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FUNCTIONAL AND POPULATION GENOMIC DIVERGENCE WITHIN AND BETWEEN TWO SPECIES OF KILLIFISH ADAPTED TO DIFFERENT OSMOTIC NICHES

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Adaptation to salinity affects species distributions, promotes speciation, and guides many evolutionary patterns in fishes. To uncover the basis of a complex trait like osmoregulation, genome-level analyses are sensible. We combine population genomic scans with genome expression profiling to discover candidate genes and pathways associated with divergence between osmotic environments. We compared transcriptome sequence divergence between multiple freshwater and saltwater populations of the rainwater killifish, *Lucania parva*. We also compared sequence divergence between *L. parva* and its sister species, *Lucania goodei*, a freshwater specialist. We found highly differentiated single nucleotide polymorphisms (SNPs) between freshwater and saltwater *L. parva* populations in cell junction and ion transport genes, including V-type H^+ ATPase. Between species, we found divergence in reproduction and osmotic stress genes. Genes that were differentially expressed between species during osmotic acclimation included genes involved in ion transport and cell volume regulation. Gene sets that were divergent in coding sequence and divergent in expression did not overlap, although they did converge in function. Like many studies using genomic scans, our approach may miss some loci that contribute to adaptation but have complicated patterns of allelic variation. Our study suggests that gene expression and coding sequence may evolve independently as populations adapt to a complex physiological challenge.

KEY WORDS: F_{st}, genome scan, ion regulation, population divergence, osmoregulation.

Different aquatic taxa are adapted to different osmotic environments, and salinity is a key factor in limiting species ranges and defining species distributions (Gunter 1945, 1950a,b; Berra 1981; Lee 1999; Lee and Bell 1999; Smith and Bermingham 2005). Aquatic habitats vary in chemical composition from freshwater with few dissolved ions to marine environments with salinities over 32 parts per thousand (Bone and Moore 2008). Fish occupy the full range of aquatic habitats, actively maintaining ions and osmolarity at certain levels to ensure proper cell and enzyme function. In hypo-osmotic (low salinity) environments, the challenge is to retain vital ions and remove excess water. Conversely, in hyperosmotic (high salinity) environments, fish need to retain water and excrete excess ions. Consequently, many species specialize in either fresh water or salt water and entire clades can be limited to one osmotic environment (Berra 1981; Nelson 2006). Within families of fish that occupy both freshwater and marine habitats, closely related species often differ in salt tolerance (Berra 1981; Dunson and Travis 1991; McKinnon and Rundle 2002; Hrbek and Meyer 2003; Huyse et al. 2004; Whitehead 2010). Hence, local adaptation of physiological traits to salinity is fundamentally important in determining niche breadth, promoting speciation, and guiding broad evolutionary patterns in fishes.

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Adaptation to salinity involves a range of organs, cell types, and enzymes, and identifying the targets of selection has proven to be a difficult task (reviewed in Perry 1997; Evans et al. 2005; Hwang and Lee 2007; Evans 2008). Most ion and osmoregulation (hereafter referred to collectively as "osmoregulation") occurs primarily in the gills with secondary help from the intestines and kidneys (Karnaky 1998; McDonald and Grosell 2006). Osmoregulation at different salinities involves changes in various ion channels and pumps (e.g., Na^+/K^+ ATPase: Perry 1997; Sakamoto et al. 2001; Marshall 2002; Scott et al. 2004), aquaporin water channels (Cutler and Cramb 2001; Hirose et al. 2003), and cell–cell junctions (Wilson and Laurent 2002). Because life in different osmotic environments requires complex alterations to physiology and life history, a top-down, discovery-based genomics approach can help identify genetic and biochemical mechanisms that underpin alternate physiologies (Barrett and Hoekstra 2011). High-throughput sequencing and gene expression studies (such as microarrays and RNA-seq) can be used to discover genes previously unaffiliated with osmoregulation and examine the relative contributions of gene expression versus coding sequence divergence in osmoregulatory evolution.

Killifish in the Fundulidae family are a model system for studying adaptation to alternate osmotic environments. Within this family of fish, many closely related species differ in both osmotic niche and physiology (Griffith 1974; Burnett et al. 2007; Whitehead 2010). In addition, osmotic environments play a role in speciation in this family by contributing to hybrid incompatibilities between species (Fuller 2008a; Kozak et al. 2012). We focus on the genus *Lucania*, which contains three closely related species (*Lucania goodei*, *Lucania parva*, and *Lucania interioris*). *Lucania* occurs phylogenetically within the genus *Fundulus* (see nuclear DNA tree in Whitehead 2010) and is closely related to *Fundulus heteroclitus*, which has a number of genomic tools including extensive EST libraries (Burnett et al. 2007) and a reference genome (www.fundulus.org).

Lucania goodei and *L. parva* co-occur and differ mainly in salinity tolerance (Table 1 and Fig. 1a). *Lucania goodei* is found almost exclusively in fresh water in Florida. *Lucania parva* is distributed throughout the southern Atlantic and Gulf Coast, where it can be found in both fresh water and salt water (Lee et al. 1981). Salinity affects multiple fitness components in *L. goodei*. As salinity increases, *L. goodei* hatching rates, growth, and survival decline (Table 1). At salinities above 15 parts per thousand (ppt), eggs and larvae have low survival (Fig. 1b), and adults produce fewer eggs (Berdan and Fuller 2012b). In contrast, hatching rates and egg/larval survival in *L. parva* remain relatively high at all salinities (Table 1 and Fig. 1b; Fuller 2008b). Previous work using qRT-PCR found divergence in gene expression of several key saltwater osmoregulatory genes between *L. goodei* and *L. parva* (Berdan and Fuller 2012a).

Table 1. Effects of salinity on distribution, survival at various life stages, and competitive interactions in *Lucania parva* and *Lucania goodei*.

	L. parva	*L. goodei*
Distribution of populations in Florida[1]	23% fresh; 46% brackish; 31% marine	93% fresh; 7% brackish
Egg hatching in dilute fungicide (methylene blue)[2–4]	High 0–40 ppt	High 0–10 ppt Low 15–40 ppt
Larval survival to eating stage[2–4]	High 0–40 ppt	High 0–10 ppt Low 15–40 ppt
Overwinter survival to adulthood[2]	Low in freshwater	Highest in freshwater
Maximum adult salinity tolerance[5,6]	> 80 ppt	20 ppt
Competitive interactions[6]	Growth suppressed in the presence of *L. goodei* in 0 ppt, but enhanced in 15 ppt	Growth suppressed in the presence of *L. parva* in 15 ppt, but enhanced in 0 ppt

[1]Fuller and Noa (2008).
[2]Fuller et al. (2007).
[3]Fuller (2008a).
[4]Fuller (2008b).
[5]Whitehead (2010).
[6]Dunson and Travis (1991).

The same osmotic challenges that have led to the divergence between *L. goodei* and *L. parva* appear to be operating on a microevolutionary scale within *L. parva*. Museum records indicate that *L. parva* has many stable populations in fresh water and salt water (Fuller and Noa 2008). Freshwater *L. parva* populations occur in unconnected, geographically distant drainages, suggesting that these populations are independently derived from brackish and saltwater populations (Fuller and Noa 2008). Preliminary evidence suggests that *L. parva* populations have adapted to fresh water: egg/larval survival in fresh water is higher in freshwater populations than in saltwater populations (Fig. 1c). Thus, we can compare genome-wide sequence divergence between freshwater and saltwater populations within *L. parva* to divergence that has occurred between *Lucania* species.

