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Cellular responses in the coral *Stylophora pistillata* exposed to eutrophication from fish mariculture

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ABSTRACT

Background: Many fringing coral reefs are exposed to anthropogenically derived eutrophication. In scleractinian corals, this type of pollution induces alterations in complex physiological functions, ranging from changes in growth rates and reproductive effort to reduction in immunity, factors that can have dire effects on coral biodiversity. The complexity of effects of eutrophication on coral physiology can be confusing and additional tools are needed to clarify these effects.

Aims: Using cellular diagnostics, we compare the physiological status of colonies of the coral *Stylophora pistillata* growing in a eutrophic environment with the status of reference colonies growing nearby in minimally polluted water. We evaluate the usefulness of the cellular diagnostics for discerning causality by comparing the results with previously observed differences in physiological parameters.

Location: Northern Gulf of Aqaba, Israel. The reference colonies grew near the Egyptian border at Taba. The colonies in eutrophic water grew 11 km to the north near net-pen fish-farms. All sites were on a sandy bottom, 19 m deep.

Methods: We used biomarkers of general metabolic condition, protein synthesis and maturation, and of oxidative stress and response to assess and compare the cellular physiological status of coral colonies and their algal symbionts from the two sites.

Results: Cellular diagnostics showed either site-specific or treatment-specific differences or both. Markers of protein synthesis and maturation showed both site- and treatment-specific differences. Host and zooxanthella Hsp60, and zooxanthella Cu/ZnSOD, were elevated in transplanted colonies compared with native and reference colonies. Differences in levels of host Hsp90 were site specific and were significantly higher in fish-farm colonies than in reference colonies. Despite this, no associated elevation in ubiquitin was apparent in these colonies. The higher concentrations of mitochondrial aconitase in fish-farm colonies corroborate the increased growth rate previously observed in these colonies. Concentrations of markers of oxidative condition were generally lower in fish-farm colonies than in the reference colonies, reflecting lower photosynthesis due to less light at the site. Levels of xenobiotic response markers showed that corals in the fish-farm environment were not detoxifying substances using

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the MXR pathway. On the other hand, CYP P450 (2-class and 6-class) levels showed clear and significant site dependence with lower levels of CYP P450 2-class, an enzyme important in lipid metabolism, reflecting the lower concentrations of lipids found in the fish-farm corals.

Conclusions: Overall, the cellular markers demonstrate clear differences between colonies from the two sites as well as differences between transplanted and naturally growing colonies, and provide evidence of differences in physiological functions in corals from those sites.

Keywords: biomarkers, cellular diagnostics, coral, fish-farm, Gulf of Aqaba (Eilat).

INTRODUCTION

For many coral reefs, clear, nutrient-poor waters are considered a prerequisite for the development of healthy coral communities. On a local scale, human economic exploitation and various types of pollution – mainly eutrophication and siltation – have exposed many fringing and offshore reefs to severe stresses (Brown, 1997). The generally sub-lethal and long-term nature of nutrient-enrichment impacts has undoubtedly contributed to the complexity of identifying specific effects, particularly because such stresses may not directly or immediately alter large-scale parameters of coral biodiversity or coral cover. In addition, the potential for synergisms among impacts that accompany eutrophication, such as increased turbidity, increased particulate matter, increased sulphide deposition, and the presence of heavy metals, enhances the ambiguity in determining causal relationships (Ferrier-Pagès *et al.*, 2000; Bongiorni *et al.*, 2003a, 2003b; Loya, 2004, 2007). In corals, eutrophication has been shown to cause physiological changes, including changes in growth and skeletal tensile strength, decreased reproductive effort, and a reduced ability to withstand disease (Kinsey and Davies, 1979; Stambler *et al.*, 1991; Marubini and Atkinson, 1999; Ferrier-Pagès *et al.*, 2000; Bucher and Harrison, 2002; Bruno *et al.*, 2003; Cox and Ward, 2003; Loya *et al.*, 2004), factors which in turn will affect biodiversity.

One example of a reef at risk from effects of anthropogenically induced eutrophication is the reef at Eilat in the Gulf of Aqaba. This reef once exhibited extraordinary high within-habitat coral species diversity, but has lately seen substantial deterioration (*sensu* Loya, 2004). Before the late 1990s, the Gulf of Eilat (Aqaba) was affected by a variety of stressors, including untreated sewage outflow from the city of Eilat at the north-western part of the Gulf. Public outcry led to the building of a sewage treatment plant (in 1995) and diversion of the sewage outflow largely stopped eutrophication from urban sources. However, the deployment of intensive *in situ* net-pen mariculture facilities at the northern tip of the Gulf replaced the sewage output (Atkinson *et al.*, 2001; Loya, 2004). Food waste and fish faeces deposited in the vicinity of the farm, or in the direction of the prevailing currents, caused substantial accumulation of organic material on the seafloor (unpublished results, Israel National Monitoring Program (INMP): http://www.iui-eilat.org.il/nmp) (Atkinson *et al.*, 2001). It was determined by a number of agencies (the INMP and the International Expert Team (IET): http://www.sviva.gov.il/) that the immediate surroundings of the fish farm site were significantly more eutrophic than areas in the southern part of the Gulf.

Changes in water quality, particularly in nutrient levels associated with fish-farms, were previously reported to affect coral reproductive physiology and growth (Koop *et al.*, 2001; Bassim and Sammarco, 2003; Bongiorni *et al.*, 2003a, 2003b; Loya *et al.*, 2004, Loya, 2007). However, there is scarce information regarding cellular responses of corals to eutrophication. The aims of the present study were to examine differences in cellular physiological parameters in colonies of the coral *Stylophora pistillata* from near these fish farms and to compare them with colonies

from a more pristine environment 11 km downstream and to the south. Using cellular diagnostic tools (see Downs, 2005), we assessed differences in cellular physiological functions. These differences were examined in light of discrepancies in reproductive effort and growth rates reported in *Stylophora pistillata* from the two sites (Bongiorni *et al.*, 2003a, 2003b; Loya *et al.*, 2004).

METHODS

Study sites and collection methods

The fish-farm site is located on a sandy bottom at 19 m depth, 150 m west of the net-pen mariculture facilities in the northern Gulf of Eilat (Aqaba). The reference site, located at the same depth (19 m) 11 km to the south and near the Taba border crossing, also has a sandy bottom. Water quality parameters collected once monthly from areas proximal to the experimental sites in the months before and during the collection period were obtained from the Israel National Monitoring Program (INMP).

