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Future Climate Change Conditions May Compromise Metabolic Performance in Juveniles of the Mud Crab *Scylla serrata*

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Abstract: Research characterising the effects of future climate change on the marine environment remains heavily focussed on that of temperate regions and organisms. Furthermore, little is known of these effects on the early life stages of many marine species. Tropical regions are already experiencing an increase in sea surface temperature and decrease in sea surface salinity, conditions favoured by pathogenic bacteria such as *Vibrio* spp. The early life stages of crabs are known to be particularly vulnerable to both the direct physiological effects of climate change and exposure to harmful microorganisms, yet there are limited data on these effects on juveniles of many tropical crustacean species. This study assessed the metabolic responses of mud crab (*Scylla serrata*) juveniles to warming and/or freshening in the presence or absence of pathogenic bacteria in southwest India. Juvenile crabs were exposed to either ambient (28 °C/30 PSU) or one of three projected climate change regimes (28 °C/20 PSU (freshening), 32 °C/30 PSU (warming), 32 °C/20 PSU (warming + freshening)) for 10 days, in either the presence or absence of the pathogenic bacteria *Vibrio parahaemolyticus*. Results show that simulated climate change conditions, especially freshening, caused a significant increase in oxygen consumption rates (MO_2), and that these were further increased when juveniles were exposed to *V. parahaemolyticus*. These results suggest that the effects of future climate change conditions could have significant implications for the conservation of wild stocks and commercial farming of this species in South Asia.

Keywords: metabolic rates; ecophysiology; oxygen consumption

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1. Introduction

The oceans continue to be subjected to the effects of climate change as a result of ongoing anthropogenic emissions [1–3]. Marine ecosystems are particularly vulnerable, as climate change is not causing ocean warming alone, but also associated increases in precipitation, causing seawater freshening [4], stratification, leading to declining seawater oxygen levels [5], and ocean acidification as the oceans continue to absorb atmospheric CO_2 [6–8]. Tropical regions of the world's oceans are projected to be some of the most impacted by these effects [9–12]. At the same time, these 'direct' climate change effects can also instigate structural changes to the microbial community at the base of the marine food chain ('indirect effects') in terms of both species' abundance and composition, including in tropical regions [13–16]. One group of marine bacteria that are favoured by these conditions are *Vibrio* spp., which prefers warm (>15 °C) environments, and are one of the most abundant aquatic bacteria groups, with some species, especially in high concentrations, pathogenic, having negative impacts through the food chain on other aquatic organisms and humans [17]. *Vibrio*-related infections are increasing worldwide affecting both humans

and aquatic animals. Reoccurring breakouts of seafood-borne illnesses caused by *Vibrio parahaemolyticus* in Peru and Alaska, where historically it has been rarely reported, and *Vibrio vulnificus* associated with oysters harvested from the Gulf of Mexico, indicate that climate anomalies such as heatwaves and El Niño-induced expansion of geographical and seasonal ranges are favouring these species [18]. Similarly, Continuous Plankton Recorder survey data from over four decades in the North Sea showed a significant increase in *Vibrios*, including cholera-inducing *V. cholera*, associated with zooplankton in years with increased sea surface temperature (SST) [19].

A growing body of literature is focusing on how these combined changes are affecting aquatic animals, including crustaceans, and what the long-term consequences will be for their fitness. Exposure to increased SST has been shown to necessitate higher metabolic requirements, including more reliance on stored energy reserves in crabs *Leptuca uruguayensis* and *Leptuca leptodactyla* [20] and *Liocarcinus depurator* [21], induce changes in immunobiological responses such as haemocyte proliferation in the crab *Carcinus aestuarii* [22], and has also been associated with changes in crustacean body size [23]. Ocean warming has also been shown to induce behavioural changes for more effective thermoregulation [20,24,25]. However, to date, most of this work has been focused on temperate marine species and systems. Meanwhile, tropical crustaceans (shrimp *Rhynchocinetes durbanensis* and hermit crab *Calcinus laevimanuhave*) have been shown to be able to employ physiological adjustments under marine heatwave conditions [26]. These climate change effects are also known to enhance the susceptibility to disease in crustaceans as temperature is an important factor influencing physiological and immunological responses to pathogens [27]. Increasing temperatures are also reported to have adverse effects on survival rates of crustacean larvae, including American lobster larvae [28], Florida stone crab larvae [29] and barnacle *Amphibalanus improvisus* larvae [30]. However, we still know relatively little about how climate change parameters, especially warming and freshening, affect the physiology of the juvenile stage of many crustacean species, while several studies have focused on the effects of ocean acidification (pCO_2) [31,32]. Juveniles are considered as potential life-history bottlenecks determining the future success of the species [33–35]. Thus, it is important to understand physiological responses to environmental changes as traits such as survival rate and growth are tightly linked to them [36,37]. Furthermore, the early developmental stages of crustaceans are an essential part of the food web, thus multi-trophic level studies are required to fully understand the consequences of direct and indirect climate change effects throughout the whole marine ecosystem [38–40].

