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Oxidative stress in abalone: The role of temperature, oxygen and L-proline supplementation

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ABSTRACT

In their natural distribution range and in on land-based mariculture facilities, *Haliotis midae* may be exposed to a relatively wide range of temperatures and oxygen levels, that can induce differential degrees of stress with concomitant metabolic responses.

In the current study juvenile *H. midae* were exposed for one month to two constant temperatures (14 °C and 19 °C), with three oxygen levels (82%, 98% and 126% oxygen saturation) at each temperature. One additional treatment was included at 19 °C, 126% oxygen, where feed was supplemented with L-proline. The results presented here suggest that long-term 19 °C exposure is more stressful to *H. midae* juveniles than a 14 °C exposure of the same duration, since exposure to higher temperature generally resulted in higher activities of the antioxidant enzymes measured but lower total antioxidant capacities. Haemolymph glucose levels responded to oxygen treatment at the 14 °C exposure, but the same response was not initiated in the 19 °C treatment, possibly because the animals exposed to the 19 °C treatment relied on proteins to a larger extent.

Increased oxygen levels at the 19 °C treatment did not cause more DNA damage or protein carbonylation compared to lower oxygen levels. Supplementing feed with L-proline at the high temperature/high oxygen treatment caused a decrease in haemolymph haemocyanin (which may exhibit catalase function) content and a slight decrease in some of the antioxidant enzyme activities, while maintaining the same total antioxidant capacity compared with the same treatment where feed was not supplemented. The overall DNA integrity profile also changed with proline-supplementation, as did the induction level of hsp70. When projecting tissue growth over a 12 month period under these conditions, we estimate that *H. midae* juveniles in this study may exhibit a significant increase in mass when their feed was supplemented with L-proline, indicating that L-proline supplementation provides some benefit to the individual. It might therefore warrant conducting long-term growth trials on land-based mariculture facilities to further investigate its potential benefit to the mariculture industry.

1. Introduction

Haliotis midae are temperate abalone, and farmed locally in on-land aquaculture facilities. They are found in coastal water along the west and south coast of South Africa and in their natural habitat, *H. midae* are found in waters with a mean temperature between 13.5 °C and 18 °C. Although the monthly average maximum temperature across their distribution range is only 21 °C they have been found to survive acute exposures to temperatures as high as 25 °C (Britz et al., 1997). Prolonged exposure to temperatures above 20 °C has been shown to cause a decline in growth rate, increased mortality rate (Britz et al., 1997) and stimulate high levels of heat shock protein 70 (hsp70), at least in adults (Vosloo and Vosloo, 2010). In most ectotherms, their thermal limit is a function of both evolutionary history and life history, and these limits can to some extent be shifted as a result of changes in acclimation temperature. This is at least partly due to the differential inducibility of stress proteins, such as heat shock proteins, at different acclimation temperatures (Tomanek, 2005).

Between the upper and lower thermal optimum limits of an organism, ectotherms typically have an optimal aerobic scope. However, as temperatures deviate outside these optimum limits, the aerobic scope decreases due to a mismatch in oxygen supply and demand, bringing about tissue hypoxia and eventually anaerobic metabolism (Pörtner, 2002). Damage to lipids, nucleic acids and proteins manifests when the capacity of antioxidants is exceeded (Abele and Puntarulo, 2004; Verberk and Bilton, 2011). Strategies to protect cells from damage or repair damage incurred are energetically costly and may channel energy away from reproduction and growth. The theory of limited thermal tolerance/acclimation capacity caused by oxygen limitation is supported by the fact that environmental hypoxia often limits (Fernandes et al., 1995; Pörtner, 2010), and hyperoxia extends (Frazier et al., 2001; Pörtner et al., 2006; Weatherley, 1973) thermal optima and tolerance ranges of ectotherms.

Damage to DNA due to high temperature (MacFadyen et al., 2004) or a mismatch between oxygen demand and delivery, can be repaired to

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some extent, but DNA damage in itself can induce further ROS (Kang et al., 2012). Unrepaired DNA can accumulate over time and result in defective DNA repair mechanisms and may amongst others result in impaired physiological function and premature aging (for review see Chen et al. (2007)). The role of heat shock proteins, and in particular hsp70, in protecting animals against heat-induced protein damage has been extensively studied in many animal groups (Airaksinen et al., 2003; Brun et al., 2008; Carpenter and Hofmann, 2000; Drew et al., 2001; Vosloo and Vosloo, 2010). However, heat shock proteins have previously been identified as a primary target of carbonylation and subsequent ubiquitination during oxidative stress in bacteria (Dukan and Nystrom, 1998, 1999), and recently, also in primates (Oikawa et al., 2009). Carbonylation results mainly from oxidative stress, and moderately carbonylated proteins are degraded, while heavily carbonylated proteins form aggregates and accumulate as unfolded proteins in cells (Dalle-Donne et al., 2006). Even though carbonylation is an irreversible process, protection against oxyradicals can be afforded by amongst others, the primary antioxidant defence system that includes superoxide dismutases, catalases and peroxidases (Nystrom, 2005). In addition, free amino acids, including L-proline, have also been found to protect cells against oxidative damage in vitro due to their ability to scavenge oxy-radicals (Paniello et al., 1988) and recently Oellermann et al. (2012) found proline to be an economic substrate for mitochondrial respiration at high temperatures.

