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Characterisation of the haemocytes of leaf oysters, *Isognomon ephippium* (Linnaeus, 1758)

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ABSTRACT

Characterisation of the immune system of bivalves can be a key to understanding their adaptation and resilience to the environment. This study is the first to characterise the haemocytes of the Family Isognomonidae. Three different types of haemocytes, granular cells, hyalinocytes and blast-like cells, were identified by light microscopy in leaf oysters *Isognomon ephippium* (Linnaeus, 1758), with relative proportions of 8.4% (±2.3%) 76.2%, (±1.9%), and 15.3% (±1.6%), respectively, determined by flow cytometry. Scanning electron micrographs illustrated the outer appearance of the major haemocyte types, including dendritic-like cytoplasmic extensions on activated haemocytes. The total haemocyte count (THC) and bacterial counts in the haemolymph of leaf oysters, as well as the bacterial load in the source water, were compared across three estuaries with differing water quality on the north coast of New South Wales, Australia. THC ranged from 3.89×10^5–2.89×10^6 cells/mL in leaf oysters and significantly differed among three sites. THC increased with nitrogen, but decreased with higher bacterial loads in the water, which in turn correlated to other water quality parameters. This study provides baseline information on the leaf oyster immune system to facilitate future monitoring of *Isognomon* health in relation to environmental stressors.

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Bivalvia; Isognomonidae; haemocyte counts; SEM; flow cytometry

Introduction

Bivalves are filter feeders which can accumulate large numbers of pathogens in their bodies (Allam and Raftos 2015). Like other invertebrate groups, bivalves lack an adaptive immune system; however, their innate immunity provides an effective physical barrier, and functional humoral and cellular components in the haemolymph prevent infection by pathogens (Song *et al*. 2010; Allam and Raftos 2015; Coates and Soderhall 2021). Haemolymph is the fluid that carries the blood cells (haemocytes), humoral proteins and nutrients through the 'open' circulatory system of bivalves (Song *et al*. 2010). The haemocytes can move freely between the tissues and haemolymph, and are capable of 'non-self' recognition using pattern recognition proteins (Coates and Soderhall 2021). Bivalves also harbour various bacteria such as *Vibrio*, *Aeromonas* and *Pseudomonas* in their haemolymph microbiome (Olafsen *et al*. 1993; Auguste *et al*. 2024).

Haemocytes are responsible for cell-mediated immune responses; mainly the phagocytic process and the release of humoral defence factors such as agglutinins, lysosomal enzymes and various antimicrobial peptides (Canesi and Pruzzo 2016; Silva Freire *et al*. 2023). Bivalve haemocytes comprise a heterogeneous cell population. Different approaches such as centrifugation, light and electron microscopy and flow cytometry have been used to characterise the morphology, cytochemistry and immunological function of haemocytes (Bachère *et al*. 1988; Aladaileh *et al*. 2007; Donaghy *et al*. 2009; Wang *et al*. 2012; Li *et al*. 2015). Three main haemocyte types have been identified in bivalves, namely granular cells, hyalinocytes, and blast-like cells (Donaghy and Volety 2011; Rebelo *et al*. 2013; Li *et al*. 2015; Vieira *et al*. 2017). While granular cells are rich in cytoplasmic granules containing immune-related molecules (Kuchel *et al*. 2010; Schmitt *et al*. 2012), hyalinocytes, also known as agranular cells, have few or no cytoplasmic granules (Aladaileh *et al*. 2007) and blast-like cells are undifferentiated haemocytes having a high nucleus/cytoplasm ratio (Sun *et al*. 2006; Li *et al*. 2015). The haemocytes of pearl oysters in the Family Pteriidae J.E. Gray, 1847 are well characterised (Wang *et al*. 2008; Kuchel *et al*. 2010; Kishore and Southgate 2015; Li *et al*. 2016; Vieira *et al*. 2017), but to date, no study has investigated the haemocytes of the sister taxon Isognomonidae Woodring, 1925.

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Total haemocyte counts (THC) have been used as an immune marker to assess environmental stress and cell-mediated responses to pathogenic infection in bivalves. Many studies have demonstrated that haemocytes can be either mobilised into the haemolymph in response to stimuli or reduced in response to certain physicochemical stressors (Coates and Soderhall 2021). Exposure to non-pathogenic foreign material can also stimulate circulating haemocytes of oysters. In Sydney rock oysters *Saccostrea glomerata* (Gould, 1850), exposure to the pesticide imidacloprid caused an increase in THC (Ewere *et al*. 2020). THC was identified as one of the hemolymph parameters impacted by pollution in a number of bivalves (Grinchenko *et al*. 2024), with significant increases in THC at polluted compared to reference sites in five species, but a significant decrease was noted in *Tetrarca boucardi* Jousseaume, 1984. Similarly, stress associated with algal blooms caused an increase in haemocyte numbers of *Crassostrea virginica* (Thunberg, 1793), especially granular cells (Hégaret and Wikfors 2005). Conversely, environmental stressors that reduce available energy and generate a metabolic deficit are more likely to reduce haemocyte numbers. For example, starvation reduced THC in *S. glomerata* (Butt *et al*. 2007) and both diel-cycling hypoxia (lowered dissolved oxygen levels overnight), and decreases in salinity led to decreased THC in *C. hongkongensis* (Lam & Morton, 2003) (Xie *et al*. 2021).

Leaf oysters, *Isognomon ephippium* (Linnaeus, 1758), of the Family Isognomonidae (MolluscaBase 2022) (previously classified under Family Pteriidae [Benthotage *et al*. 2020]), are large intertidal oysters that occur in estuarine and coastal ecosystems (Audino *et al*. 2020; Yuhara *et al*. 2021; Benthotage *et al*. 2022). Leaf oysters are native in tropical and subtropical environments, where they can form shellfish reefs (Gillies *et al*. 2018; Benthotage *et al*. 2020). Recent surveys of leaf oyster beds on the north coast of New South Wales (NSW), Australia, have revealed the different densities of leaf oysters ranging from scattered single oysters to dense oyster reefs (Benthotage *et al*. 2022). The density of leaf oyster beds and the condition index of the oysters varied between estuaries and correlated to a range of water quality parameters, as well as trace metal concentrations in sediment (Benthotage *et al*. 2022, 2023). A declining population with few remaining leaf oysters was found in the Richmond River, which is known to have poor water quality due to extensive agricultural activity (Jamal *et al*. 2024) and run-off from acid sulphate soils (EPA 2018). In comparison, dense beds with oysters in variable condition were found in the lower Tweed River, which has reasonable water quality (Tweed Shire Council 2019, 2021), and Woolgoolga Lake, an intermittently closed and open lagoon (Benkendorff *et al*. 2022). These leaf oyster populations provide a good opportunity to collect baseline information on the variation in leaf oyster immune parameters.

