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

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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.

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

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 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.

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 x 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 5,000 rpm for another 15 minutes. 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 (Roessner and Bowne, 2009). Data analysis was semi-automated. The raw data output from the GC-MS was converted to AIA format (.cdf) and analysed using Automated Mass Spectral Deconvolution and Identification Software (AMDIS) against an in-house library of 165 MCF derivatised compounds. Compounds that are not included in this library were not identified. The settings chosen for AMDIS result in more false positive identifications, in order to avoid exclusion of valid data. As the output from AMDIS returns zero values that are not suitable for statistical analysis, an R-script was used to produce data that includes trace levels of metabolites normally excluded by AMDIS. The values were generated from the maximum height of the reference ion for the compound peak. The reference ion used as a measure of abundance for each compound is generally the most abundant fragment. Unlike peak area, peak height may be affected by chromatographic disturbances such as column contamination. Early eluting peaks may sometimes be under-represented as a result. Data were checked against negative controls, and where they obviously represented contamination or artefacts they were highlighted and removed from the corrected results. Values for negative controls were not subtracted from data. Coeluting peaks were highlighted, checked and identified. Where two identifications were equally likely for one peak, both identifications have been reported. Data was normalised to the internal standard alanine-d4. The normalised values are not concentrations, but are ratios of the GC-MS response of compound peaks relative to the internal standard alanine-d4, then standardised to biomass (mg) of the original gill sample extracted.

The metabolite data was also used for the determination of biochemical pathways activity.

2.5. Statistical analyses

Methods to estimate the acute lethal temperature at which 50% (LT_{50}) and 99% (LT_{99}) of the naïve and heat-hardened mussels died followed the methods of Dunphy et al. (2013). Namely, a logistic regression (logit link) was fitted to the number of live and dead mussels at each temperature treatment used in the thermal challenge, and measures of LT_{50} or LT_{99} estimated using the inverse prediction function (based on maximum likelihood estimates) in JMP (version 10.0, SAS Institute, Carolina, USA). Significant differences among LT_{50}

estimates were identified using 95% confidence intervals. An interaction term was not included in the logistic binomial models as it resulted in unstable results in the inverse prediction function (presumably arising from the very abrupt transition between live and dead mussels at 33 -35 °C).

Two-way ANOVAs, using Type II sums of squares for the unbalanced data sets, were carried out on the log-transformed metabolite intensities and PAP*i* activity scores to assess the main effects of the *Pre-exposure* and *Temperature* treatments used in the thermal challenge, including the interaction between these two factors. Holm's adjusted p-values were calculated for the interaction *F*-tests, controlling the familywise error rate at 5% (i.e. controlling the probability of at least one false discovery among all *F*-tests for the entire set of metabolites at 5%). For those metabolites for which the interaction effect was found to be statistically significant, pairwise comparisons of means were conducted using two-sample *t*-tests to identify specific differences between pairs of means. Holm's method was also used to control the familywise error rate across *both* main effects, for the entire set of metabolites, at 5%. Consequently, only those main effect *F*-statistics with a Holm-adjusted *p*-value less than 0.025 were declared statistically significant. Pairwise comparisons between *Pre-exposure* treatment and/or *Temperature* group means were conducted only if their interaction effect was not found to be statistically significant in the ANOVA for the nominal metabolite but their main effects were. Tables of mean log-intensities, their pairwise differences (i.e. log-ratios) and standard errors of differences, plus their corresponding relative abundances (i.e. back-transformed log-ratios) and 95% confidence intervals were computed.

Comparisons of biochemical pathway activity among naïve and heat-hardened mussels exposed to the different temperatures used in the thermal challenge were achieved via secondary analysis of metabolites using the pathway activity profiling (PAP*i*) algorithm (Aggio et al., 2010). This program connects to the Kyoto Encyclopaedia of Genes and Genome (KEGG) online database (http://www.kegg.com) and uses the relative abundances of metabolites detected within each pathway to predict the activity of each metabolic pathway within individual mussels. The entire data mining, data normalisation and pathway activity predictions were performed using the PAPi R package (Aggio, 2013).

3. Results

3.1. Mortality of mussels after acute thermal challenge

Mortality of adult *P. canaliculus* following an acute thermal challenge in summer 2012 and winter 2013 is shown in Fig 1. Immersion in 35 °C water resulted in 100% mortality of mussels by the end of the acute 3 h acute challenge regardless of season, or pre-exposure treatment. The onset of mortality was rapid and usually going from 0 to 100% mortality over the space of a 2 °C increase in water temperature e.g. between 33 and 35 °C. Importantly, for both sampling periods naïve mussels experiencing 33 °C were all dead within 2-4 days after the trial ended; whereas heat-hardened mussels showed little (Winter 2013 mussels) to no (Summer 2012) mortality at this temperature. Additionally, during winter 2013 naïve mussels in the 29 and 31 °C treatments also showed a small level of heat stress mortality up to a week post trial. Modelling the mortality data with a logistic regression revealed significant seasonal and pre-exposure treatment differences (see Supplementary file S1 for details). Seasonal differences in LT_{50} values were significant for the naïve mussels, but not for the heat-hardened individuals (Table 3). Heat-hardening of adult *P. canaliculus* pushed forward their thermotolerance, with a 1.6 and 2.4 °C increase in LT_{50} compared to naïve mussels in winter and summer respectively (Table 3).

