Temperature and pCO_2: single and combined effects of climate change parameters on acid-base regulation in Louisiana red swamp crayfish (*Procambarus clarkii*)

By

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Abstract

With the global focus on ocean acidification very few studies have investigated the effect of climate change on freshwater species. The impact of individual and combined stress of elevated temperature and pCO_2 that mimic predicted levels for the year 2100 in *P. clarkii* were investigated. Measurements of hemolymph parameters, metabolic rate and differential expression of acid-base regulatory genes in the gills were conducted. Crayfish showed a decrease in hemolymph pH following 14-day acclimation to elevated temperature. Following elevated environmental pCO_2 reduced metabolic rate was observed with a successful compensatory response to the acid-base challenge. Combined temperature and pCO_2 treatments showed decreased metabolic rate, decreased hemolymph ammonia and elevated hemolymph bicarbonate and pCO_2. The results of this study suggest that dual environmental stressors, such as temperature and pCO_2, have interactive effects, potentially causing different physiological responses than single stressors. This study highlights the importance of multi-stressor studies to avoid potentially masking the true effect of climate change and impacting management decisions.

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Thesis Contributions

Some of the data presented in this thesis are currently being prepared for submission to the Journal of Global Climate Change. Metabolic rate measurements were conducted with Gwangseok Rex Yoon and hemolymph blood parameters were measured with assistance from Alex Quijada-Rodriguez, Garett Allen, Jess Gomez and Aaron Klymasz-Swartz. In addition summer student Jess Gomez measured whole animal ammonia excretion samples for the high temperature only acclimation using the gas sensitive electrode, which were collected and statistically analysed by Ashley Tripp. Aside from the aforementioned data sets, all other data in this thesis were collected and analyzed by Ashley Tripp.

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Introduction

1.1. Climate change

Since the industrial revolution the release of anthropogenic carbon dioxide (CO_2) into the atmosphere from the burning of fossil fuels has increased the partial pressures of CO_2 in marine and freshwater environments, consequently decreasing pH in these water bodies (Drever, 1982). Estimates of ocean acidification, from the hydration of CO_2 in water to carbonic acid, predict a decline in pH between 0.3 and 0.4 units by the end of the century, which equals an increase of proton concentrations by as much as 150% (Haugan & Drange, 1996; Orr et al. 2005). This is paired with an average global temperature increase of 1.5-4.5 °C as predicted by general circulation models with CO_2 doubling, creating a dual challenge to the survival of aquatic animals (Houghton et al. 1992; Mortsch & Quinn, 1996). When faced with a challenge to intracellular pH, animals can buffer their intracellular and extracellular environments, eliminate excess CO_2 from their hemolymph through their respiratory system or move acid-base equivalents between their extracellular and intracellular spaces (Henry & Wheatly, 1992; Truchot, 1983).

1.2. Climate change in seawater

1.2.1 High temperature in seawater

Due to the elevated atmospheric pCO_2 levels and the additional rise in temperature in both air and water, various physiological effects on aquatic animals have been observed. For instance, a 10°C temperature increase induced an increase in mass specific oxygen consumption

in marine Dungeness crabs (*Metacarcinus magister)*. This increase in metabolic rate to overcome the decreased oxygen content of warmer water will create a slight increase in carbon dioxide production and a greater challenge to support aerobic metabolism, which if not met will cause an increase in hemolymph lactate from anaerobic metabolism (McMahon et al. 1978; Bonvillain et al. 2012). In addition, a reduction of hemolymph pH and a corresponding decrease in hemoylmph bicarbonate was observed. However there was no change in hemolymph pCO_2 observed, likely due to the high diffusibility of carbon dioxide compared to oxygen (McMahon et al. 1978; Howell et al. 1973). Atlantic shrimp (*Xiphopenaeus kroyeri*) when acutely exposed to temperatures between 20°C and 30°C showed linear increases in both metabolic rate and ammonia excretion with increasing temperature (Carvalho & Phan, 1997). In green crabs (*Carcinus maenas*) oxygen uptake, and by proxy metabolic rate, was significantly increased (14-63%) in crabs acclimated to 10 °C and exposed to +5°C and +15°C temperature increases (McGaw & Whiteley, 2012). The consequences of living outside the optimal temperature range an animal is adapted for is lowered fitness, which can lead to physiological or behavioral changes (Weiser, 1972). When temperature exceeds the upper preferred temperature of *Carcinus maenas* it responds by leaving the water to respire in air, utilizing the higher O_2 concentrations (Newell et al. 1972; Taylor & Butler, 1978; Taylor & Wheatly, 1979). This combined with a decreased digestive efficiency with elevated temperature will influence the ability of *C. maenas* to both acquire and metabolize food (McGaw & Whiteley, 2012).

1.2.2 High pCO_2 in seawater

In general, an increase of environmental pCO_2 usually causes an increase in blood/hemolymph pCO_2 concentrations in seawater animals, allowing them to maintain an outwardly directed CO_2 gradient, that promotes the release of the gas and thereby a decrease in body fluid pH. In seawater animals this acidosis, due to an accumulation of CO_2 in the body fluids, can lead to a variety of physiological responses including the accumulation of bicarbonate in the blood/hemolymph in fish and crustaceans (Perry & Gilmour, 2006; Hans et al. 2014). Additional responses include the elimination of excess CO_2 by changing oxygen consumption rates and the retention or excretion of metabolic ammonia to regulate pH (Hans et al. 2014; Fehsenfeld & Weihrauch, 2013; Turner et al. 2015). In most fish exposed to elevated pCO_2 blood bicarbonate concentrations and acid excretion are adjusted to maintain their extracellular pH, leaving metabolic processes and respiration rate unaltered (Cameron, 1978a; Marshall & Grosell, 2006; Perry & Gilmour, 2006). In juvenile reef fishes (*Dascyllus aruanus, Pomacentrus moluccensis, Apogon cyanosoma* and *Cheilodipterus quinquelineatus*) exposure to elevated pCO_2 resulted in altered behaviour, including an inability to recognize habitat and predator odours. However, there was also no change in metabolic rate (Munday et al. 2014). For crustaceans, elevated environmental pCO_2 caused an increase in either hemolymph bicarbonate, pCO_2 or both with no change in hemolymph pH as seen in *Carcinus maenas* and *M. magister* (Fehsenfeld & Weihrauch, 2013; Hans et al. 2014) (Table.1). Ammonia excretion rates, often reflecting metabolic activity can be a more variable response towards the exposure to elevated pCO_2 levels (Langenbuch & Portner, 2002; Jobling, 1981). For instance in *M. magister* there was a significant decrease in whole animal ammonia excretion that was paired with a significant decrease in metabolic rate, also observed in the velvet swimming crab (*Necora puber*) possibly

as a result of the sedative effects of hypercapnia (Hans et al. 2014; Small et al. 2010). In contrast *C. maenas* showed increased whole animal ammonia excretion rates in response to elevated pCO_2 (Control = 54 Pa, Elevated pCO_2 = 324 Pa)(Fehsenfeld & Weihrauch, 2013). There was also no change in the % calcium of exoskeletons in the red king crab, tanner crab or velvet swimming crabs exposed to pH environments between 6.7 – 8, achieved by bubbling CO_2 (Long et al. 2013; Small et al. 2010).

Table 1. Blood parameters of decapod crustaceans exposed to elevated pCO_2 treatment.

Source	Species	Condition	Water pCO_2 (Pa)	Hemo pH	Hemo pCO_2 (Pa)	Hemo HCO_3^- (mM)
Cameron 1978b	*Callinectes sapidus*	Control	40	7.96	530	-
		44 Hour	1000	7.80	1200	-
Appelhans et al. 2012	*Carcinus maenas*	Control	66	7.82	380	8
		10 weeks	351	7.81	550	12
Truchot 1975	*Carcinus maenas*	Control	40	7.79	180	3.9
		24 Hour	310	7.71	730	13.7
Dissanayake et al. 2010	*Palaemon elegans*	Control	100	7.70	-	-
		2 weeks	310	7.55	-	-

1.2.3 The combined effect of pCO_2 and temperature in seawater

There are few studies that have investigated the impact of the combined effect of temperature and pCO_2 stressors on seawater invertebrates. Pacific sea urchins (*Echinometra sp. A*) exposed to both elevated pCO_2 (860-940 μatm) and temperature (+2-3 °C) to mimic a potential year 2100 scenario showed greater effects on decreased growth and gonad development and increased metabolic rate, in combined treatments compared to single stress treatments (Uthicke et al. 2014). Significant decrease in metabolic rate and carapace calcification was also

observed in juvenile European lobsters (*Homarus gammarus*) exposed to either a realistic ocean acidification scenario (~1100 µatm pCO_2) and a more severe model mimicking acidic water upwelling and volcanic vent inputs (~9000 µatm pCO_2) paired with a temperature increase from 10 °C to 13°C (Small et al. 2016). In the case of carapace calcification there was no change observed during single stressor elevated pCO_2 trials in both the above ocean acidification scenario in lobster and scleractinian corals held at 760 µatm at control temperatures, it was only with +3°C increase in both studies that there was significant decrease in calcification (Reynaud et al. 2003; Small et al. 2016). As seen most clearly with metabolic rate, the impact of the combination treatments can be a lessened response to either component stressors. The metabolic rate of juvenile lobsters (*Homarus gammarus*) and periwinkles (*Littorina littorea*) decreased with the combination of temperature and pCO_2, but to a lesser degree than pCO_2 stress alone (Small et al. 2016; Melatunan et al. 2011).

1.3 Freshwater

In contrast to the vast volumes and high carbonate buffering capacity of oceans, freshwater bodies are fragmented habitats with smaller water volumes and generally lowered buffering capabilities, making them potentially more vulnerable to the damaging effects of climate change (Cai, 2003; Woodward et al. 2010; Egleston et al. 2010). This variability contributes to the lack of accurate modelling for future conditions, which in turn limits experiments to determine the possible impacts of climate change on freshwater systems (Hasler et al. 2016). Living in freshwater also presents animals with a unique challenge of maintaining their internal NaCl concentrations against an external NaCl-poor environment, forcing them to work against the respective diffusion gradients (Dickson et al. 1991; Freire et al. 2008).

1.4 Climate change in freshwater

1.4.1 High temperature in freshwater

Although there is not much known, the few freshwater studies that have been conducted have shown similar patterns in physiological responses as marine animals to predicted climate change conditions. A study conducted by Chen & Kou (1996) investigating the impact of temperature exposure between 17°C and 32°C on the physiology of fasted freshwater juvenile giant prawns *Macrobrachium rosenbergii* found significant increase in oxygen consumption and ammonia excretion in direct response to a temperature increase. A similar study investigating Leichhardtian River Prawns (*Macrobrachium tolmerum*) acclimated to 16°C found metabolic rates of tail and chelae muscles were 1.8 fold higher in animals tested at 21°C (Crispin & White, 2013). Interestingly in freshwater channel catfish (*Ictalurus punctatus*) with increased temperature there was a drop in blood pH (7.9 to 7.7) and no change in either gill or kidney ammonia excretion (Cameron & Kormanik, 1982). Increasing metabolic rate with increasing temperature has also been observed in freshwater fish, as early as the larval phase in rainbow trout (*Salmo gairdneri*) (Rombough, 1988; Oliva-Teles & Kaushik, 1990). Additional studies suggest the scope of this response could be species specific with the fontane cisco (*Coregonus Fontanae*) having significantly lower standard metabolic rates than Vendace (*Coregonus Albula*) at all tested temperatures (4°C, 8°C and 15°C), associated with lower energetic costs at lower temperatures (Ohlberger et al. 2008).

1.4.2 High pCO_2 in freshwater

Blue crabs (*Callinectes sapidus*), harvested from Green Lake and the Guadalupe River in Texas, compensate for an observed respiratory acidosis after exposure to elevated pCO_2 by increasing blood bicarbonate concentrations (Cameron, 1978b). A current study on the impact of predicted climate change on freshwater acclimated Chinese mitten crab (*Eriocheir sinensis*) found that animals acclimated to a pCO_2 of 540 Pa (control pCO_2 ~110 Pa) experienced initially a decrease in hemolymph pH, that recovered by day 7. This corresponded with elevated hemolymph bicarbonate, ammonia and pCO_2, all of which remained elevated at 7 days of exposure. In addition, as seen in other calcifying invertebrates, such as *Echinometra sp. A* (Uthicke et al. 2014), a 15% reduction in carapace calcification and 50% mortality were also observed following three and two weeks of exposure, respectively (Quijada-Rodriguez, Personal Communication).

1.4.3 High pCO_2 and temperature in freshwater

To the author's knowledge, there is currently a single publication investigating the combined impact of elevated environmental pCO_2 and temperature on a freshwater invertebrate. Freshwater mussels (*Lampsilis siliquoidea*) acclimated for two weeks to either control (230 μatm) or high pCO_2 (58,000 μatm) representative of freshwater systems in the upper Midwest United States where CO_2 barriers can elevate the pCO_2 up to 100 times the usual condition, at five temperature treatments between 22°C and 34°C (Donaldson et al. 2016). This study showed that the interaction of temperature and CO_2 stress resulted in the largest mortality, as animals were unable to respond to both (Jeffrey et al. 2018). The lack of studies conducted on freshwater

invertebrates shows a large knowledge gap in how inevitable environmental change will impact important freshwater species and thereby, entire freshwater ecosystems.

1.5 *Procambarus clarkii*

Procambarus clarkii, the Louisiana red swamp crayfish is native to North-Eastern Mexico and the Southern USA, and has been introduced to all continents excluding Antarctica and Oceania. (Hobbs et al. 1989; Loureiro et al. 2015). The Atchafalaya River Basin hosts 90% of the wild crayfish that are harvested for consumption in the state of Louisiana every year, grossing US$181 million dollars annually (Isaacs & Lavergne, 2010; LSUAC, 2011). This reliance on natural populations that are subjected to changing environmental conditions puts this industry's profit at risk (Bonvillian et al. 2012). The Atchafalaya river system waters in particular experience high partial pressures of CO_2, which fluctuate from 98 Pa to a seasonal high of 222 Pa as measured in July 2009 (Huang et al. 2015). In addition, this freshwater marsh experiences considerable fluctuations in pH (6.31 - 8.52) and temperature that seasonally moves between 4.75 °C and 32.25 °C (Sabo et al. 1999). Ideal growth conditions have been found to be at a temperature range between 14 °C and 24 °C (Romaire & Lutz, 1989). As a crustacean with an annual dry season and burrowing behaviour this species is accustomed to elevated environmental ammonia and low oxygen, suggesting its robust ability to handle environmental stress (Correia & Ferreira, 1995; Bonvillian et al. 2012). However, as a calcifying organism with a calcium carbonate shell crustaceans are particularly vulnerable to the acidifying effects of climate change (Wood et al. 2008).

1.6 Carapace

The carapace is the hard exoskeleton produced by the crayfish to protect their gills and vital organs using available calcium (Ca^{2+}) and carbonate (CO_3^{2-}) from the external environment to form $CaCO_3$ (Inoue et al. 2001). Crayfish and most other crustaceans are required to moult in order to grow and this store of calcium carbonate ($CaCO_3$) in their exoskeleton is important to maintain structural integrity following ecdysis (Truchot, 1983; Cameron, 1989). Prior to moulting some of the $CaCO_3$ is reabsorbed from the exoskeleton prior to being shed (Greenaway, 1985). However, this represents the smallest fraction with, 18 % of the total body Ca^{2+} being retained from the moulted skeleton while the rest is principally taken from the environment through active uptake mechanisms in the gills (Wheatly & Gannon, 1995; Wheatly, 1999). During exposure to increased acidity crustaceans that produce a calcium shell may degrade it to free available $CaCO_3$, which will dissociate to Ca^{2+} and CO_3^{2-}, to neutralize internal acidification (Inoue et al. 2001; Cairns & Yan, 2009). The effects of increasing pCO_2 in the long term may also show a decreased ability of crayfish to make strong $CaCO_3$ shells as it increases proton concentrations and decreases the amount of CO_3^{2-} available, an already limiting factor in some freshwater environments, to make $CaCO_3$ (Kurihara, 2008; Rukke, 2002; Hammond et al. 2006). As it becomes more limiting, the potential lack of environmental $CaCO_3$ will make it hard for crayfish, having some of the highest Ca^{2+} requirements, and other calcifiers to sequester it in their shells (Kurihara, 2008; Edwards et al. 2015).

1.7 Crayfish gills

In decapod crustaceans, gills are the main organ of acid-base regulation, gas exchange and excretion (Henry et al. 2012). Crayfish have trichobranchiate gills that are feather-like in

appearance with a central stalk covered in filaments with a sail shaped lamella similar to a lobster epipodite (Fig. 1) (Dickson et al. 1991; Freire et al. 2008). This structure is highly efficient and takes advantage of both concurrent and countercurrent flow of hemolymph through the gill to optimize exchange (Burggren et al. 1974). Previous studies on the characterization of gills in *P. clarkii* have found two distinct morphologies of gill filaments in the podobranch gills; inner filaments that have thicker epithelial cells and are rich in mitochondria and surface area and outer filaments with considerably less mitochondria and surface area (Fig. 1)(Dickson et al. 1991; Dunel-Erb et al. 1997). The inner filaments are therefore thought to be responsible for active osmoregulatory NaCl uptake and the outer filaments for respiration while the location of acid-base regulatory functions are untested (Dickson et al. 1991; Henry et al. 2012).