We investigated the molecular genetic mechanisms underlying salinity adaptation in *Lucania*. We measured both gene expression and coding sequence divergence as a function of salinity because adaptive variation is likely to be underpinned by

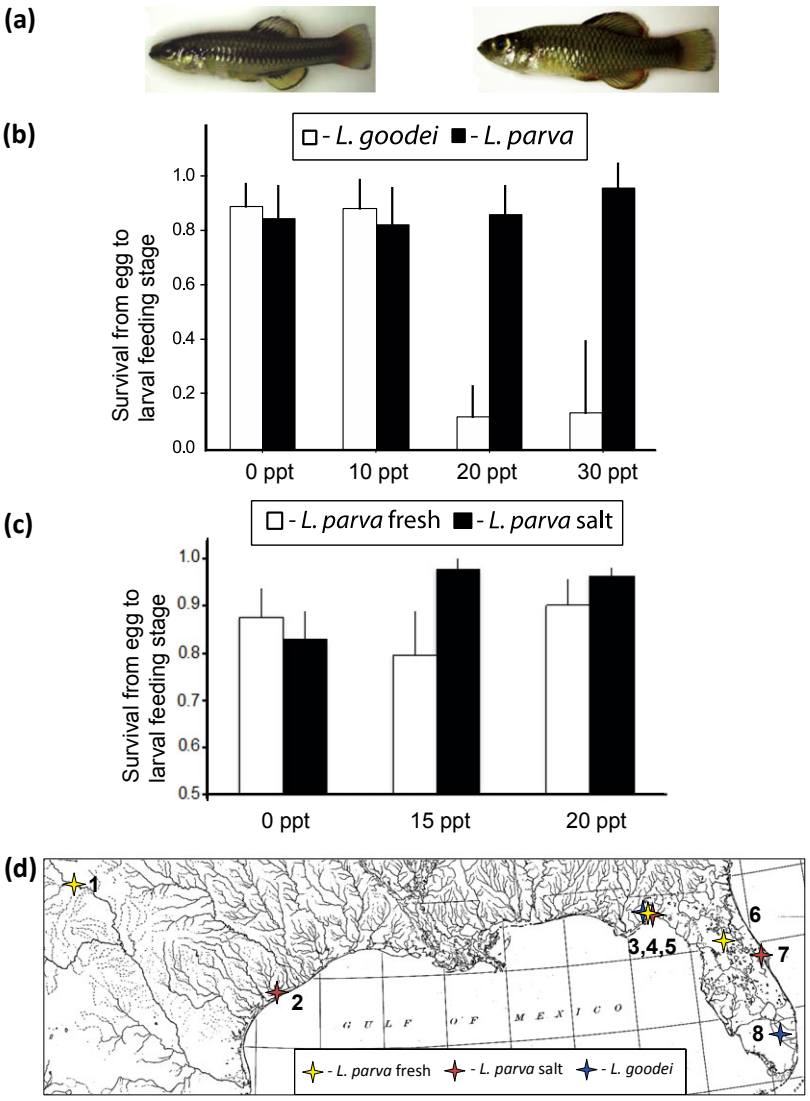

Figure 1. *Lucania* **study system. (a)** ***Lucania goodei*** **male (left) compared to** ***Lucania parva*** **male (right); (b)** ***L. goodei*** **survival (open columns) from egg to larval feeding is reduced at high salinity (20–30 ppt),** ***L. parva*** **survival (filled columns) is not (salinity-by-species interaction: $F_{3,27} = 27.79$, $P < 0.0001$; data from Fuller (2008b);** ***L. parva*** **from brackish population); (c) freshwater** ***L. parva*** **survival (open columns) is increased at low salinity and reduced at high salinity compared to saltwater** ***L. parva*** **(filled columns) from Florida Gulf populations (salinity-by-population interaction: $F_{3,21} = 5.07$, $P = 0.035$; data reanalyzed from Fuller et al. 2007); (d)** ***Lucania*** **populations used in this study, denoted by stars:** ***L. goodei*** **(blue), freshwater** ***L. parva*** **(yellow), saltwater** ***L. parva*** **(red); populations labeled by number: 1 = Pecos River (Pecos-Crockett County border, Texas); 2 = Bolivar Peninsula (Galveston County, Texas); 3 = Upper Bridge (Wakulla River, Wakulla County, Florida); 4 = Lower Bridge (Wakulla River, Wakulla County, Florida); 5 = St. Mark's (St. Mark's National Wildlife Refuge, Wakulla County, Florida); 6 = Delk's Bluff (Oklawaha River, Marion County, Florida); 7 = Indian River Lagoon (Brevard County, Florida); 8 = 26-Mile Bend, Everglades (Broward County, Florida).**

both regulatory variation and structural protein variation (Carroll 2005; Hoekstra and Coyne 2007). Physiological divergence between *L. goodei* and *L. parva* is greatest at high salinities (Fuller et al. 2007; Berdan and Fuller 2012a) and we used genome expression profiling in response to a hyperosmotic challenge to contrast functional responses between species. We identified genes that showed a common transcriptional response during acclimation to osmotic challenge in both species and genes that showed a species-dependent response. As a complement to comparative genome expression profiling, we sequenced the transcriptome of both *Lucania* species to identify regions of the genome that have diverged between different osmotic environments and may harbor targets of natural selection. We compared sequence divergence between multiple freshwater and saltwater populations within *L. parva* as well as between *L. parva* and *L. goodei*. Quantifying genome-wide sequence variation allowed us to identify potential candidate genes involved in adaptation to divergent osmotic environments and determine if consistent genomic changes

occur across micro- and macroevolutionary time scales. We asked whether candidate genes that varied in their response to osmotic challenge were the same as those identified from genomic scans. In this way, we could determine whether gene sets that are differentially expressed between species during acclimation to salinity also have heightened sequence divergence due to natural selection in different osmotic environments.

Methods

SOURCE POPULATIONS

We sampled adult *L. goodei* and *L. parva* from populations in Florida and Texas (see Fig. 1d for locations). For our transcriptome sequencing, we sampled two freshwater *L. goodei* populations: Upper Bridge (Wakulla River, Florida) and Everglades (26-Mile Bend, Everglades, Florida). We sampled six *L. parva* populations collected in pairs from three different drainages. In each drainage pair, one population was from the saltwater/estuarine terminus of the drainage and the second was from an upstream freshwater habitat. Our freshwater–saltwater pairs were Florida Atlantic Coast (Delk's Bluff, Oklawaha River—Indian River Lagoon, Atlantic Ocean; no. 6–7; Fig. 1d), Florida Gulf Coast (Lower Bridge, Wakulla River–St. Mark's, Gulf of Mexico; no. 4–5), and Texas Gulf Coast (Pecos River-Bolivar Peninsula, Galveston Bay; no. 1–2). For the expression profiling, adult *L. goodei* and *L. parva* were both collected from Lower Bridge (Wakulla River, Florida).

COMPARATIVE GENOME EXPRESSION PROFILING DURING ACCLIMATION TO OSMOTIC CHALLENGE

Lucania goodei and *L. parva* were acclimated for 2 months in fresh water (salinity = 0.2 ppt) in a common recirculating system equipped with biological, mechanical, and ultraviolet filtration at Louisiana State University. For the hyperosmotic challenge, fish were transferred to brackish water (15 ppt) in a recirculating aquarium system at a density of one fish per gallon. Brackish water was created using Instant Ocean sea salt (Spectrum Brands, Atlanta, GA). Light cycles were kept at 12L:12D, and water temperature was maintained at 22°C. Nitrogenous wastes were monitored once per day using commercial colorimetric test kits (API; Mars Fishcare Inc., Chalfont, PA). Whole gills were sampled pretransfer, and 6-, 24-, 72-, and 240-hour posttransfer. All tissues were stored in RNAlater (Ambion, Austin, TX) at −20°C. Tissues were dissected immediately following decapitation of experimental animals. Five biological replicates were included per treatment.

Gills were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA), and RNA was isolated and purified using spin columns. The MessageAmpII aRNA amplification kit (Ambion) was used to prepare antisense RNA (aRNA). Quality of total RNA and aRNA was assessed using microfluidic electrophoresis (Experion, Bio-Rad Laboratories, Hercules, CA). High-quality aRNA was coupled to Alexa Fluor dyes (Alexa Fluor 555 and 647; Molecular Probes, Eugene, OR) and competitively hybridized to custom oligonucleotide microarrays (Agilent Technologies; Agilent eArray design ID 033450) according to the manufacturer's instructions in two species-specific loops. Microarray probe oligonucleotides were designed from 6800 EST sequences from the congener *F. heteroclitus* (Whitehead et al. 2011; Whitehead et al. 2012). Each slide was incubated at 60°C for 18 hours, washed, and scanned using a Packard BioScience Scanarray Express (Perkin-Elmer, Waltham, MA). Each individual RNA sample was hybridized twice—once labeled with each of the two dyes. All data were collected from images using the program ImaGene (BioDiscovery Inc., El Segundo, CA).

