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Do saline taxa evolve faster? Comparing relative rates of molecular evolution between freshwater and marine eukaryotes

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The major branches of life diversified in the marine realm, and numerous taxa have since transitioned between marine and freshwaters. Previous studies have demonstrated higher rates of molecular evolution in crustaceans inhabiting continental saline habitats as compared with freshwaters, but it is unclear whether this trend is pervasive or whether it applies to the marine environment. We employ the phylogenetic comparative method to investigate relative molecular evolutionary rates between 148 pairs of marine or continental saline versus freshwater lineages representing disparate eukaryote groups, including bony fish, elasmobranchs, cetaceans, crustaceans, mollusks, annelids, algae, and other eukaryotes, using available protein-coding and noncoding genes. Overall, we observed no consistent pattern in nucleotide substitution rates linked to habitat across all genes and taxa. However, we observed some trends of higher evolutionary rates within protein-coding genes in freshwater taxa—the comparisons mainly involving bony fish—compared with their marine relatives. The results suggest no systematic differences in substitution rate between marine and freshwater organisms.

KEY WORDS: **Comparative method, eukaryotes, freshwater, marine, relative rates, saline.**

The major lineages of life diversified in the marine realm, yet their subsequent evolution has involved crossing the barrier among saline (SAL), freshwater (FW), and terrestrial environments multiple times independently. Of the 27 animal phyla with marine species, 16 phyla also possess FW species, although terrestrial environments may have been an intermediate stage within some of these phyla (Little 1990). The transition from marine to FW environments is relatively common in certain major animal groups (Lee and Bell 1999), whereas FW to marine movements are less common (McDowall 1997). For example, shifts from marine to FW have occurred multiple times in fish (Sezaki et al. 1999; Lovejoy and Collette 2001; Yamanoue et al. 2011; Bloom et al. 2013) and invertebrate lineages (Rousset et al. 2008; Hou et al. 2011; Graf 2013; Botello and Alverez 2013). Some groups of organisms, such as crustaceans, may be predisposed to more easily transitioning between aquatic habitat types, as supported by recent invasions of FW (Lee and Bell 1999). By contrast, eleven phyla of marine animals have failed to enter the FW environment (Little 1990). Among microorganisms, including unicellular eukaryotes, Archaea, bacteria, and viruses, switching between marine and FW appears to be relatively infrequent (Logares et al. 2009, 2010), which initially seems somewhat surprising considering their generally large population sizes, short generation times, and often large dispersal potential (Logares et al. 2007). However, many factors may constrain the rate of transition between major habitats, especially physiological barriers (Lee and Bell 1999) but also low dispersal ability and niche saturation (Logares et al. 2009).

Freshwater and SAL organisms may have different biological properties and experience different environmental pressures, which can exert influence upon their genome-wide rate of

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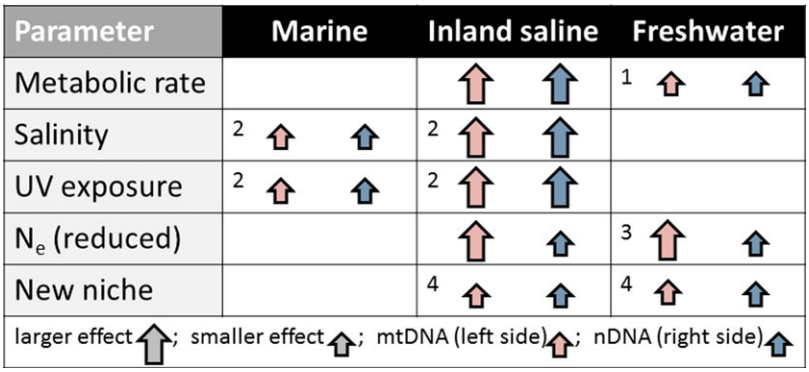

Figure 1. **Summary of expected association between biological or ecological parameters and rates of molecular evolution in the mitochondrial and nuclear genomes for marine, inland saline, and freshwater aquatic habitat categories. Presence of arrows indicate that increased molecular rates are expected in association with that parameter in the habitat; size of arrows indicate expected relative strength of effect on molecular rates, relative to other habitat categories or between mitochondrial and nuclear genomes within a habitat category. Numbers indicate the following qualifications (see text for more information): (1) metabolic rate differences based on habitat may be greater for invertebrates, (2) the effect may be more likely for smaller organisms, (3) the effect on dN rates and dN/dS ratios is expected to be greater than the effect upon dS rates, and (4) a given effect may be limited to specific genes or be approximately equivalent to the N_e parameter. These are our general hypotheses for the habitat categorizations and likely do not apply to all individual taxa analyzed.**

molecular evolution. The balance of these various pressures may produce predictable differences in molecular evolutionary rates in lineages inhabiting different aquatic realms. Saline waters have been previously associated with elevated rates of molecular evolution (Hebert et al. 2002; Wägele et al. 2003; Colbourne et al. 2006; Logares et al. 2010; discussed further below); however, this finding has not been explored on a large phylogenetic scale. In this study we consider relative molecular rates in three broad habitat categories: marine, FW, and inland SAL environments. We first introduce several primary biological and environmental parameters that are expected to generally differ among these habitat categories and to influence rates of molecular evolution (summarized in Fig. 1).

METABOLIC RATE

Because FW continental waters have lower ionic concentrations than SAL waters, transition between these habitat types requires the appropriate osmoregulatory adjustments. The greater osmotic difference that exists between FW organisms and their environment, as compared with marine organisms, is expected to have a greater metabolic cost for regulation of ion concentration and ionic pressure (Lee et al. 2012). Vertebrates are generally osmoregulators in both SAL and FW settings, whereas invertebrates are generally osmoconformers in the marine environment and osmoregulators in FW (Evans 2008). Thus, we expect FW invertebrates to have greater metabolic expenditure associated with osmotic regulation, as compared with vertebrates, than their marine counterparts. By contrast, larval-stage marine fish may have greater metabolic requirements than their FW relatives (Houde et al. 1994); however, overall for bony fish, there is no consensus on whether FW or marine species have greater metabolic costs associated with osmoregulation (Evans 2008).

Inland populations additionally experience greater chemical and physical variability of their habitat than marine organisms do, which can have implications for osmotic balance and metabolic rate. Inland SAL lakes have greater variation in their salt concentrations than the marine environment across time (Bowman 1956; Frey 1993), which can place osmotic stress on organisms inhabiting the changing environment (Herbst 2001). As well, hypersalinity in inland SAL lakes can increase the metabolic rate of organisms due to the cost of ion pumping (Hebert et al. 2002). Freshwaters and inland waters additionally have greater variation in temperatures, with temperature affecting metabolic rate (Gillooly et al. 2001).

Increased metabolic rate is expected to increase the genome-wide mutation rate over evolutionary time (Martin and Palumbi 1993). The effect may be stronger for the mitochondrial genome than the nuclear genome due to the proximity of mitochondrial DNA to DNA-damaging metabolites; however, this idea is controversial (Galtier et al. 2009). Despite possible reasons to expect a correlation between metabolic rate and molecular evolutionary rate, there is currently minimal evidence linking metabolic rate itself with general substitution rate differences among lineages (e.g., Bromham et al. 1996; Lanfear et al. 2007). Although there are many variables that differ based on habitat that could influence metabolic rate, overall we expect invertebrates in inland SAL lakes to incur the greatest relative effects on their molecular

evolution due to metabolic costs, followed by FW, and then marine invertebrates.

SALINITY

In addition to an effect mediated by metabolic rate, the salinity of the aquatic environment may directly impact the pace of molecular evolution (Hebert et al. 2002; Colbourne et al. 2006). Specifically, higher intracellular salinity is proposed to reduce the fidelity of DNA replication, as salinity impacts binding between protein and DNA and alters the performance of DNA polymerases (Favre and Rudin 1996). We expect that organisms inhabiting hyper-SAL inland waters would show the greatest molecular rate effects due to mutagenic effects of salinity itself, especially given fluctuations in salinity in the environment. We expect this effect to be genome-wide, based on observations from both nuclear and mitochondrial genomes in hyper-SAL taxa (Hebert et al. 2002). Although intracellular salinities in both FW and marine fish are maintained within a regulated range for cell metabolism (Fiol and Kültz 2007), the concentrations of many electrolytes/osmolytes in extracellular plasma are higher in marine than FW fish, for example, Ca^{2+}, Mg^{2+} (Kapoor and Khanna 2004), Na^{+}, and K^{+} (Volkenstein 1994). If salinity of environment itself leads to increased rates of molecular evolution, then marine organisms would be expected to show this effect relative to FW taxa.

