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SYMPOSIUM

Repeated Genetic Targets of Natural Selection Underlying Adaptation of Fishes to Changing Salinity

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Synopsis Ecological transitions across salinity boundaries have led to some of the most important diversification events in the animal kingdom, especially among fishes. Adaptations accompanying such transitions include changes in morphology, diet, whole-organism performance, and osmoregulatory function, which may be particularly prominent since divergent salinity regimes make opposing demands on systems that maintain ion and water balance. Research in the last decade has focused on the genetic targets underlying such adaptations, most notably by comparing populations of species that are distributed across salinity boundaries. Here, we synthesize research on the targets of natural selection using whole-genome approaches, with a particular emphasis on the osmoregulatory system. Given the complex, integrated and polygenic nature of this system, we expected that signatures of natural selection would span numerous genes across functional levels of osmoregulation, especially salinity sensing, hormonal control, and cellular ion exchange mechanisms. We find support for this prediction: genes coding for V-type, Ca^{2+}, and Na^{+}/K^{+}-ATPases, which are key cellular ion exchange enzymes, are especially common targets of selection in species from six orders of fishes. This indicates that while polygenic selection contributes to adaptation across salinity boundaries, changes in ATPase enzymes may be of particular importance in supporting such transitions.

Introduction

Salinity is arguably the single most important physical variable that structures the distribution of aquatic animals in the wild. Throughout the history of vertebrates, evolutionary transitions into novel salinity regimes have been central to diversification (Lee and Bell 1999), including the evolution of large groups of fishes and the early origins of tetrapods (Schultz and McCormick 2013). In the fishes, a hyper-diversity of freshwater (FW) species relative to available habitat (nearly half in ~0.02% of Earth's available habitat by volume) suggests that FW environments are important adaptive zones, providing opportunity for diversification (Dawson 2012; Vega and Wiens 2012). For example, in ariid catfishes, invasion of FW in Australia–New Guinea has led to adaptive morphological radiation and speciation as a result of exploitation of newly available ecological opportunities (Betancur-R. et al. 2012). In threespine stickleback (*Gasterosteus aculeatus*), adaptive phenotypic diversification in morphological and physiological traits (e.g., Bell et al. 1993; Divino et al. 2016) has resulted from the repeated invasion of marine populations into FW lakes (Bell and Foster 1994). Though important in creating

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biodiversity, the barrier between FW and seawater (SW) is formidable, with few species having crossed it (Lee and Bell 1999); indeed, the species that occupy adjacent FW and marine halohabitats (Schultz and McCormick 2013) are almost completely non-overlapping (Odum 1988), while at the same time intermediate salinity environments (e.g., estuaries) remain relatively species poor (Khlebovich 1969). Only 8–10% of actinopterygian fishes use brackish water or migrate between salinities as part of their life history (Lévêque et al. 2008; Schultz and McCormick 2013). Evolutionary transitions between osmotic environments are likely supported by adaptations in a complex suite of integrated traits, such as those associated with osmoregulatory physiology (Whitehead 2010), as well as diet (Ishikawa et al. 2019), body size and shape (Kolmann et al. 2020; Clarke 2021), defensive morphology (Bell et al. 1993), behavior (Perrott et al. 1992), and symbiotic relationships with microbiota (Lozupone and Knight 2007).

Alternative salinity environments present particularly strong and opposing physiological demands on osmoregulatory systems, and natural selection is likely to act on the processes that allow fish to sense and respond to changes in salinity. Maintaining cellular ion and osmotic concentrations within relatively narrow limits is a requirement for normal function in animals. With few exceptions, bony fishes have evolved to regulate the ion composition of their blood to approximately one-third the osmotic strength of SW (Edwards and Marshall 2012). Thus, fish in FW must counteract the passive loss of ions and gain of water to their dilute environment. They do so by actively taking up salt across the gill and excreting water *via* the kidney. In SW, by contrast, fish must counteract the passive gain of ions and loss of water to their more concentrated environment. This is accomplished first by drinking SW and absorbing both salt and water across the gut. Excess monovalent ions (Na^+ and Cl^-) are secreted by the gill, and divalent ions (Mg^+, Ca^{2+}, and $SO_4{}^+$) by the kidney. Ion uptake by the gill in FW, and secretion by the gill in SW occur through specialized ionocytes with ion transporters specific for each environment. Gill remodeling in response to changing salinity is controlled by a complex suite of osmosensory mechanisms at cellular and organismal levels (Kültz 2012) and is under hormonal control (McCormick 2011). These mechanisms, particularly with respect to ionocyte structure and function, vary among FW species (Dymowska et al. 2012; Edwards and Marshall 2012) but are more conserved among SW species (Evans et al. 2005).

Contrasts in function to maintain homeostasis between alternative salinity environments may lead to tradeoffs in performance (Brennan et al. 2015; Velotta et al. 2015), and suggest that the osmoregulatory system of many taxa may specialize on FW or SW. There is strong evidence that many FW fishes are relatively intolerant of SW, though there is little evidence for the complementary limitation of SW fishes (Schultz and McCormick, 2013). Moreover, microevolutionary-scale studies of euryhaline species that diverge in osmoregulatory function indicate that changes in gene sequence, gene expression, and organism-level function associated with water and ion exchange accompany differentiation in halohabitats among populations (Whitehead 2010, 2012; Kozak et al. 2014; Velotta et al. 2014, 2015, 2017; Brennan et al. 2015, 2018; Divino et al. 2016; McCormick et al. 2019).

In this paper, we review studies that describe intraspecific genomic differentiation associated with differing halohabitats. Over the past decade, whole-genome approaches have been applied to the identification of the genetic targets of selection that underlie transitions between marine and FW environments, primarily using euryhaline species as models (Table 1). We know of no effort to date to determine whether these targets of selection are the same in different clades. We, therefore, test whether there are common targets of selection across species, and the extent to which adaptive genetic changes that enable salinity invasions are constrained to particular loci or functional groups. In this analysis, we focus on genes that have been identified as targets of selection in microevolutionary contrasts, in which gene sequences are compared among populations that differ in halohabitat. Population-level contrasts may take the form of candidate gene studies (*e.g.,* Shimada et al. 2011) or genome-wide approaches (*e.g.,* Jones et al. 2012). Here, we favored genome-wide approaches because they allow greater scope for discovery and minimize bias regarding genes that may be important. Such population genomics studies scan for sequence differentiation between populations from different osmotic habitats and are, therefore, useful for identifying loci that support fitness in these alternate environments. Multiple comparative model systems (from multiple marine/FW clades) have been studied, thereby broadening our scope for inferring shared or divergent patterns across multiple independent FW invasions. We searched the literature for signatures of natural selection in species of fish that are distributed across a wide range of salinities or have otherwise crossed the salinity barrier. We predicted that adaptive modification of osmoregulatory physiology would span numerous genes across functional levels of osmoregulation, especially cellular ion exchange processes and the salinity sensing and hormonal control mechanisms that are key to ion flux. Though evolution of osmoregulatory physiology may be highly polygenic, we sought to test whether

Table 1 Study information for each species including in shared gene- and gene-family level analysis

Species	Common Name	Populations	Region	Halohabitat contrast or range	Citation
Clupea harengus	Atlantic herring	19	Atlantic-Baltic Sea	Gradient from 3–35 ppt	Martinez Barrio et al. (2016)[1]
Cottus asper	Prickly sculpin	4	Lower Fraser River Valley British Columbia, Canada	Lake (FW) vs. estuarine (brackish)	Dennenmoser et al. (2016)[2]
Fundulus heteroclitus	Mummichog	2	Chesapeake Bay—Potomac River	Riverine (~0ppt) vs. estuarine (~15 ppt)	Brennan et al. (2018)[3]
Gadus morhua	Atlantic cod	7	North Sea-Baltic Sea	Gradient from 6–35 ppt	Berg et al. (2015)[4]
Gasterosteus aculeatus	Threespine stickleback	49	Circumpolar	Lake (FW) vs. marine (SW)	Jones et al. (2012)[5]
Gasterosteus aculeatus	Threespine stickleback	34	Atlantic-Pacific	Lake (FW) vs. marine (SW)	Jones et al. (2012)[6]
Gasterosteus aculeatus	Threespine stickleback	5	Atlantic polar region	Lake (FW) vs. marine (SW)	Hohenlohe et al. (2010)[7]
Gasterosteus aculeatus	Threespine stickleback	9	North Sea, Jutland Peninsula, Denmark	Riverine/lake (FW) vs. marine (SW)	Ferchaud and Hansen (2016)[8]
Lucania parva	Rainwater killifish	6	Coastal Gulf of Mexico	Upstream FW site vs. estuarine terminus of repeated drainages	Kozak et al. (2014)[9]
Oncorhynchus mykiss	Rainbow trout	2	Alaska–Oregon coast	Migratory (to SW) vs. FW resident	Hale et al. (2013)[10]
Salmo salar	Atlantic salmon	9	Coastal US–Scandinavia-Russia	Anadromous vs. landlocked (FW)	Kjærner-Semb et al. (2020)[11]

