Anthropogenic CO₂-mediated freshwater acidification limits survival, calcification, metabolism, and behaviour in stress-tolerant freshwater crustaceans

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Abstract. Dissolution of anthropogenic CO₂ is chronically acidifying aquatic ecosystems. Studies indicate that ocean acidification will cause marine life, especially calcifying species, to suffer at the organismal and ecosystem levels. In comparison, freshwater acidification has received less attention rendering its consequences unclear. Here, juvenile Chinese mitten crabs, *Eriocheir sinensis*, were used as a crustacean model to investigate the impact of CO₂-mediated freshwater acidification. Our integrative approach, investigating changes in the animal's acid-base homeostasis, metabolism, calcification, locomotory behaviour, and survival rate, indicates that this economically relevant crustacean will face energetic consequences from future freshwater acidification. These energetic trade-offs allow the animal to maintain its acid-base homeostasis at the cost of reduced metabolic activity, exoskeletal calcification, and locomotion, reducing the animal's overall fitness and increasing its mortality. Results indicate that present-day Chinese mitten crab could be heavily affected by freshwater acidification like their marine counterparts and emphasizes the importance ofin understanding the long-term implications of freshwater acidification on species' fitness.

1 Introduction

Rising levels of atmospheric CO₂ partially dissolve into marine systems, causing a decrease in oceanic pH referred to as ocean acidification. In marine species, ocean acidification has been demonstrated to negatively impact development, metabolism, behaviour, and biomineralization, potentially leading to major ecosystem-level changes (Kroeker et al., 2013; Melzner et al., 2009; Tresguerres and Hamilton, 2017). It is generally believed that freshwater systems will also experience acidification (Hasler et al., 2016; Phillips et al., 2015; Weiss et al., 2018).; however, However, tthe highly variablehigh variability in biogeochemistry between freshwater systems has been a limiting factor in modellingmodelling future freshwater scenarios (Hasler et al., 2016)(Hasler et al., 2016; Phillips et al., 2015; Weiss et al., 2018). Two recent case studies on different freshwater

systems have suggested that the magnitude of CO_2 mediated acidification could be similar or even exceed predicted levels of ocean acidification (Phillips et al., 2015; Weiss et al., 2018). The potential that freshwater acidification may be of equal or greater severity than ocean acidification emphasizes the need to understand the biological responses and consequences to

35   freshwater species.

~~Amongst marine organisms, calcifying~~Calcifying species are ~~particularly~~ sensitive to acidification as dissolution of CO_2 reduces carbonate availability in parallel to pH, potentially increasing dissolution of their calcified exoskeleton (Feely et al., 2004; Roleda et al., 2012). To date~~,~~ ~~there are~~ no comprehensive studies have investigat~~ed~~ing the various physiological and behavioural effects of realistic future levels of CO_2-mediated acidification ~~in~~on calcifying freshwater invertebrates. However,

40   several studies have used high CO_2 levels beyond that relevant for potential future freshwater acidification to investigate acid-base regulation and calcification in freshwater calcifying invertebrates (Cameron, 1978, 1985; David et al., 2020; Jeffrey et al., 2018b, 2018a). Freshwater calcifying macro-organisms are largely limited to crustaceans and molluscs that comprise roughly 10% and 4% of freshwater species diversity, respectively (Balian et al., 2008). Crustaceans are arguably one of the most successful animal groups ~~having occupied~~occupying almost all ecological niches across the globe, including freshwater,

45   marine, and terrestrial habitats, making them a suitable ~~good~~ model to study global change consequences in a physiologically and ecologically robust group of species. Freshwater crustaceans occupy a key position in food webs where all crustacean life stages provide a vital food source for a wide range of juvenile and adult predators (Cumberlidge et al., 2009). Additionally, freshwater crustaceans provide vital ecological services as indicators of water quality, nutrient cycling of detritus and bioturbation of sediment (Cumberlidge et al., 2009). From an economic standpoint, freshwater crustaceans account for ~30 %

50   (2.5 million tons) of aqua-cultured crustaceans worldwide, demonstrating that this group is an important human food source (Tacon, 2020). The ecological and economic importance of freshwater crustaceans, together with the apparent sensitivity of calcifying species to acidification based on marine studies, makes it imperative to determine whether freshwater crustaceans are sensitive to anthropogenic CO_2-mediated freshwater acidification.

The Chinese mitten crab (*Eriocheir sinensis*) is one of the most important freshwater crustaceans, accounting for the third

55   largest crustacean aquaculture globally (FAO, 2018). This highly invasive catadromous species spends most of its life cycle in freshwater systems but has the physiological plasticity to migrate into marine environments where it reproduces (Veilleux and Lafontaine, 2007). The invasive status and aquacultural importance of *E. sinensis* ha~~ve~~s made it a well-studied freshwater crustacean model in biological research. Here, we used the juvenile life stage of *E. sinensis* as a freshwater crustacean model to investigate the effects of a potential future CO_2-mediated freshwater acidification scenario on acid-base regulation,

60   metabolism, calcification, behaviour, and survival rate. Native to China's Yangtze river system, the third-largest river system in the world, juvenile *E. sinensis* in this habitat already experience regular fluctuations in freshwater pCO_2 from 681 to ~~-~~3796 µatm (Ran et al., 2017), which may confer some ~~degree of~~ pre-adaptation to elevated CO_2 ~~due to~~because of life history. ~~C~~Furthermore, crustaceans are ~~generally~~ believed to be more CO_2 tolerant than other calcifying organisms such as bivalves and coral ~~due to~~because of their high metabolic activity and robust acid-base machinery, allowing for ~~a~~ more efficient

65   compensation of acid-base disturbances (Melzner et al., 2009). These combined predictors of CO_2 tolerance make *E. sinensis*

an interesting model to study the effects of future CO_2 mediated freshwater acidification, as they may already possess the adaptations necessary to deal with future freshwater acidification conditions. Therefore, we hypothesized that *E. sinensis* would be well-adapted to counteracting challenges associated with fluctuating pCO_2 resulting from anthropogenic activity and not experience detrimental physiological or behavioural impairment.

2 Methods

2.1 Animal Maintenance

Wild-caught male and female juvenile Chinese mitten crab (*Eriocheir sinensis* 10-20 g) were purchased from the Chinese mitten crab Breeding Association of Taiwan. Crabs were maintained at the Academia Sinica Institute of Cellular and Organismal Biology aquatics facility (Taipei, Taiwan) in three 120-L aquariums with flow through dechlorinated Taipei tap water (in μmol l^{-1} Na^+ 237, K^+ 16, Ca^{2+} 216, Mg^{2+} 213, Cl^- 201; Y.C. Tseng pers. comm. For methods seeSee ringer measurement methods below) on a 14:10 h light-dark cycle with temperature ranging from 23 to -25 ºC. Water parameters for these holding tanks were the same as that of the control water used in the experimental acclimations. Juvenile crabs in non-experimental holding tanks were maintained at a density of roughly 100 individuals per tank with PVC pipes for shelter and a constant flow of freshwater to prevent the build-up of metabolic wastes. Crabs were fed *ad libitum* with oatmeal and mollusc meat three times per week and monitored for activity level and the presence of disease as general health indicators. Diet was selected to maintain an omnivorous diet as seen in the wild (Czerniejewski et al., 2010) and based on what is fed by our crab supplier (Y.C. Tseng pers. comm.). Crabs were fasted for a minimum of 48 hours prior tobefore sampling to minimize the effects of dietary intake on measured parameters.

