

## REVIEW PAPER

# Co-ordination of osmotic stress responses through osmosensing and signal transduction events in fishes

T. G. EVANS

*Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950, U.S.A.*

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This review centres upon the molecular regulation of osmotic stress responses in fishes, focusing on how osmosensing and signal transduction events co-ordinate changes in the activity and abundance of effector proteins during osmotic stress and how these events integrate into osmotic stress responses of varying magnitude. The concluding sections discuss the relevance of osmosensory signal transduction to the evolution of euryhalinity and present experimental approaches that may best stimulate future research. Iterating the importance of osmosensing and signal transduction during fish osmoregulation may be pertinent amidst the increased use of genomic technologies that typically focus solely on changes in the abundances of gene products, and may limit insight into critical upstream events that occur mainly through post-translational mechanisms. © 2010 The Author

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## INTRODUCTION

### OSMOREGULATION IN OSMOREGULATORS: AN EVER-PRESENT CHALLENGE IN TELEOSTS

Regulation of intracellular and extracellular solute and water balance has become an absolute requirement for metazoans. The concentrations of intracellular electrolytes (in particular,  $\text{Na}^+$  and  $\text{K}^+$ ) are highly conserved across all kingdoms and phyla, reflecting an evolutionarily ancient optimization of the fundamental cellular machinery required to execute metabolism (Somero & Yancey, 1997; Kültz & Burg, 1998). As a corollary, osmotic stress, the result of an increase or decrease in the concentration of solutes inside an organism or cell, poses a considerable threat to basic cellular functions required for survival. Fishes and other aquatic or semi-aquatic organisms, which remain in continuous contact with environmental water, are constantly challenged to maintain plasma ion concentrations within the defined range necessary for

Present address: Department of Fisheries and Oceans, Molecular Genetics Section, Pacific Biological Station, Nanaimo, BC, V9T-6N7 Canada. Tel.: +1 250 756 7342; fax: +1 250 756 7053; email: [tyler.evans@dfo-mpo.gc.ca](mailto:tyler.evans@dfo-mpo.gc.ca)

proper cell function. This trend is exacerbated in tissues such as fish gills, which interface directly with the external aqueous environment. For example, in order to maximize oxygen diffusion, fish gills have evolved into a heavily vascularized, high-surface-area epithelium that provides only a thin barrier between the external aqueous environment and the blood. These derived characteristics concomitantly enhance the diffusion of other small molecules, such as water and ions, across gill membranes in response to osmotic gradients between the blood and the environment. As a result, the fish gill has become the dominant site for the transmembrane movement of ions, in addition to its major role in gas exchange (Evans *et al.*, 2005).

Teleost fish are osmoregulators that maintain stable plasma ion levels that are hypotonic to sea water and hypertonic to fresh water (Marshall & Grosell, 2006). The presence of this strong blood–environment osmotic gradient imparts a continuous challenge to maintain intracellular ionic homeostasis. In response, teleosts have evolved osmoregulatory systems that offset the passive-diffusive movements of ions and water across gill membranes (Fig. 1). Most of these systems rely upon predictable environmental salinities and are functionally restricted to marine or freshwater habitats (Fiol & Kültz, 2007). In marine teleosts, the diffusive loss of water and co-ordinate gain of inorganic ions are actively counteracted by secreting excess ions extracellularly across the gills. Conversely, in freshwater teleosts, where intracellular ion concentration is higher than the extracellular medium, ions are actively imported through the gills and excess water discharged *via* extremely dilute urine (Marshall & Grosell, 2006). These stenohaline (narrowly tolerant) species can only endure relatively minor changes in environmental salinity. In contrast, a smaller number of fish species inhabiting waters distinguished by fluctuating salinities have evolved exceptional osmoregulatory capabilities, and some of these euryhaline (widely tolerant) species can survive salinities ranging from fresh water to four times that of sea water (Fiol & Kültz, 2007). Examples of euryhaline fish include estuarine species, such as the goby *Gillichthys mirabilis* Cooper, the killifish *Fundulus heteroclitus* (L.), and migratory fish such as salmonids, whose life histories have evolved a marine and freshwater phase. Euryhaline species acclimatize to a range of environmental salinities through a series of complex adaptive processes that facilitate switching between hypotonic and hypertonic osmoregulatory systems (Kato *et al.*, 2005; Tang & Lee, 2007). These adaptive processes operate over a spectrum of osmotic stress levels ranging from small-scale changes in ion transport protein activity or subcellular location during periods of minor osmotic stress (Tipsmark & Madsen, 2001; Scott *et al.*, 2004) to large-scale changes that increase the abundance of specific proteins and the differentiation and turnover of particular gill cell types during more severe osmotic stress (Evans, 2002).

The ability to accurately quantify osmolality and co-ordinate a response of appropriate magnitude over a range of stress levels suggests that euryhaline fish may have evolved novel and optimized osmoregulatory mechanisms. For these reasons, euryhaline-fish gills represent ideal models to investigate the adaptive responses that occur during osmotic stress. The utility of the fish gill as a model for osmoregulation has not gone unnoticed within the scientific community, and there exists an extensive literature devoted to nearly every aspect of fish-gill osmoregulation and osmotic stress adaptation across multiple levels of biological organization (Evans *et al.*, 1999, 2005; Evans, 2002; Marshall & Grosell, 2006; McCormick & Bradshaw, 2006; Fiol & Kültz, 2007; Hwang & Lee, 2007; Kültz *et al.*, 2007). At the

