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# Effects of chronic hypercapnia and elevated temperature on the immune response of the spiny lobster, *Jasus lalandii*

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ABSTRACT

The West Coast rock lobster (WCRL), *Jasus lalandii*, inhabits highly variable environments frequented by upwelling events, episodes of hypercapnia and large temperature variations. Coupled with the predicted threat of ocean acidification and temperature change for the coming centuries, the immune response in this crustacean will most likely be affected. We therefore tested the hypothesis that chronic exposure to hypercapnia and elevated seawater temperature will alter immune function of the WCRL. The chronic effects of four combinations of two stressors (seawater $pCO_2$ and temperature) on the total number of circulating haemocytes (THC) as well as on the lobsters' ability to clear (inactivate) an injected dose of *Vibrio anguillarum* from haemolymph circulation were assessed. Juvenile lobsters were held in normocapnic (pH 8.01) or hypercapnic (pH 7.34) conditions at two temperatures (15.6 and 18.9 °C) for 48 weeks ($n = 30$ lobster per treatment), after which a subsample of lobsters ($n = 8$/treatment), all at a similar moult stage, were selected from each treatment for the immune challenge. Baseline levels of haemocytes (THC $ml^{-1}$) and bacteria (CFU $ml^{-1}$) in their haemolymph were quantified 24 h prior to bacterial challenge. Lobsters were then challenged by injecting $4 \times 10^4$ *V. anguillarum* per g body weight directly into the cardiac region of each lobster and circulating haemocyte and culturable bacteria were measured at 20 min post challenge. No significant differences in THC $ml^{-1}$ ($p < 0.05$) were observed between any of the treatment groups prior to the bacterial challenge. However lobsters chronically exposed to a combination of hypercapnia and low temperature had significantly higher ($p < 0.05$) THCs post-challenge in comparison with lobsters chronically exposed to hypercapnia and high temperature. A significant interactive effect was recorded between temperature and pH for the post-challenge THC data (two-way ANOVA, $p = 0.0025$). Lobster were very efficient at rendering an injected dose of bacteria non-culturable, with more than 83% of the theoretical challenge dose ($\sim 1.7 \times 10^5$ *Vibrio* $ml^{-1}$ haemolymph) inactivated within the first 10 min following injection. Although differences in the inactivation of *V. anguillarum* were observed between treatment groups, none of these differences were significant. Clearance efficiency was in the following order: Hypercapnia/low temperature > normocapnia/high temperature > normocapnia/low temperature > hypercapnia/high temperature. This study demonstrated that despite chronic exposure to combinations of reduced seawater pH and high temperature, the WCRL was still capable of rapidly rendering an injected dose of bacteria non-culturable.

## 1. Introduction

The South African cold water palinurid, *Jasus lalandii,* inhabits a highly variable environment, the Benguela Current Large Marine Ecosystem (BCLME), which displays pronounced variability in its physicochemical makeup in the short term (hours to days), primarily due to upwelling [1–5]. This lobster has to respond to frequent and often rapid changes in salinity, temperature, pH and oxygenation. This is not uncommon for crustaceans, which inhabit a variety of habitats and are therefore naturally exposed to a wide range of environmental parameters [6–9], giving them, to a certain degree, some physiological plasticity. Coming centuries, however, are predicted to bring new, more sustained challenges that include a decrease in ocean pH, termed "ocean acidification" and an increase in seawater temperature [10]. It is not clear, though, whether these parameters will increase or decrease over the coming years in the BCLME [11] and consequently what

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impact these long-term changes may have on the health and physiology of organisms inhabiting this environment.

The West Coast rock lobster (WCRL) supports a valuable recreational and commercial fishery in South Africa [12,13] and is a keystone benthic predator [14]. As a consequence, numerous field and aquarium based studies have been conducted to assess the effects of environmental change/stressors on a variety of biological parameters in this species, particularly growth, survival and food intake [15–19]. Detailed studies on the physiological, biochemical, molecular and particularly the immunological responses of *J. lalandii* to environmental stress are, however, lacking.

*Jasus lalandii* was observed to be capable of efficiently maintaining its acid-base balance when exposed to acute and chronic hypercapnia [13,20]. The ability of *J. lalandii* to defend itself against opportunistic bacteria and other pathogens may, however, be compromised under these conditions, especially when seawater chemistry changes together with ambient temperature. It has been postulated that disease prevalence in decapods will increase due to the projected climate scenarios [21], and several studies have observed adverse effects on the immune response of crustaceans exposed to environmental stressors, such as hypercapnia and increased temperature [7,8,22,23]; Tanner et al., 2006), suggesting that similar stressors may lead to a weakened immune defence in *J. lalandii*.

The innate immune defences of crustaceans are composed of multiple effectors/mechanisms that are capable of inactivating and eliminating potential threats [24]. Besides the physical barriers, that include the tough, chitinous exoskeleton and membranes of the digestive tract, the primary cell types that are responsible for immune surveillance and defence against foreign entities or organisms are the circulating haemocytes [24–26]. These cells are derived from haematopoietic tissue [27], such as the hepatopancreas in lobsters [28], and consist of three functional cell types, namely hyaline, semi-granular and granular cells [27,29]. The quantity of haemocytes and/or haemocyte sub-populations have been shown to vary with physicochemical and biological parameters [22,30–36]. Circulating haemocyte numbers have been shown to be abundant (10 - 80 × $10^6$ cells $mL^{-1}$ haemolymph) in healthy crustaceans [24], but can decrease significantly following stress and bacterial challenge/infection [37]; Burgents et al., 2005 ab; [38,39]. Low circulating haemocyte numbers are strongly correlated with increased susceptibility to pathogens in crustaceans [40,41].

A strong correlation exists between environmental stress and immune depression and/or disease susceptibility for molluscs [42–44], fish [7,8] and crustaceans [22,32,39,45]. Unfortunately, the majority of these correlations have only been observed following acute exposure (a few hours) to environmental stressors and results cannot be used to predict impacts following long-term or chronic exposure. Climate change will affect marine biological processes in two primary ways: ocean warming or cooling and ocean acidification [46]. There is, however, limited information on the interactive effects of temperature and hypercapnia on the physiological and immunological response of crustaceans, with, to the best of our knowledge, no information available for the recreationally and economically important west coast rock lobster, *J. lalandii*.

In the present study, we therefore aimed to address this shortfall by examining the immunological response of *J. lalandii* following chronic exposure to combinations of different temperatures and seawater pHs.

## 2. Materials and methods

### 2.1. Animal collection and maintenance

Juvenile lobsters (n = 130, w: 1.4 ± 0.75g) were collected from oyster stacks at an offshore oyster farm (Saldanha Bay Oyster Company) in Saldanha Bay (32°59′–33°05′ S, 17°56′–18°02′ E), which is a large semi-enclosed bay on the West Coast of South Africa [47]. Oyster stacks were sampled during routine maintenance operations between September and December. For transportation, lobsters were placed in plastic bags (n = 2 bags each containing ± 60 lobsters) filled with 4.5 l seawater, bubbled with oxygen before sealing and then placed into a polystyrene container with ice bricks to ensure $T_A$ remained below 20 °C. Traction for the lobsters in each bag was provided by inserting mesh cloth, which helped to minimise stress during transport. Lobsters were transported to the Aquaculture Research Aquarium of the Department of Agriculture, Forestry and Fisheries (DAFF) in Sea Point, Cape Town. Immediately upon arrival, 32 or 33 lobsters were placed into each of four glass tanks (L × W × H: 100 × 48 × 35 cm) filled with seawater (pre-acclimation tanks). All sea water utilized by the Aquaculture Research Aquarium is pumped directly from the Atlantic Ocean via pipes running into the subtidal zone of a semi-sheltered bay directly in front of the aquarium. Sea water is pumped through a drum filter and sand filters prior to entering a sump tank at the highest point in the aquarium, where after it is gravity fed to the various systems within the aquarium. Seawater flowed through the tanks at a rate of ~240 l $h^{-1}$ and seawater salinity (35‰), temperature ($T_A$ ranged from 11 to 17 °C) and pH (7.7–8.1) fluctuated per the incoming water. The tanks were constantly aerated and photoperiod was maintained on a 12 h day/night cycle.