Immune characterisation of oyster species can be vital for understanding their adaptation and resilience to fluctuating conditions within their habitats (Guo *et al*. 2015; Dang *et al*. 2023). This study aims to characterise the different types of haemocytes for the first time in *I. ephippium* (and Family Isognomonidae). To compare the baseline immunity of leaf oysters among sites, the total hemocyte counts and levels of bacteria in the haemolymph of leaf oysters were also investigated at three sites with a range of different water quality. Outcomes of this study contribute to our knowledge of the leaf oyster immune system and provide some evidence on how the haemocyte counts can vary with the varying water quality.

Materials and methods

Site selection

This study focused on subtropical populations of *I. ephippium* from estuaries in northern NSW, Australia. Leaf oyster populations have been declining or have disappeared from a number of estuaries in this region (Benthotage *et al*. 2022, personal observations). Samples were collected from the Tweed River (28° 11′17.8″S, 153°30′27.4″E), Brunswick River (28° 32′07.4″S, 153°32′42.3″E) and Woolgoolga Lake (30° 06′10.5″S, 153°11′38.5″E) (Figure 1), with one site from each estuary based on the availability of leaf oysters for sampling and previous assessments of the habitat quality (Benthotage *et al*. 2022). Leaf oysters were collected under Section 37 NSW Fisheries permit number P19/0017-1.0 and Marine Parks permit number MEAA20/327. Sampling was conducted in the first two weeks of October 2021.

Characterisation of leaf oyster haemocytes

Initial characterisation of the haemocytes was done on leaf oyster samples collected from Tweed River estuary. Ten oysters were carefully shucked, haemolymph was withdrawn immediately from the pericardial cavity by piercing the intact pericardial membrane using a syringe with a 31-G needle, and pooled (Figure 2). Fresh haemolymph from leaf oysters was loaded into a haemocytometer and live haemocytes were observed under high power (×1000 magnification) of a BHS-DIC Microscope (Olympus, Tokyo, Japan). The extracted haemolymph was also smeared onto a clean glass slide, air-dried and stained with the Hemacolor® Rapid Staining of Blood Smear kit (Sigma-Aldrich, USA) following the manufacturer's protocol. Stained slides were assessed under a BHS-DIC Microscope at ×1000 magnification (Olympus, Tokyo,

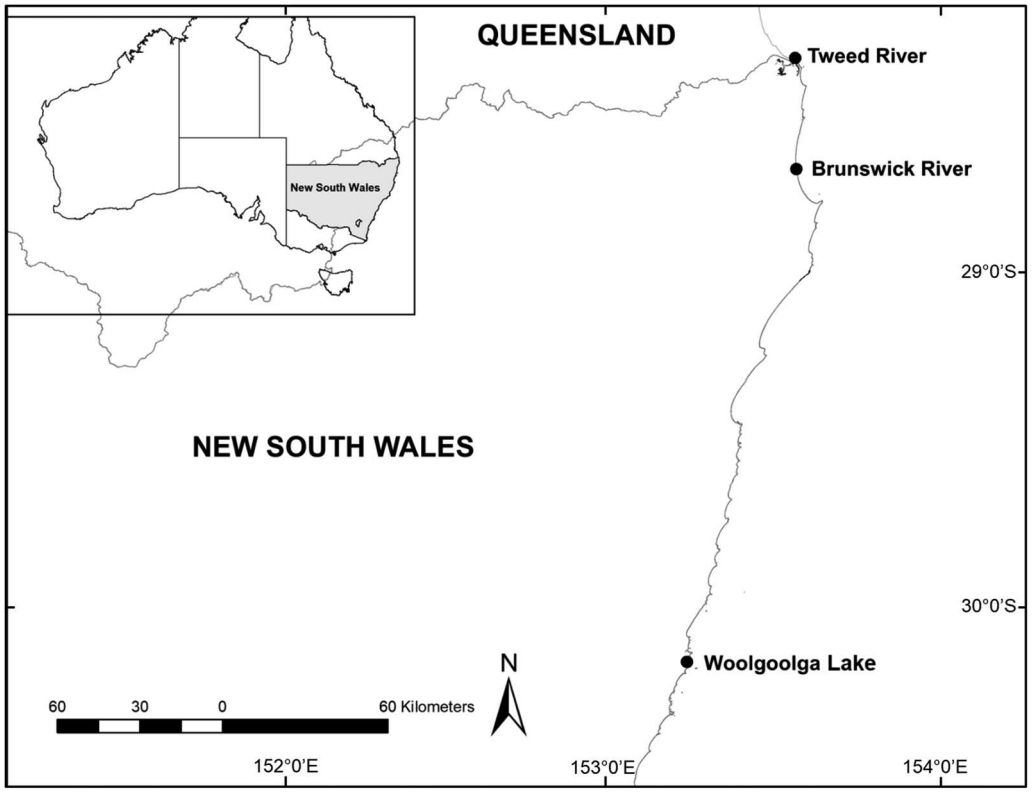

Figure 1. Map of the northern New South Wales estuaries where leaf oyster sampling was conducted.

Japan), and images were captured using a Michrome 6 Digital Microscope Camera (Tucsen, Fujian, China). In addition, haemolymph was fixed in an equal volume of 6% formalin in filtered seawater, and a drop of the solution was added to a clean glass slide and covered with a coverslip. Slides were observed under the microscope using phase-contrast to visualise the granularity of the haemocytes.

For scanning electron microscopy (SEM), approximately 1 mL of the formalin-fixed haemolymph was filtered through a Whatman Nucleopore Track-Etch Membrane filter (0.8 µm) attached to a syringe. Haemolymph cells on the filter paper were then dehydrated through a series of ethanol dilutions: 10 min in each of 30%, 50%, 70%, 85% and 95% solutions and three times for 10 min in 100% ethanol. Hexamethyldisilazane (HMDS; Sigma-Aldrich, USA) was subsequently added onto the filter paper under a fume hood and allowed to evaporate overnight. The dried filters were gold-coated using a SCD 050 Sputter Coater (Bal-Tec, USA), and imaging was done using a TM4000 Tabletop Scanning Electron Microscope (Hitachi, Japan).

After fixing the pooled haemolymph (as above) in 6% formalin in filtered seawater, leaf oyster haemocyte populations and proportions were characterised by flow cytometry. The tubes were incubated in the dark at 20 °C for 2 h with SYBR green I (Sigma-Aldrich, USA) and analysed using a FACSCalibur flow cytometer (BD Biosciences, USA) at a flow rate of 58 µL/min. Based on the size and complexity (granularity) and using forward and side scatter, gates were created to separate the different haemocyte types. Acquisition density plots were generated, and the number of events detected in each gate was used to define cell populations and the proportions. Each of the above observations and analyses were conducted in triplicate.