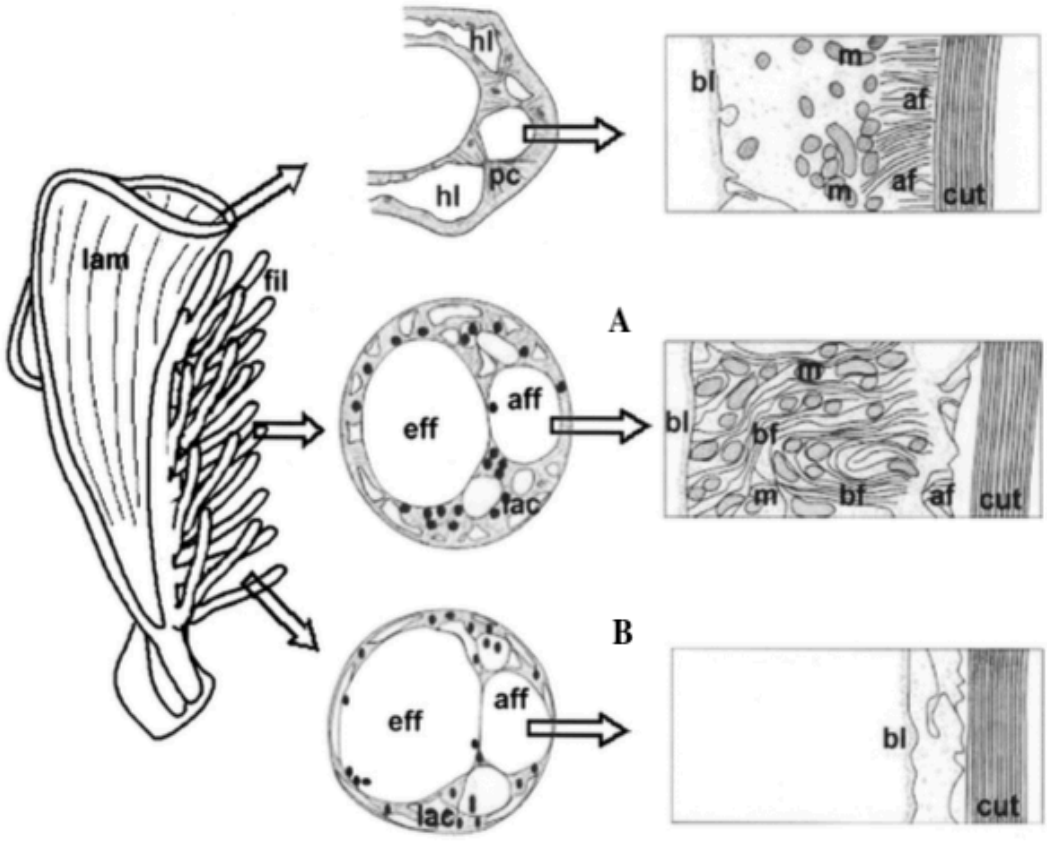


Figure 1. Schematic diagram of a single crayfish podobranch gill. Diagram is showing the two filament types where A) are the inner filaments, closest to the gill attachment in the animal that are mitochondria rich and have more surface area than B) the outer filaments, that are comparatively mitochondria poor. Indicated on the diagram is the hemolymph space (hl), pilaster cells (pc), cuticle (cut), basal lamina (bl), apical folds (af), basal infoldings (bf), lacunae (lac), mitochondria (m), the afferent vessel (aff) and the efferent vessel (eff) (Freire et al. 2008).

1.8 Freshwater gill model

Unlike the whole filaments of mitochondria rich active cell types seen in crayfish, most studied crabs have portions of the gill lamellae specialized for NaCl transport (Sarver et al. 1994). The true freshwater crab *Dilocarcinus pagei* has symmetrical anterior gills and asymmetrical posterior gills where the proximal side shows signs of actively transporting tissue, abundant mitochondria, thick epithelium, and many infoldings while the distal epithelium is thin

and is formed of apical pillar cell flanges (Onken & McNamara, 2002). Although the Na^+/ K^+-ATPase and the V-ATPase are expressed in both anterior and posterior gills, both pumps are more heavily expressed in the posterior gills (Weihrauch et al. 2004a). The posterior gills compartmentalize their osmoregulatory function by having active Na^+ uptake in the thick proximal regions and Cl^- in the thin distal regions (Onken & McNamara, 2002; Weihrauch et al. 2004a). The Cl^- absorption is thought to function using apical Cl^-/ HCO_3^- exchanger in combination with basolateral chloride channels and the apical V-ATPase, where the HCO_3^- is supplied by the actions of carbonic anhydrase (Onken et al. 1991; Henry et al. 2012) (Fig. 2). The chloride independent Na^+ uptake is accomplished using the basolateral Na^+/K^+- ATPase to maintain a negative cell potential and maintain Na^+ gradients to attract Na^+ that enters the cell through apical Na^+ channels and Na^+/ H^+ exchangers (Choe et al. 2005; Edwards et al. 2002; Zeiske et al. 1992) (Fig. 2). The anterior gills, though containing active transporters do not actively uptake NaCl (Weihrauch et al. 2004a). These have been suggested to function mainly for gas exchange with the potential to function in ammonia transport using the V-ATPase in conjunction with Rhesus proteins (Durant & Donini, 2018), carbonic anhydrase (Quijada-Rodriguez et al. 2015), and potentially the sodium/hydrogen exchanger (Weihrauch et al. 2001; Weihrauch et al. 2002) (Fig. 3).

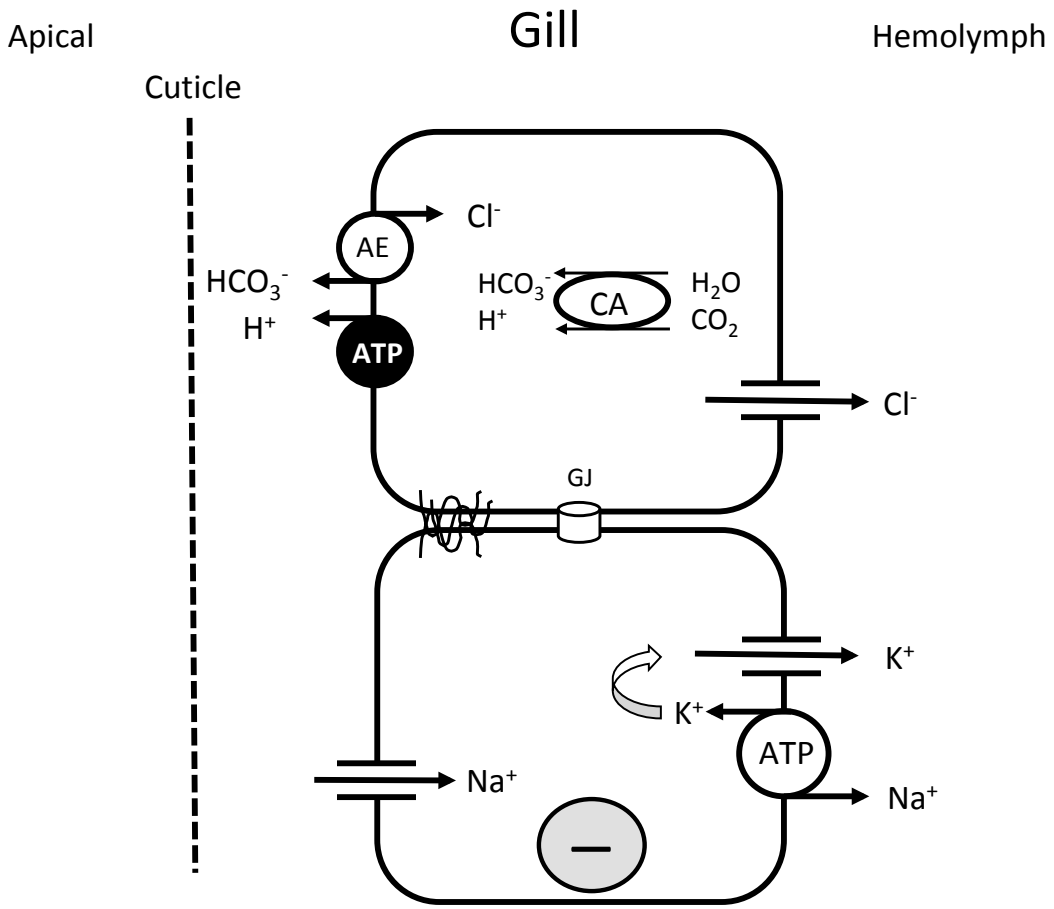


Figure 2. Model of NaCl uptake mechanisms in a freshwater crustacean gill. Model of NaCl uptake in the gills of a freshwater crustacean where negative cell membrane potential is maintained through the Na^+/K^+-ATPase and the H^+-ATPase to bring in Na though channels and utilizing HCO_3^- produced by the carbonic anhydrase to drive Cl^- uptake where these cells are connected via gap junctions (Weihrauch & O'Donnell, 2015).

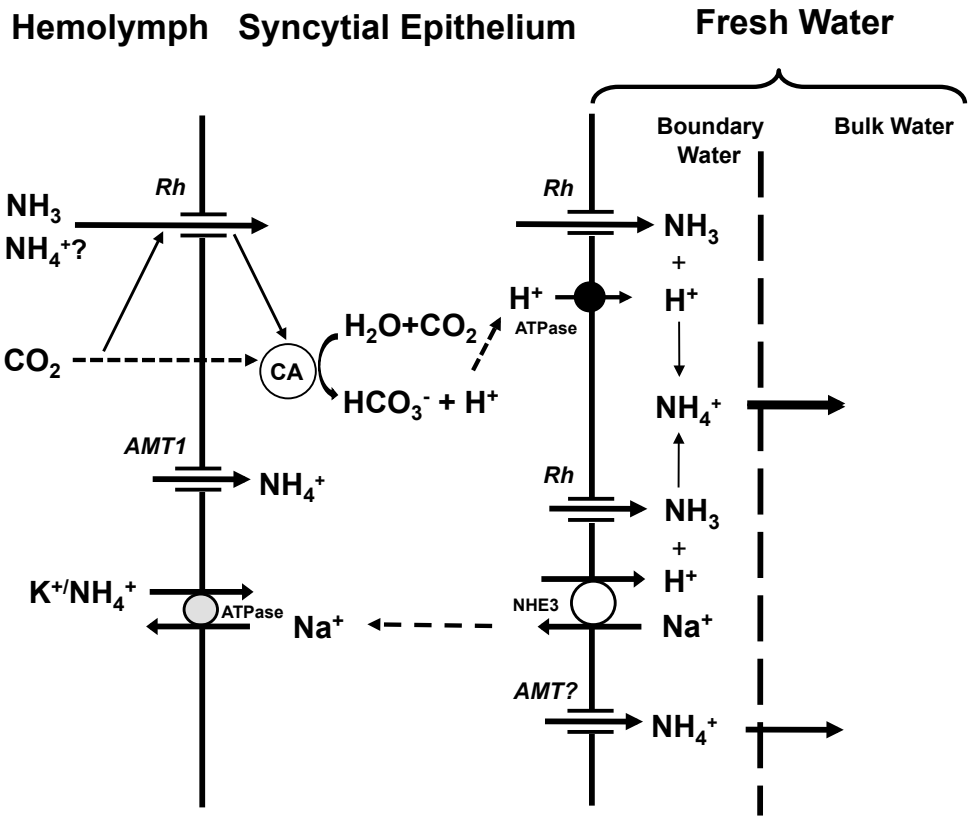


Figure 3. Model of ammonia excretion pathways in a freshwater invertebrate. Potential model of ammonia excretion in a freshwater invertebrate where ammonia (NH_3 + NH_4^+) are moved into the cell from the blood side through Rhesus proteins, Ammonia transporters (AMT) or the Na^+/K^+-ATPase and moved out by Rhesus proteins, the Na^+/H^+ exchanger into the acidified boundary layer through the work of the V-ATPase (Durant & Donini, 2018).

1.10 Objectives

The aim of this study was to determine the impact of combined and single effects of elevated pCO_2 and temperature predicted for the year 2100 on the physiology of the freshwater red swamp crayfish *P. clarkii*. As response parameters, hemolymph conditions, carapace calcification,

metabolic rate, ammonia excretion and branchial gene expression patterns of acid-base regulatory transporters/enzymes Rhesus-like protein (Rh), membrane bound carbonic anhydrase (CA1), cytoplasmic carbonic anhydrase (CA2), HCO_3^-/Cl^- anion exchanger (AE), Na^+/H^+ exchanger (NHE), Na^+/K^+- ATPase (NKA), V-Type H^+ ATPase (HAT), Na^+/ HCO_3^- co-transporter (NBC) and chloride channels (ClCh) were measured following a 14-day acclimation period.

1. Determine the distribution of transporters in the filaments of crayfish gills
2. Determine the effect of increased temperature on whole animal measurements (hemolymph parameters, metabolic rate, ammonia excretion, carapace calcification)
3. Determine the effect of increase pCO_2 on whole animal parameters
4. Determine the interaction effect between a dual temperature and pCO_2 treatment on whole animal parameters and mRNA transcript abundance of transporters in the gills.

1.11 Hypotheses

$H1_0$: The inner filaments will have a different abundance of transporters compared to the outer filaments, corresponding to their function: acid-base regulation and/or osmoregulation.

$H2_0$: Following a short term (7 days) acclimation of elevated temperature there will be an increase in metabolic rate, a decrease in hemolymph pH and an increase in hemolymph bicarbonate, which will be recovered by 14 days of exposure to treatment.

$H3_0$: Following a 7-day acclimation to elevated pCO_2 there will be an increase blood pCO_2, corresponding to a decrease in blood pH and an elevation of blood bicarbonate to combat

the acidification. An increase in metabolic rate and a decrease in carapace calcification are also expected. Recovery is expected by the end of a 14 day acclimation.

$H4_0$: In response to the combined treatment of elevated temperature and pCO_2 there is expected to be an increase in metabolic rate, a decrease in hemolymph pH, increased hemolymph pCO_2, ammonia and bicarbonate. There will be a decrease in carapace calcification and ammonia excretion.

$H5_0$: In response to 14 days acclimation to elevated pCO_2 and temperature there will be an increase in mRNA transcript abundance of transporters important for acid-base balance compared to controls to aid in maintaining homeostasis.

2. Methods

2.1 Animals

Male and female crayfish (weight = 8.1-28.77 g) were imported from Louisiana and maintained in Animal Holding Facilities (University of Manitoba, Winnipeg MB) for several years where they were acclimated to 24 °C freshwater with a 12:12 hour light/dark cycle. Crayfish were fed *ad libitum* with salmonid pellets (EWOS Vita) and duckweed (*Lemna minor*) daily. For experimental acclimations a reservoir tank filled with freshwater was controlled using an IKS Aquastar system (IKS Computer system GmbH, Germany) and was drained continuously into two 80 L aquaria, monitored with the same system. Each aquarium was constantly aerated and contained a maximum of five crayfish, identified with nail polish on their carapace (Fig. 4). Water was allowed to flow-through continuously to maintain stable water conditions and eliminate the need to do whole water changes. PVC pipes were included in the tanks to provide hiding places and reduce stress. Once moved into the acclimation tanks the crayfish were fed every two to three days with duckweed and large flake oats. All animals were starved for 48 hours prior to all experiments by removing available food. Water pH, maintained using the IKS Aquastar, was verified regularly using a pH/ATC electrode #300729.1 (Denver instruments, Gottingen, Germany) connected to a PH-ISE meter model 225 (Denver Instruments). Using the measured water temperature, pH and average total alkalinity, as determined using an assay adapted from Sarazin et al. (1999), the pCO_2 of the water was calculated using the CO2Calc program where salinity was set to zero for freshwater, and National Bureau of Standards (NBS) scale was mol/kgH_2O (Robbins et al. 2010). The below equation utilizes dissociation constants and the gas transfer velocity equation to determine pCO_2 utilizing measured pH for $\{H^+\}$ and the

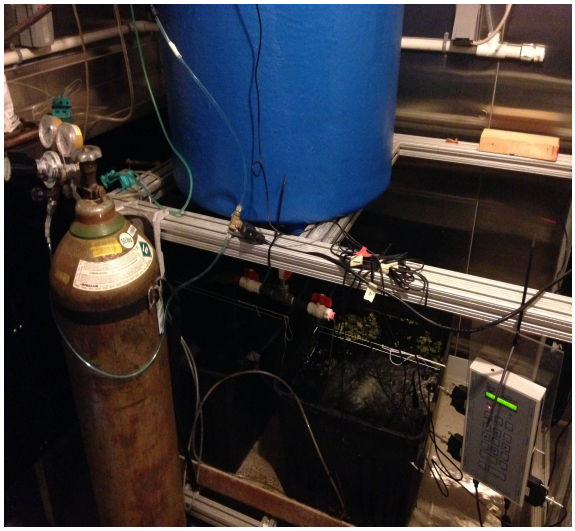
measured total alkalinity (TA) is nearly equal the carbonate alkalinity of the system (CA) (Lueker et al. 2000; Millero, 2010; Wanninkhof, 1992; Nightingale et al. 2000)

$$[CO_2] = (\{H^+\}[HCO_3^-] / K_1^* = 0.01202\ [HCO_3^-] \quad \text{(Eqn 1)}$$

$$TA \equiv 2[CO_3^{-2}] + [HCO_3^-] + [H_2BO_3^-] + 2[HBO_3^{-2}] + 3[BO_3^{-3}] + [OH^-] + [\text{organic/inorganic } H^+ \text{ acceptors}] - [H^+] \quad \text{(Eqn 2)}$$

$$CA = 2[CO_3^{-2}] + [HCO_3^-] \quad \text{(Eqn 3)}$$

Water conditions were monitored every three to four days throughout the experiment. Control temperature and pCO_2 were chosen to reflect current summer conditions in the Atchafalaya River basins (24 °C, pH of 8.03, and pCO_2 of 100 Pa) (Huang et al. 2015; USGS 2016). As pCO_2 for the Atchafalaya River basins fluctuates seasonally during the year an average value was selected for the control (Huang et al. 2015; USGS 2016). A decrease in pH of 0.5 from the control population and corresponding pCO_2 with a temperature increase of 4°C was used to simulate predicted freshwater of the year 2100 (pH of 7.5, 28°C) (Mortsch & Quinn, 1996; Philips et al. 2015; Munoz et al. 2015). Animals were acclimated to control, elevated pCO_2 and temperature, or individual treatments of elevated pCO_2 or elevated temperature for 14 days (Table. 2). Measurements of hemolymph parameters, whole animal ammonia excretion, and oxygen consumption were taken at day 7 and 14 of the acclimation. Due to hemolymph ammonia measurements, carapace calcium and qPCR requiring terminal sampling the data collected at day 7 is missing these measurements.