The lobsters were pre-acclimated in these tanks for ± 4 months prior to experimentation. During this period, lobsters were fed a mixed diet of mussel (*Chromomytilus meridionalis* and *Mytilus galloprovincialis*), sardines (*Sardinops sagax*) and Horse mackerel (*Trachurus trachurus)*, once to twice a week in the afternoon *ad libitum*. Excess food was removed the following day. Feeding was discontinued four days prior to commencement of the pre-conditioning and chronic exposure trial for the immune challenge.

### 2.2. Preparation of animals and tanks for chronic exposure

Pre-acclimated juvenile lobsters were removed from the holding tanks and each individual weighed to the nearest 0.001 g using an M-power electronic balance (Sartorius, Germany) and carapace length (*CL*) measured (tip of rostrum to mid caudo-dorsal margin of carapace) to the nearest 0.1 mm with a digital calliper (Mitutoyo Corp., Japan). Following measurement, lobsters were placed into individual plastic containers *(L × W × H: 10 × 10 × 7*.5 cm*)* pre-labelled with a unique number. This was done to allow for precise growth monitoring of each individual and to prevent cannibalism and competition for food. Each plastic container was perforated with forty-four 3 mm holes on each side to ensure sufficient water exchange with the surrounding environment. Perforation also provided better traction and assisted lobsters during moulting. Once a lobster was assigned to a numbered container, it was placed into a larger basket which was suspended in the tank *(L × W × H: 148× 100 ×* 90 cm*)* with seawater parameters replicating those of the pre-acclimation tanks. After selection of similar-sized lobsters for each treatment, according to biomass (*w*), 30 individuals were placed into each of the four treatment tanks *(L × W × H: 148× 100 ×* 90 cm*)*. Treatments consisted of a combination of: a) normocapnia/high temperature, b) normocpania/low temperature c) hypercapnia/high temperature and d) hyperpercapnia/low temperature. The measured and calculated (for methods, see Ref. [13] physicochemical seawater parameters for each of the treatments are summarised in Table 1.

The experimental pH level of 7.3 was selected according to the specific conditions that are predicted to prevail in the BCLME in future, whereas temperatures were selected from the lower and upper limits of the WCRL's tolerance range (for detailed description see Ref. [13]. The pH of the hypercapnic tanks was set as described previously [13]. This was achieved using a pH controller (7074/2, TUNZE, Germany) containing a solenoid valve (7074.111) and a pH electrode (7070.110) attached to a 9 kg $CO_2$ bottle (technical). To ensure sufficient mixing, a SP 103–2400 submersible pump (BOYU, China) was placed in each tank. Temperature, pH, total alkalinity and salinity were measured and

**Table 1**
Seawater conditions recorded during the exposure of juvenile lobsters (*Jasus lalandii*) to each respective treatment over a period of 48 weeks.

| Treatment | Salinity ‰ | $T_A$ (°C) | $A_T$ (μmol kg$^{-1}$) | pH | pCO$_2$ (Torr) | HCO$_3^-$ (mmol l$^{-1}$) |
|---|---|---|---|---|---|---|
| Normocapnic/High temp. | 35.0 ± 0.0 | 18.9 ± 0.1 | 2051 ± 11 | 8.00 ± 0.01 | 0.30 ± 0.01 | 1.71 ± 0.01 |
| Normocapnic/Low temp. | 35.0 ± 0.1 | 15.6 ± 0.1 | 2065 ± 13 | 8.02 ± 0.01 | 0.29 ± 0.01 | 1.75 ± 0.02 |
| Hypercapnic/High temp. | 35.0 ± 0.1 | 19.0 ± 0.1 | 2037 ± 23 | 7.35 ± 0.01 | 1.55 ± 0.03 | 1.99 ± 0.02 |
| Hypercapnic/Low temp. | 35.1 ± 0.1 | 15.6 ± 0.1 | 2063 ± 12 | 7.34 ± 0.01 | 1.60 ± 0.03 | 2.03 ± 0.01 |

Values are means ± S.E.

$pCO_2$, $HCO_3^-$ and $CO_3^{2-}$ calculated as outlined by Knapp et al. [13] using the appropriate constants. Temperature was maintained via a universal STC-1000 Digital Temperature Controller (DTC, AGPtek, USA) connected to two 300 W aquarium heaters (Eheim Jäger, Germany) for the treatments where high temperature was set as an environmental parameter. In treatments where low temperature was set as an environmental variable, a combination of heaters (as stated above) and a HS 66-A chiller unit (Hailea, China) were used to ensure a stable temperature throughout the trial. The latter variables were also controlled via a DTC. Each tank functioned as its own unit, being set up to be a partial recirculation system. Water exchange rates were set for approximately one full tank exchange every two days. Oxygen concentration was determined using a Multi 350i meter set (WTW, Germany) and water quality was monitored by measuring $NH_4^+$ concentration (Ammonia test kit, Sera, Germany). The latter never exceeded 0.5 mg l$^{-1}$.

After assignment of juvenile lobsters to respective treatments, water parameters were adjusted to the various experimental parameters (combinations; Table 1) over a period of two weeks. Initially, seawater parameters remained unchanged for two days (pH ranged from 8.0 to 8.1; $T_A$ ranged from 17 to 19 °C) in order to allow lobsters to acclimatize following movement. Following acclimation, pH was decreased gradually from 8.0 to 7.3 over a five-day period in treatments where the effect of hypercapnia was one of the experimental variables. Thereafter, temperature was decreased from 18 °C, at a rate of approximately 1 °C a day, to a temperature of 15.5 °C in the treatments where low temperature was an experimental variable. Once these parameters had stabilised, the 'chronic exposure' trial officially began and continued for a period of 334 days. Each lobster was fed twice a week, *ad libitum*, with fresh or frozen mussel (*Choromytilus meridionalis* and *Mytilus galloprovincialis*), depending on availability. Every evening of the day following feeding, all excess feed was removed from the containers. As the lobsters grew, feed rations were increased accordingly. When lobsters reached a size of 27 mm CL, they were transferred into larger containers (*∅ × H: 11 × 16*.3 cm*)*.

In an effort to account for potential "tank effects", approximately mid-way through the trial (day146 of exposure) treatments along with their respective water parameters were switched between tanks, i.e. lobsters' environmental conditions did not change, however, their tanks did.