ULTRAVIOLET RADIATION EXPOSURE

The movement toward more SAL environments may increase an organism's exposure to ultraviolet (UV) radiation. UV penetration is affected by dissolved organic carbon (DOC), which acts as a UV barrier but is precipitated by high salt concentrations. Within the marine realm, coastal waters—having higher DOC concentrations—have lower UV penetration than the open ocean (Tedetti and Sempere 2006). For similar DOC concentrations in continental waters, UV penetration is higher with greater salinity levels (Arts et al. 2000). Saline lake organisms may be further exposed to UV radiation, as UV radiation decreases with water depth (Tedetti and Sempere 2006), and SAL lakes tend to be shallower than marine waters. UV radiation is known to have a direct mutagenic effect on DNA, and thus exposure may lead to increased molecular evolutionary rates in both nuclear (Smith et al. 1992; Lutzoni and Pagel 1997) and mitochondrial genomes (Colbourne et al. 2006). Although individual habitats can differ from general trends in UV prevalence (e.g., translucent oligotrophic FW), we generally expect UV effects on molecular rates to be most prevalent in inland SAL organisms, followed by marine, and then FW organisms, and especially in smaller-bodied organisms.

EFFECTIVE POPULATION SIZE

Inland populations experience reduced physical stability of habitats, increased vulnerability to population bottlenecks, and higher rates of extinction and colonization, as compared to those inhabiting marine environments, which tend to be more stable in the long term (Frey 1993; Grosberg et al. 2012). Along with greater habitat subdivision across the continental landscape and generally reduced gene flow among populations within a species, this greater habitat instability is expected to reduce the effective population sizes (N_e) of inland aquatic organisms (FW or SAL) as compared with marine organisms (Whitlock and Barton 1997). Numerous studies suggest a trend of higher N_e of marine fish species than FW fish, including higher levels of gene flow (Ward et al. 1994; DeWoody and Avise 2000; Yi and Streelman 2005). Likely, not all marine species have larger N_e than related FW species, as some marine organisms are specialized to habitats occupying small geographic areas, such as on coral reefs (e.g., Underwood et al. 2012).

Smaller N_e increases the role of genetic drift relative to selection, in effect relaxing selective constraints, and is expected to lead to increased fixation of nearly neutral mutations (Ohta 1973, 1992; Woolfit and Bromham 2003). Thus, signatures of reduced N_e are expected to be increased overall substitution rates (OSRs) and increased nonsynonymous-to-synonymous substitution (dN/dS) ratios (Woolfit 2009). N_e-mediated influences upon substitution rates are expected to act genome wide but more strongly for mitochondrial DNA due to its approximately fourfold smaller N_e compared to nuclear DNA (Schmitz and Moritz 1998). As a general trend, we expect FW and inland SAL organisms to display increased molecular rates attributable to reduced N_e when compared with marine species.

DIVERSIFICATION RATE AND NOVEL NICHES

Evolutionary transitions in habitat expose transitioning lineages to novel environmental and biological conditions, which can result in increased speciation (Bloom et al. 2013). Inland waters, and especially FW, are most often the recipient environment in SAL–FW shifts. Speciation or net diversification rates may be increased in FW (Hou et al. 2011), particularly in fish; FW occupy less than 0.01% of the aquatic volume but account for over 41% of described fish species (Horn 1972). Studies testing this hypothesis within a comparative context have concluded various associations between habitat and relative diversification rates. For example, there was no difference in net diversification rates between FW and marine clades of ray-finned fishes (Vega and Wiens 2012). Higher rates of both speciation and extinction were estimated in FW versus marine silverside fish (Bloom et al. 2013). Finally, higher speciation rates were estimated for marine pufferfish than their FW or brackish relatives, contrary to predictions relating to colonization of novel habitats (Santini et al. 2013).

A link between speciation and molecular evolutionary rates (e.g., Lanfear et al. 2010) could manifest in many ways (Barraclough and Savolainen 2001), either in genome-wide or

gene-specific manners. Similarly, the direction of the habitat transition may influence molecular rate patterns. These associations could occur, for example, by a decrease in effective population size during the process of speciating or transitioning, or by increased substitutions in genes involved with the adaptation to novel niches (Rieseberg and Blackman 2010). We expect inland environments, both FW and SAL, to show the greatest molecular rate effects due to transition direction or novel niche space, as these are most often the recipient environments in FW–marine/SAL shifts.

LIFE HISTORY AND OTHER CORRELATES OF AQUATIC HABITAT TYPE

Major developmental and lifestyle changes can additionally accompany SAL–FW habitat shifts, such as changes from active-feeding to nonfeeding larvae when entering FW (Lee and Bell 1999; Kupriyanova et al. 2009). Marine fish are, on average, larger as adults than FW fish (Olden et al. 2007), although marine and FW fish larvae have similar growth rates (Houde et al. 1994). Body size itself is proposed to be negatively correlated with molecular evolutionary rates (Martin and Palumbi 1993). Multiple biological and life-history traits may differ, on average, based on habitat category, and these may covary. Given these complexities, including a large sample size of phylogenetically independent comparisons—to aid in control for confounding factors—is important for testing whether there is an association between a particular environment and rates of molecular evolution.

DO RATES OF MOLECULAR EVOLUTION DIFFER ACROSS AQUATIC ENVIRONMENTS?

Previous studies of habitat-specific molecular rates have provided evidence of trends on small taxonomic scales, with higher molecular rates observed in SAL habitats. Two studies have compared molecular rates between branchiopod crustaceans inhabiting continental SAL waters and their FW counterparts, involving a total of five evolutionarily independent habitat transitions (Hebert et al. 2002; Colbourne et al. 2006). These studies hypothesized UV exposure (Hebert et al. 2002), or UV and salinity independently (Colbourne et al. 2006), as causes for the observed higher molecular rates in SAL versus FW lake crustacean taxa. Similarly, Wägele et al. (2003) observed faster relative molecular rates in marine than in FW Asellota isopods; their study involved a single sister clade pair. In prokaryotes, Logares et al. (2010) observed 7.5 times less molecular diversity within a FW clade of the bacterial SAR11 group than in its sister marine/brackish clade. This pattern was hypothesized to be due to higher dispersal in FW, resulting in genetic homogenization, and/or to higher molecular evolutionary rates in the marine/brackish taxa. The higher rates in SAL taxa were observed across all genes in these studies, ranging from one to five genes examined, in both mitochondrial and nuclear genomes, and including coding (mitochondrial only: COI) and noncoding regions (12S, 16S, 18S, 28S). It remains unknown whether these elevated rates previously observed for continental SAL branchiopods and for several marine organisms hold for SAL environments broadly.

In this study we employ the comparative method to investigate whether a generalized relationship exists between salinity of aquatic habitat and molecular evolutionary rate. We analyzed 148 sister pairs of FW versus marine or continental SAL taxa (Table 1) for overall nucleotide substitution rates (OSRs) and dN/dS ratios in nuclear and mitochondrial noncoding and protein-coding genes. Contrary to previous expectations from studies linking SAL habitat to higher molecular rates, we observed no general habitat-linked difference in OSR when considering all genes. However, we observed some exceptions, with FW lineages having more often higher substitution rates in protein-coding genes compared with SAL lineages. Upon considering multiple mechanisms that could influence rates of molecular evolution, we could not pinpoint a clear mechanism to explain the trends observed in all genetic regions, considered together. The trends in protein-coding genes may reflect smaller effective population size and/or higher metabolic rate in FW lineages, possibly in combination with positive selection in select taxa and genes.

Materials and Methods

SOURCE STUDIES AND SPECIES CHOICE

We searched Web of Science for published studies including molecular phylogenies of aquatic eukaryotes inhabiting waters of varying salinities. The following habitat descriptions were sought within the source papers or from other works on the same taxa: "marine," "saline," "brackish," "euryhaline," and "freshwater" (search details and taxon inclusion criteria in Supporting Information). Phylogenetically independent sister clades or lineages, or paraphyletic paired lineages in some instances, were chosen so as to represent a salinity difference between sisters. Each entire source study tree, including outgroup lineages, was used in analysis. Comparisons were mainly between marine, continental SAL, or brackish versus FW categories. However, euryhaline species were occasionally used within either the "saline" (SAL) or "freshwater" (FW) category type, given a contrast in salinity of inhabited environment between the two sister lineages. We initially excluded comparisons that had already been analyzed for molecular evolutionary rate differences (Hebert et al. 2002; Wägele et al. 2003; Colbourne et al. 2006; Logares et al. 2010) so as not to pseudoreplicate results from prior studies that already tested for (and observed) habitat-specific rates. We separately analyzed all of these comparisons using the methods outlined here.

The source studies' single- or multigene tree topology was used, with preference for topologies created using maximum

Table 1. Summary of 148 freshwater–saline comparisons used in analysis.

