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# Establishing The Thermal Threshold Of The Tropical Mussel *Perna viridis* In The Face Of Global Warming

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**Abstract**

With increasing recognition that maximum oxygen demand is the unifying limit in tolerance, the first line of thermal sensitivity is, as a corollary, due to capacity limitations at a high level of organisational complexity before individual, molecular or membrane functions become disturbed. In this study the tropical mussel *Perna viridis* were subjected to temperature change of 0.4˚C per hour from ambient to 8~36˚C. By comparing thermal mortality against biochemical indices (hsp70, gluthathione), physiological indices (glycogen, FRAP, NRRT) and behavioural indices (clearance rate), a hierarchy of thermal tolerance was therein elucidated, ranging from systemic to cellular to molecular levels. Generally, while biochemical indices indicated a stress signal much earlier than the more integrated behavioural indices, failure of the latter (indicating a tolerance limit and transition to pejus state) occurred much earlier than the other indices tending towards thermal extremities at both ends of the thermal spectrum.

# Introduction

### Temperature and thermal tolerance

Species ranges are defined by a series of physical and biological limits within which survival, growth and reproduction can occur. Towards the edges of their distribution range, species perform sub-optimally and their ability to compete for resources is reduced (Gaston, 2009). The physiological mechanisms limiting and adjusting cold and heat tolerance has gained further interest in the light of global warming and associated shifts in the geographical distribution of ectothermic animals (Pörtner, 2002). Understanding how these factors interact to define species ranges is crucial to our understanding of the response and fate of marine ecosystems in the face of climate change. Recent studies have documented climate-related mortality events (e.g. Wilkinson, 1999; Hughes et al., 2003), changes in population abundances (e.g. Sagarin et al., 1999; Ottersen, 2001), shifts in species range boundaries (Root et al., 2003), and phenological shifts in the timing of reproductive and migratory events (Kingsolver et al., 2002). However, most of these studies have not involved organisms living in the tropics.

Climate change has three different outcomes depending on the physiological response of the species or population in question (Fields et al., 1993). First, if environmental changes are sufficiently small, organisms may acclimatize to those conditions. Second, if environmental conditions exceed the ability of some, but not all, of the individuals to adapt to the environmental change, then natural selection may favour some genotypes already present in the population. In this case, the species range may be unchanged, but allele frequencies may vary (Hilbish, 1985; Kirby et al., 1997). Third, if conditions are sufficiently severe, all organisms in the population will die or emigrate and the entire species range will shift (Holt, 1990). While acclimatization may imply improved organismal performance, such phenotypic plasticity may not necessarily improve fitness. For example, plasticity often involves the re-allocation of resources to one trait at the expense of another (e.g. trade-off between growth and reproduction). Furthermore, phenotypic variation can strongly influence biological

interactions within a community, often in complex and counterintuitive ways (Werner and Peacor, 2003).

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 organismic level and wider tolerance windows at lower levels of complexity. Recent studies have increased understanding of the mechanisms underlying tolerance with the first line of sensitivities becomes apparent at the highest functional level possible, typically in the integrated function of ventilation and circulation for metazoan populations (Pörtner, 2001). If failure of individual molecular mechanisms is involved whole animal limits and the limits of these individual molecular mechanisms should be identical. In consequence, sensitivity levels of molecules, organelles, cells, tissues and the intact organism need to be distinguished to demarcate the optimum, pejus (pejus = getting worse) and pessimum (switch to anaerobic respiration) ranges with respect to thermal stress, as adopted from the law of tolerance (Shelford, 1931, Frederick and Pörtner, 2000) to elucidate the thermal tolerance of a species. From an ecological perspective, pejus rather than critical conditions are likely to reflect the upper and lower tolerance limits determining species distribution. It is therefore proposed that a suite of behavioural, physiological, and biochemical indices be adopted to assess the thermal tolerance of a particular species.

**Biological indices**

A total of 7 assays were employed here to elucidate the thermal tolerance of the green lipped mussel *Perna viridis*. Mussels are particularly suitable biomonitoring organisms as they are filter-feeders, and *P. viridis* is tolerant to a wide range of salinities and temperatures (Chatterji et al., 1984; Morton, 1987) and spawns continuously throughout the year (Tham et al., 1973), enabling them to be used as biomointoring agents (Philips 1980; Tanabe 2000). By comparing thermal mortality against biochemical indices (heat shock protein 70 and gluthathione levels), physiological indices (glycogen level, Ferric Reducing Antioxidant Power test, Neutral Red Retention) and behavioural indices (clearance rate or

feeding efficacy), a hierarchy or sequence of thermal tolerance was therein elucidated, ranging from systemic to cellular to molecular levels.