### 2.3. Preparation of bacterial solution for injecting lobsters

The bacterium used in this study was a strain of *Vibrio anguillarum* 5677, which was previously isolated from diseased abalone, *Haliotis midae,* by Dr. Anna Mouton from Amanzi Biosecurity [48]. *V. anguillarum* is a gram negative, rod-shaped bacterium that is one of several major *Vibrio* spp. responsible for causing disease in a variety of marine fish and shellfish, including crustaceans [49,50]. The bacterium was transfected with a stable *Vibrio*-derived plasmid (plasmid pEVS146; provided by Prof. Eric Stabb, University of Georgia) coding for chloramphenicol (Cm) and kanamycin (Kan) antibiotic resistance. Working stocks of the bacterium and the bacterial solution for injections were prepared as described by Ref. [39]; with minor modifications. The bacterium was grown on Tryptic soya agar (TSA) plates supplemented with 2.0% NaCl (w/v), Cm (5 μg ml$^{-1}$) and Kan (100 μg ml$^{-1}$) and the inoculated plates were incubated overnight at 30 °C. Following incubation, a small number of bacteria were transferred into 5 ml of sterile HEPES solution (10 mM, pH 7.6) supplemented with 2.5% NaCl (w/v), hereafter referred to as HEPES-buffered saline. This solution was further diluted as required with HEPES-buffered saline to an optical density of 0.1 ± 0.005 at a wavelength of 540 nm, which was determined to be equivalent to a bacterial concentration of $4 \times 10^7$ colony-forming units (CFU) ml$^{-1}$. This solution was used for injecting lobsters for the bacterial clearance assays.

### 2.4. Injection of bacteria into haemolymph in J. lalandii

Bacteria were injected directly into the cardiac sinus of each lobster. This was done by carefully inserting a needle, roughly parallel to the carapace, through the exoskeleton membrane between the thorax and abdomen. Prior to injection, the injection site was swabbed clean with absolute ethanol. The heart was chosen as the site of injection to ensure rapid and even distribution of the bacterium throughout the circulatory system of the spiny lobster, as described previously for other crustaceans [39,45,51]. Each animal received an injection dose of $4 \times 10^4$ *V. anguillarum* (i.e. 1 μl of the injection preparation) g$^{-1}$ lobster, which equates to a circulating dose of approximately $1.7 \times 10^5$ bacteria ml$^{-1}$ haemolymph, based on the assumption that the haemolymph volume of *J. lalandii* accounts for approximately 23% of the body weight (Auerswald et al., *unpublished* data). This dose is below the lethal dose for *J. lalandii* and no mortalities were observed in any of the injected animals

**Table 2**
Total weight (*w*) and carapace length (*CL*) of juvenile lobsters (*Jasus lalandii*) used in bacterial challenge.

| Treatment | Initial | | Final | |
|---|---|---|---|---|
| | *w* (g) | *CL* (mm) | *w* (g) | *CL* (mm) |
| Hypercapnia/Low temp | 1.39$^a$ ± 0.21 | 13.7$^a$ ± 0.6 | 19.10$^{a,b}$ ± 2.09 | 32.6$^{a,b}$ ± 1.2 |
| Normocapnia/High temp | 1.21$^a$ ± 0.17 | 13.1$^a$ ± 0.5 | 22.49$^{a,b}$ ± 2.08 | 34.3$^{a,b}$ ± 1.2 |
| Normocapnia/Low temp | 1.31$^a$ ± 0.2 | 13.4$^a$ ± 0.7 | 17.43$^a$ ± 1.97 | 31.3$^a$ ± 1.3 |
| Hypercapnia/High temp | 1.12$^a$ ± 0.16 | 12.9$^a$ ± 0.5 | 27.64$^b$ ± 2.70 | 36.9$^b$ ± 1.3 |

Seawater parameters: Normocapnia – pH 8; hypercapnia – pH 7.3; high temperature – 19 °C; low temperature – 15.5 °C.
$^{a,b}$Values sharing the same superscript within columns do not differ significantly ($p < 0.05$).
Values are means ± S.E. (n = 8).

throughout the study period. This bacterial dose, which equated to a volume of 20–30 µl (see Table 2 for lobster weights), was administered using a 50 µl Hamilton syringe equipped with a 26-gauge x ⅝ inch needle and was injected at a slow rate to ensure sufficient dilution of the injected dose into the haemolymph flowing through the cardiac sinus. This was done deliberately to prevent localized clotting, due to possible clumping of the bacteria, around the site of injection, as described by Ref. [39].

### 2.5. Optimization of the time point for discerning the impact of stress on haemocyte count and bacterial clearance in J. lalandii

In order to determine the optimal time point for post-challenge sampling of haemolymph, where the change in circulating THCs and the number of culturable bacteria remaining in circulation following a stress could be accurately quantified, a time-course experiment was conducted. For this experiment, lobsters (n = 5, *w*: 27.3 ± 6.0 g), which had been maintained in the pre-acclimation glass tanks, while the chronic pre-conditioning experiment was underway, were transferred to separate 20 L glass aquaria containing seawater that was taken from the pre-acclimation glass tanks. Lobsters were injected with bacteria and haemolymph was subsequently sampled from the cardiac sinus of each animal at 10, 20, 40 and 120 min (Fig. 1). An additional sample of haemolymph was also taken 10 min prior to the injection of bacteria. These time intervals were preliminarily chosen since they have been shown to be optimal for measuring clearance of bacteria from the haemolymph of both mollusks [42,52] and crustaceans [51]; Burgents et al., 2005 ab; [38]. In order to inject bacteria or sample haemolymph from a lobster, the animal was carefully transferred from its holding container to a dry towel that had been placed on a laboratory bench-top; where the lobster's eyes were subsequently covered with a towel to minimize stress and prevent tail flip. Immediately following injection or sampling, which took approximately 30–40 s to complete, the animals were returned to their respective holding containers. Prior to haemolymph sampling, the extraction site was swabbed clean with absolute ethanol. For each extraction, a sterile 1 ml syringe fitted with a 26-gauge x ⅝ inch needle was used to withdraw approximately 100 µl of haemolymph from the cardiac sinus. A 50 µl subsample was immediately mixed with 450 µl of 10% neutral-buffered formalin (NBF) for the determination of total circulating haemocyte count (THC) $ml^{-1}$ (described below). Ten minutes after the initial extraction, each lobster was injected with $4 \times 10^4$ *V. anguillarum* $g^{-1}$ lobster directly into the cardiac sinus and haemolymph (~100 µl) was extracted from the cardiac sinus at 10, 20, 40 and 120 min post-injection (Fig. 1). An aliquot of this haemolymph was fixed for determination of THC $ml^{-1}$ (as described above). The remainder of the haemolymph (50 µl) was diluted in 300 µl HEPES-buffered saline and used to determine culturable bacteria (described below).

### 2.6. Assessment of baseline immune parameters

Approximately 24 h before bacterial challenge, lobsters were removed from their experimental containers and a sample of haemolymph (~100 µl) was withdrawn from the cardiac sinus, as described above (emersion time ± 45 s). This was done to measure the baseline THC $ml^{-1}$ in the haemolymph of each lobster and to determine whether any culturable bacteria were present in the haemolymph. Previous studies on crustaceans have shown that bacteria are frequently detected in the haemolymph of crustaceans collected from the wild, particularly in the warmer summer months, and recommend that lobsters with more than 5,000 bacteria $ml^{-1}$ haemolymph not be used for experimental use [51,53]. A 50 µl aliquot of the haemolymph sample was fixed in 450 µl of 10% NBF (within 20 s after haemolymph extraction) for determination of total circulating haemocyte count $ml^{-1}$ (THC) (as described below). The remaining haemolymph (50 µl) was transferred to a 1.5 ml microcentrifuge tube containing 300 µl of HEPES-buffer saline, vortexed briefly and separate 150 µl aliquots were spread plated onto TSA agar plates, supplemented with 2.0% NaCl (no antibiotics added), and incubated at 30 °C for 48 h to determine the number of live, culturable bacteria in the hemolymph (see below).

