Osmoregulation and the anthozoan-dinoflagellate symbiosis

by

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Abstract

This study investigated the responses of the temperate anemone *Anthopleura aureoradiata*, and the tropical coral *Acropora aspera* to osmotic stress and the role that free amino acids (FAAs) may play in the osmoregulatory mechanism of these anthozoan-dinoflagellate symbioses. Specimens were exposed to a range of hypo- and hyper-saline conditions for durations of 1, 12, 48 and 96 hours, whereupon respiration and photosynthetic rates were measured as physiological indicators of osmotic stress. High performance liquid chromatography was used to quantify 15 FAAs within the anthozoan host tissues to establish the response of FAA pools to osmotic stress and whether FAAs are used in an osmoregulatory capacity. Aposymbiotic specimens of *A. aureoradiata* were similarly tested to establish if the presence of symbiotic dinoflagellates alters the host's capacity to respond to osmotic stress given that the symbionts are known to release FAAs into the host cytoplasm.

In *A. aureoradiata,* significant changes in respiration were only observed with exposure to the extreme hypo-osmotic salinity of 12‰, with respiration decreasing by 67% after 1 hour of exposure. No significant changes in respiration were seen at 25, 43 or 50‰, despite a 52% decrease in respiration seen at the hyper-saline treatment of 50‰. The response of the coral *A. aspera* was markedly different, showing an increase in respiration in response to hypo-salinity (22 and 28‰). Interestingly, the most pronounced respiratory increase of up to 460% occurred in the less extreme hypo-saline treatment of 28‰. The response of photosynthesis also showed differences between the two species. In the symbiotic *A. aureoradiata,* photosynthesis declined by 61% after the 1 hour exposure to 12‰ and further decreased to 72% below control rates after 96 hours. While in *A. aspera,* photosynthesis showed no significant deviation from control levels at any of the treatment salinities.

FAA pools in both *A. aureoradiata* and *A. aspera* showed significant responses to osmotic stress. In symbiotic *A. aureoradiata,* exposure to 12‰ caused total FAA pools to decline by 50% after 1 hour, after which a seemingly stable state was reached. A hyper-osmotic treatment of 50‰ resulted in a similar trend with a more than 50% decrease after 1 hour of exposure. In *A. aspera,* the response of the FAA pool was markedly different, with the concentration increasing by up to 200% with exposure to 22‰ and by more than 260% at 28‰. Interestingly, one on the main constituents of FAA pools in *A. aureoradiata,* Taurine (15% of FAA pools at 35‰), was not present in measurable quantities within *A. aspera* host tissue.

In aposymbiotic individuals of *A. aureoradiata* exposed to extreme hypo- and hyper-saline treatments of 12 and 50‰ a significant impact on respiration was only observed at 12‰, with a 77% decrease in respiration after 96 hours. Changes in FAA pools of aposymbiotic *A. aureoradiata* were only seen after 12 hours exposure to 50‰ with a significant 26% decrease. However, the direct comparison between symbiotic and aposymbiotic *A. aureoradiata* did serve to highlight the contribution of symbiont-derived FAAs to the host pool of FAAs, with FAA pools in aposymbiotic anemones up to 41% lower than those found in symbiotic anemones.

The results seen here were not suggestive of FAAs being regulated for the explicit use as compatible organic osmolytes. Rather, changes in FAA pools showed changes consistent with other stress responses. Moreover, the response of anthozoan-dinoflagellate symbioses to osmotic stress appears to be species specific, or at least taxa specific, as the responses of respiration, photosynthesis and FAA pools were very different between the temperate anemone *A. aureoradiat*a and the tropical coral *A. aspera.* Nevertheless, differences in the respiratory response between symbiotic and apo-symbiotic anemones did indicate some influence of the dinoflagellate symbionts on the ability of the anthozoan host to mediate osmotic stress. It may therefore be that other symbiont-

derived compounds are utilised as compatible organic osmolytes (COOs), with a primary

candidate being glycerol. This warrants further investigation.

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Introduction

1.1 Symbiosis

Symbiosis has played a prodigious role in the formation of biological life on Earth, and is ubiquitous across terrestrial, aquatic and marine ecosystems. It remains an ecologically important process and can support highly productive ecosystems, such as coral reefs (Hoegh-Guldberg 1999; Moran 2006). The term symbiosis was coined in the mid-nineteenth century by German plant pathologist Heinrich Anton de Bary and broadly refers to the living together of different species (Wilkerson 2001). The term, however, does not specify the form or outcomes that any one specific symbiotic relationship may have. Since its first use, there has never been a universally agreed definition, but it is generally thought that three main categories of symbiosis exist: parasitism, commensalism and mutualism. Parasitism is where one organism benefits while the other is disadvantaged. Commensalism is where there is a noticeable positive benefit for one of the organisms, but no noticeable effect of any kind on the other. Mutualism is where both partners derive benefit from the association (Yellowlees *et al.* 2008), and is sometimes used interchangeably with the term symbiosis (Douglas 2010). Perhaps the most well known mutualism is that between corals and dinoflagellates, which is responsible for the world's most productive marine ecosystem. Other marine examples include mutualistic partnerships between various deep-sea organisms and bacteria (e.g. the squid-*Vibrio* symbiosis) (McFall-Ngai & Ruby 1991; Nyholm *et al.* 2000), and those between sponges and various heterotrophic and phototrophic bacteria (Wilkinson 1983; Webster *et al.* 2001; Taylor *et al.* 2007). These different classifications of symbiosis can appear well

defined, but symbiotic associations are best thought of as part of a shifting continuum in which specific conditions can alter the positive/negative balance of a relationship (Bronstein 1994; Thrall *et al.*. 2006). Moreover, in most cases it is impossible to firmly classify a specific interaction, as the benefits accrued by one or both partners may be difficult to determine from the negatives (Douglas & Smith 1989).

By forming a symbiosis, an organism can utilise gene-sets for all types of functions without the need to possess them itself. Indeed, in an evolutionarily stable symbiosis, both partners may start to lose certain functions or genes that are doubled up in the relationship (Moran 2003; McCutcheon & Moran 2010). This can turn what was once a facultative relationship, not necessary for the survival of either partner, into a finely balanced obligate relationship where even the smallest perturbation may disrupt the symbiosis and result in the death of one or both partners (Smith & Douglas 1987; Moran 2007). In addition, symbioses may occur between two free-living organisms living in close proximity, with one partner living on another (ectosymbiosis), or with one partner living inside the other (endosymbiosis); in both cases the smaller partner is referred to as the symbiont while the larger partner is termed the host (Douglas 1994).

1.2 Cnidarian-Dinoflagellate Symbiosis

Algal-invertebrate symbiosis in the marine environment was first described by Karl Brandt in 1883 with his discovery of chloropyll-containing yellow cells in radiolarians (Protozoa) (Brandt 1883). These "yellow cells," or zooxanthellae as termed by Brandt (Gr. *zoo* animal + Gr. *xanth* yellow + Gr. *ella* diminutive = "yellow animal cells"), were subsequently found to be unicellular phototrophic dinoflagellates of the genus *Symbiodinium* (Freudenthal 1962)*.* Members of the genus *Symbiodinium* are the most prolific of all known unicellular phototropic symbionts, of which others include cyanobacteria, rhodopyhytes,

chlorophytes and diatoms (Smith & Douglas 1987), and are found in endosymbiotic relationships with a wide range of hosts spanning both tropical and temperate latitudes (Trench 1979; Roberts *et al.* 1999). These hosts include the cnidarian classes Anthozoa (e.g. hard and soft corals, sea anemones) and Scyphozoa (jellyfish), and the molluscan classes Gastropoda and Bivalvia (e.g. giant clams), as well as sponges and Foraminifera (Trench 1993; Pawlowski *et al.* 2001). Symbiotic dinoflagellates can be found both intracellularly within the host cells and intercellularly, between cells within host tissue. Most commonly (and always in anthozoans), the dinoflagellate symbionts are found intracellularly within the cells of the host's gastrodermal layer (Muscatine & Lenhoff 1963; Farmer *et al.* 2001). Here, they are separated from the host cytoplasm by the symbiosome membrane, a multi-layered complex of symbiont-derived membranes surrounded by a single host derived vacuolar membrane, this is known as the symbiosome membrane complex. This complex is a boundary across which all host-symbiont cellular communication and nutrient transfer must occur (Wakefield & Kempf 2001).

Until 1980, all symbiotic dinoflagellates from the genus *Symbiodinium* were thought to be the same species, *Symbiodinium microadriaticum.* However this was challenged by a series of papers by Schoenberg & Trench (1980a-c) that highlighted physiological and behavioural evidence suggesting wider diversity. Subsequent work using molecular genetic techniques has uncovered a large amount of diversity within the *Symbiodinium* genus (Baker 2003). Exploration of the diversity within small subunit ribosomal DNA (18S-rDNA) led to the establishment of a classification of *Symbiodinium* into multiple clades i.e. *Symbiodinium* clades A, B, C (Rowan & Powers 1991), D (Carlos *et al.* 1999), G (Pochon *et al.* 2010) and H (Pochon *et al.* 2004). These clades are further divided into closely related sub-cladal "types" which are defined by ITS2 sequence (Veron 2000). Different physiological "types" of *Symbiodinium* have been shown to differ in their response to

environmental variables such as temperature (Iglesias-Prieto & Trench 1997; Kinzie *et al.* 2001; Perez *et al.* 2001). These differences have been attributed to the clade phylotypes, with clades being labelled with a specific phenotype, e.g. clade E as 'stress tolerant', clade A as 'invasive' (Rowan 1998) However, intra-clade variation has been found to be just as diverse in response environmental factors (Kinzie *et al.* 2001; Savage *et al.* 2002; La Jeunesse *et al.* 2003; Tchernov *et al.* 2004). A study by Tchernov *et al.* (2004) found that algae from different clades responded similarly to elevated temperature, whilst algae tested from within the same clade presented greater variation (Tchernov *et al.* 2004). This suggests that the diversity within *Symbiodinium* is far greater than is suggested be the cladal nomenclature.

Host-symbiont specificity also contributes to the diversity within the anthozoan-dinoflagellate symbiosis. The host-symbiont partnership can be both specific and flexible i.e. some members of the same host species generally harbour the same *Symbiodinium* type or clade (Rowan & Powers 1991), while other host species can host several types of *Symbiodinium* (Baker 2003). Within single coral colonies, multiple *Symbiodinium* types may also be found, at both a cladal (Baker 2003; Mieog *et al.* 2007) and sub-cladal level (Ulstrup & van Oppen 2003). In this situation, one or two of the present types are numerically dominant (Baker 2003). Specificity also varies between the symbiont types. Multiple studies have shown that different *Symbiodinium* strains vary in their ability to form stable relationships with different anthozoan hosts (Scheonberg & Trench 1980a; Coffroth *et al.*. 2001; Weis *et al.* 2001), or even in their ability to initially infect a host (Wood-Charlson *et al.* 2006).

1.2.1 Evolutionary origins and the establishment of anthozoan-dinoflagellate symbiosis

Exactly how the endosymbiotic situation arose between anthozoans and the once free-living dinoflagellates is uncertain; however some suggestions have been put forward. The most popular theory is that the symbiosis arose through the phagocytosis of ancestral dinoflagellate cells by an ancestral invertebrate (Schnepf 1992), sometime in the Triassic (Mucatine *et al.* 2005). For this to have evolved into a long-term symbiotic state, delayed or "retarded" digestion of these cells may have permitted some of the captured cells to continue to photosynthesise and release a portion of their photosynthetic products to the host cytoplasm (Schnepf 1992; Ruppert *et al.* 2004). This theory is supported by symbiont acquisition modes in modern coral-dinoflagellate symbioses. Here, zooxanthellae can be transmitted to juvenile anthozoans either vertically, where they are passed on from the maternal parent via the gamete, or horizontally, where algal cells are taken up from the surrounding environment by the planula larva or newly settled polyp. The most common mode of transmission is horizontal, with around 85% of symbiotic scleractinian corals acquiring zooxanthellae in this way (Babcock & Heyward 1986; Richmond 1997; Weis *et al.* 2001). This method, like the evolutionary theory of anthozoan-dinoflagellate symbiosis, involves the phagocytosis of free-living algae followed by the formation of the symbiosome and subsequent division, migration and colonisation of the host gastrovascular tissues by the dinoflagellates (Schwarz *et al.* 1999).

1.2.2 Metabolic Interactions

The anthozoan-dinoflagellate symbiosis is characterised largely by nutrient transfer. Photosynthetically fixed organic compounds (photosynthate) synthesised by the dinoflagellate symbiont are translocated to the anthozoan host. These are subsequently

used to support host metabolic processes including reproduction, growth, mucus production and, in the case of reef building (hermatypic) corals, calcification (Rinkevich 1989; Muscatine 1990; Anthony *et al*. 2002; Moya *et al.* 2006). The translocated photosynthate is comprised mostly of low molecular weight compounds such as glycerol, glucose, amino acids, organic acids and possibly lipids (Battey 1992; Markell & Trench 1993; Papina *et al.* 2003; Mayfield & Gates 2007).

The extent to which symbiont derived photosynthate contributes to the overall energy needs of the host is largely dependent on the prevailing environmental variables such as irradiance, temperature and nutrients. It therefore differs both locally, between individuals of the same species, and geographically, such as between tropical and temperate zones (Battey 1992; Hoegh-Guldberg 1999; Muller-Parker & Davy 2001). In symbioses under favourable and stable conditions, as can be found in the tropics, translocated photosynthate can satisfy greater than 100% of the carbon required for the host's metabolic needs (MuScatine 1981; Spencer-Davies 1984; Muller-Parker & Davy 2001), making the relationship critical to host survival (Trench 1979; Muscatine 1990).

In return for photosynthetic carbon, the host provides the algal symbionts with a stable source of carbon dioxide (CO_2) for use in photosythesis, and metabolic waste products including nitrogen, phosphorus and other elements. Host-supplied nitrogenous waste (mostly in the form of ammonium produced by amino acid deamination) is taken up by the dinoflagellate symbionts where it is incorporated in amino acids (Yellowlees *et al.* 2008). A portion of these dinoflagellate-synthesised amino acids are then transferred back to the host in a process referred to as 'nitrogen recycling' (Wang & Douglas 1998, Roberts *et al.* 1999, Roberts *et al.* 2001; Tanaka *et al.* 2006). In addition, 'nitrogen conservation' may occur, where the host preferentially uses photosynthetic carbon to support its metabolism rather than amino acids (Rees 1986; Wang & Douglas 1998). Both nitrogen

recycling and conservation are likely very significant processes in tropical seas, where nutrients are in short supply, though their relative importance remains unknown (Wang & Douglas 1998, Lipschultz & Cook 2002).

This exchange of carbon and nitrogen, as well as other nutrients about which far less is known, is thought to explain the success of coral reefs in the stable, oligotrophic seas of the tropics (Muscatine & Porter 1977). Conversely, the lack of coral reefs at high latitudes is thought, in part, to be due to the reduced photosynthetic performance of zooxanthellae in cooler and less stable waters (Muller-Parker & Davy 2001).

1.2.3 Ecological importance of anthozoan-dinoflagellate symbiosis

The anthozoan-algal symbiosis has significant ecological importance in the marine environment, with the most well known of these associations being with reef-forming corals (Davy & Cook 2001; Edmunds & Gates 2003). Such is the success of this symbiosis in the tropics, that reef-forming corals have become the foundation of these shallow-water ecosystems, promoting the most highly diverse and productive biological system in the marine environment (Hoegh-Guldberg 1999). This success can be mainly attributed to the environmental stability of tropical coastal environments and the ability of the coral-dinoflagellate symbiosis to recycle and transfer nutrients (see above). Indeed, some research suggests that zooxanthellae may transfer up to >98% of their photosynthetic products to meet the host's energetic requirements (Davy *et al..* 1996). However, because of the long-term stability and low productivity of tropical marine environments, the coral-dinoflagellate symbiosis has become highly specialised. This has led to a relationship that is thought to be largely mutualistic and obligate, and less reliant on exogenous supplies of food (Bruno *et al.* 2003).

Conversely, in less stable temperate environments, where the supply of photosythate is variable and the waters are more productive, cnidarian hosts have maintained a greater capacity for heterotrophic feeding to supplement them in times of low photosynthesis (Stachowicz 2001). Despite a reduced need for symbiosis at high latitudes, anthozoan-dinoflagellate symbioses (especially those involving sea anemones) are nevertheless often locally abundant and ecologically important members of coastal communities (Muller-Parker & Davy 2001).

Unfortunately, increases in anthropogenic perturbations are causing significant changes to the marine environment which are having a severe impact on these ecologically important anthozoan-dinoflagellate symbioses, especially upon the highly sensitive symbioses present at tropical latitudes (Douglas 2003). These perturbations in the form eutrophication and pollution, increased sedimentation due to erosion from disturbed terrestrial environments, over-exploitation, and physical disturbances from the likes of fishing activity, are all contributing to the degradation of coral reefs (Douglas 2003). However, of all anthropogenic perturbations, global climate change, for instance leading to increased worldwide incidences of coral bleaching, is perhaps the most significant threats facing the world's reefs today (Henrichson 1997; Hoegh-Guldberg 1999; Douglas 2003; Lesser 2007; Vidal-Dupiol *et al.* 2009).

1.2.4 Bleaching

Within the environmental conditions that cnidarian-dinoflagellate symbioses evolved, the partnerships are stable and beneficial for both partners. However, when environmental conditions become stressful, a breakdown in the relationship can occur. The mechanism by which the anthozoan-dinoflagellate symbiosis breaks down is termed "bleaching". This term is used due to the loss of colouration caused by either the partial or total loss of the

symbiont population as a result of expulsion or digestion, or degradation of the dinoflagellate's photosynthetic pigments (Douglas 2003).

Bleaching occurs in response to numerous external environmental stressors, including changes in salinity, high levels of visible and/or ultraviolet radiation, increased levels of sedimentation or nutrients, and pollutants (Coles & Brown, 2003). However, it is now well accepted that elevated sea surface temperatures (SST) associated with global warming, in combination with high solar radiation, are the cause of most widespread bleaching events (Hughes *et al.* 2003; Hoegh-Guldberg *et al.* 2007). In the case of coral–dinoflagellate symbioses, even slight increases of 1–2°C can result in large bleaching events (Hoegh-Guldberg *et al.* 2007). In 1998, the world's largest bleaching events, induced by increased SST, caused the loss of 16% of the world's reefs alone (Wilkinson 2000). Increases in the incidence and severity of coral bleaching events in recent times (Hoegh-Guldberg 1999) and the ever-present effects of global warming (Lesser 2007) reinforce the need for research in this field.

The photosynthetic health of the dinoflagellate symbiont has been identified as a key factor in the bleaching response due to its functional importance in nutrient transfer within the symbiosis (Weiss 2008). Indeed, it is widely thought that the bleaching process is initiated by the disruption of the symbiont photosynthetic mechanism directly caused by stressors associated with bleaching (Jones *et al.* 1998; Kerswell & Jones 2003; Philipp and Fabricius 2003; Saxby *et al.* 2003; Ralph *et al.* 2005). Stressors such as elevated temperature and high irradiance cause disruption to photosynthesis by damaging the chloroplast and photosynthetic mechanisms. The most vulnerable component is the D1 protein, which forms part of the water-splitting complex in photosystem II, situated within the thylakoid membranes (Weis 2008). The D1 protein is easily damaged, but under

normal conditions active repair processes keep the D1 protein functional (Ohad *et al.* 1994). At elevated temperature in *Symbiodinium*, the damage to the D1 protein is far greater than the normal repair mechanism can handle (Warner *et al.* 1999), with evidence suggesting that heat stress may even damage the repair mechanism itself (Takahashi *et al.* 2004). Heat and high light also directly damage the thylakoid membranes (Tchernov *et al.* 2004). This causes energetic uncoupling of electron transport in both photosystem I and II and a backup in excitation energy (Warner *et al.* 1999; Weis 2008).This excessive build up of electrons leads to the generation of multiple reactive oxygen species (ROS) in the symbiont chloroplast. ROS are highly reactive and can cause major cellular damage, including oxidizing membranes, denaturing proteins and damaging nucleic acids (Lesser 2006). As the concentration of ROS increases, the symbiont's antioxidant defence system, which includes enzymes such as the superoxide dismutase (SOD) and ascorbate peroxidase, becomes overwhelmed and ROS begin to accumulate (Franklin *et al.* 2004; Lesser 1997). Therefore, ROS can proceed to further damage photosynthetic membranes, as described above, in an escalating positive feedback loop (Lesser 2006). Furthermore, when ROS begin to concentrate in significant amounts, they diffuse into the host tissue where the damage continues and ultimately leads to bleaching (fig 1.1).
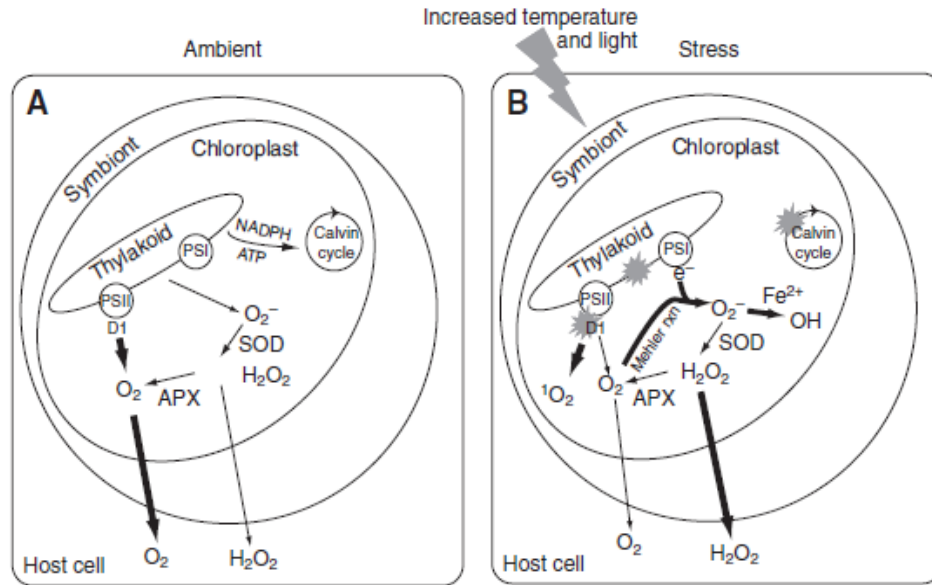
Fig. 1.1. Oxygen handling pathways in *Symbiodinium* within host cells under ambient (A), and elevated temperature and light (B) conditions. In ambient conditions, the photosynthetic apparatus, consisting of photosystem II (PSII) and photosystem I (PSI) on the thylakoid, produces large quantities of oxygen that diffuse into the host. ROS that are produced are converted back to oxygen with superoxide dismutase (SOD) and ascorbate peroxidase (APX). Under stressed conditions, damage to the photosynthetic apparatus occurs in at least three places (depicted as "flashes"): the D1 protein in PSII; in the Calvin cycle; and on the thylakoid membranes. This damage acts to generate large amounts of ROS in the form of singlet oxygen ($^1O_2$) and superoxide (O_2^-) that overwhelm the oxygen-handling pathways. O_2^- is converted to both the most highly reactive hydroxyl radical (·OH) and the more stable and highly diffusible hydrogen peroxide (H_2O_2), which can move into host tissues. Figure from Venn and colleagues (Venn *et al.* 2008).

Given the importance of the effects of increased temperature and irradiance on the bleaching response in the anthozoan-dinoflagellate symbiosis, a lot of work has focused in this direction (Iglesias-Prieto *et al.* 1992; Jones *et al.* 1998; Vidal-Dupiol *et al.* 2009). However, understanding the physiological effects of other localised stressors, such as reduced salinity from increased precipitation and runoff, is also critical for the protection of corals growing in marginal habitats (Mayfield & Gates 2007). The proceeding sections and research outlined in this thesis consider the effect of osmotic stress on the anthozoan-dinoflagellate symbiosis, and how this symbiotic partnership may work to mediate it.

1.3 The problem with salt

Very little attention has been given to understanding osmoregulation in symbiotic anthozoans and how osmotic shifts under salinity change can affect the physiology of both the host and symbiont (Mayfield & Gates 2007; Chartrand *et al.* 2009). Moreover, whether the provision of photosynthetic metabolites by the symbiont plays a role in maintaining osmotic balance within the host cell has not been studied. Indeed, if such a role exists, then it may have particular relevance to symbiosis stability under periods of photosynthetic dysfunction (e.g. thermal stress) (Mayfield & Gates 2007).

