frontiers

Supplement Material

Supplement 1: PCR Protocol for MultiplexMix Reaction

Pool	Marker	Color	Length	Primer per sample
Pool A	Sy_ty_1_Hex_202_fo	Hex (green)	175 – 250	0.5 µl
	Sy_ty_1_Hex_202_re			0.5 µl
	Sy_ty_4_Hex_123_fo	Hex (green)	100 – 150	0.125µl
	Sy_ty_4_Hex_123_re			0.125µl
	Sy_ty_6_Fam_157_fo	Fam (blue)	150 – 200	0.125µl
	Sy_ty_6_Fam_157_re			0.125µl
	Sy_ty_7_Fam_101_fo	Fam (blue)	~ 100	0.125µl
	Sy_ty_7_Fam_101_re			0.125µl
Pool B	Sy_ty_11_Hex_117_fo	Fam (blue)	100 – 125	0.125µl
	Sy_ty_11_Hex_117_re			0.125µl
	Sy_ty_22_Hex_150_fo	Hex (green)	~ 150	0.125µl
	Sy_ty_22_Hex_150_re			0.125µl
	Sy_ty_23_Fam_152_fo	Fam (blue)	125 - 150	0.125µl
	Sy_ty_23_Fam_152_re			0.125µl
Pool C	Sy_ty_16_Hex_175_fo	Hex (green)	150 – 200	0.125µl
	Sy_ty_16_Hex_175_re			0.125µl
	Sy_ty_17_Fam_229_fo	Fam (blue)	200 – 250	0.25µl
	Sy_ty_17_Fam_229_re			0.25µl
	Sy_ty_21_Hex_224_fo	Hex (green)	200 – 250	0.25µl
	Sy_ty_21_Hex_224_re			0.25µl
	Sy_ty_24_Hex_115_fo	Hex (green)	100 - 150	0.125µl
	Sy_ty_24_Hex_115_re			0.125µl

PCR Protocol for MultiplexMix Reaction:

initial primer concentration: 5pmol

Pool A

10 µl reaction volume :

- 5 µl Multiplex Mastermix
- 1.75 µl Primermix Pool A
- 2.25 µl HPLC Water
- 1 µl DNA Sample

Pool B

10 µl reaction volume :
- 5 µl Multiplex Mastermix
- 0.75 µl Primermix Pool B
- 3.25 µl HPLC Water
- 1 µl DNA Sample

Pool C

10 µl reaction volume :
- 5 µl Multiplex Mastermix
- 1.5 µl Primermix Pool C
- 2.5 µl HPLC Water
- 1 µl DNA Sample

The Multiplex PCR protocol (26 cycles)

- Initial activation 95° 15 min
- Denaturing 94° 0:30 min
- Annealing 58° 1:30 min
- Extension 72° 1 min
- Final Extension 60° 30 min
- 4° ∞

Extracted DNA from fin clips of every female *Syngnathus typhle* was sampled for 11 microsatellite loci. Microsatellite loci were pooled in 3 reactions containing 3 or 4 primer pairs. Taq polymerase and the Qiagen Multiplex PCR mix was used to amplify several loci at once in the same reaction. Reactions took place with a final volume of 10 µL with 1 µL DNA extract (template) in 96-well plates.

Supplement Table S 2: Name and function of target genes used to study gene expression. Listed are all target genes from four different functional categories: general metabolism, immune response (adaptive and innate), gene regulation (acetylation and methylation) and osmoregulation. The column 'Organism' specifies, whether the gene was targeted in female (f) or juvenile (juv) samples.

Gene	Organism	Category	Gene name	Function	Reference
BROMO	juv, f	epigenetic/ activation	Histone acetyltransferase	Histone acetylation	Beemelmanns & Roth 2016
MYST	juv	epigenetic	Histone acetyltransferase	Histone acetylation	Beemelmanns & Roth 2016
Bcell.rap	juv	adaptive	B-cell receptor-associated protein	T- and B-cell regulation activity	Roth et al. 2012
CD45	juv	adaptive	CD45 (Leukocyte common antigen)	T-cell and B-cell antigen receptor signalling	Beemelmanns & Roth 2016
HIVEP2	juv	adaptive	Human immunodeficiency virus type I enhancer 2	Transcription factor, V(D)J recombination, MHC enhancer binding	Beemelmanns & Roth 2016
HIVEP3	juv	adaptive	Human immunodeficiency virus type I enhancer 3	Transcription factor, V(D)J recombination, MHC enhancer binding	Beemelmanns & Roth 2016
IgM	juv	adaptive	Immunoglobulin light chain	Antigen/pathogen recognition	Beemelmanns & Roth 2016
lymphag75	juv	adaptive	Lymphocyte antigen 75	Antigen recognition	Birrer et al. 2012
aif	juv, f	innate	Allograft inflammation factor	Inflammatory responses, allograft rejection, macrophages activation	Roth et al. 2012
apoa1	juv	innate	Apolipoprotein A1	Antimicrobial activity	Roth et al in prep.
c1	juv	innate	Recognition subcomponent C1q	Antigen-antibody complex formation	Beemelmanns & Roth 2016
c3	juv	innate	Complement component 3	Complement system activation	Birrer et al. 2012
c9	juv	innate	Complement component 9	Membrane attack complex, bacteria lysis	Roth et al. 2012
cf	juv, f	innate	Coagulation factor II	Blood clotting and inflammation response	Birrer et al. 2012
hsp60	juv, f	innate	Heat shock protein 60 kDa	Chaperone, general stress response	Roth et al. 2012
il10	juv	innate	Interleukin 10	Macrophage activity regulation	Birrer et al. 2012
il8	juv	innate	Interleukin 8	Phagocytosis, inflammation	Beemelmanns & Roth 2016