3.2. Metabolites profile of Summer 2012 samples

A total of 50 metabolites were identified in the gills of naïve and heat-hardened mussels exposed to an acute thermal challenge at different temperatures (Table 4). A good representation of amino, fatty and various other organic acids were achieved across the metabolites identified. None of these metabolites revealed significant differences in abundances between heat-hardened and naïve mussels (see Supplementary Table S2) For both naïve and heat-hardened mussels, the temperature of the acute thermal challenge had a strong effect on metabolite relative abundance, with 35 metabolites showing differential abundance with increasing temperature (Table 4 and Supplementary Table S3). Nonetheless, a significant interaction was observed between pre-exposure treatment and temperature of the acute thermal challenge (two-way ANOVA, $F_{5, 44} = 8.59$, $P < 0.0005$). This interaction was

attributed to Succinic acid, which was significantly higher in naïve mussels experiencing 33 °C (Fig 2).

3.3. Differences in metabolic pathway activities

Analysis of outputs from the PAP*i* secondary data enrichment algorithm determined activity scores of 78 pathways (see Supplementary Table S4). Pre-exposure treatment had no significant effect on pathway activity. However, 71 pathways had significant differential activity among temperatures within the acute thermal challenge; and there was a significant interaction effect between pre-exposure treatment and the temperature used in thermal challenge on the activity of eight pathways (Table 5). This interaction effect related to mussels experiencing 33 °C where pathways in the heat-hardened mussels showed a less abrupt decline in activity compared to naïve mussels (Figure 3 and 4). Of these eight pathways, the most significant interaction term was for the neural inhibitory GABA*ergic* synapse pathway (Table 5).

4. Discussion

We provide the first reports of seasonal, and heat-hardening, effects on the thermotolerance of adult *P. canaliculus*. Our aims were to determine whether any temporal changes in heat-hardening responses were evident in this species, in addition to identifying biomarkers of heat-hardening via GC-MS and metabolomics analyses.

4.1. Seasonal variation in thermal tolerance of naïve individuals

Although small, an increase in LT_{50} of 0.5 °C was observed from winter to summer in naïve individuals of *P. canaliculus* in our study. This modest increase broadly agrees with previous records for this species, where Kennedy (1976) found an approximately 1 °C increase in LT_{50} between summer and winter acclimated mussels. However, results from both Kennedy (1976) and our study contrast with published values for other bivalves, where summertime thermotolerance is typically much greater. For example, work by Hamdoun et al. (2003) on the thermotolerance of the Pacific oyster (*Crassostrea gigas*) recorded LT_{50} values 2-3 °C higher in summer than those recorded during winter. Position on shore may explain this

290 discrepancy, as subtidal invertebrates can be less sensitive than eurythermal intertidal
291 species to seasonal drivers of thermotolerance (Hopkin et al., 2006). Thus it may be that a
292 predominantly subtidal to low-shore species such as *P. canaliculus* is less likely to show
293 dramatic shifts in thermotolerance among seasons.

294 Differences in mussel size can influence survival in thermotolerance experiments (Schneider,
295 2008). Whilst all mussels used were sexually mature, an increase in somatic growth occurred
296 in the intervening months between sampling events. This may have influenced our
297 estimates of *P. canaliculus* thermotolerance, thus caution is advised when interpreting these
298 measures.

299 *4.2. Heat-Hardening Effect*

300 When compared to naïve mussels, heat-hardening *P. canaliculus* (i.e., 29 °C for 1 hour)
301 increased LT_{50} by 2-3 °C independently of season. Furthermore, in terms of raw mortality
302 data, this difference was best evidenced at 33 °C where a much greater survival of heat-
303 hardened individuals occurred compared to naïve. Positive effects of heat-hardening on
304 thermotolerance have been reported for other mussel species e.g. *P. viridis* (Aleng et al.,
305 2015) and *M. edulis* (Huppert and Laudien, 1980), with both species able to tolerate normally
306 detrimental temperatures for much longer. In the case of *P. viridis,* heat-hardening allowed
307 up to 50% of mussels to survive 44 °C a temperature that was otherwise lethal for naïve
308 conspecifics.

309 Interestingly, such a scenario was not evident for *P. canaliculus* as regardless of pre-exposure
310 treatment, all mussels died when exposed to 35 °C. Furthermore, onset of mortality of all
311 mussels was rapid over a small response range i.e. going from 0% to 100% mortality with
312 just a 3 °C increase in temperature. Thus, whilst heat-hardening can shift the LT_{50} of *P.*
313 *canaliculus* and push upper thermal limits, there appears to be a ceiling to this protection
314 with 35 °C representing a temperature for which immersed individuals of this species
315 cannot survive. Mortality itself consists of multiple interacting variables, what is need now
316 are high resolution time series analyses to test how consistent 35 °C is as an upper thermal
317 limit for this species.