Field-based haemolymph sampling and analyses

Leaf oysters were sampled during outgoing morning low tides at Tweed River, Brunswick River and Woolgoolga Lake. Leaf oyster haemolymph was extracted on site using the same method described in the previous section 'Characterisation of leaf oyster haemolymph' (Figure 2). Haemolymph was collected by pooling from at least three leaf oysters (for each replicate) and approximately 200 µL of pooled haemolymph sample was fixed immediately in the field in an equal volume of 6% formalin in filtered seawater and taken back to the laboratory for total haemocyte counts (THC). THC was counted using a haemocytometer under a BHS-DIC Microscope at ×400 magnification (Olympus, Tokyo, Japan). In addition, at least 200 µL of fresh haemolymph was collected into sterile microcentrifuge tubes and immediately put on ice for transport to the laboratory for bacterial enumeration. Finally, approximately 200 mL of seawater from each site was collected into sterile glass bottles and kept on ice for bacterial enumeration. All sampling was conducted in triplicate.

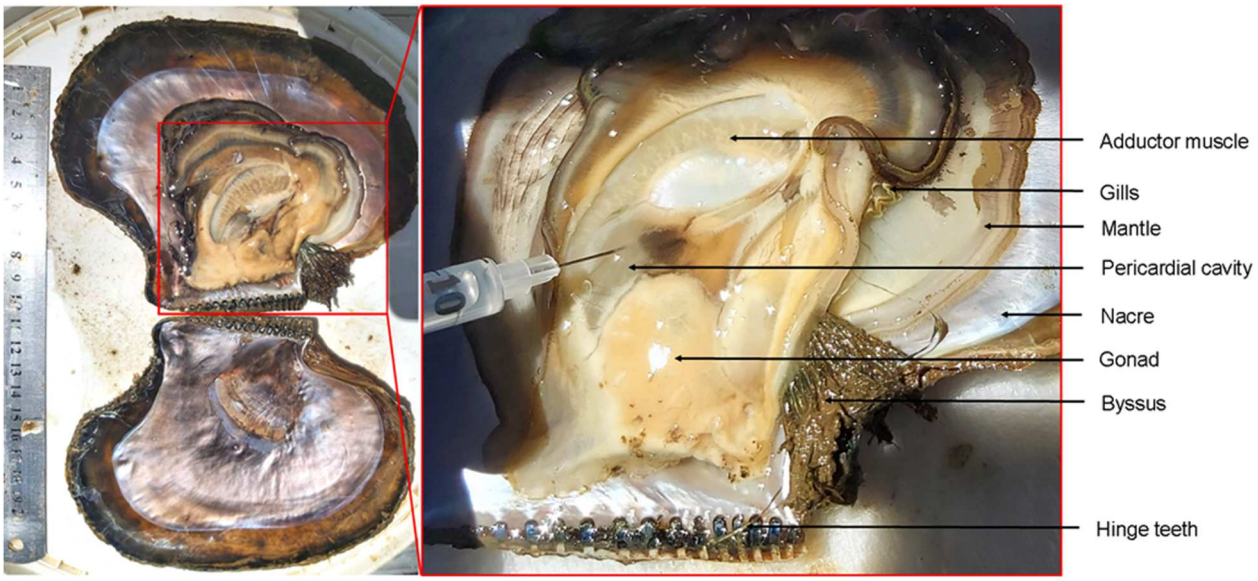

Figure 2. Haemolymph extraction from leaf oyster pericardial cavity.

Bacterial enumeration

The abundance of culturable bacteria in leaf oyster haemolymph and seawater was determined adapting a method from Ooi *et al*. (2019). Immediately after arrival in the laboratory, 100 µL of haemolymph and seawater samples were spread-plated on Zobell marine agar (Amyl Media, Dandenong, Australia). Independent plates from triplicate samples were then incubated at 28 °C for a maximum of 7 days, allowing slow-growing bacteria to colonise. The number of colonies on each plate was used to calculate the colony forming units (CFU/mL) of haemolymph and water.

In-situ water quality and water sampling

Triplicate water samples were taken in parallel to the sampling of leaf oysters at each site. Standard water quality parameters, temperature, salinity, pH and dissolved oxygen (DO%), were measured at each site using a Multiparameter Meter (HI98194 – Hanna Instruments, Rhode Island, USA). Water samples for dissolved inorganic nutrients (phosphate, nitrate, nitrite and ammonia) and dissolved organic carbon (DOC) were 0.45 µm filtered, while the samples for total organic carbon (TOC), and total nutrients (Total Phosphorous – TP; Total Nitrogen – TN), were collected without filtration. Nutrient samples (10 mL each) were frozen, and DOC and TOC samples (30 mL each) were fixed with 2 mL of 6 N sulphuric acid immediately after sampling. DOC and TOC were analysed by a TOC-L Combustion Analyser (Shimadzu, Japan), and the nutrient analyses were carried out using Quikchem Flow Injection Analysis (Lachat Instruments, USA) at the Environmental Analysis Laboratory, Southern Cross University (NATA Accreditation No. 14960). Particulate organic carbon (POC) was calculated as TOC – DOC. Dissolved inorganic Nitrogen (DIN) was calculated as the sum of dissolved nitrate, nitrite and ammonia. Particulate and dissolved organic nitrogen (PON + DON) were calculated by subtracting DIN from TN.

Statistical analysis

Data were analysed using Permutational Analysis of Variance (PERMANOVA) in the PRIMER v7 package, with Euclidean distance similarity matrices and 999 permutations of the raw data. Leaf oyster haemolymph parameters, THC and CFU, were compared among sites using one-factor PERMANOVA. When the number of permutations was $\leq$ 30, Monte Carlo tests were applied for post-hoc pairwise tests. The same one-factor PERMANOVA design was used to compare each individual water quality parameter and all parameters combined. Differences in haemolymph and water quality parameters at different sites were visually displayed using Principal Coordinates Analysis (PCO) with vector overlays based on Pearson correlation ($r > 0.8$). Distance-based linear modelling (DBLM) was used to test the relationship between normalised THC and water quality parameters. The water quality data were first tested for multi-collinearity based on Pearson's rank correlations ($r > 0.75$) (See Supplementary Table 1), and independent parameters (CFU in water, PON + DON, DIN, POC and TP) were then included in the DBLM. Marginal tests were performed for individual variables, and the Akaike information criterion (AIC) was used to select the BEST model. After testing the residuals for potential trends, correlation plots were created for individual variables that were significant in marginal tests to illustrate positive or negative relationships with THC.

