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Redox control of antioxidants, metabolism, immunity, and development at the core of stress adaptation of the oyster *Crassostrea gigas* to the dynamic intertidal environment

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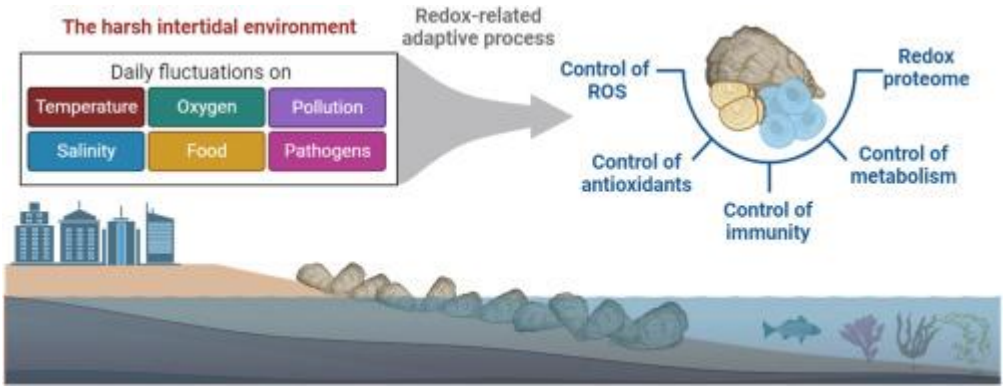
Abstract :

This review uses the marine bivalve Crassostrea gigas to highlight redox reactions and control systems in species living in dynamic intertidal environments. Intertidal species face daily and seasonal environmental variability, including temperature, oxygen, salinity, and nutritional changes. Increasing anthropogenic pressure can bring pollutants and pathogens as additional stressors. Surprisingly, C. gigas demonstrates impressive adaptability to most of these challenges. We explore how ROS production, antioxidant protection, redox signaling, and metabolic adjustments can shed light on how redox biology supports oyster survival in harsh conditions. The review provides (i) a brief summary of shared redox sensing processes in metazoan; (ii) an overview of unique characteristics of the C. gigas intertidal habitat and the suitability of this species as a model organism; (iii) insights into the redox biology of C. gigas, including ROS sources, signaling pathways, ROS-scavenging systems, and thiol-containing proteins; and examples of (iv) hot topics that are underdeveloped in bivalve research linking redox biology with immunometabolism, physioxia, and development. Given its plasticity to environmental changes, C. gigas is a valuable model for studying the role of redox biology in the adaptation to harsh habitats, potentially providing novel insights for basic and applied studies in marine and comparative biochemistry and physiology.

Graphical abstract



Highlights

► Redox biology connects environmental stimuli to biological functions. ► The intertidal species Crassostrea gigas adapts to daily stress through redox processes. ► Oyster metabolism is adapted to minimize oxidative damage in hypoxia and reoxygenation. ► Their adaptable antioxidant system is controlled by the Nrf2 and HIF-1 pathways. ► Redox biology extends to oyster immunity and development through yet understudied pathways.

Keywords : Bivalves, Oxidative stress, Glutathione, Immunometabolism, Mitochondria

Introduction

Redox biology studies the processes and mechanisms of reduction-oxidation (redox) reactions in living organisms. These events involve the transfer of electrons between molecules and are essential in various biological activities such as antioxidant defense, energy metabolism, and stress responses [1]. We can think of redox biology as one of the languages different cellular components can use to talk. It transmits chemical information by reduction/oxidation reactions starting in sensing hubs from redox systems and ending in regulatory hubs such as the nucleus and

mitochondria. These organelles can then convert such signals into responses linked to cellular metabolism and fate. Could redox biology have a role in promoting the translation of environmental changes into biological systems for stress adaptation, thereby leading to enhanced animal fitness? We will discuss this question through the lenses of the marine bivalve *Crassostrea gigas*, an oyster species used as a model organism in marine ecophysiology and ecotoxicology. As we will further discuss in detail, by living in highly dynamic intertidal environments, *C. gigas* relies on several redox regulatory processes that can promote cellular adaptation and animal resilience to multiple biotic and abiotic stress. As a result, *C. gigas* might be viewed as a viable model organism for studying antioxidant protection and oxidative stress, redox signaling events, and metabolic adaptations.

Redox biology: sensing and responding to environmental cues

For animal metabolism to function optimally, cells must be able to sense, integrate, and translate external stimuli rapidly. These are critical for animals to trigger adaptive responses under stress conditions to preserve cellular function and organismal fitness. Redox reactions are examples of ancient and well-preserved molecular/biochemical processes that occur in living cells and can regulate the pace of numerous enzyme systems and metabolic pathways [2]. As a result, the biological repercussions of redox reactions are intricately linked to cellular function and fate in response to endogenous and external stimuli (**Figure 1**), which is the central theme of redox biology [3]. This review does not expand into details on the characterization of fundamental concepts in redox biology, which have been extensively documented in recent reviews [4–7]. Instead, we will explore how redox biology can be at the core of the physiological adaptations of the oyster *C. gigas* to its dynamic intertidal environment. We expect to offer an ecological and physiological perspective on the redox biology processes that drive the unique half-terrestrial, half-marine life of *C. gigas*.

Shared Redox Sensing Processes in Metazoa

The balance between oxidizing (electron-accepting) and reducing (electron-donating) activities within a cell is called the cellular redox state. This dynamic process reflects the overall redox potential of the cellular environment at any given moment and is governed by the interaction of ROS and antioxidant mechanisms [8] (**Figure 1**). It is also crucial for maintaining cellular homeostasis and is involved in various physiological processes, from energy production to signaling pathways and regulation of gene expression. Alterations in the cellular redox state can disrupt normal cellular function and contribute to developing cellular disorders and diseases (**Figure 1**). This section will briefly explore the primary cellular redox-sensing players in animal cells. If needed, literature reviews from redox biology provide detailed information on the biochemistry of cellular redox reactions [3,9,10].

<u>Small redox-sensing molecules: glutathione, NADH, and NADPH</u>

Glutathione (GSH) is a crucial antioxidant molecule composed of three amino acids (glutamate, cysteine, and glycine) and is involved in several biological processes. As a reducing agent, GSH contributes to the defense against ROS and undergoes reversible oxidation and reduction reactions, resulting in GSH or the oxidized/disulfide form GSSG. As a result, the GSH/GSSG ratio is an essential measure of cellular redox state: under physiological conditions,

GSH levels much exceed GSSG levels, while pathologic conditions can enhance GSSG levels and lower GSH/GSSG ratios [11]. Because GSH works together with enzymes such as glutathione peroxidases (GPx) and glutathione S-transferases (GST), it supports the detoxification of ROS and electrophilic compounds that can impair the cellular redox state. As a result, a reducing intracellular environment characterized by high GSH/GSSG ratios offers improved protection against oxidative damage in proteins, lipids, and DNA and a greater capacity to metabolize and remove xenobiotics and reactive compounds. The GSH/GSSG ratio also influences redox-sensitive signaling pathways such as nuclear factor erythroid-2-related factor 2 (Nrf2), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and mitogen-activated protein kinases (MAPKs), which in turn influence cellular processes such as stress and immune responses, proliferation, differentiation, and apoptosis [12] (**Figure 1**). Finally, GSH can also trigger reversible protein glutathionylation, a post-translational modification in which GSH is covalently linked to protein cysteine residues. This process has the dual purpose of protecting cysteine from irreversible oxidation and modifying protein function in response to oxidative stress [13].

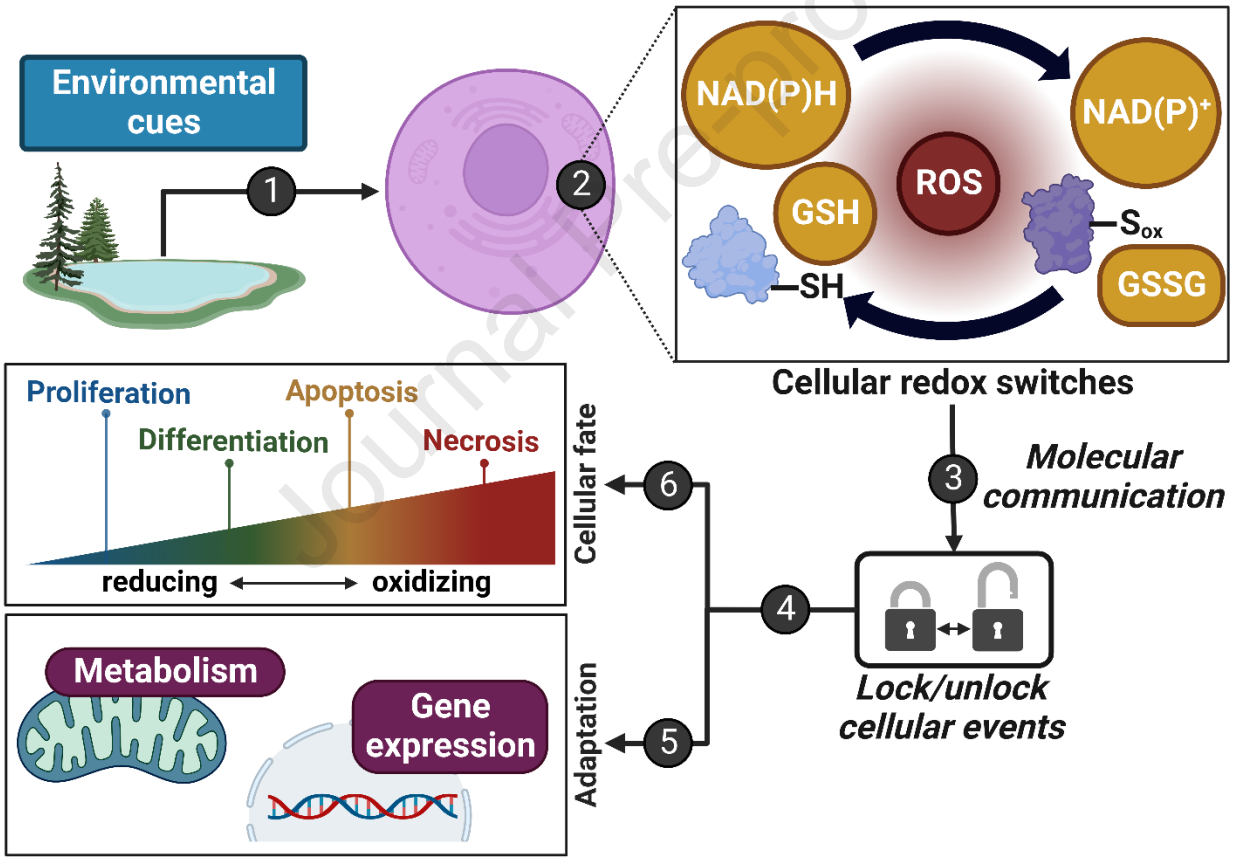


Figure 1: The role of redox biology in detecting environmental cues and translating them into nuclear, mitochondrial, and cellular fate responses. (1) Environmental stimuli or signals can influence cellular homeostasis and metabolism. (2) A portion of these modifications are facilitated by redox pairs, which serve as master cellular redox switches responsive to ROS and oxidative stress. These pairs include but are not limited to, the reduced/oxidized forms of glutathione (GSH/GSSG) and nicotinamide adenine dinucleotide (NAD(P)H/NAD(P)$^+$). Specific proteins also include cysteine residues, which contain thiol groups (-SH) prone to oxidation by ROS. (3) Cells can regulate oxidation-mediated signaling, a type of intracellular communication, by adjusting the cellular redox state. (4) This process of molecular communication can activate or suppress signaling pathways associated with different cell functions and outcomes. (5) Changes in the redox state can initiate the transcription of genes or regulate cellular

metabolism to enable the organism to adjust to a novel environmental circumstance and endure stress. (6) The cellular redox state also controls molecular processes involved in cell proliferation, differentiation, apoptosis, and necrosis.

The nicotinamide adenine dinucleotide system, including the pairings NADH/NAD^+ and NADPH/$NADP^+$, is closely linked to the cellular redox state. As reviewed by Jones and Sies [9], the NAD system employs mitochondrial dehydrogenase enzymes to regulate the catabolism of critical cellular substrates and energy supply (*e.g.* glycolysis, fatty acid oxidation, and tricarboxylic acid cycle). On the other hand, the NADPH system is intimately connected to the management of antioxidant protection and several anabolic responses of energy metabolism. Their reversible oxidizing/reducing reactions support the organization of the cellular energy metabolism and the activation or deactivation of molecular processes guiding cell fate (**Figure 1**).

As a result, the pairings GSH/GSSG, NADH/NAD^+, and NADPH/$NADP^+$ cooperate to regulate stress responses, cellular fate, and animal growth and development (**Figure 1**). Cell growth, for example, necessitates a higher reducing redox state than cellular differentiation, in which ROS are essential for the control of cellular proliferation or quiescence [3]. A more oxidative cellular redox state can cause stress/adaptive responses or apoptosis via particular cellular signaling pathways or if overly oxidized, necrosis [3] (**Figure 1**). Furthermore, such phenomena are subject to the impact of the spatial configuration of the redox pairs inside cellular microenvironments, as well as their ability to react to external stimuli through reversible dynamics [9].

Redox-sensing proteins and enzymes: the example of the thioredoxin-peroxiredoxin system

The thioredoxin-peroxiredoxin system supports the detoxification of peroxides and the control of the redox state of proteins and other molecules in cells. It comprises thioredoxin (Trx), thioredoxin reductase (TrxR), and peroxiredoxins (Prx). Trx is a small protein having two redox-active cysteine residues that operate on the reversible reduction of disulfide bonds, including the ones linked to the peroxidase activity of Prx (described below). This function also assists proteins involved in DNA synthesis, protein folding, and metabolism in maintaining their active conformation and activity, and it also causes transcription factors to translocate to the nucleus, resulting in gene expression activation. These many roles of Trx in redox reactions are supported by TrxR, an enzyme that uses NADPH as an electron donor to reduce Trx and maintain its reducing potential [14]. Prx works as a driver of peroxide control in the thioredoxin-peroxiredoxin system, with some studies indicating that they are the predominant peroxide reducers (>90%) in the cells [15]. This action depends on their peroxidatic cysteine residue, which in turn depends on reducing agents like Trx to stay active.

