Question from Steven Roberts: *With respect to an organism's phenotypic flexibility, describe in detail how environmental conditions associated with ocean acidification are recognized (sensed) by an oyster, and discuss physiological changes that would allow on oyster to maintain homeostasis. Please discuss both the sensory physiology and response in an integrative nature so that molecular mechanisms are related to whole animal biology. In terms of the response to changing conditions, please describe at least two independent processes. One process should deal directly with how pH can be maintained.*

Marine invertebrates, as environmental conformers, have sensitive sensory and response mechanisms to survive changes in the environment. Changes in temperature, salinity, and pH have proven to elicit specific reactions in a variety of physiological responses in invertebrates (Rodriguez-Lanetty et al. 2009; Lockwood & Somero 2011; Lannig et al. 2010). Through sensing environmental change and integrating responses at the molecular and whole organism levels, animals such as the Pacific oyster, *Crassostrea gigas*, are able to maintain homeostasis in a variable marine environment. Projected environmental changes to the global ocean foresee acidification and warming that will be novel stresses for contemporary biota (IPCC 2007). Oysters may or may not have the physiological resources necessary to weather these changes. Two important functions that could be under threat in a more acidic ocean are regulation of internal pH and maintenance of robust immune function. Data to date in *C. gigas* and other aquatic bivalves suggest that long-term exposure to low environmental pH may prove detrimental to both of these physiological necessities.

Animals sense changes in their environments through a variety of receptor and signaling pathways that trigger shifts in physiology and/or behavior. Extracellular fluids, such as hemolymph in *C. gigas*, allow internal cells to communicate with the environment. The exchange of extracellular and intracellular information occurs across membranes and is typically mediated by membrane-bound receptors. Once a molecule binds to a receptor, it triggers a cascade of effects within a pathway involving other enzymes and receptors, which will in turn generate signaling molecules, such as eicosanoids, gases, purines, amines, peptides, and steroids. Signaling molecules effect change as neurotransmitters, paracrine signals, hormones, neurohormones, pheromones or cytokines and elicit neurological, immune, and other responses. This entire suite of coordinated reactions results in changes to overall physiology and behavior and also involves the coordinated up- and down-regulation of genes within the pathway.

Oysters are environmental conformers, but still have a number of physiological mechanisms to protect important organismal functions from the influence of adverse environmental conditions. One of the most important systems is sensory mechanisms for detecting changes in the environment. Molluscan bivalves have a bilateral nervous system with three pairs of ganglia – cerebropleural, pedal, and cerebral – attached by nerve cords (Ruppert & Barnes 1994). They also possess a number of more specialized neurons and receptors. Molluscan veligers, as evidenced in the gastropod *Philine aperta*, have serotonergic neurons that connect to the ciliary band (Hay-Schmidt 2000). Opiate receptors in the *Mytilus edulis* pedal ganglia are highly homologous to human opiate receptors and are probably involved in modulating proinflammatory events in the immune response (Cadet & Stefano 1999). For sensing the external environment, many species have pallial tentacles with tactile and chemoreceptor cells on the mantle edge (Ruppert & Barnes 1994). Statocysts in the foot or within the pedal ganglia provide information on whole body orientation, but these are reduced in sessile oysters (Ruppert & Barnes 1994). Ocelli along the mantle or on siphons, depending on the taxon, have pigment spots that can detect light and the osphradium beneath the posterior adductor acts in chemoreception of the water in the mantle cavity (Ruppert & Barnes 1994). The ocelli in the siphon of the surf clam, *Spisula solidissima*, have multiple photoreceptive pigments that stimulate the pallial nerve in both excitatory and inhibitory ways in response to shadow or illumination (Kennedy 1960). Oysters are not encephalized and instead have a juxtaganglionar organ consisting of scattered cells in the connective tissue around the cerebral ganglion. To coordinate nervous and other physiological responses, *C. gigas* has catecholamine signaling molecules in both larval and adult forms. There is evidence of a signaling pathway in larval oysters by which environmental cues stimulate a dopaminergic pathway through epithelial receptors, which acts to increase larval sensory input to environmental factors (Bonar et al. 1990). The increased sensory input serves in finding a more suitable habitat, which then stimulates catecholamines and the adrenergic pathway to commence metamorphosis (Bonar et al. 1990). In adults, *C. gigas* release both noradrenaline and its precursor dopamine in response to mechanical stress in concentrations proportional to the intensity and duration of the stressor (Lacoste et al. 2001a). Temperature and salinity stress can elicit long-lasting catecholamine increases (Lacoste et al. 2001a). The catecholamines are probably released by a cluster of cells around the heart and are mediated by the neuropeptide acetylcholine (Lacoste et al. 2001b). This group of evidence supports that oysters have sensitive and effective neuroendocrine mechanisms that affect survival and behavior in larval and adult forms.