Eleven fragments of 2–4 cm were collected from naturally growing adult colonies (with a diameter of over 10 cm) of the coral *Stylophora pistillata* at the Taba site. Three similar sized fragments were collected from naturally growing colonies of comparable sizes at the fish-farm site (termed 'natural colonies') growing on artificial plastic substrates and elevated from the substrate in the immediate surroundings of the transplant site. Due to the low numbers of healthy adult colonies of *S. pistillata* at that site, fragments were also collected from eight healthy looking adult colonies that had been transplanted to that site (termed 'transplanted colonies') 3½ years previously. Fragments collected from the fish-farm site are collectively named 'fish-farm colonies'. The fragments were collected on consecutive days at the same time of day in August (before renewed gametogenesis) and were transported to the surface in dark bags and immediately frozen in liquid nitrogen vapours.

Sample preparation, ELISA validation, and ELISA

All chemicals for buffered solutions were obtained from EM Science (Gibbstown, NJ, USA). PVDF membrane was obtained from Millipore Corp. (Bedford, MA, USA). Antibodies against all the cellular parameters, as well as calibrant standards, were obtained from EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). Anti-rabbit conjugated horseradish peroxidase antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA).

Coral samples were ground to a powder using a liquid nitrogen-chilled ceramic pestle and mortar. Samples (~10 mg) of frozen tissue were placed in 1.8-ml microcentrifuge tubes together with 1400 µl of a denaturing buffer consisting of 2% SDS, 50 mM Tris-HCl (pH 7.8), 15 mM dithiothreitol, 10 mM EDTA, 0.5 mM desferoximine methylate, 0.001 mM sorbitol, 7% polyvinylpolypyrrolidone (w/v), 0.01% polyvinylpyrrolidone (w/v), 0.005 mM salicylic acid, 0.01 mM AEBSF, 0.04 mM Bestatin, 0.001 mM E-64, 2 mM phenylmethylsulphonyl fluoride, 2 mM benzamidine, 0.01 mM apoprotin, 5 µM α-amino-caproic acid, and 1 $\mu g \cdot 100\ \mu l^{-1}$ pepstatin A. Samples were heated at 92°C for 3 min, vortexed for 20 s, incubated at 92°C for another 3 min, and then incubated at 25°C for 5 min. Samples were centrifuged at 10,000 *g* for 10 min. Supernatant free of a lipid/glycoprotein mucilage matrix was transferred to a new tube, centrifuged at 10,000 *g* for 5 min, and again transferred to a new tube and subjected to a protein concentration assay (Ghosh *et al.*, 1988).

To ensure equal sample loading, 20 μg of total soluble protein of four randomly chosen samples were loaded onto a 12.5% SDS-PAGE gel (8 cm), the gel run until the bromophenol blue front was near the bottom of the gel, stained with a Coomassie blue solution (BB R-250) overnight, and then destained for 4 h with multiple washes of destaining solution. Equal loading was determined by visualization and optical density using a Canonscan scanner and analysis performed on a Macintosh computer using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/).

One-dimensional SDS-PAGE and western blotting for each specific antibody validated the legitimacy of using that antibody in an enzyme-linked immunosorbent assay (ELISA) for this species of coral. Five to fifteen micrograms of total soluble protein of coral supernatant was loaded onto an 8-cm SDS-polyacrylamide gel with 29:1 ratio of bis/acrylamide. TCEP 1 mM (Pierce Biotechnology, Rockford, IL, USA) was added to gels loaded with samples to be assayed with antibody to the chloroplast sHsp, invertebrate sHsp, and metallothionein. Gels were blotted onto PVDF membrane using a wet transfer system. Membrane was blocked in 7% non-fat dry milk, and incubated with the primary antibody for 1 h. The blots were washed in Tris-buffered saline (TBS) four times, and incubated in a horseradish peroxidase-conjugated secondary antibody solution for 1 h. Blots were washed again four times in TBS, and developed using a NEN Enhanced Chemiluminescence solution (PerkinElmer, Boston, MA, USA) and visualized with a Syngene GeneGnome luminescent CCD camera documentation system (Frederick, MD, USA). To characterize potential artifacts resulting from antibody non-specific cross-reactivity, blots were over-developed for at least 7 min. Calibration using a quantitative standard showed the level of detectability as 0.05 attomole of target protein.

Once validated, antibodies and samples were optimized and precision determined for ELISA using an $8 \times 6 \times 4$ factorial design (Crowther, 2000). A Beckman-Coulter Biomek 2000 with 384-well microplates was used to conduct the ELISA assays. Samples were assayed with the following EnVirtue Biotechnologies, Inc. antibodies (generated in rabbits): anti-algal glutathione peroxidase (AB-G101-P), anti-algal manganese superoxide dismutase (SOD) (AB-S100-P), anti-algal copper/zinc superoxide dismutase (AB-101-PA), anti-algal heat shock protein (Hsp) 60 (AB-H100-P), anti-algal heat-shock protein 70 (AB-H101-P), anti-algal glutathione *S*-transferase (AB-GST-PA), anti-chloroplast small heat-shock protein (AB-H104-C), anti-ubiquitin (AB-U100), anti-cnidarian heat-shock protein 70 (AB-H101-CDN), anti-cnidarian heat-shock protein 60 (AB-H100-IN), anti-aconitase (AB-ACON3), anti-cnidarian glutathione *S*-transferase (AB-G100-MU), anti-cnidarian manganese superoxide dismutase (AB-S100-MM), anti-cnidarian copper/zinc superoxide dismutase (cytosolic isoform, lot 1517), anti-cnidarian glutathione peroxidase (AB-GPX-IN), anti-cnidarian glutathione *S*-transferase (pi-isoform homologue; AB-GST-INV), anti-invertebrate anti-small heat shock protein (AB-H103), anti-cnidarian ferrochelatase (lot 1939), anti-cnidarian catalase (cytosolic homologue, lot 3113), anti-cnidarian heme oxygenase I (lot 3114), anti-cnidarian cytochrome P450 2 class (lot 1981), anti-cytochrome P450 6 class (lot 1984), and anti-MXR (ABC family of proteins; P-glycoprotein 140 and 160; AB-MDR-160). All antibodies are mono-specific polyclonal antibodies made against a synthetic 8 amino-acid residue polypeptide that reflects a specific region of the target protein. Samples were assayed in triplicate with intra-specific variation of less than 6% for all samples combined for each assay. An eight-point calibrant curve using a calibrant relevant to each antibody was plated in triplicate for each plate.

Statistical analysis

Following tests for normal distribution and equality of variances (Lavene's test), the data were examined using one-way analysis of variance (ANOVA) and a Holm-Sidak *post hoc* test. When normality failed a Kruskal-Wallis, ANOVA on Ranks was carried out. A Dunn's *post hoc* test was run when appropriate (Sokal and Rolf, 1995).

RESULTS

Water quality

Water quality parameters collected once monthly in the months before the collection date were received from Israel's National Monitoring Program (INMP). Results showed higher concentrations of ammonium, nitrates, nitrites, and phosphates at the net-pen fish-farm site than at the reference site (Figs. 1, 2).