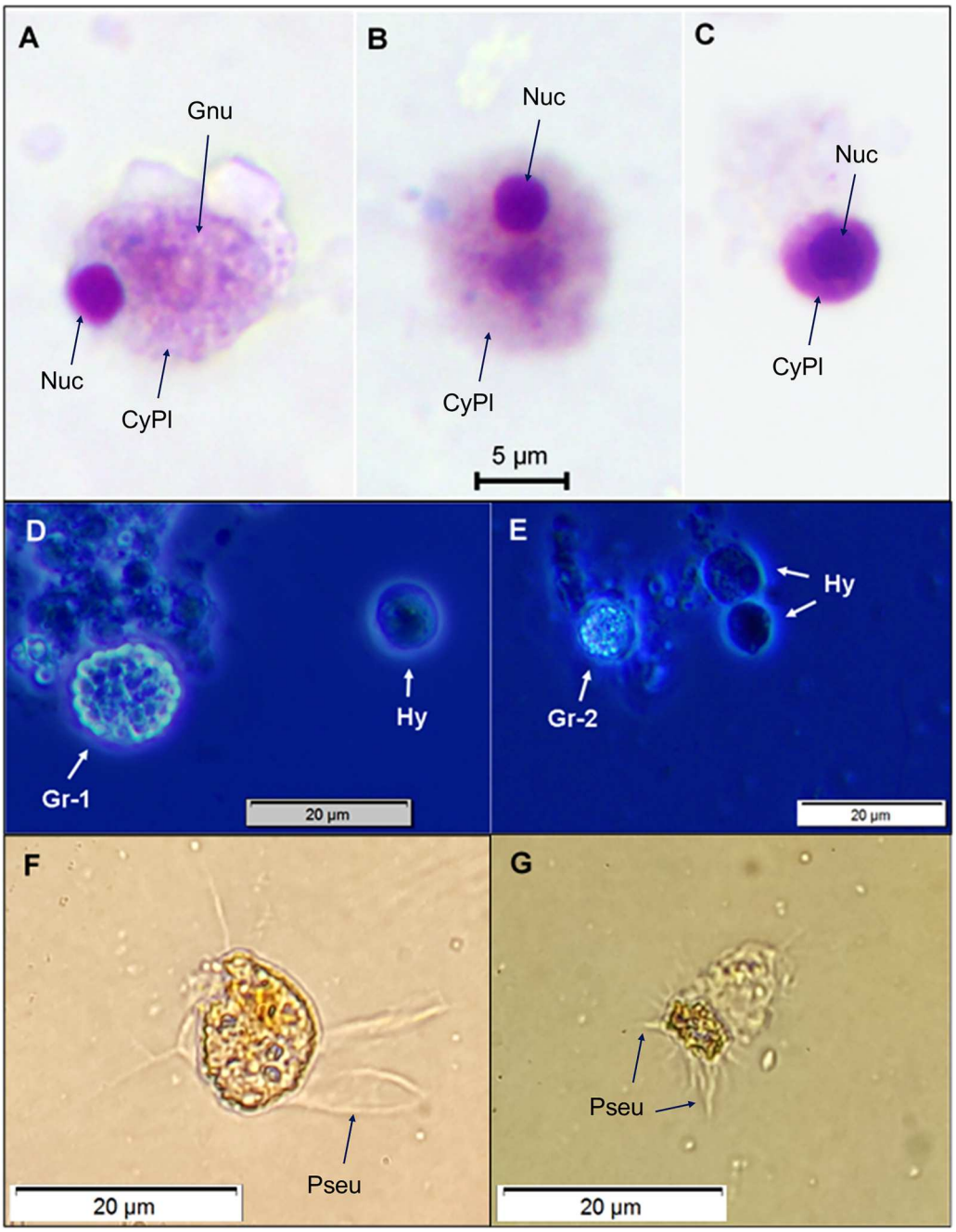

Figure 3. Light microscopy images of representative leaf oyster haemocytes. **A-C** – haemocytes stained with Hemacolor; A – Granulocyte, B – Hyalinocyte, C – Blast-like cell; CyPl – Cytoplasm, Nuc – Nucleus, Gnu – Cytoplasmic granules (Note: Figures A – C share the same scale bar). D & E – Phase-contrast light microscope images of unstained leaf oyster haemocytes; Gr-1 – larger granulocyte with large cytoplasmic granules, Gr-2 – smaller granulocyte with smaller cytoplasmic granules, Hy – hyalinocytes. F & G – Light microscope images of live leaf oyster granulocytes forming pseudopodia; Pseu – Pseudopodia.

Results

General characterisation of leaf oyster haemocytes

Light microscopy of stained and unstained haemocytes allowed the differentiation of three morphologically distinct cell types, i.e. granular cells, hyalinocytes and blast-like cells (Figures 3). Granular cells were oval or round shaped with cytoplasmic granules, which allowed differentiation from other haemocyte types (Figure 3A, D, E). Sizes of granular cells ranged from ~5 to 15 µm (Figure 3 and 4). There were larger granular cells (diameter ~15 µm) containing larger cytoplasmic granules and smaller granular cells (diameter ~ 6 µm) containing smaller cytoplasmic granules (Figure 3D, E). Hyalinocytes were similar in shape to granular cells with more hyaline cytoplasm (without granules), and cell sizes ranging from 5–12 µm in diameter (Figures 3B, D, E). Blast-like cells were round-shaped and smaller than the other two types (1–5 µm) with minimal cytoplasm (Figure 3C). Leaf oyster granular cells were also observed forming pseudopodia (Figure 3F, G). SEM confirmed the haemocyte cell types: granular cells (Figures 4A, B), hyalinocytes