Gene	Organism	Category	Gene name	Function	Reference
kin	juv, f	innate	Kinesin	Intracellular transport	Roth et al. 2012
lectpt2	juv, f	innate	Lectin protein type II	Pathogen recognition receptor	Beemelmanns & Roth 2016
tranfe	juv, f	innate	Transferrin	Bacterial growth prevention	Beemelmanns & Roth 2016
tspo	juv, f	innate	Translocator protein	Inflammatory responses, allograft rejection, macrophage activation	Roth et al. 2012
tyroprot	juv, f	innate & adaptive	Tyroproteinkinase	Cytokine receptor signalling	Beemelmanns & Roth 2016
ddpgly	juv, f	metabolism	Ddp-glycosyltransferase	Metabolizing process (natural glyosidic linkages)	Roth et al. in prep.
g6DPH	juv, f	metabolism	Glucose 6 phosphate dehydrogenase (G6PD)	Metabolizing process (pentose phosphate pathway)	Roth et al. in prep.
ptgis	juv	metabolism	Prostaglandin I2 Synthase	Lipid and fatty acid metabolism	Roth et al. in prep.
ripop	juv, f	metabolism	Ribosomal protein	Translation process	Roth et al. in prep.
TNF	juv	metabolism	Tumor necrosis factor	Lipid metabolism	Roth et al. in prep.
ubi	juv, f	metabolism	Ubiquitin	Regulatory protein labelling for degradation	Birrer, Reusch, et al. 2012
DnMt3B	juv	epigenetic	DNA methyltransferase 3b	De novo methylation	Beemelmanns & Roth 2016
JmicPhD	juv, f	epigenetic/ silencing	Lysine-specific demethylase 5B	Histone demethylation	Beemelmanns & Roth 2016
N6admet	juv	epigenetic/ activation	N(6)- adenine-specific DNA-methyltransferase 2	DNA-methyltransferase	Beemelmanns & Roth 2016
no66	juv,f	epigenetic/ silencing	Lysine-specific histone demethylase NO66	Histone demethylation	Beemelmanns & Roth 2016
TPR	juv, f	epigenetic/ activation	Lysine-specific demethylase 6A	Histone demethylation	Beemelmanns & Roth 2016
aqp3	juv	osmoregulation	Aquaporin 3	Water and small solute channel	This study
atp1a1	juv	osmoregulation	ATPase alpha 1	Na+/K+ transporting	This study
cftr	juv	osmoregulation	Cystic fibrosis transmembrane conductance regulator	Apical membrane anion channel	This study
cldn1	juv	osmoregulation	Claudin 1	Epithelial permeability regulation	This study
hsp70	juv	osmoregulation	Heat shock protein 70 kDa	Osmotic stress response	This study

Gene	Organism	Category	Gene name	Function	Reference
kcnh8	juv	osmoregulation	Voltage gated potassium channel subfamily h member 8	Cell volume regulation	This study
mapk8ip3	juv	osmoregulation	Mitogen-activated protein kinase 8 interacting protein 3	Osmosensing	This study
nkcc2	juv	osmoregulation	Na+/K+ /2Cl cotransporter (Slc12A1)	Ion transport	This study
nr3c1	juv	osmoregulation	Nuclear Receptor Subfamily 3 Group C Member 1	Glucocorticoid receptor	This study
prl	juv	osmoregulation	Prolactin	Ion uptake promotion; ion secretion inhibition	This study
prlr	juv, f	osmoregulation	Prolactin receptor	Prolactin receptor	This study
hdac1	juv	reference/ epigenetic	Histone deacetylase 1-like	Histone deacetylation	Beemelmanns & Roth 2016
hdac3	juv	reference/ epigenetic	Histone deacetylase 3-like	Histone deacetylation	Beemelmanns & Roth 2016
ash	juv	reference/epigenetic/activation	Histone methyltransferase	Histone methyltransferase	Beemelmanns & Roth 2016
calrcul	f	innate	Calreticulin	Chaperone, promotes phagocytosis and clearance of apoptotic cells	Beemelmanns & Roth 2016
ck7	f	innate & adaptive	Chemokine 7	Chemotaxis for leukocytes, monocytes, neutrophils, blood cells	Beemelmanns & Roth 2016
dnmt1	f	epigenetic/ silencing	DNA (cytosine-5)-methyltransferase 1	copies complementary marks of newly replicated DNA, maintenance methylation	Beemelmanns & Roth 2016
dnmt3a	f	epigenetic/ silencing	DNA (Cytosine-5-)-Methyltransferase 3 Alpha	de novo modifications; essential for epigenetic changes based on environmental stress	Beemelmanns & Roth 2016,
hdac6	f	epigenetic/ silencing	Histone deacetylase 6-like	Histone deacetylation (deacetylation lysine residues of core histones)	Beemelmanns & Roth 2016
hemk2	f	epigenetic/ silencing	HemK-methyltransferase family member 2	DNA methyltransferase (N6-methyladenine)	Beemelmanns & Roth 2016,
ik-cytoine	f	adaptive & innate	Ik cytokine(RED-protein)	Inhibits interferon gamma mediated downregulation of MHCII	Beemelmanns & Roth 2016
Inf	f	innate	Interferon induced trans-membrane protein 3	Negative regulation of viral entry into host cell, antiviral response	Beemelmanns & Roth 2016