Antibody validation

Antibodies against cnidarian and dinoflagellate cellular parameters did not exhibit significant non-specific cross-reactivity (Figs. 3–6), and thus could be used in an ELISA

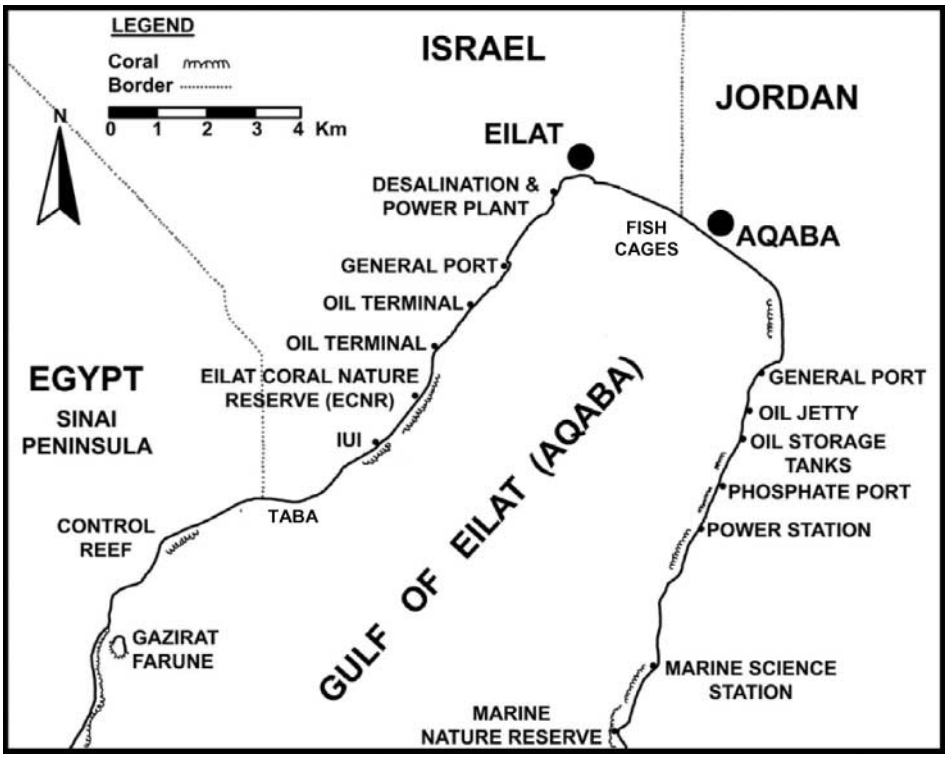

Fig. 1. Map of the northern Gulf of Aqaba (Eilat). The fish-farm site and Taba, the reference site, are marked.

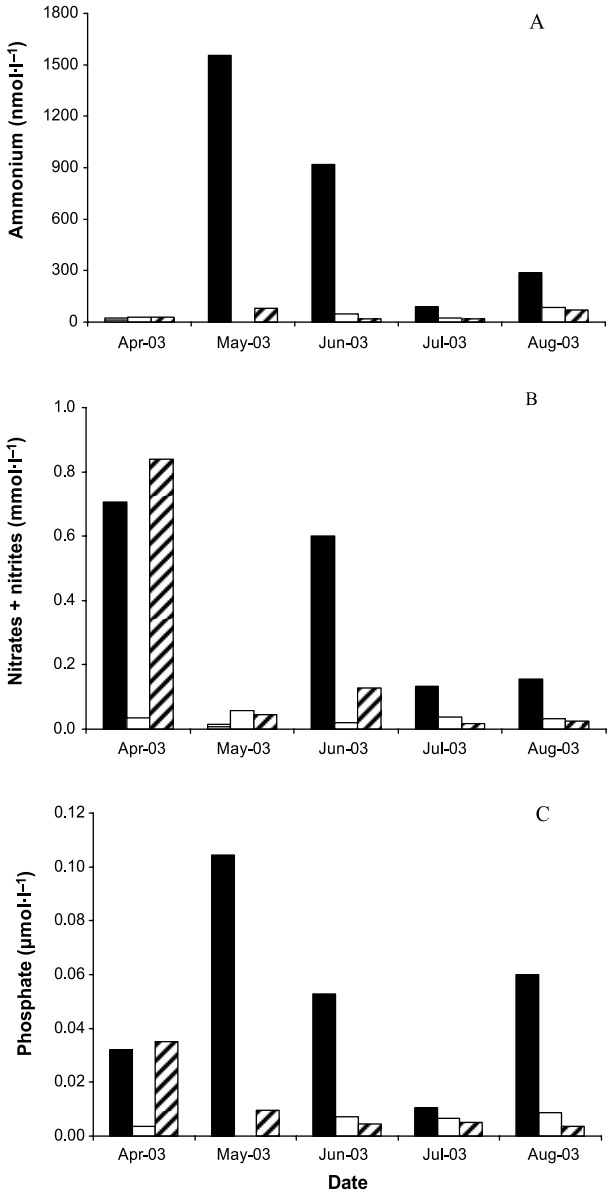

Fig. 2. Concentrations of nutrients in the study sites and in the open-sea as measured once monthly by the Israel National Monitoring Program: (A) ammonium, (B) nitrates and nitrites, (C) phosphates. The open bars are open water, hatched bars are reference site, and solid bars are the fish-farm.

format. All blots presented in these figures are over-exposed blots; a technique to characterize the potential of non-specific cross-reactivity for the ELISA. ELISA-microplate readings are based on a 4-s exposure. The sensitivity between the Syngene GeneGnome documentation system and the Bio-Tek Fluorescent/Luminescence microplate reader is approximately equal, hence the non-specific, faint bands are not detectable by the