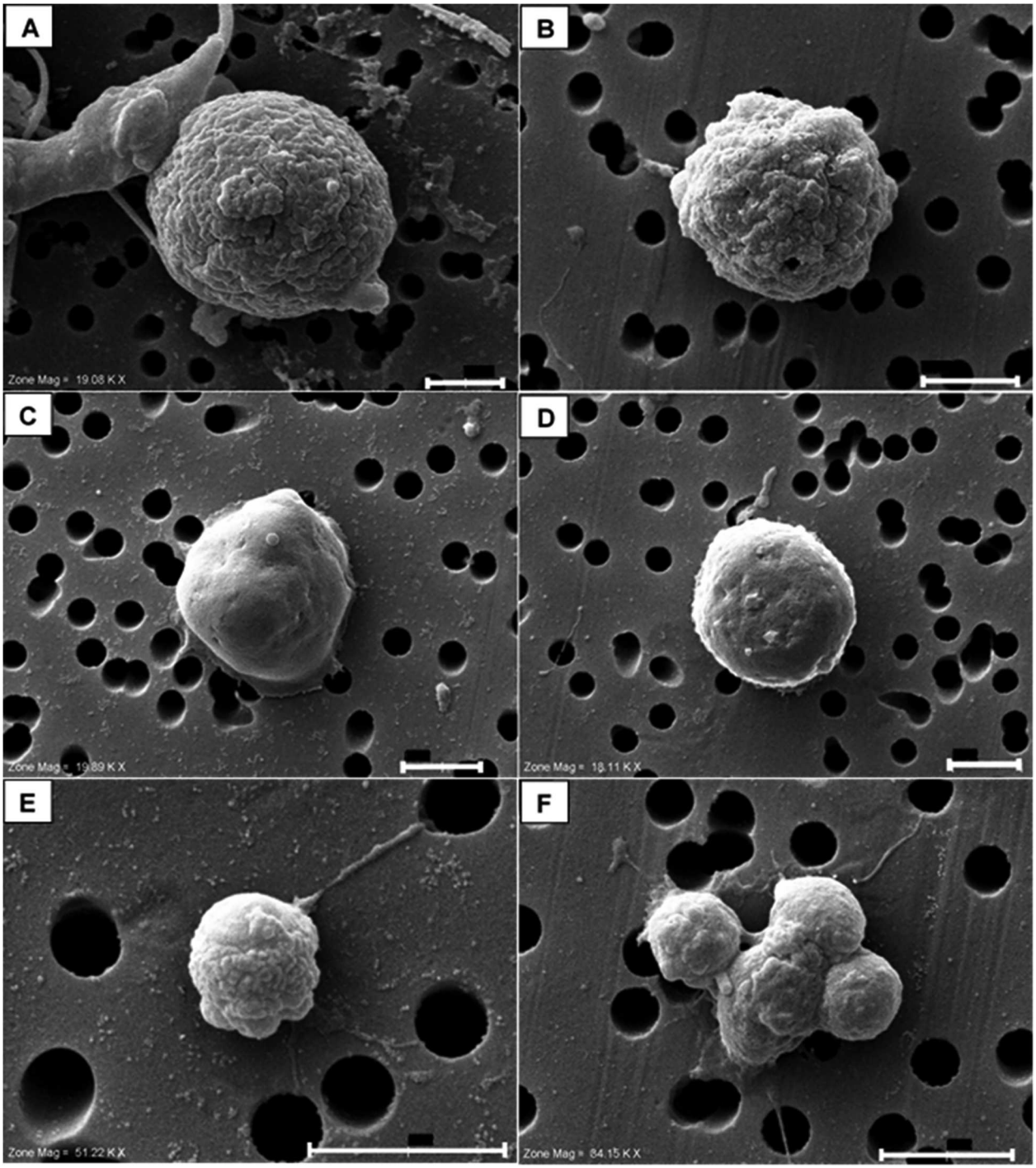

Figure 4. Leaf oyster haemocytes observed in SEM. **A & B** – granulocytes. **C & D** – hyalinocytes. **E & F** – blast-like cells (Scale bars = 2 µm)

(Figures 4C, D) and blast-like cells (Figures 4E, F). Moreover, in SEM there was an observation of short dendritic-like cytoplasmic extensions on the surface of haemocytes (Figure 5).

Acquisition density plots obtained from flow cytometry showed that the haemocyte population occurs on a continuum of cell size and texture (e.g. Figure 6). It was possible to differentiate the three cell populations. The largest cells had more complexity, consistent with granular cells. The smallest cells had lower complexity, as expected for blast-like cells, whereas the hyalinocytes fell in between these other two cell populations, a wider range of sizes and degrees of complexity. The proportions of the granular cells, hyalinocytes and blast-like cells were 8.4 (±2.3) %, 76.2 (±1.9) % and 15.3 (±1.6) % (Figure 6).

Haemocyte counts, bacterial loads and water quality parameters

The mean THC of the leaf oysters collected from Tweed, Brunswick and Woolgoolga sites were 1.25×10^6, 3.89×10^5, 2.89×10^6 cells per mL, respectively (Figure 7). One-factor PERMANOVAs revealed that THC

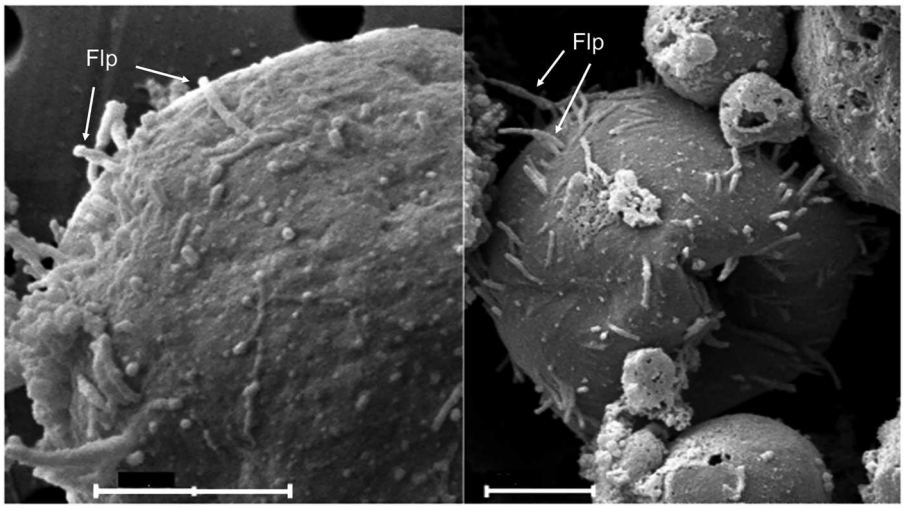

Figure 5. Formation of short dendritic-like cytoplasmic extensions (Flp) by haemocytes of leaf oysters (Scale bars = 2 µm)

and haemolymph CFU were significantly different among the three sites (THC – $MS_{2,8}$ = 3.1692, Pseudo-F = 11.443, P (perm) = 0.002 and CFU Haemolymph – $MS_{2,8}$ = 7.4689, Pseudo-F = 42.193, P (perm) = 0.004). Higher bacterial CFU was recorded in the haemolymph of leaf oysters from Tweed and Brunswick sites, whereas lower bacterial loads were seen in Woolgoolga Lake (Figure 7).

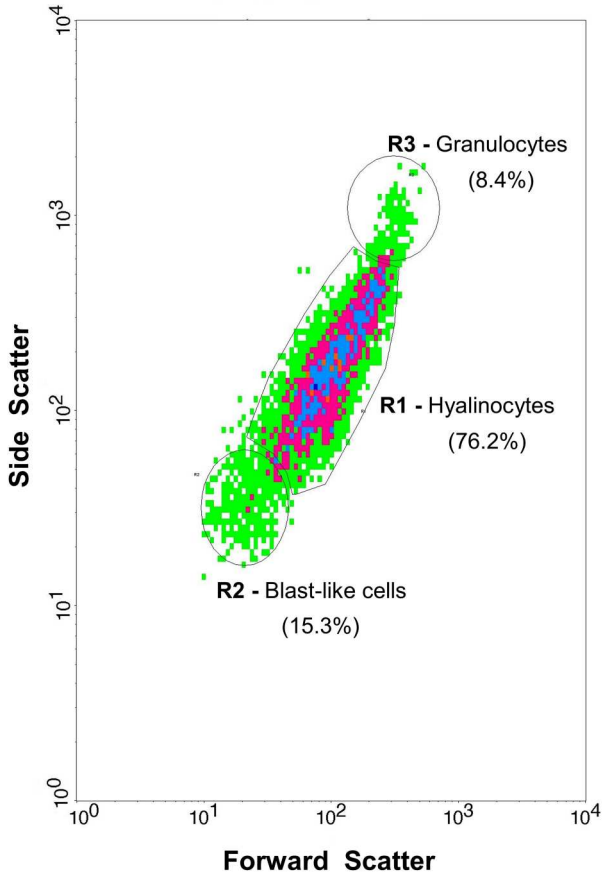

Figure 6. An acquisition density plot obtained from a representative flow cytometric analysis of leaf oyster haemolymph; Y axis: side scatter corresponding to the cellular complexity of the haemocytes and X axis: forward scatter corresponding to the size of the cells. R1, R2 and R3 represent three different cell populations.