Data were normalized using lowess and mixed models where "dye" and "array" were treated as fixed and random effects, respectively, then $\log_2$ transformed. Genes for which expression was too high (scanner PMT saturated) or too low (<2 standard deviations below background fluorescence) were filtered from further analysis. Normalized filtered data were analyzed by mixed model analysis of variance in JMP Genomics (SAS Institute, Cary, NC). The main effects were specified as "species" and "time," "dye" was specified as a fixed effect, and "array" and five replicate individuals within each treatment were specified as random effects. Because the microarray was designed for the congener *F. heteroclitus*, the main effect of species could be confounded by nucleotide substitutions leading to differential binding to probes and perceived differences in absolute levels of gene expression. Therefore, we focused on "time"-by-"species" interactions ($P < 0.05$) that reveal species differences in how expression changes during acclimation and are not confounded by substitutions. Genetic distance from *F. heteroclitus* is similar between *L. parva* and *L. goodei* (Whitehead 2010) and we expected sequence divergence between microarray probes and transcripts to be uniform between the two *Lucania* species. We found equivalent numbers of probes expressed above background fluorescence in *L. parva* (4008 probes) and *L. goodei* (4007 probes), with 3964 probes above background in both species. Gene ontology enrichment analysis was performed using DAVID Bioinformatics Resources (Huang et al. 2009).

LUCANIA TRANSCRIPTOME

For each population, we extracted RNA separately from 10 individuals (five males and five females) euthanized in an overdose of MS222. For two *L. goodei* populations and two of the *L. parva* populations (Pecos River and Indian River), tissue samples were taken from the gills (1–2 arches), dorsal fins, eyes, brain, and the gonads (ovaries or testes). For the remaining *L. parva* populations, tissue samples were taken only from the gills and gonads. In some

cases, *L. parva* tissues were obtained several months prior to RNA extraction and were stored at −80°C in RNAlater or 99% ethanol. RNA was extracted from ground tissue using β-mercaptoethanol (Carleton 2011) and QiaShredder columns (Qiagen, Valencia, CA), purified using RNeasy mini kits (Qiagen), and treated with Turbo DNase (Invitrogen, Grand Island, NY).

A pooled sample was created for each population containing equal amounts of RNA from all 10 individuals (concentrations determined via Nanodrop spectrophotometer, Thermo Fisher Scientific, Waltham, MA). The Keck Center for Comparative and Functional Genomics at the University of Illinois constructed cDNA libraries and performed 100 bp paired-end sequencing (see Supporting Information). Size-selected libraries (250–500 bp) were diluted to 10 nM, and fragments were sequenced from both ends on an Illumina HiSeq2000 using the TruSeq SBS sequencing kit version 2 (Illumina, San Diego, CA). Each population was uniquely barcoded, and up to two populations were run per lane (five lanes total). Fastq files were generated with Casava version 1.8 (Illumina). Average quality scores were above 20 for all cycles.

DE NOVO TRANSCRIPTOME ASSEMBLY

Illumina sequences were trimmed (removing the first and last 25 bp) using the FastX trimmer then assembled de novo using Velvet (Zerbino and Birney 2008). We created an assembly from all *L. parva* sequences using the paired-end option, an insert size of 250 bp, and a variety of kmer values (see Supporting Information). We found the best assembly under kmer = 25, which generated 111,628 contigs over 100 bp with an N50 = 1303 bp. We assembled the two *L. goodei* populations using the same pipeline (kmer = 25, 106,004 contigs, N50 = 618 bp). We found that 93% of the *L. goodei* contigs were represented in the *L. parva* assembly. However, 51% of the *L. parva* contigs were not represented in the *L. goodei* assembly, likely due to differences in sequencing depth between the species (one vs. four lanes). Hence, we created our reference based on the *L. parva* assembly with a total of 24,781 contigs, combining 1657 contigs from the *L. goodei* assembly (all unique contigs over 200 bp) with *L. parva* contigs that had blast matches to any of the following: (1) related teleost proteomes (Atlantic killifish: *F. heteroclitus*, Japanese medaka: *Oryzia latipes*, three-spined stickleback: *Gasterosteus aculeatus*), (2) *Fundulus* ESTs on the microarray, or (3) target genes of interest (osmoregulation and vision-related genes). We used Galaxy (Giardine et al. 2005) to filter results from blastX searches against the uniprot proteomes and retain only contigs that had only 1–2 protein matches per species (blast score ≥ 100) to avoid inclusion of contigs that were misassembled from multiple genes. Because the teleost proteomes are poorly annotated, annotations for reference contigs were obtained from the human uniprot database (blastX, blast score ≥ 100).

SNP DETECTION AND FST CALCULATION

Each population was aligned separately to our reference using BWA and its paired-end function (Li and Durbin 2009). Alignment files were processed with samtools (Li et al. 2009). For single nucleotide polymorphism (SNP) detection and F_{st} calculation, we used Popoolation2, which is designed to calculate population genetic metrics from pooled samples (Kofler et al. 2011). We calculated allele frequencies and F_{st} between populations, using a minimum coverage of 8 reads per population (to exclude SNPs from transcripts expressed at low frequency), a pool size of 10, and a sliding window of 50 bp. The mean number of reads per population for contigs with SNPs was 34.47 (range: 27.33–50.21).

This is a preliminary discovery-based approach to generate candidate SNPs, and some limitations and caveats are associated with our transcriptome data. One limitation is that the results of our F_{st} outlier analysis only apply to genes expressed in the gills, ovaries, and testes. These were the only tissues that were sampled in all populations (we sampled more tissues in a subset of populations for another project). A second caveat is that we used pooled cDNA, so variation in expression among individuals in a pooled sample may bias the estimated allele frequencies within each population. Similarly, allele-specific expression differences may bias allele frequencies within populations (Knight 2004; Palacios et al. 2009). However, we expect this bias to affect all of our populations equally. Therefore, our calculations of allele frequency *differences* between multiple population pairs should be robust to these expression differences. Previous work has verified that population differences in allele frequencies calculated in pooled genomic DNA match those calculated from individually barcoded samples (Zhu et al. 2012). However, this may not apply to pooled cDNA. To validate allele frequencies from our pooled analysis, we genotyped individuals from each population at 4545 SNPs using an Illumina Infinium Bead Chip custom designed for *Lucania* (containing 1679 candidate SNPs segregating between species, 1497 SNPs segregating between Everglades and Upper Bridge *L. goodei*, 1369 SNPs segregating between Florida Atlantic saltwater and Texas Gulf freshwater *L. parva*). Additional details of bead design and genotyping can be found in Supporting Information. We genotyped 33 *L. goodei* (16 Upper Bridge, 17 Everglades) and 39 *L. parva* (Florida Atlantic = 5 fresh, 6 salt; Florida Gulf = 8 fresh, 7 salt; Texas Gulf = 5 fresh, 8 salt) using genomic DNA extracted from caudal fin tissue (preserved in ethanol) following the Gentra PureGene protocol (Qiagen).

SPECIES AND POPULATION-SPECIFIC OUTLIER ANALYSES

To look at divergence across osmotic boundaries over different timescales, we compared the results of F_{st} outlier analyses between *L. goodei* and *L. parva* (macroevolutionary scale) to those between freshwater and saltwater *L. parva* population pairs

(microevolutionary scale). At a macroevolutionary scale, we calculated the average F_{st} between species using each *L. goodei* and each *L. parva* population (12 pairwise comparisons; a total of 20 *L. goodei* individuals and 60 *L. parva*). We used the average F_{st} rather than pooling all *L. goodei* and *L. parva* populations to control for variation in the numbers of reads per population. This also alleviated the problem of using pooled samples because outliers had to be fixed between both *L. goodei* populations and all six *L. parva* populations.

At a microevolutionary scale, we identified F_{st} outlier loci in two sets of contrasts. First, we conducted an outlier analysis between geographically distant populations that did not differ in salinity to identify loci that evolve quickly between population pairs, but are not relevant to osmoregulatory evolution. In *L. goodei*, we compared F_{st} in sliding SNP windows between Upper Bridge and Everglades populations (both freshwater). For *L. parva*, we calculated F_{st} between the most geographically distant saltwater populations (Texas Gulf salt and Florida Atlantic salt), because saltwater is the putative ancestral *L. parva* environment. Second, we compared freshwater–saltwater *L. parva* populations within drainages for each of the three pairs: Florida Atlantic, Florida Gulf, and Texas Gulf. Outliers that overlapped with those from the geographically distant saltwater *L. parva* comparison were excluded from consideration as osmotic adaptation candidates. Thus, F_{st} outliers between freshwater and saltwater pairs should capture adaptive variation across osmotic boundaries. We focused on SNPs that were outliers in two or more freshwater–saltwater pairs to detect loci that repeatedly evolve in different osmotic environments. This also helped mitigate problems associated with pooled samples because SNPs had to appear as outliers in multiple comparisons. To determine if adaptation to salinity at micro- and macroevolutionary scales involve the same core set of genes, we compared overlap between freshwater and saltwater *L. parva* outlier loci and outlier loci between species.

