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Invited review

The Stress–Immunity Axis in Shellfish

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Abstract

It is a difficult task to describe what constitutes a 'healthy' shellfish (e.g., crustacean, bivalve). Visible defects such as discolouration, missing limbs or spines, fouling, lesions, and exoskeletal fractures can be indicative of underlying issues, senescence, or a 'stressed' animal. The absence of such symptoms is not evidence of a disease-free or a stress-free state. Now, more than ever, aquatic invertebrates must cope with acute and chronic environmental perturbations, such as, heatwaves and cold shocks, xenobiotic contaminants, intoxication events, and promiscuous pathogens expanding their host and geographic ranges. With that in mind, how does one determine the extent to which shellfish become stressed *in situ* (natural) or in cultured (artificial) settings to enhance disease susceptibility? Many biomarkers – predominantly biochemical and cellular measures of shellfish blood (haemolymph) – are considered to gauge immunosuppression and immunocompetence. Such measures range from immune cell (haemocyte) counts to enzymic activities and metabolite quantitation. Stressed invertebrates often reflect degraded conditions of their ecosystems, referred to as environmental indicators.

We audit briefly the broad immune functions of shellfish, how they are modulated by known and emerging stressors, and discuss these concepts with respect to neuroendocrinology and immunotoxicology. We assert that chronic stress, alone or in combination with microbial, chemical and abiotic factors, increases the risk of infectious disease in shellfish, exacerbates idiopathic morbidity, and reduces the likelihood of recovery. Acute stress events can lead to immunomodulation, but these effects are largely transient. Enhancing our understanding of shellfish health and immunity is imperative for tackling losses at each stage of the aquatic food cycle and disease outbreaks in the wild.

Keywords: disease connectivity; haemolymph biomarkers; immunocompetence; immunosuppression; innate immunity; neuroendocrinology; microplastics

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1. Background

Providing enough food and nutrition to sustain the health of a growing global population reaching over 9 billion within the next 30 years is not without its challenges; compounded by the uncertainties that climate change brings such as stochastic weather extremes and disease outbreaks (Cann *et al.,* 2013; Fischer and Knutti, 2015; Shields, 2019). Expanding aquatic food production of finfish and invertebrates is considered a viable approach to alleviate food security concerns as they already represent the most dominant food commodities traded, and evidence of their high nutritional value is incontrovertible (Béné *et al.,* 2015 & 2016). However, disease represents the primary constraint to sustainable intensification and costs the seafood industry billions of dollars in losses annually (Stentiford *et al.,* 2017; FAO 2020). Production of aquatic foods, notably shellfish, display 'boom and bust cycles' in which several years (often decades) of exceptional growth are followed by collapse (reviewed by You and Hedgecock, 2019). Communicable diseases of shellfish (e.g., viruses, bacteria) are the cause of such collapses, and can lay waste to an entire industry. For example, white spot syndrome virus outbreaks in black tiger shrimp *Penaeus monodon* led to mass mortalities (>95%) in China and Thailand in the 1990s and 2000s, resulting in the emergence of Pacific white shrimp *Penaeus vannamei* as the dominant produce (Flegel, 2012). Too often, there is a lag in pathogen diagnosis – usually two to three years after an outbreak – therefore, the majority of research on host-pathogen antibiosis is reactive rather than proactive.

Due to a lack of standardised industry practices, substantial financial losses and compromised nutritional quality of shellfish are common occurrences (Figure 1). From conditions within culture ponds to on-site/on-vessel handling and arrival at markets, live shellfish endure emersion, acute temperature fluctuations, food deprivation and physical damage (reviewed by Fotedar and Evans, 2011). These stresses can have profound effects on the animal condition and welfare, characterised traditionally by behavioural changes, and more recently, by using immune-markers such as haemocyte counts and quantitating levels of macromolecules (total protein, glucose, L-lactate) in the haemolymph (Table 1) or other tissues like the muscle, gills and digestive glands. Establishing a reliable set of health status indicators for shellfish in natural and commercial settings would be a major asset for detecting and managing the impacts of stressors and disease control within the aquatic food sector, yet it is an

unlikely outcome (no 'one size fits all' scenario). This is made even more difficult due to the manifold impacts of existing and emerging environmental agitators, e.g., plastics, pharmaceuticals, pesticides.

The term shellfish subsumes a vast diversity of invertebrate (animal) forms and natural histories, but for the purpose of this review, we will focus mostly on commercially important, edible taxa that are wild-caught or cultured (crustaceans and molluscs). According to the Food and Agriculture Organisation (FAO), crustaceans and molluscs represent 168 out of the 598 known aqua-cultured species globally (FAO 2018 and 2020). Moreover, crustaceans and molluscs account for ~95% of cultured aquatic invertebrates, yet a knowledge deficit exists with respect to their health and immunity (FAO, 2019). Enhancing our understanding of shellfish immune defences, i.e., healthy, stressed and diseased states, aligns seamlessly with the World Health Organisations 'One Health' approach to food safety standards and the control of zoonoses (Figure 1). We present an overview of shellfish immunocompetence, how acute and chronic stressors may lead to immunosuppression, and the limitations of routine experimental measures. In doing so, we describe the complex interactions between hosts, pollutants, and their dynamic aquatic environments.

2. A brief audit of shellfish innate immunity

Many contemporary articles have reviewed the biological defences of shellfish, specific immune pathways/cascades, and effectors across tissue, cellular and molecular levels (e.g., Cerenius et al., 2010b; Wang and Wang, 2013; Coates and Nairn, 2014; Allam and Raftos, 2015; Rowley, 2016; Gerdol, 2017; Hauton, 2017; Cerenius and Söderhäll, 2018; Tassanakajon *et al.,* 2018; Liu *et al.,* 2020) – yet, few have addressed the stress-immunity axis (e.g. Adamo, 2012)

The shells of molluscs, carapaces of crustaceans, and tests of echinoids are physical barriers reinforced with minerals, proteins, chitins and pigments (e.g., melanin, naphthoquinones), which provide protection as well as limiting the number of routes available for pathogen entry (e.g., mouth, gills). The underlying epithelial barriers of exoskeletons produce antimicrobial and signalling factors to alert neighbouring tissues to damage, and to initiate coagulation and wound repair (Cerenius and Söderhäll,

2011). In crustaceans, vitellogenin-related proteins (e.g. coagulogen; Hall *et al.,* 1999) in the haemolymph plasma are crosslinked at wound sites by a group of pleiotropic proteins called transglutaminases to stop fluid loss (Martin *et al.,* 1991; Lin *et al.,* 2008; Liu *et al.,* 2011; Fagutao *et al.,* 2012; Sirikharin *et al.,* 2019). This clotting reaction is very efficient in healing wounds compared to that occurring in vertebrates (Cerenius and Söderhäll, 2011). Invading bacteria and fungi are also entrapped within these gel-like clot structures to prevent septicaemia and mycosis. The clot is further reinforced with the deposition of insoluble melanotic polymers – courtesy of phenoloxidase (PO) enzyme activities and autocatalysis of unstable quinone intermediates, which also provides a localised burst of antimicrobial oxidising and nitrosative by-products (Cerenius *et al.,* 2010a; Whitten and Coates, 2017). Similar haemostatic components have been characterised in molluscs, e.g., transglutaminase in oysters, and POs in mussels and limpets (Coles and Pipe 1994; Gueguen *et al.,* 2003; Quinn *et al.,* 2020).

