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Season-dependent effects of elevated temperature on stress biomarkers, energy metabolism and gamete development in mussels

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ABSTRACT

In coastal areas, sessile species can be severely affected by thermal stress associated to climate change. Presently, the effect of elevated temperature on metabolic, cellular and tissue-level responses of mussels was determined to assess whether the responses vary seasonally with seawater temperature and reproductive stage. Mussels were collected in fall, winter and summer, and (a) maintained at 16, 12, and 20 °C respectively or (b) subject to gradual temperature elevation for 8 days (+1 °C per day; from 16 to 24 °C in fall, from 12 to 20 °C in winter and from 20 to 28 °C in summer) and further maintained at 24 °C (fall), 20 °C (winter) and 28 °C (summer) for the following 6 days. Temperature elevation induced membrane destabilization, lysosomal enlargement, and reduced the aerobic scope in fall and summer whereas in winter no significant changes were found. Changes at tissue-level were only evident at 28 °C. Gamete development was impaired irrespective of season. Since the threshold of negative effects of warming was close to ambient temperatures in summer (24 °C or above) studied mussel populations would be vulnerable to the global climate change.

1. Introduction

Temperature is a key environmental factor affecting physiology and health of marine ectotherms including mussels due to the direct effects of temperature on the rates of all physiological and biochemical reactions and stability of macromolecules (Hochachka and Somero, 2002). Rise in seawater temperature, as expected in the context of global change, can cause severe effects in coastal ecosystems, including shift in the geographical distribution of species, phenotypical and phenological alterations, extinctions and, overall, deterioration of the ecosystem health (Walther et al., 2002). For sessile species the ability to adapt to elevated temperatures is a determining factor in survival (Schiedek et al., 2007). Mussels are sessile filter feeding ectotherms able to tolerate a wide range of environmental conditions and respond in a way that can be measured through biological effect biomarkers; hence, they are widely used as sentinels in coastal ecosystem health assessment (UNEP/RAMOGE, 1999; ICES, 2011; Marigómez et al., 2013).

Negative effects of temperature extremes on survival and fitness-related functions of ectotherms can stem from both the direct effects of temperature on stability, conformation and function of biological macromolecules (Hochachka and Somero, 2002) as well as the indirect effects due to the energy deficiency (Pörtner, 2010; Sokolova et al., 2012). In aquatic ectotherms such as mussels, moderate warming results in elevated rates of oxygen consumption in order to support an increased energy demand for cellular and organismal processes, but extreme warming can result in a reduction in aerobic scope (i.e. the amount of energy and metabolic capacity available for fitness-related functions such as growth, activity and reproduction) and/or induce metabolic depression (Pörtner, 2001, 2010). Elevated energy demand during thermal stress is commonly associated with reallocation of energy to support maintenance costs with less energy allocated to growth, storage and reproduction (Kooijman, 2010). At critical temperatures when aerobic scope disappears, marine mollusks shift to partially anaerobic metabolism which heralds time-limited survival (Sokolova and Pörtner, 2001; Anestis et al., 2010).

Lysosomal responses constitute one of the most widely accepted biomarkers for assessing health status of marine

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organisms including mussels (Regoli, 1992; UNEP/RAMOGE, 1999; Domouhtsidou and Dimitriadis, 2001; Izagirre and Marigómez, 2009; ICES, 2011; Marigómez et al., 2013). The endo-lysosomal system of mussel digestive cells is involved in intracellular digestion and pollutant accumulation and detoxification (Cajaraville et al., 1995; Domouhtsidou and Dimitriadis, 2001; Marigomez et al., 2002; Robledo et al., 2006; Izagirre et al., 2008, 2009; 2014). Lysosomal enlargement and membrane destabilization are commonly elicited by environmental stress (Moore, 1976; Tremblay et al., 1998; Garmendia et al., 2011a; Dimitriadis et al., 2012; Izagirre et al., 2014). The effect of temperature changes on lysosomal membrane destabilization, lysosomal enlargement and changes in lysosomal contents has been widely reported (Regoli, 1992; Tremblay et al., 1998; Petrovic et al., 2004; Bocchetti and Regoli, 2006; Moore et al., 2007; Dimitriadis et al., 2012; Izagirre et al., 2014). Seasonal changes in the form and function of digestive cell lysosomes have also been related to temperature, food availability and gonad development (Leiniö and Lehtonen, 2005; Izagirre et al., 2008; Lekube et al., 2014). Moreover, lysosomal membrane destabilization has been associated with the post-spawning stage suggesting physiological stress (Domouhtsidou and Dimitriadis, 2001; Garmendia et al., 2010). Environmental stress can also elicit changes in tissue morphology and functions, including changes in cell type composition in the digestive gland epithelium (e.g., basophilic cell hypertrophy and digestive cell loss), atrophy of the digestive epithelium, inflammatory responses and loss of histological integrity in digestive gland tissue (Couch, 1984; Cajaraville et al., 1992; Marigómez et al., 2006; Zaldibar et al., 2007, 2008; Garmendia et al., 2011b). Although the effects of temperature stress on lysosomal and tissue-level biomarkers have been extensively studied, variation in the biological effects of elevated temperature in the context of natural seasonal variability of environmental conditions mussels' physiology is not yet well understood limiting our ability to predict the potential effects of warming on mussel populations.