Water quality parameters such as pH, salinity, DO%, temperature, turbidity and organic matter (POC, DOC and TP) were markedly different among the sites (Table 1). Significant differences between sites were also detected after combining all the water quality physicochemical parameters and bacterial loads (CFU) in the water into one multivariate analysis (PERMANOVA: $MS_{2,8}$ = 34.006, Pseudo-F = 17.019, P(perm) = 0.003). The PCO plot showed the separation of the sites along PCO1 and PCO2, explaining 88.9% of the total variation of water quality and haemolymph parameters among the sites (Figure 8). Woolgoolga site was clearly separated from Tweed and Brunswick sites with high temperature and THC along PCO1. Brunswick site was separated from Tweed site with higher POC, PON+DON and CFU in water, whereas Tweed site was separated from the other two sites with high turbidity, total P and bacterial CFU in haemolymph. The PCO plot revealed that CFU in haemolymph was higher at sites with high salinity, DO%, pH, DOC and total phosphorus. Bacterial CFU in the water was higher at sites with high particulate organic carbon and particulate and dissolved organic nitrogen (Figure 8). Moreover, THC in leaf oysters was positively correlated with water temperature, but negatively correlated with DO%, DOC, pH, salinity, total P and bacterial loads in both haemolymph and water (Figure 8).

After removing collinear water quality parameters from the DBLM (See Supplementary Table 1), marginal tests revealed significant negative relationships between leaf oyster THC and CFU in both water (R^2 = 0.6025) and haemolymph (R^2 = 0.4284) (Figure 8 and Supplementary Figure 1). The overall BEST solution identified CFU in water, PON + DON and DIN as the main drivers of THC, which explained 77% of the variation (AIC = -6.4253, R^2 = 0.7735, RSS = 1.812). In

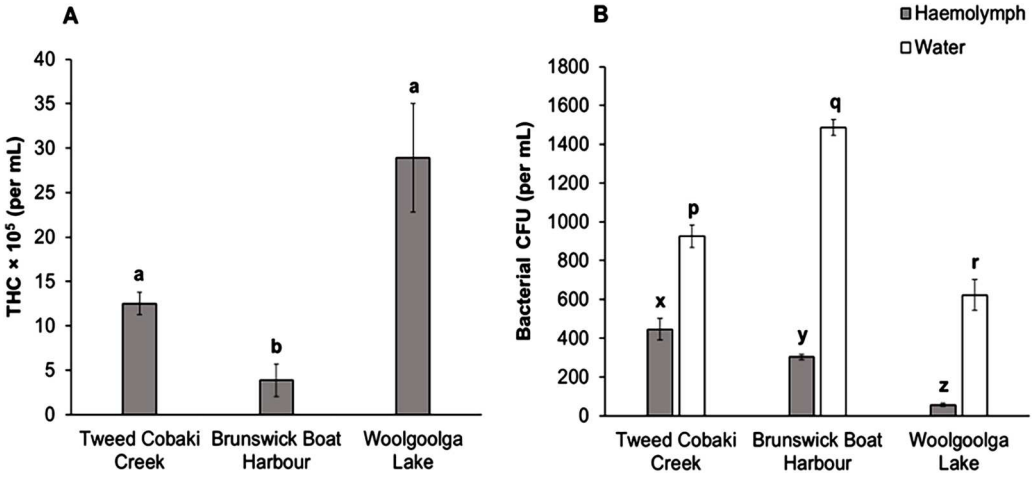

Figure 7. Total haemocyte counts (THC) (**A**) and bacterial colony forming units (CFU) of leaf oyster haemolymph and water (**B**) at three northern NSW estuaries in Australia. Error bars represent the standard error of the mean and the letters above the bars indicate statistically significant differences.

addition, DBLM marginal tests revealed CFU in haemolymph having a significant ($P < 0.05$) negative relationship with DIN ($R^2 = 0.5994$) and a significant positive relationship with TP ($R^2 = 0.7432$) (Figure 8 and Supplementary Figure 1). The overall BEST solution, explaining 88% of the variation in haemolymph CFU, identified PON + DON, DIN and CFU in water as the main drivers (AIC = -11.82, $R^2 = 0.8756$, RSS = 0.9950).

Discussion

This study provides the first characterisation of haemocytes in *I. ephippium*, a bivalve from the Family Isognomonidae. Granular cells, hyalinocytes and blast-like cells were observed in *I. ephippium* by light microscopy and SEM, with their relative proportions calculated from flow cytometry counts. Granularity, pseudopodia and dendritic-like cytoplasmic extensions were also observed in leaf oyster haemocytes. Significant differences in THC and bacterial CFU in haemolymph were observed among the three study sites, along with differences in the water quality parameters. THC was lower at sites with higher dissolved and particulate nitrogen in combination with high CFU in the water, which in turn correlated with higher DOC, pH, salinity and DO%, as well as lower water temperatures. Thus, a combination of physicochemical properties in the water may be driving the bacterial loads in the water, in addition to potential direct impacts on the haemocytes.

Light microscopy observations identified three morphologically distinct haemocyte types; granular cells, hyalinocytes, and blast-like cells in leaf oysters. Previous microscopy studies have reported the same three cell types in oysters and mussels (Kuchel et al. 2010; Donaghy and Volety 2011; Hong *et al*. 2014; Ewere *et al*. 2020), as well as other molluscs, e.g. octopus and abalone (Travers *et al*. 2008; Castellanos-Martínez *et al*. 2014). More detailed characterisation of hemocyte subpopulations may be possible by combining cell collection from flow cytometry with functional assays (e.g. Silva Freire *et al*. 2023) and molecular characterisation (e.g. Yang *et al*. 2024).

Granular cells are characterised by numerous cytoplasmic granules and are highly defensive haemocytes containing hydrolytic enzymes that assist in the intracellular killing of pathogens (Kuchel *et al*. 2010). Two types of granular cells were observed in leaf oysters, some with large cytoplasmic granules (cell diameter 15 µm) and others with smaller cytoplasmic granules (cell diameter 6 µm). Studies have also reported granular cells with large and small cytoplasmic granules in several bivalves, ranging from 4–10 µm in the pearl oyster *Pinctada imbricata* (Röding, 1798) (Kuchel *et al*. 2010), from 10.9–16.3 µm in the Iwagaki oyster *Crassostrea nippona* (Seki, 1934) (Hong *et al*. 2014) and from 4.6 to 14.4 µm in the clams *Ruditapes philippinarum* (Adams & Reeve, 1850) (Liu and Zhao 2018). In addition, SEM imaging revealed the rough and uneven outer appearance of leaf oyster granular cells, which is consistent with observations of *P. imbricata* (Kuchel *et al*. 2010) and *Pinctada fucata* (Gould, 1850) (Li *et al*. 2015) granulocytes. Overall, leaf oyster granular cells were found to be similar to the other bivalve species in terms of common morphological characteristics such as cell size, outer appearance and granularity and functional characteristics like pseudopodia formation.