Supplement 3: Description and selection of osmoregulatory genes

Osmoregulatory target genes were selected based on their function, involvement in adaptation to marine or freshwater environment, and/or inducibility upon salinity stress. The genes aquaporin 3 (*AQP3*), claudin 1 (*CLDN1*) and mitogen-activated protein kinase 8 interacting protein 3 (*MAPK8IP3*) were recently shown to alter their expression in response to different salinity treatments in alewives *Alosa pseudoharengus* (Velotta, Wegrzyn et al. 2017). *AQP3* is a member of transmembrane channel proteins transporting water and small solutes. It plays a major role in osmoregulatory organs such as the gill, kidney, oesophagus and intestine (Cutler, Martinez et al. 2007). *CLDN1* is a cell surface component of tight junction complexes (Paris, Tonutti et al. 2008) and *MAPK8IP3* is involved in osmosensing (Velotta, Wegrzyn et al. 2017). ATPase Na^+/K^+ transporting subunit alpha 1 (*ATP1α1*), cystic fibrosis transmembrane regulator (CFTR), $Na^+/K^+/2Cl^-$ cotransporter (*NKCC*) and heat shock protein 70 kDa (HSP70) were all found to be induced upon salinity changes (Hwang and Lee 2007, Taugbol, Arntsen et al. 2014, Ronkin, Seroussi et al. 2015). ATP1α1, a membrane spanning enzyme active for example in fish gills, actively transports sodium ions (Na^+) out of and potassium ions (K^+) into a cell (Cutler, Martinez et al. 2007, Hwang and Lee 2007). *CFTR* is an apical membrane anion channel secreting *Cl*$^-$, and *NKCC2* (NKCC isoform 2; also known as solute carrier family 12 member 1) is an ion cotransporter located in the membrane (Hwang and Lee 2007) Other important ion transporters like voltage gated potassium channel genes regulate cell volume and the subfamily h member 4 (*KCNH4*) gene was shown to be involved in freshwater adaptation in the three-spined stickleback *Gasterosteus aculeatus* (Taugbol, Arntsen et al. 2014). However, as no orthologous for *KCNH4* could be identified in the *S. typhle* genome, the closely related *KCNH8* was selected for this study instead (Gutman, Chandy et al. 2005). Additionally, *ATP1α1, CFTR, KCNH4* were proposed to be under selection in animals exposed to marine-freshwater gradients (Tomy, Chang et al. 2009, Taugbol, Arntsen et al. 2014) showed that expression of the prolactin receptor gene (*PRLR*) was correlated with adaptation to freshwater. Among other functions, the hormone prolactin (*PRL*) regulates water and electrolyte balance by promoting ion intake and inhibiting ion secretion (McCormick 2001, Manzon 2002). Gene nuclear receptor subfamily 3 group C member 1 (*NR3C1*) encodes glucocorticoid receptor, which stimulates ATPase Na^+/K^+ transport and proliferation and differentiation of ion transporting chloride-cells in osmoregulatory organs (Marshall, Cozzi et al. 2005). *NR3C1* expression has also been linked to salinity stress in the euryhaline fish black porgy *Acanthopagrus schlegeli* (Tomy, Chang et al. 2009) (Table S2).

Supplement 4: Design of primers for osmoregulatory genes

Transcripts for (iv) osmoregulatory genes in *S. typhle* were identified by searching for orthologous of candidate genes in transcriptomes from other teleost fish. The transcripts were taken from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore) and using the basic local alignment search tool BLAST (Altschul, Gish et al. 1990). The osmoregulatory candidate genes were identified in the *S. typhle* transcriptome and genome (Haase, Roth et al. 2013, Roth, Solbakken et al. 2020). Additionally, the protein product of the *S. typhle* gene transcript was verified in UniProt (The UniProt Consortium 2017). *S. typhle* specific primer pairs for 11 osmoregulatory genes were designed with Primer3web (version 4.1.0; for parameters see Table S1) and, finally, primer specificity was checked with BLAST against the *S. typhle* genome and transcriptome. Whenever possible, primers were designed to span Exon-Exon boundaries, visualized in alignment viewer and editor AliView (Larsson 2014).

The efficiency of potential primers was tested with quantitative real time polymerase chain reactions (qPCR) in a dilution series (1:10, 1:20, 1:40, 1:80, 1:160, 1:320). Primer specificity was checked again by visual evaluation of the melting curves. Only candidate primer pairs with efficiency between 79 - 106 % and standard curve slope (log quantity vs. threshold cycles) between -3.2 and -4.2 were chosen for the study (Table S3).

Table S4: Osmoregulatory gene primers. Primer sequences and properties of all primers osmoregulatory gene primers used in this study. Hits on scaffold, hit annotation and PCR product sequence can be found in the data archive PANGEA.

