1998; Rutherford and Lindquist, 1998; Bishop et al., 2001; Queitsch et al., 2002; Baker, 2006). Therefore, evaluating the expression of *hsp70*, *hsp90*, and *hsf1* is a good starting point for investigating the relevancy of *hsp* expression in corals under acidified conditions with quantitative analyses.

**Table 1. Summary of physical conditions in the experimental aquaria at different CO2 conditions.**

| CO2 condition | pH     | Temperature (˚C) | Salinity (%) | TA (µmol/kg) | pCO₂ (µatm) | HCO₃⁻ (µmol/kg) | CO₃²⁻ (µmol/kg) | Ω₉₄₅₆ |
|--------------|--------|-----------------|-------------|--------------|-------------|----------------|----------------|--------|
| Control (380 ppm) | 8.13 ± 0.01 | 27.0 ± 0.0 | 35.0 ± 0.0 | 2255.1 ± 3.9 | 492.3 ± 24.0 | 1776.9 ± 16.6 | 194.1 ± 6.2 | 3.0 ± 0.1 |
| 1000 ppm    | 7.89 ± 0.00 | 27.0 ± 0.0 | 35.0 ± 0.0 | 2255.9 ± 4.2 | 925.6 ± 10.8 | 1954.9 ± 3.9 | 122.1 ± 0.9 | 1.9 ± 0.0 |
stored at −80°C. Total RNA was then isolated using TRIzol Reagent (Invitrogen), following treatment with chloroform and 2-propanol. Total RNA pellets were then washed with 75% ethanol and dried. Dried total RNA pellets were resuspended in 25 μl nuclease-free water. RNA was quantified spectrophotometrically using an ND-1000 UV/visible spectrophotometer (NanoDrop technologies). TURBO DNA-free Kit (Applied Biosystems) was then used to remove contaminating DNA from the RNA preparations. cDNA was synthesized with AMV reverse transcriptase from total RNA prepared as described above, using a Takara RNA PCR kit (AMV) Ver.3.0.

Quantitative real-time PCR analysis

Real-time PCR reactions were conducted in a total volume of 20 μl comprising 10 μl of MasterMix with SYBR green (Bio-Rad), 0.6 μl of each primer (1.2 μM), 0.3 μl of sample cDNA, and 8.5 μl ultrapure water. Real-time PCR reactions were run on a Thermal Cycler Dice Real time system TP800 (TAKARA BIO INC., Shiga, Japan) under the following reaction conditions: 1 cycle of [95°C for 3 min], 45 cycles of [94°C for 30 s → 55°C for 30 s → 72°C 30 s], and 1 cycle of [95°C for 15 s → 60°C for 30 s → 95°C for 15 s]. Data were normalized against the expression of actin, which was confirmed not to vary in response to CO2 over the observational days by two-way factorial analysis of variance (ANOVA).

Primers were designed with Primer 3 (v. 0.4.0) software based on the partial sequence of each target gene of A. digitifera (Table 2).

Relativistic concentrations of mRNA for each pH treatment per observation day (x) were calculated using the following equation:

\[ x = \sum_{i=1}^{4} \frac{1}{2^{\text{cycle}+1}} \text{Ct}(\text{gene} | \text{sample}) \]

where i=anterior number (1 to 4), m=4 (there are four aquariums for each pH treatment), exCt=experimental Ct, and actCt=Ct of reference gene (actin).

Rapid amplification of 5′ and 3′ cDNA ends (RACE) and phylogenetic tree construction

The SMARTer TM RACE cDNA Amplification Kit (Clontech) was used for generating 5′ and 3′ cDNA sequences of hsp70, hsp90, and hsf1. Gene-specific 5′ and 3′ RACE and nested RACE primers for each gene were designed based on the nucleotide sequences of each gene fragment. The generated RACE-ready 5′ and 3′ cDNA, the kit reagents, and total RNA were used. First, PCR reactions were performed for each cDNA end for each gene using the universal primer (UPM) and the gene-specific RACE primers. Then, nested RACE reactions were conducted for each end of each gene by diluting the 1st PCR products as a template with the nucleotide sequence of each target gene of A. digitifera (Table 2).