Taxa group	Taxa	Source study	Loci[a]	Saline habitat type	Comparison number	
					Fig. 2	Fig. 3
Cetaceans	Toothed whales (Odontoceti, infraorder)	Hamilton et al. (2001)	**CytB**, 12S, 16S	M	1–3	1–3
Elasmobranchs	Sharks (Selachimorpha, superorder)	Vélez-Zuazo and Agnarsson (2011)	**COI**, **ND2**	M	4	4
	Whiptail stingrays (Dasyatidae, family)	Sezaki et al. (1999)	**CytB**	M	5–7	5–7
Bony fish	Sea catfishes (Ariidae, family)	Betancur-R. et al. (2012)	**CytB**, **ATP6, ATP8**, **Rag1.1**, **Rag1.2**, **Rag2**, **MYH6**, 12S, 16S, Rag1.Int	M	8–17	8–17
	Anchovies (Engraulidae, family)	Bloom and Lovejoy (2012)	**CytB**, **Rag1**, **Rag2**, 16S	M	18–24	18–24
	Terapontid grunters (Terapontidae, family)	Davis et al. (2012)	**CytB**, **Rag1.1**, **Rag1.2**, **Rag1.3**, **Rag2**, Rag1.Int1, Rag1.Int2	M+Eu, Eu	25–26	25–26
	Sardines, herrings, & relatives (Clupeoidei, suborder; Engraulidae excluded)	Lavoué et al. (2013)	**COI**, **CytB**, **ATP6**, **ATP8**, 12S, 16S	M+Eu, M, Eu	27–30	27–30
	Needlefishes (Belonidae, family)	Lovejoy and Collette (2001)	**CytB**, **Rag2**, **Tmo**, 16S	M	31–35	31–35
	Tubenose goby (*Proterorhinus,* genus)	Neilson and Stepien (2009)	**COI**, **CytB**, **Rag1**	SBr	36	36
	Fundulus (Cyprinodontiformes genus)	Whitehead (2010)	**CytB**, **Gylt**, **Rag1**	M	37–40	37–40
	Pufferfish (Teraodontidae, family)	Yamanoue et al. (2011)	**ATP6**, **ATP8**, **COI**, **COIII**, **CytB**, **ND1**, **ND2**, **ND3**, **ND4**, **ND4L**, **ND5**, 12S, 16S	M/Br/Eu	41–43	41–43
	Sculpins (Cottidae, family)	Yokoyama and Goto (2005)	12S, CR	M	44	
	New World silversides (Menidiinae, subfamily)	Bloom et al. (2013)	**CytB, ND2, Rag1, Tmo**	M	45–53	44–52

(*Continued*)

Table 1. Continued.

Taxa group	Taxa	Source study	Loci[a]	Saline habitat type	Comparison number Fig. 2	Comparison number Fig. 3
Crustaceans	Centropagidae (Copepoda family)	Adamowicz et al. (2010)	16S, 28S	M,S	54–56	
	Mysis (Mysida genus)	Audzijonytė et al. (2005)	**COI**, **CytB**, 16S, ITS2, 18S	SBr	57	53
	Gammarus (Amphipoda genus)	Hou et al. (2011)	**COI**, 18S, 28S	M	58–60	54–56
	Gammaracanthus (Amphipoda genus)	Väinölä et al. (2001)	**COI**	SBr, Br	61–62	57–58
	Palaemoninae (Decapoda subfamily)	Botello and Alverez (2013)	16S	M, M+Br	63–66	
	Typhlatya (Decapoda genus)	Hunter et al. (2008)	**COI**, **CytB**, 16S	M	67	59
Mollusks	Bivalves (Bivalvia, class)	Bieler et al. (2014)	**COI**, **H3**, 16S, 18S, 28S	M	68–70	60–62
	Cerithioidea (Gastropoda superfamily)	Strong et al. (2011)	16S, 28S	M/Br	71–73	
	Hydrobiidae (Gastropoda family)	Haase (2005)	**COI**, 16S	Br	74–76	63–65
Annelids	Earthworms, leeches, & allies (Clitellata, class)	Rousset et al. (2008)	18S	M, Br, M+Br	77–85	
Eukaryotes, other	Cyclotrichiida (Ciliophora order)	Bass et al. (2009)	18S	M	86	
	Choanoflagellatea (class)	Carr et al. (2008)	18S	M	87	
	Centrohelida (Heliozoa class)	Cavalier-Smith and von der Heyden (2007)	18S	M	88–96	
	Dinoflagellata (phylum)	Logares et al. (2007)	18S	M	97–109	
	Bicosoecida, Placididea, & relatives (within phylum Heterokonta)	Park and Simpson (2010)	18S	M, M+HS	110–112	
	Euglyphida (Cercozoa order)	Heger et al. (2010)	18S	M	113–116	
	Vannellidae (Amoebozoa family)	Smirnov et al. (2007)	18S	M, Br	117–119	
	Heterolobosea (Excavata class)	Pánek et al. (2012)	18S	M+Br, M	120–121	
	Telonemia (phylum)	Bråte et al. (2010)	18S	M	122–125	

(*Continued*)

Table 1. Continued.

Taxa group	Taxa	Source study	Loci[a]	Saline habitat type	Comparison number	
					Fig. 2	Fig. 3
Eukaryotes, algae	Thalassiosirales (Heterokontophyta order)	Alverson et al. (2007)	**psbC**, **rbcL**, 18S, 28S	M	126–131	66–71
	Raphidophyceae (Heterokontophyta class)	Figueroa and Rengefors (2006)	18S	M	132	
	Trebouxiophyceae (Chlorophyta class)	Henley et al. (2004)	18S	M/S	133	
	Chroomonas (Cryptophyta genus)	Hoef-Emden (2008)	18S, 28S	M	134–136	
	Cryptophyceae (Cryptophyta class; *Chroomonas* excluded)	Shalchian-Tabrizi et al. (2008)	18S	M	137–140	
	Haptophyta (division)	Simon et al. (2013)	18S	M, M+S	141–148	

[a]Mitochondrial protein-coding genes: ATP synthase F0 subunit 6 (ATP6) and 8 (ATP8); cytochrome c oxidase subunit I (COI) and III (COIII); cytochrome b (CytB); NADH dehydrogenase subunit 1 (ND1), 2 (ND2), 3 (ND3), 4 (ND4), 4L (ND4L), 5 (ND5). Nuclear protein-coding genes: glycosyltransferase (Gylt); histone 3 (H3); myosin heavy chain 6 (MYH6); recombination activating gene 1 (Rag1) part 1 (Rag1.1), part 2 (Rag1.2), part 3 (Rag1.3), and 2 (Rag2); toluene monooxygenase (Tmo). Chloroplast protein-coding genes: photosystem II CP43 protein (psbC); ribulose bisphosphate carboxylase large chain (rbcL). Mitochondrial noncoding genes: 12S (12S) and 16S (16S) ribosomal RNA; mitochondrial control region (CR). Nuclear noncoding genes: Rag 1 intron 1 (Rag1.Int1) and 2 (Rag1.Int2); second internal transcribed spacer (ITS2); 18S (18S) and 28S (28S) ribosomal RNA.

Saline habitat type: M, marine; Br, brackish marine; Eu, euryhaline; S, saline continental; SBr, large inland "seas" with brackish waters, HS, hypersaline; "+" (e.g., 'M+Br') signifies the individuals' habitats are known and a mixed-habitat clade was used, whereas "/" (e.g., 'M/Br') signifies the exact habitat state for each individual in a clade was not specified in the source study. Bolded gene names signify protein-coding genes. Comparisons listed are those that passed the inclusion criteria (see Supporting Information). Comparisons refer to evolutionarily independent habitat shifts; these are numbered in accordance with the ordering of results in Figures 2 and 3 within this article. Supporting Information notes where habitat information was obtained from sources other than the "source study" (for the molecular data) given here.

likelihood or Bayesian methods over maximum parsimony or neighbor joining (Hall 2005; Ogden and Rosenberg 2006). If multiple topologies were present within the same paper or taxon, we used the phylogeny that was built using the most genetic data or that included the most habitat information. We performed interspecific comparisons; however, considerations were made to maximize sample size of organisms per clade, especially in the case of unicellular organisms in which species boundaries are difficult to define (details in Supporting Information). We included the same number of terminal taxa in each sister clade to minimize the node density effect (Robinson et al. 1998), while maximizing genetic data available, between-clade salinity difference, and phylogenetic diversity (Robinson et al. 1998); after these considerations, sequences were selected from major subclades using the random number generator in R (R v2.11.1, R Development Core Team 2010). All input source study names, species names, habitat categorizations, postulated ancestral habitats, and sources of genetic data are given in the Supporting Information.

SEQUENCE DATA

Molecular data were obtained from GenBank, Dryad Digital Repository, Treebase, the source study supplementary material, online links, or directly from the source study authors (sources noted in Supporting Information), with preference for original alignments used in the construction of the phylogeny presented in the source study. Unaligned sequences were aligned using ClustalW in MEGA version 5.2 (Tamura et al. 2011) or 6.0 (Tamura et al. 2013) or using the EMBL-EBI ClustalOmega (Sievers et al. 2011) online tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). Protein-coding alignments were verified using amino acid translations to be free of stop codons and indels of 1–2 base pairs. Alignments for non-protein-coding genes, including those from the authors, were run through the online server Gblocks version 0.91b (Castresana 2000) to eliminate regions containing many gaps and uncertainty of homology, using the "less stringent" setting. Input and output files (alignment, topology, and PAML output) are available on the Dryad Digital Repository.