2.2 Freshwater acidification

For experimental acclimation, crabs were sampled upon removal from the holding tanks (0-day time point) and transferred to flow through 10-L experimental tanks (6-7 crabs per tank, 4 tanks per treatment) containing either control or acidified freshwater (Table 1) with PVC pipes added for shelter. Acidified freshwater was achieved by injection of CO_2 directly into the experimental tanks by air stone to maintain environmental pH (pH controller, Aqua-MACRO). The pH controller system used in this study required that each tank had its owna pH probe, pH controller, CO_2 tank, gas regulating solenoid and air stone, thus meaning each tank in this study was independently pH/CO_2 regulated. CO_2 bubbling rate and freshwater flow rate wereas adjusted to minimize overshooting the target pCO_2 level. Following injection of CO_2 to regulate water pCO_2 we recorded a brief pCO_2 overshoot to a maximum level of 5625 μatm resulting from direct CO_2 injection into the experimental tanks by the pH controller. Water pH, total alkalinity, and temperature were regularly measured in the experimental tanks throughout the study. Water pH (NBS scale) and temperature were measured with a pH electrode (Accumet AP55 pH/ATC electrode, Ohio, USA) connected to a portable pH meter (Accumet AP71, Ohio, USA) calibrated with pH buffers (pH 4.00, 7.00, and 10.01) traceable to NIST standard reference material (Thermofisher Orion). Water alkalinity was measured by

spectrophotometric assay on a Nanodrop 2000c (Thermo scientific, Wilminton, DE, USA) according to previously established protocols (Sarazin et al., 1999). Water pCO_2 was calculated with the CO2SYS excel add-in (Lewis and Wallace, 1998) using measured water temperature, pH and total alkalinity. Constants used for pCO_2 calculations include freshwater carbonate dissociation constants (K_1 and K_2) from Millero (1979), and $KHSO_4$ constants from Dickson (1990).

Table 1. Measured tank parameters for 7-day, 14-day and 42-day experiments of control and CO_2 acidified freshwater (FW). Measured parameters include temperature, pH (NBS scale), total alkalinity (TA), total CO2 (TCO_2), and partial pressure of CO2 (pCO_2).

	Temp (°C)	pH	TA (µmol l^{-1})	TCO_2 (µmol l^{-1})	pCO_2 (µatm)
Control 7 day	23 ± 0.15	7.41 ± 0.02	501 ± 32	547 ± 36	1299 ± 121
Acidified 7 day	23 ± 0.15	6.73 ± 0.01	430 ± 13	614 ± 18	5109 ± 157
Control 14 day	24.6 ± 0.05	7.4 ± 0.01	517 ± 4	563 ± 5	1364 ± 46
Acidified 14 day	24.5 ± 0.13	6.8 ± 0.01	429 ± 7	589 ± 7	4633 ± 87
Control 42 day	24.5 ± 0.07	7.4 ± 0.01	529 ± 6	576 ± 7	1389 ± 31
Acidified 42 day	24.4 ± 0.1	6.8 ± 0.01	433 ± 4	592 ± 4	4634 ± 58

2.3 Hemolymph Acid-Base Status

Hemolymph acid-base experiments were conducted over ~~a period of~~ seven days to determine if crabs ~~were capable of actively regulating~~ could actively regulate acid-base status in the presence of future freshwater acidification conditions. Hemolymph samples (100 µL per crab) were taken at the base of a walking leg with a sterile syringe according to previous protocols for *E. sinensis* (Truchot, 1992). Samples from 2 to ~~3~~ crabs were pooled together (200-300 µL pooled hemolymph per n value) to ~~obtain enough sample~~get a sufficient volume for downstream analys~~e~~is of ammonia, pH, and total carbon. Pooled hemolymph samples were gently mixed by slowly pipetting to avoid off gassing of CO_2 and ~~thereby~~ disrupting hemolymph acid-base parameters. Measurements of pH and total carbon were performed immediately after hemolymph collection and the remaining hemolymph was frozen at -20 °C for later analysis of ammonia. Hemolymph pH (200-300 µL samples) was measured in NBS scale using an InLab micro pH electrode calibrated with pH buffers traceable to NIST standard reference material (Thermofisher Orion). Hemolymph total carbon was measured in duplicate (50 µL per measurement) using the Corning 965 carbon dioxide analy~~z~~ser (± 0.2 mmol l^{-1} precision) calibrated with $NaHCO_3$ standards ranging from 0 to ~~2~~0 mmol l^{-1} to produce a standard curve with a minimum R^2 of 0.99. Hemolymph pCO_2 and HCO_3^- were calculated using a rearrangement of the Henderson-Hasselbalch equation with pK1 and $αCO_2$ values derived for *E. sinensis* hemolymph at 23 °C (pK1= 6.079773, $αCO_2$= 0.00031263 mmol l^{-1} Pa^{-1} (Truchot, 1976, 1992). Hemolymph ammonium was measured in triplicate (25 µL hemolymph per measurement) with a microplate reader (Molecular Devices, SpectraMax, M5) using an orthophthaldialdehyde fluorometric assay which is insensitive to amino acids and proteins (Holmes et al., 1999). Ammonia standards were made from

NH4Cl in *E. sinensis* ringer (pH 8.1) containing (in mmol l^{-1}): 185 NaCl, 16 CaCl$_2$, 6 MgCl$_2$, 7 KCl, and 13 NaHCO$_3$. The ion concentrations for the ringer were based on ion composition measurement done on 4 juvenile Chinese mitten crabs in this study (in mmol l^{-1} Na$^+$ 191, K$^+$ 7.2, Ca^{2+} 16.3, Mg^{2+} 5.9, Cl$^-$ 252). Concentrations of Na$^+$, K$^+$, Ca^{2+}, Mg^{2+} were measured by flame absorption spectrophotometry (Polarized Zeeman Atomic Absorption Spectrophotometer Z-5000, Hitachi High-Technologies, Tokyo, Japan), Cl$^-$ was measured spectrophotometrically using the mercury (II) thiocyanate method (Florence and Farrar, 1971). HCO$_3^-$ and pH values for the ringer were based on measurements taken from control crabs in this study and measured as described above.

2.4 Ammonia excretion and oxygen consumption

Ammonia excretion and oxygen consumption were measured over a seven-day acclimation to control and acidified freshwater. These two parameters were measured on individual crabs haphazardly selected from the four control and four acidified freshwater aquaria. A~~Experimental sampling of a~~mmonia excretion and oxygen consumption measurements were performed in parallel to hemolymph sampling however, crabs were first randomly selected and placed into respirometry chambers before selecting crabs for hemolymph sampling to avoid using crabs recently sampled for hemolymph. Ammonia excretion experiments were performed in plastic Tupperware filled with 200 mL of filtered control or acidified freshwater. Crabs were given 30 minutes to acclimate to the experimental chambers ~~prior to~~before initiation of water sampling, as ammonia excretion is elevated for a short time directly after handling (Hans et al., 2014). Water samples (1 mL) for ammonia analysis were collected directly after 30 and 90 minutes of being placed in the experimental chambers. Ammonia concentrations of the water at the 30 and 90--minute time points were determined using the aforementioned orthophthaldialdehyde fluorometric assay (Holmes et al., 1999). Ammonia excretion rates were calculated according to the Eq. (1):

$$Ammonia\ excretion\ rate = \frac{([Amm_{90}] - [Amm_{30}]) * V}{t * m}, \tag{1}$$

where Amm$_{90}$ is the water ammonia concentration at 90 minutes, Amm$_{30}$ is the water ammonia concentration at 30 minutes, V is the chamber volume during the flux period in litres, t is the flux time in hours, and m is the fresh weight of the crab in grams.

