Phylogenetic inference methods for Fig. 1

Phylogeny is inferred from nucleic acid sequences of a single mitochondrial locus, *16S-rDNA*. Sequences for the Atyidae were taken from von Rintelen et al. (2012), Weese et al. (2013), or the transcriptomes used here. Sequences for the Alpheidae were acquired from NCBI's GenBank. Sequence alignments were generated with Clustal Omega (Sievers et al., 2011) and the phylogeny was inferred using RAxML version 8 (Stamatakis, 2014) under the "GTRGAMMAI" model of evolution and 1000 rapid bootstrap replicates (Stamatakis et al., 2008). Values of nodal support >75 are displayed and the root set at the split between the two families.

Supplemental datasets hosted on Dryad

Supplemental File S1. Statistics and annotation for differentially expressed genes (DEGs) during salinity transfers for the anchialine shrimp species examined here. Each sheet has a set of DEGs for each transfer. Sheet names indicate species, direction of transfer, time point (for *H. rubra* only, all others are 24 hrs after transfer), and program used to identify DEGs. The first salinity in the name of the sheet (32‰ for all species except *C. rubella*) is the reference salinity. For example, in the sheet "Mmin_32_to_2_DESeq" – the first DEG (annotated as a solute carrier family member) was expressed 10.7 times higher in *M. minutus* 24 hrs after transfer from 32‰ to 2‰ than after transfer from 32‰ to 32‰ (analyses via DESeq).

Supplemental File S2. Normalized FPKM values for all contigs at the "component" level of the transcriptomes for each treatment among the anchialine shrimp species examined here. Sheet names indicate species and column names indicate treatments (treatment names are salinities examined 24 hrs after transfer, except for *H. rubra*, in which multiple time points were examined after transfer from 32‰ to 15‰).

Table S1. Accession numbers for reference transcriptomes for the anchialine shrimp species examined here. See Havird et al. (2014a) and Havird and Santos (2016) for further information.

Species	SRA Accession#	TSA Accession#
H. rubra	SRR3089133, SRR3089132	GHBK00000000
M. minutus	SRR1248250, SRR1248251	GHAO00000000
C. rubella	SRR1248099-101,SRR1248106	GHBE00000000
H. trigonophthalma	SRR1248237, SRR1248238	GHBI00000000
A. lauensis	SRR1248098, SRR1240510	GHBJ00000000
M. lohena	SRR1248247, SRR1248249	GHAP00000000

Table S2. Accession numbers for the RNA-Seq reads generated in this study.

Species	Treatment	Sample#	SRA Accession#
H. rubra	32‰ (control)	1	SRR8080057
H. rubra	32‰ (control)	2	SRR8079985
H. rubra	32‰ (control)	3	SRR8079986
H. rubra	32‰ (control)	4	SRR8079983
H. rubra	32‰ (control)	5	SRR8079984
H. rubra	32‰ (control)	6	SRR8079981
H. rubra	15‰ (3 hr)	1	SRR8079977
H. rubra	15‰ (3 hr)	2	SRR8079978
H. rubra	15‰ (3 hr)	3	SRR8079992
H. rubra	15‰ (3 hr)	4	SRR8079991
H. rubra	15‰ (8 hr)	1	SRR8079990
H. rubra	15‰ (8 hr)	2	SRR8079989
H. rubra	15‰ (8 hr)	3	SRR8079996
H. rubra	15‰ (8 hr)	4	SRR8079995
H. rubra	15‰ (48 hr)	1	SRR8079982
H. rubra	15‰ (48 hr)	2	SRR8079979
H. rubra	15‰ (48 hr)	3	SRR8079980
M. minutus	32‰ (control)	1	SRR8080045
M. minutus	32‰ (control)	2	SRR8080046
M. minutus	32‰ (control)	3	SRR8080036
M. minutus	32‰ (control)	4	SRR8080035
M. minutus	32‰ (control)	5	SRR8080034
M. minutus	32‰ (control)	6	SRR8080033
M. minutus	2‰ (24 hr)	1	SRR8080037
M. minutus	2‰ (24 hr)	2	SRR8080038
M. minutus	2‰ (24 hr)	3	SRR8080043
M. minutus	2‰ (24 hr)	4	SRR8080044
M. minutus	2‰ (24 hr)	5	SRR8080041
M. minutus	2‰ (24 hr)	6	SRR8080042
M. minutus	15‰ (24 hr)	1	SRR8080024
M. minutus	15‰ (24 hr)	2	SRR8080023
M. minutus	15‰ (24 hr)	3	SRR8080026
M. minutus	15‰ (24 hr)	4	SRR8080025
M. minutus	15‰ (24 hr)	5	SRR8080039
M. minutus	15‰ (24 hr)	6	SRR8080040
M. minutus	45‰ (24 hr)	1	SRR8080032
M. minutus	45‰ (24 hr)	2	SRR8080031

