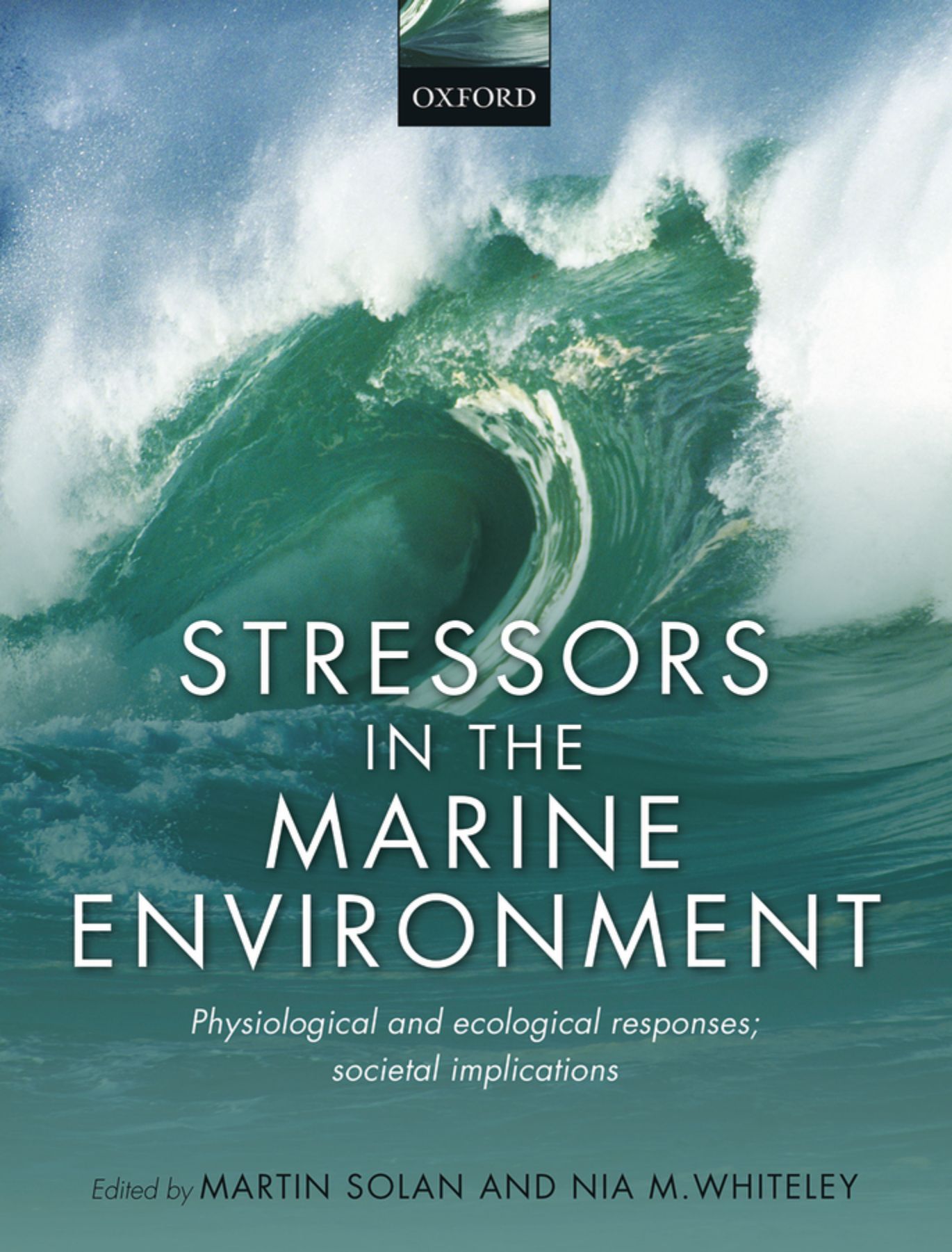
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STRESSORS
IN THE
MARINE
ENVIRONMENT
Physiological and ecological responses;
societal implications
Edited by MARTIN SOLAN AND NIA M. WHITELEY

Stressors in the Marine Environment

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Physiological and ecological responses; societal implications

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Preface

The biological composition and richness of most of the Earth's major ecosystems are being dramatically transformed—to a significant extent irreversibly—by anthropogenic activity. The oceans form a considerable sink for heat and carbon dioxide, and ozone-related increases in UV-B radiation can negatively influence many aquatic species and ecosystems. At the same time, terrestrial flooding and polar meltwaters contribute to the freshening of many coastal regions, while land run-off brings chemical pollutants and nutrients that can lead to eutrophication and the development of harmful algal blooms and hypoxic 'dead zones'. Human activities, such as offshore construction and the transport of cargo using ships, also generate novel sound fields that can affect species behaviour. Marine environments are particularly vulnerable to such changes because approximately 40% of the world's population live within 100 km of the coast, yet a significant proportion of these inhabitants also depend on the ocean for food, economic prosperity, and well-being. Consequently, the cumulative effect of multiple stressors on ecosystems is now a major source of concern to society and is fast becoming a prominent research goal, attracting interest from those tasked with managing the environment or developing environmental policy.

Understanding and predicting the combined impacts of single and multiple stressors is, however, particularly challenging because observed ecological responses are underpinned by a number of physiological and behavioural responses that are affected by the type, severity, and timing of stressors, yet integration between the traditional domains of physiology and ecology is fragmented and often focused towards a specific set of circumstances. Environmental or comparative animal physiology texts either treat the subject area system by system, moving, for example, from cardiovascular responses to nerve and muscle function, or by considering the challenges posed by specific environments, such as polar or estuarine habitats. Similarly, most general ecology-based textbooks, across a range of systems, provide only superficial considerations of environmental stressors, tending (in a marine context) to deal mainly with the adjustments required to survive or tolerate changes in temperature, salinity, oxygen, and/or pressure. Hence, the purpose of this book is to provide in a single volume an overview of the physiological and ecological responses of marine species to a wide range of potential stressors resulting from contemporary anthropogenic activity, while referencing the effects that this may have for the oceans, other systems, and for human well-being.

Recent syntheses of the available literature continue to present strong evidence that the cumulative effect of multiple stressors on a variety of marine species and habitats tends to be variable (additive, antagonistic, and synergistic effects), but they also reveal that the suite of effects brought about by individual anthropogenic forcing has been poorly represented in experimental manipulations. Indeed, it is clear from the literature that individual studies tend to refrain from considering physiological and behavioural mechanisms alongside the ecological and societal significance of their findings, and that studies tend to be either ecologically or physiologically themed. Our vision in compiling this volume was to provide a gateway to targeted and authoritative information within a discipline, while presenting alternative perspectives in relevant sister contributions. In doing so, we focus on eight stressors (salinity, hypoxia, ocean acidification, temperature, chemical pollution, nitrogen deposition, ultraviolet radiation, and noise) that are particularly prevalent in coastal and shelf sea environments, before offering perspectives on the concepts of thresholds and tipping points with just social foundations, economic valuation of stressor-mediated change, and, considering the many sectors of human society that utilize the oceans, how best to manage multiple stressors for society as a whole.

In order to cover both physiological and ecological aspects as well as the societal implications, we have brought together a range of expertise from within the marine community, including early career researchers and established leaders in the field, and combined this with internationally recognized researchers from the fields of physiological processes and ecological systems to broaden the scope and generic value of the volume. Collectively, the authors have broad interests across many different marine species (animals/plants/microbes) and habitats, and cover a range of technical expertise including environmental physiology and ecological theory, mathematical modelling, statistics, empirical and field research, social science, economics, and policy. Throughout, there is an emphasis on the species and communities that are the most affected, such as marine calcifiers in the case of ocean acidification and marine mammals in the case of noise pollution. Consideration is given to the damage caused by each pollutant, whether this is morphological or biochemical, and the means by which some species can recover, as well as the energetic implications that have far-reaching consequences by influencing the function and survival of populations and hence marine communities and ecosystems. Common themes include differences in sensitivity to specific stressors among taxa, species, and life stages, and the logistical challenges represented by introducing additional stressors to experiments, along with requirements to provide local, regional, and global perspectives. The requirement for laboratory experiments to be carried out alongside, and be informed by, field and 'natural' experiments wherever possible is also apparent. Overall, we envisage that this book will be a valuable reference for students, researchers, and those tasked with conserving or managing marine systems in both the natural and social sciences by outlining our current but patchy understanding of the complex interactions between various stressors faced by species, populations, and communities, and informing on future avenues for experimentation and observation using a combination of both laboratory and field-based studies.

