Euryhalinity in an Evolutionary Context

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Euryhalinity in an Evolutionary Context

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This chapter focuses on the evolutionary importance and taxonomic distribution of euryhalinity. Euryhalinity refers to broad halotolerance and broad halohabitat distribution. Salinity exposure experiments have demonstrated that species vary tenfold in their range of tolerable salinity levels, primarily because of differences in upper limits. Halotolerance breadth varies with the species' evolutionary history, as represented by its ordinal classification, and with the species' halohabitat. Freshwater and seawater species tolerate brackish water; their empirically-determined fundamental haloniche is broader than their realized haloniche, as revealed by the halohabitats they occupy. With respect to halohabitat distribution, a minority of species (<10%) are euryhaline. Habitat-euryhalinity is prevalent among basal actinopterygian fishes, is largely absent from orders arising from intermediate nodes, and reappears in the most derived taxa. There is pronounced family-level variability in the tendency to be halohabitat-euryhaline, which may have arisen during a burst of diversification following the Cretaceous-Palaeogene extinction. Low prevalence notwithstanding, euryhaline species are potent sources of evolutionary diversity. Euryhalinity is regarded as a key innovation trait whose evolution enables exploitation of new adaptive zone, triggering cladogenesis. We review phylogenetically-informed studies that demonstrate freshwater species diversifying from euryhaline ancestors through processes such as landlocking. These studies indicate that some euryhaline taxa are particularly susceptible to changes in halohabitat and subsequent diversification, and some geographic regions have been hotspots for transitions to freshwater. Comparative studies on mechanisms among multiple taxa and at multiple levels of biological integration are needed to clarify evolutionary pathways to, and from, euryhalinity.

1. INTRODUCTION

In the living world, transitions beget diversification. Classic cases of adaptive radiation began with colonization of a new patch of ground such as a relatively unoccupied island or lake. Changes in morphology and physiology permitting exploitation of new habitats ushered in ascendance of major groups such as tetrapods and birds. In macroevolutionary history, taxa that endured mass extinction events often expanded into newly-vacated ecospace. These homilies on diversification have a common moral, one that is close to a truism: the generalist is more likely to leave an evolutionary legacy than the specialist. In this review we endeavor to support this vague but lofty position for one group of generalists, the euryhaline fishes.

In this chapter we employ both physiological and ecological meanings of euryhalinity. Physiological euryhalinity focuses on halotolerance: it is defined as the capability of surviving in both freshwater (FW, ≤0.5 ppt) and seawater (SW, 30-40 ppt). Ecological euryhalinity focuses on halohabitat: it is defined as the occurrence in both FW and SW (and brackish water [BW, 0.5 – 30 ppt]). Ecological euryhalinity implies physiological euryhalinity; halohabitat can include both FW and SW only if halotolerance is sufficiently broad. However, the converse is not necessarily true, because a species may have a broad halotolerance but a restricted halohabitat. The distinction between the physiological and ecological facets of tolerance is the distinction between the fundamental niche, reflecting physiological capacity, and the realized niche, reflecting other ecological and historical factors (Whitehead, 2010). We begin by considering how halotolerance is characterized through empirical work, and how it is distributed among the fishes across taxa, halohabitat, and ontogenetic stage. Using the halotolerance data we designate groups of species with similar tolerance limits. We then examine the distribution of euryhalinity in terms of halohabitat among the fishes, both in deep evolutionary time at the origin of the vertebrates, as well as among the major groups of extant fishes. Finally, we review the evolutionary potential of euryhalinity, through cases of diversification arising within taxa that had the physiological capability of handling a broad range of salinity levels, occurring in habitats prone to subdivision.

2. DIVERSITY OF HALOTOLERANCE

In this section, we review how halotolerance is empirically determined and examine how halotolerance is distributed among the ray-finned fishes. Halotolerance is tested and quantified in a variety of ways, and this part of the review is intended to improve comparability among future studies. In the second part of the section we compare halotolerance limits across 141 species of ray-finned fishes; we assess variability in halotolerance limits and halotolerance breadth with respect to higher taxa and habitat groups, and we resolve ray-finned fishes into groups with similar halotolerances. Additional data on the halotolerance of fishes inhabiting extreme environments are presented by Brauner et al. (2013).

2.1. Empirical issues in halotolerance analysis

To test halotolerance limits, experimental subjects are exposed to altered salinity levels in several ways. One approach is to rear subjects from fertilization at constant salinity, and record the effect of salinity level on hatching and subsequent endpoints such as survival(e.g., Bohlen, 1999). This design rarely appears in the literature, presumably because few investigators begin work with subjects before hatching. A second approach (hereafter referred to as the 'direct' design) entails altering environmental salinity rather instantaneously.

Endpoints of different groups of subjects exposed to different salinity levels are compared for a prescribed period. A third approach (hereafter referred to as the 'gradual' design) entails an incremental change in salinity on a prescribed schedule. Endpoints are monitored as salinity changes. The direct and gradual design approaches are represented in Fig. 1.

The direct and gradual methods both have virtues. The direct method focuses on the capacity of acute responses to cope with environmental change. For some ecological inquiries, such as the effort to link halotolerance to transient changes in salinity to distribution of FW fishes in estuaries, direct transfers among salinity levels may be more appropriate than gradual alterations of salinity. The simplicity of the experimental treatment in the direct method maximizes comparability among studies. The gradual design evidently permits a better assessment of halotolerance to chronic exposure, and requires fewer fish. Differences in the magnitude of salinity change and time at a given salinity can limit comparisons among studies. Because these designs are complementary rather than duplicative, we suggest that when possible investigators should use both in assessing halotolerance limits.

Fig. 1 here

Death appears to be the only reliable end point for determination of halotolerance when a species or life stage is examined for the first time. Some studies (Peterson, 1988; Scott et al., 2007) interpret the change (or constancy) of plasma osmolality over a range of salinity levels as indicative of intolerance (or tolerance). Plasma osmolality is a valuable metric of osmoregulatory performance, but interpreting it as a metric of halotolerance makes the assumption that departure from the plasma osmolality norm is tantamount to loss of function. This assumption is unwarranted without prior empirical demonstration for the species; some euryhaline species exhibit significant changes in plasma osmolality over the range of tolerated salinity, at least temporarily (Lotan, 1971; Nordlie, 1985; Nordlie, 2009; Marshall, 2013; Shrimpton, 2013). However, once a species has been examined and thresholds for mortality have been determined, then plasma osmolality can become an acceptable substitute (for instance in the seawater challenge test widely used in studies on salmon smolts Blackburn and Clarke, 1987). Loss of equilibrium has been used in some studies (Young and Cech, 1996) with benefits of minimizing destructive use of subjects and/or permitting their use at the endpoint for determinations that require living subjects, such as plasma osmolality. However, in our experience subjects do not always demonstrate a loss of equilibrium before death due to high or low salinity exposure.

Tolerance is conventionally quantified as the central tendency of the distribution of stressor levels at which subjects succumb. It is unfortunate that many, if not most, studies investigating halotolerance do not provide statistics that summarize salinity limits. When provided, the most commonly used halotolerance statistic is referred to as the LC_{50} or LD_{50}, the concentration or dose at which half of the subjects are expected to die at a prescribed time point.

Quantifying the LC_{50} requires an estimation procedure. In many cases, the procedure is arithmetic or graphical, such as linear interpolation between two dose-mortality points to estimate the dose at which mortality was 50% (Kendall and Schwartz, 1968; Kilambi and Zdinak, 1980; Watanabe et al., 1985; Britz and Hecht, 1989; Hotos and Vlahos, 1998; Garcia et al., 1999; Fashina-Bombata and Busari, 2003). A weakness of this approach is its possible reliance on a subset of the survival data. A statistical model relating the probability of survival to salinity is a better approach. Several regression models that are employed in environmental toxicology studies also appear in the halotolerance literature. Proportion surviving at a prescribed time has been modeled by linear or multiple linear regression (de March, 1989; Lemarie et al., 2004); however, probabilities rarely are distributed so that linear regression would be appropriate. More common approaches to estimating LC_{50} involve logit models (logistic regression) and probit models (examples of probit modeling include Cataldi et al., 1999; Mellor and Fotedar, 2005); (examples of logistic regression in salinity tolerance studies include Ostrand and Wilde, 2001; Faulk and Holt, 2006). Hamilton et al. (1977) identify several shortcomings of these methods and describe the Spearman-Karber method for calculating LC_{50}, which has been used in at least one salinity tolerance study (Bringolf et al., 2005).

Methods for deriving time-independent LC_{50} estimates have not been widely used in the halotolerance literature. In most studies, particularly when the direct method is used, additional exposure time at any salinity level would result in additional mortality. Hence most LC_{50} estimates in the halotolerance literature are time-dependent; extending the prescribed time at which the effect of salinity on mortality is assessed has the effect of moderating the LC_{50} (i.e. it increases the low limit and decreases the high limit). The range of a parameter such as salinity or temperature over which the extent of mortality is time exposure-dependent is known as the lower or upper ‘zone of resistance’ and lies just beyond the ‘zone of tolerance’ within which the parameter level does not affect or induce mortality (Brett, 1956). The boundary between the zone of tolerance and the lower or upper zone of resistance is referred to as the ‘incipient lethal level’, representing the most extreme value that can be tolerated for an indefinite period. Using line-fitting methods apparently first suggested by Doudoroff (1945) and modified by Green (1965), incipient lethal salinity limits have been determined by relatively few investigators (Reynolds and Thomson, 1974; Reynolds et al., 1976; Pfeiler, 1981). Incipient lethal estimates of LC_{50} are especially valuable, because they are time-independent and are therefore most comparable among studies. We recommend that incipient lethal salinity limits be incorporated into direct design experiments. With few exceptions, the LC_{50} halotolerance limits compiled in this review are time-dependent.

2.2. Interspecific variability in halotolerance

We accumulated a dataset on halotolerance by surveying four decades of salinity exposure experiments. We used the Aquatic Sciences and Fisheries Abstracts database for references from 1971 to 2012. An initial search using the terms "salinity tolerance" or "salt tolerance" and fish or fishes for all available years yielded 995 references. We harvested from this list references that presented salinity challenge experiments and quantified tolerance endpoints, which were mortality rates except in a few occasions reporting loss of equilibrium (Young and Cech, 1996). Our search revealed surprisingly few references concerning salinity tolerance in elasmobranchs (Sulikowski and Maginniss, 2001), none on sarcopterygians, and therefore confined our analysis to studies on Actinopterygii. Our analysis is based on a set of 108 studies, reporting results published as early as 1968, on 141 species (Table S1).

We divided experimental results into groups according to life stage of the experimental subjects and according to the method used to determine tolerance limits. We categorized life stage as larva or juvenile and adult, because analyses of larvae often demonstrate pronounced changes in tolerance with development (Varsamos et al., 2001; Varsamos et al., 2005; Zydlewski and Wilkie, 2013). Studies examining tolerance through metamorphosis (Hirashima and

Takahashi, 2008) were placed among studies on larvae. Most studies involving field-collected individuals reported the size of experimental subjects if not the life stage, but in a few cases the life stage was inferred based on method of capture or other details (and in every case was identified as juvenile or adult). A small number of studies (Reynolds and Thomson, 1974; Reynolds et al., 1976) included experiments on both larvae and subsequent life stages. We categorized the experimental method as direct or gradual (Fig. 1). A few cases in which salinity was changed over a brief interval (less than 24 h, e.g., Chervinski, 1977b; Tsuzuki et al., 2000) relative to the time course of response were categorized as direct, and studies that quantified the tolerance of individuals reared at different salinity levels from early life stages (e.g., Perschbacher et al., 1990; Bohlen, 1999) were categorized as gradual. When subjects were tested at multiple temperatures, we used results from temperatures that imposed the lowest level of mortality. The aggregation by species and stage yielded 168 estimates of lower and/or upper halotolerance limits. Determination of halotolerance limits was often not possible from the results, because subjects tolerated the most extreme salinity treatments used. As was frequently the case, when survival was high in FW we imputed a lower halotolerance limit of 0 ppt. Having imputed lower limits in this way, estimates of halotolerance breadth (the range of salinity levels that can be endured) were possible in most cases; lower or upper tolerance limits could not be determined for 7 and 32, respectively, of the 168 records.

Fig. 2 here

Most species tested by the direct or gradual method tolerated FW (Fig. 2A, B). The mean lower salinity limit among direct-method experiments was 1.2 ppt (SD = 2.5) and among gradual-method experiments it was 0.19 (SD = 0.90). The most common lower tolerance limit was 0.5 ppt or below (70 of 98 species tested by direct method, 61 of 63 tested by gradual method). The highest value for lower LC_{50} estimated by the direct method was 16 ppt, observed for *Scophthalmus maximus*. The highest value for lower LC_{50} estimated by the gradual method was 7 ppt, observed for *Parablennius sanguinolentus*.

Upper tolerance limits were broadly distributed among species (Fig. 2A, B). The mean upper salinity limit among direct-method experiments was 25 ppt (SD = 16) and among gradual-method experiments it was 52 ppt (SD = 36). The upper limit was distributed in a skewed or multimodal fashion in both datasets. Among the direct-method upper tolerance limits there was a clear mode close to isotonic salinity levels, around 10 – 15 ppt (Fig. 2A). The lowest values for upper LC_{50} determined by the direct method were 6.7 and 6.8 ppt; both of these limits were observed for catfishes (*Hoplosternum thoracatum* and *Heterobranchus longifilis* respectively). The highest value for upper LC_{50} estimated by the direct method was 65 ppt, observed for *Cyprinodon dearborni*. Among the gradual-method limits there was a clear mode around 20 ppt (Fig. 2B). The lowest value for upper LC_{50} determined by the gradual method was 6.6 ppt, observed for larval *Cobitis taena*. The highest values for upper LC_{50} estimated by the gradual method were 125 ppt and 126 ppt, for *Cyprinodon variegatus* and *Mugil cephalus*.

Fig. 3 here

Halotolerance breadth varied an order of magnitude or more among species. Estimates of breadth determined by the direct method (Fig. 3A) varied from 6.7 ppt (*Hoplosternum thoracatum* and *Heterobranchus longifilis*) to 59 ppt (*Leuresthes sardina* larvae; mean = 23, SD = 14). The values for breadth determined via direct challenge were distributed around a pronounced single mode at 10-15 ppt; the distribution for larval-stage subjects was comparable to that for later ontogenetic stages. Estimates of tolerance breadth determined by the gradual method (Fig. 3B) were about twice as long as estimates determined by the direct method, varying from 6 ppt (*Cobitis taena* larvae) to 125 ppt (*Cyprinodon variegatus*; mean = 50, SD = 34). The values for breadth determined via gradual salinity increases had a lower mode centered around 20 ppt.