Surprisingly, increasing levels of peroxides can overoxidize the peroxidatic cysteine of Prxs, rendering it temporarily inactive and mediating peroxide-mediated signaling in eukaryotes [15]. As recently reviewed, Prxs might be seen from two perspectives [16]. One potential implication is that the inactivation of Prxs by overoxidation plays a significant role in the "direct oxidation" of other protein thiols. This process involves inhibiting the thiol peroxidase activity of Prxs and a rise in localized H_2O_2 concentrations, resulting in the subsequent oxidation of protein thiols. According to the second viewpoint, the capacity of Prxs to readily react with H_2O_2 gives them a major competitive advantage over other proteins. The cysteine-SOH generated by this reaction can interact with target redox-regulated proteins, enabling the transfer of oxidizing

equivalents. This property of Prxs to act as a "redox relay" allows for the regulated stimulation of protein oxidation rather than acting as an antioxidant to block it. Thus, the distinct properties of Prx place them in the center of major physiological processes associated with ROS and redox biology, including cell regulatory events (cell proliferation, angiogenesis, senescence, and apoptosis), immunology, circadian rhythms, aging, and stress response [17].

Other redox-sensing proteins include oxidoreductases like glutaredoxin and sulfiredoxin, which may catalyze the oxidoreduction of protein thiols and disulfides in either direction depending on the cellular redox status [18]. The information available on the role of these enzymes in *C. gigas* and other bivalves is limited (if not virtually absent), and therefore, they will not be addressed in this review.

Redox-sensing transcription factors

Nrf2 is probably the most well-studied transcription factor with redox-sensing activity that can trigger cellular redox-regulatory systems. Under normal conditions, Nrf2 interacts with the Kelch-like ECH-associated protein 1 (Keap1), an adaptor protein that prevents Nrf2 from being translocated from the cytosol to the nucleus by triggering its degradation through the ubiquitin/proteasome system. Increased amounts of ROS or electrophiles can change Keap1 structure by targeting Keap1 cysteine residues, inhibiting Nrf2 degradation. Upon translocation to the nucleus, Nrf2 can trigger the expression of cellular proteins essential for antioxidant defense mechanisms. These proteins include those involved in NADPH synthesis and glutathione and thioredoxin metabolism. Consequently, Nrf2 aids in the restoration of the cellular redox state [19]. Moreover, the Nrf2 protein is crucial in regulating several cellular processes, including immune responses, metabolism, proteostasis, iron homeostasis, and the activation of multidrug resistance proteins. This highlights the significance of its redox-sensing function in facilitating diverse cellular stress responses and adaptive mechanisms [20].

Hypoxia-inducible factor 1 (HIF-1) is another transcription factor with redox-sensing activity. HIF-1 coordinates several adaptive responses that increase cell survival and function in oxygen-depleted conditions. The oxygen-sensitive component HIF-1α is hydroxylated and targeted for degradation by the proteasome in normoxic conditions. Under hypoxic conditions, altered mitochondrial activities increase ROS levels, which block the prolyl-hydroxylases that target HIF-1, resulting in HIF-1 stability and activation [21]. Oxidative stress or redox unbalance can trigger the attack of cysteine residues from HIF-1α or prolyl-hydroxylases by ROS, resulting in HIF-1 stabilization even in normoxic circumstances (pseudohypoxia) [22,23]. HIF-1 will target genes that encode proteins involved in various adaptive responses, including glycolysis, angiogenesis, erythropoiesis, antioxidant and immune responses, and cell survival.

Other transcription factors, such as Activator Protein 1 (AP-1), NF-κB, MAPKs, and Janus kinase-signal transducer and activator of transcription (JAK-STAT), are also sensitive to changes in the cellular redox state, being activated by ROS and oxidative stress and controlling cell proliferation, apoptosis, inflammation, and immune responses [24,25]. Some of these pathways will be briefly discussed in the section *Linking redox biology with immunology and immunometabolism*.

The Pacific oyster *C. gigas*: a promising model for redox biology studies

The oyster *Crassostrea* (also described as *Magallana*) *gigas* (Thunberg, 1793), commonly known as the Pacific oyster, has emerged as a captivating biological model for research in aquatic ecosystems due to its intricate ecological interactions, adaptability, and sensitivity to environmental changes. Its worldwide interest as an aquaculture resource has also boosted significant research to understand their biology. Such was this interest that *C. gigas* was the first mollusk species to have its genome sequenced, which revealed the over-representation of genes involved in the defense against biotic and abiotic stress [26]. Indeed, this oyster is a super-tolerant organism that supports various environmental conditions and can adapt its metabolism to rapid dynamic changes in O_2, temperature, pH, and salinity depending on the tidal rhythms (**Figure 2**). *C. gigas* has also been broadly used as a model in research on the impacts of global changes, such as ocean warming, acidification, and pollution [27]. Their sensitivity to these stressors provides scientists with a valuable tool to gauge the health of aquatic ecosystems and predict the potential impacts of future climate changes [28]. Beyond ecological roles, oysters provide a platform for genetic and physiological studies. Their sequenced genome [26] and kinome [29] facilitate investigations into the molecular basis of adaptation, disease resistance, and responses to changing environmental conditions. These studies enhance our understanding of oyster biology and provide broader insights into potentially conserved or unique molecular mechanisms (from genes to proteins) driving adaptation to various environmental stress factors.

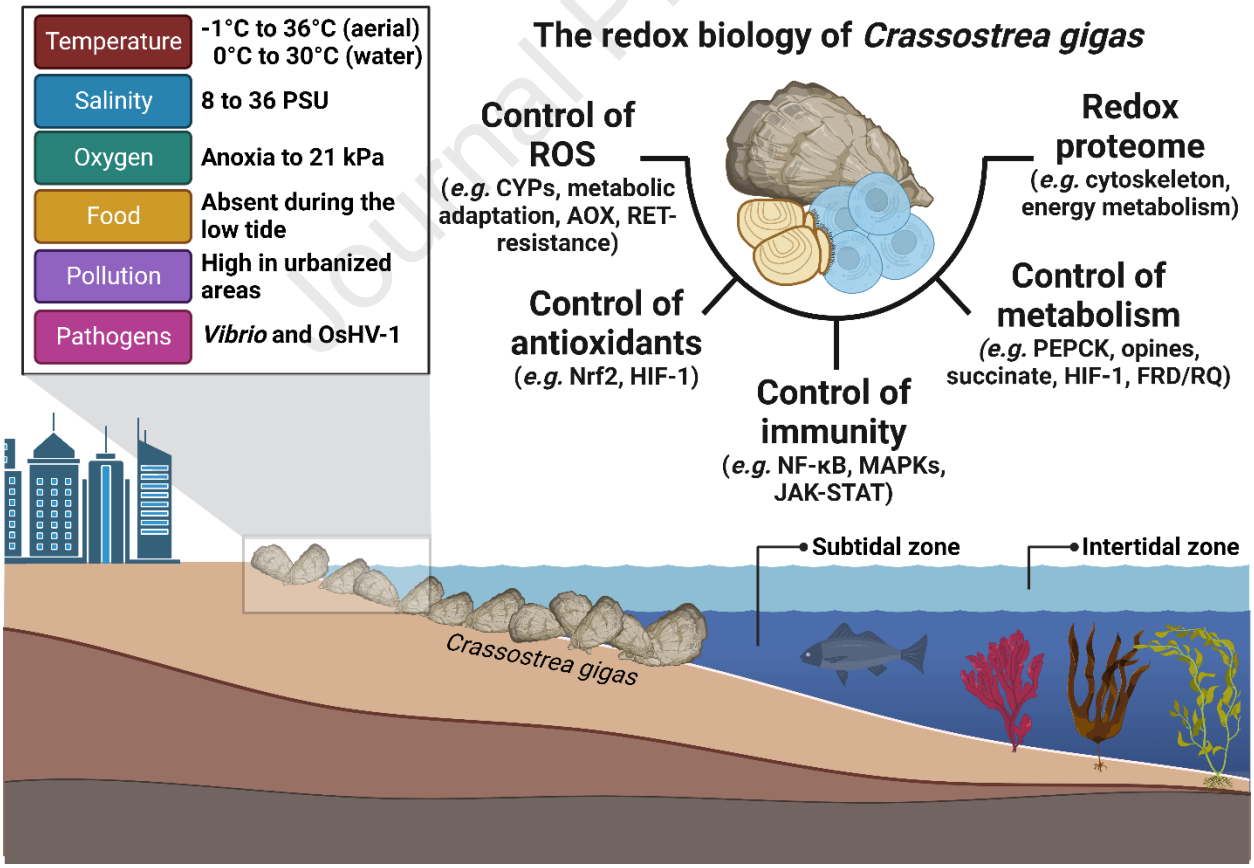


Figure 2: Environmental factors that impact the survival and development of oysters *Crassostrea gigas* in coastal habitats. *C. gigas* encounters significant fluctuations in abiotic variables due to its habitat in intertidal or

shallow subtidal zones. High tides facilitate the influx of water, oxygen, and nutrients, while low tides subject organisms to atmospheric exposure and heightened fluctuations in temperature. The estuarine zones have diurnal variations in salinity, and pollution poses an additional stressor to the habitat of *C. gigas*. This offers a unique set of challenges to maintaining cellular homeostasis, in which the redox biology of *C. gigas* will play a crucial role and perhaps provide valuable insights into the adaptations of animals to extreme conditions. The top left box data are from Corporeau et al. [30] and the ECOSCOPA network in France [31]. Abbreviations: AOX: alternative oxidase; CYPs: cytochrome P450s; FRD: fumarate reductase; PEPCK: phosphoenolpyruvate carboxykinase; RET: reverse electron transfer; RQ: rhodoquinone. Oysters on the top right are shown in their adult stages together with their cells and larvae (D-larvae stage).

Coastal environments are characterized by the increasing interactions between streams, rivers, lakes, wetlands, and estuaries as it gets closer to the coast. Unique among coastal ecosystems are the intertidal zones. They face high environmental variability at both the spatial (tidal) and temporal (seasonal and daily) levels, with gradients of biotic and abiotic variables such as temperature, salinity, nutrients, ultraviolet radiation, microorganisms, and rainfall fluctuations [32,33] (**Figure 2**). Such fluctuations can happen within minutes or hours, depending on the vertical position on the coast, tide height, day/night cycle, and air exposure. For example, in coastal zones from the Brittany region in France, intertidal bivalves can face significant and rapid dynamic temperature fluctuations within minutes or hours, ranging from below zero to above thirty degrees Celsius [30,33] (**Figure 2**). The salinity in the intertidal zone usually is closer to that of saltwater (35 ppt). However, it can decrease dramatically during heavy rain or freshwater input periods and varies significantly in estuaries (**Figure 2**). The dynamic nature of the intertidal zone is also characterized by changing water levels during tidal cycles, exposing sessile organisms to fluctuating periods of heat and UV exposure as well as food and oxygen availability (**Figure 2**). Besides all these natural variations, anthropogenic stressors also particularly impact the intertidal ecosystem. This is because coastal regions are densely populated and rapidly developing, thus negatively impacting the intertidal ecosystem and its inhabitants with unintentional or purposeful chemical discharge, sewage and wastewater discharge, plastic and marine debris, and deposition of atmospheric pollutants (**Figure 2**). Considering all the variables described above, the marine intertidal zone can be considered one of the most dynamic and unpredictable environments on our planet. Nevertheless, the oyster *C. gigas* can live in various intertidal habitats distributed among over 50 countries, from sheltered bays to more exposed rocky shores.

The redox biology of the oyster *C. gigas*: molecular mechanisms for living on the edge of stress.

One of the most studied and remarkable adaptive features of *C. gigas* is its high tolerance to periodic hypoxia/reoxygenation episodes. When exposed to air, as is typical during low tides, valves close, and *C. gigas* rapidly depletes the dissolved oxygen available within their tissues to undetectable levels (the physiological oxygen levels within *C. gigas* tissues during immersion or emersion are discussed in greater detail in the section *The importance of physioxia to in vitro research using invertebrate cell models*). Thus, cells must quickly switch between aerobic and anaerobic metabolism, thanks to the impressive metabolic flexibility of this organism as a facultative anaerobe and hypoxia-tolerant species.

The high tolerance of intertidal bivalves to oxygen deprivation is dependent on crucial redox reactions within pathways of facultative anaerobic energy metabolism in mitochondria: the opines and aspartate-succinate pathways during the early phase of oxygen deprivation and the phosphoenolpyruvate branchpoint during prolonged anaerobiosis [34] (**Figure 3**). The opine pathway is essential for terminating anaerobic glycolysis and producing NAD^+, thus restoring the NADH/NAD^+ redox state. This enables the process of glycolysis to partially sustain cellular ATP synthesis via the use of carbohydrate reserves, such as glycogen. The opines pathway relies on cytoplasmic opine dehydrogenases, which catalyze the reductive condensation of pyruvate with an amino acid (*i.e.,* arginine, glycine, alanine, β-alanine, and taurine), generating the opines. A major suggested advantage of this pathway is that anaerobic opine end products are less acidic than lactate, thus preventing high levels of tissue acidification similar to the ones in mammals. During the early stages of hypoxia, a portion of the pyruvate that is not utilized in the opines pathway is diverted to the aspartate-succinate pathway for malate synthesis (**Figure 3**). This process involves the conversion of pyruvate to alanine and aspartate to malate. This process is facilitated by the enormous amount of free amino acids in bivalve tissues used for osmoregulation [34]. Alanine can be further utilized for protein synthesis, while malate is elegantly used to sustain mitochondrial energy metabolism under anaerobic conditions, which will be discussed below. Malate synthesis can also occur through the glucose-succinate pathway during the prolonged phase of hypoxia. For this process to occur, the phosphoenolpyruvate branch point assumes a pivotal function by deviating from pyruvate synthesis and facilitating the production of oxaloacetate through the increased activity of phosphoenolpyruvate carboxykinase (PEPCK) [35]. Oxaloacetate may thereafter be used by the glucose-succinate pathway to generate malate inside the cytosol (**Figure 3**). Therefore, both the early and later phases of hypoxia produce cytosolic malate, which is then incorporated by the mitochondria.

Once the malate generated by the aspartate-succinate or glucose-succinate pathways enters the mitochondria, it is converted into either acetate (oxidative branch) or succinate (reductive branch) (**Figure 3**). The malate oxidative branch is essential for forming NADH inside mitochondria and generating ATP via substrate-level phosphorylation. NADH can then feed mitochondrial complex I, allowing proton pump and ATP synthase-mediated ATP production. This process can occur in the absence of oxygen, where fumarate appears as the ultimate electron acceptor instead of oxygen, thanks to the malate reductive branch (**Figure 3**) [34]. Redirection of electrons from complex I towards the enzyme fumarate reductase is achieved using rhodoquinone. Thus, both the early and late phases of hypoxia rely on anaerobic energy metabolism in mitochondria through malate dismutation, eventually synthesizing succinate and acetate as final end-products. In addition, succinate can be further metabolized into propionate, with the extra generation of ATP [34, 36, 37] in the case of the late phase of hypoxia (**Figure 3**). Interestingly, the anaerobic energy metabolism in bivalves does not seem to promote lactate fermentation, as several studies failed to detect lactate accumulation in different tissues of bivalves under hypoxic conditions [37]. Although less effective in ATP synthesis than aerobic metabolism (about 5 ATPs per glucose), these anaerobic energy metabolism pathways are more advantageous than lactate fermentation, enabling bivalves to live and flourish in intense and prolonged oxygen deprivation settings.

