TITLE: Acclimation to cyclic hypoxia improves thermal tolerance and copper survival in the caridean shrimp *Palaemon varians*

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KEYWORDS: acclimation to hypoxia; temperature tolerance; copper survival; Caridea

Abstract:

In response to the continuous variation of environmental parameters, species must be able to adjust their physiology to overcome stressful conditions, a process known as acclimatization. Numerous laboratory studies have been conducted to understand and describe the mechanisms of acclimation to one environmental stressor (e.g. cyclic hypoxia), but currently our understanding of how acclimation to one stressor can change tolerance to a subsequent stressor is limited. Here, in two different experiments, we used the shrimp *Palaemon varians* to test how, following 28-days acclimation to cyclic hypoxia (mimicking a cyclic hypoxic regime currently found in its natural habitat), critical thermal maximum (CT_{max}) and sensitivity to copper (Cu^{2+}) exposure ($30mgL^{-1}$) changed in comparison to shrimp acclimated to normoxic conditions and then exposed to thermal stress or Cu^{2+}. Acclimation to cyclic hypoxia improved both CT_{max} (~1˚C higher than controls) and survival to acute Cu^{2+} exposure (~30% higher than controls) and induced significant gene expression changes (i.e. up-regulation of heat shock protein 70 – *HSP70*, hypoxia inducible factor – *HIF*, phosphoenolpyruvate carboxykinase – *PEPCK*, glucose 6-P transporter – *G6Pt*, metallothionein – *Mt,* and down-regulation of hemocyanin – *Hem*) in animals acclimated to

cyclic hypoxia. Our results demonstrate how acclimation to cyclic hypoxia improved tolerance to subsequent stressors, highlighting the complexity of predicting organismal performance in variable (i.e. where multiple parameters can simultaneously change during the day) environments.

Introduction:

Acclimatization is a biological process that triggers physiological adjustments to meet the demands of the body in changing environments (Liknes and Swanson, 2011). This fundamental ability to acclimate is found in all eukaryotes and the physiological changes (e.g. a shift towards anaerobic metabolism during hypoxia (Bridges and Brand, 1980)) can also imply a functional change. For example, we recently showed that acclimation to cyclic hypoxia induced morphological changes in the gills of the shrimp *Palaemon varians* by increasing gill lamellar surface area; since the diffusivity of gases across compartments is directly proportional to the surface area of the compartments (as stated by Fick's law), an increase in lamellar surface area (i.e. functional change) translates in an increased gas exchange capacity that is useful to counteract the impact of cyclic hypoxia (Peruzza et al., 2018). However, real world habitats are characterized by co-variation of multiple environmental parameters (Richards, 2011; Sperling et al., 2016). In this context, acclimatization to one environmental condition can affect (by improving or reducing) the ability to tolerate other environmental conditions (McBryan et al., 2016; Todgham and Stillman, 2013). This is extremely important, especially for species living in changing environments, such as coastal or estuarine habitats, where multiple parameters can change during the day (Richards, 2011; Sperling et al., 2016).

In these changing environments species can experience multiple stressors in a simultaneous (i.e. when stressors are in phase) or sequential way (i.e. when stressors are out of phase); this is very important because the physiological response of organisms depends on the type, intensity and relative timing of each stressor (Gunderson et al., 2015). The timing of occurrence of stressors is important because it can determine the type and strength of interactive effects elicited by the stressors. For example if the cellular mechanisms used to defend against two stressors are shared, exposure to one stressor may prime the body resulting in an improved tolerance to a subsequent different stressor (Todgham and Stillman, 2013). Cyclic hypoxia, temperature and exposure to Copper (Cu^{2+}) are three stressors for which there is the potential for interaction through overlap in the physiological mechanisms

affected (i.e. oxygen supply/demand), and thus their impact in multi-stressor studies should be addressed. In this context the timing of multistressor exposure might be extremely important: in fact a long term pre-exposure to one stressor could enhance performance under another subsequent stressor due to the overlap in physiological mechanisms. To date, the majority of multi-stressor studies are focussed on the simultaneous exposure to two or more stressors, while stressors applied sequentially are rarely assessed. In order to shed light into the effects of sequential exposure to two stressors we investigated, in two different experiments, how exposure to cyclic hypoxia affected subsequent thermal tolerance and sensitivity to Cu^{2+} in the ditch shrimp *Palaemon varians*, a species commonly found in salt marshes of Northern Europe.

Temperature is considered one of the major environmental factors that affects the overall biology of organisms (Burleson and Silva, 2011; Peruzza et al., 2015); with climate warming, extreme thermal events are predicted to increase in frequency, severity and duration and consequently will constitute a greater threat to marine species (Galli et al., 2017). The critical thermal maximum – CT_{max} – indicates a species upper thermal limit (Verberk et al., 2018) and is usually associated with the temperature where one or more vital physiological functions terminate. Recently Verberk et al. (2016) reviewed the literature to understand how exposure to acute hypoxia affects CT_{max} in arthropods and demonstrated that without prior acclimation to hypoxia, CT_{max} was reduced in 59% of the cases. However, in the literature there is no report of how acclimation to cyclic hypoxia might subsequently affect CT_{max} in invertebrates. In this context we hypothesised that, in *P. varians*, acclimation to cyclic hypoxia would increase CT_{max} thanks to the increase in lamellar surface area (mentioned above), which translates in an improved gas exchange (i.e. more oxygen available for the body) thus sustaining vital physiological functions beyond the thermal limit of non-acclimated animals.

Another important stressor in coastal habitats is constituted by heavy metals such as Cu^{2+}. In fact, because of its toxicity to marine life, Cu^{2+} is globally used as antifoulant in vessels and for this reason it has been identified as the greatest threat to aquatic ecosystems in UK and China (Donnachie et al., 2014; Johnson et al., 2017; Su et al., 2017). Unfortunately, Cu^{2+} can leach from antifouling paints into the water where its concentration can substantially vary throughout the year, especially in coastal areas (Johnson et al., 2017; Jones and Bolam, 2007), hence it constitutes an additional stress to marine life. Cu^{2+} induces anatomical and cytological damage to the gills of crustaceans that impairs the ability of gills to take up oxygen from the environment, resulting in the development of Cu^{2+}-induced

internal hypoxia (Soegianto et al., 1999; Spicer and Weber, 1992). Several studies have assessed the co-occurrence of Cu^{2+} and acute or chronic hypoxia on survival of marine species (Fitzgerald et al., 2016; Sappal et al., 2016; Spicer and Weber, 1992) and results generally show an increased toxicity, mainly due to gill damage, following exposure to both stressors. However, to the best of our knowledge, there is currently no assessment on the effects of Cu^{2+} toxicity after acclimation to cyclic hypoxia. Given the morphological changes to the gills reported above we hypothesised that acclimation to cyclic hypoxia increases tolerance to Cu^{2+}. This would be accomplished by delaying the development of Cu^{2+}-induced internal hypoxia that results from tissue damage following Cu^{2+} exposure.