M. minutus	45‰ (24 hr)	3	SRR8080030
M. minutus	45‰ (24 hr)	4	SRR8080029
M. minutus	45‰ (24 hr)	5	SRR8080028
M. minutus	45‰ (24 hr)	6	SRR8080027
C. rubella	25‰ (control)	1	SRR8080049
C. rubella	25‰ (control)	2	SRR8080050
C. rubella	25‰ (control)	3	SRR8080051
C. rubella	25‰ (control)	4	SRR8080052
C. rubella	25‰ (control)	5	SRR8080053
C. rubella	32‰ (24 hr)	1	SRR8079975
C. rubella	32‰ (24 hr)	2	SRR8079970
C. rubella	32‰ (24 hr)	3	SRR8080059
C. rubella	32‰ (24 hr)	4	SRR8079972
C. rubella	32‰ (24 hr)	5	SRR8079971
C. rubella	32‰ (24 hr)	6	SRR8080058
C. rubella	2‰ (24 hr)	1	SRR8080054
C. rubella	2‰ (24 hr)	2	SRR8080055
C. rubella	2‰ (24 hr)	3	SRR8080056
C. rubella	2‰ (24 hr)	4	SRR8079974
C. rubella	2‰ (24 hr)	5	SRR8079973
C. rubella	2‰ (24 hr)	6	SRR8079976
C. rubella	15‰ (24 hr)	1	SRR8080009
C. rubella	15‰ (24 hr)	2	SRR8080010
C. rubella	15‰ (24 hr)	3	SRR8080015
C. rubella	15‰ (24 hr)	4	SRR8080016
C. rubella	15‰ (24 hr)	5	SRR8080047
C. rubella	15‰ (24 hr)	6	SRR8080048
H. trigonophthalma	32‰ (control)	1	SRR8079999
H. trigonophthalma	32‰ (control)	2	SRR8080000
H. trigonophthalma	32‰ (control)	3	SRR8080005
H. trigonophthalma	32‰ (control)	4	SRR8080006
H. trigonophthalma	32‰ (control)	5	SRR8080018
H. trigonophthalma	32‰ (control)	6	SRR8080017
H. trigonophthalma	2‰ (24 hr)	1	SRR8080003
H. trigonophthalma	2‰ (24 hr)	2	SRR8080004
H. trigonophthalma	2‰ (24 hr)	3	SRR8079997
H. trigonophthalma	2‰ (24 hr)	4	SRR8079998
H. trigonophthalma	15‰ (24 hr)	1	SRR8080003
H. trigonophthalma	15‰ (24 hr)	2	SRR8080004
H. trigonophthalma	15‰ (24 hr)	3	SRR8079997

H. trigonophthalma	15‰ (24 hr)	4	SRR8079998
H. trigonophthalma	15‰ (24 hr)	5	SRR8080001
H. trigonophthalma	15‰ (24 hr)	6	SRR8080002
H. trigonophthalma	45‰ (24 hr)	1	SRR8080020
H. trigonophthalma	45‰ (24 hr)	2	SRR8080019
H. trigonophthalma	45‰ (24 hr)	3	SRR8080022
H. trigonophthalma	45‰ (24 hr)	4	SRR8080021
A. lauensis	32‰ (control)	1	SRR8080014
A. lauensis	32‰ (control)	2	SRR8080007
A. lauensis	32‰ (control)	3	SRR8080008
A. lauensis	2‰ (24 hr)	1	SRR8080011
A. lauensis	2‰ (24 hr)	2	SRR8080012
A. lauensis	2‰ (24 hr)	3	SRR8080013

Table S3. Experimental details of salinity transfers for the anchialine shrimp species examined here, including the number of gills pooled for each sample to create a biological replicate, the reference salinity, experimental salinities, time points sampled (in hours), and sample sizes for each treatment.

Species	# Gills Pooled	Ref. Salinity, *n*	Exp. Sal., Time, *n* (T_1)	Exp. Sal., Time, *n* (T_2)	Exp. Sal., Time, *n* (T_3)
H. rubra	3-6	32, 6	15, 3, 4	15, 8, 4	15, 48, 3
A. lauensis	3-6	31, 3	2, 24, 3	NA	NA
H. trigonophthalma	4-7	32, 6	2, 24, 4	15, 24, 6	45, 24, 4
C. rubella	2	25, 5	2, 24, 6	15, 24, 6	32, 24, 6
M. minutus	3-5	32, 6	2, 24, 6	15, 24, 6	45, 24, 6

Table S4. Number of RNA-seq reads for each sample.

Species	Treatment	Sample#	#Reads
H. rubra	32‰ (control)	1	17,309,744
H. rubra	32‰ (control)	2	28,219,448
H. rubra	32‰ (control)	3	13,242,426
H. rubra	32‰ (control)	4	13,180,762
H. rubra	32‰ (control)	5	17,417,620
H. rubra	32‰ (control)	6	287,564
H. rubra	15‰ (3 hr)	1	14,807,309
H. rubra	15‰ (3 hr)	2	9,961,560
H. rubra	15‰ (3 hr)	3	12,142,010
H. rubra	15‰ (3 hr)	4	13,453,505
H. rubra	15‰ (8 hr)	1	8,853,129
H. rubra	15‰ (8 hr)	2	10,056,737
H. rubra	15‰ (8 hr)	3	12,030,020
H. rubra	15‰ (8 hr)	4	14,053,186
H. rubra	15‰ (48 hr)	1	11,307,909
H. rubra	15‰ (48 hr)	2	11,598,040
H. rubra	15‰ (48 hr)	3	15,907,727
M. minutus	32‰ (control)	1	5,054,417
M. minutus	32‰ (control)	2	12,075,372
M. minutus	32‰ (control)	3	8,176,451
M. minutus	32‰ (control)	4	7,168,838
M. minutus	32‰ (control)	5	8,604,546
M. minutus	32‰ (control)	6	7,615,409
M. minutus	2‰ (24 hr)	1	10,851,158
M. minutus	2‰ (24 hr)	2	9,194,533
M. minutus	2‰ (24 hr)	3	9,809,365
M. minutus	2‰ (24 hr)	4	6,966,642
M. minutus	2‰ (24 hr)	5	8,356,222
M. minutus	2‰ (24 hr)	6	8,006,300
M. minutus	15‰ (24 hr)	1	10,205,829
M. minutus	15‰ (24 hr)	2	10,930,627
M. minutus	15‰ (24 hr)	3	10,898,302
M. minutus	15‰ (24 hr)	4	10,809,391
M. minutus	15‰ (24 hr)	5	7,855,714
M. minutus	15‰ (24 hr)	6	10,669,014
M. minutus	45‰ (24 hr)	1	9,975,425
M. minutus	45‰ (24 hr)	2	3,231,459
M. minutus	45‰ (24 hr)	3	4,736,963
M. minutus	45‰ (24 hr)	4	5,504,044
M. minutus	45‰ (24 hr)	5	3,982,154