In most vertebrates, events of hypoxia and reoxygenation within tissues are extremely deleterious, as they are generally followed by a high production of ROS and cell damage [38,39]. In contrast, adaptive mechanisms prevent increased ROS levels upon reoxygenation in bivalves.

Upon re-immersion and reoxygenation, opines and alanine accumulated due to anaerobic metabolism are not excreted but might undergo additional metabolic processes that do not produce ROS (**Figure 3**). Accumulated succinate in *C. gigas*, on the other hand, will support aerobic metabolism through the tricarboxylic acid (TCA) cycle and complex II activity (**Figure 3**). In mammals, elevated succinate concentrations serve as a source of energy for complex II, resulting in the buildup of ubiquinol (the reduced form of ubiquinone). This, in turn, triggers reverse electron transfer to complex I, leading to a substantial generation of ROS [40]. However, in *C. gigas*, increased ROS levels do not occur despite high succinate accumulation, demonstrating a surprising resistance to this reverse electron transfer [41]. The ability of *C. gigas* to survive in intertidal habitats characterized by regular hypoxia and reoxygenation cycles is partially attributed to this crucial protective mechanism of resistance. In such situations, succinate has been identified as a potentially effective energy source, enabling the organism to thrive under stressful circumstances [41].

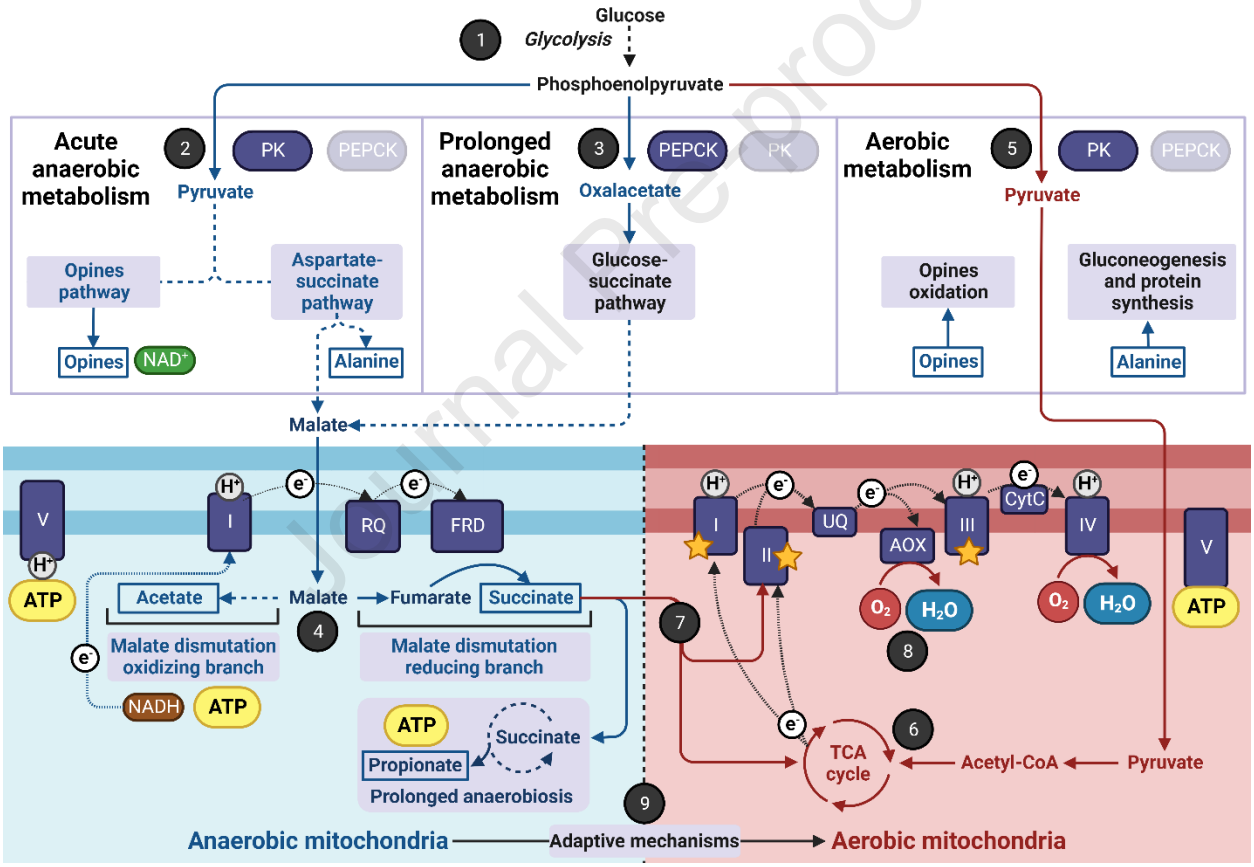


Figure 3: Brief overview of bivalve anaerobic and aerobic energy metabolism and ROS production. (1) The phosphoenolpyruvate branchpoint is reached by glucose in glycolysis. (2) Hypoxia causes early anaerobic metabolic activities to synthesize pyruvate using pyruvate kinase (PK). The opine pathway ferments pyruvate to regenerate NAD^+. Pyruvate can also be transformed into alanine and aspartate into malate by the aspartate-succinate pathway. (3) Under prolonged environmental hypoxia, anaerobic metabolism produces malate from phosphoenolpyruvate and initiates the glucose-aspartate route due to a higher ratio of phosphoenolpyruvate carboxykinase (PEPCK) to PK activity. (4) Cytosolic malate from aspartate-succinate or glucose-succinate pathways enters mitochondria and is converted into fumarate and later succinate via fumarate reductase (FRD). Rhodoquinone (RQ) replaces ubiquinone (UQ) in accepting electrons from complex I and donating them to FRD. Another portion of malate will be turned into acetate, creating NADH and ATP. NADH enables complex I activity and promotes both a proton gradient and ATP synthase function in the absence of oxygen and the transfer of electrons to the RQ/FRD system. ATP is also produced

by succinate-propionate conversion during prolonged anaerobic metabolism. (5) In aerobic conditions, PK synthesizes pyruvate for aerobic mitochondrial metabolism. (6) The aerobic metabolism can also employ accumulated succinate to power the TCA cycle or complex II activities. (7) Aerobic metabolism uses pyruvate to power the electron transfer chain through electrons from the TCA cycle. (8) Alternative oxidase (AOX) reduces oxygen to water, controlling oxygen levels, oxidative metabolism, and ROS generation. (9) Adaptive processes drive the transitions between anaerobic and aerobic mitochondrial metabolism during cycles of hypoxia and reoxygenation, which are discussed in the text. Stars show ROS-forming sites in aerobic mitochondria. A box marks anaerobic metabolism end-products. Dashed lines indicate that a metabolic pathway is summarized, and not all steps are shown. Dashed lines with e- denote electron transfers. H^+ indicates the pumping of protons. For clarity, the stoichiometry and the inclusion of all substrates or cofactors associated with ATP/NADH/NAD^+ have been omitted. Further abbreviations: (I) Complex I; (II) Complex II; (III) Complex III; (IV) Complex IV; (V) ATP synthase; (CytC): cytochrome C. Figure adapted from [34,36]. See [34] for details on the biochemical pathways summarized here.

Sources of ROS in C. gigas: in vitro studies with hemocytes or isolated mitochondria

ROS are a group of chemically reactive molecules containing oxygen and are produced naturally as byproducts of metabolic processes within living organisms. ROS are produced within cells through various metabolic events, including mitochondrial respiration, enzymatic activities, and stress responses. Many investigations on the generation and metabolism of ROS in bivalves are based on *in vitro* studies with the circulating blood cells known as hemocytes [42–46]. The great interest in studying these cell types lies in their pivotal role in the immune defense and maintenance of oyster health. They can be readily sampled in a non-lethal manner and maintained under laboratory conditions for short periods. Their diverse functions encompass pathogen recognition, phagocytosis, immune signaling, encapsulation, tissue repair, detoxification, nutrient transport, wound healing, ion regulation, and waste clearance [47–49]. In *C. gigas*, ROS production in unstimulated hemocytes (no contact with pathogens) was thought to be dominated by the NADPH-oxidase pathway (80-85% of total ROS production), which is associated with the "respiratory burst" phenomenon, with the remaining fractions attributed to a physiological constitutive output of oxidant molecules [42].

Further research with *C. gigas* found that most of the previously assumed NADH-oxidase-dependent ROS production was instead linked to the activities of the mitochondrial respiratory complexes I and III in hemocytes [46] (**Figure 3**). The same study discovered that blocking complex I with rotenone had no effect on mitochondrial membrane potential or ROS generation rates, whereas inhibiting complex III with antimycin A reduced both values. In mammals, inhibiting complex III with antimycin A can enhance mitochondrial ROS production, attributed to increased mitochondrial superoxide anion production by electron backflow to complex I [50,51]. The presence of an alternative oxidase (AOX) (**Figure 3**) as a non-energy conserving branch in the respiratory chain of *C. gigas* can act as an additional terminal oxidase before transferring electrons to complex III [52], preventing the reverse electron transfer to complex I and increased ROS levels in this species. According to the *C. gigas* hemocyte cell model, mitochondrial generation of ROS in this species is strongly associated with complex III and the forward electron transfer pathway [53].

In vitro investigations using isolated mitochondria from *C. gigas* tissues (*e.g.,* gills and digestive gland) provide additional and crucial information on the molecular processes directing mitochondrial ROS generation in this species, particularly concerning its hypoxia/reoxygenation tolerance. The production of ROS in isolated mitochondrial preparations of marine invertebrates is about an order of magnitude lower than in mammalian studies and is sensitive to mild uncoupling

of H^+ motive force [53]. This is true for *C. gigas* partly because of its low body temperature, oxygen turnover rates, and mitochondrial densities as an ectothermic organism in contrast to mammals. Additionally, internal systems regulating the formation of ROS further contribute to this phenomenon. Under hypoxic conditions, *C. gigas* can reduce oxygen consumption by mitochondrial respiration and enhance the efficiency of aerobic energy production, both indicators of metabolic depression [54]. *C. gigas* can also inhibit β-oxidation and amino acid oxidation, as well as prevent the conversion of pyruvate to acetyl-CoA, reducing the electron input and the ROS formation by the electron transfer system [55] (see also the adaptive metabolic response to hypoxia in **Figure 3**). It can also reduce the respiration rate of complex I, complex II, and complex IV and improve the control of mitochondrial iron load during hypoxia, all mechanisms to prevent ROS formation upon reoxygenation [55]. Increased expression of AOX during hypoxia is another adaptive mechanism of *C. gigas*, which can redirect about 10-15% of the oxygen flow towards this alternate pathway and decrease ROS production by detouring from complex III [54]. *C. gigas* also responds to hypoxia by increasing the content of NADH-oxidizing enzymes and decreasing the abundance of NADH-generating enzymes to control the mitochondrial redox state through a high $NADH/NAD^+$ ratio [55]. Therefore, the use of metabolic depression, global repression to halt energy-consuming transcription, and regulation of mitochondrial complexes are crucial for the great resistance to hypoxia/reoxygenation observed in this particular species [56].

As previously reviewed [36], bivalves may also produce ROS in the endoplasmic reticulum. On the other hand, the production of ROS by peroxisomes in bivalves is currently unclear, even though this organelle is a significant producer of hydrogen peroxide, superoxide anion, and nitric oxide [57]. The endoplasmatic reticulum is an organelle linked to protein folding and assembly, and ROS can be produced as a byproduct of this action or by the catabolic activity of cytochrome P-450 (CYPs). CYPs are particularly relevant since they catalyze phase I detoxification processes of both endogenous substances and xenobiotics, generating ROS (although at much lower levels than mammals). By inhabiting coastal zones with constant anthropogenic stresses, bivalves are exposed to many xenobiotics known to be metabolized by, or that increase the expression of CYPs [58]. Interestingly, *C. gigas* has 121 protein-coding CYP genes, which is a large number when compared to other organisms like scallops (*Chlamys farreri*, 88 CYP genes), copepods (*Tigriopus japonicus*, 52 CYPs), fruitflies (*Drosophila melanogaster*, 85 CYPs), zebrafish (*Danio rerio*, 94 CYPs), and humans (57 CYP genes) [59]. This suggests an increase in the *C. gigas* CYP family, which may contribute to CYP variety and responses to environmental stress [26,59]. Environmental contaminants such as sanitary sewage [60], toxins found in harmful algal blooms [61,62], polycyclic aromatic hydrocarbons [63], or environmental conditions such as lower seawater pH (ocean acidification) [64] and osmotic stress (salinity) [65] can all increase the expression or activity of different CYP isoforms in *C. gigas*, influencing oxidative metabolism and ROS production in other tissues and cellular compartments. It is crucial to recall that CYPs may also be present in mitochondria (the second most prevalent group of CYPs in *C. gigas*), which could contribute to mitochondrial ROS generation in response to natural or anthropogenic environmental stress.

Redox-controlling pathways in C. gigas: the Nrf2 and HIF-1 signaling pathways

Control of the antioxidant system is significant for intertidal bivalves like *C. gigas*, which can boost antioxidant efficiency (increased protein levels of SOD, CAT, PRx, GPx) in response to heat and oxygen stress while living in areas where they would be exposed to air for 80% of the

time [66]. Moreover, according to the literature, one of the most prevalent molecular mechanisms of reaction to hazardous circumstances in bivalves is the amplification of antioxidant defenses [67–70]. In *C. gigas*, higher production of hydrogen peroxide due to cadmium exposure is linked to increased gene expression of SOD, CAT, and GPX in the gills [71]. Higher reproductive investment (increased production of germ cells) can also increase the gene expression or activity of antioxidant enzymes such as GPx and extracellular and mitochondrial SOD [72]. This is most likely owing to the high reproductive allocation of bivalves (gonads can take up to 70% of the animal volume) and increased metabolic rate, respiratory activity, and mitochondrial ROS production during reproductive development [72–74]. Seasonal variations are also seen to modulate antioxidant levels with decreased antioxidant capacity during the winter (lower temperatures and limited food availability). In other bivalves such as mussels, amplification of antioxidants such as GR, GPx, SOD, and TrxR has been detected in parallel to increased levels of hydroperoxides and lipid peroxidation during zinc exposure [75]. Together, these studies provide evidence of coordinated signaling pathways triggering antioxidant adaptive mechanisms in intertidal bivalves.