Hyalinocytes were identified as the dominant cell population in leaf oyster haemolymph (78%). The observed size range of leaf oyster hyalinocytes is 5–12 µm, which is consistent with the hyalinocyte sizes of other bivalve species such as *Pteria hirundo* (Linnaeus, 1758) (7.2–13.1 µm) (Vieira *et al*. 2017), *C. nippona* (6–15 µm) (Hong *et al*. 2014) and *R. philippinarum* (3.4–13.4 µm) (Liu and Zhao 2018).

Table 1. Water quality data (±Standard Deviation) of the sampling sites at three northern NSW estuaries.

	PERMANOVA results					
Parameter	$MS_{2,8}$	Pseudo-F	*P* (perm)	Tweed Cobaki Creek	Brunswick Boat Harbour	Woolgoolga Lake
CFU in water (per mL)	575480	51.229	**0.006**	926.67 (±58.12)[a]	1486.67 (±40.55)[b]	623.33 (±78.81)[c]
pH	0.0787	44.547	**0.035**	8.09 (±0.05)[a]	8.11 (±0.02)[a]	7.82 (±0.05)[b]
Salinity (ppt)	186.69	4274.2	**0.031**	35.25 (±0.06)[a]	35.20 (±0.31)[a]	21.56 (±0.17)[b]
Dissolved Oxygen (%)	81.323	481.52	**0.003**	94.30 (±0.20)[a]	97.43 (±0.21)[b]	87.27 (±0.65)[c]
Temperature (°C)	24.513	774.1	**0.005**	19.72 (±0.03)[a]	19.60 (±0.01)[b]	24.61 (±0.31)[c]
Turbidity (NTU)	3.8491	21.176	**0.044**	4.37 (±0.41)[a]	2.36 (±0.60)[b]	4.27 (±0.14)[a]
Particulate organic carbon (µmol/L)	49188	7.9334	**0.03**	26.10 (±22.76)[a]	247.33 (±134.21)[b]	25.00 (±8.33)[a]
Dissolved organic carbon (µmol/L)	8773.1	29.921	**0.011**	338.89 (±12.73)[a]	361.11 (±20.97)[a]	258.33 (±16.67)[b]
Particulate and dissolved organic nitrogen (µmol/L)	2386.1	5.5174	0.059	19.60 (±0.68)[a]	75.53 (±1.62)[b]	53.83 (±35.98)[ab]
Dissolved inorganic nitrogen (µmol/L)	4.1088	4.5873	0.069	2.79 (±1.26)	4.60 (±0.89)	4.98 (±0.52)
Total phosphorous (µmol/L)	0.1503	13	**0.043**	1.72 (±0.18)[a]	1.61 (±0.002)[ab]	1.29 (±0.00)[b]

Note: Superscript letters indicate significant differences among the sites.

Hyalinocytes contain very few or no cytoplasmic granules and are usually more morphologically heterogeneous than granular cells (Aladaileh *et al*. 2007). Leaf oyster hyalinocytes had more or less smooth and even cell surface according to light microscopy and SEM imaging. Similar observations have been reported for hyalinocytes of pearl oysters, *P. imbricata* (Kuchel *et al*. 2010) and *P. fucata* (Li *et al*. 2015). Blast-like cells are typically very small agranular cells with a large nucleus and scanty cytoplasm lacking organelles like Golgi complex and endoplasmic reticulum (Sun *et al*. 2006; Li *et al*. 2015). They were previously thought to be a special type of hyalinocyte (Sun *et al*. 2006); however, they were later identified as undifferentiated haemocyte stem cells, freely circulating in the haemolymph (Donaghy *et al*. 2009). In leaf oysters, we observed these in the size range of 1–5 µm by light microscopy and SEM. The stem cell nature of the blast-like cells explains the gradient of cell size and complexity observed using flow cytometry.

In SEM, we observed the formation of dendritic-like cytoplasmic extensions on the surface of some haemocytes. Kuchel *et al*. (2010) have reported similar surface structures in *P. imbricata* haemocytes and called them 'filopodia'. It appears that hyalinocytes in leaf oysters also possess similar structures, which can be described as cytoplasmic extensions. These extensions can form interconnecting bridges that shorten and thicken to bring the haemocytes closer together (Fisher 1986).

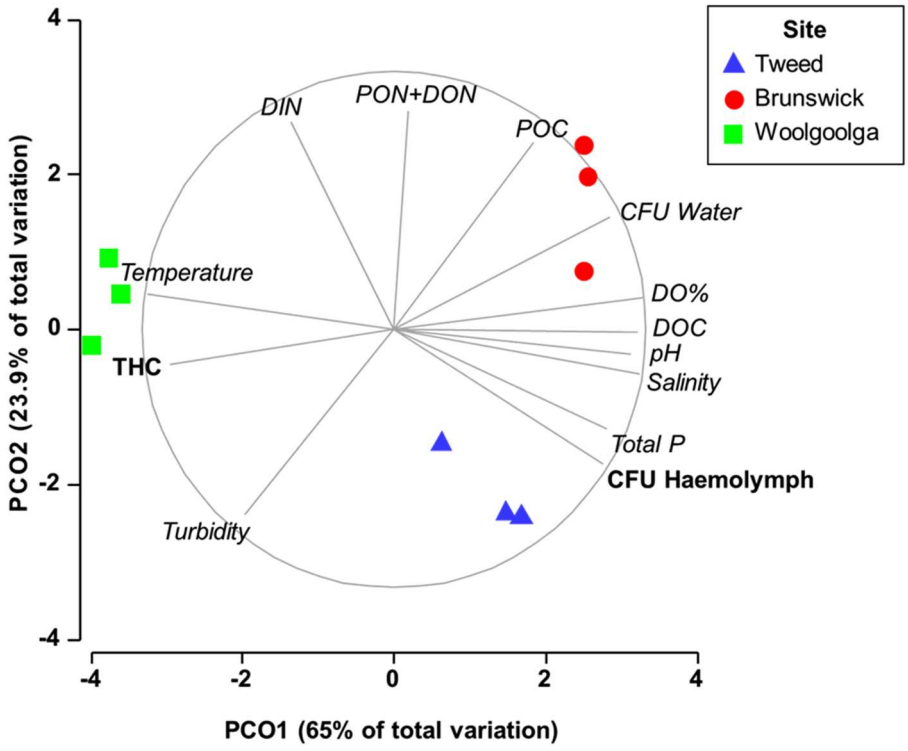

Figure 8. Principal Coordinates Analysis (PCO) plot showing the variation of water quality variables and haemolymph parameters of leaf oysters. Data were normalised and resemblance matrix was based on Euclidean distance. Vector overlays represent the main drivers of the variation (DIN – dissolved inorganic nitrogen, PON + DON – particulate and dissolved organic nitrogen, POC – particulate organic carbon, CFU – bacterial colony forming units, DO% – dissolved oxygen, DOC – dissolved organic carbon).