Halotolerance breadth varied by order. Species in orders within the Otophysi all exhibited low values for breadth. The median value for breadth determined via the direct method for fishes in the Cypriniformes and Siluriformes was 13 ppt and 10 ppt respectively (Fig. 4A). Breadth values for species in other well-represented orders were variable and the breadth distributions were comparable to each other (median values 20 to 30 for the direct method).

Fig. 4 here

We aligned halotolerance to halohabitat for each species. There have been few efforts to determine whether laboratory-determined salinity tolerance correlates with field limits, i.e. whether the fundamental niche and realized niche correspond. Kefford et al. (2004) found that direct-transfer experiments underestimated halohabitat breadth; early life stage and adult fish were often field-collected in salinity levels higher than direct-transfer experiments indicated they could tolerate. Gradual-method determinations of tolerance were better predictors of field distribution among the Australian fishes examined by Kefford et al. (2004). To test for correspondence of fundamental and realized haloniche among the species in our review, we downloaded data on halohabitat from FishBase (download 22 February 2012); every species in the database is listed as present or absent in FW, SW, and BW. We encoded species in the halotolerance dataset as FW if they were present only in FW, SW if they were present only in SW, and BW if their halohabitat included BW; some of these species are diadromous and some are nonmigratory.

Halotolerance limits and breadth varied among FW, BW and SW fishes, but the experimentally-determined fundamental haloniche was typically broader than the realized haloniche. Lower and upper halotolerance limits were lowest in FW species, intermediate in species whose halohabitat included BW, and highest in SW species (Table 1). On average, BW fish tolerated salinity ranging from FW to nearly full-strength SW when subjected to direct testing, and up to about 2 times SW when subjected to gradual testing. The mean upper tolerance limit for FW fish was about half-strength SW when determined by the direct method. However, FW fishes were able to tolerate SW when subjected to gradual salinity increases. Halotolerance studies therefore indicate that FW fishes generally have the capacity to survive in BW or SW. The mean lower tolerance limit for SW fish was higher than FW but well below the salinity of isotonicity, indicating that SW fishes also have the capacity to survive in BW. Halotolerance breadth also varied as expected by the habitat occupied (Fig. 4B). Quartile values of breadth distribution were lower among FW fishes than BW and SW fishes, but some FW fishes had breadth values as high as fishes in the other groups. Breadth values for BW and SW fishes were generally from 20 ppt to 50 ppt when determined by the direct method and were more than 70 ppt for BW fishes when determined by the gradual method. Hence, in contrast to previous findings (Kefford et al., 2004), the empirically-determined fundamental haloniche is broader than the realized haloniche.

Table 1 here

A comparable amount of variability in halotolerance breadth was explained by taxon and halohabitat, while less was explained by ontogenetic stage. Analyses of variance including all three effects (representing taxon by order), explained one half to two-thirds of the variance in halotolerance breadth determined both by the direct and gradual methods (Table 2). Taxon

Table 2 here

and habitat were significant ($p < 0.05$) in both full models and stage was not. To compare the contributions of the three effects to variance in halotolerance breadth, we examined changes in R^2 when each effect was eliminated from the full model, and the value of R^2 when each effect was by itself in a one-way model. Taxon explained more variance than halohabitat, from one-third more to twice as much. Ontogenetic stage was a weak predictor in both datasets; there is a significant stage effect only in analysis of the gradual dataset in which it is the sole predictor (mean breadth for larvae and juveniles + adults = 16 [N = 5] and 53 [N = 48], respectively). We conclude that the degree of euryhalinity is predicted both by the present habitat of the species and by the evolutionary history of the species (i.e. the ancestral halohabitat) represented by the taxon.

We conducted cluster analyses to define groups of species with similar halotolerances. Our goal was to define a range of halotolerances that distinguish euryhaline from stenohaline fishes. In principle, stenohaline SW species should be intolerant of salinity substantially below isosmotic levels (9-10 ppt) and stenohaline FW species should be intolerant of salinity substantially above isosmotic levels. Euryhaline species should have the lower halotolerance limits of stenohaline FW species and the upper halotolerance limits of stenohaline SW species. The variables used for clustering were the upper halotolerance limit, and in the direct method dataset, halotolerance breadth (in the gradual method dataset there was perfect collinearity between upper tolerance limit and tolerance breadth). We conducted clustering by the centroid method because we expected our clusters to be of unequal size (variable number of species per group) and dispersion (variable range of tolerance breadth). Because results for larvae were different from those for juveniles and adults, we restricted the cluster analysis to experiments employing only juveniles and adults. If there were multiple determinations for a species we did not average them. Inferential tools are not well established in cluster analysis and we did not attempt to assess the significance of cluster groupings. In the direct method dataset, we identified two disparate groups that we designate as empirically stenohaline and euryhaline; the tolerance limits of species by group are listed in Table S2 (stenohaline tolerance breadth 7-35 ppt, euryhaline tolerance breadth 43-58 ppt) The groups are clearly separated based on centroid distance: the distance between clusters when the dataset is divided in two (standardized distance = 1.8) is large relative to the distance separating clusters at the next split in the tree (standardized distance = 0.7). In the gradual method dataset, we identified three groups that we designate as stenohaline FW and two levels of euryhaline: euryhaline-FW and euryhaline. The tolerance limits for each species are listed by group in Table S3 (stenohaline FW tolerance breadth 9-46 ppt, euryhaline-FW tolerance breadth 55-80 ppt, euryhaline tolerance breadth 99-125 ppt). The division into groups is more subtle in the gradual dataset: the centroid distance between clusters does not change as dramatically as the number of clusters increases from two (standardized distance = 1.25) to three (standardized distance = 0.75).

Our analysis and conclusions are unavoidably biased by the selection of species that have been subjected to tolerance tests. Tolerance tests such as these are often directed at revealing limits in broadly tolerant species; indeed, many of the studies in our review were motivated in some way to discern the limits of species known to be euryhaline, because of an interest in the culture or the ecology of the species. Most marine fishes that have been tested, even those that are not regarded as estuary-dependent, can be regarded as tolerance-euryhaline: they have halotolerance limits well below isotonic salinity levels and a broad tolerance breadth. Only a few studies that we have found suggest that an SW species is limited to salinity levels above that at which it is expected to be isotonic, and we hope that more studies on SW fishes will be designed to test whether this limit is more prevalent than the existing literature suggests.

3. EVOLUTIONARY TRANSITIONS IN EURYHALINITY

Is euryhalinity a basal condition in fishes? How is it distributed phylogenetically; is there an evident phylogenetic signal among higher taxa, suggesting that gain or loss of broad tolerance occurred in deep nodes of the ‘fish tree’, or alternatively is broad tolerance distributed uniformly among major fish groups, suggesting that lineages routinely switch from broadly to narrowly tolerant and back again? To develop answers to these questions, in this section we examine the debate over the environment in which the earliest fishes evolved, and we analyze how habitat-euryhalinity is distributed among broad taxonomic groups of extant fishes. We also review recent studies that have used phylogenetically-informed analysis techniques to map salinity tolerance or halohabitat as a character.

3.1. Euryhalinity and halohabitat transitions in early fishes

Consideration of how euryhalinity was temporally and phylogenetically distributed among the earliest vertebrates must begin with the question of the halohabitat in which the first vertebrates evolved. Overall evidence supports the hypothesis that the earliest fishes were SW and stenohaline, followed by euryhalinity in some lineages and diversification in FW as well as SW (Evans et al., 2005). Early discussions (e.g. Smith, 1932; see also Vize, 2004) favored an

FW origin, based on the predominance of a glomerular kidney in extant vertebrates and the intermediate concentration of inorganic ions in body fluids. Neither of these functional characters has proven to be decisive evidence for habitat of origin. Filtration by the glomerulus drives ionoregulatory functions of the kidney (particularly of divalent ions) in SW as well as FW habitats. Furthermore, lower ionic concentration of plasma can plausibly be a derived rather than ancestral condition via selective advantages of more precisely tuned system of reactive tissues relying on membrane potentials (Ballantyne et al., 1987). Recent papers propose alternate scenarios in which the earliest vertebrates were estuarine or euryhaline. Ditrich (2007) suggests that vertebrates originated as osmoconformers in BW. According to his argument, protovertebrate kidney tubules functioned to maintain ion homeostasis and to recover metabolically important solutes but would not have been capable of high-rate ionic exchange necessary for osmoregulation or urea retention. Ditrich's proposal has the substantial difficulty that it confers a requirement for stenohalinity on an organism in an estuary, which is likely to have highly variable salinity. Griffith (1987) proposes an anadromous life history for the protovertebrate, citing ancestral features of the kidney that he regards as evidence for hyperosmoregulation, and adaptive explanations for virtually all features shared by basal and derived fishes in terms of the advantages these features confer during migration. Molecular phylogenetic analysis also provides support for the euryhaline origin hypothesis. In contrast to morphologically-based phylogenies, which identify stenohaline SW hagfish as basal to all other fishes, molecular analyses resolve jawless fishes as a monophyletic group (Heimberg et al., 2010). This placement implies that stenohalinity in the hagfish may be a derived condition, in which case the ancestral condition could be euryhalinity. An ecological difficulty of the euryhaline origin scenario is the harshness of fluvial habitats during the Cambrian; in the absence of banks stabilized by terrestrial or aquatic plants, waters would have been turbid, carried high sediment loads, and been completely unproductive. An additional count against the alternative scenarios is that the recent fossils illuminating the earliest emergence of Cambrian vertebrates or their precursors have been found in coastal SW deposits (Hagedorn 2002).

Transitions among halohabitats were frequent during the Paleozoic diversification of fishes, suggesting that physiological and ecological barriers were not difficult to surmount. Halstead (1985) discerned a proliferation of endemic genera and species upon colonization of brackish and FW habitats in several major groups (e.g., thelodonts, cephalaspids). Diversification was less clearly associated with paleohabitat transition in other groups (e.g., Janvier et al., 1985 on osteostracans). Friedman and Blom (2006) assessed the paleoenvironment of basal actinopterygians using cladistic methods. They, like others, cautioned that paleoenvironmental reconstruction is subject to many uncertainties, especially for Paleozoic fossils for which there are no extant phyletic analogues. They proposed an SW origin for the clade based on earliest upper Silurian deposits in Sweden and China, and early Devonian SW diversification. Middle Devonian deposits record the appearance of actinopterygians in FW. Their evidence suggested four separate penetrations of FW, leading them to conclude that “the assembly of the earliest freshwater ecosystems was dominated not by unique, isolated ‘seedings’ of these novel environments by primitively marine clades, but instead by iterative and relatively frequent colonization events”. Other analyses indicated that there were many transitions to FW, supported by multiple instances of genera that occurred in both FW and SW water deposits (Schultze and Cloutier, 1996); similarly, 53 trace fossil Paleozoic genera occurring in both marine and nonmarine deposits have been charted (Maples and Archer, 1989). Finally, ancestral-state reconstruction based on a molecular phylogeny of ray-finned fishes indicates that all extant ray-finned fishes are descended from an FW or BW ancestor (Vega and Wiens, 2012), indicating that a complex history of transitions between SW and FW is embedded in the evolutionary history of this diverse group. To summarize, the halohabitat of the most recent common ancestor of all vertebrates was probably SW or BW, and that of the most recent common ancestor of ray-finned fishes was probably FW or BW. Euryhalinity may have played a significant role in Paleozoic diversification of fishes.

3.2. Euryhalinity among extant fishes

Halohabitat use is distributed heterogeneously among broad taxa of fish, as is the case for other aquatic Metazoa. Hutchinson (1960), commenting on animal phyla that have FW and SW representatives, noted that “the distribution [of freshwater species] in the taxonomic system is highly irregular, suggesting a great degree of superdispersion of the physiological characters that pre-adapt marine organisms to entrance into freshwaters [sic].” Similarly, Nelson (2006) documented that the FW fishes are concentrated in certain orders. The likelihood of diadromy or euryhalinity is also known to vary taxonomically and phylogenetically. Diadromy is more prevalent among basal fishes (McDowall, 1988; but see Dodson, 1997 for critique of McDowall’s assignment of diadromy to taxa). Gunter (1967) suggested that euryhalinity is more pronounced in basal fishes, without quantifying the heterogeneity.

In this section, we summarize data on the phylogenetic distribution of halohabitat use among broad taxa of ray-finned fishes. Ballantyne and Fraser (Ballantyne and Fraser, 2013) demonstrate that euryhalinity and FW tolerance has evolved multiple times in the Elasmobranchii. To our knowledge, no detailed description of the phylogenetic distribution of halohabitat in the Actinopterygii has been previously published; however there have been several efforts to characterize halohabitat into distinct estuarine zones (Bulger et al., 1993) or to define euryhaline fish functional groups (Elliott et al., 2007), and the predominant halohabitat of fish families has been described by Evans (1984). We focus on the Actinopterygii because it contains the vast majority of extant fish species, has arguably a greater heterogeneity in halohabitat use than the Chondrichthyes or the Sarcopterygii, and because this confines the analysis to an osmoregulatory physiology strategy. As described in Section 2.2., we downloaded data on halohabitat use from FishBase (download 22 February 2012). We will here refer to any species that is found in BW as halohabitat-euryhaline. Within this set there are subsets of habitat-euryhalinity: there are species that are found in SW and BW, species that are found in FW and BW, species that are found in all three halohabitats, and species that are found in only BW. We term species occurring in both SW and FW as 'halohabitat-amphihaline'. The original application of 'amphihaline' to a species that migrates between FW and SW (Fontaine, 1975), has been trumped by the common usage and more precise etymology of 'diadromous'.

FishBase presently recognizes 30,972 separate species or subspecies. Subspecies (N = 397) are recognized in 153 species, within 24% of which halohabitat varies among subspecies. For this analysis we treated all subspecies as if they were species and will henceforth refer to them as such. Additional information that we downloaded from FishBase are entries on migratory behavior (e.g., amphidromous, oceanodromous). Migratory behavior has presently been recorded for about 3818 species of Actinopterygii, of which about 50% are listed as non-migratory. Because the taxonomic distribution of species for which migratory behavior has been recorded is uneven, any association between euryhalinity and migratory behavior should be regarded as tentative.