The present work aimed to study the cellular, biochemical and molecular biomarkers in the foot muscle, mantle tissue and haemocytes of *Perna viridis* and determine whether there is a hierarchy in their response to thermal stress. In the context of global warming the use of biochemical, cellular, molecular and physiological biomarkers could help in understanding the hierarchy in the cellular damage and dysfunction during thermal stress and in determining the threshold of temperatures inducing cell dysfunction. Also, such data may contribute in the understanding of how 'environmental signals' (e.g. air, surface and water temperatures) might translate into signals at the scale of the organism or cell (Pörtner and Farrell, 2008; Helmuth, 2009; Helmuth et al., 2010; Hofmann and Todgham, 2010).

## Materials and methods

### Animal collection

The green lipped mussels were purchased from a local fish farm in Singapore and were kept in 28±0.5ºC, 20‰ artificial seawater for at least 24 hours before experimentation. The mussels (20 mussels per treatment temperature, shell length 7±1 cm) were subjected to a heating or cooling regime of 0.4ºC per hour from 28 ºC until the target temperatures of 8, 12, 16, 20, 24, 28, 32, and 36ºC were reached, and subsequently incubated at the respective target temperatures for 24 hours. A further 14 ºC and 38 ºC thermal regimes were examined for survivorship due to the sudden drop of 100% survival at 16 ºC to 15% survival at 12 ºC and to ascertain total mortality above 36 ºC (50% survival). At the end of the incubation period, mortality was assessed by gentling probing the mussel with a blunt seeker. Failure of the mussel to close its shell was scored as functional mortality, which was used to determine upper 50% lethal ($UTL_{50}$) and lower 50% lethal ($LTL_{50}$) limits. Of the surviving mussels, 5 were then individually transferred to glass beakers containing 500ml of 28±0.5ºC, 20‰ artificial seawater inoculated with algae feed (Instant Algae, Shellfish Diet 1800) for clearance rate assay while 5 were sacrificed for physiological and biochemical assays:

haemolymph was quickly drawn from each individual mussel which would then be dissected: 0.1g of wet weight tissue from the mantle was used for glutathione assay while 0.1g of wet weight tissue from the foot muscle was used for hsp70 and glycogen analysis separately. Dissected tissue were immediately kept in -80ºC freezer until the time of assay. Approximately 1ml of haemolymph was drawn from each mussel, which was then divided into two portions: 500µl for Neutral Red Retention assay (NRRT) and 500µl for Ferric Reducing Antioxidant Power assay (FRAP). These were then processed and analysed immediately.

**Clearance rate**

For each clearance rate setup, 1ml of microalga feed was added to 500ml artificial seawater in a beaker. Two 200µl aliquots were taken from the beaker, once immediately before and once 2 h after introducing the mussels. The microalga cells were then enumerated using a haemocytometer and the density of the cells calculated. Clearance rate per test mussel (CR, $Lh^{-1}$) was calculated with the formula below (Coughlan, 1969)

$$CR = \frac{60V[(\ln C_0 - \ln C_1)]}{nt1000}$$

where V is the volume of the water in the chamber (ml), $C_0$ the initial concentration of microalgae (cells $ml^{-1}$), $C_1$ the final concentration of microalgae (cells $ml^{-1}$), n the number of test organisms in each dish and t the time between $C_0$ and $C_1$ (=2 h). Five mussels were used for each target temperature ($\sum N = 30$) with one mussel in each clearance rate set up.

**Neutral Red Retention Time (NRRT)**

The NRRT assay was modified from that described by Lowe and Pipe (1994). A volume of 0.5ml of haemolymph was drawn from the posterior adductor muscle of each *Perna viridis* and added to 0.3ml of physiological saline solution (Hepes 0.5%, NaCl 2.5%, $MgSO_4$ 1.3%, KCl, 0.08%, $CaCl_2$ 0.15%, w/v, pH 7.4) in siliconised Eppendorf tubes (Sigma Aldrich, T3406). An aliquot of 40µl of the cell suspension was applied onto a poly-L-lysine (Sigma Aldrich, P8920) coated glass slide and incubated for 30 min in a light proof humidity chamber before neutral red working solution (2% w/v, PBS buffer) was added. The slides

were incubated for 15 min in the chamber, covered with a coverslip and systematically examined under the light microscope at 15 min intervals for the first hour, and 30 min intervals thereafter, at 600X magnification. The end point was reached when 50% of the cells in view had leaked dye from their lysosomes into the cytosol (Pipe, 1990).

