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The impact of holding stressors on the immune function and haemolymph biochemistry of Southern Rock Lobsters (*Jasus edwardsii*)

Ryan D. Day*, Quinn P. Fitzgibbon, Caleb Gardner

Fisheries and Aquaculture Centre, Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, Tasmania, 7001, Australia

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ABSTRACT

Lobsters are fished world-wide due to their status as a high value, luxury seafood. A large proportion of the product is sold via live export, with lobsters subject to a range of stressors during holding post-capture. Improving the current understanding of the immune response to these stressors assists in improving efficiency and reducing loss in the chain between capture and consumption. In this study, the immune status of four treatment groups of Southern Rock Lobster (*Jasus edwardsii*) were studied: controls recently landed from a fishing boat, lobsters displaying advanced shell necrosis, lobsters in an unexplained moribund state and lobsters held in a processing facility for 10 weeks in standard conditions (i.e. high density, fasted). A total of 15 immune parameters and 19 haemolymph biochemical parameters were assayed. Phenoloxidase activity was only sporadically observed in haemocyte lysate and was consistently observed at a low level in the plasma with no difference between treatments for either. Haemocyte lysate prophenoloxidase activity was detected in most individuals, with no differences found between treatments. Prophenoloxidase in the plasma showed the highest level of activity, with the shell necrosis treatment demonstrating an elevated activity level relative to the other three treatments. Cell viability was not affected in any treatment. Lobsters with shell necrosis had a reduced capacity for phagocytosis, a significantly higher total haemocyte count, fewer hyalinocytes and more granulocytes and semigranulocytes. Fasted lobsters showed an opposite shift, with significantly more hyalinocytes compared to the other treatments and very few granulocytes and semigranulocytes. The balance of a range electrolytes, minerals metabolites and enzymes were affected in shell necrosis and fasted treatments, raising them as potential markers for immunocompromised lobsters. Multivariate analysis of all assayed parameters showed that all individuals in the necrosis treatment showed a similar, distinct immune response and that the fasted treatment, along with one control and one moribund individual, showed a separate intermediate response. The remainder of the control and moribund lobsters demonstrated a distinct “non-response” in comparison. These results offer a characterisation of the physiological response to common challenges during post-capture holding of rock lobsters, demonstrating the differential response to pathogenic bacterial infection, long term fasting, non-specific moribundity and the stress of capture and transport.

1. Introduction

The crustacean immune system is a nonspecific, innate system, with responses including clotting, recognition of foreign particles, phagocytosis, melanisation and cytotoxicity mediated by the functions of circulating haemocytes. Three main crustacean haemocyte cell types are widely recognised: hyalinocytes, semigranulocytes and granulocytes [1]. Hyaline cells are small and spindle shaped, lacking cytoplasmic granules and are considered the primary phagocytic cell type [2]. To deal with foreign particles that are too large to be phagocytosed, the crustacean immune system has a chemical response – the phenoloxidase (PO) system. This system is mediated by the granulocytes, which contain a large number of cytoplasmic granules that contain prophenoloxidase (proPO), the zymogen precursor to PO, and the semigranulocytes, which contain a variable number of small cytoplasmic granules containing PO activating enzymes. When the PO system is initiated through the detection of lipopolysaccharides, β-1,3-glucans or peptidoglycans [3], the granulocytes and semigranulocytes degranulate and exocytose proPO and the activating enzymes, respectively [4]. Factors released as a part of the activated PO system function

Abbreviations: SRL, Southern rock lobster; PO, phenoloxidase; ProPO, prophenoloxidase; HLS, haemocyte lysate supernatant; CL, carapace length (mm); CTL, control treatment; NEC, necrosis treatment; MOR, moribund treatment; FAST, fasted treatment

* Corresponding author.
E-mail address: ryan.day@utas.edu.au (R.D. Day).

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to aggregate semigranulocytes onto the foreign cell, creating a nodule and encapsulating the cell, and then it is destroyed through the generation of cytotoxic quinones and melanin [2].

Understanding the immune function of crustaceans can be an important tool for enhancing the value of wild catch fisheries. Crustaceans are subjected to a range of stressors as they pass through the supply chain, including injury or limb loss, emersion, changes in water quality and fasting during transport and holding. Of particular interest are rock lobster (Palinuridae) fisheries, as these are considered a high value luxury seafood commodity and are fished throughout the tropical and temperate seas around the world, with an annual global value of over US$2 billion [5]. Much of this value is reliant on the supply of live product to overseas markets. Australia has four rock lobster fisheries, the Western Rock Lobster (*Panulirus cygnus*), the Tropical Rock Lobster (*Panulirus ornatus*), the Eastern Rock Lobster (*Sagmariasus verreauxi*) and the Southern Rock Lobster (SRL; *Jasus edwardsii*). The latter industry is important in the Australian states of South Australia, Tasmania and Victoria, comprising 28%, 11% and 27% of those states' total gross fishery revenue, for a combined US$160 million from an annual catch of 3000 tonnes [6]. The annual total allowable catch for the industry is strictly regulated via a quota system which is intended to maximise rents from the fishery (seen as payments from fishers to quota owners) rather than tonnage of catch [7]. Rents (and economic yield) currently equate to more than 60% of the revenue, which is achieved by maintaining small catches, high catch rates, efficient harvesting and high market price [8], all of which is enhanced by reducing mortalities. Fewer mortalities effectively raises the productivity of the system which means that harvesting is more efficient. Mortalities in the supply chain also affect price because the SRL industry is reliant on live export to sell over 75% of the annual catch, predominantly to China [9]. In this market the presence of mortalities has greater impact than the direct loss of product because of the effect of reputational damage and also by limiting the ability of processors to hold product to time peaks in market demand.

To improve the understanding of how catching and processing affects the immune function of SRL held in processing facilities, the present study compared a range of immune and biochemical parameters between lobsters that experienced one of several common stresses. First, a cohort of lobsters recently caught, transferred from a fishing boat and processed through a facility served as a control to which other treatments could be compared. Having this control group go through the process of transport, grading and purging at the facility was important to reflect the treatment that all lobsters entering into a processing facility will have undergone. Second, a cohort of lobsters showing advanced shell necrosis was analysed to provide a positive control. Shell necrosis is a common bacterial infection that presents initially as black (e.g. melanised) pitting of the shell surface that can progress to large lesions penetrating into the body [10]. Third, a cohort of lobsters held in standard conditions at a processing facility for 10 weeks was evaluated to determine the effect of long-term fasting on immune function. Although 10 weeks of holding represented an extreme situation within current industry practices, SRL have shown considerable ability to survive fasting, with one study reporting the loss of 35% of stock following 30 weeks of fasting [11]. Finally, a cohort of moribund lobsters that appeared healthy when originally processed into the facility and did not demonstrate any outward signs that explained their decline in health were analysed. To compare these treatments, a range of haemolymph parameters were assessed including the pH and refractive index, phenoloxidase and prophenoloxidase activities in both the haemocytic and plasma fractions of the haemolymph, haemocyte viability, phagocytic capacity, total and differential haemocyte counts and protein concentrations of the haemolymph. In addition, haemolymph biochemistry panels previously used to assess stress in SRL [12,13] were performed, quantifying concentrations of electrolytes and metabolites. Comparing these parameters between lobsters experiencing known and unknown stressors was done to provide a more thorough understanding of immune function in SRL, particularly in regards to the immunological stresses they face following capture and holding.

2. Methods

2.1. Animals and experimental conditions

All laboratory work was conducted at the University of Tasmania's Institute for Marine and Antarctic Studies (IMAS) site at Taroona, Tasmania. For lobsters obtained from processing facilities, lobsters were packaged at the facility according to industry standard procedures into a polystyrene box and covered with wood wool and transported to IMAS. Upon arrival, lobsters were placed into a tank of seawater obtained from the flowthrough system (temperature *ca.* 16 °C) and allowed to acclimate for 1 h prior to sampling with a battery operated air pump providing aeration (Aqua One 250C, 150 L/h). Following sampling, lobsters were euthanised via immersion in an ice/seawater slurry.