The open ocean has a stable salinity of ~35 parts per thousand (‰) (Thomas & Dieckmann 2002). However, after heavy rainfall events, salinities on shallow inshore reefs can drop as low as 14 to 10‰ (Van Woesik *et al.* 1995; Devlin *et al.* 1998). Such extreme osmotic conditions generally return to normal within hours or days due to tidal flow, however long-term exposure to osmotically stressful conditions is not uncommon. Devlin *et al.* (1998) documented decreased salinities of 28-32‰ for almost a month on the central Great Barrier Reef (GBR). Even more extreme long-term hypo-saline conditions were noted on the GBR after Tropical Cyclone Joy, where surface salinities of between 7-10‰ were recorded for 15 days on the reefs surrounding the Keppel Islands (Van Woesik *et al.* 1995). At the other end of the scale, some coral reefs and other anthozoan-dinoflagellate symbioses are exposed to elevated salinities. Coral reefs in the Middle East, such as the Persian Gulf, the northern Red Sea, the Gulf of Aquba and the Gulf of Suez are exposed to average salinities well in excess of 40‰ (Sheppard *et al.* 1992; Kleypas *et al.* 1999; Coles 2003). Such high salinities arise due to a combination of low water movement, high evaporation and lower freshwater input (Sheppard *et al.* 2009). Extensive coral communities have been found existing in salinities of up to 45‰ in the central Red Sea,

however these communities are less diverse than those seen at normal oceanic salinities, with coral diversity steeply declining between 41 and 50‰ (Sheppard 1988).

Low salinities have been shown to affect many physiological processes in marine organisms, such as intracelluar transport, feeding rates, respiration, excretion and osmoregulatory capacity (Normant & Lamprecht 2006). In symbiotic anthozoans specifically, hypo-salinity caused by intensive rainfall events and increased runoff can affect coral growth and reproduction (Flaxneld *et al.* 2010; Humphrey *et al.* 2008) and can even lead to local coral bleaching events (Coles and Jokiel 1978; Egaña & DiSalvo 1982; Van Woesik 1995; Kerswell & Jones 2003) and death (Flaxneld *et al*. 2010).

Along with increases in temperature, it has been predicted that certain areas may experience increased precipitation with increasing atmospheric CO_2 through the increased number, intensity and duration of cyclone and hurricane events (IPCC 2007, Webster *et al.* 2005). Although coral bleaching events caused by osmotic stress may be relatively minor in comparison to those caused by other stressors, especially temperature, osmotic stress can work synergistically with such stressors to multiply their effects (Flaxneld *et al.* 2010). This was demonstrated by Berkelmans & Oliver (1999), who saw that nearshore corals exposed to lower salinities experience greater degrees of bleaching during periods of increased SST than did corals that lived further offshore. This theory was substantiated by Flaxneld *et al.* (2010), who undertook multi-stress experiments on the coral *Tubinaria mesenterina*. These authors demonstrated greater decreases in respiratory and photosynthetic rates when *T. mesenterina* was exposed to both temperature and salinity stressors, than when exposed to just one of these stressors at a time; multiple stresses also resulted in higher incidences of bleaching and death of corals.

A large number of studies have examined the cellular mechanisms associated with osmotic stress in vertebrates and higher invertebrates (Harris & Aladin 1997; Oritz 2001; Normant & Lamprecht 2006,). However, there have been few studies that have examined the physiological responses of symbiotic anthozoans to this environmental variable (Downs *et al.* 2009). The few studies that have been conducted concentrated mainly on the effects of hypo-salinity on photosynthesis or the general respiratory physiology of corals (e.g. Muthiga & Szmant 1987; Alutoin *et al.* 2001; Manzello & Lirman 2003; Kershwell & Jones 2004; Chartrand *et al.* 2009). In the proceeding sub-sections, I will introduce some fundamental principles of osmoregulation before outlining what is known about osmoregulation in anthozoan-dinoflagellate symbiosis. The potential links between osmoregulation and dysfunction/bleaching will then be explained.

1.4 Osmoregulation

The cells of all organisms require a stable environment within the limit of their specific physiological constraints to achieve optimal metabolic function. Conversely, most environments have some degree of dynamic variability. To achieve a level of stability in environments of changing extracellular osmolarity, cells with semi-permeable membranes must perpetually bring intracellular and extracellular osmolarity into equilibrium. This mechanism is known as osmoregulation. However, osmotic stress can occur when a shift in the osmolarity of the extracellular environment is so great that osmoregulation surpasses basal levels and becomes energetically expensive (Hochachka & Somero 2002). High levels of osmotic stress can perturb cellular function through excessive volume and osmolyte change that can compromise both cellular structure and macromolecule function (Lang *et al.* 1998).

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In the marine environment, organisms adopt two major strategies to deal with these dynamic changes in external osmolarity: osmoregulation and osmoconformation. Marine vertebrates and higher invertebrates are largely osmoregulators. This strategy relies on maintaining the concentration of osmotically active molecules (osmolytes), both within interstitial body fluid and within cells, at a constant level within the range of optimal metabolic function, regardless of the external osmolarity (Mayfield & Gates 2007). Conversely, most marine invertebrates are osmoconformers, maintaining intracellular osmolyte concentrations in balance (i.e. iso-osmotic) with the external marine environment (Shick 1991; Kerswell & Jones 2003).

The term osmoconformer is slightly misleading as it alludes to passive free flow of water and osmolytes across the cell membrane, with little control by the cell itself. However, these organisms still dedicate a substantial amount of energy to regulate internal cellular osmolarity (Somero & Yancey 1997; Mayfield & Gates 2007). There are numerous physiological cellular mechanisms that osmoconforming cells use to mediate internal osmolarity whilst trying to maintain optimal conditions for cellular function (Oren 2002; Kultz *et al.* 2007).

Allowing water and inorganic ions to flow freely across the cell membrane is the most energetically inexpensive method that osmoconformers can use to balance internal osmolarity with the external environment. However, for anthozoans and other invertebrates, this strategy can be unfavourable despite the low energetic cost (Oren 1999; Oren 2002). This is because extreme and variable concentrations of inorganic ions, such as those found in seawater (e.g. Na^+, K^+, Cl^-), can negatively impact macromolecular and enzymatic function, causing cellular dysfunction (Lang 1998; Oren 1999). Furthermore, the uptake or loss of excessive water can have negative impacts on the

cytoskeleton and membrane structure as cells shrink or expand in response to changes in water volume (Mayfield & Gates 2007). Water availability and internal inorganic solute concentration may also negatively affect a large number of biochemical reactions if not maintained at their optima (Atkinson 1969; Mayfield & Gates 2007). For example, cellular hypotonicity caused by hypo-saline conditions can damage a number of different organelles and cellular components (Maeda & Thompson 1986; Jahnke & White 2003), with mitochondria being amongst the most susceptible. Changes in osmolarity can disrupt mitochondrial electron transport and alter NADH redox capacity, producing an increase in reactive oxygen species (ROS); this alters cellular metabolism and causes cellular damage (Ballantyne & Moon 1986; Shivakumar & Jayaraman 1986). Osmotic shifts can also cause adverse changes in endoplasmic reticulum structure and lysosomal function in plant and animal cells (Nicholson 2001). Photosynthetic organelles of organisms such as zooxanthellae can also be affected, as hypotonicity can inhibit photosynthetic electron transport and catalytic activity in the chloroplast, so reducing photosynthetic capability (Allen 1977; Lockau 1979; Asada 1999).

Due to the nature of osmosis, sudden changes in the salinity of the external medium will guarantee changes in intracellular water volume and solute concentration. This happens despite the detrimental effects that these processes may have, at least in the short term. It is when this stress is too extreme or energetically expensive for other mechanisms to re-establish a homeostatic balance that the organism can be irreparably damaged or die. In euryhaline environments, the survival of osmoconforming invertebrates depends on mechanisms that can negate these detrimental effects. The most typical mechanism amongst osmoconforming invertebrates is the adjustment of the internal water potential using solutes compatible with the structure and function of essential macromolecules. These compatible solutes effectively function in the same osmoregulatory capacity as

inorganic ions, but do not have the same negative impacts (see above) (Yancey *et al.* 1982; Blakwell & Gilmour 1991; Shick 1991; Roberts *et al.* 2001).

1.5 Compatible organic osmolytes

The first suggestion of intracellular compatible solutes being involved in osmoregulation was by Brown & Simpson (1972), who discovered that osmophilic yeasts accumulated intracellular glycerol when exposed to hypertonic conditions. Compatible solutes that are used in an osmoregulatory capacity are known as compatible organic osmolytes (COOs). COOs are organic molecules that can be synthesised or selectively taken up by most marine invertebrate cells. They fluctuate in response to external osmotic stress while causing minimal disruption to cellular function (Mayfield & Gates 2007). These compounds are ubiquitous across the animal kingdom because they are able to accumulate inside cells in significant quantities while still permitting the efficient functioning of enzymes (Brown 1990; Oren 1999; Kültz 2005). A diverse range of small molecules are known to be used as COOs, including polyols such as glycerol and arabitol, sugars and sugar derivatives (sucrose, trehalose, glucosyl), amino acids and derivatives, and quaternary amines such as glycine and betaine (Oren 1999, 2002; Yancey *et al.* 2010); this list is steadily growing. Controlling the levels of internal COO pools in response to external osmotic change reduces the need for water and salts to cross the cell membrane, ameliorating the detrimental effects that long term changes in ionic concentration and water volume incur (see above) (Oren 1999; Mayfield & Gates 2007). As such, in many organisms COOs are used as the primary osmoregulatory mechanism. For example, in the crab *Callinectes sapidus,* FAAs can account for up to 70% of the intracellular osmotic pressure in the muscle cells, nerve tissue and blood (Gerard & Giles 1972). COOS may also serve a secondary role in stabilising proteins and other cellular components against the denaturing effects of high inorganic ion concentrations (Yancey 2005). This cytoprotective

role is not fully understood, but it is thought to arise from the formation of hydration shells around proteins when COO concentrations are increased. These shells force proteins to fold up more compactly, so increasing the stability of protein folding and reducing the exposure of the peptide-bond backbone to unfavourable interactions with solutes (Kempf & Bremmer 1998; Bolen & Baskakov 2001; Yancey 2010).

Despite the benefits of using COOs, the strategy does come with drawbacks. Synthesis and maintenance of osmotically significant pools of COOs can be energetically expensive (Oren 1999, 2002). The cost of producing compatible solutes is at least an order of magnitude greater than for the use of inorganic solutes, both in terms of energy (protons absorbed per osmol generated) and water (H_2O lost per osmol generated) (Oren 1999). Because of the energetic costs, rapid changes achieved through multiple biochemical pathways can affect the metabolic rates of an organism as cellular processes are altered. Thus the rapid response of metabolic processes such as respiration and photosynthesis is critical to the survival of an organism exposed to frequent variations in environmental salinity (Hoegh-Guldberg & Smith 1989; Blackwell & Gilmore 1991; Normant & Lamprecht 2006). However, compared with inorganic ions, the regulation of COOs is a slower process, with the time taken to reach isosmotic balance ranging from hours to days (Lang *et al.* 1998).

The accumulation of COOs within a cell under hyper-osmotic stress can be achieved by increased selective uptake, synthesis, or by limiting degradation or release. It has been shown that when FAAs are readily available in the external medium, marine invertebrates can preferentially take these up (e.g. through Na^+-coupled transport) for use as COOs due to reduced energetic cost (Häussinger *et al.* 1991; Hochachka & Somero 2002).

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However, when not readily available in the external medium, control of FAA pools through protein synthesis or proteolysis (the breaking down of proteins) is necessary. One protein molecule has the same osmotic value as one Na^+ ion, however each of the hundreds of amino acids that make up a protein also have the equivalent value when they are in a free form (Mayfield & Gates 2007). Thus the breakdown or synthesis of a protein could potentially alter the osmotic value associated with that protein by up to at least three orders of magnitude. Shrinkage of vertebrate cells due to hyper-osmotic conditions has been shown to both stimulate proteolysis (Häussinger *et al.* 1991) and inhibit protein synthesis (Saransaari *et al.* 1992), so controlling intracellular FAA concentrations. Conversely, cell swelling due to hypo-osmotic conditions has been found to inhibit proteolysis and stimulate protein synthesis, thus reducing the pool of FAAs by incorporating them into proteins (Lang *et al.* 1998). However, it must not be assumed that all FAAs are equally as effective as COOs. Gerard & Giles (1972) found that the non-essential amino acids (taurine, aspartate, glutamate, proline, glycine, alanine and serine) play a more important role in osmoregulation than do the essential ones (valine, methionine, isoleucine, leucine, phenylalanine and tyrosine). Additionally, not all FAAs are even 'compatible', with arginine and lysine in particular thought to disturb cellular function (Oren 2001). The ubiquitous and dynamic nature of FAAs, along with other key COOs such as glycerol, has led to their wide use in osmoregulation across phyla (Kültz 2005). As such, they may well play an important osmoregulatory role in the anthozoan-dinoflagellate symbiosis.

1.6 Osmoregulation in anthozoan-dinoflagellate symbiosis

Anthozoans, like many marine invertebrates, maintain free amino acid (FAA) and glycerol pools for osmoregulation (Farmer & Reeve 1978; Shick 1991). Indeed, many studies suggest that FAAs may play a dominant role in anthozoan osmoregulation (Gerard & Gilles

1972; Farmer & Reeve 1978; Bowlus & Somero 1979; Law 1991; Gates *et al.* 1995; Lang *et al.* 1998).

In the absence of substantial quantities of FAAs in the external medium, cells must increase FAA concentrations by either synthesising them *de novo* or by breaking proteins down into their constituent amino acids. While it was previously believed that anthozoans could only obtain amino acids from heterotophic or autotrophic sources (Fitzgerald & Szmant 1997), more recent studies suggest that anthozoans may be able to synthesise amino acids from ammonium through the glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT) pathways (Fitzgerald & Szmant 1997; Swanson & Hoegh-Guldberg 1998; Roberts *et al.* 2001). However, the speed at which this synthesis takes place, and how the GS and GOGAT pathways respond to osmotic stress has yet to be quantified. Glycerol is another commonly utilised COO which is thought to be important in anthozoan osmoregulation. Like FAAs in non-symbiotic anthozoans, glycerol can be obtained either from the external medium or heterotrophically (Deaton & Hoffmann 1988; Mayfield & Gates 2007). However, only minimal work has looked at the use of glycerol as a COO in anthozoan osmoregulation.

Symbiotic dinoflagellates, like most unicellular photoautrophs, also use COOs in response to osmotic stress (Wegmann 1971; Stewart & Lee 1974; Measures 1975). Unlike free-living unicellular photoautotrophs, the host's intracellular medium provides a barrier from direct contact with external osmotic changes. However, large external osmotic changes may still be felt by symbiotic dinoflagellates, due the initial influx of inorganic salts or water into the host cell before the host's osmoregulatory mechanisms can be effectively initiated. These changes may be enough to cause decreases in photosynthetic health and in extreme cases can cause cellular apoptosis (Warner *et al.* 1999; Kerswell & Jones 2003;

Downs *et al.*. 2009). Studies on the osmoregulatory mechanisms of unicellular algae, however, have focused mainly on *Dunaliella* sp., a unicellular green alga that is genetically distant from the genus *Symbiodinium* (Wegmann 1971, Ben-Amotz & Avron 1973; Borowitzka & Brown 1974; Marengo *et al.* 1985; Goyal 2007). In unicellular algae, glycerol is thought to be the major contributor to the regulatory response to osmotic change. Goyal (2007) demonstrated how osmotic stress induces changes with respect to glycerol incorporation into the intracellular pool in *Dunaliella* sp. Under hyper-osmotic stress, glycerol production via either photosynthesis or the breakdown of stored starch products increases, resulting in glycerol accumulation in the cell cytoplasm. Conversely, under hypo-osmotic conditions, glycerol synthesis in *Dunaliella* sp. is reduced and existing glycerol pools diminish, either as a result of their breakdown or the incorporation of glycerol into other products; there is no evidence of glycerol release into the surrounding medium in this species. However, Blackwell & Gilmour (1991) demonstrated active transport of glycerol and other osmolytes into the external medium in the green alga *Chlorococcum submarinum* under hypo-saline conditions. These studies, though showing the general use of COOs, suggest that the precise mechanisms employed by unicellular photoautotrophs vary between species. This limits the assumptions that can be made about the mechanism(s) that zooxanthellae may employ to deal with osmotic stress.

Given that glycerol is one of the major constituents of the photosynthate translocated from symbiotic dinoflagellates to the host cytoplasm (Muscatine 1967; Lewis & Smith 1971; Battey & Patton 1984), potential exists for glycerol to be concentrated in the host's intracellular pool in an osmoregulatory capacity (Gates *et al.* 1995; Gates & Edmunds 1999). However, in anthozoan cells, glycerol is metabolised quickly, so making temporal changes in response to osmotic stress difficult to quantify (Mayfield & Gates 2007). FAAs are another compatible solute, synthesised by symbiotic dinoflagellates and transferred to

the host in substantial amounts (Gates 1995). Given the suggestion that FAAs play a dominant role in anthozoan osmoregulation (Gerard & Gilles 1972; Lang 1998), photosynthetically-derived FAAs could have considerable potential to be used by the host in an osmoregulatory role.

The physiological scenario in the anthozoan-dinoflagellate symbiosis is very different from that experienced by comparable organisms when in isolation. Hosts are not only responsible for maintaining their own osmotic balance in the face of osmotic stress, but they must also provide an osmotically stable medium for their endosymbionts. This is necessary to maintain the delicate nutritional balance upon which they depend, by providing optimal conditions for symbiont photosynthesis and photosynthate transfer (Mayfield & Gates 2007). Indeed, when considering the intracellular osmoregulatory mechanisms within the anthozoan-dinoflagellate symbiosis, looking at either organism in isolation is insufficient. Mayfield & Gates (2007) presented two theories regarding osmoregulation in the intact symbiosis: a) the dinoflagellate symbionts contribute to the osmotic balance of the host through the transfer of photosynthetically-derived compatible solutes; and b) any disruption to the transfer of these symbiont-derived solutes causes osmotic stress within the host cells as intracellular pools of COOs become depleted. Furthermore, they suggested that internal osmotic stress caused by photoinhibition is a key mechanism in the bleaching process (Fig 1.2). This internal osmotic stress has the potential to trigger any number of mechanisms that could lead to bleaching, including exocytosis and apoptosis (programmed cell death) of host cells. In addition, the internal osmotic stress experienced by the host may cause added stress to the symbiont through mitochondrial ROS production. This may cause further disruption to photosystem II and create a positive feedback loop that prompts an increased bleaching response. Before it is possible to examine the suggested roles of osmotic stress in the

bleaching mechanism of symbiotic anthozoans, the extent of the osmotic relationship between the symbiotic partners must first be examined. How and to what extent do symbiotic dinoflagellates contribute to the osmoregulatory mechanism of host anthozoans?
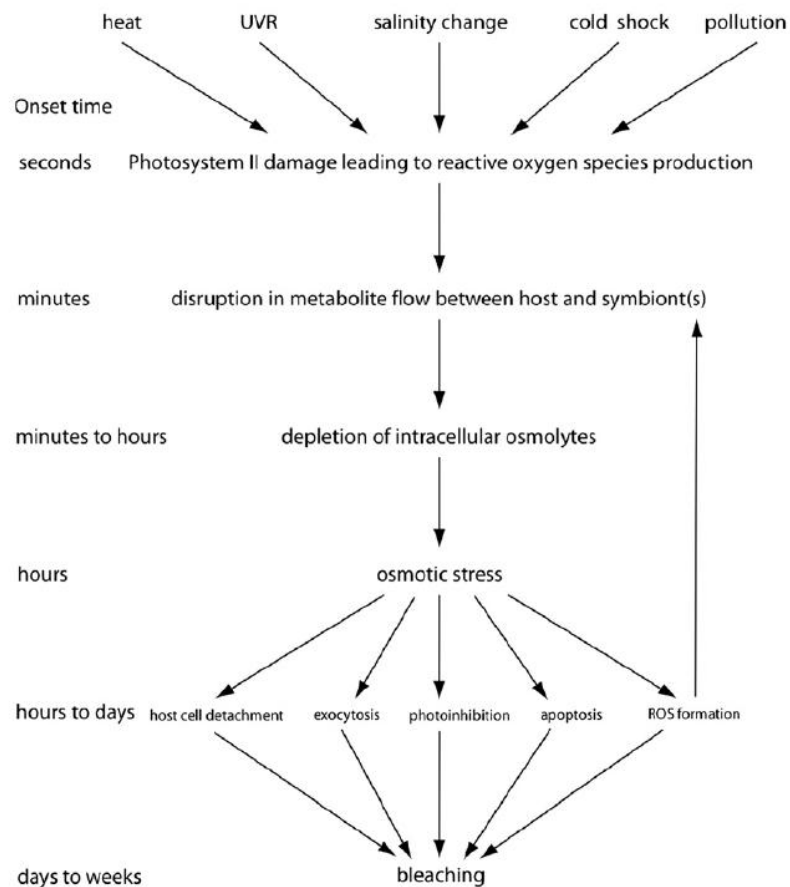
Fig 1.2: A flow chart decribing how multiple -stressors could elicit coral bleaching as a result of an osmotic stress response. The time of onset of each proposed event is on the left axis. From Mayfield and Gates (2007).

1.7 Aims and specific objectives

Given the suggestion by Mayfield & Gates (2007) that internal osmotic stress may play a key role in the bleaching mechanism, the osmotic interaction between the anthozoan and dinoflagellate partners needs to be determined. The major difficulties involved with measuring the glycerol content of anthozoan cells, as a result of the rapid metabolism of this compound, rendered it beyond the scope of this thesis. Instead, this study aimed to measure the influence of the symbiotic dinoflagellates on the FAA pools and osmoregulatory capacity of host Anthozoa, in this case a temperate sea anemone and a tropical coral.

The specific objectives were:

1) To determine if symbiotic and aposymbiotic (i.e. symbiont-free) anthozoans are equally susceptible to osmotic stress, by using respiration rate as a physiological indicator.

2) To measure changes in the intracellular FAA pools of both symbiotic and aposymbiotic anthozoans in response to osmotic stress. Hence, to establish the influence of the symbiotic state on these pools and infer any role that they might play in determining the metabolic stress responses seen in Objective 1.

3) To compare and contrast both metabolic and FAA-pool responses to osmotic stress between a temperate symbiotic sea anemone (*Anthopleura aureoradiata*) and a tropical reef-building coral (*Acropora aspera*). Hence, to establish if the responses observed are applicable to more than a single anthozoan-dinoflagellate symbiosis.

2

Methods

2.1 Experimental organisms

2.1.1 Temperate sea anemone

The sea anemone *Anthopleura aureoradiata* was used as a model temperate cnidarian-dinoflagellate symbiosis. Commonly known as the mudflat anemone, *A. aureoradiata* is small (<2 cm oral disc diameter) and found throughout New Zealand on both rocky and muddy shores. However, *A. aureoradiata* is most typically found on intertidal mudflats, where it attaches to the shells of the burrowing cockle *Austrovenus stutchburyi* (Fig. 1C&D). Anemones used in this study were found in <5 cm depth of mud in the mid-shore region of Pauatahanui Inlet, Porirua, New Zealand (Fig. 2.2). Anemones were collected at low tide, when they retract beneath the surface of the mud, by digging up cockle shells sitting just beneath the mud surface and detaching any anemones found on their shells.

2.1.1.1 Symbiotic anemones

A stock of *A. aureoradiata* was collected from the mudflat at the western end of Pauatahanui Inlet during low tide (Fig 2.2 B). Specimens were transferred to the laboratory at Victoria University where they were cleaned of all mud and detritus in 1 µm filtered seawater (FSW). Once clean, anemones were maintained in 35‰ artificial seawater (ASW) (see ASW recipe in Appendix) in 4 L glass bowls and maintained at 15°C ± 1°C in a temperature-controlled room. ASW was used to eliminate exogenous sources of free amino acids (FAA) that can be readily taken up by marine invertebrates (Gomme

2001). A 12-hour light cycle at 70-110 µmol photons $m^{-2} s^{-1}$ was provided by 16 low heat

5W LED bulbs in a parallel circuit light bank. Water was changed and bowls cleaned of any

surface detritus or algal/bacterial growth twice weekly. Anemones were acclimated under

these conditions for a period of at least three weeks before being placed under

experimental conditions. Anemones were fed with *Artemia* sp. nauplii every three days

during the acclimation period prior to experimental treatment.