The increased input of anthropogenic CO2 into the earth’s atmosphere causes a concomitant increase in oceanic dissolved CO2 creating a more acidic environment for marine organisms. The Intergovernmental Panel on Climate Change predicts a decrease in average global ocean pH of 0.14-0.35 units by the end of the century from contemporary values of about pH 8 (IPCC 2007). All life stages of *C. gigas* are susceptible to these changes in oceanic pH and will need mechanisms to tolerate a more acidic environment. As larvae, *C. gigas* stays above the halocline (Kennedy in *The Eastern Oyster*), which is the body of water that is in equilibrium with atmospheric CO2 and where the concentrations of anthropogenic CO2 are the highest (Sabine et al. 2004). Adults are sessile and so subject to any environmental change without many options for behavioral modification. Adult oysters in the intertidal will also be impacted by increased acidity of surface waters and by the ocean-wide shoaling of the aragonite and calcite saturation horizons and acidic upwellings (Feely et al. 2004; Hauri et al. 2009). Acidic waters undersaturated for calcite and aragonite (polymorphs of calcium carbonate) will affect not only the pH balance of *C. gigas* but also potentially contribute to shell dissolution. Dissolved CO2 will more readily diffuse across marine invertebrate animal epithelia and membranes and reach equilibrium in intra- and extracellular spaces (Fabry et al. 2008). Carbon dioxide can then interact with internal fluids, decreasing the pH, which can only be counteracted by passive buffering of the extracellular and intracellular fluids, ion exchange/transport, CO2 transport on respiratory pigments (not available to oysters), and metabolic suppression (Fabry et al. 2008). In experimental exposures to decreased pH, Sydney rock oyster larvae, *Saccostrea glomerata*, have pitted and deformed shell deposition from dissolution and inhibited calcification (Watson et al. 2009). Juvenile eastern oysters, *C. virginica*, at low pH for 20 weeks demonstrated increased mortality, decreased body and shell condition, and increased standard metabolic rate in response to the stress (Beniash et al. 2010). Adult *C. virginica* increased expression of carbonic anhydrase in the mantle tissue, the site of calcification (Beniash et al. 2010). Carbonic anhydrase is an enzyme that converts CO2 into bicarbonate (HCO3-) for mineralization. These detrimental effects of increased pCO2 and low pH at all life stages of the oyster suggest that future scenarios may overwhelm the capabilities of their physiological response. Exploration of the impacts of an acidic environment on the maintenance of internal pH and of proper immune function in *C. gigas* and other bivalves provides further evidence that projected future climate change may be an intolerable stressor.