Martin Solan and Nia M. Whiteley

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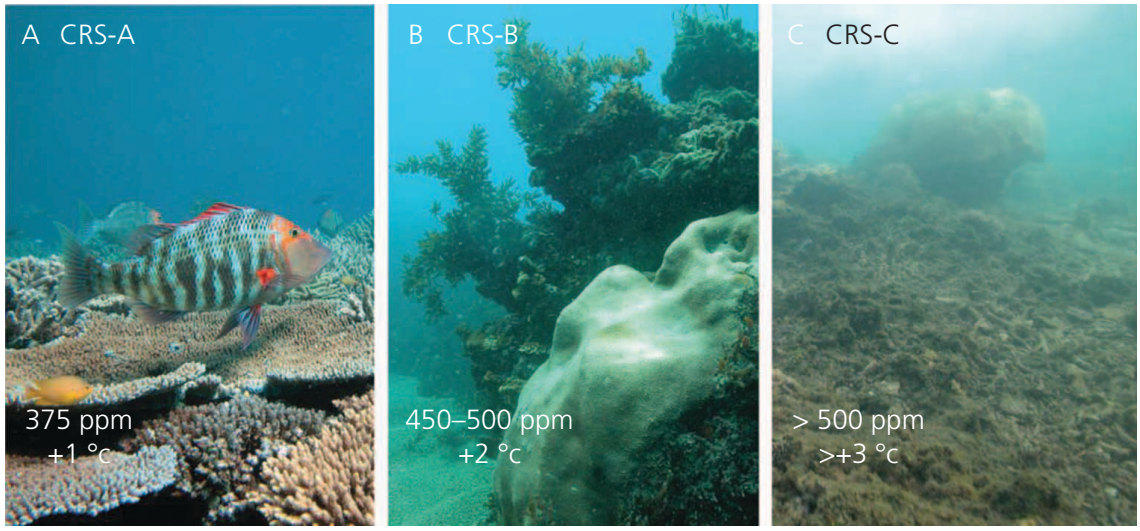

Plate 1 Ecological structures predicted to form in place of the coral reefs for three different scenarios of global climate change, the Coral Reef Scenario (CRS)-A, CRS-B, and CRS-C. The typical anticipated ecological structures are illustrated using extant examples of reefs from the Great Barrier Reef. The atmospheric CO_2 concentration and temperature increases are shown for each Coral Reef Scenario (note that these conditions do not refer to the values measured at the photographed locations). CRS-A scenario assumes that the atmospheric CO_2 concentrations have stabilized at ~380 ppmv (note that as of September 2013, the atmospheric CO_2 levels have already passed that point, reaching ~395 ppmv). CRS-B scenario assumes an increase in CO_2 levels to approximately 500 ppmv, which is slightly below the predictions of a conservative IPCC B1 scenario for the year 2100, at ~550 ppmv. CRS-C scenario assumes an increase of CO_2 to levels above 500 ppmv. For comparison, a moderate IPCC A2 emission scenario predicts atmospheric CO_2 levels of ~800 ppmv by the year 2100, and the current trajectory of CO_2 increase indicates that it is a conservative estimate likely to be exceeded. (A) Reef slope communities at Heron Island. (B) Mixed algal and coral communities associated with inshore reefs around St. Bees Island near Mackay. (C) Reefs not dominated by corals illustrated by an inshore reef slope around the Low Isles near Port Douglas. Reprinted from Science, Vol. 318, by Hoegh-Guldberg et al. 'Coral reefs under rapid climate change and ocean acidification', pp. 1737–1742, Copyright 2007, with permission of the American Association for the Advancement of Science (See also Figure 3.1 on page 37).

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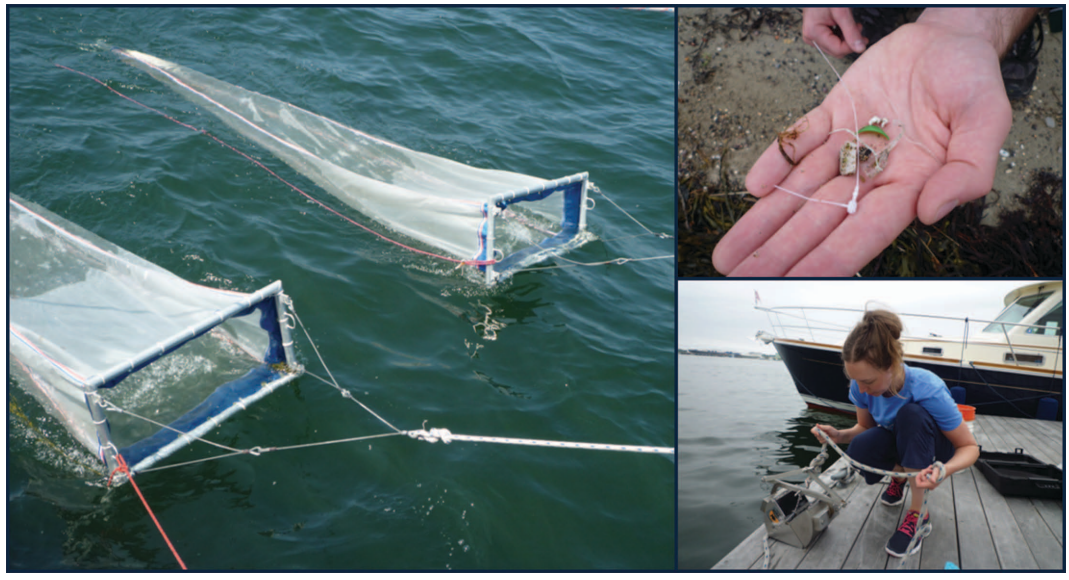

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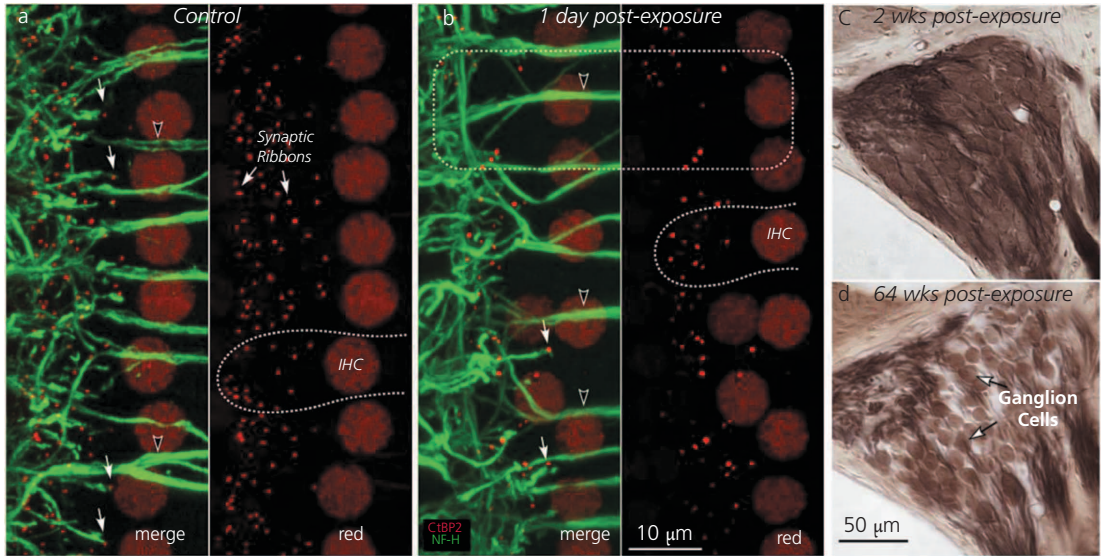

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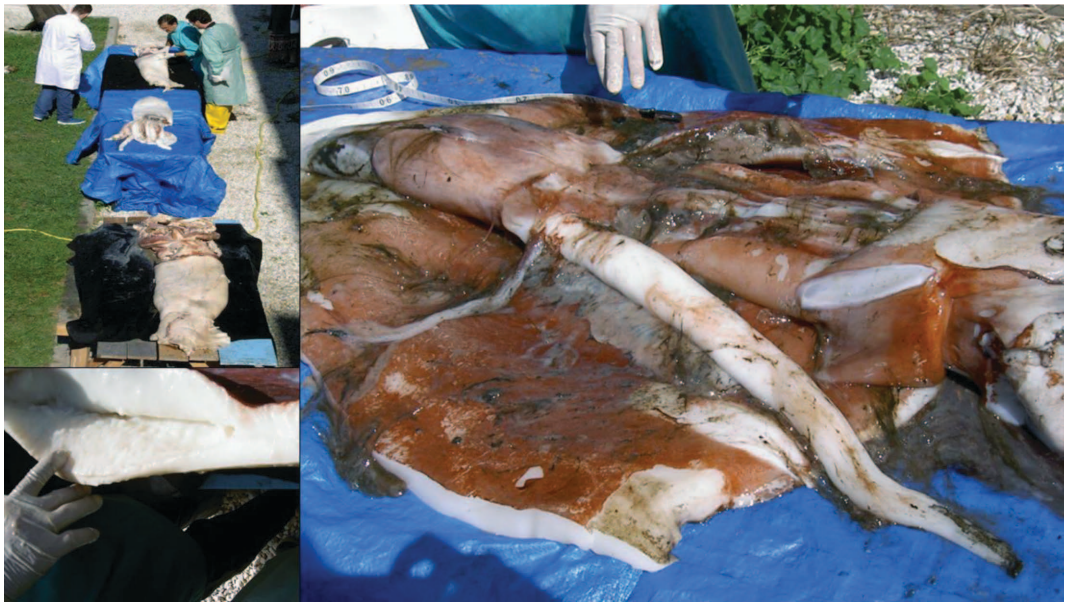