In order to determine how acclimation to one environmental condition can affect tolerance to a subsequent stressor we used the Atlantic ditch shrimp, *Palaemon varians*. *P. varians* is a detritivore species involved in the mechanical breakdown of refractory organic matter such as plant fibres but it is also a predator that actively feeds on invertebrates such as nematodes, polychaetes, motile mysids, and mosquito larvae (Aguzzi et al., 2005). For these reasons *P. varians* plays a fundamental role in its ecosystem in the transfer of nutrients and energy among the various trophic levels of the ecosystem. We acclimated adult *P. varians* to cyclic hypoxia (or normoxia) for 28 days, a time corresponding to two intermoult cycles (Peruzza et al., 2018) that allowed gill adjustments to take place. During acclimation we assessed the expression of metabolic and stress biomarkers to monitor the changes in the expression of these genes during the process of acclimation. Then, after acclimation, we assessed, in two independent experiments, adult tolerance to a subsequent thermal stress (by means of CT_{max}) or adult survival after exposure to copper (Cu^{2+}, 0 and 30mg Cu^{2+} L^{-1}) in order to test whether acclimation to cyclic hypoxia could improve tolerance to a subsequent stress exposure.

Materials and Methods:

Animal collection and acclimation to cyclic hypoxia:

Adult *Palaemon varians* were collected from Lymington salt marsh (UK, location: 50°44′19.8″ N and 50°44′22.2″ W) with hand-nets in Spring 2017. Once in the lab animals were kept in tanks with recirculating seawater (salinity 33psu). They were slowly acclimated by increasing temperature +1 ˚C every other day from the field temperature of 14 ˚C until 22 ˚C (representing a temperature frequently experienced by *P. varians* in its habitat during the summer (Peruzza et al., 2018)). Once at 22 ˚C, animals were kept at this temperature for at

least one week before the experiments. To perform the 28-day acclimation, two flow-through experimental systems were built, one for each acclimation treatment (i.e. cyclic hypoxia and normoxia). Each experimental system was composed of three 12 L aquaria in which cyclic hypoxia or normoxia conditions were independently obtained. In all experiments, the maximum density was 18 animals per tank.

Animals from the cyclic hypoxic treatment were exposed to an average water pO_2 of ~2.7 ±0.9 kPa from 0230 to 0930 hr and to normoxic conditions (water pO_2 ~21 kPa) for the rest of the day for 28 days (see (Peruzza et al., 2018) for a detailed explanation on the experimental system and how cyclic hypoxia was obtained), while animals acclimated to normoxia were kept in normoxic conditions (water pO_2 ~21 kPa). A similar daily cycle of pO_2 has been previously reported during the summer in the habitat where the population used in the experiments was collected (Peruzza et al., 2018). A pO_2 level of 4.5 kPa is, at 22 ˚C, the critical oxygen tension – p_{crit} (Peruzza et al., 2018). Briefly, hypoxia was achieved by bubbling N_2 in the experimental tanks and water pO_2 was continuously monitored (one measurement every 5 seconds) and logged with Microx TX 3 (PreSens Precision Sensing GmbH) sensors. The flow of N_2 and water were set in order to obtain the desired hypoxic conditions and the continuous flow of water prevented the formation of anoxic zones in the aquaria.

To monitor the expression of genes during acclimation to cyclic hypoxia, 8 shrimps from each treatment (i.e. cyclic hypoxia and normoxia) were randomly sampled at 4 time points (i.e. days 0, 1, 7 and 28). Sampling was always performed within one hour from the end of the daily hypoxic period. Sampled animals were immediately snap frozen in liquid N_2 and their cephalothorax stored at -80 ˚C for further analysis. We chose to focus our analyses on the cephalothorax because this region contains all the major organs of these animals, while the tail contains almost only muscle tissue.

Assessment of CT_{max}:

CT_{max} was assessed after 28-days of acclimation. For both acclimation treatments the assessment of CT_{max} was carried out in normoxic conditions. The reason for this choice is that as we recently showed (Peruzza et al., 2018), *P. varians* continuously faces a co-variation of oxygen and temperature in its habitat that result in hypoxic and thermal stress, however these stressors do not occur simultaneously; in fact, hypoxia develops during the night when temperatures are decreasing, while temperature increases during the day when

normoxic conditions are found (Peruzza et al., 2018). After acclimation to cyclic hypoxia or normoxia, individual animals (n= 6 per treatment) were placed (five hours after the end of the daily hypoxic period) in a 500 mL beaker with 250 mL of fully-aerated and filtered sea-water at 22 ˚C inside a water bath (Thermo Electron Corporation Haake W46 water bath). Beakers were not sealed and water was mixed manually to prevent deoxygenation. During the thermal stress there was no substantial decrease in oxygen saturation in the beakers (i.e. oxygen did not drop below 80%). Temperature was monitored to the nearest 0.1 °C (Microx TX 3, PreSens). From 22 ˚C the temperature was increased at a constant rate of 0.33 ˚C min^{-1} (New et al., 2014; Ravaux et al., 2012). Behaviour in response to temperature was recorded with a GoPro Hero 3+ black camera and analysis was performed in single blind assessment (i.e. the operator did not know which video recording represented control and cyclic hypoxia-acclimated shrimp). CT_{max} was defined as the temperature at which Loss of Equilibrium was observed (Oliphant et al., 2011; Ravaux et al., 2012). LOE was defined as the water temperature at which the shrimp rested on the bottom in either an “upside-down” or a “side-ways” position for more than 2 s (New et al., 2014; Ravaux et al., 2012). After the test animals were immediately snap frozen in liquid N_2 and their cephalothorax stored at -80 ˚C for gene expression (GE) analysis. Animals were not sampled before thermal ramping at the starting temperature.