2.1.1.2 Aposymbiotic anemones

Stock colonies of aposymbiotic *A. aureoradiata* were produced over a period of 6 months.

Symbiotic anemones, collected as per section 2.2.1.1, were maintained in 500ml glass

bowls (with no more than 30 individuals per bowl) in complete darkness and periodically

cold shocked over a month to invoke a bleaching response. Cold shocking was performed

twice per week by incubating anemones at 0.5-2.5°C±1°C for periods of 12 to 24 hours.

Care was taken during feeding and cleaning (as per section 2.2.1.1) to expose the

anemones to as little light as possible. The symbiotic state of the anemones was

periodically checked using fluorescence microscopy to estimate the abundance of

Symbiodinium cells within the anemone tissues. After approximately 6 months, anemones

were judged to be aposymbiotic by the lack of any notable chlorophyll auto-fluorescence

(Fig. 2.1 B).
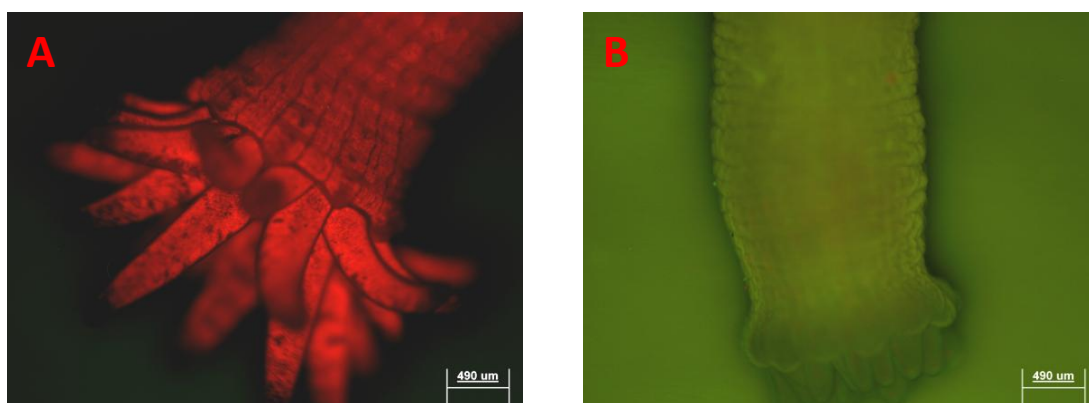
Fig 2.1: Fluorescence microscopy used to to determine the symbiotic status of *Anthopleura aureoradiata*. (A) A fully symbiotic anemone. (B) An aposymbiotic anemone showing no signs of symbiont autoflouresence after 6 months of cold shocking and maintainence in the dark. Photos by Will Arlidge

Fig 2.2: *Anthopleura aureoradiata* collection site, Pauatahanui Inlet, Pauatahanui, New Zealand. (A) Porirua Habour, south-west coast of the North Island. The harbour is made up of two inlets: Porirua Inlet (1) and Pauatahanui Inlet (2)(maps courtesy of Google Earth 5.2, 2010). (B&C) Collection site: mudflat at the western end of the Pauatahanui Inlet. (D) *A. aueroradiata* on the shell of an exposed cockle, *Austrovenus stutchburyi* (indicated by red arrows)(photos by Shyam Morar). (E) *A. aureoradiata* in the laboratory.
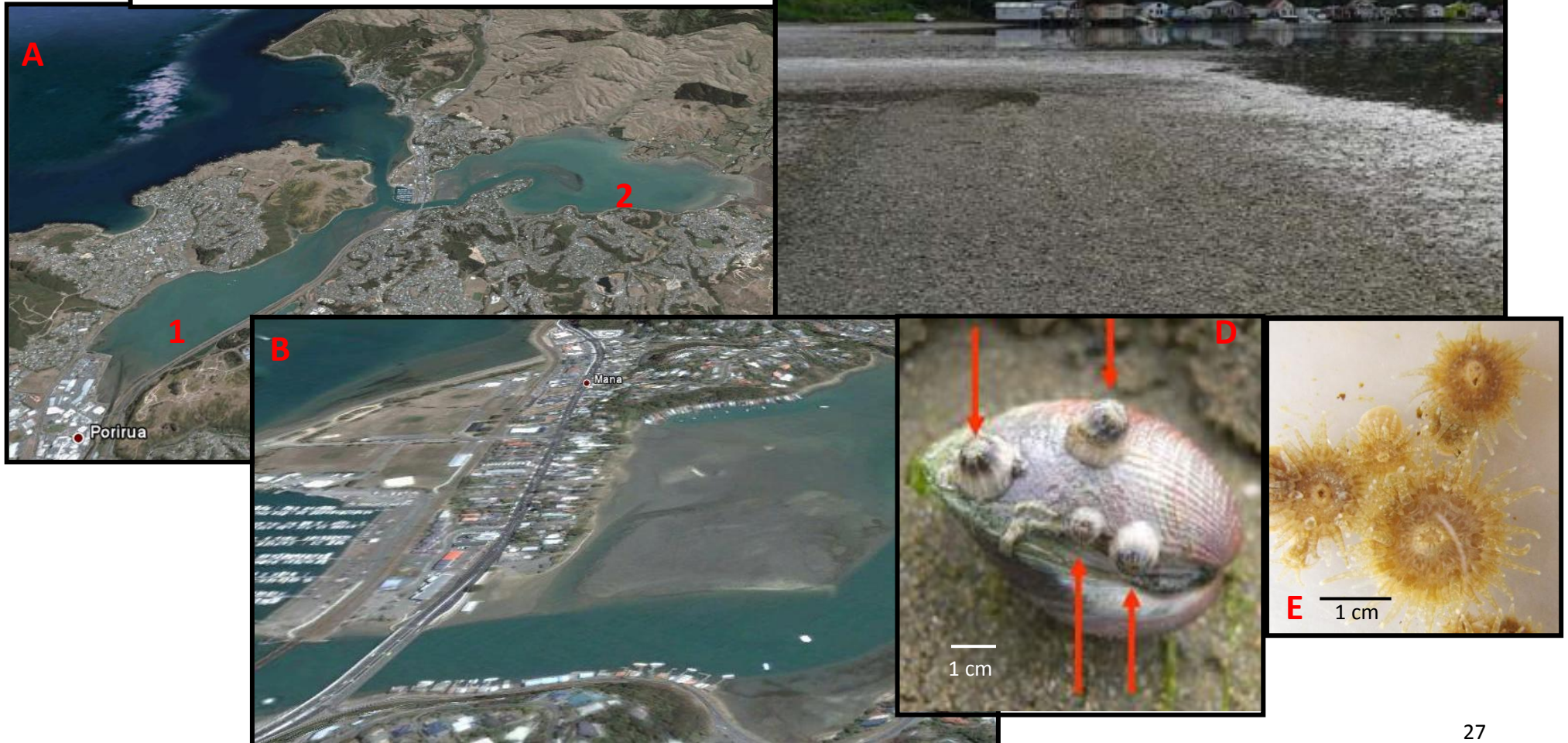
2.2.2 Tropical Coral

Coral fragments were collected from the Heron Island reef flat adjacent to the Heron Island Research Station, within the Great Barrier Marine Park Authority (GBRMPA) scientific zone SR-23-2009 (GBRMPA zoning map MPZ17-Gladstone) (Fig 2.3 C). Samples were collected at low tide, when the reef-flat and reef-crest could be accessed by wading. Eighty colonies were haphazardly chosen within the collection site. No more than two fragments were taken from a single colony to increase the chance of genetic diversity across specimens, while fragments were not collected from adjacent colonies within a 4 m radius to reduce the chance of sampling from colony clones. Coral fragments 3-4 cm in length were selected haphazardly from the upper portions of colonies, at least 25 cm below the low tide mark. Fragments were removed from branch tips using secateurs, placed into a nally bin of fresh seawater, and then transferred to the lab within an hour.

In the laboratory, coral fragments were maintained in glass aquaria, in which they were hung from PVC piping via 0.2-mm nylon monofilament lassoed around their middle (Fig. 5). Aquaria were stationed on a wet table and fed with a constant flow of seawater at a rate of approximately 9 L min^{-1} from an overhead supply pumped directly from the reef flat. A 12-h light/12-h dark cycle, with an irradiance of 90-120 µmol photons m^{-2} s^{-1}, was provided by two 58W fluorescent bulbs housed in waterproof casings that lay directly across the glass aquaria (Fig 2.4 A & B). Water temperature varied between 23 and 26 °C, subject to diurnal and tidal cycles on the reef flat. Room temperature was maintained at 24°C with a Mitsubishi air conditioner. Coral fragments were acclimated in aquaria for a minimum of two weeks before experimental use.

An exogenous source of carbon and nutrients was continuously supplied via the unfiltered flow-through water that came directly from the reef flat; this supplemented the

Fig 2.3: Coral reef study site at Heron Island on the Great Barrier Reef, Australia. (A) The Great Barrier Reef, eastern coast of northern Queensland, Australia. (B) Heron Island situated on the southern end of the Great Barrier Reef. (C) *Acropera aspera* collection site on the reef flat off the southern side of Heron Islad (All maps courtesy of Google Earth 5.2, 2010)

photosynthetic carbon derived from the coral's endosymbionts. The ready availability of particulate food in the seawater was demonstrated on occasion when the reef experienced cyanobacterial blooms; in these events, high concentrations of cyanobacteria were immediately evident within the laboratory aquaria. Aquaria were cleaned at least every three days.
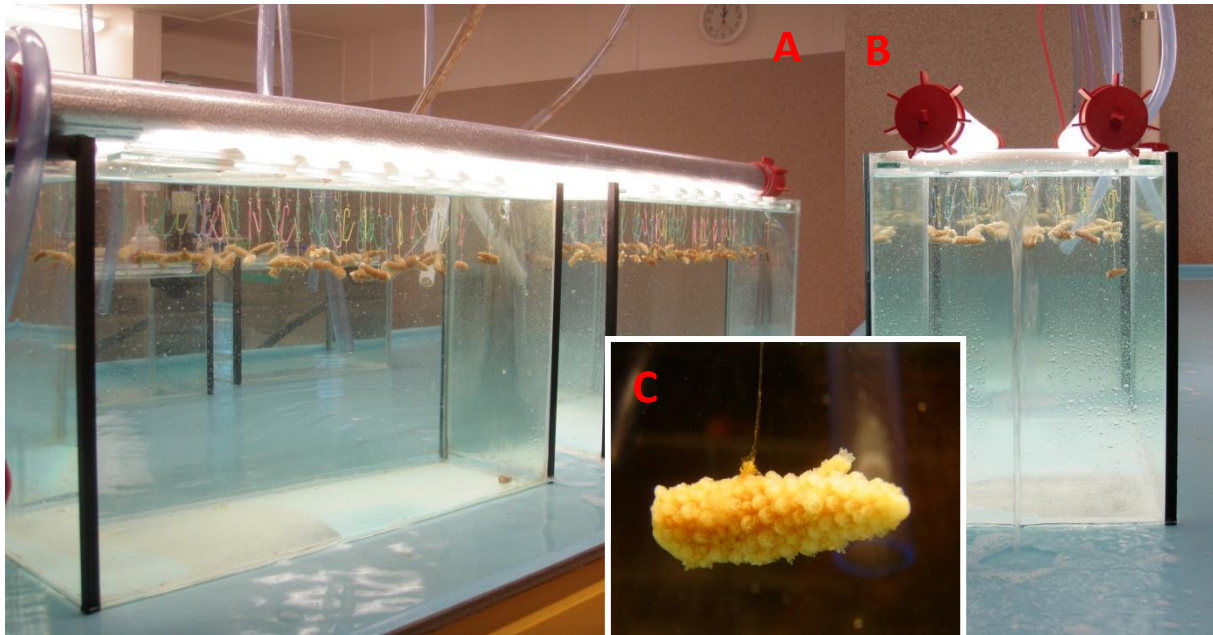
Fig 2.4: Acclimation tanks for *Acropora aspera* specimens. (A&B) Glass aquaria with fragments suspended from PVC tubing into constant flow-through seawater pumped from the reef flat. (C) Close up of suspended *A. aspera* fragment. (photos by Michael Cowlin)

2.3 Salinity exposure experiments

2.3.1 Temperate anemones

The temperate anemone *A. aureoradiata* was exposed to five different salinities for four different periods. Salinity treatments included two hypo-saline treatments of 12 and 25‰, two hyper-saline treatments of 45 and 50‰, and a control of 35‰ (±1‰ for all salinities). Extreme salinities were selected from the results of a pilot study to assess the most extreme hypo- and hyper-saline treatments that *A. aureoradiata* could be exposed to for 96 hours with less than 25% mortality (data not presented). Intermediate salinities

were arbitrarily selected to be 10‰ on either side of the control. Four different exposure times were used per salinity treatment: 1 h (initial exposure), 12 h, 48 h and 96 h (n = 4 for each salinity/time exposure combination).

Treatment salinities were prepared using artificial seawater (ASW) (see ASW recipe in appendix). ASW pH was adjusted to 8.1± 0.05 with 1 M sodium hydroxide (NaOH) solution (pH measured using Metler Toledo pH meter, calibrated with pH buffer solutions from Sigma, Germany). Experimental treatments were prepared in 1 L glass bowls in a temperature-controlled room maintained at 15°C ± 1°C. A 12-h light/12-h dark cycle at 70-110 µmol photons m^{-2} s^{-1} was provided by 16 low heat 5W LED bulbs. Treatment tanks were cleaned and the water was replaced every 24 hours during the course of the experiment.

At the commencement of the 12, 48 and 96 hour exposure times, anemones were transferred directly into their treatment salinities. Due to constraints of accurately measuring the O_2 consumption by such small anemones, each replicate 'individual' was in fact made up of three individual anemones to ensure enough biomass to register significant changes. The size of the anemones used for each replicate was kept as constant as was possible. The use of multiple individuals for each replicate was compensated for by the standardization of results to μg^{-1} protein per replicate. At the conclusion of each replicate's exposure time, anemones were transferred to a respiration chamber (section 2.4), with the exception of the one-hour treatments where anemones were maintained in the respiration chamber from the outset; this avoided excessive short-term disturbance. After one hour of settlement, respiration rates were assessed immediately.

2.3.2 Tropical Coral

Like the temperate anemones, coral fragments were exposed to five different salinities over four exposure times. Due to the lower resilience of tropical corals to environmental stress than is typical of temperate symbiotic cnidarians, the salinities to which corals were exposed were less extreme than those used with temperate anemones. Treatments used were the hypo-saline treatments of 22 and 28‰, two hyper-saline treatments of 42 and 48‰, and a control of 35‰ (all ±1‰)
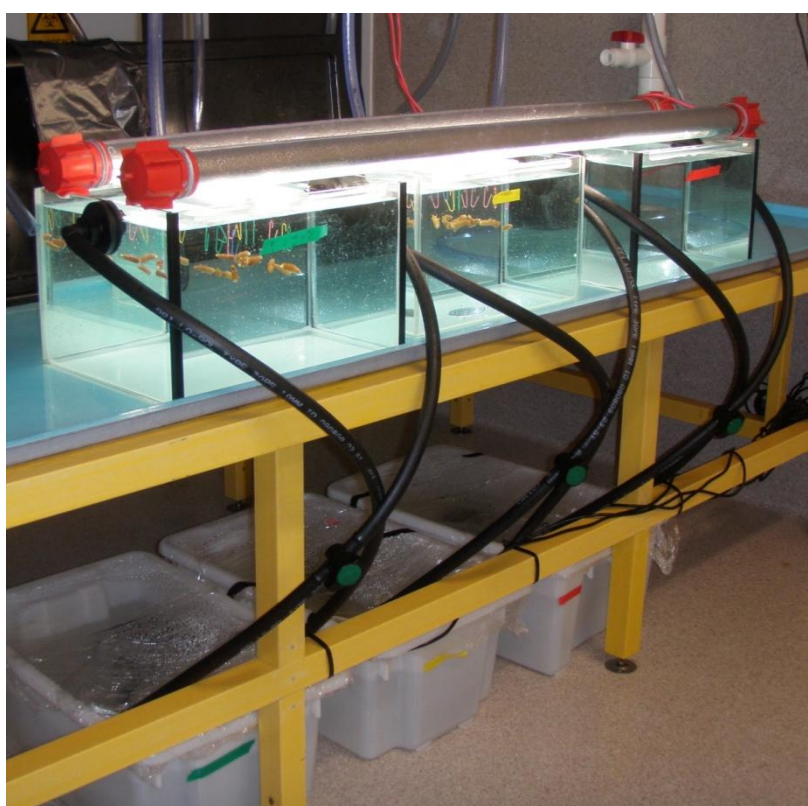
Fig 2.5: Experimental set up for *Acropora aspera* salinity treatments. 25-L glass aquaria + 50-L sumps were used to create recirculating closed water systems. *Green* = control, *yellow* = intermediate and *red* = extreme salinities

Corals require a high water flow and volume when maintained in aquaria. To achieve this, 75-L closed recirculating systems were used in the experimental set-ups (Fig 2.5). Each treatment system consisted of a 50-L nally bin sump, with 25-L glass aquaria connected by 19-mm ID black polythene inlet and outlet pipes. Water flow was provided by Pondmate PM1500P power heads (QMAX 1500 L.h^{-1}) and set to a flow rate of 3.3 L^{-1} min^{-1} (higher

flow rates caused too much disturbance in these relatively small aquaria). The sumps were located under a wet table to reduce light-induced fouling and were enclosed with cling-film to reduce evaporation. The system showed no visible signs of algal growth over the maximum treatment period of 96 hours (Cowlin pers. obs.).

Treatment tanks were situated on the same wet table as acclimation tanks (see section 2.2.2), with a room temperature at a constant 24°C ± 1°C. Treatments tanks were maintained on a 12-h light/12-h dark cycle with a light intensity of 90-120 µmol m^{-2} s^{-1}. Due to limited access to salts and distilled water on Heron Island, pure ASW salinity treatments could not be used, as they had been in the temperate anemone experiment. Instead, treatments were prepared by adding distilled water or salts (see ASW recipe in appendix) to freshly collected seawater until the desired salinity was reached. Water pH was then adjusted to pH 8.1 ± 0.05 using 10% v/v NaOH or HCl. Salinities were checked twice daily and pH 8.1 distilled water was added to compensate for any evaporation.

At the commencement of the experiment, acclimated coral fragments for the 12-, 48- and 96-hour treatments were transferred directly into the treatment tanks and hung from PVC piping (Fig 2.5). In contrast, corals in the 1-h incubations were exposed to their treatment salinity directly in the respiration chamber (see Section 2.4).

2.4 Respirometry

At the conclusion of each salinity exposure, the respiration and photosynthetic rates of the cnidarian-dinoflagellate symbiosis were measured. This was done to quantify the effects of salinity on the overall physiological health of the symbiosis.

2.4.1 Temperate Anemones

At the conclusion of the salinity treatment, anemones were placed into a 12.4-ml glass respiration chamber filled with ASW at the treatment salinity. The chamber contained an internal, raised mesh floor, under which a magnetic stir-bar was placed to ensure thorough mixing throughout the chamber during oxygen measurements. Chambers were placed on a submersible magnetic stirrer in a temperature-controlled water bath and maintained at 15°C ± 1°C. Anemones were settled for 20 minutes in the light followed by 20 minutes pre-incubation in darkness. After the settlement period, chambers were sealed with acrylic O-ringed lids and respiratory and photosynthetic rates measured. Oxygen concentration within the chamber was measured every 30 seconds via an optical mini-sensor oxygen meter (Fibox 3, Presens, Germany), with the probe inserted through the acrylic lid of the chamber.

Respiratory rates were measured in the dark, with the chamber enclosed in aluminium foil, for 30 minutes or until a constant decrease in O_2 content was observed for at least 15 minutes. Photosynthetic rates were measured by removing the aluminium foil and exposing the chamber to a photosynthetically-saturating irradiance of ~250 µmol photons $m^{-2} s^{-1}$ via a single 35w tungsten halogen bulb. Readings were concluded after a constant rate of O_2 evolution had been recorded for at least 15 minutes.

On conclusion of the O_2 flux measurements, anemones were immediately homogenised in 3 ml of 35 ‰ ASW using a 15-ml hand-held glass tissue homogeniser. This was done for 5-10 minutes, after which the homogenate was made up to 6 ml with additional ASW. Host and zooxanthellar fractions were separated by centrifugation. Homogenate was centrifuged at 1400 x g for 5 minutes, followed by 5 minutes at 2000 x g. Host tissue supernatant was pipetted off and the algal pellet was re-suspended in 3 ml ASW and

centrifuged for a further 5 minutes at 2000 x g. The remaining supernatant was pipetted off and added to the previously collected supernatant. Host supernatant was topped up to 6 ml and the algal pellet was re-suspended in a final volume of 3 ml ASW. Both fractions were frozen at -20°C until further analysis (section 2.5 & 2.6).

2.4.2 Tropical Coral

Coral respiration and photosynthesis were measured using the same overall method as that used for the temperate anemones (section 2.4.1), but with several modifications. The respiration chamber used for coral fragments was 65 ml in volume and fragments were hung from the lid of the chamber above the magnetic spin bar (Fig. 2.6 B). A temperature-controlled water bath was not used as the room was maintained at the desired temperature. For photosynthetic measurements, light was provided by an Olympus LG-PS2 fibre optic light, positioned to provide 400 μmol photons m^{-2} s^{-1} incident on the coral surface (Fig. 2.6 A). On completion of the oxygen flux measurements, tissue was removed from coral fragments using a compressed air-gun attached to a dive cylinder. The resulting tissue slurry was collected in 3 ml 35 ppt ASW and homogenised using a handheld 15-ml glass tissue homogenizer for 5-10 minutes or until no large particles could be seen. The resulting tissue homogenate was centrifuged at 1400 x g for 5 minutes directly followed by 5 minutes at 3000 x g, whereupon supernatant was pipetted off. The algal pellet was then re-suspended in 3 ml ASW and centrifuged for a further 5 mins at 3000 x g. The supernatant was pipetted off and added to the original supernatant. The animal supernatant was topped up to 6 ml with ASW and the algal pellet was suspended in a final volume of 3 ml of ASW. Two x 1 ml subsamples of both fractions were frozen at -70°C for transit back to Victoria University of Wellington.
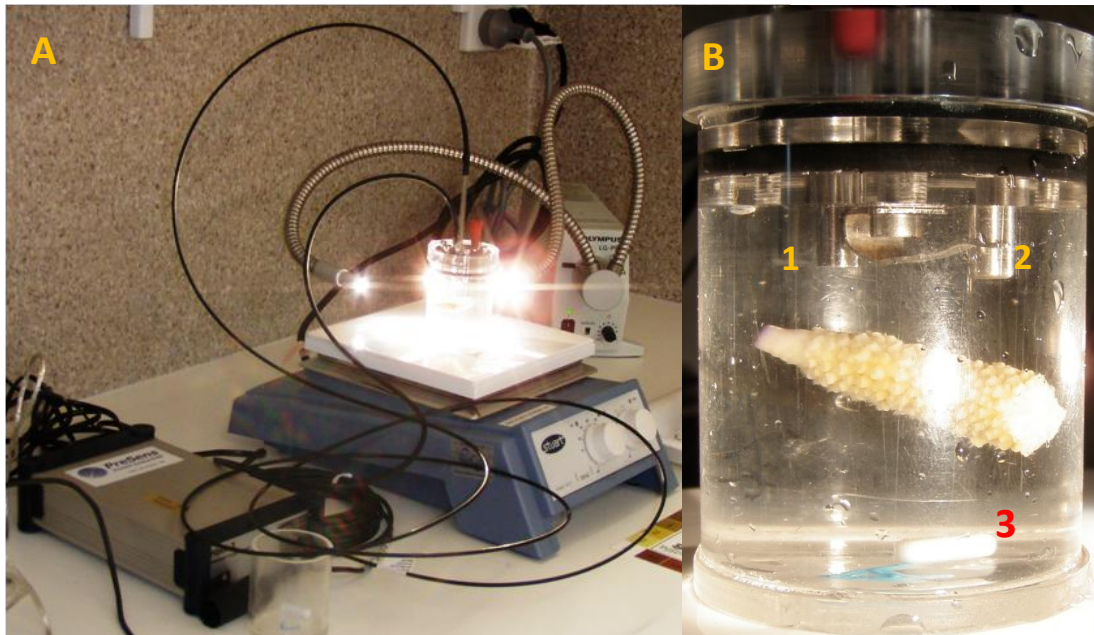
Fig 2.6: Respirometry set-up for tropical coral fragments. A. Complete set-up with oxygen minisensor, magnetic stirrer, chamber and fibre optic light. B. Respirometry chamber with suspended coral fragment; 1 = oxygen electrode; 2 = temperature sensor; 3 = magnetic spin bar.