**Ferric Reducing Antioxidant Power assay (FRAP)**

Total antioxidant status was determined by measuring the combined reducing power of the electron-donating antioxidants present (i.e. ferric reducing antioxidant potential) after Griffin and Bhagooli (2004). 500µl of mussel haemolymph was centrifuged at 1000rpm for 5 mins and 50µl supernatant extracted for analysis. A stoichiometric excess of the oxidant ferric tripyridyltriazine ($Fe^{III}$-TPTZ) was added to each 50 µl of haemolymph sample (200 µl of 10 mM in 300 mM sodium acetate, pH 3.6) and its reduction to the ferrous form ($Fe^{II}$) was measured spectrophotometrically after 30 min incubation (room temperature) at 593 nm.

**Glycogen analysis**

About 0.1g of foot muscle wet tissue was excised from each individual and dissolved in 400µl KOH (30%, w/v) at 100˚C. Absolute ethanol (1 ml) was added to each sample and cooled on ice for 2 hours before centrifuging at 4000 rpm for 10 minutes, supernatant discarded, and the residue reconstituted using 1 ml of distilled water. 1 ml of Anthrone reagent (0.05% Anthrone, w/v, Fluke 10740, into 3:7 deionised water: sulphuric acid) was added to 50µl of each sample and the absorbance was read off the spectrophotometer at 620 nm after 10mins of boiling at 100˚C.

**Heat Shock Protein analysis (Hsp70)**

A total of five independent samples were analysed for each target temperature. Between 0.1–0.2 g of foot muscle (wet weight) was excised from each individual animal and homogenized in a Tris-based lysis buffer for protein extraction. 30 µg of total protein from the protein sample was then separated by means of a 10% T: 2.6% C Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Hsp70 was analysed with a monoclonal anti-hsp70 antibody (Stressgen, SPA-810, USA). Intensity of the hsp70 bands was quantified

using the ECL™ detection system and hyperfilm (GE Healthcare, Amersham Hyperfilm, Singapore).

**Total Glutathione analysis (GSH)**

Total glutathione analysis was performed using the Glutathione detection kit (Enzo Life Science, ADI-900-160) and executed in accordance to the user manual. In gist, samples (0.1g wet weight of *Perna viridis* mantle tissue) were deproteinised by homogenizing in ice cold 5% (w/v) Metaphosphoric acid (20 ml/g tissue) and subsequently centrifuged at 12000 x g for 10 minutes at 4˚C to obtain the supernatant. For total glutathione (GSSG + GSH) determination, 150µl mixture of glutathione reductase (Enzo Life Science, Cat#80-1668) and reaction mix (Enzo Life Science, #80-1667) was added to 50µl of sample extract and absorbance was immediately read off at 405 nm using a microplate reader at 1 minute intervals over 10 minutes. To oxidized GSSG determination, 1µl of 2M 4-vinylpyridine was added to 50µl of sample and incubated at room temperature for 1 hr. Glutathione reductase and reaction mix were then similarly added to each sample as described above and absorbance read off at 1min interval for 10 minutes (405 nm). Total reduced glutathione (GSH) was then obtained by subtracting the amount of oxidized GSSG from total glutathione levels (GSSG + GSH).

The shell length of individual mussels used in each temperature trial were normally distributed and compared with one-way analysis of variance and posthoc SNK tests. The 95% confidence intervals for upper and lower temperatures when 50% mortality ($ULT_{50}$, $LLT_{50}$) occurred were estimated from sigmoid curves fitted separately to the upper and lower 6 data points (Sigmaplot 12, Systat software Inc. -3 parameter sigmoid fit). Curves were fitted separately to upper and lower limits as the sudden drop in survival at these limits was not well described by any of the curves that could fit across the full temperature range. Significant differences were accepted when 95% confidence intervals did not overlap.

For the bioassays (sans heat shock protein production), temperatures for upper 50% ($CT_{max}$) and lower 50% ($CT_{min}$) where index performance changed by 50% compared to that

of control animals at 28˚C were calculated by probit analysis. Individual bioassay responses were compared with one-way analysis of variance and posthoc SNK tests.