We also performed F_{st} estimation using Infinium bead genotype data. Because we had individual genotypes, we used the hierfstat package in R to calculate F_{st} at all possible SNPs with segregating alleles for each species/population comparison (Goudet 2005). SNP bead probe source sequences were aligned with our transcriptome reference using blastn. Genomic SNPs were then paired with the correct 50 bp window from the pooled analysis and we compared F_{st} values calculated on individual genotypes to the pooled data. Using the Infinium genotype data, we conducted two GO enrichment analyses on outlier loci: (1) between species (33 *L. goodei*, 39 *L. parva*) and (2) between all freshwater and all saltwater *L. parva* (18 freshwater, 21 saltwater individuals).

We identified F_{st} outliers based on empirical distributions of divergent loci (Beaumont and Balding 2004; Narum and Hess 2011). This approach has been used to identify putative targets of selection in *Drosophila* (Kolaczkowski et al. 2011), humans (Akey et al. 2002), *Arabidopsis* (Turner et al. 2010), and dogs (Akey et al. 2010). This empirical approach is particularly useful for species for which demographic parameters cannot be estimated and make simulation-based F_{st} outlier approaches difficult (as is the case for *Lucania* species). However, this empirical approach does reduce representation of adaptive variation from standing variation or from recessive variation (Teshima et al. 2006).

Our empirical outlier cutoff value for all analyses was defined as loci in the top 5% of the F_{st} distribution for each population/species comparison (Akey et al. 2002; Kolaczkowski et al. 2011; Flight and Rand 2012). The top 5% of all F_{st} values were identified using the quantile function in R version 2.15 (R Core Development Team 2010). We created an annotated universal gene list from the human uniprot blast matches for all contigs with SNPs. We performed GO enrichment analysis in R using the Bioconductor GOStats package, conditional correction, and a *P*-value cutoff of 0.05 (Falcon and Gentleman 2007). We performed enrichment analysis separately on different population/species comparisons.

Results

COMPARATIVE GENOME EXPRESSION DURING ACCLIMATION TO OSMOTIC CHALLENGE

After quality control filtering, 4051 genes were included in the statistical analysis of genome expression, where 3964 genes were expressed above background in both species (Table S1). A large gene set (429 genes) showed a conserved response between species during the acclimation time-course following osmotic challenge (Fig. S1). However, 310 genes (7.8%) had species-specific response patterns to the hyperosmotic challenge (significant species-by-time interaction). Coregulated genes were clustered into six sets with different patterns of regulation variation between species (Fig. 2). Sets *a* and *b* include genes that were up- or downregulated in the same direction in both species, but to a different degree. Sets *c* and *d* include genes that were up- and downregulated during acclimation only in *L. goodei*, and sets *e* and *f* include genes that were up- and downregulated only in *L. parva*. The genes sets that were expressed primarily in *L. goodei* (*c* and *d*) were not enriched for any gene ontology functions. However, genes that were selectively expressed in *L. parva* in response to osmotic challenge were enriched for the GO category "ion transport," including subunits of the Na^+/K^+ ATPase (NKA: downregulated in *L. parva*). Divergent expression between species during the acclimation time-course also occurred in: serine/threonine protein kinase (SGK1: upregulated in *L. parva*) which is involved in the regulation of diverse ion channels and pumps (including NKA: Lang et al. 2010), malcavernin (CMM2: transiently downregulated in *L. goodei*) which is involved in hyperosmotic challenge-induced p38 MAPK activation (Fong et al. 2007), osmotic

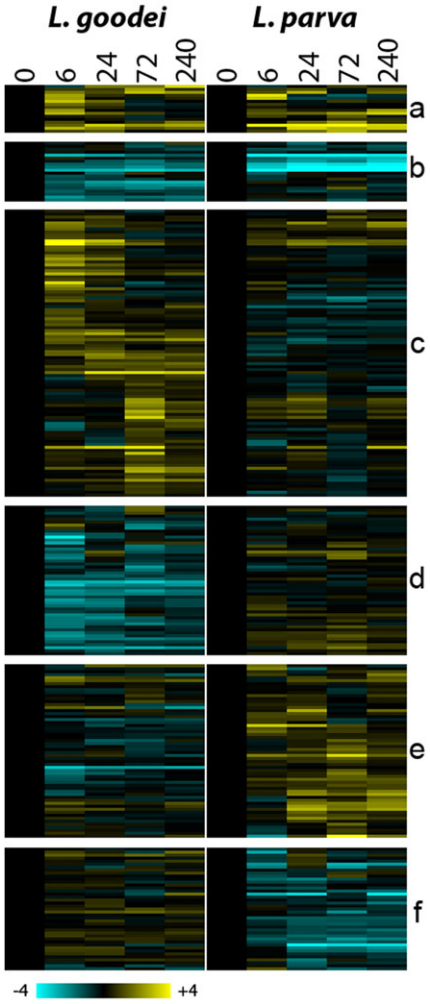

Figure 2. Expression patterns for genes with species-specific transcriptional responses during the time-course of acclimation to hyperosmotic challenge (significant species-by-time-course interaction) for *Lucania goodei* and *Lucania parva*. Columns are treatment means for pre-transfer (time 0) and each time-point post-transfer (6 hours, 24 hours, 72 hours, and 240 hours). Rows represent individual genes. Cell color indicates fold level of up- or downregulation (yellow or blue, respectively) relative to the pretransfer control. Genes are organized into sets with similar differences between species. Sets (a) and (b) include genes that are expressed in the same direction (up or down) between species, but that differ in degree of up- or downregulation, or in time-course pattern, between species. Sets (c) and (d) include genes that are preferentially up- or downregulated in *L. goodei*. Sets (e) and (f) include genes that are preferentially up- or downregulated in *L. parva*.

stress transcription factor (OSTF1: transiently upregulated in *L. goodei*) which is a known component of the osmotic response in fishes (Fiol and Kultz 2005), inositol monophosphatase (IMPA1) which is involved in the synthesis of the osmolyte inositol (Michell 2008), and aquaporins (AQP1 and AQP3: both downregulated in *L. parva*) which are involved in osmoreception and cell volume regulation during osmotic acclimation (Watanabe et al. 2005, 2009; Chen et al. 2011).

TRANSCRIPTOME DIVERGENCE BETWEEN LUCANIA SPECIES

We identified 215,704 SNPs that were segregating among our eight populations. F_{st} was calculated over 48,129 intervals (50 bp in length) with at least one segregating SNP from 8365 contigs. These 8356 contigs mapped to 4454 genes with functional annotations. Distribution and average F_{st} values for population/species pairs are shown in Table 2 and Figure 3.

Between species, 2760 of the 50 bp windows had one or more SNPs that were completely fixed and identified as outliers between species (top 5% of F_{st} distribution: average $F_{st} = 1$). These outlier SNPs were in 1656 contigs that mapped to 1339 genes with GO annotations. We found that these outliers had significant GO enrichment in 130 biological processes, 7 cellular components, and 23 molecular functions (Table S2). A number of these potential genes were involved in regulation of Ras GTPase activity (40 genes), a key component of signal transduction. Signal transduction was significantly enriched (three biological processes, three molecular functions), including "receptor signaling protein serine/threonine kinase activity," which may be involved in ion transport regulation (Lang et al. 2010). Twenty-nine of our outliers were potential genes involved in sexual reproduction and gamete development (four enriched biological processes). In particular, we found outlier SNPs in the estrogen receptor and several spermatogenesis genes (Table 3). We also found that water transport and the glucocorticoid receptor signaling pathway were significantly enriched biological processes. The glucocorticoid receptor contained two outlier SNPs. This receptor mediates cortisol-induced changes in expression of ion transport genes (Kiilerich et al. 2007). An outlier SNP was also present in the gene for aldehyde dehydrogenase family 7 protein, which is involved in hyperosmotic stress (Brocker et al. 2010).