To our knowledge, the first study investigating a redox-controlling pathway in bivalves was with *C. gigas* [76]. In this study, the expression of Nrf2 and target genes were evaluated in different tissues after acute exposure to waterborne curcumin [76], a classic inducer of the Nrf2 pathway in vertebrates [77]. The findings suggest that (i) bivalves have an oxidative stress response mechanism comparable to vertebrates, with increased GSH production and enhanced activity of antioxidant enzymes associated with the GSH system (**Figure 4**). Surprisingly, antioxidant amplification was observed in the gills but not in the digestive gland, indicating that tissues in direct contact with the environment have a prompt and sensitive Nrf2 pathway. Later, it was shown that the Nrf2 pathway in *C. gigas* gills (but not the digestive gland) is responsive to tert-butylhydroquinone, another common Nrf2 activator [78]. While these were mechanistic studies on the tissue-specific responsiveness of the Nrf2 pathway, later studies found that other bivalves showed Nrf2-like antioxidant responses under pro-oxidant conditions such as after exposure to polycyclic aromatic hydrocarbons [79–82], toxic microalgae and cyanobacteria or their toxins [80,83–88], and other types of stressors such as stock densities in aquaculture conditions [89]. According to investigations using interfering RNA against Keap1 or Nrf2 [67,90], the regulation of antioxidants from the Trx system by the Nrf2 pathway has already been confirmed in the freshwater bivalve *Cristaria plicata*. As can be seen, most study on the Nrf2 pathway in bivalves focuses on how Nrf2-targets respond to environmental stresses. The current state of bivalve research on the control of Nrf2, the identification of potential Nrf2 target genes, and the effects of Nrf2 activation on animal ecophysiology, particularly in *C. gigas*, is currently lacking in both breadth and depth.

Recent research suggests that the Nrf2 pathway of *C. gigas* interacts with other critical signaling pathways involved in cellular defense systems (**Figure 4**), as already indicated in different model organisms. The aryl hydrocarbon receptor (AhR) pathway, which reacts to polycyclic aromatic hydrocarbons and other polyhalogenated aromatic chemicals, can boost the expression of several phase I and phase II detoxification pathways and antioxidant enzymes. The AhR and Nrf2 pathways in *C. gigas* and other bivalves can interact via two primary pathways: (i) AhR modulates the expression of detoxification genes associated with ROS production (*e.g.*, CYPs), thereby activating or deactivating Nrf2 activity, (ii) AhR modulates the MAPK and protein kinase C (PKC) signaling pathways, which may act as downstream regulatory pathways for the

AhR pathway by phosphorylation/activation of Nrf2 [81,85]. The selective autophagy receptor p62/sequestosome 1 (p62) is another pathway linked to regulating the Nrf2 pathway in bivalves. It is a classical selective autophagy receptor, but it also has roles in the ubiquitin-proteasome system, cellular metabolism, signaling, and apoptosis [91]. In mammals, oxidative stress can trigger p62 to interact with and inhibit Keap1 activity or activate the autophagy-induced degradation of Keap1, freeing the Nrf2 for nuclear translocation and activating antioxidant responses [92]. In bivalves, p62 has been shown to lack a domain responsible for binding to Keap1. However, it has increased expression by Nrf2 activation and can also lead to its own expression and the expression of other genes regulated by Nrf2, thus cooperating with the Nrf2 pathway to confer improved antioxidant efficiency [93]. In addition, bivalves contain two Keap1 orthologs (Keap1a and Keap1b): Keap1a seems to be analogous to the ortholog Keap1 of vertebrates, while Keap1b could have been gradually formed during the evolution of invertebrates but lost critical cysteines residues linked to Nrf2 inactivation (cysteines analogous to Cys-273 and Cys-288 of mammalian Keap1) [87]. While some of these studies do not involve *C. gigas*, these regulatory processes may be conserved across bivalve species and deserve special attention from the scientific community to understand better the control and ecophysiological implications of a master regulator of the antioxidant system such as the Nrf2 pathway in *C. gigas* and other bivalve species.

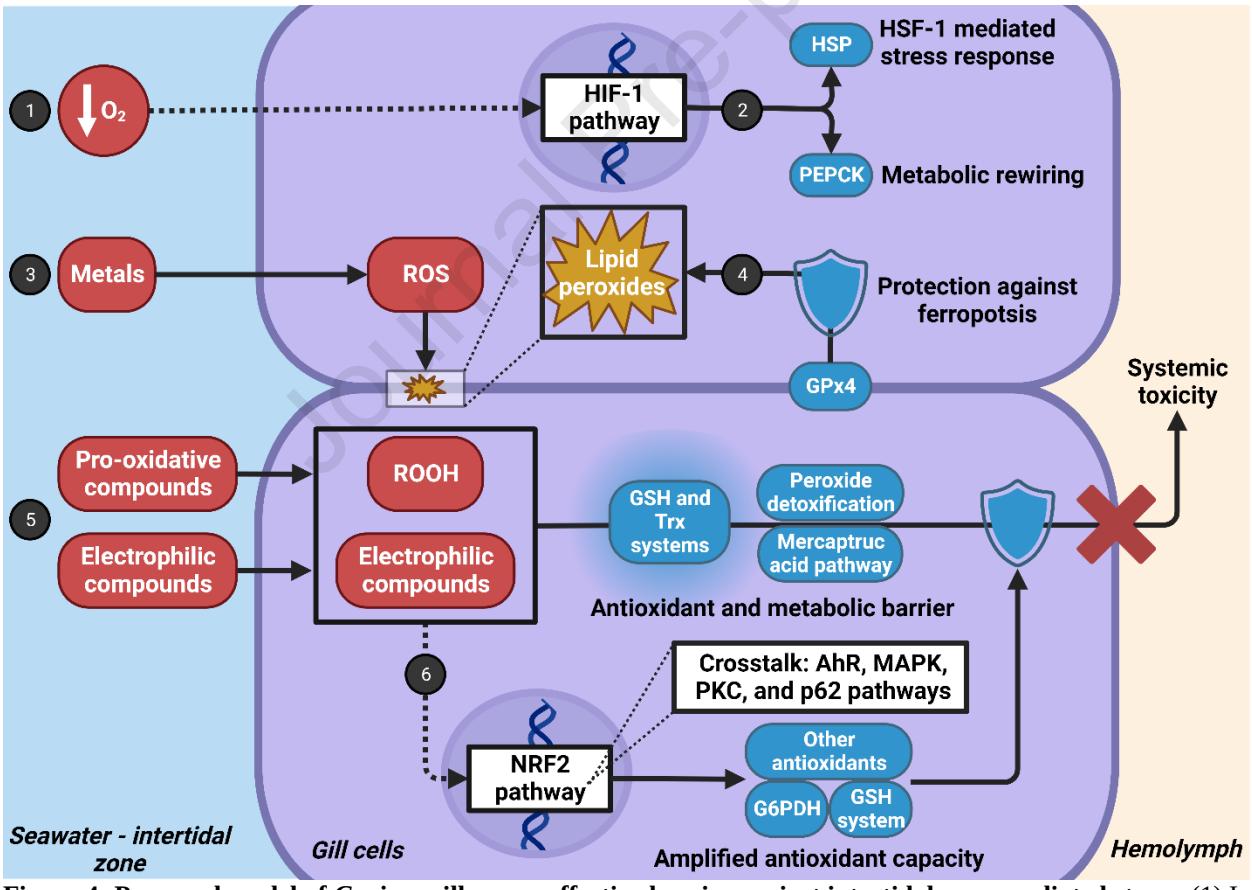


Figure 4: Proposed model of *C. gigas* gills as an effective barrier against intertidal zone-mediated stress. (1) In the gills, hypoxia can activate the HIF-1 pathway. (2) This causes cytoprotective responses via the heat shock factor 1 (HSF1) and metabolic adaptation to anaerobiosis via elevated PEPCK expression. (3) Metal exposure can raise ROS levels, which may damage lipid membranes and initiate a cascade of lipid peroxidation, potentially leading to cell death. (4) Metal-induced increases in the activity of GPx4 may protect against the production of lipid peroxides and inhibit the activation of the ferroptosis pathway. (5) Pro-oxidative and electrophilic chemicals can be swiftly cleared

by the GSH and Trx systems via peroxide detoxification and the mercapturic acid pathway in the gills, preventing them from reaching the hemolymph and the rest of the organism. These chemicals can also activate the Nrf2 pathway, which increases antioxidant capacity and gill protection. The Nrf2 pathway can also interact with other signaling pathways, broadening the *C. gigas* redox biology network toward other cellular activities. Dashed lines represent the activation of gene expression mediated by cellular protective pathways. Abbreviations: G6PDH: glucose 6-phosphate dehydrogenase; GPx4: phospholipid hydroperoxidase; GSH: glutathione; HSF-1: heat shock factor 1; PEPCK: phosphoenolpyruvate carboxykinase; Trx: thioredoxin.

The HIF-1 signaling pathway was already described in bivalves and is essential in regulating numerous elements of bivalve oxidative and energy metabolism, notably in adaptive responses to hypoxia. Interestingly, the oxygen-responsive HIF-1 subunit α (β subunit is expressed constitutively) is abundantly expressed in the gills of bivalves, both under normoxic and hypoxic conditions, displaying the key physiological role of this tissue in oxygen sensing and gas exchange [94]. But other tissues also express HIF-1α, and all tissues in bivalves respond to oxygen levels in a HIF-1-dependent manner [94]. For example, the hypoxia-tolerant eastern oysters *Crassostrea virginica* exhibit a high abundance of HIF-1α transcripts, comparable to the transcript abundance of commonly used housekeeping genes like β-actins. Furthermore, in the presence of hypoxia, the expression of HIF-1α in the gills can be further enhanced [95]. In the study conducted by [96], it was shown that a total of six HIF-1α isoforms are present in *C. gigas*. These isoforms were shown to play a crucial role in controlling the organism's response to oxygen levels and temperature stress. Previous studies have demonstrated that *C. gigas* HIF-1α can identify and bind to hypoxia response elements located in the promoter region of phosphoenolpyruvate carboxykinase [95,97]. This interaction leads to a redirection of phosphoenolpyruvate, derived from glycolysis, towards malate dismutation and mitochondrial anaerobic metabolism, as discussed in the section *The redox biology of the oyster C. gigas: molecular mechanisms for living on the edge of stress* (**Figure 3**). Moreover, HIF-1α can attach to hypoxia response elements located in the promoter region of the heat shock factor 1 in *C. gigas*, therefore initiating a heat shock response mechanism that is adaptive in nature [96] (**Figure 4**). The observed upregulation of several heat-shock proteins in response to heat stress in *C. gigas* (14 isoforms of HSP70 and three isoforms of HSP20) may be attributed to their role as a cytoprotective mechanism [98]. Typically, this cytoprotective strategy is observed concurrently with the decrease in oxidative metabolism and the transition to anaerobic metabolism [99,100], with the consequent buildup of alanine, succinate, propionate, acetate, and opines [101] (refer to **Figure 3**). Although *C. gigas* can undergo metabolic suppression via the HIF-1 pathway in response to hypoxic circumstances, there is no change in the generation of mitochondrial ROS in the gills after 24 hours of hypoxia [102]. However, it should be noted that under similar circumstances, there is an increase in ROS production in hemocytes [103], the circulating immune cells of bivalves. Both transient hypoxia lasting 24 hours and prolonged hypoxia lasting six days do not result in the oxidation of thiol-containing amino acids in *C. gigas*. This can be attributed to the higher level of metabolic depression, adaptive anaerobic metabolism, accumulation of cytoprotective compounds, and reduced accumulation of pro-oxidant metabolites observed in this highly hypoxia-tolerant bivalve species [104]. Despite such advances in understanding the role of HIF-1 in oysters and bivalves, it is apparent that there is a demand for additional studies on the regulation of HIF-1 concerning the adaptive response of hypoxia tolerance in the redox biology of *C. gigas*.

ROS scavenging systems in C. gigas

The antioxidant system in *C. gigas* has been extensively studied as a cellular defensive mechanism, particularly in response to environmental pollutants and stressors. These biological responses are widely applied to ecotoxicological and biomonitoring studies as they are considered significant indicators of physiological stress and can be associated with organismal and environmental health [105,106]. As filter feeders, bivalves can uptake and accumulate substantial quantities of environmental contaminants, including metals, organic pollutants, and microplastics occurring in the water, food, or sediment of contaminated coastal environments [107–109]. Thus, it is unsurprising that they have been employed as model organisms in ecotoxicological research and governmental initiatives to monitor water quality [110]. In this context, antioxidants may be consumed or depleted due to exposure to pollutants with pro-oxidant properties or enhanced to restore the cellular redox equilibrium and protect against oxidative damage [111]. Therefore, the antioxidant system can offer a comprehensive overview of the cellular stress state, possible adaptive responses to pro-oxidant environmental stimuli, and mechanisms of toxicity associated with ROS and oxidative metabolism in bivalves living in contaminated areas.