The oxygen consumption rate was measured by closed-system respirometry in custom-made 3 L glass respiration chambers containing filtered (0.2 μm) freshwater. To achieve the correct experimental CO$_2$ tension, respirometry chambers were submerged in large 18 L buckets of filtered freshwater and a~~which used a~~ pH controller (Aqua-MACRO) ~~to measure pH and inject CO$_2$ to maintain the desired CO$_2$ tension~~was used to regulate the injection of CO$_2$ as described above for experimental tanks. Crabs were transferred to the submerged respirometry chambers and given 15 minutes to adjust to fully oxygenated respiration chambers before being sealed. Chambers were placed horizontally, allowing for lateral crab movement in the chamber, and oxygen saturation was ~~continuously~~ measured continuously every 15 seconds for 30 minutes at 23 °C. The oxygen sensor (PreSens oxygen micro optode, type PSt1, PreSens Precision Sensing GmbH, Regensburg, Germany) was attached to the top of the chamber and connected to an OXY-4 mini multichannel fibre~~r~~ optic oxygen transmitter (PreSens

Precision Sensing GmbH, Regensburg, Germany). Oxygen saturation was ~~never allowed to drop below~~ always maintained above 80%.~~and R~~respiration chambers without a crab were used to determine any potential background bacterial respiration for each trial. Preliminary trials demonstrated that crab movement and ventilation rate in the chamber was sufficient to mix the water within the chamber and prevent oxygen stratification, as indicated by a linear decline in oxygen availability. While this approach allows for the measurement of oxygen consumption, ~~there are some limitations which~~some limitations must be considered. ~~Due to logistical constraints, we were unable to use an~~Logistical constraints prevented the use of an intermittent flow respirometry approach where the animal could have been given a long amount of time to acclimate to the respirometry chamber. This technical limitation means that the reported measurements ~~in this study~~cannot be considered a resting metabolic rate as the handling stress, brief air exposure and transfer to a novel environment may have influenced the animal's metabolic rate. However, we would like to point out that in previous trials from our lab using an intermittent flow respirometry setup on green crabs *Carcinus maenas*, crayfish *Procambarus clarkii*, and lobsters *Homarus americanus*, that crustaceans placed in respirometry chambers will stabilize oxygen consumption to a resting rate in under 30 minutes (Gwangseok R. Yoon pers. comm).

2.5 Carapace calcification

To assess carapace calcification, changes in the calcium content relative to carapace mass was measured at one, two, three and six weeks of high CO_2 exposure according to previously established protocols (Spicer and Eriksson, 2003). In brief, a piece of carapace (ca. 2.5 cm^2, 15.2 ± 0.4 mg) was removed from the dorsal carapace. The weighed piece of carapace was digested in HNO_3 (13.1 N) at 60°C for 16 hours. Digested samples were then diluted to a final HNO_3 concentration of 2 % v/v. The carapace Ca^{2+} content was measured by atomic absorption spectrophotometer (Z-8000; Hitachi). Standard solutions from Merck (Darmstadt, Germany) were used to make the Ca^{2+} standard curve.

2.6 Locomotory Behaviour Assay

A 24 x 24 cm square, novel, opaque tank was used in the open field test to assess changes in movement of juvenile crabs after a seven-day exposure to control and freshwater acidified conditions. Acclimated crabs were transferred to the novel tank containing control or acidified freshwater and given 5 minutes to acclimate, as done in previous crustacean behavioural studies (Robertson et al., 2018). After acclimation, crab activity was recorded with a digital camera (UI-3240CP Rev.2, Ids, Germany) for 5 minutes (300 seconds) and videos of the movement were processed with the image analysis Ethovision XT motion tracking software (v. 7.0, Noldus, Netherlands). In this study, ~~4~~four factors were measured; distance covered (cm), velocity (cm/s), movement (time in movement, seconds) and mobility (time in mobile state, seconds). We defined movement as the duration for which the central body point (whole body) was changing location. Mobile state was defined as the duration in which crabs exhibited any movement, even if the center point of the animals remained in the same location, for example, appendage movement.

2.7 Statistical analysis

Statistical analyses were conducted using JMP Pro 16 (Cary, NC, USA) and GraphPad Prism 8.4.2 (San Diego, CA, USA). Data were analyzed for outliers by the ROUT test with a Q value of 1 %. For all data, heterogeneity of variance was tested by Levene's test and normal distribution of residuals by Shapiro-Wilk test. Two transformations were done so that data could meet the assumptions of normal distribution and homogeneity of variance. A Johnson SB transformation was applied to hemolymph pCO_2 data and a square root transformation was applied to ammonia excretion rate data. In this study, hemolymph parameters, ammonia excretion, and oxygen consumption data were analyzed by a two-way ANOVA post hoc Dunnett's test. For Dunnett's test, comparisons were made to the zero-day control with time and pCO_2 values as fixed factors. Carapace calcification data were analyzed by a two-way ANOVA post hoc Tukey HSD with time and pCO_2 values as the fixed factors. Behavioural data displayed a high degree of co-linearity between dependent variables, violating the assumptions of the MANOVA test. Therefore, we analyzed behavioural data using a student's t-test except for appendage movement time. Appendage movement time data violated assumptions of student's t-test, so were analyzed by the Wilcoxon test. Survival curves were analyzed for significant differences by the Mantel-Cox test. The survival curve hazard ratio was determined by the Mantel-Haenszel test. For all data sets, p values ≤ 0.05 were considered significant. Data are presented as mean ± standard error (SEM). Statistical output results are written in text or summarized in Table 2.