## Results

There was no significant difference in the shell length of mussels used in the different temperature trials ($F_{(96,7)}$=0.489, $p$ = 0.84). $ULT_{50}$ and $LLT_{50}$ was 35.6 – 36.2˚C and 13.3 – 13.9˚C respectively (Fig 1). Clearance rates of thermally stressed mussels were significantly lower compared to the clearance rate of control mussels kept at 28˚C ($F_{(30,5)}$=52.544, $p$ < 0.001, Fig 2A) where clearance rate of mussels kept at 28˚C were significantly higher than the other thermal treatments. A similar trend was observed in the NRRT of the mussels where dye retention time of control mussels were significantly longer than that of thermally stressed mussels ($F_{(30,5)}$=73.867, $p$ < 0.001, Fig 2A). Glycogen levels of mussels kept in 28˚C was also significantly higher than that of the other mussels ($F_{(30,5)}$=12.243, $p$ < 0.001, Fig 2B). Differences between the thermally stressed groups were however not that demarcated. FRAP levels of mussels kept in 28˚C was also significantly higher than that of the other mussels ($F_{(30,5)}$=37.074, $p$ < 0.001, Fig 2B). GSH levels also reflected this trend where control animals (28˚C) had a significantly higher GSH level compared to the thermally stressed animals ($F_{(30,5)}$=294.796, $p$ < 0.001, Fig 2B). Hsp70 was not significantly induced for animals kept in thermal regimes 12 to 28˚C, but a significant induction was observed at 32˚C which was observed to be attenuated again at 36˚C, although hsp70 levels at 36˚C was still significantly higher than that for mussels kept between 12 to 28˚C ($F_{(30,5)}$=22.417, $p$ < 0.001, Fig 3). $ULT_{50}$ and $LLT_{50}$ were significantly higher and lower than $CT_{max}$ and $CT_{min}$ of the indices respectively (Figure 4). No significant difference was observed for $CT_{min}$ for clearance rate, NRRT, glycogen, NRRT and glutathione, but $CT_{max}$ of clearance rate was significantly lower than that of the other biological indices. $CT_{max}$ of glutathione was in turn significantly lower than that of NRRT and FRAP.

## Discussion

The biological indices generally followed a thermal sensitivity (dysfunction) hierarchy of systemic to cellular to molecular levels. As observed from the $CT_{max}$ results, clearance rate had the lowest tolerance to heat stress (i.e. most sensitive in terms of capacity failure), followed by GSH and glycogen levels, whereas FRAP and NRRT had lower thermal sensitivities and were able to function at a more extreme temperature as compared to clearance rate. This follows the symmorphosis concept put forth by Pörtner (2002) where oxygen limitation is the unifying principle for tolerance (Taylor and Weibel, 1981): exposure to extreme heat or cold appears to involve elimination of aerobic scope and finally oxygen deficiency. At more extreme temperatures, only time limited passive survival is supported by anaerobic metabolism or the protection of molecular functions by heat shock proteins and anti-oxidative defence (Pörtner et al., 2005). As a corollary, the first line of thermal sensitivity is due to capacity limitations at a high level of organizational complexity, i.e. the integrated function of the oxygen delivery system, before individual, molecular or membrane functions become disturbed. Thus a sequence of sensitivities prevails within metazoan organisms, with the highest sensitivity at the organismic level and wider tolerance windows at lower levels of complexity. Interpretively, we would expect to see a disturbance or hiatus of higher levels of functional integration like animal behavior (foraging), reproduction or growth before central functions and co-ordinations (e.g. oxygen carrying circulation) would be disturbed, whereupon onset of thermal limitation at low and high pejus (=getting worse) thresholds occurs (Shelford, 1931), with progressively limited capacity of circulatory and ventilator mechanisms. Thereafter, organellar membranes and compartmental coordination would be affected before cellular membranes, molecular function and metabolic complexes become disturbed.

With this in mind, it thus follows that the clearance rate, which essentially measures the breathing efficiency of the mussel by way of measuring the volume of water drawn through its siphon, be affected at the lowest temperature of elevated heat stress. GSH, by

definition of measuring reserve reduced glutathione, would expectedly have a high GSSG to GSH ratio in stressed animals, hence the low observed $CT_{max}$. However, a low GSH level, while indicative of stress, does not necessarily translate to a diminished anti-oxidative power, since every GSSG and NADPH reaction releases two GSH molecules for free radical scavenging functions. As temperature rises further beyond pejus levels, individual tolerance becomes progressively time limited. Spontaneous activity is reduced as aerobic scope falls such that food uptake is finally prevented towards the critical temperature. Survival becomes a function of time, depending on individual resistance to starvation.

Oxidative stress will also increase when oxygen limitation set in at pejus. Oxygen radicals are primarily produced by mitochondria at between 1% and 3% of their rate of oxygen consumption (Sohal and Weindruch, 1996) naturally. Thus at higher temperatures, increase of metabolic rate will be coupled with an associated increase in production of oxygen radicals. This, in conjunction with hypoxia, results in excess production of oxygen radicals due to facilitated auto-oxidation of haem groups in mitochondria (Boveris, 1977). As such, free radical scavenging activities becomes increasingly crucial beyond pejus thermal threshold, and would understandingly, have a lower thermal sensitivity as compared to higher biological functions.