Catalase and superoxide dismutase in *C. gigas*

The proportional importance of main ROS-detoxifying enzymes to their antioxidant system, including catalase (Cat), superoxide dismutase (SOD), and GPx, varies across bivalves and other invertebrate species. The particular activities of SOD and GPx in invertebrates appear to be lower compared to vertebrates, with a decrease of around 1-2 orders of magnitude [112]. In contrast, the activity of Cat in invertebrates remains similar to that observed in many vertebrates [112]. Elevated Cat activity or gene expression has been seen in *C. gigas* following exposure to microcystins [113,114], metals [71], high temperatures [115], pharmaceuticals [116], pesticides [117,118], and pathogens [119]. In the context of SOD, it is observed that invertebrates possess a significantly more intricate SOD repertoire, characterized by the presence of multiple forms of cytosolic (Cu/Zn-SOD), mitochondrial (Mn-SOD), and extracellular (extracellular Cu/Zn-SOD) enzymes. According to a recent study [120], the proliferation of SOD genes in bivalves has resulted in 8-13 distinct SODs in some oyster species, accompanied by changes in both their structural characteristics and encoded molecules. *C. gigas* was reported to have a total of 13 putative SOD enzymes (compared to 3 isoforms in humans), including a Mn-SOD and a cytosolic Cu/Zn-SOD with SOD activity and all the conserved amino acid residues that are expected to function as ligands for metal ions. The remaining 11 genes comprise intracellular and extracellular Cu/Zn-SODs composed of one, two, or four Cu/Zn-SOD domains. The multi-domain SODs have been seen exclusively in aquatic species and winged insects. These SODs possess Cu^{2+} ligands, which may confer SOD activity, or lack metal ion ligands, resulting in the absence of SOD activity. However, the precise roles of these multi-domain SODs remain uncertain. In contrast, the single-domain SODs exhibited an active SOD center encompassing ligands capable of binding Cu^{2+}, Zn^{2+}, both Cu^{2+} and Zn^{2+}, or no metals. Thus, *C. gigas* contains one mitochondrial and one cytosolic form of SODs with SOD activity and several other intracellular or extracellular SODs probably lacking SOD activity that can function as antioxidants by facilitating metal transport [120]. Like Cat, the cytosolic SOD in *C. gigas* exhibits increased enzymatic activity when exposed to various environmental stressors. These stressors encompass microcystin and toxins derived from microalgae [113], metals [121], organic contaminants [122], and pesticides [118], all of which possess pro-oxidant properties. On the contrary, the information on the role of the MnSOD in *C.*

gigas redox biology is still minimal. We also propose reassessing the available data on Cu/Zn-SOD mRNA transcripts to environmental stresses is warranted in light of these 12 different Cu/Zn-SOD isoforms in *C. gigas* [120]. Although Cat and SOD are enzymes usually recognized as a primary and first defense mechanism against ROS in bivalves, it is not uncommon to see unchanged activity or gene expression in response to stress.

The glutathione system in *C. gigas*

The glutathione system comprises many key components, including GSH (with its production enzymes), GPX, GR, and GST (for further details, see the section *Small redox-sensing molecules: glutathione, NADH, and NADPH*). We have found that the GSH system in the gills of *C. gigas* is regulated by the Nrf2 pathway (**Figure 4**). As previously discussed, this pathway in *C. gigas* was proven to be activated in response to well-known Nrf2 inducers, including curcumin, tert-butyl hydroquinone, and chlorodinitrobenzene [76,78,123]. We have also shown that increases in the levels of GSH, as well as the activities of GR, GST, and GPx in the gills of *C. gigas,* have been associated with a seven-fold enhancement in tolerance and survival against organic peroxides, namely cumene hydroperoxide [76]. In contrast, we observed that reductions in GSH levels and the enzymatic activities of GR and TrxR in the gills of *C. gigas* lead to an aggravation of the pro-oxidative impacts caused by the redox-cycling quinone known as menadione [124]. Additionally, it results in a 30% reduction in the *in vivo* detoxification rate of cumene hydroperoxide and a four-fold rise in the mortality rate associated with menadione. In mussels, we have also identified that inhibition of both the GSH and Trx/Prx system can increase the mortality to cumene hydroperoxide by 2-4 fold and decrease their cumene hydroperoxide detoxification capacity up to 15 fold [125]. It is noteworthy to mention that cumene hydroperoxide is metabolized by both the GSH/GPx and Trx/Prx systems [126,127], but not Cat, being an interesting tool for mechanistic studies of these two systems. Besides their protective role against chemicals, we have also demonstrated the importance of GSH pools in oyster resistance to bacterial infections [128]. GSH and GST can also convert the gills of *C. gigas* into a metabolic barrier that protects against waterborne electrophiles. This is achieved by rapidly transforming lipophilic and electrophilic compounds into more water-soluble compounds through the mercapturic acid pathway, ultimately leading to their excretion [125]. Thus, it appears that the gills of bivalves exhibit a vital mechanism that involves the efficient functioning of the GSH system [123–125], along with a remarkably reactive Nrf2 pathway [76,78,123] (**Figure 4**). As an illustration, when entering the circulatory system of *C. gigas*, the electrophilic chemical chlorodinitrobenzene has the potential to induce cellular toxicity and impair immunological function by inhibiting the hemocyte antioxidant system [129]. Thus, one crucial significance of the GSH system in tissues in direct contact with seawater lies in its ability to provide resistance against waterborne electrophiles and peroxide-forming compounds (**Figure 4**). This can prevent a systemic formation of harmful adducts or oxidative damage in DNA, RNA, and proteins within the oyster.

Based on this model, it may be inferred that the GSH/ system may have a wide range of influence in the redox biology of *C. gigas*. (i) It can neutralize hydrogen peroxides, larger hydroperoxides, and lipid peroxides generated during normal or pro-oxidant conditions, such as quinone exposure (**Figure 4**). (ii) It can interact with electrophilic compounds, including menadione and chlorodinitrobenzene, thus impacting the excretion rate of these compounds (**Figure 4**). (iii) Increased activity of GPx and consumption of GSH can lead to a higher

consumption rate of NADPH and a temporary alteration in the cytosolic NADPH/$NADP^+$ ratio, resulting in a more oxidizing environment. Such change in the cellular redox state, in turn, could initiate additional adaptive responses within the cellular system [130,131]. For instance, coordinated amplification responses of the GSH system and the glucose-6-phosphate dehydrogenase (G6PDH) enzyme have been seen in the gills of oysters from polluted regions in Brazil [132,133]. Increased G6PDH activity can increase the synthesis of NADPH, which in turn supports the antioxidant activity of the GSH system and can help to restore the intracellular redox state through the NADPH/$NADP^+$ ratio (**Figure 4**).

Many studies indicate that the GSH/GPx system is upregulated by environmental stressors in *C. gigas*, such as exposures to metals [131], toxins from harmful microalgae [130,134], prolonged hypoxia [135], and organic contaminants [136], which could be related by the activation of redox signaling pathways including the Nrf2 and HIF-1 pathways. The GSH synthesis pathway, which consists of glutamate-cysteine ligase and glutathione synthase, is seen to be increased in conjunction with GPx, GR, and GST in the gills of *C. gigas* that have been exposed to cadmium [130]. This is an example of a synchronized overexpression and protective mechanism that could limit GSSG accumulation inside the cell. The identification of the functional role of a phospholipid hydroperoxide GPx in protecting against metal-induced lipid peroxidation in mussels exposed to metals such as cadmium, copper, lead, and iron was established in the early 2000s [137]. However, the identification of the GPx isoform GPX4 (phospholipid hydroperoxidase) as a participant in ferroptosis, an iron-dependent lipid peroxidation process that results in cell death, was only a relatively recent discovery in bivalves (*C. gigas*) [138]. GPx4 is an enzyme known as phospholipid hydroperoxidase, which plays a crucial role in protecting cells against the peroxidation of membrane lipids. This protective function is significant as it helps prevent the spread of lipid peroxidation processes that might result in mitochondrial damage and ferroptosis [139] (**Figure 4**). This discovery presents a novel area of research focused on characterizing the impacts of metal exposure and homeostasis in bivalve organisms inhabiting metal-contaminated coastal environments around the globe. The inhibitory effect of GSH on the propagation of lipid peroxidation resulting from ROS generated during the metabolism of polycyclic aromatic hydrocarbons in *C. gigas* has also been demonstrated [140]. Additionally, we have observed that the inhibition of GR by exposure to ionic zinc or zinc oxide nanoparticles might result in elevated oxidative effects in *C. gigas* and other bivalves. These effects include lipid peroxidation, protein oxidation, and disruption of mitochondrial function [75,141,142].

The available literature on the redox dynamics of the GSH/GSSG pair in *C. gigas* is scarce despite its significance as a fundamental indicator of cellular redox status. It is recognized that elevated water temperatures have the potential to enhance mitochondrial oxidative metabolism and glycogen store utilization in *C. gigas* [143]. These changes suggest an increased metabolic rate and the potential formation of mitochondrial ROS. Consequently, a decline in the GSH/GSSG ratio was observed in *C. gigas* as water temperatures increased [143], indicating an increased degree of oxidation within the intracellular milieu. The decrease in the GSH/GSSG ratio as a result of elevated temperatures is also observed in other oyster species, including *C. virginica* [144]. Indeed, the reproductive period of *C. gigas* extends throughout the summer, during which elevated temperatures trigger a catabolic metabolic state and increased oxidative metabolism to provide supplementary energy required to fulfill the reproductive energy requirements. Consequently, *C. gigas* exhibits an increased antioxidant capacity in somatic cells during reproductive investment [72]. This is crucial in mitigating the ROS generated as a byproduct of gonadic maturation.

However, it remains uncertain whether the GSH/GSSG ratio plays a role in connecting the cellular redox state with the antioxidant system and oxidative metabolism during the summer season in this species. It is noteworthy that elevated trophic conditions and higher temperatures have been seen to enhance the reproductive effort and respiratory rate of *C. gigas*. This can be attributed to regulating energy balance and reproduction in response to nutrition availability. This phenomenon is associated with increased mortality throughout the summer [74]. The organism's resistance or vulnerability to summer mortality, characterized in part by high or low antioxidant capacity and control or energy balance, is influenced by the balance between ROS generated from reproductive investment and the organism's antioxidant response [74,145,146]. However, the question remains unanswered as to whether animals with a more remarkable ability to tolerate summer mortality events worldwide can also effectively regulate the GSH/GSSG ratio and swiftly adjust their GSH metabolism to maintain a more reducing redox state. These adaptive responses are crucial in integrating the field of redox biology into animal ecophysiology and stress resistance.

The Trx/Prx system in *C. gigas*

The Trx/Prx system in *C. gigas* and other bivalves remains minimally characterized, unlike the GSH/GPx system. Previous studies have undertaken mechanistic investigations on the inhibition of TrxR in *C. gigas* and Perna perna mussels using chlorodinitrobenzene [124,125,129]. However, it should be noted that chlorodinitrobenzene can also deplete GSH and inhibit GR, limiting the ability to analyze the particular protective effects of the Trx/Prx system. However, the findings suggest that the chlorodinitrobenzene leads to a reduction in antioxidant activity, resulting in a decrease in the rate at which organic hydroperoxides are detoxified *in vivo* and an increase in vulnerability to further pro-oxidant insults (**Figure 4**). A correlation has also been detected between reduced TrxR activity and elevated levels of DNA damage and protein thiol oxidation in mussels that were subjected to copper exposure [147]. Bivalves have been observed to enhance the activity, protein levels, and gene expression of TrxR, Prx4, and Prx5 in reaction to various stimuli, including exposure to metals [75], pesticides [118], pathogens [119], reduced seawater pH [148], and hypoxia [149]. The expression of Prx6 is also positively linked with environmental contamination in populations of *C. gigas* residing along the French Atlantic coast [150]. The findings indicate that the *C. gigas* possesses a functioning Trx/Prx system, influenced by various abiotic and biotic stimuli, and can protect against oxidative damage (**Figure 4**). However, it is imperative to conduct mechanistic investigations to identify potential areas of overlap and distinct functions of the Trx/Prx and the GSH/GPx systems in this model species.

Protein thiols sensitive to oxidative stress in marine bivalves

Protein thiols, predominantly characterized by cysteine residues, exhibit high sensitivity to alterations in the intracellular redox condition. They function as crucial regulatory components in various biological activities, encompassing enzyme activity, gene expression, cellular signaling, and antioxidant defense mechanisms. The redoxome of protein thiols consists of many proteins, including enzymes, transcription factors, chaperones, and receptors [151]. The redox-active cysteines of proteins can undergo reversible oxidative modifications, including the formation of disulfide bonds, S-glutathionylation, and S-nitrosylation, in response to changes in the cellular environment. Hence, the thiol redoxome offers a unique viewpoint of the molecular processes that govern rapid physiological responses to intrinsic and environmental stimuli before alterations in mRNA or protein levels occur.

In light of the insufficiency of current research referring to the redox proteome of *C. gigas*, we will discuss this topic by considering the available information concerning other species of bivalves. The global assessment of protein thiol oxidation in bivalves, which excludes protein identification by mass spectrometry, indicates that many contaminants with pro-oxidative properties (*e.g.*, pesticides, metal nanoparticles, polycyclic aromatic hydrocarbons, quinones, and metals) lead to oxidative modifications in the proteins of marine bivalves [141,147,152–155]. It is well characterized that approximately 90% of proteins in eukaryotes include at least one cysteine residue. However, the vast majority of cysteine residues are present in non-cytosolic proteins and are involved in creating disulfide bridges. Free and redox-reactive thiol groups are estimated to constitute only roughly 1.4% of the overall protein content (abundance) in marine bivalves, similar to the values obtained for fungi (2.5%) and bacteria (1.4%) [156,157]. Nonetheless, among the 19 amino acids, cysteine residues have the most significant degree of conservation, indicating a crucial role in protein structure and redox activity [158]. Despite their overall low abundance in terms of total protein content, protein thiol levels are approximately 2.5 times more abundant than non-protein thiols (*e.g.*, GSH and free cysteine) in *C. gigas* [124]. Hence, although redox-sensing cysteines are observed in only a limited number of proteins, such proteins represent a significant pool of redox-reactive thiols within the cellular environment. Notably, proteins can utilize the redox chemistry of cysteine not only for defense against oxidative stress but also for redox signaling under sub-stress conditions. Altogether, the provided data supports the concept that cysteine residues are highly prevalent in the proteome of bivalves and can be considered a central hub for cellular redox processes. Such characteristic renders the redox proteome a particularly appealing omic approach for mechanistic studies integrating physiological responses to environmental stress, as already highlighted by the literature [156].

More comprehensive examinations of the redox proteome of bivalves have effectively identified the presence of reversible or irreversible oxidation of cysteine residues and the formation of carbonyl groups within the protein structures of bivalves that have been exposed to sublethal concentrations of various toxicants. Most of these studies primarily used in-gel proteomics and fluorescent labeling techniques targeting thiols and carbonyl groups. As an example, the impact of nanoparticles (zinc oxide, titanium dioxide, C60 fullerene, or their combinations) was investigated on the redox state and carbonyl content of many proteins in the clam species *Ruditapes philippinarum* [159] (Table 1). The exposure to these nanoparticles was proven to induce oxidative stress, which leads to the oxidation of proteins involved in protein metabolism, energy metabolism, and cytoskeleton maintenance. Silver nanoparticles can also alter the thiol status of proteins linked to digestion, extracellular matrix, and stress responses in mussels *Mytilus galloprovincialis* (Table 1) [160]. In mussels *Mytilus edulis*, exposure to the pharmaceutical diclofenac also caused a significant increase in the oxidation of protein thiols [161]. This exposure resulted in structural alterations in cellular pathways associated with cell stress, energy metabolism, and cellular signaling. The redox proteomic of mussels *M. edulis* has also been investigated in response to copper oxide nanoparticles [162] and gold nanoparticles [163–165]. Increased protein thiol oxidation, ubiquitination, and carbonylation are detected in the gills after exposure to gold nanoparticles, indicating the pro-oxidant activity of this nanomaterial. Copper oxide nanoparticles oxidize thiols of proteins linked to the cytoskeleton, energy metabolism, and the antioxidant system (Table 1). The use of model pro-oxidant compounds, such as the quinone menadione, indicates the susceptibility of protein thiols to the formation of protein disulfide bridges in mussels *M. edulis* [166], which seems to be driven in part by the mixed disulfide formation activity of GST pi. The redox regulation of protein disulfide isomerase (PDI) was also detected after exposure to

menadione. This indicates that the participation of redox mechanisms in bivalves extends to include endoplasmic reticulum oxidoreductases, mitochondria, and NADPH oxidases, all of which are associated with the redox role of protein disulfide isomerase [167]. Such findings provide only a preliminary understanding of the cellular mechanisms affected by the oxidative impacts of environmental stresses. Further studies are needed linking these redox protein modifications to physiological responses and ecological relevance.