2.5 Host protein and zooxanthella quantification

Host protein content and zooxanthellar number were used to standardise the respirometry readings for both temperate and tropical samples. Respiration rates were expressed as μg C respired μg^{-1} protein h^{-1}. Photosynthetic rates were expressed as μg C fixed 10^{-3} zooxanthellae h^{-1}. Host protein content was also used to normalize free amino acid (FAA) concentrations (see below).

Protein content was assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Host tissue homogenate (2 μl) was pipetted on to the Nanodrop and protein content was quantified using the Protein A280 method supplied with the ND 1000 software (version 3.1.0).

Quantification of zooxanthellae was determined from subsamples of the suspended zooxanthellar pellet, to normalise oxygen evolution in the light per 1000 cells. Ten cell

counts per coral fragment or sea anemone were performed using a haemocytometer (Improved Neubauer, Germany).

2.6 Free amino acid quantification using high performance liquid chromotography (HPLC)

Free amino acid content of cnidarian host tissue was assessed using High Performance Liquid Chromotography (HPLC). FAAs were derivatised using the *o*-phthalaldehyde (OPA) method (Jarret *et al.* 1985, Roberts *et al.* 2001). HPLC method, including system set up and reagents, was modified from Green *et al.* (2009) and Woodward & Henderson (2007) to suit the Ageilent 1200 series system at Victoria University (section 2.6.2). Precolumn derivitisation was carried out using a preprogrammed online autosampler injection protocol taken from Greene *et al.* (2009). The use of a pre-programmed injection protocol allowed for reduced preparation time and standard derivitisation across all samples.

2.6.1 HPLC sample preparation

A low molecular weight fraction from the host tissue supernatant was prepared using the method described in Roberts *et al.* (2001). Sub-samples (300 µl) were added to an equal volume of 5% v/v trichloroacetic acid (TCA) and left overnight at 4°C to allow proteins to precipitate. Precipitated proteins were removed from samples by centrifugation at 11,337 x g for 10 min. The low molecular weight supernatant was then extracted with an equal amount of diethyl ether to remove TCA; the resulting diethyl ether layer was removed by pipette. This was repeated three times to ensure that all TCA had been removed. One x 100 µl sample of the low molecular weight fraction was then loaded into a 0.1 ml conical glass insert, sealed in a 6 ml amber HPLC vial, and loaded into the HPLC auto-sampler at 10°C for FAA analysis.

2.6.2 HPLC system and method

2.6.2.1 HPLC System:

HPLC was performed on an Agilent Technologies 1200 Series system consisting of the following components: G1322A Degasser; G1311A Quaternary pump; G1329A Autosampler; G1316A thermostatted column compartment (TCC); G1315B diode array detector (DAD); and G1321A fluorescence detector (FLD). Agilent ChemStation software was used for system control and post-run analysis. The column used was a Phenosphere-NEXT 3μ C18, 150 x 4.6 mm, supplied by Phenomenex (USA).

2.6.2.2 FAA Standards:

Standard solutions of FAAs were obtained from Agilent Technologies (USA). Standards contained 1 nmol μl^{-1} for each of 17 primary amino acids: L-alanine, L-arginine, L-aspartic acid, L-cystine, glycine, L-histidine hydrochloride monohydrate, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyronsine & L-valine. Stock standards were diluted with double-distilled water (DDH_2O) to give five final concentrations of 1 nmol, 250 pmol, 100 pmol, 25 pmol & 10 pmol μl^{-1}. This concentration gradient was used to construct an FAA standard curve.

2.6.2.3 Reagent and buffer solutions:

Reagent: OPA/MPA reagent was prepared by adding 10 mg orthophthaldildehyde (OPA) and 10 mg 3-mercaptopropionic acid (MPA) to 1 ml borate buffer. A new solution of the OPA/MPA reagent was made fresh at the beginning of each week due to reagent degradation (Jarret *et al.*, 1986).

Buffer: Borate buffer: 0.4 M, pH 10.2 (Agilent, USA)

Eluent system:

The eluent system consisted of two mobile phases:

Mobile Phase A: 1.4 g anhydrous Na_2HPO_4 + 3.8 g $Na_2B_4O_7.10\ H_2O$ in 1 L DDH_2O giving a 10 mM phosphate and 10 mM borate solution. pH was adjusted to 8.15 with 1.5 ml of concentrated HCl. Sodium azide was also added at 8 ppm to prevent microbial growth.

Mobile Phase B: methanol:acetonitrile:water (9:9:2). HPLC grade. For additional details see table 1 and Fig 2.7.

2.6.2.4 Instrument configurations

Flow rate: 1.5 ml min^{-1}

Gradient timtable:

Time (min)	% Mobile phase A	% Mobile phase B
0.0	98.0	2.0
0.5	98.0	2.0
16.0	53.0	47.0
16.1	0.0	100.0
19.5	0.0	100.0
19.6	98.0	2.0
21.0		STOP

Table 1: Eluent system solvent gradient. The changing gradient of the solvents causes the change in retention time of derivitised amino acids
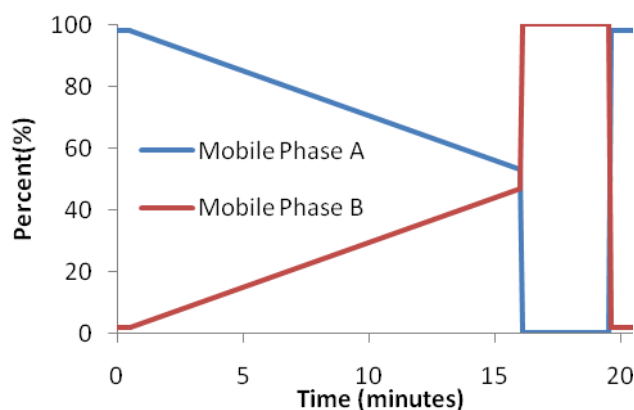
Fig. 2.7: Graph of eluent system solvent gradient shown in Table 1.

TCC: set to 40°C

DAD: PW 0.01 min; slit 4 nM;
Signal A: 338, 10 nm; Ref 390, 20 nm
Signal B: 262, 16 nm; Ref 324, 8 nm
Signal C: 338, 10 nm; Ref 390, 20 nm
Signal D: 230, 16 nm; Ref 360, 100 nm

FLD:
Ex 230 nm; Em 450 nm; Filter 295 nm

Autosampler injection protocol:

1. DRAW 2.5 μl from borate vial
2. DRAW 1.0 μl from sample vial
3. MIX 3.5 μl "in air", max speed, 5x
4. DRAW 0.0 μl from H_2O vial (needlewash)
5. DRAW 0.5 μl from OPA/MPA vial
6. MIX 4.0 μl "in air", max speed, 10x
7. DRAW 0.0 μl from water vial
8. DRAW 32 μl from injection diluent vial
9. MIX 20 μl "in air", max speed, 8x
10. INJECT
11. WAIT 0.1 min
12. VALVE BYPASS

2.7 Statistical Methods

All statistical analyses were carried out with SPSS© 17.0 for Windows (2008). The statistical methods used were employed consistently across samples for symbiotic and aposymbiotic *Anthopleura aureoradiata* and *Acropera aspera*. For each of the three variables of interest, respiration, photosynthesis and Total FAA pools, results were separated by time, and a univariate one-way ANOVA performed to test differences between salinities at each exposure period. A further breakdown of the differences found in the ANOVA tests was performed using post hoc Tukey tests to establish which salinities were significantly different from the control group. Univariate one-way ANOVAs separating results by salinity were also used to establish any significant changes occurring *within* each salinity treatment across exposure periods. Multivariate one-way ANOVAs were used to ascertain changes in the 15 individual free amino acids. Again results were separated by exposure time, with differences between salinities tested at each exposure time. Differences between salinity treatments and control groups were again established using post hoc Tukey tests.

To establish significance differences between aposymbiotic and symbiotic *A. aureoradiata,* comparisons between the respiration rate and total FAA pools were undertaken using a univariate one-way ANOVA between each corresponding salinity/exposure time treatment.

3

Results

3.1. Symbiotic anemones &corals

3.1.1. Respiration rates

Respiration rates of anemones and coral fragments were measured to assess the metabolic response to osmotic stress.

3.1.1.1. Anthopleura aureoradiata

Under control conditions (35‰),the respiration rate of *A. aureoradiata* averaged 0.37 ± 0.007 µg C µg^{-1} protein h^{-1}and did not vary significantly with exposure time (1, 12, 48 and 96 hours). The respiration rate of *A. aureoradiata* decreased with increases in osmotic stress (Fig. 3.1).

In the hypo-saline treatments, 1-hour exposure to a salinity of 12‰ resulted in a significant, 67% decrease in the respiration rate (Tukey test *post hoc* one-way ANOVA, $p<0.01$). However, the respiration rates of anemones exposed to 12‰ for 12 and 96 hours were not statistically different from the control rates (Tukey test *post hoc* one-way ANOVA, $p>0.05$). In comparison, after 1-hour exposure to a salinity of 25‰, a 20% decrease in the respiration rate was seen, further decreasing to 39% below control rates after 96 hours exposure; however, these decreases were not significant (Tukey test *post hoc* one-way ANOVA, $p>0.05$).

In the hyper-saline treatments, exposure to a salinity of 43‰ did not elicit any major changes in respiration until ≥48 hours, when rates were reduced by 36% at 48 hours and 39% at 96 hours. At a salinity of 50‰, the respiration rate was 48% lower than control rates at 1 hour, and, similarly, 52% lower at 12 hours. However, none of these trends were significant due to high levels of variability in the data (Tukey test *post hoc* one-way ANOVA, $p > 0.05$).
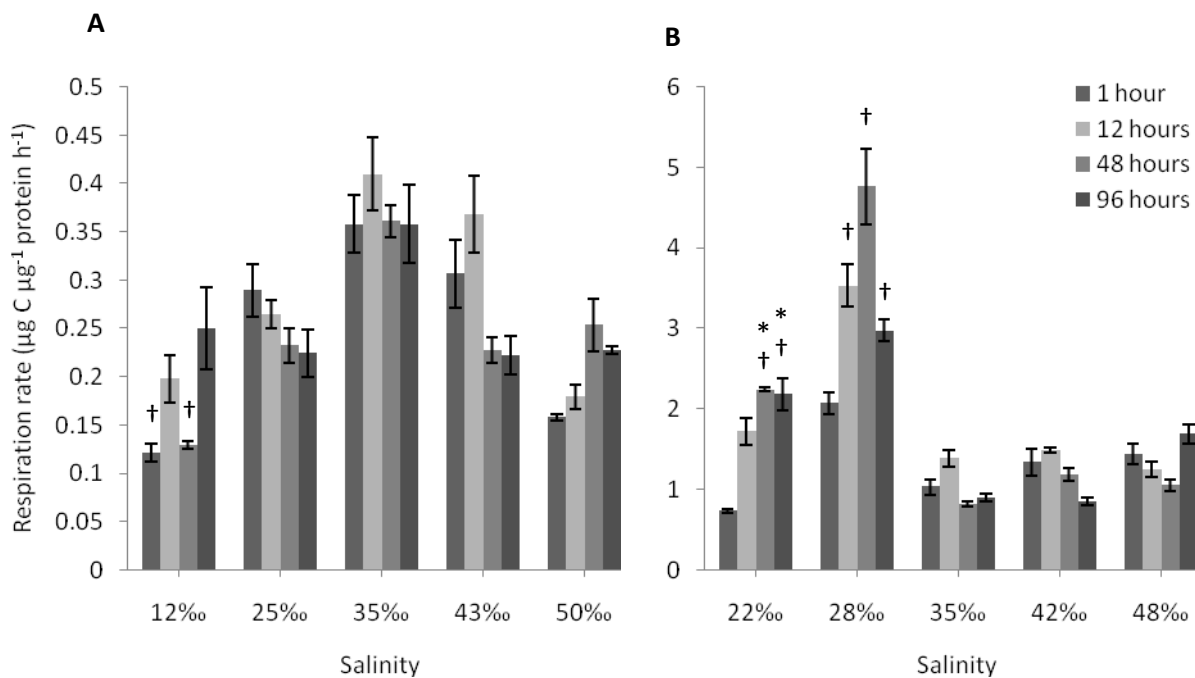
Fig. 3.1: Respiration rate (µg C µg^{-1} protein h^{-1}) for a) *Anthopleura.aureoradiata* at hypo-saline salinities of 12 and 25‰, hyper-saline salinities of 43 and 50‰, and the control salinity of 35‰. b) *Acropora aspera* at hypo-saline salinities of 22 and 28‰, hyper-saline salinities of 42 and 48‰, and the control salinity of 35‰. Both experiments were run for 1, 12, 48 and 96 hours. Values are means ± SE. n = 4 per each salinity/time treatment. † *indicates significant difference from the control at the corresponding timepoint (p<0.05), * incates significant difference from 1-hour exposure time of the same treatment.*

3.1.1.2. Acropora aspera

Acropora aspera showed significant changes in respiration rate with salinity treatment. However, contrary to *A. aureoradiata*, *A. aspera* exhibited a significant increase in respiration rate when exposed to extreme hypo-saline conditions (22‰) and an even greater increase under intermediate hypo-saline conditions (28‰). In contrast, hyper-saline conditions elicited no significant change in respiration rate, either at the

intermediate (42‰) or extreme (48‰) salinities (one-way ANOVA, p>0.05 for both treatments).

Respiration rates of *A. aspera* exposed to 22‰ steadily increased with increasing exposure time (two-way ANOVA $F_{[3,11]}$ 5.687, P<0.05), resulting in a 211% increase above the control rates after 96 hours (Tukey test *post hoc* one-way ANOVA, P<0.001). The response when exposed to a salinity of 28‰ was even greater: respiration increased immediately, with a greater than 200% increase after 1 hour exposure. After 48 hours, the respiration rate increased to 460% greater than the control rate, while at 96 hours the respiration rate decreased by 37% but was still significantly higher than the respiration rate after 1 hour exposure (Tukey test *post hoc* one-way ANOVA, P<0.05). The respiration rates in both hyper-saline treatments were not significantly different from the rates in the control treatment (at the corresponding time points), or within treatments over time.

When compared, the respiration rate of *A. aspera* at the control salinity was, on average, about 2.8 times higher than the respiration rate of *A. aureoradiata* at this same salinity.

3.1.2 Photosynthesis

Photosynthetic rates of both *A. aureoradiata* and *A. aspera* were measured to assess the impact of osmotic stress on the photosynthetic productivity of their symbiotic dinoflagellates.

3.1.2.1. Anthopleura aureoradiata

In *A. aureoradiata*, photosynthetic rate varied significantly with salinity (two-way ANOVA, $F_{[4,79]}$ 13.945, P<0.001). A general trend of decreased photosynthesis with increasing osmotic stress was seen in both hypo- and hyper-osmotic treatments (fig.3.2).
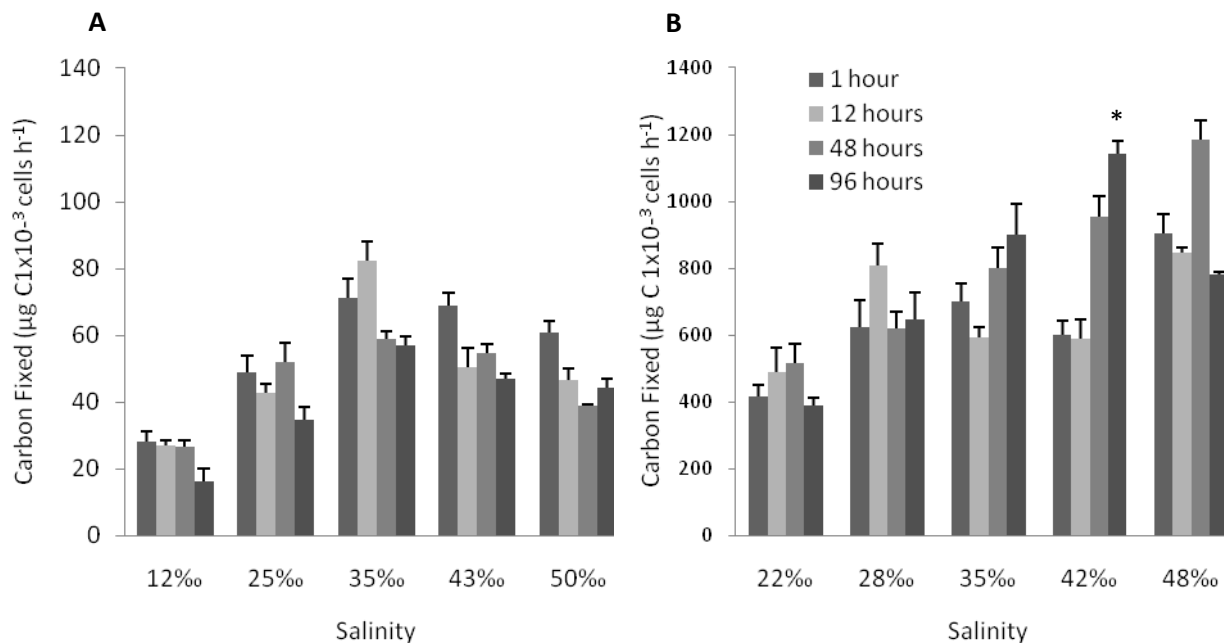
Fig 3.2: Average photosynthetic rate (µg C 1×10^{-3} cells h^{-1}) for a) *Anthopleura aureoradiata* at hypo-saline salinities of 12 and 25‰, hyper-saline salinities of 43 and 50‰, and the control salinity of 35‰. b) *Acropora aspera* at hypo-saline salinities of 22 and 28‰, hyper-saline salinities of 42 and 48‰, and the control salinity of 35‰. Both experiments were run for 1, 12, 48 and 96 hours. Values are means ± SE. n = 4 per each salinity/time treatment. † *indicates significant difference from the control at the corresponding timepoint (p<0.05), * incates significant difference from 1-hour exposure time of the same treatment.*

At 12‰, photosynthesis showed a sharp, 61% decrease after just one hour of exposure, with a further decrease with increasing exposure time (though these temporal decreases were not quite significant, Tukey test *post hoc* one-way ANOVA, P>0.05 for all treatments). Exposure to a salinity of 12‰ for 96 hours resulted in the photosynthetic rate being 72% below control levels (Tukey test *post hoc* one-way ANOVA, P<0.001). Anemones exposed to a salinity of 25‰ exhibited a decrease in photosynthesis of around 32% after one hour of exposure, with a further decrease between 48-96 hours to 39% lower than the control; however these rates were not significantly different from the corresponding control values (Tukey test *post hoc* one-way ANOVA, P>0.05). The hyper-saline treatments of 43% and 50‰ resulted in no significant difference between the treatments and their corresponding controls, despite a large 44% decrease at the 12-hour exposure period.

3.1.2.2. Acropora aspera

The cell-specific photosynthetic rate (µg C fixed 1×10^{-3} zooxanthellae h^{-1}) of *A. aspera* at the control salinity was >10-fold greater than the photosynthetic rate of *A. aureoradiata* at the control salinity.

An overall trend of decreased photosynthesis in hypo-saline conditions and increased photosynthesis in hyper-saline conditions was seen (fig. 3.2b). However, this did not translate into significant changes from the control treatment at any of the individual time-points (Tukey test *post hoc* one-way ANOVA p>0.05). Within treatments only coral exposed to 42‰ showed a significant change in photosynthetic rate between exposure times (two-way ANOVA, $F_{[3,15]}$ 7.939, P<0.005), with an increase of 190% between 1 and 96 hours.

3.1.3 Free amino acid pools

3.1.3.1. Anthopleura aureoradiata

Total FAA pools:

In *A. aureoradiata*, total free amino acid pools showed significant changes with salinity. Overall there were pronounced decreases in the concentration of total FAAs at both the hypo-saline treatment of 12‰ and the hyper-saline treatment of 50‰. After just 1-hour of exposure to a salinity of 12‰, the total FAA concentration decreased by more than 50% (Tukey test *post hoc* one-way ANOVA, P<0.001). The total FAA concentration remained at similarly depressed levels (34-57% below controls; Tukey test *post hoc* one-way ANOVA, P<0.005 for all treatments) for the remainder of the experiment. Similarly, exposure to a salinity of 50‰ resulted in a 53% decrease in the total FAA concentration after just 1 hour (Tukey test *post hoc* one-way ANOVA, P<0.001), and remained significantly lower throughout all other exposure periods (Tukey test *post hoc*
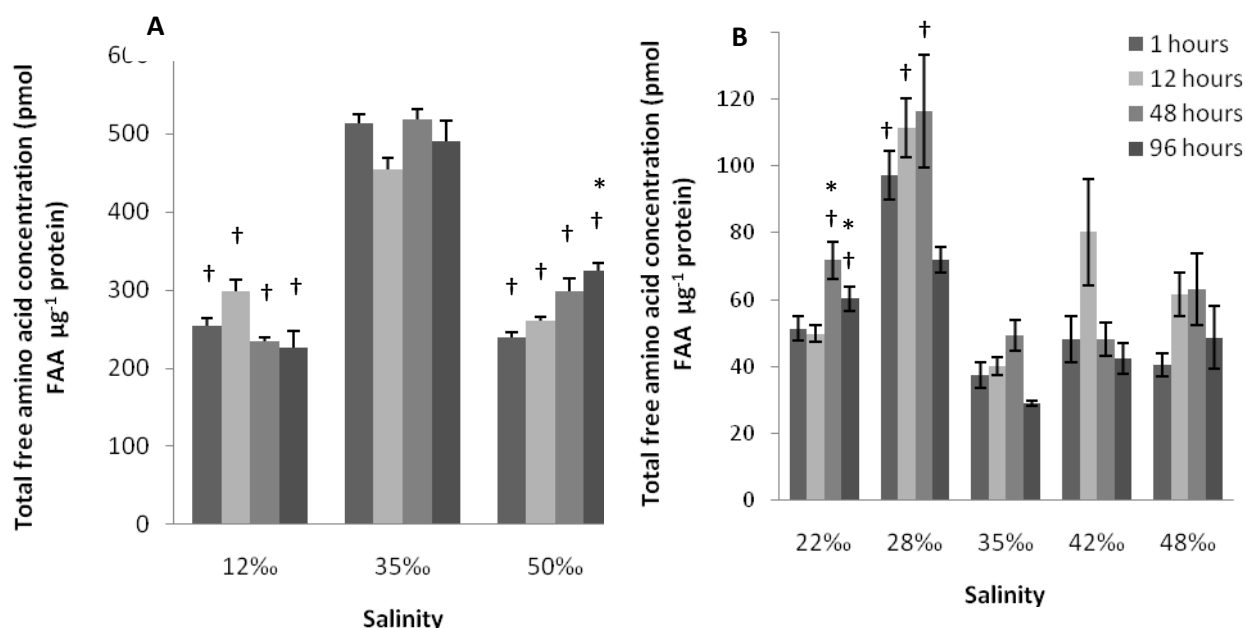
Fig 3.3: FAA pool concentrations (pmol FAA . μg^{-1} protein) for a) *Anthopleura aureoradiata* at hypo-saline salinities of 12 and 25‰, hyper-saline salinities of 43 and 50‰, and a control salinity of 35‰. b) *Acropora aspera* at hypo-saline salinities of 22 and 28‰, hyper-saline salinities of 42 and 48‰, and a control salinity of 35‰. Both experiments were run for 96 hours. Values are means ± SE. n = 4 per each salinity/time treatment. † *indicates significant difference from the control at the corresponding timepoint (p<0.05), * incates significant difference from 1-hour exposure time of the same treatment.*

one-way ANOVA, P<0.05). However the slight recovery in the total FAA pools with time was significant (two-way ANOVA $F_{[3,16]}$ 3.567, P<0.05).