ESTIMATION OF RELATIVE RATES OF MOLECULAR EVOLUTION

The program *baseml* within the package PAML version 4.4 (Yang 2007) was used to estimate relative OSRs, and the program *codeml* was used to calculate dN/dS ratios. Each sister lineage or clade was coded differently, so that a rate would be estimated for each separately from their point of divergence onward. There were often multiple sister clades coded in the tree, with the rest of the lineages assigned to the background rate category (e.g., seven sister clades would have 15 rate categories coded in the tree). Due to different species being available for the various genes within the same sister pair, and to examine patterns for each gene, we estimated relative rates for each gene separately. For OSRs, models of molecular evolution for each gene were estimated in MEGA using the source topology, and the best model by Bayesian Information Criterion without +G or +I parameters was used in PAML.

We combined the relative rates (OSRs or dN/dS ratios) obtained for the separate genes for each sister pair to analyze overall molecular patterns across multiple phylogenetically independent habitat transitions. Concatenation of gene sequences was not possible due to different species having different genes available. Clade-wise rates are not provided by PAML for clades assigned rate classes. We, therefore, created a Python script to calculate estimates for dN and dS substitution rates for clades of interest from *codeml* outputs through averaging rates for sister lineages and adding internal lineages, starting from the lineage tips (script also provided on Dryad repository). With these values per gene, we concatenated the dN rates, dS rates, and dN/dS ratios across all genes per sister comparison by adding estimated substitution counts for individual genes (Mitterboeck and Adamowicz 2013). Total branch lengths were also similarly calculated for protein-coding genes using these estimated dN and dS substitution counts. We display the dN/dS ratios as relative by using a formula (1 – smaller/larger rate) and signing the metric, based on which habitat displayed the larger ratio (SAL > FW positive, FW > SAL negative; as in Wright et al. 2006). However, for OSRs, relative rates are provided, and we could not concatenate the rates in the same way across genes using absolute counts of estimated substitutions. Instead, for each individual gene within a sister pair, the FW and SAL relative rates were divided larger over smaller. We subtracted 1 from these ratios, because if the sister rates were equal, a ratio of 1 would be produced; a distribution centered around zero was preferable for further analysis. This difference from 1 was next signed based on direction. Based upon sequence length, a weighted average of the signed differences across genes was calculated for each sister pair. To ensure that the displayed ratios are intuitive to interpret, we revert the summarized differences to be relative, that is, by adding 1 to the positive and subtracting 1 from the negative overall result.

We examined each gene for each sister pair against our minimum inclusion criteria, separately for OSRs and dN/dS ratios, to avoid including genes lacking information and those producing extreme rate differences (details in Supporting Information). Therefore, the genetic data represented by each sister pair may not be exactly the same between OSR and dN/dS analyses.

ANALYSIS OF RELATIVE RATES OF MOLECULAR EVOLUTION IN SALINE VERSUS FRESHWATERS

We tested for an overall habitat-related trend in the summarized relative OSRs and dN/dS ratios by two-tailed binomial tests

(all tests are two-tailed) with null expectation of 50% positive values (SAL > FW) and 50% negative values (FW > SAL). Statistical tests were performed in R (R v2.13.0, R Development Core Team 2010). Similarly, we tested specific gene categories. For OSRs we grouped the genes into two broad categories, "protein-coding" and "noncoding," as well as into five more specific categories: mitochondrial protein-coding, nuclear protein-coding, mitochondrial noncoding, nuclear noncoding, and chloroplast protein-coding. For dN/dS ratios, we grouped the genes into three categories of protein-coding genes: mitochondrial, nuclear, and chloroplast. Where we tested multiple gene categories, we consider the *P*-values without correction. We additionally repeated the above tests excluding summarized relative OSRs that were close to equal (<5% deviation between the two values).

We furthermore parsed the data into three major groupings of organisms: vertebrates (cetaceans, elasmobranchs, bony fish), invertebrates (crustaceans, mollusks, annelids), and "microeukaryotes" (we include algae and other small/unicellular eukaryotes). These categories were delineated based on general expected differences in body size, population size, and osmoregulation mechanism or capacity. Note that gene categories were differentially represented by these groupings. We tested for patterns within each gene category for these three independent organismal groupings by binomial test, whenever the gene category was represented by six or more sister pairs. We corrected for the number of tests per gene category (up to three organismal groupings) by sequential Bonferroni correction (Holm 1979).

Summarized relative rates of protein-coding genes were additionally subjected to Wilcoxon signed-rank tests in which the minimum sample size was 10; this was possible for protein-coding genes because branch length information was available. The data were verified via the procedures of Welch and Waxman (2008) and Garland et al. (1992) to prevent inclusion of low-information-content pairs or greater rate difference with more divergent pairs (excluded rate pairs given in Supporting Information). A Wilcoxon signed-rank test, with null expectation of a median value of zero, was performed on the rate differences standardized by the square root sum of branch lengths.

We examined the concordance in habitat-linked relative rates across the genome by testing whether the directions (i.e., SAL > FW, FW > SAL) of the protein-coding mitochondrial and nuclear relative rates matched significantly more often than expected by chance, by binomial test. Additionally, individual genes represented by six or more sister pairs were tested for consistently higher rates in either habitat category by binomial test; multiple testing of individual genes were corrected by sequential Bonferroni correction (Holm 1979). Finally, we performed a binomial test on relative rates of inland-SAL versus FW comparisons alone.

TESTS FOR LINK WITH DIRECTION OF HABITAT SHIFT

Because the act of transitioning into a novel environment may influence the relative molecular rates, we tested whether relative rates differed by transition direction. The direction of the habitat transition was inferred based upon either the postulated ancestral habitat information or phylogenetic mapping of habitats in the source studies. Comparisons were not included here if ancestral information was equivocal. We tested for directional patterns in OSRs and dN/dS ratios within each habitat direction category (i.e., FW to SAL, or SAL to FW) by binomial test, and between transition directions using Fisher's exact test.

RE-ANALYSIS OF DATA FROM PREVIOUS STUDIES

To test the conservativeness of our methods, we re-analyzed the data from previous studies that reported habitat-specific differences in relative rates of molecular evolution. We analyzed: the five crustacean comparisons present and overlapping in Hebert et al. (2002) and Colbourne et al. (2006), as well as re-analyzed the *Daphnia* comparisons using an updated phylogenetic hypothesis (Adamowicz et al. 2009); the single comparison from Raupach et al. (2004) in isopods, which contained the same transition and gene as Wägele et al. (2003); and a single comparison from Logares et al. (2010) in SAR11 bacteria. We analyzed these studies consistent with our above methods, including choosing a balanced number of terminal taxa for our sister comparisons.

Results

NO DIFFERENCE IN FRESHWATER VERSUS SALINE RATES WHEN CONSIDERING ALL GENES TOGETHER

One-hundred forty-eight comparisons, each representing an independent transition in habitat state, had at least one gene that passed the inclusion criteria for OSR analysis (all comparisons are given in Table S1.1). In total, across these sister pairs, 396 pairs of relative rates, each representing a single gene for OSRs, were included in subsequent statistical testing. Seventy-one sister pairs had at least one protein-coding gene that passed the inclusion criteria for dN/dS ratios; these contained a total of 236 pairs of dN/dS ratios for single genes.

We present the results as follows: number of comparisons with *SAL* (SAL clade) rate greater (positive direction), number of comparisons with *FW* (FW clade) rate greater (negative direction), number of sister pairs exhibiting equal rates (if present), total *N* used for binomial test, *P*-value from binomial test. Bonferroni correction was applied to *P*-values stated below, in cases in which more than one set of data (a set having six or more data points) existed for that category of test.