For phylogenetic relationships of major taxa, we followed Nelson (2006) for the placement of orders basal to the teleosts, and Wiley and Johnson's (2010) analysis of teleost clades. Relationships among derived Acanthopterygii are poorly resolved, and we aggregated 30 orders (most of which are monophyletic but some of which are not monophyletic yet are widely regarded as taxa, e.g. "Perciformes") into Division Percomorphacea. We retained several polytomies (Ateleopodiformes + Stomiatiformes + Eurypterygia [not shown, consisting of Aulopiformes and more derived orders]; Percopsiformes + Gadiformes + Acanthopterygii) because further aggregation would have obscured substantial phylogenetic detail. In addition we aggregated Hiodontiformes and Osteoglossiformes into Osteoglossomorpha because the former has only two species. Assignment of species to each major taxon was done as follows: placement in family was done according to FishBase; family placement in higher taxa was done if possible according to Wiley and Johnson (2010) or according to Nelson (2006).

A minority of species are habitat-euryhaline. There are 2844 species (about 9% of the total) that include BW in their halohabitat (Table 3). The largest category of euryhaline species is found in BW and SW but not FW (4.2% of all Actinopterygii). Roughly a quarter of these species may be diadromous; most species for which there are migration behavior entries in FishBase are listed as non-migratory, oceanodromous (migrating in SW only), or oceano-estuarine (migrating between SW and BW). About 2% of all Actinopterygii are

Table 3 here

amphihaline, and these species are almost exclusively diadromous. Another 2% of actinopterygians use BW and FW but not SW, and about 80% of these species for which there are migration behavior records are listed as non-migratory or potamodromous. Remarkably few species are found in only BW (0.3% of Actinopterygii). The apparently high percentage of species in the BW-only category that are diadromous must be viewed with caution as the number of migration behavior records is low. Over all categories, 60% of species that are halohabitat-euryhaline may be diadromous.

Habitat-euryhalinity appears primarily among the most basal and the most derived taxa in the Actinopterygii. At least half of the species are euryhaline in the basal clades Acipenseriformes, Lepisosteiformes, Elopiformes, Albuliformes, and Clupeiformes (Fig. 5). No more derived clade has a similarly high proportion of species that are euryhaline; nonetheless, most of the species that are euryhaline (n=2030) are in the derived and speciose clade Percomorphacea. Although comprising only 12% of the clade, the halohabitat-euryhaline percomorphs are 71% of all the halohabitat-euryhaline fishes. There is significant heterogeneity among orders in the proportion of species that are euryhaline (test of independence of euryhalinity and order, chi-square = 4360, df = 59, p < 0.0001).

Fig. 5 here

Among the habitat-euryhaline species, the representation of euryhalinity subsets varies among the clades. Euryhaline species are most commonly FW + BW in predominantly FW clades, such as Cypriniformes and Characiformes (Fig. 6). Conversely, euryhaline species are most commonly SW + BW in SW clades such as Clupeiformes. The predominant type of euryhalinity in a clade mirrors the predominant type of stenohalinity in the clade: the correlation between the proportion of the clade's euryhaline species that inhabit

Fig. 6 here

both FW and BW and the proportion of species in a clade that inhabit only FW is significant (n = 12 clades that have species occurring in FW and BW; r = 0.78, p = 0.003). Similarly, the correlation between the proportion of the clade's euryhaline species that inhabit both SW and BW and the proportion of species in a clade that inhabit only SW is significant (n = 16 clades that have species occurring in SW and BW; r = 0.86, p < 0.0001). Two clades deviate notably from the strong association between predominant stenohalinity habitat and predominant euryhalinity habitat. In Salmoniformes, 70% of the species are stenohaline-FW but only 12% of the euryhaline species are confined to FW and BW, reflecting the high proportion in this group that occurs in all waters. Conversely, in Lepisosteiformes 42% of the species are stenohaline FW but all of the remaining species are confined to FW and BW, i.e., species in this clade do not inhabit SW.

Habitat-euryhalinity varies among taxa within the most derived clade, currently recognized as the Percomorphacea. For each major taxon within the Percomorphacea, we estimated the percentage of species within each of the habitat use categories (Table 4). With the exception of Elassomatiformes, orders within series Smegmamorpharia (also comprising Mugiliformes, Synbranchiformes, Gasterosteiformes, Atheriniformes, Beloniformes, and Cyprinodontiformes) are relatively euryhaline: by order the species that inhabit BW ranges from 7% (Cyprinodontiformes) to 76% (Mugiliformes), and overall 16% of smegmamorph species occur in BW, versus 11% among species in the remaining orders. For the most part, a comparable percentage of smegmamorph fishes are euryhaline-SW, euryhaline-FW, and euryhaline-all waters. The most euryhaline among the remaining orders are Carangiformes, Gobiiformes, Scombriformes, and

Table 4 here

Batrachoidiformes. Relative to smegmamorphs, these euryhaline species are more likely to be euryhaline-SW.

Family-level variability in the tendency to be halohabitat-euryhaline is strong among Percomorphacea. Family-characteristic halohabitat use has long been recognized (Myers, 1938; Gunter, 1967) but has not been quantitatively assessed. To test the degree to which family is predictive of halohabitat use, we estimated the proportion of species that are euryhaline within each percomorph genus. The identity of higher taxonomic levels (order, and family nested within order) accounted for 35% of the variance in arcsine-transformed proportion of species that are euryhaline, whereas order alone explained only 5.5%. We conclude that the pronounced variability among percomorph higher taxa in patterns of halohabitat use is largely the result of shared ecology and physiology among species at an intermediate familial level of evolutionary relationship. Diversification at this level would have arisen primarily in the aftermath of the Cretaceous-Palaeogene extinction, when there was a sharp expansion in the number of extant fish families and a burst of morphological diversification among the percomorphs (Friedman, 2010). A satisfying concordance is suggested here between physiological, ecological and morphological diversification.

3.3. Evolutionary diversification upon transitions in halohabitat

Low prevalence notwithstanding, euryhaline species are potent sources of evolutionary diversity. A broadly-tolerant physiology and wide range of occupied habitats heighten the likelihood of a transition to a new habitat and a more specialized regime, potentially giving rise to new species, i.e. cladogenesis. In particular, euryhaline species are subject to landlocking, wherein a population becomes restricted to FW. In this section we review studies that provide conceptual or empirical insights into the cladogenetic potential of euryhalinity in fishes. We identify taxa and regions that are well-represented in recent literature on transitions and discuss the evolutionary processes associated with transitions.

As is often the case in evolutionary science, an early contemplation on the diversifying potential of broad salinity tolerance can be found in the publications of Charles Darwin. Considering the puzzling distributions of some FW fish groups whose distribution includes multiple continents, he wrote (1876) “Salt-water fish can with care be slowly accustomed to live in fresh water; and, according to Valenciennes, there is hardly a single group of which all the members are confined to fresh water, so that an SW species belonging to a fresh-water group might travel far along the shores of the sea, and could, it is probable, become adapted without much difficulty to the fresh waters of a distant land.” There is a clear connection between this thought and subsequent dispersalist explanations for the distribution of “secondary FW species” that may occasionally enter SW such as gar, synbranchids, cichlids, and cyprinodontids (Myers, 1938). Although widely adopted, the distinction between primary FW species, which spend their entire lives in FW, and secondary FW species has been criticized on the grounds that it is circular (i.e. if a taxon is widely distributed it must be capable of coastal or marine dispersal Rosen, 1974), and is not in fact predictive of a group’s dispersal abilities (e.g., Sparks and Smith, 2005).

In more recent years, a series of insightful reviews have commented on the diversifying potential of either diadromous or estuarine life cycle or habitat. Lee and Bell (1999) briefly reviewed literature on postglacial (Pleistocene and recent) transitions to FW in invertebrates and diadromous fishes, emphasizing how recent invasions provide opportunity to examine mechanisms involved in habitat transitions. McDowall (2001) described the paradoxically homogenizing and diversifying role of diadromous migration, on the one hand promoting gene flow and on the other hand yielding landlocking, isolation and cladogenesis. Other authors have considered the diversifying potential of estuarine fishes. Bamber and Henderson (1988) hypothesize that "selection for plasticity has preadapted estuarine and lagoonal teleosts with the ability to invade fresh waters. The evolutionary history of fish has included repeated invasions from the estuary to fresh waters, followed by adaptive radiation." Bilton et al. (2002) generalize on this perspective both taxonomically (i.e. extend their review to all estuarine animals) and dynamically; they note that the estuarine habitat is itself spatially subdivided, potentially restricting gene flow and enhancing spatial differentiation in population genetic structure. Features that are explicitly or implicitly common to these discussions are adaptive change associated with shifts in halohabitat, speciation by allopatric, parapatric or sympatric mechanisms, and repetition over space and/or time promoting adaptive radiation. Furthermore, virtually all studies on diversification in euryhaline fishes and their descendents (Table 5) allude to the role that changes in sea level have played in altering the habitat configuration of fishes living on the continental margin.

Table 5 here

Some anadromous fishes and their landlocked derivatives furnish several model systems of diversification in evolutionary biology. Salmonids show high fidelity and local adaptation to natal sites (Hendry et al., 2003b), whereas other anadromous species show little tendency for homing and have weak geographic population structure (Shrimpton, 2013). Modifications in landlocked populations of salmon and threespine stickleback have illustrated the nature and pace of adaptive change, and the predictability of adaptive change has been highlighted in stickleback. There have been extensive recent reviews of diversification in salmonids and stickleback (McKinnon and Rundle, 2002; Kinnison and Hendry, 2003), and we will not consider them here in comparable detail.

Physiological and/or behavioral characteristics make some euryhaline taxa particularly susceptible to changes in halohabitat and subsequent differentiation (Table 5), such as silversides (Atheriniformes). The New World has multiple examples of atherinid species flocks or adaptive radiations arising from habitat transitions (Barbour, 1973; Beheregaray and Levy, 2000; Beheregaray and Sunnucks, 2001; Beheregaray et al., 2002; Bloom et al., 2009; Heras and Roldan, 2011) and species pairs in overlapping halohabitats (Fluker et al., 2011). In the Old World, the cosmopolitan species *Atherina boyeri* is known to be differentiated according to halohabitat (Klossa-Kilia et al., 2007). Australian coast atherinids have also diversified in halohabitat (Potter et al., 1986). As indicated above, Bamber and Henderson (1988) suggest that underlying this readiness to transition to FW habitat is a high intrinsic level of phenotypic plasticity in the family.

Two euryhaline-migratory species of *Galaxias* (*G. truttaceus* and *G. brevipinnis*), a southern hemisphere genus of salmoniform, have undergone repeated transitions to FW, giving rise to species complexes in Tasmania and New Zealand's South Island (Table 5). *G. auratus* and *G. tanycephalus* inhabit lake clusters in separate drainage basins of Tasmania, and are each extremely similar to *G. truttaceus* with which they form a well-defined clade (Ovenden et al., 1993). *G. truttaceus* itself has several landlocked populations (Ovenden and White, 1990). Resolution of relationships among the three nominal species and reconstruction of the isolating events have been hampered by bottleneck- or founder effect-induced reductions in genetic diversity of the landlocked species and genetic variability of the migratory progenitor (Ovenden et al., 1993), but it appears that all landlocking events occurred in the last 100,000 years. Phylogenetic resolution and paleoreconstruction has been more successful for the more diverse *G. vulgaris* New Zealand complex of landlocked species, which arose from the diadromous *G. brevipinnis*. A well-resolved phylogeny for the group indicates that nine stenohaline-FW species arose from three separate losses of migration—this conclusion required the assumption that migration was the basal condition for the group, which is supported on other lines of evidence (Waters and Wallis, 2001a). Time since divergence estimates and geological evidence indicates that a 2- to 4- million year old (Pliocene) uplift of the South Island's mountain range was the process that isolated previously migratory populations from the sea (Waters and Wallis, 2001b).

The FW habitat is plesiomorphic for taxa in larger FW groups, such as the catfishes. Two catfish families, Ariidae and Plotosidae, consist largely of euryhaline-SW species. Phylogenetic analysis securely places this as the derived halohabitat within the Siluriformes, and was independently derived for each family or superfamily in each case (Sullivan et al., 2006). Transition to FW occurred 10-15 times within the Ariidae, yielding 16 partially- or fully-FW genera (Table 5) that are located in every region where marine ariids are found (Betancur-R, 2010). In this group, the proclivity to evolve FW habitat occupation appears to reflect a tendency to stenohalinity that was not lost in the SW ancestors.

The Anguilliformes provide an example of a large group in which euryhaline taxa evolved from stenohaline-SW ancestors. A recent phylogenetic analysis of the Anguilliformes strongly supports an SW origin of this group. Catadromy (hence developmental amphihalinity) evolved once in the order, and is a synapomorphy for the family Anguillidae and its single genus *Anguilla* of 16 species, all of which are catadromous (Inoue et al., 2010).

Another family-wide analysis, for the pufferfishes Tetraodontidae, finds that the derived FW lineages, occurring repeatedly on different continents, are well dispersed across the phylogeny (Yamanoue et al., 2011). Habitat optimized on the phylogenetic tree indicates that the coastal SW habitat is ancestral for the family (Table 5). Stenohaline FW puffers occur on South America, southeast Asia and Central Africa; divergence time estimates suggest that the transition to FW occurred first in Asia (Eocene, up to 78 mya) and most recently in South America (Miocene or more recent). These transitions have given rise to 29 species in four genera.

Taxon-wide data on salinity tolerance are much harder to come by than taxon-wide data on halohabitat, and thus the study by Whitehead (2010) on the frequency, distribution and timing of transitions in tolerance euryhalinity within the killifish genus *Fundulus* is unique and valuable (Table 5). For the most part (23 species), this study was able to use salinity tolerance data that had been collected using the gradual experimental design; data on halohabitat were used for two additional species, providing character data for about 75% of the species in the genus. Upper salinity tolerance data (all species were tolerant of FW) resolved into three groups, consisting of relatively stenohaline (limit 20-26 ppt), intermediate (60-75 ppt) and tolerant (80-115 ppt). Mapping of physiological characters on the phylogeny indicated that the tolerant physiology is basal and that there have been five independent transitions to less-tolerant states. The effort to reconstruct ancestral physiology was challenged by high transition rates among character states, so that the state of deep nodes could not be attributed with confidence. This problem is likely to arise frequently in such analyses, in groups that underwent rapid diversification (i.e. an adaptive radiation) associated with changes in salinity tolerance.