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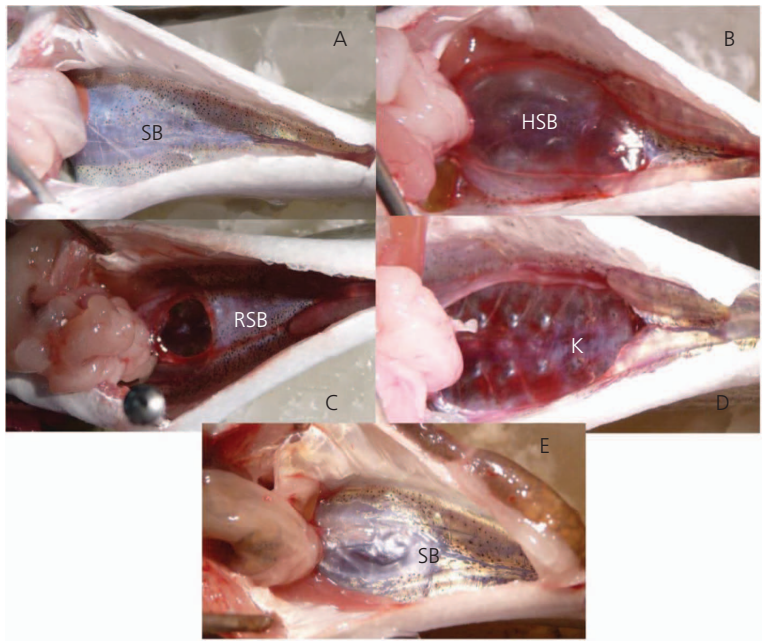

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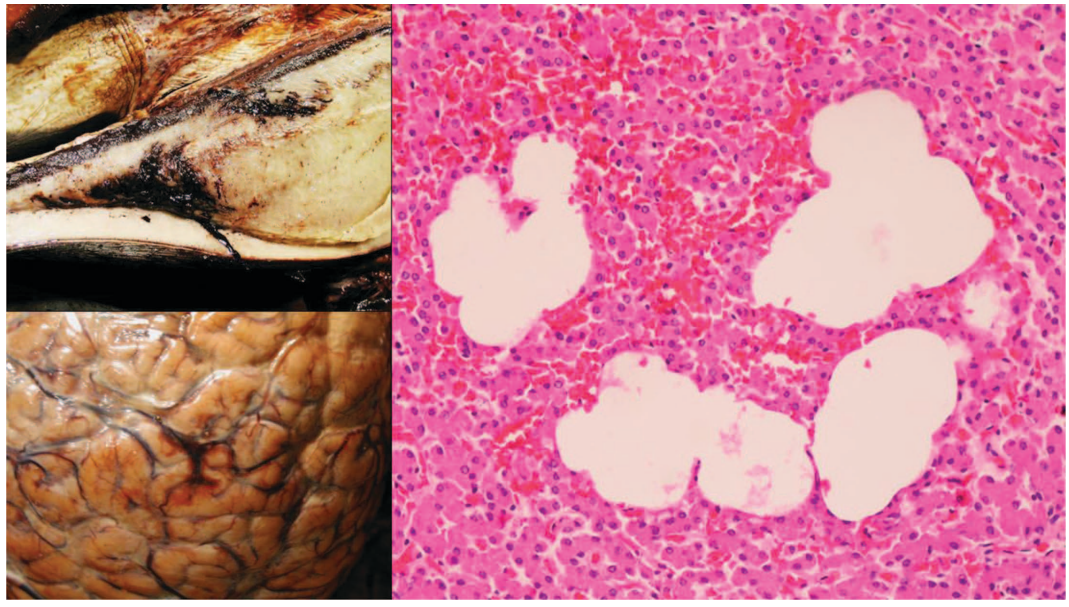

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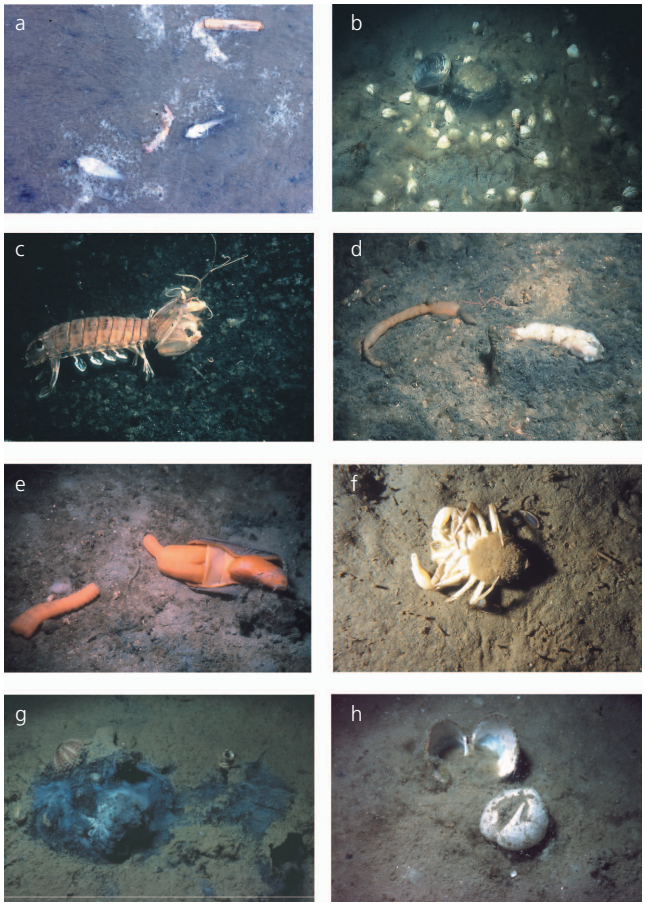

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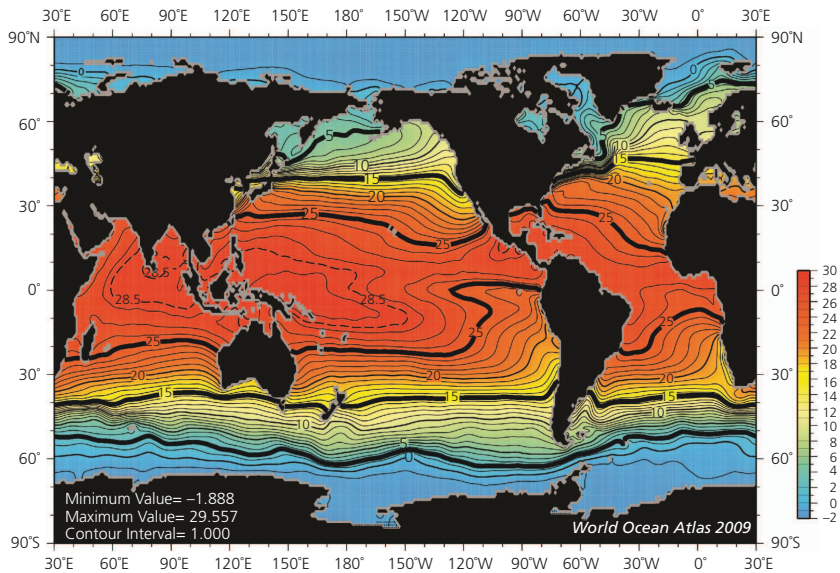

Plate 10 Mean annual sea surface temperature (°C) climatology on a one-degree latitude-longitude grid. Bold lines indicate limits to major thermal biogeographic zones: tropical (> 25 °C), subtropical (25–15 °C), temperate (15–5 °C in the northern hemisphere, or 15–2 °C in the southern hemisphere), and polar (< 5 °C in the northern hemisphere or < 2 °C in the southern hemisphere) (Lalli and Parsons, 1997). Adapted from Locarini et al. (2010) (See also Figure 12.1 on page 214).