[1]Selected genes in genomic regions meeting two criteria: (1) contingency χ^2 tests of allele frequency differences in binned marine (Atlantic Ocean) or brackish (Baltic Sea) populations, and (2) significant in *Bayenv2* (Günther and Coop 2013) test of allele frequency association with salinity gradient.
[2]Selected genes called from F_{ST} outlier tests between paired estuary vs. lake population comparisons in *Bayescan* (Foll and Gaggiotti 2008).
[3]Multivariate approach combined F_{ST} and π (nucleotide diversity) used to calculate *P*-value distribution of which top 1% outliers were called as selected.
[4]Selected genes called from F_{ST} outliers in *Bayescan* (Foll and Gaggiotti, 2008) or *LOSITAN* (Antao et al. 2008).
[5]Selected genes called with combined Self-Organizing Map and Hidden Markov Model approach.
[6]Selected genes significant in *Bayenv* (Coop et al. 2010) test for allele frequency associations between marine and lake populations.
[7]Selected genes called in regions of significant pairwise marine vs. lake F_{ST} using goodness-of-fit *G* statistic.
[8]Pairwise F_{ST} outlier approach using Gaussian kernel smoothing across each chromosome and applying sliding window parameter of 150,000 bp.
[9]Selected genes called as top 5% outliers from empirical F_{ST} distribution. To be conservative, we restricted analysis here to only those loci that were under selection in at least two out of the three drainages tested.
[10]Selected genes called from F_{ST} outliers in *LOSITAN* (Antao et al. 2008).
[11]Selective sweep regions identified in 100 kb sliding windows using pairwise difference in allele frequency as dAF statistic (Carneiro et al. 2014).

phylogenetically independent adaptations to divergent salinities were or were not constrained to a small subset of key genes.

Methods

We searched the literature for genome-wide scans for selection in populations that span a FW–SW boundary or are distributed across a natural salinity gradient. Within each study, we identified annotated genes predicted to be under selection across populations, and then compared these results across species to identify selected genes shared among them. We repeated this analysis after assigning each selection gene to a gene family (see below for details) to test whether shared selection was more common at higher functional levels. We used the search term "*selection scan and genom∗ and fish and salinity*" in Google Scholar. Additional studies were found by scanning reference sections of papers identified in our search as well as from our own knowledge of the literature and by contacting colleagues. We manually filtered this literature for studies that met three criteria: (1) studies that estimate the historical signature of natural selection (Barrett and Hoekstra 2011) in populations of fish distributed across brackish (> isosmotic, ∼10 ppt) or SW to low salinity (< isosmotic) or FW; (2) studies that estimated selection genome-wide, as opposed to those based on a predetermined set of candidates; and (3) studies for which gene annotations could be translated to human orthologs for direct comparison. We identified a total of 13 studies that fit these criteria across eight species of teleost fish (Table 1). A total of four of these studies were conducted on threespine stickleback, and we combined genes from each of these data sets, which together represented populations from across the species' circumpolar distribution. Each of the other species was represented by a single study (Table 1). In some studies, salinity was not measured or presented (Table 1). In those cases, we followed the authors categorization of halohabitat (as FW, brackish, or SW) and where not noted by the authors, interpreted halohabitat as follows: "lake" or "riverine" populations as FW, "estuarine" populations as brackish water, and "marine" or "anadromous" as having at least the adult portion of the life cycle in SW.

Species comparisons of selected genes

For ease of interpretation, we refer to genes identified under selection as "selected genes." We based the determination of selected genes on the criteria set by the authors of each study (Table 1). All studies identified single nucleotide polymorphisms (SNPs) across populations (at least two population-level comparisons) and used these data to identify selected genes based on allele frequency differences or associations of allele frequency and environmental variation (Table 1). A list of all selected genes for each study can be found online (Supplementary Table S1).

We examined common targets of selection among species using the *multiple list comparator* function in *molbiotools* (molbiotools.com). We refer to these common targets at the gene level as "shared-selected genes." Prior to assessment of shared selection, all selected genes were converted to human gene names and their corresponding symbols using the *g:Orth* function (https://biit.cs.ut.ee/gprofiler/orth) in the online version of *gProfiler* (Reimand et al. 2016). This function provides orthologous gene mappings based on data from the Ensembl database (ensembl.org). Readers who wish to find species-specific gene names or Ensembl IDs for species represented in this study can run *g:Orth* in reverse by querying the human gene names listed in Supplementary Tables S1 and S2 online. Additionally, we determined the gene family to which each selected gene belonged (thus designated as a "selected gene family") and examined sharing at the family level, referring to common targets at the gene family level as "shared-selected gene families." Gene family information was determined according to the Human Genome Gene Nomenclature Committee (HGNC) downloaded from genenames.org. Gene families are derived by duplication from a single ancestral protein, often followed by sub- or neo-functionalization and as such, tend to have shared biochemical functions (Gray et al. 2016). Genes in the HGNC list were only considered if they have active gene symbols and at least one family specified. Sharing relationships at gene- and gene-family levels among all eight species were visualized using UpSet diagrams, a tool for visualizing set intersections in a graph-based set layout (Lex et al. 2014).

Finally, we searched our observed set of selected genes for candidate genes and gene families known to be involved in osmotic and ion regulation in fishes. This set of candidate genes was curated by the authors (with the assistance of Dr. William Marshall, St. Francis Xavier University, Nova Scotia, Canada) based on our knowledge of the physiology and literature, and this list is available online (Supplementary Table S2). The focus of this candidate list was on proteins (and the genes coding for them) that are known to contribute to functional complexes that govern ionoregulation in the epithelial tissues, primary the gills, but also the brain, gut, and kidney. We categorized candidate genes by their purported function, including the ATPase pumps, secondary ion cotransporters and channels, structural junction proteins, osmosensors and water channels, and regulatory pathways including

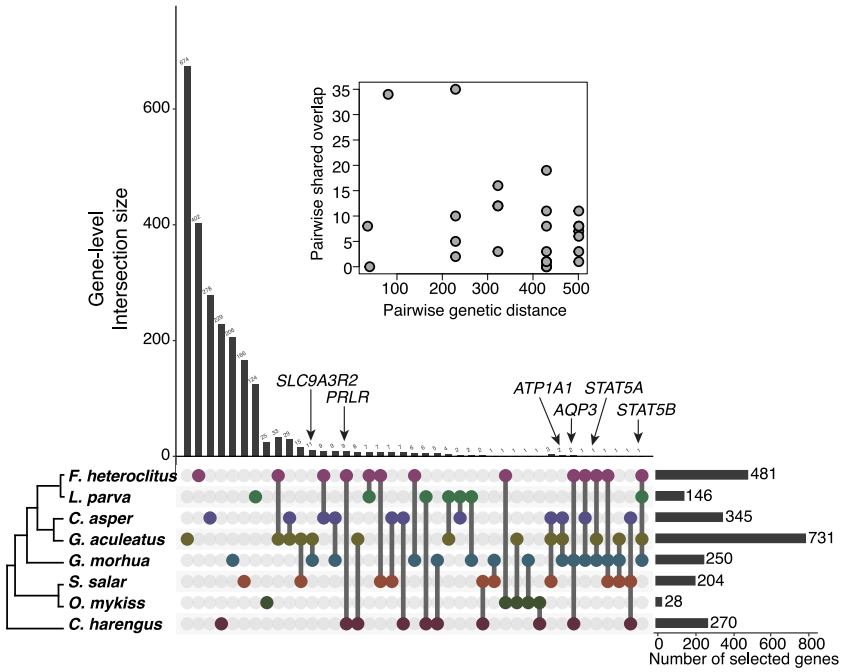

Fig. 1 Gene-level UpSet diagram showing shared overlap (and non-overlap) in "selected genes" among eight species (rows). Intersection size represents the number of genes present in each contrast (columns). The first eight columns represent those selected genes unique to each species (i.e., not shared with any other species). Gray lines connecting grid points within each column indicate the specific contrast between two or more species. Horizontal bars show the total number of selected genes for each species. Arrows point to contrasts containing shared genes from our reference set of candidate osmoregulatory loci (Supplementary Table S2). Phylogenetic tree created from data in Deepfin database. Inset shows that there is no significant relationship between phylogenetic relatedness (genetic distance) and the extent of shared overlap in selected genes between any two pairs of species