Across 148 sister comparisons (Fig. 2), neither the SAL nor FW habitat category had higher OSRs, considering all genes

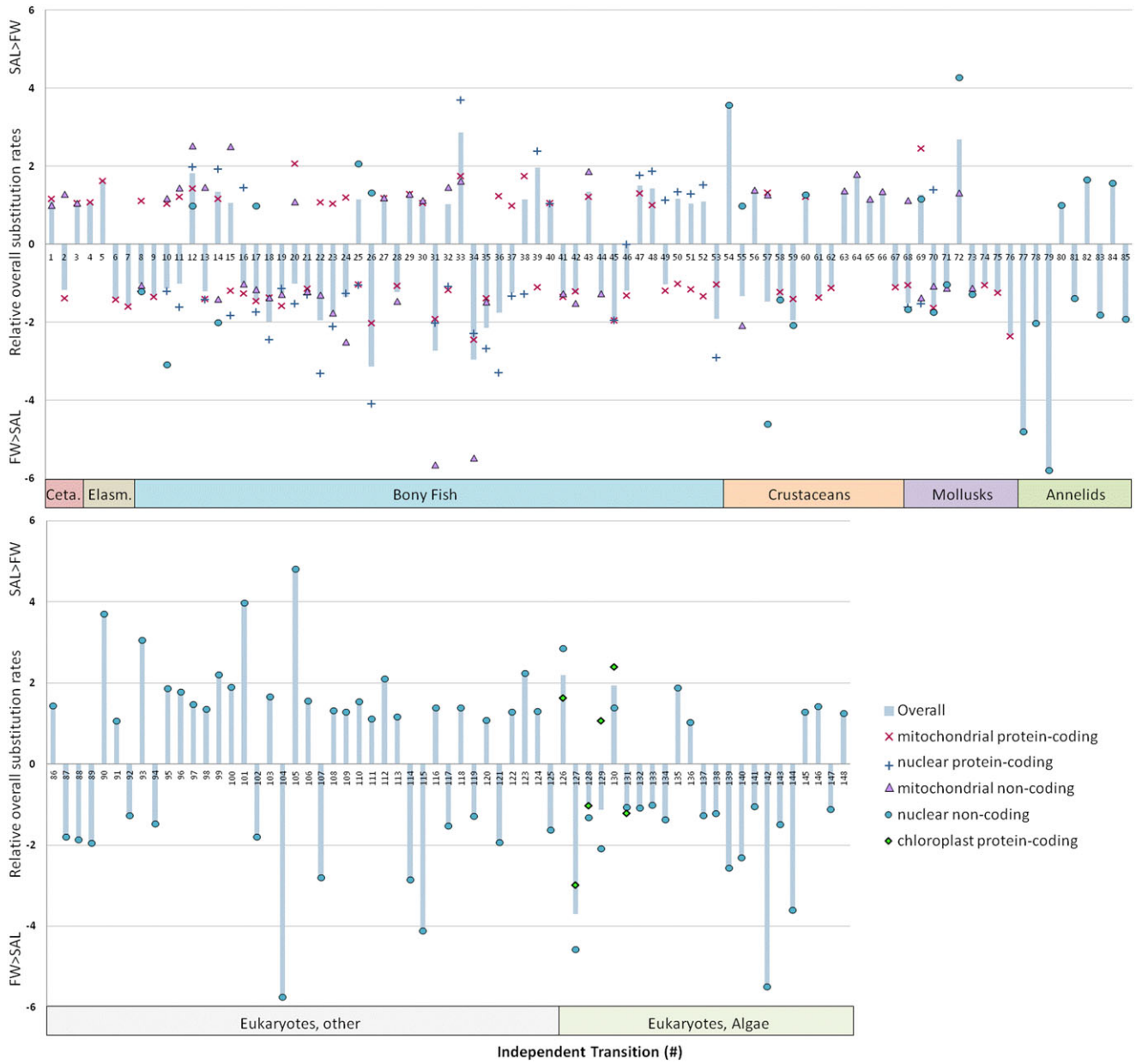

Figure 2. **Relative saline: freshwater (SAL:FW) overall substitution rates (OSRs) across 148 comparisons. The bars represent the relative OSRs summarized across all genes analyzed for each phylogenetically independent habitat transition. The symbols represent the separately summarized mitochondrial and nuclear (protein-coding and noncoding separately) and chloroplast protein-coding gene results. Those bars or symbols above the *x*-axis indicate comparisons in which the summarized SAL relative rate was greater than the FW rate, and those below the *x*-axis indicate where the summarized FW rate was greater than the SAL. Note: Shown are "relative rates" (starting at 1) with direction, and so there are no values between –1 and +1. The overall SAL rate was greater than FW in 67 sister pairs; the FW rate was greater than the SAL rate in 81 sister pairs ($P = 0.29$). Taxa labels: Ceta., cetacean; Elasm., elasmobranch.**

together (67, 81, 148, $P = 0.29$; Fig. 4A). The median relative OSR was –1.04 across all genes (i.e., the FW lineage rate was >4% higher than the SAL rate in half of the sister pairs). Across the 71 sister comparisons analyzed for dN/dS ratios (Fig. 3), neither habitat category more often had higher dN/dS ratios when considering all genes together (33, 38, 71, $P = 0.64$; Fig. 4B), with the median relative ratio being –1.05.

HIGHER MOLECULAR RATES IN FRESHWATER LINEAGES IN PROTEIN-CODING GENES

Protein-coding genes tended to have faster OSRs in FW taxa. Among the gene categories tested for OSRs (Fig. 4A), all protein-coding genes together (26, 45, 71, $P = 0.032$) and nuclear protein-coding genes (13, 26, (1), 39, $P = 0.053$) displayed generally higher rates in the FW taxa. The median relative OSR was −1.10

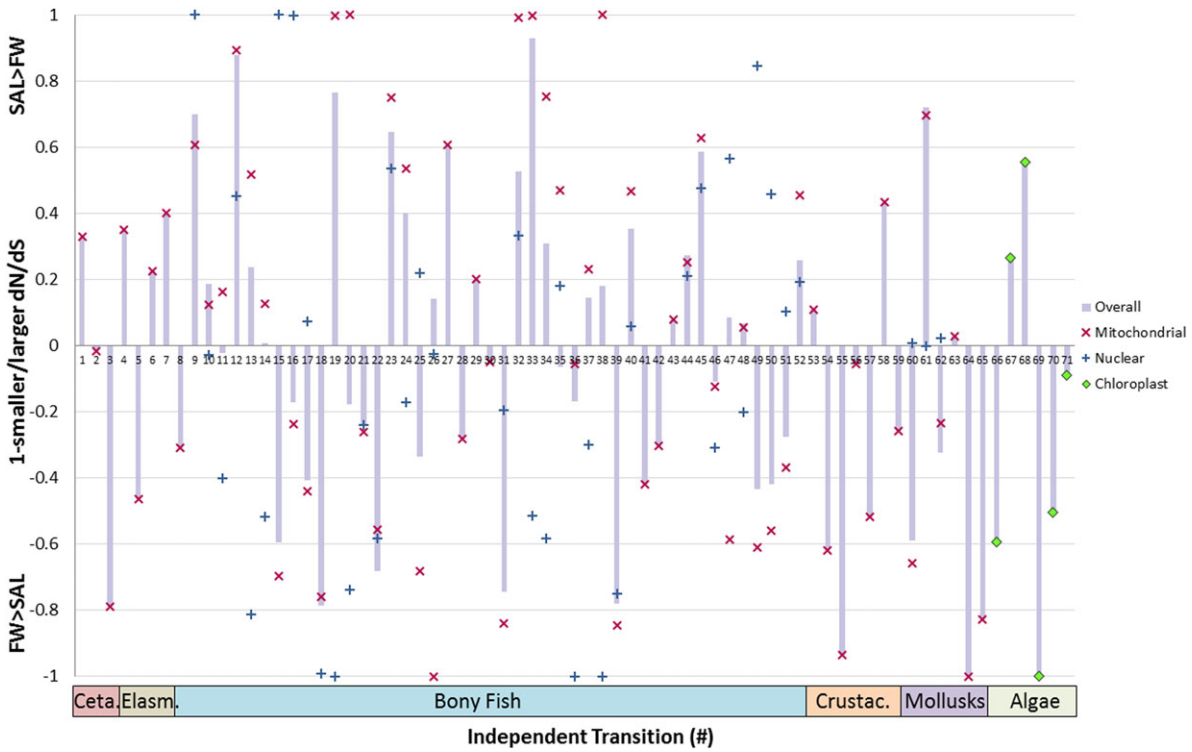

Figure 3. **Relative saline: freshwater (SAL:FW) dN/dS ratios across 71 comparisons. For ease of viewing, we display 1 minus the smaller dN/dS ratio over the larger dN/dS ratio. Bars represent the overall dN/dS ratios across all genes for a given sister pair, and symbols represent the mitochondrial, nuclear, and chloroplast gene results. Those bars or symbols above the *x*-axis indicate comparisons in which the SAL dN/dS ratio was greater than the FW, and those below the *x*-axis indicate where the FW dN/dS ratio was greater than the SAL. The overall dN/dS ratios were higher in the SAL clade in 33 comparisons and higher in the FW clade in 38 comparisons ($P = 0.64$). Taxa labels: Ceta., cetacean; Elasm., elasmobranch; Crustac., crustacean; Algae, eukaryotes, algae.**

across all protein-coding genes and −1.27 across the nuclear protein-coding genes. Mitochondrial protein-coding OSRs had a weaker tendency toward higher rates in FW taxa, whereas noncoding and chloroplast genes exhibited no difference in rates between habitat categories. These trends remained when excluding sister pairs displaying near-equal relative rates (Supporting Information).

dN and dS rates were each not significantly linked to habitat across the 71 comparisons (both 32, 39, 71, $P = 0.48$). Among the three protein-coding gene categories, dN/dS ratios (Fig. 4B), dN, and dS rates each exhibited relatively even results between habitats (Table 2 and Fig. S2).