Table 2. Primers for quantitative real-time PCR.

| Gene   | Sense        | Antisense     |
|--------|--------------|---------------|
| hsp70  | ATCAAATCCTCTC-AGCCAGGT | AGCGGATCTAAAG-GGAAACC |
| hsp90  | CTTTTCGTTGAAA-GGTCTACGC | GGAATGATGTCC-TCGAGTT |
| hsf1   | CAGGCTTCCAG- TTTACA | TTCCTAACAACAC-ACCGGAA |
| Actin1 | GCAGAAAGGAATT- GCTGCTC | CACATCGTGTGGA-AAGTT |

Expression of hsf1 under elevated CO2

Expression of hsf1 under elevated CO2 was significantly higher than that of the other two other genes throughout the experiment. Expression of hsf1 on Day 1 was more than 50-fold higher than that of the other genes and nearly 5-fold more than that of the other observation days for hsf90 (Tukey HSD test, P<0.05). Expression of hsf1 was relatively low throughout the experiment. The expression of hsf1 was bit higher on Day 5 (6 dpf) than on the other days.

Expression of hsp70 under elevated CO2

Expression of hsp70 was not significantly different between CO2 conditions throughout the period of observation (ANOVA, FCO2 conditions × Day = 1.1438, p=0.351, Fig. 4). Expression under the elevated CO2 condition during the first 2 days of observation (Day1 [2 dpf] and Day3 [4 dpf]) was relatively higher than that under the control condition and the other days under the elevated CO2 condition.

Expression of hsp90 under elevated CO2

Expression of hsp90 did not differ considerably between CO2 conditions throughout the experiment, whereas expression was somewhat higher in the control condition than in the elevated CO2 condition (Table 4) (Fig. 5). Expression was significantly higher on Day 1 than on the other days (Tukey HSD test, P<0.05), and then decreased on Day 3, and maintained a low level over the following days.

Expression of hsf1 under elevated CO2

Expression of hsf1 did not vary significantly between CO2 conditions throughout the observation (ANOVA, FCO2 conditions × Day = 0.8503, p=0.48, Fig. 6). The expression patterns were not consistent between CO2 conditions. In the elevated CO2 condition, expression showed a downward trend over time, whereas expression under the control condition was erratic.
Fig. 2. See next page for legend.
showing a tendency to decrease during the first two days, then an increase at Day 5, and a decrease at Day 7.

Discussion

In the present study, we evaluated the expression of hsp70, hsp90, and hsf1 to determine whether these genes are involved in the tolerance response against acidified conditions during the

Table 3. Two-way factorial ANOVA on the gene expression of larvae of Acropora digitifera in the control CO2 condition (380 ppm).

| Source          | Df | Sum of squares | F   | p     |
|-----------------|----|----------------|-----|-------|
| Gene            | 2  | 0.57723        | 28.1457 | < 0.0001 |
| Day             |    | 0.17905        | 5.8206 | 0.0024 |
| Gene x Day      | 6  | 0.35831        | 5.8239 | 0.0003 |

Fig. 2. Phylogenetic trees of hsp70, hsp90, and hsf1 amino acid sequences. (A) hsp70, (B) hsp90, and (C) hsf1. [Hv] Heliothis viriplaca, [Dm] Drosophila melanogaster, [Bf] Branchiostoma floridae, [Pv] Perna viridis, [Mg] Mytilus galloprovincialis, [Cf] Chlamys farreni, [Ai] Argpecten iradians, [Ch] Chironex fleckeri, [Ga] Gallus gallus, [Mm] Mus musculus, [Dr] Danio rerio, [Xl] Xenopus laevis, [Pa] Pongo abelii, [Hs] Homo sapiens heat shock 70 kDa protein 1-like, [Ss] Sus scrofa, [Hs1] Homo sapiens heat shock 70 kDa protein 1-like, [Hs] Homo sapiens, [Ss] Sus scrofa, [Bt] Bos taurus, [Ec] Equus caballus, [Rn] Rattus norvegicus, [Ll] Loa loa, [Bm] Brugia malayi, [Pd] Pocillopora damicornis, [Sp] Stylophora pistillata, [Sk] Saccoglossus kowalevskii, [Bg] Biomphalaria glabrata, [Oe] Ostrea edulis, [Nv] Nematoistella vectensis, [Pt] Portunus trituberculatus, [Me] Metapeneaus ensis, [Dy] Drosophila yakuba, [Se] Spodoptera exigua, [Sf] Spodoptera frugiperda, [Sn] Sesamia nonagrioides, [Lm] Locusta migratoria, [Cg] Cricetulus griseus, [Pt] Pan troglodytes, [Md] Monodelphis domestica, [Xt] Xenopus tropicalis, [Am] Alligator mississippiensis, [Mn] Mscaca mulatta, [Dk] Dendronephthya kuzingani, [Ame] Ailuropoda melanoleuca, [Bi] Boa indicus, [Ce] Cervus eldi, [Ss] Sus scrofa, [Dr] Danio rerio, [Ha] Haliothis asinine, [Mb] Mamestra brassicae, [Hn] Hydra magnipapillata.