Of the individual amino acids, arginine, alanine and taurine were found in the highest concentrations, comprising 16%, 15% and 15%, respectively, of the total FAA pool under control conditions. Histidine, valine and cytosine were the least abundant, comprising 1.5%, 0.5% and 0.35%, respectively of the total FAA pool. Eleven of the 15 individual amino acids examined in this study exhibited similar decreases in concentration with exposure to both hypo-and hyper-saline treatments, reflecting the overall decreases in the total FAA pool seen in these treatments. These were aspartate, glutamate, serine, histidine, glycine, threonine, alanine, tyrosine, cystine, valine and isoleucine. The remaining amino acids, arginine, taurine, methionine and phenylalanine, showed varying responses to both treatments and exposure time.

Individual FAAs of interest:

A number of the individual FAAs showed significant responses to a change in salinity. The significant responses (Tukey test *post hoc* one-way ANOVA, P<0.05) of these FAAs will be described in detail here; non-significant responses (P>0.05) are not described.

After 1 hour at a salinity of 12‰, glutamate showed an 84% decrease in concentration. However, the concentration slowly increased with increasing exposure time, with concentrations that were 36% lower than the control after 96 hours (Tukey test *post hoc* one-way ANOVA, P<0.001). After 96 hours at 12‰, glutamate accounted for 6.6% of the total FAA pool. Glutamate concentration also declined with exposure to 50‰, when it was 80-85% lower than the control at all time points (Tukey test *post hoc* one-way ANOVA, P<0.005 in all cases).

At a salinity of 12‰, arginine levels increased significantly by 32% after 12 hours (Tukey test *post hoc* one-way ANOVA, P<0.05) exposure, though they subsequently decreased after 48-96 hours, showing no significant difference from control concentrations (Tukey test *post hoc* one-way ANOVA, P>0.05). As a result of this initial increase, arginine accounted for >35% of the total FAA pool at 12‰, compared to only 16% at the control salinity. Similarly, at a salinity of 50‰, arginine rapidly accumulated in the anemone's tissue (Tukey test *post hoc* one-way ANOVA, P<0.001), resulting in an increase of 16% after 1 hour and 83% after 96 hours (Tukey test *post hoc* one-way ANOVA, P<0.05); by this time, arginine comprised 50% of the total FAA pool.

In the hypo-saline 12‰, tyrosine showed a significant, 50% decrease after an hour of exposure (Tukey test *post hoc* one-way ANOVA, P<0.005), decreasing further to 90% below the control concentration after 48 hours. However, after 96 hours, the

concentration increased back to near-control levels (Tukey test *post hoc* one-way ANOVA, P>0.05). At a salinity of 50‰, there was an initial decrease of 67% in the concentration of tyrosine after an hour (Tukey test *post hoc* one-way ANOVA, p<0.001), though this recovered slightly to around 45-50% lower (Tukey test *post hoc* one-way ANOVA, p=0.001) at 12-96 hours exposure.

At both 12 and 50‰, cystine concentrations fell below detection levels, effectively giving a ~100% decrease, after 48 and 96 hours exposure (Tukey test *post hoc* one-way ANOVA, P<0.001). This was most likely due to the levels of cystine being only just detectable with the methods used in this study; control concentrations were already below 2.5 pmol per µl.

Valine concentrations showed decreases greater than most other FAAs at both 12 and 50‰. At 12‰, the valine concentration decreased by 92% after the first hour (Tukey test *post hoc* one-way ANOVA, P<0.001) and maintained similarly reduced concentrations over all exposure periods. This trend was repeated when exposed to a salinity of 50‰, however the concentration at 12 hours was only 79% lower than the control (Tukey test *post hoc* one-way ANOVA, P<0.001).

Taurine levels did not change in either the hypo- or hyper-saline treatments (Tukey test *post hoc* one-way ANOVA, P>0.05 for all treatments). However, of note, this led to an increase in the relative importance of taurine given that the total FAA concentration declined; taurine comprised ~25% of the FAA pool at both 12 and 50‰ versus 15% at the control salinity.
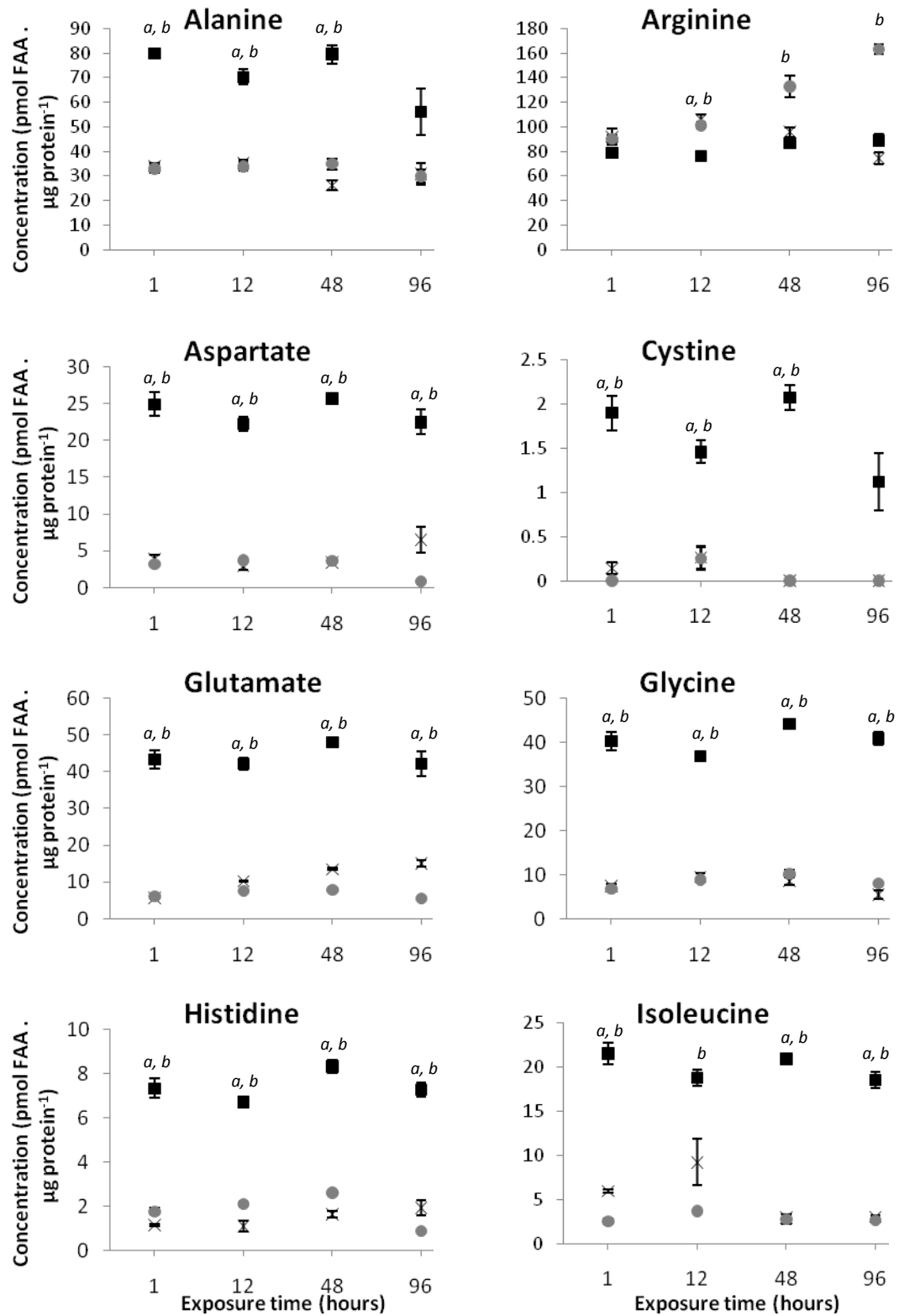
Fig 3.4: Quantities (pmol g^{-1} protein) of individual free amino acids in symbiotic *Anthopleura aureoradiata*. X = 12‰ hypo-saline treatment, ● = 50‰ hyper-saline treatment, ■ = 35‰ control salinity. Values are means ± SE. n = 4 per each salinity/time treatment. Significant variation from control (P<0.05) is indicated by *a* for 12‰ and *b* for 50‰.
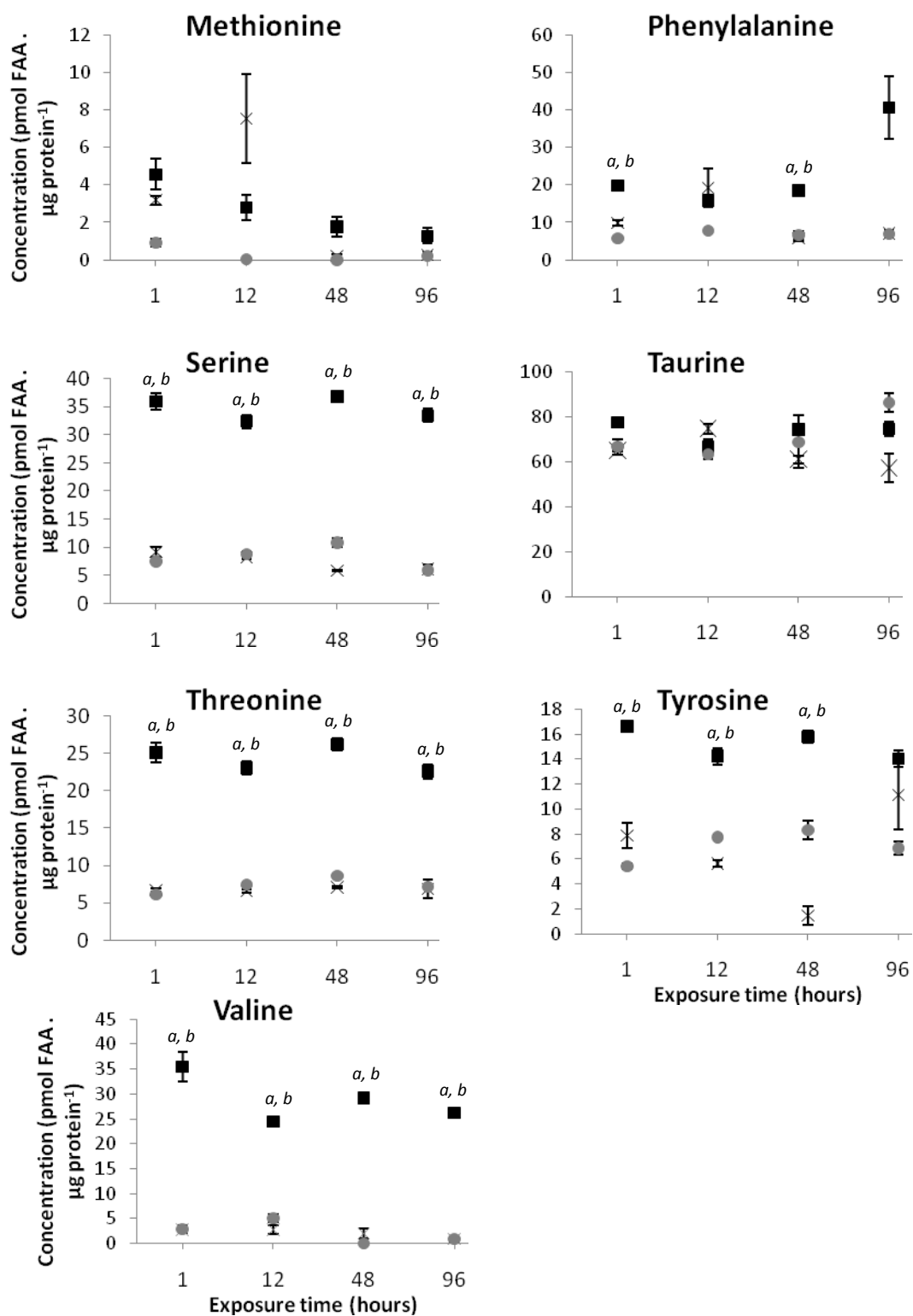
Fig 3.4: *Continued, see previous page for detail.*

3.1.3.2. Acropora aspera

In *A. aspera*, total free amino acid (FAA) pools showed significant changes with salinity (two-way ANOVA $F_{[4,76]}$ 12.251, $P<0.001$). Exposure to a salinity of 28‰ resulted in a significant increase in the concentration of the total FAA pool from that seen in the control treatment and all other treatments. After an initial one-hour exposure to 28‰, FAA pool concentrations increased to 260% those seen at the control salinity (97.17 pmol μg^{-1} protein versus 37.57 pmol μg^{-1} protein, respectively (Tukey test *post hoc* one-way ANOVA, $P<0.05$). This increased FAA concentration persisted until, at the 96-hour time-point, FAA pools decreased to a concentration of 71 pmol μg^{-1} protein; this concentration was not significantly different from the control ($P>0.05$). In contrast, total FAA pool concentrations in the other salinity treatments did not significantly differ from those in the control.

Of the 15 individual amino acids examined under control conditions, glutamate, aspartate and glycine were present at the highest concentrations, representing 28%, 20% and 15% of the total pool, respectively. Histidine, valine and cytosine were the least abundant, representing 1.5%, 0.5% and 0.35%, respectively, of the total FAA pool. Ten of the amino acids measured showed similar responses to hypo-osmotic conditions of 28‰, with aspartate, serine, histidine threonine, arginine, alanine, tyrosine, valine, methionine and isoleucine all exhibiting significant increases (Tukey test *post hoc* one-way ANOVA, $P<0.05$)(Fig 3.5). The remaining 5 amino acids examined demonstrated no significant responses to any hypo- or hyper-osmotic treatments ($P>0.05$).

Individual FAAs of interest:

Significant changes in response to hyper- or hypo-salinity were measured in ten individual FAAs. These being aspartate, serine, histidine, arginine, alanine, tyrosine, valine, methionine, isoleucine and threonine (Fig 3.5); it is possible that small changes to other FAAs were masked by a high degree of variability in the control data and the concentrations sometimes being close to the detection limits of the HPLC.

Aspartate showed dramatically increased concentrations with exposure to 28‰ of between 200 and 380% across all time points, however because of high variation between samples significance was only found after 12 hours of exposure (Tukey test *post hoc* one-way ANOVA, P=0.005).

Like the trends seen for aspartate, serine also showed large increases with time of between 280 and 370% with exposure to 28‰. However this was only significant in the 12-hour exposure treatment (Tukey test *post hoc* one-way ANOVA, P<0.01)

Histidine was only rarely detected at control levels, but was detected more frequently under hypo- and hyper-saline treatments, across all exposure times. However, due to large variability in the data, a significant increase above the control levels was only recorded after 96 hours of exposure to 28‰, (Tukey test *post hoc* one-way ANOVA, P<0.005).

Arginine was not present at detectable concentrations in all but one control sample. Exposure to 28% resulted in increased concentrations of arginine or between 4.5 to 13.3 pmol μg^{-1} protein, however as this FAA remained undetected in some samples, significant

increases were seen only in the 96-hour treatments (Tukey test *post hoc* one-way ANOVA, P=0.025).

The concentrations of alanine, like other FAAs seen here, also showed a dramatic increase of up to 6.6 times control concentrations across all exposure periods, but was only significantly higher at 12 and 48 hours (Tukey test *post hoc* one-way ANOVA, P<0.005 and 0.05 respectively).

Like arginine, tyrosine was not present at detectable concentrations in all but one control sample. Exposure to 28% resulted in increased concentrations of arginine, averaging between 1.83 to 4.37 pmol μg^{-1} protein. These increases were only significant at the one hour time point (Tukey test *post hoc* one-way ANOVA, P<0.001)

Valine concentrations only showed significant changes after 1-hour exposure to 28‰ (Tukey test *post hoc* one-way ANOVA, P<0.005), with all longer exposure periods showing levels similar to control concentrations (Tukey test *post hoc* one-way ANOVA, P>0.005).

Methionine concentrations followed suit with the other FAA changes seen here, with significant changes only seen with exposure to 28‰. Increases of 292% and 330% were seen after one hour (Tukey test *post hoc* one-way ANOVA, P<0.005) and 48 hours (Tukey test *post hoc* one-way ANOVA, P<0.05).

Isoleucine was detected only rarely under control conditions. However, exposure to a salinity of 22‰ for 1 hour elicited a significant increase in the concentration of isoleucine (Tukey test *post hoc* one-way ANOVA, P<0.01); this concentration dropped back to just above the lower detection limits at the subsequent time-points.

Threonine concentrations increased to between 2.5 and 11.5 times the control concentrations under both hypo-saline treatments across all time exposures. However, the only significant difference seen was a 430% increase after an hour exposure to 28‰ (Tukey test *post hoc* one-way ANOVA, P=0.038).

Another amino acid worthy of note was Phenylalanine. This amino acid showed the opposite trend of decreasing its concentration by 50% to 100% (lower than detectable levels) at both 22‰ and 28‰. However, these trends were not significant.

Of particular interest was the complete lack of the amino acid taurine in *A. aspera*, in marked contrast to the high concentrations of taurine measured in *A. aureoradiata*.
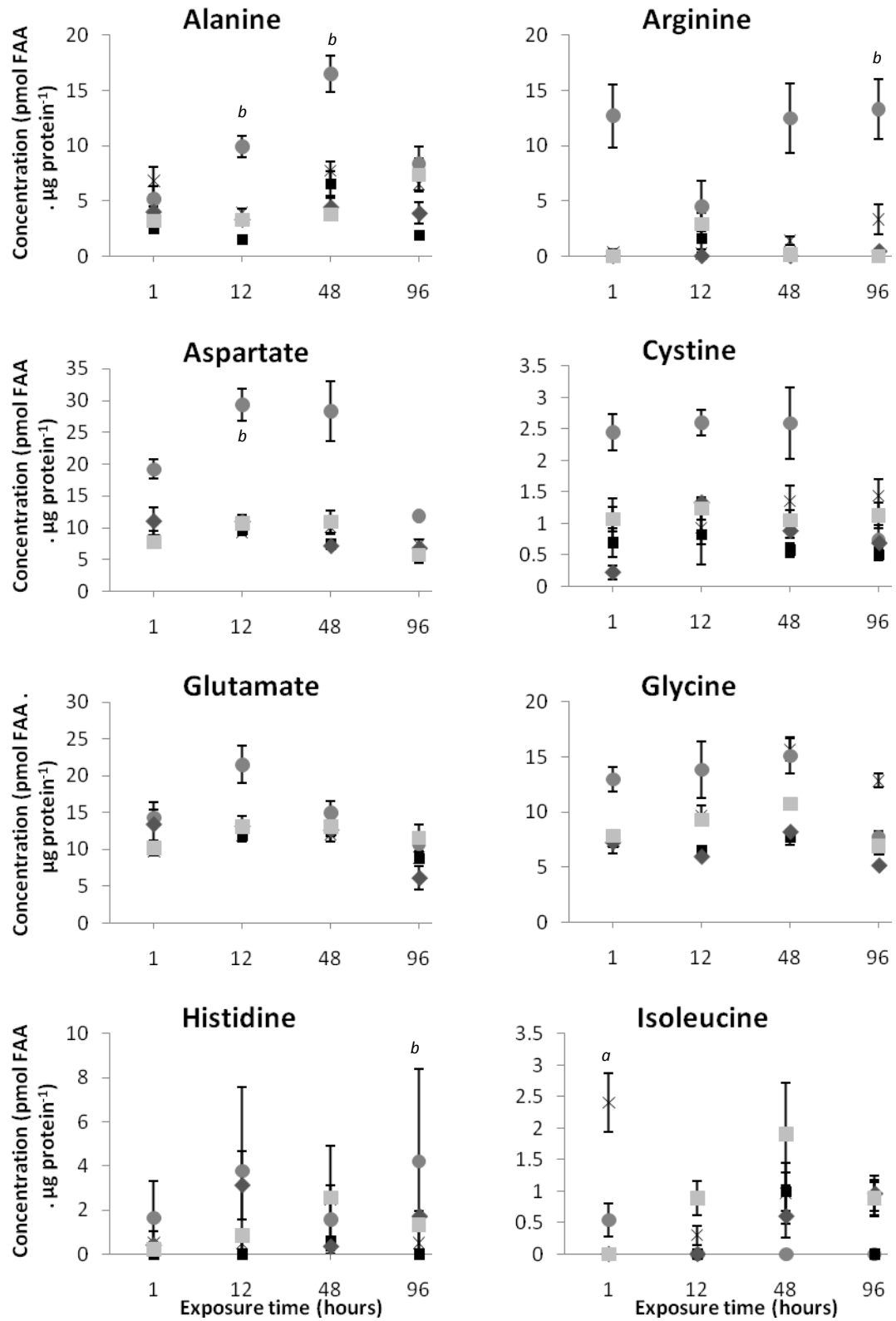
Fig 3.5: Quantities (pmol g^{-1} protein) of individual free amino acids in symbiotic *Acropora aspera*. X = 22‰ hypo-saline treatment, ● = 28‰ hypo-saline treatment, ◆ = 42‰ hyper-saline treatment, ■ = 48‰ hyper-saline treatment, ■ = 35‰ control salinity. Values are means ± SE. n = 4 per each salinity/time treatment. Significant variation from control is indicated by † P<0.05.
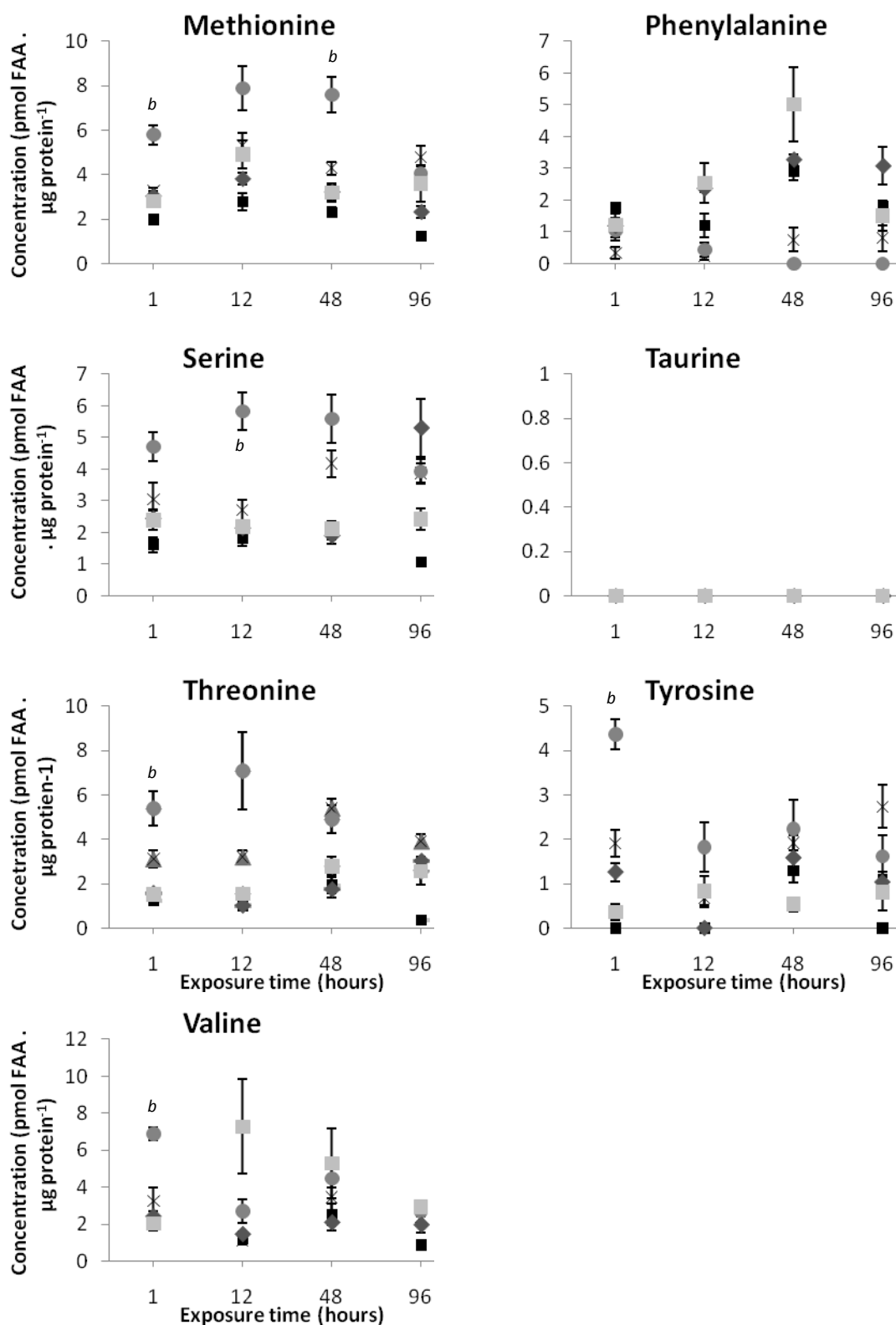
Fig 3.5: *Continued, see previous page for detail.*

3.2. Aposymbiotic vs. symbiotic Anthopleura aureoradiata

Aposymbiotic individuals of *A. aureoradiata* were subjected to identical salinity treatments as were the symbiotic anemones described above. This was done to elucidate whether the presence of symbiotic dinoflagellates had an effect on the anthozoan host's response to osmotic stress.