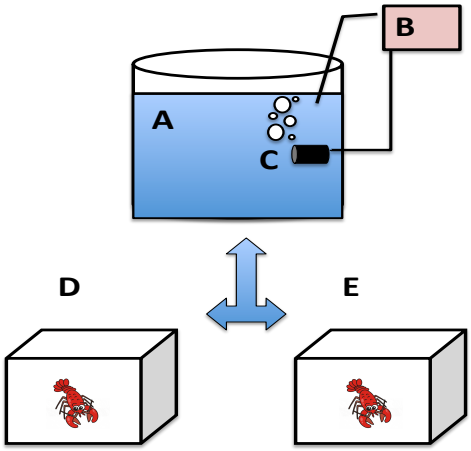


Figure 4. Experimental tank set-up. Left panel: Image of the actual environmental chamber set up showing the reservoir tank that is controlled by an Aquastar system, keeping the water at a set pH by bubbling CO_2 in before it is drained down into the two tanks containing crayfish. Right panel: Diagram showing tank set up in environmental chamber where a reservoir tank (A) is monitored with an Aquastar system (B) that maintains pH within a set range by bubbling CO_2 (C) before it drains continuously into two tanks (D & E) containing crayfish a maximum of five crayfish per tank.

Table 2. Experimental tank water conditions during acclimation of *P. clarkii* to control, high pCO_2, high temperature or high pCO_2 and temperature. Water conditions averaged from measurements taken from tanks with crayfish exposed to control, individual temperature and pCO_2 treatments or combined. Values are given as mean ± SEM (N=4).

	Control	High Temp	High pCO_2	High pCO_2 + Temp
Temperature [°C]	24.1 ± 0.2	28.1 ± 0.3	23.9 ± 0.2	28.2 ± 0.1
pH	8.1 ± 0.008	8.1 ± 0.01	7.5 ± 0.03	7.53 ± 0.02
pCO_2 [Pa]	92.2 ± 2.6	70.8 ± 4.8	228 ± 16.8	272.2 ± 7.6

2.3 Hemolymph parameters

Hemolymph was removed from the base of the walking legs of the crayfish using a sterile syringe. Hemolymph pH was determined immediately upon removal from the animal using an InLab Micro pH electrode (Mettler Toledo, Ohio, USA). Samples were sonicated on ice to remove clots prior to being run through a Corning 965 TCO2 Analyzer (Corning Limited Halstead, Essex, England) to determine total carbon (C_T). Preliminary tests confirmed there was no change in hemolymph C_T with sonication. Using the measured hemolymph pH and C_T the pCO_2 and $[HCO_3^-]$ were calculated using the following equations (Truchot 1976):

$$pCO_2 = CT/((10^{pH - pK1} * \alpha CO_2) + \alpha CO_2) \quad \text{(Eqn 4)}$$

$$[HCO3^-] = 10^{pH-pK1} * \alpha CO_2 * pCO_2 \quad \text{(Eqn 5)}$$

where pK1 is the first dissociation constant of carbonic acid and αCO_2 is the solubility coefficient of carbon dioxide. Assuming a hemolymph salinity of 12.3 ppt (~369 mOsm) pK1 and αCO_2 were determined for the different acclimation temperatures (24°C and 28°C) (Millero et al. 2002; Weiss 1974). A αCO_2 value of 0.00033 (mmol/I*Pa) and a pK1 of 5.98 were used for crayfish held at 24°C. For crayfish at 28°C a αCO_2 of 0.00029 (mmol/I*Pa) and pK1 of 5.94 were used. Additional sonicated hemolymph was stored at -20°C until ammonia could be analysed. Hemolymph was deprotinated to remove the potential for proteins to release ammonia when the solution is alkaline, overestimating the hemolymph ammonia, using a modified protocol derived from Veauvy et al. (2008). 300 µl of hemolymph was combined with equal volumes of 6% perchloric acid and was incubated for 10 minutes on ice. Deprotinated homogenate was centrifuged (14,000 RMP 4°C, 5 minutes) and neutralized with 0.6 volumes of

2.5 mol L^{-1} $KHCO_3$. Following neutralization, homogenate was centrifuged (14,000 RMP, 4°C, 5 minutes) and supernatant removed and diluted to a final hemolymph dilution of 1 in 10. Samples were analyzed using an ammonia electrode as previously described by Weihrauch et al. (1998). Standard curves were made using crayfish ringers made without ammonia that was deprotinated and diluted to the same degree as the samples.

2.4 Resting metabolic rate

Whole body metabolic rate was measured with intermittent flow respirometry (Loligo systems, Diele, Denmark) and AutoResp^{TM} software version 2.3.0 to assess resting metabolic rate (RMR) as previously described by Yoon (2017). Animals were weighed to nearest 1 mg and placed in metabolic chambers. Flow rate was adjusted to exchange water sufficiently, but not cause any stress to the animals. A black curtain surrounding the metabolic chambers was used to minimize any external visual stress. Water surrounding respirometry chambers was paired to acclimation temperature and pH that animals were acclimated to and monitored using an Aquastar to determine pH drift (~ 0.3). Water was changed between trials to keep conditions stable. Each cycle of measurement was computerized with 360 seconds of flow through, 60 seconds of waiting and static measurement of 900 seconds. Oxygen saturation was not allowed to decrease below 80% throughout the experiment to prevent metabolic suppression (Marsh & Manahan, 1999; Chabot et al. 2016). Animals were acclimated in chambers for one hour (three cycles) following which RMR was measured for an hour (three measurements). Biological oxygen demand (BOD) was measured following each trial and was subtracted from the oxygen consumption values for each animal. After each experiment, all the equipment was disinfected with 1:40 diluted OxiVir (Diversey, North Carolina, USA) to minimize the bacterial oxygen

consumption in the following experiments due to equipment contamination. Oxygen consumption was averaged over three measurements and used to calculate metabolic rate, expressed as μmol O_2 $g^{-1}h^{-1}$.

2.5 Whole animal ammonia excretion

Whole animal ammonia excretion rates were determined by placing starved (48 h) crayfish in constantly aerated containers with 200 mL of freshwater. Crayfish were weighed prior to the experiment and conditions were maintained at experimental acclimation temperatures using a water bath (Table. 1). The background ammonia for each acclimation was determined using the same set up without an animal. A 10 ml water sample was removed from each container after 60 minutes and 120 minutes. Samples were immediately frozen at -20 °C until analysis using a gas-sensitive NH_3 electrode (Orion 9512; Thermo Scientific, Cambridgeshire, England) (Weihrauch et al. 1998).

2.6 Carapace calcification

Carapaces were removed following sacrificing of animals and were stored at -80 °C until analysis. Carapaces were freeze dried, powdered, and dissolved in 65% nitric acid (~65°C for 3-4 days) following the methods used by Spicer and Eriksson (2003). Samples were diluted 1 in 201 by placing 50 μl of carapace in nitric acid solution in 10 ml H_2O and were analyzed for relative Ca^{2+} concentrations using ion chromatography (Metrohm 850 Professional IC; Herisau, Switzerland).

2.7 Silver staining

Following the silver staining protocol used by Dickson et al (1991) to determine different regions of the gill live crayfish were rinsed in deionized water and then submerged for 30 minutes in 0.05% w/v $AgNO_3$ adjusted to 25 °C using a water bath and heater. $AgNO_3$ was rinsed off with deionized (DI) water and crayfish were sacrificed to remove the carapace. The crayfish with exposed gills was submerged for one hour in Kodak Microdol-X-developer (Kodak Canada Inc.) diluted 3:1 with water. Gills were removed, washed in DI water and placed in distilled water with two drops (~30 µl) of NH_4S, after which they were imaged using a Canon 70D camera with 55-135 mm lens.

2.8 Tissue preparations for RNA isolation

Crayfish were placed on ice for 15-20 minutes after which their head and carapace were removed to expose the gills. Podobranch gills one through six from both the right and left side were cut at the base with RNase-free scissors, placed in RNAlater® (Ambion, Fisher Scientific, Ottawa, ON, Canada) and were stored at 4°C overnight to allow RNA preserving agent to permeate the tissues. RNA isolation protocol was used the day following dissection and isolated RNA was stored at -20 °C until further processing.

2.9 qPCR

Gills stored in RNAlater overnight were separated and kept in solution to protect the RNA while the branchial inner and outer filaments were removed utilizing silver staining image as a guide (Fig. 5). Filaments were centrifuged (2500 x g, 5 min, 4°C) using 40 µm nylon mesh

sterile cell strainers (Fisherbrand®, Edmonton, AB, Canada) to remove RNAlater after which RNA was isolated using E.Z.N.A® Total RNA kit 1 (OMEGA bio-tek, Norcross, GA, USA). To improve RNA quality it was run through OneStep™ PCR inhibitor removal kits (Zymo Research, Irvine, CA, USA) and afterwards re-precipitated using 2/5 volume of RNA of 1.5 M sodium acetate (Sigma Life Science, St Louis, MO, USA), equal volumes 99.5% isopropanol (Acros organics, NJ, USA) and RNA grade glycogen (final concentration 1 µl/µl) (Thermo Scientific, Carlsbad, CA, USA) to improve yield. RNA was cleaned with ice cold 70% ethanol and resuspended with RNAstorage (Ambion, Fisher Scientific, Ottawa, ON, Canada) following protocols provided by Thermoscientific for the glycogen. Successful removal of genomic DNA by DN*ase* treatment (DNase 1, Invitrogen, Carlsbad, CA, USA) was confirmed using PCR (35 cycles) employing G Protein Suppressor primers (see Table 3) and a successful treatment was indicated by the absence of a PCR product. For all samples cDNA was synthesized from 0.28 µg DN*ase* treated total RNA using iScript cDNA synthesis kits (Biorad, Hercules, CA, USA). Primers were designed using known sequences of genes in GenBank and the *P. clarkii* transcriptome. All primer pairs (Table. 3) were tested to confirm a single product of predicted size was produced. The amplicons were extracted using an E.Z.N.A gel extraction kit (Omega Bio Tek, Winooski, VT, USA) and sequenced to confirm product at "The Centre for Applied Genomics" (Toronto, ON). Optimal annealing temperature for each primer pair was determined utilizing a gradient PCR. A standard curve was generated for each qPCR using a serial dilution series of whole crayfish gill cDNA. An R^2 value of >0.98 and an efficiency of >90% and <110% was required of all PCR conditions. Real-time PCR assays were run using cDNA (2 µl of 0.28 µg cDNA diluted one in four), primers (final concentration of 400 nM) and SSO FastEvaGreen Supermix (Biorad, Hercules, CA, USA) in a 12µl assay. In addition a melt curve analysis was

performed for each qPCR run to confirm single products. As the internal reference gene the G Protein Suppressor, a gene involved in carapace maintenance was used, as it did not show any significant difference across tissues and treatments (Gao et al. 2015)(Fig. 6). G-Protein suppressor primers were tested using qPCR with all treatment and control tissues to verify there was no difference in amplification of product as a result of treatment to determine suitability as an internal standard (Fig. 6).

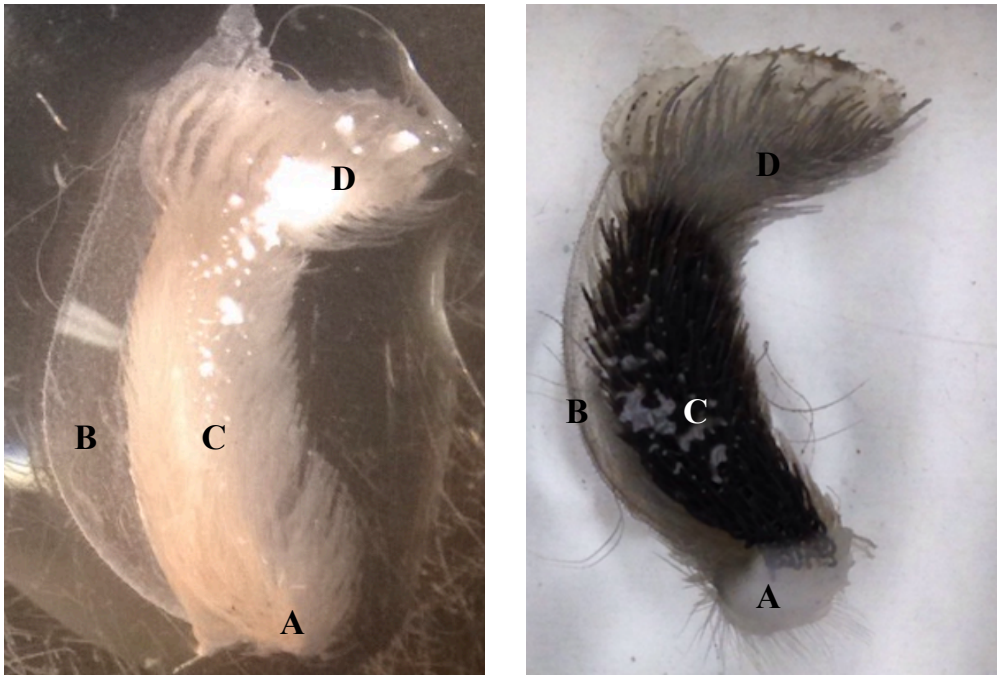


Figure 5. Image of a *P. clarkii* podobranch gill. Left panel: A control crayfish gill showing attachment in the animal (A) lamella (B) darkly stained inner filaments (C) and lightly stained outer filaments (D) as determined using the silver stained image (Right panel) for tissue dissection.

Table 3. Employed primers for qPCR. Primer sequences designed and used for quantitative PCR of G-protein suppressor (GPro), sodium hydrogen exchanger (NHE), membrane bound carbonic anhydrase (CA1), cytoplasmic carbonic anhydrase (CA2), Rhesus-like protein (Rh), Cl^- /HCO_3^- anion exchanger (AE), sodium potassium ATPase α –subunit (NKA), and the V- (H+) ATPase subunit B (HAT) sodium bicarbonate co-transporter (NBC) and chloride channel (ClCh). All primers are reported as 5' to 3'.

Transcript	Primer Sequence (5' → 3')	Product Size (bp)	Annealing Temp (°C)
GPro F	TAGTGGGAGACTGTGGACTATG	119	54
GPro R	GAGGTTGAACACCGCTAACA	119	54
NHE F3	TTAGCCAGATCAAGATGCAGG	192	60
NHE R3	CCTAATGTACGGGACCTCAATG	192	60
CA1 F	TCCACTACAACGCAGCTTAC	168	55
CA1 R	GGGATGTTCGCTGAGTTGA	168	55
CA2 F	GATCGTACTGGACATATGGCG	152	54
CA2 R	CATCCTTGTGAGAGTTGTCCC	152	54
Rh F	CTGGAATCATTGGCTGCATTG	197	54
Rh R	CACCAATCAAATTCTCGCTCTC	197	54
AE F2	GTGTCATCTTTGCCCTGTTTG	153	54
AE R2	CATGAGAGTGTGGATTGGCA	153	54
NKA F2	ACCATGACCGCAAAATTCTC	183	54
NKA R2	AGAGGAAGGCAGCCAGTGTA	183	54
HAT F	TGAAAGCTGTAGTGGGTGAAG	197	54
HAT R	CGTATTCCAGCCTCAACTCTT	197	54
NBC F	AGGAGGAGATGAAGATGCGA	181	55
NBC R	ATGATTGGGGTGAGACAAGC	181	55
ClCh F	TTGGCTCTGTCCTTGGTTTAG	139	60
ClCh R	CCGTCCGTGAACATCTTCTTTA	139	60

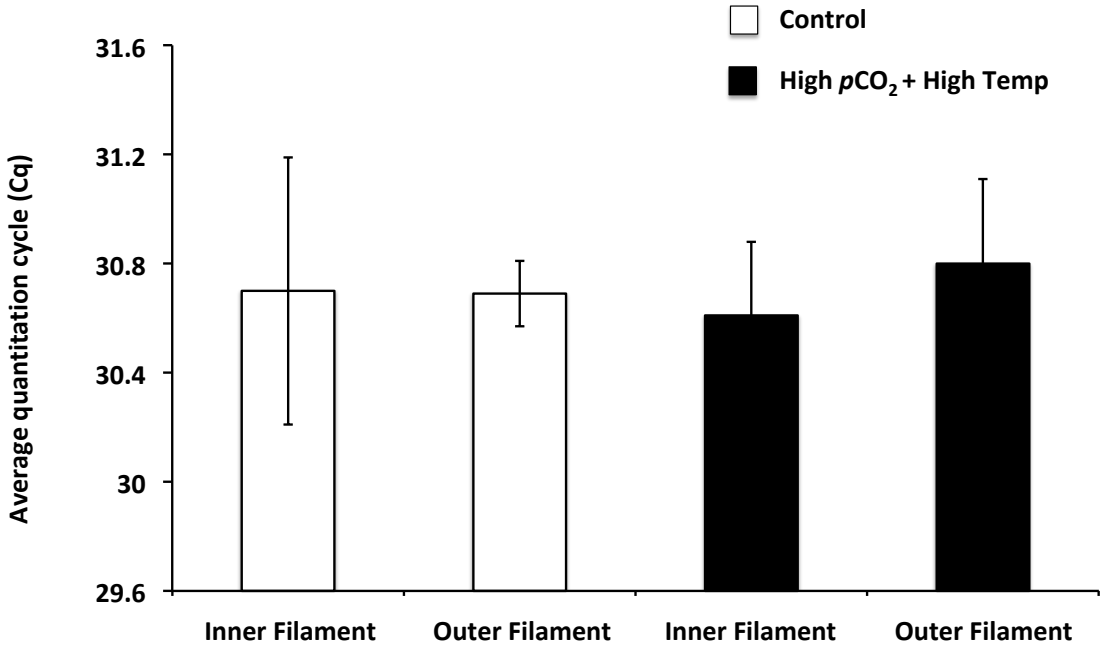


Figure 6. Average quantitation cycle (Cq) of gill tissues from control and high pCO2 and temperature acclimations with GPro primers. There was no significant different between tissues and treatments as determined by a two way ANOVA (p-value >0.05) (N=8).