NRRT, being a measure of integrity of the organellar membrane of lysosomes, would therefore have a wider and higher tolerance to temperature, as would the anti-oxidative powers of FRAP in animal plasma. Apart from chaperone molecules that confer thermal tolerance, arguably this would be a last line of defence for cellular functionality. Heat shock protein production was not included in the $CT_{max}$ analysis for this protein is usually only induced rapidly against extreme thermal events (Tomanek and Somero, 1999 & 2000).

As observed in this study, hsp70 was only significantly induced at 32ºC, followed by attenuation at 36ºC. Considering that the test organisms *Perna viridis* will only experience these temperature ranges during very limited time periods (versus 24 hours incubation in this study), hsp70 is probably activated as a last resort “hardening” mechanisms for time limited stress events, e.g. hypoxia events (Feder and Hofmann, 1999 for review). Moreover, at more

extreme temperatures, hsp synthesis actually stops (see Tomanek and Somero, 1999). Thus while hsp70 would be a good candidate for assessing short term acute stress, inherently its indicative range might be already near the mortality limit of the test animal.

Glycogen serves as a secondary long term energy storage in animal cells and can be used as bioindicators of stress levels and energy reserve (Leung and Furness, 2001; Palais et al., 2011) representing the readily mobilisable storage form of glucose for most organisms. A glycogen reduction was observed here for heat stressed animals, and as a metabolic complex, the relatively higher tolerance and wider range of working temperature of this index still adheres to the principle of a hierarchical sensitivity of behavioural, physiological, cellular and molecular responses to stress (Pörtner, 2002).

Thermal stress is widely cited as the dominant physical stress in intertidal habitats (Garrity, 1984 and Helmuth and Hofmann, 2001) and is reported to cause mortality events on both temperate (Lewis and Bowman, 1975; Harley and Helmuth, 2001; Helmuth et al., 2006) and tropical shores (Williams and Morritt, 1995, Firth and Williams, 2009). In the study here, it is evidential that despite the hardiness of the *Perna viridis* (Morton 1987), it actually has a very narrow range of thermal optimal window. Despite a survival thermal range of 13.6 to 36.0ºC, feeding, and therein growth, is affected a mere 1.8ºC increase in ambient temperature ($CT_{max}$ compared to ambient 28ºC). This mirrors the findings of Nguyen et al. (2011) and Lai et al. (2011) that tropical marine animals are possibly the most vulnerable and likely to be the first affected under current global warming and climate change conditions.

Conversely, while many studies focus on the effects of warm thermal stress on the physiological and behavioural responses of organisms (Somero, 2002, Jones et al., 2009, Denny et al., 2011 and Sorte et al., 2011), the effects of cold thermal stress are often neglected. In this study here, none of the biomarkers showed differences in sensitivity to cold stress. This is probably due to the difference in ecological relevance of cold tolerance in *Perna viridis* and basic mechanism failure for high and low temperatures. At high temperatures, excessive oxygen demand causes insufficient oxygen levels in the body fluids, whereas at low temperatures the aerobic capacity of mitochondria becomes limiting for

ventilation and circulation (Pörtner, 2001). Acclimation to seasonal cold is well known to cause a rise in mitochondrial density or mitochondrial aerobic capacity in fish (e.g. St.-Pierre et al., 1998; Guderley, 1998) and recently discovered in marine invertebrates (*Arenicola marina*, Pörtner et al., 2007). This is to cope with the limited capacity of mitochondria to produce energy in the cold leading to the loss of function and scope (Pörtner et al., 1998, 2000). For the eurythermal *Perna viridis* which rarely experience seasonal cold, the energy cost of significant metabolic cold adaption will be extremely high (Pörtner et al., 2000, 2001) and energetic budgeting for cold adaption will not be very significant for the tropical mussel. Thus the $CT_{Min}$ of all the indices are relatively close to the 22ºC range, probably explaining why the geographical range of the mussel has thus far being limited to the regions with warmer waters (Urian et al., 2011).

It is well documented that climate warming on the scale of decades can alter the composition of marine communities by facilitating the poleward spread of warm-adapted species (Southward et al., 1995, Sagarin et al., 1999, Stachowicz et al., 2002 and Mieszkowska et al., 2007). While studies here suggest that the *Perna viridis* has reduced acclimatory ability, and are particularly susceptible to increases in temperatures, range shift of this invasive species (Power et al., 2004) into colder regions might occur with a global increase in sea and air temperatures. Understanding the mechanisms underlying these changes is of critial importance to enable us to predict how ecosystems will change into the future.

