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Measure of stress response induced by temperature and salinity changes on hatched larvae of three marine gastropod species

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ABSTRACT

To better understand the cascade of molecular reactions leading to delayed development and mortality of early life stages of marine intertidal gastropods, in response to temperature and salinity changes associated with climate change, three biomarkers: total antioxidant capacity, lipid peroxidation and lysosomal stability were investigated on hatched larvae. Encapsulated embryos of three marine gastropod species (*Bembicium nanum*, *Siphonaria denticulata* and *Dolabrifera brazieri*), which have already proven responsive to thermal and osmotic variations, were exposed to six combinations of temperature (22 °C and 30 °C) and salinity (25‰, 35‰ and 45‰) until the larvae hatched. Time to hatching was affected by salinity and temperature in all three species. High salinity (45‰) generally retarded the hatching process although the response was species-specific for temperature. Total antioxidant capacity and lipid peroxidation were also highly species-specific with the general trend showing that these biomarkers were adversely affected by high temperature (30 °C) at salinities of 25‰ and 45‰. *Bembicium nanum* lysosomal destabilisation increased significantly with an increase in temperature and salinity (30 °C and 45‰) and this was associated with delayed development and increased mortality. Investigations on the additional biomarker, lysosomal stability, gave a clearer picture of the numerous and complex molecular and cellular mechanisms leading to mortality and underdevelopment in response to environmental stress for this species. As few differences were observed in the enzymatic biomarkers total antioxidant capacity and lipid peroxidation between hatched larvae and the previously investigated encapsulated embryo response to thermal and osmotic stress, it is suggested that further studies could be undertaken using embryos encapsulated in egg masses, as it is less time consuming than working on hatched larvae.

1. Introduction

The fragile balance between UV exposure, temperature, salinity and pH of the aquatic environment will inevitably change as a consequence of climate change (Langdon, 2000; IPCC, 2001; Meier, 2002; Harley et al., 2006). UV radiation as well as temperature (30 °C) and salinity (25‰ and 45‰) extremes, simulating global warming associated changes, have already been shown to induce increased mortality and delayed development on early life stages of the three gastropod species *Bembicium nanum*, *Siphonaria denticulata* and *Dolabrifera brazieri* (Przeslawski et al., 2005). We have investigated the response to temperature and salinity changes of the encapsulated embryos of the same three species of gastropods, establishing a cascade of molecular reactions, through total antioxidant capacity deficit and enhanced lipid peroxidation leading to embryonic mortality for both *B. nanum* and *S. denticulata* (Deschaseaux et al., 2010). Increased mortality in *D. brazieri* was not always linked to oxidative stress, suggesting that other internal mechanisms might have been responsible for increasing mortality, at least for this species (Deschaseaux et al., 2010).

To further investigate the cellular and molecular consequences of temperature and salinity induced oxidative stress on the early life stages of these gastropods, the destabilisation of lysosomal membranes was investigated. Lysosomes are intracellular organelles involved in many essential functions including degradation of cellular waste products and in cellular defence by sequestering metals and other contaminants. The destabilisation of the lysosomal membrane has been shown to be an effective method for evaluating organism cell damage, in response to toxicity, in a variety of taxa (Lowe et al., 1992; Lowe et al., 1995; Ringwood et al., 2003; Moore et al., 2006) and a correlation between lysosomal destabilisation and salinity and temperature changes has previously been demonstrated in mature molluscs. Increasing salinity, for example, induced autophagocytosis or apoptosis in digestive cells of the blue mussel *Mytilus edulis*, stimulating the lysosomal–vacuolar system (Pipe and Moore, 1985), autophagy being a complex mechanism which is particularly important under environmental stress conditions and which consists of a degradation of cellular constituents (Klionsky and Emr, 2000;

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Pollard and Earnshaw, 2002). In addition, water temperature changes have been reported to affect the lysosomal membrane integrity in hemocytes of the blacklip abalone *Haliotis rubra* (Wang et al., 2006).

This paper presents supplementary investigations on the molecular cascade of reactions leading to embryonic mortality as a consequence of temperature and salinity changes for *B. nanum*, *S. denticulata* and *D. brazieri*. In parallel with biomarkers previously measured on the encapsulated embryos, total antioxidant capacity and lipid peroxidation (Deschaseaux et al., 2010), a method for lysosomal destabilisation has been adapted to the gastropod early life stages by examining hatched larvae instead of embryos within the egg mass. This paper also describes step by step the measures undertaken to improve and adapt lysosomal stability measurement to gastropod egg masses and therefore constitutes time-saving information for future research. This new approach also allows comparisons between embryonic and larval stress responses to salinity and temperature changes.