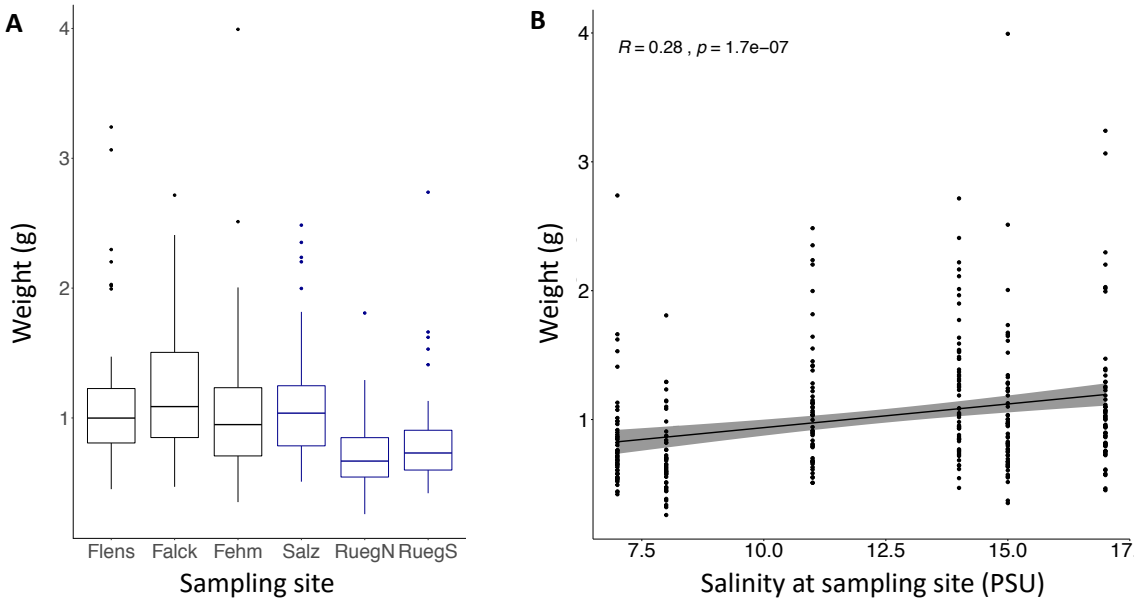
Gene	Target	Primer Sequence (forward/reverse)	PCR product length	melting temperature	efficiency [%]
aqp3	Aquaporin 3	CTTCCAGATCCGCAACCTACTG	145	60.74	105.8
		GGCAAAGTTAACCGTCAAGAACA		59.93	
atp1a1	ATPase alpha 1	GCTGGGAAGAGGGAAAGATGAT	170	59.83	85.5
		GGTCGGTTCCGTACTTCCTATG		60.22	
zcldn1	Claudin 1	AACGACAACACCAAAGCTTACG	145	59.97	89.4
		ATGATCTCCACCATGACGTACG		59.97	
mapk8ip3	Mitogen-activated protein kinase 8 interacting protein 3	CACGAAATCAAAGACGCCAAGT	130	60.03	89
		GAAGCTGTTTGTTCTCCGTCAG		59.78	
prl	Prolactin	TGGTTTTGTCCTCTTCCGCTAA	182	59.89	88.6
		GATGTCATTGCCGCCTCTGTA		60.47	
prlr	Prolactin receptor	CGGCTGGATCACACTCATCTAT	188	59.7	83.9
		TTTGGGACTCAGAGCTCCATTC		60.03	
nr3c1	Nuclear Receptor Subfamily 3 Group C Member 1	TCCCGTCAACAGGAAGTCTTTT	163	59.83	73.3
		ACGTTGGCAGTGATAGAAGAGG		60.09	
cftr	Cystic fibrosis transmembrane conductance regulator	AAGTTGGACCTCACTGACGTTT	125	60.09	86.4
		CGCATCAAACTGGGCTTCTTC		60.14	
kcnh8	Voltage gated potassium channel subfamily h member 8	CTCATCTTTGCACTCGTCAACC	181	59.84	79.2
		GCTGGCAGTTAAACAACGACAT		60.03	
hsp70	Heat shock protein 70 kDa	TGAGGGCGTTGATTTCTACACA	124	59.96	81.6
		GCCCTTGTCCATCTTAGCATCT		60.16	
nkcc2	Na+/K+ /2Cl- cotransporter (Slc12A1)	CTTCATCCTACTGGCGGCTATT	145	59.96	98
		CGACTCTTGATTCCTGAAAGCC		59.32	

Table S5: Sample size of target gene expression for females, including sampling site, salinity category at origin, salinity acclimation in the lab and number of processed females

Origin	Origin salinity	Acclimation salinity	Number of females
Flensburg	High	High	14
	High	Low	15
Falkenstein	High	High	14
	High	Low	14
Fehmarn	High	High	12
	High	Low	13
Salzhaff	Low	High	12
	Low	Low	15
Ruegen North	Low	High	11
	Low	Low	12
Ruegen South	Low	High	12
	Low	Low	11

Table S6: Pairwise F_{ST} of pipefish from different sampling sites

Comparison	FST
Falck-Fehm	0.0239
Falck-Flens	0.0162
Falck-Salz	0
Falck-RuegN	0.0064
Falck-RuegS	0
Fehm-Flens	0
Fehm-RueN	0.0102
Fehm-RuegS	0.0015
Fehm-Salz	0.0031
Flens-ReugN	0.0033
Flens-RuegS	0.0079
Flens-Salz	0
RuegN-RuegS	0
RuegN-Salz	0



Supplement figure S7: Weight of adult pipefish. The weight of pipefish (y-axis) is plotted for different sampling site (x-axis) (A), and correlated with the salinity at the different sampling sites (B).

Supplement table S7A: Analysis of variance (ANOVA) for length of adult pipefish

Fixed factors are *Sex*, *Origin salinity*, *Acclimation salinity* and *Sampling site* nested in *Origin salinity*.