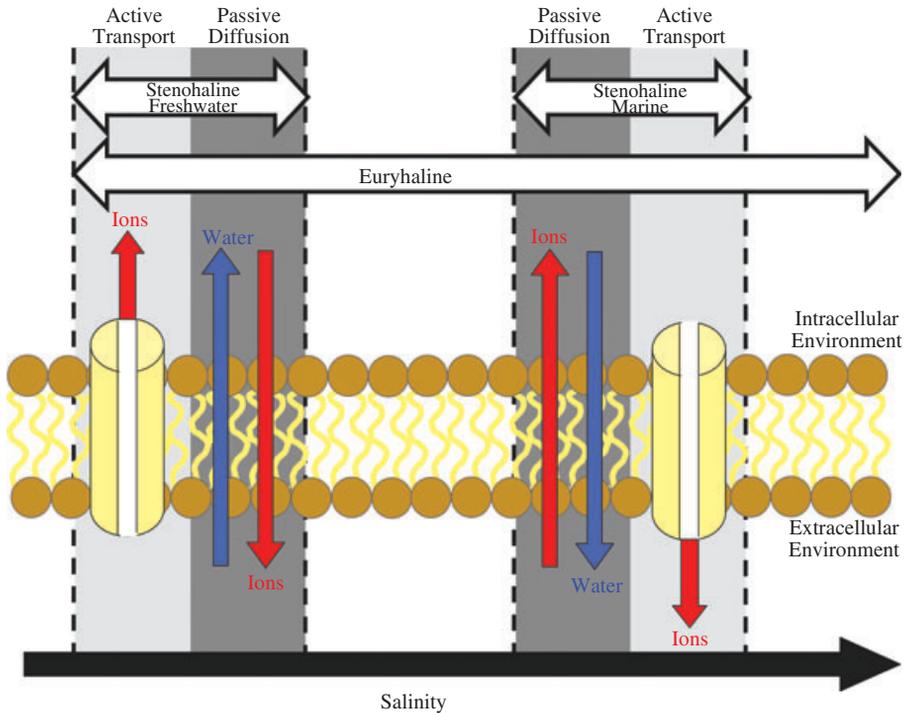


FIG. 1. Continuous osmoregulation in teleost fish. Teleost fish are osmoregulators that maintain stable plasma ion levels that are hypotonic to sea water and hypertonic to fresh water. The presence of this strong blood–environment osmotic gradient imparts a continuous challenge to maintain ionic homeostasis. In marine teleosts (right side), the diffusive (darkly shaded area) loss of water (blue arrows) and co-ordinate gain of inorganic ions (red arrows) is actively counteracted by secreting excess ions extracellularly across gill membranes through ion transport proteins (lightly shaded area). Conversely, in freshwater teleosts, where intracellular ion concentration is higher than the extracellular medium (left side), ions are actively imported through the gills (lightly shaded area) and excess water eliminated through extremely dilute urine (not shown). These stenohaline species can only endure relatively minor changes in environmental salinity (white arrows). Euryhaline species adapt to a much wider range of environmental salinities through a series of adaptive processes that facilitate switching between hypotonic and hypertonic osmoregulatory systems.

molecular level, previous research has focused heavily on the characterization of the so-called osmotic effector proteins, whose synergistic actions work to restore intracellular ion homeostasis. Canonical osmotic stress effector proteins include ion transporters that shuttle ions intra or extracellularly and organic osmolyte producing enzymes that minimize damaging changes in cell volume. Although the identity and function of these and other effector molecules have been well described, knowledge of the upstream mechanisms that regulate effector protein expression and orchestrate adaptive responses is much more limited. Appropriately, the purpose of this review will be to integrate recent data surrounding molecular osmosensing and signal transduction into a framework of cellular and physiological responses to osmotic stress. A major objective will be to describe how these upstream regulatory processes co-ordinate both small and large-scale responses to osmotic stress in fish gills. In

the concluding sections, the potential role of osmosensory signal transduction in the evolution of euryhalinity is examined, and experimental approaches that are likely to motivate future research in this area are described. From a broader perspective, emphasizing the importance of osmosensing and signal transduction events, which occur largely through post-translational events, may be warranted as 'omic'-based tools that typically detect only changes in the abundance of gene products that continue to drive research in the area.

## CO-ORDINATION OF OSMOTIC STRESS RESPONSES THROUGH OSMOSENSING AND SIGNAL TRANSDUCTION

### DIRECT COUPLING OF OSMOSENSORS TO OSMOTIC EFFECTORS

The effects of prolonged osmotic imbalance can be severe, damaging cellular macromolecules, disturbing cell volume, altering intracellular macromolecular density and modifying stringent intracellular inorganic ion concentrations (Kültz & Burg, 1998; Deane *et al.*, 2002; Kültz, 2005). Time lags between the onset of stress and the re-establishment of cellular homeostasis extend the time that cells are exposed to adverse conditions and increase the probability of such cellular damage. Consequently, euryhaline fish have evolved mechanisms to rapidly modulate the activity of pre-existing osmotic effector molecules and minimize temporal delays between the onset of stress and the corresponding adaptive responses. One means to achieve these goals is by linking adaptive changes in osmotic effector proteins directly to osmosensing mechanisms. Translationally controlled tumour protein (TCTP) is a highly conserved protein whose structure in yeast reveals homology with a family of small chaperone proteins (Thaw *et al.*, 2001) and whose expression is osmotically regulated in fish gills (Evans & Somero, 2008). Recent data have shown that TCTP interacts with the catalytic  $\alpha$  subunit of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, acting as a repressor of activity (Kim *et al.*, 2008). The dual role of TCTP as a molecular chaperone and an inhibitor of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity suggests a regulatory role during osmotic stress, whereby increased activity in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPases is tied to protein damage occurring from osmotic stress. In this mechanism, osmotic stress-induced protein damage triggers an increased requirement for molecular chaperones to assist in the folding of denatured proteins. TCTP is subsequently drawn away from the  $\alpha$  subunit of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, relieving its inhibitory functions and promoting increased  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity (Evans & Somero, 2008, 2009). This potential role in osmosensory signal transduction is supported by the fact that other molecular chaperones, such as heat shock proteins, are known to play protective roles during osmotic stress in fishes (Smith *et al.*, 1999), and that protein damage has been identified as a regulator of osmotic stress responses (Lamitina *et al.*, 2006).

Acute exposure to hyper or hypo-osmotic stress induces rapid changes in cell volume, resulting in cell shrinkage and swelling, respectively. Because of the extensive associations between the cytoskeleton and the cellular membranes, cytoskeletal organization is markedly affected by perturbations in cell volume and cytoskeletal proteins have been implicated as putative osmosensors (Pedersen *et al.*, 1999, 2001). Several types of ion transport proteins exhibit direct functional interactions with