Cu^{2+} exposure:

Adult *P. varians* were acclimated for 28-days to cyclic hypoxia or normoxia, as described above. Within 5 hours from the end of the acclimation, survival of acute Cu^{2+} exposure was tested in normoxic conditions. To perform Cu^{2+} exposure, a stock solution of $CuSO_4 \cdot 5H_2O$ (Sigma-Aldrich, USA https://www.sigmaaldrich.com) was prepared using distilled water and analytical reagent grade compounds, in accordance with Brown et al. (2017). The stock solution was used to obtain an exposure concentration of 30 mg Cu^{2+} L^{-1} in 10 L plastic aquaria, based on available lethal copper toxicity data in palaemonids (96 h lethal concentration to 50% of individuals: 37.0 mg L^{-1} in *Palaemonetes pugio* at 22 ˚C, (Curtis and Ward, 1981)). For each acclimation treatment adults were placed in 10 L plastic aquaria (n= 7 per aquarium) and were exposed to seawater without Cu^{2+} (Cu^{2+} treatment: 0 mg Cu^{2+} L^{-1}, n= 20 in total) or artificial seawater spiked with Cu^{2+} (Cu^{2+} treatment: 30 mg Cu^{2+} L^{-1}, n= 21 in total) and incubated at 22 ˚C. Mortality was assessed every 24 hours. After

6 days, surviving animals were immediately snap frozen in liquid N_2. The cephalothorax was used for GE analysis.

Gene expression:

Total RNA was extracted from cephalothorax of all samples using a TRI-Reagent™ (Sigma-Aldrich, USA) protocol according to the manufacturer's recommendations, and its quality and concentration were assessed with NanoDrop™ spectrophotometer (Thermo Fisher Scientific) and Bioanalyzer (Agilent technologies, USA). Subsequently, 1 µg of RNA was DNase treated and reverse transcribed and cDNA was used to perform qPCR reactions according to Peruzza et al. (2018). All primer sets (Supplementary Table 1) were compliant with the MIQE guidelines (Bustin, 2010). geNorm analysis from qBase+ software (Biogazelle, Belgium) was used to test candidate reference genes. After assessing endogenous refence genes (ERGs) stability, the geometric mean of the two reference genes was used to normalise gene of interest expression. The best normalisation strategy was achieved using elongation factor 1-alfa (*eef1A*) and ribosomal protein L8 (*rpl8*) as ERGs. Normalised relative quantities (NRQs) were calculated using qBase+ software and were then plotted and subjected to statistical analysis. The expression of common stress-associated genes (e.g. Heat shock protein 70 (*HSP70*), crustacean Hyperglycaemic Hormone (*cHH*) and Hypoxia inducible factor (*HIF*)) and metabolic genes (e.g. lactate dehydrogenase (*ldh*), phosphoenolpyruvate kinase (*PEPCK*) and Glucose-6-phosphate transporter (*G6Pt*)) was quantified in animals after acclimation to 28-days of cyclic hypoxia or normoxia and following thermal stress. Following Cu^{2+} exposure, the expression of genes coding for common detoxifying proteins (e.g. Metallothionein (*Mt*) and superoxide dismutase *SOD*) was assessed together with the expression of stress-associated genes (i.e. *HIF*, *HSP70* and *cHH*) and the respiratory pigment Hemocyanin (*Hem*).

Statistical analysis:

All data were tested for normality with Kolmogorov-Smirnov or Shapiro test. CT_{max} data were analysed using unpaired t-test. GE data from acclimation to cyclic hypoxia and from CT_{max} were analysed using Mann-Whitney or pairwise Wilcoxon rank sum test. Survival of animals exposed to Cu^{2+} was tested using Log-rank (Mantel-Cox) test. The effects of cyclic hypoxic acclimation and Cu^{2+} exposure on genes were assessed using a

Two-way ANOVA with "acclimation treatment" and "Cu^{2+} dose" as factors; *post-hoc* Tukey's multiple comparisons test were further used.

For all tests, statistical significance was identified at p-value < 0.05. Data shown in figures are presented as means ± SD for normally distributed data, while they are presented as Tukey's box and whiskers plot when data did not comply with normal distribution.

Results:

Gene expression during acclimation:

During acclimation to cyclic hypoxia we assessed the expression of six genes (Fig. 1), three biomarkers of stress: heat shock protein 70 (*HSP70*), crustacean hyperglycaemic hormone (*cHH*), hypoxia inducible factor (*HIF*); and three metabolic enzymes: lactate dehydrogenase (*Ldh*), phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6P transporter (*G6Pt*). At day 1 *HSP70*, *HIF* and *PEPCK* were up-regulated in cyclic hypoxic acclimated animals (Wilcoxon rank sum test, p-value = 0.033, 0.016 and 0.028 respectively). At day 7 *PEPCK* was up-regulated in cyclic hypoxic acclimated animals (Wilcoxon rank sum test, p-value = 0.016), in comparison to normoxic acclimated animals. At day 0 and day 28 no statistical difference between cyclic hypoxic and normoxic acclimated animals could be observed.

CT_{max} and gene expression:

CT_{max} was behaviourally assessed by measuring LOE in animals. LOE differed between animals acclimated to normoxic conditions and animals acclimated to cyclic hypoxia for 28 days (unpaired t-test = 9.548, p-value < 0.0001, df=10, Figure 2), with animals acclimated to cyclic hypoxia having a ~1 ˚C higher thermal tolerance in comparison to animals acclimated to normoxia.

GE analysis performed on animals immediately after CT_{max} showed that the expression of key enzymes of the gluconeogenic pathway *PEPCK* and *G6Pt* was higher in animals acclimated to cyclic hypoxia (Mann-Whitney U = 5, p-value = 0.03, n = 12 and Mann-Whitney U = 5, p-value = 0.04, n = 12, respectively, Fig. 3). However, no difference was detected in the expression levels *HSP70* (Mann-Whitney U= 15.5, p-value = 0.27, n = 14, Fig. 3), *cHH* (Mann-Whitney U = 23, p-value = 0.90, n = 14), *HIF* (Mann-Whitney U = 18, p-value = 0.45, n = 14) and *Ldh* (Mann-Whitney U = 22, p-value = 0.77, n = 14).

Acute 96-h Cu^{2+} L^{-1} exposure:

Survival was not statistically different between the hypoxic- and normoxic-acclimated groups at 0 mg Cu^{2+} L^{-1} (Mantel-Cox test, χ^2: 1.11, df=1, p-value=0.29; Fig. 4). At 30 mg Cu^{2+} L^{-1} survival was statistically higher (+ 28%) in the hypoxic-acclimated animals in comparison to normoxic-acclimated animals (Mantel-Cox test, χ^2: 4.2, df=1, p-value =0.04, Fig. 4A). Animals exposed to 30 mg Cu^{2+} L^{-1} showed a "blackening" in the ventral region of the cephalothorax, where the gills are located (Fig. 4B), a common symptom as a consequence of Cu^{+2} exposure.