Full model – Adult length	Df	Sum Sq	Mean Sq	F value	p
Sex	1	8.82	8.824	2.4	0.123
Origin salinity	1	182.00	182.004	49.4	**< 0.001***
Acclimation salinity	1	3.19	3.189	0.9	0.353
Sex: Origin salinity	1	1.44	1.443	0.4	0.531
Sex: Acclimation salinity	1	0.10	0.105	0.0	0.866
Origin salinity:Acclim salinity	1	27.27	27.269	7.4	**0.006***
Origin salinity:Sampling site	4	166.46	41.616	11.3	**< 0.001***
Sex: Origin salinity:Acclimation salinity	1	2.52	2.525	0.7	0.409
Residuals	320	1179.79	3.687		

Reduced model	Df	Sum Sq	Mean Sq	F value	p
Origin salinity	1	182.0	182.0	49.4	**< 0.001***
Acclimation salinity	1	3.2	3.2	0.8	0.353
Origin salinity:Acclimation salinity	1	26.7	27.3	7.4	**0.007**
Origin salinity:Sampling site	4	165.0	41.6	11.3	**< 0.001***
Residuals	320	1179.8	3.7		

Supplement table S7B: Post hoc test Tukey HSD for body length of adult pipefish contrasting different salinity histories, i.e. salinity origin and acclimation salinity.

	Estimate	Std. Error	t value	Pr(>|t|)
*H*L – *H*H == 0	-0.72	0.30	-2.3	0.083
*L*H – *H*H == 0	-2.08	0.32	-6.4	**< 0.001***
*L*L – *H*H == 0	-1.66	0.32	-5.2	**< 0.001***
*L*H – *H*L == 0	-1.36	0.32	-4.3	**< 0.001***
*L*L – *H*L == 0	-0.94	0.31	-3.0	**0.014**
*L*L – *L*H == 0	0.41	0.33	1.2	0.583

Supplement table S7C: Post hoc test Tukey HSD for body length of adult pipefish contrasting sampling sites.

	Estimate	Std. Error	t value	Pr(>|t|)
Fehm – Falck == 0	-0.7373	0.3566	-2.067	0.307
Flens – Falck == 0	-0.1271	0.3537	-0.359	0.999
RuegN – Falck == 0	-2.8133	0.3765	-7.471	**< 0.001***
RuegS – Falck == 0	-2.1409	0.3684	-5.811	**< 0.001***
Salz – Falck == 0	-0.4729	0.3666	-1.290	0.790
Flens – Fehm == 0	0.6102	0.3537	1.725	0.516
RuegN – Fehm == 0	-2.0760	0.3765	-5.513	**< 0.001***
RuegS – Fehm == 0	-1.4036	0.3684	-3.810	**0.002***
Salz – Fehm == 0	0.2644	0.3666	0.721	0.979
RuegN – Flens == 0	-2.6862	0.3738	-7.187	**< 0.001***
RuegS – Flens == 0	-2.0138	0.3656	-5.508	**< 0.001***
Salz – Flens == 0	-0.3459	0.3637	-0.951	0.933
ReugS – RuegN == 0	0.6724	0.3877	1.734	0.510
Salz – RuegN == 0	2.3404	0.3860	6.063	**< 0.001***
Salz – RuegS == 0	1.6680	0.3781	4.412	**< 0.001***

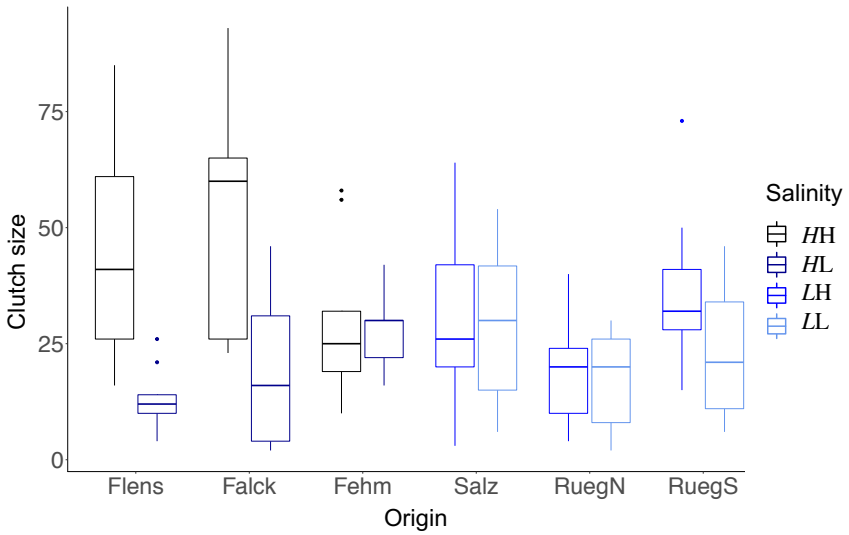
Supplement table S8A: Analysis of variance (ANOVA) for brood size of adult pipefish

Fixed factors are *Origin salinity*, *Acclimation salinity* and *Sampling site* nested in *Origin salinity*.