Once noxious agents make their way into the haemocoel (body cavity), they must contend with the coordinated efforts of cellular and humoral immunity. Circulating haemocytes (or coelomocytes) tend to be a heterogeneous population of immune cells that recognise non-self (exoplasmic) moieties of pathogens (Jiravanichpaisal *et al.,* 2006; Gerdol *et al.,* 2018). Pathogen-associated molecular patterns (PAMPs) – bacterial peptidoglycans (lipopolysaccharides, lipoteichoic acids), fungal and algal β-glucans, and viral nucleic acids – are intercepted by pathogen recognition proteins (PRPs) dissolved within the lymph and/or spanning the membranes of haemocytes (Cerenius and Söderhäll, 2018). Peptidoglycan-binding proteins (Wei *et al.,* 2012b; Vaseeharan, 2012), C-type lectins (Wang *et al.,* 2011; Wei *et al.,* 2012a), β-glucan binding proteins (Cerenius *et al.,* 1994; Zhao *et al.,* 2009; Itoh et al., 2010), serine-protease homologues (Sriphaijit *et al.,* 2007; Zhang *et al.,* 2009), members of the immunoglobulin superfamily (Dscam, FREPs) amongst many other PRPs and their transcriptional variants have been functionally characterised in shellfish (Cooper and Alder, 2006; Ghosh *et al.,* 2011). Signal transduction cascades, arising from the activation of cellular receptors, trigger the translocation of transcription factors into the nucleus to switch-on the expression of immune-associated mRNAs. There are vast numbers of studies dedicated to immune activation (Humphries and Yoshino, 2003 [molluscs]; Li and Xiang, 2013 [shrimp]). The Toll, immune deficiency (Imd) and Jak/STAT pathways as well as their ligands are best characterised and appear to be

highly conserved among invertebrates – at least for arthropods (Palmer and Jiggins, 2015).

Despite the immune cell heterogeneity observed among shellfish (e.g., crustaceans have three types, whereas sea urchins have at least four), phagocytosis, encapsulation, nodule formation, and cytotoxic degranulation events are conserved responses. Pathogens can be ingested and destroyed intracellularly through respiratory burst (Bell and Smith, 1994), immobilised in large numbers through the formation of haemocyte palisades (usually melanised) and starved of oxygen and nutrients. Concurrently, immune factors such as lysozyme, antimicrobial peptides and proteases are released to neutralise virulence factors and compromise the surface of microbes making them leaky (reviewed by Jiravanichpaisal *et al.,* 2006). Vast numbers of antimicrobial peptides have been characterised, especially for crustaceans (reviewed by Smith and Dyrynda, 2015; Tassanakajon *et al.,* 2018), and their expression are not limited to haemocytes. Acute phase proteins such as lysozyme are found invariably in crustaceans and molluscs – with a clear role in bacterial wall degradation and lysis (muramidase activity; Sotelo-Mundo *et al.,* 2003; Bachali *et al.,* 2002; Xue *et al.,* 2010; Gopalakrishnan *et al.,* 2011).

The proPhenoloxidase (proPO) activation cascade is emblematic of the innate immune response of many aquatic and terrestrial invertebrates, as it facilitates the early steps of melanisation (reviewed by Cerenius *et al.,* 2008). Melanotic polymers are employed by the host to immobilise microbial intruders (microbiostatic), and the toxic by-products of catalytic activities inflict damage to microbes and parasites – as determined in crustaceans (Cerenius *et al.,* 2010a), chelicerates (Coates and Talbot, 2018), and gastropods (Quinn *et al.,* 2020). The proPO cascade is a striking example of the intersection between humoral and cellular defences. PAMP detection by haemocyte-bound receptors trigger the release of proPO via exocytosis and the extracellular proPO zymogen is cleaved proteolytically at the *N*-terminus to activate the enzyme (Jearaphun*t et al.,* 2014). Activated POs help to produce melanins for microbiostatic and microbicidal purposes, in addition to wound repair. Recently, Sirikharin *et al.* (2020) demonstrated a novel link between the cleaved *N*-terminal peptide of proPO and increased haematopoiesis in crayfish (*Pacifastacus leniusculus*). The release of proPO from haemocytes tends to be followed by

apoptosis, and so the liberated *N*-terminal fragment may in fact be a cryptide tasked with signalling for new haemocytes to replace those spent in the immediate inflammatory response.

3. Environmental conditions and markers of stress and immunity

Natural populations of shellfish, especially those of commercial value, are under constant threat of overexploitation and environmental degradation. Gross shifts in external conditions from heatwaves and cold shocks (climate change) to pollutants and marine intoxication episodes render populations vulnerable. Both biotic and abiotic stresses set-off a complex chain of costly cellular and molecular events in order to maintain homeostasis (i.e., allostasis), which over time are known to weaken biological defences in vertebrates and invertebrates alike (Ottaviani and Franceschi, 1996; Table 2). When we consider 'stress' in vertebrates, we think of the release of neuroendocrine chemicals (e.g., catecholamines and glucocorticoids), which target diverse tissues to redirect resources to address the stressor (Ottaviani and Franceschi, 1996; Malham *et al..,* 2003; Adamo, 2012). Immune-regulation is inextricably linked to neuroendocrine signalling and the management of stress (Webster *et al.,* 2002) – alas, the neuroendocrine-stress-immunity axis is poorly understood in shellfish by comparison to finfish and humans. A notable exception is the co-option of catecholaminergic neurotransmitters as essential substrates for PO-mediated immunity in shellfish.

The term 'phenoloxidase' (PO) may incorporate four functionally distinct proteins, namely catecholoxidase, tyrosinase, laccase, and haemocyanin (Coates and Costa-Paiva, 2020). All have demonstrated a capacity to oxidise L-DOPA and dopamine into quinones (DOPAchromes), with haemocyanin-derived phenoloxidase (HC-d PO) also able to generate products from (nor)epinephrine (Jaenicke and Decker, 2008; Coates and Talbot, 2018). Catecholamines are generated from amino acids, phenylalanine → L-tyrosine → L-DOPA → dopamine, norepinephrine and epinephrine. Beyond immunity, POs (amongst other enzymes) use these biogenic amines for developmental processes, cuticle hardening post-ecdysis (crustaceans), and shell biomineralization (bivalves; Sun *et al.,* 2015). Exposing shellfish to acute or chronic stress leads to increases in catecholamine concentrations within the haemolymph,

e.g., epinephrine levels doubled in *P. vannamei* forced to flee for 1 minute (Aparicio-Simon *et al*. 2010). Emersion (air exposure), temperature (17 to 28ºC) and salinity (31 to 20 ppt) changes also stimulated increases in haemolymph levels of biogenic amines in the scallop *Chlamys farreri* (Chen *et al.,* 2008). In a recent study, silencing of the gene encoding dopamine beta-hydroxylase (DBH) in *P. vannamei*, or chemical inhibition of its activity using disulfiram, led to decreases in immune markers (haemocyte numbers, phagocytic activity, PO activity), and enhanced susceptibility to *Vibrio alginolyticus* (Cheng *et al.,* 2017).

Acute stress events can lead to transient spikes in haemolymph catecholamine levels or even immune-stimulation (Table 2). Interestingly, 'stress management' has been trialled as a disease control strategy for finfish aquaculture (Sung *et al.,* 2011)**,** wherein brief temperature shocks lead to the upregulation of heat shock proteins that are linked to induced thermotolerance and cross tolerance (protection against other stressors). Regarding shellfish, there now exists a sizable body of literature describing so called 'immune-dysfunction' associated with chronic stress (reviewed by Le Moullac and Haffner, 2000; Mydlarz *et al.,* 2006; Ellis *et al.,* 2011). Perhaps it is more accurate to say that chronic stress affects immune-vigour, which we will define as the strength available to fight infection and how effective that force is to enable recovery. Variations in temperature, pH, dissolved gasses (hypoxia/hypercapnia), salinity and nutrient over-enrichment (e.g., ammonia-N) impose major physiological burdens (including immune-vigour) on shellfish when they fall outside of their tolerance ranges (Table 3) – such parameters are exacerbated by over-crowding in managed culture/capture settings (e.g., penaeid shrimp).

