Anthropogenic CO_2-mediated freshwater acidification limits survival, calcification, metabolism, and behaviour in stress-tolerant freshwater crustaceans

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Abstract. Dissolution of anthropogenic CO_2 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 CO_2-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 in understanding the long-term implications of freshwater acidification on species' fitness.

1 Introduction

Rising levels of atmospheric CO_2 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; however, the highly variable biogeochemistry between freshwater systems has been a limiting factor in modelling future freshwater scenarios (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 freshwater species.

Amongst marine organisms, 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 investigating the various physiological and behavioural effects of realistic future levels of CO_2-mediated acidification in calcifying freshwater invertebrates. Freshwater calcifying macroorganisms 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 almost all ecological niches across the globe including freshwater, marine, and terrestrial habitats making them a 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 % (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 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* has 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, 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 681-3796 µatm (Ran et al., 2017), which may confer some degree of pre-adaptation to elevated CO_2 due to life history. Furthermore, crustaceans are generally believed to more CO_2 tolerant than other calcifying organisms such as bivalves and coral due to their high metabolic activity and robust acid-base machinery allowing for a more efficient 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 see ringer measurement methods below) on a 14:10 h light-dark cycle with temperature ranging from 23-25 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 to 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 own 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 was 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).

	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 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 2-3 crabs were pooled together (200-300 µL pooled hemolymph per n value) to obtain enough sample for downstream analysis 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 -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 analyser (± 0.2 mmol l^{-1} precision) calibrated with $NaHCO_3$ standards ranging from 0-20 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 ºC (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 NH_4Cl 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. Experimental sampling of ammonia excretion and oxygen consumption 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 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):

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

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 which used a pH controller (Aqua-MACRO) to measure pH and inject CO_2 to maintain the desired CO_2 tension. 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 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 fiber optic oxygen transmitter (PreSens Precision Sensing GmbH, Regensburg, Germany). Oxygen saturation was never allowed to drop below 80% and 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 must be considered. Due to logistical constraints, we

were unable to use 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 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 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 15 (Cary, NC, USA) and GraphPad Prism 8.4.2 (San Diego, CA, USA). Data were analysed for outliers by ROUT test with a Q value of 1 %. For all data heterogenicity of variance was tested by Levene's test and normal distribution of residuals by Shapiro-Wilk test. Two transformations were done in this study 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 were as a square root transformation was applied to ammonia excretion rate data. In this

study hemolymph parameters, ammonia excretion, and oxygen consumption data were analysed by a two-way ANOVA post hoc Dunnett's test where comparisons were made to the zero-day control with time and pCO_2 values as fixed factors. Carapace calcification data was also analysed by a two-way ANOVA however, as we had no zero-day control a post hoc Tukey HSD with time and pCO_2 values as the fixed factors. As behavioural data displayed a high degree of co-linearity between dependent variables the assumptions of a MANOVA test were not met and therefore we analysed this data set by student's t-test with the exception of appendage movement time that did not meet parametric assumptions so was analysed by Wilcoxon test. Survival curves were analysed for significant differences by the Mantel-Cox test and hazard ratio was determine 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), with a 50 % mortality in crabs held in the acidified freshwater compared to 15 % mortality in control freshwater. 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.