cortisol, peptide, and protein hormones and their receptors, as well as known second messengers. We focused this analysis on the gene family level (e.g., the Na^+ and K^+-transporting ATPases), since in most cases the precise role of specific loci within gene families, especially recently derived paralogs, has not been elucidated across all species. We refer to this candidate set hereafter as our "reference set" of osmoregulation genes/families.

Statistical analysis

A phylogenetic tree was created with *phytools* (Revell 2012) in R v. 4.1.1 using data from the Deepfin database (Betancur-R et al. 2017). We assessed whether the degree to which shared-selected genes or selected gene families was correlated to phylogenetic relatedness by regressing the number of shared-selected gene or gene families between each pair of species against their pairwise genetic distance. Pairwise genetic distance was calculated in *phytools*, using the *cophenetic.phylo* function, which computes the pairwise distances between the pairs of tips from a phylogenetic tree using its branch lengths. We expected to find a significant negative relationship if evolutionary relatedness influenced the extent of sharing.

Results and discussion

Selected genes were largely unique to individual species (Fig. 1). For example, of the more than 800 selected genes identified in threespine stickleback, 674 (84%) are under selection in this species only and not in any of the others we analyzed (Fig. 1). Species in which more total selected genes were identified tended to have a greater number of selected genes that are not shared with any other species (Fig. 1). Across all species, phylogenetic relatedness does not contribute to the extent of sharing of selected genes between pairs of species ($r = -0.3$; $P > 0.05$; Fig. 1). A total of 13 genes were identified to be under selection in at least three of eight species (Supplementary Table S3 online). Several of these shared selected genes are associated with osmoregulatory processes. One of them, *STAT5B*, is shared by four species, the most of any single gene (Fig. 1). This gene is a second messenger that translates FW stimuli into ion uptake and exchange at the gill (Bollinger et al. 2018). A total of three additional reference set osmoregulatory genes (Supplementary Table S2) were found to be shared-selected genes (*ATP1A1*, *AQP3*, and *STAT5A*; Fig. 1); all are described in more detail below. Most of the shared selected genes code for proteins with functions that are difficult to interpret in the context of

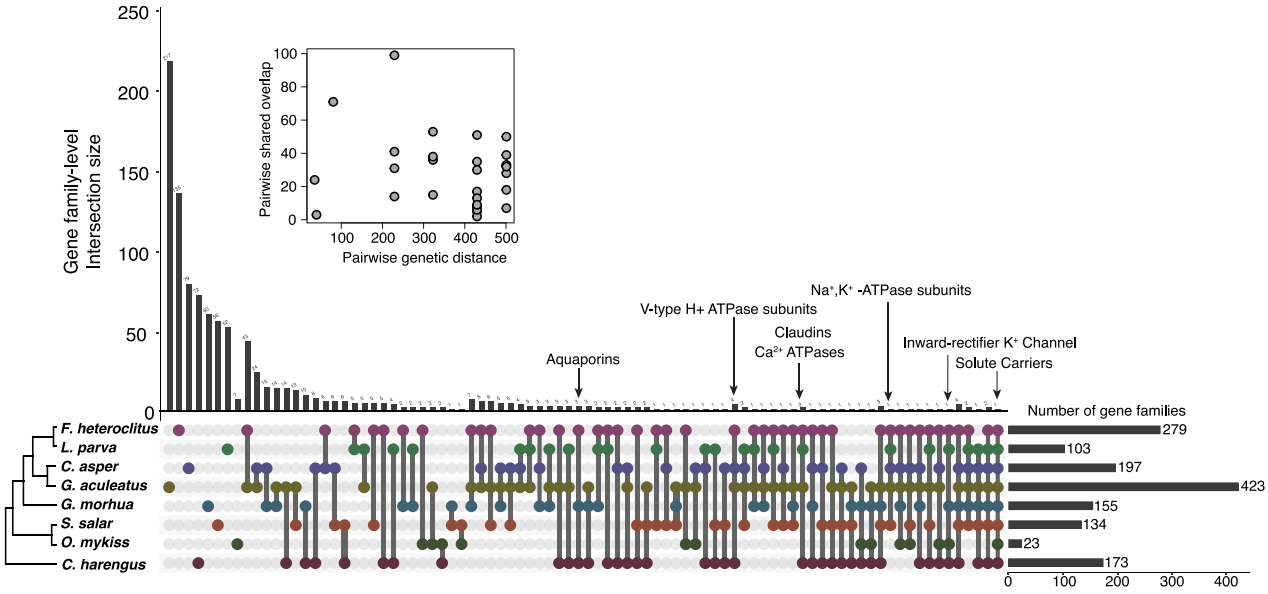

Fig. 2 Gene family-level UpSet diagram showing overlap (and non-overlap) of families with at least one "selected gene" in any of the eight species (rows). Intersection size represents the number of gene families present in each contrast (columns). The first eight columns represent those families unique to each species (i.e., not shared with any other species). Gray lines connecting grid points within each column indicate the specific contrast between two or more species. Horizontal bars show the total number of gene families for each species. Arrows point to contrasts containing shared gene families from our reference set of candidate osmoregulatory loci (Supplementary Table S2). Inset shows that there is no significant relationship between phylogenetic relatedness (genetic distance) and the extent of shared overlap in selected gene families between any two pairs of species

evolution across salinity boundaries (Supplementary Table S3). One gene, thyroid hormone receptor *THRA*, may play a role in ion secretion in SW by supporting—or interacting with—the growth hormone (GH)/insulin-like growth factor I (IGF-1) axis, which exerts a strong influence on SW acclimation ability in euryhaline species (McCormick 2001; see below for more details). In summary, a small fraction of the many genes identified as under selection is shared across multiple species, with even fewer of these having interpretable functional roles in the transition across salinity boundaries.

The finding that most selected genes are unique to individual species is not surprising for two reasons: (1) given that 230 million years (MY) of evolution separates them (timetree.org), we may expect little convergence at the genetic level, as the degree of parallelism tends to decrease at lower mechanistic levels (see below; Bolnick et al. 2018) and with decreasing phylogenetic relatedness (Conte et al. 2012); (2) the species studied here are undoubtedly subject to a wide range of selection pressures that vary considerably in degree across the environments to which they have adapted: these include variation in salinity, but also differences in temperature regime (Barrett et al. 2011) and temperature variability (Lee and Bell 1999), shifts in food resources (Ishikawa et al. 2019), and changes in patterns of migration (Dalziel et al. 2012; Velotta et al. 2018). Differences in environmental pressures should yield species-specific adaptations arising from selection at different genomic targets.

Limited sharing of selected genes also suggests that selection across salinity boundaries may act at the level of core functional pathways that regulate physiological function rather than being constrained to any specific genes or loci. Considering this, we assigned selected genes to gene families in order to assess the degree of convergence among genes that share similar functions or are otherwise related (e.g., subunits of large proteins, tissue-specific paralogs, or duplicated genes that serve a related function). We found a greater degree of shared overlap at the family-level compared to the gene-level (Fig. 2). A single large family, the solute carriers (~ 400 members in the human genome), has at least one selected gene in all eight species (Fig. 2). Among these shared gene families are a number in the reference set, i.e., involved in ion and osmotic regulation (summarized in Fig. 3); of 31 reference set gene families, nearly half are shared-selected gene families.