DIVERGENCE BETWEEN GEOGRAPHICALLY DISTANT POPULATIONS

To determine which loci evolve quickly between populations due to processes other than adaptation to salinity, we compared divergence between our two most geographically distant populations in each species. Between our two *L. goodei* populations (Fig. 3b), we identified 2402 outlier SNP windows ($F_{st} \geq 0.421$). We found 73 enriched biological processes, 13 cellular components, and 16 molecular functions (Table S3). We only found enrichment of two potential osmoregulatory molecular functions (coupled ATPase activity, protein serine/threonine kinase activity) and one reproductive biological process (C21-steroid hormone metabolic process). For *L. parva*, we identified outlier SNPs between our two most distant saltwater populations: Florida Atlantic salt and

Table 2. Genome-wide F_{st} estimated between all *Lucania* populations across all 48,129 SNP windows. Freshwater–saltwater *Lucania parva* drainage comparisons bolded.

	Upper Bridge *L. goodei*	Everglades *L. goodei*	Florida Atlantic *L. parva* Fresh	Florida Gulf *L. parva* Fresh	Texas Gulf *L. parva* Fresh	Florida Atlantic *L. parva* Salt	Florida Gulf *L. parva* Salt	Texas Gulf *L. parva* Salt
Upper Bridge *L. goodei*	X	0.09263	0.3924	0.3720	0.4481	0.4003	0.4143	0.3725
Everglades *L. goodei*		X	0.3951	0.3789	0.4513	0.4018	0.4198	0.3789
Florida Atlantic *L. parva* Fresh			X	0.1127	0.1748	**0.1285**	0.1720	0.1110
Florida Gulf *L. parva* Fresh				X	0.1629	0.1085	**0.1243**	0.0406
Texas Gulf *L. parva* Fresh					X	0.1401	0.2236	**0.1619**
Florida Atlantic *L. parva* Salt						X	0.1722	0.1057
Florida Gulf *L. parva* Salt							X	0.1245
Texas Gulf *L. parva* Salt								X

Texas Gulf salt (Fig. 3c). We identified 2405 outlier SNPs ($F_{st} \geq 0.441$) in 1632 contigs. GO enriched categories included immune function (eight enriched biological processes) and signal transduction (GTPase activity: Table S4). None of these enriched GO categories in the geographically distant *L. parva* comparison included any osmoregulatory functions. These saltwater–saltwater *L. parva* outlier contigs were removed from *L. parva* drainage comparisons to control for rapid evolution and genetic drift.

DIVERGENCE BETWEEN FRESHWATER AND SALTWATER *L. PARVA*

To identify candidate loci involved in adaptation to osmotic environments, we compared SNPs that were differentiated between freshwater and saltwater *L. parva* populations within a drainage. Outliers in each drainage were identified using a cutoff based on all three drainages ($F_{st} \geq 0.635$, top 5% of all three drainages). We had 1701 outlier SNP windows between the Florida Atlantic freshwater and saltwater populations (262 completely fixed SNPs, $F_{st} = 1$: Fig. 3d); 1400 outlier SNP windows (211 fixed) between the Florida Gulf populations (Fig. 3e); and 4122 outlier SNP windows (1017 fixed) between Texas Gulf populations (Fig. 3f). There were 5374 SNP windows unique to the freshwater–saltwater analysis (after removal of 1344 SNP windows also present among the saltwater–saltwater outliers). These contigs corresponded to 2114 annotated genes. Many of the GO-enriched categories were related to ion transport (Table S5). Enriched molecular functions included ATPase activity, sodium ion transmembrane transporter activity, solute:cation symporter activity, inorganic cation transmembrane transporter activity, and inorganic anion exchanger activity. Outlier ion transport genes are listed in Table 3.

We found 189 SNP windows that were shared outliers in at least two drainages (and which were not present in saltwater–saltwater outliers). These contigs corresponded to 163 annotated genes. Fourteen of these were outliers in all three drainages (Table 3), and 149 were shared in only two drainages (Fig. 4a). Of 19 enriched GO molecular functions, 7 were related to ion transport including ligand-gated channel activity, inorganic anion exchanger activity, cation channel activity, ion transmembrane transporter activity, and monovalent inorganic cation transmembrane transporter activity (Table S6). Four ion-related biological processes were also enriched. Cell junctions were an enriched cellular component and several tight junction proteins, including claudins, were among the outlier loci (Table 3).

Contigs that had outlier SNPs between freshwater and saltwater population pairs often contained a species-specific SNP (Fig. 4b). Sixty-four of the 163 genes (39.26%) that contained outliers in at least two freshwater–saltwater drainages contained at least one SNP window with high differentiation between species ($F_{st} = 1$). This number of shared outliers is significantly higher than the random expectation of shared genes (outliers in at least two drainages = 3.66% of the total annotated genes; species outliers = 30.06% of our total genes; shared random expectation = 1.11%; Fisher's exact test, $P < 0.0001$). These shared genes included electrogenic sodium bicarbonate cotransporter, V-type H^+ ATPase, insulin receptor, and several transmembrane proteins (Table S7). Among ion transport gene outliers in at least one freshwater–saltwater drainage, 61% also contained outlier SNPs between species (19 of 31; Table 3).

POOLED SEQUENCE DIVERGENCE VERSUS INDIVIDUAL SEQUENCE DIVERGENCE

We found similar levels of divergence between species when comparing *L. goodei* and *L. parva* individuals genotyped on Infinium bead chips (33 *L. goodei*, 39 *L. parva*). Average F_{st} across 3481 SNPs was 0.386 ± 0.006, similar to the mean F_{st} of 0.38 from the pooled analysis (*t*-test assuming unequal variance: $t_{4,881} = 0.33$, $P = 0.74$).

We found there was a significant positive correlation between F_{st} estimates between species in the pooled analysis and in the Infinium data (SNPs aligned within 50 bp, Pearson $r = 0.49$, $P < 0.0001$, $N = 299$; Fig. S2). F_{st} estimates from the pooled analysis

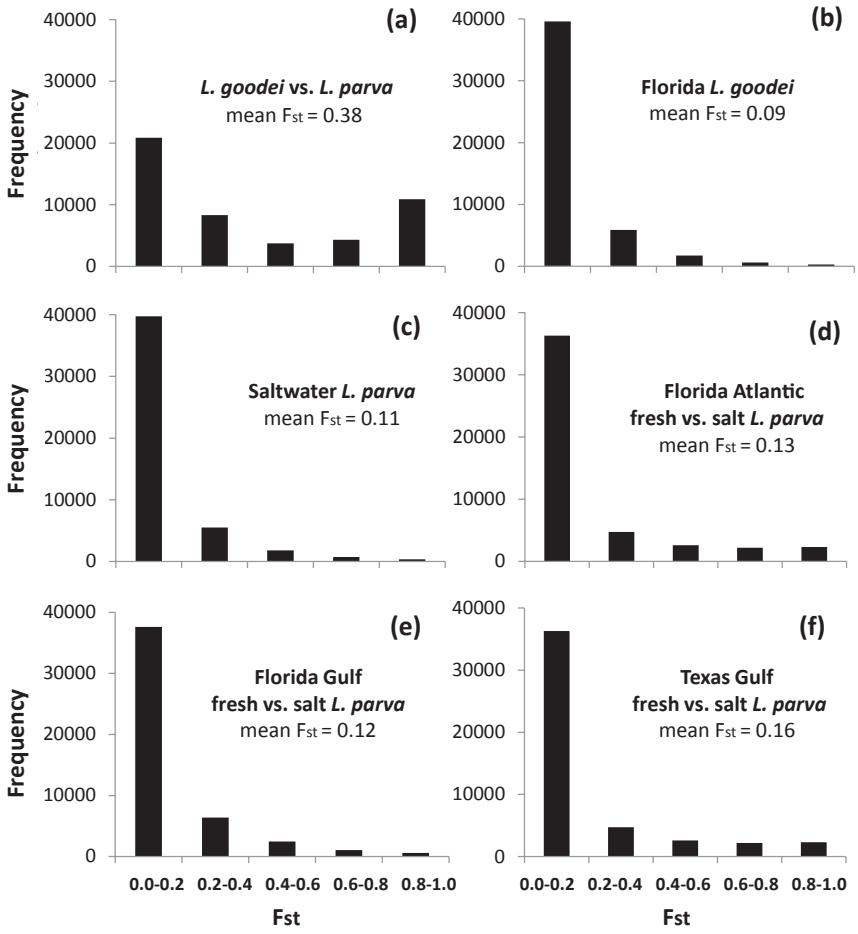

Figure 3. Distribution of F_{st} of SNP sliding windows. Histogram showing (a) average F_{st} between *Lucania goodei* and *Lucania parva* populations; (b) F_{st} between *L. goodei* populations (Upper Bridge and Everglades); (c) F_{st} between most distant saltwater populations (Florida Atlantic salt and Texas Gulf salt); (d) F_{st} between freshwater and saltwater *L. parva* from Florida Atlantic drainage; (e) F_{st} between freshwater and saltwater *L. parva* from Florida Gulf drainage; and (f) F_{st} between freshwater and saltwater *L. parva* from Texas Gulf drainage.

tended to be slightly higher than estimates from the individual genotype data, but this difference was not significant (pooled mean: $F_{st} = 0.48 \pm 0.02$; individual mean: $F_{st} = 0.45 \pm 0.02$; paired t-test: $t_{298} = 1.61$; $P = 0.11$). Among aligned SNPs, 19 were identified as outlier loci between species in the pooled analysis ($F_{st} = 1$) and 16 of these pooled outliers (84%) also showed high divergence in the genotype data: 14 had $F_{st} = 1$ and an additional two had $F_{st} > 0.97$. Thus, outlier loci showed high correspondence between analyses.