The mud crab *Scylla serrata* is an economically important species in the Indo-Pacific region with increasing importance in the aquaculture sector [41–43]. The ecological importance of mud crabs has been rarely studied, yet, inhabiting mangroves and estuaries, mud crabs at their different life stages are both prey and predator in a complex multi-trophic system [44]. In Australia, *S. serrata* has been considered an environmental indicator species in a harbour health report card [45]. In this study, we assessed the metabolic performance of juvenile mud crabs (*Scylla serrata*) to warming and/or freshening and the interaction of these with climate change induced changes in the marine microbial community.

2. Materials and Methods

2.1. Experimental Design and Setup

To assess the direct and indirect effects of climate change on mud crab juveniles, a multivariate experimental design approach was used which included two different levels of seawater temperatures, and two salinities with/without exposure to *V. parahaemolyticus*. The two seawater temperature levels correspond to the mean monthly SST in the Arabian Sea (+28 °C) [46] and to an increase of 4 °C in line with projected warming trends for SST in the region [2,15,16]. Juvenile mud crabs tolerate a wide salinity range, thus two salinity levels were chosen which correspond to the optimum sea surface salinity (SSS) (30 PSU) and the lowest salinity in which the survival rate is not impacted (20 PSU) [47,48]. Juvenile crabs were also exposed to the pathogenic bacteria *Vibrio parahaemolyticus* (10,000 cells/mL/day).

V. parahaemolyticus is a common marine and estuarine pathogen that can be both harmful to the host (e.g., fish [49], shrimps [50] and crabs [51–53]) and humans, causing acute gastroenteritis by consuming contaminated marine products [54]. The pathogenic *V. parahaemolyticus* strain was retrieved from frozen (−80 °C), maintained at the Nitte University Centre for Science Education and Research, and sub-cultured and cultivated in tryptic soy broth (TSB) containing 1% NaCl. After determining viable cell count, an appropriate volume of bacteria containing broth was added to the water to achieve the correct concentration of 10,000 cells/mL/day per treatment in the microorganism group.

Juvenile crabs (crablets, 3.92 ± 1.98 g body mass, approximately 30 mm carapace width) were obtained from the mud crab hatchery of the Rajiv Gandhi Centre for Aquaculture in Tamil Nadu, India in January 2020. Crablets were transported to the laboratory at the Nitte University Centre for Science Education and Research (NUCSER) by road (~26 h). Crablets were regularly sprayed with water and the survival rate was 99%. Upon arrival, crablets were acclimated to laboratory conditions at ambient temperature and salinity for at least three days before being exposed to the experimental conditions. Crablets were exposed to the four climate change treatments for 10 days with or without microorganism exposure: 'control' (28 °C + 30 PSU), 'warming' (32 °C + 30 PSU), 'freshening' (28 °C + 20 PSU) or 'warming + freshening' (32 °C + 20 PSU). To achieve the desired temperature of +32 °C by day zero for the groups exposed to warming, the water temperature was increased by +1 °C per day by using stick heaters in the acclimation period. Similarly, salinity was decreased by 3 PSU per day by adjusting seawater with filtered freshwater to achieve the necessary salinity for the groups exposed to freshening.