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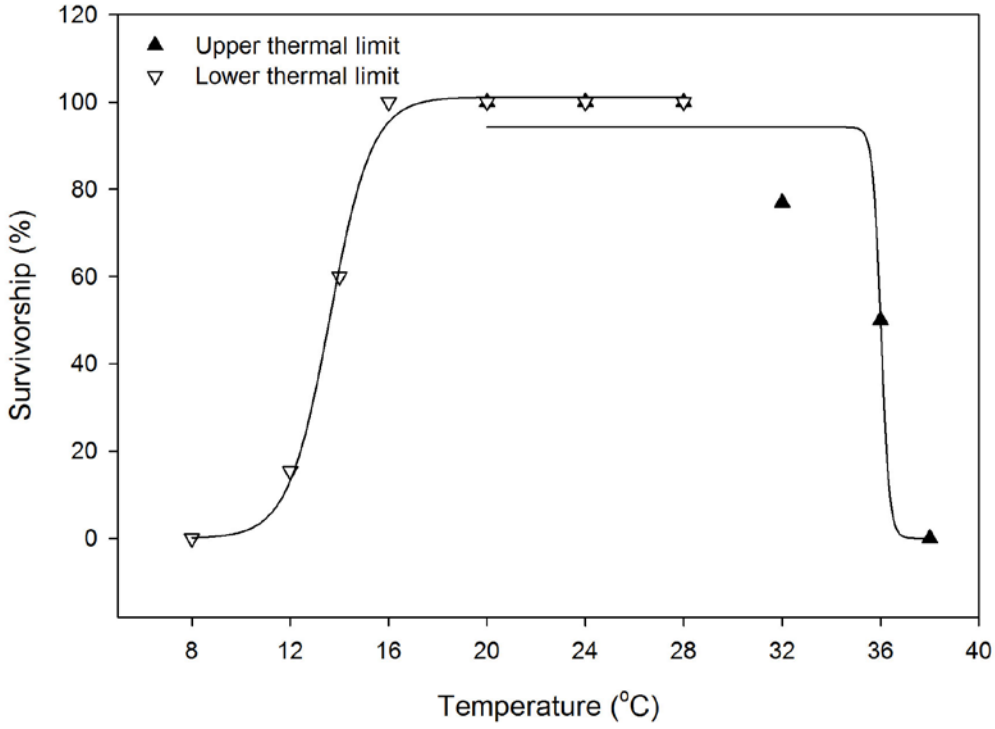


Figure 1 Thermal dependency of survival of *Perna viridis*. Percentage of mussels that survived at each temperature is shown together with upper and lower fitted 3 parameter sigmoid curves.