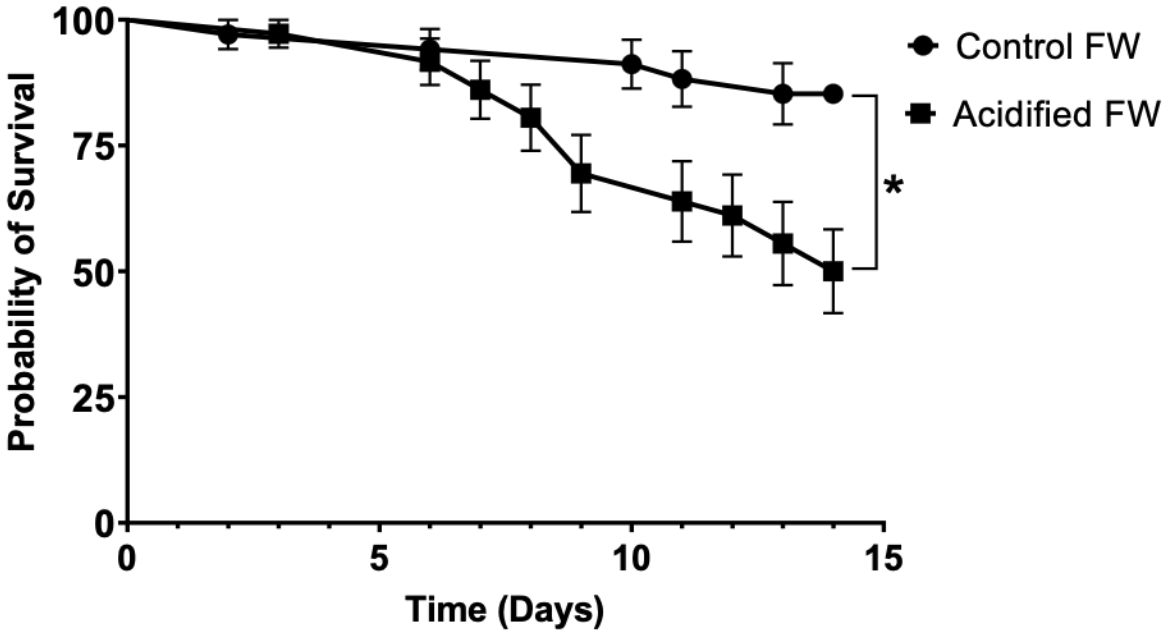

Figure 1. Survivorship curves of juvenile Chinese mitten crab, *Eriocheir sinensis*, over 14 days of exposure to control (pH 7.4, 1364 µatm pCO_2) or CO_2-acidified (pH 6.8, 4633 µatm pCO_2) freshwater. Data are presented as probability of survival +/- SEM. (N=34 for control freshwater and N=36 for acidified 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 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 acidosis was maintained for two days with full recovery occurring 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); however, no 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.