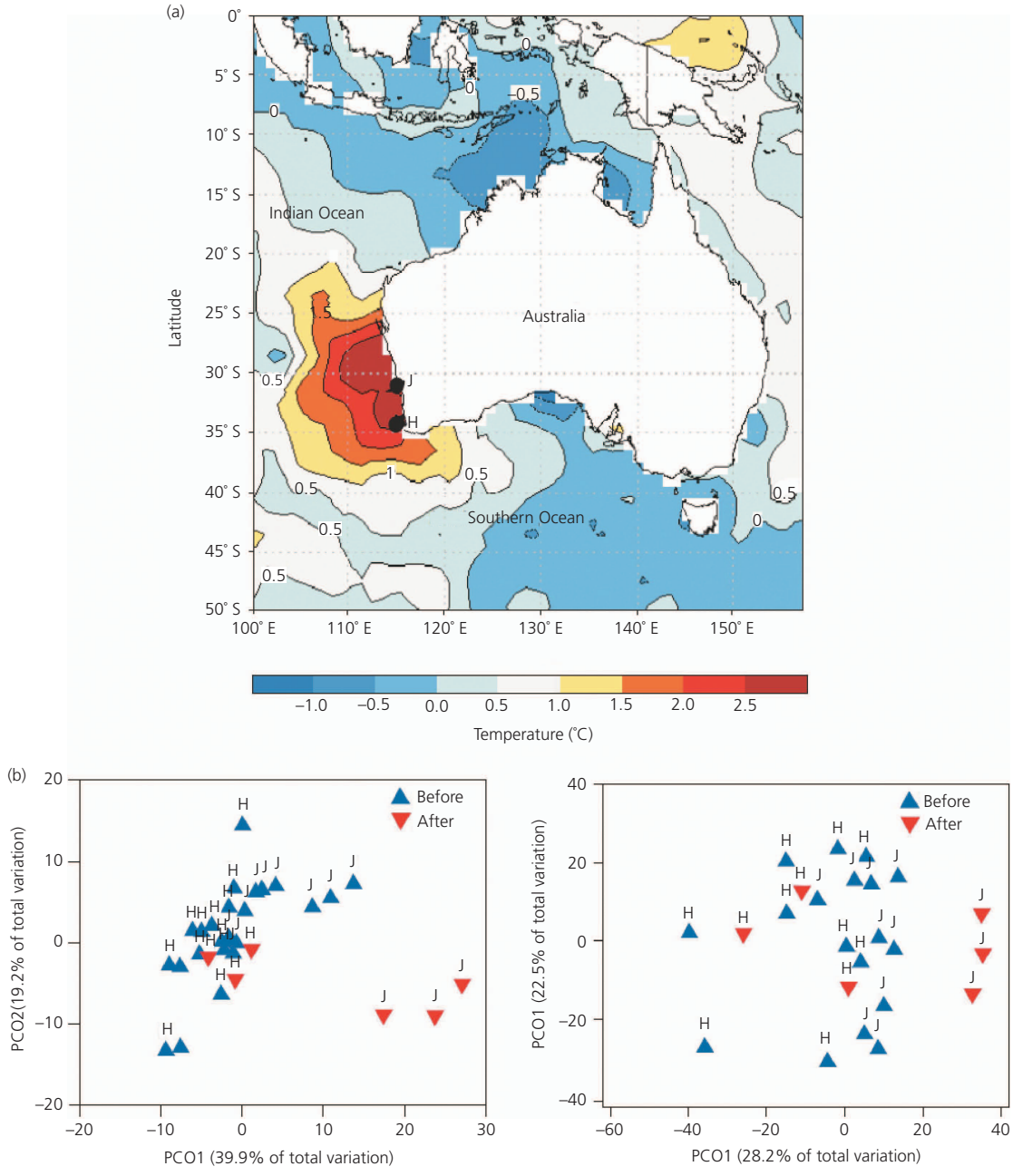

Plate 11 (a) The 2011 heat wave in the southeast Indian Ocean (relative to 1971–2000 baseline). Increased warming was observed (> 2.5 °C) along the west coast of Australia; position of Jurien Bay (J) and Hamelin Bay (H) indicated. (b, c) The ecological structure of marine communities before and after the heat wave of 2011. Principal coordinates analysis of (b) benthic (invertebrates and macroalgae) and (c) fish community structure on the rocky reefs at each study location, before and after the 2011 warming event. PCO1 and PCO2 are the first and second principal coordinate axes, indicating percentage of variation explained by each axis. Reproduced from Wernberg et al. (2013) (See also Box 12.2 Figure 1 on page 220).

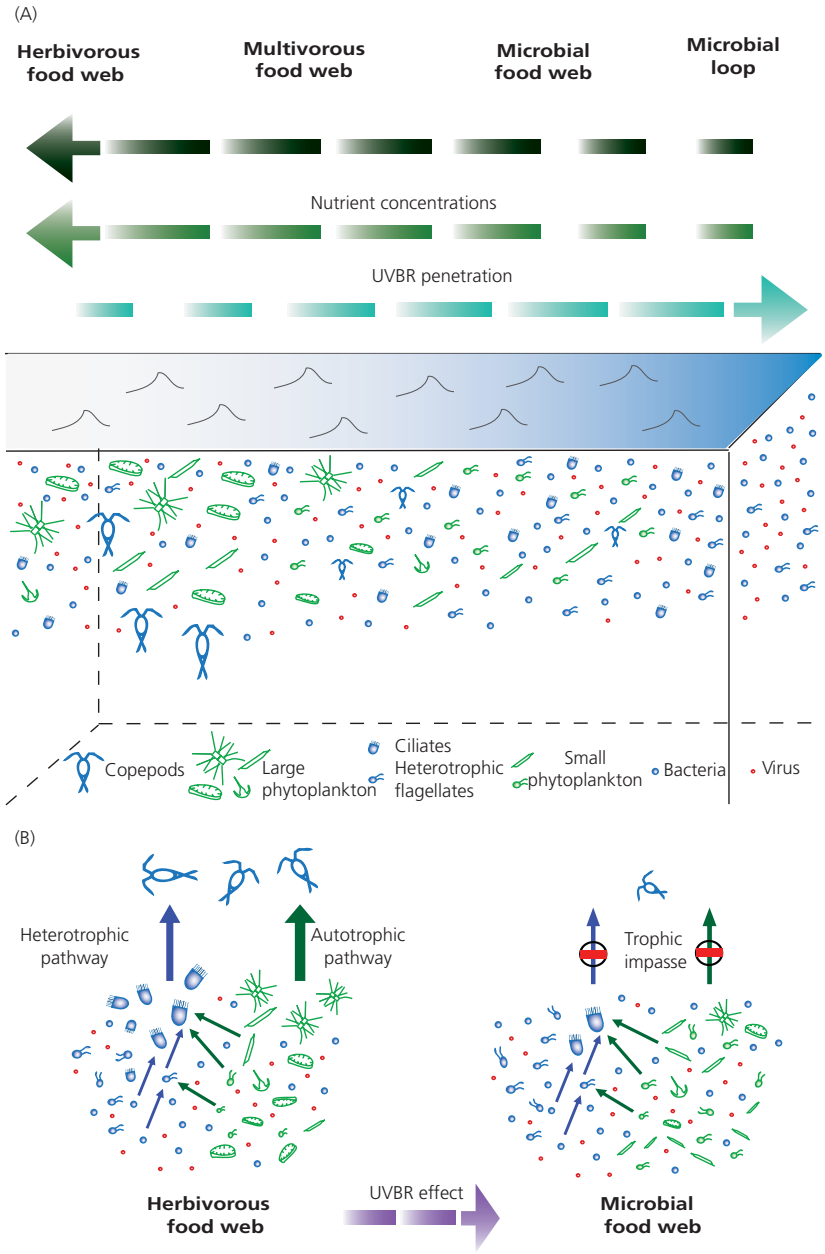

Plate 12 (A) Schematic representation of the continuum between the herbivorous, multivorous, and microbial food webs and the microbial loop. The UVBR penetration is usually higher when the microbial food web or the microbial loop are dominant because of the lower dissolved and particulate matter present within the water column due to oligotrophic conditions. On the other hand, the herbivorous food web is usually more present when nutrients concentrations are higher. In addition, the production of the system, the sedimentation of organic matter and the export of organic carbon to higher trophic levels are higher when the system is dominated by the herbivorous food web. In the herbivorous food web, large copepods graze on large phytoplankton cells, so that predators are mostly metazoans. In the multivorous food web, both copepods (metazoans) and ciliates (protozoans) graze on both large and small prey. In the microbial food web, all components are microorganisms and small phytoplankton and heterotrophic bacteria are predated by protozoans (ciliates and flagellates). Finally, in the microbial loop all components are heterotrophic microorganisms (bacteria, flagellates, and ciliates). It should be noted that viruses are present in all types of food webs. (B) Based on the results of Mostajir et al. (1999) and Ferreyra et al. (2006), UVBR seem to drive planktonic communities from being dominated by a herbivorous food web to being dominated by a microbial food web and, therefore, towards less productive systems with less food transfer to higher trophic levels. The green and blue arrows represent 'predation' (See also Figure 15.3 on page 266).