3.2.1. Respiration rates

3.2.1.1 Aposymbiotic anemones

The respiration rate in aposymbiotic anemones was only significantly impacted when the anemones were exposed to the hypo-saline treatment of 12‰. In this treatment, the respiration rate after an hour was similar to that in the control, but with increasing exposure time a steady decrease in respiration was observed (fig 3.6). After 96 hours at 12‰, respiration was 77% lower than the control rates (Tukey test *post hoc* one-way ANOVA, P<0.005).

3.2.1.2 Aposymbiotic vs. symbiotic anemones

Overall, the respiration rate of aposymbiotic *A. aureoradiata* under control conditions was 45% lower than in symbiotic *A. aureoradiata* (two-way ANOVA, $F_{[1,31]}$ 21.40, P<0.001). There were no significant differences between the respiration rates of the symbiotic and aposymbiotic anemones in the either the hypo- or hyper-saline treatments (two-way ANOVA, $F_{[1,31]}$ 21.40, P=0.198 and P=0.098 respectively).
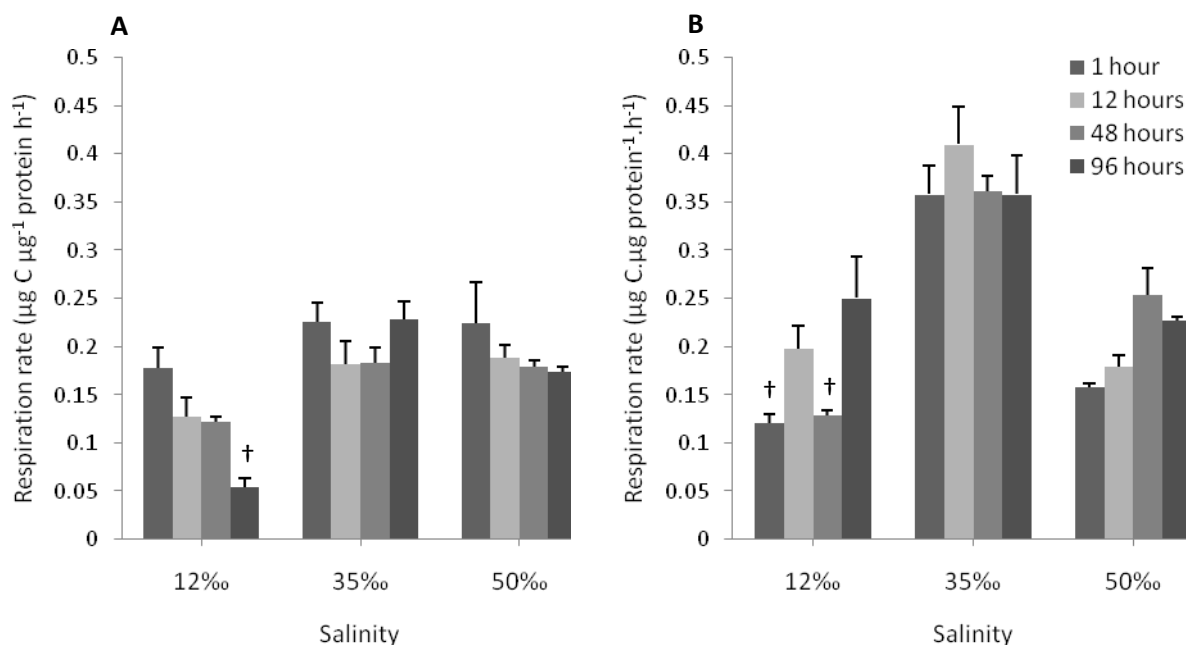
Fig 3.6: Respiration rates (µg C µg^{-1} protein h^{-1}) of a) aposymbiotic and b) symbiotic individuals of *Anthopleura aureoradiata* at hypo-saline treatment salinities of 12 and 25‰, hyper-saline treatments of 43 and 50‰, and the control salinity of 35‰ for 1, 12, 48 and 96 hours . Values are means ± SE. n = 4 per each salinity/time treatment. † *indicates significant difference from the control at the corresponding timepoint (p<0.05).*

3.2.2 Free amino acid pools

No significant changes from control concentrations of total FAAs were seen when aposymbiotic *A. aureoradiata* were exposed to 12‰. However significant changes in FAA concentrations were seen over time in the 12‰ treatment, with significantly lower total FAA pool concentrations at 48 and 96 hours than at 12 hours (Tukey test *post hoc* one-way ANOVA, P<0.05 and P<0.005, respectively). In contrast, the concentration of total FAAs did not change with time at salinities of 35‰ or 50‰. The only significant difference seen between the control and treatment salinities was at 12 hours exposure to 50‰ (Tukey test *post hoc* one-way ANOVA, P<0.5).

Under control conditions, the FAA pools of aposymbiotic *A. aureoradiata* were up to 41% lower than the FAA pools of symbiotic *A. aureoradiata,* with concentrations in the control treatment being significantly different at 48 and 96 hours (one-way ANOVA, P<0.005 and P<0.01 respectively). Comparison of FAA pools under treatment salinities revealed an

opposite trend. At 12‰, exposure periods of 1, 12, and 48 hours were significantly higher than symbiotic anemones exposed for 96 hours (One-way ANOVA, P<0.05, P<0.05 and P<0.001 respectively). A difference between the 50‰ treatments was only seen at 12 hours with symbiotic FAA pool concentrations being 26% lower than the pool concentrations in aposymbiotic anemones (One-way ANOVA, P<0.05)
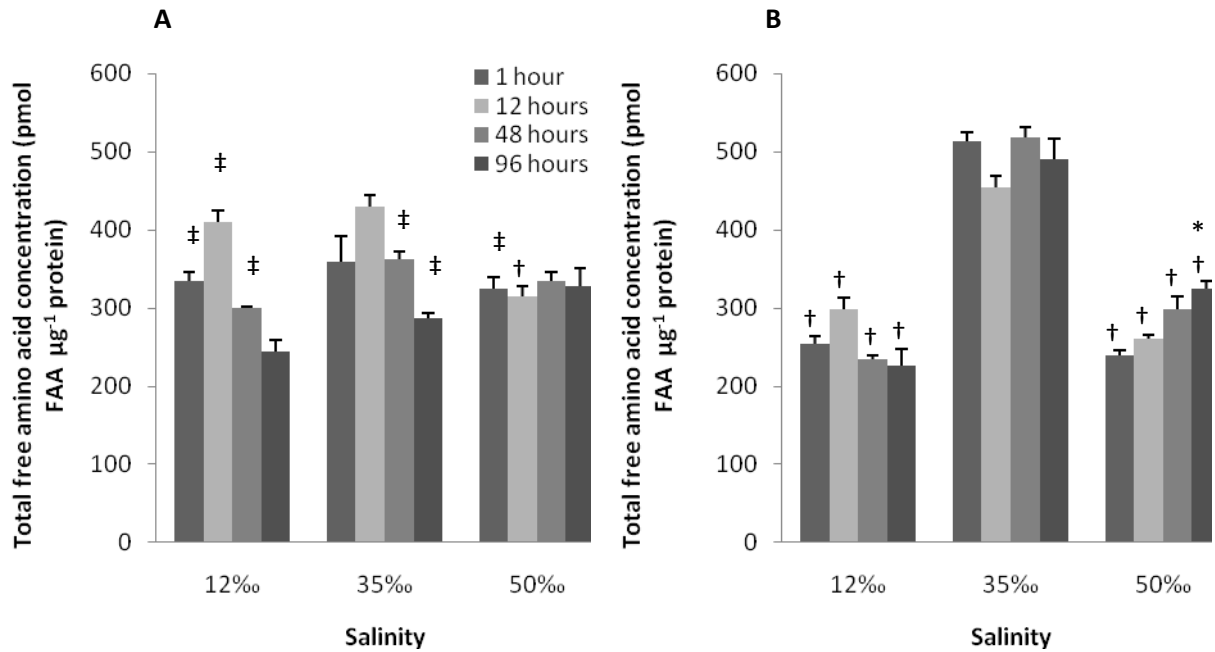
Fig 3.7: FAA pool concentrations (pmol FAA μg^{-1} protein) for a) aposymbiotic and b) symbiotic *Anthopleura aureoradiata* at treatment salinities of 12 and 50‰, and the control salinity of 35‰. Both experiments were run for 1, 12, 48 and 96 hours. Values are means ± SE. n = 4 per each salinity/time treatment. † *indicates significant difference from the control at the corresponding timepoint (p<0.05), * indicates significant difference from 1-hour exposure time of the same treatment, ‡ indicates significant differnce from symbiotic treatments*

The concentration of individual FAAs within aposymbiotic *A. aureoradiata* under control conditions was highly variable. This high variability was possibly due to the smaller size of the aposymbiotic anemones compared to the symbiotic ones, which in some cases led to FAA levels being close to or below the detection limits of the HPLC. Nevertheless, aposymbiotic *A. aureoradiata* exhibited a similar FAA pool profile as did the symbiotic anemones. Taurine, alanine and arginine represented the highest proportion of the total FAA pool, contributing 23%, 21% and 19%, respectively. At the other end of the scale, methionine and histidine contributed just 1.6% and 0.8% of the total FAA pool,

respectively, while cystine was only detected on a couple of occasions and therefore was not present or was present at levels below the lower detection limit in the majority of cases. Some FAAs peaked at 12 hours in the controls, though these peaks were possibly artefacts caused by the sensitivity of the method used, as they were not apparent for the more concentrated FAAs.

Individual FAAs of interest in aposymbiotic A. aureoradiata:

A number of the individual FAAs responded to changes in salinity. The significant responses ($P<0.05$) of these FAAs will be described in detail here; non-significant responses ($P>0.05$) are not described.

Aspartate decreased in concentration when exposed to salinities of 12‰ and 50‰. At 12‰, aspartate levels decreased by 91% after 48 hours (Tukey test *post hoc* one-way ANOVA, $P<0.05$). Exposure to 50‰ only caused a significant decrease after 12 hours, when it was 77% lower than in the control (Tukey test *post hoc* one-way ANOVA, $P<0.001$), while after 96 hours aspartate levels were below detectable limits.

Glutamate only showed a significant (76%) decrease from the control after exposure to 50‰ for 12 hours (Tukey test *post hoc* one-way ANOVA, $P<0.001$). Although no significant change from control glutamate concentrations was found with exposure to 12‰, there were some significant changes between exposure periods; with concentrations at 12 hours higher than at all other exposure times at this salinity (Tukey test *post hoc* one-way ANOVA, $P<0.001$).

Serine showed reduced concentrations at both 12‰ and 50‰. However the only significant differences from the control were seen after 48 hours at 12‰, with a 48%

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decrease, and after 12 hours exposure to 50‰, with a 66% decrease (Tukey test *post hoc* one-way ANOVA, P<0.02 and P<0.001, respectively).

Histidine concentrations decreased significantly from control levels after 48 hours at both 12‰ and 50‰ (Tukey test *post hoc* one-way ANOVA, P=0.001 and P=0.005, respectively); other differences between the treatment and control salinities may have been masked by the large variation in the control samples (two-way ANOVA $F_{[3,16]}$ 16.082, P<0.001).

Glycine levels were more or less stable with time at 50‰ (Tukey test *post hoc* one-way ANOVA, P>0.05), but were significantly lower than in the control treatment at 12 hours due to the variability in the controls (Tukey test *post hoc* one-way ANOVA, P=0.001). Exposure to 12‰ for 48 hours showed a 47% decrease in glycine (Tukey test *post hoc* one-way ANOVA, P<0.05) but no other significant change at any other time point.

Threonine concentrations decreased significantly, by 76%, after 12 hours exposure to 50‰ (Tukey test *post hoc* one-way ANOVA, P<0.001).

Arginine concentrations responded to exposure to both 12‰ and 50‰ with concentrations increasing to 177% the control levels after 48 hours in both treatments (Tukey test *post hoc* one-way ANOVA, P<0.05 for both). After 96 hours exposure to 12‰, arginine concentrations declined 146% from control concentrations, and at 50‰ they increased more than 2 fold, but these changes were not significant.

The only significant change seen in the FAA alanine was a 43% decrease after 48 hours exposure to 12‰ (Tukey test *post hoc* one-way ANOVA, P<0.01).

Exposure to 12‰ caused valine concentrations to fall below detectable levels after 48 hours (Tukey test *post hoc* one-way ANOVA, P<0.05) and 96 hours (Tukey test *post hoc* one-way ANOVA, P<0.05). A similar trend was seen with exposure to 50‰, when concentrations fell below detectable levels after 12 hours (Tukey test *post hoc* one-way ANOVA, P<0.01) Large decrease were also seen after 48 and 96 hours at 50‰, but these were not significant.

There was a note worthy 80% increase in tyrosine concentrations after 12 hours exposure to 12‰, but this change was not significant due to the highly variable control group (Tukey test *post hoc* one-way ANOVA, P>0.05).
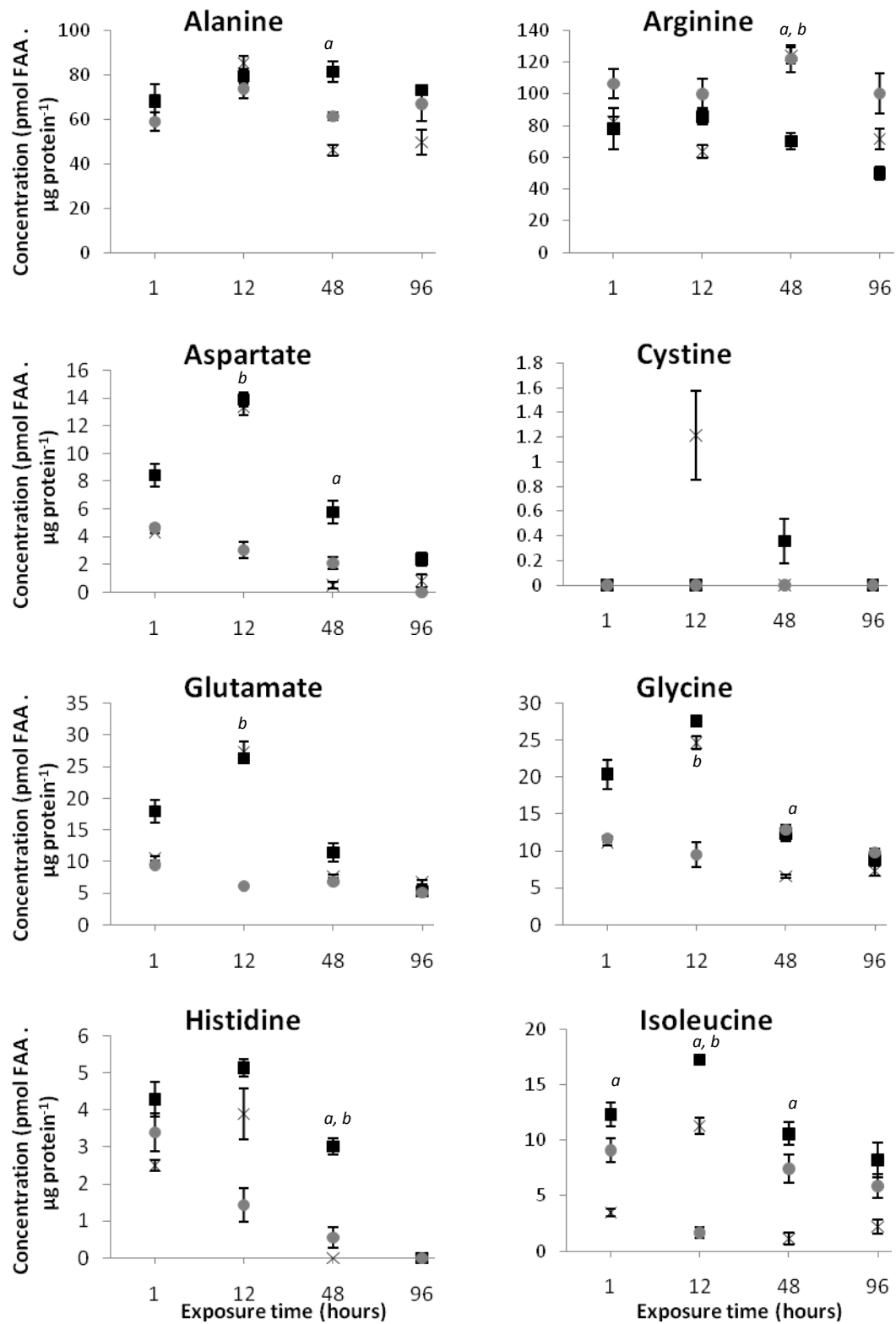
Fig 3.8: Quantities (pmol g^{-1} protien) of individual free amino acids in aposymbiotic *Anthopleura aureoradiata*. X = 12‰ hypo-saline treatment, ⬤ = 50‰ hyper-saline treatment, ⬛ = 35‰ control salinity. Values are means ± SE. n = 4 per each salinity/time treatment. Significant variation from control is indicated by † P<0.05.
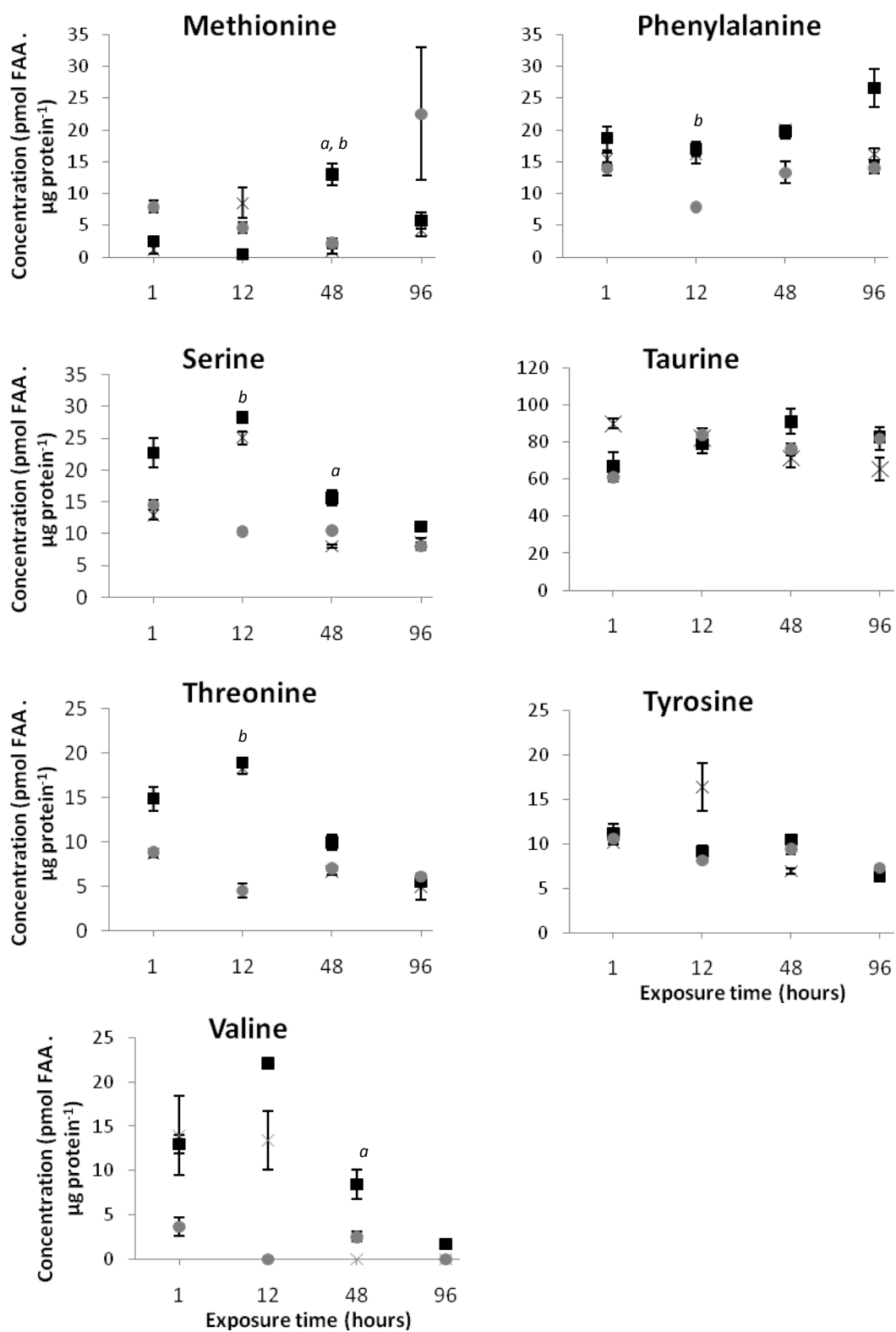
Fig 3.8: *Continued, see previous page for detail.*

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Discussion

In this study, it was shown that exposure to acute changes in external osmolarity can induce changes in the respiration, photosynthesis and FAA pool concentrations of symbiotic anthozoans. This study is the first to examine the osmotic response of FAA pools within the host tissue of the intact anthozoan-dinoflagellate symbiosis. However, the responses seen in both *Anthopleura aureoradiata* and *Acropora aspera* were not suggestive of FAAs being regulated for the explicit use as compatible organic osmolytes. Rather, changes in FAA pools showed changes consistent with other stress responses. Moreover, the response of anthozoan-dinoflagellate symbioses to osmotic stress appears to be species/taxa specific, as the responses of respiration, photosynthesis and FAA pools were very different between the temperate anemone *A. aureoradiat*a and the tropical coral *A. aspera.*

4.1. The effects of osmotic stress on respiration

When faced with changes in osmotic conditions, osmoconforming organisms must adjust their physiology in order to mediate physiological stress (Kinne 1971). Using respiration as an indication of metabolic rate, both *A. aureoradiata* and *A. aspera* showed a metabolic response to osmotic stress. However, this response differed between both species and symbiotic state.

Variability in the metabolic response to osmotic stress, both between and within species of marine invertebrates, is well documented (Vernberg & Vernberg 1972; Muthiga &

Szmant 1987; Moberg 1997; Lirman & Manzello 2009). The types of metabolic response to osmotic stress were described by Vernberg & Vernberg (1972) as: (a) an increase in respiration regardless of the direction of salinity change (either hypo- or hyper-saline conditions); (b) a decrease in respiration regardless of direction of salinity change; (c) an increase in respiration if salinity is lowered but a decrease if salinity is increased; and (d) no change in respiration. Furthermore, Vernberg & Vernberg (1972) also noted at that, if osmotic stress is persistent, a new steady state of metabolic rate is achieved after an initial transitory phase. The new steady state and the extent of the transitory phase depend on the intensity of osmotic stress and the outright ability of the organism to acclimate to the prevailing osmotic conditions.

4.1.1 Symbiotic anthozoans

The respiration rate of *Acropora aspera* at the control salinity was 2.8 times greater than that of *Anthopleura aureoradiata*. This is consistent with a general trend for tropical species to have higher metabolic rates than do temperate species due to the warmer temperatures they experience (Johnston *et al.* 1991; Gillooly *et al.* 2001).