Collectively, the redox proteomics analysis of bivalves suggests a broad generalization that the effects of environmental pro-oxidants on post-translational modifications of proteins might potentially disturb the cellular architecture and catabolic pathways responsible for energy production (**Figure 5**). Other biological functions can also be affected but appear more species-, contaminant-, and tissue-specific.

754
755 **Table 1: Summary of studies on redox alterations and carbonylation of proteins in marine bivalves exposed to**
756 **different contaminants.** Abbreviations: DG: digestive gland; G: gills; NPs: nanoparticles; SH: thiol; C=O: carbonyl
757 group; PS-SX: intermolecular disulfide bridge.

Protein	Effect	Exposure	Tissue	Species	Reference
Antioxidant and biotransformation systems					
Cu-Zn Superoxide dismutase	↑ C=O	Copper oxide NPs 1 hour	G	*M. edulis*	[162]
Glutathione transferase Pi	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Cellular signal					
Arginine kinase	↓ SH	Diclofenac 7 days	G	*M. edulis*	[161]
Cytoskeleton					
Actin	↓ SH	Copper oxide NPs 1 hour	G	*M. edulis*	[162]
Actin-1	↑ SH	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
Actin-1	↓ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Actin-15A	↑ C=O	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
Actin-15A	↑ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Actin-15B	↑ SH	Titanium dioxide NPs 7 days	DG	*R. philippinarum*	[159]
Actin-15B	↑ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Actin-15B	↑ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Gelsolin	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Tropomyosin	↑ C=O	Copper oxide NPs 1 hour	G	*M. edulis*	[162]
Tubulin alpha chain	↑ C=O	Copper oxide NPs 1 hour	G	*M. edulis*	[162]
Tubulin alpha-1 chain	↑ C=O	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]

Tubulin alpha-2 chain	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Tubulin alpha-3C/D chain	↑ C=O	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
Tubulin beta chain	↓ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Tubulin beta chain	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Tubulin beta-4 chain	↓ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Tubulin beta-4 chain	↓ SH	Fullerene C60 NPs 7 days	DG	*R. philippinarum*	[159]
Digestion					
Trypsin	↓ SH	Silver NPs 12 hours	DG	*M. galloprovincialis*	[160]
Protease serine 1	↓ SH	Menadione 24 hours	G	*M. edulis*	[166]
Energy metabolism					
ATP synthase subunit alpha	↑ C=O	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
ATP synthase subunit alpha	↓ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Enolase	↑ SH	Zinc oxide NPs 7 days	G	*R. philippinarum*	[159]
Enolase	↑ SH	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
Enolase	↓ SH	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
Enolase	↓ SH	Diclofenac 7 days	G	*M. edulis*	[161]
Enolase	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Glyceraldehyde-3-phosphate dehydrogenase	↑ SH	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
Triosephosphate isomerase	↓ SH	Copper oxide NPs 1 hour	G	*M. edulis*	[162]
Extracellular matrix organization					
Collagen-like protein 7	↑ SH	Silver NPs	DG	*M. galloprovincialis*	[160]

		12 hours			
Shell myostracum collagen-like protein 1	↑ SH	Silver NPs 12 hours	DG	*M. galloprovincialis*	[160]
Metal ion binding					
Heavy metal binding protein	↓ SH	Menadione 24 hours	G	*M. edulis*	[166]
Transferrin	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
mRNA processing					
RNA binding protein	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Protein metabolism					
26S Proteasome regulatory subunit 8	↑ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Calreticulin	↓ SH	Menadione 24 hours	G	*M. edulis*	[166]
Cysteine-tRNA ligase	↑ SH	Mixture of NPs 7 days	G	*R. philippinarum*	[159]
GDP dissociation inhibitor	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Proteasome subunit alpha type-6	↑ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Protein disulphide isomerase	↓ SH	Menadione 24 hours	G	*M. edulis*	[166]
Protein disulphide isomerase	PS-SX	Menadione 24 hours	G	*M. edulis*	[166]
Serine/threonine-protein phosphatase PP1-beta catalytic subunit	↑ C=O	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Stress response and homeostasis					
78 kDa glucose-regulated protein	↓ SH	Mixture of NPs 7 days	DG	*R. philippinarum*	[159]
Caspase 3/7-4	↓ SH	Diclofenac 7 days	G	*M. edulis*	[161]

HSP70	↓ SH	Diclofenac 7 days	G	*M. edulis*	[161]
HSP gp96	↓ SH	Menadione 24 hours	G	*M. edulis*	[166]
Predicted peptidyl-prolyl cis-trans isomerase	↓ SH	Silver NPs 12 hours	DG	*M. galloprovincialis*	[160]

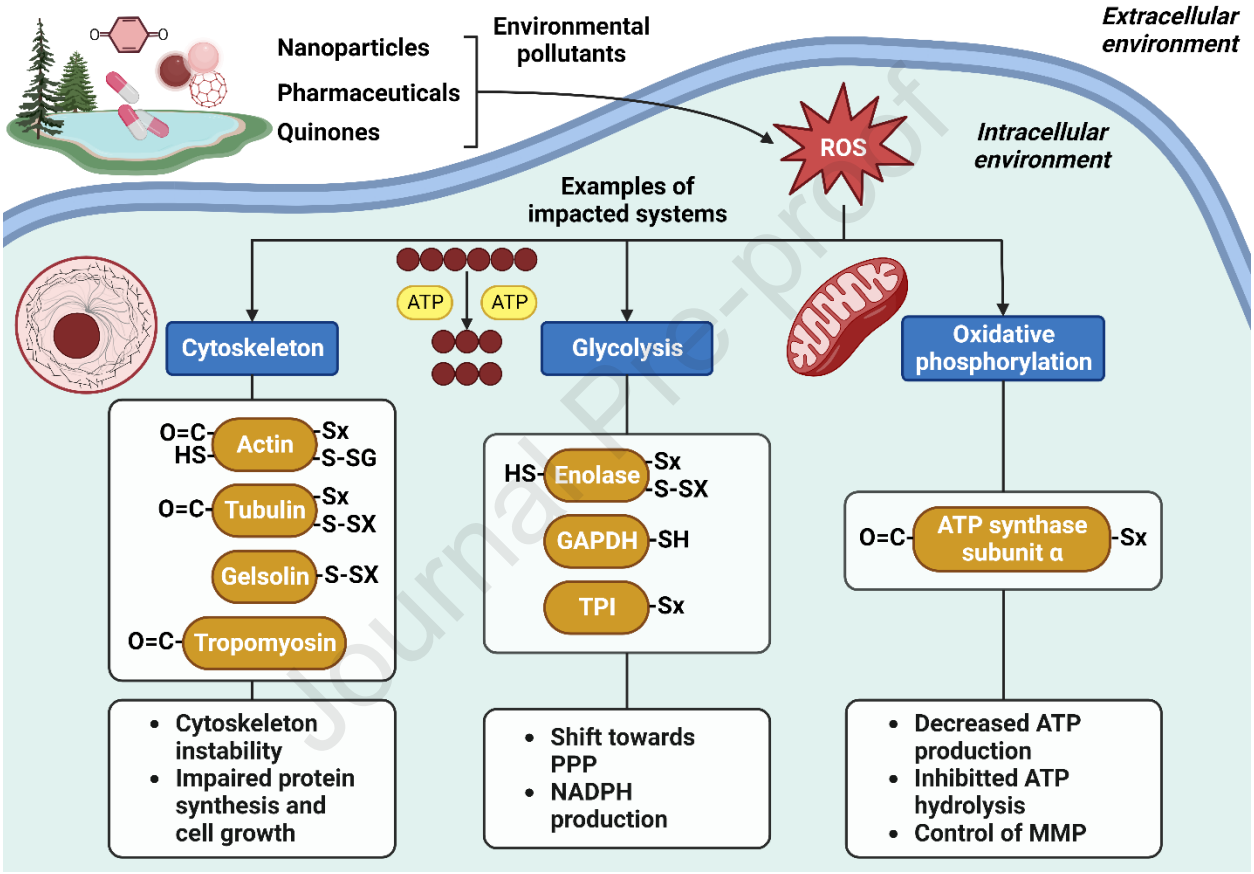


Figure 5: The effects of protein oxidation caused by environmental contaminants on cell structure and functions in marine bivalves. Exposure to environmental pollutants can lead to an excessive generation of ROS, which can induce protein oxidation, altering the structure and functionality of proteins. These oxidations can function as redox switches, therefore modulating the activation or deactivation of particular biological functions. The image illustrates three primary systems impacted in marine bivalves when exposed to pollutants, as identified using redox proteomics analysis (see Table 1 for detailed information). These systems include the cytoskeleton and energy generation processes such as glycolysis and oxidative phosphorylation. The boxes denote each of the different types of oxidation that have been identified (see the abbreviation below), together with potential cellular consequences. Abbreviations: -SH denotes greater levels of reduced thiols; -Sx denotes greater levels of thiol oxidation; -C=O denotes greater levels of carbonyl groups; PS-SX denotes greater levels of intermolecular disulfide bridges; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MMP: mitochondrial membrane potential; PPP: pentose phosphate pathway; TPI: triosephosphate isomerase.

The oxidation of protein thiols in the plasma of oysters *Crassostrea brasiliana* is seen when exposed to complex combinations, such as seawater polluted by municipal sewage. This

exposure results in significant thiol oxidation of the three most prevalent plasma proteins: dominin (homolog of extracellular Cu/Zn-SOD), segon, and actin [168]. The vulnerability of dominin to model oxidants at the low micromolar concentration range, including hydrogen peroxide, cumene hydroperoxide, and hypochlorous acid, has been shown in *in vitro,* and two cysteine residues were identified as potential sites of oxidation according to *in silico* studies [168]. Studies such as this, which include mechanistic approaches to examine the impact on the redox status of significant markers within the redox proteome of bivalves, remain limited. For example, it was also seen that the gills of mussels *M. edulis* are more susceptible to the pro-oxidative activity of hydrogen peroxide, with actin as a marked target for glutathionylation [169]. This critical antioxidant mechanism prevents overoxidation of actin, giving a redox-sensing activity to the cytoskeleton and thus a cross-talk between redox status and cellular processes governed by cytoskeleton arrangement, such as protein synthesis [170]. Additionally, it was shown that gills in mussels *M. edulis* are found to be particularly vulnerable to protein carbonylation [171]. However, contrary to expectations in the light of mechanisms described in mammals, protein carbonylation did not significantly indicate protein ubiquitination and subsequent proteasomal degradation. This same study has also demonstrated that protein ubiquitination is a very sensitive and early biomarker of oxidative stress in bivalve species, especially for proteins found in lower abundance. These studies indicate that bivalves use abundant cytosolic (actin) and plasma (dominin) proteins as highly responsive redox sensors. In contrast, less abundant proteins can be targets of a more generalized oxidative attack (protein carbonylation). The studies of these proteins, coupled with the assessment of protein glutathionylation and ubiquitination may serve as supplementary indicators for identifying novel biomarkers associated with oxidative stress and redox homeostasis in marine bivalves.

Emerging fields and research gaps

The field of redox biology in *C. gigas* continues to provide several research gaps and challenges that require being addressed in the forthcoming years. This species has the potential to make significant advancements in marine bivalves, serving as a valuable model for ecotoxicological and ecophysiological investigations. While there are many areas where further study is needed, this discussion will focus on three significant topics attracting substantial attention in the scientific community, particularly concerning other model species: immunometabolism, physioxia, and developmental biology.

Linking redox biology with immunology and immunometabolism in oyster research

Redox signaling plays a pivotal role in immune responses, regulating immune cell activation, proliferation, differentiation, and effector functions. On the other hand, dysregulation of redox signaling can contribute to immune-related diseases. Recently, immunometabolism has emerged as a critical area of research, revealing mitochondria and other metabolic pathways as significant modulators of immune cell function. The interplay between redox biology, immunology, and immunometabolism has become a promising area of study in the health and disease of vertebrates. However, it is a practically unexplored field for marine invertebrates.

Redox reactions can directly influence many aspects of the immune response, which has been extensively reviewed [18]. The most well-known example of the role of redox reactions in immune defense involves the production of ROS by immune cells, such as macrophages, neutrophils, and dendritic cells as part of their antimicrobial function, aiding in the destruction of pathogens and infected cells [172]. Another classic example involves the NF-κB, a key transcription factor regulating immune and inflammatory responses. ROS can activate the NF-κB signaling pathway by modulating the activity of enzymes involved in its activation (IκB kinases) [173]. Besides ROS, the antioxidant enzyme Trx activates the NF-κB by increasing its DNA binding activity [174]. The NF-κB example demonstrates that ROS and oxidoreductase enzymes can directly affect immune signaling. Other key pathways linking redox mechanisms and immune responses involve the above-discussed HIF-1 pathway [175] as well as the MAPKs, the AP-1, the cyclic GMP-AMP synthase, and stimulator of interferon genes (cGAS-STING), and the JAK-STAT pathways [176–178]. Genes associated with most of these pathways were already described in *C. gigas* [179,180], revealing that redox-regulated immune responses are potentially well-conserved between bivalves and mammals and play essential roles in the oyster adaptation to their harsh intertidal environment (**Figure 2**).