Gene expression following Cu^{2+} exposure:

GE analysis, by means of Two-way ANOVA, revealed an effect of Cu^{2+} dose on the expression of metallothionein (*Mt),* superoxide dismutase (*SOD), HSP70, HIF* and *Hem* (Fig. 5 and Supplementary Table 2) and a significant effect of the hypoxia acclimation treatment on the expression of *Mt* and *Hem*. Tukey's multiple comparisons test revealed a statistical difference in the expression levels of *Mt* and *Hem* between cyclic hypoxic acclimated and normoxic acclimated animals exposed to 30 mg Cu^{2+} L^{-1} (Fig. 5).

Discussion:

Species are regularly exposed to variations of the physiochemical conditions in their habitat that frequently lead to stressful conditions (Richards, 2011). In every environment there are various types of stressors (e.g. hypoxia, acidification) and the timing with which they are experienced by animals (i.e. simultaneously or sequentially) can be extremely important in relation to how organisms respond to such stressors. In fact, pre-exposure to one stressor may harden animals and render them less vulnerable to a second stressor. In this work our aim was to investigate how acclimation to one stressor affected the responses to another subsequent stressor and we showed that acclimation to cyclic hypoxia improved both thermal tolerance and Cu^{2+} survival in a decapod species.

In order to adjust to stressful conditions, cells are able to elicit a series of responses: cellular stress responses (CSR) and cellular homeostasis responses (CHR) (Kultz, 2005; Kültz, 2003; Morris et al., 2013). While CSRs are transient and triggered by macromolecular damage, CHRs are permanent (i.e. until environmental conditions change) and triggered by

296 stressor-specific sensors (e.g. the transcription factor TonEBP/NFAT5, that during osmotic
297 stress activates osmoprotective genes in mammalian cells) (Kultz, 2005). Our work revealed
298 that during acclimation to cyclic hypoxia *HSP70*, *HIF* and *PEPCK* genes were up-regulated
299 in the initial stages of acclimation (i.e. at day 1 and, only for *PEPCK*, day 7) in cyclic
300 hypoxic animals but then by day 28 there was no difference between acclimation treatments
301 in the expression of all the examined biomarkers. All these biomarkers, or the corresponding
302 enzymes, have been previously reported as up-regulated upon exposure to short-term hypoxia
303 (i.e. < 7 days) (Alter et al., 2015; Bridges and Brand, 1980; Brown-Peterson et al., 2011;
304 Brown-Peterson et al., 2008), while none of the abovementioned genes was found up-
305 regulated in chronic exposures to hypoxia (Brouwer et al., 2007); hence their up-regulation
306 could be interpreted as part of the metabolic/CSR responses triggered in the initial stages to
307 adjust the body to the stressful conditions.
308 Subsequently, we investigated how the previous acclimation to cyclic hypoxia would
309 alter shrimp tolerance to heat stress and we found that previous acclimation improved *P.*
310 *varians* thermal tolerance by ~1 ˚C (mean CT_{max}: 37.65 ±0.2 ˚C for animals acclimated to
311 cyclic hypoxia and 36.4 ±0.3 ˚C for animals acclimated to normoxia). Arguably this
312 difference might be explained by the observed increase in gill surface area (that we
313 previously reported, see Peruzza et al. (2018)) resulting from acclimation to cyclic hypoxia in
314 *P. varians*. In fact, this morphological change would provide a greater efficiency in gas
315 exchange thus sustaining respiratory functions and oxygen requirements of the body for a
316 longer time when animals are facing heat shock, thereby increasing their thermal tolerance.
317 To the best of our knowledge, this study constitutes the first report of increased thermal
318 tolerance following acclimation to cyclic hypoxia in a marine invertebrate, while some
319 examples exist for fish acclimated to chronic hypoxia. In fact Burleson et al. (2002) and
320 Burleson and Silva (2011) reported that 7-d exposure to chronic hypoxia increased CT_{max} in
321 the channel catfish *Ictalurus punctatus* by providing the animals a greater heart rate, systolic
322 pressure and ventilation. In a similar way, McBryan et al. (2016) demonstrated that 6-weeks
323 acclimation to warm temperatures was accompanied by an increase in gill respiratory surface
324 area and resulted in increased hypoxia tolerance in the killifish *Fundulus heteroclitus*. Finally
325 Grimes et al. (2020a) reported a decrease in oxygen uptake and an increased oxygen
326 consumption during chronic (i.e. less than 7 days) exposure to hypoxia in the bearded
327 fireworm *Hermodice carunculata,* and further they reported an increased number of branchial
328 filaments in worms kept for 7 days in hypoxia (Grimes et al., 2020b) but unfortunately they
329 did not assess CT_{max} in worms after hypoxic exposure. Not all species demonstrate an

increase in CT_{max} following acclimation to hypoxia. Motyka et al. (2017) reported no change in CT_{max} of the steelhead trout *Oncorhynchus mykiss* after 3-months of chronic hypoxic. In this context then, the ability of the species to acclimate (by adjusting their physiology) to different environmental conditions seems to be the key factor for the observed increase in performance (i.e. CT_{max} or hypoxia tolerance).

On the other hand, several studies have focussed on the interaction between acute hypoxia and CT_{max} in crustaceans, reporting either no effect on CT_{max} (e.g. in the giant tiger shrimp *Penaeus monodon* (Ern et al., 2015)), or a reduced CT_{max} following acute hypoxia (e.g. in the European crayfish *Astacus astacus* (Verberk et al., 2016)). Given the fact that morphological adjustments to the gills of crustaceans can take place only during moult (since gills are covered with cuticle), these aforementioned short-time exposures to hypoxia may not have lasted long enough to trigger the functional changes (i.e. increase in gill surface area after moulting) that we have observed with a 28-days exposure to cyclic hypoxia.

GE changes after thermal stress showed that *PEPCK* and *G6Pt* genes were up-regulated in animals acclimated to cyclic hypoxia in comparison to animals acclimated to normoxia. These two genes encode for important metabolic enzymes: the first catalyzes an irreversible step of gluconeogenesis and it is thought to be essential in glucose homeostasis, the second produces Glucose from Glucose-6-phosphate and is essential for control of blood glucose levels (Parker, 2004). The response of many aquatic animals to pollutants and stress is to induce hyperglycaemia (Lorenzon, 2005) and, in crustacean, this has been widely reported to result from exposure to several environmental stressors (Lorenzon et al., 1997; Manfrin et al., 2016). In the light of this it can be hypothesised that the up-regulation of *PEPCK* and *G6Pt* could have contributed to attenuate the thermal stress. However, due to the absence of biological samples before the thermal stress, the relative contributions of cyclic hypoxia and temperature to changes in GE could not be specifically identified.