Crustaceans and molluscs are ectotherms; therefore, physiologic processes are influenced heavily by temperature fluctuations of the surrounding waters. Temperature can also impact pathogenicity and disease outcomes – for example, crayfish (*Pacifastacus leniusculus, Astacus astacus*) challenged via intramuscular injection with white spot syndrome virus survived (100%) for 45 days when maintained at either 4 or 12ºC, but all died at 22ºC (Jiravanichpaisal *et al.,* 2004). Moribund shrimp could avoid *in exitus* when transferred to 16ºC. This lack of viremia at hypothermic conditions is likely caused by an inability of the virus to bind to haematopoietic tissue and replicate within cells due to cell cycle arrest (Korkut et al., 2018). Higher water temperatures

cannot hold as much oxygen as colder water. Sub-optimal oxygen levels reduce the ability of shellfish to fight infection. Emersion, hypoxia and haemolymph acidosis restrict haemocyte-associated respiratory burst – in a manner similar to mammalian neutrophils – and delays wound healing (Allen *et al.,* 1997; Coates and Decker, 2017; Table 3). Hypoxia often co-occurs with hypercapnia and haemolymph acidosis as CO_2 will bind to water in the haemolymph to form carbonic acid (H_2CO_3.). Increased salinities above an iso-osmotic threshold (~24-25 psu) can reduce the pH of crab haemolymph, whereas drastic decreases in salinities can cause alkalosis (Whiteley *et al.,* 2001). Such changes in haemolymph gases and pH balance are known to modulate shellfish immune-vigour, mostly to the detriment of the host (Table 3). Water temperature increases (+4^{o}C) and pH decreases (-0.4 units) in line with climate change predictions for 2100 interfere with cellular immunity – such deteriorations in condition have been recapitulated in several high value shellfish: Norway lobster (*Nephrops norvegicus*; Henroth *et al.,* 2012), blue mussels (*Mytilus edulis*; MacKenzie *et al.,* 2014), Pacific oyster (*Crassostrea gigas*; Clark et al., 2013; Wang *et al.,* 2016), and common cockles (*Cerastoderma edule*; Ong *et al.,* 2017).

Maintaining suitable levels of ammonia (and other nutrients) in shrimp culture ponds is important for two reasons: 1) it provides phytoplankton with a source of nitrogen, thereby increasing the levels of dissolved oxygen in the water and acting as a food for the shrimp, and 2) excessive levels of ammonia-N in culture ponds correlates broadly with reduced survival rates of penaeid shrimp (de Lourdes Cobo *et al.,* 2014; reviewed by Zhao *et al.,* 2020). Ammonia is an immune-modulator (see Table 3) and levels between 14 and 20 mg L^{-1} can cause oedema, haemocyte infiltration, necrosis and melanisation of shrimp gill tissue (Fregoso-López *et al.,* 2017), with the latter being observed in the antennal gland alongside pyknotic nuclei (Fregoso-López *et al.,* 2018). Moreover, Hostins *et al.* (2019) reported on the manipulation of C/N ratios in ponds using biofloc systems in order to reduce the risk of acute hepatopancreatic necrosis disease (AHPND). Shrimp raised in heterotrophic bioflocs for 21 days showed enhanced resistance to *Vibrio parahaemolyticus*, the causative agent of AHPND.

When determining the impacts of the aforementioned environmental stressors on shellfish, immune-makers such as PO activity, haemocyte counts and protein levels are possibly useful, however, the reader should be aware that these fluctuate

seasonally and are impacted by moult cycles, biomineralization and reproductive status (Hauton *et al.,* 1997; Terwilliger *et al.,* 2006; Cao *et al.,* 2007). Therefore, basic assays should not be used alone as markers of immunocompetence and other approaches including measuring actual disease resistance (i.e., LD_{50}) in challenge trails together with a panel of cellular (e.g. phagocytosis, microbial clearance dynamics) and humoral (e.g. antimicrobial peptides, lysozyme activity) measures are needed for high confidence diagnoses and prognoses. Recent efforts have focussed on assessing the usefulness of the shrimp gut microbiome as an indicator of health and disease, particularly in managed culture settings (reviewed by Holt *et al.,* 2020). While this approach is appealing, it is costly, technically challenging, and has not been developed for high-throughput application.

There remains a lack of standardisation for PO enzyme assays. This is not surprising considering the diversity of study species (aquatic versus terrestrial; e.g., Huang *et al.,* 2010), and the many ways in which one can measure PO activity: 1) direct spectrophotometric readings of active enzyme within extracted haemolymph (usually with the addition of excess substrate), 2) total enzyme available (i.e., PO, proPO, HC-d PO) through the addition of an activator such as SDS or (chymo)trypsin, and 3) staining of cells for calculating the proportion of PO-positive haemocytes. Authors tend to make little effort to distinguish between them. We are compelled to state that PO assays are misused repeatedly, basing superficial interpretations of immune-capacity on residual activity in serum after accidental activation and lack of effort to either separate or stabilise haemocytes and cell lysates prior to measurements (Söderhäll and Smith, 1983). Furthermore, increased PO activity in the haemolymph is not a compensatory mechanism for haemocyte loss – circulating cell numbers are reduced because they have ruptured (undergone apoptosis) to release the enzyme now being detected.

4. Environmental disruptors: pesticides, pharmaceuticals and (micro)plastics

The detrimental impacts of pollutants (e.g. oil spills), nanomaterials, dredge spoils and heavy metals (e.g., cadmium, lead, copper) on shellfish health and immunity have been characterised and reviewed extensively by ecotoxicologists (Smith et al., 1995; Dyrynda *et al.,* 1998 and 2000; Galloway and Depledge, 2001; Renault, 2015). There

has been much focus on the validation of certain aquatic invertebrates as environmental indicators or sentinels (e.g., bivalves and echinoids), which are said to reflect conditions *in situ* such as pollutant contamination (e.g., heavy metals, pharmaceuticals). Wootton et al. (2003) tell a cautionary tale regarding the generalisation of single species for inferring immunosuppression. The authors exposed three bivalves, mussels (*Mytilus edulis*), cockles (*Cerastoderma edule*) and razor clams (*Ensis siliqua*), to increasing doses (50 - 400 µg L^{-1}) of polycyclic aromatic hydrocarbons and measured several immune activities. Despite *M. edulis* being used as a common bioindicator, there was substantial variation in the haemocyte-associated responses (e.g., superoxide generation and lectin staining), with mussels being distinct to the others. In contrast to mussels, all cockles and razor clams died within 14 days exposure to the highest dose of 400 µg L^{-1}. As such, the use of a single species to represent broad groups like crustaceans or molluscs is not recommended.

Below, we recount shellfish immunotoxicology in relation to contemporary issues. We should like to impress upon the reader that assays of oxidative damage alone, such as, superoxide dismutase activity and malondialdehyde levels (listed in Table 1), offer little insight into the direct impact of toxins on immune functioning. Toxins often interfere with haemocyte viability, membrane and cytoskeleton stability leading to cellular swelling or lysis. Such changes can be measured and viewed in haemocytes *ex vivo* to identify putative mechanisms.

Pesticides such as organophosphates and neonicotinoids make their way into aquatic environments from agricultural run-off and aerial (drift) sprays. While concentrations of these pesticides can be lethal in extreme cases, more often they accumulate within shellfish and lead to chronic idiopathy. For example, exposure of American lobsters (*Homarus americanus*) to environmentally relevant concentrations of chlorpyrifos, 0.5 - 0.82 µg L^{-1}, led to the inhibition of acetylcholinesterase activity (within 24 - 48 hours) and interfered with moulting and growth (Taylor *et al.,* 2019). Malathion and endosulfan alone, and in combination, reduced survival of *P. vannamei* by <4% over 96 hours using concentrations at 1, 10 and 50% the LC_{50} for each pesticide (78 and 0.2 µg L^{-1}, respectively; Bautista-Covarrubias *et al.*, 2020). Nonetheless, malathion use alone was associated with compromised PO activity (-60%), and reduced

haemocyanin levels by ~40% when combined with endosulfan (from 5 hours post exposure).

Two-week incubation of Sydney rock oysters (*Saccostrea glomerata*) in the presence of imidacloprid – one of the most widely used pesticides globally – at environmentally relevant concentrations (0.01 and 0.5 mg L^{-1}) led to increased catalase and glutathione-s-transferase activity in the digestive gland and gills (Ewere *et al.,* 2019a). Intriguingly, Ewere *et al.* (2019b) recorded negative impacts of imidacloprid on feeding rate and gill acetylcholinesterase activity in *S. glomerata* exceeding environmentally-relevant concentrations (>2 mg L^{-1}) within 24 hours. Lower doses of 0.5 and 1 mg L^{-1} began to affect oysters adversely after 4 days exposure. Concentrations as low as 0.01 mg L^{-1} can stimulate the expression of detoxification (superoxide dismutase) and stress (heat shock) proteins in the haemolymph (Ewere et al., 2020). Dondero et al. (2010) also described drastic alterations in the digestive gland proteome of mussels (*Mytilus galloprovincialis*) after neonicotinoid treatment. Imidacloprid use in integrated aquaculture continues to be popular for finfish and macro-crustaceans (Hong *et al.,* 2020). A sublethal dose of imidacloprid (5 µg L^{-1}) increased superoxide dismutase activity and the expression of heat-shock proteins (60, 70 and 90) in Chinese mitten crab (*Eriocheir sinensis*), but was inhibitory at a much higher concentration (500 µg L^{-1}). Catalase and GST activities decreased in a dose-dependent manner with imidacloprid (Hong *et al.,* 2020). The authors describe a switch in the gut bacterial microbiome from symbionts to pathobionts after pesticide exposure.

