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Consequences of acclimation on the resistance to acute thermal stress: Proteomic focus on mussels from pristine site

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ABSTRACT

Climate change constitutes an additional threat for intertidal species that already have to cope with a challenging environment. The present study focuses on the blue mussel *Mytilus edulis* and aims at investigating the importance of thermal acclimation in heat stress response. Microcosm exposures were performed with mussels submitted to an identical acute thermal stress following two thermal summer acclimations standing for present or future temperature conditions. Gill proteomes were analyzed by 2DE and 96 differentially expressed proteoforms were identified. Our results show that cell integrity appears to be maintained by the rise in molecular protective systems (*i.e.* Heat Shock Proteins), and by the reallocation of energy production via a switch to anaerobic metabolism and the setting up of alternative energy pathways. Finally, our results indicate that the response of mussels to acute thermal stress is conditioned by the acclimation temperature with an improved response in organisms acclimated to higher temperatures.

1. Introduction

Since the early 2000', studies dealing with climate change were multiplied by ten (PubMed, 2015) and mostly focused on the impact of temperature increase or ocean acidification on organisms, at different scales ranging from population status to biochemical processes. According to the last IPCC report (IPCC, 2014), the main observable effect of the anthropic footprint is a global rise in aquatic and terrestrial temperatures. Additional modifications are projected such as an increase in the sea level, large-scale ice sheet meltings and a higher occurrence of extreme meteorological events. Among those, heat wave phenomena are expected to arise more frequently and for longer durations. A recent report estimates that, in 2100, the air temperature would be 2–4 °C higher in the coastal zone of Normandy. Moreover, 5–10 days of heat waves per year are predicted, a value that contrasts with the 0–2 days of extremely elevated temperatures observed during the 1975–2005 period (Ouzeau et al., 2014).

Intertidal environments host habitats submitted to periodic biotic and abiotic modifications affecting temperature, oxygen, food availability and salinity. Changes occur either daily, with tidal flows, or for longer periods in a context of global change. Among organisms inhabiting shores, the blue mussel *Mytilus edulis* constitutes an important key species and a model for ecophysiologic studies (see Mussel Watch concept: Goldberg, 1986). *M. edulis* is a sessile bivalve living in beds all along the tidal zone and exhibiting a high tolerance in contrasted conditions. Bivalves are ectothermic organisms and, therefore, do not regulate their body temperature. At low tide, mussels exposed to solar radiation can experiment temperatures higher than 35 °C for several hours. Besides, Davenport and Woolmington (1982) demonstrated the occurrence of rapid drop in oxygen content after valve closing in *M. edulis* organisms. To cope with this challenging environment combining heat stress and emersion-induced hypoxia, mussels have developed adaptive mechanisms that maintain cell integrity and energy supply in anaerobiosis. First, glycolytic rate can be multiplied by 12 to keep ATP production similar to that observed in normoxia (Grieshaber et al., 1994). Moreover, mussels can use phosphorylated guanidinium compounds, also known as phosphagens, for rapid ATP formation. Secondly, bivalves exposed for long periods to high

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terrestrial temperatures are able to open their valves. This gaping process is assumed to provide oxygen while mussels are exposed to severe hypoxia rather than to refresh the body by evaporation (Nicastro et al., 2012; Widdows et al., 1979). At the molecular level, induction of heat shock proteins has been associated with resistance to multiple stresses. In *Mytilus* gender, the expression of HSP 70 and isoforms have been widely studied in response to thermal stress in acute or acclimation contexts (Anestis et al., 2010, 2007; Buckley et al., 2001; Chapple et al., 1998, 1997; Hofmann and Somero, 1995; Péden et al., 2014; Roberts et al., 1997).

Proteomics is a powerful tool in environmental studies (Lemos et al., 2010). By freezing a wide range of proteins at t-time, it may reveal the potential integration and complexity of the response to stress. Hence, recent proteomic studies on *Mytilus* sp. were focused on the impact of contaminants (Apraiz et al., 2009; Campos et al., 2012; Gomes et al., 2014; Letendre et al., 2011), oxidative stress (Sheehan and McDonagh, 2008; You et al., 2014), and thermal stress (Fields et al., 2012; Tomanek, 2012, 2011; Tomanek and Zuzow, 2010). Most of these publications used two-dimensional gel electrophoresis (2DE) for identification of differentially expressed proteins. Since it has been introduced by O'Farrell in the mid-1970s (O'Farrell, 1975), this gel-based method progressively gave way to shotgun proteomics (Rabilloud, 2012). However, 2DE stands for a meaningful technique, especially when dealing with non-model organisms which genomes are partially known (Armengaud et al., 2014; Rocher et al., 2015).

In a context of global changes, the question of intertidal organism resistance to acute thermal stress was raised. Here, we developed an experimental approach based on a closed climatic microcosm that mimics intertidal summer conditions in Normandy: a tidal cycle (6 h emersion − 6 h immersion), a photoperiod (16 h light, 8 h dark) and a constant water temperature (18 °C). By changing the air temperature, mussels were acclimated during emersion periods to (1) conditions representative of local average temperatures in the 2010s summers and (2) of future conditions based on estimated temperatures in the 2100s according to the A1B IPCC scenario (IPCC, 2007). As heat wave phenomena are expected to dramatically increase in Normandy coast, we chose to expose these differentially conditioned mussels to their thermal limits (+35 °C). Gills proteomes were analyzed in order to decipher adaptive mechanisms set up by mussels challenged with global changes in our experimental conditions.

