Journal of Thermal Biology 74 (2018) 37–46

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Metabolomic analysis of heat-hardening in adult green-lipped mussel (*Perna canaliculus*): A key role for succinic acid and the GABA*ergic* synapse pathway

B.J. Dunphy[a,*], K. Ruggiero[b], L.N. Zamora[c], N.L.C. Ragg[c]

[a] School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[b] Department of Statistics, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[c] Cawthron Institute, Private Bag 2, Nelson 7042, New Zealand

ARTICLE INFO

Keywords:
Greenshell™ mussel
Acute thermal challenge
Thermal tolerance
Anaerobic metabolism

ABSTRACT

We evaluated the thermotolerance (LT_{50}) of adult green-lipped mussels (*Perna canaliculus*) following an acute thermal challenge in the summer of 2012 and the winter of 2013. Mussels were grouped into two treatments, naïve (N, no prior heat treatment) and heat-hardened (HH = 1 h at 29 °C, 12 h recovery at ambient) before being immersed for 3 h in water of varying temperature, i.e. Ambient (Control), 25, 29, 31, 33, and 35 °C with subsequent mortality monitored for 30 days. As expected, naïve mussels were less thermotolerant than heat-hardened i.e. Summer LT_{50}, N = 31.9, HH = 33.5 °C; Winter LT_{50}, N = 31.4, HH = 33.8 °C. Moreover, at 33 °C no heat-hardened mussels died compared to 100% mortality in naïve specimens. At 35 °C all mussels died regardless of treatment.

For the 'Summer' mussels, metabolite abundances in gill tissues of both naïve and heat-hardened mussels were quantified. For mussels at 33 °C, succinic acid was significantly higher in naïve mussels than heat-hardened mussels, indicating perturbations to mitochondrial pathways in these thermally stressed mussels. Additionally, analysis of biochemical pathway activity suggested a loss of neural control i.e. significantly reduced GABA*ergic* synapse activity, in naïve vs. heat-hardened mussels at 33 °C. Taken together these findings suggest that heat-hardening improves mussel survival at higher temperatures by delaying the onset of cellular anaerobic metabolism, and by maintaining inhibition of neural pathways. Such results offer new perspectives on the complex suite of sub-cellular stress responses operating within thermally stressed organisms.

1. Introduction

In marine ectotherms, seawater temperature is an important environmental cue, capable of modifying essential biological processes such as metabolism, reproduction, growth, behaviour, immune response, and survival (Angilletta, 2009; Pörtner, 2002). Species usually dwell within a tolerance range of temperatures (or thermal windows) in which they are able to cope with changes in temperature whilst carrying out normal physiological processes (Pörtner and Farrell, 2008; Sokolova et al., 2012). However, beyond these thermal limits, survival is compromised depending on factors such as the magnitude, rate, and duration of any temperature change (Monaco and Helmuth, 2011; Pörtner and Farrell, 2008).

The thermal tolerance of a species can be increased for a short time by means of an acute exposure to a sub-lethal temperature i.e., heat-hardening (Bowler, 2005), a process known to botanists for decades (Feldman, 1968). Heat-hardening is a transient phenomenon that enhances survival to a higher temperature for a few hours to days (Hoffmann et al., 2003). Such situations can also occur in nature, with the increasing temperatures of consecutive spring low tides on the intertidal shore known to naturally "heat-harden" invertebrates in preparation for the peak temperature associated with extreme low tides (Hamdoun et al., 2003; Pasparakis et al., 2016).