Four treatments of lobsters were compared in this study: control (CTL), shell necrosis (NEC), fasted (FAST) and moribund (MOR). CTL lobsters (n = 5 males, 113 ± 1 mm mean carapace length (CL) ± SEM and 695 ± 23 g mean mass ± SEM) were obtained from a local lobster processing facility 1 day after they had been landed from a fishing vessel to represent the immune function of lobsters in the wild with a brief acclimation time to recover from the stress of capture and transport. NEC lobsters (n = 5 males, 116 ± 2 mm CL and 789.9 ± 26.7 g mass) displayed advanced shell necrosis following 6 months holding in tanks at IMAS and were used to serve as a positive control to analyse the response of lobsters suffering a challenge to the immune system. FAST lobsters (n = 5 males, 111 ± 2 mm CL and 662.7 ± 26.3 g mass) were obtained from a local processing facility following 10 weeks of holding in conditions standard to the industry: 12 °C water temperature, contained crates at high density (approximately 20 lobsters per m^2) and not fed for the duration of holding. This treatment was included to evaluate the impact of long term holding and fasting on the immune function of lobsters. Finally, MOR lobsters (n = 3 males, 112 ± 0.3 mm CL and 661.3 ± 11.5 g mass) were opportunistically collected following two weeks of holding at the late stages of a mortality event at a local processor to characterise whether moribund lobsters demonstrated any immune response that may be indicative of a pathogenic causative agent. These lobsters had no outward signs of injury or infection that would suggest a cause for their state and had previously been assessed with a high vitality score at landing to the processing facility. CTL and MOR lobsters were sampled in August and September 2017 and were identified as intermoult based on the lack of epibiotic growth on their carapaces indicating they had previously moulted. NEC lobsters were known to have not moulted based on observations during their holding at IMAS and thus were considered to be approaching pre-moult status. FAST lobsters were sampled in May 2018 and were considered to be approaching pre-moult status based moult timing in *J. edwardsii* [14].

2.2. Collection of haemolymph

Lobsters were removed from their holding tank, placed ventrum up on a bench and haemolymph was drawn from the sinus at the base of the posterior walking leg using a pre-chilled 1 ml syringe fitted with a 22 ga needle. A sample of haemolymph was measured for pH using a handheld pH probe (Testo, 205) and refractive index (Brix) using a digital refractometer zeroed with distilled water (Hanna Instruments, HI96801). Haemolymph was aliquoted at a 1:1 ratio into pre-chilled citrate-EDTA anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6; [15,16]) for the phenoloxidase, viability and phagocytosis assays, at a 1:1 ratio into pre-chilled *N*-ethylmaleimide anticoagulant (NEM; 0.2 M *N*-ethylmaleimide

in 0.45 M NaCl [16]) or at a 1:2 ratio into Lillie's formal calcium (1.3 M formalin, 126 mM calcium acetate) for total and differential cell counts.

2.3. Phenoloxidase and prophenoloxidase assay

Spectrophotometric assays of PO (EC 1.14.18.1) and proPO were performed on haemolymph lysate and plasma following the microplate method described by Hernández-López [17] and Perdomo-Morales et al. [16] by measuring the formation of dopachrome through the reaction of phenoloxidase with L-dihydroxyphenylalanine (L-DOPA). The PO assay was optimised for haemolymph and plasma volume, substrate concentration and duration of steady-state kinetic reaction. The Total proPO (PO + proPO) assay was optimised for haemolymph and plasma volume, substrate concentration, trypsin concentration for activation, trypsin activation incubation time and duration of steady-state kinetic reaction. All assays were performed in triplicate with sodium cacodylate buffer (10 mM sodium cacodylate, 450 mM NaCl, 10 mM $CaCl_2$, 26 mM $MgCl_2$, pH 7.8) added to bring to total reaction volume in the well to 200 µl. Calculation of proPO activity was determined for HLS and plasma by subtracting PO activity from Total PO activity for each fraction.

Haemocyte lysate was prepared by first centrifuging the haemolymph suspension in citrate-EDTA at 800 × *g* at 4 °C for 10 min. The supernatant was discarded and the cell pellet was washed twice with sodium citrate-EDTA. The cell pellet was then resuspended using 0.45 M NaCl, pH 7.8 and transferred into a glass Dounce tissue grinder that had been chilled on ice. The homogenate was then transferred to a microfuge tube and centrifuged at 10,000 × *g* at 4 °C for 10 min. The resulting haemocyte lysate was removed from the cell pellet and use for PO and proPO assays.

Haemolymph plasma was prepared as described by Perdomo-Morales et al. [16], with NEM used as an anticoagulant to avoid isoelectric precipitation of proteins in the plasma. After collection, the haemolymph-NEM suspension was centrifuged at 800 × *g* at 4 °C for 10 min and the supernatant removed from the cell pellet for use in PO and proPO assays.

For the PO assays, 50 µl haemolymph lysate or 25 µl plasma were reacted with 15 mM L-DOPA (concentration in well) and the formation of dopachrome was determined by reading optical density at 492 nm absorbance for 20 min. For proPO assays, 50 µl haemolymph lysate and 25 µl plasma were activated using an in-well concentration of 0.1% trypsin, with the reaction incubated at room temperature for 20 min. Then, L-DOPA was added at a concentration in the well of 15 mM and the reaction was read at 492 nm for 20 min. One unit of PO activity (U) was expressed as the increase of 0.001 in optical density at 492 nm per minute.

2.4. Cell viability

Cell viability was determined using a Live/Dead stain kit (Molecular Probes, Inc.) containing ethidium homodimer-1 (EthD-1) and calcein-AM dyes to identify dead and live cells, respectively. Eth-D1 is capable of penetrating the compromised cell membranes of dead cells to bind to nucleic acids and fluorescing red (> 600 nm), whereas live cells exclude the dye and no fluorescence is produced. Extracellular calcein-AM does not fluoresce, but fluoresces green (530 nm) when it permeates cell membranes and is hydrolysed within the cell. The assay was optimised for dye concentration to ensure intense EthD-1 fluorescence and minimal calcein-AM fluorescence in cells killed with 70% methanol and minimal EthD-1 fluorescence and intense calcein-AM fluorescence in a sample containing live cells. Incubation time was then optimised to ensure haemocytes had an adequate amount of time to take up the dyes.

Haemolymph diluted 1:1 in citrate-EDTA was centrifuged at 800 × *g* at 4 °C for 10 min. The plasma supernatant was removed from the cell pellet and discarded, then the cell pellet was resuspended in an equivalent volume of citrate-EDTA buffer. The cell suspension was plated in a black-walled microplate in triplicate and 50 µl each of 28 µM EthD-1 and 20 µM calcein-AM were added. The plate was incubated in the dark for 60 min. A subsample of the cell suspension was incubated with 70% methanol for 30 min to kill the cells, then this sample was plated and incubated in the same manner. Plates were read for fluorescence at 530/645 nm for EthD-1 and 485/530 for calcein AM. The dead cell sample was used as a known sample against which the percentage of dead cells could be quantified.

2.5. Phagocytosis

To test the competency of haemocytes, the phagocytic capacity was measured following the method of Owesen et al. [18]. A commercial preparation (BioParticles, MolecularProbes, Inc.) of yeast zymosan particles labelled with fluorescein-5-isothiocyanate (FITC) were diluted in citrate-EDTA buffer with 2 mM sodium azide to a concentration of 1×10^7 particles ml^{-1}. Haemolymph was diluted 1:1 using citrate-EDTA, allowing for 20–50 zymosan particles per haemocyte. Haemocytes and zymosan particles were added to wells of a black walled microplate and incubated in the dark at room temperature for 75 min, after which 100 µl 0.4% trypan blue was added and incubated for a further 10 min to quench extracellular fluorescence. The plate was then read for fluorescence at 485/528 nm. The average relative fluorescence units (RFU) from five measurements per individual and the fluorescence of a zymosan positive control without haemocytes were used to calculate the phagocytic index: $PI\% = [(RFU_{haemocytes}/RFU_{zymosan}) \cdot 100]$.

2.6. Total and differential haemocyte counts

Haemolymph diluted 1:2 in Lillie's formol calcium was used for total haemocyte counts (THC) and differential haemocyte counts (DHC). For THCs, haemocytes were counted using an improved Neubauer counting chamber at 40× magnification. For DHCs, haemolymph smears were prepared on slides, fixed with methanol, stained using May-Grünwald Giemsa and 200 cells per slide were identified as hyalinocytes, semi-granulocytes or granulocytes.

2.7. Protein assays

Protein content of the haemolymph lysate and plasma components used in the phenoloxidase assay and the whole haemolymph in citrate-EDTA anticoagulant used in the phagocytosis assay were determined using the Bradford method [19]. Haemolymph lysate was used undiluted, plasma was diluted by a factor of 1:280 and whole haemolymph was diluted by a factor of 1:400. For each sample, 5 µl was plated, to which 250 µl Bradford reagent (Sigma Aldrich) was added. The plate was incubated at room temperature for 10 min and then read for absorbance at 600 nm. Protein content of these samples was determined against a bovine serum albumin standard curve and expressed as mg ml^{-1}.