3 Results

3.1 Probability of Survival

The effect of freshwater acidification on survival was determined by generating survival curves for crabs in control and acidified freshwater (Fig. 1). There was a significant difference in the probability of survival between the control and acidified freshwater environments (Mantel-Cox log rank test, $X^2_1 = 9.41$, p=0.0022, Fig. 1). Crabs in the acidified freshwater tanks had a 50% mortality, compared to 15 % mortality in control freshwater tanks. Calculation of the Mantel-Haenszel hazard ratio indicates that crabs in acidified freshwater have a 3.68 times greater probability of mortality than the crabs held under control conditions.
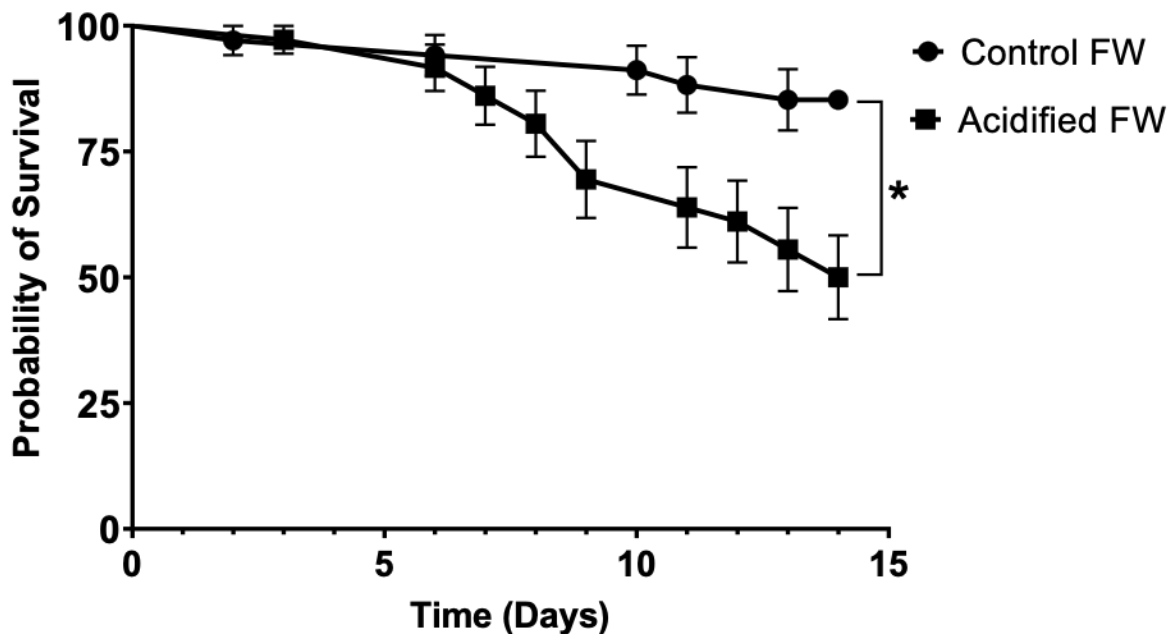
Figure 1. Survivorship curves of juvenile Chinese mitten crab, *Eriocheir sinensis*, over 14 days of exposure to control (pH 7.4, 1364 μatm pCO₂) or CO₂-acidified (pH 6.8, 4633 μatm pCO₂) freshwater. Data are presented as probability of survival +/- SEM. (N=34 for control freshwater and N=36 for acidified freshwater). Statistical significance was assessed by Mantel-Cox test * indicating significant difference between probability of survival between control and freshwater acidified crab populations.

3.2 Acid-base status

Chinese mitten crab maintained in control freshwater showed no changes in hemolymph pH, bicarbonate, pCO_2, or ammonia throughout the experimental time course (Fig. 2; Table 2). In contrast, acidified freshwater had a significant effect on hemolymph pH, bicarbonate, pCO_2, or ammonia (Fig. 2; Table 2). Exposure to acidified freshwater induced a respiratory acidosis indicated by a decline in hemolymph pH (pH 8.11 ± 0.015 to 8.03 ± 0.0019) and an increase in hemolymph pCO_2 (404 ± 23 Pa to 486 ± 26 Pa; 1 μatm = 0.101325 Pa) within the first six hours of exposure (Fig. 2a, c). ~~This~~ Hemolymph acidosis was maintained for two days. ~~with full~~ ~~R~~ recovery of hemolymph pH ~~occur~~ occurred ~~ring~~ by day seven, ~~of exposure when hemolymph pH returned to control levels,~~ although hemolymph pCO_2 remained elevated (499 ± 20 Pa). Recovery of hemolymph pH coincided with increases in hemolymph HCO_3^- (16.7 ± 0.78 mmol l⁻¹) and ammonia (136 ± 2.9 μmol l⁻¹; Fig. 2b, d).~~;~~ ~~N~~however, ~~n~~o significant changes in hemolymph HCO_3^- and ammonia were observed until seven and two days of exposure, respectively, suggesting a delayed extracellular pH regulatory response.
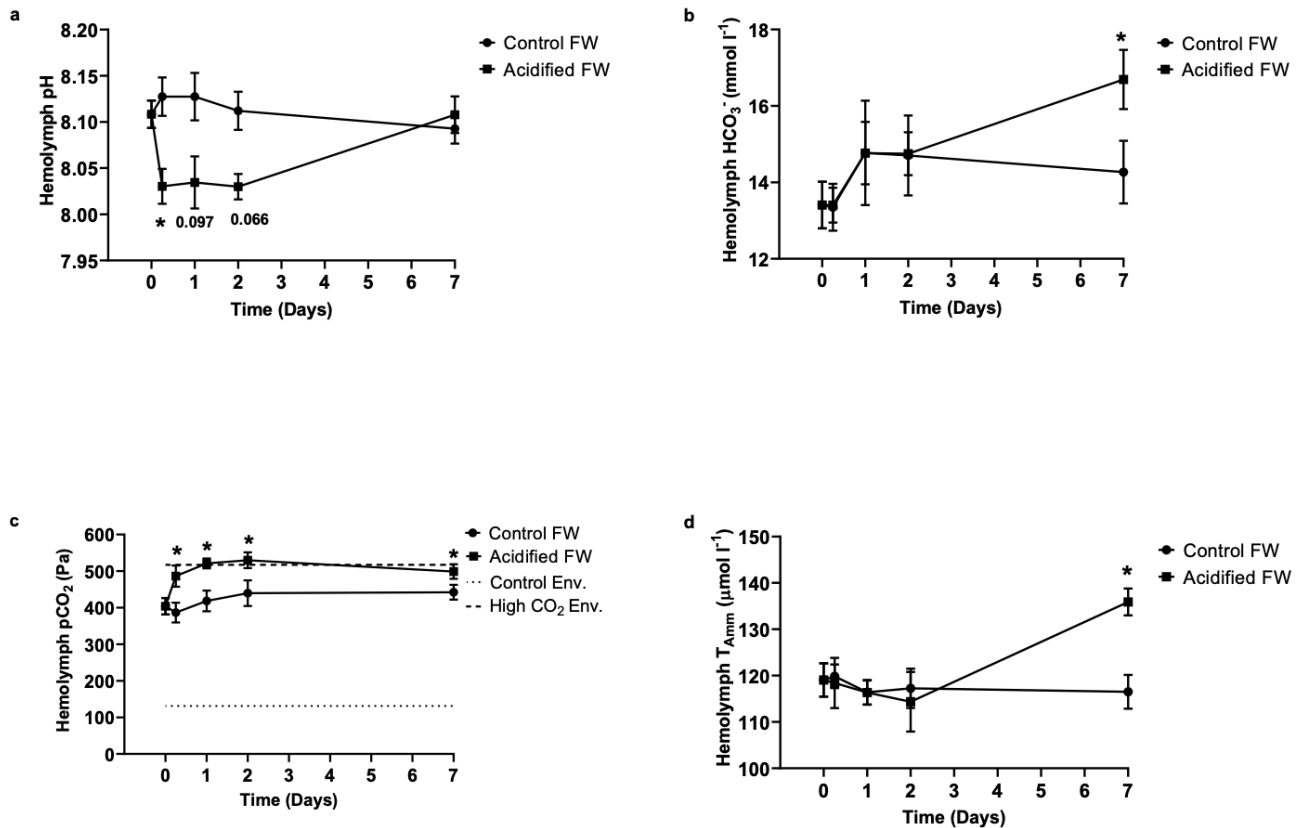
Figure 2. Changes in extracellular (a) pH, (b) HCO₃⁻, (c) pCO₂, and (d) ammonia of juvenile Chinese mitten crab, *Eriocheir sinensis*,
during a 7-day time course of exposure to control (pH 7.41, 1299 μatm pCO₂) or CO₂-acidified (pH 6.73, 5109 μatm pCO₂)
freshwater. Data are presented as mean +/- SEM. (N=6-14, 2-3 crabs pooled per N value). Statistical significance was assessed by
two-way ANOVA followed by a post-hoc Dunnett's test with * indicating significant difference from day zero measurements. P-
values near but not <0.05 are written above corresponding data point.