Pharmaceuticals, notably antidepressants and antibiotics, developed for humans and livestock are now common in aquatic environments – due to domestic and industrial effluents of wastewaters. Antibiotics that are “normally” present in water bodies may affect the resistance of shellfish (and other aquatic organisms) to bacteria, fungi and viruses (reviewed by Fong and Ford, 2014). Prolonged exposure (three weeks) of a crustacean (*Pacifastacus leniusculus*) to environmentally relevant concentrations of the antibiotic sulfamethoxazole (100 ng L^{-1} and 1 µg L^{-1}) used frequently in aquaculture, heightened susceptibility to white spot syndrome virus (Hernandez-Pérez *et al.,* 2020). Sulfamethoxazole exposure led to the down-regulation of an AMP (Crustin 3) in haemocytes, as well as decreased haemocyte numbers depleted of granular cells. The antidepressant drug fluoxetine – which has been detected in

aquatic systems – had distinct immunosuppressive effects on both cellular and humoral factors in blood cockles (*Tegillarca granosa*) between 1 and 100 µg L^{-1} (Shi *et al.,* 2019). The authors concluded that diminished haemocyte numbers and viability, phagocytic capacity (linked to altered levels of intracellular Ca^{2+}) and NFκB signalling leave this shellfish prone to infection. In a follow-on study, Shi *et al.* (2020) co-exposed *T. granosa* to the antidepressant sertraline and microplastics (500 nm - 30 µm diameter) as they are often found contaminating the same environments. Again, haemocyte counts, and phagocytosis levels decreased alongside elevated levels of apoptosis, lipid peroxidation and acetylcholinesterase activity. The combination of pharmaceutical/microplastics worsened the symptoms of immunosuppression when compared to either stressor alone.

Microplastics (<5 mm) have been contaminating, and accumulating in, aquatic environments for decades. Not only can they impose a cumulative physiological burden on shellfish, but they act as fomites in water – providing a platform for microbes, viruses and microeukaryotes (both opportunists and pathogens) to 'hitch a ride'. Exposure of the common shore crab (*Carcinus maenas*) to polypropylene rope microfibres (<5 mm in length) over a 4-week period had significant detrimental effects on food consumption and the available energy budget (Watts *et al.,* 2015). In the same species, Watts *et al*. (2016) noted a transient impact on oxygen consumption after ingesting polystyrene latex microspheres for one hour but returned to normal by 16 hours. Several recent laboratory studies on single and repeated exposures of shellfish to microplastics depict their pernicious effects on health and immunity. According to Détrée and Gallardo-Escárate (2018), 18-day exposure of mussels (*Mytilus galloprovincialis*) to polyethylene microbeads induced differential expression of immune genes in the mantle (e.g., mytilin 4, galectin) and digestive gland (e.g., mytilin 1, C-type lectin, defensin) at the apparent expense of energy reserves and growth performance (~70% reduction compared to the control). Subjecting Chinese mitten crabs (*Eriocheir sinensis*) to microplastics (0.04 - 40 mg L^{-1}) over 21-days (Liu *et al.,* 2019) interfered with haemocyanin levels, as well as PO and lysozyme activities in the haemolymph in a time- and dose-dependent manner. Haemocyanin and lysozyme mRNAs levels fell by >90%. Conversely, increased mRNAs of the cell-death associated gene caspase and the immune-signalling regulator MyD88 were recovered from haemocytes. The authors noted a shift in intestinal microbial composition from a

Firmicutes/Bacteroidetes-dominated community to *Fusobacteria/Proteobacteria* at the highest dose tested. Whether dysbiosis was caused by the microplastics themselves or indirectly from the host response to microplastics was not determined (Liu *et al.,* 2019). After 52 days exposure to polyethylene microplastics, blue mussels (*M. edulis*) demonstrated diminished attachment strength by >50% and fewer byssal threads, as well as alterations to immune proteostasis and detoxification (amongst other proteins involved in metabolism; Greene *et al.,* 2019). In a separate study, the number of dead haemocytes increased 3-fold as did the generation of ROS in mussels replete with polystyrene microspheres along the gastrointestinal tract after 7-days post-exposure (Paul-Pont *et al.,* 2016). When microplastics were combined with fluoranthene – a polycyclic aromatic hydrocarbon – gross changes in histopathology were recorded, e.g., tissue abnormalities, lipofuscin accumulation and haemocyte infiltration (Paul-Pont *et al.,* 2016). Exposure of cockles (*T. granosa*) to microplastics alone (500 nm to 30 µm), and in combination with persistent organic pollutants (benzo[*a*]pyrene and 17β-oestradiol), displayed immunomodulatory properties (e.g., haemocyte counts, phagocytic capacity), including the inhibition of Toll-like receptor expression (Tang *et al.,* 2020).

It is evident from the aquarium trials discussed above, and those available in the broader literature, that pesticides, pharmaceuticals and microplastics are environmental insults. Shellfish expend energy attempting to detoxify and metabolise them, and in doing so, they are weakened and likely immunocompromised. Studies favour the use of pure chemicals and plastics, whereas in natural settings there exists complex mixtures of metabolites and degenerated fragments (embrittlement) due to photo-lysis/oxidation (bond rearrangements), mechanical erosion, biological and chemical weathering from microbes and aquatic macrophytes (Katagi, 2006; Fatta-Kassinos et al., 2011). Furthermore, plastic sizes (micro versus nano) differ in their immuno- and cytotoxicity – as determined recently in mussels (Cole et al., 2020). Some plastic resins like polyethylene also absorb pollutants such as polychlorinated biphenyls and polycyclic aromatic hydrocarbons (Rochman et al., 2013), and increases the risk of toxicosis when ingested. There is clearly a 'One Health' perspective to the microplastics problem – although pertinent experimental evidence of immune-pathological effects from shellfish through to humans is absent. Tolerance levels and the adverse effects of pesticide and pharmaceutical burdens remain

unknown for many shellfish– commercial or otherwise – and it is unclear to what extent biomagnification occurs over trophic levels (Zenker et al., 2014; Rocha et al., 2018). Further speculation is beyond the scope of this review.

5. A One Health case study: toxin transfer between dinoflagellates, shellfish and humans

Harmful algal blooms and their toxins are increasing in frequency, severity and distribution, being linked to climate change (Pearl and Paul, 2012; Trainer *et al.,* 2020). Elevated water temperatures and decreased pH will impact the accumulation, retention and clearance kinetics of toxins by shellfish. For example, Braga *et al.* (2018) exposed mussels (*Mytilus galloprovincialis*) to saxitoxin producing dinoflagellates (*Gymnodinium catenatum*) – the causative agents of paralytic shellfish poisoning – under different warming and acidification regimes. Although the environmental extremes seemed to lower toxicity levels compared to mussels kept under current climate conditions, the intoxication episode was prolonged.

The acute pathological symptoms of the five major shellfish poisoning syndromes, namely diarrheic, amnesic, neurotoxic, paralytic and azaspiracid, are well defined (Botana, 2016), but their chronic impacts on human health are poorly understood. Consumption of contaminated shellfish flesh, mostly bivalves, manifests as gastrointestinal disorders (cramps, vomiting, diarrhoea), but *in extremis* can lead to memory loss, paralysis and death. There have been cases of crustaceans testing positive for marine toxins (e.g., Oikawa *et al.,* 2002) – but these are rare in comparison to their molluscan counterparts. Only in the last decade has it become clear that dinoflagellate-derived toxins cause harm to shellfish tissues, not just humans (Figure 2). These toxins can be transferred across trophic levels – from fish to birds to marine mammals (James *et al.,* 2010) – with shellfish potentially acting as both reservoirs and vectors. Passage through the different hosts also leads to a myriad of toxin-derived metabolites.