The total number of 256 crablets per microorganism exposure (no microorganisms or *Vibrio*) were randomly divided into four groups of 64 animals assigned to one of the climate change experimental regimes including control. These 64 individuals were then once again randomly distributed in four 15 L buckets filled with 8 L filtered (5 µm) and aerated seawater with pipe fragments to serve as a refuge to reduce cannibalism (Figures S1 and S2). Each bucket consequently contained 16 animals at a density of two animals per litre. Those four buckets of the same treatment were placed into larger tanks with water, which were heated with stick heaters to reach experimental temperature. Temperature and salinity were recorded twice a day and adjusted if needed. Crablets were fed once a day with chopped prawns ad libitum and any leftovers were removed. Dead animals, if any, were removed daily. At the end of the exposure period (day 10), crabs of each bucket containing 16 animals were randomly assigned to one of the three groups. Group one crablets were used for the determination of oxygen consumption, and groups two and three for other analyses not discussed in this paper.

2.2. Determination of Metabolic Rates

Standard oxygen uptake (MO_2) after 10 days of exposure period was determined using closed chamber respirometry. Respirometry chambers (volume = 245 mL) containing a magnetic flea and a plastic mesh to prevent contact between the crablet and the magnetic flea, were filled with aerated and clean (filtered through a 0.22 µm filter) seawater at the respective experimental temperature and salinity. A crablet was added to each chamber and sealed while submerged in the water to prevent air bubbles. Before placing the chambers onto magnetic stirrers (Remi Laboratory Instruments, Mumbai, India) for preventing oxygen stratification with the chamber and ensuring adequate mixing, they were loosely covered with aluminium foil to minimise the disturbance to the animal. To establish resting MO_2, crablets were allowed to settle in the chambers for 40 min. Planar optode spots (diameter 0.5 cm; PreSens Precision Sensing GmbH, Regensburg, Germany) were glued to the inside of each chamber to allow oxygen levels to be measured. Oxygen levels in the chambers were measured every 5 min for a period of 1 h using a Fibox 4 oxygen meter (PreSens Precision Sensing GmbH). The decline in pO_2 over the measurement period was linear and was not allowed to fall to hypoxic levels. Background respiration was taken into account by running a series of blanks, and the average value across them was subtracted

from the original MO_2 value. MO_2 was expressed as μmol O_2 $h^{-1} \cdot g^{-1}$. After completing the measurements of MO_2, crablets were removed from the chambers and weighed. Crablet volume was also obtained by displacement.

2.3. Data Analysis

For each analysis, firstly, the dataset was checked for outliers using the inter-quartile range rule multipliers 1.5 and 3.0. Levene's test was then used to test for equality of variances and the Kolomogorov-Smirnov and Shapiro-Wilk tests were used to test if the data were normally distributed.

A two-way analysis of covariance (ANCOVA) was used to investigate the effect of climate change and microorganism exposure on oxygen consumption with crab body mass as a covariate. Although two-way ANOVA is a parametric test, it is regarded as a robust and powerful test that can be used for non-normally distributed data, as was the case here [55]. Post-hoc Bonferroni tests adjusted for covariates were conducted to identify differences between groups. One-way ANOVA was used to test whether survival rates were impacted by climate change or microorganism exposure. Two-way ANOVA was used to test whether exposure to microorganisms has a significant effect on oxygen consumption if the crab body mass is not seen as an essential covariate. All analyses were conducted in SPSS v.25.

3. Results

3.1. Survival Rates

Survival rates were greater than 85% in most treatment groups, and the lowest survival rate was 66.66% in the group exposed to *V. parahaemolyticus* and warming (Table S1). There were no statistically significant differences in survival rates between groups ($p > 0.05$). No significant mortality was observed as expected; the temperature and salinity of simulated climate change were chosen to potentially induce physiological stress, but not cause mass mortality. One replicate (a tank of 16 crabs) from the no microorganism exposure group and two replicates from the *Vibrio* exposure group were lost due to technical issues at the start of the exposure and thus were excluded from the total number of crabs used in the experiment.