We previously demonstrated that adult *H. midae* find short-term exposure to 19 °C stressful and they increase hsp70 levels at those exposures. However, they were found to acclimate to 19 °C after prolonged exposure (Vosloo and Vosloo, 2010). We also demonstrated that adult *H. midae* are sensitive to subtle changes in environmental oxygen levels at constant acclimation temperature, but that juveniles are largely unaffected due to their increased antioxidant status (A. Vosloo et al., 2013). In the current study we aim to describe some aspects of the antioxidant status of juveniles of the same species to subtle changes in environmental oxygen levels at two different temperatures. We specifically measured total antioxidant capacity and activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. In addition, we measured protein carbonylation and DNA integrity at higher temperature. Haemocyanin concentration was measured because of its dual role in carrying oxygen and acting as an antioxidant enzyme. Because oxidative stress is known to cause carbonylation of proteins, we assessed the usefulness of proline as feed additive in reducing the expected oxidative stress at combined high temperature and high oxygen exposures. We aimed to keep the temperatures and oxygen levels realistic and well within the daily averages experienced by these animals in their natural habitat. Based on the discussion above, we expect elevated oxidative stress at elevated temperature and oxygen treatments above and below saturation, and that L-proline supplementation will relieve some oxidative stress at the high temperature, high oxygen treatment.

2. Materials and methods

2.1. Animals, exposures and sampling

Juvenile abalone were collected from the grow-out platform, and subsequently acclimated and exposed in an on-farm laboratory at Irvin & Johnson, Abalone Division, South Africa as described in A. Vosloo et al. (2013). We exposed animals in the current study the juveniles from the A. Vosloo et al. (2013) study conducted at 16 °C, therefore the experimental setup is similar. The exposures for this study consisted of two temperatures (13.80 ± 0.07 °C and 18.96 ± 0.01 °C), which will hereafter be referred to as 14 °C and 19 °C. Within each temperature, dissolved oxygen levels were maintained at 6.40 ± 0.07; 7.70 ± 0.01; 9.59 ± 0.09 mg L^{-1} respectively, which will be referred to as 82%, 96% and 126% of oxygen saturation in seawater. Oxygen levels lower and higher than saturation were maintained by supplementing atmospheric air pure nitrogen or oxygen respectively. The tanks were cleaned twice a week and were provided with 0.015 g formulated abalone feed (Abfeed S34, Marifeed (Pty) Ltd.) per gram wet body mass as described before (A. Vosloo et al., 2013). One additional treatment, where animals exposed to 19 °C and high oxygen, was set up where animals were supplied with Abfeed S34, supplemented with 10.0 g L-proline per kilogram dry feed. This concentration was reported by Vosloo and Van Rensburg (2009) to be the optimal concentration for protecting abalone against dehydration during live export and no other concentrations were therefore tested in the current study. The batches of Abfeed S34, with and without L-proline were produced on the same day and the L-proline was introduced upon mixture of the supplemented feed, before extrusion.

The treatments were maintained for one month before samples were collected to measure the parameters discussed below. Haemolymph was sampled from the pedal sinus using a one millilitre syringe, fitted with a 27 gauge needle. Gill and foot muscle tissue was collected after shucking the animal. All haemolymph and muscle samples were snap-frozen in liquid nitrogen and transported on dry-ice to the University of KwaZulu-Natal. Aliquots from the haemolymph samples were kept iced for analysis.

2.2. Haemolymph glucose levels and haemocyanin content

Haemolymph glucose levels were determined colorimetrically with a glucose assay kit from Megazyme Inc. as described previously (A. Vosloo et al., 2013). Haemocyanin content was determined spectrophotometrically in aerated haemolymph samples as described by Behrens et al. (2002).

2.3. Antioxidant enzyme activity and total antioxidant capacity

Proteins were extracted from gill tissue in 100 mmol L^{-1} Na-phosphate buffer of pH 7.5, supplemented with 0.1 mol L^{-1} phenylmethylsulphonyl fluoride (Regoli and Principato, 1995). Activities of the antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase in gill tissue were measured with commercially available kits (Fluka and Sigma). The total antioxidant capacity of gill tissue was measured using the ferric reducing/antioxidant potential (FRAP) assay as described by Griffin and Bhagooli (2004), using 20 μg of the same proteins extracted for the antioxidant enzyme activities. The incubation time for the FRAP assay was extended to 20 min as suggested in Berker et al. (2007).

2.4. Protein carbonylation and hsp70 immunodetection

Proteins were extracted from foot muscle tissue in 32 mmol L^{-1} Tris–HCl and 2% SDS supplemented with protease inhibitors (Drew et al., 2001). Protein carbonylation was measured in 0.5 mg of protein extract using a colorimetric assay (BioVision, California, USA) and hsp70 immunodetection was performed in 150 μg of the extracted proteins, as described before (A. Vosloo et al., 2013), using commercial anti-hsp70 and anti-α-tubulin antibodies (Sigma).

2.5. DNA integrity

The comet assay was performed in haemolymph cells as described before (A. Vosloo et al., 2013). One hundred comets per animal were analysed for the percentage of DNA in the tail and the Olive tail moment (Casp 1.2 software, SourceForge®, 2006).

2.6. Expected cumulative growth

The specific growth rates (100 × [ln final weight − ln initial weight] / experimental period in days) for animals from each of the three oxygen treatments at 14 °C and 19 °C were calculated as per