2.10 Statistics

Statistical analyses were performed using Prism version 6.00 statistical software (GraphPad Prism 6.00). The Shapiro-Wilk normality test was used to verify values have a Gaussian distribution (α =5%). Grubb's test was used to determine outliers, which were removed from analysis. All data sets were checked for homogeneity of variance using Levene's test. Normally distributed data were analyzed using a two-way ANOVA with Dunnett's post hoc analysis, where temperature and pCO_2 were set as independent factors. All data that didn't meet any assumptions of the ANOVA were analyzed using a general linear model (GLM) where average control values were set as the intercept in R (R Core Team, 2013). Paired T-tests were used to compare repeated measurements using the same individuals. All data are presented as mean ± SEM and were considered statistically significant for all p-values < 0.05. Trends in data with P-values between 0.1 and 0.05 are indicated with ◆

3. Results

3.2 Effect of increased temperature

3.2.1 Hemolymph parameters

Following 7 days of acclimation to elevated environmental temperature (+4°C) there was no change in the measured hemolymph parameters; pH, pCO_2, and [HCO_3^-] compared to controls (Fig. 7). By the end of a 14-day acclimation to a temperature increase to 28 °C there was a significant decrease in hemolymph pH by 0.1 to 7.45 ± 0.024 compared to controls (7.54 ± 0.013) (two way ANOVA p=0.025)(Fig. 8).

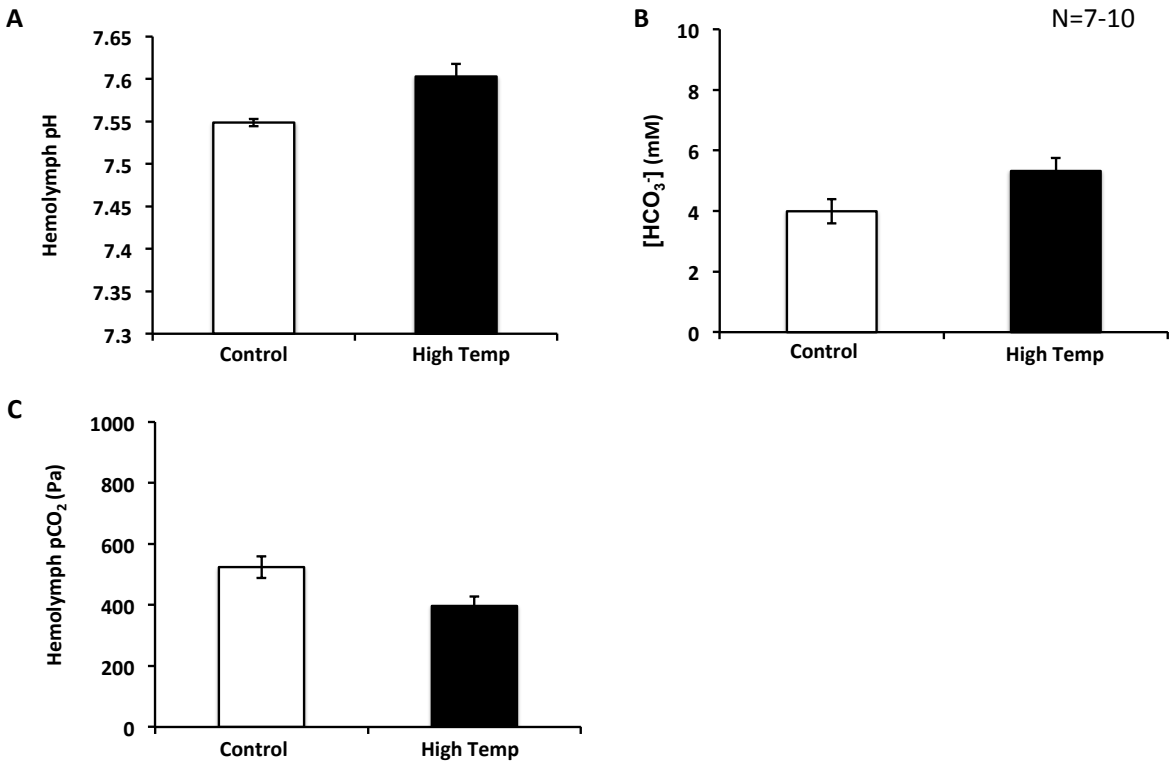


Figure 7. Hemolymph parameters following a 7-day acclimation to elevated temperature. Hemolymph pH (A), [HCO_3^-] (B), and pCO_2 (C) of *P. clarkii* exposed to 7 days of elevated temperature (28°C) compared to control (24°C). Values are presented as mean ± SEM (N=7-10). Significance is indicated by * for p-values < 0.05 as determined by a two way ANOVA.

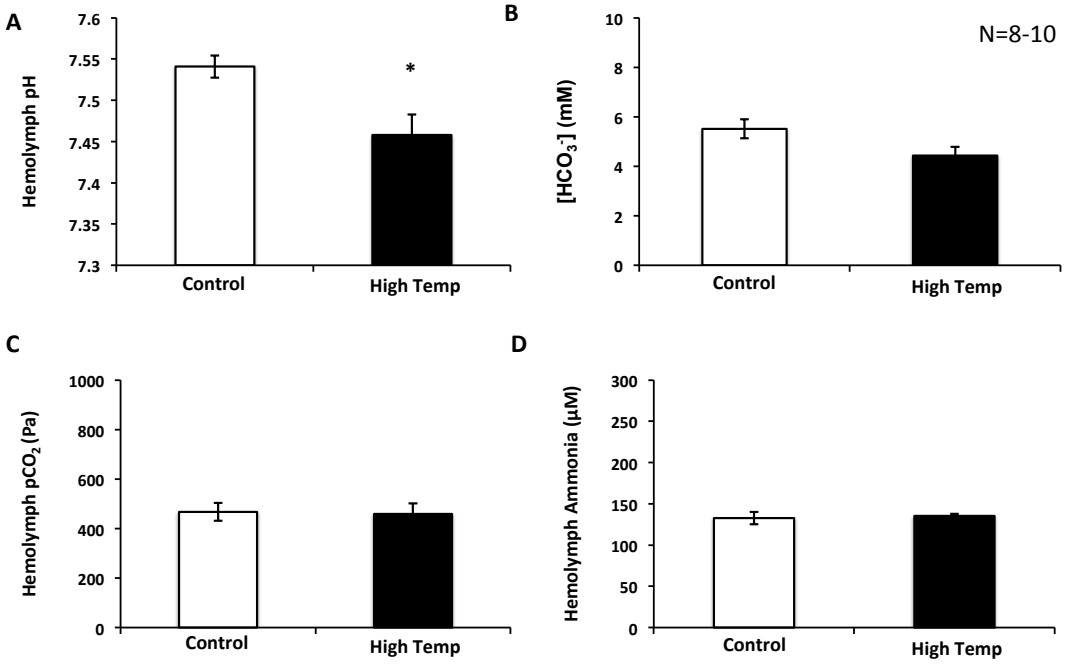


Figure 8. Hemolymph parameters following a 14-day acclimation to elevated temperature. Hemolymph pH (A), $[HCO_3^-]$ (B), pCO_2 (C), and ammonia concentration (D) of *P. clarkii* exposed to 14 days of elevated temperature (28°C) compared to control (24°C). Values are presented as mean ± SEM (N=8-10). Significance is indicated by * for p-values < 0.05 as determined by a two way ANOVA.

3.2.2 Metabolic rate and ammonia excretion

7 days of elevated temperature exposure caused a significant increase, nearly double, in metabolic rate (7.63 ± 0.72 µmol O_2 $g^{-1}h^{-1}$) compared to controls (3.53 ± 0.30 µmol O_2 $g^{-1}h^{-1}$)(two way ANOVA $p < 0.0001$) (Fig. 9). Following a 14-day exposure to elevated temperatures crayfish showed a recovery of metabolic rate to a level not statistically different from controls (3.08 ± 0.38 µmol O_2 $g^{-1}h^{-1}$) (Fig. 10). There was no change in whole animal ammonia excretion between control and high temperature acclimated crayfish.

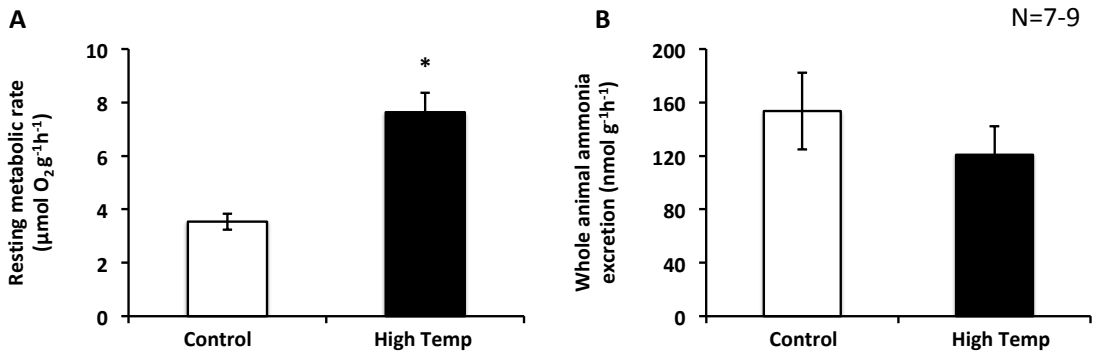


Figure 9. Metabolic rate and ammonia excretion following a 7-day acclimation to elevated temperature. Resting metabolic rate (N=7-9)(A) whole animal ammonia excretion (N=7-9)(B) in crayfish exposed to elevated temperature (28°C) compared to control (24°C) for 7 days. Significance (P-value < 0.05) was determined using a two way ANOVA and is indicated by *.

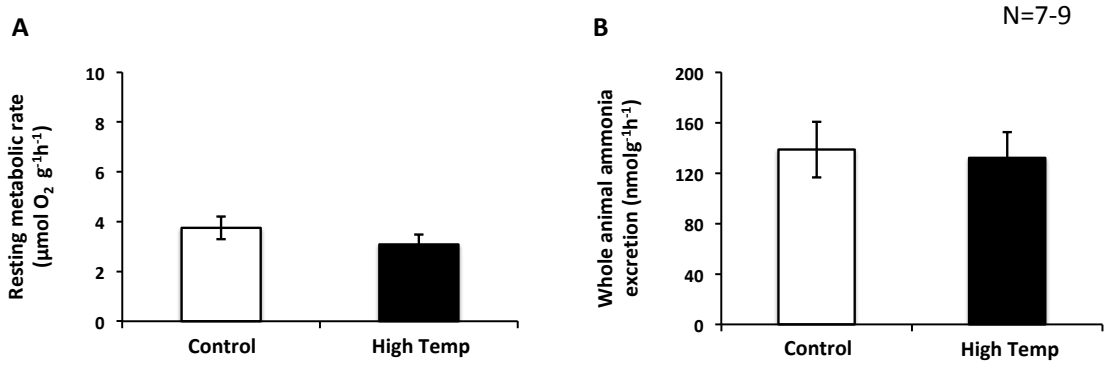


Figure 10. Metabolic rate and whole animal ammonia excretion following a 14-day acclimation to elevated temperature. Resting metabolic rate (N=7-9)(A) whole animal ammonia excretion (N=7-9)(B) in crayfish exposed to elevated temperature for 14 days (28°C) compared to control (24°C). Significance (P-value < 0.05) was determined using a two way ANOVA and is indicated by *.

3.2.3 Carapace calcification

Following a 14 day acclimation to elevated temperature (~28 °C) there was no change in carapace calcification (Ca^{2+}) (Fig. 11).

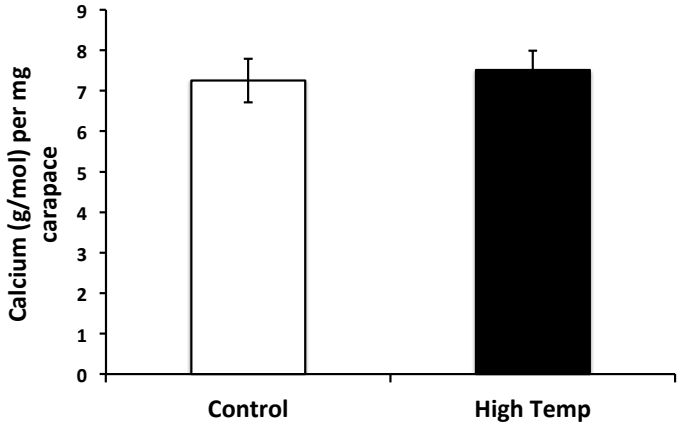


Figure 11. Carapace calcium (Ca^{2+}) content following a 14-day acclimation to elevated temperature. Carapace calcium content in control and high temperature acclimated crayfish following 14 days exposure. Values are presented as mean ± SEM (N=8) and significance was determined using a two way ANOVA.

3.3 Effect of increased pCO_2

3.3.1 Hemolymph parameters

7 days of exposure to elevated environmental pCO_2 (~230 Pa) caused a significant increase in hemolymph bicarbonate concentration from 3.99 ± 0.40 mM in controls to 6.57 ± 0.62 mM in treatment (two way ANOVA p=0.0066) (Fig. 12). Following a 14-day acclimation to elevated environmental pCO_2 there was a significant increase in hemolymph ammonia was observed from 132.70 ± 7.47 µM in controls to 182.12 ± 19.13 µM in treatment animals (two way ANOVA p=0.0067) (Fig. 13) There was no significant effect of the 14 day acclimation to elevated pCO_2 on hemolymph pH, pCO_2, and $[HCO_3^-]$.

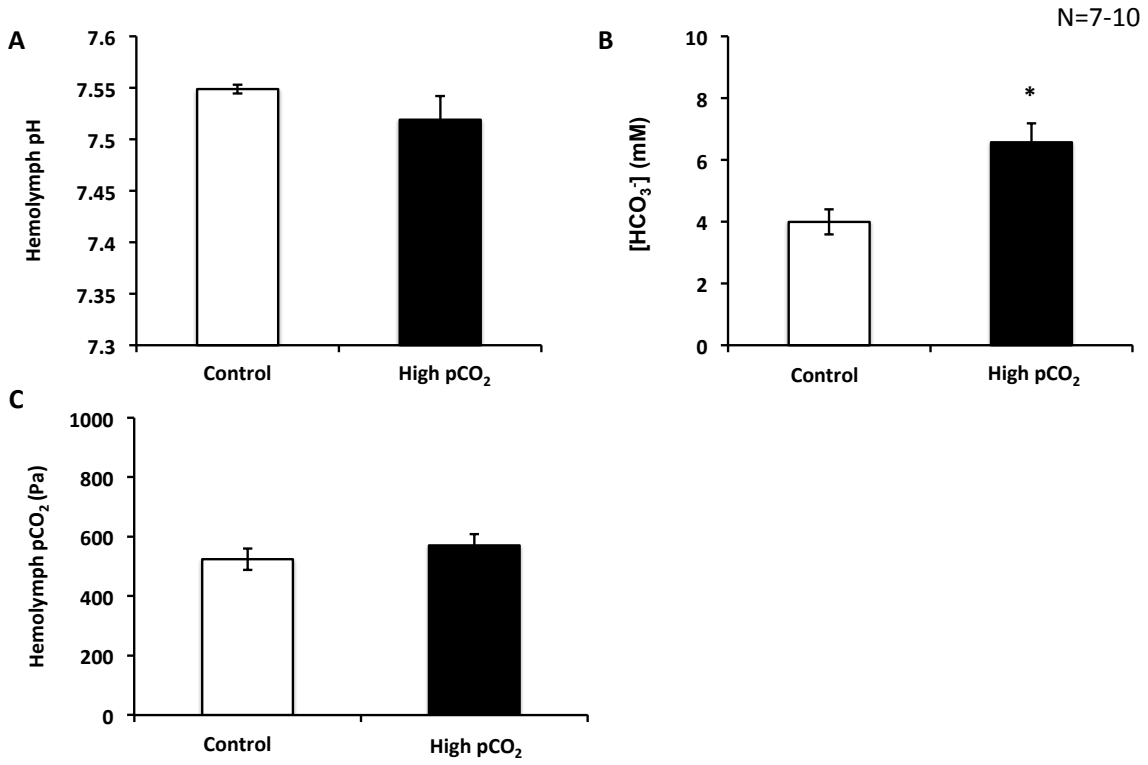


Figure 12. Hemoylmph parameters following a 7-day acclimation to elevated pCO_2. Hemolymph pH (A), $[HCO_3^-]$ (B), pCO_2 (C), and ammonia concentration of *P. clarkii* exposed to 7 days of elevated pCO_2 (~230 Pa) compared to control (~90 Pa). Values are presented as mean ± SEM (N=8-10) and significance is indicated by * for p-values < 0.05 as determined by a two way ANOVA.