Inoculation of clam (*Ruditapes decussatus*) and mussel (*M. galloprovincialis*) haemocytes with okadaic acid (up to 500 nM) – the causative agent of diarrheic shellfish poisoning – induced DNA damage, promoted cell death, and reduced levels of phagocytosis (Prado-Alvarez *et al.,* 2013; Prego-Faraldo *et al.,* 2015). Exposure of

mussels (*M. galloprovincialis, Perna viridis*) to food contaminated with okadaic acid or dinoflagellates capable of producing the toxin (*Prorocentrum lima*) induced the up-regulation of stress associated genes (Manfrin *et al.* 2010) and proteins (Huang *et al.* 2015), representing detoxification and REDOX balance, apoptosis, cellular structure and function. Cytoskeletal dysfunction was reported for *P. lima* exposed mussels (Huang *et al.,* 2015), and likely explains the mechanism of reduced functionality of haemocytes observed in the studies mentioned above. Similarly, *in vitro* exposure of mussel (*Mytilus chilensis*) haemocytes to saxitoxin interfered with the expression of key immune genes (C-type lectin, toll-like receptors) and those encoding antioxidant enzymes (e.g., catalase; Astuya *et al.,* 2015). An investigation into oysters (*Crassostrea gigas*) and mussels (*Perna perna*) during, and 30-days after, a bloom event in Brazil (Simoes *et al.,* 2015) suggested that okadaic acid is an immune-modulator, but the measured immune parameters including total haemocyte counts and PO activity were not consistent between the species or sample locations. Mussels accumulated 10-fold more toxin than oysters and suffered an >50% reduction in the hemogram, whereas tissue histology depicted gross haemocyte infiltration of the oyster gastrointestinal tract during the event (in response to tissue damage). It is now apparent that shellfish, notably bivalves, are not passive accumulators of toxins. A of growing body of literature describes many negative impacts of harmful algal blooms (HABs) and toxins on these commercially sensitive species; from okadaic acid induced DNA fragmentation, tissue inflammation and digestive gland distortion (loss of tubular architecture) in *C. gigas* (McCarthy *et al.,* 2014; de Jesus Romero-Geraldo *et al.,* 2016), to hemocytopenia, immune dysfunction and stress response mobilisation in scallops (*Argopecten irradians;* Chi *et al.,* 2016 and 2018). It remains unclear whether these intoxication events lead to enhanced susceptibility to bacterial and viral diseases, as HABs and toxins appear to antagonise would-be pathogens (reviewed by Lassudrie *et al.,* 2020).

6. Concluding remarks

A majority of publications reporting on the detrimental impacts of specific stressors on shellfish innate immunity often omit a key (microbial) challenge experiment, yet claim the animals are immunosuppressed. There is no consensus for a universal marker of immunosuppression – with the exception of depleted circulating haemocyte numbers, but this does not provide any information on the mechanism of interference. The use

of other so-called health indicators, such as lysozyme activity, total protein levels, ammonia accumulation, etc., requires reflection as they present inconsistent trends across the literature, and have functions beyond immunity and stress. What is clear is that oxidative damage arising from xenobiotic contamination or immune reactivity (high levels of PO activity) can be inferred reliably from MDA levels and/or enzymatic detoxicants (e.g., superoxide dismutase). Shellfish exposed to individual or multiple stresses respond by targeting resources to maintain homeostasis, which can be reallocated from the immunity budget. In the absence of further resource acquisition, the increased metabolic demand alone will leave them vulnerable to disease. Coping with stress while maintaining immunity is costly, and prolonged up-regulation of immune activities increases the likelihood of collateral damage to the host from cytotoxic by-products.

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Competing interests

We have no conflicts of interest, financial or otherwise.

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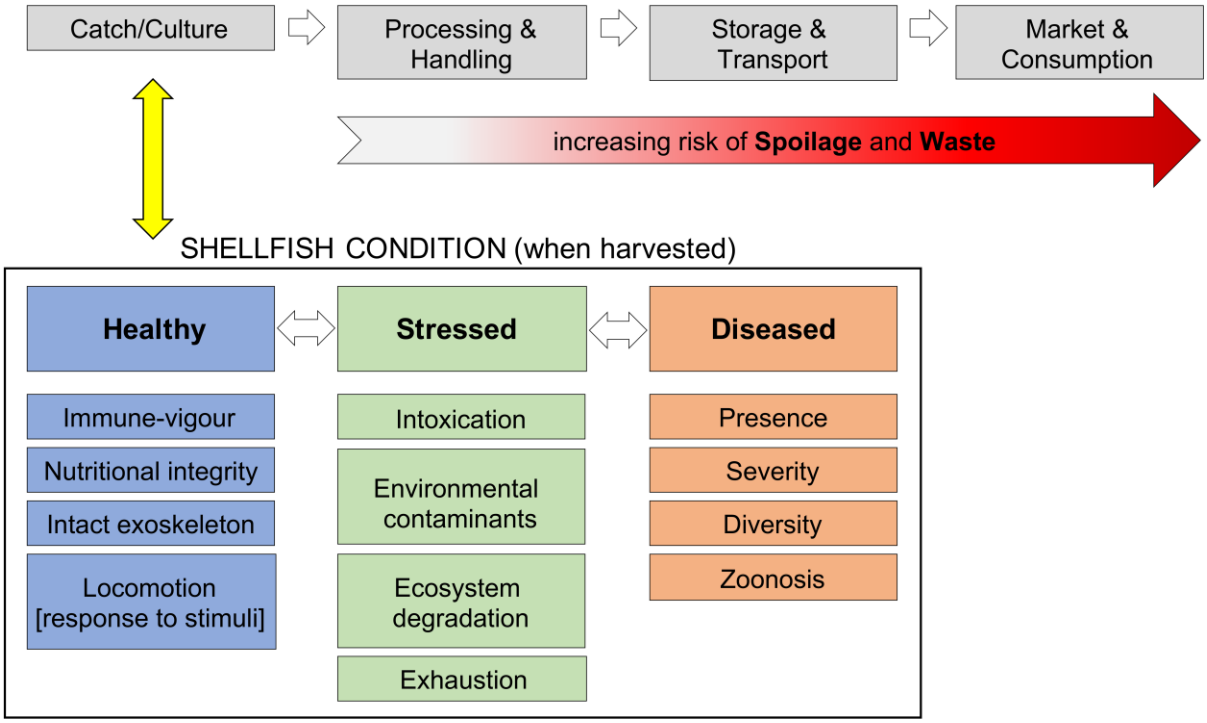

Figure 1 Integration between the food cycle and condition status of shellfish. At each stage in the food cycle (grey boxes), the condition of shellfish (i.e., food commodity) can be impacted negatively by stresses (e.g., temperature fluctuations, emersion, physical damage) and can worsen pre-existing morbidities from production. As the shellfish are processed from vessel or containment (e.g., ponds) to plate, these stressors and the associated harm they cause can accumulate, thereby increasing the risk of spoilage and waste, e.g., toxin levels above regulatory limits (see Figure 2), necrotic tissue, muscle atrophy, changes in organoleptic profiles (*ante-* and *post-mortem* quality; Gornik *et al.,* 2010), and unsightly discolouration (hyperpigmentation; Coates and Nairn, 2013). Environmental contaminants include plastics, pesticides, and pharmaceuticals.