Species are regularly exposed to variations in their habitat and may also encounter pollutants such as Cu^{2+}. In the current study, acclimation to cyclic hypoxia significantly increased the acute survival (i.e. 96h) of animals exposed to 30mg Cu^{2+} L^{-1} in comparison to controls, which were acclimated to normoxia. In these conditions, 71% of the hypoxic-acclimated animals survived, in contrast to 43% of the normoxic-acclimated animals. To the best of our knowledge, this work constitutes the first assessment of how acclimation to a daily cyclic hypoxic regime is able to alter the impact of Cu^{2+} on survival of crustaceans.

It has been hypothesized that Cu^{2+} exposure might lead to the development of internal hypoxia due to histological alterations at the gills (Malekpouri et al., 2016; Spicer and Weber,

1992). A previous study showed an increase in the diffusion barrier thickness at the gills resulting in a respiratory impairment in *Cancer pagurus* exposed to sub-lethal concentrations of Cu^{2+} (0.4 mg L^{-1}) for 7 days (Spicer and Weber, 1992), while another study showed that gills of *Penaeus japonicus* turned black after exposure to 1mg Cu^{2+} L^{-1} (Soegianto et al., 1999). In the current study, confirmation of damage to the gills was evident since the gills of animals from both groups (i.e. normoxic acclimated and cyclic hypoxic acclimated) turned black after exposure to 30mg Cu^{2+} L^{-1}. Previously, Spicer and Weber (1992) studied the effects of acclimation to Cu^{2+} on hypoxic tolerance of edible crab *Cancer pagurus.* They concluded that crabs acclimated to Cu^{2+} and subsequently exposed to hypoxia suffered a respiratory impairment that was primarily due to an increase in the diffusion barrier thickness at the gills, following cytological damage from Cu^{2+}. In light of our data, we could speculate that the higher survival observed in the cyclic hypoxic group might be explained by the changes to the gills (i.e. greater lamellar surface area that can improve diffusion of O_2) induced from acclimation to cyclic hypoxia, as previously mentioned. In fact, it can be hypothesised that this change could offset the damage to the gills as a consequence of Cu^{2+}, and therefore prevent (or at least slow) the onset of Cu^{2+}-induced internal hypoxia. Additional evidence in support to this hypothesis comes from our GE data. In fact, *Hem* was not up-regulated in animals acclimated to cyclic hypoxia while it was up-regulated in normoxic acclimated animals exposed to Cu^{2+}; this difference could suggest a possible mechanism to counteract the onset of hypoxia (resulting from gill damage) in normoxic acclimated animals, as reported in *Daphnia magna* (Gorr et al., 2004).

At cellular level, free metal ions such as Cu^{2+} are able to damage cellular machinery by increasing the production of Reactive Oxygen Species (ROS), such as superoxide (O_2^-) (Ahearn et al., 2004; Barata et al., 2005). Among the mechanisms that cells have evolved to sequester free metal ions and protect from ROS, Mt and SOD are probably the most studied (Ahearn et al., 2004; Coyle et al., 2002; Rhee et al., 2011). Overall, the expression of *Mt* and *SOD* decreased in both treatments exposed to Cu^{2+}, in a similar way to previous studies (Ren et al., 2011; Sappal et al., 2016). In fact Ren et al. (2011) demonstrated that the Chinese mitten crab, *Eriocheir sinensis*, exposed to different Cu^{2+} concentrations, regulate transcription of *Mt*, with maximum transcript numbers at intermediate Cu^{2+} concentration and minimum levels at higher concentration. Therefore, in the current study, the observed decrease in *Mt* could result from the exposure to the relatively high level of Cu^{2+} (30mg L^{-1}), which was just below the reported LD_{50} of 37mg L^{-1} for *P. pugio* (Curtis and Ward, 1981), but sufficient to trigger a stress response by upregulating *HSP70*. Interestingly, within the

groups exposed to Cu^{2+}, the expression of *Mt* was higher in hypoxic-acclimated animals in comparison to normoxic-acclimated animals, thus potentially accounting for an increased detoxification activity.

Conclusions:

Coastal ecosystems can be characterized by a continuous co-variation of multiple environmental parameters and can be subjected to contamination from heavy metals such as Cu^{2+}. This study identified that, following acclimation of adult *P. varians* to a cyclic hypoxic regime currently experienced in its habitat, animals showed an increased CT_{max} (~1 ˚C higher) in comparison to animals acclimated to normoxic conditions. In a separate experiment, an increased survival rate (~30% higher than controls) was observed when animals (acclimated to cyclic hypoxia) were exposed to 30mg Cu^{2+} L^{-1} coupled with an upregulation of *Mt* (detoxifying protein) in comparison to controls. In this work it was proven that complex interactions/responses can be revealed when considering the effects of exposing animals to subsequent stressors, in particular when examining long-term exposure to stressor conditions that can trigger acclimation responses. As such this work reinforces the need to understand complexity in the physiological responses to multi-factorial experiments, bearing in mind that acclimation to one stressor (i.e. cyclic hypoxia) can play a fundamental role in the physiological response to another stressor.

Acknowledgements:

This study was supported by a PhD scholarship to LP from the Graduate School of the National Oceanography Centre, Southampton. Authors would like to thank Dr Alastair Brown for his advice and guidance on conducting copper toxicity experiments.

Data Accessibility:

All raw data have been deposited in the Dryad Digital Repository.

Author Contributions:

LP planned, with input from CH the experimental work. LP performed all experimental work and analysed the data. LP wrote the manuscript with input from CH and ST.

Conflict of Interest:

Authors declare no conflict of interest.