Kuchel *et al*. (2010) reported that small granular cells project multiple actin-rich filopodia, but we observed these cytoplasmic structures extending from the haemocytes that appeared to be small hyalinocytes (diameter around 6 µm). This is consistent with the fact that hyalinocytes play a major role in haemocyte aggregation and wound healing (Aladaileh *et al*. 2007). Haemocyte aggregation typically does not occur in bivalves unless they are stimulated/activated (Fisher 1986). Contact and friction with foreign objects, or mechanical agitation such as withdrawal into a syringe, often serve as stimuli (Takatsuki 1934). In addition, bacterial infection will cause cell aggregation and this can result in a coincidental drop in the number of circulating haemocytes (Parisi *et al*. 2008; Vieira *et al*. 2017). Consequently, high bacterial loads in the haemolymph could stimulate a drop in the THC. This was observed in our distance-based linear model for THC with water quality parameters, where marginal tests revealed a significant negative correlation between THC and haemolymph bacterial CFU.

Flow cytometry has previously been used to investigate the haemocyte subpopulations in bivalve haemolymph (Donaghy *et al*. 2010; Wang *et al*. 2012; Hong *et al*. 2014). Studies on some bivalves such as *C. gigas* (Thunberg, 1793), *Perna viridis* (Linnaeus, 1758) and *Ostrea chilensis* (Küster, 1844) have observed a continuum of cell size and complexity in density plots (Donaghy *et al*. 2010; Donaghy and Volety 2011; Rolton *et al*. 2020), similar to what we observed in leaf oyster haemocytes, whereas other studies reported separated distinct haemocyte populations in *C. ariakensis* (Fujita, 1929) and *C. nippona* (Donaghy *et al*. 2009; Hong *et al*. 2014). Percentages of granular cells, hyalinocytes and blast-like cells in *C. gigas* collected from the field were 8.7 (±7.0) %, 79.0 (±5.6) % and 10.7 (±5.8) %, respectively (Donaghy *et al*. 2010), which are consistent with the proportions we observed in leaf oysters; 8.4 (±2.3) %, 76.2 (±1.9) % and 15.3 (±1.6) %, respectively. However, other studies have found a lower dominance of the hyalinocyte population; for example percentages of granular cells, hyalinocytes and blast-like cells were 21.8 (±8.5) %, 57.9 (±9.8) % and 16.3 (±5.1) %, respectively in the Suminoe oyster *C. ariakensis* (Donaghy *et al*. 2009), 20.0 (±1.0) %, 66.8 (±1.9) % and 5.7 (±0.6) %, respectively in *C. nippona* (Hong *et al*. 2014), and 38%, 46% and 15% respectively in *S. glomerata* (Aladaileh *et al*. 2007). There were only granular cells and hyalinocytes observed in the green-lipped mussel *P. viridis* with proportions of 59.3 (±5.2) % and 40.8 (±5.2) %, respectively (Donaghy and Volety 2011). Therefore, it is possible that the proportion of haemocyte populations vary depending on the bivalve species. However, all these studies just provide a snapshot of the haemocyte populations taken from one population in time and space.

Across three different estuaries, we found variation in the THC of the leaf oysters ranging between 3.89×10^5–2.89×10^6 cells per mL. Previous studies have reported THCs; 2.0×10^5–1.08×10^6 cells per mL in *C. nippona*, 7.06×10^5 cells per mL in *C. ariakensis* (Donaghy *et al*. 2009), 4.9×10^6 cells per mL in *P. fucata* (Li *et al*. 2015), 4.01×10^5 cells per mL in *C. gigas* (Donaghy *et al*. 2010). THC of oysters can also vary with the season (Soudant *et al*. 2004), sudden temperature changes (Hégaret *et al*. 2003), pathogenic invasions (Labreuche *et al*. 2006), pesticide exposure (Ewere *et al*. 2020) and reproductive status (Brokordt *et al*. 2019). Along with significant differences in THC of leaf oyster populations between estuaries, we found significant differences in the bacterial load in the haemolymph and the water, as well a number of water quality parameters between the three estuaries. Multivariate analysis using DBLM and PCO plots revealed that a range of physicochemical parameters could be contributing to differences in bacterial loads in the water and coincidently, the THC of leaf oysters. The PCO plot revealed that bacteria loads were highest at sites with high salinity, DO%, pH and DOC, with bacteria in the water higher at sites with high particulate organic carbon and dissolved and particulate nitrogen. These factors combined explained most of the variation in THC, which could relate to indirect effects of water quality and nutrient availability on the bacteria, as well as direct effects of bacteria causing THC aggregation.

THC in leaf oysters was positively correlated with water temperature, but negatively correlated with DO%, salinity and bacterial loads. Temperature has been previously shown to impact THC in bivalves. Upon exposure to 15°C, 20°C and 25°C, THC of three bivalve species, *C. gigas*, *Mytilus galloprovincialis* (Lamarck, 1819) and *Katelysia rhytiphora* (Lamy, 1935), significantly increased at 25°C (Rahman *et al*. 2019). Exposure of the clam *Chamelea gallina* (Linnaeus, 1758) to 30°C significantly increased the THC compared to the lower temperatures of 20°C and 25°C (Monari *et al*. 2007). Temperature stimulates the growth of some marine pathogens like *Vibrio* (Larsen 1984; Billaud *et al*. 2022), but in our study we found temperature was negatively correlated with total bacterial loads, which could be driven by lower salinity at the site with higher temperature. Bacterial CFUs in leaf oyster haemolymph did not correspond directly to bacterial loads in water (e.g. CFU water was highest in Brunswick River but CFU haemolymph was higher in Tweed River). Previous studies have demonstrated that the internal microflora of some bivalves can be different from their surrounding water (Prieur *et al*. 1990; Beleneva *et al*. 2003). This could be attributed to the ability of filter-feeding bivalves to selectively eliminate some bacteria like *E. coli*, as has been observed in *Anodonta cygnea* (Linnaeus, 1758)

(Antunes *et al*. 2010). Single point sampling of THC counts and CFUs in leaf oysters, along with water quality assessment, can be useful for assessing the immediate state of health of local populations. However, longer-term monitoring is required for spatial comparison of the different populations to assess their immune health across a range of different weather conditions and during different times in the reproductive cycle.

This study provides baseline information on the haemolymph and cell mediated immune system of *I. ephippium* in the Family Isognomonidae. We were able to identify and characterise three types of haemocytes, granular cells, hyalinocytes and blast-like cells that play important roles in bivalve immunity. Granular cells that are large in size form pseudopodia and are responsible for phagocytosis and intracellular killing of foreign pathogens, as well as shell regeneration. The largest population of leaf oyster haemocytes, the hyalinocytes, showed less internal cell complexity, but the formation of dendritic-like cytoplasmic extensions indicates important roles in cell aggregation and wound healing. Blast-like cells, known as undifferentiated stem cells, were smallest in size and develop into granular cells and hyalinocytes. As well as the water quality parameters, haemocyte counts and bacterial loads were significantly different among the three estuaries. These parameters show complex relationships indicating a range of direct and indirect effects on leaf oyster haemocytes. Outcomes of this study provide important information that contributes to knowledge of the leaf oyster immune system.

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