Fig. 3. Expression of hsp70, hsp90, and hsf1 in the larvae of cultured Acropora digitifera in the control CO2 condition (380 ppm) at 27°C over 7 days. (A) Overall view of the expression, (B) Enlarged view of y-axis from 0 to 0.1 of Fig. 3A. Error bars indicate mean and standard error (N=4).

Fig. 4. Expression of hsp70 in the larvae of cultured Acropora digitifera in the control and elevated CO2 conditions at 27°C over 7 days. Error bars indicate mean and standard error (N=4).
larval stage in corals. Larvae are considered to have a number of cellular defense mechanisms to buffer the effects of environmental stressors (Goldstone et al., 2006; Hamdoun and Epel, 2007). If the expression of hsps and hsf change in acidified conditions, these stress proteins are likely to be involved in the stress response to acidified conditions (Feder and Hofmann, 1999; Dahlhoff, 2004).

In contrast to our hypothesis, the expression of hsps in coral larvae may not be affected by an external pH change. Our findings demonstrated that the expression levels of hsp70, hsp90, and hsf1 in larvae of Acropora digitifera were poorly differentiated between the control and acidified conditions (Figs 4, 5, and 6). These findings indicate that the pH treatments do not induce heat shock proteins to refold some denatured cytoplasmic proteins by an acid/base imbalance due to external pH changes. Similar to our results, Hauton et al. (Hauton et al., 2009) reported no significant effect of acidified seawater on the expression of hsp70 in the neritic amphipod Gammarus locusta, suggesting that physiologic buffering mechanisms are present in the tissues of G. locusta to cope with a decrease in external pH. Larvae of A. digitifera should be able to cope with the pH decrease that is expected to occur in this century at the cellular level.

Our next step will be to investigate effects of multiple environmental factors simultaneously to clarify the effects of acidified seawater on hsp expression in coral larvae, which was beyond the scope of the present study. For example, expression of hsp70 in sea urchin larvae with a simultaneous temperature increase differs among pCO2 conditions (380 ppm–970 ppm) (O’Donnell et al., 2009). Therefore, in sea urchin larvae, the stress response to thermal stressors is altered according to the acidified conditions.

Although the expression of hsps and hsf1 did not change significantly under the acidified conditions, their expression was significantly different among genes under control conditions.

Hsp70, Hsp90, and HSF1 function differently at early life stages, including the larval stage. For example, Hsp70 is toxic in early development under ambient or non-stressful conditions (Krebs and Feder, 1997), as opposed to its beneficial effects on survival under stressful conditions (Feder et al., 1992). A low abundance of hsp70 at larval stages was previously reported for the fruit fly and the silver sea bream (Velazquez et al., 1983; Deane and Woo, 2003), such as the coral A. digitifera (Fig. 3). This may explain the comparatively low expression of hsp70 in the present study.

Contrary to the toxic characteristics of Hsp70 in early life stages, Hsp90 is among the most abundant proteins in the cytosol of eukaryotic cells (Mayer and Bukau, 1999), and the expression of hsp90 is observed from very early developmental stages under normal unstressed conditions (e.g. Ali et al., 1998; Deane and Woo, 2003; Manchado et al., 2008). Hsp90 is also considered to control cell growth and differentiation (e.g. Krone et al., 1997; Mayer and Bukau, 1999; Deane and Woo, 2003; Krone et al., 2003) and to be involved in hormonal control, which regulates developmental changes in early development of larvae (e.g. Deane and Woo, 2003). Correspondingly, relatively higher expression levels of hsp90 were detected in the present study, suggesting the importance of Hsp90 during early developmental stages.

In response to a multitude of stress conditions, HSF1 acquires DNA binding activity to the heat shock element, leading to the accumulation of Hsps (Wu, 1995; Morimoto, 1998). For instance, the accumulation of Hsp70 leads directly to the binding of Hsp70 to the HSF1 activation domain, and results in the repression of heat shock-induced transcription. This reaction subsequently converts HSF1 trimers to monomers, losing HSF1 DNA binding and then reading, to attenuate the heat shock response (Shi et al., 1998). Thus, Hsp70 may have a role in regulating HSF1 deactivation (Abravaya et al., 1992; Shi et al., 1998). As another example, Hsp90 has a role in maintaining HSF1 in an inert state (Ali et al., 1998; Zou et al., 1998; Bhardwaj et al., 1999). Therefore, these processes may account for the expression profiles of the three target genes in the present study; a relatively high expression of hsp90 and substantially low expression of hsp70 and hsf1 throughout the experiment.