Figures:

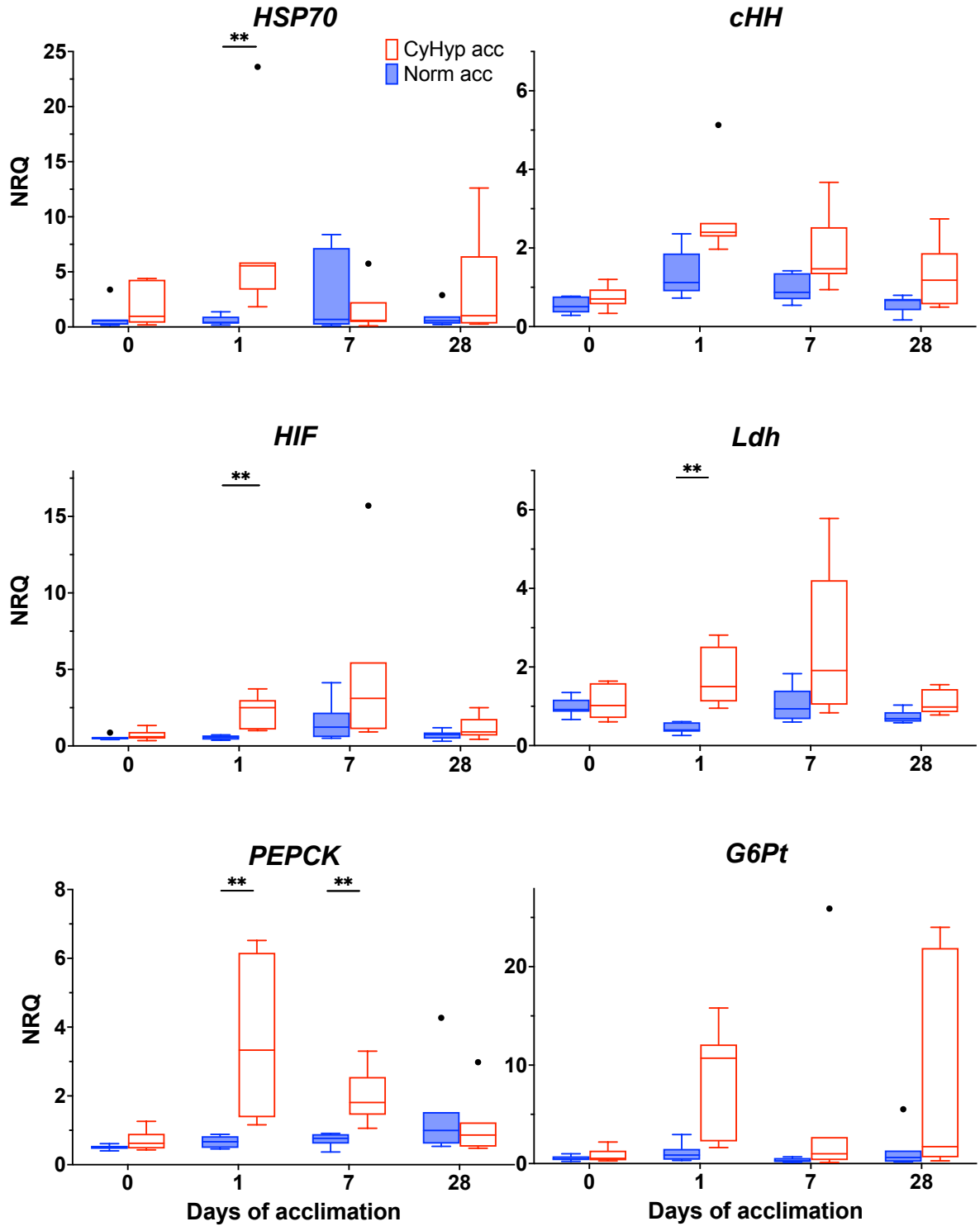


Figure 1: Gene expression (as Normalised Relative Quantities, NRQ) in adult *P. varians* during acclimation to cyclic hypoxia (red, empty boxes) or normoxia (blue, filled boxes). Tukey boxplots showing first, second, third and fourth quartile of the distribution (n=7 animals per each treatment). "**" indicates statistical difference (Wilcoxon rank sum test, p-value < 0.01). HSP70: heat shock protein 70; cHH: crustacean hyperglycaemic hormone;

442 HIF: hypoxia inducible factor; Ldh: lactate dehydrogenase; PEPCK: phosphoenolpyruvate
443 carboxykinas; G6Pt: glucose 6-P transporter.

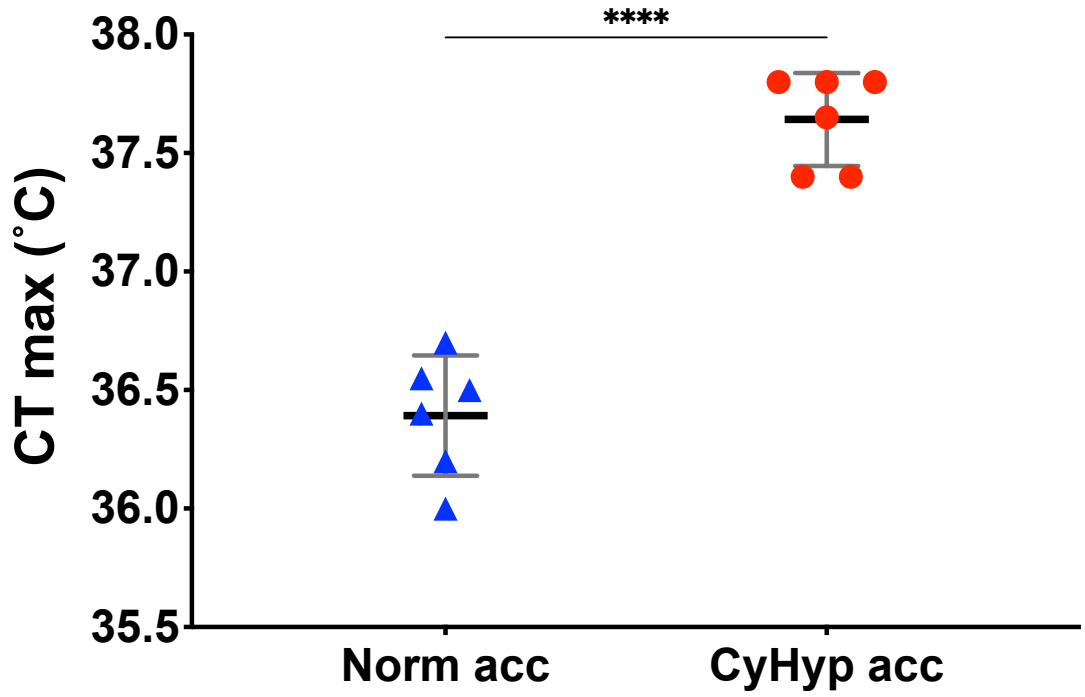


Figure 2: CT_{max} in the different acclimation treatments (e.g. Cyclic hypoxic and Normoxic-acclimated animals). Each dot represents one individual replicate shrimp, the black and grey bars indicate the mean and SD of each group, respectively. "****" indicates statistical difference (unpaired t-test, p-value <0.0001).