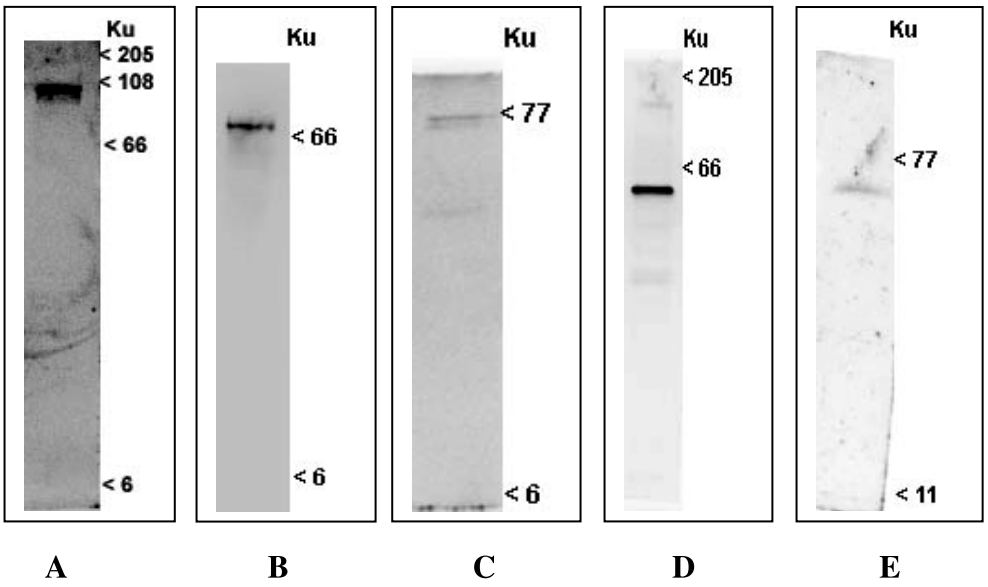

Fig. 3. Protein metabolic condition parameters. Coral were homogenized and subjected to SDS-PAGE (5 µg total soluble protein per lane), western blotting, and assayed with polyclonal antibody against each target protein. The migration rate for each band is indicated with the following blots: A = Hsp90 (85–98 Ku); B = Hsp70 cnidarian (70–75 Ku); C = Hsp70 dinoflagellate (70–78 Ku); D = Hsp60 cnidarian (58–65 kU); E = Hsp60 dinoflagellate (60–65 Ku).

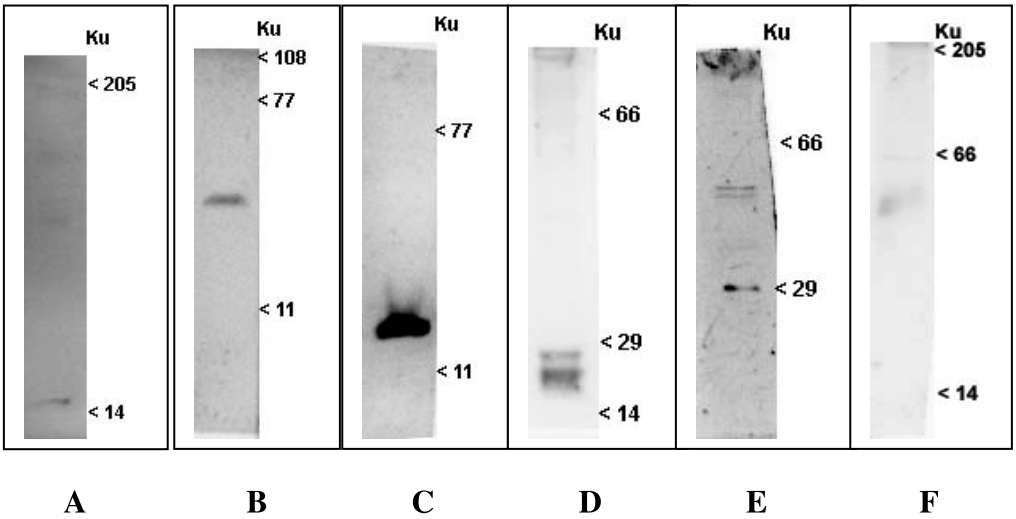

Fig. 4. Oxidative stress parameters. Coral were homogenized and subjected to SDS-PAGE (5 µg total soluble protein per lane), western blotting, and assayed with polyclonal antibody against each target protein. The migration rate for each band is indicated with the following blots: A = Cu/ZnSOD cnidarians (20 Ku); B = Cu/ZnSOD dinoflagellate (38 Ku); C = GPX cnidarians (19 Ku); D = MnSOD cnidarians (24 Ku); E = GPX dinoflagellate (29 Ku and 45–50 Ku); F = catalase cnidarians (50–55 Ku).

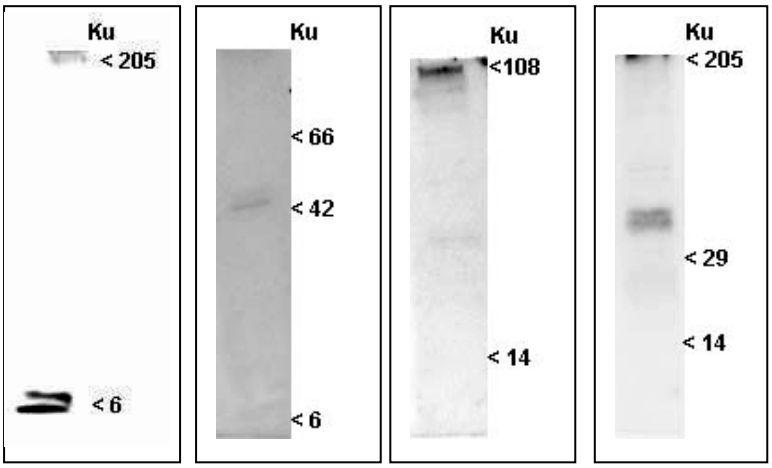

Fig. 5. Metabolic condition parameters. Coral were homogenized and subjected to SDS-PAGE (5 μg total soluble protein per lane), western blotting, and assayed with polyclonal antibody against the following parameters: A = metallothionein I cnidarians (~5 Ku); B = ferrochelatase cnidarians (40 Ku); C = aconitase cnidarians (108 Ku); D = Heme oxygenase I cnidarians (35 Ku).

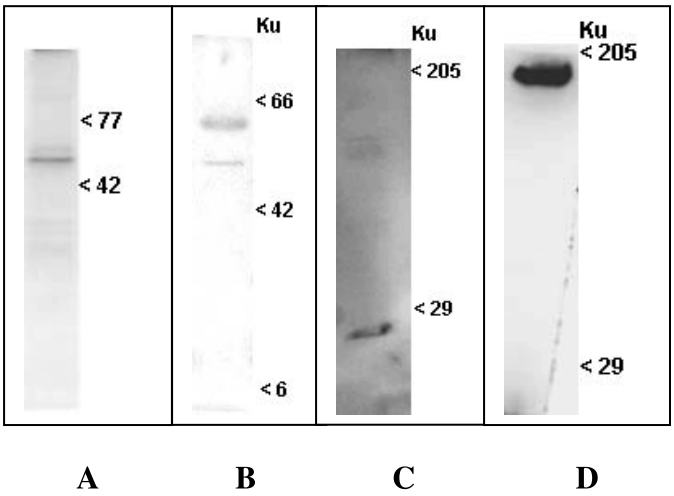

Fig. 6. Xenobiotic response parameters. Coral were homogenized and subjected to SDS-PAGE (5 μg total soluble protein per lane), western blotting, and assayed with polyclonal antibody against the following parameters: A = cytochrome P450 2-class cnidarians (50–55 Ku); B = cytochrome P450 6-class cnidarians (50 and 58 Ku); C = GST cnidarians (25 Ku); D = MXR, both cnidarian and dinoflagellate isoforms (160 and 180 Ku).

microplate reader. Because of the evolutionary conservation of ubiquitin and MXR, these antibodies detected proteins from both species in the coral homogenate (Figs. 3 and 5; ubiquitin data not shown); consequently, an ELISA measurement using this antibody detects the total concentration of these proteins.