### 2.7. Basic experimental protocol

After 48 weeks of exposure to the seawater conditions described above (Table 1), individual lobsters (n = 8) from each treatment group were selected for the immune challenge (Table 2). Two criteria were utilized for this selection, namely an approximate calculation (estimated from last moult) of the moult stage ($C – D_{1''}$) and lobster weight (mean representative weight for the specific treatment). Moult stage, in particular, was assessed as it has been shown to affect a number of immune parameters in crustaceans and their susceptibility to infection [33,54]. Each selected lobster was then weighed (blotted dry before weighing) to nearest 0.001 g to allow for accurate determination of the volume of *Vibrio* or saline to be injected in the challenge trial. All lobsters used for the immune challenge were in a similar range of moult stages and there was no significant difference in mean weight of lobsters between treatments. At 24 h prior to injection/challenge, a sample of haemolymph (100 µl) was withdrawn from the cardiac sinus to determine the baseline immune parameters, namely THC and CFU $ml^{-1}$, of each individual crab, as described above. These individuals were

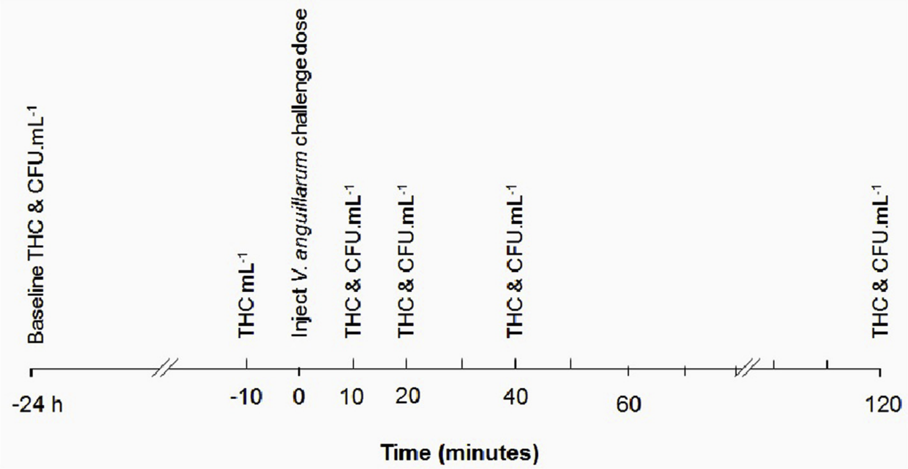

**Fig. 1.** Schematic diagram of the preliminary experimental design to determine the optimal time for bacterial challenge. The illustration indicates the timing of the *Vibrio anguillarum* injection and haemolymph withdrawals along with subsequent analysis.

**Table 3**
Seawater parameters measured and calculated in the vessels used for the challenge trials.

| Treatment | Salinity ‰ | Temp (°C) | pH | $A_T$ (μmol kg$^{-1}$) | $pCO_2$ (Torr) | **$HCO_3^-$ (mmol l$^{-1}$)** |
|---|---|---|---|---|---|---|
| Normocapnia/High temp. | 35.0 ± 0.00 | 18.9 ± 0.03 | 8.04 ± 0.03 | 1948 ± 8 | 0.25 ± 0.02 | 1.60 ± 0.02 |
| Normocapnia/Low temp. | 34.9 ± 0.13 | 15.7 ± 0.00 | 8.07 ± 0.03 | 1962 ± 15 | 0.24 ± 0.02 | 1.64 ± 0.03 |
| Hypercapnia/High temp. | 34.9 ± 0.13 | 19.1 ± 0.04 | 7.36 ± 0.04 | 1956 ± 11 | 1.49 ± 0.15 | 1.91 ± 0.02 |
| Hypercapnia/Low temp. | 34.9 ± 0.1 | 15.5 ± 0.1 | 7.32 ± 0.04 | 1967 ± 9 | 1.61 ± 0.16 | 1.94 ± 0.01 |

Values are means ± S.E.

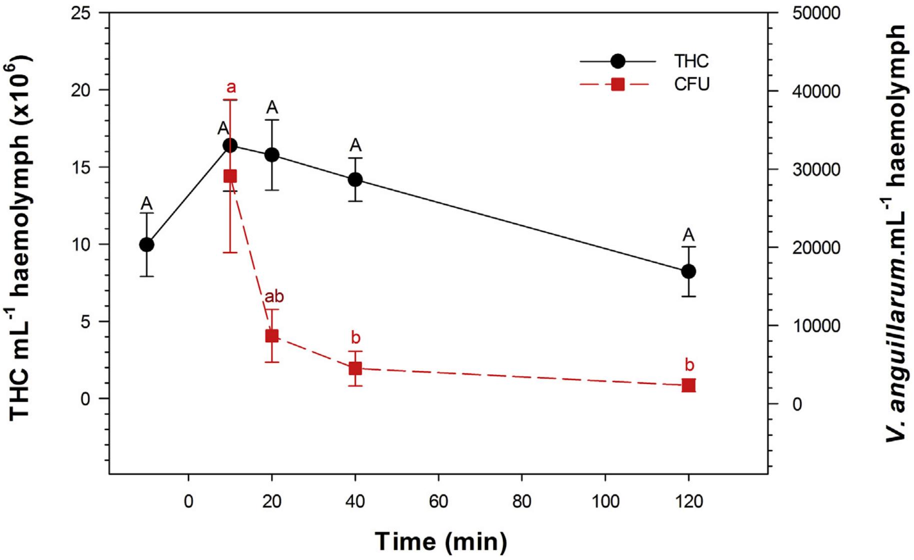

**Fig. 2.** Total haemocyte count (THC) and the amount of colony forming units (CFU) of culturable *Vibrio anguillarum* present in the haemolymph of *Jasus lalandii* over several time points. Values are mean ± S.E. (n = 5). Baseline THC was measured at time point (−10) and then 10, 20, 40 and 120 min post-challenge with 4.6 × $10^7$ *V. anguillarum* g$^{-1}$ lobster. A significant time-point effect on THC was detected ($p = 0.049$), but significant differences were not detected in pairwise comparisons (Holm-Sidak) between individual time-points. Lobsters where sampled at 10, 20, 40 and 120 post-challenge to determine the number of culturable bacteria remaining in circulation. The recovery of culturable bacteria was significantly affected by time ($p < 0.001$; one-way ANOVA). Different letters indicate a significant difference ($p < 0.05$) between values (Holm-Sidak multiple comparison).

then placed back into experimental treatments and allowed to recover for 24 h.

For the duration of the challenge trial, each lobster was transferred in their respective individually marked containers to a separate vessel *(∅ × H: 23.5 × 25 cm)* containing seawater that was taken from the respective treatment tank (emersion time of < 5 s, Table 3). Each lobster then received a challenge dose of 4 × $10^4$ *V. anguillarum* (i.e. 1 μl of the injection preparation) g$^{-1}$ lobster. At 20 min post-injection, a sample of haemolymph (100 μl) was withdrawn from the cardiac sinus of each lobster to determine the THC ml$^{-1}$ in the haemolymph and the number of culturable *V. anguillarum* remaining in haemolymph circulation (according to the methods describe above and below). The 20 min time-point was chosen as preliminary experiments (described above) indicated that it would be optimal for measuring the impact of a stress on the chosen immune parameters. Haemolymph obtained from saline injected crabs (conducted randomly throughout the experimental period) were included as negative controls to ensure that there was no growth of bacteria on the selective media other than the injected *V. anguillarum*.