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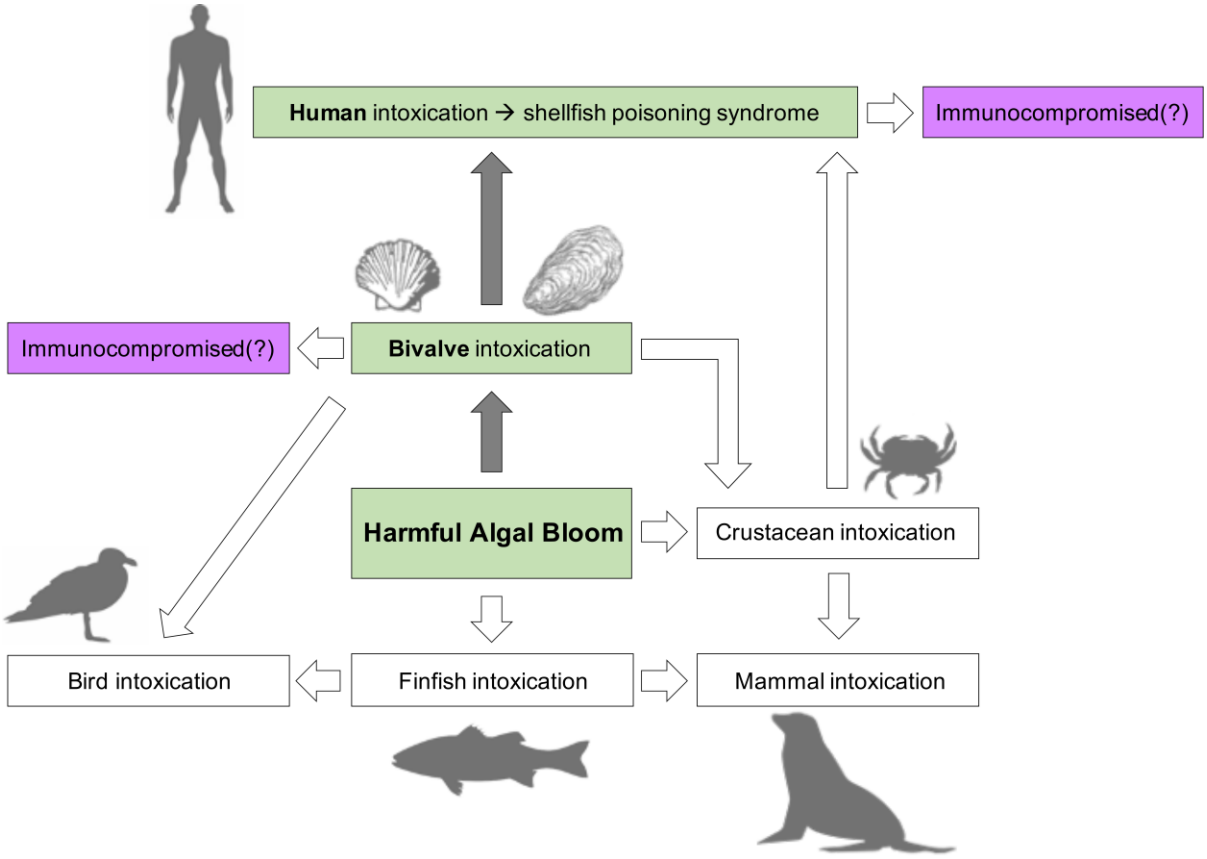

Figure 2 Transfer of shellfish poisoning toxins between trophic levels. Harmful algal blooms can lead to the release of toxins that accumulate in the tissues of marine animals, notably bivalves (grey arrow). Human consumption of these contaminated (intoxicated) bivalves can lead to a series of illnesses referred to as shellfish poisoning syndromes (grey arrow). The white arrows signify additional (but not all) routes of toxin transfer. James *et al.* (2010) inspired this figure.

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1387 **Table 1 Common biochemical and cellular measures of shellfish condition**
1388

	Indicator (stress, immunity, disease)	Example references (not exhaustive)
Ammonia	It is predominantly a by-product of protein catabolism. Excessive excretion by gills in stressed crustaceans can lead to reduced levels of ammonia. Over-accumulation in the haemolymph – due to 'stress' – has been linked to immunosuppression.	Reviewed by Zhao *et al.* (2020)
Antimicrobial activity (of haemolymph)	Several assays exist to determine the extent of microbe killing efficiency from the acellular fraction of haemolymph. There are many soluble immune factors in the haemolymph, and reduced capacity to clear microbes is associated with immunocompromised animals (usually linked to reduced PO and lysozyme activities).	Le Moullac and Haffner (2000); Fotedar and Evans, (2011); Gopalakrishnan et al. (2011)
Catalase [EC 1.11.1.6]	Detoxification-associated enzyme that catalyses the conversion of hydrogen peroxide (H_2O_2) to water and O_2.	Zhang *et al.* (2011); Ben-Khedher *et al.* (2013)
Clotting time (haemostasis)	Clotting (haemolymph gelation) is a rapid response in healthy invertebrates to avoid hypovolemia post-wounding. Increased clotting time is considered a sign of stress, and also increases the risk of septicaemia, viremia and mycosis.	Smith *et al. (*1995); Jussila *et al.* (2001); Vijayavel, *et al.* (2005); Fotedar *et al.* (2006);
Esterase (non-specific) activities	These represent diverse enzymes that can be involved in the detoxification of xenobiotics	Wootton *et al.* (2003); Matozzo and Marin (2010)
Glucose and glycogen	Elevated levels of glucose (hyperglycaemia) and depleted levels of glycogen suggest a switch to using energy stores to fuel the physiological demands of 'coping' with stress and immune-stimulation, or may be manipulated by pathogenic agents, as seen in *Hematodinium*-infected crabs.	Hall and van Ham (1998); Stentiford *et al.* (2001); Rosas *et al.* (2004); Ivanina *et al.* (2013)
Glutathione (disulphide) reductase [EC 1.8.1.7] and Glutathione-s-transferase [EC 2.5.1.18]	-GR is an enzyme that reduces glutathione disulphide to glutathione, which is an important chemical antioxidant. -GST is a detoxification-associated enzyme that conjugates the reduced form of glutathione to xenobiotics.	Revathy *et al.* (2012); Rodrigues *et al.* (2012); Ben-Khedher *et al*. (2013); Chi *et al.* (2018)
$_L$-lactate	Elevated levels in the haemolymph and muscle suggest acidosis caused by fatigue (anaerobiosis), or, compromised oxygen tension caused by microbial infection. Can also be linked to oxygen-transport protein levels (see below).	Baldwin *et al.* (1992); Rosas *et al.* (2004); Schock *et al.* (2010); Albalat *et al.* (2016)
Lysozyme [EC 3.2.1.17]	Immune-enzyme deployed to degrade bacterial cells (via muramidase activity). Elevated levels can be associated with stress or microbial presence, whereas depleted levels are found often in immunocompromised shellfish.	Oliver and Fisher (1999); Yao et al. (2008); Gopalakrishnan *et al.* (2011); Cotou *et al.* (2013); Ivanina *et al.* (2014)
Malondialdehyde (MDA)	This enol derivative is a key marker of oxidative stress. Accumulation of MDA is indicative of lipid peroxidation (namely fatty acids).	Chaufan *et al.* (2006); Funes *et al.* (2006); Ben-Khedher *et al.* (2013)
Phenoloxidase(s) - LAC [EC 1.10.3.2] - CO [EC 1.10.3.1]	Conversion of simple mono- and di-phenolic substrates into quinones – the precursors of melanin. One of the most common measures of invertebrate immunity. Elevated or excessive levels are associated	Perazzolo *et al.* (2002); Tanner *et al.* (2006); Kuchel *et al.* (2012); Johnson *et al*. (2016);

- TY [EC 1.14.18.1]	with stress and disease. Low levels can be correlated with depleted proteins and immunocompromised animals.	Luna-Acosta et al. (2017); Albalat et al. (2019)
Superoxide dismutase [EC 1.15.1.1]	Detoxification-associated enzyme that partitions highly reactive superoxide (O_2^-) radicals into 'harmless' dioxygen (O_2)	Chaufan et al. (2006); Funes et al. (2006); Ren et al.., (2015); Chi et al. (2018)
Total and Differential immune cell counts [phagocytosis, respiratory burst]	Alongside PO measurements, haemocyte counts are used to gauge health. Decreased haemocyte numbers (haemocytopenia) are often associated with stress (and infectious disease, parasitism). In these circumstances, haemocytes usually show high levels of apoptosis and reduced phagocytic capacity.	Le moullac and Haffner (2000); Perazzolo et al. (2002); Wootton et al. (2003); Goimier et al. (2006); Perez and Fontanetti, 2011; Coates et al. (2012)
Total protein levels (and differential levels)	Depleted levels of total protein within the haemolymph (hypoproteinaemia) are associated with compromised shellfish. Elevated protein levels often reflect immunocompetence. The presence and abundances of specific proteins or polypeptides can provide additional information, e.g., oxy-haemocyanin levels (immunity and respiration), antimicrobial peptides (immunity), heat shock proteins (temperature stress), hypoxia-inducible factors (hypercapnia) etc.	Rosas et al. (2004); Goimier et al. (2006); Lorenzon et al. (2011); Coates et al. (2012)