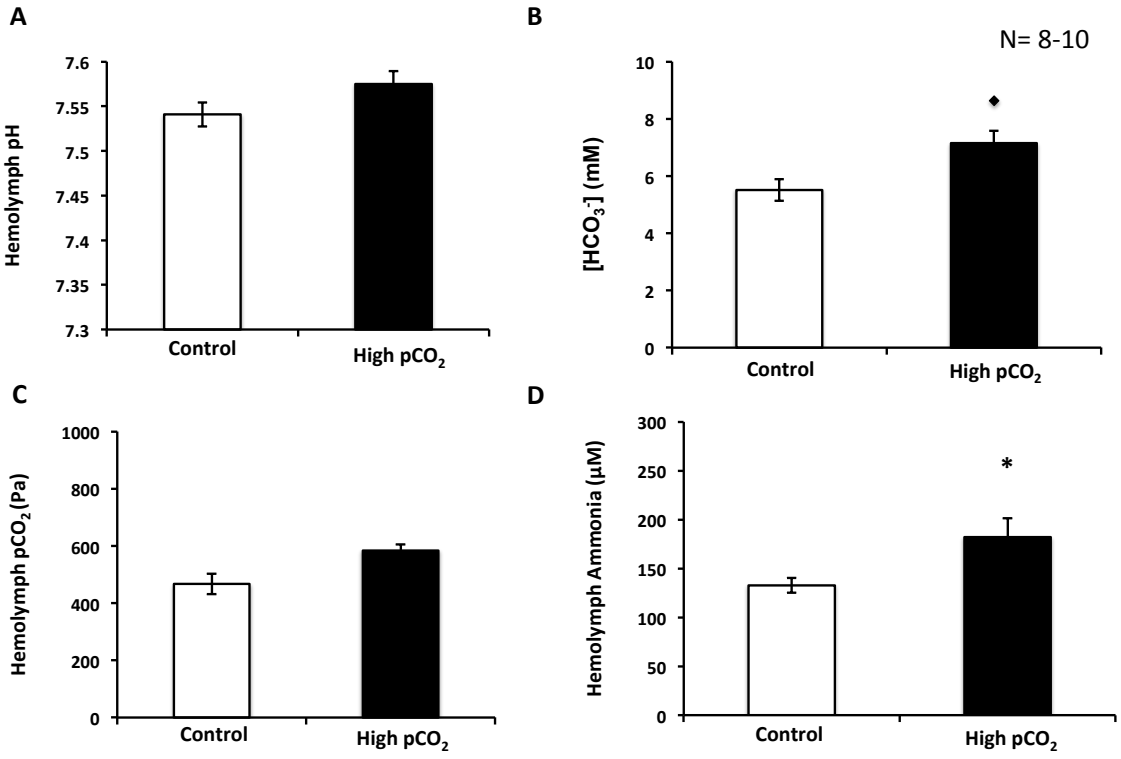


Figure 13. Hemolymph parameters following a 14-day acclimation to elevated pCO_2. Hemolymph pH (A), $[HCO_3^-]$ (B), pCO_2 (C), and ammonia concentration of *P. clarkii* exposed to 14 days of elevated pCO_2 (~230 Pa) compared to control (~90 Pa). Values are presented as mean ± SEM (N=8-10) and significance is indicated by * for p-values < 0.05 as determined by a two way ANOVA. P-values between 0.1 and 0.05 are indicated with ◆.

3.3.2 Metabolic rate and ammonia excretion

A 7-day acclimation to elevated environmental pCO_2 caused a substantial decrease in whole animal ammonia excretion rates (50.38 ± 8.12 $nmol g^{-1}h^{-1}$) compared to control levels (153.57 ± 28.85 $nmol g^{-1}h^{-1}$) (two way ANOVA p=0.022). Resting metabolic rate at day 7 of treatment was lower than control (2.06 ± 0.38 µmol O_2 $g^{-1}h^{-1}$ and 3.54 ± 0.30 µmol O_2 $g^{-1}h^{-1}$ respectively) but was not statistically significant (two way ANOVA p= 0.12)(Fig. 14). By 14 days of exposure a significant decrease in metabolic rate was observed with 1.15 ± 0.17 µmol O_2

$g^{-1}h^{-1}$ compared to controls (3.75 ± 0.46 µmol O_2 $g^{-1}h^{-1}$)(two way ANOVA p = < 0.0001). Although whole animal ammonia excretion rates were decreased from 138.72 ± 22.12 $nmolg^{-1}h^{-1}$ to 79.18 ± 13.52 $nmolg^{-1}h^{-1}$ in the high pCO_2 acclimated crayfish this difference was no longer significant after 14 days (two way ANOVA p=0.23) (Fig. 15).

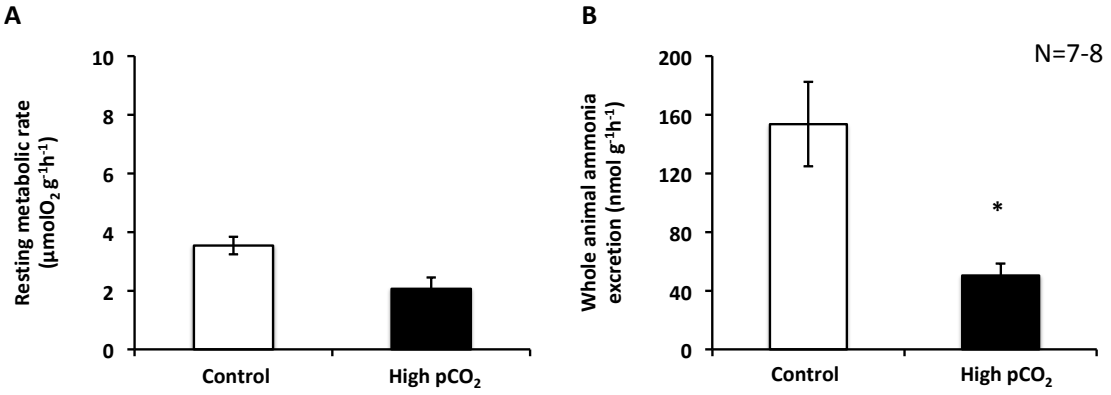


Figure 14. Metabolic rate and ammonia excretion following a 7-day acclimation to high pCO_2. Resting metabolic rate (N=8)(A) whole animal ammonia excretion (N=8-9)(B) in crayfish exposed to pCO_2 for 7 days (~230 Pa) compared to control (~90 Pa). Significance (P-value < 0.05) was determined using a two way ANOVA and is indicated by *.

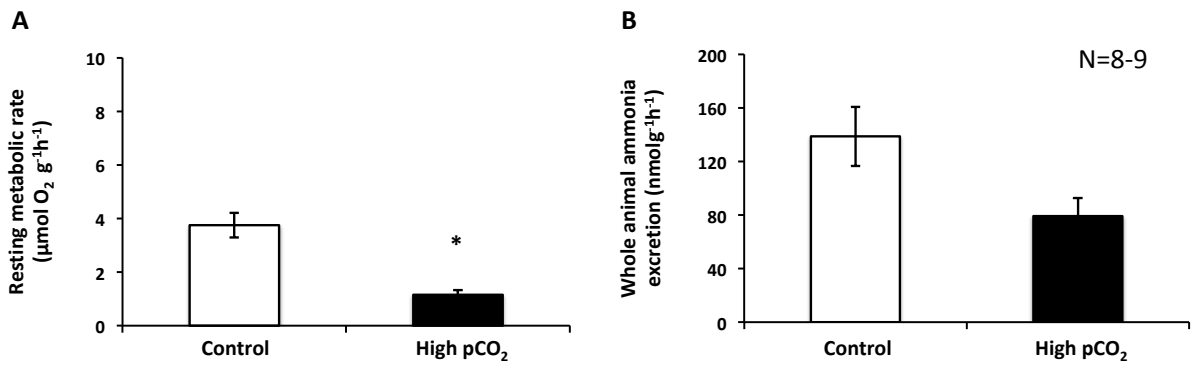


Figure 15. Metabolic rate and ammonia excretion following a 14-day acclimation to elevated pCO_2. Resting metabolic rate (N=8)(A) whole animal ammonia excretion (N=8-9)(B) in crayfish exposed to elevated pCO_2 for 14 days (~230 Pa) compared to control (~90 Pa). Significance (P-value < 0.05) was determined using a two way ANOVA and is indicated by *.

3.3.3 Carapace calcification

There was no significant effect of a 14-day acclimation to elevated environmental pCO_2 (~230 Pa) on the Ca^{2+} per mg carapace (Fig. 16).

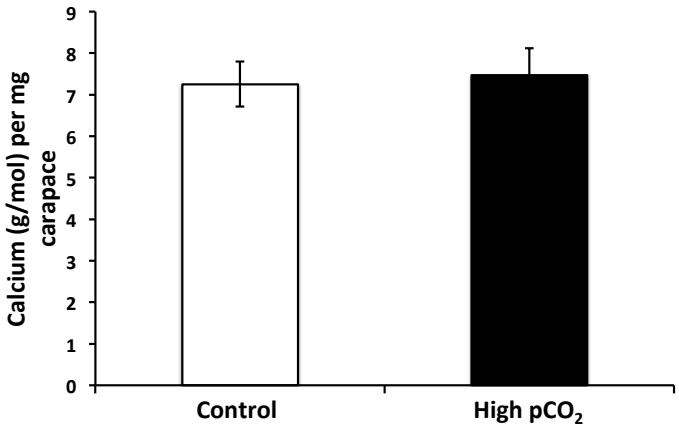


Figure 16. Carapace calcium (Ca^{2+}) content following a 14-day acclimation to elevated environmental pCO_2. Carapace calcification in control and high pCO_2 acclimated crayfish following 14-day exposure. Values are presented as mean ± SEM (N=7-8) and significance was determined using a two way ANOVA.

3.4 Effect of high pCO_2 and temperature

3.4.1 Hemolymph parameters

Day 7 of dual stressor treatment showed a significant decrease in hemolymph pH (7.46 ± 0.03) and an increase in pCO_2 (799.14 ± 78.18 Pa) and [HCO_3^-] (7.68 ± 0.77 mM) compared to controls (7.55 ± 0.004/ 524.20 ± 35.98 Pa/ 3.99 ± 0.40 mM; two way ANOVA p= 0.024, 0.0026, 0.0002, respectively)(Fig. 17). Following 14 days of treatment, pH returned to control levels; control (7.54 ± 0.01) and treatment (7.47 ± 0.04) but there was still significant elevation in hemolymph pCO_2 (821.64 ± 83.65 Pa) and bicarbonate (7.89 ± 1.03 mM), and a decrease in ammonia (47.85 ± 3.22 μM) compared to control animals (467.28 ± 35.72 Pa/ 5.51 ± 0.38 mM/ 132.71 ± 7.47 μM; two way ANOVA p= <0.01, 0.020, <0.01 respectively) (Fig. 18).

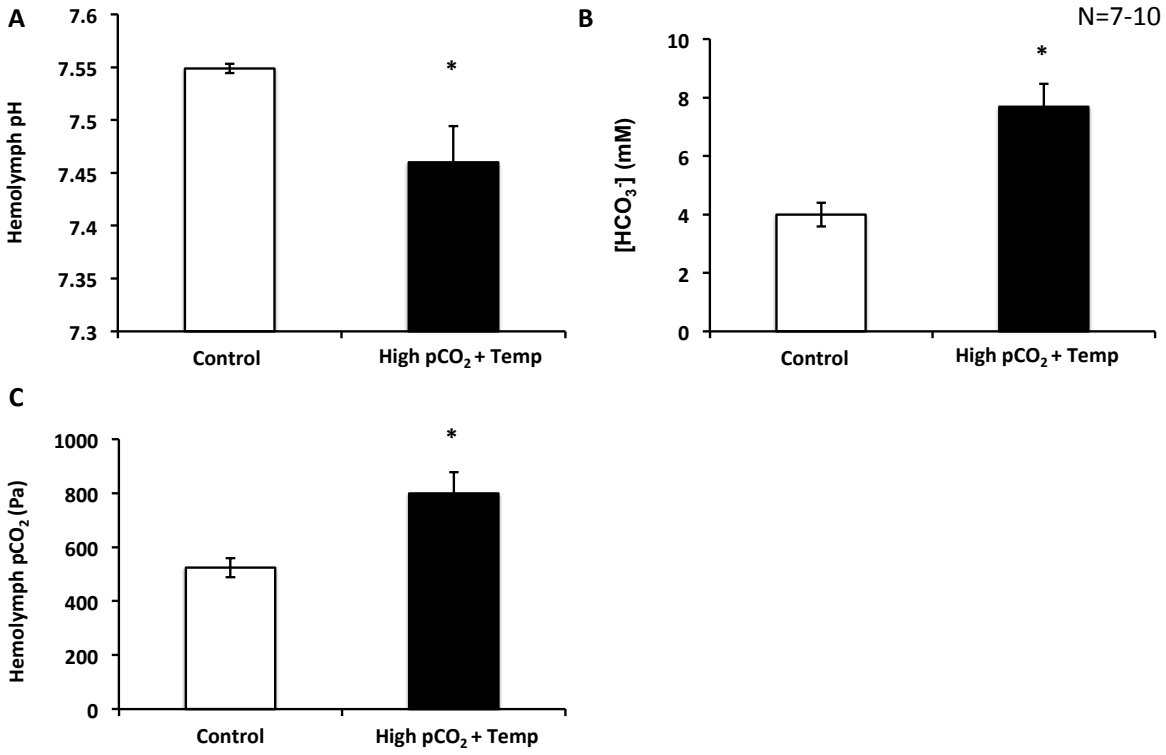


Figure 17. Hemolymph parameters following a 7-day acclimation to elevated pCO_2 and temperature treatment. Hemolymph pH (A), $[HCO_3^-]$ (B), pCO_2 (C), and ammonia concentration of *P. clarkii* exposed to 7 days of elevated pCO_2 and temperature (~270 Pa, 28°C) compared to control (~90 Pa, 24°C). Values are presented as mean ± SEM (N=7-10). Significance is indicated by * for p-values < 0.05 as determined by a two way ANOVA.

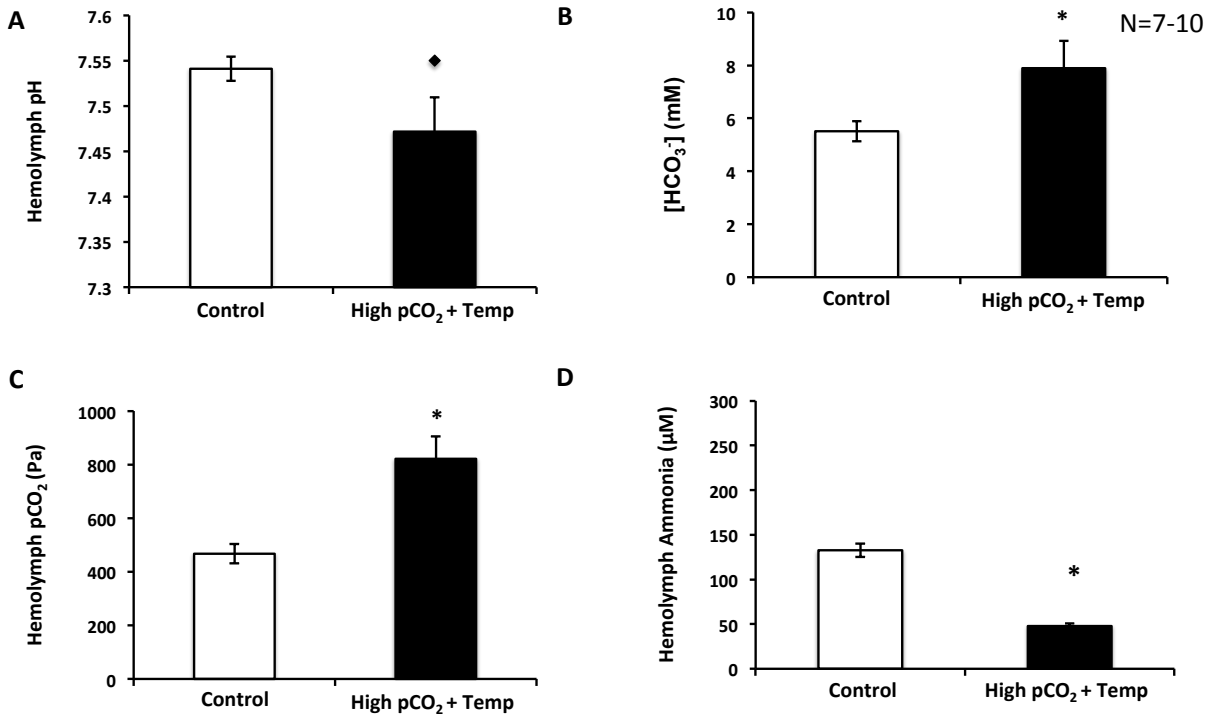


Figure 18. Hemolymph parameters following a 14-day acclimation to elevated environmental pCO_2 and temperature. Hemolymph pH (A), $[HCO_3^-]$ (B), pCO_2 (C), and ammonia concentration of *P. clarkii* exposed to 14 days of elevated pCO_2 and temperature (~270 Pa, 28°C) compared to control (~90 Pa, 24°C). Values are presented as mean ± SEM (N=7-10). Significance is indicated by * for p-values < 0.05 as determined by a two way ANOVA. P-values between 0.1 and 0.05 are indicated with ◆

3.4.2 Metabolic rate and ammonia excretion

At day 7 of exposure to elevated pCO_2 and temperature there was no significant change in metabolic rate or whole animal ammonia excretion (Fig. 19). There was a significant decrease in metabolic rate from 3.75 ± 0.46 µmol O_2 $g^{-1}h^{-1}$ to 2.09 ± 0.31 µmol O_2 $g^{-1}h^{-1}$ following a 14 day acclimation to elevated pCO_2 and temperature (two way ANOVA p= 0.0078) (Fig. 20). There was no net change in whole animal ammonia excretion.

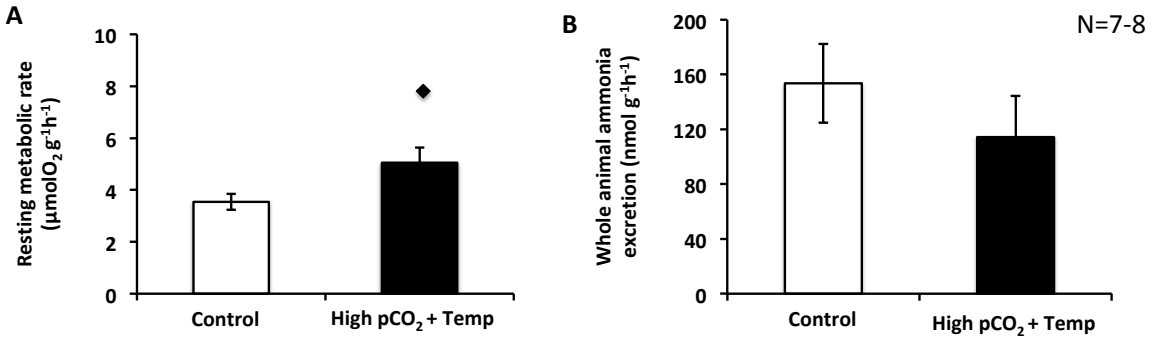


Figure 19. Metabolic rate and ammonia excretion following a 7-day acclimation to elevated pCO_2 and temperature. Resting metabolic rate (N=8)(A) whole animal ammonia excretion (N=9)(B) in crayfish exposed to of elevated pCO_2 and temperature for 7 days (~270 Pa, 28°C) compared to control (~90 Pa, 24°C). Significance (P-value < 0.05) was determined using a two way ANOVA and is indicated by *. P-values between 0.1 and 0.05 are indicated with ◆.