### 2.8. Total haemocyte counts

The total number of circulating haemocytes of the fixed blood cells was counted with a haemocytometer and a light microscope (100× magnification). Three separate aliquots (10 μl each) of the diluted haemocyte suspension were counted and averaged for each lobster and the values expressed as the mean ( ± SE) THC ml$^{-1}$ haemolymph for lobsters in each treatment group.

### 2.9. Selective plating

In order to determine the number of culturable *V. anguillarum* remaining in the haemolymph of each lobster 20 min following injection, selective plating was used. Immediately following haemolymph withdrawal and dilution, separate aliquots (150 μl) of the diluted haemolymph samples from each lobster were spread plated onto TSA plates supplemented with 2.0% NaCl (w/v), Cm (5 μg ml) and Kan (100 μg ml). All plates were incubated for 48 h at 30 °C, at which point the number of bacterial colonies were counted and recorded. Counts were averaged from the duplicate plates and data expressed as the mean ( ± SE) culturable *V. anguillarum* ml$^{-1}$ haemolymph for lobsters in each treatment group.

### 2.10. Moult stage determination

Moult stage determination was carried out according to a modified method from Marco [55]; described in Knapp et al. [13]. The moult stages of *Jasus lalandii* are divided into nine stages and sub-stages (A, B, $C_{1\text{-}4}$, $D_{0,\,1',\,1'',\,1''',\,2,\,3}$) that are based on the development of the setae and the degree of pleopod epidermal retraction [56].

### 2.11. Statistical analysis of data

All statistical analysis was conducted using SigmaPlot Version 12.0 (Systat Software Inc., 2011).

For the lobsters that were challenged initially to assess their response to an introduced dose of *V. anguillarum*, the assumptions of normality and equal variance were met and therefore one-way ANOVA was conducted on THC and CFU (log-transformed) data to assess whether significant differences ($p < 0.05$) were present between individual time points (Fig. 2). The Holm-Sidak method was used for all post hoc multiple comparisons and significance was assigned to *P* values of 0.05 for both one-way ANOVA and Holm-Sidak analysis.

To determine whether there was a significant difference in the number of circulating haemocytes between treatment groups before and following injection with *Vibrio anguillarum*, a two-way ANOVA was conducted on log transformed THC data. To determine whether there were significant changes between pre- and post-bacterial THC data within each treatment group, paired t-tests were conducted. Where assumptions of normality and equal variance were met, a two-way ANOVA was conducted to assess whether the number of CFUs or percent change in THCs (relevant to the baseline; −10 min) was significantly ($p < 0.05$) affected by temperature and pH and whether an interaction existed between the main effects (temperature and pH). The CFU data was log transformed prior to statistical analysis, whereas all percent growth data were arc sin square root-transformed. Where assumptions for normality and equal variance were not met, a Kruskal-Wallis test was performed. The Holm-Sidak method was used for all post hoc multiple comparisons and significance was assigned to $P$ values of 0.05 for both two-way ANOVA and Holm-Sidak analysis.

To determine whether there was a significant difference in the number of circulating haemocytes of lobsters in moult stages C or D exposed to each of the four treatments, a one-way ANOVA was conducted on log transformed THC data. Where assumptions for normality and equal variance were not met, a Kruskal-Wallis test was performed. The Holm-Sidak method was used for all post hoc multiple comparisons and significance was assigned to $P$ values of 0.05 for both one-way ANOVA and Holm-Sidak analysis. To determine whether there was a significant difference in the number of circulating haemocytes between moult stages within a treatment, paired *t*-test was conducted on log transformed THC data. Significance was assigned to $P$ values of 0.05 for the latter test.

## 3. Results

### 3.1. Effect of time on circulating haemocyte numbers and recovery of culturable V. anguillarum in haemolymph

The mean density of circulating haemocytes in the haemolymph of non-challenged lobsters in the pre-acclimation tanks (prior to chronic stress) ranged from $4 - 10 \times 10^6$ cells ml$^{-1}$ (n = 5). The bacterial injection significantly affected THC ($p = 0.049$; one-way ANOVA), however no significant differences were noted between any of the individual time points in the post hoc pairwise comparisons (Holm-Sidak multiple comparison). At 10 min following injection of *V. anguillarum*, circulating haemocyte numbers increased by 65% above pre-injection levels, to $7 - 20 \times 10^6$ THC ml$^{-1}$ haemolymph, but then declined steadily thereafter, returning to pre-bacterial challenge levels at 120 min (Fig. 2). The recovery of culturable bacteria from haemolymph circulation decreased significantly with time ($p < 0.01$; one-way ANOVA). Juvenile lobsters were capable of rapidly rendering the injected dose of *V. anguillarum* non-culturable, with more than 83% of the theoretical challenge dose of bacteria (~$1.7 \times 10^5$ *V. anguillarum* ml$^{-1}$ haemolymph) inactivated within the first 10 min following injection. By 20 min, less than 5% of the injected *V. anguillarum* was recovered and thereafter bacterial numbers declined gradually to $2343 \pm 798$ CFU ml$^{-1}$ haemolymph at 120 min.

### 3.2. Impact of chronic exposure to pH and temperature on circulating haemocyte numbers

Pre-challenge (baseline) average circulating haemocyte numbers of lobsters in the four treatment groups ranged between $6 – 8 \times 10^6$ THC ml$^{-1}$ haemolymph (Fig. 3). Temperature and pH had no significant effect on baseline circulating haemocyte numbers (Fig. 3; two-way ANOVA, $p > 0.05$) and there was no statistically significant interaction between the main effects ($P = 0.662$). Conversely, a significant interactive effect was recorded between temperature and pH for the post-challenge THC data (two-way ANOVA, $p = 0.0025$). Following bacterial challenge (20 min post-injection), circulating haemocyte numbers of lobster exposed to hypercapnia/low temperature increased significantly above pre-injection levels (paired *t*-test, $p = 0.049$) and the THC of lobsters in this group was significantly higher than THC of lobster in the hypercapnia/high temperature treatment (two-way ANOVA, $p = 0.003$). However, since a significant interactive effect (two-way ANOVA, $p = 0.0025$) was recorded between temperature and pH for all treatments post-challenge, the main factor contributing towards the significant increase in haemocyte density in the latter treatment could not be determined. On average, circulating haemocyte numbers increased by between 49 and 60% in three of the treatments (hypercapnic/low temp, normocapnic/high temp, normocapnic/low temp), when compared to baseline levels, except for lobsters chronically exposed to a combination of hypercapnia and high temperature; where a 7% reduction in THC ml$^{-1}$ haemolymph was recorded (Fig. 4). These latter changes were however not significant (two-way ANOVA, $p > 0.05$).