Some areas such as the Amazon have been hotspots for transitions to FW, which can often be explained by large-scale events such as marine incursions that acted on multiple euryhaline groups simultaneously. The Amazon basin is richly endowed with FW derivatives of SW fishes, offering at least 39 genera in 17 largely-SW families within 14 orders. In an effort to clarify timing and mechanism of origins of these groups, Lovejoy et al. (2006) tested predictions arising from hypothesis that Miocene marine incursions, which established a large system of brackish lakes, promoted transition. Their analysis, combining phylogeny, geology, the fossil record and biogeography, supported the Miocene incursion model for multiple groups including potamotrygonids, engraulids, belonids, hemirhamphids and sciaenids (Table 5). A genus of puffers also occurs in the Amazon Basin and an independent analysis of time of divergence for the FW species from its sister taxon is consistent with the Miocene marine incursion model (Yamanoue et al., 2011).

The Mediterranean Basin is another region with a large number of SW- or euryhaline-to-FW transitions that are attributable to geological history. In comparison to the rest of Europe, the Mediterranean Basin has a relatively large number of fish species and a high degree of endemism; endemism is especially high in the eastern portion of the region as represented by Greek collection sites (Ferreira et al., 2007). Including introduced and diadromous species, 135 to 162 fish species inhabit Greece's FW (Bobori and Economidis, 2006; Oikonomou et al., 2007). At least 13 of these species represent relatively recent transitions from SW to FW habitats (Table 5). FW species that are clearly derived from euryhaline relatives include two species of landlocked shad (*Alosa macedonica* and *A. vistonica* Bobori et al., 2001), a blenny (*Salaria fluvatilis* Zander, 1974), and nine species in five genera of goby (Economidis and Miller, 1990; Miller, 1990). Differentiation of the euryhaline silverside *Atherina boyeri* in Hellenic lakes has already been noted. *Pungitius hellenicus* is a critically-endangered species of stickleback that is endemic to a small region of FW springs and associated wetlands (Keivany et al., 1997) and is the only member of its genus to be stenohaline. Many of these transitions can be attributed to the dynamic history of salinity transitions in the region. The most detailed reconstruction of diversification upon transition to FW in the Mediterranean basin has been outlined for gobies (Economidis and Miller, 1990; Miller, 1990). In this reconstruction, separation between the ancestor of *Economidichthys* + *Knipowitschia* and *Pomatoschistus* occurred during the middle-Miocene closure of the brackish Sarmatic Sea, an event that represented the onset of Ponto-Caspian endemism. Separation between *Economidichthys* and *Knipowitschia* occurred during the late-Miocene Messinian salinity crisis.

Post-glacial changes in the distribution of surface FW and the elevation of landmasses are primarily responsible for the landlocking of euryhaline species at high latitudes and some cases of lower-latitude landlocking. Most of the existing stenohaline FW populations of threespine stickleback were isolated from ancestral SW habitat as a result of glacial retreat and isostatic rebound, wherein landmasses rose in elevation when relieved of masses of ice (Bell and Foster, 1994). Changes in sea level during and after the Pleistocene created lagoons and promoted diversification of silversides in southern Brazil (Beheregaray et al., 2002).

A high incidence of FW derivations in some regions may be attributable to ecological, in addition to or instead of, physical-geographic factors. The Usumacinta River of Mexico and

Guatemala harbors multiple independent incidences of FW derivation. High calcium concentration in the water of this karstic region may essentially lower the physiological hurdle that must be surmounted for colonization from BW (Lovejoy and Collette, 2001). In general the extent of diversification that occurs in FW following colonization by SW forms will be dependent on factors such as the diversity of habitats, the intensity of competition from already-established FW species (Miller, 1966; Betancur-R et al., 2012), and the availability of refuge from predation (e.g. widespread albeit seasonal areas of hypoxic water in the Amazon Anjos et al., 2008).

While we have emphasized cases of euryhaline differentiation associated with transitions to another halohabitat, diversification within a euryhaline halohabitat has also been documented. A species complex of gynogenetic unisexual silversides arose from repeated hybridizations between female *Menidia peninsulae* and males of a congener, probably *M. beryllina* (Echelle et al., 1989). It is likely that the unisexual complex arose early in the divergence between the two euryhaline parental species, because gynogenetic lines will arise when parental species differ in regulation of meiosis but do not differ to the extent that hybrid offspring would have markedly lower fertility or viability. Such diversifying contact between populations early in the speciation process is quite consistent with arguments summarized above regarding why estuarine environments could serve as incubators of evolutionary novelty.

3.4. Adaptation upon transitions in halohabitat

Intraspecific divergence in morphology, behavior, physiology, and life history occurs between euryhaline forms and their counterparts in FW and SW. Adaptive morphological and behavioral changes are associated with changes in predator regime and prey field (McKinnon and Rundle, 2002; Bell et al., 2004; Palkovacs and Post, 2009) and reproductive substrates (Beheregaray and Levy, 2000). Morphometric analysis has revealed body shape differences between euryhaline and stenohaline forms (Klepaker, 1993; Gelmond et al., 2009; Fluker et al., 2011). Change in water chemistry (particularly lower availability of calcium) can also affect body form via direct effects (phenotypic plasticity) and heritable effects on ion uptake and deposition; although the role of water chemistry in selecting heritable differences in calcium regulation is implied by studies demonstrating growth differences between armor gene alleles in stickleback (Barrett et al., 2008), to our knowledge this has not been further tested. Differences between euryhaline and FW forms have been found in salinity tolerance (Dunson and Travis, 1991; Foote et al., 1992; Plaut, 1998; Purcell et al., 2008; McCairns and Bernatchez, 2010), expression patterns of loci associated with osmoregulation (Nilsen et al., 2007; McCairns and Bernatchez, 2010; Whitehead et al., 2011), and gene sequence in osmoregulation loci or regions closely associated with such loci (implicating positive selection for change in coding regions-- Hohenlohe et al., 2010; DeFaveri et al., 2011; Czesny et al., 2012). At least in threespine stickleback, life history trait changes upon landlocking include reduction in clutch mass (g), clutch size (number of eggs), and reproductive allocation (proportion of body mass devoted to reproduction-- Baker et al., 2008). A shift in reproductive timing has been observed: lacustrine populations of *Galaxias truttaceus* shifted from autumn spawning to spring spawning (Ovenden and White, 1990), ostensibly in response to strong overwinter mortality selection on early life stages. Within the salmonids there is an apparent evolutionary progression to acquiring salinity tolerance earlier in development (McCormick, 2013).

Where they are in contact, euryhaline and FW populations or sister species may be reproductively isolated, providing a necessary condition for speciation. Morphological changes associated with the transition facilitate prezygotic isolation, particularly in species with intersexual selection. An FW (*Lucania goodei*) and a euryhaline (*L. parva*) species of killifish co-occur in some locations in Florida (USA). Genetic differences between the species are small, and prezygotic (behavioral) isolation maintains the species boundary; no loss of viability in hybrids has been found despite demonstrable differentiation between the species in salinity tolerance (Fuller et al., 2007). Conversely, in a contact zone between euryhaline and FW forms of stickleback where hybrids are common, prezygotic isolation appears to be weak but genetic evidence suggests there is a robust postzygotic barrier (Honma and Tamura, 1984; Jones et al., 2006).

4. CONVERGENCE AND EURYHALINITY

Euryhalinity has arisen multiple times within the ray-finned fishes. The fossil record indicates that there were multiple independent transitions to FW halohabitat within the Actinopterygii, each of which required prior capability of functioning in intermediate salinity levels. The phylogenetic distribution of halohabitat types among extant fishes indicates that euryhalinity was pervasive, if not common, among basal ray-finned fishes. Hence it seems that euryhalinity was an ancestral condition or was readily derived. The phylogeny suggests that subsequent lineages were less euryhaline, however. Only a few orders branching from intermediate reaches of the actinopterygian tree are thoroughly euryhaline. Percomorphs present some increased affiliation with BW and some orders within the Percomorphacea are quite estuarine. Therefore, judging from the macroevolutionary pattern of halohabitat use, euryhalinity happened multiple times: euryhaline ostariophysans arose from stenohaline FW ancestors and euryhaline percomorphs arose from stenohaline SW ancestors. The dataset on physiological tolerance also supports the multiple-origin model for euryhalinity. Most ostariophysans have demonstrably narrow values for tolerance breadth, but the capacity for dealing with brackish and salt water appears in some derived families (although to our knowledge no euryhaline ostariophysans, such as the marine catfishes, have been subjected to salinity tolerance testing, it is virtually certain that this would demonstrate that they are derived outliers from their order's distribution). The picture is not so clear for the percomorphs, because we know little about the tolerance limits of the stenohaline SW haloniche, which is the inferred ancestral condition. With existing data, we cannot determine whether the phylogenetic pattern of halohabitat use for the percomorphs and their precursors reflected a stenohaline SW physiology, in which case the physiological capacity to handle brackish and FW was derived independently of more basal actinopterygians, or alternatively if the physiological capacity for euryhalinity was maintained in spite of the stenohaline habitat use.

Comparative studies on the mechanisms of euryhalinity among multiple taxa and at multiple levels of biological integration are needed. We have a good grasp on how changing salinity levels are physiologically accommodated for model species of most major fish taxa (Edwards, 2013), yet even within this limited representation there is evident variability in response mechanisms (Marshall, 2013; Zydlewski and Wilkie, 2013). Differences in the genetic and physiological mechanisms of euryhalinity should reflect phylogenetic legacies and will shed light on alternative evolutionary pathways to broad halotolerance.

5. CONCLUSION AND PERSPECTIVES

Comparatively little attention has been directed at evolutionary changes and consequences associated with the salt-fresh habitat transition in vertebrates, relative to the aquatic-terrestrial transition. The movement of fishes (and/or their predecessors) into FW, which required the capability of dealing with a broad range of salinity levels, had substantial macroevolutionary repercussions. Implications for colonization of land and the origin of Tetrapoda aside, the colonization of FW habitats initiated a quantum leap in diversification. Despite the vanishingly small amount of FW habitat relative to SW habitat, extant FW fish species diversity is comparable to SW fish diversity (Horn, 1972) and within-species genetic differentiation is greater in FW fish species than SW fish species (Ward et al., 1994). This disparity is attributable to the greater restrictions of gene flow among locations in FW habitats, the greater spatial heterogeneity of habitat, and the lower productivity of FW, which reduces sustainable population size and increases the potency of genetic drift.

Euryhalinity has accordingly been nominated as a key innovation (Lee and Bell, 1999), meaning a trait whose evolution enables exploitation of new adaptive zone, triggering cladogenesis (Galis, 2001). Does the evolution of halotolerance consistently promote diversification or adaptive radiation into new halohabitats? This question can be addressed by mapping physiological capability on phylogenies as in Whitehead (2010). Given high variability among families, a comparative analysis that spanned several closely-related families would be valuable. Does diversification go both ways? The evolutionary history of ariid catfish (Betancur-R, 2010) is unique, at least to date, in documenting bidirectional diversification.

A peculiar feature of euryhalinity meriting further study, in the context of the thesis that it has played a significant role in the diversification of vertebrates, is its apparent rarity. If it is indeed a potent generator of biological diversity, it is also transitional—it ushers in a round of cladogenesis seemingly resulting in stenohaline taxa. The rarity of euryhaline species may reflect substantial fitness costs of plasticity (or costs of migration, in the case of diadromous fishes) that are exceeded by benefits under special circumstances, so that traits promoting euryhalinity are rapidly lost if they are not under strong selection. Thorough study of the circumstances in which the benefits of broad salinity tolerance exceed the costs will require analysis of biotic interactions such as competition, because the outcome of interactions in one set of abiotic conditions may be reversed under another set (Dunson and Travis, 1991). Another factor contributing to the rarity of euryhaline species is the rarity and mobility of estuarine habitat, owing to its restriction to a narrow and dynamic coastal zone and changing sea levels. Any particular estuary is geologically young (McLusky, 1989). Habitat rarity and mobility are both features that could limit its inhabitants to a short evolutionary lifespan.

Euryhalinity is a graded feature that shows variability in its upper and lower limits among teleosts (Figure 4). Based on our effort to summarize it, the salinity tolerance literature does not support a simple expectation that the transition from stenohalinity to euryhalinity (or the reverse) is quantized, requiring only the addition or deactivation of a single switch that activates ion absorption or secretion, water uptake or elimination. How is physiological capability tuned to environmental demands—does halotolerance breadth reliably indicate the range of salinity to which a population is exposed? Which genetic and physiological components of the response to changing salinity are most decisive in limiting capability?

Judging from broad phylogenetic view of halohabitat, euryhalinity was a lost trait for a considerable period of actinopterygian evolution, and then was rediscovered. Does this reflect physiological capacity? In particular, are most SW fishes stenohaline? How do pathways promoting broad tolerance differ among major groups that independently underwent transition among halohabitats? In other words, how do the genetic and physiological bases for evolutionary euryhalinity vary among broad taxa? We hope that more analyses using the phylogenetically-rigorous comparative approach will incorporate measures of salinity tolerance to determine whether broad tolerance of species inhabiting FW or SW plays a role in the evolution of euryhalinity. In other words, can euryhaline species evolve as easily from stenohaline species with narrow halotolerance as from those with broad halotolerance?

Our presently limited view of FW colonization events in the fossil record is bound to improve. In early vertebrate evolution it seems that the boundary between SW and FW was easily breached. We know little about the business of the early euryhaline fishes. Were they migrants? What habitats did they frequent? Did occupation of FW precede or coincide with Devonian rise of terrestrial plants?

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Table 1. Upper and lower halotolerance limits of actinopterygian fishes by halohabitat in ppt. Table provides the mean, range of values, standard error and number of species for the lower and upper tolerance limits in each of three halohabitat categories: freshwater (FW), brackish water (BW), and saltwater (SW). Tolerance limits determined using the direct method and those determined using the gradual method are presented separately.