2.8. Biochemical analysis

Haemolymph sampling for biochemical analysis has been described previously in reference (Fitzgibbon et al., 2017). Briefly, two 1.5 ml samples collected using pre-chilled syringes and dispensed into centrifuge tubes. They were centrifuged at 10,000 × *g* for 5 min at 4 °C, after which the supernatant was removed using a pipette, transferred to a 2 ml cryotube, frozen and stored in a −80 °C freezer until they were shipped in a dry shipper charged with liquid nitrogen to the Diagnostic Services laboratory at the Atlantic Veterinary College, University of Prince Edward Island, Canada, and analysed using a Cobas c501 automated biochemistry analyzer (Roche Diagnostics Corporation, Indianapolis, IN, USA) for a full blood profile consisting of the electrolytes (mmol L^{-1}) sodium (Na), chloride (Cl), potassium (K),

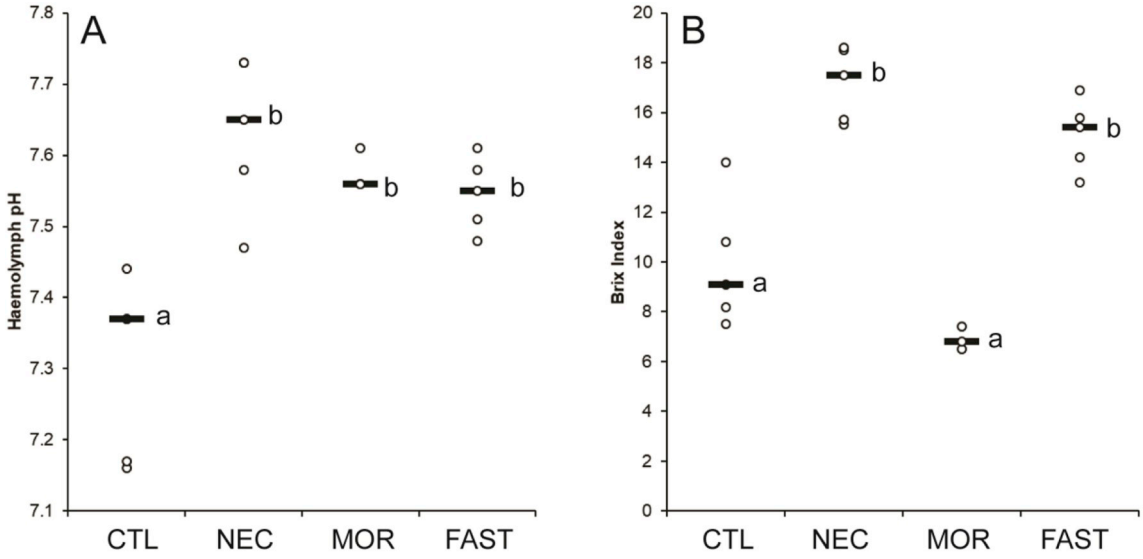

Figure. 1. pH and refractive (Brix) index of lobster haemolymph. A) Univariate scatterplot showing mean and distribution of haemolymph pH of control (CTL), shell necrosis (NEC), moribund (MOR) and fasted (FAST) treatments; B) Univariate scatterplot showing mean and distribution of haemolymph refractive index expressed in % Brix of CTL, NEC, MOR and FAST treatments. Each circle indicates an individual within a treatment with the treatment mean indicated by a horizontal bar. Means sharing a letter are not statistically different.

magnesium (Mg) and bicarbonate (bicarb); minerals (mmol L^{-1}) calcium (Ca) and phosphorus (P); metabolites (mmol L^{-1}) glucose (Gluc), lactate (Lact), cholesterol (Chol), triglyceride (Trig), total protein (TP, in g L^{-1}), urea, and uric acid (Uric, in μmol L^{-1}); enzymes (U L^{-1}) lipase (LIP), amylase (AMY), alanine (ALT) and asparate (AST) aminotransferases, alkaline phosphatase (ALP), sorbital (SDH) and glutamate (GDH) dehydrogenases, and gamma-glutamyl transferase (GGT).

2.9. Statistical analysis

All results from the measurement of haemolymph parameters are presented as univariate scatter plots that show the treatment mean and individual distributions as suggested for low sample size data by Weissgerber et al. [20].

All haemolymph parameters were checked for normality and equality of variance using Shapiro-Wilks tests and Levene's tests, respectively, transformed as needed and then compared using analysis of variance (ANOVA) followed by Tukey HSD post-hoc multiple comparison tests for significant results ($\alpha = 0.05$). All analyses were performed in R v. 3.4.1.

Data were compared using multivariate analysis in Primer-6 with PERMANOVA+. First, data were normalised to account for the varying units and scales of the immune and biochemical parameters comprising the data. A resemblance matrix using Euclidian distance was then used to generate a multidimensional scaling plot (MDS). Complete linkage CLUSTER analysis was performed to determine the relationships between individuals and the similarity between treatments was analysed using ANOSIM.

3. Results & discussion

3.1. Physical and biochemical parameters

The lobsters in each treatment did not vary significantly in carapace length ($F(3,14) = 2.03$, $P = 0.156$), but did in weight ($F(3,14) = 6.08$, $P = 0.007$), as NEC lobsters had a significantly greater weight than CTL, MOR and FAST treatments. This is not surprising, given the long holding time (6 months) prior to sampling for this treatment, in which they were fed a nutrient rich diet of mussels on a regular basis. In comparison, CTL lobsters were freshly caught from the wild, MOR were had been held fasted within the processing facility for 2–3 weeks and FAST were fasted for 10 weeks during holding at the processing facility.

Haemolymph pH (Fig. 1a) in lobsters from the CTL treatment, with a mean of 7.30 ± 0.06, was significantly lower ($F(3,14) = 11.411$, $P < 0.001$) than that of lobsters from the other three treatments, which was 7.55 ± 0.02 in FAST lobsters, 7.57 ± 0.02 in MOR lobsters and 7.63 ± 0.05 in NEC lobsters. In a previous study, the haemolymph pH of healthy *J. edwardsii* maintained in tanks under ideal conditions ranged from 7.62 ± 0.03 (14 days holding) to 7.50 ± 0.03 (120 days holding), indicating that the FAST, MOR and NEC lobster treatments were within normal range and not exhibiting any signs of compromised acid-base balance [12]. The relatively acidic pH of the CTL treatment reflects a well characterised response indicative of a lack of full recovery from the stresses resulting from capture and transport to the processing facility. In previous studies on a range of lobster species, clearance of the built-up lactate that depressed haemolymph pH required *ca.* 96 h of holding after periods of emersion [13,21,22].

Brix index (Fig. 1b) was also significantly different ($F(3,14) = 28.07$, $P < 0.001$), as CTL and MOR lobsters had similar means, whereas NEC and FAST lobsters had values 1.7 and 1.5 times greater than CTL lobsters and 2.2 and 2 times greater than MOR, respectively. The comparatively high Brix values of NEC and FAST lobsters can be explained by an increase in haemolymph protein levels due to their pre-moult status compared to the inter-moult status of the other three treatments [23,24], and offers further support for the superior nutritional condition of the NEC treatment [23,25].

3.2. Phenoloxidase system

Phenoloxidase (PO) and its intermediate products are highly reactive and toxic, requiring tight control of the cascade system that initiates an immune response [26], thus, high levels of PO activity are not generally expected to be found in the haemolymph. Furthermore, in contrast to most other crustaceans, in which PO/proPO activity is located in the haemocytes [2], there is some debate over whether this holds true for rock lobsters, with conflicting reports of activity solely in the plasma [17] or in both the plasmatic and haemocytotic fractions [16]. In the present study, PO was found to be largely absent from the haemolymph lysate (HLS; Fig. 2a) with no detectable PO activity in 72% of the individual lobsters assayed. Specifically, no CTL individuals