### 3.3. Impact of chronic exposure to pH and temperature on recovery of culturable V. anguillarum

Twenty-four hours prior to the bacterial challenge, haemolymph obtained from the WCRL that had been plated on TSA without antibiotic supplementation showed no (94% of lobsters) or low (6% of lobsters) numbers of circulating bacteria per ml$^{-1}$ haemolymph. One lobster from the hypercapnic, low temperature treatment and one from the hypercapnic, high temperature treatment group had approximately 1,000 bacteria ml$^{-1}$ hemolymph. However, measured immune parameters (THC ml$^{-1}$ and bacterial clearance ability) were not different ($P < 0.05$) when compared with data collected from other lobsters within their respective treatments. As a consequence, these animals were not omitted from further analysis.

Twenty minutes post-injection, lobsters had reduced the number of injected bacteria from the calculated circulating dose (approximately $1.7 \times 10^5$ bacteria ml$^{-1}$ haemolymph) to a range of ~7,600 to 11 200 CFU ml$^{-1}$ haemolymph, a reduction of more than 93% (Fig. 5). The ability of lobsters to render an injected dose of *V. anguillarum* non-culturable was depended on the levels of both sea water temperature and pH, with a significant interactive effect (two-way ANOVA, $p = 0.039$) recorded between the main effects. The ability of lobsters to render bacteria non-culturable in each of the treatment groups ranked as follows: Hypercapnia/low temperature > normocapnia/high temperature > normocapnia/low temperature > hypercapnia/high temperature, however no significant differences in bacterial clearance between treatment groups were observed (two-way ANOVA, $p > 0.05$ for all factors) (Fig. 5).

### 3.4. Moult stage

Lobsters utilized in the immune challenge from each experimental group were in a similar range of moult stages. These stages included the inter-moult stage C and pre-moult stage D, with the stages/sub-stages varying as follows within each treatment: In the hypercapnic/low temperature treatment, six lobsters were at stage $D_0$, one at $D_1$, and one at $D_1''$. In the normocapnic/high temperature group, four lobsters were at stage $D_0$, and four at $D_1$. In the normocapnic/low temperature treatment, three lobsters were at stage C, four at $D_0$, one at $D_1$. In the hypercapnic/high temperature treatment, one lobster was at stage C, three at $D_0$, three at $D_1$ and one at $D_1''$. The juvenile WCRLs used in the bacterial challenge trial provided a good representation of the population within each treatment with regards to moult stage, where the majority of the lobsters were shown to be in moult stages C and D (Fig. 6); with lobsters in pre-moult assigned predominantly to sub-stages $D_0$ and $D_1$ (data not shown). The corresponding THCs for lobsters from each experimental group assigned to moult stages C and D were also shown to be similar, with no statistically significant difference recorded between treatments for each moult stage (one-way ANOVA, $p > 0.05$) or between moult stages within a treatment (paired *t*-test, $p > 0.05$) (Fig. 6).

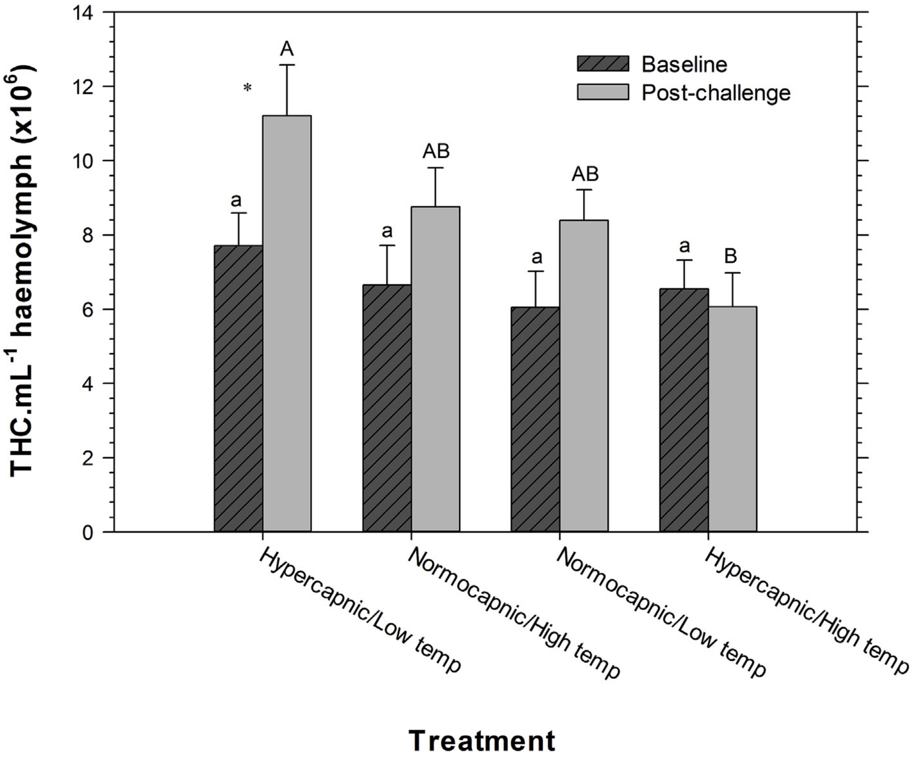

**Fig. 3.** Mean baseline total haemocyte count (THC) per mL haemolymph in juvenile *Jasus lalandii* compared to post-challenge THC for each respective treatment. Values are mean ± S.E. (n = 8). Different letters indicate a significant difference ($p < 0.05$) between treatments pre- and post-challenge with *Vibrio anguillarum* (Holm-Sidak multiple comparison). An asterisk denotes a significant difference (paired *t*-test, $P < 0.05$) between pre-and post-challenge THC data for a specific treatment.

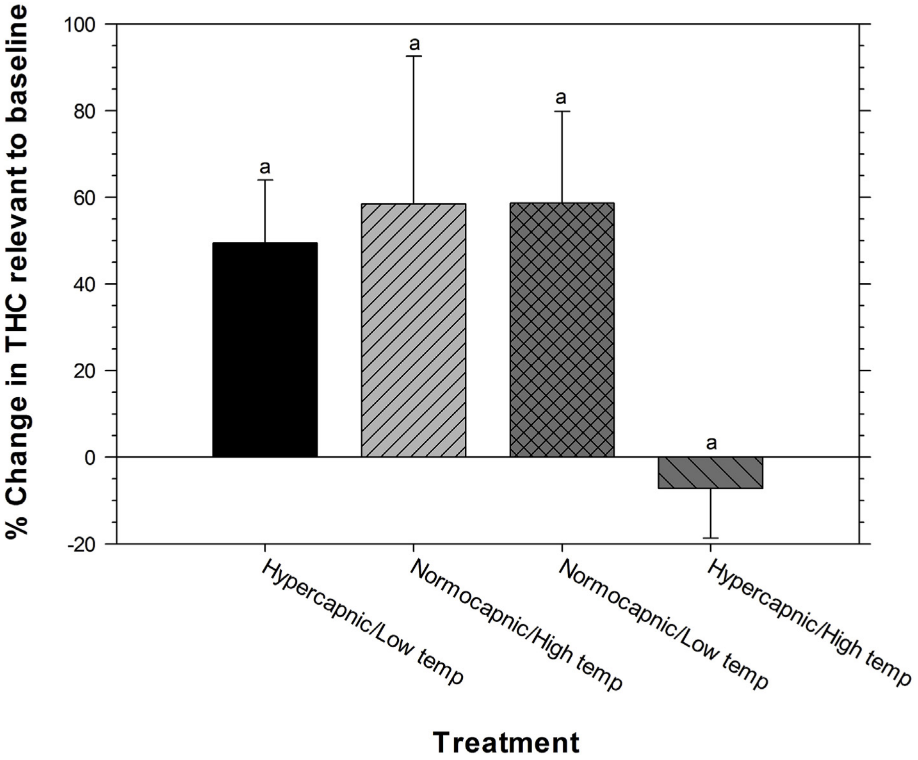

**Fig. 4.** Mean percentage change in the total haemocyte count (THC) of *Jasus lalandii* in each treatment at 20 min post-challenge with a dose of *Vibrio anguillarum*. Values are mean ± S.E. (n = 8). Different letters indicate a significant difference ($p < 0.05$) between treatments (Holm-Sidak multiple comparison).