Enzyme-linked immunosorbent assay (Table 1)

Protein metabolic condition

Host. Neither cnidarian Hsp70 nor ubiquitin was significantly different between the reference and fish-farm sites. Hsp90 was significantly higher in both transplanted and native colonies at the fish-farm site compared with the reference site. Hsp60 was significantly higher in colonies transplanted to the fish-farm site when compared with the reference site (Fig. 3, Table 2).

Symbiont. Zooxanthellae Hsp70 and Hsp60 were significantly elevated in corals from the fish-farm site compared with the reference site (Fig. 3, Table 2).

Oxidative stress and response

Host. Cu/ZnSOD was significantly lower in fish-farm colonies than in reference colonies (Fig. 4, Table 3). There was no significant difference in cnidarian MnSOD between the transplanted fish-farm colonies and reference colonies but MnSOD was significantly lower in the native fish-farm colonies compared with the reference population (Table 3). Cnidarian glutathione peroxidase (GPX) was not significantly different in the various treatments, although levels were lowest in the native fish-farm population. Catalase was not significantly different between the transplanted and reference colonies, although the natural colonies exhibited a significantly lower expression of this protein (Table 3).

Symbiont. Algal Cu/ZnSOD was significantly elevated in the transplanted colonies compared with those at the reference sites, but not at the natural site (Fig. 4, Table 3). Algal MnSOD was not significantly different in the transplanted and reference colonies, although this marker was significantly lower in the natural than the reference colonies. Algal GPX was significantly lower in both types of fish-farm colonies compared with the reference colonies (Table 3).

Metabolic condition

Cnidarian ferrochelatase did not differ significantly with treatment. Metallothionein was significantly depressed in the natural colonies. Heme oxygenase was significantly lower in native colonies from the fish-farm sites when compared with those from the reference site. There was a significantly higher concentration of aconitase in the fish-farm colonies compared with the reference colonies (Fig. 5, Table 4).

Xenobiotic response

There were no significant differences in GST or MXR among any of the three populations (Fig. 6, Table 5). Both fish-farm populations had significantly reduced CYP P450 2-class and had significantly elevated CYP P450 6-class compared with the reference colonies (Table 5).

DISCUSSION

A clear stratification is apparent in the Northern Gulf of Aqaba (Eilat) following winter upwelling events and depending on water temperatures (INMP, 2004). Results of the Israel National Monitoring Program showed that during the period of stratification there are

Table 1. The biological significance of each diagnostic biomarker assay (adapted from Downs *et al.*, 2001)

Molecular assay	Biological significance
Protein metabolic condition	
Heat-shock protein 90 (Hsp90)	An ATPase enzyme that, together with other co-chaperones, is essential for the activation of specific proteins (Pearl and Prodromou, 2000). One isoform functions in the binding with specific steroid hormone receptors, affecting the ability of the steroid hormone receptor to initiate a signal cascade (Pratt and Dittmar, 1998). Other isoforms play a role in the regulation of heat-shock and stress-inducible transcription factors, or support and protect cytoskeletal elements (spindle fibres), joining chromosomal centromeres and centrosomes during mitosis and meiosis
Heat-shock proteins 60 and 70 (Hsp60 and Hsp70)	Essential components for cellular function, during both normal and stressed conditions. They regulate protein structure and function under normal physiological conditions as well as during and following stress by renaturing denatured proteins into active states in an ATP-dependent manner. Hsp60 and Hsp70 concentrations increase in response to stress, specifically in response to increased protein synthesis and denaturation. Hsp70 helps shuttle nascent proteins and is crucial to their maturation (Ellis, 1996; Hartl, 1996)
Ubiquitin	A protein found in most phyla, used in marking proteins for rapid degradation. Increased concentrations are an indication of increased protein degradation, and hence increased protein turnover. The level of ubiquitin is an index of the structural integrity of the protein component of the superstructure of the cell
Oxidative stress	
Copper/zinc superoxide dismutase (Cu/ZnSOD)	An important enzyme in the anti-oxidant defence pathway and occurs only in the cytosol of animals. Found both in the cytosol and chloroplast of algae. This protein is constitutively expressed, but becomes elevated in response to an increase of reactive oxygen species
Manganese superoxide dismutase (MnSOD)	Localized to the mitochondria in eukaryotic cells. Accumulates in response to oxidative stress. Involved in one of the main anti-oxidant defence pathways. Increased concentrations have been linked to increased longevity, increased tolerance to ischaemic/reperfusion events, and increased tolerance to factors that induce oxidative stress
Glutathione peroxidase (GPX)	Important in cellular antioxidant defence systems. Detoxifies hydroperoxides and organic peroxides, including the peroxides of free (but not esterified) fatty acids and other lipids. This enzyme is most active (60–75%) in the cytoplasm of eukaryotic cells, but also in the mitochondria (25–40%)
Catalase	The principal enzyme that catalyses hydrogen peroxide into water and oxygen. Increased H_2O_2 in the cytosol will elicit an accumulation of this protein

Table 1. (*Continued*)

Molecular assay	Biological significance
Metabolic condition	
Metallothionein (MET)	Cysteine-rich, low molecular-weight proteins that bind a variety of metals (for a review, see Klassen *et al.*, 1999). May act as an anti-oxidant, a factor to ensure a reservoir of zinc, protect against metal toxicity, but predominantly acts as a regulator of mitochondrial function (Klaassen *et al.*, 1999; Ye *et al.*, 2001)
Ferrochelatase (FC)	An enzyme that inserts ferrous iron into protoporphyrin IX to form heme. In invertebrates, it is able to utilize several different metals and porphyrin substrates. Cellular detoxification pathways and essential cellular metabolism require heme or porphyrin-based substrates. If an organism is going to up-regulate metabolic or xenobiotic detoxification pathways, it will need to increases its heme production, and to up-regulate ferrochelatase
Aconitase	A zinc/iron-sulphur protein that acts in iron regulation and in the Krebs cycle. Up-regulation of this protein is usually indicative of an increased demand on respiration products, while down-regulation of this protein is indicative of a toxic pathology, either by reactive oxygen species or alkylating agents (Senft *et al.*, 2002)
Heme oxygenase I (HO)	Also known as Hsp32, HO is up-regulated in response to oxidative damage. Heme oxygenase degrades heme to biliverdin, free iron, and carbon monoxide
Xenobiotic response	
Multi-drug resistance protein (MXR)	P-glycoprotein members of a superfamily of proteins that act as channels and transporters of solutes across membranes. They play a role in xenobiotic detoxification. Currently, it is believed that P-glycoproteins effectively process certain xenobiotics to exit the cell
Glutathione *S*-transferase (GST)	Usually associated with detoxification by conjugation of genotoxic and cytotoxic xenobiotic electrophils derived from drugs, carcinogens, and environmental pollutants. During a xenobiotic challenge, glutathione can be conjugated to a xenobiotic by GST, representing a major detoxification pathway (Sies, 1999). Additionally, GST detoxifies DNA hydroperoxides, playing an important role in DNA repair
Cytochrome P450 2-class	Best known for its role in xenobiotic transformation in vertebrates and some invertebrates (Gassman and Kennedy, 1992; Snyder, 2000). It has a number of physiological roles, including metabolizing endogenous compounds such as fatty acids, lipid hydroperoxides, and ketone bodies into aldehydes (Ronis *et al.*, 1996; Lieber, 1997)
Cytochrome P450 6-class	The major inducible xenobiotic response protein in most phyla of invertebrates and is the primary protein responsible for insecticide resistance in Insecta