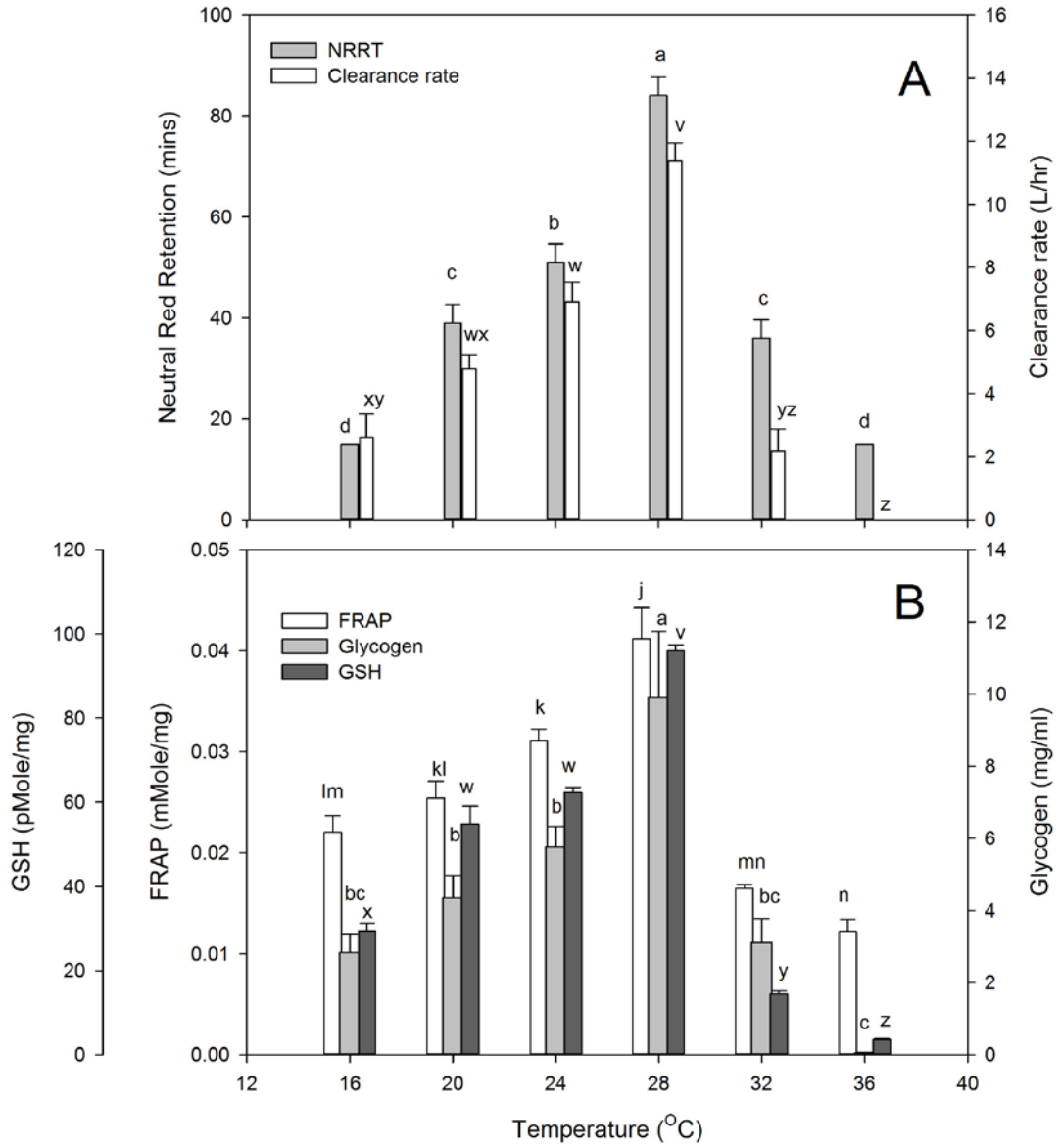


Figure 2 Thermal dependency (Mean ± SE) of *Perna viridis* biological functions and processes. (A) Clearance rate of the mussels at each temperature is shown together with their Neutral Red retention time. Means with different letters (a, b, c & d for NRRT and v, w, x, y & z for clearance rate) are significantly different at $p < 0.05$, SNK test. (B) Antixoidant levels (Ferric reducing power and GSH) are shown together with the glycogen levels at each temperature. Means with different letters (a, b, c for glycogen, j, k, l, m & n for FRAP and v, w, x, y, z for GSH) are significantly different at $p < 0.05$, SNK test. Mussels at 36°C were not feeding.

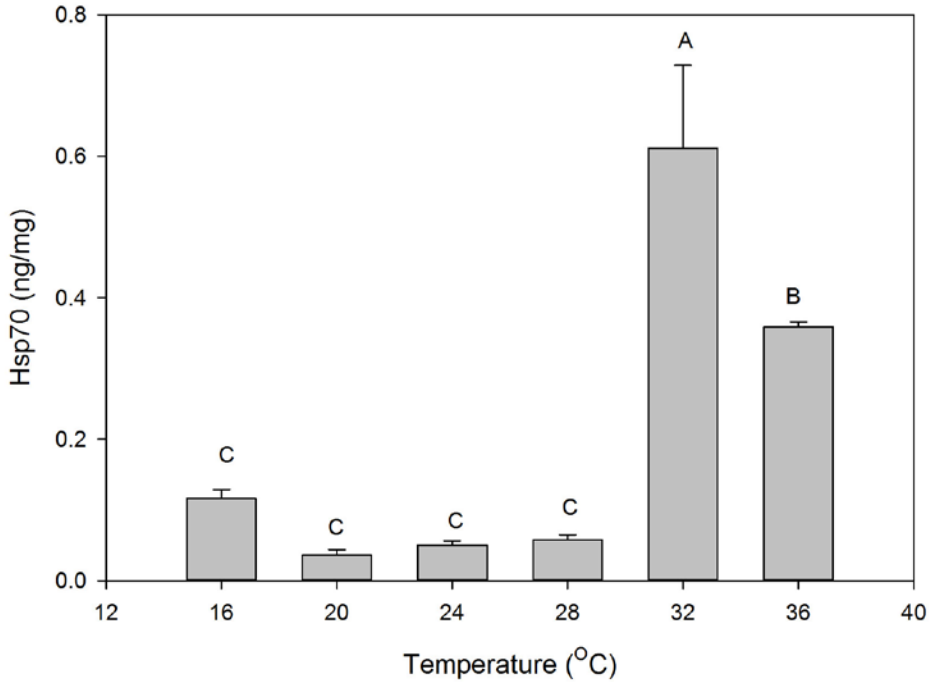


Figure 3 Heat shock protein production (Mean ± SE) of *Perna viridis* with heat stress. Hsp70 at 32ºC and 36ºC were significantly higher than the other temperature exposures. Means with different letters (a, b, c) are significantly different at $p < 0.05$, SNK test.