In conclusion, our findings are the first to demonstrate the expression patterns of hsps and hsf1 during larval development in coral by observing the transcription levels of two Hsps and an HSF of coral larvae under acidified conditions. These findings

| Source                  | Df | Sum of squares | F    | p     |
|-------------------------|----|----------------|------|-------|
| pCO2 condition          | 1  | 0.05319        | 0.5169 | 0.479 |
| Day                     | 3  | 3.26178        | 10.5663 | < 0.001 |
| pCO2 condition × Day    | 3  | 0.07171        | 0.2323 | 0.872 |

Fig. 5. Expression of hsp90 in the larvae of cultured Acropora digitifera in the control and elevated CO2 conditions at 27°C over 7 days. Error bars indicate mean and standard error (N=4).

Fig. 6. Expression of hsf1 in the larvae of cultured Acropora digitifera in the control and elevated CO2 conditions at 27°C over 7 days. Error bars indicate mean and standard error (N=4).
suggest that coral larvae are able to buffer the external pH changes that are expected to occur by the end of this century.

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References

Albright, R., Mason, B., Miller, M., and Landgong, C. (2010). Ocean acidification compromises recruitment success of the threatened Caribbean coral Acropora palmate. Proc. Natl. Acad. Sci. USA 107, 20400-20404.

Ali, A., Bharadwaj, S., O’Carroll, L., and Ovseenk, N. (1998). HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. Mol. Cell. Biol. 18, 4949-4960.

Abravaya, K., Myers, M. P., Murphy, S. P., and Morimoto, R. I. (1992). The human heat shock protein hsP70 interacts with HSFG, the transcription factor that regulates heat shock gene expression. Genes Dev. 6, 1153-1164.

Baker, M. E. (2006). The genetic response to Snowball Earth: role of HSP90 in the Cambrian explosion. Geobiology 4, 11-14.

Bharadwaj, S., Ali, A., and Ovseenk, N. (1999). Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 in vivo. Mol. Cell. Biol. 19, 8033-8041.

Bishop, C. D., Bates, W. R., and Brandhorst, B. P. (1998). Cell specific expression of HSP90 and induction of metamorphosis in ascidian larvae. Dev. Biol. 198, 381.

Bishop, C. D., Bates, W. R., and Brandhorst, B. P. (2001). Regulation of metamorphosis in ascidians involves NO/GMP signaling and HSP90. J. Exp. Zool. 289, 374-384.

Caldeira, K., and Wickett, M. E. (2005). Ocean model prediction of chemistry changes from carbon dioxide emission to the atmosphere and ocean. Geophys. Res. Lett. 32, C0904, doi: 10.1029/2004GL020671.

Dahlhoff, E. P. (2004). Biochemical indicators of stress and metabolism: applications for marine ecological studies. Annu. Rev. Physiol. 66, 183-207.

Deane, E. N., and Woo, N. Y. S. (2003). Ontogeny of thyroid hormones, cortisol, hsp70 and hsp90 during silver sea bream larval development. Life Science 72, 805-818.

Doney, S. C., Fabry, V. J., Feely, R. A., and Kley, P. J. (2009). Ocean acidification of the other CO2 problem. Science 328, 193-196.

Dupont, S., Harenhand, J., Thorndyke, W., Peck, L., and Thorndyke, M. (2008). Near-future level of CO2-driven ocean acidification radically affects larval survival and development in the brittlestar Ophiothrix fragilis. Mol. Mar. Biol. Biotechnol. 17, 320-323.

Ebenstein, A. H., and Petron, A. (2010). The impact of climate change on the world’s marine ecosystems. Science 328, 1523-1528, doi: 10.1126/science.1198930.

Hauton, C., Tyrrell, T., and Williams, J. (2009). The subtle effects of sea water acidification on the amphipod Gammarus locusta. Biogeochemistry 109, 1479-1489.

Krebs, R. A., and Feder, M. E. (1997). Delimited consequences of Hsp70 overexpression in Drosophila melanogaster larvae. Cell Stress Chaperones 2, 60-71.

Krone, P. H., Sass, J. B., and Lele, Z. (1997). Heat shock protein gene expression during embryonic development of the zebrafish. Cell. Mol. Life Sci. 53, 122-129.

Krone, P. H., Evans, T. G., and Blecha, N. R. (2003). Heat shock gene expression and function during zebrafish embryogenesis. Semin. Cell Dev. Biol. 14, 267-274.

Kurihara, H., and Shirayama, Y. (2004). Effects of increased atmospheric CO2 on sea urchin early development. Mar. Ecol. Prog. Ser. 274, 161-169.