Table 2. Protein metabolic conditions

Cellular parameter	Reference	Fish-farm transplanted	Fish-farm native	*P*
Hsp90 cnidarian (fmol · ng^{-1} TSP)	277 ± 49^{a}	644 ± 59^{b}	598 ± 49^{b}	<0.001
Hsp70 cnidarian (fmol · ng^{-1} TSP)	180 ± 100^{a}	164 ± 61^{a}	135 ± 61^{a}	N.S.
Hsp70 zooxanthellae (fmol · ng^{-1} TSP)	107 ± 74^{a}	207 ± 39^{b}	263 ± 178^{b}	<0.01*
Hsp60 cnidarian (pmol · ng^{-1} TSP)	17.2 ± 6.6^{a}	32.1 ± 9.6^{b}	12.6 ± 5.5^{a}	<0.01
Hsp60 zooxanthellae (pmol · ng^{-1} TSP)	96 ± 37^{a}	203 ± 71^{b}	99 ± 74^{a}	<0.05
Ubiquitin (pmol · ng^{-1} TSP)	471 ± 44^{a}	491 ± 44^{a}	483 ± 34^{a}	N.S.

Note: Entries in the table give treatment means ± 1 standard deviation.
Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Tukey-Kramer HSD method.
* Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Kruskal-Wallis multiple comparison test.

Table 3. Oxidative stress

Cellular parameter	Reference	Fish-farm transplanted	Fish-farm native	*P*
Cu/ZnSOD cnidarian (pmol · ng^{-1} TSP)	0.145 ± 0.046^{a}	0.0453 ± 0.394^{b}	0.051 ± 0.090^{b}	<0.001
Cu/ZnSOD zooxanthellae (pmol · ng^{-1} TSP)	0.180 ± 0.102^{a}	0.650 ± 0.313^{b}	0.276 ± 0.0513^{a}	<0.01
MnSOD cnidarian (pmol · ng^{-1} TSP)	0.790 ± 0.378^{a}	0.547 ± 0.202^{a}	0.099 ± 0.230^{c}	<0.01
MnSOD zooxanthellae (pmol · ng^{-1} TSP)	208.88 ± 62.80^{a}	$163.76 \pm 47.84^{a,c}$	$99.10 \pm 52.39^{b,c}$	<0.05
GPX cnidarian (pmol · ng^{-1} TSP)	32.53 ± 23.59^{a}	40.307 ± 17.39^{a}	18.2 ± 11.89^{a}	N.S.
GPX zooxanthellae (pmol · ng^{-1} TSP)	0.253 ± 0.209^{a}	$0.142 \pm 0.040^{a,c}$	$0.0618 \pm 0.0194^{b,c}$	<0.05*
Catalase (pmol · ng^{-1} TSP)	39.26 ± 16.23^{a}	$29.43 \pm 7.10^{a,c}$	$17.12 \pm 5.544^{b,c}$	<0.05

Note: Entries in the table give treatment means ±1 standard deviation.
Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Tukey-Kramer HSD method.
* Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Kruskal-Wallis multiple comparison test.

elevated concentrations of nutrients in the water column, which was particularly true in the immediate vicinity of the fish-farms compared with the study site to the south (INMP, 2004). High levels of nutrients result in increased algal blooms (INMP, 2004) that competitively excluded corals and/or affected coral physiology (Costa *et al.*, 2000; Koop *et al.*, 2001; Loya, 2004). In the Northern Red Sea, the reproductive season of *Stylophora pistillata*

Table 4. Metabolic conditions

Cellular parameter	Reference	Fish-farm transplanted	Fish-farm native	*P*
Metallothionein (pmol · ng^{-1} TSP)	16.44 ± 9.73^{a}	$10.95 \pm 5.885^{a,b}$	2.489 ± 2.61^{b}	<0.05
Ferrochelatase (pmol · ng^{-1} TSP)	26.25 ± 14.17^{a}	17.86 ± 10.42^{a}	11.612 ± 6.86^{a}	N.S.
Aconitase (pmol · ng^{-1} TSP)	0.509 ± 0.0982^{a}	0.706 ± 0.092^{b}	0.71 ± 0.048^{b}	<0.001
Heme oxygenase (pmol · ng^{-1} TSP)	52.558 ± 33.18^{a}	34.15 ± 11.59^{a}	7.59 ± 2.99^{b}	<0.05*

Note: Entries in the table give treatment means ±1 standard deviation.
Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Tukey-Kramer HSD method.
* Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Kruskal-Wallis multiple comparison test.

Table 5. Xenobiotic response

Cellular parameter	Reference	Fish-farm transplanted	Fish-farm native	*P*
MXR (pmol · ng^{-1} TSP)	2.192 ± 0.886^{a}	2.417 ± 0.6476^{a}	1.268 ± 0.246^{a}	N.S.
GST (pmol · ng^{-1} TSP)	0.666 ± 0.101^{a}	0.702 ± 0.1195^{a}	0.716 ± 0.0873^{a}	N.S.
Cyp 450 class 2 (pmol · ng^{-1} TSP)	3.641 ± 1.010^{a}	1.91 ± 0.8494^{b}	0.707 ± 0.635^{b}	<0.0001
Cyp 450 class 6 (pmol · ng^{-1} TSP)	2.86 ± 0.6865^{a}	15.60 ± 5.941^{b}	16.33 ± 5.5075^{b}	<0.001*

Note: Entries in the table give treatment means ± 1 standard deviation.
Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Tukey-Kramer HSD method.
* Treatment means with different superscript letters differed significantly at $\alpha = 0.05$ using the Kruskal-Wallis multiple comparison test.

coincides with the cooling of the water column and with the upwelling events (Rinkevich and Loya, 1979; Loya, 2004).