The present investigation is aimed at determining whether the biological effects of elevated temperature in mussels (*Mytilus galloprovincialis*) depend on the seasonal variation in seawater temperature and/or the reproductive stage of the mussels. For this purpose, the effects of gradual temperature rise (+8 °C) on mussel health and metabolic status were assessed in three different seasons. Among general stress biomarkers, we focused on the structural changes in lysosomes (Marigómez et al., 2005; Izagirre and Marigómez, 2009), intracellular neutral lipid accumulation (Cancio et al., 1999; Marigómez and Baybay-Villacorta, 2003), changes in cell type composition, thinning of the digestive gland epithelium and integrity of the digestive gland tissue (Zaldibar et al., 2007; Brooks et al., 2011; Garmendia et al., 2011b). Metabolic adjustments to temperature changes were examined in the posterior adductor muscle by determining the activities of the enzymes pyruvate kinase and phosphoenolpyruvate carboxykinase, which control the glycolytic flux to aerobic vs. anaerobic pathways, respectively (De Vooys, 1980; Greenway and Storey, 1999).

2. Materials and methods

2.1. Materials

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma–Aldrich (St. Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

2.2. Animal collection and maintenance

Mussels, *M. galloprovincialis*, (3.5–4.5 cm shell length) were collected from the intertidal zone in Mundaka at the estuary of Urdaibai (Biosphere Reserve, UNESCO 1984) (43°22′N, 2°40′W; Bay of Biscay) in summer (July 2009), fall (November 2009) and winter (early March 2010). Mussels were transported to the laboratory within 1 h of collection, and placed in flow-through tanks (45 L) with aerated natural filtered (0.45 μm) seawater (33‰ salinity). A different seawater experimental temperature was established for each season (16 °C, 12 °C and 20 °C in fall, winter and summer respectively) depending of the temperature at the source at the time of collection (surface seawater temperature data at Bilbao station (43.40°N 3.13°W, 53 m depth) were obtained from www.puertos.es >Oceanografía y Meteorología >Redes de Medida>Red Costera; access dates: July 2009–April 2010). Food was provided as cultured live cells of algae *Isochrysis* spp. (20.000 cells/mL, 4 μm size) by continuous administration (1 L/day). Mussels were allowed to acclimate to laboratory conditions for 5 days. No mortality was observed during this period.

2.3. Experimental design

After acclimation, mussels were randomly divided into two groups. In the first, temperature was increased 1 °C per day during 8 days (from 16 °C to 24 °C in fall, from 12 °C to 20 °C in winter from 20 °C to 28 °C in summer) and maintained at 24 °C in fall, 20 °C in winter and 28 °C in summer for 8 days (ESM1, Fig. S1). The second group was used as experimental control, with the initial temperature (16, 12 or 20 °C) being kept constant over the experimental period. Mussels ($n = 15$) were retrieved from both experimental groups at days 0, 1, 4, 8 (during temperature rise) and at day 14 (after acclimation to elevated temperature; 24, 20 or 28 °C). For each treatment, mantle ($n = 15$), posterior adductor muscle (PAM; $n = 5$) and digestive gland ($n = 5$) tissues were collected. PAM tissue and one half of each digestive gland were placed into separate cryovials, flash-frozen in liquid nitrogen and stored at −80 °C until analyses. The second half of the digestive gland and the mantle were fixed in 4% formaldehyde containing 0.1 M phosphate buffer for 24 h. Blind-coded labels were used in order to avoid operator's subjectivity.

2.3.1. Lysosomal biomarkers

2.3.1.1. Lysosomal Membrane Stability (LMS) test. The lysosomal membrane stability test (LMS) (Marigómez et al., 2005) was performed according to standard procedures (ICES, 2004). Serial tissue sections (10 μm thick) of frozen digestive gland were cut in a Leica CM 3000 cryostat (Leica Instruments) at a cabinet temperature of −24 °C. For each season, all the samples were stained simultaneously to reduce processing variability. Prior to staining, slides were air-dried at 4 °C for 20 min and then brought to room temperature. The sections were pre-treated in 0.1 M citrate buffer (pH 4.5) at 37 °C in a shaking water bath for different time intervals (0, 3, 5, 10, 15, 20, 30, and 40 min) to determine the lysosomal destabilization time. Further on, the sections were incubated at 37 °C for 20 min, in 0.1 M citrate buffer (pH 4.5) containing 2.5% NaCl, 0.04% naphthol AS-BI N-acetyl-b-D glucosaminide dissolved in 2-methoxyethanol (Merck Biosciences) and 7% of Polypep®. The sections were then rinsed in a saline solution (3% NaCl) at 37 °C for 2 min and transferred to a 0.1 M phosphate buffer (pH 7.4) containing 0.1% Fast Violet B diazonium salt, at room temperature for 10 min. The slides were rinsed in running tap water for 5 min, fixed in Baker's formol calcium containing 2.5% at 4 °C for 10 min, rinsed in distilled water and mounted in Kaiser's glycerin gelatin.