3.3 Metabolism

Metabolic changes were quantified through ~~an individuals'~~ ammonia excretion rate ~~as an indicator of potential shifts in protein catabolism and their oxygen consumption rate as an indicator of changes in aerobic metabolism.~~ and oxygen consumption rate. Ammonia excretion rate was used as an indicator of potential shifts in protein catabolism. Oxygen consumption rate was used as an indicator of changes in aerobic metabolism. Control crabs exhibited steady oxygen consumption rates and ammonia excretion rates throughout the measured time course (Fig. 3; Table 2). ~~In contrast, crabs~~ Crabs exposed to freshwater acidification experienced a significant reduction in oxygen consumption rate within six hours that was maintained throughout the ~~remainder~~ rest of the time course (Fig. 3a; Table 2). Ammonia excretion rates were also significantly affected by ~~acidified~~ freshwater acidification (Fig. 3b; Table 2.). Initially, ammonia excretion rates were unchanged until the second day of exposure

On the second day of exposure, ammonia excretion rates doubled

245 and remained elevated for the duration of the seven-day time course (Fig. 3b).
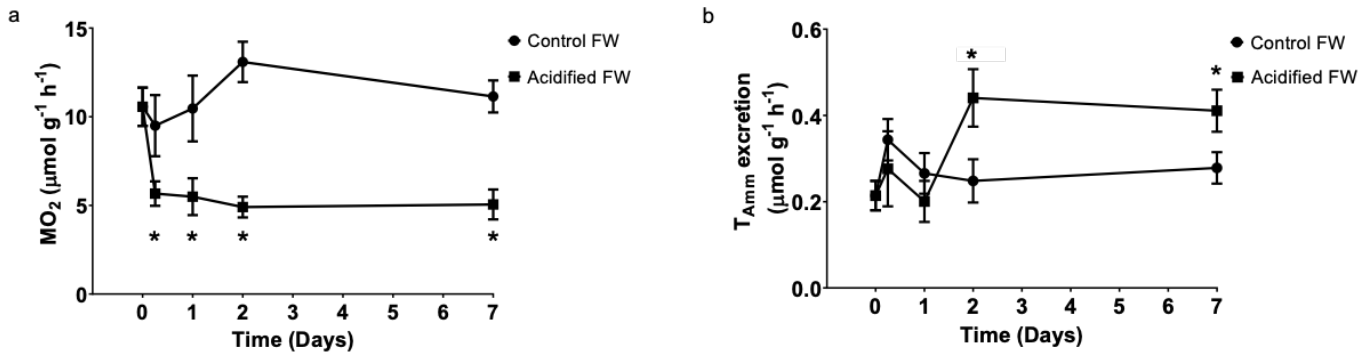
Figure 3. Changes in whole animal (a) oxygen consumption rate (MO$_2$) and (b) ammonia excretion rate of juvenile Chinese mitten crab, *Eriocheir sinensis*, during a 7-day time course of exposure to control (pH 7.41, 1299 μatm pCO$_2$) or CO$_2$-acidified (pH 6.73, 5109 μatm pCO$_2$) freshwater. Data are presented as mean +/- SEM. (N=5-6 for oxygen consumption and N=7-12 for ammonia excretion). Statistical significance was assessed by two-way ANOVA followed by a post-hoc Dunnett's test. Significant differences from day zero measurements are indicated by *. P-values near but not <0.05 are written above corresponding data point.

3.4 Carapace calcification

Changes in calcification were quantified as the change in the crab's exoskeletal calcium content following exposure to

255 freshwater acidification conditions. Calcification was measured several times over a six-week acclimation, as several studies on marine crustaceans report changes in calcification after 20+ days of acclimation (Long et al., 2013; Ries et al., 2009; Taylor et al., 2015). Overall, there was a significant time, pCO$_2$ and interactive time and pCO$_2$ effect on calcification (Table 2). Post hoc analysis suggests there were no significant changes in carapace calcification in the first two weeks of exposure to freshwater acidification (Fig. 4). However, after three and six weeks of exposure, a significant decline in carapace calcium

260 content to 84.1 ± 2.9 % and 85.2 ± 3.3 % of control crab levels was observed (Fig. 4).
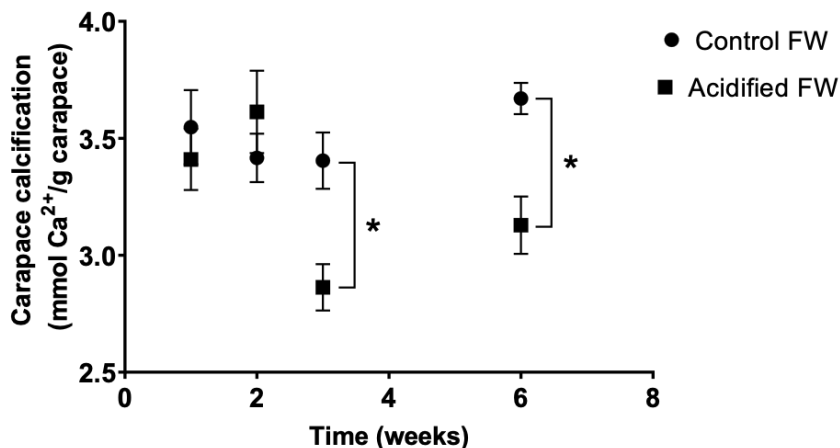
Figure 4. Changes in carapace calcium content of juvenile Chinese mitten crab, *Eriocheir sinensis*, over a 6-week exposure to control (pH 7.4, 1389 µatm pCO_2) or CO_2-acidified (pH 6.8, 4634 µatm pCO_2) freshwater. Data are presented as mean +/- SEM. (N=6-12). Statistical significance was assessed by two-way ANOVA followed by a post-hoc Tukey HSD test with * indicating significant difference between control and acidified FW crabs for each respective week.

Table 2. Statistical results of two-way ANOVAs from hemolymph acid-base parameters, oxygen consumption, ammonia excretion and carapace calcification experiments. For hemolymph acid-base measurements response variables were hemolymph pH, HCO_3^-, pCO_2, or ammonia with time and CO_2 as fixed independent variables. For whole animal experiments response variables were oxygen consumption rate and ammonia excretion rate with time and CO_2 as fixed independent variables. For carapace calcification experiments, the response variable was carapace calcium content with time and CO_2 as fixed independent variables. P-values below 0.05 are considered statistically significant and are bolded.