	Lower limit				Upper limit			
Habitat	Mean	(Range)	SE	N	Mean	Range	SE	N
Direct								
BW	1.11	(0-16)	0.37	53	27	(8-60)	2.4	37
FW	0.1	(0-1)	0.04	32	18	(7-65)	2.2	33
SW	4.5	(1-9)	0.79	10	49	(38-64)	4.9	5
Gradual								
BW	0	(0-0)	0	38	68	(7-126)	6.4	32
FW	0.1	(0-1)	0.1	21	33	(7-109)	5	23
SW	7.0	(7-7)	.	1	.	(.-.)	.	0

Table 2. Predictors of halotolerance. Table presents the results of analyses of variance on halotolerance breadth estimates derived from experiments using the direct method and the gradual method, testing the effect of ontogenetic stage (larva or juvenile+adult), taxonomic order, and halohabitat (5 levels: FW, SW, or BW plus FW and/or SW). The table presents the results of multiple models: the R^2 of the full model including all three effects, the decrease in R^2 when each effect is dropped from the full model, and the R^2 of the model including each effect by itself. The R^2 values of significant single-effects models are indicated in bold.

	R^2 of full model	Reduction in R^2 when dropped	R^2 of one-way
Direct method			
	0.55		
Stage		0.020	0.000053
Order		0.25	**0.43**
Habitat		0.12	**0.29**
Gradual method			
	0.64		
Stage		0.019	**0.085**
Order		0.32	**0.35**
Habitat		0.26	**0.26**

Table 3. Halohabitat use of Actinopterygii. Table provides the number of species (N(spp)) and an estimate of the percent of those species that are diadromous (with the number of species on which this estimate is based in parentheses), for each halohabitat category. Halohabitat categories are encoded according to whether a species is found (indicated by +) or is absent (indicated by -) in brackish water (BW), freshwater (FW) and/or saltwater (SW), such that a species occurring in all waters is encoded as +BW+FW+SW.

Halohabitat	N(spp)	Diadromous
+BW+FW+SW	732	93% (527)
+BW+FW-SW	727	20% (212)
+BW-FW+SW	1293	28% (288)
+BW-FW-SW	92	67% (9)
-BW+FW+SW	0	
-BW+FW-SW	14391	4% (1330)
-BW-FW+SW	13737	1% (1452)

Table 4. Halohabitat use of Percomorphacea. For each order, table provides the number of species (N(spp)) and an estimate of the percent of those species that occur in: brackish and FW (% EuryFW), brackish and saltwater (% EurySW), brackish, FW and saltwater (% EuryAW), brackish water only (% EuryBW), FW only (% StenoFW), and saltwater only (% StenoSW). Orders are arranged to reflect phylogeny insofar as it can be presently resolved (see Wiley and Johnson, 2010); names in quotes indicate groups for which there is no evidence of monophyly.

order	N(spp)	% EuryFW	% EurySW	% EuryAW	% EuryBW	% StenoFW	% StenoSW
Elassomatiformes	7	0	0	0	0	100	0
Mugiliformes	82	8.5	20	45	2.4	3.7	21
Synbranchiformes	120	11	0	0	0	89	0
Gasterosteiformes	351	3.7	10	5.7	0.28	8.3	72
Atheriniformes	334	6.3	8.4	5.7	1.2	64	14
Beloniformes	273	8.8	9.2	8.8	0.73	25	47
Cyprinodontiformes	1231	5.7	0.57	0.89	0.16	93	0.081
Acanthuriformes	116	0	15	2.6	0	0	83
Anabantiformes	195	4.1	0	0	0	96	0
Batrachoidiformes	81	0	15	3.7	0	6.2	75
Blenniiformes	906	0.33	3.6	0.22	0.55	0.22	95
"Caproiformes"	18	0	0	0	0	0	100
Carangiformes	160	0	38	2.5	0	0	59

Table 4 (cont’d)

Cottiformes	1191	0.34	1.9	0.76	0.084	7.0	90
Dactylopteriformes	7	0	14	0	0	0	86
Gobiesociformes	362	0.28	3.9	0.28	0.28	2.8	93
Gobiiformes	1943	6.3	10	10	2.7	21	50
Icosteiformes	1	0	0	0	0	0	100
Labriformes	2688	2.2	1.3	0.11	0.074	59	37
Lophiiformes	347	0	0.29	0.29	0	0	99
Nototheniiformes	148	0	0	0	0	0	100
"Ophidiiformes"	517	0.39	0.97	0	0.19	0.97	97
"Perciformes"	2889	2.0	11	3.7	0.035	15	69
Pholidichthyiformes	2	0	0	0	0	0	100
Pleuronectiformes	782	1.3	9.2	3.5	0.13	3.7	82
Scombriformes	162	0	19	0.62	0	0	80
Scorpaeniformes	1314	0.15	4.6	0.38	0	0.61	94
Stromateiformes	66	0	9.1	0	0	0	91
Tetraodontiformes	432	1.9	9.3	1.9	0.46	6.7	80
"Trachiniformes"	294	0	3.1	0.68	0	0.34	96

Table 5. Evolutionary transitions in euryhalinity. Table presents a selection of studies that documented diversification within a taxon in halohabitat or salinity tolerance. For each taxon, table provides the order and family, the ancestral and derived halohabitat (saltwater: SW; brackish water: BW; saltwater, and brackish water: EurySW; saltwater, brackish water and freshwater: EuryAW; diadromy indicated by (D); brackish water and freshwater: EuryFW; freshwater: FW), the time at which the transitions occurred, and the taxonomic level (diversification within species: intraspecific; transition giving rise to new species or genera: interspecific, intergeneric, etc.).

Order	Family	Taxon	Ancestral halohabitat	Derived halohabitat	Timing of transition	Taxonomic level of diversification	References
Angulliformes	Angullidae	*Anguilla*	SW	EuryAW (D)		Interspecific	(Inoue et al., 2010)
Atheriniformes	Atherinidae	*Atherina boyeri*	EurySW	BW, FW		Intraspecific	(see also Francisco et al., 2006; Klossa-Kilia et al., 2007)
Atheriniformes	Atherinidae	*Chirostoma, Poblana spp.*	EuryAW	FW	Plio-Pleistocene	Interspecific and intergeneric	(Barbour, 1973; Bloom et al., 2009)
Atheriniformes	Atherinidae	*Menidia beryllina*	BW	FW		Intraspecific	(Fluker et al., 2011)

Table 5 (cont'd)

Atheriniformes	Atherinidae	*Menidia clarkhubbsi*	BW	BW		Intraspecific (clonal)	(Echelle et al., 1989)
Atheriniformes	Atheriniopsidae	*Odontesthes argentinensis*	SW	BW		Intraspecific	(Beheregaray and Levy, 2000; Beheregaray and Sunnucks, 2001;
Atheriniformes	Atheriniopsidae	*Odontesthes perugiae complex*	BW	FW	Pleistocene	Interspecific	(Beheregaray et al., 2002)
Atheriniformes	Atheriniopsidae	*Odontesthes spp*	SW, EurySW	FW	Pleistocene	Interspecific	(Heras and Roldan, 2011)
Atheriniformes	Cyprinodontidae	*Aphanius spp.*	EurySW	EuryFW, FW	Miocene	Interspecific	(Kosswig, 1967)
Beloniformes	Belonidae	*Belonion, Potamorrhaphis, Pseudotylosurus, Xenontodon*	EurySW	FW		Interspecific and intergeneric	(Lovejoy and Collette, 2001)
Blenniiformes	Blenniidae	*Salaria fluviatilis*	EurySW	FW	Miocene	Interspecific	(Kosswig, 1967; Zander, 1974; Plaut, 1998)
Clupeiformes	Clupeidae	*Alosa pseudoharengus*	EuryAW (D)	FW	Recent	Intraspecific	(Palkovacs et al., 2008; Post et al., 2008; Palkovacs and Post, 2009)

Table 5 (cont'd)

Atheriniformes	Atherinidae	*Menidia clarkhubbsi*	BW	BW		Intraspecific (clonal)	(Echelle et al., 1989)
Clupeiformes	Clupeidae	*Alosa spp.*	EuryAW (D)	FW		Interspecific	(Bobori et al., 2001)
Clupeiformes	Engraulidae	*Anchovia surinamensis*	EurySW	EuryFW	Miocene	Interspecific	(Lovejoy et al., 2006)
Clupeiformes	Engraulidae	*Jurengraulis juruensis*	EurySW	FW	Miocene	Interspecific and intergeneric	(Lovejoy et al., 2006)
Cyprinodontiformes	Fundulidae	*Fundulus spp.*	EurySW	FW		Interspecific	(Whitehead, 2010)
Gasterosteiformes	Gasterosteidae	*Gasterosteus aculeatus*	SW, euryAW (D)	FW	Pleistocene to present	Intraspecific	(Klepaker, 1993; McKinnon and Rundle, 2002; Bell et al., 2004; Gelmond et al., 2009)
Gobiiformes	Gobiidae	*Economidichthys, Knipowitschia, Orsinogobius, Padogobius, Proterorhinus*	SW, EurySW	FW	Miocene	Interspecific and intergeneric	(Economidis and Miller, 1990; Miller, 1990)

Table 5 (cont'd)

Myliobatiformes	Pomatotrygonidae	*Pomatotrygon, Paratrygon, Plesiotrygon*	SW	FW	Miocene	Interspecific and intergeneric	(Lovejoy et al., 2006)
Perciformes	Sciaenidae	*Plagioscion, Pachypops, Pachyurus, Petilipinnis*	EurySW	EuryFW, FW	Miocene	Interspecific and intergeneric	(Lovejoy et al., 2006)
Salmoniformes	Galaxiidae	*Galaxias auratus, G. tanycephalus, G. truttaceus*	EuryAW (D)	FW	Pleistocene to recent	Interspecific and intraspecific	(Ovenden and White, 1990; Ovenden et al., 1993)
Salmoniformes	Galaxiidae	*Galaxias vulgaris* complex	EuryAW (D)	FW	Pliocene	Interspecific	(Waters and Wallis, 2001b; Waters and Wallis, 2001a)
Salmoniformes	Salmonidae	*Oncorhynchus, Salmo, Salvelinus spp.*	EuryAW (D)	FW		Intraspecific	(Hendry et al., 2003a)
Siluriformes	Ariidae	*Notarius, Catharops, Potamarius, Arius, Cephalocassis, Hemiarius, Neoarius, Potamosilurus, Cinetodus, 'Sciades', Brustarius, Pachyula, Doiichthys, Nedystoma, Nempteryx, Cochlefelis*	EurySW	FW		Interspecific and intergeneric	(Betancur-R, 2010)

Table 5 (cont'd)

Tetraodontiformes	Tetraodontidae	*Auriglobus, Carinotetraodon, Colomesus, Tetraodon*	EurySW, EuryAW	FW	Miocene to recent	Interspecific and intergeneric	(Yamanoue et al., 2011)

Fig. 1. Approaches to halotolerance testing of fishes. A) Schematic of experimental testing via direct and gradual changes in salinity. In direct testing, subjects are transferred instantaneously at time t0 from the salinity of acclimation (here represented as 0.5 ppt) to one of several levels of treatment salinity (0.5 ppt as control, 10, 20 and 30 ppt). In gradual testing, salinity is changed in small increments, here represented as 5 ppt increases every 24 h. Some points are offset for clarity. B) Results from direct testing. Figure represents a typical example of survivorship curves for each of several salinity treatments. Points at 100% survival are offset slightly for clarity. Data from Guo et al. (1995). C) Results from gradual testing. Figure represents a typical example of change in survival rate at each salinity as salinity is progressively altered. Data from Nordlie and Walsh (1989).

Fig. 2. Lower and upper salinity tolerance limits of actinopterygian fishes. Histograms represent the frequency of estimated tolerance limits by species. Typical salinity values for freshwater (FW), salinity at which fish are isotonic (IW), and seawater (SW), are indicated on each x axis. Note that the x axis scales of the two panels differ. A) Tolerance limits, in classes of 5 ppt, determined via direct method. B) Tolerance limits determined by gradual method.

Fig. 3. Halotolerance breadth. Each species is represented by a line which extends between the lower and upper tolerance limit along the scale on the x axis. In each plot species are sorted by tolerance breadth. A) Tolerance breadth determined via direct method. B) Tolerance breadth determined by gradual method. Note difference in range of x axes.

Fig. 4. Halotolerance breadth of selected groups. Lower, middle and upper lines of boxes represent the quartiles of each distribution, whiskers represent the 10th and 90th percentiles, and points represent observations outside the 5th and 95th percentiles. Results were plotted if sample size for a group was 9 or greater A) Well-represented orders in the dataset. Group of distributions on the left were estimated in direct experiments, group on the right in gradual experiments. Ather: Atheriniformes; Cdont: Cyprinodontiformes; Cypri: Cyprinodontiformes; Perci: Perciformes; Silur: Siluriformes. B) Grouping species by habitat. Group of distributions on the left were estimated in direct experiments, group on the right in gradual experiments. FW: fresh water; SW: marine; BW: estuaries.

Fig. 5. Phylogenetic relationships and halohabitat use of Actinopterygian fishes. Pie chart for each terminal taxon in the phylogeny represents the proportion of species in the taxon that occur in salt water only (stenohaline-saltwater: StSW), fresh water only (stenohaline-freshwater: StFW), or occur in brackish water (Eury). Area of each pie chart is scaled to represent the number of species in the taxon ($\log_{10}$ scale); the smallest pie, for Amiiformes, represents one species, whereas the largest pie for Percomorphaea represents 17,020 species.

Fig. 6. Phylogenetic relationships and halohabitat use of euryhaline Actinopterygian fishes. Terminal branches are labeled with pie charts if taxon has species occurring in brackish water. Pie chart represents proportion of species in taxon that occur in saltwater and brackish water (euryhaline-saltwater: EurySW), saltwater, brackish water and freshwater (euryhaline-all waters: EuryAW), and freshwater and brackish water (euryhaline-freshwater: EuryFW). For clarity, species occurring in brackish water only are represented as EuryAW. Area of each pie chart is scaled to represent the number of euryhaline species in the taxon ($\log_{10}$ scale); the smallest pies represent one species and the largest pie for Percomorphaea represents 2030 species.