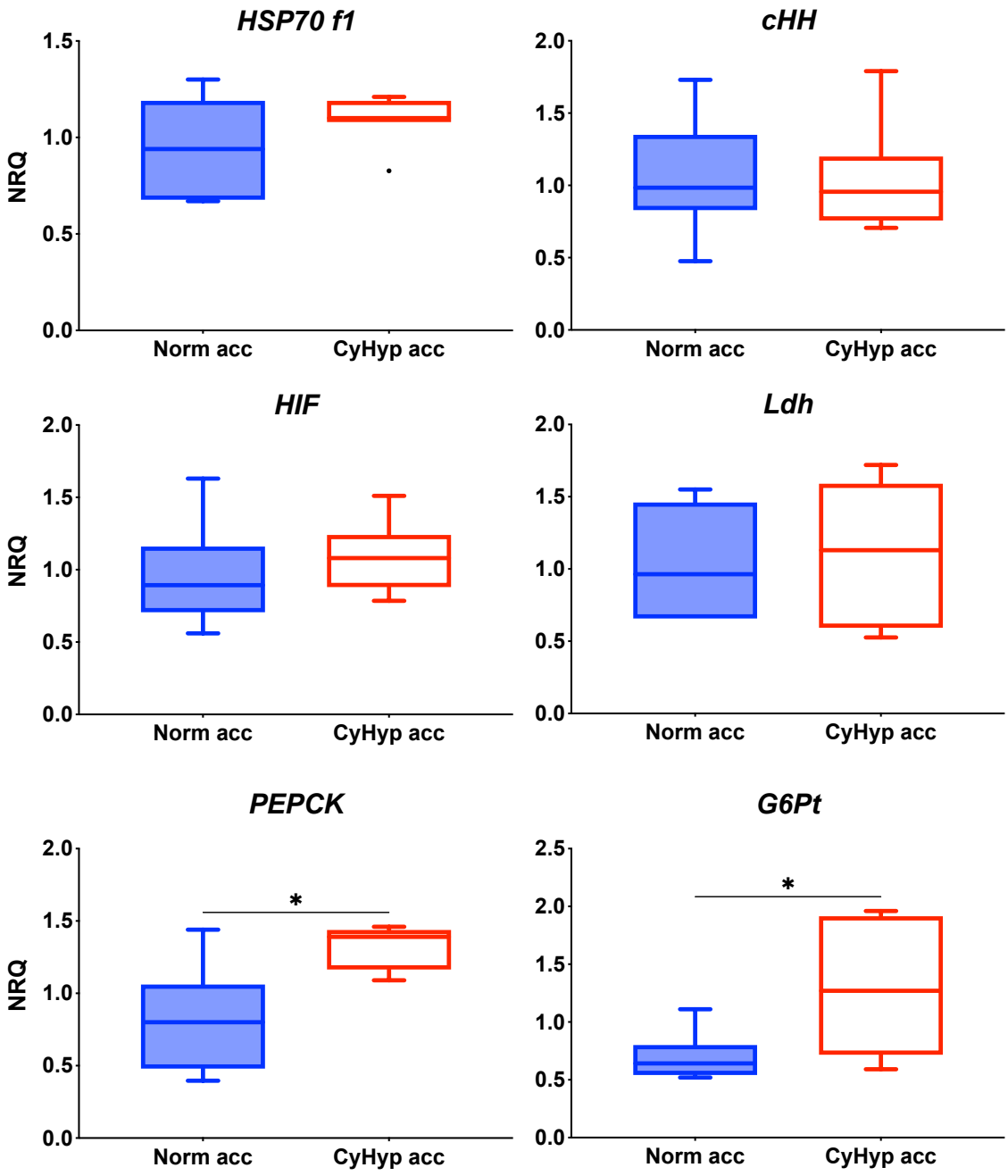
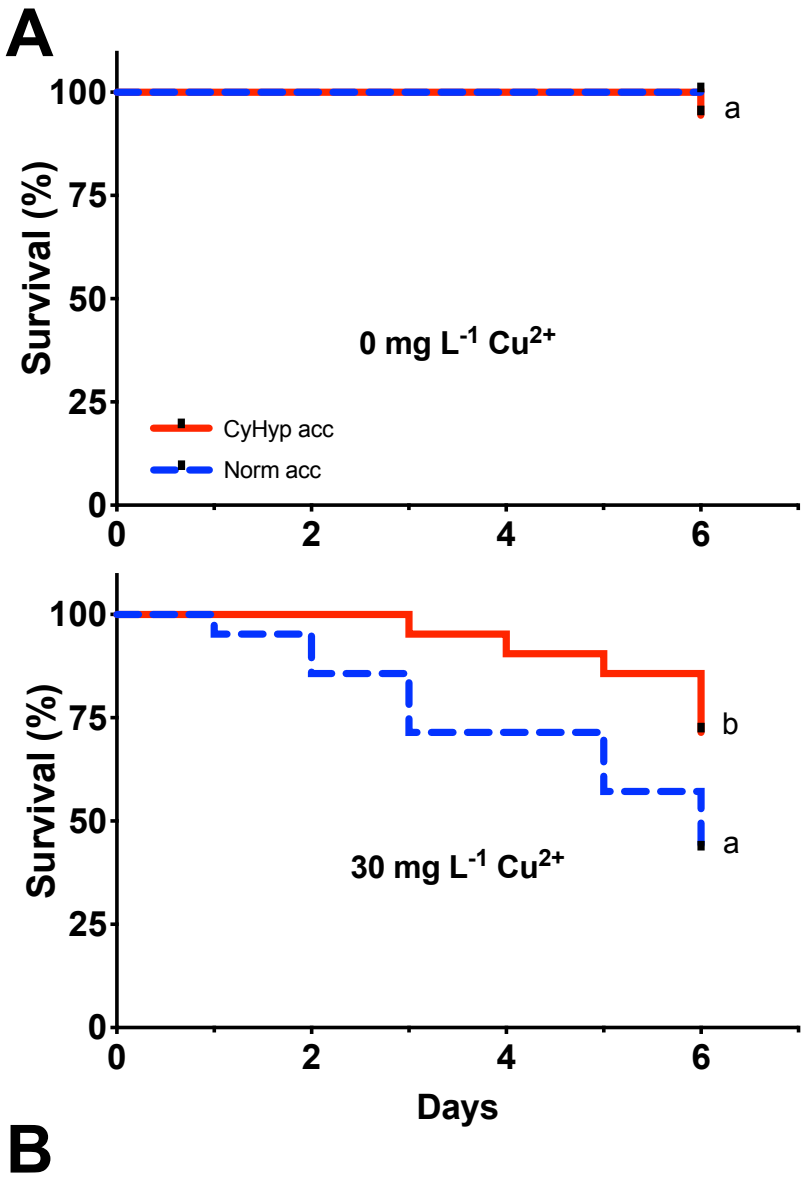


Figure 3: Gene expression in adult *P. varians* immediately after thermal stress test in the two acclimation treatments. Tukey boxplots showing first, second, third and fourth quartile of the distribution (n=6-7 animals per each treatment). "*" indicates statistical difference (Mann-Whitney p-value < 0.05). HSP70: heat shock protein 70; cHH: crustacean hyperglycaemic hormone; HIF: hypoxia inducible factor; Ldh: lactate dehydrogenase; PEPCK: phosphoenolpyruvate carboxykinas; G6Pt: glucose 6-P transporter.