PART I

Physiological Responses

CHAPTER 1

Effects of salinity as a stressor to aquatic invertebrates

Chris Hauton

1.1 Introduction

The homeostases of ionic composition and cell volume regulation are fundamentally important prerequisites for successful persistence, growth, and development of aquatic species. Ion regulation is directly necessary to maintain optimal electrostatic interactions of enzymes and substrates and receptors and their ligands (Dubyak, 2004; Fernandez-Reiriz et al., 2005), as well as to maintain ion gradients across membranes, protein phosphorylation, and genomic integrity (Kültz, 2005) and the transduction of impulses in nerve cells (Silver et al., 1997). Cellular ionic homeostasis is also essential for the maintenance of cellular osmotic potential, as cell cytoplasm ion concentrations can be altered to permit the uptake of necessary organic molecules that have their own osmotic potential. Maintenance of cell and tissue volume within an organism is essential as dramatic changes in volume can disrupt cell membrane integrity and cell structure. In addition, excess water in cells can have a fundamental impact on protein function and performance within the cell (Lang, 2007).

Deviations from the maintenance of cell ionic concentration or volume can lead to stress. At a cellular level, stress can be defined as the impact of: 'environmental force(s) on macromolecules,' (Kültz, 2005). If not corrected, this can result in the manifestation of stress response at the level of the organism. Organismal stress has been variously defined in the literature previously, but remains a challenging concept. Barton (2002) has defined organismal stress as: 'a non-specific response of a body to any demand placed upon it such that it cases an extension of a physiological stated beyond a normal resting state,' which reflects the integrated and longer–term response of the organism to perturbation. Both definitions are of value to this chapter, which considers both cellular and whole organism integrated responses to changes in environmental salinity that have been reported in the literature.

For open ocean species these demands are limited as the salinity of the world's oceans is generally within the range of 34.6–34.8 (Worthington, 1981). However, for neritic and estuarine species deviations from this mean can be extreme, extending from salinities of 0 to above 40 and subject to change on a tidal or even hourly basis (McAllen and Taylor, 2001). Nonetheless, these environments are some of the most productive across the world and support shellfish production for many of the world's nations (Field et al., 1998). The importance of marine invertebrate shellfish fisheries and aquaculture within these challenging and changing aquatic environments has been the motivation for a considerable body of research on the direct and indirect impacts of salinity on marine invertebrates. This chapter will focus on the impacts of changes in salinity as a primary stressor of aquatic invertebrates, ranging from freshwater crustaceans to fully marine corals and echinoderms.

For coastal marine and freshwater aquatic species homeostasis is complex as their body surfaces are not completely impermeable to the external environment, an environment which often has a different osmotic potential to the organism's internal (cellular/tissue) environment. In considering osmoregulatory capacity, species can be defined along a continuum from stenohaline—having a narrow tolerance range for environment salinity—to euryhaline—tolerating a wide range of environment salinity (Schmidt-Nielsen, 1997). Species can also be considered along a spectrum from complete osmoconformers, where the body fluid osmolarity matches the external environment, to complete osmoregulators in which the organism actively controls the osmolarity of the body fluids irrespective of the external osmolarity. In general terms the majority of marine invertebrates are stenohaline in habit

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and a minority are euryhaline, although coastal and estuarine environments are dominated by euryhaline species. Within the euryhaline group most are osmoconformers that can only control their osmolarity at a cellular level or by behavioural modification. Euryhaline osmoregulators are mainly comprised of the Crustacea, and this group actively regulate the osmolarity of their body fluids (Davenport, 1985; see also Henry, 2001).

1.2 An overview of the mechanisms for osmotic control

Davenport (1985) and Lang (2007) have provided comprehensive reviews of the mechanisms for osmotic control in cells and, specifically, in marine fauna. Dubyak (2004), Henry et al. (2012), and McNamara and Faria (2012) have also provided excellent detailed accounts of cellular molecular mechanisms of ion homeostasis. Complete details of mechanisms for cellular osmotic control are beyond the scope of this chapter to review. In brief, however, maintenance of the cell osmolarity and cell volume through regulation of the free amino acid pool (FAAP) and ion exchange are features of all cells adjusting to a new extracellular osmotic environment, whether that be the osmoconforming extracellular fluid of a bivalve such as the mussel *Mytilus edulis*, or the more regulated extracellular fluid of a crustacean osmoregulator, such as the European shore crab *Carcinus maenas*. Both intracellular ion regulation and regulation of the FAAP can be used to regulate cell volume (Fig. 1.1). However, a major constraint on varying ionic concentrations intracellularly is that very quickly this can have significant detrimental impacts on enzyme interactions and metabolic pathways. As a result, intracellular osmotic pressure is substantially created (< 60–70%) and regulated using organic molecules (Yancey et al., 1982; Davenport, 1985; see also Deaton and Pearce, 1994, and other papers in that special issue). Control of cell volume by FAAP regulation is considered in further detail in Section 1.4, which considers the cellular homeostatic response to salinity stress.

In addition to the maintenance of cell volume, osmoregulating organisms regulate the ionic composition and osmolarity of the extracellular fluids relative to the changing environmental conditions. As described in Davenport (1985), most aquatic invertebrates are hyperosmotic regulators which maintain their osmolarity above that of the environment at low salinities, becoming iso-osmotic at high salinity. Ionic regulation in osmoregulating crustaceans is achieved via membrane-bound ion exchange pumps that are concentrated within the gill epithelial, and in many species predominantly within the posterior gills (Neufeld et al., 1980; Henry and Cameron, 1982; Boettcher et al., 1995).

A large suite of ion regulatory pumps and channels have been identified, mainly from crustaceans, including Na^+/K^+-ATPases, V-type proton- ATPases, bicarbonate-ATPases, K^+ and Cl^- channels, Na^+ channels, Cl^-/bicarbonate exchangers, $Na^+/K^+/2Cl^-$ co-transporter, Ca^{2+}-pumps, Na^+/Ca^{2+} exchangers, and carbonic anhydrases (CA) (Henry, 1984; Henry et al., 2012; and McNamara and Faria, 2012). Increased activity of membrane-associated Na^+/K^+-ATPases and CAs, as well as the gene transcription of new Na^+/K^+-ATPases (e.g. Towle et al., 2001) and CAs (e.g. Henry et al., 2003) have been generally associated with acclimation to low salinity environments in crustaceans (e.g. Pacific white shrimp *Litopenaeus vannamei*, Palacios et al., 2004 and Sun et al., 2011; shore crab *Pachygrapsus marmoratus*, Jayasundara et al., 2007; tiger shrimp *Penaeus monodon,* Pongsomboon et al., 2009; and shore crab *Carcinus maenas*, Towle et al., 2011).

Changes in gene transcription and protein expression do not necessarily have the same temporal profile however. A detailed study of *Carcinus maenas* by Jillette et al. (2011) demonstrated that although there were rapid (<1 week) changes in gene expression of two isoforms of carbonic anhydrase in the posterior gills in response to hypo- and hyperosmotic acclimation, changes in gill carbonic anhydrase enzyme activity had a different time course. On exposure to low salinity (to a salinity of 15, from a control salinity of 32) there was a significant (approximately fourfold) increase in enzyme activity in the posterior gills within one week. However, it took a period of four weeks for that enzyme activity to return to baseline levels once the crabs were returned to the control conditions (salinity 32). These differential time courses identify the importance of establishing changes in physiology at multiple levels of biological organization and not relying on a single measure, for example gene transcription alone.