the cytoskeleton, and it has long been speculated that the actin-based cytoskeleton might be involved in ion transporter regulation during osmotic stress (O'Grady *et al.*, 1987; Short *et al.*, 1998). Experimental evidence demonstrates that the osmosensory properties of the cytoskeleton may be directly coupled to ion transporter activity. Treatment of Ehrlich ascites cells with cytochalasin (a chemical agent that inhibits actin polymerization) produced membrane blebs that lack actin and myosin, but in which the  $\text{Na}^+ - \text{K}^+ - \text{Cl}^-$  co-transporter is permanently activated under isotonic conditions (Jessen and Hoffmann 1992; Hoffmann *et al.*, 1994; Pedersen *et al.*, 2001). Similar results have been reported for the  $\text{Cl}^-$  and  $\text{K}^+$  channels and the  $\text{Na}^+ - \text{H}^+$  exchanger (Schwiebert *et al.*, 1994; Orłowski & Grinstein, 1997; Hibino *et al.*, 1999). Short actin filaments have also been shown to directly regulate the  $\text{Na}^+$  channel and cystic fibrosis transmembrane conductance regulator (CFTR) activity (Cantiello & Prat, 1996). In addition, phosphorylation of cytoskeletal elements can affect  $\text{Na}^+ - \text{K}^+ - \text{Cl}^-$  co-transporter activity independent of the phosphorylation state of the co-transporter itself. For example,  $\text{Na}^+ - \text{K}^+ - \text{Cl}^-$  co-transporter activity can be stimulated through the phosphorylation of goblin, a protein similar to the cytoskeletal element ankyrin (Pewitt *et al.*, 1990). Ankyrin has been shown to be osmotically regulated in the highly euryhaline fish *G. mirabilis* during hypo-osmotic stress (Evans & Somero, 2008). Analogous to the mechanism described for TCTP-mediated regulation of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, these data provide examples where the osmoregulatory actions of effector proteins may be directly linked to the osmosensing properties of the cellular cytoskeleton, providing an efficient and rapid means to modulate the activity of endogenous osmotic effector proteins.

#### AMPLIFICATION AND DISTRIBUTION OF OSMOREGULATORY CUES VIA CELL SIGNALLING CASCADES

Although directly coupling osmosensors to osmotic effector proteins provides a very straightforward means to initiate adaptive reactions, larger scale osmoregulatory mechanisms are not as linear and operate by linking molecular osmosensors to cell signalling pathways. Cellular signalling pathways are activated by ligand-receptor binding and are propagated through a number of transducer proteins *via* progressive phosphorylation or dephosphorylation events. Through this system, osmosensory signals are still quickly transduced within cells but can be amplified and distributed to a greater variety of downstream effectors with relevance to osmotic stress (Kültz, 2007). For example, the scaffold-adaptor 14-3-3 proteins serve as major convergence points for osmosensory signals and are characterized by their ability to regulate multiple downstream signal transduction pathways and diverse effectors (Fu *et al.*, 2000; Kültz *et al.*, 2001; Koskinen *et al.*, 2004). In the euryhaline teleost *F. heteroclitus*, 14-3-3 proteins are suspected of modulating ion transport during osmotic stress in several ways, including through the activation of the  $\text{H}^+$ -ATPase, which in turn energizes ion transport *via* the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, by decreasing protein kinase C activity, which in turn can inhibit  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in the gill epithelium (Crombie *et al.*, 1996) and by binding calmodulin, which inhibits the  $\text{Ca}^{+2}$  activated  $\text{Cl}^-$  channel (Chan *et al.*, 2000; Kültz *et al.*, 2001).

Serum and glucocorticoid-regulated kinase isoform 1 (SGK-1) is an intracellular signalling molecule with an established role in ion homeostasis and whose activity is strongly regulated by osmotic stress in fish gills (Evans & Somero, 2008; Shaw

*et al.*, 2008). The foremost function of SGK-1 is a stimulatory effect on sodium transport *via* the epithelial Na<sup>+</sup> channel, where it serves as a convergence point for multiple regulators of sodium transport (Pearce, 2001; Loffing *et al.*, 2006). SGK-1 likely modifies the activity of Na<sup>+</sup> channels *via* phosphorylation (Diakov & Korbmayer, 2004). SGK-1, however, also appears to influence the activities of other ion transporters, such as the K<sup>+</sup> channel, the Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> co-transporter and the CFTR, by regulating their phosphorylation state (Lang & Cohen, 2001; Pearce & Kleyman, 2007; Shaw *et al.*, 2008). This central role in osmotic stress signalling cascades allows SGK-1 to integrate numerous signalling inputs and to modify the activity of multiple downstream osmotic stress effector proteins. This type of signal amplification and distribution is probably an important aspect of more extensive responses to osmotic stress in fish.

Cell signalling events analogous to those described for SGK-1 and 14-3-3 proteins appear repeatedly during osmotic stress signal transduction. For example, the Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> co-transporter can be phosphorylated on at least five sites, strongly suggesting its activity is dependent on the activities of multiple signalling proteins (Flatman, 2002). Similarly, osmotically induced casein kinase can count aquaporin 4 and the K<sup>+</sup> channel as substrates (Meggio & Pinna, 2003). The CFTR is phosphorylated by protein kinase A (Marshall *et al.*, 1995; Singer *et al.*, 1998), while Lyn tyrosine kinase can directly phosphorylate the anion exchanger in skate *Raja erinacea* Mitchill erythrocytes (Perlman & Goldstein, 2004). Ste20/sps1-related proline-alanine-rich protein kinase (PASK) co-immunoprecipitates with Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> co-transporters, implicating phosphorylation as a prime candidate for its regulation (Dowd & Forbush, 2003). Vast amounts of circumstantial evidence also exist demonstrating ion transport to be dependent on the activity of kinases, including mitogen-activated protein kinases (MAPK), focal adhesion kinase, myosin light-chain kinase, protein kinase C and others (Marshall *et al.*, 2005, 2008).

The functions of 14-3-3 proteins, SGK-1 and other osmotically regulated signalling proteins illustrate that osmotic stress-regulated signalling cascades can serve to amplify osmosensory signals and subsequently modify the functional properties of a number of downstream osmotic effector proteins. In addition, protein-protein interactions between the cellular cytoskeleton or molecular chaperones and ion transport proteins provide a direct mechanism to link changes in the activity of osmoregulatory effector proteins to osmosensing events. An important caveat to these types of systems, however, is that re-establishing ion homeostasis exclusively through modulating the activity of existing ion transport proteins is limited by their endogenous levels within cells and the rate at which these proteins can shuttle ions intra or extracellularly. Therefore, while responses of this magnitude are appropriate for modest or relatively slow changes in environmental salinity, or for temporarily preserving cellular functions while large-scale responses are initiated, this system alone will become overwhelmed during cases of severe osmotic stress. Yet, many species of euryhaline fish are able to overcome extreme changes in environmental salinity, indicating the presence of more profound osmoregulatory strategies. For example, the estuarine goby *G. mirabilis* can acclimatize to salinities ranging from freshwater to three times that of ambient sea water (Nagahama *et al.*, 1973; Owens *et al.*, 1977; Loretz, 1979), whereas the Mozambique tilapia *Oreochromis mossambicus* (Peters) can live in fresh water as well as in water with salinities up to four times that of sea water (Fiol & Kültz, 2007). Adapting to such

severe osmotic stress requires large-scale, genomic-based responses that involve a network of interacting proteins working in concert to modify diverse cellular processes, such as gene expression, protein subcellular localization and cell proliferation and turnover. These more comprehensive modifications are likely to build upon earlier and more rapid changes in the activity of pre-existing osmotic effectors but appear to be orchestrated by distinct osmosensing and signal transduction events.