We performed a GO enrichment analysis on species outliers from the Infinium SNP data and compared this to the enrichment analysis using the pooled samples. For the Infinium data, there were 594 outliers between species ($F_{st} = 1$), 360 corresponding to annotated genes. Only 45 of these were shared with the pooled outlier analysis due to novel genes being present on the bead. Similar to the results of the GO enrichment analyses on the pooled data, Infinium outliers between species were significantly enriched for reproductive genes (such as those involved in spermatid development and ovulation). However, no osmoregulatory categories were enriched (Table S8).

Within species, we also found that mean F_{st} values at aligned SNPs were equivalent between pooled and individual data in all population comparisons (*L. goodei*: paired t-test $t_{146} = -0.69$, $P = 0.49$; Florida Atlantic: $t_{94} = -1.14$, $P = 0.26$; Texas Gulf: $t_{117} = -1.51$, $P = 0.13$), except the Florida Gulf (paired t-test $t_{94} = 5.55$, $P < 0.0001$; Fig. S2). Gulf populations were not used in bead design and it is likely that alternate alleles from these populations were not included on the bead.

We identified 103 F_{st} outliers (top 5%: $0.1683 \leq F_{st} \leq 0.36$) between freshwater and saltwater *L. parva* populations using the Infinium SNP data; 81 contigs corresponded to annotated genes. Ion transport and ATPase activity were enriched biological processes and molecular functions (Table S9). Outlier SNPs were found in transcripts potentially encoding V-type H^+ ATPase (subunits B, D, H), voltage-gated K^+ channel (subunit β), Na^+/K^+ ATPase (subunit α), and FXYD domain-containing ion

Table 3. Summary of potential gene outliers between freshwater and saltwater *Lucania parva* compared to between *L. parva* and *Lucania goodei*.

Potential gene name	Function	Outlier, number of freshwater –saltwater drainages	Outlier between species
Electrogenic sodium bicarbonate cotransporter	Ion transport	3	Y
Anion exchange protein 2	Ion transport	2	N
ATP-sensitive inward rectifier potassium channel 15	Ion transport	2	N
FXYD domain-containing ion transport regulator 6 precursor	Ion transport	2[1]	N
Solute carrier family proteins	Ion transport	2-no.35 1-no.10,12,15,23, 25,30	No. 12,15,22, 25,35
V-type H^+ ATPase	Ion transport	2- subunit S1 1-subunit A,C,D[1]	subunit A
Band 3 anion transport protein	Ion transport	1	N
Calcium channel flower homolog	Ion transport	1	Y
Calcium-transporting ATPase type 2 C member	Ion transport	1	Y
Canalicular multispecific organic anion transport 2	Ion transport	1	N
Hydrogen/chloride exchange transporter 7	Ion transport	1	N
Plasma membrane calcium-transporting ATPase 1	Ion transport	1	N
Probable cation-transporting ATPase 13 (A and B)	Ion transport	1	Y
Sacroplasmic/endoplasmic reticulum calcium ATPase 2	Ion transport	1	Y
Short transient receptor potential channel 4	Ion transport	1	Y
Sodium bicarbonate cotransporter 3	Ion transport	1	Y
Sodium channel modifier 1	Ion transport	1	N
Sodium/calcium exchanger 1	Ion transport	1	N
Sodium/hydrogen exchanger 8	Ion transport	1	Y
Sodium/potassium/calcium exchanger	Ion transport	1	Y
Sodium/potassium-transporting ATPase subunit β-1	Ion transport	1	N
Transient receptor potential cation channel (subfamily M)	Ion transport	1	Y
Two pore calcium channel protein 1	Ion transport	1	Y
Voltage-gated hydrogen channel 1	Ion transport	1	Y
Chloride intracellular channel protein 2	Ion transport	0	Y
Potassium voltage-gated channel subfamily H	Ion transport	0[1]	Y
Sodium channel and clathrin linker 1	Ion transport	0	Y
Solute carrier organic anion transporter family member 5A	Ion transport	0	Y
Claudin 4	Cell junction/adhesion	2	N

Table 3. Continued.

Potential gene name	Function	Outlier, number of freshwater –saltwater drainages	Outlier between species
Claudin 10	Cell junction/adhesion	2	N
Claudin 17	Cell junction/adhesion	1	N
Tight junction protein ZO-3	Cell junction/adhesion	1	Y
Gap junction β-4 protein	Cell junction/adhesion	0[2]	Y
Occludin	Cell junction/adhesion	0[2]	N
Glucocorticoid receptor	Osmotic/salt stress	1	Y
Aldehyde dehydrogenase 7 (A1)	Osmotic/salt stress	1	Y
Bardet-Biedl syndrome 4 protein	Microtubule attachment to centromere	3	N (protein 7 present)
Calcium homeostasis endoplasmic reticulum protein	Calcium release into the cytosol	3	N
Epidermal growth factor receptor kinase	Cell proliferation	3	N
FAD-dependent oxidoreductase domain-containing protein 1	Mitochondria	3	Y
Insulin receptor	Glucose homeostasis	3	Y
Protein BANP	Cell cycle	3	Y
Putative deoxyribonuclease TATDN2	Activation of signaling protein activity involved in unfolded protein response	3	Y
SAM and SH3 domain-containing protein	Regulation of immune cell proliferation	3	Y
Transmembrane protein 170B	None	3	N
Trimethylguanosine synthase	RNA capping	3	N
U3 small nucleolar RNA-associated protein	rRNA processing	3	Y
Uncharacterized protein 4	None	3	Y
Uncharacterized protein C14orf28	None	3	N
Estrogen receptor	Reproduction	1	Y
MORC family CW-type zinc finger	Spermatogenesis	1	Y
Round spermatid basic protein 1-like	Spermatogenesis	1	Y
Anti-Mullerian hormone	Spermatogenesis	0	Y
Katanin p60 ATPase-containing subunit A-like 1	Spermatogenesis	0	Y
RING finger protein 114 (zinc finger protein 313)	Spermatogenesis	0	Y
Piwi-like protein 2	Spermatogenesis	0	Y
Zonadhesin-like	Egg–sperm interaction	0	Y

[1]Freshwater–saltwater outlier Infinium bead chip analysis.

[2]Yes but in saltwater–saltwater *L. parva* outliers and removed.

transport regulator 6 precursor. Both V-type H^+ ATPase and FXYD domain-containing ion transport regulator 6 were also outliers in multiple drainages in the pooled transcriptome data (Table 3).