Further, longer term, acclimation to chronic salinity change has been shown to require the significant synthesis of new protein pumps, in addition to the increased activity of existing pumps (e.g. Towle et al., 2001; Henry et al., 2003). Lovett et al. (2006b) determined the expression profile of Na^+/K^+-ATPase during acute and chronic hypoosmotic stress in the blue crab *Callinectes sapidus*. These authors reported that

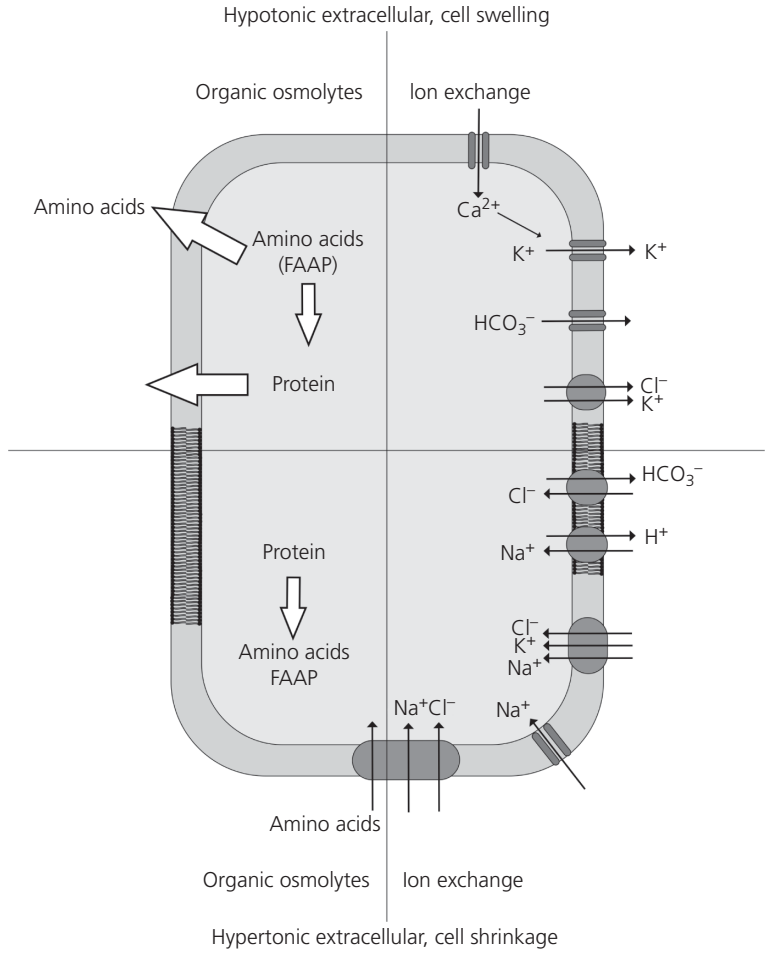

Figure 1.1 Overview of ionic and organic mechanisms involved in intracellular volume regulation in animal cells, showing an increase in ion uptake and the FAAP in response to hyperosmotic stress, and a reduction in the FAAP and excretion of ions in response to hypoosmotic stress. Adapted from Davenport (1985) and Lang (2007).

crabs exposed to dilute seawater for over 18 days showed a 300% increase in Na^+/K^+-ATPase specific activity and a 200% increase in Na^+/K^+-ATPase protein levels; chronic exposure to low salinity resulted in the synthesis of new enzyme (Lovett et al., 2006b). Of significance to subsequent discussion is the fact that the sustained activity of membrane-bound ATPases and the transcription and translation of new enzymes represents a significant energetic commitment from the individual in the form of ATP. Na^+/K^+-ATPase activity is a major demand on maintenance metabolism (3–40%; Leong and Manahan, 1997) and so osmoregulatory adjustments clearly represent a significant energetic cost to the individual.

Further evidence for the energetic cost of chronic osmoregulation in crustaceans is provided by data showing that, within a species, gill ultrastructure is plastic and can be modified in response to salinity change. Changes in the apical border of gill epithelial cells, mitochondria, and cytoplasmic lacunae have been recorded in amphipods *Gammarus duebeni* grown in high salinity environments (Shires et al., 1994), and proliferation of mitochondria-rich cells in the gills of *C. maenas* transferred to low salinity environments for 4–7 days (Compère et al., 1989). Such tissue reorganization in response to environmental challenge will present a significant energetic cost to the individual. The implications of increased metabolic demands of

osmoregulation for whole organism respiration rates are considered further in Section 1.5.

It should not be overlooked that osmoconformers, and some osmoregulators, can also employ behavioural modifications to control tissue osmolarity; this is especially the case for mobile species. Behavioural avoidance is widespread in mobile species (Davenport, 1985) such as the homarid lobsters (Charmantier et al., 2001) and the sandhopper *Talitrus saltator* (Fanini et al., 2012), which preferentially burrows into high salinity sediments. Nonetheless, behavioural control of osmolarity is also common among the bivalve and gastropod molluscs. These groups can either close their valves, clamp down on to the substrate, or seal themselves inside the shell behind the opercular plate. For example, bivalves such as the mussel *Mytilus edulis* close their valves in response to low salinity water (below a salinity 20; Davenport, 1985). Suspension feeding in the gastropod *Crepipatella dilatata* is also arrested at salinities below 20 and is associated with the isolation of the mantle cavity from the environment, a mechanism that also can be employed to create a brood chamber for developing embryos (discussed in Section 1.7; Chapparro et al. (2008)). Alternately, deep burrowing species such as the clam *Mya arenaria* (Davenport, 1985; Deaton, 1992) and the lugworm *Arenicola marina* (Shumway and Davenport, 1977) can retract deeper into their burrow or retreat behind a mucus boundary to avoid exposure to salinity stress from surficial waters. While effective, prolonged exposure to low salinity environments through valve closure can lead to anaerobic metabolism, reduced feeding rates, and, subsequently, impacts to growth (Poulain et al., 2011). Behavioural regulation of osmotic pressure is reviewed more fully in Davenport (1985).

In summary, neritic, estuarine, and freshwater invertebrates are presented with significant challenges to osmotic control that are acute, but temporary, as well as chronic. To meet these challenges all species have evolved mechanisms for behavioural avoidance and intracellular volume regulation. In addition, and mainly a characteristic of the Crustacea, some groups have evolved ion regulatory mechanisms which permit them to also regulate the osmolarity and ionic composition of their extracellular fluids, relative to a changing external osmolarity. All of these mechanisms: (1) behavioural, (2) cell volume control via intracellular amendment of the FAAP and ionic regulation, and (3) regulation of the extracellular fluid in osmoregulators, require the provision of energy in the form of ATP. This requirement underlies the majority of the 'stress responses' which are developed through this chapter.

1.3 The cellular stress response (CSR) to salinity perturbation

At a cellular level responses to environmental perturbation can be divided into two stages. Early responses to stress or insult focus on the counteraction and repair of stress-induced damaged, increased tolerance against further stress damage, and apoptosis or maintenance of the cell cycle (Kültz, 2005). This evolutionarily conserved response, the 'cell stress response (CSR)' is ubiquitous across the different Kingdoms of Life and is triggered as a result of non-specific macromolecular damage. The CSR is a transient state and is followed by the cellular homeostatic response (CHR), which is a semi-permanent state that remains until the environmental conditions of the cell change again (Kültz, 2005). While the CSR is a conserved response, the CHR has the potential to be species-, cell- and even stressor-specific. The homeostases of ion balance and cell volume described in Section 1.2 are components of the CHR.

Of the 44 proteins with known functions in the CSR, a number include members of different molecular weight (MW) families of heat shock proteins (HSPs), including the 60 kDa, 70 kDa, and 90 kDa families, which variously: (a) assist with the refolding of denatured proteins, or (b) chaperone irreversibly damaged proteins for polyubiquitination and degradation at the proteasome (Hochstrasser, 1996). Up-regulation of HSP gene expression in response to high salinity stress has been reported in many aquatic invertebrates (e.g. the ascidian *Styela plicata*, Carmen Pineda et al., 2012; the Chinese mitten crab *Eriocheir sinensis*, Sun et al., 2012; and the estuarine copepod *Eurytemora affinis*, Xuereb et al., 2012). In contrast, in the osmoconforming echinoderm *Apostichopus japonicus* both hyper- and hypoosmotic stress have been shown to increase the expression of 70 kDa HSP (HSP70) proteins (Dong et al., 2008), although the temporal profile of expression differed at different salinities.

However, notable differences do exist, for example the euryhaline osmoregulating European shore crab *Carcinus maenas* did not show elevated HSP70 gene expression in response to salinity stress (Towle et al., 2011) and Werner and Hinton (2000) reported data from field collections and laboratory experiments which identified a *decrease* in the expression of HSP70 proteins in the Asian clam *Potamocorbula amurensis* at extremely low salinities. One requirement for the action of heat shock proteins is the supply of energy in the form of ATP, which drives the conformational changes required in the molecule to support function

(Mayer, 2010); this again represents a cost to the individual which can translate into longer term impacts identified later in this chapter.

While the measurement of HSP expression in response to salinity stress—either in terms of gene transcription or protein concentration by Western blot—is common within the literature, Morris et al. (2013) have recently questioned its widespread continued use, in light of many uncertainties over what is being measured (heat shock protein versus heat shock cognates) and inconsistent responses reported from different populations of the same species, especially in field studies. Morris et al. (2013) have instead argued in favour of the monitoring of stressor specific responses, triggered as part of the cellular homeostatic response (CHR—described in Section 1.4), as being more informative.