Two-way ANOVA

Dependent Variable	Independent Variable	df	df_{error}	F ratio	p-value
Hemolymph pH	Time	4	84	1.36	0.25
	CO_2	1	84	15.63	**0.0002**
	Time x CO_2	4	84	3.8	**0.0068**
Hemolymph HCO_3^-	Time	4	84	2.85	**0.028**
	CO_2	1	84	0.94	0.33
	Time x CO_2	4	84	0.83	0.51
Hemolymph pCO_2	Time	4	84	3.22	**0.016**
	CO_2	1	84	16.24	**0.0001**
	Time x CO_2	4	84	1.74	0.15
Hemolymph Ammonia	Time	4	74	2.52	**0.048**
	CO_2	1	74	1.41	0.24
	Time x CO_2	4	74	3.33	**0.015**

O_2 consumption	Time	4	47	2.04	0.1
	CO_2	1	47	40.95	**<0.0001**
	Time x CO_2	4	47	3.39	**0.016**
Ammonia excretion	Time	4	76	3.01	**0.023**
	CO_2	1	76	0.7	0.4
	Time x CO_2	4	76	2.91	**0.027**
Carapace Ca^{2+}	Time	3	68	3.98	**0.011**
	CO_2	1	68	8.59	**0.0046**
	Time x CO_2	3	68	4.33	**0.0074**

3.5 Locomotory Behaviour Assay

An open field test was used to quantify locomotory behavioural changes over a five-minute recording period in a novel arena (Table 3). Crabs exposed to acidified freshwater ~~on average moved less distance in the novel arena~~ covered less distance than crabs in control freshwater (student's t-test, t_{35}=-2.5, p=0.017, Table 3). Crabs in acidified freshwater also had a lower velocity than crabs in control freshwater after the seven-day exposure (student's t-test, t_{35}=-2.37, p=0.024, Table 3). Movement and mobility were also quantified.~~,~~ M~~where m~~ovement was defined as the crab changing its relative location in the arena.~~ and~~ M~~m~~obility was defined as the movement of body appendages even if the crab's location did not change. There was a significant decrease in movement (student's t-test, t_{35}=-2.55, p=0.015, Table 3) and mobility (Wilcoxon test, Z=2.08, p=0.037, Table 3) following the seven-day exposure to acidified freshwater.

Table 3. Changes in locomotory behaviour of juvenile Chinese mitten crab, Eriocheir sinensis, after a seven-day exposure to control (pH 7.41, 1299 µatm pCO₂) or CO₂-acidified (pH 6.73, 5109 µatm pCO₂) freshwater. Data are presented as mean +/- SEM. (N=18-19). Statistical significance was assessed by student's t-test or Wilcoxon test for mobility time with * indicating significant difference between control and acidified FW treatments.

	Distance moved (cm)	Velocity (cm s^{-1})	Movement time (s)	Mobility time (s)
Control FW	761 ± 46	2.53 ± 0.15	148 ± 7	215 ± 5
Acidified FW	601 ± 45*	2.04 ± 0.15*	119 ± 9*	179 ± 14*

4 Discussion

Anthropogenically driven aquatic acidification has the potential to negatively impact both freshwater and marine life. Meta-analyses of biological responses to ocean acidification suggest that marine crustaceans generally experience minimal consequences to pCO₂ tensions (~1000 µatm) predicted to occur by the year 2100 (Kroeker et al., 2013; Melzner et al., 2009;

Wittmann and Pörtner, 2013). Acidificationwith further acidification to levels expected for the year 2300 (~2000 µatm) negatively impacting impacts about half of the studied marine crustaceans (Wittmann and Pörtner, 2013)(Kroeker et al., 2013; Melzner et al., 2009; Wittmann and Pörtner, 2013). In contrast, the biological responses of any freshwater invertebrate to realistic future CO_2 mediated freshwater acidification remains unknown. In the present study, we aimed to demonstrate the physiological and behavioural consequences of a possible future CO_2 mediated freshwater acidification scenario on a juvenile freshwater crustacean, the Chinese mitten crab, *Eriocheir sinensis*. Our results suggest that freshwater juvenile Chinese mitten crab experience significant impairment of metabolism, calcification, locomotory behaviour and survival when exposed to a potential future freshwater acidification (4633-5109 µatm pCO_2). While extracellular acid-base status is successfully regulated, the The high energetic demands to sustain essential physiological processes such as acid-base regulation may be causingcause energetic reallocation that leads to a trade-off that impairs several physiological processes and alters animal fitness.

4.1 Plausibility of Freshwater Acidification Conditions

Modelling of future CO_2 mediated freshwater acidification for the year 2100 is nearly non-existent, making the plausibility of the pCO_2 levels used in this study difficult to assess. The control pCO_2 levels used in this study reflect the average pCO_2 measured in 13 stations along the mainstem of the Yangtze River system (excluding Nanjing station which is at the mouth of the river and influenced by coastal upwelling) (Ran et al., 2017). The future freshwater acidification conditions used in this study represents a 3500+ µatm increase in pCO_2 from control levels. This increase isand roughly 1000+ µatm higher than the highest average level recorded by the 13 stations along the mainstem of the Yangtze river (Ran et al., 2017). While future CO_2 mediated acidification models are not available for the Yangtze River, the relationship between changes in freshwater pCO_2 in other freshwater systems as a response to changes in atmospheric pCO_2 may provide indications of plausible future increases in pCO_2. Weiss et al. (2018) tracked changes in pCO_2 of four freshwater bodies in Germany between 1981-2015 and reported that freshwater pCO_2 increased by an average of 561 µatm over this time period while atmospheric pCO_2 increased by ~60 µatm from 340 to 399 µatm (National Oceanic and Atmospheric Administration; www.esrl.noaa.gov/gmd/dv/iadv). This relationship suggests that for every 1 µatm increase in atmospheric pCO_2, these freshwater bodies increased by 9.35 µatm. Since atmospheric pCO_2 is projected to rise to approximately 985 µatm by the year 2100 (IPCC, 2013) this would mean that freshwater pCO_2 in these systems could rise by as much as 5469 µatm. Assuming this relationship is accurate, the pCO_2 levels used in this study would be within a range that could feasibly occur in the Chinese mitten crab's native environment by the year 2100. Further, it should be noted that while freshwater systems average pCO_2 levels of 3100 µatm (streams and rivers) and 1410 µatm (lakes), the pCO_2 levels used for acidified freshwater in this study are within ranges that can already be seen in freshwater systems globally (Raymond et al., 2013). Ffor example, the Mackenzie, Mississippi, Ohio and Elbe rivers suggesting that acidification scenario used in this study is conceivable for freshwater (Cole and Caraco, 2001; Raymond et al., 2013).

4.2 Probability of Survival

Sensitivity to aquatic acidification is quite variable in marine crustaceans. In mid to high intertidal and burrowing species including porcelain crabs (*Petrolisthes cinctipes*, *Petrolisthes manimaculus*, and *Porcellana platycheles*), burrowing shrimp (*Upogebia deltaura*), and barnacles (*Semibalanus balanoides* and *Elminius modestus*), minimal changes in survival probability are reported at pCO_2 tensions ranging from 1395 ~~to~~ -2707 µatm (Donohue et al., 2012; Findlay et al., 2010; Page et al., 2017). Presumably, the variability in CO_2 levels experienced in burrows and intertidal zones has driven the evolution of adaptation for greater CO_2 tolerance in these groups of crustaceans. We predicted that juvenile Chinese mitten crab would also have an elevated CO_2 tolerance and face minimal changes in survival probability ~~due to freshwater acidification as the natural habitat of these crustaceans,~~ because the Yangtze river~~, is known to fluctuate~~ normally fluctuates by as much as 3000 µatm (Ran et al., 2017). Despite being a freshwater organism with strong ionoregulatory capabilities, ~~to deal with environmental acidification~~ our results ~~alarmingly~~ show a sharp decrease in survival rate of Chinese mitten crabs over ~~a 14-day period of~~14 days of exposure to 4633 µatm pCO_2 (Fig 1). Such rapid decreases in survival have also been observed in non-burrowing crustaceans or crustaceans that do not inhabit high intertidal regions including brine shrimp (*Artemia sinica*), red king crab (*Paralithodes camtschaticus*), and low intertidal long-clawed porcelain crab, (*Pisidia longicornis*), exposed to 1500, 1637, and 5821 µatm pCO_2, respectively (Long et al., 2013; Page et al., 2017; Zheng et al., 2015). It might be tempting to conclude that low survival in Chinese mitten crabs compared to tolerant mid to high intertidal and burrowing marine crustaceans is simply due to the greater pCO_2 tensions used in the present study (4633 µatm)~~.,~~ ~~H~~however, in the intertidal broad-clawed porcelain crab (*Porcellana platycheles*) pCO_2 levels of 5821 µatm have been shown to not affect the probability of survival after 24 days of exposure ~~even higher levels (5821 µatm) have been shown to have no effect on the probability of survival in a tolerant intertidal broad-clawed porcelain crab (*Porcellana platycheles*) after a 24-day exposure~~ (Page et al., 2017). It should also be mentioned that for all mortalities in this experiment there were no obvious signs of disease and intact bodies of deceased crabs were collected, suggesting that the elevated CO_2 treatment and not disease or cannibalism was the reason ~~of~~ for increased mortality. Therefore, the low survival rates in the present study suggest a high susceptibility to acidification and ~~are not consistent with~~contradict our hypothesis that inhabiting a highly fluctuating CO_2 environment would confer tolerance to future freshwater acidification.