#### ADJUSTING THE BIOAVAILABILITY OF OSMOREGULATORY HORMONES AND THEIR RECEPTORS

Major osmoregulatory adaptations in fish gills are driven largely by the concerted actions of a myriad of hormones that, through an association with cellular signalling cascades, modulate an array of cellular processes that include changes in the abundance of osmotic effectors and altering the size, distribution and number of ion-transporting cells in the gill epithelium. Major players include corticosteroids, prolactin, growth hormone and insulin-like growth factor 1 (IGF-1) (Evans, 2002). Although a description of the precise actions of these osmoregulatory hormones are beyond the scope of this review, it is of relevance that their ability to drive large-scale adaptive processes during osmotic stress is probably refined through precise osmosensing and signal transduction events.

Cortisol, the major corticosteroid in fish, is considered a master regulator of gill chloride cell differentiation and function during osmoregulation and operates in synergy with both prolactin and IGF-1 (McCormick, 2001). The physiological actions of cortisol are exerted in part through binding to the glucocorticoid receptor, which can regulate gene expression by acting as a ligand-dependent transcription factor (Cato *et al.*, 2002; Greenwood *et al.*, 2006). The functional properties of the glucocorticoid receptor are strongly influenced by interactions with chaperone proteins (Grad & Picard, 2007). FK506-binding protein 51 (FKBP-51) is a molecular chaperone comprising one component of a heterocomplex of proteins maintaining the glucocorticoid receptor in a constitutively inactive state in the absence of ligand binding (Westberry *et al.*, 2006; Zhang *et al.*, 2008). FKBP-51 expression is influenced by osmotic stress in fish and may act as an osmosensory protein that regulates the availability of glucocorticoid receptors during salinity changes (Evans & Somero, 2008, 2009). Through this mechanism, denatured proteins resulting from increased or decreased intracellular osmolality may recruit the chaperone activity of FKBP-51 away from the glucocorticoid receptor, relieving its inhibitory functions and promoting ligand binding and subsequent transcriptional events. This mechanism is consistent with current knowledge regarding the regulation of the glucocorticoid receptor by molecular chaperones (Grad & Picard, 2007), and reminiscent of heat-shock factor regulation, an environmental stress-induced transcription factor responsible for the synthesis of heat-shock proteins (Voellmy, 2004).

In addition to regulating the availability of hormone receptors, emerging evidence suggests that the bioavailability of the osmoregulatory hormones themselves may be modulated by osmotically sensitive protein–protein interactions. These data suggest that hormone-binding partners may function as cellular osmosensors or form part of signalling cascades that modulate hormone activity. In euryhaline salmonids, IGFs in extracellular fluids are found complexed with specific high-affinity binding proteins

(IGFBP) (Shimizu *et al.*, 1999). IGFBPs have been suggested to mediate the efflux of IGFs from the vascular space to the cell surface, thereby promoting interactions between ligands and receptors, and to prevent proteolytic degradation of IGF, consequently prolonging their half-lives (Reinecke *et al.*, 2005; Wood *et al.*, 2005). The induction of IGFBP-1 mRNA in hyper-osmotically stressed gill tissue in a euryhaline fish implies that IGFBPs may be playing a similar role during osmotic stress responses in fish (Evans & Somero, 2008, 2009). This potential role is strengthened by the prominent functions of IGFs during adaptive responses to osmotic stress in fish (Duan, 1997). The regulatory actions of IGFBPs may provide a means to more accurately control IGF-1 availability to cellular receptors and ensure an adaptive response of proper magnitude is mounted.

Prolactin is a pituitary polypeptide hormone that mediates its cellular effects through transmembrane domain prolactin receptors. One of the earliest known functions of prolactin in teleost fish was its role in ion uptake as a freshwater-adapting hormone (Sakamoto & McCormick, 2006). Gene expression, synthesis, secretion and plasma levels of prolactin increase during freshwater acclimatization (Manzon, 2002), and prolactin regulates osmotic balance by decreasing water permeability and increasing ion retention on osmoregulatory surfaces (Sakamoto & McCormick, 2006). In a mechanism that parallels the regulatory role of IGFBPs, recent evidence strongly suggests that the bioavailability of prolactin in euryhaline fish is regulated through an association with a specific, evolutionarily conserved prolactin-releasing peptide (PrRP) (Moriyama *et al.*, 2002; Seale *et al.*, 2002). PrRP presumably functions as an osmosensory signalling molecule during hypo-osmotic stress, acting as a ligand for a G-protein-coupled receptor that, through subsequent signalling events, regulates prolactin release (Fujimoto *et al.*, 1998; Moriyama *et al.*, 2002). PrRP likely acts as a local regulator of prolactin secretion from the pituitary and operates directly on prolactin-producing cells to control prolactin availability to its receptors in the gill during freshwater acclimatization in fish (Sakamoto *et al.*, 2003, 2005; Kwong & Woo, 2008).

## REGULATING OSMOTIC STRESS-SPECIFIC TRANSCRIPTION FACTORS

Transcription factors form integral components of signalling cascades by transmitting environmentally regulated cellular signals to the genetic machinery in the nucleus. In fish gill tissue, numerous ion and water transporters are regulated at the mRNA level by salinity, indicating that transcriptional regulation is an important means of modulating effector-protein expression during osmotic stress (Tipsmark *et al.*, 2002). Although knowledge of transcriptome regulation during osmotic stress adaptation in fish is far from comprehensive, recent data have demonstrated the existence of transcription factors that may play critical roles in promoting adaptive changes in gene expression (Fiol & Kültz, 2005; López-Bojorquez *et al.*, 2007). Importantly, these transcription factors appear to be regulated exclusively by osmotic stress and probably evolved to meet the unique homeostatic challenges faced by cells during osmotic imbalance.

Osmotic stress transcription factor 1 (OSTF1) is a transcriptional regulator that is transiently up-regulated at an early stage in fish gill epithelial cells exposed to hyperosmotic stress (Fiol & Kültz, 2005; Fiol *et al.*, 2006; McGuire *et al.*, 2010).