Fig. 1

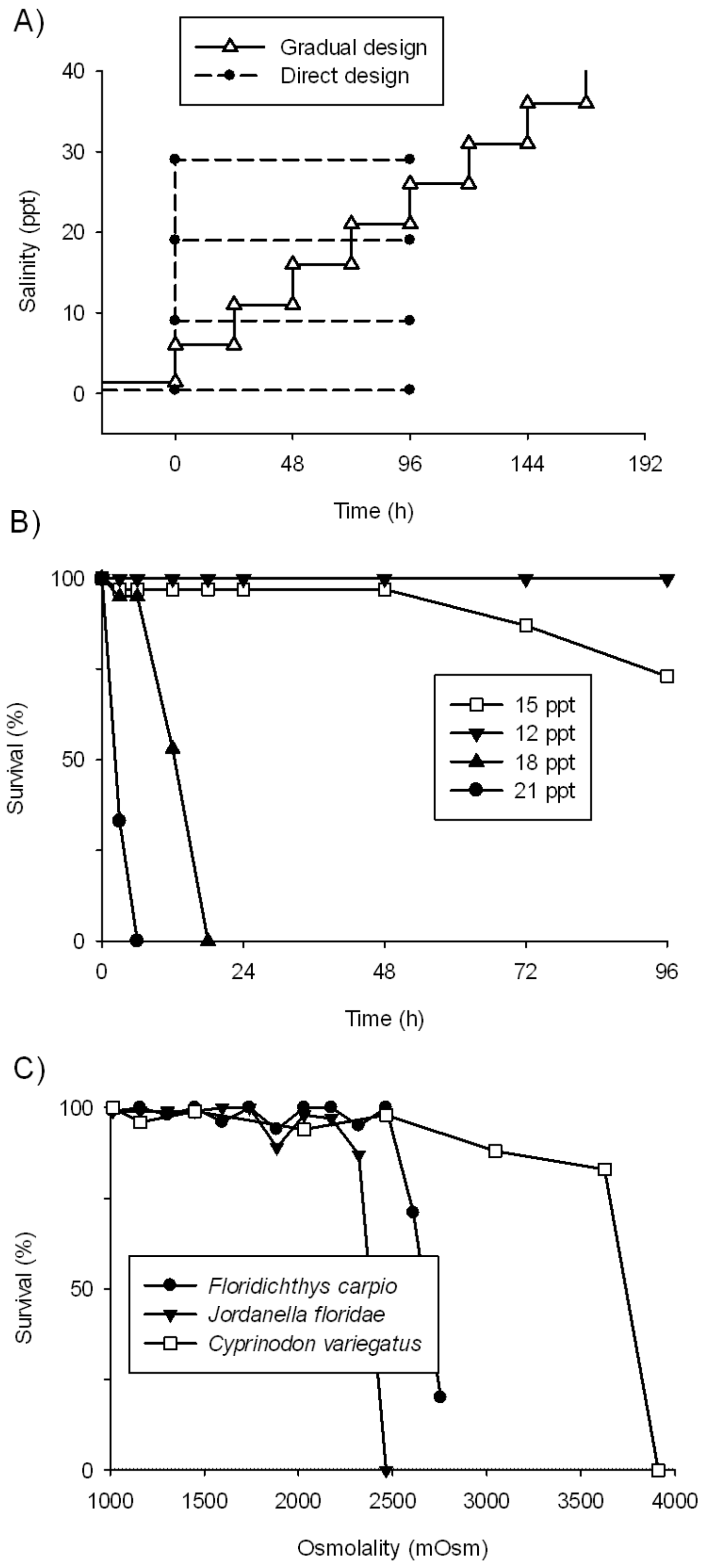
A)
Gradual design
Direct design
Salinity (ppt)
0
10
20
30
40
0
48
96
144
192
Time (h)
B)
Survival (%)
0
50
100
15 ppt
12 ppt
18 ppt
21 ppt
0
24
48
72
96
Time (h)
C)
Survival (%)
0
50
100
Floridichthys carpio
Jordanella floridae
Cyprinodon variegatus
1000
1500
2000
2500
3000
3500
4000
Osmolality (mOsm)

Fig. 2

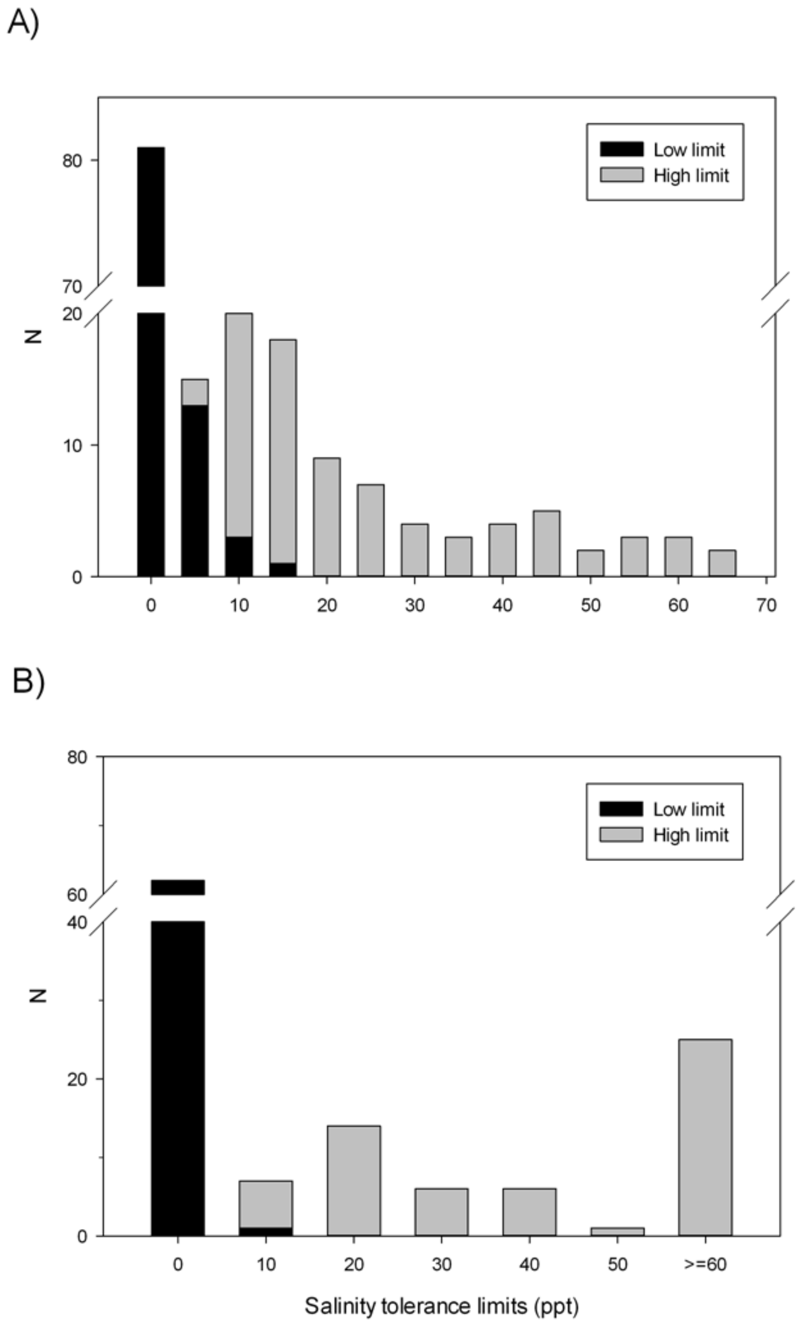
A)
80
70
20
10
0
N
Low limit
High limit
0
10
20
30
40
50
60
70
B)
80
60
40
20
0
N
Low limit
High limit
0
10
20
30
40
50
>=60
Salinity tolerance limits (ppt)

Fig. 3

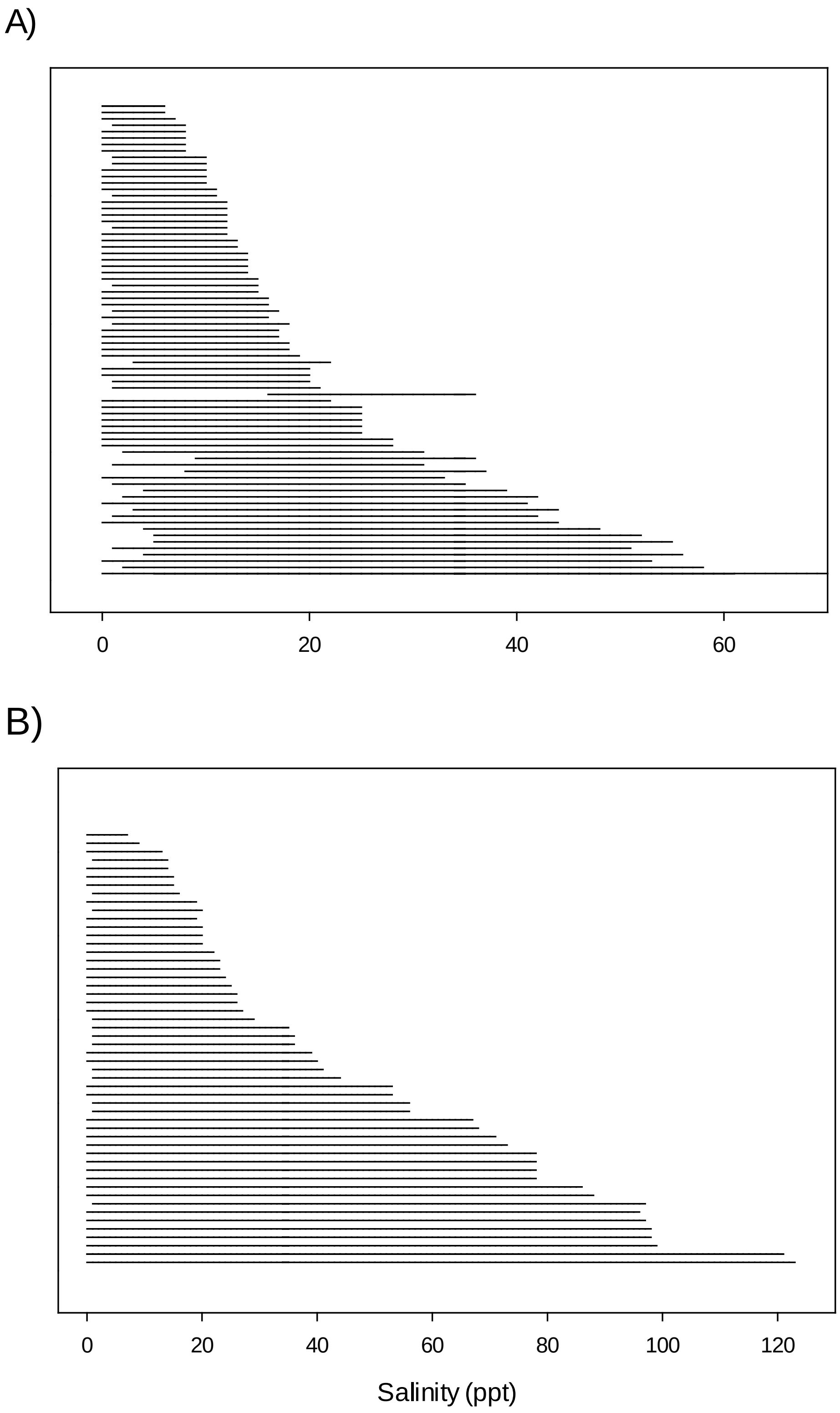

Fig. 4

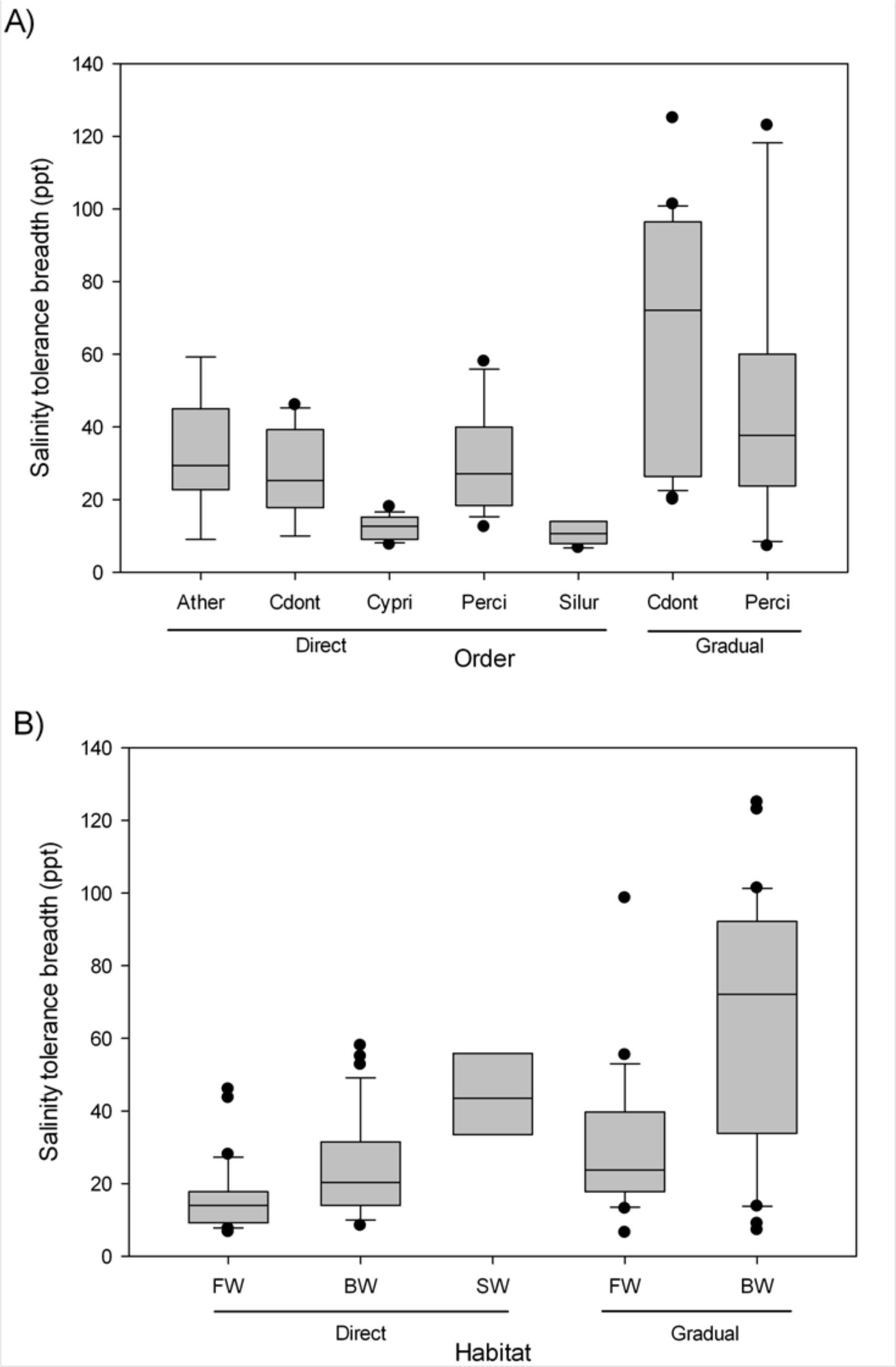
A)
Salinity tolerance breadth (ppt)
0
20
40
60
80
100
120
140
Ather
Cdont
Cypri
Perci
Silur
Cdont
Perci
Direct
Gradual
Order
B)
Salinity tolerance breadth (ppt)
0
20
40
60
80
100
120
140
FW
BW
SW
FW
BW
Direct
Gradual
Habitat