Full model	Df	Sum Sq	Mean Sq	F value	p
Origin salinity	1	5.0	5.0	2.0	0.160
Acclimation salinity	1	41.2	41.2	15.0	**0.001***
Av male length	1	14.6	14.6	5.8	**0.017***
Origin salinity:Acclimation salinity	1	25.6	25.6	9.0	**0.003***
Origin salinity:Sampling site	2	9.1	4.6	1.8	0.168
Origin salinity:Av male length	1	0.4	0.4	0.1	0.705
Acclimation salinity:Av male length	1	1.3	1.3	0.5	0.474
Origin sal: Acclim sal:Av male length	1	31.7	31.7	12.6	**0.001***
Residuals	109	273.6	2.5		

Supplement table 8B: Post hoc test Tukey HSD for clutch size of adult pipefish salinity histories. The italic letter represents the origin salinity (*H* - High, *L* - Low) and the second letter indicates the salinity level in the lab (H - High, L - Low).

	Estmate Std.	Error	t value	p
***H*L - *H*H == 0**	-22.4	4.6	-4.8	**< 0.001***
***L*H - *H*H == 0**	-14.0	4.5	-3.1	**0.010***
***L*L - *H*H == 0**	-16.5	4.5	-3.6	**0.003***
*L*H - *H*L == 0	8.4	4.8	1.7	0.512
*L*L - *H*L == 0	5.8	4.8	1.2	0.762
*L*L - *L*H == 0	-2.5	4.7	-0.5	0.976



Supplement Figure 8C: Clutch size is reduced at low salinity for all sampling sites

Number of juveniles (clutch size) is shown for pipefish from different origins (sampling site). Italic letters and colors indicate the salinity level at the origin of (*H*: 15 PSU, black & dark blue; *L*: 7 PSU blue & light blue). The 2nd letter indicates the salinity during breeding in the lab (H: 15 PSU, black and blue; L: 7 PSU, dark & light blue).

Table S9: PERMANOVA statistics of female gene expression for all sampling sites. A PERMANOVA was applied to gene expression (-ΔCt values) of all 155 samples for all genes and gene groups, including immune genes, which are comprised of the innate, adaptive and complement genes, as well as genes involved in metabolism, osmoregulation and epigenetics, e.g. methylation or histone modification. Results are based on euclidian distance matrices with 1000 permutations. Significant p-values are in bold. K is a factor for body size, integrating total length and weight of individuals(weight/(length^3).

	All genes			**Immune**		**Innate**		**Adaptive**		**Complement**	
	Df	F Model	Pr (>F)	F	Pr	F	Pr	F	Pr	F	Pr
Origin salinity	1	**2.6**	**0.017***	**3.1**	**0.010***	**4.0**	**0.002***	2.1	0.115	0.8	0.428
Acclimation salinity	1	1.3	0.193	1.3	0.207	1.5	0.149	0.7	0.500	0.8	0.423
K	1	1.4	0.155	1.4	0.182	1.0	0.290	0.1	0.841	2.8	0.070
Origin salinity: Acclimation salinity	1	0.7	0.667	0.8	0.569	0.9	0.469	0.3	0.788	0.3	0.729
Origin salinity:Sampling site	4	1.1	0.342	1.2	0.244	1.3	0.163	1.4	0.173	0.8	0.590
Residuals	146										

	Epigenetic			**Activating**		**Silencing**		**Metabolism**	
	Df	F	Pr	F	Pr	F	Pr	F	Pr
Origin salinity	1	0.6	0.760	0.5	0.719	0.6	0.630	1.3	0.289
Acclimation salinity	1	1.6	0.139	1.6	0.920	**2.9**	**0.044***	0.3	0.878
K	1	1.1	0.224	1.1	0.376	1.4	0.152	1.9	0.086
Origin salinity: Acclimation salinity	1	0.4	0.899	0.4	0.722	0.3	0.905	0.6	0.594
Origin salinity:Sampling site	4	0.8	0.778	0.8	0.645	0.7	0.747	1.0	0.477

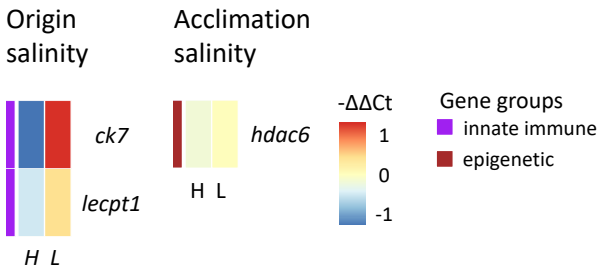


Figure S9A: Gene expression patterns of pipefish females.

Non-hierarchical gene expression heatmap for genes, which are significantly differentially expressed (-ΔΔCt) in response to *origin salinity* or *acclimation salinity*. Genes are sorted by gene groups, which are assigned to different colors (purple: innate immune, brown: epigenetic).