1389

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1390 **Table 2 Evidence for neuroendocrine – stress – immunity axis in shellfish**
1391

	Stressor [acute vs chronic]	Indicator	Immunomodulation	Disease outcome	References
Crustaceans					
Carcinus maenas	Salinity (4 – 45 psu)	Cholinesterase activity	Cholinesterase activity increased alongside higher (and substantially reduced) salinities. Activities from enzymatic detoxicants (glutathione-s-transferase, peroxidase and glutathione reductase) were significantly higher at salinity extremes.	Not tested	Rodrigues et al. (2012)
Macrobrachium rosenbergii	Direct injection of norepinephrine	Norepinephrine	Between 2- and 8-hours post inoculation, haemocyte numbers, respiratory burst and phagocytosis, and phenoloxidase activity decreased	Enhanced susceptibility to *Lactococcus garvieae*	Chang et al. (2011)
Macrobrachium rosenbergii	Temperature (22 - to 28 to 34^{o}C)	Norepinephrine	Hyaline cell numbers and granular cell PO activity increased within 2 hours, with some upregulation of phenoloxidase-associated gene	Not tested	Chang et al. (2015)
Penaeus vannamei	Direct injection of CHH	Crustacean hyperglycaemic hormone (CHH)	Increased total haemocyte counts, phenoloxidase activity and serum protein levels	Enhanced resistance to *Vibrio harveyi*	Wanlem et al. (2011)
	-Temperature (28 to 22^{o}C)	Elevated levels of norepinephrine	Decreased haemocyte numbers, oxidative burst and phenoloxidase activity; increased levels of apoptosis and caspase-3 activity	Enhanced susceptibility to *Vibrio alginolyticus*	Cheng et al. (2006); Chang et al. (2009)
	-Direct injection of norepinephrine				
	Salinity (16 to 31 ppt)	CRH, ACT, dopamine, norepinephrine	Hormone levels in shrimp held at lower salinities over 12 hours increased significantly. Conversely, total haemocyte counts, PO activity, crustin and C-type lectin expression, phagocytic capacity and hemagglutination activities decreased significantly	Not tested, however, haemocyte numbers were the only parameter not to recover after 12 hours.	Zhao et al. (2016)
Molluscs					
Chlamys farreri	Bacterial challenge	Dopamine, epinephrine and norepinephrine	Superoxide dismutase, catalase and lysozyme activities increased alongside all catecholamines	Blocking of adrenoreceptors repressed both catalase and lysozyme activities.	Zhou et al. (2011)

			monitored within 6 to 12 hours post inoculation of V*ibrio anguillarum*		
Crassostrea gigas	Temperature (held at 28^{o}C)	DBH monooxygenase activities; catecholamine metabolic processes	Approximately 40% decrease in phenoloxidase activity compared to the control	No differences in bacterial (*Vibrio splendidus*) colony forming units in the haemolymph compared to the control.	Liu et al. (2017)
	Mechanical disturbance*	Elevated norepinephrine and dopamine from 5 to 60 minutes	Acute (within 5 - 15 minutes) decline in haemocyte numbers, migratory and phagocytic capacity, and ROS production	Enhanced susceptibility to *Vibrio splendidus* [exacerbated by noradrenaline and adrenocorticotrophic injections]	Lacoste et al. (2001; 2002)
Haliotis tuberculata	Mechanical* disturbance	Elevated norepinephrine and dopamine from 5 to 60 minutes	Acute (within 5 - 15 minutes) decline in haemocyte numbers, migratory and phagocytic capacity, and ROS production	Not tested	Malham et al. (2003)
Saccostrea glomerata	-Salinity -Temperature, -Physical disturbance	norepinephrine	Within 1 – 2 hours post noradrenaline injection (70 ng); phenoloxidase activity, haemocyte (total and differential) numbers, and phagocytic capacity all declined	Not tested	Aladaileh et al. (2008)

*Shaking animals for 15 minutes to mimic handling practices on shellfish farms. ACT, adrenocorticotropic hormone; CRH, corticotrophin-releasing hormone; DBH, dopamine beta-hydroxylase (converts dopamine to norepinephrine)

1412 **Table 3 Environmental drivers of immunomodulation in shellfish**
1413

Species	Immune-status (unchanged, suppressed, stimulated)	References (not exhaustive)
Ammonia		
Chlamys farreri [bivalve]	Within one-hour, SOD and superoxide anion levels increased dramatically after ammonia-N (20 mg L^{-1}) exposure. By 24 hours, MDA levels increased, as did gene expression for HSPs 70/90, IDH, and GST. Cellular energy allocations across several tissues (e.g., muscles) and glycogen were depleted within 12-24 hours. Enhanced susceptibility to *Vibrio anguillarum.*	Wang *et al.* (2012)
Corbicula fluminea [bivalve]	Exposure to 10 – 25 mg L^{-1}, over 48 hours, led to significant changes in gene expression, including down regulation of TLR4, IL7 and IL1, and upregulation of TLR2, HSP70 and NF-κB.	Zhang *et al.* (2019)
Haliotis diversicolor [gastropod]	Abalone were injected with *Vibrio parahaemolyticus* (1.6 x10^5 per animal) and then placed in tanks with increasing concentrations of ammonia, 0.01 – 10mg L^{-1}. Increasing ammonia concentrations correlated positively with mortality. Exposing abalone to 3 mg L^{-1} ammonia (without bacterial challenge) led to decreases in phagocytic and phenoloxidase activities within 24 hours, reduced haemocyte numbers after 72 hours.	Cheng et al. (2004a)
Panulirus homarus [crustacean]	Increasing ammonia concentration up to 3 mg L^{-1} resulted in significant reductions in haemocyte numbers and phenoloxidase activities.	Verghese *et al.* (2007)
Penaeus monodon [crustacean]	High levels can lead to enzyme inhibition (e.g., chitinase), moulting and growth interference, and immunosuppression (phenoloxidase activity, antimicrobial potency of the haemolymph).	Zhao *et al.* (2020)
Penaeus schmitti [crustacean]	5 mg L^{-1} dissolved ammonia ed to a ~66% reduction in haemocyte numbers by 72 post-exposure, and remained low by 168 hours	Rodriquez-Ramos *et al.* (2008)
Penaeus vannamei [crustacean]	Exposure to 10 mg L^{-1} dissolved ammonia over 48 hours led to significant decrease in phenoloxidase and bactericidal activities, decreased oxy-haemocyanin levels, as well as increases in haemolymph glucose and L-lactate levels	Cui *et al.* (2017)
Portunus trituberculatus [crustacean]	Levels of ammonia in excess of 5 mg L^{-1} (over 48 hours) led to fewer circulating haemocytes, reduced phagocytic capacity, decreased haemolymph bacteriolytic activity. Temporarily, reduced crustin (AMP) and lysozyme gene expression, and α-macroglobulin activity (6 - 12 hours post exposure),	Yue *et al.* (2010)
Ruditapes philippinarum [bivalve]	Exposure to 0.1 – 0.5 mg L^{-1}, over3 to 21 days, disrupted mitochondrial membrane potential of haemocytes, and gill tissue. Additionally, higher levels of cell death (apoptosis) were observed in gill histology.	Cong *et al.* (2019)
Hypoxia & Hypercapnia		
Callinectes sapidus [crustacean]	Crabs were more susceptible to *Vibrio campbellii* when held in tanks under hypoxia/hypercapnia (PO_2 = 4 kPa; CO_2 = 1.8 kPa) compared to those held under normoxia (PO_2 = 20.7 kPa; CO_2 <0.06 kPa) for 24 hours. Fluctuations in haemocyte counts appeared similar across the two conditions. In another study,	Macey *et al.* (2008); Tanner *et al.* (2006)