SOME TRENDS WITHIN ORGANISMAL GROUPINGS

For OSRs, the vertebrate nuclear protein-coding genes had the most pronounced direction (12, 24, (1), 36; $P = 0.065$; Table 2); here, only one organismal category (vertebrates) had large enough sample size, and so no Bonferroni correction was applied. These were all bony fish comparisons. Mitochondrial protein-coding genes had more often, but not significantly, higher relative dS rates in FW for vertebrates (19, 33, 52, $P = 0.070$, $P = 0.14$ corrected). Within these major taxonomic groupings, some other trends existed. Of the nuclear protein-coding OSR comparisons, the silverside fish group (Bloom et al. 2013) appears to have a different tendency than other fish (supported by a Fisher's exact test, $P = 0.036$), having a majority of comparisons SAL > FW (6, 3, 9, $P = 0.51$). Excluding the silversides, and considering all remaining sister pairs, the nuclear protein-coding OSRs are strongly more often higher in FW across all taxa (7, 24, 31, $P = 0.0033$) and for bony fish alone (6, 22, 28, $P = 0.0037$). Within "micro-eukaryotes," the algae and other eukaryotes (Fig. 2) also appear to have different tendencies in OSRs across genes (there was mainly one gene available for those comparisons), with algae (7, 16, 23, $P = 0.093$) and other eukaryotes (26, 14, 40, $P = 0.081$) differing by Fisher's exact test ($P = 0.010$).

CODING VERSUS NONCODING GENES

The lack of a habitat-linked pattern in relative rates of evolution within noncoding genes does not appear to be due to a difference in availability of sequences among taxa. For comparisons having both protein-coding and noncoding OSR data, protein-coding gene rates were again more often higher in FW, whereas

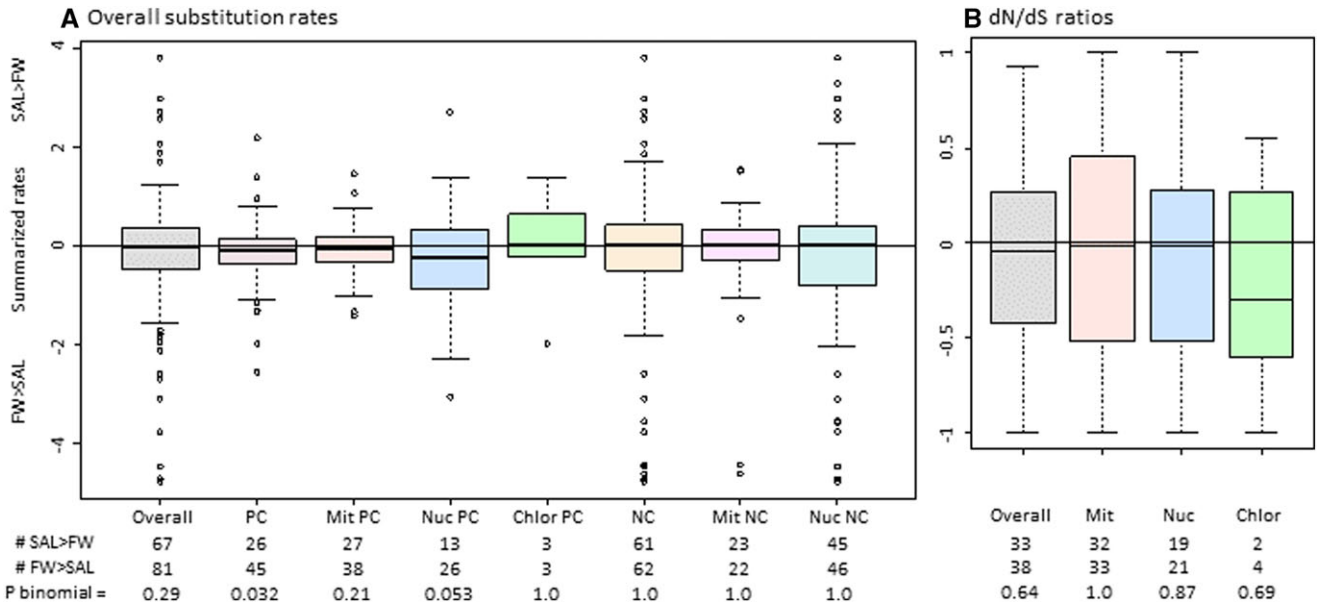

Figure 4. **Summarized overall substitution rates (OSRs) and dN/dS ratios by gene category. Rates within gene categories are summarized separately for each comparison, where the number of data points is the number of independent sister comparisons. These boxplots show the distribution of relative rates shown in Figures 2 and 3. The first boxplot in (A) "overall" is the distribution of bar heights in the "overall" gene category in Figure 2; the first boxplot in (B) is the distribution of bar heights in the "overall" gene category in Figure 3; and other boxplots represent the distribution of symbols in those figures. Data among boxplots are not independent because plots overlap in SAL-FW sister pairs; that is, multiple gene types are often available for the same sister pair. *P*-values are from two-tailed binomial tests. The greatest SAL–FW differences are observed for OSR protein-coding genes overall ($P = 0.032$) and nuclear protein-coding genes ($P = 0.053$). Some results shown here overlap with those presented in Table 2. Gene categories: Nuc, nuclear; Mit, mitochondrial; Chlor, chloroplast; PC, protein-coding; NC, noncoding.**

noncoding genes showed no association to habitat (protein-coding: 16, 30, 46, $P = 0.054$; noncoding: 20, 26, 46, $P = 0.46$). This was similar for bony fish comparisons alone (protein-coding: 8, 22, 30, $P = 0.016$; noncoding: 13, 17, 30, $P = 0.58$).

LITTLE EVIDENCE FOR GENOME-WIDE EFFECT OF HABITAT UPON RATES

Directions of relative rates (e.g., SAL > FW or FW > SAL) were not strongly consistent between nuclear and mitochondrial protein-coding genes from the same taxa. The nuclear and mitochondrial relative rates were higher in the same habitat in about half of the sister pairs (OSRs: 21 of 39, and dN/dS ratios: 17 of 40), which would be the random expectation (Supporting Information).

NO SINGLE GENE DRIVING THE PATTERN IN PROTEIN-CODING GENES

Individual genes did not show significantly higher rates in either habitat. The most consistent habitat-linked pattern was in the recombination activating gene 2 (Rag 2), with generally higher rates in FW lineages (dN/dS ratios 6, 16, 22, $P = 0.052$ uncorrected, $P = 0.52$ corrected). Those genes with the most data points (>40 sister pairs) for OSRs were 18S, 16S, and CytB; for dN/dS ratios, the gene with the highest data availability was CytB. Each of these genes had relatively even directional results (Supporting Information). The majority of individual genes more often had the FW lineage as having the higher rates (OSRs $P = 0.015$, dN/dS $P = 0.36$); however, gene relative rates are not independent as several genes are from the same organisms.

NO CONSISTENT PATTERN IN CONTINENTAL SALINE VERSUS FRESHWATER COMPARISONS

Only eight comparisons included inland SAL habitats as the "saline" category. These inland habitats vary in size and salinity and include inland brackish "seas." For these eight comparisons alone, neither habitat had the higher OSRs (2, 6, 8, $P = 0.29$) or dN/dS ratios (2, 1, 3, $P = 1.0$). Two of the continental SAL taxa were most similar to the types of comparisons used in previous literature—crustacean comparisons from small SAL lakes. These two comparisons did not have consistently higher rates in the SAL taxa (OSRs: 1, 1, 2, $P = 1.0$).

NO RELATIONSHIP BETWEEN ANCESTRAL HABITAT AND OVERALL RATES

There was no significant trend in either habitat having higher relative OSRs or dN/dS ratios when the comparisons were divided

Table 2. All relative rates, including dN and dS rates, by taxonomic breakdown.