3.2. Metabolic Rates

Eleven samples were identified as outliers, thus excluded from further analyses. Data did not meet assumptions for homogeneity of variances (Levene's test, $p > 0.05$), yet was normally distributed according to Kolomogorov-Smirnov ($p > 0.05$), but not according to Shapiro-Wilk ($p = 0.004$). In the preliminary analysis, the term 'tank' as a random factor was found not to have a significant effect on the oxygen consumption, and, thus, was excluded from further analyses. Crab body mass was found to be statistically significant, therefore was included as a covariate. The two-way ANCOVA removing/accounting for crab body mass showed that simulated climate change had a significant effect ($p = 0.000$, $F_{(3,91)} = 7.984$) on the oxygen consumption rate, while the combination of microorganism exposure together with climate change did not have any effect ($p = 0.966$, $F_{(3,91)} = 3.409$). Microorganism exposure alone was not statistically significant if the p-value of the significance level α is considered to be 0.05. However, p-value = 0.068 ($F_{(1,91)} = 3.409$) indicates a trend thus is recognised as significant. Crab body mass as a covariate had a statistically significant effect on oxygen consumption ($p = 0.000$, $F_{(1,91)} = 112.380$). Pairwise comparisons, Bonferroni post-hoc tests, based on means and adjusted for crab body mass, showed a statistically significant difference between ambient and freshening groups ($p = 0.001$), between freshening and warming ($p = 0.024$), and between freshening and warming + freshening ($p = 0.000$). Yet, there were no statistically significant differences between ambient and warming and between ambient and warming + freshening groups ($p > 0.05$). However, the mean oxygen consumption of juvenile mud crabs not adjusted for crab body mass showed

increased oxygen consumption in all simulated climate change groups exposed to *Vibrio* compared to juvenile crabs not exposed to pathogenic bacteria (Figure 1).

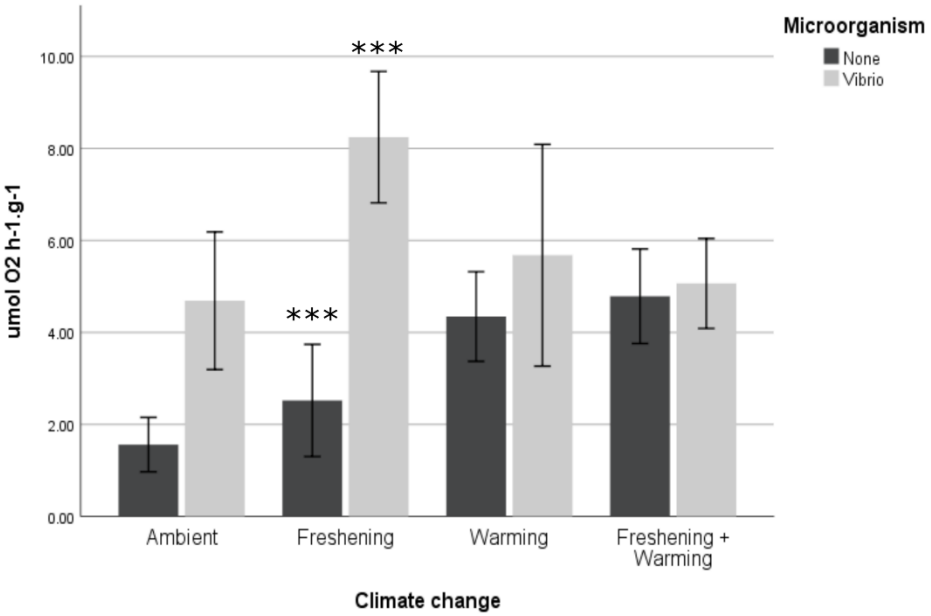

Figure 1. The mean oxygen consumption of juvenile mud crabs ± SD in simulated climate change conditions and microorganism exposure. Three asterisks indicate $p \leq 0.001$ between ambient and simulated climate change groups.