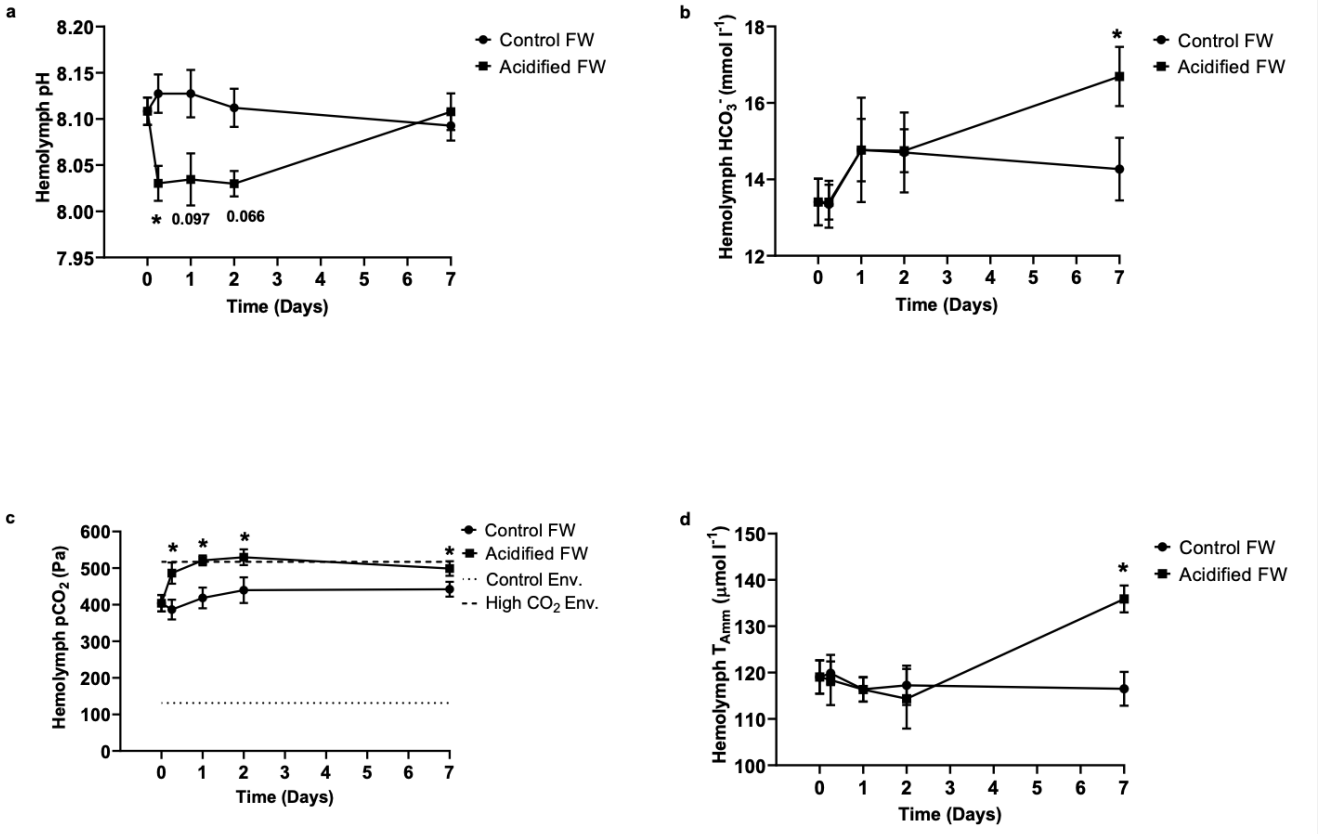

Figure 2. Changes in extracellular (a) pH, (b) HCO_3^-, (c) pCO_2, and (d) ammonia of juvenile Chinese mitten crab, *Eriocheir sinensis*, during a 7-day time course of exposure to control (pH 7.41, 1299 µatm pCO_2) or CO_2-acidified (pH 6.73, 5109 µatm pCO_2) freshwater. Data are presented as mean +/- 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. Control crabs exhibited steady oxygen consumption rates and ammonia excretion rates throughout the measured time course (Fig. 3; Table 2). In contrast, crabs exposed to freshwater acidification experienced a significant reduction in oxygen consumption rate within six hours that was maintained throughout the remainder of the time course (Fig. 3a; Table 2). Ammonia excretion rates were significantly affected by acidified freshwater (Fig. 3b; Table 2.). Initially excretion rates were unchanged until the second day of exposure at which point excretion rates nearly doubled and remained elevated for the duration of the seven-day time course (Fig. 3b).

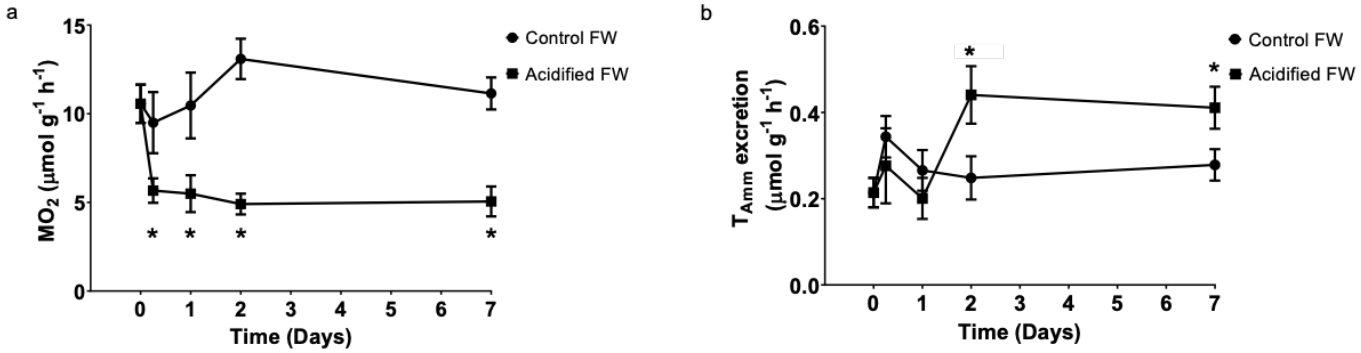

Figure 3. Changes in whole animal (a) oxygen consumption rate (MO_2) and (b) ammonia excretion rate of juvenile Chinese mitten crab, *Eriocheir sinensis*, during a 7-day time course of exposure to control (pH 7.41, 1299 µatm pCO_2) or CO_2-acidified (pH 6.73, 5109 µatm pCO_2) freshwater. Data are presented 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 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 content to 84.1 ± 2.9 % and 85.2 ± 3.3 % of control crab levels was observed (Fig. 4).