Although heat-hardening can induce the production of protective heat shock proteins (Hsp's), the associated increase in thermal tolerance may not be solely attributed to their expression. This is because the stress response is a complex process involving adjustments to behaviour, tissue and organ function in addition to metabolite fluxes (Bilyk et al., 2012; Bowler, 2005; Hoffmann et al., 2003; Jensen et al., 2010). This is perhaps best illustrated in stenothermal Antarctic fish who demonstrate a heat-hardening response (Bilyk et al., 2012) yet lack inducible expression of heat shock proteins (Hofmann et al., 2005). For many marine ectotherms, studies describing heat-hardening have focussed either on whole organism responses (e.g. mortality, cardiac function), or Hsp70 expression, with changes in metabolite fluxes (metabolomics) largely overlooked despite their importance in

* Corresponding author.
E-mail address: b.dunphy@auckland.ac.nz (B.J. Dunphy).

https://doi.org/10.1016/j.jtherbio.2018.03.006
Received 14 November 2017; Received in revised form 20 February 2018; Accepted 9 March 2018
Available online 13 March 2018

describing the thermotolerance of freshwater aquatic insects (Verberk et al., 2013).

Metabolomics approaches have their advantages as simultaneous identification and quantification of multiple small molecular weight molecules are achieved. This provides biomarkers which are intrinsically linked with the physiology of the animal, allowing a more in-depth understanding of how the organisms respond to external changes in their living environment (Viant, 2007, 2008). Metabolomic analysis of heat-hardened *Drosophila* showed significant changes in metabolite profiles, with shifts in acetate profiles indicative of heat-hardened individuals (Malmendal et al., 2006). Whilst metabolomics has been used to describe toxicological effects (Kwon et al., 2012), larval quality (Young et al., 2015) and heat stress responses (Dunphy et al., 2015) in mussels, no metabolomics analyses describing heat-hardening in mussels (nor any marine invertebrate for that matter) could be found.

Lastly, metabolomics datasets also allow secondary analyses of metabolite set and pathway enrichment using various algorithms, e.g. Pathway Activity Profiling (PAP*i*), Metabolites Biological Role (MBRole) and Metabolites Pathway Enrichment Analysis (MPEA), which can then map changes in the activity of biochemical pathways in response to experimental perturbations (Booth et al., 2013). These algorithms provide an integrated snapshot of organismal response at the next level of biological organisation i.e. pathway as opposed to metabolite; however many are tailored to specific model organisms, making selection of an appropriate algorithm crucial (Aggio et al., 2010).

Thus, we provide the first investigation of heat-hardening in green-lipped mussels (*Perna canaliculus* Gmelin, 1791), a species of distinct ecological and economic importance in New Zealand (Gui et al., 2016). We set out to determine whether temporal changes in heat-hardening responses were evident in this species and to what extent. Moreover, comparison of metabolomics profiles between naïve and heat-hardened mussels was undertaken in order to identify candidate biomarkers and biochemical pathways involved in the heat-hardening process.

2. Materials and methods

2.1. Origin of adult mussels

Green-lipped mussels (*P. canaliculus*) used in this study were part of an ongoing selective breeding program and are maintained on culture longlines within Kauauroa Bay, Marlborough Sounds, New Zealand. During the summer (December) 2012 (SST = 18–20 °C for approx. two months), and winter (June) 2013 (SST = 11–12 °C again for 3 months), a subset of mussels were brought back to the Cawthron Institute's Glenhaven aquaculture park and acclimated for one month in a flow-through system under ambient light conditions with water supplied by outdoor bloom ponds (8.1–8.3 pH, 1.0 ± 0.5 μg L^{-1} Chl-*a*) allowing ad libitum feeding of the mussels. Each cohort of mussels (summer 2012, winter 2013) underwent one month of acclimation to the laboratory conditions, after which 240 adult mussels were washed, tagged and their live mass and shell length were recorded (Table 1). Visual inspection of tissues during shucking for metabolomics sampling showed that all mussels were reproductively active with gonadal tissue present.

Table 1
Biometrics (mean ± S.D.) of naïve and heat-hardened adult *P. canaliculus* used in acute thermal challenge trials.