The rapid induction of OSTF1 suggests that its activity may be largely regulated by post-translational modifications or other signalling events transduced through existing cellular proteins. OSTF1 contains phosphorylation and binding sites for DNA-protein kinases, cyclin-dependent kinases and MAPK, suggesting its activity is probably influenced by these major signalling pathways (Fiol & Kültz, 2005). MAPK are prominent players during osmotic stress responses in fish (Kültz & Avila, 2001) and regulate major cellular outcomes such as cell proliferation, cell growth and differentiation and cell death (Karin, 1998; Cowan & Storey, 2003). These data combined with the putative phosphorylation sites for cyclin-dependent kinases, key regulators of the cell cycle, suggest that OSTF1 may ultimately influence the proliferation or turnover of specific gill cell types during large-scale responses to osmotic stress. OSTF1 expression is also initially dependent on mRNA stabilization mechanisms that permit a rapid increase in steady-state mRNA. mRNA stabilization is generally associated with the binding of stabilizer proteins to the 3' untranslated region of the mRNA (Guhaniyogi & Brewer, 2001), and OSTF1 mRNA may interact with an as yet unidentified protein that plays a prominent role during osmosensory signal transduction in fish. This type of regulation is characteristic of inducible transcription factors and other immediate early genes and is commonly used during rapid responses to various forms of environmental stress (Fiol *et al.*, 2006).

Tonicity response element-binding protein transcription factor (ORE-BP) is an osmotically regulated transcription factor responsible for the up-regulation of a suite of osmotic effector proteins. In fish cells, the transcriptional activity of ORE-BP appears to be dependent on cell signalling events that govern the nucleocytoplasmic trafficking of ORE-BP in an osmotic stress-specific manner (Tong *et al.*, 2006; López-Bojorquez *et al.*, 2007; Xu *et al.*, 2008). In euryhaline *F. heteroclitus* and rainbow trout *Oncorhynchus mykiss* (Walbaum), ORE-BP is exclusively localized to the nucleus during hypo-osmotic stress, whereas during hyperosmotic stress, ORE-BP remains in the cytoplasm (Orozco *et al.*, 2002; López-Bojorquez *et al.*, 2007). Cytoplasmic ORE-BP sequestered from DNA would remain transcriptionally inactive, whereas nuclear translocation stimulated by hypo-osmotic stress and osmosensory signal transduction would promote ORE-BP–DNA interactions and lead to transcriptional changes in a set of hypo-osmotic stress-specific genes. Reversible phosphorylation catalysed by the signalling protein casein kinase appears to play a major role in regulating the bi-directional shuttling of ORE-BP between the nucleus and the cytoplasm in response to changes in osmolality (Xu *et al.*, 2008). Casein kinase expression has been shown to be regulated by osmotic stress in euryhaline fish gills (Evans & Somero, 2008), and these data indicate a significant role for casein kinase signalling in the co-ordination of large-scale osmotic stress responses in fish gills.

## STABILIZING TRANSLATION DURING OSMOTIC STRESS

Osmotic stress-specific transcription factors are an ideal way to regulate expression in an explicit set of genes. Some euryhaline fish, however, may have evolved mechanisms to further ensure adaptive changes in gene expression by stabilizing the translation of osmotically regulated transcripts. Euryhaline Atlantic salmon *Salmo salar* L. undergo directed physiological changes at specific points in their life cycle to permit the transition from a fresh water to a marine environment. Altered gene expression plays a central role in facilitating these changes, and translation of these

important gene products in a sub-optimal cellular environment may be challenging. Translation in *S. salar* at this stage, however, may be aided through the action of an osmotic stress-specific RNA-binding protein expressed exclusively in the gill (Pan *et al.*, 2004). The putative protein, dubbed salmon glycine-rich RNA-binding protein (SGRP), is predicted to facilitate the continuous translation of transcripts required for adaptation to elevated salinity. SGRP expression is induced only in response to osmotic stress and is insensitive to heat stress, cold stress or sodium arsenite. These data suggest SGRP evolved to function exclusively during salinity transitions and is an important component of osmotic stress-specific sensing and signal transduction pathways. Treatment of gills with chemical inhibitors of MAPK signalling did not affect expression of SGRP during osmotic stress in *S. salar*, indicating that this central osmotic stress signalling pathway may not be involved in SGRP regulation (Pan *et al.*, 2004). Further experimentation is required to determine the precise nature of this potentially significant regulatory protein during osmotic stress responses in fish.

#### INTEGRATION INTO LARGE AND SMALL-SCALE OSMOTIC STRESS RESPONSES

The examples described above illustrate the wide range of molecular specializations that euryhaline fish possess to properly co-ordinate an appropriate adaptive response to a range of environmental salinities. The ability to respond to varying degrees of osmotic stress with functionally distinct cellular and physiological mechanisms implies a means to very accurately quantify environmental osmolality. One plausible hypothesis is that osmoregulatory cues emanate from multiple cellular osmosensors, each with different sensitivity ranges (Fiol & Kültz, 2007). As stress levels increase, more and more osmosensors are triggered. These molecular osmosensors are then coupled to a discrete set of signal transducers (*e.g.* hormone receptors, cell signalling pathways, osmotic stress-specific transcription factors) and effector mechanisms (*e.g.* ion-transporting proteins) that function during particular magnitudes of osmotic stress and act through diverse pathways to initiate cellular, physiological and behavioural responses that ultimately restore osmotic homeostasis. The osmosensory signal transduction events described in this review are congruent with this hypothesis. For example, relatively acute osmosensors may initiate pathways that alter the activity of endogenous ion transporters through phosphorylation (*e.g.* 14-3-3 proteins and SGK-1) and direct links to osmosensing proteins (*e.g.* TCTP and the cytoskeletal proteins). Adaptive responses of this magnitude serve a number of purposes by providing a rapid means of re-establishing ionic balance during periods of relatively minor osmotic stress, transiently stabilizing ion balance while large-scale responses take form and minimizing temporal delays between the breaching of stress thresholds and the onset of an adaptive response. Large-scale adaptive responses that occur during periods of severe osmotic stress may then use an alternative set of molecular osmosensors linked to different signal transduction pathways that involve changes in hormone and hormone receptor bioavailability (*e.g.* IGF1Ps, FKBP-51 and PrRP), the activity of osmotic stress-specific transcription factors (*e.g.* OSTF1 and ORE-BP) and the translation of nascent osmotic effector proteins (*e.g.* SGRP). The development of this type of progressive response may stem from a need to balance the quantity of cellular resources directed towards an adaptive response with the intensity of osmotic stress encountered. As a consequence, smaller changes

in osmolality are met with an appropriate small-scale response, whereas more severe cases of osmotic stress demand a larger investment of resources to properly restore cell homeostasis. Indeed, osmoregulatory processes are energetically expensive activities (consuming 20–68% of the total energy expenditure in fish; Morgan & Iwama, 1991; Boeuf & Payan, 2001) and have probably led to sizeable selective pressures to optimize energy expenditure during osmotic stress adaptation (Fig. 2).