TRANSCRIPTOME SEQUENCE DIVERGENCE VERSUS GENOME EXPRESSION DIVERGENCE

Our goal was to determine whether genes that were differentially expressed between species in response to osmotic challenge also showed high levels of sequence divergence between freshwater and saltwater *L. parva* populations. We found matches for 73.1% of the gene expression probe sequences within our assembled transcriptome (2961 genes). Of the 310 genes which showed significant species-dependent expression in response to the salinity challenge, 122 matched contigs with segregating SNPs. But we found no genes that were outliers in two or more freshwater–saltwater drainages which also showed species-dependent expression differences. Despite this lack of overlap in genes, we found three GO

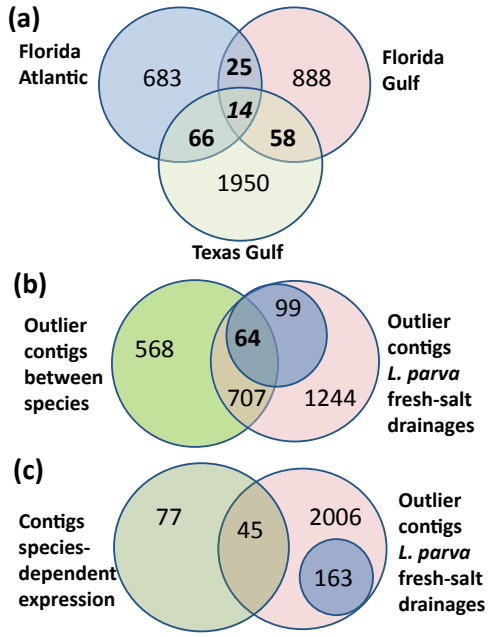

Figure 4. Venn diagrams of shared annotated contigs with SNPs. (a) Annotated contigs with outlier SNP windows shared between freshwater and saltwater drainages in *Lucania parva*: Florida Atlantic (blue), Florida Gulf (red), Texas Gulf (green). (b) Annotated outlier contigs found between species (green), freshwater–saltwater *L. parva* within a drainage (red) and shared between two or more freshwater–saltwater drainages (blue inset circle). (c) Contigs with species-dependent expression during acclimation to osmotic challenge (green), outlier contigs between freshwater and saltwater *L. parva* drainages (red), and shared between two or more freshwater–saltwater drainages (blue inset circle).

enrichment terms were shared between the two analyses: monovalent inorganic cation transmembrane transporter activity, hydrolase activity, and nitrogen compound biosynthetic/metabolic process. All of these were in set *f* (downregulated in *L. parva*). A comparison of genes with species-dependent expression to outlier genes from at least one of our freshwater–saltwater *L. parva* drainages yielded 45 genes (Fig. 4c and Table S10). We found overlap in enriched GO categories of ATPase activity, purine ribonucleotide metabolic process (ATP synthesis), inorganic cation transmembrane transporter activity, and hydrolase activity (all set *f*).

Discussion

In this study, we identified genes containing outlier SNPs that were highly differentiated between species and compared these with genes containing outlier SNPs that had consistently diverged between freshwater and saltwater populations. We found repeated differentiation of potential genes known to be important in osmoregulation, such as ion transport genes. Between species, we also found high differentiation in genes related to reproduction. This pattern of divergence in reproductive genes did not emerge within species. We compared our outlier genes to those genes that showed species-dependent expression patterns during a salinity challenge but we did not find that genes that were differentially expressed were likely to be highly diverged in transcribed sequence.

POPULATION/SPECIES DIVERGENCE IN OSMOREGULATORY GENES

Freshwater and saltwater *L. parva* differ in their osmotic environments, and this was reflected in our genomic data. We found a number of contigs that contained highly differentiated SNPs that were associated with ion transport. We found differentiation in many of these same genes between *L. parva* and *L. goodei*, which also differ radically in their salinity tolerance. This pattern is unlikely to be due to drift or selection on other characteristics, as genes involved in osmoregulation were not identified as outlier SNPs between the two *L. goodei* populations or between the two distant, saltwater populations of *L. parva*.

Both freshwater–saltwater and between species comparisons suggest the importance of V-type H^+ ATPase and electrogenic sodium bicarbonate cotransporter to osmoregulatory divergence. We found several highly differentiated SNPs in the subunits of V-type H^+ ATPase, with freshwater *L. parva* alleles diverging from saltwater populations and differentiation between *L. parva* and *L. goodei*. V-type H^+ ATPase is a key contributor to osmotic balance in freshwater in both osmoconforming invertebrates and osmoregulating vertebrates. Freshwater Atlantic killifish (*F. heteroclitus*) were found to have high concentrations of V-type H^+ ATPase protein in gill tissue at low salinity using immunocytochemistry (Katoh et al. 2003). Lysosomal V-type H^+ ATPase is a consistent outlier locus in genome scans between freshwater and saltwater stickleback populations (*G. aculeatus*: Hohenlohe et al. 2010; Jones et al. 2012). In invertebrate copepods (*Eurytemora affinis*), V-type H^+ ATPase activity in individuals from freshwater populations is consistently higher than individuals from saltwater populations when the animals are in fresh water (Lee et al. 2011a). Adaptation to fresh water in selection experiments also results in rapid increases in V-type H^+ ATPase activity in copepods under freshwater conditions (Lee et al. 2011a). Given the importance of V-type H^+ ATPase in freshwater osmoregulation, it would be interesting to compare differences in expression of this gene between *L. goodei* and *L. parva* in a hypo-osmotic challenge, rather than the hyperosmotic challenge we used in this experiment. A previous study found that V-type H^+ ATPase expression in *Lucania* tended to be higher in fresh water, although this result was not statistically significant (Berdan and Fuller 2012a).

We also found differences between species and freshwater–saltwater populations in electrogenic sodium bicarbonate cotransporter (NBCE1). The importance of this gene to osmoregulation appears to vary among fish species. It is involved in sodium ion balance in freshwater rainbow trout gills (Parks et al. 2007) and in freshwater acclimation in Japanese eels (Tse and Wong 2011). However, it does not appear to play an important role in zebrafish osmoregulation (Lee et al. 2011b). Most research has focused on the role of this transporter in the intestinal tract of marine teleosts (Chang et al. 2012), but we did not include any intestinal tissues in our samples. Our results suggest that this may be another key gene involved in freshwater osmoregulation in *Lucania* gills.

We found that Na^+/K^+ ATPase showed significant changes in expression between species in response to a hyperosmotic challenge. During acclimation, Na^+/K^+ ATPase had decreased expression in *L. parva* over time compared to *L. goodei*. However, Na^+/K^+ ATPase did not show consistent coding sequence divergence between or within species. Outlier SNPs in Na^+/K^+ ATPase were only found between Texas Gulf freshwater-saltwater *L. parva* and in the Infinium data between freshwater and saltwater *L. parva*. Previous work in Atlantic killifish has also found differences in NA^+/K^+ ATPase expression between freshwater and saltwater adapted populations (Scott et al. 2004; Scott and Schulte 2005). In Atlantic killifish, V-type H^+ ATPase has been shown to colocalize with Na^+/K^+ ATPase in the basolateral membrane of mitochondrial rich cells, suggesting that these two transporters work together to facilitate the uptake of sodium ions in low sodium environments (Katoh et al. 2003). However, our findings suggest that selection may be operating differently on these related ionoregulatory ATPase genes, perhaps because Na^+/K^+ ATPase also plays a prominent role in osmoregulation at high salinity (Karnaky et al. 1976; Perry 1997; Evans et al. 2005).

Changes in cell junction genes also appear to be important to osmoregulatory evolution in *L. parva*. In freshwater–saltwater *L. parva* comparisons, we found differentiation in several tight junction genes, including claudins. Claudins play a key role in permeability changes in the gills, because more tight junctions are required for osmoregulation in freshwater (Tipsmark et al. 2008b; Tipsmark and Madsen 2012). Past research has also shown that claudins are vital to gill remodeling under changing salinities (Tipsmark et al. 2008a,b; Sandbichler et al. 2011).

Salinity affects multiple aspects of the environment including osmolarity. Between species, we found differences in several stress response genes, including the glucocorticoid receptor. This receptor may play a role in the regulation of ion channel and Na^+/K^+ ATPase gene expression in gills (Kiilerich et al. 2007), but may also be involved in more general stress responses (Lethimonier et al. 2000). We also found that genes involved in fat cell differentiation were divergent between species and between multiple freshwater and saltwater *L. parva* populations, which is interesting given that thermal tolerance appears to differ between the species (*L. goodei* is more cold tolerant in low salinities; Fuller et al. 2007). As with many genomic studies, we have focused our attention on genomic patterns that correspond to important biological differences between the two species, such as those involved in osmoregulation and reproduction. But there are many genes that our outlier analysis identified as consistently diverged between freshwater and saltwater for which we have no biological story. Genetic drift or hitchhiking may contribute to some of these outlier SNPs. Another possibility is that salinity itself may result in selection on traits other than osmoregulation (such as traits associated with pathogen resistance, diet, or predator avoidance; Fuller and Noa 2008; Kozak et al. 2012).