	Status	Shell Length (mm ± SD)	Whole Weight (g ± SD)
Summer 2012	Naïve	75.1 ± 8.7	35.17 ± 10.1
	Heat-hardened	76.1 ± 9.3	36.8 ± 11.5
Winter 2013	Naïve	90.8 ± 14.1	45.9 ± 16.2
	Heat-hardened	98.5 ± 9.9	53.2 ± 17.5

2.2. Heat-hardening and acute thermal challenge

For both the winter and summer periods, half of the 240 mussels were allocated to the Heat-hardening treatment, while the remaining 120 mussels were allocated to the Naïve treatment. Mussels were allocated at random to mesh bags (n = 10 per bag, with two bags per treatment) and assigned to a pre-exposure treatment i.e. naïve or heat-hardened. Heat-hardened mussels were prepared by dipping in 29 °C water for 1 h, allowed to recover in acclimation tanks for 12 h (to replicate natural tidal cycle) and then exposed to an acute thermal challenge to estimate their thermal limits. While the naïve mussels, were taken immediately to the acute thermal challenge without prior exposure to high seawater temperatures.

For the acute thermal challenge in both summer and winter methods used followed those reported in (Dunphy et al., 2015), namely six 200 L tanks were prepared, each of which was held at a set temperature treatment (i.e. Ambient control, 25, 29, 31, 33 and 35 °C) with static water flow and air stones to maintain aeration and prevent thermal stratification (see Table 2 for actual temperature values). A subsample (i.e., 20 mussels per tank for each treatment) of either the heat-hardened and naïve mussels were immersed directly in each of the tanks for three hours (i.e., acute thermal challenge) and then allowed to recover in ambient conditions for 30 days. During the recovery time, mortality was monitored daily for 30 days after cessation of acute thermal challenge. Mussels were considered dead when gaping individuals were unable to adduct valves following 10 rapid squeezes i.e. the British Standard Squeeze method (Dunphy et al., 2015).

2.3. Gill sample collection for metabolomics analysis

To evaluate metabolite profiles and metabolic pathway differences between naïve and heat-hardened mussels, gill samples were harvested during the Summer 2012 trial for later analysis. Immediately following the 3 h acute temperature challenge at the different temperatures treatments, five mussels from each temperature treatment were shucked and had their gill tissues rapidly dissected and snap frozen in liquid nitrogen, before being stored at − 80 °C until metabolite profiles could be ascertained using GC-MS analysis.

2.4. Metabolites identification and quantification

Identification and quantification of metabolites in the gill samples followed the methods described in (Dunphy et al., 2015). Metabolites were extracted from frozen gill tissue by first grinding in liquid nitrogen, using a mortar and pestle. Approximately 100 mg of the ground tissue was then transferred into a 50 mL centrifuge tube containing ice cold MeOH:H_2O solution (2.5 mL, 50% [vol/vol], − 30 °C). An internal standard, d4-alanine (20 μL), was added before each tube was vortex-mixed for 3 × one minute intervals, ensuring samples remained chilled via holding tubes in a − 30 °C ethanol bath between mixing cycles. Samples were centrifuged at − 20 °C for 15 min at 5000 rpm. The supernatant was collected and placed in a 50 mL polypropylene tube. Another 2.5 mL of the cold methanol solution was added to the remaining tissue pellet and mixed for 30 s using a vortex mixer before being centrifuged again at − 20 °C and 5000 rpm for another 15 min. The resulting supernatant was collected and added to the first supernatant in the 50 mL tube. Bidistilled water (8 mL at 4 °C) was added to the tube containing 5 mL of supernatant. Using a vortex mixer, three short (~one second) mixing cycles were applied to the sample before placing it in a freeze dryer for 24 h. Following this, samples were volatilised and a metabolite profile was obtained using non-target Gas Chromatography-Mass Spectrometry (GC-MS) analysis on a Thermo Agilent 7890B GC coupled to a 5977AC inert mass spectrometer with a split/splitless inlet. This is a complementary analytical method for metabolomics, as it allows for the determination and quantification of as many identified or unidentified metabolite compounds as possible