Although molecular mechanisms linking redox biology and immune responses have been investigated for over five decades [18], the role of redox reactions on immunometabolism is an emerging field in immunology. Immunometabolic pathways have been identified over the past two decades by linking proliferation, differentiation, and functions of immune cells to cellular metabolism. Because redox reactions are at the core of metabolic pathways, it is no surprise the increasing number of vertebrate studies that demonstrate that metabolic shifts and redox state are tightly intertwined with immune responses [181]. For example, the redox-regulated NF-κB signaling pathway is involved in metabolic rewiring by altering mitochondrial respiratory capacity in myeloid immune cells, which is critical for balancing proinflammatory and anti-inflammatory responses [182]. In another example, M1 macrophage differentiation induced by lipopolysaccharide is associated with a metabolic shift towards glycolysis. This induction of glycolysis is strongly dependent on the redox-sensitive transcription factor HIF-1α, which in turn can act through its downstream effectors to decrease OXPHOS [181].

Understanding the intricate relationship between redox, immune, and metabolic pathways is pivotal for environmental stress studies. Mainly because, in impacted ecosystems, animals are concomitantly exposed to several stress factors, including abiotic (*e.g.*, temperature, oxygen, salinity, and pH variations; food deprivation; and chemical pollutants) and biotic factors (*e.g.*, opportunistic and pathogenic microbes). In combination, such factors directly impact both host energy demands and cellular defense pathways, adding another level of complexity to redox and immunometabolic responses. Thus, it is essential to highlight that some redox-mediated immunometabolic adaptations to specific stress conditions may alter the resistance to others. For example, Mello et al. [183] were probably among the first to interrogate the effects of an environmental toxicant on immunometabolic pathways using an invertebrate model, the nematode *Caenorhabditis elegans*. Exposure of nematodes to the pesticide rotenone, a known complex I inhibitor and inducer of oxidative stress [184], revealed that environmentally-induced mitochondrial dysfunction altered the expression of several immune-related genes, including genes involved in immunometabolic pathways (*i.e.*, HIF-1), and altered the resistance of subsequent bacterial challenges [183]. Rotenone-exposed worms were more resistant to the pathogen *Pseudomonas aeruginosa*, but more resistant to *Salmonella enterica*. Numerous studies

with the oyster *C. gigas* have brought up the effects of different stress factors on parameters of the antioxidant, immune, and metabolic responses. However, most of such studies interrogate these parameters independently. Nonetheless, interpreting these studies through a more integrative approach reveals evidence of the close relationship between immunometabolic and redox pathways in *C. gigas*. For example, [30] showed that harsh intertidal environmental conditions (oxygen and food deprivation and high thermal amplitude at high intertidal levels) modify immune and metabolic responses, revealing upregulation of several immune-related proteins and down-regulation of OXPHOS components and mitochondrial regulatory proteins [30]. In another study using the same methodological design (same study site comparing oysters maintained at different intertidal levels), oysters from the harsh intertidal condition (high shore) had increased levels of antioxidant proteins such as SOD, CAT, Prx, and a methionine sulfoxide reductase (Msr) [66]. Interestingly, oysters raised at the high shore were also more resistant to the Pacific Oyster Mortality Syndrome (POMS) [30], and demonstrated postponed age-related telomere shortening [66].

POMS is a polymicrobial disease initiated by a viral (OsHV-1 µVar) infection followed by lethal bacteremia [185]. Environmental variables, such as temperature, food availability, and pollutants, may influence POMS's establishment and/or development by altering oyster metabolism and physiology [186–188]. Thus, POMS represents an exceptional opportunity to study how oysters cope with environmental stress through redox and immunometabolic adaptive mechanisms. A recent study of genetic and epigenetic differences between resistant and susceptible oyster populations evidenced the co-regulation of metabolic and immune-related genes implicated in the resistance to POMS [180]. Although the authors focused mainly on presenting and discussing pathways and genes directly linked to immunity, the analysis of oyster genetic variation revealed metabolism as a critical biological process associated with the resistant phenotype to POMS. Moreover, the major pathways implicated in POMS resistance reported in this manuscript (*i.e.*, NF-κB, JAK-STAT, and cGAS-STING) are all known redox-regulated immunometabolic pathways [180,182].

Altogether, these studies are examples of the pivotal role of redox and immunometabolic processes in protecting oysters against diseases and aging. Future studies should focus on unraveling such regulatory mechanisms, which can promote new strategies to boost oyster health for aquaculture and coastal environment sustainability and provide discoveries in biology and medicine.

The importance of physioxia to in vitro research using marine invertebrate cell models

The natural microenvironment of cells in an organism is characterized by a range of oxygen levels specific to each tissue. Traditional *in vitro* culture conditions using standard incubators often expose cells to ~18 kPa or 18% O_2 (near the 21 kPa of atmospheric oxygen at sea level), which considerably deviates from their physiological norm (physioxia). This discrepancy can trigger aberrant cellular behaviors, leading to skewed results that fail to reflect accurate biological responses [189–191]. For this reason, very recently, significant efforts have been undertaken to recapitulate better the *in vivo* physiological oxygen microenvironment (physioxia) of cells for *in vitro* experiments in biomedical research.

Although there is a significant amount of studies investigating the responses of oysters *C. gigas* to different oxygen levels, the importance of oxygen availability has been primarily disregarded when using this model *in vitro*. Cells from different oyster tissues have been successfully cultivated *in vitro* as primary cultures for several days, such as gills, digestive gland, mantle, heart, adductor muscle, gonad, and blood cells (hemocytes) [192,193]. However, to our knowledge, none of the studies have attempted to maintain these cells under physiological normoxia. Besides the need for appropriate equipment to accurately control oxygen conditions in vitro, one of the most significant challenges is the knowledge gap regarding physiological oxygen levels within *C. gigas* tissues. We found only three studies in the literature reporting physiological oxygen levels of *C. gigas*. The earliest studies are from Jones et al. (1993 and 1995) [194,195], which report maximum O_2 levels in the oyster blood (hemolymph) equivalent to around 12 kPa [194] or 17 kPa [195] under laboratory conditions at 13 °C. Allen and Burnett [196], on the other hand, revealed O_2 levels in the hemolymph of ~7 kPa in oysters submerged at 18 °C, ~4 kPa in oysters emersed for four hours at 22 °C, and ~3 kPa in oysters emersed for four hours at 30 °C. Using a real-time fiber-optic oxygen micro-sensor (OxyLiteTM, Oxford Optronix), we have recently quantified physiological oxygen levels within different tissues of *C. gigas*. We found that most oyster tissues presented, on average, ~5 KPa and maximum levels of ~12 kPa O_2 at 13 °C when animals opened their valves and actively filtered seawater under laboratory conditions. These levels dropped to zero kPa in less than 5 minutes after the animals were removed from the water, revealing the active oxygen consumption by oyster tissues and the need for a rapid change to hypoxic/anoxic conditions. Surprisingly, opened oysters that seemed to be vigorously filtering would sometimes show undetectable quantities of oxygen. (D. Mello and C. Corporeau, unpublished data; **Figure 6**). The discrepancy between our results and those reported by Allen and Burnett [196] regarding the O_2 levels found in closed oysters could be related to the quantification method. In our case, the minimally invasive oxygen micro-sensor (350 µm tip diameter) was introduced within oyster tissues and reported real-time oxygen levels without opening a notch within the oyster shell. On the other hand, Allen and Burnett [196] made a notch in the shell, extracted hemolymph with a glass needle, and recorded single-read oxygen levels by injecting the hemolymph into a thermostated chamber of a Po2 electrode. Such techniques might have contaminated the sample with ambient oxygen. Nonetheless, all these results are in accordance with the fact that, similarly to humans, the oxygen levels within oyster tissues are much lower than the body's surroundings (note that seawater has O_2 levels of ~21 kPa, similarly to atmospheric air at sea level). As a result, when oyster cells are transplanted to *in vitro* settings, they are exposed to significantly greater oxygen levels (hyperoxia) than their physiological norm (physioxia) (**Figure 6**).

The underlying differences between the physiology and redox response of cells cultured in physioxia and hyperoxia have been investigated using vertebrate models. As expected, several redox-regulated pathways respond differently when cells are cultured under physioxia. Such responses were already elegantly recently reviewed by Keeley and Mann [189]. As an example, pre-adapting human primary endothelial cells to physioxia (5 kPa O_2) significantly attenuates the expression of Nrf2 target genes upon exposure to a classic Nrf2 inducer as compared to cells continually cultured in standard CO_2 incubators (hyperoxia; 18 kPa) [198]. Additionally, ROS production by isolated mitochondria depends on oxygen levels, with ROS generation being diminished at lower O_2 levels [199].

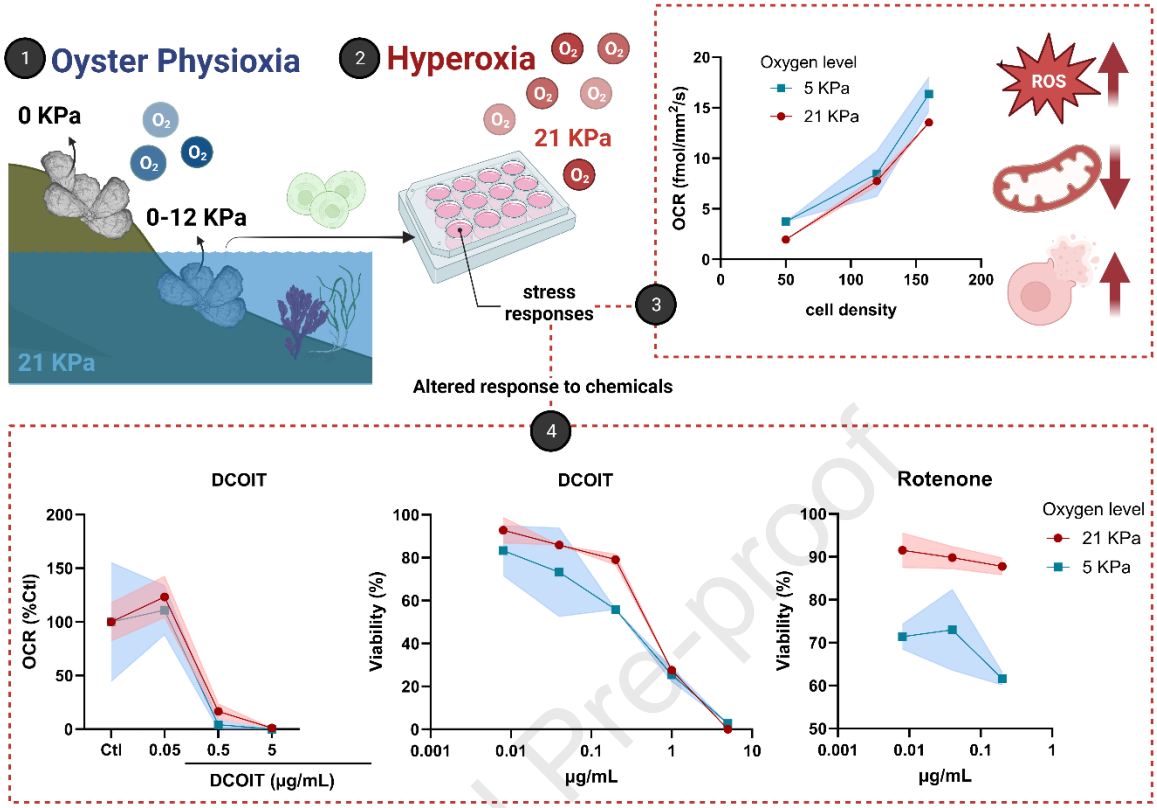


Figure 6. Importance of physioxia for *in vitro* studies using bivalve cell culture models. (1) The oyster Crassostrea gigas and many other bivalve species in the intertidal zone are subjected to oxygen deprivation during low tides. No measurable oxygen level exists throughout oyster tissues, including the muscle and heart cavity, less than five minutes after air exposure (emersion). When oysters are underwater (immersion), they can open and filter oxygen from the water (21 KPa) through their gills. In this condition, we have detected oxygen levels ranging from 0 to 12 KPa, but most frequently, we have detected around 5 KPa within oyster tissues, particularly the cardiac cavity. (2) When oyster cells are collected to obtain primary cultures, they are exposed to atmospheric air conditions (21 KPa), corresponding to much higher oxygen levels than they were physiologically exposed in the animal (hyperoxia). (3) This leads to the induction of stress and adaptive responses, such as differential mitochondrial metabolism, ROS production, and cell survival (oxygen consumption rates - OCR - results by D. Mello, G. Mann and C. Corporeau, unpublished data; membrane potential, ROS and cell survival results by Donaghy et al. [197]. (4) Such adaptive mechanisms can alter the *in vitro* response of cells to environmental contaminants, including biocides such as DCOIT and rotenone (results by D. Mello, G. Mann, and C. Corporeau, unpublished data). Data are shown as average and standard deviation (colored areas).

One study was found in the literature reporting the effects of oxygen on the functioning of *C. gigas* cells *in vitro*. Primary cultures of oyster hemocytes were briefly exposed (90 min) to 85% less oxygen and displayed decreased ROS production, increased mitochondrial membrane potential, and reduced cell death [197]. Using undisturbed measurements of mitochondrial respiration (Resipher, Lucid Scientific) on primary cultures of *C. gigas* hemocytes, we have found that cells presented a 32 % higher oxygen consumption rate when cultures were maintained at 5 kPa O_2 as compared to 21 kPa O_2 (D. Mello, G. Mann, and C. Corporeau, personal unpublished data: **Figure 6**). These findings reveal that, similarly to vertebrate *in vitro* models, cultivating oyster cells at different oxygen levels also influences the functioning and physiology of the cell. Therefore, it is crucial to regulate cellular microenvironmental parameters, specifically oxygen

levels, to accurately simulate natural microenvironmental conditions within the organ when utilizing *in vitro* invertebrate cell models. This is necessary for a comprehensive understanding of *in vivo* physiological responses and redox mechanisms in response to environmental changes, which have often been neglected in previous studies.