## 4. Discussion

The results of this study revealed, for the first time, that 1) juvenile *J. lalandii* are very efficient in clearing (inactivating) an injected dose of bacteria from their haemolymph circulation and that this clearance efficiency is independent of treatment; and 2) that chronic exposure to a combination of high temperature and hypercapnia led to a significantly lowered circulating total haemocyte count (THC) in juvenile WCRL, post-bacterial challenge, compared with lobsters exposed to low temperature and hypercapnia.

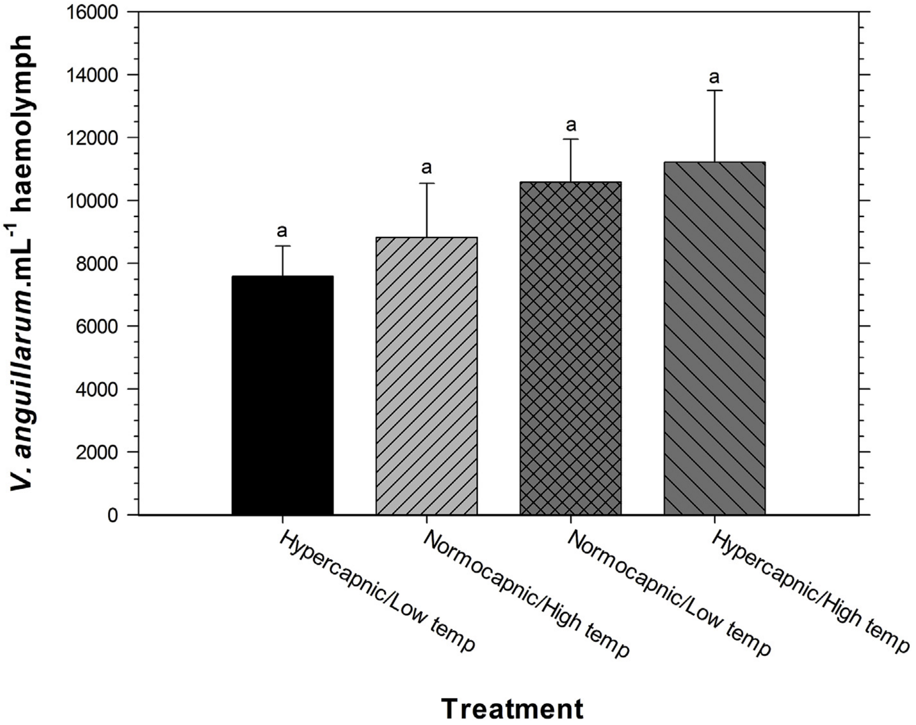

**Fig. 5.** Mean number of culturable bacteria remaining in haemolymph circulation of Jasus lalandii in each treatment at 20 min post-challenge with *Vibrio anguillarum*. Values are mean ± S.E. (n = 8). Different letters indicate a significant difference ($p < 0.05$) between treatments (Holm-Sidak multiple comparison).

### 4.1. *Pre-trial evaluation of immune response*

The initial, pre-trial investigation of this study into two aspects of the innate immune response of juvenile WCRL provided some important insight on circulating haemocyte numbers and the overall immune response (ability to inactivate an injected dose of bacteria) following chronic exposure to hypercapnia and elevated seawater temperature. Total haemocyte counts vary amongst and within species due to a number of factors, such as moult stage, diet and temperature [33,57]. The basal circulating THCs determined in this study (Fig. 2) are in a similar range to those reported for other lobster species, including the Western rock lobster (*Panulirus cygnus*) and the Mediterranean spiny lobster (*Palinurus elephas*); where counts of $\sim 12 \times 10^6$ and $10 \times 10^6$ cells ml$^{-1}$, respectively, have been reported [58,59]. Shortly

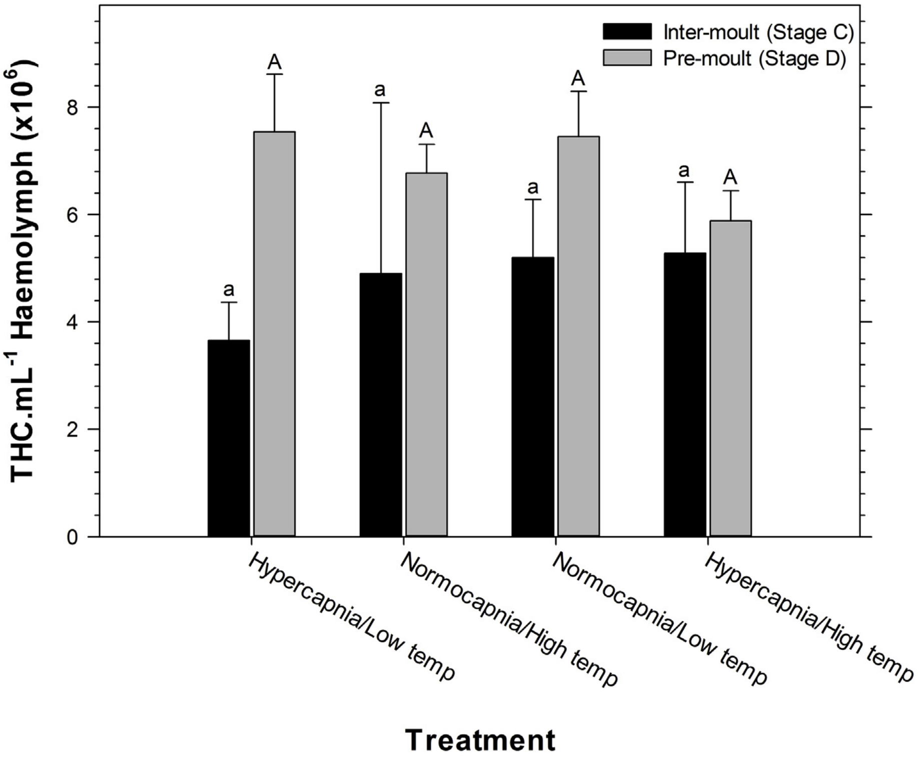

**Fig. 6.** Mean total haemocyte count (THC) of *Jasus lalandii* in moult stages C (inter-moult) and D (pre-moult) within each treatment (entire chronic exposure cohort, n = 97 lobsters). Values are mean ± SE. Different letters indicate a significant difference ($p < 0.05$) in THC between treatments (Holm-Sidak multiple comparison) for lobster assigned to moult stages C or D.

after administering the challenge dose of *V. anguillarum* in the present study, circulating haemocyte concentrations initially increased, until approximately 15 min post-challenge, and then decreased continuously thereafter for the remainder of the trial. A similar response has been reported for the American lobster (*Homarus. americanus*) and Norwegian lobster (*Nephrops norvegicus*) following a bacterial challenge [60,61]. The initial increase in the number of circulating THCs and subsequent decrease thereafter have been attributed to the rapid migration of haemocytes to the site of injection followed by adherence of haemocytes to bacteria, ensuring rapid bacterial inactivation/degradation and wound sealing [26]; as observed in the Ridgeback prawn (*Sicyonia ingentis*) and American lobster (*H. americanus*, [62]. The loose clumps of bacteria and haemocytes then get lodged in narrow spaces, especially in the fine vasculature of the gills [53,62]; Martin et al., 1993), after which clumps are melanised and eliminated when the exoskeleton is shed [63]. Another site in which bacteria are accumulated and then degraded is the hepatopancreas [64].