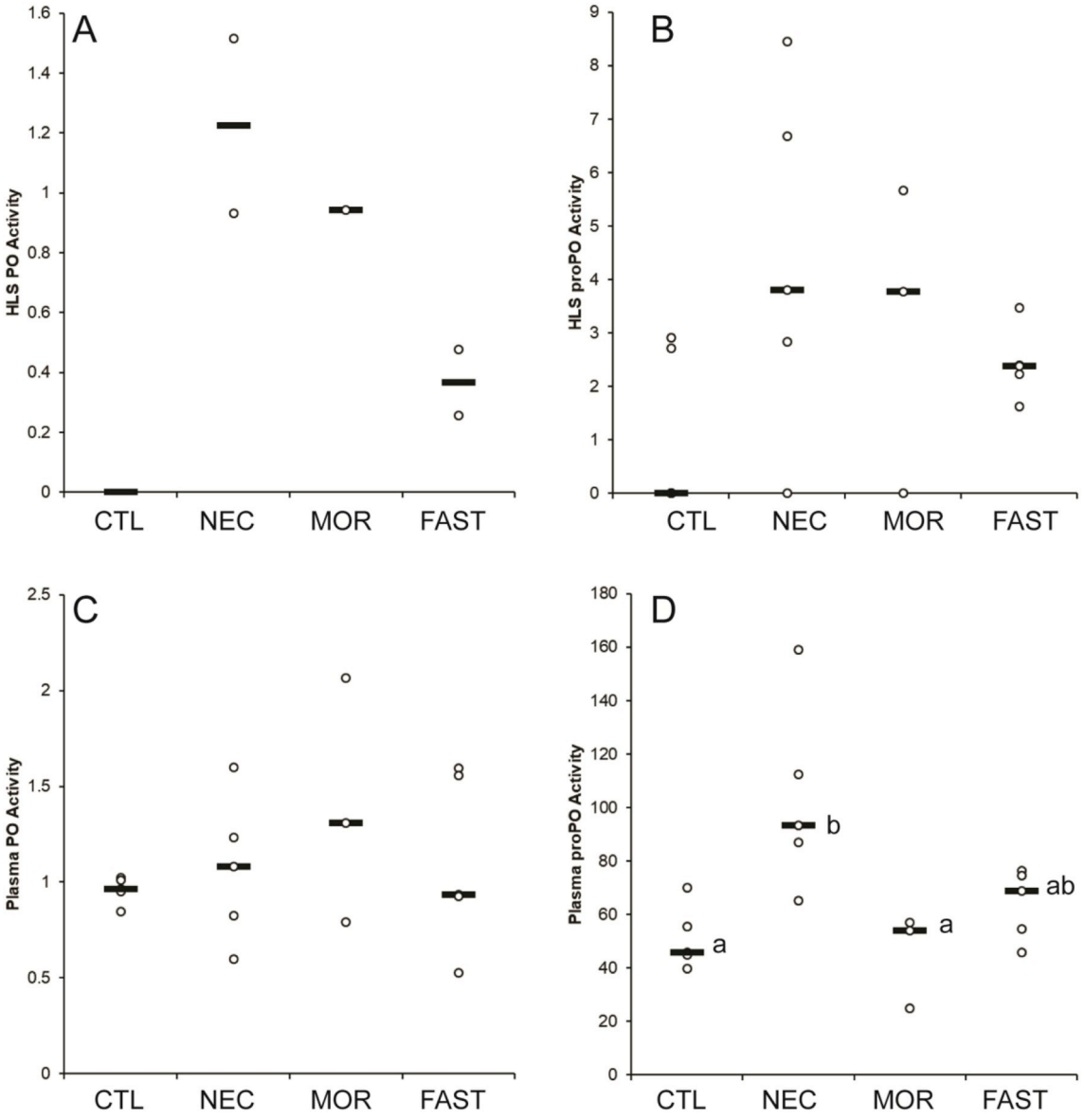

Fig. 2. Phenoloxidase system activity in haemocytic and plasma fractions of lobster haemolymph. A) Phenoloxidase (PO) activity, expressed in U (change in 0.001 optical density min^{-1} at 495 nm), in the haemolymph lysate supernatant (HLS); B) pro-PO activity following trypsin activation in the HLS; C) PO activity in the plasma; and D) pro-PO activity following trypsin activation in the plasma of control (CTL), necrosis (NEC), moribund (MOR) and fasted (FAST) treatments. Each circle indicates an individual within a treatment with the treatment mean indicated by a horizontal bar. Means sharing a letter are not statistically different.

displayed activity, one MOR lobster showed modest activity and two individuals each from NEC and FAST treatments showed detectible activity. Due to the frequency of no detected activity, these data were not statistically analysed. Trypsin activated proPO activity was more prevalent in the HLS (Fig. 2b), with only 28% of the lobsters assayed in this study showing no detectible activity, including three CTL lobsters, one NEC lobster and one MOR lobster. No statistical difference in activity level was found between treatments ($F(3,14) = 1.76$, $P = 0.20$).

PO was consistently present in the plasma fraction (Fig. 2c) of the haemolymph at a similar, modest level in all four treatments ($F(3,14) = 0.72$, $P = 0.55$), which is again not surprising due to its reactivity. This detectible activity was likely the result of auto-activation during the assay due to the sensitivity of the proPO system mediating cells [27]. ProPO had the strongest activity in the plasma, with activities that differed significantly ($F(3,14) = 6.59$, $P = 0.005$), as NEC lobsters had more than double the activity of both CTL and MOR lobsters, demonstrating an upregulation of the cells that mediate the proPO system in response to the necrosis (see section 3.4). ProPO activity comprised 93–100% of the Total PO (PO + proPO) activity in the HLS by treatment and 96–99% of Total PO activity in the plasma by treatment, thus Total PO results (not shown) were similar to that of plasma proPO, with no difference between treatments in the HLS ($F(3,14) = 1.55$, $P = 0.25$) and a significantly higher activity in NEC lobsters compared to both CTL and MOR lobsters ($F(3,14) = 6.6$, $P = 0.0053$).

PO/proPO expression was not as uniform as previously suggested, with activity either in the plasma or in the haemocytes. Rather, activity suggested a facultative function, in which HLS from lobsters facing a challenge to the immune system demonstrated PO/proPO activity whereas healthy lobsters from the CTL treatment did not. Further study is needed, as *J. edwardsii* has previously been reported to demonstrate proPO activity in both healthy and tail-fan necrosis infected treatments, with the former demonstrating an activity level 50% greater than the latter, which the authors attributed to the TFN infected lobsters being immunocompromised [28]. This runs counter to the findings presented here, both with regard to the presence of proPO in healthy SRL and

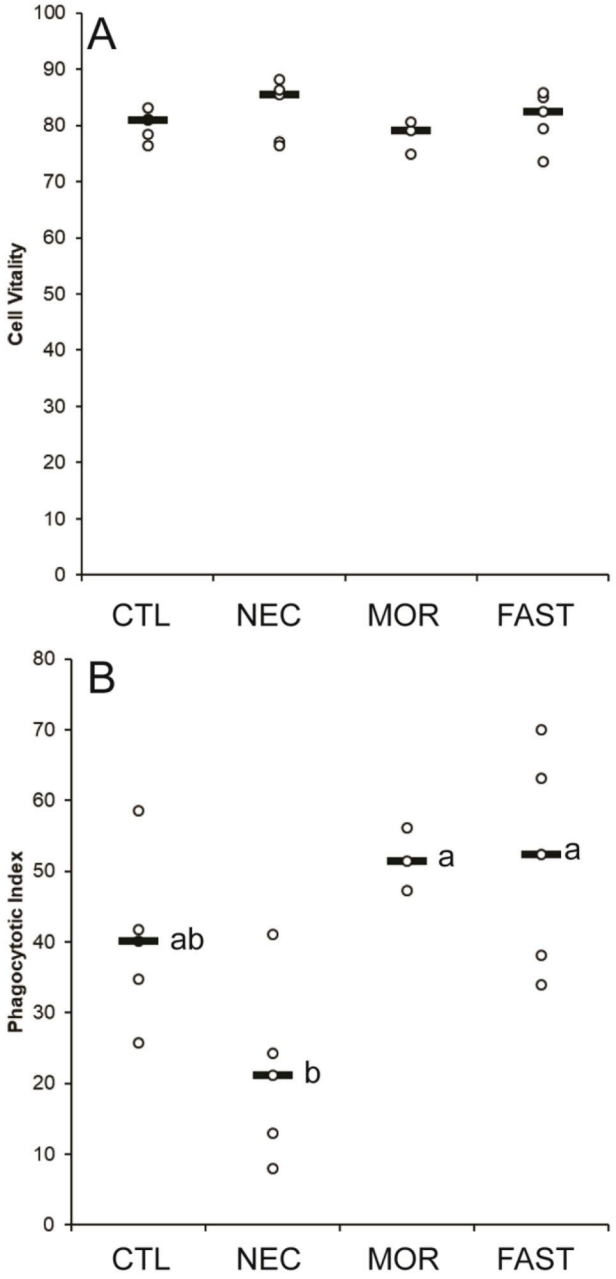

Fig. 3. Cell function in lobster haemolymph. A) Cell vitality, measured as a percentage of live cells, and B) Phagocytotic index, measured as a percentage of phagocytosed particle, of control (CTL), necrosis (NEC), moribund (MOR) and fasted (FAST) treatments. Each circle indicates an individual within a treatment with the treatment mean indicated by a horizontal bar. Means sharing a letter are not statistically different.

suggestion of reduced capacity in SRL with bacterial infections.