Fig. 5

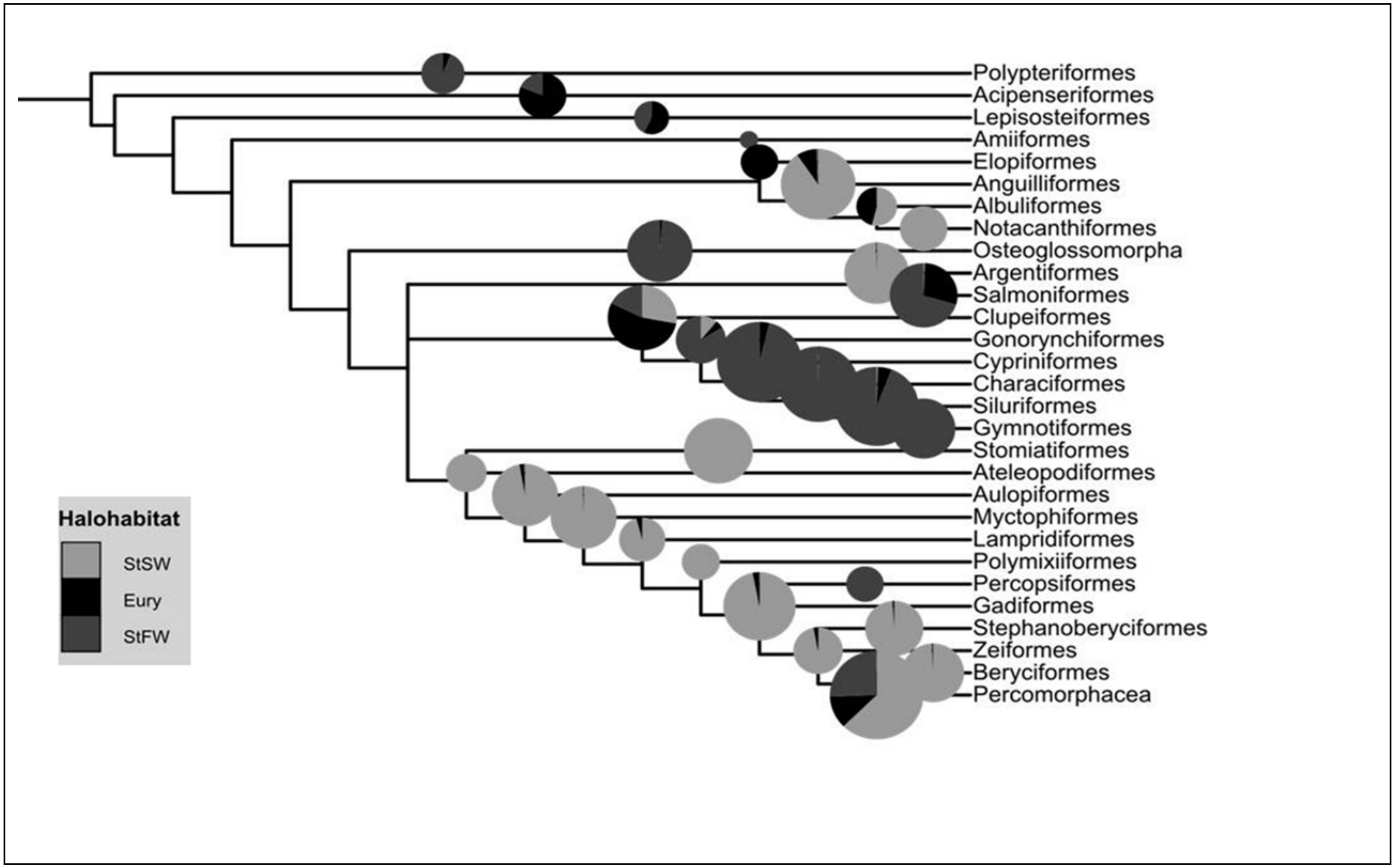
Polypteriformes
Acipenseriformes
Lepisosteiformes
Amiiformes
Elopiformes
Anguilliformes
Albuliformes
Notacanthiformes
Osteoglossomorpha
Argentiformes
Salmoniformes
Clupeiformes
Gonorynchiformes
Cypriniformes
Characiformes
Siluriformes
Gymnotiformes
Stomiatiformes
Ateleopodiformes
Aulopiformes
Myctophiformes
Lampridiformes
Polymixiiformes
Percopsiformes
Gadiformes
Stephanoberyciformes
Zeiformes
Beryciformes
Percomorphacea
Halohabitat
StSW
Eury
StFW

Fig. 6

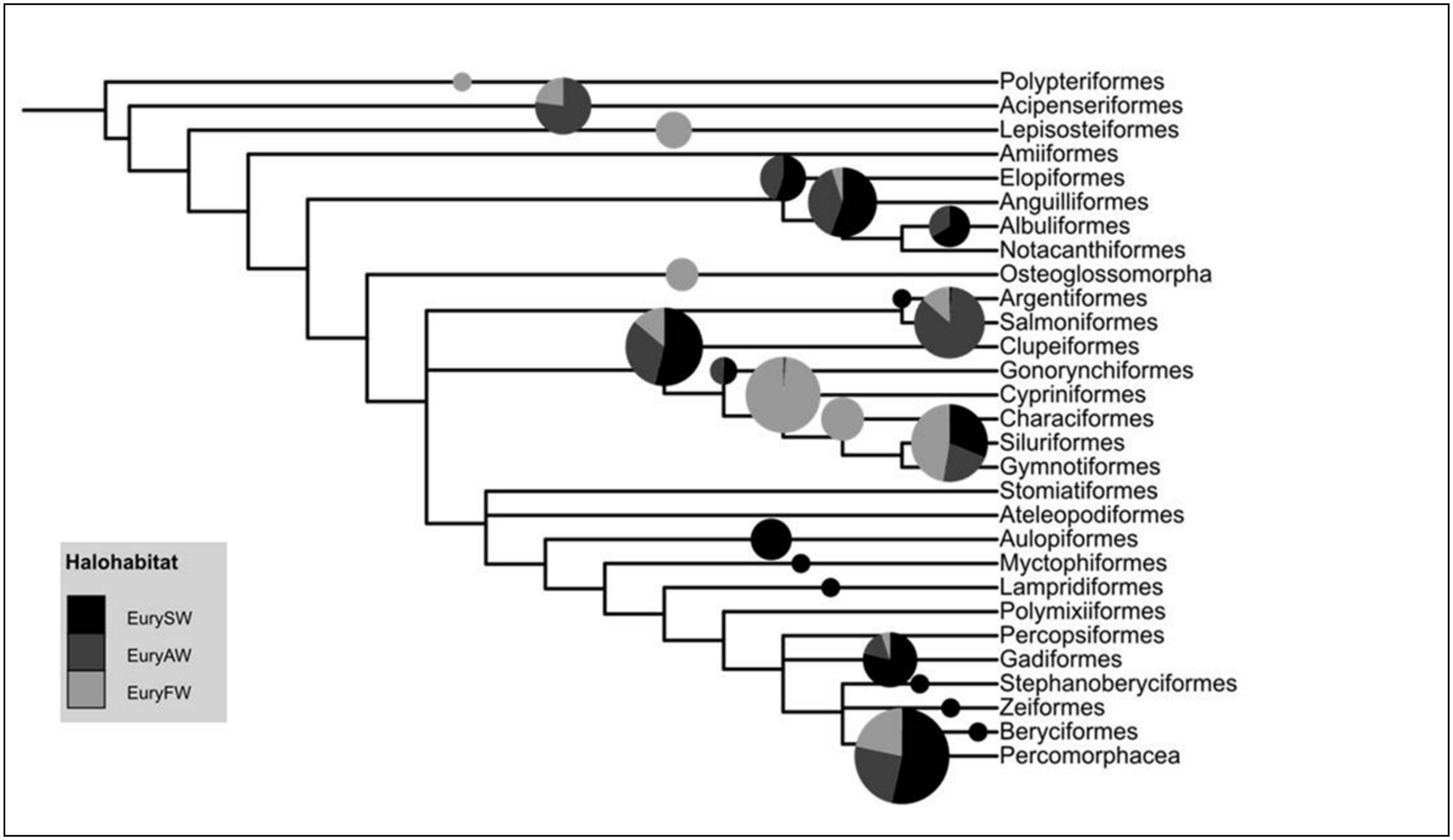
Polypteriformes
Acipenseriformes
Lepisosteiformes
Amiiformes
Elopiformes
Anguilliformes
Albuliformes
Notacanthiformes
Osteoglossomorpha
Argentiformes
Salmoniformes
Clupeiformes
Gonorynchiformes
Cypriniformes
Characiformes
Siluriformes
Gymnotiformes
Stomiatiformes
Ateleopodiformes
Aulopiformes
Myctophiformes
Lampridiformes
Polymixiiformes
Percopsiformes
Gadiformes
Stephanoberyciformes
Zeiformes
Beryciformes
Percomorphacea
Halohabitat
EurySW
EuryAW
EuryFW

Table S1. Data on halotolerance by order, family and species. For one or more reference on each species, table provides the number of tolerance limits determined by ontogenetic stage of the subjects and experimental approach (direct in left hand column and gradual in right hand column for each stage, e.g. the top row indicates that 2 direct limits were determined for larvae and 4 direct limits were determined for juveniles + adults, in *Acipenser naccarii*).

Order	Family	Species	Limits estimated				Reference
			Larva		Juv&Ad		
			Dir	Grad	Dir	Grad	
Acipenseriformes	Acipenseridae	*Acipenser naccarii*	2		4		(Cataldi et al., 1999)
		Huso huso			1		(Farabi et al., 2007)
Albuliformes	Albulidae	*Albula sp*	2		6		(Pfeiler, 1981)
Atheriniformes	Atherinidae	*Chirostoma promelas*	2	2			(Martinez-Palacios et al., 2008)
		Craterocephalus stercusmuscarum				2	(Williams and Williams, 1991)
		Leuresthes sardine	4		2		(Reynolds and Thomson, 1974)
		Leuresthes tenuis	2		2		(Reynolds et al., 1976)
		Menidia beryllina			2		(Hubbs et al., 1971)
		Odontesthes bonariensis			2		(Tsuzuki et al., 2000)
		Odontesthes hatcheri			2		(Tsuzuki et al., 2000)

	Melanotaeniidae	*Melanotaenia splendida*	2	2	(Williams and Williams, 1991)
Characiformes	Characidae	*Astyanax bimaculatus*		1	(Chung, 1999)

Table S1 (cont'd)

Clupeiformes	Clupeidae	*Clupea harengus*	1				(Yin and Blaxter, 1987)
Cypriniformes	Cobitidae	*Cobitis taenia*		2			(Bohlen, 1999)
	Cyprinidae	*Aristichthys nobilis*			2		(Garcia et al., 1999)
		Barbus callensis			2		(Kraiem and Pattee, 1988)
		Carassius auratus			2	2	(Jasim, 1988)
					1	2	(Schofield and Nico, 2009)
					2		(Threader and Houston, 1983)
		Catla catla			2		(Ghosh et al., 1973)
		Ctenopharyngodon idella			2		(Chervinski, 1977b)
						4	(Kilambi and Zdinak, 1980)
					2		(Maceina and Shireman, 1979)
		Cyprinus carpio			2		(Abo Hegab and Hanke, 1982)
					2		(Geddes, 1979)
		Danio rerio			2		(Dou et al., 2006)
		Hybognathus placitus			2		(Ostrand and Wilde, 2001)
		Hypophthalmichthys molitrix			2		(Chervinski, 1977b)
		Labeo rohita			2		(Ghosh et al., 1973)
						2	(Pillai et al., 2003)
		Notropis buccula			2		(Ostrand and Wilde, 2001)
		Notropis oxyrhynchus			2		(Ostrand and Wilde, 2001)

Table S1 (cont’d)

		Pogonichthys macrolepidotus		2	(Young and Cech, 1996)
		Ptychocheilus lucius	2		(Nelson and Flickinger, 1992)
		Puntius conchonius	2		(Nazneen and Begum, 1981)
		Puntius sophore	2		(Nazneen and Begum, 1981)
		Rutilus rutilus	2		(Schofield et al., 2006)
Cyprinodontiformes	Aplocheilidae	*Aplocheilus panchax*	2		(Nazneen and Begum, 1981)
	Cyprinodontidae	*Adinia xenica*		2	(Nordlie, 1987)
		Cyprinodon dearborni	1	1	(Chung, 1982)
		Cyprinodon rubrofluviatilis	2		(Ostrand and Wilde, 2001)
		Cyprinodon variegatus		1	(Jordan et al., 1993)
				2	(Nordlie and Haney, 1993)
		Floridichthys carpio		2	(Nordlie and Haney, 1993)
		Jordanella floridae		2	(Nordlie and Haney, 1993)
	Fundulidae	*Fundulus catenatus*		2	(Griffith, 1974)
		Fundulus chrysotus	2	2	(Crego and Peterson, 1997)
				2	(Griffith, 1974)
		Fundulus cingulatus		2	(Griffith, 1974)
		Fundulus confluentus		2	(Griffith, 1974)
		Fundulus diaphanus		2	(Griffith, 1974)
		Fundulus grandis			(Crego and Peterson, 1997)

		2	(Perschbacher et al., 1990)

Table S1 (cont'd)