Below we provide details on shared-selected genes and gene families that are involved in osmoregulatory function. We organized these genes and gene families into their relevant functional groups (assigned *a priori*; Supplementary Table S2) to emphasize that natural selection has acted on a diverse set of osmoregulatory functions that span several levels of organization, from

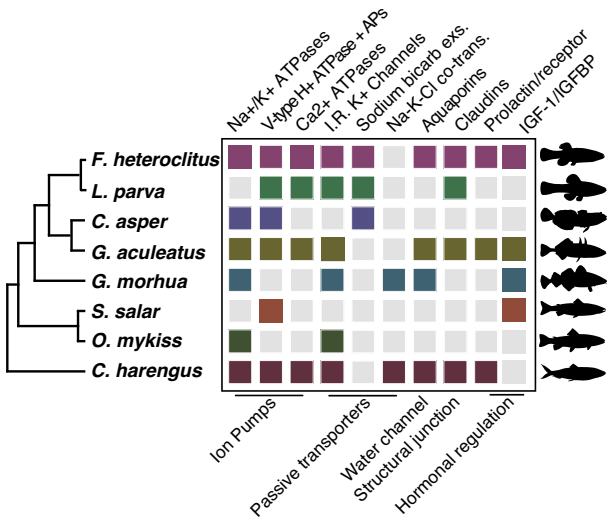

Fig. 3 Shared-selected genes/gene families in the candidate reference set related to ion- and osmoregulation studied. Results may differ from the above figures since evidence for selection was more loosely interpreted and candidate gene studies were included. Groupings are based on functional knowledge of proteins involved in osmoregulation (Supplementary Table S2).

osmotic sensing (Kültz 2012) and hormone signaling to ion exchange (McCormick 2011).

Active ion pumps are repeatedly targeted by selection

Active ion pumps are transmembrane enzymes that transform energy in the form of ATP to power transepithelial ion exchange and the maintenance of internal ion and water balance.

Na^+, K^+- ATPases

The Na^+, K^+- ATPase (NKA) pump is a key component of active ion transport in almost all transport epithelia. The role of NKA in osmoregulation, particularly in driving ion exchange at the gill, is well-established (Fig. 4). In the FW gill, for example, NKA maintains low internal Na^+ that helps promote transcellular Na^+ uptake (Evans et al. 1999). In SW, NKA provides both ion and electrical gradients used by co-transporters Na^+, K^+, Cl- co-transporter (NKCC), and the apical chloride channel CFTR, for transcellular Cl^- and paracellular Na^+ secretion (Edwards and Marshall 2012).

We found that *ATP1A1*, which codes for the catalytic α subunit of NKA, is under selection in three species (Fig. 1). *ATP1A1* is also potentially a selected gene in Atlantic herring, but this was indicated only when a less conservative modeling approach was considered (Martinez Barrio et al. 2016). This gene has been a repeated target of selection in FW populations of threespine stickleback over their circumglobal distribution (Shimada et al. 2011; Jones, Grabherr et al. 2012; DeFaveri and Merilä 2014; Nelson and Cresko 2018). The glycoprotein β subunit of NKA, which forms the heterodimer, thereby regulating the amount of NKA in the plasma membrane, is under selection in two other species (mummichog [*Fundulus heteroclitus*] and rainbow trout [*Oncorhynchus mykiss*]; Fig. 3; Supplementary Table S1). Overall, six species in our analysis exhibit a signature of selection in at least one subunit of NKA, if the less-conservative result for *ATP1A1* in Atlantic Herring (*Clupea harengus)* is included (Fig. 3).

Expression of alternative NKA α subunit paralogs under different salinities has been found in several teleosts (Richards et al. 2003; Dalziel et al. 2014) resulting in "FW isoforms" and "SW isoforms"—NKAα1a and NKAα1b, respectively (McCormick et al. 2009). Such "isoform switching" as it has been dubbed (Richards et al. 2003) may itself be the result of selection for broad salinity tolerance, such as in migratory salmon. Although the isoforms appearing in disparate groups have evolved independently, Dalziel et al. (2014) found sequence similarities among them indicating parallel evolutionary change: one-quarter of the amino acid substitutions that occur in the salmonid FW and SW NKAα paralogs also occur in either Mozambique tilapia (*Oreochromis mossambicus*) or climbing perch (*Anabas testudineus*), which have evolved independently from salmonids since the early Jurassic (Hughes et al. 2018).

Some progress has been made in clarifying the functional significance in the sequence differences between FW and SW NKAα paralogs. Using amino acid substitution into mammalian NKA, Jorgensen (2008) suggested that the FW isoform "promotes binding of Na^+ over K^+ from the cytoplasm, to reduce the Na^+/ATP ratio" relative to the SW isoform. Thus, selection may target mutations that alter Na^+ and K^+ kinetics or efficiencies to meet the demands of ion regulation in FW vs. SW. In particular, the need for differential electrical gradients across ionocytes may be greater in SW than in FW. It is of interest to note that zebrafish (*Danio rerio*), a stenohaline FW species, have evolved five NKAα1a isoforms that are present in functionally distinct gill ionocytes. Thermodynamic modeling studies suggest that a mutation in a zebrafish-specific NKAα isoform results in transfer of a single Na^+ and K^+ per ATP (Esbaugh et al. 2019) in dilute FW; that this isoform is preferentially upregulated in ion-poor FW suggests that changes to its thermodynamic properties allow it to compensate for the adverse electrochemical gradient imposed by this extremely dilute environment (Esbaugh et al. 2019). This work suggests that salinity-based selection on NKA may promote a more energetically efficient ion exchange process. Moreover, gene duplication and neofunctionalization of NKAs in teleosts (Dalziel et al.,

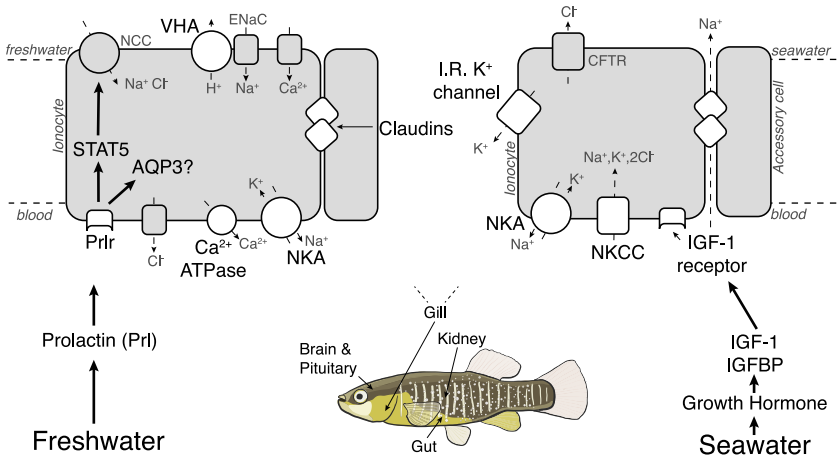

Fig. 4 A simplified diagram of the cellular mechanisms of ion transport involved in FW (left diagram) and SW (right diagram) osmoregulation at gill ionocytes. White-filled proteins represent those coded for by shared-selected genes, while gray represents proteins important to the pathway, but for which selection was not detected. Although osmoregulation involves the gills, gut, kidney, and brain, we focus here on the gills because it is the primary osmoregulatory tissue in fishes. Many genes repeatedly targeted by natural selection have known functions in gill osmoregulation and much is known about the precise mechanisms by which these candidate proteins influence ion homeostasis. FW ionocyte diagram represents generalized transport features and does not reflect one species or cell type in particular (Dymowska et al. 2012; Edwards and Marshall 2012). VHA = V-type H^+ ATPase; ENaC = Epithelial sodium channel; STAT5 = Signal transducer and activator of transcription 5; AQP3 = aquaporin 3, Prlr = Prolactin receptor; NKA = Na^+, K^+- ATPase, I.R. K^+ Channel = Inward-rectifier K^+ channel; NKCC = Na^+, K^+, Cl^- Co-transporter; IFG-1 = Insulin-like growth factor 1; and IGFBP = IGF binding protein. Note the presence of an unidentified or uncharacterized apical Ca^{2+} channel and basolateral Cl^- channel in FW.