RECONCILING GENOME EXPRESSION PROFILING AND POPULATION/SPECIES DIVERGENCE

Comparative genomic expression profiling of both species during acclimation to an osmotic challenge identified a set of genes with conserved expression and a set of genes with species-dependent expression. Those with species-dependent expression are the most likely candidates for functional osmotic divergence between species. Many of these genes are involved in ion transport and regulation (NKA, SGK1), osmolyte synthesis (IMPA1), osmotically induced cell signaling (CMM2, OSTF1), and cell volume regulation (AQP1, AQP3). Several of these genes are also the targets of evolved expression divergence between populations of Atlantic killifish that are physiologically adapted to alternate osmotic environments (e.g., IMPA1, OSTF1, AQP3; Whitehead et al. 2011), indicating that expression of some genes repeatedly diverge in their response to osmotic challenge in killifish taxa with different osmotic tolerances (Whitehead et al. 2013).

In general, there was little overlap between the candidate genes identified by expression profiling and by our scan of transcriptome sequence variation. None of the genes that were outliers in two or more freshwater–saltwater *L. parva* comparisons showed divergence in expression in response to a hyperosmotic challenge, including claudins and V-type H^+ ATPase. Previous comparisons of protein sequence and expression divergence in genes subject to directional selection have yielded mixed results. Some have found a strong overlap between genes showing sequence and expression divergence (Holloway et al. 2007) whereas others found a lack of overlap (Good et al. 2006; Jeukens et al. 2010). However, what distinguishes our study from these previous ones is that we focus on genes that vary not in constitutive expression levels between species, but in their pattern of transcriptional response to an environmental variable (salinity) that is relevant for differences in the fundamental niches of the taxa being compared. As such, the genes we identify are more likely to underlie variability in a physiologically relevant adaptive response.

The lack of overlap between expression and sequence divergence could be explained by a number of alternate hypotheses. Osmoregulatory evolution may require fine-tuning of sequence of some relevant genes and expression responses of other relevant genes. For example, expression of tight junction proteins including claudins appears to be consistently called upon during osmotic acclimation in diverse *Fundulus* killifish species. This pattern of expression response tends to be highly conserved between populations that have adapted to alternate osmotic environments (Whitehead et al. 2011, 2012, 2013). Here, we found that claudin gene sequence has diverged between freshwater and saltwater *L. parva*. Thus, evolutionary variation in the expression of these genes in response to osmotic challenge may be tightly conserved even though their sequence may evolve.

In addition, physiological divergence between *Lucania* species may occur mainly through the loss of high salt tolerance and plasticity of gene expression in *L. goodei*. This loss of hyperosmotic plasticity in fresh water could be selectively neutral. If this is the case, we would not expect overlap in divergently expressed genes and loci with signatures of positive selection. Instead, genes conferring high-salinity tolerance would slowly accumulate deleterious mutations in freshwater populations. These genes would be unlikely to bear the signature of strong directional selection. The biogeography of *Lucania* suggests that *L. goodei* evolved from a euryhaline ancestor (Burgess and Franz 1978; Whitehead 2010) and likely faced selection to cope with freshwater conditions. Similarly, *L. parva* has invaded freshwater multiple times. Hence, the relevant comparison may be between sets of genes that are differentially expressed during hypo-osmotic shock and those identified in our population genomic scans.

Another possible explanation for the lack of overlap between genes in our sequence and expression analyses is that our genomic scans were not sensitive enough to pick up genes that evolved from standing variation. A limitation of histogram-based F_{st} analyses is that they are likely to miss these types of adaptive outliers (Teshima et al. 2006). Work in other freshwater–saltwater systems suggests that standing variation in marine populations may play an important role in repeated colonization of freshwater (Colosimo et al. 2005; Lee et al. 2011a). As genomic resources and population demography information increase for *Lucania*, future work could use more sophisticated genomic scans and determine how standing variation may contribute to adaptation in freshwater *Lucania* populations.

Although there was little overlap in candidate genes between our sequence and expression analyses, the functional types of genes identified were similar. Sequence outliers and expression outliers tended to converge around genes with common function; both sets of candidate genes were enriched for ion transport. This is consistent with adaptation targeting functional variation associated with ion transport in response to divergence in osmotic environments.

SCALING UP: POPULATION/SPECIES DIVERGENCE AND REPRODUCTIVE ISOLATION

The major patterns of genomic differentiation between *L. goodei* and *L. parva* found in this study reflect patterns of reproductive isolation between these species. F_2 hybrids between species suffer decreased hatching success at high salinities (Fuller 2008a). The presence of this extrinsic isolation suggests that divergence in osmoregulatory genes between species may lead to decreased ability of hybrids to osmoregulate at high salinity. Consistent with this, we identified species-specific SNPs in a number of ion transport proteins. Similarly, there is preliminary evidence for reduced fitness of hybrid eggs between freshwater and saltwater *L. parva* populations (Kozak et al. 2012) and in this study, we showed that freshwater and saltwater populations of *L. parva* have highly differentiated SNPs in a number of the same ion transport proteins that have differentiated between *L. goodei* and *L. parva*. Thus, our genome-wide scan provides the groundwork for identifying genes that contribute to reduced hybrid fitness and incompatibilities between populations and species.

Hybrid males between *Lucania* species also have greatly reduced fertility. F_1 hybrid males resulting from a cross between a female *L. parva* and a male *L. goodei* have less than 50% of the fertilization success of the reverse F_1 hybrid cross and parental males (Fuller 2008a). We found a large number of species-specific SNPs in genes associated solely with reproductive traits, including spermatogenesis. This supports the idea that genes involved in gamete production have differentiated between species and contribute to male sterility. Even though ovaries and testes were one of the primary tissues sampled, reproductive genes were not overrepresented in within species comparisons: within *L. goodei* (Upper Bridge versus Everglades) or within *L. parva* (multiple population comparisons). Therefore, this pattern of divergence in reproductive genes between species is likely real.

Summary

Osmoregulation is a complex trait and involves changes in multiple phenotypes. Our data showed consistent divergence across multiple drainages and multiple time scales in osmoregulatory genes. Rapid sequence divergence appeared repeatedly in ion transport, water balance, and cell junction proteins. Between *Lucania* species, there was also striking divergence in reproductive genes that is consistent with observed hybrid sterility. Ongoing QTL studies of salinity tolerance using newly developed linkage maps for *Lucania* (Berdan 2012), coupled with emerging genomics resources for this group of fishes, promise to enrich

Lucania and related *Fundulus* species as models for studying the evolution of osmotic tolerance.

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DATA ARCHIVING

Dryad doi:10.5061/dryad.hv75h. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-1854. The custom Agilent *Fundulus heteroclitus* EST microarray design was also deposited in the ArrayExpress database under accession number A-MEXP-2336. The transcriptome work archived in Genbank (bio-project ID: SRP029261; accession numbers: SRX340119, SRX340220, SRX340250, SRX340824, SRX340826, SRX340836, SRX340838, SRX340853).

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Supporting Information

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

Figure S1. Expression patterns for genes with responses during the time-course of acclimation to hyperosmotic challenge that are conserved between species (significant time-course effect, but no significant species-by-time-course interaction).

Figure S2. Correlation between F_{st} calculated in pooled samples and F_{st} calculated using individual genotypes (Infinium SNP bead chip data).

Table S1. Gene expression during the hyperosmotic challenge.

Table S2. GO term enrichment for outlier contigs between species (*Lucania goodei* vs. *Lucania parva*).

Table S3. GO term enrichment for outlier contigs between *Lucania goodei* populations.

Table S4. GO term enrichment for outlier contigs between most distant *Lucania parva* saltwater populations (Florida Atlantic salt and Texas Gulf salt).

Table S5. GO term enrichment for outlier contigs between freshwater and saltwater *Lucania parva* drainages (at least one drainage; saltwater–saltwater shared contigs removed).

Table S6. GO term enrichment for outlier contigs present two of more freshwater–saltwater *L. parva* drainages (only those categories with a count greater than one reported).

Table S7. Outlier loci shared between multiple freshwater and saltwater drainages in *L. parva* and between species.

Table S8. GO term enrichment for outlier contigs present between species in using genomic DNA and Infinium genotyping.

Table S9. GO term enrichment for outlier contigs present between all freshwater and saltwater *Lucania parva* using genomic DNA and Infinium genotyping.

Table S10. Genes with species-dependent expression differences and sequence divergence in freshwater–saltwater populations.