M. minutus	45‰ (24 hr)	6	3,372,354
C. rubella	25‰ (control)	1	2,674,085
C. rubella	25‰ (control)	2	8,067,453
C. rubella	25‰ (control)	3	9,682,383
C. rubella	25‰ (control)	4	9,968,370
C. rubella	25‰ (control)	5	11,735,923
C. rubella	32‰ (24 hr)	1	7,370,129
C. rubella	32‰ (24 hr)	2	8,626,450
C. rubella	32‰ (24 hr)	3	7,374,356
C. rubella	32‰ (24 hr)	4	9,359,303
C. rubella	32‰ (24 hr)	5	9,645,797
C. rubella	32‰ (24 hr)	6	5,926,832
C. rubella	2‰ (24 hr)	1	8,832,310
C. rubella	2‰ (24 hr)	2	8,242,345
C. rubella	2‰ (24 hr)	3	11,231,925
C. rubella	2‰ (24 hr)	4	7,735,644
C. rubella	2‰ (24 hr)	5	6,637,090
C. rubella	2‰ (24 hr)	6	7,585,178
C. rubella	15‰ (24 hr)	1	7,316,050
C. rubella	15‰ (24 hr)	2	8,540,739
C. rubella	15‰ (24 hr)	3	6,858,329
C. rubella	15‰ (24 hr)	4	8,140,018
C. rubella	15‰ (24 hr)	5	6,366,371
C. rubella	15‰ (24 hr)	6	5,836,129
H. trigonophthalma	32‰ (control)	1	1,614,404
H. trigonophthalma	32‰ (control)	2	5,587,233
H. trigonophthalma	32‰ (control)	3	6,360,563
H. trigonophthalma	32‰ (control)	4	1,749,659
H. trigonophthalma	32‰ (control)	5	3,622,528
H. trigonophthalma	32‰ (control)	6	6,130,579
H. trigonophthalma	2‰ (24 hr)	1	6,697,038
H. trigonophthalma	2‰ (24 hr)	2	9,733,476
H. trigonophthalma	2‰ (24 hr)	3	8,750,497
H. trigonophthalma	2‰ (24 hr)	4	10,388,199
H. trigonophthalma	15‰ (24 hr)	1	6,113,205
H. trigonophthalma	15‰ (24 hr)	2	7,626,859
H. trigonophthalma	15‰ (24 hr)	3	6,546,097
H. trigonophthalma	15‰ (24 hr)	4	8,063,859
H. trigonophthalma	15‰ (24 hr)	5	9,435,719
H. trigonophthalma	15‰ (24 hr)	6	8,309,449
H. trigonophthalma	45‰ (24 hr)	1	7,550,015
H. trigonophthalma	45‰ (24 hr)	2	9,401,152
H. trigonophthalma	45‰ (24 hr)	3	7,998,025

H. trigonophthalma	45‰ (24 hr)	4	8,454,334
A. lauensis	32‰ (control)	1	9,459,177
A. lauensis	32‰ (control)	2	9,692,546
A. lauensis	32‰ (control)	3	10,062,111
A. lauensis	2‰ (24 hr)	1	9,466,449
A. lauensis	2‰ (24 hr)	2	9,731,540
A. lauensis	2‰ (24 hr)	3	11,707,499