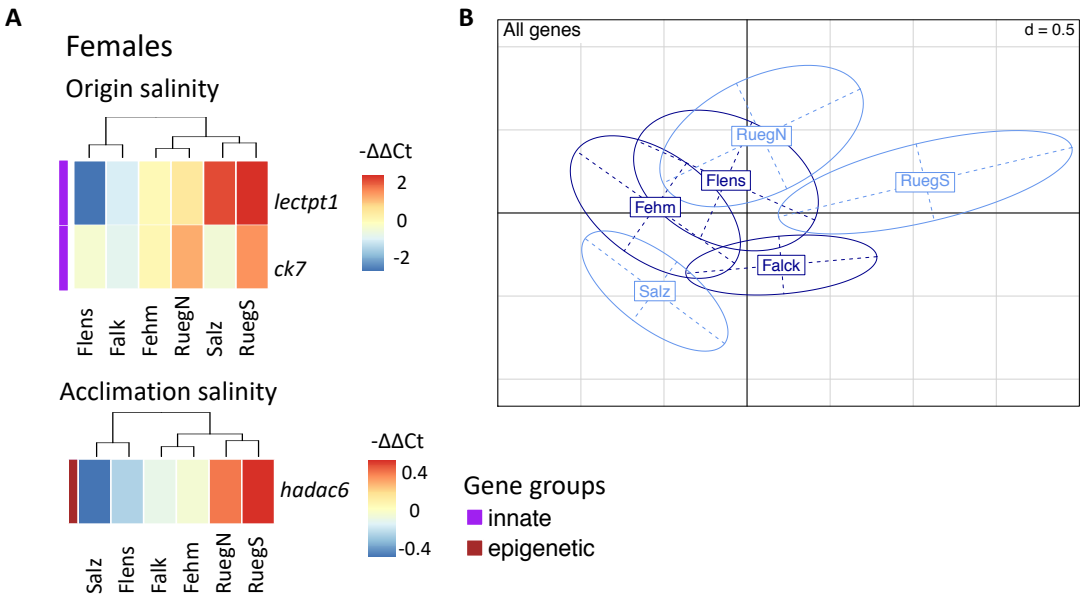


Figure 9B: Gene expression patterns of pipefish females.
A: Hierarchical gene expression heatmap for genes which are significantly differentially expressed (-ΔΔct) due to genetic background (*origin salinity*) or phenotypic plasticity (*acclimation salinity*). Genes are sorted by gene groups which are assigned to different colors (purple: innate immune, brown: epigenetic)
B: Principal component analysis (PC1: 27.5%; PC2: 19.8%) of all genes visualizing the salinity origin effect.

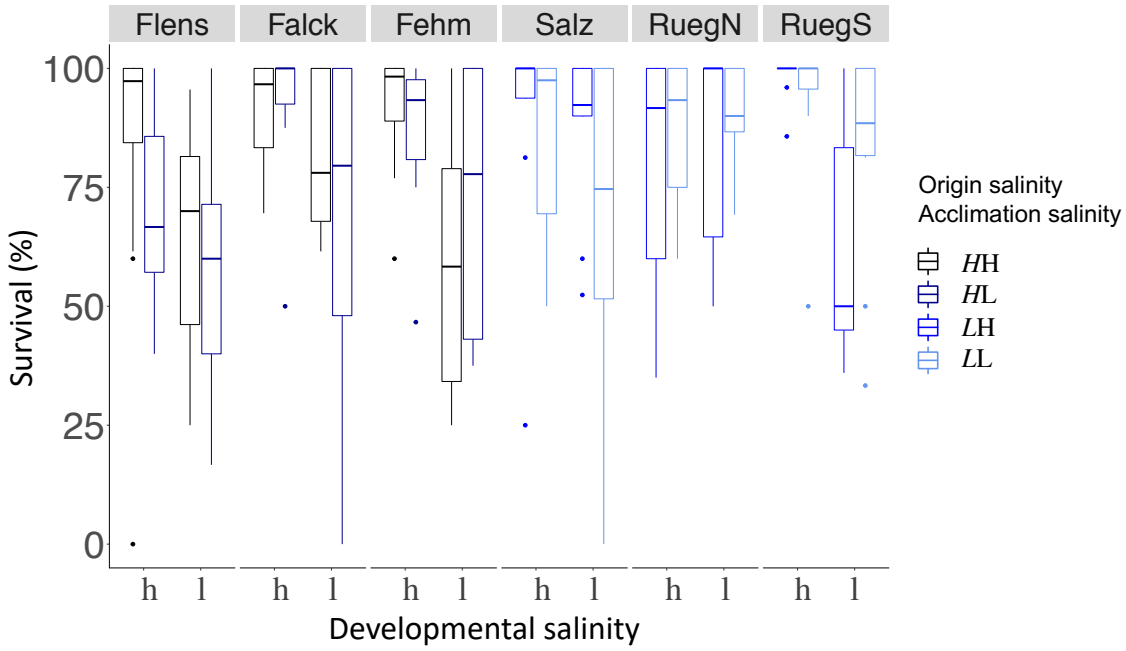


Figure S10: Juvenile survival 10 days post hatch

The percentage of juveniles that survived the first 10 days post hatch (%) are plotted on the y-axis. The x-axis indicates the *developmental salinity* (h – high/15 PSU, l – low/7PSU). Panels denote the *sampling sites*. Italic letters and colors specify the *origin salinity* (*H*: 15 PSU, black & dark blue; *L*: 7 PSU blue & light blue). The 2nd letter indicates the *acclimation salinity* (H: 15 PSU, black and blue; L: 7 PSU, dark & light blue).

Table S10A: Analysis of Deviance Table (Type II tests) for juvenile survival in the first 10 days post hatch. To test for differences between treatments we used a generalized linear model (glm). Survival was measured as ratio of dead and alive fish, i.e. dead fish were noted in one column, alive one in an other (using cbind function). Fixed factors are comprised of *origin salinity*, *acclimation salinity*, *developmental salinity* and *origin salinity:sampling site*.

	df	Chisq	p
Origin salinity	1	2.6	0.108
Acclimation salinity	1	1.4	0.236
Developmental salinity	1	**192.8**	**< 0.001***
Origin salinity:Acclimation salinity	**1**	**6.1**	**0.013***
Origin salinity:Developmental salinity	1	3.8	0.050
Acclimation salinity:Developmental salinity	1	2.8	0.094
Origin salinity:Sampling site	4	24.1	< 0.001
Origin salinigy:Acclimation salinity:Developmental salinity	1	0.0	0.970

Reduced model	Df	Chisq	p
Origin salinity	1	2.6	0.108
Acclimation salinity	1	1.4	0.236
Offspring salinity	1	**192.8**	**< 0.001***
Origin salinity: Acclimation salinity	**1**	**6.1**	**0.013***
Origin salinity:Developmental salinity	1	3.8	0.050
Acclimation salinity: Developmental salinity	1	2.8	0.094
Origin salinity:Sampling site	4	**24.1**	**< 0.001***

Table S10B: Post hoc test, Tukey HSD for juvenile survival for significant origin salinity: Acclimation salinity interaction. Italic letter indicates origin salinity (H – High or L – low) and second letter acclimation salinity (H - High or L - low).