2014) should provide ample substrate on which selection can act. In the future, researchers should apply comparative thermodynamic modeling experiments as in Esbaugh et al. (2019) to those genotypes targeted by selection in other species, such as those with FW- and SW-adapted populations that we include in our analysis, to more thoroughly test hypotheses about enzyme efficiency and energetic tradeoffs in alternative halohabitats.

V-type H^+-ATPases

We found that paralogous subunits and components of the V-type H^+-ATPase ion pump (VHAs) are commonly selected in six of the eight species examined here, in all but rainbow trout and Atlantic cod (*Gadus morhua*; Fig. 3). At the gene level, no single VHA subunit (of which there are 13 in humans) is a shared-selected gene (Supplementary Table S1), but V-type ATPase transporting subunits are a shared-selected gene family in four species (Fig. 3). In rainwater killifish (*Lucania parva*) and Atlantic salmon (*Salmo salar*), selection is detected not on VHA subunits themselves, but on their accessory proteins (Fig. 3; Supplementary Table S1). In addition to those included in this analysis, VHAs have been identified as targets of selection across salinity boundaries between sister species of killifish, *Lucania goodei vs. L. parva* (Kozak et al. 2014), and in the copepod *Eurytemora affinis* (Lee 2021). In *E. affinis*, selection on VHAs is associated with upregulation of mRNA expression and enzyme activity levels in FW-invading populations (Lee et al. 2011). Moreover, in laboratory experiments, selection on FW tolerance leads to rapid upregulation of VHA activity (Lee et al. 2011), providing additional support for its role in adaptive salinity acclimation. In fishes, higher levels of gill VHA expression and activity have been detected in FW-invading populations, including landlocked populations of the ancestrally anadromous alewife *Alosa pseudoharengus* (Velotta et al. 2017), and FW lineages of mangrove rivulus *Kryptolebias marmoratus* (Dong et al. 2021).

The role of VHAs in ion uptake in fishes is not well understood. Several models suggest that it works to power cation transport across the gill apical membrane (Fig. 4) by generating a proton gradient that is coupled to Na^+ transport, though this mechanism is not well defined across fishes (Kumai and Perry 2012). VHA may be a primary mechanism facilitating cation uptake in invertebrates, which would explain why it is a target of selection in *E. affinis* and the rapidity with which it evolves (Lee 2021). Adapting to FW habitats appears to entail enhanced activity and/or expression of VHA to enhance ion uptake. While NKA is likely the rate-limiting ion pump in SW, thermodynamic considerations suggest that it may be insufficient as the sole driver of ion uptake at very low salinities (Lee 2021). As such, it is plausible that increases in VHA activity or efficiency would follow FW invasion; the functional effects of se-

lection on ion transport should be characterized across a wider variety of taxa.

Ca^+ ATPases

Repeated selection on plasma membrane Ca^{2+} ATPases (*ATP2B*) subunits was detected in four species (Figs. 2 and 3). Genes coding for four different subunits of the Ca^{2+} ATPase pump (*ATP2B1,2,3,4*) were selected genes in mummichog, rainwater killifish, Atlantic herring, and threespine stickleback (Supplementary Table S1). Given that intracellular Ca^{2+} in vertebrate cells is low, uptake of Ca^{2+} from the environment is thought to occur passively at the apical ionocyte membrane *via* a Ca^{2+} channel (Marshall 2002). Once in the ionocyte, Ca^{2+} is actively pumped into intracellular fluid by a basolateral Ca^{2+} ATPase (Marshall 2002). Selection on Ca^{2+} ATPases is likely an adaptation to improve transport in ion-poor FW environments, where Ca^{2+} is often limiting. FW-adapted populations of ancestrally marine threespine stickleback repeatedly evolve pelvic spine reduction, as energy required for calcium uptake trades off with growth (Giles 1983; Bell et al. 1993). Thus, FW adaptation may involve selection on Ca^{2+} ATPases to increase Ca^{2+} transport efficiency and utilization for bone development.

Few passive ion co-transporters and channels are under selection

Na^+, K^+, and Cl^- co-transporter

Passive ion channels and co-transporters leverage the electrochemical power provided by ATPase pumps to move ions across epithelia. The Na^+, K^+, Cl^- co-transporter (NKCC), in particular, is a key solute carrier family protein involved in cell volume regulation and epithelial ion transport. A total of two major paralogs are known: NKCC1 has a basolateral cellular distribution and NKCC2 is present on the apical surface. NKCC2 has an important role in ion and water absorption by the gut of SW fish as the apical entry point of Na^+ and Cl^-. NKCC1 is critical for salt secretion by the gill of SW teleost fish, where its major function is to transport Cl^- from the blood into the cell (Pelis et al. 2001). Once in the cell, Cl^- is secreted on a downhill electrical gradient (created by NKA) through the apical Cl^- channel CFTR (Edwards and Marshall 2012). Thus, NKA, NKCC1, and CFTR form a tripartite functional unit for salt secretion by the SW gill (Fig. 4) and most other salt secretory tissues. We found that NKCC1 is a selected gene in Atlantic cod (Berg et al. 2015) and NKCC2 is a selected gene in Atlantic herring (Martinez Barrio et al. 2016). It is conspicuous that NKCC proteins are rarely targets of selection, and CFTR is never implicated in selection in any species. However, considering their importance primarily for salt secretion, an adaptive role for altered function in FW fishes may not be likely since hypo-osmoregulatory demands may not impose directional selection on the function of these proteins. Alternatively, they may be somewhat released from adaptive constraint, or alternatively still, negative pleiotropy may constrain their evolution altogether.

Sodium-bicarbonate exchangers

We found evidence of selection on sodium-bicarbonate exchangers in species two killifish species (Kozak et al. 2014; Brennan et al. 2018) and prickly sculpin (*Cottus asper*; Dennenmoser et al. 2016; Fig. 3). Sodium-bicarbonate exchangers are thought to be present on gill ionocytes in FW and play a role in ion uptake. Salinity-specific paralogs of sodium-bicarbonate exchangers have been found in the desert Amargosa pupfish (*Cyprinodon nevadensis amargosae;* Lema et al. 2018). In Atlantic salmon, there appears to be two major paralogs in the gill (*nbce1.2a* and *2b*), expression of which is downregulated after exposure to SW (Jason Breves, personal communication). While these data suggest that sodium-bicarbonate is involved in osmoregulation in FW, much more work is required to establish their localization and function. Research on species where salinity-specific selection has been demonstrated may be especially fruitful, toward clarification of the functional significance of selected differences across habitats.

Inward-rectifier K^+ channels

The inward rectifier K^+ channel (Kir), thought to be important in cellular K^+ recycling at gill ionocytes (Evans et al. 2005; Fig. 4), is a poorly understood but widely shared-selected gene. We identified five species in which this small and highly specialized group of K^+ channels are under selection (Figs. 2 and 3). In teleost fishes, Kir channels serve diverse cellular functions, which includes, in addition to K^+ recycling, maintenance of resting potential and K^+ excretion. Recycling of K^+ at the ionocyte basolateral membrane is likely to be critical to hypo-osmoregulation in SW, as the actions of both active (NKA) and passive (NKCC) Na^+ and Cl^- transport across membranes require a supply of K^+ in the body fluid (Evans et al. 2005). The precise role of Kir channels in teleost osmoregulation is unclear; several studies have found that expression of Kir transcripts were upregulated at the gill after salinity change, and have localized such expression to the basolateral membrane of gill ionocytes (Suzuki et al. 1999; Furukawa et al. 2012, 2014). The repeated selection on Kir genes suggests a widespread role in ion secretion and hypo-osmoregulation, and that functional allele frequency variation in Kir may underlie repeated adaptation to