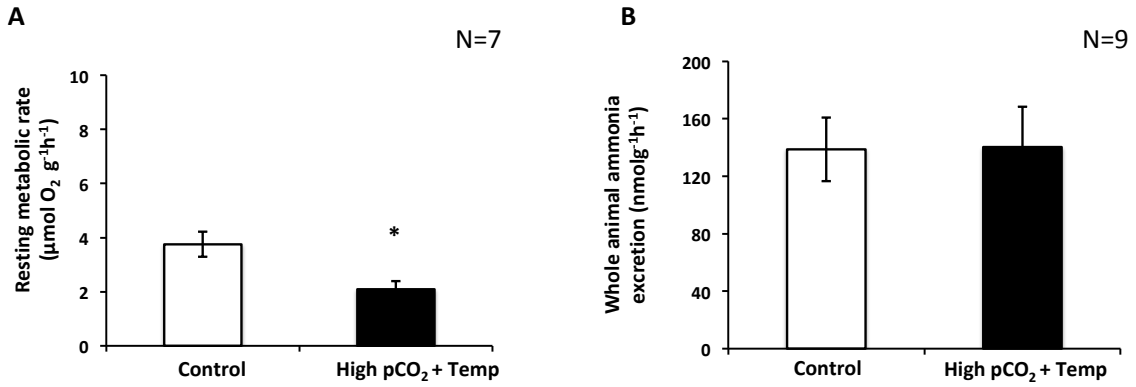


Figure 20. Metabolic rate and ammonia excretion following a 14-day acclimation to elevated environmental pCO_2 and temperature. Resting metabolic rate (N=8)(A) and whole animal ammonia excretion (N=9)(B) in crayfish exposed to of elevated pCO_2 and temperature for 14 days (~270 Pa, 28°C) compared to control (~90 Pa, 24°C). Significance (P-value < 0.05) was determined using a two way ANOVA and is indicated by *.

3.4.3 Carapace calcification

There was no change in the calcium per mg of carapace in crayfish exposed to elevated pCO_2 and temperature after 14 days (6.72 ± 0.46 (g/mol) calcium per mg carapace) compared to controls (7.25 ± 0.54 (g/mol) calcium per mg carapace)(two way ANOVA P=0.82) (Fig. 21).

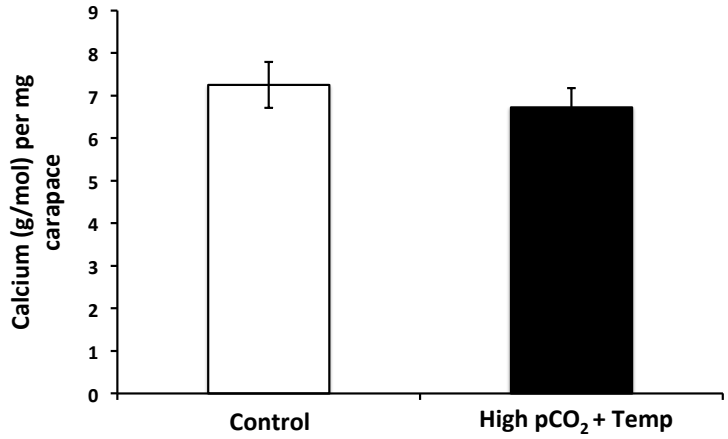


Figure 21. Carapace calcium content following a 14-day acclimation to elevated environmental pCO_2 and temperature. Carapace calcification in control and high temperature and high pCO_2 acclimated crayfish following 14-day exposure. Values are presented as mean ± SEM (N=8) and significance was determined using a two way ANOVA.

3.5 Identification of chloride rich filaments by silver staining

In order to determine the regions of the gill, as determined by different cell types, silver staining was performed. The results of the silver staining protocol on the gills of *P. clarkii* showed very dark silver staining of the inner, chloride rich, filaments and more lightly stained outer filaments in all of the podobranch gills from both the right and left side of the animal (Fig. 22).

Figure 22. Images of silver stained control crayfish gills. Podobranch gills (one to six) taken from the right and left side of a control crayfish following silver staining protocol (See methods).

3.6 Quantitative PCR

3.6.1 Inner filaments versus outer filaments

The results of the qPCR analysis of the inner and outer gill filaments of control crayfish show the variation in mRNA transcript abundance of various genes. There was higher, nearly double, expression of Rh1 and NHE in the inner filaments compared to the outer filaments (Fig. 23). Interestingly, the anion exchanger (HCO_3^-/Cl^-) was seven to eight times higher expressed in the outer filaments compared to the inner filaments. In addition, the Na^+/K^+-ATPase was also significantly elevated in the outer filaments. There was no significant difference in the mRNA

transcript abundance of both the membrane bound and cytoplasmic carbonic anhydrase between the inner and outer filaments.

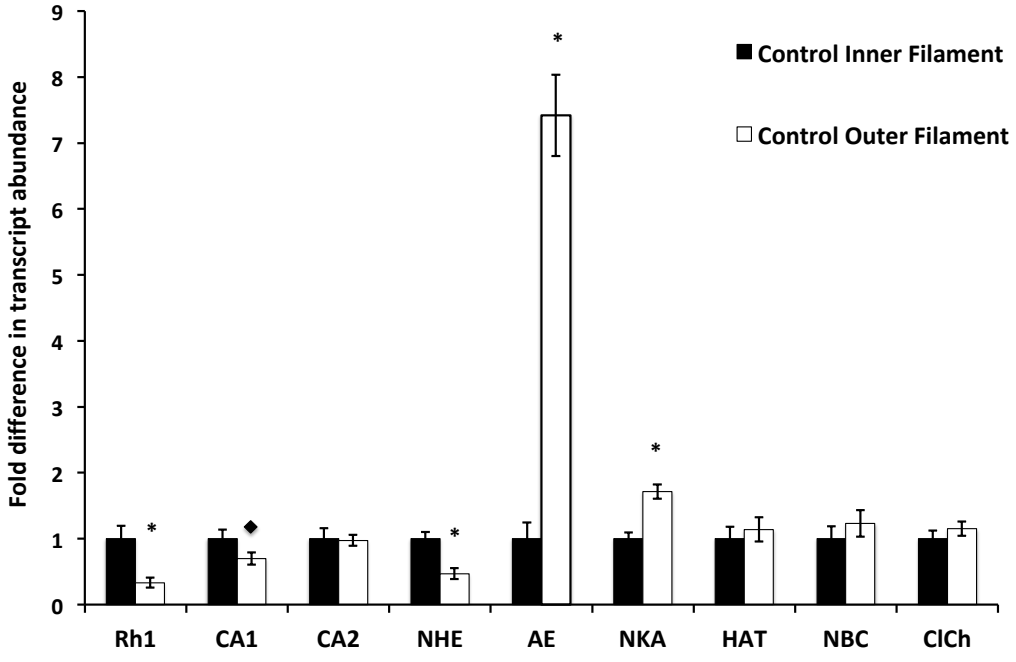


Figure 23. Fold difference in transcript abundance between control *P. clarkii* inner and outer filaments. Fold difference in transcript abundance of Rhesus protein (Rh1), carbonic anhydrase 1 and 2 (CA1, CA2) sodium hydrogen exchanger (NHE), anion exchanger (AE), sodium potassium ATPase (NKA), V-Type H+-ATPase (HAT), sodium bicarbonate co-transporter (NBC) and chloride channels (ClCh) the inner and outer filaments of control crayfish gills. Data has been normalized using the G-protein suppressor (GPro) and is presented as mean ± SEM. Significance between inner and outer filaments is indicated by * (two-tailed paired T-test; $P<0.05$; N=7-8). P-values between 0.1 and 0.05 are indicated with ◆.

3.6.2 The effect of high pCO_2 and high temperature

The acclimation of crayfish to elevated pCO_2 and temperature induced changes in the mRNA transcript abundance in the gills. There was a significant down regulation of membrane bound carbonic anhydrase (CA1) and a significant increase in cytoplasmic carbonic anhydrase

(CA2) in both inner (Fig. 24) and outer filaments (Fig. 25) in response to high pCO_2 and high temperature. There was no significant change in Rh1, NHE, AE, NKA, and HAT in either the inner or outer filaments with exposure to treatment.

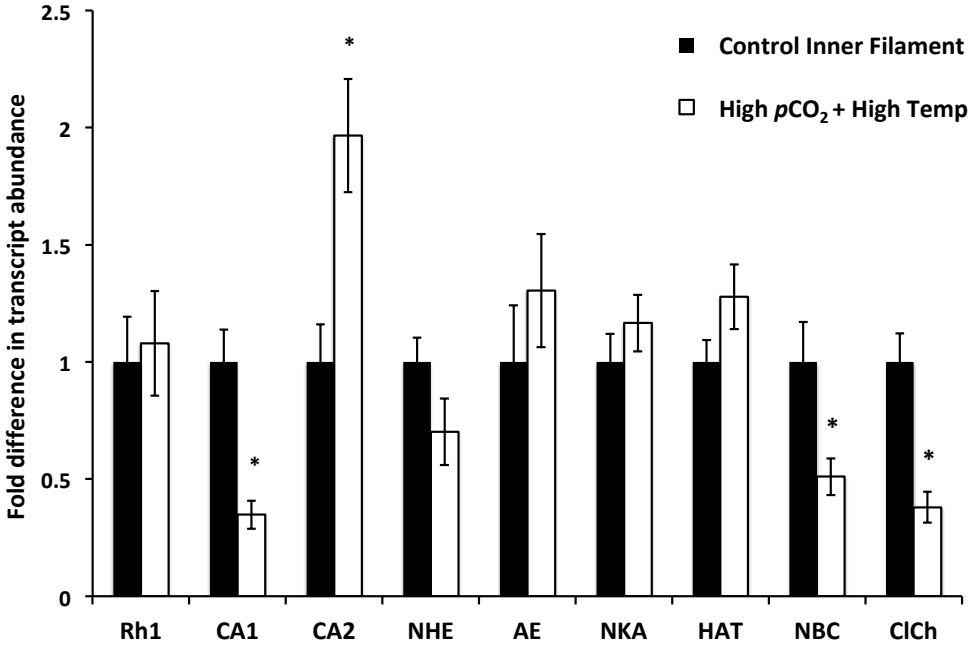


Figure 24. Fold difference in transcript abundance between control and high pCO_2 and temperature in inner (darkly stained) filaments. Fold difference in transcript abundance of Rhesus protein (Rh1), carbonic anhydrase 1 and 2 (CA1, CA2) sodium hydrogen exchanger (NHE), anion exchanger (AE), sodium potassium ATPase (NKA), V-Type H+-ATPase (HAT), sodium bicarbonate co-transporter (NBC) and chloride channels (ClCh) in the inner gill filaments of control (~90 Pa, 24°C) and high pCO_2 and temperature (~270 Pa, 28°C) acclimated crayfish. Data has been normalized using the G-protein suppressor (GPro) and is presented as mean ± SEM. Significance between inner and outer filaments is indicated by * (two-tailed T-test; $P<0.05$; N=7-8).

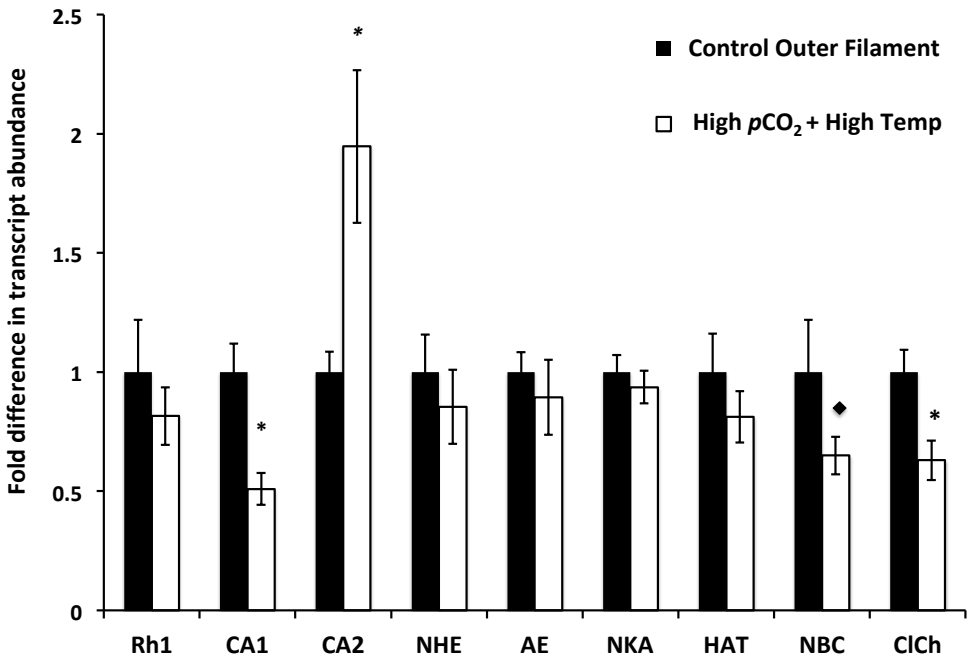


Figure 25. Fold difference in transcript abundance between control and high pCO_2 and temperature in outer (lightly stained) filaments. Fold difference in transcript abundance of Rhesus protein (Rh1), carbonic anhydrase 1 and 2 (CA1, CA2) sodium hydrogen exchanger (NHE), anion exchanger (AE), sodium potassium ATPase (NKA), V-Type H+-ATPase (HAT), sodium bicarbonate co-transporter (NBC) and chloride channels (ClCh) in the outer gill filaments of control (~90 Pa, 24°C) and high pCO_2 and temperature (~270 Pa, 28°C) acclimated crayfish. Data has been normalized using the G-protein suppressor (GPro) and is presented as mean ± SEM. Significance between inner and outer filaments is indicated by * (two-tailed T-test; $P<0.05$; N=6-8). P-values between 0.1 and 0.05 are indicated with ◆.

3.7 Notes on methods

There were several setbacks experienced in the execution of this study. The use of environmental chambers, allowing for the absolute control of room temperature, lighting and other factors was thought to allow for the most constant environment during acclimations. Nevertheless there were issues of water temperature being several degrees lower than set air

temperature, changes in water quality and limitations of the sensitivity of the aquastar that were resolved through daily testing and adjustment of water flow, CO_2 flow, adjustment of aquastar set points and air bubbled into the system, to adjust pH.

There were additional flaws with long-term storage (six months or more) of tissues in RNAlater® (Ambion, Fisher Scientific, Ottawa, ON, Canada) at -80 °C for future RNA isolations. RNAlater® solution guidelines instruct that tissues are safe to store at room temperature for one week or at -20°C or -80°C indefinitely without compromises in RNA quality. However, there was a decline in RNA purity with long term storage as determined by the 260/280 and 260/230 values when nanodropped (Table. 4). This could potentially be due to increased penetration of preserving compounds into the tissues that are then not effectively washed out with isolation techniques. The best results for RNA isolations of gill tissues were found when tissues were dissected and immediately placed in RNAlater overnight at 4 °C after which they were dissected and RNA was isolated (Table. 5). It is because of poor purity and time constraints that qPCRs conducted on the single stressor treatments, temperature and pCO_2, have been excluded from the data set.

Table 4. Results of RNA isolations from gill tissues stored in RNAlater at -80°C for 6 months. Tissues were sampled from animals following a 14 day acclimation to elevated environmental pCO_2 and temperature and were isolated using E.Z.N.A® Total RNA kit 1 (OMEGA bio-tek).

Crayfish	Tissue	RNA Concentration (µg/µl)	260/280	260/230
1	Gill inner filament	0.1117	2.13	1.33
1	Gill outer filament	0.0112	2.16	0.03
2	Gill inner filament	0.0904	2.14	0.85
2	Gill outer filament	0.0570	2.17	0.15
3	Gill inner filament	0.0192	2.21	0.06
3	Gill outer filament	0.0079	2.58	0.03
4	Gill inner filament	0.1015	2.15	0.37
4	Gill outer filament	0.0572	2.19	0.15

Table 5. Results of RNA isolations from gill tissues stored in RNAlater at 4°C for 24 hours. Tissues were sampled from animals following a 14 day acclimation to elevated environmental pCO_2 and temperature and were isolated using E.Z.N.A® Total RNA kit 1 (OMEGA bio-tek). Results are prior to reprecipitation protocol (see appendix Table S4).

Crayfish	Tissue	RNA concentrations (µg/µl)	260/280	260/230
1	Gill inner filament	0.1637	2.12	0.83
1	Gill outer filament	0.0609	2.06	0.71
2	Gill inner filament	0.2798	2.13	1.46
2	Gill outer filament	0.1167	2.09	0.93
3	Gill inner filament	0.3026	2.11	1.15
3	Gill outer filament	0.2068	2.12	0.52
4	Gill inner filament	0.0975	2.09	0.66
4	Gill outer filament	0.0279	2.10	0.20

4. Discussion

4.1 Effect of temperature

Although the temperature treatment of +4 °C in this current study is predicted to accompany a climate change scenario for the year 2100 the starting point for control acclimations (24°C) was decided based on average surface water temperatures during the hottest time of the year at Morgan City, located towards the southern end of the River Basin and flood zone (USGS 2016). This was an estimate on the lower end of the average temperature the animals would experience in the Atchafalaya river basin but may not represent the upper end of their thermal tolerance or the maximum temperature measured in the basin, which is closer to 32°C (Chucholl, 2011; Sabo et al. 1999). This decision towards a realistic year 2100 might not have exposed them to the maximum temperature they could potentially experience, however it did have control animals acclimated to the upper end of their ideal range (14-24°C), so although the response to elevated temperature is mild it is in response to a more realistic temperature scenario (Romaire & Lutz, 1989).