### THE RELEVANCE OF OSMOSENSORY SIGNAL TRANSDUCTION TO THE EVOLUTION OF EURYHALINITY

At the molecular level, osmoregulatory adaptations in euryhaline fish typically culminate through shifts in the abundance and activity of ion and water-transporting proteins. For example, hyperosmotic challenge in coho salmon *Oncorhynchus kisutch* (Walbaum), *S. salar* and brown trout *Salmo trutta* L. results in a gradual adjustment of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and  $\text{Na}^+-\text{K}^+-\text{Cl}^-$  co-transporter expression in gill epithelial cells (Tipsmark *et al.*, 2002). Exposure to hyperosmotic stress also increased  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and  $\text{Na}^+-\text{K}^+-\text{Cl}^-$  co-transporter expression in Japanese eel *Anguilla japonica* Temminck & Schlegel (Tse *et al.*, 2007), and pufferfish *Tetraodon nigroviridis* Marion de Procé (Tang & Lee, 2007). Hyposaline water has been shown to increase  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in the gills of *F. heteroclitus* (Scott *et al.*, 2004) and gilt-head sea bream *Sparus aurata* L. (Laiz-Carrión *et al.*, 2005). Collectively, these and many other data strongly suggest that maintaining osmotic homeostasis during either hypo or hyperosmotic conditions is largely dependent on the proper regulation of these effector molecules. As a corollary, one plausible explanation for the limited osmoregulatory capabilities of stenohaline species is an inability to properly execute the osmosensing and signal transduction events required to modify effector protein activity and expression during osmotic stress.

Knowledge surrounding the molecular adaptations, or lack thereof, that underlie stenohaline lifestyles in fish is limited. The paucity of data that does exist suggests that stenohaline fish do indeed possess genes encoding osmotic effector proteins, such as the CFTR,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and  $\text{Na}^+-\text{K}^+-\text{Cl}^-$  co-transporter, but are unable to actively regulate their expression or activity during osmotic stress. For example, the stenohaline longhorn sculpin *Myoxocephalus octodecimspinosus* (Mitchill) can tolerate short-term exposure to low salinity water for days but are unable to fully acclimatize because they cannot regulate ion transporter density or activity. This regulatory deficiency eventually results in a lethal loss of ions. Interestingly, *M. octodecimspinosus* do express constitutive levels of the CFTR,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and  $\text{Na}^+-\text{K}^+-\text{Cl}^-$  co-transporter in gill cells, but the abundances of these proteins do not respond to hypo-osmotic stress (Hyndman & Evans, 2009). In a similar vein, immunological data demonstrate that the putative cell type that is responsible for active sodium absorption in euryhaline elasmobranchs is present in marine stenohaline elasmobranchs. These data imply that the inability of marine stenohaline elasmobranchs to survive in fresh water is not due to a lack of specific ionoregulatory cell types but rather the inability to properly regulate their abundance or turnover during osmotic stress (Choe *et al.*, 2007). Finally, in the stenohaline freshwater catfish *Heteropneustes fossilis* (Bloch), exposure to hyperosmotic stress did not influence the activity of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, causing significant elevations

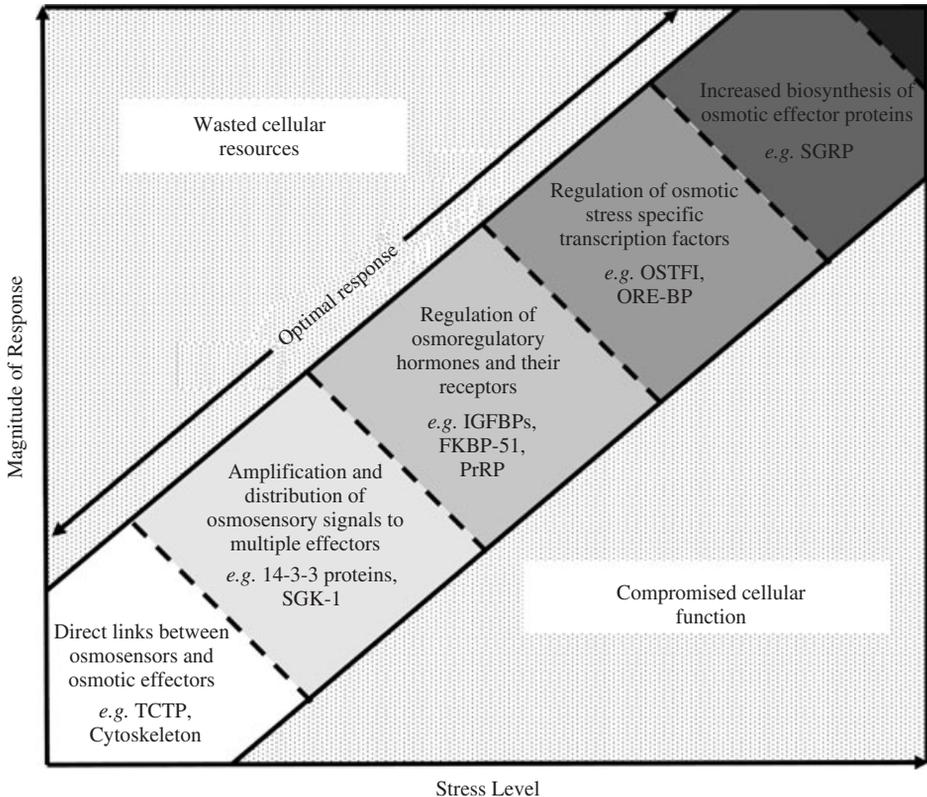


FIG. 2. Co-ordination of osmotic stress responses by osmosensing and signal transduction events. Adaptive responses to osmotic stress in euryhaline fish are co-ordinated through distinct osmosensing and signal transduction events appropriate for specific osmotic stress levels (*i.e.* the intensity of the stressful environmental stimulus). Minor osmotic stress can be overcome rapidly through modifying the activity of the existing ion transport proteins (bottom left panel) and may be achieved by directly linking osmosensors to osmotic effectors (*e.g.* TCTP and the cytoskeleton). As osmotic stress levels increase, osmosensory signals may be amplified and distributed to a greater variety of osmotic effectors through cell signalling cascades (*e.g.* SGK and 14-3-3 proteins). If osmotic imbalances persist, large-scale adaptive mechanisms are required. These extensive responses are orchestrated by osmotic stress-specific transcription factors (*e.g.* OSTF-1 and ORE-BP) and hormone-regulated signalling pathways (*e.g.* FKBP-51, IGFBPs and PrRP) that alter the abundance of osmotic stress effector proteins (*e.g.* facilitated by SGRP) and increase the turnover and differentiation of specific gill cell types in an effort to enhance the overall capacity of ion transport. The development of this type of progressive response that integrates rapid small-scale responses with slower acting large-scale adaptive mechanisms may stem from a need to balance the quantity of cellular resources directed towards an adaptive response with the level of stress encountered. Under this system, minor changes in intracellular osmolality are met with an appropriate small-scale response, whereas more severe cases of osmotic stress demand a larger investment of resources to properly restore cell homeostasis. Underestimating the severity of stress would conserve cellular resources but leave cells vulnerable to osmotic stress-related damage and compromise cell function (area under panels). In contrast, overestimating the degree of stress unnecessarily diverts cellular resources away from growth and reproduction (area above panel).

in plasma osmolality. Additional experimental evidence suggested that *H. fossilis* gills were unable to reverse polarity from ion uptake in a freshwater environment to ion excretion at elevated salinities (Sherwani & Parwez, 2008).