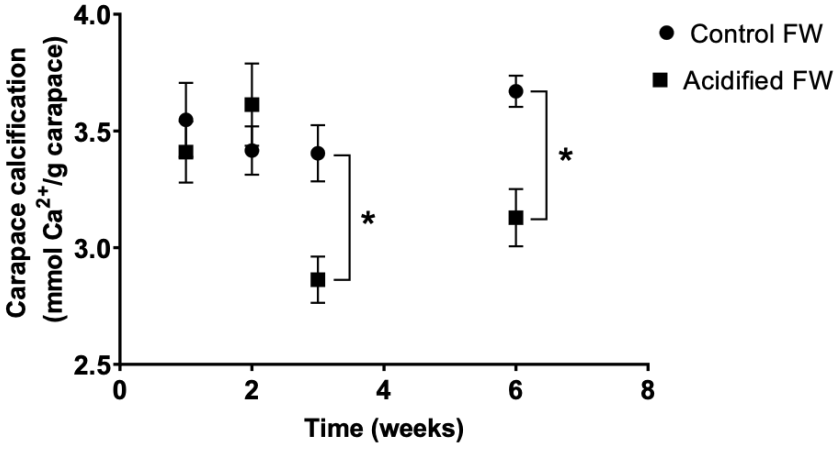

Figure 4. Changes in carapace calcium content of juvenile Chinese mitten crab, *Eriocheir sinensis*, over a 6-week exposure to control (pH 7.4, 1389 µatm pCO_2) or CO_2-acidified (pH 6.8, 4634 µatm pCO_2) freshwater. Data are presented as mean +/- SEM. (N=6-12). Statistical significance was assessed 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. 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 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, where movement was defined as the crab changing its relative location in the arena and mobility 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 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 pCO_2) or CO_2-acidified (pH 6.73, 5109 µatm pCO_2) 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 pCO_2 tensions (~1000 µatm) predicted to occur by the year 2100 with further acidification to levels expected for year 2300 (~2000 µatm) negatively impacting about half of the studied marine crustaceans (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 high energetic demands to sustain essential physiological processes such as acid-base regulation may be causing energetic reallocation that leads to a trade-off that impairs several physiological processes and alter 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 and 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 1µatm increase in atmospheric pCO_2, these freshwater bodies increased by 9.35µatm. 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 for 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 1395-2707 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, the Yangtze river, is known to fluctuate 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 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), however, 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 increased mortality. Therefore, the low survival rates in the present study suggest a high susceptibility to acidification and are not consistent with 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 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 magister*) and velvet crab (*Necora puber*) 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); however, measurement 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 20 % of an animal's energetic budget (Milligan and McBride, 1985), 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 through reallocation of energy budgets or through modification of metabolism to increase energy supplies. In fact, in marine brittle star *Amphiura filiformis* exposure to CO_2 tensions ranging from 1000-8000 µatm for 40 days caused an increase in metabolic rate (increased energy budget) 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 Gammarus*) remained unchanged when exposed to 1100 and 8000 µatm CO_2; however, 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 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 present study is observed when an organism is unable to 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 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 oxygen consumption is a viable strategy used by many organisms to survive short-term periods of environmental stress (Guppy and Withers, 1999), 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).

In addition to 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 (decrease in O:N ratio) indicates that juvenile Chinese mitten crab 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 pH homeostasis (Hu et al., 2014; Michaelidis et al., 2005). While potentially beneficial for 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 muscle wastage an effect seen in brittle star during heightened energetic demands of ocean acidification (Wood et al., 2008). Further, the fact that 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 a greater

reliance on protein catabolism during energetically constricted times a 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. Generally, 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., 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, *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 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 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 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 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 occur from elevated environmental CO_2 (For review of neural effects of aquatic acidification see Tresguerres and Hamilton, 2017). Additionally, with a potential reduction in overall energy availability, crabs may be reducing 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 their survival as reduced movement would make these crabs more vulnerable to predation, reduce migatory capabilites and reduce foraging ability.

5 Conclusion

In conclusion, we found impairment of survival, metabolism, calcification, and locomotion with exposure to a potential future CO_2 mediated freshwater acidification scenario. Overall, 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 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 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 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 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.

Author Contributions

A.R.Q.R designed the study, performed experiments, analyzed data and wrote the manuscript. P-L.K, P-H.S, and M-T.H. performed experiments. G.J.P.A analyzed 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.

Competing Interests

The authors declare that they have no conflict of interest

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