2. Methods

2.1. Egg mass collection

Egg masses of three different species (*B. nanum*, *S. denticulata*, and *D. brazieri*) were collected from the New South Wales coast, Australia: Bellambi (34°21′57″S, 150°55′29″E), and North Wollongong (34°25′27″S, 150°54′51″E). The egg masses of the three species are all gelatinous but vary in size and shape (Przeslawski et al., 2005). *D. brazieri* egg masses are flattened continuous ribbons deposited in a characteristic zig-zag pattern containing 75 μm diameter eggs, *B. nanum* egg masses are composed of several discrete compartments containing 175 μm diameter eggs, while egg masses of *S. denticulata* are spiralled cylindrical ribbons containing 125 μm diameter eggs. *B. nanum* and *S. denticulata* are mid-shore species which lay egg masses on rock platforms exposed to full sun, usually in small shallow pools. Eggs of *D. brazieri* are usually found in deeper rock-pools beneath boulders low on the shore.

2.2. Experimental treatment

Four replicates of fresh egg masses of each species were exposed to six treatments, consisting of three salinities (25‰, 35‰, and 45‰) and two temperatures (22 °C and 30 °C) until hatching. For each replicate, several egg masses from the same species were split into pieces of approximately the same size and randomly assigned to the six different containers (8.5 cm × 10 cm) corresponding to each treatment combination (22 °C/25‰, 22 °C/35‰, 22 °C/45‰, 30 °C/25‰, 30 °C/35‰, and 30 °C/45‰) so all treatment tanks contained a mixture of the same combination of egg masses. Previous work has demonstrated that the splitting of egg masses in this fashion increases the rate of development, but does not change mortality (Przeslawski et al., 2005). The three experimental salinities were artificially prepared by adding aquarium salt to deionised water (Age of Aquariums, premium marine salt) and temperature was maintained by placing the containers in two different incubators (Clayson-PRTCL programmable control system). A twelve-hour cycle of light/dark (4000 lx, full spectrum without UV) simulating daylight and night time conditions was established inside the incubators. The containers were sealed and a hole drilled in the lids so they could be continually aerated. To prevent salinity increase from evaporation, the original water level was marked on each container at the start of the experiment and water levels were maintained by the addition of deionised water. Salinity, temperature, and pH were monitored daily. Once the egg masses had hatched, total antioxidant capacity and lipid peroxidation were measured for each species and lysosomal destabilisation was measured for *B. nanum* only.

2.3. Measurement of biomarkers

2.3.1. Hatching time

To evaluate the degree of hatching with time, four stages of hatching were determined and defined as follows:

0	no hatching
1	beginning of hatching with a number of larvae <10
2	hatching with a number x of larvae $10<x<100$ (not considered numerous enough to be collected for lysosomal destabilisation, lipid peroxidation or total antioxidant capacity analysis)
3	high degree of hatching with a number of larvae >100

If egg masses had not reached the stage 3 of hatching after 7 days of exposure, and hatched larvae were laying in the bottom of the container, the experiment was stopped and larva were collected at this point for further analysis. In this case, time to hatching was recorded as 7 days.

2.3.2. Biomarker analysis

2.3.2.1. Sample preparation. When stage 3 of hatching was reached, the remaining salt water in the tanks containing the larvae was filtered through a 100 μm screen filter for *B. nanum*, a 75 μm filter for *S. denticulata*, and a 40 μm filter for *D. brazieri*, the screen size being determined by the larvae size. Larvae were then removed from the filter with a spatula and collected in a microcentrifuge tube for total antioxidant capacity and lipid peroxidation analysis.

2.3.2.2. Tissue lysate preparation. A buffer of 5 mM potassium phosphate, 0.9% sodium chloride, 0.1% glucose, pH 7.4, 4 °C was used for the tissue preparation. 250 μl of buffer was added to each microcentrifuge tube and larvae were crushed with a motorised pellet pestle (Motor Sigma-Aldrich Z359971.1EA).

Another 250 μl of Buffer was added to each microcentrifuge tube before sonication at 40 V for 15 s, and centrifugation at 10,000 × *g* 15 min at 4 °C. Supernatant was used for the total antioxidant capacity, lipid peroxidation and protein analyses. All samples were stored at −80 °C until analysis.

2.3.2.3. Protein assay. Protein concentration of tissue lysates was measured using the FluoroProfile™ protein quantification assay, based on the detection and quantification of total protein determined by fluorescence intensity (Sigma Aldrich, USA). Samples were compared to a Bovine Serum Albumin standard. Final total antioxidant capacity and thiobarbituric reactive substances results were expressed as mg^{-1} of the protein concentration of each sample.

2.3.2.4. Total antioxidant capacity assay. The total antioxidant capacity (TAOC) was measured using an assay based on the ability of the tissue lysate antioxidant system to inhibit the oxidation of ABTS® (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metmyoglobin in the presence of hydrogen peroxide. This was compared with the antioxidant capacity of a standard, Trolox (Cayman Chemicals). The amount of $ABTS^{\bullet+}$ produced was measured by the suppression of absorbance at 750 nm and is proportional to the final total antioxidant capacity concentration, expressed in millimolar Trolox equivalents.

2.3.2.5. Thiobarbituric acid reactive substances assay. The Thiobarbituric Acid Reactive Substances (TBARS) assay was used to measure lipid peroxidation by measuring the malondialdehyde (MDA) concentration in each tissue lysate. The end product of lipid peroxidation, MDA, forms a 1:2 adduct with TBARS and produces a colour reaction