3.3. Haemocyte numbers and function

Following stimulation of an immune response in crustaceans, the number and proportion of haemocytes can vary, generally with a decrease in overall cell numbers and a shift in type dependent upon the response followed by a substantial increase in cell populations as haemocytes are generated through haematopoiesis [29]. Crustaceans have also demonstrated similar shifts in haemocyte populations in response to a range of extrinsic and intrinsic factors including environmental stress, moult cycle and nutritional state. Thus, the number, cell type proportion and function of haemocytes can be powerful indicators of immune status in lobsters.

Haemocyte viability (Fig. 3a), calculated from the proportion of cells demonstrating ethidium homodimer-1 fluorescence compared to a sample of cells permeabilised by incubation in 70% methanol, showed that all treatments had a similar level of live and dead cells with 80–83% live cells and no significant difference between treatments ($F_{(3,14)} = 1.32$, $P = 0.31$), indicating that haemocyte viability does not appear to show sensitivity to the stressors investigated here. These results are generally on par with previous measurements of haemocyte viability in rock lobster, which reported about 10% dead haemocytes [28,30], with the difference in dead cells likely an artefact of the more extensive handling required by the microplate assay used in the present study compared to the results of flow cytometry assays.

Phagocytotic index (Fig. 3b) was significantly different between treatments ($F_{(3,14)} = 5.85$, $P = 0.008$), with NEC showing a reduced capacity for taking up fluorescent-labelled zymosan particles compared to MOR and FAST lobsters. Decreases in phagocytotic capacity are not uncommon in crustaceans [31], but neither fasting nor the unidentified stresses affecting the MOR lobsters had an impact in the present study, as neither treatment was found to differ significantly from CTL lobsters.

Total haemocyte counts (Fig. 4a) were significantly different between treatments ($F_{(3,14)} = 4.83$, $P = 0.016$), as NEC lobsters were found to have significantly higher THC than both MOR and FAST treatments with over 3-fold more haemocytes.

The THC levels for all four treatments reflect previously reported ranges for SRL [12,13] and rock lobsters more broadly [32,33]. However, THCs of the CTL and NEC treatments were considerably higher than the values reported by Zha et al. [28], in which a control SRL cohort were reported to have < 40% of the number of haemocytes of the controls in the present study and the TFN-infected treatment only 15% of the NEC treatment reported here. Crustaceans tend to show a high level of haemocyte variation between individuals, both in terms of total number and proportion of cell type, with a range of biotic and abiotic influences identified [29,33]. As such, the substantial differences between the Zha et al. [28] study and the present study may be attributed to some combination of variations in season, moult cycle, nutrition, weather, stress or a range of other factors.

Another substantial difference in the response to pathogenic bacterial infection in SRL reported by Zha et al. [28] was that TFN-infected lobsters were found to have a significantly reduced haemocyte count compared to controls, whereas in the present study, necrosis infection resulted in a significantly greater count. The general crustacean response to immune insult is characterised by an interplay between cell synthesis, or haematopoiesis, and the loss of cells through immune response functions including cell autolysis, phagocytosis or proPO system activation. This response leads to a rapid decrease in THC followed by a subsequent stimulation of haematopoiesis and haemocyte proliferation, termed a 'left shift' response [34–36], which was seen in the present study in the NEC treatment. In contrast, the relatively low THC in MOR and FAST treatments reflected the comparatively long holding time without feeding [37,38], as haematopoiesis requires energy expenditure [39] and may be facultative during times of poor nutrition [37,40].

Differential haemocyte counts (Fig. 4a–d) varied significantly between the four treatments for the percentage of hyalinocytes ($F_{(3,14)} = 25.84$, $P < 0.001$), semigranulocytes ($F_{(3,14)} = 29.64$, $P < 0.001$) and granulocytes ($F_{(3,14)} = 4.19$, $P = 0.026$). For hyalinocytes, CTL and MOR had a similar proportion of cells, NEC had fewer than all other treatments and FAST had more than all other treatments. Correspondingly, NEC had more semigranulocytes than all other treatments, FAST had fewer semigranulocytes than all other treatments and CTL and MOR were similar. For granulocytes, FAST had the lowest proportion and significantly fewer than NEC. CTL and MOR had a similar, intermediate number of granulocytes.

Previous studies have reported a wide range proportions of the three cell types in healthy rock lobsters, with a haemocyte composition at an

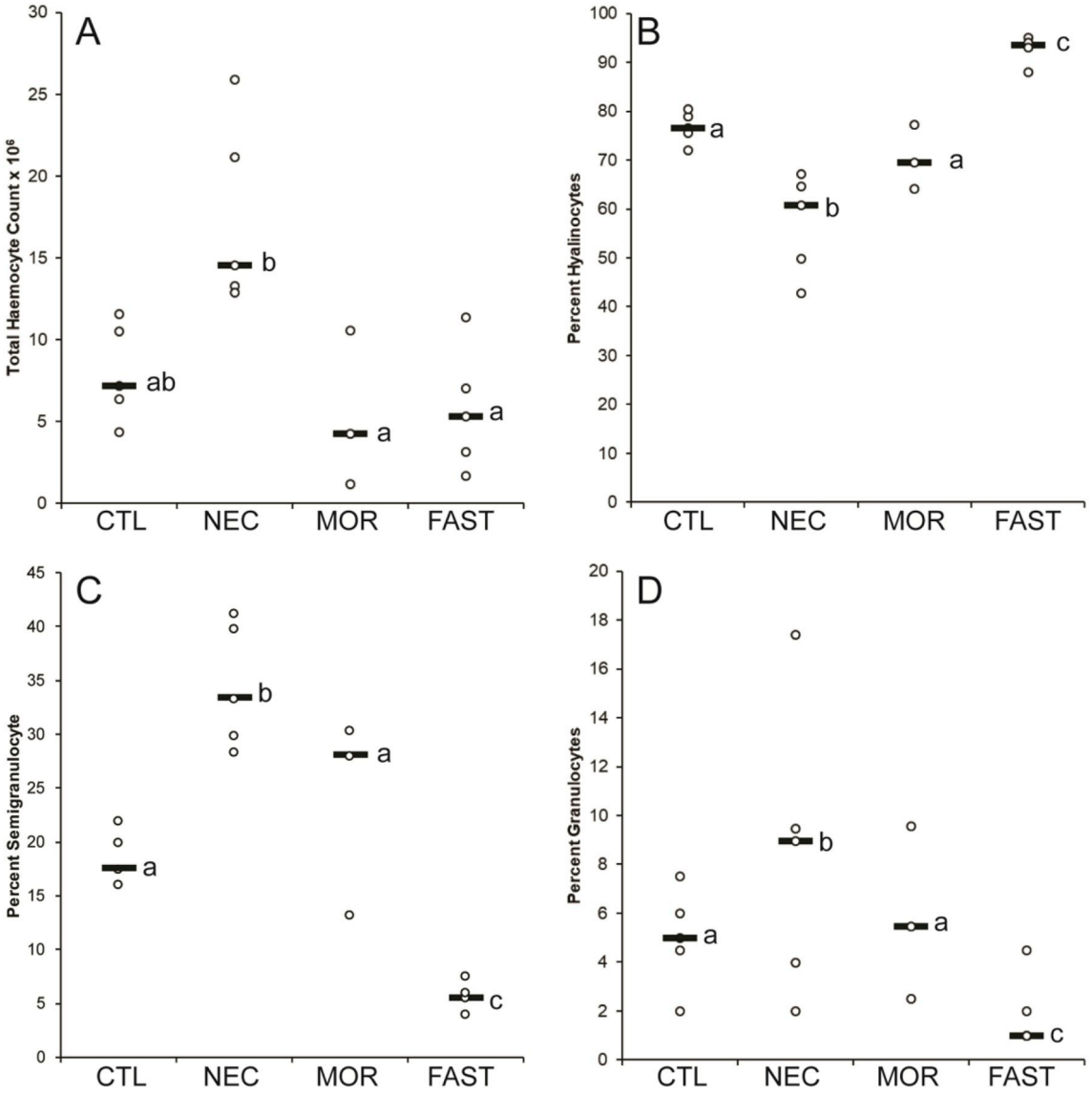

Fig. 4. Haemocyte numbers in lobsters. A) Total haemocyte counts; B) Percentage of hyalinocytes; C) Percentage of semigranulocytes and D) Percentage of granulocytes in control (CTL), necrosis (NEC), moribund (MOR) and fasted (FAST) treatments. Each circle indicates an individual within a treatment with the treatment mean indicated by a horizontal bar. Means sharing a letter are not statistically different.

approximately 60% hyalinocytes (H), 30% semigranulocytes (SG) and 10% granulocytes (G) ratio reported in the California rock lobster (*Panulirus interruptus*) [41], a roughly 60%:5%:35% H:SG:G ratio in the European rock lobster (*Palinurus elephas*) [42] and in Western Rock Lobster (*Panulirus cygnus*), a range from 75%:20%:5% H:SG:G [21] to 30%:60%:10% H:SG:G [32,43]. In lobsters subject to stress, these ratios generally shifted to a more haemocyte dominated ratio (70%:4%:26% H:SG:G) in *P. elephas* [42] and in *P. cygnus* with 80%:15%:5% H:SG:G [21] and 35%:52%:13% H:SG:G [32].