A second component of the CSR is the expression of proteins and enzymes of various antioxidant pathways. Intracellular reactive oxygen species (ROS)—including superoxide anions and hydrogen peroxide—are produced continuously as a by-product of routine respiration within the mitochondria. However, cellular ROS production can be increased in response to environment perturbations, such as salinity stress (Paital and Chainy, 2012) or uptake of pollutants, when cellular homeostasis cannot be restored (reviewed by: Luschak, 2011; Galluzzi et al., 2012). Extracellular ROS production can also be increased in response to pathogen infection, accompanying phagocytosis to break down the cell membranes of invading pathogens (described in Section 1.8). Unregulated production of ROS causes harm to the host, which was initially considered in the 'free radical theory of aging' (Harman, 1956), although the significance of this idea has since been questioned, especially for the case of marine invertebrates (Buttemer et al., 2010).

The damaging effects of an excess production of ROS are controlled through the expression of diverse antioxidant protective pathways, including the redox enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH), as well as the peroxiredoxins (Prxs). The rapid action of all of these proteins protects cells and tissues from the damage induced by environmental insult and also inappropriate immune responses. Copper and zinc conjugated SODs are widespread in the cytoplasm of many eukaryotic cells while manganese-conjugated SODs are found within the mitochondria. SOD enzymes catalyse the dismutation of superoxide anions ($O_2^{\bullet -}$) into oxygen and hydrogen peroxide, while catalases further breakdown hydrogen peroxide to water and oxygen. Peroxiredoxins, which contain a redox-active cysteine residue, also detoxify hydrogen peroxide to water. Glutathione (GSH) functions by providing reducing equivalents for key antioxidant defence enzymes and can also scavenge hydroxyl radicals directly. High levels of glutathione disulphide (GSSG), the oxidized disulphide form of GSH, accumulate during the ROS detoxification processes and it is therefore necessary to recycle GSSG back to the reduced glutathione form, requiring the enzyme glutathione reductase (GR).

Antioxidant responses to the production of free radicals associated with salinity exposure have been reported in marine invertebrates, but as with the HSPs, responses have been shown to vary as a function of species, population, and tissues studied. For example, Paital and Chainy (2010) have reported extensive changes in the antioxidant pathway in the mud crab *Scylla serrata*. In response to an increase in environmental salinity from 10 to 35, they reported a decline in activity of superoxide dismutase (SOD) in abdominal muscle, contrasting with an increase in activity of the catalase enzyme and no change in the activity of glutathione peroxidase (GPx). In the hepatopancreas, however, SOD and CAT activities eventually decreased, while GPx and GR activities consistently decreased with increasing salinity. In gill tissue, SOD activity initially fell before increasing as salinity increased, while decreases in CAT and GPx activities were noted after 21 days (Fig. 1.2). Freire et al. (2011) compared the antioxidant response of the swimming crabs *Callinectes danae* (euryhaline) and *C. ornatus* (stenohaline) in response to hyper-and hypoosmotic stress. *C. danae* displayed higher baseline activities of GPx (in hepatopancreas and muscle) and CAT (in hepatopancreas, muscle, anterior and posterior gills) than *C. ornatus,* which only demonstrated activation of these enzymes when exposed to hypersalinity (40). Rodrigues et al. (2012) also reported little perturbation in the antioxidant response of the euryhaline European shore crab *Carcinus maenas* exposed for seven days in the salinity range of 4–45. Differential responses have also been reported between species of bivalves. Zanette et al. (2011) reported that in the Pacific oyster *Crassostrea gigas*, salinity perturbations of between 35 and 9 did not produce major changes in the gill CAT or GST activity.

From field studies, Philipp et al. (2012) reported population level differences in the expression of genes coding for antioxidant enzymes between populations of the oceanic quahog *Arctica islandica*, a species which can have an extremely long lifespan (> 500 years; Treaster et al., 2014). Philipp et al. (2012) compared

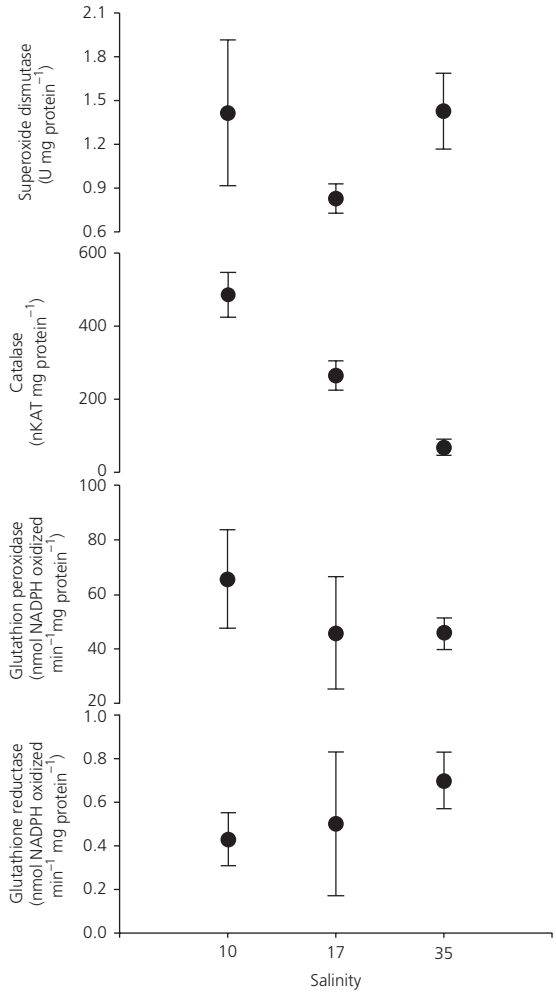

Figure 1.2 Activity of selected antioxidant enzymes within the gill tissue of the mud crab *Scylla serrata* after 21 days acclimation to different salinity, showing the mean ± SD. Figure plotted from data presented in Paital and Chainy (2010) (their Table 3).

the expression of genes for antioxidant enzymes in German Bight quahogs (maximum lifespan (MSLP) of ~150 years; typical environmental salinity 33) with those of the Baltic Sea (MSLP ~40 years; typical salinity 20–25). These authors concluded that the existence of populations of shorter lifespan quahogs within the variable environment (including salinity) of the Baltic was not a result of metabolic rate depression but a consequence of 'stress-hardening'; an increase in their ability to up-regulate the expression of genes coding for antioxidant enzymes at times of stress. However, Basova et al. (2012) have also argued that the long lifespan of German Bight clams might be a function of an initial low rate of ROS formation, resulting from a low metabolic rate combined with a high damage repair (antioxidant) capacity.

Antioxidant responses to salinity perturbation have also been shown to vary as a function of the nature of the salinity change, whether acute or chronic. Cailleaud et al. (2007) measured glutathione-S-transferase GST activity in the calanoid copepod *Eurytemora affinis* sampled from the Seine Estuary in France and reported that activity was maximal during acute exposures to salinities within the range of 5–15, while long-term exposure resulted in maximal GST activity at a salinity of 5.

A complication in all of these data is that, as with HSP expression, the temporal component of antioxidant response, and potentially the magnitude of response, can vary as a function the type of measurement taken. The immediate antioxidant responses to perturbations in salinity rely on the activity of existing mature proteins, while longer-term exposures are likely to require increases in gene transcription to maintain or increase the capacity of the antioxidant pathways. As an example, Seo et al. (2006) have reported that expression of the gene encoding for glutathione reductase was significantly increased and sustained from 6 h after exposure to high salinity stress (24 and 40, controls acclimated to 18) in the copepod *Tigriopus japonicus*, while expression to low salt stress (0 and 12) resulted in a down-regulation in the expression. Van Horn et al. (2010) have reported also that in the flatback mud crab *Eurypanopeus depressus* the gene coding for peroxiredoxin was transcribed initially at low levels in the gill, hypodermis, and hepatopancreas of crabs under non-stressed conditions and was only elevated about threefold in gills after 48 h exposure to hypoosmotic stress (acclimation salinity 30, exposure salinity of 10).

1.4 The cellular homeostatic response (CHR) and maintenance of cell volume

Once the initial damage from osmotic stress has been contained the cellular homeostatic response (CHR) regulates cell processes to achieve acclimation to the new extracellular environment. This includes the maintenance of cell volume and hydration by the regulation of cell osmolarity. Osmolarity, and therefore cell volume, in both osmoregulators and osmoconformers can be achieved intracellularly through adjustments to ionic regulation (in part) and the free amino acid

pool (FAAP). Intracellular amino acids contribute to the intracellular osmotic pressure and, by adjusting the concentration of the FAAP, changes in this osmotic pressure can be achieved to regulate the osmotic water flux between the intracellular and extracellular compartments. Adjustments to the FAAP (Fig. 1.1) occur via the catabolism and anabolism of intracellular protein (e.g. Gaspar Martins and Bianchini, 2009), by the *de novo* synthesis of amino acids in high salinity environments, or by the expulsion of FAA from the cells for deamination and excretion (e.g. Rosas et al., 1999). Ultimately the osmoregulatory CHR can significantly affect organism excretion rates (e.g. Tirard et al., 1997; Shinji and Wilder, 2012).