318 In order to accurately map temperature effects (particularly for the metabolomics analyses),
319 we elected to use immersed mussels to reduce body temperature variations due to

evaporative cooling that may occur in emersed mussels. Whether such immersed upper thermal limits accurately reflect the emersed condition needs further investigation. This is especially pertinent in light of work by Petes et al. (2007) where significant mortality of *P. canaliculus* was recorded in intertidal beds when temperatures briefly reached 36 °C.

4.3. Metabolites profile of Summer 2012 samples

The tolerance benefits conferred from heat-hardening have often (but not always) been linked to the production of heat shock protein (Hsp) chaperones (Feder and Hofmann, 1999). Our metabolomics data provides a complementary approach to Hsp analyses and suggests succinic acid (the anion of which is succinate) is indicative of the heat-hardening process. Located within the mitochondria, succinic acid is a tricarboxylic acid that has an important role in energy production via the Krebs/TCA cycle. Metabolomic studies typically interpret increased abundances of a metabolite as indicative of a perturbed biochemical pathway e.g. faulty succinate dehydrogenase activity (Aggio et al., 2010). Complicating this interpretation is that succinate will accumulate in mussels under anaerobic conditions as the mitochondrial pathways work in reverse i.e. succinate is catalysed from fumarate via fumarate reductase (Zandee et al., 1985). In fact, anaerobiosis and thermotolerance are linked with lower thermotolerance capacity being linked to internal hypoxia within heat stressed animals i.e. oxygen and capacity limitation of thermal tolerance (OCLTT) (Pörtner, 2012). For *Mytilus edulis*, thermally induced mortality was related to a sharp increase in mantle succinate indicating insufficient oxygen reaching mitochondria in these thermally stressed mussels (Zittier et al., 2015).

Metabolomic analyses of heat-stressed stonefly (*Dinocras cephalotes*) nymphs (Verberk et al., 2013) and naïve *P. canaliculus* adults (Dunphy et al., 2015) revealed greater levels of organic acids (succinic, lactic, fumaric acid) and amino acids (proline, alanine, asparagine) associated with anaerobic metabolism as temperature stresses increased. For many taxa, the accumulation of succinate is indicative of severe pathological conditions i.e. ischaemia related to stroke and cardiac infarction in humans (Chouchani et al., 2014), thus whether the delay in succinic acid upregulation in heat-hardened mussels is due to more robust pathways (as metabolomics might suggest) or a delayed switch to anaerobiosis needs to be uncovered. This could take the form of thermal tolerance trials undertaken in hypoxic and

hyperoxic water conditions combined with measurements of ventilatory and cardiac performance as per the methods of Pörtner et al. (2006).

4.4 Differences in metabolic pathway activities

Secondary enrichment analysis of metabolite abundance data revealed a further set of thermal stress biomarkers in *P. canaliculus*. As might be expected, acute temperature shock had a significantly negative effect on pathway activity. Perhaps of greater interest to our study are the eight pathways that showed differential expression among naïve and heat-hardened mussels experiencing 33 °C. Although all mussels showed reduced activity scores in these pathways, the deleterious effects of thermal stress was not as pronounced in heat-hardened individuals with carbohydrate (Butanoate and Citrate cycle), amino acid (alanine, aspartate, glutamate, phenylalanine and tyrosine) and energy (Oxidative phosphorylation) metabolism being significantly more perturbed in naïve mussels.

Given that succinic acid abundance distinguished naïve and heat-hardened mussels it is unsurprising that the citrate acid cycle pathway is indicative of thermal stress effects among our mussels. However, the GABA*ergic* synapse pathway appears particularly important in distinguishing heat-hardened mussels from naïve. This pathway is reliant on GABA, which is typically an inhibitory neurotransmitter but can have an excitatory role in mollusc nervous systems. Nonetheless, bolstering the activity of the GABA*ergic* synapse pathway during thermal stress indicates that retention of neural function is correlated with the heat-hardening process. Similar results were found in preparations of rat brain, where heat-hardened brain slices modulated GABA*ergic* related synaptic transmission when experiencing experimental heat stress (Kelty et al., 2002). Thus our findings demonstrate the role the central nervous system has in enduring thermal stress in a more basal animal taxon.

4.5 Conclusions

Taken together, our metabolites and mortality data correlate well. A schematic summary of our findings is provided in Figure 5 which shows that heat-hardened mussels experiencing 33 °C exhibit greater survivorship than naïve mussels and this was associated with no elevation of a key a metabolite, succinic acid. Moreover, preservation of neural function (among others) appears key at the biochemical pathway level, thereby enabling homeostasis

to be maintained under otherwise lethal thermal conditions. Such mechanisms indicate the way in which heat-hardened *P. canaliculus* appear to buy more 'thermal headroom'. But it is apparent such measures can only operate over a narrow temperature range, as once temperatures reach 35 °C such defensive mechanisms are rapidly surmounted and mortality is assured. Whilst the onset of anaerobic metabolism is correlated with reduced thermotolerance, further work is necessary to interpret whether the delayed increase of succinic acid in heat-hardened mussels represents an increase in oxygen delivery or an adjustment for more thermally stable enzymes (or both).

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Appendix A. Supplementary material. Data associated with this article can be found in the online version.