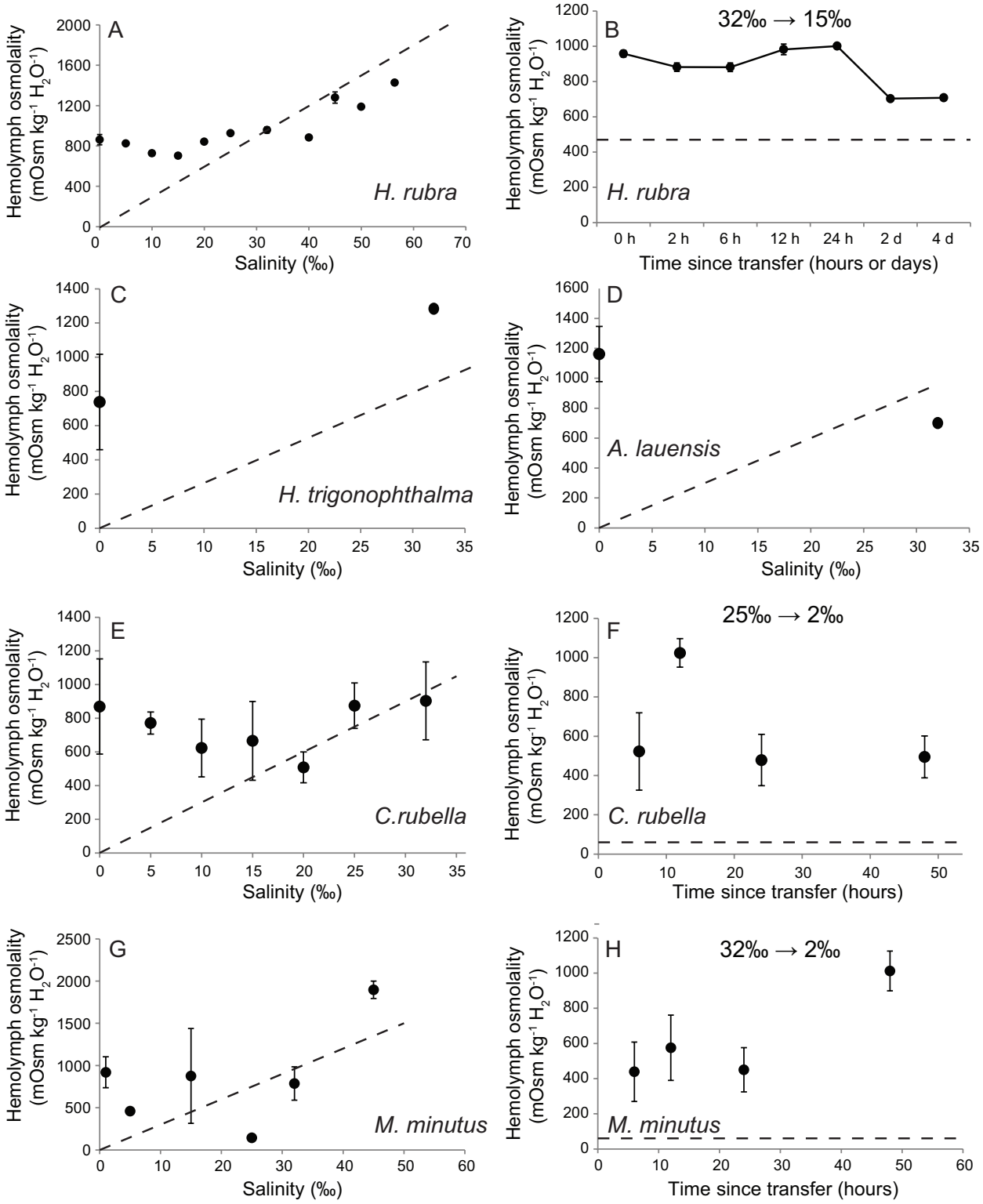

Fig. S1. Hemolymph osmolality during salinity transfer for the anchialine shrimp species examined here. A) and B) are from *H. rubra* (data from Havird et al. 2014b), C) is from *H. trigonophthalma*, D) is from *A. lauensis*, E) and F) are from *C. rubella*, and G) and H) is from *M.*

minutus. For transfers to multiple salinities (A, C, D, E, and G), hemolymph osmolality was measured after shrimp had acclimated for at least 2 days. For transfers where hemolymph osmolality was measured at multiple time points after transfer, the type of transfer is indicated. Dashed lines represent the iso-osmotic line – points near these suggest shrimp are acting as osmoconformers, while points away from them suggest shrimp are acting as osmoregulators. Note error bars for the four species from Japan are especially large, likely due to variation introduced during sample shipment (see main text); values should not be considered as quantitative absolutes, but rather as qualitative support for the general observation that most euryhaline crustaceans act as osmoconformers in marine waters and osmoregulators in fresh waters. Sample sizes: n = 3-6 for *H. rubra*, n = 1-3 for *M. minutus*, n = 3-4 for *C. rubella*, and n = 1-2 for *H. trigonophthalma* and *A. lauensis*. Error bars show ± SEM.