There appears to be a continuum of immune response to stress in rock lobsters that manifests as a trade-off between hyalinocytes and the two granular cell types. At one end of the response continuum, FAST lobsters showed an almost complete shift toward hyalinocytes, which comprised *ca.* 90% of the haemocytes in this treatment, at the cost of semigranulocyte (5%) and granulocyte (5%) proportions. It seems likely that this response was underpinned by the balancing of the energetic costs of immune system response against the constraints of long term fasting, but it was not possible to draw any solid conclusions due to the paucity of data on the metabolic costs of immune responses in invertebrates in general [44] and, more specifically, the lack of data on the effect of long term fasting on DHC in crustaceans.

At the opposite end of the spectrum, as seen in some *P. cygnus* results [21,32] and in the NEC lobsters in the present study, the response to stress or immune challenge was characterised by a preponderance of the two granular cell types and a decrease in hyaline cells. Indeed, a strong negative correlation ($R^2 = 0.95$) was found between hyalinocytes and semigranulocytes in the present study across all treatments (Fig. 5a). The relationships between hyalinocytes and granulocytes (Fig. 5b) was also negative, but weaker ($R^2 = 0.57$; Fig. 5b). This trade off, in which hyalinocytes are downregulated and granular cells are upregulated, drove a shift in immune function from the hyaline cell mediated phagocytosis toward a granulocytic proPO system focused response. The strong proPO activity of the NEC treatment supported this hypothesis, given that the granular cell types are the storage site for the PO precursor and mediator of its activation. In addition, the significantly fewer proportion of hyalinocytes in the NEC lobsters relative to the MOR and FAST treatments implied a significantly reduced capacity for phagocytosis [2,37]. Although NEC lobsters had the lowest proportion of hyalinocytes of the four treatments in this study, their substantially greater THC means they had more in number than the

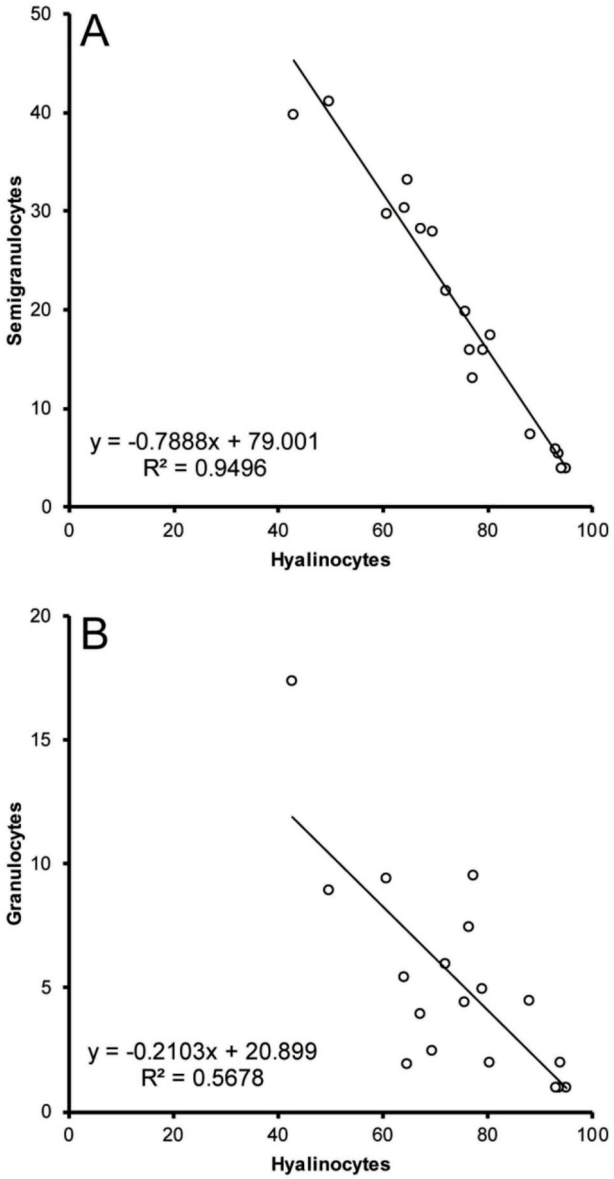

Fig. 5. Relationships between cell types. A) Linear regression of semigranulocytes against hyalinocytes across all four treatments demonstrating the strong relationship between the two cell types, B) Linear regression of granulocytes against hyalinocytes across all four treatments demonstrating the weak relationship between the two cell types.

other treatments, yet these cells were less "active" in phagocytosis than those of other treatments.

There is some evidence to suggest that hyaline cells are an intermediate step in the differentiation of phagocytic semigranular cells and that a second sub-type of non-phagocytic semigranular cell develops into granular cells [35,45]. Differentiation would presumably be mediated by some yet to be identified haemolymph-borne factor, which would explain both the trade-off relationship of the cells and the functional shift from phagocytosis to proPO despite the relatively large number of hyaline cells. This cell differentiation hypothesis still requires further experimental validation and is based primarily on work in two crustacean taxa (the black tiger prawn *Peneaus monodon* and the freshwater crayfish *Pacifastacus leniusculus*). Both the prawn and the crayfish have haemocyte compositions dominated by semigranular and granular cells, with semigranulocytes serving as the primary phagocytes rather than the hyaline cells, so it is unclear what implication that may have for the hyalinocyte dominated rock lobster system. Thus far,

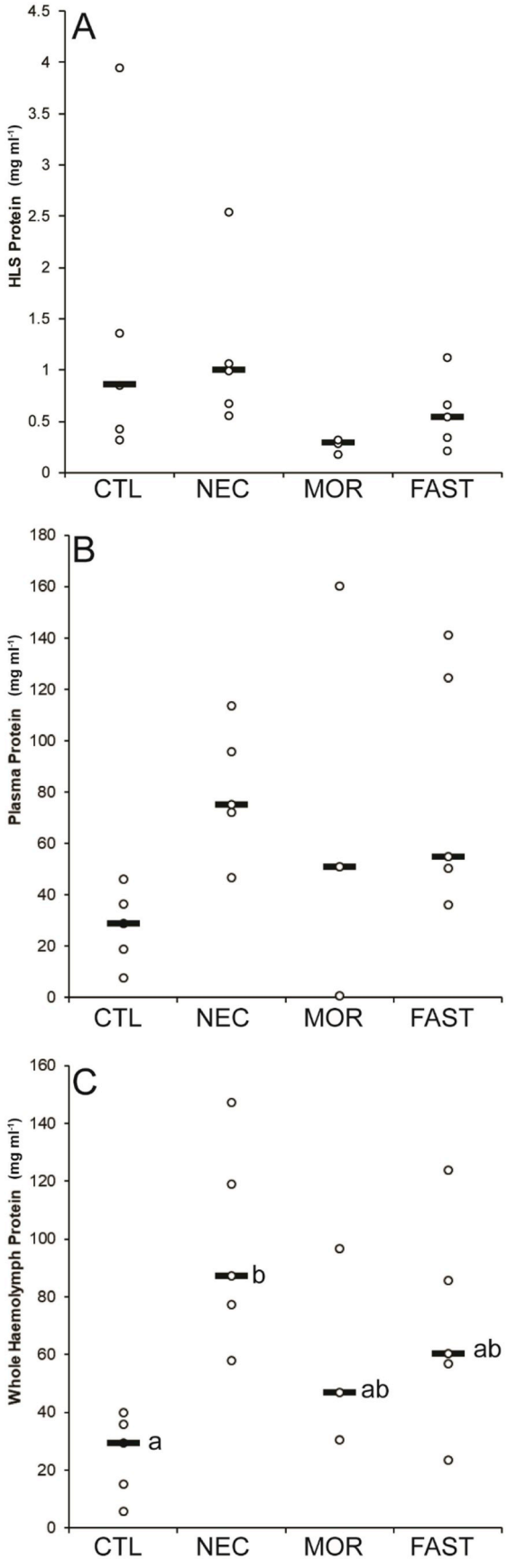

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Fig. 6. Protein concentration of the lobster haemolymph and its constituents. Protein concentration, expressed in mg/ml, in the A) haemocyte lysate suspension, B) plasma and C) whole haemolymph in control (CTL), necrosis (NEC), moribund (MOR) and fasted (FAST) treatments. Each circle indicates an individual within a treatment with the treatment mean indicated by a horizontal bar. Means sharing a letter are not statistically different.

rock lobster hyalinocytes and semigranulocytes have both been reported to demonstrate phagocytic [46] and cytolytic capabilities [47], implying that these cells may be different stages of the same lineage, although the specific function and overall interplay between haemocytes in the immune response of rock lobsters remains unclear.