Vitae

Dr Brendon Dunphy (BSc, MSc hons, PhD): Brendon is a lecturer at the University of Auckland where he researches the ecophysiology of a diverse range of marine taxa. He is particularly interested in mechanistic insights into the resilience of marine animals and studies a diverse range of invertebrate and vertebrate species.

Dr Katya Ruggiero (PhD) is a statistician at the University of Auckland whose research lies at the interface of statistics and biology. Her works spans the statistical design and analysis of experiments using high-throughput biological data collection platforms, principally those involving current trancriptomic, proteomic and metabolomic technologies, bioinformatics, developing algorithms for the improvement of GC-MS data quality, through to introducing cutting-edge multivariate statistical methods to studying whale morphology. She has made significant methodological contributions to these fields."

Dr Norman Ragg (BSc hons, MSc, PhD) is a senior research scientist at the Cawthron Institute, where he specialises in the characterisation of stress and physiological performance in commercially important mollusc species. Norman's current research priorities focus on husbandry and breeding strategies to build resilience to a changing ocean.

Dr Leonardo N. Zamora (BSc, PhD) is a researcher at the Cawthron Institute, interested on the understanding of the biology and physiology of marine invertebrates, particularly commercially valuable mollusc and echinoderm species

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List of tables

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

Table 2. Nominal and actual temperature levels at which adult naïve and heat-hardened mussels **(***Perna canaliculus***)** were exposed to an acute thermal challenge for 3 h.

Table 3. Mortality estimates of LT_{50} and LT_{99} of naïve and heat-hardened *P. canaliculus* adults after a 3 h acute thermal challenge. Similar superscripts denote nonsignificant relationships within a mortality estimate i.e. where confidence intervals do not overlap.

Table 4: Metabolites extracted from mussel gill tissues for both naïve and heat-hardened *P. canaliculus* after a 3 h acute thermal challenge during Summer 2012. Metabolites were determined via GC-MS analyses. Note: metabolites in *italics* exhibited differential expression compared to ambient (i.e., controls) with arrows indicating either an increase (↑) or decrease (↓) in abundance of that metabolite.

Table 5: ANOVA table comparing output from PAP*i* secondary enrichment analyses on biochemical pathway activity of thermally stressed green-lipped mussels (*P. canaliculus*) that were either heat-hardened or naïve before treatment.

	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

	Experimental target temperature for thermal challenge					
	Ambient (Control)	**25 °C**	**29 °C**	**31 °C**	**33 °C**	**35 °C**
Actual temperature Summer 2012(mean °C ± S.D.)	19.4 ± 0.2	25.1 ± 0.2	28.5 ± 1.6	31.3 ± 1.2	32.6 ± 1.7	35.1 ± 1.7
Actual temperature Winter 2013(mean °C ± S.D.)	12.8 ± 0.5	27 ± 0.6	30.4 ± 0.16	31.0 ± 0.4	33.1 ± 0.1	35.6 ± 0.2

	Status	LT_{50} (± 95% C.I.)	LT_{99} (± 95% C.I.)
Summer 2012	Naïve	31.9 ± 0.1[a]	33.8 ± 0.4[a]
	Heat-hardened	33.5 ± 0.4[b]	35.44 ± 0.4[b]
Winter 2013	Naïve	31.4 ± 0.2[c]	33.3 ± 0.4[a]
	Heat-hardened	33.8 ± 0.4[b]	35.7 ± 0.4[b]

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Amino Acids	**Amino Acid Derivatives**	**Fatty Acids**
Alanine ↑	*Glutathione* ↑	13,16-Docosadienoic acid
Asparagine ↑	*S-Adenosyl-L-methionine* ↑	*Arachidonic acid* ↓
Aspartic acid ↑	**Amino Acid Metabolic Intermediates**	cis-11,14-Eicosadienoic acid
	D-2-Aminoadipic acid ↑	
beta-Alanine ↓	L-2-Aminoadipic acid	*cis-11-Eicosenoic acid* ↑
Cysteine ↑	*Creatinine* ↑	*Docosahexaenoic acid* ↓
Glutamic acid ↑	**Organic Acids**	*Dodecanoic (Lauric) acid* ↑
Glycine ↑	Adipic acid	*gamma-Linolenic acid* ↑
Histidine	Benzoic acid	Heptadecanoic acid
Isoleucine ↑	cis-Aconitic acid	*Linoleic acid* ↑
Leucine ↑	*Citraconic acid* ↑	Myristic acid
L-Ornithine	Decanoic acid	Octadecanoic (Stearic) acid
Lysine	*Fumaric acid* ↑	*Oleic acid* ↑
Methionine ↑	Glutaric acid	Palmitelaidic acid
Phenylalanine ↑	*Itaconic acid* ↑	Palmitic acid
Proline ↑	*Lactic acid* ↑	*Palmitoleic acid* ↑
Serine ↑	*Malonic acid* ↑	Pentadecanoic acid
Threonine ↑	Nicotinic acid	**Secondary Metabolites**
Tyrosine ↑	Oxalic acid	p-Toluic acid
Valine ↑	Pyroglutamic acid	Quinic acid
Non-standard Amino Acids	*Succinic acid* ↑	**Alkaloids**
4-Amino-n-butyric acid (GABA) ↑		Benzothiazole