4. Discussion

Crustacean fisheries and aquaculture are one of the fastest-growing sectors, contributing to food security and economic growth [56,57]. Meanwhile, marine heatwaves and overfishing have caused declines in crustacean catch in some areas e.g., [58,59]. Thus, understanding the impacts of climate change is essential both for conservation management and for the food production sector. It is particularly important to understand the impacts on early life stages, as juveniles generally are more susceptible to any changes. However, there are limited data on the effects of climate change on crustacean juveniles, in particular, on physiological responses, with studies to date mainly focusing on survival rates and development [29,60,61]. *Scylla serrata* is a useful model species in which to assess the effects of these climate change parameters as they spend their early life stages in marine environments and their adult life in estuarine environments [44] and are thus particularly vulnerable to future climate change [62].

Our study showed that simulated climate change conditions (especially freshening) significantly increased oxygen uptake in juvenile mud crabs, indicating higher energy levels are needed to regulate physiological processes that maintain homeostasis. The statistically significant increase in oxygen consumption in crabs exposed to freshening is worrying as tropical regions are projected to experience increased monsoon rainfall [9]. Mud crabs tolerate wide temperature and salinity ranges; however, these depend on the life stage and geographical location, but the optimum temperature and salinity for juvenile mud crabs are between 25 °C and 30 °C and 15 and 35 PSU, respectively [47,48]. Lowered salinity and elevated temperature have been shown to increase oxygen uptake in juvenile *S. serrata*, indicating that the energy expenditure of juvenile mud crabs is lower at 25 PSU and 30 PSU compared to 15 PSU and 20 PSU [63]. It has also been found that decreased salinity (4 PSU) induced high activity of Na^+/K^+-ATPase in posterior gills of juvenile *S. serrata* [64], which is an important osmoregulatory response of euryhaline estuarine crustaceans when exposed to low salinities [65], showing that ions are being actively pumped from water into the haemolymph. Adult *Scylla olivacea* exhibited an identical response when exposed to salinities at 5, 10 and 15 PSU [66], indicating that osmoregulation

is consistent at all development stages. Another study on *S. olivacea* showed that exposure of adult mud crabs to freshwater (0 PSU) rapidly increased oxygen consumption, which then gradually declined after 12 h and became stable after 4 days [67]. Thus, it is evident that freshening induces stress in animals, yet the cell processes of mud crabs are well adapted to varying salinities and have various osmoregulatory mechanisms to reduce any damage. The high salinity gradient in estuaries also can manifest in increased salinity and lower concentrations of dissolved oxygen [68]. Paital and Chainy [69] hypothesised that hypersalinity (35 PSU) is more likely to cause severe oxidative stress than freshening.

Although our study did not identify warming as a statistically significant factor, it is known that both high (40 °C) and low (15 °C) temperatures induce oxidative stress that can lead to weakened cellular respiratory functions in *S. serrata* [70]. A study on mitochondrial metabolite levels of populations of *S. paramamoisan* from the south and north of China also showed that northern populations adapt better to seasonal temperature variations [71]. In addition, habitat can also define physiological responses. Fiddler crabs from unvegetated areas increased their oxygen consumption consequently adjusting their metabolic rate as a response to a higher temperature, while crabs from vegetated areas that are used to having a refuge were more vulnerable to warming under laboratory conditions [20]. These findings underline the importance of research on temperature effects on different species in different geographical locations. Furthermore, it has been shown that tidal pools in the tropics can act as ecological traps for molluscs, crustaceans and fish as the water temperature can exceed the upper thermal limit of animals [72] and mud crabs are often found in relatively shallow waters [44]. Warming also affects crustacean behaviour, increasing time spent in burrows and decreasing their activity and feeding time [20,73,74]. Although behavioural observation and quantification of feed leftovers were not part of this study, crablets exposed to warming, as well as warming + freshening left more uneaten feed and were seemingly less active. However, mud crabs are mainly nocturnal, thus a different experimental design would be beneficial in future to assess the impact of increased temperature on their behaviour. Besides, this study was relatively short (10 days), but some of the most extreme marine heatwaves can last up to 160 and even 250 days [75], therefore studies exposing juvenile crabs to these conditions for longer and assessing their transformation to sub-adults are also necessary. Mud crabs are known for their aggressive and cannibalistic behaviour especially if there is a discrepancy in size vs. development stage, [44], however, it is difficult to determine the impact of this on survival rates. An experimental design involving keeping crabs in individual containers could exclude these behavioural factors and account for moulting, which was observed in this study but not accounted for. Juvenile crabs were observed in this study using the pipe fragments to hide in or under, thus providing more refuge options could be another approach. The most holistic approach for further studies would be to include behavioural observations and limit factors that could cause mortality, yet that is a restrain of such laboratory experiments as it does not imitate the conditions of natural habitats.