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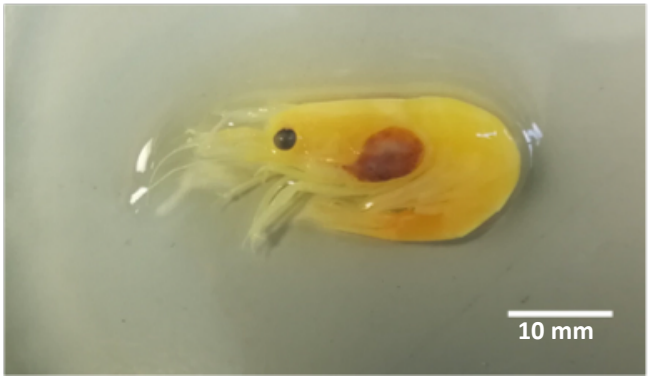

Figure 4: A) Survival of adult *P. varians* exposed to acute Cu^{2+} toxicity. n=21 per each combination of acclimation treatment and Cu^{2+} dose. Different letters indicate statistical

468 difference (Mantel-Cox test, p-value < 0.05). B) *P. varians* exposed to Cu^{2+} and showing a
469 "blackening" in the ventral region of the cephalothorax where gills are located. Scale bar =
470 10mm.
471
472

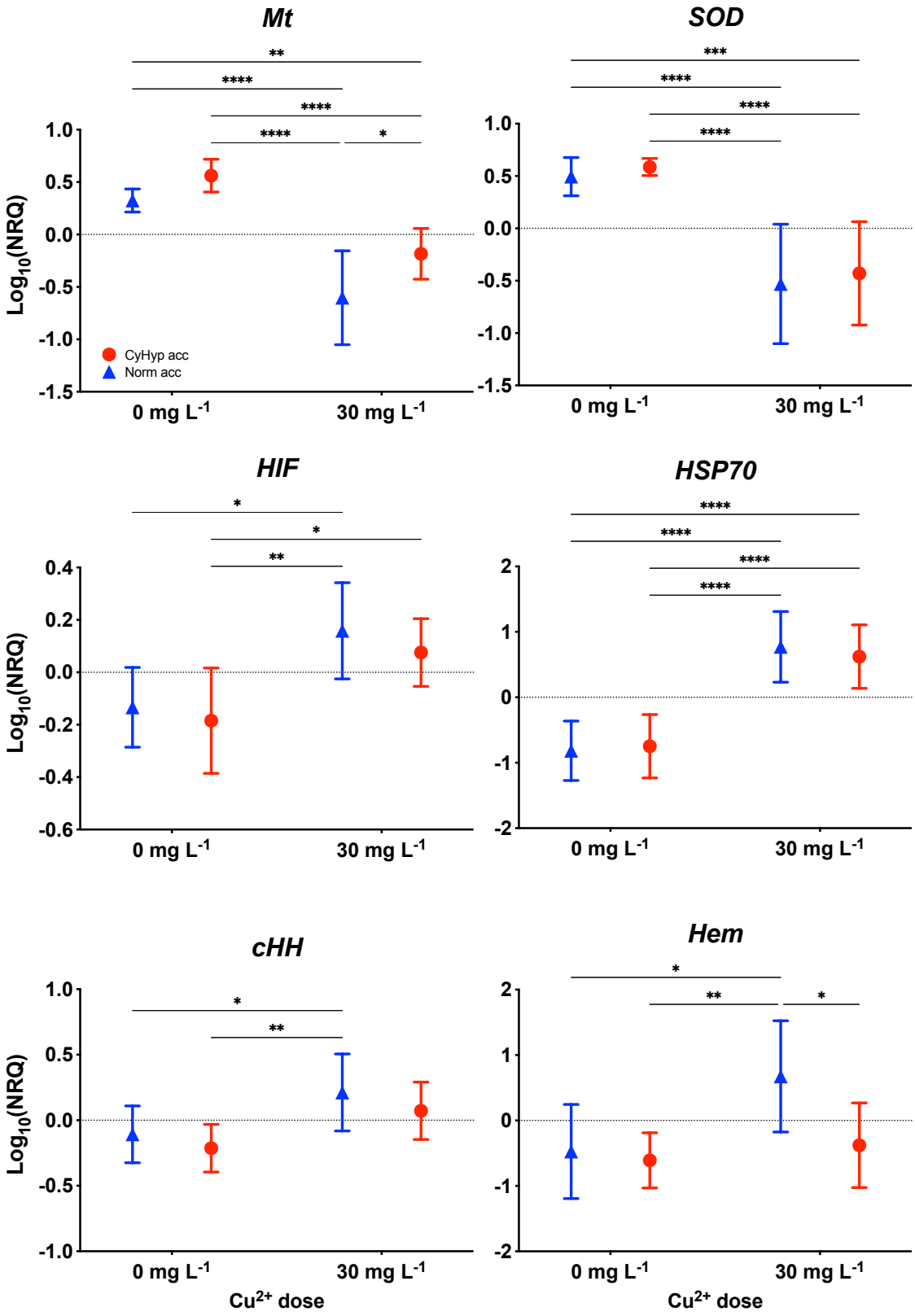

Figure 5: Gene expression in adult *P. varians* exposed to acute Cu^{2+} toxicity (mean ±SD, n=7-9 animals per each treatment per each time point). Stars indicate statistical difference

following Two-way ANOVA test (Tukey's multiple comparisons test, *: p-value <0.05; **: p-value <0.01; ***: p-value <0.001; ****: p-value <0.0001). Mt: metallothionein; SOD: super oxide dismutase; HIF: hypoxia inducible factor; HSP70: heat shock protein 70; cHH: crustacean hyperglycaemic hormone; Hem: hemocyanin.

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