In the present study, we evaluated differences in cellular biomarkers of coral colonies from the two sites as well as in native and transplanted colonies (Table 1). To minimize any possible masking effect of additional environmental and physiological parameters, the cellular biomarkers were evaluated in the corals before peak upwellings and mixing events and before gametogenesis. The results were assessed and evaluated in light of previously reported differences in growth and reproduction in the corals from the two sites (Bongiorni *et al.*, 2003a, 2003b; Loya *et al.*, 2004). Overall, the data indicate significant differences in physiological status of the coral populations at the two sites, as well as differences between transplanted and native colonies at the fish-farm site.

Protein metabolic condition

In general, protein metabolic condition markers showed a variety of effects. Some markers showed no significant differences between sites. For example, cnidarian Hsp70, a cytosolic chaperonin that reflects both a cognate Hsp70 and the inducible Hsp70 (Table 1), was not significantly different in corals from both sites (Table 2). Similarly, levels of ubiquitin, an

identification tag on proteins that are slated for proteolytic degradation within the cytosol, were not significantly different in corals from either sites or treatments. The lack of significant differences in these two markers between sites suggests that colonies from the fish-farm site were not experiencing a protein denaturing or protein damaging stress. On the other hand, markers of protein synthesis and maturation did show site-specific and treatment-specific differences. Hsp60 is a mitochondrial chaperonin and functions to mature nuclear-encoded, mitochondrial-imported proteins into their active state (Ellis, 1996; Hartl, 1996). The observed up-regulation of cnidarian Hsp60 in colonies transplanted to the fish-farm site may be a sign that protein metabolic equilibrium is being altered (Tables 1, 2). In addition, Hsp90 was significantly higher in all of the colonies from the fish-farm environment. Hsp90 is an ATPase enzyme that, in conjunction with other co-chaperones, is essential for the activation of specific proteins (Buchner, 1999). To date, Hsp90 has been reported to play several seemingly independent, physiological roles, including maintenance of protein structure and function, and maturation of estrogen receptor (Table 1) (Rutherford and Lindquist, 1998; Lange *et al.*, 2000; Bouhouche-Chatelier *et al.*, 2001). In the coral *Paramuricea clavata*, levels of Hsp90 correlated positively with water temperature and negatively with levels of lipids and carbohydrates in the coral tissues (Rossi *et al.*, 2006). The higher concentration of this enzyme in fish-farm colonies suggests that there is an increased demand on protein maturation, and/or may be an indication of decreased lipid reserves in the tissues of the fish-farm corals (see Bongiorni *et al.*, 2003a, 2003b; Loya *et al.*, 2004). Taken as a whole, these results reflect an increase in protein production and maturation indicative of the active tissue growth reported previously by Bongiorni *et al.* (2003a, 2003b).

In contrast, zooxanthella Hsp70 and Hsp60 were significantly elevated in the fish-farm colonies compared with the reference colonies (Table 1). However, a higher concentration of these two proteins does not necessarily imply a stressed condition for the zooxanthellae, since both proteins can be up-regulated in response to a shift in protein metabolic condition as a result of increased protein synthesis (Hemmingsen, 1990). Given that in fish-farm *Stylophora* colonies there was an increased level of chlorophyll a content per zooxanthella (Y. Loya, unpublished results) and that there is a high nitrogen/phosphate content in the water (Atkinson *et al.*, 2001; Loya, 2004, 2007), the most likely interpretation is that this shift was a result of increased metabolic demands of growth and protein production in the zooxanthellae.

Oxidative stress and response

Markers of oxidative response showed an array of differences between the treatments. For the most part they were significantly lower in the fish-farm than in the reference colonies (Table 3). The lower cnidarian Cu/Zn superoxide dismutase (Cu/ZnSOD), MnSOD, and catalase levels in colonies from the fish-farm site may indicate that they are experiencing less of an oxidative assault than colonies from the same depth at the reference site. This may have been a reflection of less light at the fish-farm site (see Downs *et al.*, 2002; Levy *et al.*, 2006).

The significantly lower concentrations of cnidarian MnSOD in the natural colonies compared with the reference colonies may reflect increased cell proliferation in the former. Recent studies have shown that a reduction in MnSOD correlates with increased rates of cell proliferation in some mammalian tumours (Cullen *et al.*, 2003) (Table 1). Assuming that this enzyme has a similar role in invertebrates, it is possible that the lower levels of MnSOD are a reflection of the increased cell proliferation that accompanied the accelerated growth (Bongiorni *et al.*, 2003a, 2003b) in the native corals. The discrepancy between the transplanted and

native colonies may be explained by the fact that transplanted colonies were moved to the site as adults that had already reached a near maximum size and thus growth was not an issue. These speculations, however, still merit further study.

Cu/ZnSOD, MnSOD, and GPX function similarly in the alga as in the host, although there are distinct gene/protein structure differences (Bowler *et al.*, 1992). Algal MnSOD and GPX levels were overall lower in the fish-farm colonies than the reference colonies, indicating a reduced oxidative stress load associated with the fish-farm site. On the other hand, algal Cu/ZnSOD was significantly elevated in the transplanted but not the natural colonies compared with the reference colonies. This might be because the zooxanthella population in the transplanted colonies is being adversely affected by something in the environment that is not an issue for the native zooxanthella population. Such a result may be possible if different zooxanthella populations, clades, species or subspecies inhabit the native colonies from the fish-farm but not the transplanted site. Recent studies have shown differences in algal populations inhabiting stressed environments (Baker *et al.*, 2004). Furthermore, in the Gulf of Eilat different clades have been reported to inhabit *S. pistillata* at different depths (A. Daniel, personal communication). It is thus possible that zooxanthella clades in natural colonies from each site are adapted to the site and therefore do not exhibit any differences in oxidative state, whereas those that had been transplanted along with their host do.

Metabolic condition

Metallothionein is a key regulator of mitochondrial function, which acts both as a modulator of the electron-transport chain and of aconitase in the Krebs cycle (Simpkins *et al.*, 1998; Ye *et al.*, 2001; Feng *et al.*, 2005). A lack of any difference in metallothionein between the reference and the transplanted colonies indicates that there are no overt adverse consequences to mitochondrial function in the transplanted colonies (Table 4). The significantly lower level of metallothionein in the natural colonies may reflect the higher growth rate of these colonies (see Bongiorni *et al.*, 2003a, 2003b), since some isoforms of this enzyme have been found to act as growth inhibitors (Jacob *et al.*, 2002). Furthermore, lower levels of metallothionein can result in higher levels of aconitase, which is also consistent with increased respiration rates (Ye *et al.*, 2001). Aconitase is an iron-sulphur protein that acts in iron regulation and in the Krebs cycle. Up-regulation of this protein is usually indicative of an increased demand on respiration products, while down-regulation of this protein is indicative of a toxic pathology, either by reactive oxygen species or alkylating agents (Senft *et al.*, 2002). The significantly higher concentration of aconitase together with the lower concentrations of metallothionein in the fish-farm population compared with the reference one suggests that there is an increased demand on Krebs cycle products, perhaps indicating the increased respiration that accompanies increased growth.