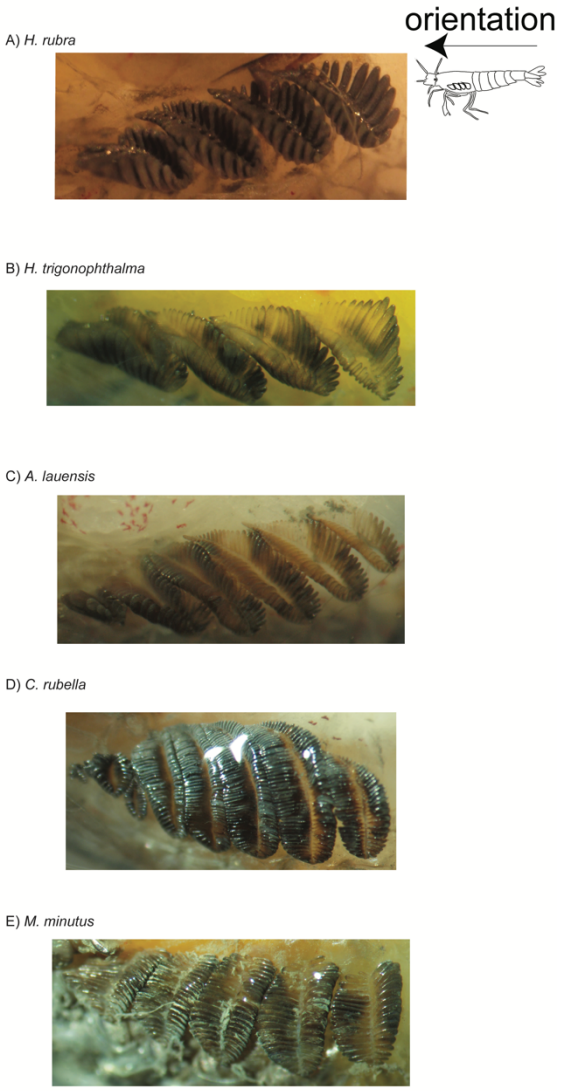

Fig. S2. Gill morphology of the anchialine shrimp species examined here as revealed via a representative photograph of gills stained with $AgNO_3$ for an individual acclimated to low salinity.

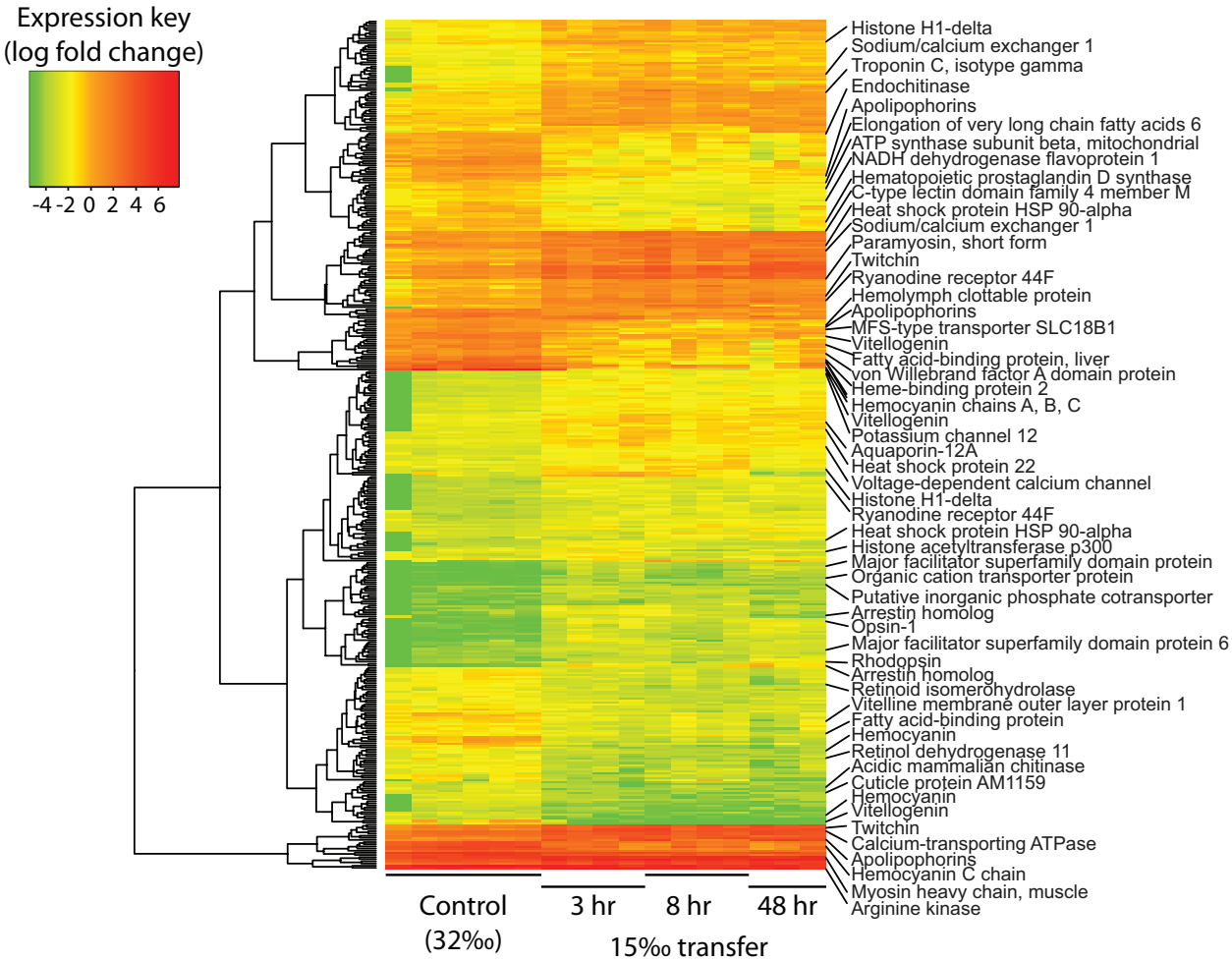

Fig. S3. Heatmap of differentially expressed genes (DEGs) from gill tissue of *Halocaridina rubra* during salinity transfer from 32‰ to 15‰. DEGs were identified via DESeq and log-fold changes in relative expression are displayed in shades of green (low) to red (high). Genes were clustered using an unsupervised hierarchical method based on their expression patterns (shown to the left). Annotation for genes of interest are indicated to the right.