Primary bivalve cell cultures are typically used in ecotoxicology studies. Thus, a significant drawback when using traditional *in vitro* hyperoxic conditions lies in the fact that the real effects of toxicants may be skewed. Depending on the mechanism of toxicity of a particular compound, it is hypothesized that cells could either be more sensitive due to a stress-on-stress effect or more resistant, as defense pathways might be upregulated to maintain redox equilibrium and cell homeostasis. The influence of oxygen levels on the impacts of chemicals has only recently begun to be investigated in vertebrate models. In one of the few studies, for example, pulmonary cells cultured at 13 kPa oxygen were shown to be more susceptible to copper oxide nanoparticles than cells cultured at 21 kPa [200]. This research indicates that cells exposed to an oxygen concentration of 21 kPa exhibited elevated intracellular concentrations of GSH. This provides evidence in favor of the hypothesis that subjecting cells to atmospheric oxygen conditions leads to developing defensive antioxidant mechanisms, which can alter their vulnerability to pro-oxidative compounds. In agreement with these findings, some of our preliminary results also reveal a higher susceptibility of oyster cells to chemicals at physioxia. When *C. gigas* hemocytes were exposed to DCOIT, the biocidal component in the commercial antifouling product SeaNine 211, they showed greater sensitivity at 5 KPa than at 21 KPa oxygen. (**Figure 6**; D. Mello, G. Mann, and C. Corporeau, unpublished data). At 5 KPa, DCOIT caused a higher dose-dependent inhibition of cellular OCR and promoted a 2-fold decrease in the LC50 (from 0.41 µg/mL at 21 KPa to 0.21 µg/mL at 5 Kpa) (**Figure 6**). Likewise, 5 KpA rendered the cells more vulnerable to the herbicide and Complex I inhibitor rotenone (**Figure 6**). These early findings emphasize the significance of conducting *in vitro* chemical hazard prediction under physioxia settings. This can build new paths in toxicity testing and considerably contribute to chemical regulation and the management and preservation of the aquatic environment.

Redox control of developmental biology in bivalves

Probably one of the most intriguing and fascinating roles of redox biology lies at the beginning of the life of an organism. ROS play a direct role in several biological processes related to early life, including spermatogenesis and oogenesis, fertilization and early embryonic development, morphogenesis, angiogenesis, and cell migration [201]. Mammalian development is regulated by a limited set of evolutionarily conserved pathways involved in intercellular and intracellular signal transduction, such as Wnt/beta-catenin, integrin, receptor tyrosine kinase, JAK/STAT, and Notch. As already reviewed, these pathways have demonstrated sensitivity to cellular redox state [202]. While the detailed discussion of the developmental program of marine bivalves is beyond the scope of this text (for further information, refer to relevant studies or reviews such as [203–205]), it is crucial to emphasize the importance of regulating embryogenesis in response to environmental conditions. This significance arises from the external fertilization and development processes of these organisms. As a result, rather than considering the development of marine bivalves in isolation, it makes sense to analyze it in connection to their environment.

As previously mentioned, environmental stimuli can influence cellular redox sensing hubs, such as GSH/GSSG, NADPH/$NADP^+$, and protein thiols. These hubs play a role in triggering adaptive responses, which may lead to deviations in gene expression programs that are often linked with growth and differentiation. In addition, the development rate may be described as the pace at which organisms transition from a growth stage characterized by a gradual increase in metabolic flux to a final state of differentiation or senescence [202]. As stated before (refer to Figure 2), intracellular redox changes can initiate the transition from proliferation to differentiation, namely a shift from a predominantly reducing to a moderately oxidizing cellular redox state. There is still minimal information on the antioxidant system and redox status of *C. gigas* (as well as bivalves in general) during cell fertilization, embryogenesis, and larval development. Below, we present some of the few studies linking redox and development in bivalves.

One key characteristic of marine organisms is the high levels of polyunsaturated fatty acids, including during the early life stages, which are the substrates of lipid peroxidation. Thus, enzymes and low molecular weight antioxidants are essential to elevate the resistance of cell membranes in developing embryos against oxidative stress [206]. GSH concentrations often exhibit enhancement in the gonadal tissues of bivalves, particularly during the reproductive period. The depletion of GSH from the gonadal tissue enhances the susceptibility of C. virginica oyster embryos to the harmful effects of metals [207]. The GSH levels, the GSH/GSSG ratio, and the activities of CAT and SOD in oocytes positively correlate with fertilization and developmental success in mussels of the species Perna canaliculus. Conversely, markers indicative of oxidative stress, such as lipid hydroperoxides and protein carbonyl, display a negative correlation [208]. Some transcriptomic studies during bivalve development provide insights into redox reactions during development. For example, in clams *R. philippinarum*, transcriptomic analysis indicates the enrichment of genes related to the GSH metabolism during the transition from the gastrula to the trochophore larvae stage, when organogenesis occurs [203]. The findings from transcriptome investigation of the mussel Mytilus coruscus also indicate that the synthesis of glutathione (GSH) is notably elevated throughout the later stages of development, namely during metamorphosis and in juvenile individuals. This observation aligns with the increased resistance to oxidative stress exhibited by individuals in these later stages. This mechanism is similar to findings made in mammals, where the synthesis of GSH initiates after gastrulation to counterbalance the substantial ten-fold decrease in GSH levels that takes place from fertilization to the blastocyst stage [209]. Similar findings are also seen in an aquatic vertebrate species presenting external development, the zebrafish (Danio rerio). The redox ontogeny of GSH exhibits a decline in the GSH/GSSG ratio during the cellular proliferation stages until gastrulation [210] and, subsequently, a gradual restoration of this ratio occurs as the more significant phases of organogenesis and differentiation have already occurred. This regulation seen in zebrafish seems to be influenced, at least in part, by the Nrf2 pathway [211]. In invertebrates, Nrf2 also plays a pivotal role in regulating development, as demonstrated in C. elegans and Drosophila melanogaster [212]. It would be interesting to address whether there are conserved or unique temporal redox responses and regulation dynamics during embryonic and larval development in bivalves.

As seen above, the intersection of redox and developmental biology in marine bivalves is an intriguing area of study, like in other model organisms, but remains predominantly unexplored. As a result, the field remains open for researching these associations and addressing significant research gaps, which might lead to breakthroughs in fundamental knowledge and practical applications in areas like aquaculture, marine conservation, and comparative biology. Below is a

list of suggested subjects for additional research on this topic. Due to its wide distribution, accessibility of its genome, utilization of standardized developmental techniques, and commercial significance, we propose that the oyster *C. gigas* has excellent potential as a model organism for addressing many of these research gaps.

(i) Role of ROS and antioxidants in development: examining the mechanisms underlying the generation of ROS and the regulation of antioxidants during the early developmental stages of marine bivalves has the potential to clarify the molecular processes dictating the beginning of life in these organisms. This can be supported by the use of transcriptomic, proteomics, and redox proteomics for identifying essential regulatory genes and pathways, alongside the advancement of novel tools and techniques, including real-time imaging of redox sensors to describe the redox dynamics occurring in embryonic development comprehensively.

(ii) Metabolic shifts during development: characterize the metabolic shifts that occur during the transition from embryonic, larval, and juvenile stages in bivalves. How do changes in metabolism relate to redox status, and how do they support rapid and proper growth and development?

(iii) Environmental stress and development: examine the effects of environmental stressors, such as pollution, temperature fluctuations, and ocean acidification, on the redox biology of developing bivalves. What is the influence of these stressors on the redox balance and developmental mechanisms, and what approaches may organisms employ to mitigate these consequences and maintain reproduction and population growth? Can the field of redox biology provide insights into the observations obtained from embryotoxicity assessments?

(iv) Epigenetic regulation in development: examine the impact of epigenetic alterations on the link between redox biology and developmental processes in marine bivalves. Epigenetic modifications can exhibit lasting implications for development and transgenerational effects and are potentially susceptible to modulation by the cellular redox state.

(v) Nutritional Influences: In light of the potential future changes in coastal planktonic communities and the continuous advancements in commercial diets, it is essential to investigate the impacts of natural and commercial diets on the dynamics of redox cofactors and their subsequent effects on development.

(vi) Adaptations to extreme environments: investigate how bivalves in extreme environments, such as hydrothermal vents or polar regions, adapt their redox biology to support development. These extremophiles may hold unique insights into redox-dependent developmental processes.

(vii) Phylogenetic comparisons: conduct comparative studies across different bivalve species to identify conserved and species-specific aspects of redox regulation during development. This can help elucidate evolutionary trends.

(ix) Applied research: explore practical applications of the knowledge gained in this field, such as establishing strategies to promote sustainable aquaculture or monitoring the impact of environmental changes on bivalve populations using redox biology.

Concluding remarks

The redox biology of marine bivalves in the intertidal zone is fascinating because it shows how well they have adapted to the harsh and ever-changing conditions of the shore. In the model *C. gigas, the* production of ROS, modulation of the antioxidant system via the Nrf2 and HIF-1 pathways, and regulation of energy metabolism are all intertwined with this adaptation. The diurnal fluctuations in temperature and oxygen levels provide a considerable challenge for intertidal bivalves. Nevertheless, *C. gigas* has developed intricate ways to regulate oxidative stress and thrive in this environment. The Nrf2 pathway plays a significant role in this protective mechanism, governing the antioxidant responses and controlling the excessive production of ROS. Notably, its efficacy is more pronounced in the gills, a tissue in direct contact with the environment, demonstrating the importance of redox control in response to external stimuli. Concurrently, the HIF-1 pathway is crucial in orchestrating the adaptive reactions to a state of oxygen deprivation. By regulating gene expression related to oxygen transport and energy metabolism, the HIF-1 facilitates the utilization of oxygen and energy resources in bivalves. The simultaneous activation of the NRF2 and HIF-1 pathways illustrates the complex coordination necessary for organisms to adapt and thrive in the intertidal zone's dynamic oxygen concentrations.

The ability of *C. gigas* to efficiently transition between aerobic and anaerobic metabolic pathways, as well as the use of pathways such as the phosphoenolpyruvate branchpoint and opine systems, also enables the regulation of ROS production. Additionally, the existence of cytoplasmic opine dehydrogenases serves as a crucial factor in avoiding the intense acidification that occurs through lactate fermentation and in the preservation of the $NADH/NAD^+$ redox equilibrium, thereby contributing to the cellular redox state during the early phase of hypoxia. Using succinate as a vital energy source over prolonged durations of hypoxia emphasizes the remarkable metabolic flexibility of *C. gigas*. Thus, intertidal bivalves demonstrate a notable adaptation to manage variations in oxygen levels in their surroundings by efficiently sustaining vital cellular functions and cellular redox state while minimizing the excessive generation of ROS.

However, *C. gigas* and other intertidal bivalves are confronted with the increasing burden of environmental pollution, alongside the other constraints posed by their intertidal habitat. It is worth mentioning that pollutants can cause substantial modifications in the redox-regulated proteins linked, for example, to the cytoskeleton and energy metabolism in these animals. These disturbances frequently result in cytoskeletal component oxidative damage, compromising cell division, protein synthesis, intracellular transport, and tissue integrity. They can also result in alterations in the metabolic efficiency of bivalves, with prompt energy diversions towards antioxidant reactions. More in-depth investigations of the redox proteome of marine bivalves are still needed since they may give insight into far-reaching consequences for their survival and the general health of the ecosystem.

Without question, redox biology is a dynamic and growing field of research, abundant with promising avenues of investigation, particularly in the context of marine bivalves. We point, for example, to some fascinating topics demanding our attention and more research. In the world of marine bivalves, the precise consequences of biotic and abiotic factors on immunometabolism remain unknown. Understanding how these interesting species use the power of their redox systems to modulate immunological functions and metabolic pathways might lead to new

discoveries about their resilience and adaptive capacities. Moreover, *in vitro* research have long been a cornerstone of scientific investigation, and their use in the study of marine bivalves is no exception. However, adopting oxygen levels that nearly replicate the physiological circumstances encountered by these species in their native environments is pivotal, especially within the context of the dynamic intertidal environment. This change toward mimicking specific environmental circumstances has the potential to deliver more accurate and ecologically relevant insights on how marine bivalves manage with variable oxygen levels, oxidative stress, and metabolic adjustments in real-world scenarios. Finally, the precise link between cellular redox state and the crucial stages of embryonic and larval development in bivalves remains largely unknown. These early life stages are especially sensitive to environmental stresses, and identifying how the redox biology affects embryogenesis and larval survival might have far-reaching ramifications for population dynamics and the sustainability of marine resources.

The subject of redox biology in marine bivalves is like a Pandora's box, with an abundance of discoveries just waiting to be revealed. As we continue to explore these untouched regions, we are sure to gain important insights not just into these unique species, but also into how redox biology relates to life in our oceans. Such novel findings should be both thrilling and informative, potentially expanding our understanding of distinct and shared ancestral mechanisms for adaptation to stress and dynamic environments.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal affiliations that might have impacted this work.

Supervisor's supporting statement

"I am delighted to support the invitation from Free Radical Biology & Medicine to Drs Danielle F. Mello and Rafael Trevisan to submit an Early Career Researcher Invited Review. I had the privilege of supervising both of them during their master's and doctoral studies at the Laboratório de Defesas Celulares, Federal University of Santa Catarina, Brazil. Throughout their academic journey, Drs. Mello and Trevisan consistently demonstrated exceptional scientific growth, independent and creative thinking, scientific maturity, and profound expertise in the fields of bivalve redox biology, immunity, physiology, and bivalve-environment interactions. Their

remarkable skills and dedication undoubtedly position them as promising and accomplished scientists.

Drs. Mello and Trevisan possess extensive knowledge of bivalve biology, encompassing diverse aspects such as the exploration of practical environmental issues like poisoning by harmful algal toxins and the prevalence and effects of bivalve pathogens. Additionally, they have conducted meticulous mechanistic studies, delving into the relevance of endogenous antioxidants, with a particular focus on glutathione, glutathione peroxidase, and peroxiredoxin backup systems, for oyster physiology and immunity. Notably, they were the pioneers in investigating the Nrf2 response in bivalves, providing unequivocal evidence regarding the crucial role of antioxidant defenses in the protection and survival of oysters.

The exceptional qualities of this text lie in its ability to offer the readers of FRBM valuable information on relatively understudied animal models while also paving the way for exciting new discoveries. Without a doubt, this review will captivate the attention of FRBM readers, inspiring further exploration of bivalves' redox biology and broadening the scope of the journal's research endeavors."

Alcir Luiz Dafre Ph.D.

Professor at the Federal University of Santa Catarina, Brazil.

"I strongly support the invitation from Free Radical Biology and Medicine to Dr. Danielle Mello and Dr. Rafaël Trevisan to submit an Early Career Researcher Invited Review. They play key roles in advancing the field of redox biology, particularly to investigate adaptation to environmental stress and pollution in marine invertebrates. They provide new insights into the original mechanisms of redox signaling molecules and pathways in the oyster, a marine species champion for metabolic adaptation."

Charlotte Corporeau Ph.D.

Researcher at the French Research Institute for Exploitation of the Sea (IFREMER)

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Highlights

- Redox biology connects environmental stimuli to biological functions.
- The intertidal species Crassostrea gigas adapts to daily stress through redox processes.
- Oyster metabolism is adapted to minimize oxidative damage in hypoxia and reoxygenation.
- Their adaptable antioxidant system is controlled by the Nrf2 and HIF-1 pathways.
- Redox biology extends to oyster immunity and development through yet understudied pathways.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: