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Thermal acclimation in clownfish: An integrated biomarker response and multi-tissue experimental approach

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ABSTRACT

The effects of increased temperature were tested in *Amphiprion ocellaris*, using a cellular diagnostics approach (in several tissues) combined with an organismal approach (body condition). Clownfish were exposed to a one month experiment following two temperature treatments: control (26 °C) and elevated temperature (30 °C). Fish were sampled at 0, 7, 14, 21 and 28 days for (1) assessment of stress biomarkers (catalase, lipid peroxidation, glutathione-*S*-transferase, superoxide dismutase, acetylcholinesterase, heat shock protein 70 kDa and ubiquitin – in brain, gills, liver, intestine and muscle), (2) estimation of integrated biomarker response index based on the biomarkers tested and (3) assessment of Fulton's K index. Results show all biomarkers except acetylcholinesterase responded consistently and significantly to elevated temperature across tissue types suggesting they are suitable indicators of thermal stress in *A. ocellaris*. Biomarker levels were tissue-specific, and in addition, the most reactive tissues to temperature were muscle, gills and liver which suggest that highly oxygenated tissues seem to be the most responsive under thermal stress. The most responsive sampling times to increased temperature were T7 and T28: thermal stress was observed after 7 days of exposure (biomarker levels increased), then a pattern of decrease in biomarker levels towards the end of the experiment was observed, which may suggest fish were able to acclimate to exposure conditions. This indicates that *A. ocellaris* probably lives far from its upper thermal limit and is capable of adjusting the protein quality control system and enzymes' activities to protect cell functions under elevated temperatures. The temperature treatment did not significantly influence body condition of the animals but biomarkers were negatively correlated to wet body weight. This suggests that thermal acclimation incurs at some energetic cost. In conclusion, these results suggest that this coral reef fish species presents a significant acclimation potential under ocean warming scenarios of +4 °C.

1. Introduction

Temperature fundamentally affects all aspects of physiology by influencing reaction rates, as well as the physical properties of biological molecules (Hochachka and Somero, 2002). Hence, thermal stress is widely proposed as the dominant physical stress in intertidal and shallow water habitats and is reported to have pervasive effects on marine fish on both temperate (Helmuth et al., 2006) and tropical shores (Firth and Williams, 2009). It is hypothesized that, for a complex organism, a hierarchical series of tolerance prevails, ranging from systemic to cellular to molecular levels (Weibel et al., 1991), with highest sensitivity at the organism level and wider tolerance windows at lower levels of complexity (Goh and Lai, 2014). Because seasonal temperature variations are minimal in tropical areas, marine fish inhabiting such habitats are expected to display narrow tolerance ranges of temperature once their thermal limits may be close to their optimal temperature (Deutsch et al., 2008). In consequence, changes in environmental temperature may lead to the poor maintenance of physiological homeostasis, resulting in stress (Long et al., 2012).

However, no specific criteria have been established as to what constitutes optimum conditions for tropical marine ecosystems, and baseline data are often not available. While there is a growing appreciation that warmer temperatures will indirectly affect tropical reef fish communities through the degradation of reef habitats from mass coral bleaching (Pratchett et al., 2008), much less

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is known about the direct effects that increasing water temperature will have on coral reef fish (Nilsson et al., 2010). In particular, research is needed to determine suitable tropical test species that are or relate to potential impacts on keystone species, as well as appropriate test conditions (Botté et al., 2012). Studies are necessary to characterize and shed more light on the physiological and biochemical behavior of molecules in reef fish, under normal conditions to enable further analysis of changes induced by stress exposure (Assis et al., 2012).

Recent research on cardinalfish and damselfish has shown that exposure to elevated temperatures can result in increased O_2 demand and therefore increase basic maintenance costs for the organism (Clarke, 2003). This can reduce aerobic scope of reef fish (Nilsson et al., 2009) and key aspects of individual performance, such as growth (Munday et al., 2008a,b) and fecundity (Donelson et al., 2010). In fact, hyperthermic stressful conditions can trigger a cascade of metabolic alterations in individuals (Romero, 2004) and when it exceeds the individual capacity for acclimation, it can lead to health disorders due to enzyme inactivation, protein unfolding and degradation, DNA damage and lipid peroxidation (Livingstone, 2003). Hence, fish (like other organisms) have evolved defense systems and cytoprotective mechanisms which are employed for the maintenance of cell viability and functional activity (Tang et al., 2014).

The response to thermal stress thus comprises both enzymatic and nonenzymatic pathways in a process called cellular stress response (CSR) (Kaviraj and Gupta, 2014). On one way, enzymatic pathways involve antioxidant enzymes whose primary purpose is to quench or reduce the flux of reactive oxygen species (ROS) produced during oxidative metabolism (Lesser, 2011). On the other way, nonenzymatic processes involve the induction of protective proteins that act as molecular chaperones and prevent protein unfolding and aggregation which would render them nonfunctional. In the first case, the enzymes superoxide dismutase (SOD; converts O_2^- to $H_2O_2 + O_2$), catalase (CAT; reduces H_2O_2 to H_2O) and glutathione *S* transferase (GST, phase II enzyme that conjugates hydro-peroxides with glutathione) are used by cells to maintain their oxidative status and are also the biomarkers of choice to appraise oxidative stress (Di Giulio et al., 1995). In addition lipid peroxides (LPO) are used as markers of cell membrane damage (Ferreira et al., 2015a), and the activity of the enzyme acetylcholinesterase (AChE, terminates synaptic transmission) can be used as a measure of neurotoxicity (Ferreira et al., 2015a). In the second case, induced heat shock proteins (Hsps) act in the refolding of denaturated structural and cellular proteins and ubiquitin (Ub) signals nonfunctional proteins for degradation in the proteosome (Coles and Brown, 2003). Variations in the expression of stress proteins and antioxidants are hence used as biomarkers of effect once they are a direct measure of changes in the degree of reversible/irreversible macromolecular damage and their expression and accumulation in cells represents a quantifiable response to sublethal thermal stress exposure (Vieira et al., 2014).

However, the application of biomarkers in environmental assessment is limited without an integrated system to overcome difficulties in relating information and in categorizing the severity of stressors and induced changes in the health status of the organisms (Cravo et al., 2012). In these circumstances, a simple method is needed to summarize biomarker responses and simplify their interpretation in biomonitoring programs (Beliaeff and Burgeot, 2002). One solution is the use of a methodology that integrates the responses of different biomarkers into a single value or graph, allowing information to be summarized in the form of a multivariate data set. Among these indices, the Integrated Biomaker Response (IBR, Beliaeff and Burgeot, 2002) along with star plots, are amongst of the most used in field and laboratory studies (Serafim et al., 2012). This should provide a more valid basis for interpretation of ecological surveys.

The main objective of this study was to measure the cellular stress response in the tropical reef fish species *Amphiprion ocellaris* under exposure to elevated temperature. In particular, the research aims were (1) to evaluate the effects of elevated temperature in *A. ocellaris* through the quantification of molecular biomarkers i.e. CAT, LPO, SOD, GST, AChE, Hsp70 and Ub at two different temperatures (26 °C – control, and 30 °C – elevated temperature), along one experimental month (sampling at 0, 7, 14, 21 and 28 days), in several tissue types (brain, gills, liver, intestine and muscle); (2) to use a multi-biomarker integrated approach (IBR and star plots) for the evaluation of the effect of elevated temperature on organismal health and ability to acclimate and (3) evaluate the effects of elevated temperature on body condition of *A. ocellaris*.

2. Materials and methods

2.1. Test organism and acclimation procedure

The model species used in this study was the clownfish *Amphiprion ocellaris* (Cuvier 1830). Juvenile fish (n = 45) were obtained from Tropical Marine Centre Iberia hatchery (Loures, Portugal). Upon arrival at the laboratory fish were placed in one indoor re-circulating water system (total volume of 2000 L), comprised of six 70 L polyvinyl tanks supplied with aerated sea water, one common sump and an external skimmer and UV filter. Inflow of clean water in each tank was 300 mL min^{-1}. Fish were randomly distributed across tanks (n = 10 individuals per tank) and allowed to acclimate at 26 ± 0.5 °C for 2 weeks.

2.2. Experimental design and sampling

All experimental procedures followed ethical guidelines in national and international legislation, namely the European Food Safety Authority recommendations (three of the authors possess FELASA level C accreditation). The experimental design (Fig. EMS1) consisted of two temperature treatments: 26 °C ± 0.5 °C (control, physiological optimum for *A. ocellaris*) and 30 °C ± 0.5 °C (elevated temperature, chosen to reflect current IPCC projections for 2100 in tropical areas (IPCC, 2013)), with the duration of one month and samplings at 0, 7, 14, 21 and 28 days. For each treatment there were 3 replicate tanks (35 × 35 × 55 cm each), with 10 individuals per tank. All tanks were provided with a filter (ELITE 9 Underwater Mini-Filter Hagen, 220 L h^{-1}) and a thermostat heater (ELITE 200W). To keep environmental parameters constant throughout the experiment, a monitoring scheme was employed. Each tank was provided with a Petco thermometer with suction cup to monitor temperature continuously. Other parameters i.e. salinity (kept at 35), pH (kept at 8 ± 0.01), ammonia (kept at 0 mg L^{-1}) and nitrites (kept under 0.3 mg L^{-1}), were monitored twice a week using a hand-held refractometer (Atago, Japan), a digital pH probe (model HI9025, Hanna Instruments, USA), and Tetra Test Kits (Tetra Ammonia Test Kit and Tetra Nitrites Test Kit, USA), respectively. A period feeding regime (twice a day) was implemented. Fish were fed with fresh food (mixture of shrimp and mussels in proportion 1:1) and dry food (Tropical Super Spirulina Forte Mini Granulat). At each sampling day 5 animals from each treatment were chosen randomly (Fig. ESM2) sacrificed by cervical dissection and then measured and weighed. Internal organs (brain, gills, liver, intestine and muscle) were then extracted and frozen at −80 °C until further analyses (Fig. ESM3).