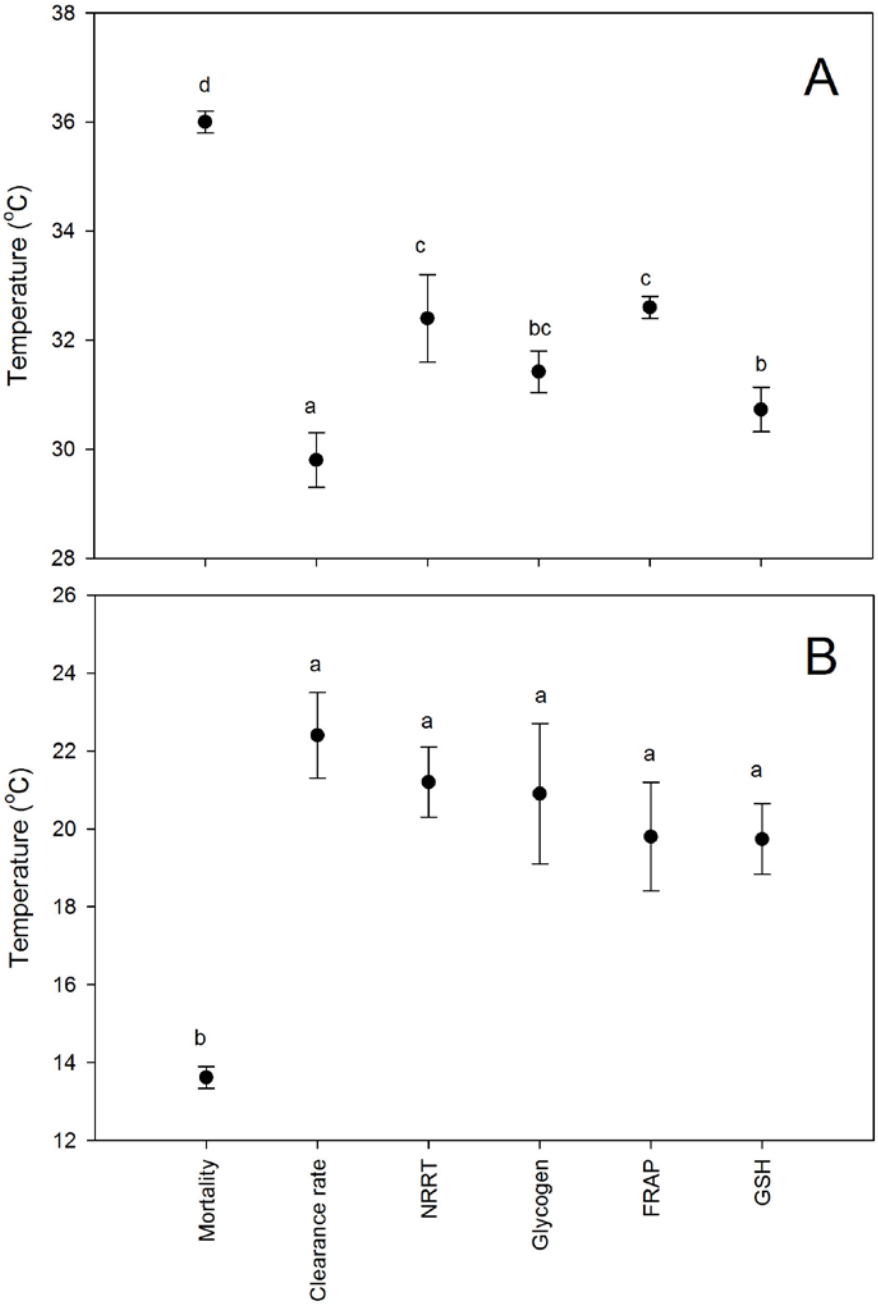


Figure 4 $LT_{50}$ (mortality) and CT (biological indices) plots of *Perna viridis*. (A) $ULT_{50}$ and $CT_{max}$ for clearance rate, NRRT, glycogen, FRAP and GSH. (B) $LLT_{50}$ and $CT_{min}$ for clearance rate, NRRT, glycogen, FRAP and GSH. Error bars depict 95% confidence intervals. Means with different letters (a, b, c & d) are significantly different as the 95% confidence intervals do not overlap.

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