The presence of osmotic effector proteins in these stenohaline fish species, despite their insensitivity towards osmotic stress, lends support to the hypothesis that increased tolerances towards osmotic stress occur in part through the modification of osmosensing and signal transduction events that enable changes in the spatial and temporal activity or abundance of downstream effector proteins rather than the evolution of novel osmotic stress effector proteins themselves. By this logic, the synergistic actions of upstream osmosensing and signal transduction events, such as those described for SGK-1, TCTP, FKBP-51, IGF1Rs and others, make appealing candidates as contributors to the molecular basis of euryhalinity in fish. This hypothesis is compatible with a recent analysis of stress response evolution that indicated most of the variation in stress responsive genes (in both sequence and gene content) lies in environmental sensing and signal transduction genes, while the least variation is in the effector proteins that carry out the response (Singh *et al.*, 2008). One possible explanation for this trend is that osmotic effector proteins may be more directly linked to other fundamental cell processes distinct from osmoregulation, making their functional properties difficult to manipulate because an improvement towards one function would be offset by impairments in others (Waxman & Peck, 1998; Hartwell *et al.*, 1999). For example, the Na<sup>+</sup>, K<sup>+</sup>-ATPase not only serves as an important effector protein in the maintenance of osmotic homeostasis but also plays fundamental roles in establishing the chemical gradients that propagate electric signals and drive the secondary active transport of other essential molecules (Jorgensen *et al.*, 2003). As a result, it may be difficult to direct Na<sup>+</sup>, K<sup>+</sup>-ATPases exclusively towards osmoregulatory functions without corresponding effects on important processes unrelated to osmoregulation. As an alternative, natural selection has focused on appropriately regulating effector proteins during osmotic stress.

A possible caveat to the theory that euryhalinity in fish may be dependent on osmosensing and signal transduction involves the presence of Na<sup>+</sup>, K<sup>+</sup>-ATPase isoforms that are differentially regulated in a salinity-dependent manner. These studies demonstrate divergent functions for Na<sup>+</sup>, K<sup>+</sup>-ATPase  $\alpha$  isoforms during freshwater and saltwater acclimation and hint at the evolution of novel osmotic stress effector proteins. Euryhaline *O. mykiss* possess five Na<sup>+</sup>, K<sup>+</sup>-ATPase  $\alpha$  isoforms, with mRNA levels of  $\alpha$ 1a and  $\alpha$ 1b isoforms decreasing and increasing during hyperosmotic stress, respectively (Richards *et al.*, 2003). This phenomenon has also been documented in Pacific and Atlantic salmon species during acclimatization to new salinities (Bystriansky *et al.*, 2006; McCormick *et al.*, 2009). The possession of genes encoding  $\alpha$ 1a and  $\alpha$ 1b isoforms, however, does not preclude euryhalinity. In stenohaline populations of land-locked Arctic char *Salvelinus alpinus* (L.) that are unable to acclimatize to sea water, expression of the Na<sup>+</sup>, K<sup>+</sup>-ATPase  $\alpha$ 1a isoform quickly decreases in response to elevated salinities, but levels of the  $\alpha$ 1b isoform thought to facilitate hyperosmotic stress adaptation in euryhaline species remain unchanged (Bystriansky *et al.*, 2007). A similar situation has been described for land-locked stenohaline populations of *S. salar* (Nilsen *et al.*, 2007). These data suggest that the osmotic stress-specific functions of  $\alpha$ 1a and  $\alpha$ 1b Na<sup>+</sup>, K<sup>+</sup>-ATPase isoforms are still dependent on upstream regulatory cues for their salinity-specific expression, and therefore, reinforce the potential importance of osmoregulatory signal transduction in the evolution of euryhalinity.

The apparent significance of upstream regulatory processes to the evolution of euryhalinity should provide additional impetus for more detailed research surrounding

osmosensing and signal transduction, which have received considerably less attention than osmotic effector proteins. The need to further investigate the role of osmosensing and signal transduction during osmotic stress responses may be especially relevant in the context of the increased use of high-throughput technologies that continue to drive future research in the area (Douglas, 2006; Steinberg *et al.*, 2008). Used in isolation, these genomic-based tools may limit insight into osmosensing and signal transduction because these events are dominated by processes that occur post-translationally and would be undetected by these methods. In the proceeding sections, experimental approaches and future directions that may best address this issue are discussed.

## **FUTURE DIRECTIONS: SYSTEMS PHYSIOLOGY AND REDIRECTING KROGH'S PRINCIPLE**

### **RE-ESTABLISHING A COMPARATIVE APPROACH**

Researchers investigating osmotic stress responses in fish have typically relied on Krogh's principle for the selection of their study organisms. That is, for every problem, there exists an organism uniquely suited for its analysis (Krogh, 1929; Gracey & Cossins, 2003; Strange, 2007). This mantra has directed osmoregulatory research in fish towards strongly euryhaline species, with the aim of gaining insight into the processes underlying their extraordinary ability to tolerate osmotic fluctuations. Although this approach has proved successful in developing a framework for osmoregulatory processes and osmotic stress responses in fish, it has done little to expose the molecular mechanisms that separate euryhaline fish from their stenohaline counterparts. A large contributing factor to this dilemma stems from an apparent lack of comparative studies that concurrently investigate osmotic stress responses in euryhaline and stenohaline species. Although some recent studies have provided an excellent starting point by demonstrating that euryhalinity may have emerged by modifying interactions among upstream regulatory proteins during osmosensing and signal transduction events (Hyndman & Evans, 2009), the actual protein players involved or regulatory processes governing these interactions remain largely obscured or are based solely on conjecture.