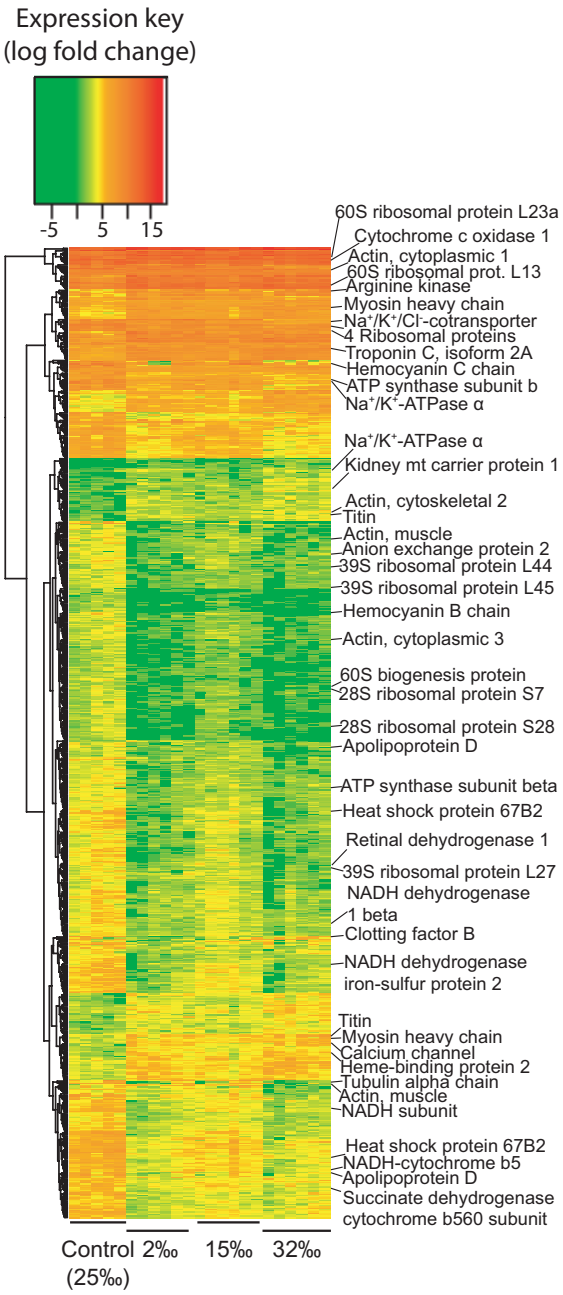

Fig. S4. Heatmap of differentially expressed genes (DEGs) from gill tissue of *Caridina rubella* 24 hrs after salinity transfer from 25‰ to either 2‰, 15‰, or 32‰. DEGs were identified via DESeq, with the exception of the 15‰ transfer, which was based on DESeq2 analyses since no DEGs were initially identified via DESeq. Coloring, clustering, and labeling are as in Fig. S3.