4.3 Physiological Responses

Juvenile Chinese mitten crab effectively recovered extracellular pH following respiratory acidosis resulting from freshwater acidification by the accumulation of extracellular HCO_3^- as a buffer (Fig. 2). Compensation of acid-base homeostasis under freshwater acidification was not surprising given that strong acid-base regulatory capabilities are typically seen in highly active organisms such as fish, cephalopods and crustaceans (Melzner et al., 2009). Similar recovery of extracellular pH to elevated environmental CO_2 has also been observed in Dungeness crab (*Metacarcinus magiste*r) and velvet crab (*Necora pube*r) exposed to even higher pCO_2 tensions (10000+ µatm; Pane and Barry, 2007; Spicer et al., 2007). In contrast, green crab (*Carcinus*

maenas) and blue crab (*Callinectes sapidus*) have been shown to not fully compensate extracellular pH at 10000+ µatm CO_2
levels (Cameron, 1978; Fehsenfeld and Weihrauch, 2016)~~.;~~ ~~Hh~~owever, measurement~~s~~ in these species were only done over 48
hours and more time may have been required for the animals to recover, as seen in our study, where recovery was only observed
after seven days. The compensatory responses to acidosis in crustaceans generally includes respiratory CO_2 excretion, H^+
excretion typically through Na^+/H^+ or NH_4^+ exchange and accumulation of extracellular HCO_3^- as a buffer, where HCO_3^- is
derived through either branchial Cl^-/HCO_3^- exchange and, to a lesser degree, from calcified structures (e.g. exoskeleton)
(Wheatly and Henry, 1992). In freshwater crustaceans, acid-base regulation occurs mainly within the gills (Henry et al., 2012),
where the Na^+/K^+-ATPase and H^+-ATPase generate the electrochemical gradients that drive ion exchange (Leone et al., 2017).
The Na^+/K^+-ATPase alone may already account for ~~more than~~over 20 % of an animal's energetic budget (Milligan and
McBride, 1985). The ~~, therefore, an~~ increase in ion transport that must occur to re-establish and maintain acid-base homeostasis
in the face of freshwater acidification could pose an increased energetic demand. In fact, in sea urchin larvae pCO_2 tensions of
800 µatm have been shown to ~~potentially~~ double ion transport ATP demands (Pan et al., 2015). It is therefore conceivable that
the energetic cost for long-term maintenance of acid-base homeostasis under freshwater acidification may come at substantial
energetic cost, which could have negative implications on other physiological parameters and thereby animal fitness.

Heightened energetic demands to maintain crucial physiological processes during exposure to environmental CO_2 acidification
~~could be met~~can occur through reallocation of energy budgets or through modification of metabolism to increase energy
supplies. In fact, in the marine brittle star, *Amphiura filiformis*, exposure to CO_2 tensions ranging from 1000 to ~~-~~8000 µatm for
40 days caused an increase in metabolic rate (increased energy budget)(Wood et al., 2008). This metabolic change~~which~~ was
postulated to fuel increased calcification observed in this species (Wood et al., 2008). In contrast, the metabolic rate of juvenile
European lobster (*Homarus g~~G~~ammarus*) remained unchanged when exposed to 1100 and 8000 µatm CO_2 (Small et al., 2020).~~;~~
~~however,~~However, in *H. gammarus,* branchial Na^+/K^+ ATPase activity was increased, demonstrating a reallocation of energy
supplies despite maintaining an unchanged energy budget (Small et al., 2020). Unlike ~~in~~ juvenile European lobster and brittle
star, juvenile Chinese mitten crabs experienced a decrease in oxygen consumption (potentially decreased energy budget).
Despite reductions in oxygen consumption, crabs ~~were still able to~~could still re-establish extracellular pH through HCO_3^-
accumulation, suggesting a potential reallocation of energy supplies to essential ionoregulatory processes.

Typically, a reduction in oxygen consumption, as seen in the present study, is observed when an organism ~~is unable to~~cannot
compensate for a reduction in extracellular pH (Pörtner et al., 2004). While in juvenile Chinese mitten crabs, this could be the
case at the initial two days of the time course, by day seven extracellular pH was fully compensated yet, oxygen consumption
rates were reduced. It is known that high environmental pCO_2 levels can trigger an accumulation of compounds such as
adenosine that can lead to reduced oxygen consumption as observed in the peanut worm, *Sipunculus nudus* (Reipschläger et
al., 1997). A similar mechanism could conceivably be in place that led to reduced oxygen consumption in the Chinese mitten
crab as a strategy to conserve energy supplies to promote survival upon exposure to short-~~-~~term stressors like high
environmental pCO_2 levels. Such an adaptation may be present in Chinese mitten crab as these crabs would regularly
experience short-term fluctuations in environmental CO_2 of their natural habitat. In fact, in the Mediterranean mussel (*Mytilus*

galloprovincialis)~~ a~~ chronically reduced oxygen consumption rates lasting up to 90 days have been observed to allow survival following exposure to ocean acidification (5026 µatm pCO_2, Michaelidis et al., 2005). ~~While reducing~~Reducing oxygen

390   consumption is a viable strategy used by many organisms to survive short-term periods of environmental stress (Guppy and Withers, 1999). However, ~~,~~it is a less viable long-term strategy as reduction in metabolic rate reduces energy availability for costly physiological processes such as calcification and protein synthesis which would ultimately affect growth and reproductive success as reported in freshwater pink salmon (*Oncorhynchus gorbuscha*) and marine amphipod (*Gammarus locusta*) (Borges et al., 2018; Ou et al., 2015).