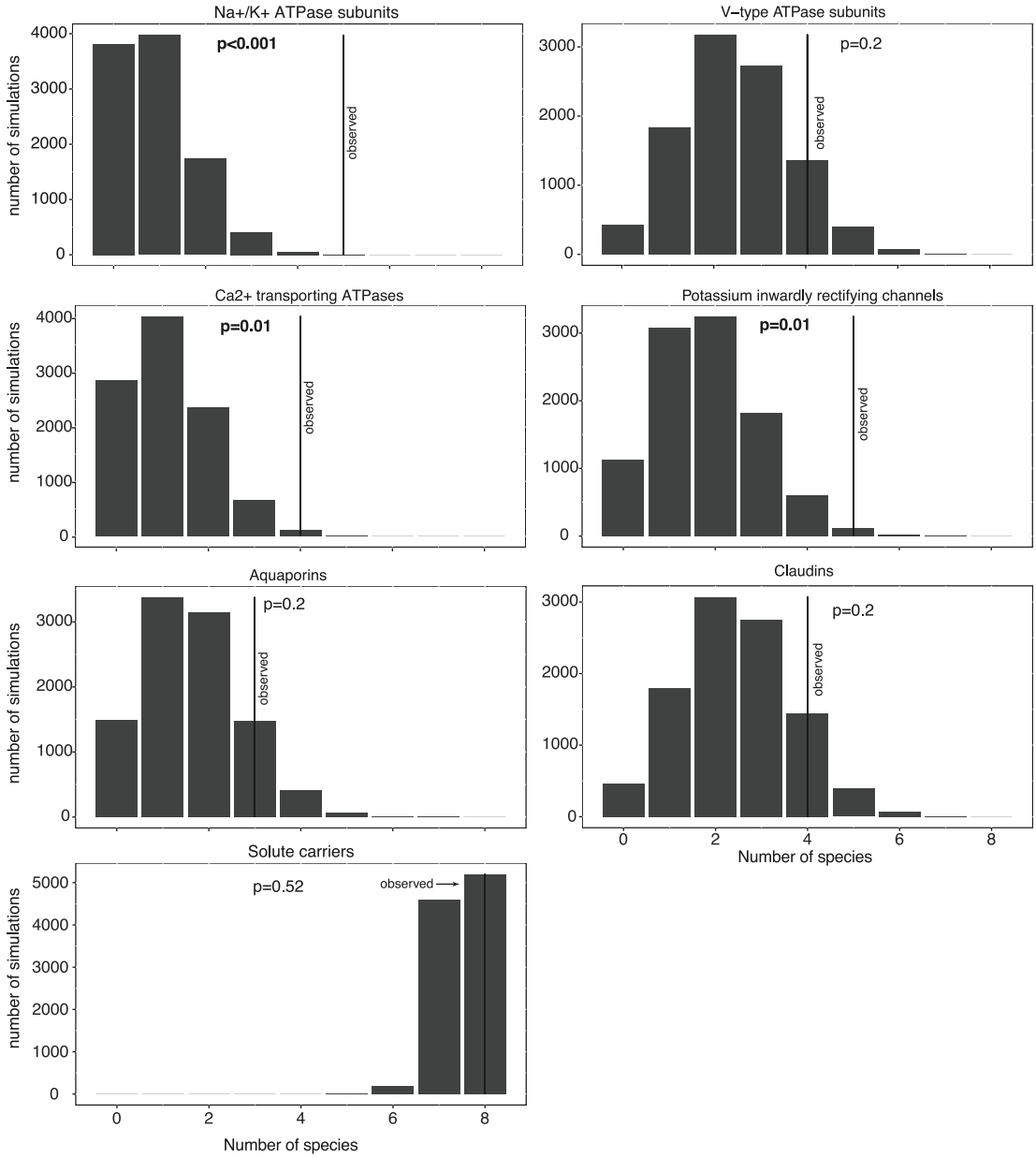

Fig. 5 Simulation results for gene families in reference set of candidate osmoregulatory genes. We used randomization tests to produce a null distribution of shared selected gene-family overlap across species. *P*-values determined as the proportion of randomized shared selected families greater than the observed (plotted as vertical line)

novel salinity environments. Whether natural selection on Kir allows for more efficient K^+ recycling and ion exchange across the teleost gill is not known and should be the subject of future research.

Aquaporin 3

We found that *AQP3* (Aquaporin 3) is a shared-selected gene in three species in our formal gene-level analysis (Fig. 1), and a fourth including one study of candidate genes in *G. aculeatus* (Fig. 5; Shimada et al. 2011). Aquaporins act as important transepithelial fluid transporters and cell volume regulators in vertebrates and many paralogs of aquaporin have been identified in fishes (e.g., up to 42 in salmonids; Finn et al. 2014). *AQP3* appears to play an important role in hyper-osmoregulation. Localized to the gill, gene expression of *AQP3* is upregulated upon transfer from SW to FW (Brennan et al. 2015; Breves et al. 2016; Ellis et al. 2019), and expression is higher in FW-derived lineages of mangrove rivulus (Dong et al. 2021). Regulation of branchial *AQP3* expression by the "freshwater-adapting" hormone prolactin suggests that it may in turn be an important regulator of cell volume (Ellis et al. 2019) and/or a modulator of osmosensitivity (Breves et

al. 2016), which would be critical for adaptation to FW. Whether and how selection on *AQP3* directly results in improved osmoregulatory capacity will be an exciting subject of future research.

Many genes in a large family of structural junction proteins are under selection

Claudins comprise a shared-selected family of structural junction proteins (Figs. 2 and 3) that influence epithelial permeability (Fig. 4). Early morphological studies indicated that there were "tight junctions" between ionocytes and other cells in FW, and "loose junctions" between ionocytes and accessory cells in SW (Evans et al. 2005). These observations are reflective of greater passive ion flux levels in SW relative to FW fish (Evans 1984) and morphological and physiological differences are likely attributable to differential expression of different tight junction proteins (which include claudins, as well as occludins, and MARVEL proteins) between FW and SW (Bagherie-Lachidan et al. 2008; Tipsmark et al. 2008a, 2008b). While the uptake of Na^+ and Cl^- in FW and the secretion of Cl^- in SW are transcellular (across cell membranes), the secretion of Na^+ in SW is paracellular (between cells), implicating a role for tight junction proteins. It is generally thought that basolateral Na^+, K^+-ATPase moves Na^+ into the space between ionocytes and accessory cells; there, Na^+ can be secreted along a favorable downhill electrical gradient, because the blood has a slight positive charge relative to SW. The efficiency of this process could be enhanced if paracellular pathways were Na^+ selective. Marshall and co-workers have recently found a strong candidate for a Na^+ selective tight junction protein (claudin 10) in the euryhaline mummichog (Marshall et al. 2018). Among the junction proteins we would, therefore, anticipate that *CLDN10* is a focus of selective change, and it is indeed a selected gene in in two taxa, rainwater killifish (Kozak et al. 2014) and Atlantic herring (Martinez Barrio et al. 2016). A total of three other claudin genes are selected in other species (Supplementary Table S1).

Regulatory proteins commonly under selection include hormones and their receptors, as well as key FW second messenger protein

Prolactin

We found that the gene coding for the hormone prolactin (*Prl*) is under selection in threespine stickleback, while its receptor (*Prlr*; Fig. 1) is under selection in two other species (Fig. 3; Supplementary Table S1). Prolactin is produced in the pituitary and has multiple roles, including regulation of reproduction, milk production, and immune response. Prolactin is intricately involved in FW acclimation in many euryhaline teleosts (Takei and McCormick 2013; Breves et al. 2014). In estuarine species mummichog and tilapia (*O. mossambicus*), removal of the pituitary prevents normal ion uptake in FW, which can be restored by injection of prolactin (Breves et al. 2010). Prolactin upregulates the FW isoform of NKA (NKA1α) and the apical Na^+, Cl^- cotransporter NCC (Breves et al. 2010; Fig. 4). It also upregulates apical NCC transporter in the stenohaline FW zebrafish upon exposure to low ion content FW (Breves et al. 2013). Prolactin also regulates the ionic permeability of the gill in several species, perhaps *via* influence on specific claudins (Tipsmark et al. 2016). Its importance is less clear in FW acclimation of anadromous species such as Atlantic salmon and Pacific salmon (*Oncorhynchus spp.*), where removal of the pituitary does not affect FW survival or plasma ion levels (Björnsson and Hansson 1983).