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KEGG Map pathway type	Pathway	p value of interaction (treatment x temp)
Amino acid metabolism	Alanine, aspartate and glutamate metabolism	0.01
	Phenylalanine metabolism	0.006
	Tyrosine metabolism	0.006
Chemical structure transformation maps	Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid	0.03
Carbohydrate metabolism	Butanoate metabolism	0.002
	Citrate cycle (TCA cycle)	0.006
Energy metabolism	Oxidative phosphorylation	0.006
Nervous system	GABAergic synapse	0.0003

Figure captions

Fig. 1. Cumulative mortality of heat-hardened (filled symbols) and naïve (white symbols) green-lipped mussels (*P. canaliculus)* following an acute thermal challenge (3 h immersion) in water baths containing seawater of different temperatures during the summer of 2012 and winter of 2013 and then 30 days recovery in ambient seawater. Note for heat-hardened mussels experiencing 35 °C, symbols are offset for ease of visibility.

Fig. 2. Expression of succinic acid in gill tissue of naïve and heat-hardened adult *P. canaliculus* that experienced a 3 h acute thermal challenge during the Summer of 2012 at different temperatures above ambient (20 °C). Succinic acid spectra were obtained via GC-MS and relative abundances determined using AMDIS software. Results of two-way ANOVA indicated by asterisks (** = $P<0.0005$).

Fig. 3. PAP*i* activity scores of amino acid (and derivatives) metabolism pathways determined in naïve and heat hardened mussels exposed to an acute thermal challenge for 3 h during the Summer of 2012 at different temperatures above ambient (20 °). Data are mean activity score (± S.E.) Note: activity scores for mussels experiencing 25, 29 and 31 °C not shown for clarity. Results of two-way ANOVA indicated by asterisks (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$) which show differences between treatments at 33 °C.

Fig. 4. PAP*i* activity scores of carbohydrate metabolism (Butanoate metabolism), energy metabolism (Citrate cycle, Oxidative phosphorylation), and Nervous signalling (GABAergic synapse) metabolic pathways determined in naïve and heat hardened mussels exposed to an acute thermal challenge for 3 h during the Summer of 2012 at different temperatures above ambient (20 °). Data are mean activity score (± S.E.) Note: activity scores for mussels experiencing 25, 29 and 31 °C not shown for clarity. Results of two-way ANOVA indicated by asterisks (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, ****$p<0.0005$) which show differences between treatments at 33 °C.

Fig. 5. Schematic depiction summarising our findings on the responses of naïve (blue line) and heat-hardened (red line) green-lipped mussel (*P. canaliculus*) when experiencing a hypothetical heat-stress event.

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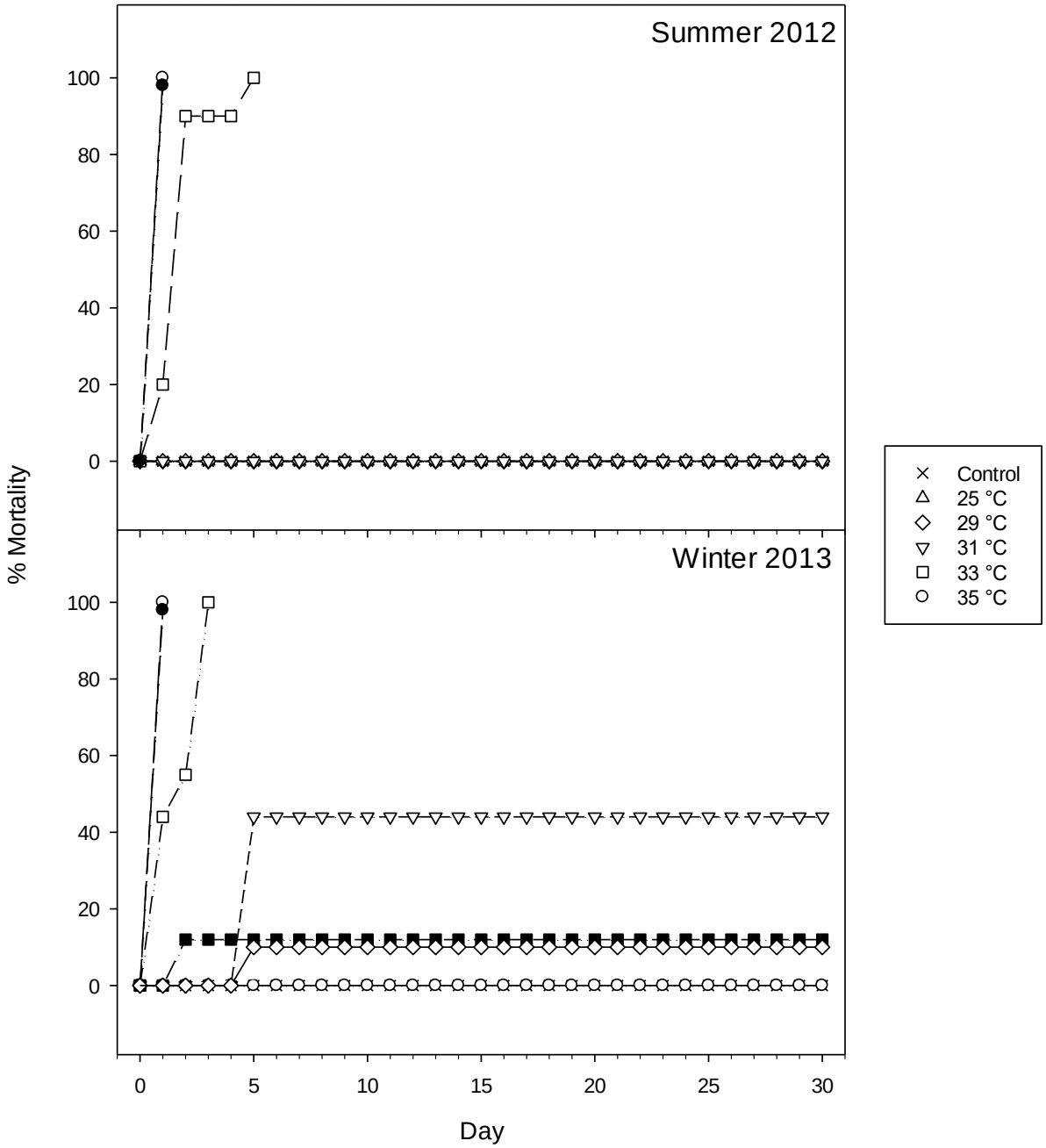