		Overall substitution rates								dN/dS ratios—Protein-coding only															
			Protein-coding				Noncoding			All protein-coding genes				Mitochondrial				Nuclear				Chloroplast			
Sister pairs[a]	Direction	All	All	Mit	Nuc	Chl	All	Mit	Nuc	dN	dS	dN/dS	BLn	dN	dS	dN/dS	BLn	dN	dS	dN/dS	BLn	dN	dS	dN/dS	BLn
All:1–148	SAL > FW (+)	67	26	27	13	3	61	23	45	32	32	33	31	31	28	32	28	16	18	19	17	2	3	2	2
	FW > SAL (–)	81	45	38	26	3	62	22	46	39	39	38	40	34	37	33	37	23	21	21	23	4	3	4	4
	Binomial *P*	0.29	**0.032**	0.21	**0.053**	1.0	1.0	1.0	1.0	0.48	0.48	0.64	0.34	0.80	0.32	1.0	0.32	0.34	0.75	0.87	0.43	0.69	1.0	0.69	0.69
	Wilcoxon *P*	–	**0.038**	0.12	**0.063**	–	–	–	–	**0.054**	0.48	0.76	0.14	0.24	0.33	0.86	0.46	0.44	0.60	0.80	0.61	–	–	–	–
Vert:1–53	SAL > FW (+)	22	20	24	12	–	16	15	4	25	21	27	19	26	19	28	18	15	17	17	16	–	–	–	–
	FW > SAL (–)	31	32	28	24	–	18	17	3	27	31	25	33	26	33	24	34	21	19	20	21	–	–	–	–
	Binomial *P*	0.27	0.13[b]	0.68	**0.065**	–	0.86	0.86	1.0	0.89	0.21	0.89	**0.070**[c]	1.0	**0.070**[b]	0.68	**0.036**[b]	0.41	0.87	0.74	0.51	–	–	–	–
	Wilcoxon *P*	–	**0.046**[b]	0.24	**0.075**	–	–	–	–	0.19	**0.021**[b]	0.73	**0.054**[b]	0.48	0.11	0.63	**0.087**	0.48	0.60	0.80	0.65[d]	–	–	–	–
Invert:54–85	SAL > FW (+)	12	3	3	1	–	12	8	8	5	8	4	10	5	9	4	10	1	1	2	1	–	–	–	–
	FW > SAL (–)	20	10	10	2	–	14	5	13	8	5	9	3	8	4	9	3	2	2	1	2	–	–	–	–
	Binomial *P*	0.22	**0.092**[c]	**0.092**[b]	1.0	–	0.85	0.58	0.38	0.58	0.58	0.27	**0.092**[b]	0.58	0.27	0.27	**0.092**	1.0	1.0	1.0	1.0	–	–	–	–
	Wilcoxon *P*	–	0.22	0.24[b]	–	–	–	–	–	0.19	**0.092**	0.17	**0.064**	0.22	**0.064**[b]	0.15	**0.077**[b]	–	–	–	–	–	–	–	–
Micro:86–148	SAL > FW (+)	33	3	–	–	3	33	–	33	2	3	2	2	–	–	–	–	–	–	–	–	2	3	2	2
	FW > SAL (–)	30	3	–	–	3	30	–	30	4	3	4	4	–	–	–	–	–	–	–	–	4	3	4	4
	Binomial *P*	0.80	1.0	–	–	1.0	0.80	–	0.80	0.69	1.0	0.69	0.69	–	–	–	–	–	–	–	–	0.69	1.0	0.69	0.69
Bony fish[e]:8–53	SAL > FW (+)	18	17	20	12	–	13	12	4	21	18	23	16	22	16	24	15	15	17	17	16	–	–	–	–
	FW > SAL (–)	28	28	25	24	–	18	17	3	24	27	22	29	23	29	21	30	21	19	20	21	–	–	–	–
	Binomial *P*	0.18	0.14[b]	0.55	**0.065**	–	0.47	0.46	1.0	0.77	0.23	1.0	**0.072**[c]	1.0	**0.072**[b]	0.77	**0.036**[b]	0.41	0.87	0.74	0.51	–	–	–	–
	Wilcoxon *P*	–	**0.032**[b]	0.23	**0.075**	–	–	–	–	0.23	**0.027**[b]	0.77	**0.076**[b]	0.61	0.16	0.65	0.25	0.48	0.60	0.80	0.65[d]	–	–	–	–

[a] Sister comparisons as numbered in the second right-most column in Table 1 and in Figure 2.

[b] Corrected *P*-value would be the given *P*-value multiplied by 2.

[c] Corrected *P*-value would be the given *P*-value multiplied by 3.

[d] The Wilcoxon signed-rank *P*-value is for the opposite direction (i.e., FW > SAL or SAL > FW) than the direction of the binomial test.

[e] Bony fish category *P*-values were corrected by the same factors as vertebrate *P*-values (as shown).

P-values presented in the table are raw (those less than 0.1 are bolded). In cases in which correction for multiple tests was performed (see Methods), significance value is indicated with a footnote. Vert, vertebrates; Invert, invertebrates; Micro, micro-eukaryotes; Chl, chloroplast; Mit, mitochondrial; Nuc, nuclear; BLn, branch lengths calculated from dN and dS substitution counts.

based on direction of the habitat shift (FW to SAL or SAL to FW directions; Supporting Information).

RE-ANALYSIS OF PREVIOUS STUDIES REPORTING HABITAT-SPECIFIC RATE DIFFERENCES

All studies re-analyzed (Hebert et al. 2002; Wägele et al. 2003/Raupach et al. 2004; Colbourne et al. 2006; Adamowicz et al. 2009; Logares et al. 2010) gave higher molecular rates in the SAL habitat category as compared with the FW habitat category for each of the comparisons when considering all genes together (Supporting Information), in accordance with the results presented in those original studies. In a minority of the individual genes (from the studies of Hebert et al. 2002 and Colbourne et al. 2006), the FW lineage was calculated as having the higher rate. These minor differences from the original study results could be due to our use of a balanced number of tip lineages for each habitat category as well as to our method of estimating branch lengths.

SOME VARIATION IN RESULTS WITH DIFFERENT MOLECULAR MEASURES

Two methods of estimating relative branch lengths were used for protein coding genes—(1) relative OSRs from PAML *baseml* (Figs. 2, 4, and Table 2), and (2) branch lengths in PAML *codeml* from estimated dN and dS substitution counts (Table 2 BLn columns). Branch lengths from *baseml* have been used more commonly in the literature (e.g., Woolfit and Bromham 2005; Bromham et al. 2013), whereas substitution counts can be useful to allow concatenation of rates across genes in which different species are available (Mitterboeck and Adamowicz 2013). However, here, the raw relative rates (before any summary across genes) from these two methods corresponded only 78% of the time in terms of which habitat had the higher estimated rate in over 200 individual gene pairs tested (Supporting Information). Part of the difference in methods could stem from differing weighting of substitution types; we observed from simple datasets the latter method may estimate more substitutions at synonymous sites compared to nonsynonymous sites (as compared with the former method), although this would need rigorous testing. Further difference between these methods can be added when summarizing rate estimates across multiple genes per sister pair, as our summary of relative OSRs may more equally weigh different gene results, whereas summary using substitution counts may give more weight to those genes that are evolving faster (Bromham and Leys 2005). Although vertebrates had consistently higher FW than SAL habitat rates using both methods, discrepancies existed within invertebrates (Table 2), and due to this we do not delve into biological interpretation of the (nonsignificant) invertebrate results. We focus interpretation on the OSRs, which were available for all gene types, along with the dN and dS rates alone.

Discussion

Our results indicate that prior findings of higher molecular rates in continental SAL lakes are not mirrored by an overall pattern of higher rates in SAL environments when including the marine realm. Rather, we observed no general difference in relative rates between habitats, with some exceptions of higher rates in FW for protein-coding genes—specifically for vertebrates and bony fish as a subset. Although we did not directly measure differences among our included taxa in terms of key parameters that may influence their relative rates—relative salinity, UV, metabolic rate, effective population size, or diversification—here, we discuss the findings in the context of possible causative factors. After considering these various parameters and their influence on molecular rates, we cannot attribute the observed weak trend in protein-coding genes combined with lack of trends overall to a single cause.

The genes included in our analysis represent a small and biased, yet suitable, portion of the genome. The genes were those chosen for previous phylogenetic studies of the source taxa, likely selected with the intention of them being more conserved in function and sequence, providing phylogenetic resolution. Therefore, most of these genes are unlikely to have large differences in molecular rates due to positive selection based on habitat. For the purposes of our analyses investigating genome-wide rate differences, we were interested in patterns in molecular rates due to mutation rate differences or differences in the relative influence of genetic drift (vs. selection). We are not addressing specific genes under positive selection nor mechanisms of habitat adaptation here.

NO GENERAL DIFFERENCE BETWEEN FRESHWATER AND SALINE RATES

The more conserved nature of the genes included could contribute to the observed lack of higher rates in either FW or SAL organisms. However, this does not specifically explain the lack of trends as some of the same genes analyzed here have previously displayed systematic rate variability in association with environmental or biological parameters (e.g., COI, 16S, 18S; e.g., Hebert et al. 2002; Mitterboeck and Adamowicz 2013). Furthermore, our methods do not appear to be conservative, as we obtained the same trends as the original authors upon re-analyzing studies reporting higher molecular rates in SAL environments (Hebert et al. 2002; Wägele et al. 2003; Colbourne et al. 2006; Logares et al. 2010).

In this study, the noncoding DNA regions, including rRNA genes and several introns, do not follow the same trends as protein-coding genes. The included ribosomal gene regions may be more conserved across taxa as compared with the protein-coding sequences; however, our application of minimum exclusion criteria as well as visual inspection of alignments suggest that these

sequences exhibit variability and substantially so in some cases, even after removing regions of uncertain homology. Both higher coding (e.g., COI) and noncoding (e.g., 16S and 18S) rates have been observed in studies investigating the hypothesis of mutation rate differences relating to habitats or organismal traits (e.g., Hebert et al. 2002; Bromham et al. 2013). Given the larger number of independent comparisons used here, we expected patterns in both coding and noncoding loci if consistent differences existed in genome-wide influences on molecular rates across habitats (e.g., N_e or mutation rate).