Other players in the prolactin signal cascade are under selection across salinity boundaries. Binding of prolactin to its surface receptors, for example, results in dimerization and cross-phosphorylation and subsequent activation of the JAK/STAT pathway, which then goes on to influence gene transcription and phosphorylation of target proteins. In both mammals and teleosts JAK2 and STAT5 (Fig. 4) are particularly important for prolactin signaling (Breves et al. 2014). This specificity along with the importance of prolactin in regulating the SW to FW acclimatization may explain why STAT5, along with prolactin and its receptor, have been the targets of natural selection in divergent halohabitats (Figs. 1 and 3). It will be of great interest to determine the functional significance of selective changes on prolactin regulation and signaling. In particular, it remains an open question as to how selection acting on a pleiotropic regulator, a hormone in this case, can have specific effects in one pathway without altering the functional properties of another in potentially maladaptive ways (Schweizer et al. 2019). This may help explain why selection is detected on prolactin itself in one species (Hohenlohe et al. 2010; Jones, Grabherr et al. 2012), but only its receptor in two separate species (Martinez Barrio et al. 2016; Brennan et al. 2018).

GH/IGF-1

Similar to what we found in the prolactin cascade, selection on genes associated with GH included not only the hormone IGF-1, but also its receptor and associated binding proteins that aid in its transport (Fig. 3). The GH/IGF-1 axis is involved in SW acclimation in a number of teleost species (Björnsson et al. 1987; McCormick et al. 1991), and there appears to be an important interaction with cortisol for many of its osmoregulatory effects (reviewed in Takei and McCormick 2013). Likewise, GH/IGF-1 axis may also interact with thy-

roid hormone in the support of SW acclimatization in some species (McCormick 2001), which is especially interesting because we also found a thyroid hormone receptor (*THRA)* to be a shared selected gene in three species (Supplementary Table S3). The GH/IGF1 axis is specifically involved in the proliferation and differentiation of SW-type gill ionocytes and abundance of NKA, NKCC, and CFTR (Richman and Zaugg, 1987; Takei and McCormick 2013). Plasma GH levels that are critical for induction of the parr-smolt transformation (which includes increases in SW tolerance) are much lower in landlocked than in anadromous populations of Atlantic salmon, as is salinity tolerance (Nilsen et al. 2008; McCormick et al. 2019). IGF-1 shows evidence for selection in Atlantic salmon and threespine stickleback, as does IGF-1 receptor in Atlantic cod, and IGF binding proteins (IGFBP) in Atlantic cod, threespine stickleback, and mummichog (Fig. 3). A candidate gene analysis on threespine stickleback found that IGFBP5 in particular was differentially expressed between marine and stream ecotypes, was associated with plasma Na^+ in a Quantitative Trait Locus (QTL) analysis, and was located in a region of high population-genomic differentiation (Kusakabe et al. 2017). There are five to seven major IGFBP paralogs in most vertebrates, and these are especially important for the signaling of IGF-1, with some promoting and others inhibiting the availability and/or binding of IGF-1 with its receptor. The expression of some IGFBPs have been shown to respond to salinity (Breves et al. 2017). Since the physiological action of IGFBPs in response to salinity is still unclear, it will be useful to evaluate their role (particularly the paralogs IGFBP1-3,5) especially in those species showing evidence of selection at these loci.

Simulation of family-level convergence

Our analysis revealed genes and gene families that have selectively differentiated between populations across salinity boundaries in multiple species of teleost fishes. The probability that a gene family was identified as a shared-selected gene family is, however, dependent on the number of genes in the family. In the human genome, for example, there are over 400 solute carriers, while there are just 10 Na^+, K^+ transporting ATPase subunit genes. To examine the impact of this bias, we conducted a randomization procedure for each reference set gene family outlined above (and in Fig. 2). The purpose of the randomization procedure was to determine the null expectation of shared selection for a given gene family, against which our observed value could be tested. In a single iteration of this procedure, for each species, we randomly selected genes from the HGNC list at a sample size equal to the number of genes in each species' selected gene set. For each candidate family, the number of shared-selected gene families was then determined. We repeated this randomization procedure 10,000 times, creating a null distribution of shared selection. A *P*-value for each reference set gene family was determined as the proportion of randomized shared selected families greater than the observed value.

Our randomization procedure revealed that Na^+, K^+ ATPase subunits ($P < 0.001$), Ca^{2+} ATPases ($P = 0.01$), and inward rectifier K^+ channels ($P = 0.01$) were observed in more species than expected by random chance alone ($P < 0.05$; Fig. 5). Although we found selected-genes of the solute carrier family of proteins in each species, such widespread sharing as a selected-gene family was not greater than expected under the null; it occurred about 50% of the time in our simulations ($P = 0.5$; Fig. 5). The high frequency with which studies across species have detected selection in NKAs, Ca^{2+} ATPases, and Kirs suggests a level of functional convergence within these families during adaptation to new salinity environments, potentially reflecting their repeated importance in maintaining osmotic homeostasis in the face of novel salinity regimes.

Conclusions

Our analysis of the literature suggests that selection on individual genes across salinity boundaries is not commonly shared across teleost species, but that several processes are subjected to widespread selection as indicated by selected-gene family sharing patterns (Figs. 3–5). In particular, there is widespread selection on pathways involved in cellular ion exchange at various levels, including their hormonal control and secondary regulation (Fig. 4). The dominant pattern that emerges from this analysis is one of polygenic adaptation; population-level selection across salinity boundaries likely occurs across many loci involved in the complex, multi-tissue processes that differ in demands, functions, and constraints on osmoregulatory structures and functions. Variation in the precise gene or locus targeted by selection is undoubtedly a result of the complex interplay between relatedness and evolutionary history, differences in availability of standing genetic variation, epistasis and pleiotropy (Losos 2011; Storz 2016), differences in physiology, and characteristic features of the habitat (Paccard et al. 2020), including salinity regime, to which each species in our analysis has adapted.

In general, convergent adaptation can emerge or deteriorate at different levels of biological organization (Arendt and Reznick 2008). For example, at the physiological level it is apparent that blood plasma osmolality tends to converge at lowered values among fishes that independently invaded FW. Other features

also converged, such as gill ionocytes with large apical surface areas and loss of the drinking reflex (Smith 1932). However, at the cellular level and below, convergence may be less evident. For example, the molecular makeup of FW ionocytes, including the proteins that interact to establish electrochemical gradients, differs among some FW species (Dymowska et al. 2012), increasing the potential suite of genetic targets by which selection may act, and thus decreasing the potential for convergence at genetic and molecular levels. Indeed, the extent of convergence tends to deteriorate at decreasing mechanistic levels, from pathways to genes to the individual mutations that lead to functional change (Bolnick et al. 2018). The degree to which convergence is evident is a reflection of factors affecting the pace of trait evolution such as negative pleiotropy, functional redundancy among causative genes, and the availability of—and interactions between—relevant genetic variation (Bolnick et al. 2018). Convergence is also more likely among closely related taxa, because more recent shared ancestry may support shared genetic variation and shared constraints (Conte et al. 2012). In our analyses, we include globally distributed species that span a range of phylogenetic distances. Though several species are clustered within the same family (e.g., Atlantic salmon, rainbow trout, and mummichog and rainwater killifish), the last common ancestor of all species was 230 MYA. One might anticipate that selected genes are more likely to be shared among species within families, than among species that are more distantly related. In general, though, we do not detect a higher level of convergence among closely related species (Fig. 1), and, most importantly, we find that convergence is sometimes detected between distantly related species. This suggests that shared genetic variation does not explain the patterns of convergence that we detect, but rather that adaptive osmoregulatory changes may be limited to functional pathways, and even in some cases particular genes and gene families (Figs. 1–3), that regulate and mediate key osmoregulatory processes.