4.1.1.1 Anthopleura aureoradiata

In the sea anemone *A. aureoradiata,* significant changes in respiration were only observed with exposure to the most extreme hypo-osmotic level of 12‰. The response to 12‰ was rapid, with respiration decreasing markedly after only 1 hour of exposure. However, after 96 hours of exposure a complete recovery of respiration was observed. Exposure to salinities of 25, 45 and 50‰ resulted in no significant changes in respiration due to large intra-treatment variability in respiration. Problems involving the variability of respiration quantification in anthozoans have been previously noted as an issue (Muthiga & Szmant 1987; Lirman & Manzello 2009) and highlight that, although non-significant, the larger

changes observed in the present study may still be of interest. Most apparent is the decrease in respiration seen with exposure to the most extreme hyper-saline treatment of 50‰, where 12 hours of exposure resulted in respiration rates that were 52% lower than control levels. Similarly, respiration in the coral *A. aspera* responded markedly to hypo-salinity (22 and 28‰), though of note the response was an increase in respiratory rate rather than a decrease. Interestingly, the most rapid increase occurred in the less extreme hypo-saline treatment of 28‰, though in both cases the respiration rate increased more than two-fold over the course of the experiment.

The results for *A. aureoradiata* emulate those of Muthiga & Szmant (1987) who noted substantial decreases in respiration of the coral *Siderastrea siderea* when exposed to both increases and decreases of 14‰, while changes of only ±10‰ elicited no significant response. Such reductions in respiration rate have been described as an important cellular response to osmotic stress in corals (Vernberg & Vernberg 1972; Moberg 1998; Ferrier-Pagès *et al.* 1999). Lower respiration rates decrease overall metabolic cost of cellular maintenance, in turn increasing the energetic capacity of a cell to resist changes in the environment (Vernberg & Vernberg 1972; Koehn & Bayne 1989). Thus a sustainable decrease in respiration in response to a change in salinity suggests an ability of an organism to acclimate to the prevailing osmotic conditions. This suggests that *A. aureoradiata* has an ability to acclimate to the levels of hypo- and hyper-osmotic stress employed in this study.

However, there is evidence to suggest that the higher levels of osmotic stress tested here may be close to the acclimatory threshold for *A. aureoradiata*, as increased exposure times at the more extreme salinities reduced the capacity to maintain these lower and presumably beneficial respiration rates. Alternatively, this trend may be interpreted as a

recovery of the respiration rate after a transitionary response while physiological acclimation took place (Vernberg & Vernberg 1972). However, the health of anemones after 96 hours exposure to 12‰ was apparently poor, i.e. non-responsive to tactile stimulation and degradation of tissue beginning to show (M. Cowlin, pers. obs.), so the elevated respiration rate was more likely linked to cellular degradation and death.

Exposure to osmotic stress beyond the scope of an organism's ability to acclimate causes direct damage to cellular structures. Changes in cell volume due to osmotic stress result in disruption to cellular functions (Muthiga & Szmant 1987), while alteration of ion concentrations may inhibit enzyme function, both of which result in changes to the metabolic rate (Rankin & Davenport, 1981; Lobban & Harrison 1994; Kerswell & Jones 2003). Mitochondria, the key site of cellular metabolism, are the cell structures most highly susceptible to osmotic damage. Exposure of mitochondria to hypo-saline conditions can disrupt electron transport, alter NADH redox capacity, increase reactive oxygen species (ROS) production (Ballantyne & Moon, 1986; Shivakumar & Jayaraman, 1986; Martinez *et al.* 1995; Downs *et al.* 2009), and decrease the activity of mitochondrial enzymes that play a key role in amino acid metabolism (Moyes *et al.* 1986; Devin *et al.* 1997). Under moderate conditions, all the above effects can work to decrease the respiration rate. However, respiratory decreases caused by such physiological disruption are unsustainable if osmotic stress is beyond the cell's ability to repair damage or if it persists longer than an organism can afford to make such repairs; in such cases, the stress will eventually lead to cell death. Once this has occurred, the organism is subject to microbial decomposition of necrotic tissue that elevates the respiration rate measured (Kristensen *et al.* 1991) and can erroneously give the impression that the organism has achieved stabilisation of physiological stasis. This was likely the case with *A. aureoradiata* after 96 hours under osmotic stress.

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4.1.1.2 Acropora aspera

The response of *A. aspera* was very different to that seen in *A. aureoradiata*, with the increased respiratory rate under hypo-salinity likely a genuine physiological acclimatory response rather than an artefact caused by tissue necrosis, as the response was seen only at the moderately hypo-saline salinity of 28‰. This response was different to that seen previously in corals (see above), but of note has been reported in other types of invertebrates (Kinne 1964; Normant & Lampprecht 2006). For example, increased respiration with exposure to hypo-osmotic stress has been demonstrated in the euryhaline crab *Callinectes sapidus* (Findley *et al.* 1978; Guerin *et al.* 1992), with the increased respiration attributed to enhanced activity and the energetic demands on cellular repair mechanisms. Increased respiration is also accompanied by increased ammonia excretion (Guerin & Stickle 1992; Normant & Lamprecht 2006) which suggests that the greater energetic demands may be attributed to higher rates of amino acid catabolism as a mechanism to regulate osmotic balance (Regnault 1987; Garton & Berg, 1989; Chen & Kou 1996; Jiang *et al.* 2000). Whether such physiological events occur in *A. aspera* is unknown, however.

In addition to physiological responses to osmotic change at the cellular level, it is also important to consider behavioural responses of the organism. Indeed, such responses could partly be responsible for the initial sharp decrease in respiration seen in symbiotic *A. aureoradiata* when exposed to 12‰; contraction was also observed in all symbiotic and aposymbiotic specimens of *A. aureoradiata* exposed to extreme hypo- and hyper-saline salinities. Retraction of the anthozoan tentacles and oral disc reduces the surface area of tissue in contact with the surrounding environment, reducing the influx/efflux of water and/or solutes with sudden changes in external osmotic pressure (Shick 1991). However, retraction also reduces gas exchange with the external environment, and may affect

oxygen fluxes (Muthiga & Szmant 1987); increased mucus secretion under osmotic stress may have similar impacts on water, solute and gas fluxes (Bursey & Harmer 1979; Mayfield & Gates 2007; Coles *et al.*. 2009). Retraction in response to osmotic stress has been observed in many anthozoan species, including the anemones *Anthopleura elegantissima* (Englebretson & Martin 1994) and *Metridium senile* (Shumway 1978), as well as the scleractinian corals *Siderastrea siderea* (Muthiga & Szmant, 1987), *Porites astreoides* (Lirman, 2009) and *Porites furcata* (Manzello & Lirman, 2003). The importance of this response *versus* cellular acclimation in *A. aureoradiata* could not be determined here and warrants further study.

Polyp retraction could also explain the response of *A. aspera* to hypo-salinity. When exposed to 22‰, this coral showed a slower and less pronounced increase in respiration than when exposed to 28‰. Polyp retraction occurred in all coral nubbins when exposed to 22‰ for either 1 hour or 12 hours, which for the reasons described above may have done enough to mitigate any metabolic changes at these time-points; alternatively, contraction may have reduced gas exchange to such a point that any metabolic changes where masked. In contrast, exposure to 22‰ for periods of greater than 12 hours caused the coral's tissue to swell, so counteracting polyp retraction. This could explain the increased respiration rate at the 48 and 96 hour time-points.

The differing metabolic responses of *A. aspera* and *A. aureoradiata* to salinity stress may reflect their differential capacities to cope with osmotic stress. The differences seen here could likely be due to the different habitats in which these two species are found. *A. aspera* is adapted to a relatively stable tropical environment, where light, temperature and salinity change little over the year (though there is still the potential for high/low salinity on the reef flat), while *A. aureoradiata* is a temperate species that inhabits rocky

shores and mudflats, where light, temperature and salinity all have the potential to vary considerably on a daily basis and with season. The greater tolerance of the temperate *A. aureoradiata* was reflected by the anemone's ability to deal with salinities as low as 12‰ compared a lower threshold of about 22‰ in *A. aspera*. Such differences in the ability to cope with osmotic stress may also exist between species that inhabit that same environment, as was found between the tropical corals *Pocillopora damicornis* and *Porites lutea* (Moberg *et al.* 1998). However, given that the treatment salinities were altered for each species to give comparable levels of osmotic stress, the different responses in respiration seen between species is presumably the effect of different osmotic strategies rather than an artefact of host origin.

4.1.2 Aposymbiotic A. aureoradiata

In comparison to the symbiotic state, aposymbiotic specimens of *A. aureoradiata* respired at a different rate at the control salinity and exhibited a different response to the various salinity treatments. The biomass-specific respiration rate at the control salinity was 45% lower for aposymbiotic *A. aureoradiata* than for symbiotic anemones. This lower respiration rate was likely due to the lack of dinoflagellate symbionts in the host, which contribute to the overall respiratory consumption of the holobiont (Harland & Davies 1995).

Aposymbiotic individuals of *A. aureoradiata* were also exposed to the extreme hypo- and hyper-saline treatments of 12 and 50‰. A significant impact was observed at 12‰. Unlike in symbiotic *A. aureoradiata*, there was no initial change in the respiration rate after 1 hour of exposure to 12‰. Rather, respiration showed a steady decrease with increasing exposure time, resulting in the respiration rate being 77% lower than for the control after 96 hours. Although dramatic, all anemones at this point were fully retracted but intact,

while in contrast most symbiotic *A. aureoradiata* at the same time-point displayed some level of visible tissue degradation coinciding with the increased rate of respiration. These observations, consistent with the prediction of Moberg *et al.*. (1998) for cnidarians, suggest that aposymbiotic individuals of *A. aureoradiata* are able to physiologically acclimate to this hypo-saline treatment for at least 96 hours without major cellular damage. In comparison, exposure to the hyper-saline treatment of 50‰ elicited no significant or otherwise marked change in the respiration rate across all time-points. The lack of a response suggests an inability to acclimate to this level of hyper-osmotic stress, as has been suggested for other cnidarians (Vernberg & Vernberg 1972; Moberg *et al.* 1998).

Comparison with the respiratory response of symbiotic individuals of *A. aureoradiata* suggests that the absence of symbiotic dinoflagellates may both reduce the osmoregulatory capacity of *A. aureoradiata* under hyper-osmotic conditions and increase its ability to withstand hypo-saline conditions. The explanation for this may lie in the absence of photosynthate (e.g. free amino acids, FAAs) translocated from the symbiotic dinoflagellates. This is discussed in detail in section 4.3.

4.2. The effect of osmotic stress on photosynthesis

A reduction in photosynthesis is the most commonly documented effect of salinity stress on anthozoan-dinoflagellate symbiosis (reviewed by Coles & Jokiel 1992), with decreases often directly proportional to the magnitude or duration of the salinity change (Muthiga & Szmant 1987). However, the impacts of salinity stress appear to be highly species-specific (Lirman 2009). Like respiration, the response of photosynthesis to osmotic stress seen in both *A. aureoradiata* and *A. aspera* was varied.

4.2.1 Anthopleura aureoradiata

In the anemone *A. aureoradiata*, photosynthesis was only significantly impacted with exposure to the hypo-saline treatment of 12‰. At this level of hypo-osmotic stress, photosynthetic rate decreased significantly within 1 hour of exposure, with further reductions seen over 96 hours. No significant impact on photosynthesis was detected in any of the other salinity treatments. However, as with respiration at 50‰, the highly variable oxygen flux measurements at this salinity may have masked any significant trend with respect to photosynthesis. Indeed, the 44% decrease in photosynthetic rate after 12 hours exposure to 50‰ suggests that there was in fact a negative impact.

These results agree with a number of studies that have described a reduction in the photosynthetic rate of symbiotic dinoflagellates *in hospite* following exposure to hypo-saline conditions (Muthiga & Szmant 1987; Moberg *et al.* 1997; Ferrier- Pages *et al.* 1999; Kerswell & Jones 2003; Manzello & Lirman 2003; Downs 2009); hypo-salinity also causes reduced rates of photosynthesis in a range of other microalgal species (Jahnke & White 2003). In more recent research, photosynthetic productivity is generally assessed by looking at the quantum efficiency of photosystem II (PSII), expressed as F_v/F_m, otherwise

termed "photosynthetic health". Kerswell & Jones (2003) found up to a 50% reduction in the photosynthetic health of the coral *Stylophora pistillata* with exposure to salinities of lower than 26‰ for 12 hours. Like Kerswell & Jones' (2003) study, most previous experimental work has focused solely on effects of hypo-salinity (Moberg *et al.* 1997; Ferrier-Pages *et al.* 1999; Manzello & Lirman 2003; Kerswell & Jones 2003), however work by Muthiga & Szmant (1987) examined the effect of both hypo- and hyper-salinity. They demonstrated that exposure of the coral *Siderastrea siderea* to both an increase and decrease of 14‰ resulted in a 67% and 39% decrease in the photosynthetic rate, respectively; similar to the results seen here in *A. aureoradiata.* It should be noted, however, that increased photosynthesis with exposure to moderate hypo-saline conditions has also been seen in a range of different free-living algae (diatoms, dinoflagellates, green algae) (Qasim *et al.* 1972).

Decreases in photosynthetic rate are generally thought to represent sub-lethal effects of bleaching and/or decreasing photosynthetic health of the symbionts (Coles & Jokiel 1992; Fang *et al.* 1995; Kerswell & Jones 2003). Due to photosynthetic measurements in the current study being standardized to the total concentration of symbiont cells, the decreases observed here represent a loss of photosynthetic capacity per symbiont cell, rather than a reduction in the number of symbionts via bleaching. Such reductions in photosynthetic rate under hypo-osmotic conditions have been attributed to both behavioural and physiological responses (Chartrand *et al.* 2009). Host tissue retraction in anthozoans is a widely observed behavioural response to osmotic stress, thought to reduce direct contact with the osmotically stressful external medium (Shick 1984). Additionally, tissue retraction reduces gas exchange and increases internal shading, both of which may significantly impact photosynthetic productivity of the dinoflagellate symbionts (Muthiga & Szmant 1987; Moberg 1997; Ferrier-Pages *et al.* 1999). Although

such a behavioural response may contribute to an observed impairment of photosynthesis, direct physiological disturbance of photochemical pathways of the symbiotic dinoflagellates, particularly PSII, may play a greater role (Muthiga & Szmant 1987; Moberg *et al.* 1997; Kerswell and Jones 2003).

The mechanisms by which reduced salinities lead to reductions in the photochemical efficiency of PSII *in hospite* are uncertain (Chartrand *et al.* 2009). However, work on isolated (*in vitro*) symbiotic dinoflagellates and other free-living single-celled algae may help to elucidate potential mechanisms. Dinoflagellates isolated from the corals *Pocillopora damicornis* and *Montipora verrucosa* have been found to show similar responses to those *in hospite,* with decreased PSII efficiency upon exposure to hypo-saline conditions (Sakami 2000). The free-living green alga *Chlorococcum submarinum* also shows a similar response to that seen here in *A. aureoradiata*, with the rates of photosynthesis decreasing upon exposure to both hypo- and hyper-osmotic conditions (Blackwell & Gilmour 1991). The actual biochemical impact of osmotic stress upon PSII is multifaceted and complicated. Work on the green alga *Dunaliella tertiolecta* (Jahnke & White 2003) suggests that reductions in PSII efficiency under hypo-osmotic conditions occur as a result of increased cell size, and increased levels of anti-oxidants in the thylakoid and envelope membranes of the chloroplast. Alternatively, research on other marine algae demonstrates that ionic alterations caused by hypo-osmotic stress may impair PSII electron flow by decreasing the availability of critical ions required as cofactors in photosynthesis (Gross *et al.* 1969; Simon *et al.* 1999), while hypo-saline conditions may also affect electron transport by directly impacting upon the electron acceptor molecule in PSII (Xia *et al.* 2004).

4.2.2 Acropora aspera

The response of photosynthesis in the coral *A. aspera* differed from that seen in the *A. aureoradiata.* Photosynthesis showed no significant deviation from control levels when exposed to any of the treatment salinities, but the rates were significantly different between the extreme hypo- and hyper-saline conditions. *A. aspera* exposed to 12‰ showed a significantly lower rate of photosynthesis than when exposed to 48‰, with the control rate being midway between the two.

To the author's knowledge, an increase in photosynthesis under extreme hyper-saline conditions has not previously been demonstrated in free-living micro- and macro-algae (Macler 1998) or in symbiotic dinoflagellates (Lirman 2009). Such a response has only been documented in some species of seagrass, which display an increased photosynthetic rate when exposed to intermediate hyper-saline conditions, at similar irradiances to those used in this study (Torquemada *et al.* 2005). However, Torquemada *et al.* (2005) offered no physiological reasoning as to why such increases may have occurred. The increases seen in the present study could have potentially arisen from the expulsion of symbiotic dinoflagellates into the external medium via bleaching (Marcus & Thorhaug 1981; Engebretson & Martin 1994; Titlyanov *et al.* 2000; Kerswell & Jones, 2003), so releasing them from any inhibitory factors that may occur *in hospite*, whether under normal conditions (e.g. internal shading, or nitrogen and carbon limitation)(Weis *et al.* 1993; Cook *et al.* 1992, 1994)or under osmotic stress (e.g. increased ROS production, reduced gas exchange) (Muthiga & Szmant 1987; Downs *et al.* 2009).

4.2.3 Causes and implications of altered photosynthetic productivity of symbiotic dinoflagellates

In all of the experimental studies cited above, osmotic stress was applied directly to the photoautotrophs in question; however the extent to which symbiotic dinoflagellates are exposed to direct changes in external osmolarity while *in hospite* is unknown. The alteration of the host's internal osmotic pressure may be enough to cause direct osmotic stress upon the symbiont. However, the osmoregulatory mechanisms of the host species in question and the extent to which COOs are utilised complicates this osmotic situation, as the intracellular matrix of the host may provide a buffer to changes in salinity that would otherwise be experienced with direct contact to the extracellular environment (Mayfield & Gates 2007). This intricate osmotic scenario is further convoluted by other stress responses of the host, such as a stress-induced increase in ROS production (Downs *et al.* 2009). ROS produced by the disruption of host mitochondrial electron transport (see section 4.1) can freely diffuse into the dinoflagellate symbiont's cytoplasm, whereupon they can cause oxidative damage to the symbiont thykaloid membranes and PSII (Mayfield & Gates 2007); this exacerbates any negative effects on photosynthesis that may already be taking place from direct osmotic stress upon the symbiont cells. The separation of symbiotic dinoflagellates from the external environment by the host's tissues makes the response of symbiont photosynthesis to osmotic stress highly dependent on both the behavioural and physiological responses of the host. It is therefore not surprising that the impact of osmotic stress on the photosynthetic performance of symbiotic dinoflagellates varies between different host species (Lirman 2009), as demonstrated here by the different responses of *A. aureoradiata* and *A. aspera*. It is also highly likely that differences in *Symbiodinium* type hosted in these two species may affect the response of photosynthesis to osmotic stress. Different physiological "types" of *Symbiodinium* have

been shown to differ in their response to environmental variables such as temperature and irradiance (Iglesias-Prieto & Trench 1997; Rowan *et al.* 1997; Kinzie *et al.* 2001; Perez *et al.* 2001). These differences have been attributed to the clade phylotypes (Rowan 1998). Although not typed in this study, in the anemone *A. aureoradiata* the most prevalent *Symbiodinium* likely belongs to clade A (Phillips 2006) while *A. aspera* is mostly likely dominated by clade C, as are the majority of coral species on the southern Great Barrier Reef (LaJeunesse *et al.* 2003). Clade A has been characterised as a weedy type that is more tolerant of high stress environments (Rowan 1998) while clade C is known to be susceptible to acute changes in environmental stressors such as increased irradiance (Rowan *et al.* 1997). This suggests that *Symbiodinium* within *A. aspera* may be less tolerant of stressful environments. However, as mentioned above, the differing responses of the host to osmotic stress also influence the response of *Symbiodinium in hospite,* so the role of symbiont type in determining the responses seen here is difficult to ascertain.

Despite some differences in photosynthetic response across the range of salinities, both species displayed a similar trend of decreased photosynthesis under hypo-saline conditions, a response that has been commonly reported for symbiotic anthozoans (Muthiga & Szmant 1987; Moberg *et al.* 1997; Ferrier- Pages *et al.* 1999; Kerswell & Jones 2003; Manzello & Lirman 2003; Downs 2009). The reduced rate of photosynthesis seen under hypo-osmotic stress in both *A. aureoradiata* and *A. aspera*, and under hyper-osmotic stress in *A. aureoradiata*, has important implications not only for the overall health of the intact symbiosis, but also potentially to the osmotic status of the host (Mayfield & Gates 2007). It is widely regarded that an impairment of photosynthesis is an important component of the bleaching response (Iglesias-Prieto *et al.* 1992; Jones *et al.* 1998; Warner *et al.* 1999; Kerswell & Jones 2003; Downs *et al.* 2009), and that the majority of experiments examining hypo-osmotic effects on coral–algal symbioses have

shown at least some degree of bleaching (Mayfield & Gates 2007). Kerswell & Jones (2003) demonstrated that bleaching caused by hypo-osmotic stress was preceded by a severe decline in the photosynthetic health of the symbionts. Although the precise biochemical mechanism that triggers the bleaching response remains unknown, it has been suggested that it may be linked to a stress-induced increase in ROS production (Weis 2008) and/or intracellular changes caused by the reduction in translocated photosynthetically-derived products (photosynthate) (Mayfield & Gates 2007). The transfer of photosynthate represents a major source of carbon and amino acids for host metabolic requirements (Gates & Edmunds 1999; Mayfield & Gates 2007), and thus a reduction in the amount of photosynthate available will negatively impact the nutritional state of the host. Major components of photosynthate such as FAAs and glycerol are compatible molecules that may also be used in an osmotic capacity by the host (Mayfield & Gates 2007). Thus, the impairment of photosynthesis under osmotic stress may cause a reduction in the amount of these translocated molecules and hence reduce COO pools within the host's tissues, further unbalancing the host's capacity to osmoregulate in response to its external environment.

4.3. The effect of osmotic stress on free amino acids

It is widely accepted that FAAs, as compatible organic osmolytes, play an important role in the osmoregulatory response of many marine invertebrates (Lang *et al.* 1998; Hochachka & Somero 2002). However, given the numerous other ubiquitous molecules that can also be used as COOs, the importance of FAAs in this role may be highly variable across taxa (Yancey 2010). In the anthozoan-dinoflagellate symbiosis, the dinoflagellate provides a large proportion of FAAs that the anthozoan host requires to meet its metabolic needs (Roberts *et al.* 2001). However, this source of readily available FAAs may also be utilized by the host when responding to changes in the external osmolarity. When exposed to hypo-osmotic stress, osmoconforming marine organisms must reduce the osmotic potential of cells, so avoiding excessive detrimental swelling caused by hypotonicity (Jahnke & White, 2003). This is achieved by reducing the concentration of both inorganic and organic osmolytes within the cells, with the role of FAAs in this response well documented in many types of marine invertebrates (Lang *et al.* 1998). Conversely, under hyper-osmotic conditions, an accumulation of COOs occurs to avoid hyper-tonicity, or cell shrinkage (Lang *et al.* 1998). The accumulation of FAAs to balance internal osmotic pressure with that of the external medium has been observed in a range of osmoconforming organisms (Measures 1975; Kasschau *et al.* 1984; Chen *et al.* 1994; Lang *et al.* 1998). Here, I examine how the FAA pools of *A. aureoradiata* and *A. aspera* vary in response to osmotic stress, and discuss the extent to which FAAs may be used in the osmoregulatory response of the anthozoan-dinoflagellate symbiosis.

4.3.1. Response of FAAs to osmotic stress

4.3.1.1 Anthopleura aureoradiata

Total FAAs

In symbiotic *A. aureoradiata*, the response of FAA pools did not entirely conform to the aforementioned trends that would be expected if FAAs were playing an integral role in the osmotic response. However, exposure to a hypo-osmotic salinity of 12‰ resulted in a decreased concentration of FAAs that was suggestive of osmoregulation. After just 1 hour of exposure to this salinity, total FAA pool concentrations declined by 50%, after which a seemingly stable state was reached.