Xenobiotic response

Markers of xenobiotic response (e.g. cnidarian GST MXR) did not show a site-specific response, indicating that it is likely that the corals in the fish-farm environment were not detoxifying substances using the MXR pathway. On the other hand, cytochrome P450 2- and 6-class both showed site-specific expression and can be regarded as 'site-dependent' markers that might provide insight into what is affecting the populations found near the fish-farms. Cytochrome P450 2-class is known for its role in xenobiotic transformation in

vertebrates and some invertebrates (Gassman and Kennedy, 1992; Snyder, 2000) and plays a role in metabolizing endogenous compounds such as fatty acids, lipid hydroperoxides, and ketone bodies into aldehydes (Ronis *et al.*, 1996; Lieber, 1997). Both fish-farm coral populations had significantly reduced cytochrome P450 2-class levels compared with the reference colonies (Table 4). Several mechanisms can explain this reduced expression. One possible mechanism is that a xenobiotic is present in the fish-farm environment and is acting as a cytochrome P450 2-class suppressant. A second possibility is that there is a reduction in fatty acids available for metabolism. Fatty acid metabolism has an important role in oocyte maturation and hormonal control in a number of marine invertebrates (e.g. Pacey and Bentley, 1992), and is known to play a role in oocyte maturation and act as a sperm attractant in a number of coral species (Coll *et al.*, 1994). Thus a reduction in fatty acid metabolism would have an effect on the coral's reproductive function. Indeed, previous studies describing reproductive effort of *S. pistillata* colonies from the same sites showed that both native and transplanted colonies from the fish-farm site developed more testes and more but smaller oocytes and had lower lipid concentrations in their tissues than colonies from the reference site (Bongiorni *et al.*, 2003a; Loya *et al.*, 2004).

A third mechanism, and not a mutually exclusive possibility, is that these corals may have been affected by hormones in the medium or by hormonal mimetics that may have altered their metabolic condition (i.e. endocrine disruption) (see Tarrant *et al.*, 2003). Steroid hormones were detected in coral eggs, in sexually mature polyps, and also surrounding medium of spawning corals (Atkinson and Atkinson, 1992; Tarrant *et al.*, 1999; Twan *et al.*, 2003, 2006), suggesting they have a role in the reproductive activities of these organisms. Moreover, in some coral species, sex steroid concentrations vary seasonally and between the sexes, with peaks occurring before gamete release (Slattery *et al.*, 1997, 1999; Tarrant *et al.*, 1999; Pernet and Anctil, 2002; Twan *et al.*, 2003, Tarrant 2005). Although steroid receptors have not been identified in cnidaria, nuclear receptor genes have been identified, strengthening the premise that steroids play a role in coral physiology (Grasso *et al.*, 2001; Tarrant *et al.*, 2008).

Cytochrome P450 is known to play a role in steroidogenesis in vertebrates and invertebrates alike (Honkakoski and Negishi, 2000; Snyder, 2000) and indeed was found to show seasonal fluctuations, increasing coincidental with the coral's reproductive season (Garcia *et al.*, 2005). The gene for cytochrome 19 was not found in the sea anemone *Nematostella vectensis* (Goldstone, 2008), although a temperature-dependent aromatase activity was demonstrated in the corals (Twan *et al.*, 2003). In light of these reports, it is possible that the lower levels of cytochrome P450 2-class found in both the fish-farm populations compared with the reference population may have been a reflection of the reproductive state observed in these colonies (Bongiorni *et al.*, 2003a, 2003b; Loya *et al.*, 2004). In gastropods, low levels of cytochrome P450 caused by a variety of xenobiotics was accompanied by masculinization (Santos *et al.*, 2002). If cytochrome P450 2-class acts similarly in corals, the lower levels found in fish-farm colonies may explain the 'masculinization' (i.e. increased spermaries) that has been reported previously in these colonies (Bongiorni *et al.*, 2003a, 2003b; Loya *et al.*, 2004). It may also explain the smaller average oocyte size in these colonies when compared with reference colonies (see Loya *et al.*, 2004). These differences in reproductive effort may also be reflected by the site-specific differences in Hsp90, an enzyme that is also known to play a role in steroid receptor stabilization and activation (Bouhouche-Chatelier *et al.*, 2001; Stein *et al.*, 2001).

In addition, both fish-farm coral populations had significantly elevated levels of cytochrome P450 6-class compared with the reference population (Table 4). Cytochrome P450 6-class is known to oxidize and be up-regulated by pesticides such as aldrin, dieldrin,

diazinon, chlorpyrifos, deltamethrin, and a wide range of pyrethroid compounds (Dunkov *et al.*, 1997; Scott and Wen, 2001). Although the role of this enzyme is still unknown in cnidaria, this cytochrome P450 class is known to be largely responsible for pesticide resistance in insects (Scott and Wen, 2001). Hyper-accumulation of this protein in insects is usually indicative of a specific xenobiotic exposure, such as pesticides. Assuming a similar role in cnidaria, this may indicate that a xenobiotic is affecting these corals.

CONCLUSIONS

The present study provides evidence of significant differences in physiology of *Stylophora pistillata* colonies from the two sites as well as between transplanted and native colonies. The identification of site-specific differences in protein metabolism and xenobiotic response suggest that overall there are 'site-driven' differences in physiology at the cellular and whole organism level. The differences in biomarkers also correlated with site-specific differences, showing decreased reproductive effort and increased coral growth (Bongiorni *et al.*, 2003a, 2003b; Loya *et al.*, 2004). Results also showed that native colonies of *S. pistillata* at the fish-farm site seemed to be faring better than transplanted colonies. This may be explained by local selective processes acting on native colonies that result in survival of individuals best adapted to that environment (Zvuloni, 2003) and as a consequence in a reduction in genetic diversity at the eutrophic sites.

The physiological differences described between the native and previously transplanted colonies may indicate that transplantation can affect coral physiology for even long periods of time following the event. This must be taken into account when attempting to use transplantation methods for replenishing degrading reefs.

Unfortunately, very little is still known of the role of many of the enzymatic pathways in cnidarians in general, and in scleractinians in particular. To validate environmentally caused changes, it will be necessary to clarify the role of these cellular parameters in normal metabolic processes in reef-building corals. This must be carried out using controlled experiments taking into account genetics as well as physiological parameters such as age and reproductive state. Understanding the roles of these enzymes in normal physiology will also provide information as to the choice of relevant biomarkers that will provide an early warning system for assessing deviation from the norm and thus ascertaining the health of a coral reef.

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