395   ~~In addition to~~Besides reduced oxygen consumption, freshwater acidification led to an increase in extracellular concentrations and excretion of ammonia, a metabolic product of protein catabolism. Elevated excretion of ammonia may function as an excretable acid equivalent to assist the maintenance of pH homeostasis, a mechanism suggested for the brackish water green crab (*Carcinus maenas*) and hydrothermal vent crab (*Xenograpsus testudinatus*) (Allen et al., 2020; Fehsenfeld and Weihrauch, 2013). Furthermore, the previously mentioned ~~observed~~ reduction in oxygen consumption and increased ammonia excretion

400   (decrease in O:N ratio) indicates that juvenile Chinese mitten crab~~s~~ have a greater reliance on protein catabolism as an energy source under elevated environmental CO_2. Similar decreases in oxygen consumption and increases in ammonia excretion have been observed in the Mediterranean mussel (*M. galloprovincialis*, 5026 µatm pCO_2, 15-90 days) and brittle star (*A. filiformis*, 6643 µatm pCO_2, 28 days), where catabolism of amino acid such as glutamine may provide metabolic bicarbonate to further ~~assist in sustaining~~ help sustain pH homeostasis (Hu et al., 2014; Michaelidis et al., 2005). While potentially beneficial for

405   sustaining acid-base status, elevated protein catabolism requires a consistent source of protein through either a high protein diet or increased food consumption which if not met could ~~result in~~cause muscle wastage an effect seen in brittle star during heightened energetic demands of ocean acidification (Wood et al., 2008). ~~Further, the fact that~~Interestingly, feeding rate has been shown in juvenile European lobster (*H. gammarus*) and green crab (*C. maenas*) to decline as a result of elevated environmental CO_2 ~~makes~~making a greater reliance on protein catabolism during energetically constricted times a potentially

410   precarious situation for juvenile Chinese mitten crab (Appelhans et al., 2012; Small et al., 2020).

Carapace calcification is an energetically costly process related to growth and predation defence in crustaceans that ~~could be impaired by~~ freshwater acidification and the associated metabolic changes could impair. ~~Generally, decapod~~Decapod crustaceans are believed to be the least susceptible of calcifying organisms to aquatic acidification as their exoskeletal $CaCO_3$ exists in the more stable calcite form providing greater resilience to dissolution in contrast to bivalves and corals (Ries et al.,

415   2009). Indeed, the marine crustacean carapace is well protected from aquatic acidification mediated dissolution with reports of either no change or an increase in calcification being typically observed (Kroeker et al., 2013; Ries et al., 2009; Whiteley, 2011). However, in the present study, juvenile Chinese mitten crab had reduced levels of carapace calcification as reflected by a lower carapace calcium content after three and six weeks of exposure (Fig. 4). While not as common, examples of reductions in carapace calcification have been observed in marine crustaceans, including several porcelain crabs and the tanner crab,

420   *Chionoecetes bairdi* (Long et al., 2013; Page et al., 2017). In crustaceans, ~~it has been suggested that~~ carapace dissolution may occur to support extracellular pH buffering that normally occurs through branchial HCO_3^- uptake by providing an alternative

source of HCO_3^- (Cameron, 1985; Defur et al., 1980). In the present study, extracellular pH was recovered long before carapace dissolution was apparent, therefore it is less likely that the carapace is mobilized as a source of HCO_3^-. Instead, reductions in carapace calcium content most likely reflect an alteration in the rate of calcification or acid mediated dissolution of the carapace. As carapace formation and maintenance is an energetically expensive process requiring careful ion regulation by numerous organs, the aforementioned changes in whole animal energetics due to freshwater acidification could have negative implications on animal fitness either by weakening the exoskeleton or impairing post-moult calcification which ~~in turn~~ can hamper growth and leave animals vulnerable to predation.

4.4 Behavioural Responses

Elevated freshwater pCO_2 altered locomotory behaviour in juvenile Chinese mitten crabs. Crabs in acidified freshwater covered less total distance during movement and did so at a lower velocity. No studies have previously examined changes in crustacean distance covered in the presence of elevated environmental CO_2. However, reduced speed of movement has also been report~~ed~~ in Shiba shrimp (*Metapenaeus joyneri*) exposed to CO_2 levels of 9079 µatm; however, unlike in Chinese mitten crab, this shrimp did not experience a reduction in ~~resting/standard metabolic~~oxygen consumption rate correlated with locomotory impairment (Dissanayake and Ishimatsu, 2011). While not measured in our study, in Shiba shrimp there was a reduction in aerobic scope, which would likely lead to reduced aerobic performance and ~~thereby~~ reduced movement (Dissanayake and Ishimatsu, 2011). Similar alterations in aerobic scope could partially be behind the reductions in velocity seen in juvenile Chinese mitten crab, however, this is entirely speculative and there are many cases where elevated CO_2 does not alter aerobic scope (Lefevre, 2016). In addition to moving slower, Chinese mitten crab spent less time moving their entire body throughout the novel arena and less time moving only their appendages while staying at a fixed location. Reduced movement time and appendage movement ~~was~~were also seen in the hermit crab (*Pagurus bernhardus*) exposed to 12000 µatm CO_2 (de la Haye et al., 2011). In contrast, the isopod (*Paradella dianae*) experienced no change in swim time or crawling time when exposed to 2085 µatm CO_2 despite a measured metabolic depression (Alenius and Munguia, 2012). Differences in the effect of CO_2 on movement time may ~~be a result~~result from~~of~~ the CO_2 levels employed, but further studies on a greater variety of species are required to determine potential patterns for crustaceans. It is plausible that overall locomotory behaviour is reduced in this study due to alterations in neurological function resulting from ionic imbalances or other CO_2-mediated effects that ~~are known to~~may occur from elevated environmental CO_2 (For review of neural effects of aquatic acidification see Tresguerres and Hamilton, 2017). ~~Additionally, with~~With a potential reduction in overall energy availability, crabs may ~~be reducing~~reduce energy expenditure through locomotion to conserve energy stores for physiological processes more crucial to surviving the physiological distress caused by freshwater acidification. The overall reductions in locomotion observed in juvenile Chinese mitten crab could have negative consequences ~~on~~for their survival, as reduced movement would make these crabs more vulnerable to predation, reduce migratory capabilities and reduce foraging ability.

5 Conclusion

In conclusion, we found impairment of survival, metabolism, calcification, and locomotion with exposure to a potential future
455   CO_2 mediated freshwater acidification scenario. ~~Overall, energy~~Energy availability was reduced despite heightened ionoregulatory energetic demands. Changes in the animals' energy budgets likely result in a greater dependency on protein catabolism as an energy source to allow for extracellular pH recovery at the cost of reducing their exoskeletal calcification and locomotion. We found that despite successful acid-base compensation, survivals rates declined with ~~a~~ 3.8 times greater probability of mortality under acidified freshwater conditions. While our study suggests negative impacts of freshwater
460   acidification, these results should be assessed with caution as the assumed acidification levels are based on a relationship between changes in atmospheric CO_2 and freshwater CO_2 which remains to be more effectively modelled. Nevertheless, this study shows that despite inhabiting an environment that experiences regular fluctuations in pCO_2 the Chinese mitten crab may be at risk to future freshwater acidification. This ~~and~~ emphasizes the importance of modelling acidification in freshwater systems to accurately assess biological consequences of global change. Based on our findings that a physiologically robust
465   species displays sensitivity to future freshwater acidification, further research investigating the effect of ~~future~~ freshwater acidification on a wide range of freshwater species from all phyla ~~is~~ are required to better identify the effects of anthropogenic CO_2 accumulation on freshwater ecosystems.

Data Availability

Data are available at the following link http://dx.doi.org/10.6084/m9.figshare.13888034.

470 **Author Contributions**

A.R.Q.R designed the study, performed experiments, analysed data and wrote the manuscript. P-L.K, P-H.S, and M-T.H. performed experiments. G.J.P.A analysed data and assisted with writing. P-P.H. provided financial support and analytical tools. Y-C.T. assisted in designing the study, writing the manuscript, and provided financial support and analytical tools. D.W. assisted in designing the study, writing the manuscript, and provided financial support and analytical tools.

475 **Competing Interests**

The authors declare that they have no conflict of interest

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