In response to hypo-osmotic stress, the reduced concentration of intracellular FAA pools can be achieved by a variety of cellular mechanisms, most commonly the catabolic breakdown of FAAs (Haussinger *et al.* 1990), active (Hochachka & Somero 2002) or passive efflux into the external medium (Jackson & Strange 1993), or the incorporation of FAAs into proteins (Lang *et al.* 1998):

(1) Catabolic breakdown of FAAs: The increased catabolism of FAAs under hypo-osmotic stress has been demonstrated in many marine invertebrates (Haberfield *et al.*. 1975; Mangum *et al.* 1976; Regnault 1987; Garton & Berg 1989; Normant & Lampprecht 2006). For example, in the prawns *Penaeus japonicus* (Dalla Via 1986) and *P. monodon* (Fang *et al.* 1992), hypo-saline conditions resulted in decreases in the intracellular FAA pools of up to 50% that were attributed to the increased catabolism of FAAs (Chen *et al.* 1994). The catabolism of FAAs in response to hypo-osmotic stress is also marked by large increases in respiration and nitrogenous waste efflux, generally as ammonium (Gillies 1973; Chen 1994). Ammonium excretion was not measured in this study, however decreased

respiration rates were not observed in *A. aureoradiata*, suggesting that the loss of FAAs is not the result of their increased catabolism.

(2) FAA efflux: The efflux of intact FAAs is an energetically less expensive mechanism than catabolic breakdown of FAAs for reducing cellular FAA pools. The active expulsion of FAAs in response to hypo-osmosity occurs via ion membrane transporters such as the Na^+-coupled amino transport systems A, N and ASC (Broer *et al.* 1999), or the as yet unresolved Ca^{2+}-coupled transport mechanism that has been described across phyla (Rohloff *et al.* 2003; Amado *et al.* 2011). Additionally, hypo-osmotic conditions may trigger the passive efflux of FAAs via leakage across the cell membrane towards the lower external solute concentration, as the lipid bi-layer stretches with increased hypotonicity and structural integrity is compromised (Hoffman & Lambert 1983).

(3) Protein synthesis: Increased hypo-tonicity may inhibit proteolysis, and stimulate the synthesis of osmotic stress response proteins such as HSPs (Lang *et al.* 1998; Kültz 2005) as well as FAA membrane transporter proteins (Alfieri *et al.* 2004; Fiol *et al.* 2006). Under stressful conditions most organisms up-regulate a host of stress response proteins (HSPs) as part of the cellular response for redox regulation, DNA damage sensing and repair, molecular chaperoning, and metabolic regulation (Kültz 2005). Of the 44 known universal HSPs, transcription rates of 31 have been demonstrated to be up-regulated in response to a diverse range of stressors, including both hypo- and hyper-osmotic stress (Gasch *et al.* 2000). Such a sharp increase in protein synthesis has the potential to deplete standing FAA pools as they are incorporated into the up-regulated HSPs (Lang *et al.* 1998; Kültz 2005).

The response of FAAs to hypo-osmotic stress observed here is suggestive of an osmotic response, with the potential mechanisms including those discussed above. However, given the complex osmotic situation in the anthozoan-dinoflagellate symbiosis, FAA pools in the host may also be affected by the response of the algal symbionts to the osmotic stress. It has previously been shown that when external sources of FAAs are readily available, marine invertebrates will preferentially take these up (i.e. through Na^+-coupled transport) for use as COOs instead of maintaining energetically expensive heterotrophic mechanisms (Häussinger *et al.* 1992; Hochachka & Somero 2002). The supply of FAAs via translocation from symbiotic dinoflagellates is essentially a regular external source of FAAs and thus has the potential to reduce the demands for heterotrophic acquisition of FAAs (McCutcheon & Moran 2010). Here, however, the decrease in the FAA pools seen with exposure to 12‰ is consistent with a decrease in the photosynthetic productivity of the symbiont dinoflagellates with an associated reduction in the translocation rate of amino acids and other photosynthetic products (Gates 1999; Mayfield & Gates 2007). Therefore, while it is suspected that a combination of various osmoregulatory mechanisms likely contributes to the trends seen here under hypo-osmotic stress, the similar declines in the total FAA pool seen under hyper-osmotic stress (see below) also suggest a potential impact of salinity stress on the photo-physiological performance of the dinoflagellate symbionts rather than direct regulation of FAA pools by the host.

Exposure to the hyper-osmotic treatment of 50‰ resulted in a similar decline in the total FAA pool to that seen at 12‰. After 1 hour of exposure to this hyper-saline treatment, the total FAA concentration decreased by more than 50%; this reduced level was maintained until the 96 hour time-point, when a slight but significant recovery was noted. These results conflict with those found in other marine osmoconformers that are known to use FAAs as dynamic COOs (Thurston *et al.* 1980; Kasschau *et al.* 1984; Chen *et al.*

1994), in which FAA concentration are increased to balance the internal cellular osmotic

pressure with the increased external osmolarity. For example, the sea anemone

Bunodosoma cavernata, a species that has a similar salinity tolerance to *A. aureoradiata*,

increased its intracellular pool of the amino acid alanine by 28 times when the salinity was

increased from 26‰ to 40‰ over 96 hours (Kasschau *et al.* 1984). This accumulation of

FAAs can be achieved by increased amino acid synthesis (Roberts *et al.* 2001), increased

FAA uptake from the external environment (Hochachka & Somero 2002), and/or down-

regulation of other processes through which FAAs may be lost (Lang *et al.* 1998) such as

amino acid catabolism (Chen *et al.* 1994). It therefore seems that *A. aureoradiata* does

not use FAAs for osmoregulation under hyper-saline conditions (at least, not in any

noticeable way). To my knowledge, this is the first time such a response to osmotic stress

has been reported in an invertebrate, and it is conceivable that, as discussed above for

hypo-osmotic stress, the reduction in FAA concentration could result from a negative

impact on the photo-physiological performance of the dinoflagellate symbionts. This

requires further investigation.

Individual FAAs

The FAA pools of most anemones are frequently dominated by just a few amino acids

(Roberts *et al.* 2001). The situation in *A. aureoradiata* was no different, with the major

FAAs being taurine, alanine and arginine. The decrease in the total FAA pool

concentration seen in *A. aureoradiata* when exposed to either extreme hypo- or hyper-

osmotic conditions was driven by decreased concentrations of 12 of the 15 amino acids

quantified: alanine, aspartate, cystine, glutamate, glycine, histidine, isoleucine,

phenylalanine, serine, threonine, tyrosine, and valine, all of which showed similar

decreases in their concentrations. However, two of the main FAAs present, taurine and

arginine, did not follow this trend. Taurine did not change significantly under either hypo-

or hyper-salinity, while arginine showed no change or significantly increased its concentration in response to both hypo-and hyper-salinity.

Taurine has been found to be one of the dominant FAAs in a range of different anthozoan species, e.g. *Anthopleura xanthogrammica* (Male & Storey 1983), *Bunodosoma cavernata* (Kasschau *et al.* 1984), *Haliplanella lineate* (Shick 1976), *Metridium senile* (Deaton & Hoffman 1988), *Aiptasia pulchella* (Wang & Douglas 1998) and *Anemonia viridis* (Roberts *et al.* 2001). However, unlike the results of this study, taurine has been found to account for the majority of the change in the total FAA pool in response to both hypo- and hyper-osmotic stress (Thurston *et al.* 1980; Deaton & Hoffmann 1988; Law 1995). Indeed, in *M. senile,* reduced concentrations of taurine accounted for nearly the entire decline in the total FAA pool when the anemone was exposed to hypo-saline conditions for two weeks (Deaton & Hoffmann 1988). Conversely, in mammalian brain cells under hyper-saline conditions, taurine has been found to account for more than 50% of the increase in the total FAA pool (Thurston *et al.* 1980). Taurine is a metabolically inert (Bishop *et al.* 1978), non-proteinogenic (protein-forming) amino acid (Deaton & Hoffmann 1988). The sustained concentrations of taurine, in contrast to most other FAAs, in the current study might therefore be consistent with the depletion of other, proteinogenic FAAs for HSP and transporter protein synthesis, as discussed earlier. Alternatively, however, levels of hypotaurine may have increased in response to stress-induced ROS production; hypotaurine plays a key role as a cellular antioxidant that scavenges ROS hydroxyl radicals ($^{\cdot}$OH) and hypochlorous acid (HOCl) (Aruoma *et al.* 1988; Yancey 2005). Unfortunately though, the quantification method used in this study does not differentiate between taurine and its sulphinic acid intermediary, hypotaurine (Chaimbault *et al.* 2004), so no conclusions can be made with respect to hypotaurine production.

Arginine also responded differently to most other FAAs; it either increased in concentration or did not change at all. Unlike taurine, arginine is a proteinogenic amino acid, however arginine plays another important cellular role as it is the precursor to nitric oxide (NO) (Trapido-Rosenthal *et al.* 2005). NO is a ubiquitous molecule and has a diverse range of roles in eukaryotic cells; in particular, it is thought to be an important cell signalling molecule. Interestingly, in cells under osmotic stress, NO has been linked to the up-regulation of HSPs (Domitrovic *et al.* 2003), perhaps explaining why arginine concentrations need to be maintained within the osmotically-stressed cell at the expense of other FAAs. This putative role of arginine could be especially important in a phototrophic symbiosis, where the potential for oxidative stress arising from osmotic damage to the chloroplast is exacerbated (as it is under thermal bleaching), and warrants further research.

4.3.1.2. Acropora aspera

Total FAAs

The response of the total FAA pool in the coral *A. aspera* was markedly different to that seen in *A. aureoradiata.* Significant changes occurred only under hypo-saline conditions, with exposure to 22‰ leading to a more than doubling in the total concentration of FAAs after 96 hours. The greatest response however, was seen at the intermediate hypo-osmotic treatment of 28‰. Exposure to this salinity prompted an immediate response and the FAA concentration increased by 260% after only 1 hour of exposure and thereafter remained consistently higher than in the control treatment. The increased concentration of total FAAs was driven by increases in 9 of the 15 amino acids examined, with increases in aspartate, serine and alanine showing the most pronounced increases; the other 6 FAAs did not change their concentrations.

The increased concentration of the total FAA pool with exposure to hypo-salinity was contrary to what would be considered a direct osmoregulatory response by FAAs. As discussed above (section 4.3.1.1), if FAAs were being used directly in an osmoregulatory capacity, a decrease in FAA concentration in response to low salinity would be expected. Such a response has not been seen before in other systems and it is unknown why it occurred here. Nevertheless, the accumulation of FAAs in the anthozoan host tissue could be achieved by a variety of mechanisms: increased *de novo* synthesis of FAAs (Male & Storey 1983; Roberts *et al.* 2001), the inhibition of synthesis and increased catabolism of proteins (Lang *et al.* 1998), increased supply of translocated photosynthate, or, if available, the uptake of FAAs from the external environment (Hochachka & Somero 2002). It is known that the synthesis of non-essential and possibly essential amino acids can take place within anthozoan tissues (Fitzgerald & Szmant 1997; Swanson & Hoegh-Gulberg 1998; Roberts *et al.* 1999). Indeed, in the anemone *Anthopleura xanthogrammica* the synthesis of glutamate has even been found to increase under hyper-osmotic stress (Male & Storey 1983). However, it is believed that the level of synthesis that takes place within anthozoan tissues is limited when compared to the amount of amino acids obtained via translocation from the dinoflagellate symbionts (Swanson & Hoegh-Guldberg 1998; Roberts *et al.* 2001; Yellowlees *et al.* 2008). This might suggest that the increased FAA concentration seen here was more likely the result of an increase in photosynthesis by the symbionts, though no such increased productivity was apparent; this does not preclude an increase in the translocation rate though, which was not measured here. Alternatively, one of the other physiological processes mentioned above could have caused the increase. In particular, the increased respiration rate, which is similar to that seen under hypo-osmotic stress in other invertebrates, would be consistent with increased proteolysis (the breakdown of proteins) (Haussinger *et al.* 1991; Lang *et al.* 1998). This

catabolic response has typically only been seen under hyper-osmotic stress, with hypo-saline conditions generally seen to cause inhibition of proteolysis, increased protein synthesis (Stoll *et al.* 1992) and catabolism of FAAs (Haussinger *et al.* 1991). Why this might have occurred in *A. aspera* is unknown, though it could reveal a hyper-sensitivity to reduced salinity (which could override any osmoregulatory mechanisms) in this tropical species that was not apparent in the temperate *A. aureoradiata*.

Individual FAAs

The composition of the total FAA pool in *A. aspera* was different to that seen in *A. aureoradiata*. In *A. aspera*, the FAA pool was dominated by glutamate, aspartate and glycine, which represented 68% of the total FAAs quantified and are known to be major contributors to the FAA pool in other anthozoans (Pierce & Minasian 1973; Male & Storey 1983; Roberts *et al.* 2001). However, *A. aspera* appears to lack taurine. This is not to say that taurine was completely absent in *A. aspera*, as it might have been present at concentrations below the lower detection limit of the HPLC. Such low levels of taurine are unusual when compared to levels in other anthozoan species, where this amino acid forms a dominant portion of the total FAA pool (Shick & Storey 1983; Kasschau *et al.* 1984; Deaton & Hoffman 1988; Wang & Douglas 1998; Roberts *et al.* 2001). However, if hypo-osmotic stress does cause increased proteolysis, as suggested previously, it is not surprising that taurine levels do not increase from their possibly very low levels, as taurine is not a constituent of proteins (Deaton & Hoffmann 1988). The prevalence and response of arginine in *A. aspera* also differed from that seen in *A. aureoradiata*. Under control conditions arginine was rarely detectable, though like the majority of other amino acids showed increases with exposure to 28‰. This suggests that the importance of arginine, and thus NO production, (see above) in the osmoregulatory stress response may be limited, or at the least is species/taxa specific.

The trends seen in *A. aspera* and the lack/low levels of taurine were unexpected, and prevent any strong conclusions regarding the possible role of FAAs in the osmoregulation of this coral. However, while these results do suggest that the FAA pools in *A. aspera* respond to changes in the external salinity, it seems more likely than not that changes in FAA concentrations offer only limited (at most) protection from osmotic stress.

4.3.1.3. *Aposymbiotic vs Symbiotic* Anthopleura aureoradiata

In stark contrast to symbiotic *A. aureoradiata* and even *A. aspera*, the exposure of aposymbiotic *A. aureoradiata* to both a hypo-saline (12‰) and a hyper-saline (50‰) treatments resulted in only minimal changes to the total FAA pool. Exposure to 12‰ did not result in a significant change, while FAA concentrations at 50‰ were only significantly different (lower) from the controls at the 12-hour time-point. The most likely explanation for this inconsistent trend is that it is an artifact caused by the relatively small size of some of the aposymbiotic anemones in the control treatment; aposymbiotic anemones suffer from a lack of nutritional input from symbiotic dinoflagellates and are typically small. This small size meant that the FAA pool size was sometimes close to the lower detection limits of the HPLC and hence introduced a degree of variability/error. This interpretation is supported by the fact that the FAA concentration was much more stable in the 50‰ treatment than it was in its corresponding control treatment.

Despite this high variability, the FAA pools in aposymbiotic *A. aureoradiata* under control conditions were up to 41% lower than FAA pools in symbiotic anemones. This difference suggests that the higher concentration seen in symbiotic individuals may be due to a constant supply of symbiont-derived FAAs translocated to the host's cells. The drastic reduction in the size of FAA pools in symbiotic anemones when exposed to both hypo-and hyper-saline treatments, alongside reduced photosynthetic production, is consistent with

this interpretation, and highlights the potentially significant impact that osmotic stress can have on the symbionts and hence the functional biology of the symbiosis.

In contrast to the decline seen in the symbiotic anemones, FAA concentrations in aposymbiotic *A. aureoradiata* did not decline drastically under osmotic stress. Indeed, FAA levels in the first 48 hours of exposure to 12‰ and after an hour of exposure to 50‰, were in fact higher than in symbiotic *A. aureoradiata* under the same treatments. The reasons for this difference are unknown, but it has previously been noted that the availability of compatible solutes from exogenous sources (e.g. symbiont-derived COOs) may reduce the transcription of genes that encode components for the uptake and synthesis of osmoprotectants (Verheul *et al.* 1997; Kempf & Bremer 1998). It could therefore follow that, in the absence of dinoflagellate symbionts, the aposymbiotic anthozoan may maintain osmoprotectant mechanisms that keep FAA pools more stable. Conversely, symbiotic anemones acclimated to optimal environmental conditions may reduce the maintenance of such an energetically expensive mechanism due to a steady supply of symbiont derived COOs (Fiol *et al.* 2006a).

Comparison of the respiratory responses seen in symbiotic *versus* aposymbiotic individuals of *A. aureoradiata* suggests that the absence of symbiotic dinoflagellates may both increase the osmoregulatory capacity of *A. aureoradiata* under hypo-osmotic conditions and reduce its ability to withstand hyper-saline conditions. The explanation for this may lie in the absence of COO-containing photosynthate in the aposymbiotic anemones. Under hypo-saline conditions, the respiratory response of aposymbiotic individuals of *A. aureoradiata* demonstrated a greater ability to reduce energetic costs compared to their symbiotic counterparts. In symbiotic *A. aureoradiata* the continuing transfer of photosynthate, even though potentially reduced, may conflict with the host cell's need to reduce internal COOs, thus prolonging osmotic stress and water influx.

Conversely, under hyper-saline conditions, the *lack* of transferred photosynthate in aposymbiotic anemones may increase the energetic cost of maintaining/increasing COO pools in response to hyper-osmotic stress. However while respiration rates showed no adaptive response, there was also increase which may be expected with cell having to spend more energy to actively increase COO pools. However, while respiration rates showed no adaptive response, there was an increase which may be expected as the cell has to spend more energy to actively increase COO pools.

4.3.2 The role of FAAs as osmolytes in Anthopleura aureoradiata *and* Acropora aspera

It is well established that the use of COOs in marine invertebrates is a ubiquitous mechanism for dealing with changes in the external osmolarity. Indeed, given the euryhaline environments in which both *A. aureoradiata* and *A. aspera* live, these species must utilise COOs (Brown & Simpson 1972). Although many previous studies have suggested that FAAs may play a dominant role in anthozoan osmoregulation (Gerad & Giles 1972; Farmer & Reeve 1978; Bowlus & Somero 1979; Law 1991; Gates 1995; Lang *et al.* 1998) this current study highlights that the direct osmoregulatory role of FAAs may not always be important, or at the very least is species/taxa dependent. There is some indirect evidence, however, that specific FAAs (i.e. taurine, arginine) might play a role in moderating the impacts of osmotic damage to the cell.

Another reason to question the role that FAAs play in the osmoregulatory response of both *A. aureoradiata* and *A. aspera* is the overall concentration of the total FAA pool in the host's tissues. In previous studies that have experimentally demonstrated an osmoregulatory role for FAAs in other organisms, FAA concentrations were at least an order of magnitude greater than those measured here (i.e. nmol - µmol per µg protein *vs*.

pmol per μg protein) (Measures 1975; Kasschau *et al.* 1984; Dalla Via 1986; Deaton & Hoffmann 1988; Fang *et al.* 1992; Chen 1994; Swanson & Hoegh-Guldberg 1998). Additionally, during osmotic stress, total osmolyte concentrations can change by several hundred millimoles (Blackwell & Gilmour 1991), which is in marked contrast to the very small changes (hundreds of *picomoles*) seen here under a wide range of salinities. These trends all suggest that FAAs likely contribute little to the osmotic pressure of the host anthozoan's cells.

However, the potential still exists for symbiont-derived COOs other than FAAs to influence the osmoregulatory capacity of the host. Thus, future work should focus on the other main constituents of the symbiont's translocated photosynthate that are known to be utilised as COOs. Glycerol, considered to be the primary form in which photosynthetically-fixed carbon is translocated by symbiotic dinoflagellates (Muscatine 1967), is a primary candidate (Gates & Edmonds 1999). Glycerol is accumulated at high concentrations in salt-tolerant algae. For example, in the salt tolerant *Dunaleilla* sp., dynamic pools of glycerol respond to osmotic stress (Wegmann 1971; Ben-Amotz & Avron 1973; Borowitzka & Brown 1974), while glycerol accounted for 42% of the internal balance to the external salt concentration in the green alga *Chlorococcum submarinum* (Blackwell & Gilmour 1991). Additionally, Yancey *et al.* (2010) suggested that betaines and dimethylsulfoniopropionate (DMSP) may play a major role in the osmoregulation of symbiotic cnidarians. These authors demonstrated that these compounds contributed more to the osmotic potential of cnidarian host cells (in seven different cnidarian host species) than did other common osmolytes, including taurine.

In this study, like many others before (e.g., Muthiga & Smantz 1987; Kershwell & Jones 2003; Manzello & Lirman 2004; Downs 2009), acute changes in salinity were applied to induce hyper- and hypo-osmotic treatments, by directly transferring the experimental

organisms from control to treatment salinities. This approach has been recently criticised in Chartrand *et al.* (2009), as acute exposure does not allow for the acclimatory responses that may occur under more natural situations, where changes in salinity may be more gradual. Thus, a stepwise approach to altering salinity may have been a more robust methodology to assess the osmotic response of FAAs in the anthozoan-dinoflagellate symbiosis. The drastic changes in salinity to which the corals and anemones were exposed here could certainly go some way to explaining the large and rapid reductions seen in the rates of both respiration and photosynthesis, though only by repeating this work with more gradual changes in salinity will we be able to truly ascertain whether the two approaches yield different results.

In summary, although exposure to acute changes in external osmolarity did induce changes in the FAA pool of symbiotic anthozoans, the responses seen in both *A. aureoradiata* and *A. aspera* were not suggestive of FAAs being regulated for the explicit use as compatible organic osmolytes. Rather, changes in FAA pools showed changes consistent with other stress responses. Moreover, the response of anthozoan-dinoflagellate symbioses to osmotic stress appears to be species or taxa specific, as the responses of respiration, photosynthesis and FAA pools were very different between the temperate anemone *A. aureoradiat*a and the tropical coral *A. aspera*; it is conceivable that this difference was related to the very different environments (i.e. stable tropical *versus* variable temperate) inhabited by these cnidarians, but whether this is truly a latitudinal difference requires further investigation with more species. The comparison between symbiotic and non-symbiotic *A. aureoradiata* did serve to highlight the contribution of symbiont-derived FAAs to the host pool of FAAs, however in the absence of symbionts the host's total FAA pool was not significantly impacted by either hypo- or hyper-osmotic stress. Of note though, the respiratory data did suggest that the presence of symbiotic

dinoflagellates does influence the host's capacity to tolerate osmotic stress, but the underlying reasons are unknown. Mayfield & Gates (2007) recently suggested that internal osmotic stress caused by photoinhibition of the dinoflagellate symbionts may be a key mechanism in coral bleaching and, while FAAs may not play an important role, further work should be focused on other known symbiont-derived compounds known to be utilised as COOs, such as glycerol.

5

Appendix

5.1. Artificial seawater (ASW)

Anemone treatments

Artificial seawater (ASW) was made by adding a seven salt recipe to distilled fresh water (DH_2O). Different salinities were achieved by altering the amount of salts (table 5.1)

Coral experiments:

Due limited salt supplies on Heron Island, salinity treatments were achieved by altering the salinity of seawater obtained from the reef

For 22 and 28‰ treatments, DH_2O was added to seawater (35‰) until desired salinity was achieved. Salts were added to seawater to achieve the 42 and 48‰ treatments. No $NaNO_3$ was added to coral treatments as it was assumed ambient seawater would contain sufficient concentrations of nitrogen.

Salts	Anemone treatments Salinity					Coral treatments Salinity	
	12‰	25‰	35‰	45‰	50‰	42‰	48‰
NaCl	119.49	175.71	246.00	316.29	351.43	49.2	91.37
KCl	3.25	4.79	6.70	8.61	9.57	1.34	2.48
CaCl.2H$_2$O	6.61	9.71	13.60	17.49	19.43	2.72	5.05
MgSO$_4$.7H$_2$O	30.55	44.93	62.90	80.87	89.86	12.58	23.36
MgCl.6H$_2$O	22.63	33.29	46.60	59.91	66.57	9.32	17.30
NaHCO$_3$	0.86	1.26	1.76	2.27	2.52	0.3525	0.65
NaNO$_3$	10.00	10.00	10.00	10.00	10.00	-	-

Table 5.1: Artificial seawater recipe: Anemone treatments are total amounts of salts added to fresh DH_2O. Coral treatments are the amount of salts added seawater to achieve increased salinity. All amounts are in grams

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