2. Material and methods

2.1. Sample collection and microcosm exposure

Adult mussels (shell length 37.5 mm ± 3 mm) were collected during July 2013 in tidal zone of "La Pointe du Chicart, Yport (49°44′28"N, 0°17′53"E, water temperature 15 °C). Gills of twenty mussels were directly dissected and snap frozen in liquid nitrogen in order to constitute the control group (CT). The other mussels were randomly split in two climatic chambers reproducing tidal variations (6h immersion − 6h emersion) and diurnal cycles (16 h day − 8h night) as explained in Fig. 1. Prior to thermal exposure, mussels were acclimated to setting water temperature (18 °C) by 1 °C/day steps. Animals were fed daily with Shellfish Diet 1800 (Reed Mariculture, USA). Mussels were exposed for 7 days either to present thermal scenario (present conditioning: CX group) or to predictive A1B IPCC scenario (future conditioning: FX group) as defined in Fig. 1. Briefly, present and future scenarios consist of daily variations of the air temperature from 16.9 °C to 21.2 °C and from 18 °C to 26.2 °C, respectively, during emersion phases. Finally, both groups were exposed to an identical acute thermal stress for 3 days (daily variation of the air temperature from 20 °C to 35.2 °C). Mussels were sacrificed at day 10 and gills were dissected and snap frozen for proteomic analysis.

Mussel mortality was followed by daily observations. Dead animals were removed and not replaced. At the end-point, the rate of mortality was 5.6 and 5.8% in groups CX et FX respectively.

2.2. Protein extraction

Gills were grind using Precellys® Homogenizers in a 1:4 ratio of lysis buffer (9 M urea, 2% CHAPS, 65 mM dithioerythritol, 0.02% pharmalyte 3-10NL (GE Healthcare)). Lysates were removed, sonicated 30 s in 3" Cup-Horn (Maximum power: 100%, Qsonica, France) and finally supernatants were collected after a centrifugation (10 000 *g*; 20 °C; 20 min). Protein concentrations were measured according to the method of Bradford with bovine serum albumin as a standard.

2.3. 2D electrophoresis gels

Eight biological replicates were performed for each group for a total of 24 gels (8 CT, 8 CX and 8 FX). For isoelectrofocusing, proteins were separated along 3–10 non-linear pH gradient strips (GE Healthcare, France) in Multiphor apparatus (Amersham Pharmacia Biotech). A content of 750 μg of proteins were loaded on dry strips for overnight rehydratation. The electric parameters were as follow: 500 V for 0.01 h (gradient), 500 V for 5 h, 3500 V for 5 h (gradient) and 3500 V for 9.5 h. Strips were then incubated in an equilibration buffer (Tris buffer 0.5 M pH 6.8 with 6 M urea, 1% SDS, 26% glycerol) containing 30 mM dithiotreitol for 15 min and in an equilibration buffer containing 245 mM iodoacetamide for 5 min. For SDS-PAGE electrophoresis, 24 large gels were used (12% acrylamide, 20 cm × 20 cm × 1.5 cm) and ran in parallel in Protean plus Dodeca-Cells (Bio-Rad).

2.4. Gel analysis and statistic treatment

Gels were stained with blue colloidal, scanned using the GS-800™ Calibrated Densitometer (Bio-Rad) and exported from Quantity One as raw file (16-bits.tif). Images were analyzed using Delta 2D (Decodon, Gmbh). One gel exhibits migration impairment and was rejected prior to analysis. The 23 gels were fused in a unique master gel. Detected spots were subsequently transferred back to each of the 23 gels. For each spot, the normalized volume was expressed as percentages of the total volume of all spots of the gel. Normalized volumes were compared between control group and the two acclimation groups following a Student t-test. Spots with a p-value below 0.01 were considered as differentially expressed.

2.5. Protein identification by liquid chromatography and electrospray ionization MS/MS

Proteins of interest were excised manually from CBB-stained 2D gel and submitted to in-gel digest by trypsin (Promega France). After digestion, peptides were dried on speedvac, resuspended in 15 μL of 3% (v/v) acetonitrile and 0.1% (v/v) formic acid and then analyzed with a nano-LC1200 system coupled to a Q-TOF 6520 mass spectrometer equipped with a nanospray source and an HPLC-chip cube interface (Agilent Technologies). A 30-min linear gradient (3–80% acetonitrile in 0.1% formic acid), at a flow rate of 370 nL/min, was used to separate peptides on polaris-HR-Chip C18 column (150 mm long × 75 μm inner diameter). Full autoMS1 scans from 200 to 1700 *m/z* and autoMS2 from 59 to 1700 *m/z* were recorded. In every cycle, a maximum of 5 precursors sort by charge state (2 + preferred and single-charged ions excluded) were