Even though *C. gigas* is an osmoconformer and poikilotherm, it may possess more physiological regulatory mechanisms for changes in environmental pH. Decreased environmental pH is first experienced extracellularly, since that is the milieu in contact with the environment. Buffering of these changes is mostly accomplished by increases in HCO3- and other ions and is more efficient in the contained and already more acidic intracellular compartments (Pörtner et al. 2004). Accumulation of HCO3- may in fact be a prerequisite for recovery of intracellular pH (pHi) during hypercapnic acidosis (Pörtner et al. 2000). Regulation of pHi recovery in invertebrates is mediated by a number of ion transports and exchangers: Na+/K+-ATPase, H+-ATPase, and Na+-dependent Cl-/HCO3- anion exchanger (Pörtner et al. 2000). Even with all these mechanisms in place, however, maintenance of pHi in invertebrates is both costly and difficult to achieve. In the isolated body wall musculature of the marine worm *Spinculus nudus*, the recovery of pHi at extracellular pH of 7.5 both took longer and had a delayed response when compared to pH of 7.9 (Pörtner et al. 2000). Also the differential use of transporters during acidosis caused an increased ATP demand in the worm, which was offset by metabolic depression, a response that could have other negative physiological impacts (Pörtner et al. 2000). Adult *C. gigas* shows evidence of acidosis and buffering when exposed to a low pH of 7.68 over 55 days. Over this time period, extracellular pCO2 increased and hemolymph pH decreased by about 0.5 units compared to control at pH ~8.1 (Lannig et al. 2010). In order to buffer these changes, circulating concentrations of HCO3-, Na+, K+, and Ca2+ increased, probably evidence of shell dissolution to combat changes in internal pH (Lannig et al. 2010). Similarly to the worm, the oysters underwent metabolic depression as evidenced by decreased hemolymph pO2 and decreased body condition index (Lannig et al. 2010). Gluconeogensis (indicated by decreased alanine and ATP) took over for glycolysis in the mantle because acidified conditions inhibit the latter while increased succinate in the gills and hepatopancreas indicated onset of anaerobic metabolism (Lannig et al. 2010). These costly metabolic changes may have paid off since there was only 4.3% mortality in the CO2-exposed group over the 55 days (Lannig et al. 2010). The studies by Lannig et al. (2010) and Pörtner et al. (2000) prove that marine invertebrates, including *C. gigas*, have capable but costly pH regulatory mechanisms. The depressed metabolic rate and concomitant decrease in body condition could have significant impacts on fitness and long-term survival. Oyster reproduction is affected by environmental stress (Thompson et al. in *TEO*) and the results outlined above indicate cellular and physiological stress. If long-term acidification causes ongoing decreases in body condition index, then oysters may have fewer resources available for spawning resulting in decreased reproductive output or larvae without enough nutrient resources.

Further studies in other bivalves implicate more extensive changes to physiology in acidified conditions. One of the causes of decreased body index in bivalves exposed to acidic conditions may be the direct use of proteins to buffer internal pH changes. Mussels, *Mytilus edulis*, exposed to low pH over 8 weeks degraded proteins probably to provide more HCO3- for pHi regulation (Thomsen & Melzner 2010). *M. edulis* also shows the ability to halt the decrease of extracellular pH (pHe). During exposure to pH 7.4 for three months, pHe dropped 0.2 units within two days as hemolymph pCO2 rose over four days (Michaelidis et al. 2005). However, a linear increase in [HCO3-] and [Ca2+] in the hemolymph over those first four days of exposure successfully staved off further decreases in pHe for the remainder of the experiment, although these compensatory mechanisms came at the cost of slower shell growth (Micahelidis et al. 2005). Over 120 days of exposure to pH of 7.78, the clam *Laternula elliptica* increased O2 consumption and basal metabolic rate and showed increased *heat-shock protein 70* (*hsp70*) gene expression in mantle tissue (Cummings et al. 2010). Heat-shock protein 70 is a molecular chaperone that is up-regulated in response to a wide variety of environmental stresses. The main role of heat-shock proteins is to repair damaged proteins that are unfolding due to stress or mark those that are beyond repair for ubiquitination. Up-regulation of *hsp70* in the clam stress response to low pH suggests that acidic conditions also cause protein damage. The sensitivity of marine invertebrates, when compared to vertebrates, in low pH environments may be because they have a low capacity to acid-base regulate pHe since they are hypometabolic (Pörtner et al. 2008).