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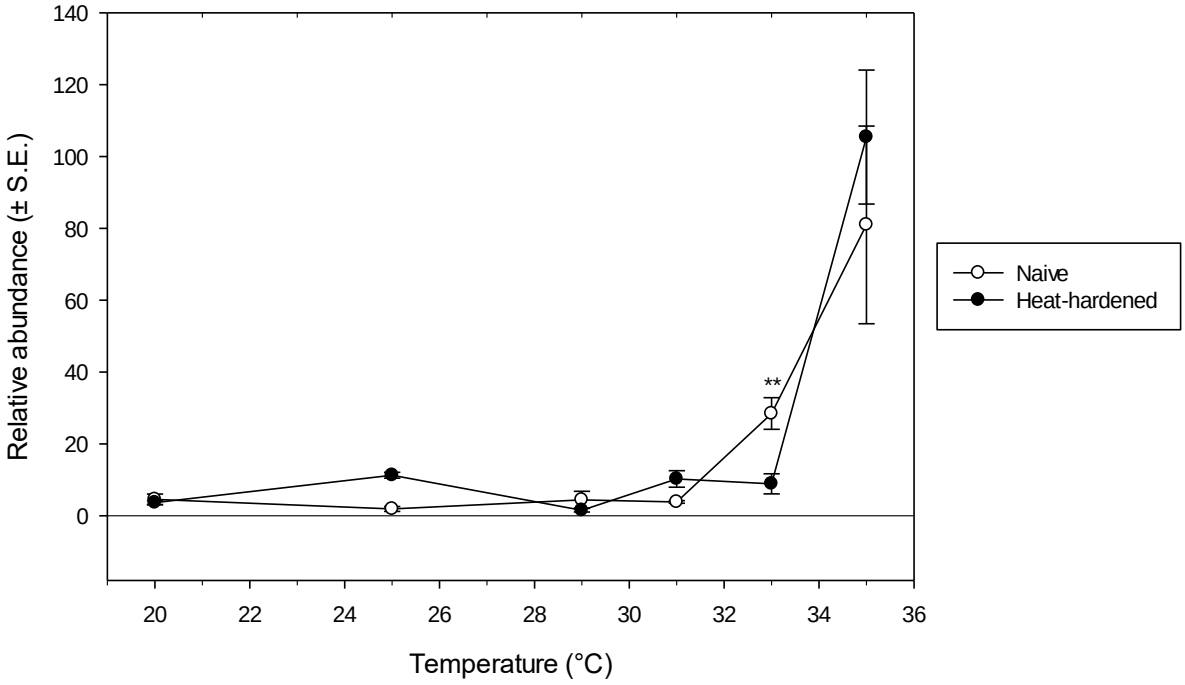
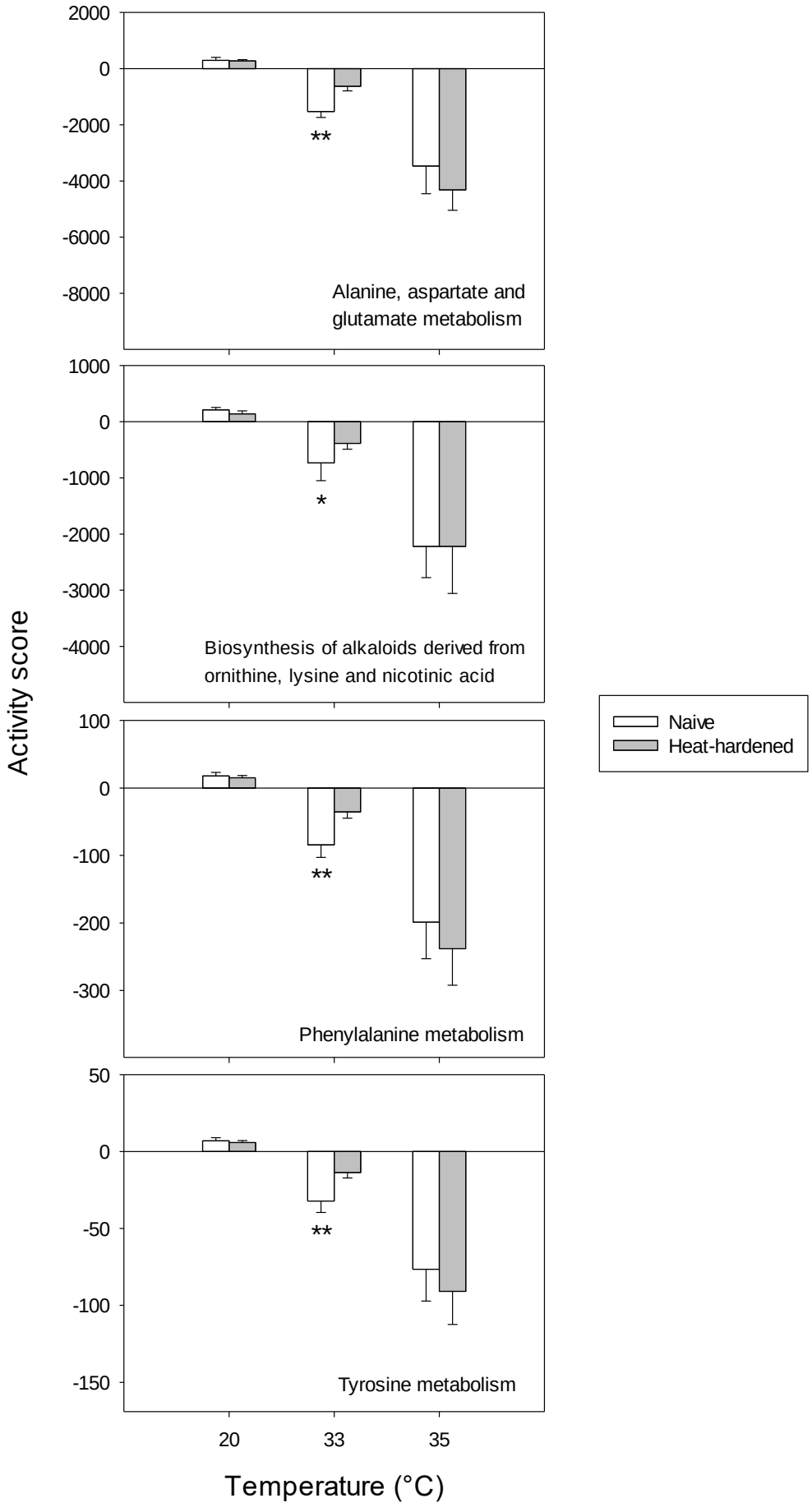