Furthermore, although a large proportion of our total number of taxonomic comparisons had only one (noncoding) gene available, comparisons with multiple noncoding genes (in vertebrates) also did not show significant trends for those genes. Upon considering these points, it is unclear why there were no trends in noncoding genes, whereas patterns in protein-coding genes existed. The trends observed in this exploratory study would need further detailed investigation to fully delve into all causal mechanisms; nevertheless, here we consider the possible parameters acting to produce the observed trends in protein-coding genes.

MUTATION RATE DIFFERENCES VERSUS N_E EFFECT

Patterns in nonsynonymous-to-synonymous (dN/dS) ratios can be useful in indicating differences in positive or relaxed selection between lineages. Generally, synonymous sites should be freer to vary and approximate relative mutation rates, whereas nonsynonymous sites can indicate selection or an impact of effective population size (N_e).

For vertebrates, the slightly more often higher protein-coding OSRs and dS rates in FW taxa suggest more often higher mutation rate in FW than SAL lineages. Higher molecular rates in coding and noncoding mitochondrial loci have been observed in fish living in warmer waters—warmer due to either depth or latitude differences—compared with fish living in cooler waters (Wright et al. 2011). This observation was proposed to be due to higher metabolic rate in warmer waters. Higher mutation rates related to metabolism or temperature is a possible contributing influence on molecular rates in FW, whether due to some inherent difference between FW and SAL environments or to a latitudinal bias in the occurrence or sampling of FW versus SAL lineages (not tested here).

Higher protein-coding OSRs in FW may also be due to reduced N_e in FW. However, the effect of differences in N_e would be expected mainly in dN/dS ratios and dN rates, and furthermore, mitochondrial genes would be expected to show greater N_e effects than nuclear genes. Here, nuclear protein-coding dN relative rates are not strongly higher in FW, whereas the mitochondrial protein-coding dN relative rates showed no statistical pattern. Thus, N_e alone does not seem like a likely mechanism for the protein-coding patterns that are strongest in nuclear genes.

POSITIVE OR RELAXED SELECTION

Relaxed or positive selection on relevant genes may be associated with entering a new ecological niche, such as upon transitioning between habitats or lifestyles (e.g., Shen et al. 2009). We did not explicitly investigate positive selection: it may act on only one or a few sites (Hughes 2007) and thus be difficult to detect using gene-wide measures of molecular rates. The most consistent habitat association for a single gene was observed in the recombination-activating gene 2 (Rag2), which plays a role in the adaptive immune response (Jones and Gellert 2004). Many evolutionary changes may be selected for upon changing environments (Jones et al. 2012), including immune function (e.g., Sun et al. 2013). It is possible that positive selection may have impacted patterns of molecular evolution for some individual genes included in our study.

Relaxed selection can act upon gene categories, such as mitochondrial protein-coding genes involved with energy production. Higher dN/dS ratios, likely due to relaxed selection, have been observed in mitochondrial (but not nuclear) genes of specific groups of fish, insects, and birds with hypothesized lower energy requirements (Shen et al. 2009; Mitterboeck and Adamowicz 2013; Strohm et al. 2015). Given that here we did not observe trends in mitochondrial gene dN/dS ratios based on habitat, there may not be strongly different selection regimes related to metabolic costs between FW and marine fish.

TRANSITION DIRECTION AND THE SPECIATION-MOLECULAR EVOLUTION LINK

Our primary analyses considered habitat occupancy; however, the act of transitioning may additionally influence the rates of molecular evolution (e.g., McMahon et al. 2011). The comparisons included in this study reflect the reality of the FW environment being more often the newly colonized environment in SAL–FW shifts. The trends of higher FW molecular rates are consistent with the hypothesis of a link between transition direction and molecular rates. However, the sample size for the reverse direction transition (FW to SAL) was small, and no significant trends based on transition direction were detected. Further work investigating the distinction between rates associated with a trait versus trait shift direction would improve our predictive power for molecular patterns across the tree of life.

The results for bony fish do not seem likely due to the speciation-molecular rate hypothesis. Our obtained nuclear relative rates from Bloom et al. (2013) were higher in the marine environment, but they observed a greater speciation rate for FW than marine fish. Additionally, we observed greater molecular rates in FW pufferfish, whereas Santini et al. (2013) demonstrated higher speciation rate in marine lineages. However, this remains an area for further investigation using a larger sample size, particularly given that such a link has been demonstrated in several other

taxonomic groups, such as plants (Barraclough and Savolainen 2001).

LACK OF CONSISTENCY AMONG MOLECULAR MEASURES AND GENE TYPES SUGGESTS MULTIPLE INFLUENCES

The results do not support a single strong genome-wide influence as the cause of the observed habitat-linked trend in protein-coding genes; the protein-coding gene trends were present more strongly when rates were estimated across all sites than when dN or dS estimates were examined separately; also, nuclear and mitochondrial protein-coding relative rates were not found to be concordant in direction. Rather, there may be multiple causes of habitat-linked differences in overall rates, likely varying among taxa. We suggest a possible combination of general influences in vertebrates or bony fish that match some of the nuances in the protein-coding results, but may be present weakly or only for certain taxa: (1) lower effective population size in FW, leading to higher dN rates or dN/dS ratios genome wide (note: expected to be greater effect for mtDNA than nDNA. This difference was not observed, but matches with expectation #3); (2) higher metabolic rate in FW producing greater mutation rate, leading to mainly higher dS rates genome-wide (note: possible greater effect for mtDNA, which was consistent with results); (3) if higher metabolic rate is present in FW, then this may also lead to tighter selective constraints specifically on mtDNA, thereby reducing the dN or dN/dS ratios of mitochondrial genes in FW taxa; and (4) possible positive selection in some cases, for example, in nuclear genes related to immune response. These potential influences, excepting gene-specific positive or relaxed selection, however, do not explain the lack of trend in noncoding genes. Given the complexity of the results overall—relative rates varying among genes and the subtle habitat-linked trend in some gene types and taxa—more detailed investigation of select taxa is needed to determine the genetic consequences of these aquatic habitat transitions.

A COMPARISON OF MARINE PATTERNS VERSUS CONTINENTAL SALINE LAKES

Prior studies reported strongly higher molecular rates in continental SAL versus FW branchiopod crustaceans, in contrast to our findings including the marine realm. This difference may arise due to differences in salinity, UV exposure, environmental variability, or possibly due to unique biological features of branchiopod crustaceans. The conductivities of the inland SAL environments in previous studies (Hebert et al. 2002; Colbourne et al. 2006) ranged broadly (from 20,000 to 100,000 uS/cm), whereas sea water has a conductivity nearer the lower end of this range (45,000 uS/cm). On the other hand, parallels do exist between our study of mainly marine SAL taxa and previous studies of inland SAL taxa. In both cases, the higher rates are observed in the more recently colonized environment, and the new environment is also postulated to be more variable or harsh than the ancestral environment. Thus, if environmental variability or transition direction play a role in influencing rates of molecular evolution, such a mechanism would harmonize the findings of previous studies with the results from our study.

Conclusions

Through a comparative overview, we have demonstrated that the SAL and FW environments have generally equal relative rates of molecular evolution across a range of taxa, with some exceptions of faster rates in FW for protein-coding genes. Some of the causes of the previously observed patterns of higher rates in inland SAL lakes versus FW may be common to inland FW versus marine waters as well. This study contributes to a growing body of knowledge of the environmental and biological correlates of molecular rate variability.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1.1. 150 comparisons with genetic data analyzed.
Table S1.2. Summary of individual gene relative rates for overall substitution rates (OSRs) analysis.
Table S1.3. Summary of dN/dS ratios for individual genes.
Table S2.1. All molecular rates including overall substitution relative rates, dN/dS ratios, dN and dS rates, habitat information, genetic data sources, postulated ancestral habitat states, genes included in each comparison, and all rates summarized by gene category.
Table S2.2. Testing subsets of data: Comparing protein-coding versus noncoding relative rates, excluding near-equal summarized overall substitution rates, and data points included in Wilcoxon signed-rank tests.
Table S2.3. Testing direction of habitat transition versus relative rates.
Table S2.4. Testing concordances in relative rates between mitochondrial versus nuclear protein-coding genes.
Figure S1. Distribution of 402 pairs of OSR relative rate ratios (difference from 1:1 ratio between sister lineages shown), for all sister pairs for which both lineages had branch lengths greater than zero.
Figure S2. Relative dN/dS ratios, dN rates, and dS rates across 71 comparisons, including branch lengths (calculated from the dN and dS rates).