In addition to relatedness, the degree of convergence tends to weaken at broader geographical scales, reflecting increased environmental variation and a more variable landscape of selection (Paccard et al. 2020). In our analysis, the scale and range of the environmental salinity gradient across which population diverge varies among species (Table 1); in some species, for example, upper salinity levels are less than full strength SW (e.g., mummichog), while in others, minimum salinity is higher than typical FW values (e.g., Atlantic cod and Atlantic herring). Despite this, population genomic analyses reveal local adaptation with respect to salinity (Table 1), suggesting that salinity gradients are a strong selection pressure. Although variation in the steepness of the salinity gradient likely contributes to our result that most selection is species-specific (Fig. 1), we do find common selected genes among species that differ widely in salinity gradient. NKA, for example, is under selection in nearly every species examined (Fig. 3), including those whose populations vary from full-strength SW to FW (e.g., threespine stickleback; Jones et al. 2012a, 2021b) as well as marine species whose lower range limit does not exceed 6 ppt (e.g., Atlantic cod; Berg et al. 2015).

Despite the general pattern that adaptive modification of many genes is important for physiological adaptation to FW, it does appear that selection targets ion pumps at a high rate, higher than most other functional categories of osmoregulatory proteins (Figs. 3 and 4) except for the inward-rectifying K^+ channel (Fig. 3). At least one NKA gene, one VATP gene, and one Ca^{2+} ATPase gene is a shared target of natural selection in all species examined here (although we did not detect shared selection over random chance for VATP family of genes). Selection commonly occurred on ATPase pumps, which likely reflects their central role in ion exchange (often serving to set-up critical electrochemical gradients) as well as the high energetic costs associated with their activity. In lake resident charrs (*Salvelinus spp.)* for example, NKA is under relaxed selection compared to anadromous species across the salmonid tree (Schneider et al. 2019). As noted above, ATPase pumps are also major functional targets of selection in copepods that have invaded FW (Lee 2021). Nevertheless, repeated selection on osmoregulatory function is not limited to ATPase pumps, but rather spans many functional levels of organization. Motivated by these findings, future research should focus on the gene families identified here to better understand how selection on functional genetic variation reshapes the ability to osmoregulate in novel salinity environments.

Analytical limitations and considerations

Our assessment of shared selection is subject to several limitations that we outline below. First, identification of selected genes was limited to a single study in all cases except threespine stickleback (Table 1). Lack of standardization across these studies (e.g., sequencing effort, library preparation, and method of inferring selection) may limit identification of shared selected genes; even within species, for example, different programs for detecting selection can yield variable results (e.g., Shimada et al. 2011). Second, for several species, few populations are compared (e.g., Brennan et al. 2018), which decreases our ability to draw species-level inferences, as variation in population genetic structure and ecological differences are not taken into account. Third, our

inference of selected genes is limited by species-specific gene annotations and translation to human orthologous genes in the *g:Profiler* database. This necessarily limited our analysis to those species for which orthologous translations have been made. Moreover, because gene family characterization was based on homology to the human genome, we cannot be certain that family-level functions are strictly conserved in fish species in our analysis, except in specific cases where function is known from empirical data. Finally, our finding that phylogenetic relatedness does not contribute to the extent of convergence (Figs. 1 and 2) may have been affected by large variation among species in the number of selected genes. This variation may be attributable to differences in the extent of selection in different species, but it may just as easily be attributable to study-specific analytic differences based on sample sizes, numbers of populations compared, differences in sequencing, and/or variation in population genomic approaches to SNP discovery and estimation of allele frequency variation and thus, selection. As a result of these limitations, we feel as though at best our analysis is an underestimate of the extent to which selected genes are shared among taxa. In light of this, what emerges from our analysis is a summary of broad patterns in the literature and the current state of the field. Our hope is that this work spurs more interest in understanding the genomic basis of adaptation to alternative salinity regimes such that the broad patterns can be further illuminated, and the details more precisely dissected.

In this paper, we chose to take a population-level genome scan approach, identifying studies that used allele frequency variation as a metric of selection. Genome-wide approaches may also include comparative transcriptomics and quantitative genetics, and population genomic approaches. Comparative transcriptomics is informative for indicating the cellular pathways that diverge to support alternate osmoregulatory strategies (Whitehead et al. 2011; Gibbons et al. 2017; Velotta et al. 2017; Jeffries et al. 2019), but has limited ability to identify the underlying genetic loci that were subject to directional or diversifying natural selection. Quantitative genetics studies (e.g., QTL, GWAS: Genome-Wide Association Studies) are powerful for associating genetic with phenotypic variation but cannot in and of themselves identify variation that is adaptive in different environments; allele-trait associations do not necessarily reveal patterns of variation characteristic of selection (as do genome-wide scans for selection). Moreover, few quantitative genetic association studies have been conducted on osmoregulatory traits (e.g., Brennan et al. 2018). The challenge, however, with genome-wide scans for selection such as those we reviewed is that statistical methods do not explicitly associate selected loci with particular adaptive traits (e.g., with adaptive osmoregulation vs. adaptive morphology), thereby limiting the functional inferences that can be drawn. We chose to focus on population-level genome scans for their ability to detect extreme allele frequency divergence, and thus signatures of past natural selection. In the future, however, we encourage research that allows for more detailed functional inference such as those directly integrating population genomic scans for selection with comparative transcriptomics and quantitative genetic association studies.

Our search of the literature identified studies of population genomic signatures of selection across salinity boundaries in 8 teleostean species. In most cases, ancestral populations are marine (Atlantic cod and Atlantic herring) or euryhaline/diadromous (mummichog, rainwater killifish, threespine stickleback, prickly sculpin, and Atlantic salmon; Table 1), the direction of population movement is into FW from more saline ancestral states. This asymmetry in our analysis likely reflects a real asymmetry in the history of vertebrates, rather than a reporting bias in the literature; overwhelmingly, ancestral marine lineages have invaded FW habitats and diversified there, compared to the other way around (Betancur-R et al. 2015; Rabosky 2020).

Areas for future research

Given the history of selection we identify here, functional differences across loci related to physiological regulation of ion and water balance are expected to influence mechanisms and signaling pathways for ion transport, subsequently affecting physiological performance and fitness in different salinity environments. The connection between mutation, allele frequency, physiology, and performance or fitness, however, is rarely tested. To move forward, we suggest combining studies of selection scans as presented here, with genome-wide association studies to pinpoint loci involved in adaptative differentiation of osmoregulatory function more precisely (e.g., Brennan et al. 2018). Once the loci of selection are identified, studies that detail how mutation leads to functional changes in osmoregulation and/or related physiological processes will be critical, though we acknowledge that this research is technically complicated (Schweizer et al. 2019; Ivy et al. 2022). Nevertheless, as whole genome sequencing becomes more reliable and cost-effective, researchers should move toward a more holistic understanding of how mutation influences gene expression, cellular bio-

chemistry, and whole-organism performance in species that have made these important ecological transitions across salinity barriers.

Future studies should also seek to distinguish between genetic adaptations that are structural from those that are regulatory (Hoekstra and Coyne 2007; Wray 2007). For example, a mutation may be adaptive because it alters the protein sequence such that the protein functions differently in alternate environments. In contrast, a mutation may be adaptive because it alters the regulation of a gene rather than the function of its product. For example, a regulatory mutation may affect the timing of gene expression during development, in which tissues it is expressed, whether its protein is localized to basolateral or apical membranes within a cell, or whether it is up- or down-regulated in response to environmental change (Carroll 2000). Because protein sequence mutations may alter function thereby affecting all of the pathways in which that protein participates, protein sequences are considered subject to greater evolutionary constraint than *cis*-regulatory mutations (e.g., King and Wilson 1975). However, genes can escape these pleiotropic constraints through duplication followed by sub/neo-functionalization (Ohno 1970). Gene family expansion can, therefore, provide raw resources for adaptive specialization. Future studies should consider the influence of gene loss and duplication during adaptation to alternate osmotic environments. Genome scan data alone are often insufficient for specifying the gene, the gene region (regulatory or coding), or especially the actual mutation, that provides adaptive advantage. This is because genome scans provide evidence for selection that sometimes spans a large genomic region that captures coding and non-coding sequence and may capture many genes. Distinguishing whether the adaptive target is difficult, because of linked selection (Smith and Haigh 1974; Burri 2017), where identifying the precise mutation that is adaptive is usually not possible. Additional fine-mapping studies, gene expression studies, and functional manipulation studies (e.g., transcript knock-down or injection, or gene knock-out) are often necessary to achieve this level of insight.

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Supplementary data

Supplementary data available at *ICB* online.

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