While decreases in the FAAP are qualitatively associated with acclimation to low salinity, considerable variation has been reported in the dominant amino acids involved in different species, and also in the temporal profile of different amino acids. Glycine, proline, and taurine were reported as quantitatively the most important amino acid osmolytes in crustaceans (Bishop and Burton, 1993) but decreases in glycine, taurine, proline, *and* alanine were reported as responsible for a reduction in the FAAP in *Palaemon elegans* following acclimation from a salinity of 40 to 10 (Dalla Via, 1989).

In bivalves, taurine and glycine have been reported to be two major contributors to the FAAP, at least in gill tissues. In the oyster *Crassostrea gigas*, decreases in taurine concentration were a major contributor to the overall decrease in FAAP during acclimation from a salinity of 30 to 7 (Lee et al., 2004). In the osmoconforming blood worm *Glycera dibranchiata* red coelomocytes have been identified to regulate cell volume during hypoosmotic stress (498 milliosmoles (mOsm) compared to an acclimation osmolality of 996 mOsm) by reducing the intracellular concentration of the FAAP, principally by reductions in the amino acids proline, asparagine, and also again taurine (Costa and Pierce, 1983).

In hyperosmotic environments amino acids are either synthesized *de novo* or are produced by the catabolism of cellular protein, thus producing an increase in the FAAP and intracellular osmotic pressure (Fig. 1.1). Proline and alanine were the primary contributors to increases in the FAAP pool in the copepod *Tigriopus californicus* (Burton, 1991) while the red swamp crayfish *Procambarus clarkii* largely accumulated D- and L-alanine together with glycine, L-glutamine, and L-proline in both muscle and hepatopancreas on transfer from fresh water to salinities of 17 and 25 (Fujimori and Abe, 2002).

Similar responses have also been reported in osmoconforming bivalves. In the brackish water bivalve *Rangia cuneata*, glutamic acid, glycine, alanine, and arginine constituted 70–80% of the total muscle FAAP, and concentrations were elevated by as much as 300% during acclimation to seawater (Henry et al., 1980; Otto and Pierce, 1981); however, they also demonstrated that not all amino acids were regulated in response to salinity stress. Amino acids such as serine varied little after 42 days exposure to increasing salinity (Fig. 1.3). In the clam *Meretrix lusoria* hyperosmotic conditions (150% sea water) led to the accumulation of alanine in adductor muscle, gills, and midgut gland (Okuma et al., 1988), while in the commercially important Pacific oyster *Crassostrea gigas* the accumulation of taurine and glycine drove increases in the FAAP within 48 h of being exposed to increased salinities from 30 to 39 (Lee et al., 2004).

Conflicting data have raised questions over whether the accumulation of amino acids and other organic osmolytes in response to hyperosmotic conditions is a rapid process that results only from the activity of existing enzymes or changes in cell permeability, or rather if amino acid synthesizing enzymes must first be translated from mRNA. The fourfold accumulation of proline in response to hyperosmotic stress in the copepod *Tigriopus californicus* could be inhibited by the protein synthesis inhibitor cycloheximide, leading to the conclusion that this required the synthesis of one or more enzymes of the proline biosynthetic pathway (Burton, 1991). However, Deaton (2001) concluded that

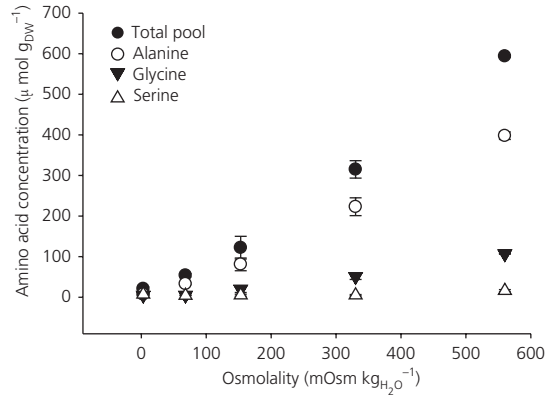

Figure 1.3 Changes in the intracellular free amino acid concentration of selected amino acids in the foot muscle of the brackish water bivalve *Rangia cuneata* acclimated to different salinities for 42 days, showing the mean ± SEM. Figure plotted from data presented in Otto and Pierce (1981) (their Table 1).

gene transcription and translation were *not* responsible for the increase from 45 to 150 µmol g dry weight of betaine^{-1} in the gills of the ribbed mussel *Geukensia demissa* within 12 h of transfer from 250 to 1000 mOsm (Deaton, 2001).

It is of relevance to this book that while trends in the FAAP established from laboratory manipulations broadly appear consistent across diverse invertebrate phyla, outcomes of laboratory manipulations of salinity are not necessarily replicated in field studies, where multiple stressors may interact in complex ways. Kube et al. (2006) identified a complex pattern of cell volume regulation via the FAAP in different populations of the bivalves *Macoma balthica* and *Mytilus* spp. along their European distribution. They classified these patterns into a northern Baltic type, a southern Baltic type, and an Atlantic/Mediterranean type. These three types differed in the relative importance of two amino acids: alanine and taurine. Kube et al. (2007) further developed this study and concluded that salinity was not the main factor in determining FAAP concentration; the seasonal patterns of FAAP components varied as a complex function of environmental conditions (salinity and temperature) and physiological state of the bivalve (glycogen content and reproductive stage).

1.5 The energetic and metabolic requirements of osmotic control and the consequences for organism fitness

From the overview provided in Sections 1.3 and 1.4 it is apparent that, in many cases, the initial CSR and subsequent regulation of cell volume by the FAAP, as well as the regulation of cellular and extracellular ionic composition using membrane-bound ATPases, require an energy source which is obtained by the hydrolysis of the phosphoanhydride bonds in adenosine triphosphate (ATP) and adenosine diphosphate (ADP). As such, this requirement can be directly measured as alterations in the adenylate energy charge (AEC) of a tissue (Atkinson and Walton, 1967). The AEC is proportional to the mole fraction of ATP plus half the mole fraction of ADP (as ATP contains two high energy phosphoanhydride bonds whereas ADP contains one) and is given by the equation:

$$\frac{[\mathrm{ATP}]+0.5\,[\mathrm{ADP}]}{[\mathrm{ATP}]+[\mathrm{ADP}]+[\mathrm{AMP}]}$$

Rainer et al. (1979) reported a 17% reduction in mean AEC following a reduction in salinity from 35 to approximately 10 for the gastropod *Pyrazus ebeninus* and the bivalves *Anadara trapezia* and *Saccostrea commercialis*. Nevertheless, while the AEC has been applied as an index of salinity stress in experimental studies on invertebrates (e.g. Matsushima et al., 1984), its validity in the study of stress in organisms sampled directly from the field has been questioned (Veldhuizentsoerkan et al., 1991), as the gross requirement for ATP is an integrated function of responding to multiple environmental conditions simultaneously, again reflecting the challenge of interpreting stress indices in a multi-stressor environment. A further practical limitation to the use of AEC as a measure of stress in field populations is that it is very difficult, if not impossible, to determine *in situ*. Tissues for AEC determination must be flash frozen using a tissue clamp as the relative concentrations of the different adenosine pools can be altered over timescales of seconds. The handling stress imposed on an organism as it is removed from the field, returned to the laboratory, and then sampled will, inevitably, confound any measurement of relative abundance of ATP, ADP, and AMP.

To provision ATP for ion and volume regulation, osmoregulation places increased demands on oxidative metabolism that increases the requirement for oxygen, the ultimate electron acceptor within the mitochondrial electron transport system, as well as substrates of glycolysis and the tricarboxylic acid cycle. These demands are reflected in the mobilization of carbohydrate and lipid (e.g. Telahigue et al., 2010; Martins et al., 2011) and even protein reserves. These requirements can be summarized by the determination of the Cellular Energy Allocation (De Coen and Janssen, 1997). The aim of the CEA is to quantify the available energy reserves and consumption within a cell to produce a single integrated measure of metabolic status. The CEA is calculated from the following equations:

$$\mathrm{CEA} = \frac{E_\mathrm{a}}{E_\mathrm{c}}$$

$$\text{where: } E_\mathrm{a}\ (\text{available energy}) = E_{\mathrm{Carbohydrate}} + E_{\mathrm{Lipid}} + E_{\mathrm{Protein}} (\mathrm{mJ\ mg^{-1}WW})$$

$$\text{and: } E_\mathrm{c}(\text{energy consumption}) = \text{ETS activity (mJ mg}^{-1}\ \mathrm{WW\ h^{-1}})$$

A decrease in the CEA indicates either a reduction in available energy or a higher energy expenditure, both of which will reduce the energy available for growth, reproduction, or other processes such as the