The rapid decrease in culturable bacteria from circulation is attributed to the inactivation, degradation and/or transport of bacterial cells to other tissue compartments, as reported for Atlantic blue crab *Callinectes sapidus* [65] as well as the penaeid shrimp (*S. ingentis*), where the bacteria *Bacillus ceres* and *Aerococcus viridans* (challenge dose $1 \times 10^6\,\text{ml}^{-1}$) were completely removed from circulation within 10 min [66].

### 4.2. Effect of treatment on immune response

The effect(s) of hypercapnia and elevated temperature on innate immunity has been reported for several crustaceans, but these environmental stressors have mostly been investigated in isolation. Increased temperature has led to contrasting effects on circulating THCs, with some studies reporting an increase in THC, for example in the sand fiddler crab (*Uca pugilator*), mole crab (*Enierita asiatica*), green crab (*Carcinus maenas*) and giant freshwater prawn (*Macrobrachium rosenbergii,* [30,67–69], whereas others have noted a decrease in THC [70,71]. Differences recorded between species, and within species, have been attributed to differences in natural temperature ranges [30] and acclimation conditions [71]. While there is a far greater body of literature on the impacts of temperature on innate immunity, the effects of hypercapnia on the immune response of marine crustaceans have often been simulated in combination with hypoxia [39,42,45,51], making it difficult to determine the effects of hypercapnia alone on the immune system. Of the limited studies reporting on the effects of hypercapnia alone, various changes in the immune system have been observed, including decreased haemocyte functionality [72,73] and cell numbers, reduced bacteriolytic- and antibacterial activity, and increased phenoloxidase activity [74].

In contrast to the above-mentioned reports, the present study investigated the combined impact of chronic exposure to two environmental factors on the innate immune response of lobster. We demonstrated that, prior to bacterial challenge, chronic exposure to a combination of hypercapnia and elevated seawater temperature had no significant impact on circulating haemocyte numbers in the WCRL. Similar findings were reported by Hernroth et al. [22]; who demonstrated that exposure of Norwegian lobster (*N. norvegicus*) to a range of temperatures, ranging from 5 to 18 °C, for a four-month period did not affect circulating THCs. The initial THCs observed in the lobsters from their respective treatment groups were however lower than THCs recorded for pre-trial lobsters in this study (Figs. 2 and 3). This difference may be caused by a combination of different moult stages and diet, as both are known to affect THCs [33,57].

When *J. lalandii* is exposed to decreased seawater pH, there is an associated decrease in haemolymph pH [13]. This is the results of the increased $pCO_2$ of the seawater and the need to maintain a gradient between the internal and external environmental $pCO_2$, thereby ensuring $CO_2$ removal [13]. In organisms that are not able to maintain extracellular pH during hypercapnic exposure, such as the sea star (*Asterias rubens*), green sea urchin (*Strongylocentrotus droebachiensis*), and the Norway lobster (*N. norvegicus*), the innate immune response is depressed [22,73,75]. Dupont and Thorndyke [75] suggested a relationship between cellular immune response and extracellular pH, because in both species of polar echinoderm tested (*S. droebachiensis and L. polaris*), acidosis of the coelomic fluid was associated with 1) an increased number of coelomocytes in both species and 2) a reduction in vibratile cells in *S. droebachiensis*. Several days prior to the bacterial challenge in the present study, haemolymph acid-base parameters were assessed and we demonstrated that lobsters are capable of maintaining haemolyph pH within a relatively narrow range (pH 7.72–7.77) across treatments following chronic (48 weeks) exposure to hypercapnia and elevated seawater temperature [13]. We also showed that by 20 min post-injection of bacteria, the number of culturable bacteria remaining in haemolymph circulation was similar for all lobster, irrespective of treatment, and similar to bacterial numbers recorded for lobsters in the pre-trial investigation (Figs. 2 and 5). Based on this observation, we propose that the ability of *J. lalandii* to preserve its *in vivo* pH homeostasis ensures efficient and rapid clearance of *V. anguillarum* (Fig. 5). A reduction in phenol oxidase activity, a key pathway in the innate immune defence of crustaceans, was observed in Atlantic blue crab (*C. sapidus*) following exposure to low pH (Tanner et al., 2006). A reduction in the production of reactive oxygen species by oyster (*Crassostrea virginica*) haemocytes was also observed following exposure to low pH. The findings of these studies support the notion that low pH can have a detrimental impact on the innate immunity of invertebrates, especially following acute exposure to pH and when homeostasis is not maintained.

After lobsters were challenged with a known dose of *V. anguillarum,* the number of circulating haemocytes increased slightly (but not significantly) above pre-challenge levels in three of the treatments (hypercapnic/low temp, normocapnic/high temp, normocapnic/low temp) following a similar trend to that observed for lobsters in the pre-trial experiment of this study. Conversely, WCRL in the hypercapnic, high temperature treatment had a decreased THC relative to pre-challenge levels (Fig. 3). There are several factors which may have contributed to the observed result. 1) Growth of lobsters in this treatment group was shown to be greater than lobsters in the other treatments (Knapp et al., *in press*) and it is postulated that this enhanced growth may be the result of an increase in metabolism. 2) Acid-base regulation for such an extended period (48 weeks) would be energetically more expensive for those lobsters subject to hypercapnic conditions [76]. 3) Impaired aerobic scope has been demonstrated in crustaceans following bacterial challenge and is the result of rapid accumulation of haemocyte-bacterial aggregates (nodules) in the fine vasculature of the gills within a short period (10 min) [62,77]. In the Atlantic blue crab a 43% reduction in oxygen uptake was observed [77] following bacterial challenge. It is postulated that the reduced oxygen uptake can lead to an increased energy cost associated with maintaining haemolymph $pO_2$ and a subsequent impairment of behavioural and other physiological processes, including immune defence [78]. As a consequence of the factors mentioned above and the additional role of haemocytes in nutrient transport [79,80], it is therefore hypothesised that the decreased post-challenge THC in the hypercapnic, high temperature treatment is due to the migration of haemocytes from the haemolymph to tissues to fuel the above mentioned ongoing processes [80,81], thereby reducing the number of haemocytes available to mount an immune response.

## 5. Conclusion

To the best of our knowledge, this is the first study whereby immune parameters of *J. lalandii* were tested after chronic exposure to a combination of environmental stressors. The study revealed that this species is very efficient at rendering an injected dose of bacteria non-culturable, irrespective of the treatment tested. Although THC was lower in the hypercapnic/high temperature treatment compared with the hypercapnic/low temperature treatment, this did not significantly affect the ability of *J. lalandii* to defend itself against bacterial infection. This study however, needs to be followed up with an assessment of the impact of acute exposure to these variables (independently and in combination), especially considering the increased incidents of red tides/low oxygen events along the South African coastline where this species is abundant.

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