Typically, high-throughput technologies such as microarrays or proteomics represent ideal approaches when searching for environmentally regulated molecules. Such profiling experiments constitute an unbiased screen of genes, proteins or metabolites influenced by environmental perturbation (Gracey & Cossins, 2003). Although transcriptomic-based approaches have been effective in detecting a sub-set of osmoregulatory events that occur through changes in gene expression, a detailed interpretation of these data only reinforce the potential importance of osmosensing and signal transduction events that occur in the absence of changes in gene expression (Evans & Somero, 2008). Thus, a major obstacle, and one principle underlying the lack of information on osmosensing and signal transduction events to date, is the absence of a comprehensive, large-scale screening tool to identify osmotically regulated post-translational processes. One potential solution lies in adopting modified proteomic-based approaches that are becoming increasingly tractable for the analysis of post-translational modifications and protein–protein interactions (Mann & Jensen, 2003; Seo & Lee, 2004; Fu *et al.*, 2009). Newly developed protocols allow

the detailed study of protein complexes by concurrently identifying genuine protein partners, detecting variable composition of complexes upon cellular disturbances and localizing phosphorylation sites (Pflieger *et al.*, 2008). Integrating this technology into future studies of osmotic stress responses would represent a significant advance in the field. Another possible solution lies in employing the so-called systems-level approaches, which permits the functional characterization of individual genes but still capitalizes on the data-generating power of genomic and proteomic technologies.

## SYSTEMS PHYSIOLOGY

Although a precise definition of systems physiology is difficult, a major thread of this philosophy attempts to interpret the consequences of gene expression within the context of other molecules and ultimately the entire intact organism (Ideker *et al.*, 2001; Dow, 2007; Gracey, 2007; Strange, 2007). One means to meet these criteria is to incorporate functional assessments of individual genes of interest by manipulating expression upwards or downwards, and subsequently determining the biological consequences at various levels of organization (Strange, 2007). In this way, researchers can assess the actual stress-specific functions of genes rather than interpreting function from gene expression patterns or gene ontology databases. Implementing this type of approach may be better suited for investigations into osmosensory signal transduction in fish. For example, the functional contributions of the osmosensory proteins discussed previously could be assessed by first disrupting their expression and subsequently re-evaluating organismal performance during osmotic stress using a suite of cellular and physiological level analyses such as Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme activity assays, monitoring the intracellular concentrations of ions or hormones or generating survivorship curves. Importantly, functional assessments at the molecular level could still exploit the power of genomic- and proteomic-based gene expression tools in order to determine the effect of the manipulated gene on the expression of other genes. These molecular level assessments could be used to generate and refine osmotic stress gene regulatory networks and to identify candidate gene hubs whose activity is disproportionately important to the function of the gene network (*i.e.* osmotic stress adaptation). Genes of critical importance could then be targeted in more directed experiments aimed at investigating their specific functional properties.

An important caveat to systems-based studies is that experiments of this nature are dependent on a number of experimental tools. Most notable are extensive genetic resources, such as large amounts of sequence information, and the availability of mutant strains and targeted gene manipulation capabilities. Using these requirements as a foundation in applying Krogh's principle, the number of suitable experimental fish models to implement a systems-based approach to osmotic stress responses is restricted to just a few key species (Cossins & Crawford, 2005). The zebrafish *Danio rerio* (Hamilton) is a stenohaline freshwater teleost, accompanied by extensive resources including a fully sequenced genome, hundreds of readily accessible mutant strains, established strategies for targeted gene manipulation and commercially available microarray platforms. Parallel time-course analyses of osmotic stress responses in the stenohaline *D. rerio* and genomics or proteomics enabled euryhaline fish (such as *F. heteroclitus*, *S. salar*, *O. mykiss*, *G. mirabilis*, *S. aurata* and others; Douglas, 2006) would lay much of the groundwork in identifying molecules

that regulate osmotic stress tolerances. Subsequent experiments could address the functional effects of specific genes during osmoregulation in *D. rerio* by capitalizing on the large number of previously generated and easily accessible mutant strains, of which genes encoding signalling proteins are prominent (Haffter & Nüsslein-Volhard, 1996; Haffter *et al.*, 1996; Amsterdam *et al.*, 2004; Amsterdam, 2006). These types of experiments could also be expanded through the use of targeted gene knockdown strategies such as the microinjection of morpholino-modified anti-sense oligonucleotides (Summerton, 1999; Ekker & Larson, 2001; Heasman, 2002). Although studies of this nature are complicated by inherent discrepancies in comparing transcriptomic or proteomic profiles between species, the fact that euryhalinity appears to have evolved several times throughout fish evolution (possibly through divergent mechanisms) and the ability of inbred laboratory strains to induce stress responses equivalent to their wild counterparts, these studies would nonetheless provide testable hypotheses to be addressed through more directed experiments in non-model species. From this perspective, studies on model and non-model species are viewed as complementary, with non-model species best used in the discovery phase of research, whereas model species then offer the tractability to better pinpoint underlying molecular mechanisms (Gracey & Cossins, 2003).

## CONCLUSION

At the molecular level, achieving ion homeostasis during osmotic stress is contingent upon a cell's ability to recognize and quantify environmental osmolality and arrange an appropriate response. Integral are the co-ordinated activities of osmosensors, which activate suitable signal transduction pathways, signal transducers, which relay molecular messages to specific target molecules, and effectors, which work in concert to restore homeostasis (Fiol & Kültz, 2007). Effector mechanisms involved in osmotic adaptation of a great many fish species have been identified and characterized in detail. In contrast, only modest attention has been directed towards the molecular osmosensing and signal transduction events leading up to their activation. Recent efforts, however, have begun to shed light on the identity and expression of molecules that function during the early phases of osmotic stress adaptation. These data indicate that osmosensory signal transduction has far-reaching effects during osmotic stress responses and orchestrate cellular and physiological responses across a spectrum of stress levels. Emerging evidence posing a link between osmosensory signal transduction and the evolution of euryhalinity underscores this importance and suggests that upstream regulatory events may represent plastic and evolutionarily accessible components of osmoregulatory networks. As new technologies increase our ability to dissect the functional basis of osmosensing and signal transduction pathways *a priori*, insights into the molecular mechanisms underpinning osmotic stress adaptation and euryhalinity are certain to emerge. Adopting the so-called systems approaches may be the key to establishing unified principles that underlie osmotic stress adaptation in a broader range of fish species.

Topics addressed in this review have relied heavily upon a vast network of data generated by others, and I apologize that not all of that hard work could be recognized here. The quality of this manuscript was enhanced by helpful comments from G. N. Somero (Stanford University) and by two anonymous reviewers. This work was funded by the Partnership for the

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