Kurihara, H., Kato, S., and Ishimatsu, A. (2007). Effects of increased seawater pCO2 on early development of the sea urchin. Transresids. Aquat. Biol. 1, 91-98.

Manchado, M., Sanis-Leiton, E., Infante, C., Ponce, M., Asensio, E., Crespo, A., Zunasti, E., and Cañavate, J. P. (2008). Molecular characterization, gene expression and transcriptional regulation of cytosolic HSP90 genes in the flatfish Senegalese sole (Solea senegalensis). Gene 416, 77-84.

Mayer, M. P., and Bukau, B. (1999). Molecular chaperones: The busy life of Hsp90. Curr. Opin. Cell Biol. 11, R322-R325.

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. 12, 3788-3796.

Michaelidis, B., Ouzounis, C., Paleras, A., and Pörtner, H. O. (2005). Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels (Mytilus galloprovincialis). Mar. Ecol. Prog. Ser. 293, 109-118.

Morita, M., Nishikawa, A., Nakajima, I., Ichgi, A., Sakai, K., Takamura, A., and Okuno, M. (2006). Eggs regulate sperm flagellar motility initiation, chemotaxis, and inhibition in the coral, Acropora digitifera, A. gemmifera, and A. tenuis. J. Exp. Biol. 209, 4574-4579.

O’Donnell, M. J., Hammond, L. M., and Hofmann, G. E. (2009). Predicted impact of ocean acidification on a marine invertebrate: elevated CO2 alters response to thermal stress in sea urchin larvae. Mar. Biol. 156, 439-446.

O’Donnell, M. J., Todgham, A. E., and Hofmann, G. E. (2010). The impact of climate change on the echinoderm Crassostrea gigas. Aquat. Biol. 14, R322-R325.

Pelletier, S., Lewis, E., and Wallace, D. W. R. (2006). MS Excel Program Developed for CO2 System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee.

Pirkkala, L., Nyka¨nen, P., and Sistonen, L. (2001). Roles of the heat shock factors in regulation of the heat shock transcription factor in regulation of the heat shock transcription factor in regulation of gene expression in larvae. Mol. Ecol. Prog. Ser. 398, 157-171.

Piechórski, L., Nneyan, P., and Sistonen, L. (2001). Roles of the stress-sensitive complex with HSF1. Mol. Biol. 35, 65-71.

Posada, D., and Buckley, T. R. (2004). Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. Stat. Biol. 53, 793-808.

Posada, D., and Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Quetsch, C., Sangster, T. A., and Lindquist, S. (2002). Hsp90 as a capacitor of phenotypic variation. Nature 419, 618-624.

Ravina, J., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., Riebesell, U., Shepherd, J., Turley, C., and Watson, A. (2005). Ocean acidification due to increasing atmospheric carbon dioxide. Policy document 12/05. Royal Society.

Reischläger, A., and Pörtner, H. O. (1996). Metabolic depression during environmental stress: the role of extra- versus intracellular pH in Scapharca putata. J. Exp. Biol. 199, 1801-1807.

Rutherford, S. L., and Lindquist, S. (1998). Hsp90 as a capacitor for morphological evolution. Nature 396, 336-342.

Shi, Y., Mosser, D. D., and Morimoto, R. I. (1998). Molecularchaperones as HSF1-specific transcriptional repressors. Gene 19, 654-666.

Shinzato, C., Shoguchi, E., Kawashima, T., Hamada, M., Hisata, K., Fujii, M., Fujiwara, M., Koyanagi, R., Ito, T., et al. (2011). Using the Acropora digitifera genome to understand coral responses to environmental change. Nature 476, 320-323.

Sorger, P. K. (1991). Heat shock factor and the heat shock response. Cell 65, 363-366.

Suzuki, A., and Morimoto, R. I. (2010). Effects of acidification on early life stages of scleractinian corals (Genus Acropora). Fish. Sci. 76, 93-99.

Swofford, D. L. (2003). PAUP*: Phylogenetic Analysis And Other Methods Version 4 0 Beta. Sunderland, USA: Sinauer Associates.

Todgham, A. E., and Hofmann, G. E. (2009). Transcriptomic response of sea urchin larvae Strongylocentrotus purpuratus to CO2-driven seawater acidification. J. Exp. Biol. 212, 2579-2594.

Velazquez, J. M., Sonoda, S., Bogaschewsky, G., and Lindquist, S. (1983). Is the major heat shock protein of the sea urchin a stress sensitive complex with HSF1? Curr. Biol. 9, 471-480.