	decreasing water oxygen levels from 15 to 1% O_2 suppressed phenoloxidase activity by 33 to 70%, respectively.	
Carcinus aestuarii [crustacean]	Oxygen levels were reduced from 8.3 to over a 4 mg O_2 L^{-1} 1-hour period. Haemolymph glucose levels went from ~40 mg/dL under normoxia to ~140 mg/dL under hypoxic conditions. Haemocyte numbers went from ~7 x10^6 mL haemolymph (normoxia) to <5 x10^6 mL (hypoxia).	Qyli *et al.* (2020)
Chlamys farreri [bivalve]	Bivalves were held in tanks containing varying oxygen levels, 2.5 to 8.5 mg L^{-1}, for 21 days. Haemocyte numbers declined dramatically at the lowest dissolved oxygen concentration (survival and growth rate were also poor). Below 4.5 O_2 mg L^{-1} , superoxide dismutase levels increased significantly.	Chen *et al.* (2007)
Panulirus homarus [crustacean]	Dissolved oxygen 1 and 5 mg L^{-1}. Hypoxia led to significant reductions in haemocyte numbers and phenoloxidase activities.	Verghese *et al.* (2007)
Penaeus stylirostris [crustacean]	Exposure to hypoxia (1 mg O_2 L^{-1}) for 24 hours significantly reduced haemocyte numbers (notably granular cells) and respiratory burst. PO activity increased under hypoxia, which is unusual considering oxygen is essential for phenolic conversion into quinones. Hypoxic conditions increased mortality from 32 to 48% in shrimp challenged intramuscularly with *Vibrio alginolyticus*.	Le Moullac *et al.* (1998)
Salinity		
Apostichopus japonicus [echinoderm]	Salinity range, 20 – 35 ppt, for 72 hours. Phagocytosis levels peaked at 0.5 to 1-hour post exposure to all salinities, with little difference thereafter. Detoxification (SOD, CAT) elements tended to be higher at 25 and 35 ppt.	Wang *et al.* (2008)
Haliotis diversicolor [gastropod]	Abalone were injected with *Vibrio parahaemolyticus* and then transferred to tanks containing different salinities (20 to 35 ppt). Within 48 hours, all animals held at 20 ppt were died. Haemocyte numbers increased alongside salinities (in the absence of bacteria), whereas phenoloxidase activity, phagocytic activity and respiratory burst decreased at 20 to 35 ppt.	Cheng *et al.* (2004b)
Macrobrachium rosenbergii [crustacean]	Haemocyte numbers decreased from by ~50% when salinity was reduced from 15 ppt to 0 ppt. Interestingly, phenoloxidase activity did not change much across the entire salinity range.	Cheng and Chen (2000)
Macrocallista nimbosa [bivalve]	Clams were acclimated to 30 ppt, then transferred to tanks with salinities ranging from 18 to 38 ppt for one week. Low salinity levels, 18 and 21 ppt, reflected reduced haemocyte numbers in the haemolymph – haemocytes also had enlarged lysosomes and decreased phagocytic capacity. Overall, animals there were more clam deaths at lower salinities as well as oxidative stress.	Jauzein *et al.* (2013)
Panulirus homarus [crustacean]	Salinity range, 20 – 45 ppt. Total haemocyte counts, and respiratory burst were significantly reduced at salinity extremes (20 and 40 ppt). Phenoloxidase activity increased alongside salinity.	Verghese *et al.* (2007)
Temperature, & pH		
Apostichopus japonicus [echinoderm]	Temperature exposure range, 0ºC to 32ºC, for 72 hours. Could not tolerate 32ºC for more than 12 hours – mortality. Phagocytosis levels remained consistent at 0ºC but varied greatly in excess of 16ºC. Detoxification elements (SOD, CAT) increased significantly within 3 - 12 hours exposure to 32ºC.	Wang *et al.* (2008)
Carcinus aestuarii [crustacean]	Crabs were housed at 4, 17 and 32ºC for one week. Temperature extremes (4 and 30ºC) led to significant reductions in haemocyte numbers but increases in PO activity in cell-free plasma. Interestingly, haemocyte proliferation was significantly increased compared to crabs held at 17ºC.	Matozzo *et al.* (2011)

	Crabs were housed at 4, 17 and 32ºC for one week. Haemolymph glucose levels went from ~40 mg/dL (at 17ºC) to ~100 mg at the other two temperatures. Haemocyte numbers reduced from ~6.5 x10^6 mL haemolymph (at 17ºC) to ~4 x10^6 mL at the two other temperatures.	Qyli *et al.* (2020)
Homaraus americanus [crustacean]	Female lobsters were maintained in aquaria that mimicked current environmental conditions (16ºC, pH8), future warming (20ºC, pH8), future acidification (16ºC, pH7.6), and combined stressors (20ºC, pH7.6) for 42 days. Animals from the warming conditions (alone or combined with reduced pH) displayed fewer haemocytes within the haemolymph and succumbed to infection by *Aerococcus viridans* var *homari* (causative agent of gaffkaemia) five days earlier than those maintained under current environmental conditions. These animals also lost twice as many claws when infected – enhancing their risk of predation.	Harrington *et al.* (2020)
Homarus gammarus [crustacean]	Lobsters were held at 4, 8 and 12ºC for 6 months under starved and fed conditions. PO activity generally increased with temperature but was significantly higher in starved lobsters at 12ºC. Under these conditions, the largest decrease in haemocyte numbers was also observed. Haemolymph protein levels were not a good indicator of differences in immune-capacity.	Albalat *et al.* (2019)
Limulus polyphemus [chelicerate]	Temperature range, 8ºC to 23ºC, in captivity over 56 days led to apparent immunosuppression: decreased cell (amoebocyte) counts, protein (haemocyanin) levels, and phenoloxidase-like activity (derived form haemocyanin).	Coates *et al.* (2012)
Macrobrachium rosenbergii [crustacean]	Haemocyte numbers dropped from > 6 x10^6 mL^{-1} in shrimp held at pH 7.5/7.7 to <4.5 x10^6 mL^{-1} in those held at either pH 4.6/5 or 9/9.5. Phenoloxidase activities dropped by 20 to 40% when held in either more acidic or alkaline conditions.	Cheng and Chen (2000)
Mactra veneriformis [bivalve]	Increasing temperature from 10ºC to 30ºC, increased circulating haemocyte numbers, but phagocytic capacity was compromised as well as reduced lysozyme activity.	Yu *et al.* (2009)
Mytilus edulis [bivalve]	Six months exposure to combined temperature increase (ambient +4ºC) and pH decrease (ambient -0.4 units) in line with future climate change predictions revealed temperature to be the more potent immune-modulator. Haemocyte numbers dropped by ~1 x10^6 mL^{-1} between ambient and +4ºC, and -0.4 pH units. Acidification alone impacted negatively phagocytosis rates. Melanin accumulation, lipofuscin deposition and haemocyte tissue infiltration all increased under the experimental conditions.	MacKenzie *et al.* (2014)
Panulirus homarus [crustacean]	Modifying water pH from 8 to 5 or 9.5 led to significant reductions in haemocyte numbers and phenoloxidase activities within the haemolymph.	Verghese *et al.* (2007)
Penaeus vannamei [crustacean]	Transfer of shrimp from 27/28ºC to 32/34ºC enhanced susceptibility to *Vibrio alginolyticus.* Haemocyte counts, phagocytosis, respiratory burst and phenoloxidase activity all decreased significantly at 32ºC. Higher temperatures are also known to increase virulence of vibrio, and alongside an immune-compromised host, accounts for the high rates of mortality observed.	Cheng *et al.* (2005)

	Acute (30 minutes) temperature shock, 28ºC to 38ºC, led to the enhanced mRNA levels of heat shock protein (*hsp70*) and immune (*proPO* and *haemocyanin*) genes but did not increase resistance to bacterial challenge (*Vibrio harveyi*)	Loc *et al.* (2013)

CAT, catalase; GST, glutathione-s-transferase; HSP, heat shock proteins; IDH, isocitrate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase; Toll-like receptor;