140
120
100
80
60
40
20
0
Relative abundance (± S.E.)
**
20
22
24
26
28
30
32
34
36
Temperature (°C)
Naive
Heat-hardened

608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-4000
-6000
-8000
**
Alanine, aspartate and glutamate metabolism
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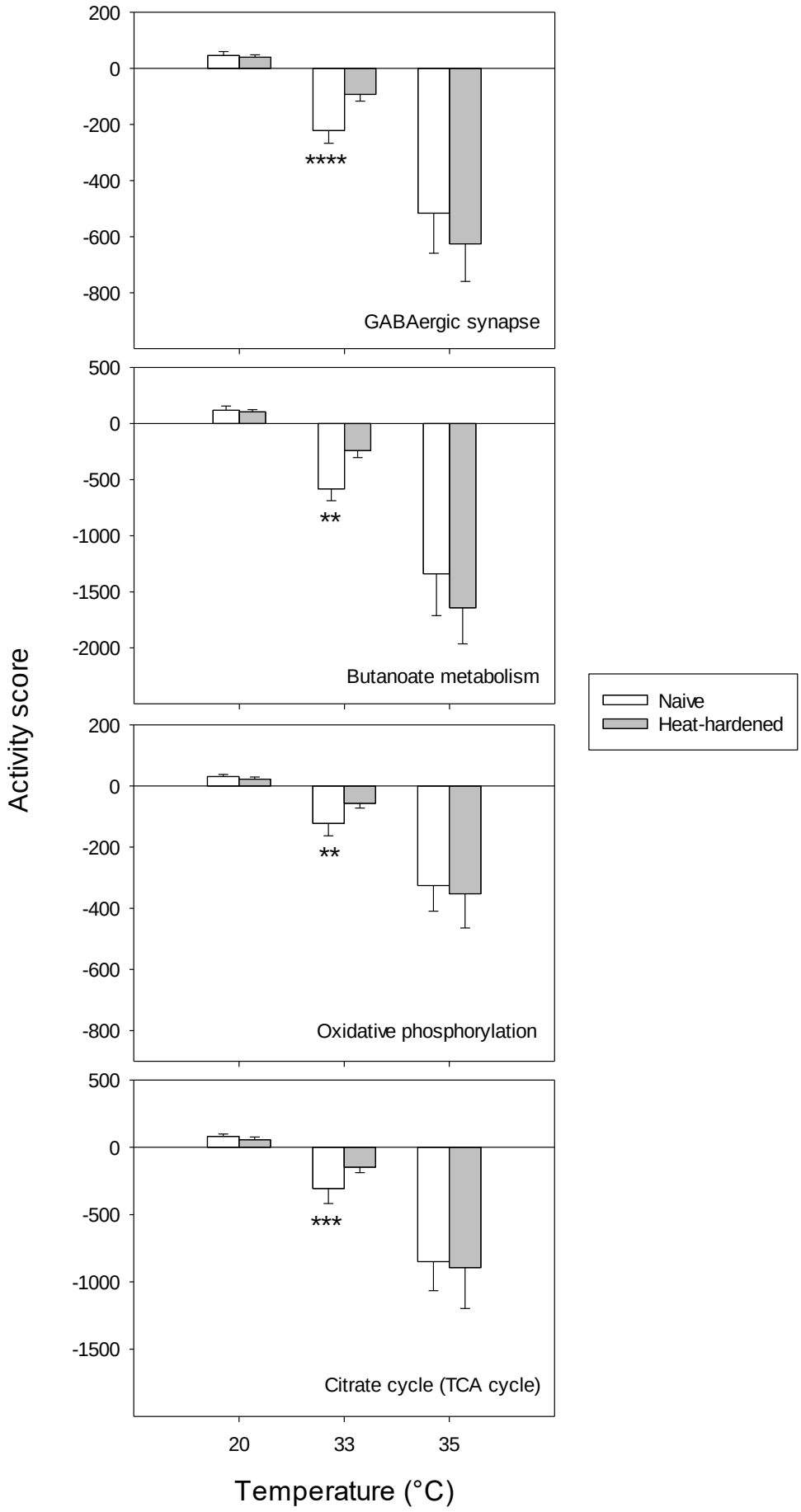
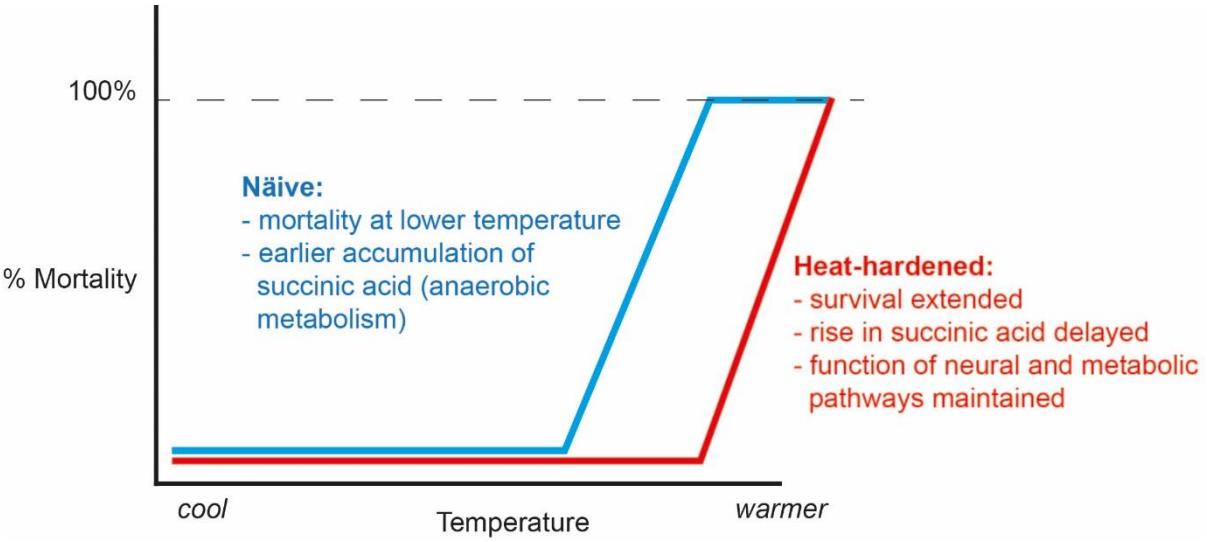
0
-1000
-2000
-3000
-4000
*
Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid
100
0
-100
-200
-300
**
Phenylalanine metabolism
50
0
-50
-100
-150
**
Tyrosine metabolism
Activity score
20
33
35
Temperature (°C)
Naive
Heat-hardened

609


200
0
-200
-400
-600
-800
****
GABAergic synapse
500
0
-500
-1000
-1500
-2000
**
Butanoate metabolism
200
0
-200
-400
-600
-800
**
Oxidative phosphorylation
500
0
-500
-1000
-1500
***
Citrate cycle (TCA cycle)
Activity score
Naive
Heat-hardened
20
33
35
Temperature (°C)

610

611


100%
% Mortality
Näive:
- mortality at lower temperature
- earlier accumulation of succinic acid (anaerobic metabolism)
Heat-hardened:
- survival extended
- rise in succinic acid delayed
- function of neural and metabolic pathways maintained
cool
Temperature
warmer

612