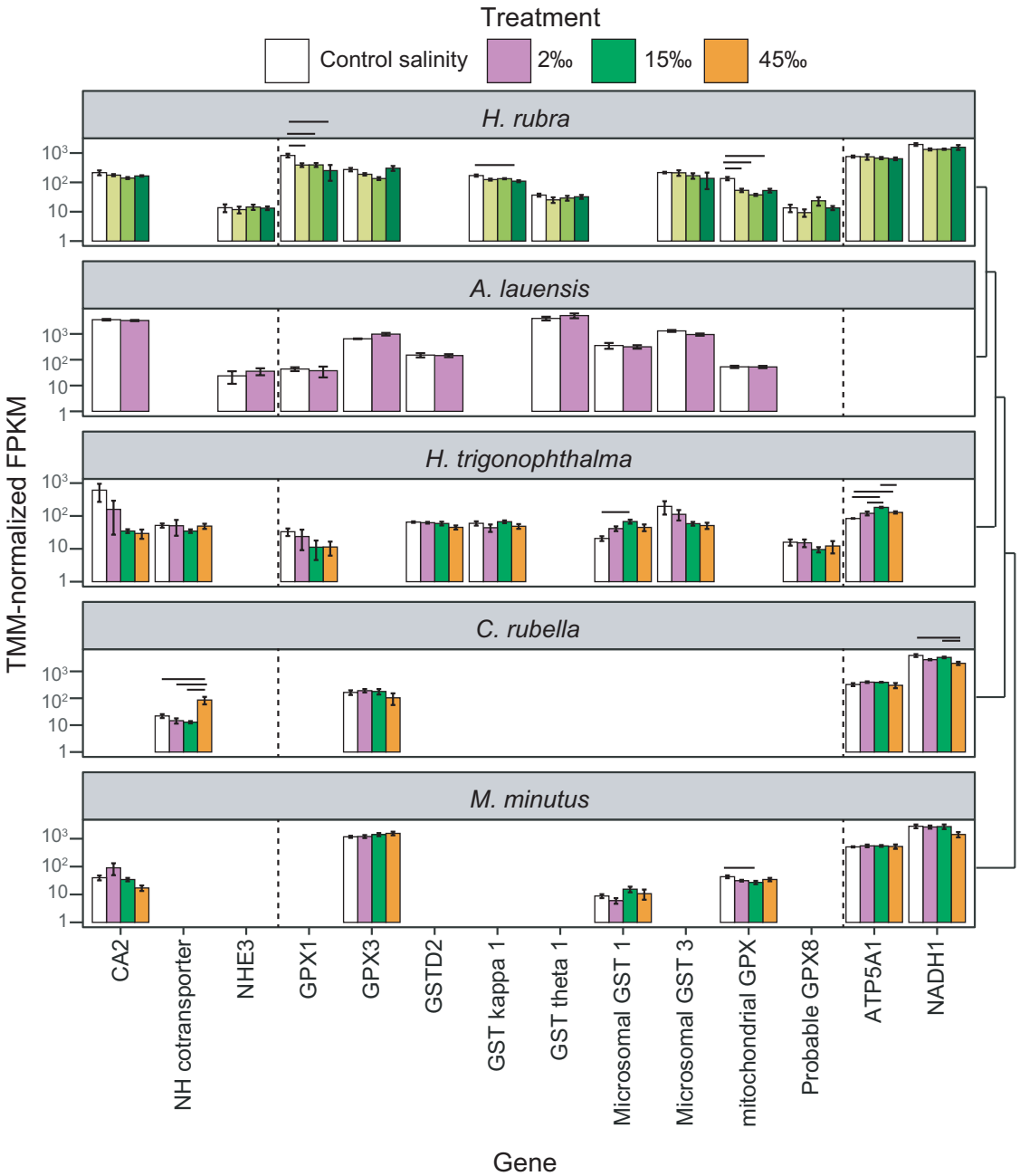

Fig. S5. Salinity-induced gene expression measured via TMM-normalized FPKM values for specific genes of interest identified and recovered from gill tissue in at least two of the five anchialine shrimp species examined here (see Fig. 4 for genes of interest identified in all five species). Bars above treatments indicate significant differences ($P < 0.05$) as inferred using one-way ANOVA with Tukey post-hoc analyses, although caution should be used in interpreting these analyses with regard to whether the gene was identified as a DEG (see main text, other supplemental files). Abbreviations for ion transporters and accessory enzymes: carbonic anhydrase (CA), Na^+/HCO_3^- cotransporter (NH cotransporter), Na^+/HCO_3^- exchanger (NHE). Abbreviations for genes involved in general stress response: glutathione peroxidase (GPX) and glutathione s-transferase

(GST). Abbreviations for OXPHOS subunits: ATP synthase (ATP) and NADH dehydrogenase (NADH).