Increased temperature created a significant increase in metabolic rate by 7 days of exposure (Fig. 9). This relationship has been well documented in both freshwater and saltwater crustaceans including; river prawns (*Macrobrachium rosenbergii*), Atlantic shrimp (*Xiphopenaeus kroyeri*) and green crabs (*Carcinus maenas*) exposed between five and 20 days (Crispin & White, 2013; Carvalho & Phan, 1997; McGaw & Whiteley, 2012). By 14 days of high temperature acclimation, metabolic rate recovered back to control levels and there was a decrease in hemolymph pH, by 10 times the $[H^+]$, likely due to metabolic acidosis (Fig. 8). Hemolymph pCO_2 levels, in contrast to pH, tend to remain constant with increased temperature,

also observed in the present study (Cameron & Batterton, 1978; Truchot, 1973). The lack of increase in bicarbonate and ammonia implies the decrease in hemolymph pH is uncompensated for as there is no evidence of a response as HCO_3^- is typically used to compensate for temperature related acidosis through regulation at the gills (Henry et al. 2012).

The impact of elevated temperature on acid-base status of catfish (*Ictalurus punctatus*) held at 24°C and tested between 10°C and 30 °C showed a significant drop in blood pH from 7.9 to 7.7 with increased temperature from 15°C to 31°C, thought to be caused by an increase in blood pCO_2 with changing rates of oxygen consumption. In addition there was no apparent change in blood total carbon to act as a buffer. With a 10°C increase in temperature there was a consistent decrease in hemolymph pH in *Callinectes sapidus*, *Cancer magister*, *Carcinus maenas*, and *Uca pugilator* with some changes observed in hemolymph pCO_2, which generally increases, and HCO_3^- which generally decreases (Howell et al. 1973; McMahon et al. 1978; Truchot, 1973). In the current study there was no significant increase in calculated blood pCO_2, suggesting the source of the pH change is from metabolic acidosis (Fig. 8). In addition there was no change in ammonia excretion by the gills or kidney in catfish, similar to the whole animal ammonia excretion rates of *P. clarkii*, which were unchanged with increasing temperature (Cameron & Kormanik, 1982) (Fig. 10).

4.2 Effect of pCO_2

Hemolymph pCO_2 was not significantly altered in crayfish exposed to elevated environmental pCO_2 for either 7 or 14 days. In control animals there is a tendency towards high internal pCO_2, with as much as five times higher levels in the hemolymph (~500 Pa) over the external environment (~92 Pa) (Fig. 12). This high internal concentration is thought to be maintained to ensure an outwardly directed gradient for metabolic CO_2 excretion (Melzner et al.

2009). With an increase in the external environment pCO_2 by ~200 Pa there was still a three fold outwardly directed pCO_2 gradient in place (Fig. 13). By keeping high internal pCO_2 concentrations *P. clarkii* is able to maintain homeostasis and an outwardly directed gradient, even while experiencing fluctuations in their environment. This is representative of the Atchafalaya river basin in Louisiana that shows large seasonal fluctuations in pCO_2 between 98 and 222 Pa (Huang et al. 2015).

In contrast to hemolymph pCO_2 hemolymph bicarbonate levels were also significantly elevated following 7 days of treatment and remained high into the 2nd week (Fig. 13). The acid-base challenge presented by elevating environmental pCO_2 appears to be successfully compensated for. This is evident by the stable hemolymph pH and elevated bicarbonate and ammonia, an important acid and base equivalent, showing adequate buffering (Fig.13). Potentially the most damaging long term impact of elevated pCO_2 is the reduction available energy through the decrease in metabolic rate observed in crayfish by day 14, and seen in several other studies (Fig. 15). In other crustaceans like the Dungeness crab *M. magister*, the velvet swimming crab (*Necora puber*), deep sea tanner crab (*Chionoectes tanneri*), molluscs like the Mediterranean mussel (*Mytilus galloprovincialis*) and the echinoderm sea urchin *Psammechinus miliaris* all exhibit a reduction in metabolic rate in response to hypercapnia (Hans et al. 2014; Small et al. 2010; Pane & Barry, 2007;Michaelidis et al. 2005; Miles et al. 2007). Although *P. clarkii* survived this short-term stressor we could speculate that their response may hamper long-term survival and other energetic processes like reproduction and growth, potentially impacting the survival chances of the species (Heuer & Grosell, 2014;Ganser et al. 2015). Additional work is needed to determine the long-term population level effects of treatments such as elevated environmental pCO_2 to investigate if metabolic rate is ever recovered and if not what that means

for the survival of the animal. With species of importance to the aquaculture industry, including *P. clarkii*, the impact of elevated pCO_2 is of particular interest. Fish have been shown to excrete 10 times more CO_2 than they do NH_3 (Randall & Wright, 1989). This combined with nitrification from biofilters makes the accumulation of CO_2 inevitable in single pass water flow systems used in aquaculture (Sanni & Forsberg, 1996; Fivelstad et al. 1998) (Table. 6). This can regularly expose animals to elevated pCO_2, having a variety of effects including gill damage and reduced growth in Atlantic salmon smolts (Fivelstad & Binde, 1994).

Table 6. pCO_2 measurements in a variety of aquaculture species.

Study	Species	pCO_2 (Pa)	pH
Fivelstad et al. 2003	Atlantic salmon (*Salmo salar*)	~360-759	5.5-6.0
Challener et al. 2013	Edible sea urchin (*Lytechinus variegatus*)	430	7.41
Good et al. 2010	Rainbow trout (*Oncorhynchus mykiss*)	~1172	7.17
Moran & Stottrup, 2010	Atlantic cod (*Gadus morhua*)	380- 850	-

4.3 The combined effect of pCO_2 and temperature

Elevated pCO_2 in the hemolymph as a result of the combined pCO_2 and temperature treatment causes a decrease in hemolymph pH, an acid-base challenge to the intracellular and extracellular medium. The ability of an animal to maintain acid-base balance in their blood/hemolymph is a critical measure of their ability to survive stressors as pH is maintained within a very tight physiological range for proper protein function etc. In dogs, a representative mammalian system, hypothermia induces major shifts in pH showing a severe drop of more

than 0.6 units, ultimately leading to death by cardiac failure (Osborn, 1953). In rainbow trout (*Salmo gairdneri*), as few as six minutes of extreme exercise resulted in 40% mortality over 24 hours due to higher blood metabolic acid loads in non-survivors, proposed to indicate a greater intracellular acidosis (Wood et al. 1983). In green crabs (*Carcinus maenas*) exposed to combination treatments of copper and dilute salinity elicited the most severe acidosis, compared to single stress treatments, in crabs where pH dropped from control levels of 7.84 to 7.58 following 7 days of exposure (Weeks et al. 1993). In that study hemolymph pH was used as a biomarker for physiological status where the crabs observed to be closest to death or in otherwise poor condition had the lowest hemolymph pH. Although crayfish are remarkably acid tolerant, with a pH LC50 of 2.8 after 96 hours in *P. clarkii* compared to an LC50 of 4.1-4.5 following 167 hours in rainbow trout, they still experience a hemolymph acidosis of the same magnitude from the acid exposure (Morgan & McMahon, 1982; Neville, 1979). This exposure to sulphuric acid (N_2SO_4) dropped hemolymph pH from control levels of 8.2 to 7.5 after four days, resulting in 50 % mortality (Morgan & McMahon, 1982). The acidosis in *P. clarkii* was partially compensated for at day 7 and fully compensated for by day 14 with elevations in bicarbonate, returning the pH to control levels even with the elevated pCO_2 still present in the hemolymph (Fig. 18). This change in pH might be due to the increase in internal pCO_2, despite the internal condition being already elevated above treatment, to maintain the same outwardly directed gradient as seen in controls.

In addition, there was an observed decrease in metabolic rate in the combination treatments but to a lesser extent than it was seen in the single stress pCO_2 treatment (Fig. 20). A similar outcome was observed in the periwinkle *Littorina littorea* where metabolic rate was

decreased by 31% in elevated pCO_2 exposure and decreased only 15% in the combined treatment of elevated pCO_2 and temperature to mimic the year 2100 (Melatunan et al. 2011). In the present study elevated pCO_2 caused a 70% decrease in metabolic rate while elevated pCO_2 and temperature created only a 44.3 % decrease in metabolic rate from controls (Fig. 20). This result further illustrates the potential interactive effects of multi-stressor treatments that are not simply additive, but may result in a modulated response that is closer to control treatments than either component stress when applied singularly.

Related to the decreased metabolic rate there was an observed decrease in hemolymph ammonia, as a result of decreased metabolic ammonia production and no change in whole animal ammonia excretion (Fig. 20). The decrease in metabolic rate as a result of lowered oxygen consumption with no change in whole animal ammonia excretion would mean a lower total ammonia and overall lowering of the oxygen to nitrogen ratio (O:N) compared to controls. In general a reduction in O:N ratio can indicate a shift in the energy source used for metabolism (Uthicke et al. 2014). In marine mussels high O:N ratios indicate a healthy mussel that is utilizing carbohydrate and lipid catabolism for energy while a lowered O:N ratio can indicate a switch to breaking down proteins in response to starvation (Bayne et al. 1985). In contrast, freshwater mussels seemed to show that low O:N ratios are the result of dietary protein catabolism rather than body proteins (Ganser et al. 2015). A study examining the effect of dietary protein and energy levels on growth in white leg shrimp (*Litopenaeus vannamei*) and northern white shrimp (*Litopenaeus setigerus*) juveniles found that O:N ratios were reduced with high protein use but also that the requirement for dietary protein for optimum growth decreased with age (Rosas et al. 2001). In the present study crayfish were maintained on a diet of duckweed and oats and were starved (48 hours) prior to all measurements. Although this diet appears to be

low in protein oats contain 4 grams of protein for every 30 grams ingested and duckweed has been utilized as a protein feedstuff in common carp diets to replace commercially used diets (32% protein) (Yilmaz et al. 2004). A diet containing 20% duckweed showed no significant difference in carp growth or performance than those fed traditional protein diets (Yilmaz et al. 2004). With rising costs of traditional protein sources for fishmeal substituting plant proteins has been used as a solution to decrease fish aquaculture production costs, where as much as half of the dietary protein can be easily replaced with plant protein (Hardy, 2010). In addition although food was removed two days before all experiments this would not place animals in a full starvation mode. In studies on shrimp *Penaeus japonicas*, *Penaeus duorarum* and *Crangon crangon* following 28 days of starvation it was determined that glucose was preferentially used, depleting glycogen stores from the hepatopancreas, followed by tail muscle lipids and lastly protein (Cuzon et al. 1980; Schafer, 1968; Cuzon & Ceccaldi, 1973). Due to the lack of true starvation in the present study and the moderate amount of protein in the diet the slightly lowered O:N ratio suggests a utilization of dietary proteins over other sources. However, the lowered hemolymph ammonia indicates lowered ammonia production overall, conflicting with the above interpretation. In this case the varied response in the hemoylmph ammonia and whole animal ammonia excretion suggest that the O:N ratio may not be representative of what is happening in this system.

4.4 Carapace calcification

There was no observed change in carapace calcification following 14 days of all of the tested treatments (Fig.11, 16, 21). This implies that the elevated hemolymph bicarbonate seen in various treatments, elevated pCO_2 and pCO_2 with temperature, is not the product of shell

degradation but has instead been either harnessed from the external environment, difficult in a freshwater low in ions, or it is the product of internal CO_2 conversion that has been retained through regulated base secretion. This result also suggests that these animals are still capable of the regular carapace maintenance required to keep up shell integrity even if they are not actively degrading it themselves for the available carbonate. There are multiple factors potentially impacting shell integrity including environmental pH, time, temperature, and species resilience though based on the literature some may hold higher importance than others (Kurihara, 2008). In studies on juvenile red king crabs, tanner crabs, and velvet swimming crabs exposed to pH treatments between 8 and 6.7 for 200 days for the first two species or 30 days respectively showed no change in shell calcification in any of the animals or treatments (Long et al. 2013; Small et al. 2010). Similar findings were observed in juvenile European lobsters and corals where single treatments of elevated environmental CO_2 over 5 weeks at control temperatures has no impact on calcification but with as little as +3 °C temperature increase in the case of the corals caused as much as a 50% decrease in calcification (Small et al. 2016; Reynaud et al. 2003). The above results suggest that temperature paired with an acid-base stress strongly influence calcification, however the environmental availability of calcium carbonate may play an even larger role as all of the above studies were conducted on seawater organisms. In freshwater acclimated Chinese mitten crabs (*Eriocheir sinensis*) exposed to only elevated pCO_2 treatment (534 Pa, pH=6.74) there was a significant decrease in carapace calcium content in as little as three weeks of exposure (Quijada-Rodriguez, personal communication). The above study, conducted on an equally successful invasive species as *P. clarkii*, was exposed for longer periods to a higher pCO_2 treatment than the present study, potentially explaining the lack of change in carapace calcium content observed (Gutierrez-Yurrita et al. 1998; Chucholl, 2011).

4.5 Gene expression in the gills

The following data presented should be considered with some scepticism as one of the findings show higher abundance of the Na^+/K^+-ATPase in the mitochondria poor outer filaments than the mitochondria rich inner filaments, which directly contradicts the literature and previous work on this organism. This result is also contradictory because of the abundance of mitochondria are in the inner filaments, as confirmed by silver staining and ultrastructural analysis, which would be needed to supply ATP to power the pumps (Dickson et al. 1991; Siebers et al. 1982; Towle & Weihrauch 2001; Henry et al. 2012). Previous work on *P. clarkii* has found both increased Na^+/K^+-ATPase activity and more negative transepithelial potentials in the inner mitochondria rich filaments, suggesting higher mRNA for the Na^+/K^+- ATPase would be expected (Dickson et al 1991). In work completed on other species such as *D. pagei* and *C. maenas* higher abundance of NKA corresponds to more active tissues, containing the majority of the gill transporters (Weihrauch et al. 2004a; Fehsenfeld & Weihrauch, 2013). In fish the NKA has also been identified as being essential in creating the Na^+ gradient in the cell to push H^+ excretion using the apical Na^+/H^+ exchanger, which was more highly expressed in the mitochondria rich regions in the present study (Choe et al. 2005; Edwards et al. 2002). There are additional studies though, such as those completed by Hans et al. (2018) where there is equal mRNA abundance of the NKA in both the mitochondria rich and mitochondria poor regions in the book gills of the horseshoe crab (*Limulus polyphemus*). A limitation of qPCR is that mRNA does not always correlate to protein or enzyme activity; post-translational processes could ultimately determine how much of the detected mRNA becomes functional proteins in the cell. In the case of the V-type H^+- ATPase more mRNA was found in the posterior over the anterior

gills of *Dilocarcinus pagei* but the HAT activity was not significantly different across the two gill types (Weihrauch et al. 2004a). Although it would be an advantage in the ion poor freshwater environment that this animal survives in to utilize the Na^+/K^+- ATPase (NKA) to drive cell membrane potential and utilize its transporters to maintain osmoregulation in all the gill tissues, this result suggests the shortcomings of this data. The NKA also aids in ammonia excretion and acid-base regulation as the NKA can move ammonia in place of the K^+ due to similar size and charges, as seen in other crustaceans and the freshwater ribbon leech (Skou 1960; Weihrauch et al. 1999; Quijada-Rodriguez et al. 2015). Additional work is needed to determine if this discrepancy in mRNA is reflected in the protein and activity level of the NKA in the gill filaments of crayfish, potentially through the use of scanning ion electrode technique (SIET), western blotting for proteins, or enzyme assays.

As a result of the discrepancy in the NKA distribution in the gills all the data should be further confirmed with additional studies but assuming the qPCR data correctly reflects the arrangement in the gill cells the results of qPCR analysis of the inner, mitochondria rich, filaments and outer mitochondria poor, filaments of the crayfish podobranch gills show some division of gene expression and function. The inner filaments had increased gene transcription abundance for the Na^+/H^+ exchanger (NHE) and Rhesus protein (Rh1), the ammonia and proposed CO_2 transporter (Perry et al. 2010; Khademi et al. 2004). This complement of transporters suggests a paired Na^+ uptake/ ammonia excretion mechanism at work in the inner filaments (Weihrauch et al. 1999). This mechanism has been observed in several other haline crustaceans using inhibitor studies. The application of Na^+/H^+ exchanger inhibitor amiloride showed a 29% inhibition in ammonia excretion across the gill, demonstrating its importance in the release of NH_4^+ (Weihrauch et al. 1999). In contrast the outer filaments appear specialized for

Na^+ independent chloride uptake, as evident by the high transcription abundance of the Cl^-/ HCO_3^- exchanger (AE). This arrangement is similar to those found in the posterior gills of the true freshwater crab *Dilocarcinus pagei*, where the thick proximal mitochondria rich epithelium is responsible for active Na^+ uptake while the distal epithelium, thin and mitochondria poor, brings in chloride via the proposed apical Cl^-/ HCO_3^- exchanger (Weihrauch et al. 2004a; Onken et al. 1991;Tresguerres et al. 2008).

There was equal mRNA abundance of the membrane-bound and cytoplasmic enzyme carbonic anhydrase, V-type H^+-ATPase (HAT), Na^+/ HCO_3^- (NBC) co-transporter and chloride channels, suggesting they are of the same relative importance in all of the tissues measured. Several of the transporters that are equally expressed serve multiple functions and would potentially be useful in filaments completing separate functions. For example, both isoforms of carbonic anhydrase are important for acid-base regulation as well as providing the counter-ions (HCO_3^- and H^+) needed for both Na^+ and Cl^- uptake, completed in different filament types as found by this study (Serrano & Henry, 2008; Siebers et al. 1994;Whiteley et al. 2001). The HAT could also be important in all gill tissues for its ability to alter the pH environment of the boundary layer of gills in freshwater, due to less buffering, to facilitate NH_3 trapping for ammonia excretion and for its supporting role in conjunction with the NKA in cell membrane potential to aid osmoregulation against outwardly directed gradients (Larsen et al. 2014; Weihrauch et al. 2012;Weihrauch et al. 2001; Henry et al. 2012). The HAT is found exclusively apically to fulfil this function and has been found in other freshwater species including *Dilocarcinus pagei*, and *Eriocheir sinesis* (Weihrauch et al. 2004a; Onken & Putzenlechner, 1995).