Fundulus heteroclitus		4[1]	(Griffith, 1974)
Fundulus jenkinsi		2	(Griffith, 1974)
Fundulus kansae	2	2	(Stanley and Fleming, 1977)
Fundulus luciae		2	(Griffith, 1974)
Fundulus majalis		2	(Griffith, 1974)
Fundulus notatus		2	(Griffith, 1974)
Fundulus notti	2	2	(Crego and Peterson, 1997)
Fundulus olivaceus		2	(Griffith, 1974)
Fundulus pulvereus		2	(Griffith, 1974)
Fundulus rathbuni		2	(Griffith, 1974)
Fundulus sciadicus		2	(Griffith, 1974)
Fundulus seminolis	2	2	(DiMaggio et al., 2009)
		2	(Griffith, 1974)
Fundulus similis			(Crego and Peterson, 1997)
Fundulus stellifer		2	(Griffith, 1974)
Fundulus waccamensis		2	(Griffith, 1974)
Fundulus zebrinus		2	(Griffith, 1974)

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[1] Two limits reported in this paper for *Fundulus swampinus*, which is a synonym of *Fundulus heteroclitus*

Table S1 (cont'd)

				2		(Ostrand and Wilde, 2001)
		Lucania goodei		2		(Dunson and Travis, 1991)
		Lucania parva			2	(Dunson and Travis, 1991)
	Poeciliidae	*Gambusia affinis*		2	2	(Chervinski, 1983)
		Poecilia latipinna		2		(Nazneen and Begum, 1981)
					2	(Nordlie and Walsh, 1989)
		Poecilia reticulata		6		(Shikano and Fujio, 1998)
		Xiphophorus helleri		2		(Dou et al., 2006)
Esociformes	Esocidae	*Esox lucius*		2		(Jacobsen et al., 2007)
					2	(Jørgensen et al., 2010)
Gadiformes	Gadidae	*Gadus morhua*		1		(Provencher et al., 1993)
			1			(Yin and Blaxter, 1987)
Gasterosteiformes	Gasterosteidae	*Gasterosteus aculeatus*		1		(Campeau et al., 1984)
		Gasterosteus wheatlandi		1		(Campeau et al., 1984)
	Syngnathidae	*Hippocampus kuda*		1		(Hilomen-Garcia et al., 2001)
Mugiliformes	Mugilidae	*Chelon labrosus*		1		(Chervinski, 1977a)
				1	1	(Hotos and Vlahos, 1998)
		Liza aurata		2		(Chervinski, 1975)
		Liza haematocheila	2			(Bulli and Kulikova, 2006)
		Liza saliens		2	1	(Chervinski, 1977a)

Table S1 (cont’d)

		Mugil cephalus	1	1	(Hotos and Vlahos, 1998)
Osmeriformes	Osmeridae	*Hypomesus nipponensis*		2	(Swanson et al., 2000)
		Hypomesus transpacificus		2	(Swanson et al., 2000)
	Retropinnidae	*Retropinna semoni*		2	(Williams and Williams, 1991)
Perciformes	Ambassidae	*Chanda commersonii*	2		(Rajasekharan Nair and Balakrishnan Nair, 1984)
		Chanda thomassi	2		(Rajasekharan Nair and Balakrishnan Nair, 1984)
	Anarhichadidae	*Anarhichas lupus*	1		(Le Francois et al., 2003)
	Blenniidae	*Parablennius sanguinolentus*		1	(Plaut, 1999)
		Salaria fluviatilis		1	(Plaut, 1998)
		Salaria pavo		1	(Plaut, 1998)
	Centropomidae	*Centropomus parallelus*	1		(Tsuzuki et al., 2007)
	Cichlidae	*Hemichromis letourneuxi*	2	2	(Langston et al., 2010)
		Oreochromis aureus		2	(Lutz et al., 2010)
			2		(Watanabe et al., 1985)
		Oreochromis mossambicus		2	(Lutz et al., 2010)

Table S1 (cont'd)

	Oreochromis niloticus				2	(Lemarie et al., 2004)
				2		(Li and Li, 1999)
				2		(Li et al., 2008)
					2	(Lutz et al., 2010)
				2		(Watanabe et al., 1985)
	Sarotherodon melanotheron				2	(Lemarie et al., 2004)
				2		(Li et al., 2008)
Eleotridae	*Dormitator maculatus*				2	(Nordlie et al., 1992)
	Hypseleotris klunzingeri				2	(Williams and Williams, 1991)
Gobiidae	*Boleophthalmus boddaerti*			2		(Ip et al., 1991)
	Gobiosoma robustum			2		(Schöfer, 1979)
	Luciogobius pallidus	2				(Hirashima and Takahashi, 2008)
	Microgobius gulosus			2		(Schöfer, 1979)
	Rhinogobius sp1		2			(Hirashima and Tachihara, 2000)
	Rhinogobius sp2		2			(Hirashima and Tachihara, 2000)
Lutjanidae	*Lutjanus argentimaculatus*					(Estudillo et al., 2000)
Moronidae	*Dicentrarchus labrax*			1		(Dalla Via et al., 1998)
				1		(Marino et al., 1994)
		2				(Varsamos et al., 2001)
Osphronemidae	*Trichogaster trichopterus*			2		(Dou et al., 2006)

Table S1 (cont'd)

	Percichthyidae	*Maccullochella peelii peelii*	2				(Mellor and Fotedar, 2005)
	Percidae	*Perca fluviatilis*		2			(Bein and Ribi, 1994)
	Rachycentridae	*Rachycentron canadum*	2				(Faulk and Holt, 2006)
	Sciaenidae	*Cynoscion nebulosus*	3				(Banks et al., 1991)
	Serranidae	*Centropristis striata*			1		(Young et al., 2006)
	Siganidae	*Siganus rivulatus*					(Saoud et al., 2007)
	Sparidae	*Acanthopagrus butcheri*				1	(Partridge and Jenkins, 2002)
	Teraponidae	*Bidyanus bidyanus*			2		(Guo et al., 1995)
Pleuronectiformes	Paralichthyidae	*Paralichthys californicus*					(Madon, 2002)
		Paralichthys dentatus					(Malloy and Targett, 1991)
		Paralichthys lethostigma		1			(Cai et al., 2007)
			1				(Daniels et al., 1996)
					2		(Smith et al., 1999)
		Paralichthys olivaceus	2				(Wang et al., 2000)
		Paralichthys orbignyanus				1	(Sampaio et al., 2007)
	Pleuronectidae	*Microstomus achne*			1		(Wada et al., 2007)
		Platichthys bicoloratus			1		(Wada et al., 2007)
		Platichthys flesus			1		(Arnold-Reed and Balment, 1991)
			1				(Yin and Blaxter, 1987)

Table S1 (cont'd)

		Platichthys stellatus		1		(Takeda and Tanaka, 2007)
				1		(Wada et al., 2007)
		Pseudopleuronectes yokohamae		1		(Wada et al., 2007)
		Verasper variegatus		1		(Wada et al., 2007)
	Scophthalmidae	*Scophthalmus maximus*		2		(Mu and Song, 2005)
Salmoniformes	Salmonidae	*Coregonus nasus*		3		(de March, 1989)
		Oncorhynchus tshawytscha		1		(Taylor, 1990)
		Salvelinus alpinus		1		(Dempson, 1993)
				1		(Staurnes et al., 1992)
Scorpaeniformes	Cottidae	*Cottus asper*			1	(Henriksson et al., 2008)
		Leptocottus armatus			1	(Henriksson et al., 2008)
Siluriformes	Callichthyidae	*Callichthys callichthys*	2			(Mol, 1994)
		Hoplosternum littorale	2			(Mol, 1994)
		Megalechus thoracata	2			(Mol, 1994)
	Clariidae	*Clarias gariepinus*	2			(Britz and Hecht, 1989)
				2		(Odo and Inyang, 2001)
		Clarias lazera		2	2	(Chervinski, 1984)
					2	(Clay, 1977)
		Heterobranchus longifilis		2		(Fashina-Bombata and Busari, 2003)
	Ictaluridae	*Ictalurus catus*		2		(Kendall and Schwartz, 1968)

		Ictalurus furcatus	2		(Allen and Avault, 1971)
		Ictalurus punctatus	2		(Allen and Avault, 1971)
		Pylodictis olivaris	4	2	(Bringolf et al., 2005)
Synbranchiformes	Synbranchidae	*Monopterus albus*	2	2	(Schofield, 2003)
Tetraodontiformes	Tetraodontidae	*Sphoeroides greeleyi*			(Prodocimo and Freire, 2001)
		Sphoeroides testudineus			(Prodocimo and Freire, 2001)

Table S2. Halotolerance groups defined by cluster analysis, direct method experiments. For two named halotolerance groups identified by centroid cluster analysis, table provides species, the lower and upper LC_{50} halotolerance limits, and halotolerance breadth.

Group	**Species**	**Lower**	**Upper**	**Breadth**
Euryhaline	*Albula sp*	2.9	52	49
	Albula sp	3.3	59	56
	Albula sp	5.2	63	58
	Cyprinodon rubrofluviatilis	0	46	46
	Fundulus kansae	0.4	44	44
	Fundulus zebrinus	0	43	43
	Gobiosoma robustum	0	55	55
	Leuresthes sardina	5	58	53
	Microgobius gulosus	2	60	58
Stenohaline	*Acipenser naccarii*	0	15	15
	Acipenser naccarii	0	22	22
	Ambassis ambassis	0.45	31	31
	Ameiurus catus	0	14	14
	Aplocheilus panchax	0	10	10
	Barbus callensis	0.5	16	15

Bidyanus bidyanus	0	17	17
Boleophthalmus boddarti	1.7	31	29
Carassius auratus	0	12	12
Carassius auratus	0	16	16
Catla catla	0	12	12
Clarias gariepinus	0.042	13	12

Table S2 (cont'd)

Stenohaline (cont'd)	*Clarias gariepinus*	0.14	11	11
	Coregonus nasus	0	16	16
	Ctenopharyngodon idella	0.5	10	9.5
	Ctenopharyngodon idella	0	15	15
	Cyprinus carpio	0	17	17
	Cyprinus carpio	0	15	15
	Danio rerio	0	12	12
	Esox lucius	0	12	12
	Fundulus chrysotus	0	26	26
	Fundulus nottii	0	17	17
	Fundulus seminolis	0	28	28
	Gambusia affinis	0.4	22	21
	Hemichromis letourneuxi	0	25	25
	Heterobranchus longifilis	0	7	7
	Hybognathus placitus	0	16	16
	Hypophthalmichthys molitrix	0.5	8.8	8.3
	Hypophthalmichthys nobilis	0	7.6	7.6
	Ictalurus furcatus	0	14	14
	Ictalurus punctatus	0	14	14

Labeo rohita	0	11	11
Leuresthes tenuis	8.6	38	29

Table S2 (cont'd)

Stenohaline (cont'd)	*Lucania goodei*	0	25	25
	Melanotaenia splendida	0.3	21	21
	Menidia beryllina	0.8	36	35
	Monopterus albus	0.2	17	17
	Notropis buccula	0	18	18
	Notropis oxyrhynchus	0	15	15
	Odontesthes bonariensis	0	25	25
	Odontesthes hatcheri	0	25	25
	Oreochromis aureus	0	20	20
	Oreochromis niloticus	0	20	20
	Oreochromis niloticus	0	14	14
	Oreochromis niloticus	0	20	20
	Parambassis thomassi	0	23	23
	Poecilia latipinna	0	10	10
	Poecilia reticulata	0	34	34
	Poecilia reticulata	0	23	23
	Poecilia reticulata	0	27	27
	Ptychocheilus lucius	0	13	13
	Puntius conchonius	0	8.4	8.4

Puntius sophore	0	8.4	8.4
Pylodictis olivaris	0	15	15
Pylodictis olivaris	0	10	10
Rutilus rutilus	0	14	14
Sarotherodon melanotheron	0	34	34
Scophthalmus maximus	16	38	22
Trichopodus trichopterus	0	17	17
Xiphophorus helleri	0	20	20

Table S3. Halotolerance groups defined by cluster analysis, gradual method experiments. For three named groups identified by centroid cluster analysis, table provides species, the lower and upper LC_{50} halotolerance limits, and tolerance breadth.

Group	**Species**	**Lower**	**Upper**	**Breadth**
Euryhaline FW	*Dormitator maculatus*	0	75	75
	Fundulus chrysotus	0	65	65
	Fundulus diaphanus	0	70	70
	Fundulus grandis	0	80	80
	Fundulus jenkinsi	0	74	74
	Fundulus seminolis	0	60	60
	Fundulus waccamensis	0	55	55
	Gambusia affinis	0.4	59	58
	Hemichromis letourneuxi	0	55	55
	Jordanella floridae	0	80	80
	Lucania parva	0	80	80
	Poecilia latipinna	0	80	80
	Retropinna semoni	0.3	59	58
Euryhaline	*Adinia xenica*	0	100	100
	Cyprinodon variegatus	0	125	125
	Floridichthys carpio	0	90	90
	Fundulus confluentus	0	99	99

	Fundulus heteroclitus	0	114	114
	Fundulus kansae	0.4	99	99
	Fundulus luciae	0	101	101
	Fundulus majalis	0	99	99
	Fundulus pulvereus	0	101	101
	Fundulus zebrinus	0	89	89
	Sarotherodon melanotheron	0	123	123

Table S3 (cont'd)

Stenohaline	*Carassius auratus*	0	14	14
	Carassius auratus	0	12	12
	Clarias gariepinus	0.14	11	11
	Clarias gariepinus	0.12	23	22
	Craterocephalus stercusmuscarum	0.3	44	43
	Ctenopharyngodon idella	0	16	16
	Ctenopharyngodon idella	0	14	14
	Esox lucius	0	14	14
	Fundulus catenatus	0	24	24
	Fundulus chrysotus	0	20	20
	Fundulus cingulatus	0	23	23
	Fundulus heteroclitus	0	27	27
	Fundulus notatus	0	20	20

Fundulus nottii	0	28	28
Fundulus olivaceus	0	24	24
Fundulus rathbuni	0	26	26
Fundulus sciadicus	0	24	24
Fundulus seminolis	0	23	23
Fundulus stellifer	0	21	21
Hypomesus nipponensis	0	27	27
Hypomesus transpacificus	0	19	19
Hypseleotris klunzingeri	0.3	38	38
Labeo rohita	0	9	9
Melanotaenia splendida	0.3	30	30
Monopterus albus	0.3	14	14
Oreochromis aureus	0.4	38	38
Oreochromis mossambicus	0.4	47	46
Oreochromis niloticus	0	46	46

Table S3 (cont'd)

Oreochromis niloticus	0.4	26	26
Pogonichthys macrolepidotus	0	19	19
Pylodictis olivaris	0	16	16