3.4. Protein concentration

In the plasma fraction of the haemolymph, measurements of protein levels have frequently been investigated as an indicator of physiological condition. Haemocyanin, the protein that binds oxygen, is the most abundant constituent comprising > 60% of total protein levels, with coagulogen, apohaemocyanin, hormones and lipoproteins accounting for the remainder [48], with water uptake, which dilutes the blood, and utilisation of protein as a metabolic reserve during starvation [49] as the primary mechanisms underlying changes in protein concentration.

Neither HLS ($F(3,14) = 3.00$, $P = 0.0664$) nor plasma ($F(3,14) = 1.94$, $P = 0.169$) protein concentration were found to differ between treatments (Fig. 6a and b). Whole haemolymph protein (Fig. 6c) did differ significantly ($F(3,14) = 5.46$, $P = 0.011$), as NEC lobsters had significantly higher protein levels than CTL lobsters. No difference was found between MOR or FAST lobsters and the other treatments.

Although haemolymph protein concentration is frequently used as a measure of stress in crustaceans, there is evidence to suggest that in rock lobsters, it is not as sensitive as other parameters, including metabolites, THC, DHC and refractive index [43,49–50]. Previous work showed that tail fan necrosis did not significantly impact protein concentration in either the HLS or plasma of *J. edwardsii* [28], suggesting that the relatively high protein concentration in the whole haemolymph of NEC lobsters seen in the present study was caused by comparatively high nutritional condition relative to lobsters in the other treatments [49,52].

3.5. Haemolymph biochemistry

Amongst electrolytes and minerals (Table 1), Na concentration showed a significant difference between treatments ($F(3,14) = 11.32$, $P < 0.001$), with the NEC treatment lower than CTL and MOR treatments. Neither K ($F(3,14) = 1.83$, $P = 0.189$) nor the ratio between Na:K ($F(3,14) = 0.85$, $P = 0.487$) were significantly different between treatments. The concentration of Cl differed significantly between treatments ($F(3,14) = 10.41$, $P < 0.001$), with the NEC treatment again having significantly lower levels than CTL and NEC and FAST lower than CTL. The relationship between treatments was similar in Na and Cl, suggesting a common mechanism drove the change. Indeed, decreases in Na and Cl concentration have previously been linked to moribundity in SRL following emersion [13], suggesting that the NEC and FAST treatments were under physiological stress. There were significant differences in the concentration of haemolymph bicarbonate between treatments ($F(3,14) = 12.71$, $P < 0.001$), with FAST lobsters showing significantly lower levels than all three other treatments and MOR lobsters showing significantly higher bicarbonate than CTL. Bicarbonate has also previously been shown to respond to emersion stress in SRL [13], increasing as a means to buffer falling haemolymph pH as acidic metabolic products build up. However, the MOR lobsters did not have acidic pH levels, making the underlying cause of the observed increase unclear. FAST lobsters had a significantly lower Mg concentration ($F(3,14) = 4.08$, $P = 0.028$) than both CTL and NEC treatments. Increases in Mg concentrations have been linked to poor condition following transport in lobsters and during moulting [23,53]. There was no significant difference in the concentration of Ca ($F(3,14) = 0.692$, $P = 0.572$), Phos ($F(3,14) = 1.352$, $P = 0.298$) or osmolality ($F(3,14) = 1.97$, $P = 0.165$) between treatments.

Total protein was significantly different between treatments ($F(3,14) = 12.11$, $P < 0.001$), with both NEC and FAST lobsters showing significantly elevated levels compared to the CTL and MOR treatments. The measurement of albumin, which in crustaceans more accurately reflects the level of bromocresol green dye-binding high molecular weight proteins [54] (primarily haemocyanin) than actual albumin, which they lack [55], was found to significantly differ between treatments ($F(3,14) = 23.32$, $P < 0.001$), with NEC lobsters higher than CTL and MOR treatments and FAST higher than CTL. Globulin levels, calculated as total protein minus "albumin" [54], were also significantly different ($F(3,14) = 9.28$, $P = 0.001$), with NEC and FAST treatments showing levels around double those of CTL and MOR. However, the albumin:globulin ratio did not significantly differ between treatments ($F(3,14) = 2.80$, $P = 0.08$), suggesting that the change in both parameters was correlated, such as through changes in haemolymph hydration.

Uric acid, a metabolic waste product that is converted to ammonia for secretion, was significantly elevated in NEC lobsters ($F(3,14) = 4.62$, $P = 0.02$) relative to CTL lobsters. Glucose concentration did not differ significantly ($F(3,14) = 0.87$, $P = 0.48$). Cholesterol levels were substantially higher ($F(3,14) = 14.83$, $P < 0.001$) in the NEC treatment than in the other three treatments by a factor of at least 1.7 (relative to FAST) and as much as 2.5 (MOR). Triglyceride concentrations differed significantly ($F(3,14) = 9.22$, $P = 0.001$), with NEC and FAST treatments showing more than twice the concentration of both CTL and MOR treatments. Triglycerides and cholesterol, which occur in a low concentration (3% and 4%, respectively) in rock lobster haemolymph compared to phospholipids (88%) [56] nonetheless function in important roles, with the former serving as a metabolic energy reserve and the latter as a structural component for cell membranes [57]. Both triglycerides and cholesterol respond rapidly to the onset of fasting, making them a useful indicator of nutritional condition in clawed and rock lobsters [23,57]. Thus, it is not surprising that the well-fed NEC lobsters had a high concentration of uric acid, cholesterol and triglycerides that corresponded to those reported for well-fed lobsters in other studies [23,57], though the elevated levels of triglyceride in the FAST lobsters is indeed surprising and cannot be explained.

Although a range of haemolymph enzymes were assayed, only two showed significant differences between treatments, as γ-glutamyl transferase activity levels ($F(3,14) = 5.06$, $P = 0.014$) showed that NEC and MOR treatments had elevated levels relative to CTL and glutamate dehydrogenase activity levels were significantly higher in NEC and FAST relative to CTL and MOR ($F(3,14) = 14.89$, $P < 0.001$). Both of these enzymes are commonly used in vertebrates (particularly humans) as an indicator of tissue damage and have previously been shown to increase in stressed SRL, with GGT levels correlated to mortality [13], suggesting they may be useful indicators of health status.

3.6. Multidimensional scaling analysis

A multidimensional scaling plot incorporating the 15 haemolymph and immune parameters and 19 haemolymph biochemical parameters quantified in this study was generated to determine the similarity of the individuals within each treatment and the similarity (or lack thereof) between each treatment. Part of the rationale for this was that many of the mechanisms driving changes in biochemistry and haemolymph cytology are unclear in rock lobsters and this scaling enabled all data to be pooled for exploring changes between treatments.

The resultant MDS plots showed a 2D Stress of 0.10 (Fig. 7), indicating a good fit and a 3D stress = 0.06, indicating an even better fit.

Table 1
Biochemical analysis of the electrolytes and minerals, metabolites and enzymes of the haemolymph of lobsters. Mean ± SEM values are given for each treatment. For each parameter, statistically different means as determined using ANOVA are indicated by differing letters. See Section 2.8 for details on parameter abbreviations.