The basis of the invertebrate immune system is the hemocytes within the hemolymph, which is the main circulatory fluid and is impacted by environmental changes. Bivalve hemolymph pCO2 increases and pH decreases upon exposure to hypercapnic conditions (Michaelidis et al. 2005; Lannig et al. 2010). Relatively few studies have been done on the direct effects of ocean acidification on hemocyte function and the hemolymph, but a number of studies on the effects of salinity and temperature stress lend support to how environmental change affects immune function. Changes to hemocyte function from environmental stress have the potential to make an organism more susceptible to disease-related mortality. Hemocytes are instrumental in recognizing foreign material through pathogen-associated molecular patterns and the recognition of non-self (Medzhitov & Janeway 2000). *C. gigas* has a number of the genes necessary for receptors to recognize pathogens, indicating the evolutionary importance of an efficient innate immune system (Bardiotti et al. 2007; Gagnaire et al. 2007; Gueguen et al. 2003; Gonzalez et al. 2005; Zhang et al. 2011). In *M. edulis*, exposure to pH between 6.5 and 7.8 for 60 days caused leaky lysosomes (Beesley et al. 2008). Disruption of lysosome function could lead to increased permeability to substrates, activation of cell death, cytolytic damage, and effects on immunity such as phagocytosis, endocytosis, and autophagy (Beesley et al. 2008). A number of lysosomal enzymes are associated with the hemolymph – acid phosphatase, lipase, aminopeptidase, and lysozyme – and are released during phagocytosis (Cheng in *TEO*). In *C. gigas* exposed to pesticide and then a bacterial challenge, lysozyme mRNA was down-regulated compared to the non-pesticide exposed control (Gagnaire et al. 2007). Lysosomes and their associated enzymes are important in oyster immune function and risk functional inhibition in low pH environments. Enzymes in general can be pH sensitive, with each enzyme having pH and temperature optima at which they function best. Also in *M. edulis*, phagocytosis of hemocytes was decreased at low pH/high CO2 after 16 and 32 days of exposure (Bibby et al. 2008). The decrease in phagocytic activity may have been a result of shell dissolution increasing hemolymph Ca2+, an important signaling molecule for hemocytes (Bibby et al. 2008).

Exposures of oysters to other stressors provide insight into how environmental stress can affect hemocyte function. Hemocyte migration is temperature-dependent and salinity affects the movement of granulocytes, the most phagocytically active hemocyte (Cheng in *TEO*). After exposure to the environmental contaminant tributyltin and hypoxia, *C. virginica* had greater *Perkinsus marinus* infection loads than controls, an indication of reduced immune competency (Anderson et al. 1998). In a comparison of mussels exposed to different temperatures and environmental copper levels and then challenged with the bacteria *Vibrio tubiashii*, Parry and Pipe (2004) found that the immunocompetence of *M. edulis* was affected differently by the various stressors and their levels. However, various combinations of temperature, copper, and pathogen resulted in inhibition to phagocytic activity, circulating hemocytes, and generation of superoxide radicals (Parry & Pipe 2004). Low salinity stress and high temperature showed initial inhibitory effects on hemocyte phagocytic activity in the European flat oyster *Ostrea edulis* (Fisher et al. 1987). After 24 hours of exposure to either low salinity or high temperature, the hemocytes showed signs of acclimation and recuperation of phagocytic activity (Fisher et al. 1987). The stresses used in Fisher et al. (1987) were relatively mild, 15 ppt for salinity and 250C for temperature, but the ability of the hemocytes to sense and acclimate to the change in environment suggests that there is potential for acclimatization, at least in the short term.

Pacific oysters and other marine invertebrates are able to detect ambient changes in environmental pCO2 and respond with specific physiological mechanisms. For maintenance of internal body pH, bivalves are able to control pH to a certain degree, but at a great metabolic cost. The cost to body condition and metabolism may have further detrimental effects if oysters are exposed to additional environmental stressors. Under all climate change scenarios, it is guaranteed that there will be associated increases in global mean temperature and pollution as the oceans acidify (IPCC 2007). These changes may very well prove to be overly taxing on the physiological response of *C. gigas* and could lead to death of individuals and population extirpations. Ocean acidification also affects immune function in bivalves and could make oysters more susceptible to disease as lysosomes, hemocytes, and other components of immune function are compromised. Ocean acidification will most likely affect multiple systems and functions within the oyster. Current populations do not appear to have the extensive response necessary for such a long-term stressor to which they are not adapted. The potential for survival of oyster populations in the face of acidifying oceans will be in their potential to quickly adapt via microevolution, dependent upon genetic variability for traits associated with low pH tolerance or resistance.

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