Not all species will be negatively affected by projected future climate conditions. Some, such as bacteria and algae thrive at increased temperatures and different climate change induced salinities. However, those thriving are often pathogenic bacteria [13] adding yet another pressure on animals and humans already exposed directly to warming and freshening. The role of climate change in emerging and re-emerging bacterial diseases affecting humans has been widely acknowledged [76], yet less attention has been brought to how more abundant pathogenic bacteria especially in tropical regions will affect the physiology and health status of aquatic animals. Although the calculated measure of probability was higher than the conventional, yet the arbitrary level of significance of 0.05 [77], there is a noticeable trend of increased metabolic rates in animals exposed to *V. parahaemolyticus*, which requires further investigation. In addition, crabs exposed to *Vibrio* were on average 62% smaller compared to the groups not exposed to microorganisms despite random selection (varying from 23% in warming + freshening groups to 108% in warming groups). The crabs were selected by hand randomly but were still subject to

unconscious human bias and this resulted in placing animals of the same size together as a group. Mud crab *Scylla serrata* juveniles, especially in aquaculture settings, are also susceptible to *Vibrio*-caused bacterial shell disease, which, although not lethal itself, can cause perforations in the shell leading to secondary infections [78].

The highest oxygen uptake indicating potential oxidative stress and high energy consumption was observed in crabs exposed to freshening and *V. parahaemolyticus*, yet interestingly metabolic rates were not significantly affected by the presence of pathogenic bacteria in animals exposed to warming and warming + freshening compared to the same simulated climate change conditions not exposed to microorganisms. The optimum growth conditions of *V. parahaemolyticus* are 20–25 PSU and 30–35 °C [79], thus the simulated climate change conditions should not have affected bacterial colonies. However, the interaction between environmental conditions and time of exposure has been reported to impact the scale and intensity of infections in blue crabs, *Callinectes sapidus* [80]. Thus, in any future study, the severity of *Vibrio* infection should be determined by performing crab histopathology or quantifying the uptake of viral DNA with the help of real-time PCR (qPCR) at the end of the experiment. Previously it has been demonstrated that *V. parahaemolyticus* amplified significant negative effects caused by warming and freshening on metabolic and immunobiological status of mussels, *Perna viridis*, simultaneously increasing toxin-pathogen load that could adversely affect seafood consumers [15]. Although mud crab juveniles are not filter feeders, by being exposed to free-living bacteria and potentially feeding on infected molluscs and fish, pathogenic bacteria could compromise their physiological health. Further research is also necessary to assess the toxin-pathogen load in adult mud crabs in relation to food safety.

It is essential to understand the impacts of ocean warming and associated environmental conditions on mud crabs and other tropical crustacean fisheries and aquaculture as they are a significant livelihood resource for many people. They also play an important role in food security, being a source of protein. Besides, inhabiting estuaries and mangroves, juvenile and adult mud crabs are a part of a complex and rich natural ecosystem playing their part in maintaining its balance. Thus, data obtained from studies such as ours, characterising the physiological responses to temperature increases and salinity changes in a tropical marine crab species can be used to inform local communities and policymakers in planning for future climate change conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse10050582/s1, Figure S1: Schematic of the experimental set-up. Each larger tank was assigned to one treatment—ambient, freshening, warming or warming + freshening—and contained four 8L buckets with 16 animals; Figure S2: Photos of the experimental set-up. (A) Each tank was assigned to one treatment as indicated in Figure S1 and the experimental temperature was obtained by warming the water in the tank with the help of stick-heaters; (B) Each tank contained four replicates, each of them was aerated and the water was changed daily; Table S1: Survival rates and average body mass (±SD) for *Scylla serrata* juveniles after 10 days of exposure to different simulated climate change conditions and microorganisms.

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