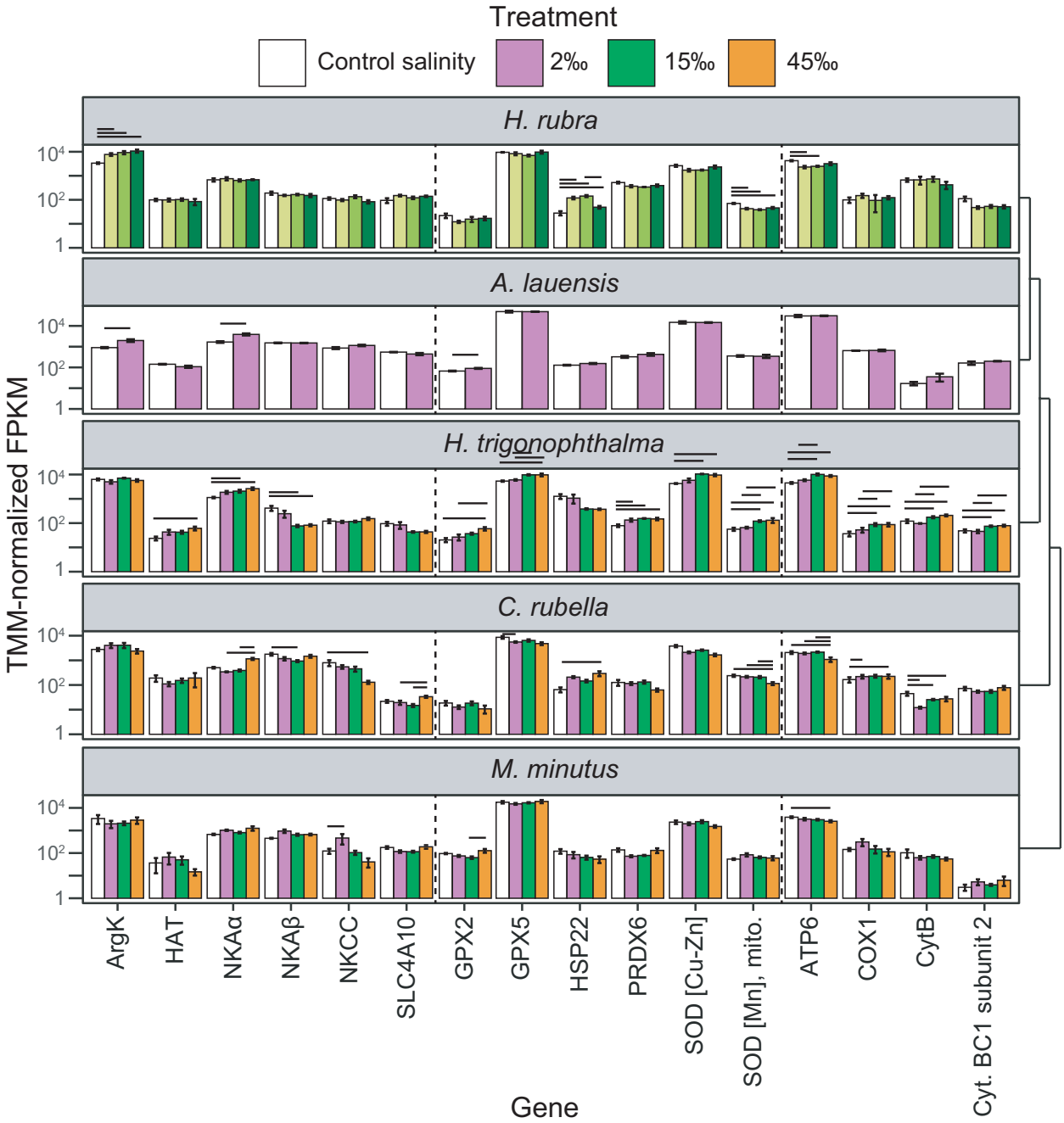

Fig. S6. Salinity-induced gene expression measured via TMM-normalized FPKM values for specific genes of interest shared in all five anchialine shrimp species examined here. Categories of genes (left to right): ion transporters, stress-related, and OXPHOS subunits. Note that for *C. rubella* 32‰ was investigated instead of 45‰. Shading used for *H. rubra* indicates that only 15‰ was investigated, but at three time points (3, 8, and 48 hrs are indicated with light to dark shading). Error bars show ± SEM. Bars above treatments indicate significant differences ($P < 0.05$) as inferred using one-way ANOVA with Tukey post-hoc analyses, although caution should be used in interpreting these

analyses with regard to whether the gene was identified as a DEG (see main text, other supplemental files).

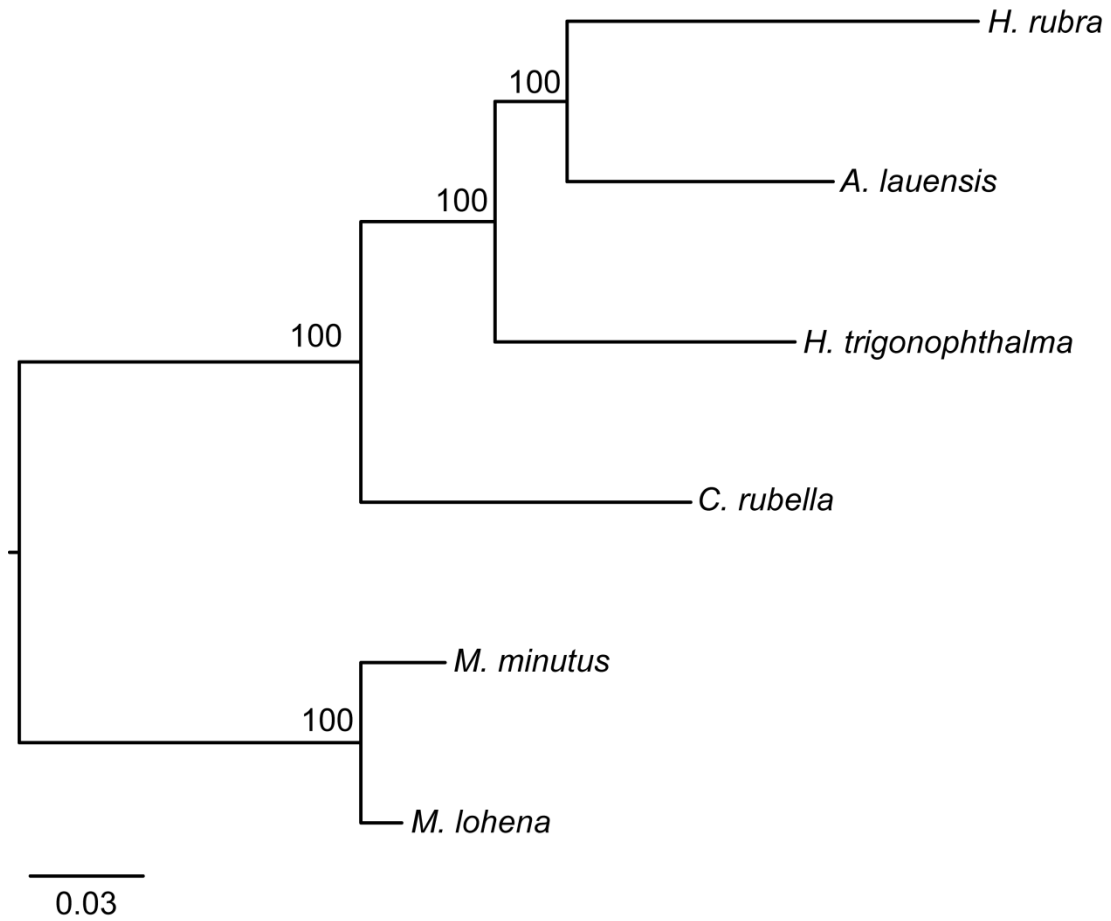

Fig. S7. Inferred evolutionary relationships based on phylogenomic data among the anchialine shrimp species examined here. Sequence alignments from amino acid sequences of orthologs described here and previously (Havird et al., 2014a) were individually generated with Clustal Omega (Sievers et al., 2011) and subsequently concatenated. Analysis was limited to orthologs recovered from all five species as well as the anchialine alpheid *Metabetaeus lohena* (Havird et al., 2014a), with the final concatenated alignment based on 758 orthologs. This phylogeny was inferred via maximum likelihood analysis and rooted as described in Fig. 1.

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