4.5.1 The effect of high pCO_2 and temperature

Exposure to elevated pCO_2 and temperature for 14 days had several effects on the relative gene transcript abundance of transporters in the gill. There was a significant down-regulation in membrane bound carbonic anhydrase in favour of an up-regulation in cytosolic carbonic anhydrase. This suggests a preferential regulation of the intracellular environment using the cytosolic carbon anhydrase. A limitation of the present study is that using qPCR techniques does not allow for localization of the membrane bound carbonic anhydrase on the basolateral, apical or both membranes, making it difficult to say what impact the down-regulation is having on the gill tissues and surrounding systems. Carbonic anhydrase is the enzyme that catalyzes the conversion of carbon dioxide in water into carbonic anhydrase that dissociates into HCO_3^- and H^+ due to its pKa (6.36), making it valuable during acid-base stress, such as elevated environmental pCO_2 (Truchot, 1983; Henry & Wheatly, 1992; Liu et al. 2015). In the white shrimp *Litopenaeus vannamei* there was a 5-fold increase in expression of the cytoplasmic carbonic anhydrase after 24 hours in pH 7.4 water (control pH=8.2)(Liu et al. 2015). While the major responder to decreased pH is the cytoplasmic carbon anhydrase in white shrimp the membrane bound carbonic anhydrase was found to respond to high pH stress (Liu et al. 2015). An increase in cytoplasmic carbonic anhydrase was also observed with low pH exposure (6.2) in a freshwater crayfish species (*Cherax quadricarinatus*) after 24 hours in treatment (Ali et al. 2017). In addition, there was a significant increase in NKA after just six hours of exposure to low pH that was still significantly high after 24 hours. This disruption in ion regulation caused by low pH demonstrates the effect acid-base imbalance might have on osmoregulation (Ali et al. 2017).

There was also a significant decrease in the gene transcript abundance of both the Na^+/ HCO_3^- co-transporter and the chloride channel in the present study. There is a lack of expressional studies looking at ion channels in invertebrates limiting the scope of the chloride channel findings however the Na^+/ HCO_3^- co-transporter has been proposed to be important for acid-base regulation (Tresguerres et al. 2008). This down-regulation could be in an attempt to alter the concentration gradients to discourage the movement of HCO_3^- out of the cell to be retained for its buffering capacity against acidic hemolymph pH stress. Although this change was seen in both inner and outer filaments for the outer filaments in particular, that are very high in mRNA for the anion exchanger (HCO_3^- /Cl^-), the down regulation of basolateral chloride channels and the Na^+/ HCO_3^- co-transporter would mean a decrease in the amount of chloride leaving the cell and a decrease in Na^+ and HCO_3^- coming in. This change would effectively increase the amount of chloride in the cytoplasm and decrease HCO_3^-, slowing the outwardly directed gradient of HCO_3^- facilitated by the HCO_3^- /Cl^- anion exchanger (AE). The benefit of this proposed mechanism would be an increase in internal HCO_3^-, to buffer against pH stress, with the cost of decreased intracellular Cl^- concentrations. In response to an acid-base stress NaCl transport processes may be modified in the hemolymph through the regulation of transporters for H^+ and HCO_3^- and their counter ions Na^+ and Cl^-, important for osmoregulation and normal functioning (Henry & Wheatly, 1992). Several other studies have found a correlation between external pH stress and decreased internal concentrations of Na^+ and Cl^-, either through the use of transporters for acid-base balance or through increased membrane permeability. In freshwater crayfish *P. clarkii* and *O. rusticus* low pH exposure was found to increase internal K^+ concentrations and decrease internal $Cl^-$and Na^+ (Wood & Rogano, 1986; Zanotto & Wheatly, 1993).

Interestingly all the changes in gene transcription abundance observed were seen in both the inner and outer filaments in the same pattern and rough magnitude. This would support equal responsiveness to acid-base stress in both filament types, and all parts of the gill. This contrasts with the findings determined by Fehsenfeld & Weihrauch (2013) in *Carcinus maenas* where individual gills showed changes in mRNA levels that were not mirrored in any other gills. In addition the gills that showed the greatest response to the acid-base stress, as evident by an up-regulation in the Rhesus protein and carbonic anhydrase were the anterior gills. The Anterior gills were thought to be utilized primarily for gas exchange, while the posterior gills are used primary for osmoregulation and associated with the most activity and transporters (Fehsenfeld & Weihrauch, 2013).

Almost as interesting as the genes that were changed by treatment, were the surprising finding that the Rhesus protein, an important transporter for acid-base regulation and ammonia excretion was unchanged in both inner and outer filaments in response to elevated pCO_2 and temperature after a 14-day acclimation. The importance of the Rhesus proteins has been investigated in several models demonstrating its importance in ammonia and proposed CO_2 transport that can be utilized to maintain acid-base homeostasis (Adlimoghaddam et al. 2016; Nawata et al. 2010; Perry et al. 2010; Khademi et al. 2004). This lack of change in the Rh might be due to the time scale of the present study being long enough that the response had returned to control levels, since the response time of some transporters occurs as soon as 6 hours after exposure and can be recovered back to control levels as soon as 24 hours following treatment (Ali et al. 2017). In green crabs exposed to elevated environmental pCO_2 Rhesus proteins are down regulated in anterior gills 4 and 5 measured at day 7 of the acclimation (Fehsenfeld & Weihrauch, 2013). In crustaceans there has been one identified Rhesus isoform however in some

species including *C. elegans* and *L. polyphemus* there are two expressed Rhesus protein isoforms and 3 in *A. albopictus* (Weihrauch et al. 2004b; Martin et al. 2011; Adlimoghaddam et al. 2016; Hans et al. 2018; Wu et al. 2010). In the horseshoe crab Rhesus protein 1 is higher expressed in mitochondria rich areas of the book gills while Rh2 is higher in the mitochondria poor areas. This supports the findings of the current study where Rh1 was higher expressed in the inner, mitochondria rich, filaments. Although primers were designed to target a second rhesus protein due to difficulty with the detectability of the Rh2 in the filaments of the crayfish gills its transcription abundance could not be reliably determined using qPCR. This is similar to the findings of Adlimoghaddam et al (2015), where the Rhr1 in *C. elegans* has mRNA expression levels 10 times higher than Rhr2. Rhr1, localized on the basolateral side, is considered to be a housekeeping ammonia excretion gene while less is known about the Rhr2 that is apically localized with the potential to exist on the basolateral side as well (Adlimoghaddam et al. 2016). Neither appeared to change significantly in *C. elegans* exposed chronically to pH 5.5, suggesting limited involvement in acid-base regulation, and the lack of change in the present study (Alimoghaddam et al. 2016).

In addition to the Rh1 there was no observed changes in NHE, AE, NKA, and HAT in response to elevated environmental pCO_2 and temperature treatments. This suggests that either these genes are not directly involved in acid-base balance, or they potentially have been recovered to control levels by the time the measurement was taken (14 days). Freshwater animals have the advantage of being osmoregulators, who actively maintain internal ion (Na^+ /Cl^-) concentrations against an outwardly directed gradient, over seawater osmoconformers that may not have the machinery in place to immediately respond to a sudden environmental change. In studies conducted on the active ammonia excretion across three species of crabs the freshwater

acclimated Chinese mitten crabs have the greatest capacity to resist influx of large inwardly directed gradients over SW crabs *Cancer pagurus* and brackish water acclimated *Carcinus maenas*. This lack of membrane permeability was attributed to the success of this animal against passive Na^+ and Cl^- loss to the environment and the high capacity for uptake in the gills, increasing their ability to maintain internal homeostasis, compared to seawater crabs that are isosmotic to the environment (Weihrauch et al. 1999).

5. Conclusions

This study elucidates the interactive effects of elevated environmental temperature and pCO_2 for a potential year 2100 based on freshwater modelling. As seen with metabolic rate the potential interactive effects of multi-stressor treatments are not always additive, but may result in a modulated response that is closer to control treatments than either component stress when applied singularly. The largest impact of dual stressor treatments on the physiological responses, such as metabolic rate and blood parameters, suggests the inadequacy of single stressor studies to act as a proxy for climate change, which we know will combine temperature and carbon dioxide as is already evident in seawater studies. Investigation into the gene transcription abundance of the filamentous crayfish gills show mitochondria rich filaments are most likely responsible for paired Na^+ uptake and ammonia excretion while the mitochondria poor outer filaments contain more machinery for chloride uptake. Both inner and outer filaments though distinctly different both responded in the same way to an acid-base stress suggesting equal involvement. The animals in this study were able to survive this short-term stress but whether or not there will be long-term consequences; such as shell degradation to buffer against internal pH stress, increasing mortality or lowering fitness having reproductive consequences, is unknown. Although *P. clarkii*

had many advantages as a model species including its availability, long history in the literature for comparison and economic importance it is also highly invasive and lives in a constantly fluctuating environment. For the purposes of this study this durability allowed for the combination treatments to be examined without any mortalities observed. As a crayfish species more centrally located toward the equator it may also be less impacted than more northern populations that experience less variability and might be at increased risk.

Future Directions:

1. Further investigation into the disparity between the NKA mRNA measured and the mitochondria abundance.
2. There are several other tissues that may be of particular interest during a pH challenge such as the antennal gland, and hepatopancreas.
3. Long-term studies are needed to determine what, if any, the costs will be for reproduction and long-term survival of *P. clarkii*.
4. More research is needed to investigate dual stressor treatments on important freshwater invertebrates; some species may be at more risk such as native northern crayfish populations.

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Appendices

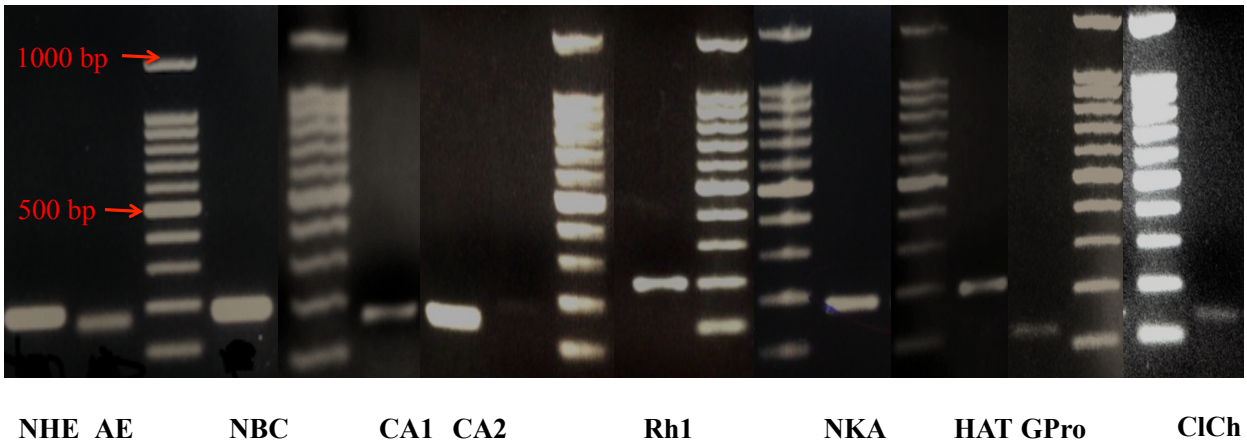

Figure S1. Images of PCR products using primers designed for qPCR. Genes are Na^+/H^+ exchanger (NHE; 192 bp), HCO_3^-/Cl^- anion exchanger (AE; 153 bp), Na^+/HCO_3^- co-transporter (NBC; 181 bp), Membrane bound carbonic anhydrase (CA1; 168 bp), cytosolic carbonic anhydrase (CA2; 152 bp), Rhesus protein (Rh1; 197bp), Na^+/K^+-ATPase (NKA; 183 bp), V-Type H^+ -ATPase (HAT; 197 bp), G-protein suppressor (GPro; 119 bp), and the chloride channel (ClCh; 139 bp)

Table S1. Genbank accession numbers utilized to design primers for qPCR. G-protein suppressor (GPro), sodium hydrogen exchanger (NHE), membrane bound carbonic anhydrase (CA1), cytoplasmic carbonic anhydrase (CA2), Rhesus-like protein (Rh), Cl^-/HCO_3^- anion exchanger (AE), sodium potassium ATPase α –subunit (NKA), and the V- (H^+) ATPase subunit B (HAT) sodium bicarbonate co-transporter (NBC) and chloride channel (ClCh)

Transcript	Genbank Accession #
G-Protein Suppressor 2 (GPro)	GARH01022697.1
Sodium hydrogen exchanger (NHE)	GARH01011635
Membrane bound carbonic anhydrase (CA1)	GARH0100502
Cytoplasmic carbonic anhydrase (CA2)	GBEV01003363
Rhesus-like protein (Rh)	GARH01042958.1
Cl^-/HCO_3^- anion exchanger (AE)	GBEV01060512
Sodium-potassium ATPase α - subunit (NKA)	GARH01042137
V-H^+-ATPase subunit B (HAT)	GBEV01003402
Sodium Bicarbonate co-transporter (NBC)	GARH01021665
Chloride channel (ClCh)	MF385059.1

Table S2. Efficiency and R^2 of standard curves produced with the following qPCR primers. Including G-protein suppressor (GPro), sodium hydrogen exchanger (NHE), membrane bound carbonic anhydrase (CA1), cytoplasmic carbonic anhydrase (CA2), Rhesus-like protein (Rh), Cl^-/HCO_3^- anion exchanger (AE), sodium potassium ATPase α –subunit (NKA), and the V- (H+) ATPase subunit B (HAT) sodium bicarbonate co-transporter (NBC) and chloride channel (ClCh)

Transcript	Efficiency (%)	R^2
G-Protein Suppressor 2 (GPro)	90	0.99
Sodium hydrogen exchanger (NHE)	100.3	0.984
Membrane bound carbonic anhydrase (CA1)	90.2	0.98
Cytoplasmic carbonic anhydrase (CA2)	105.2	0.98
Rhesus-like protein (Rh)	102.6	0.98
Cl^-/HCO_3^- anion exchanger (AE)	104.8	0.985
Sodium-potassium ATPase α -subunit (NKA)	95.3	0.98
$V\text{-}H^+$-ATPase subunit B (HAT)	90.1	0.98
Sodium Bicarbonate co-transporter (NBC)	106.9	0.98
Chloride channel (ClCh)	105.6	0.98

Table S3. Results of control *P.clarkii* gill RNA isolations before and after reprecipitation. RNA isolations were performed on gill inner filaments (IF) and outer filaments (OF) utilizing E.Z.N.A® Total RNA kit 1 (OMEGA bio-tek, Norcross, GA, USA) and concentrations were determined using a Nanodrop, where 260/280 of > 2 and 260/230 of > 1.80 were preferred.

Sample	[RNA] (μg/ μl)	260/280	260/230	Reprecipitated [RNA] (μg/ μl)	260/280	260/230
Cray 1 IF	0.0962	2.14	1.90	0.0937	2.12	2.04
Cray 1 OF	0.1120	2.09	0.56	0.0820	2.08	2.15
Cray 2 IF	0.3633	2.12	1.62	0.3227	2.13	2.27
Cray 2 OF	0.3400	2.11	2.02	0.2913	2.11	2.25
Cray 3 IF	0.5726	2.12	1.94	0.4403	2.12	2.25
Cray 3 OF	0.1716	2.12	1.27	0.1707	2.13	2.17
Cray 4 IF	0.0886	2.20	0.17	0.0731	2.12	1.94
Cray 4 OF	0.1014	2.09	0.82	0.0822	2.09	2.03
Cray 5 IF	0.0758	2.14	1.12	0.0681	2.14	1.92
Cray 5 OF	0.0825	2.08	1.52	0.0645	2.11	1.86
Cray 6 IF	0.0829	2.12	1.79	0.0664	2.15	2.04
Cray 6 OF	0.1534	2.09	2.09	0.1280	2.09	2.20
Cray 7 IF	0.3051	2.12	0.53	0.3431	2.12	2.24
Cray 7 OF	0.2379	2.11	1.49	0.2096	2.13	2.23
Cray 8 IF	0.0237	2.25	1.93	0.0244	2.17	1.61
Cray 8 OF	0.0595	2.10	1.00	0.0487	2.08	1.86

Table S4. Results of high pCO_2 and temperature acclimated *P.clarkii* gill RNA isolations before and after RNA reprecipitation. RNA isolations were performed on gill inner filaments (IF) and outer filaments (OF) utilizing E.Z.N.A® Total RNA kit 1 (OMEGA bio-tek, Norcross, GA, USA) and concentrations were determined using a Nanodrop where 260/280 of > 2 and 260/230 of > 1.80 were preferred.

Sample	[RNA] (μg/ μl)	260/280	260/230	Reprecipitated [RNA] (μg/ μl)	260/280	260/230
Cray 1 IF	0.1637	2.12	0.83	0.1035	2.10	2.07
Cray 1 OF	0.0609	2.06	0.71	0.0433	2.01	1.78
Cray 2 IF	0.2798	2.13	1.46	0.1975	2.11	2.16
Cray 2 OF	0.1167	2.09	0.93	0.0752	2.06	2.05
Cray 3 IF	0.3026	2.11	1.15	0.2288	2.12	2.19
Cray 3 OF	0.2068	2.12	0.52	0.1564	2.10	2.11
Cray 4 IF	0.0975	2.09	0.66	0.0648	2.02	1.75
Cray 5 IF	0.0869	2.19	0.14	0.0556	2.02	1.82
Cray 5 OF	0.0333	2.12	0.19	0.0228	2.07	1.80
Cray 6 IF	0.2564	2.14	1.34	0.0506	2.15	1.99
Cray 7 IF	0.3002	2.14	1.80	0.4609	2.1	2.12
Cray 7 OF	0.0674	2.08	0.63	0.0737	2.04	1.71
Cray 8 IF	0.1379	2.08	0.58	0.0892	2.02	1.77
Cray 8 OF	0.1106	2.13	1.30	0.1131	2.06	1.75