	Na mmol/L	K mmol/L	Na:K	Cl mmol/L	Osm mOsm/kg	Bicarb mmol/L	Ca mmol/L	Phos mmol/L	Mg mmol/L
CTL	508 ± 5 a	7.0 ± 0.5	74 ± 5	513 ± 9 a	1027 ± 5	3.5 ± 0.4 a	16.08 ± 0.42	0.44 ± 0.16	12.34 ± 1.02 a
NEC	470 ± 6 b	6.1 ± 0.2	78 ± 2	450 ± 9 b	980 ± 18	3.6 ± 0.2 ab	17.40 ± 1.36	0.80 ± 0.20	12.33 ± 2.18 a
MOR	509 ± 7 a	6.4 ± 0.1	80 ± 2	512 ± 16 ac	1029 ± 16	5.1 ± 0.8 b	16.39 ± 1.35	0.46 ± 0.44	9.51 ± 1.52 ab
FAST	487 ± 5 ab	6.1 ± 0.2	81 ± 3	473 ± 7 bc	993 ± 23	2.3 ± 0.1 c	17.95 ± 1.01	0.90 ± 0.03	6.86 ± 0.03 b

	TPro g/L	Alb g/L	Glob g/L	A:G	Uric μmol/L	Gluc mmol/L	Chl mmol/L	Trig mmol/L	GGT U/L	GD U/L
CTL	45 ± 9 a	4 ± 1 a	41 ± 9 a	0.12 ± 0.04	20 ± 2 a	1.0 ± 0.2	0.41 ± 0.06 a	0.30 ± 0.07 a	1.2 ± 0.5 a	6.8 ± 0.7 a
NEC	100 ± 5 b	14 ± 1 b	86 ± 4 b	0.17 ± 0.01	34 ± 2 b	0.7 ± 0.1	1.00 ± 0.02 b	0.81 ± 0.04 b	5.4 ± 1.3 b	23.0 ± 5.0 b
MOR	42 ± 19 a	6 ± 1 a	35 ± 18 a	0.25 ± 0.07	21 ± 1 ab	0.6 ± 0.1	0.39 ± 0.18 a	0.23 ± 0.14 a	6.6 ± 1.8 b	9.3 ± 3.0 a
FAST	84 ± 5 b	9 ± 1 b	75 ± 4 b	0.12 ± 0.01	24 ± 4 ab	0.6 ± 0.1	0.59 ± 0.05 a	0.91 ± 0.18 b	3.0 ± 0.7 ab	23.4 ± 3.2 b

ANOSIM showed a significant difference between treatments (Global $R = 0.659$, $P < 0.001$; where values of R range from 0 to 1 with 0 representing complete similarity and 1 representing complete dissimilarity), with pairwise comparison showing a high level of dissimilarity between CTL and NEC treatments (R statistic = 0.900). NEC and FAST showed the next highest level of dissimilarity ($R = 0.728$), followed by CTL and FAST ($R = 0.692$). MOR and FAST were moderately dissimilar ($R = 0.662$), as were NEC and MOR (0.662). CTL and MOR showed a low degree of dissimilarity ($R = 0.231$).

Complete linkage CLUSTER analysis identified two distinct groupings of lobsters in this study (indicated in green on Fig. 7): the first comprised of all five NEC lobsters, all five FAST lobsters plus one each of MOR and CTL lobsters and the second comprised of all four CTL and two MOR lobsters. Within the former cluster, two further clusters were identified, with the first made up entirely of the five NEC lobsters and the second made up of the five FAST lobsters and one each of CTL and MOR treatments. This indicates that the immune function and haemolymph biochemistry of NEC lobsters, the treatment under the greatest immune challenge, showed a distinct response from the other treatments in the study. FAST lobsters, which were similarly under a comparatively high degree of stress, also demonstrated a response in immune and biochemical parameters, but one that was distinct from that of the NEC treatment. The inclusion of the one individual each from the CTL and MOR treatments in the FAST cluster indicates that these individuals were demonstrating a similar response. The grouping of two MOR individuals with the CTL treatment and the grouping of the third with the FAST lobsters rather than the NEC treatment leads to the conclusion that the moribund lobsters were not suffering some pathological condition, rather, one was likely demonstrating a response characteristic of fasting whereas the other two were in a moribund state due to some other influence, such as stress from being caught or processed, water quality factors or some other impact.

4. Conclusions

The aim of this study was to investigate how the immune system of the Southern Rock Lobster would reflect the challenges presented by recent arrival at a processing facility, advanced shell necrosis, long term fasting in industry standard processing facility conditions and being in a moribund state. The shell necrosis treatment served as a positive control, characterising a distinct immune response to a pathogenic infection. This response was typified by an increase in haemocyte numbers and a shift in haemocyte type to a higher proportion of semigranulocytes and granulocytes. Along with this change came a decrease in phagocytosis capacity but an increase in phenoloxidase activity. The fasted treatment was also found to demonstrate an immune response, though the mechanism was quite different. Although fasted lobsters did not demonstrate a change in haemocyte numbers, there was a marked shift in cell type composition, with a high level of hyalinocytes at the cost of semigranulocytes and granulocytes, though this shift did not correspondingly improve phagocytosis capacity or hinder phenoloxidase production.

Of the lobsters in an unexplained moribund state, two of the three sampled showed a “non-response” and were grouped with the control treatment in the multivariate analysis. It appears that these lobsters were not suffering any sort of pathogenic infection that might elicit a response similar to the bacterial infection of the necrosis treatment and suggests that these lobsters were moribund due to some other cause, such as water quality.

The results of this study indicate that phenoloxidase is indeed present in the plasma, though some activity was observed in the haemocytes as well, particularly in the more immune-stimulated treatments. Of the parameters measured in this study, total and differential haemocyte counts appear to have the most predictive power in regard to health status. Staff can be easily trained to take these measurements, which do not require specialised equipment, potentially making them a

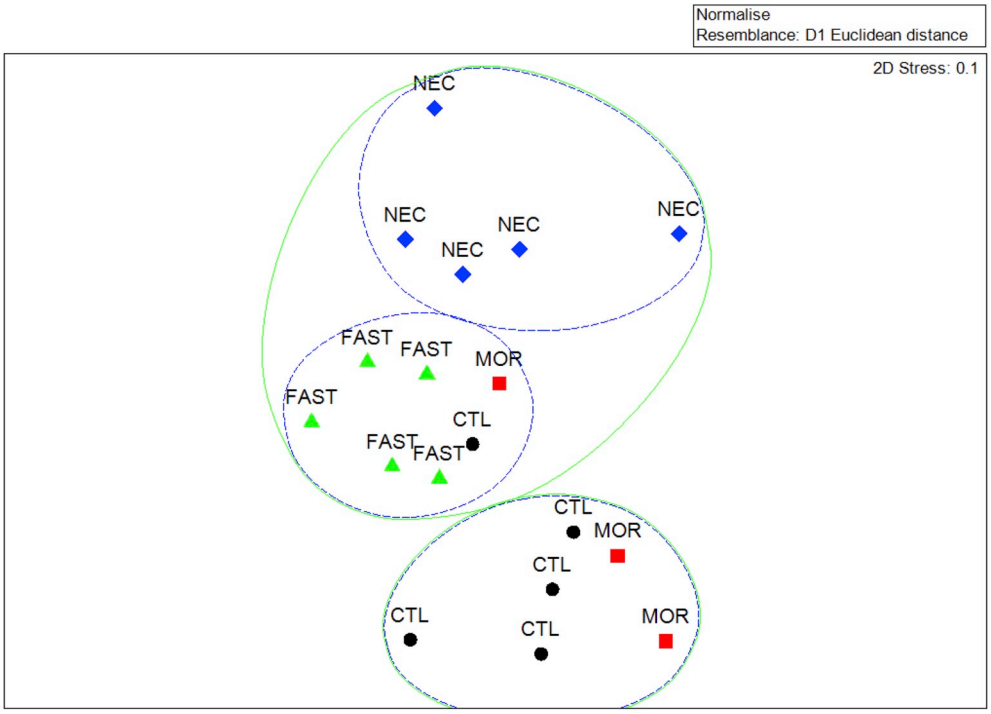

Fig. 7. Immune function in lobsters. Multidimensional scaling (MDS) plot analysing the 15 haemolymph physical and immune parameters and the 19 biochemical parameters analysed in the four treatments in this study. The distance between any two individuals on this plot indicates their relative similarity, with increasingly similar points more close together. The 2D stress indicates a good fit and CLUSTER analysis identifies two major groups, encircled in solid green. Within the "response" cluster, two further groups were identified, encircled in dashed blue, with one comprised entirely of the NEC treatment and the other comprised of the FAST treatment with one individual each of MOR and CTL. Within the separate "non-response" treatment is the remainder of the CTL and MOR treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

powerful tool within the supply chain of lobster fisheries. Other biochemical parameters, such as electrolyte and mineral ion levels, bicarbonate concentration and GGT activity, appeared to be well correlated to immune status, but require further study to form a conclusive link between values and health status, as well as identifying the mechanisms that drive changes.

Author contributions

QPF and CG conceptualised and acquired funding for the research project. RDD and QPF designed the methodology. RDD performed the investigation and conducted the analysis. RDD drafted the manuscript. QPF and CG reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2019.03.043.

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