	Estimate	Std. Error	z value	Pr (>|z|)
*H*L - *H*H	0.27	0.13	2.1	0.148
*L*H - *H*H	-0.00	0.11	-3.6	1.000
*L*L - *H*H	-0.11	0.12	-5.0	0.783
*L*H - *H*L	-0.27	0.14	-3.6	0.218
***L*L - *H*L**	**-0.38**	**0.14**	**-3.4**	**0.043***
*L*L - *L*H	-0.11	0.13	-4.7	0.842

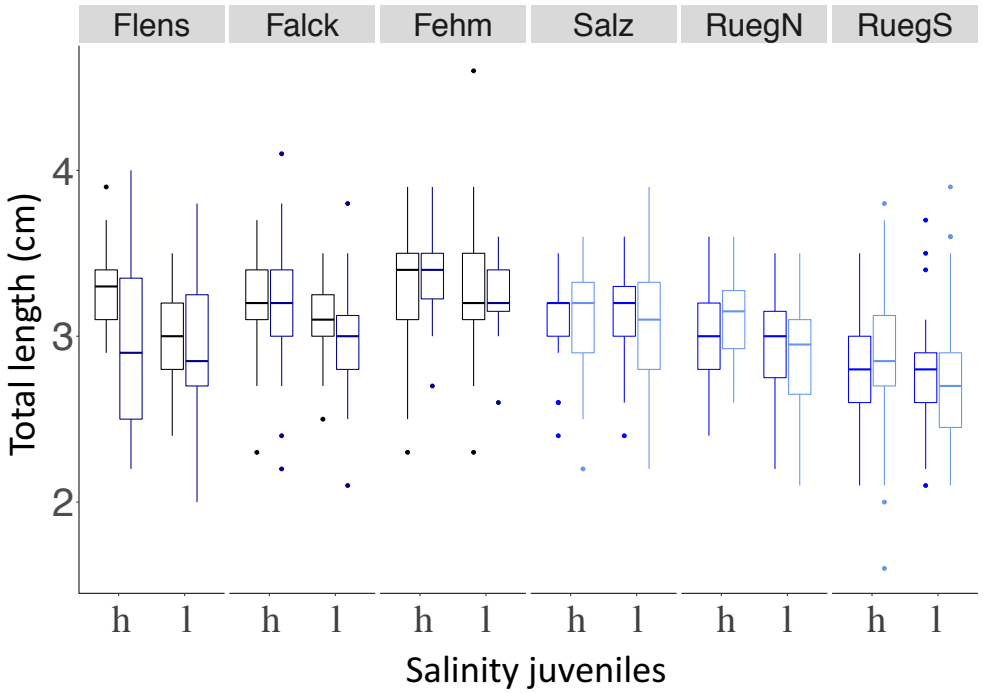


Figure S11: Juvenile size 10 days post hatch

Juvenile size (cm) 10 days post hatch is plotted on the y-axis. The x-axis indicates the *developmental salinity* (h – high/15 PSU, l – low/7PSU). Panels denote the *sampling sites*. Italic letters and colours specify the *origin salinity* (*H*: 15 PSU, black & dark blue; *L*: 7 PSU blue & light blue). The 2nd letter indicates the *acclimation salinity* (H: 15 PSU, black and blue; L: 7 PSU, dark & light blue).

Table S11: Analysis of variance (ANOVA) for juvenile size first 10 days post hatch Fixed factors are *Sex*, *Origin salinity*, *Acclimation salinity* and *Sampling site* nested in *Origin salinity*.

	Df	Sum Sq	Mean Sq	F value	p
Origin salinity	1	10.4	10.4	86.2	**< 0.001***
Acclimation salinity	1	0.3	0.3	2.2	0.136
Developmental salinity	1	2.1	2.1	17.4	**< 0.001***
Origin salinity:Acclimation salinity	1	0.2	0.2	2.2	0.192
Origin salinity:Deveoplemtal salinity	1	0.3	0.3	0.72	0.136
Acclimation salinity:Develop salinity	1	0.2	0.2	1.4	0.235
Origin salinity:Sampling site	4	10.0	2.5	20.9	**< 0.001***
Origin salinity:Acclimation salinity :Developmental salinity	1	0.1	0.1	1.2	0.270
Residuals	782	94.0	0.1		

Reduced model	Df	Sum Sq	Mean Sq	F value	p
Origin salinity	1	10.3	10.4	85.9	**< 0.001***
Acclimation salinity	1	0.3	0.3	2.2	0.136
Developmental salinity	1	2.1	2.1	17.4	**< 0.001***
Origin salinity:Sampling site	4	10.0	2.5	20.7	**< 0.001***
Residuals	786	94.8	0.1		

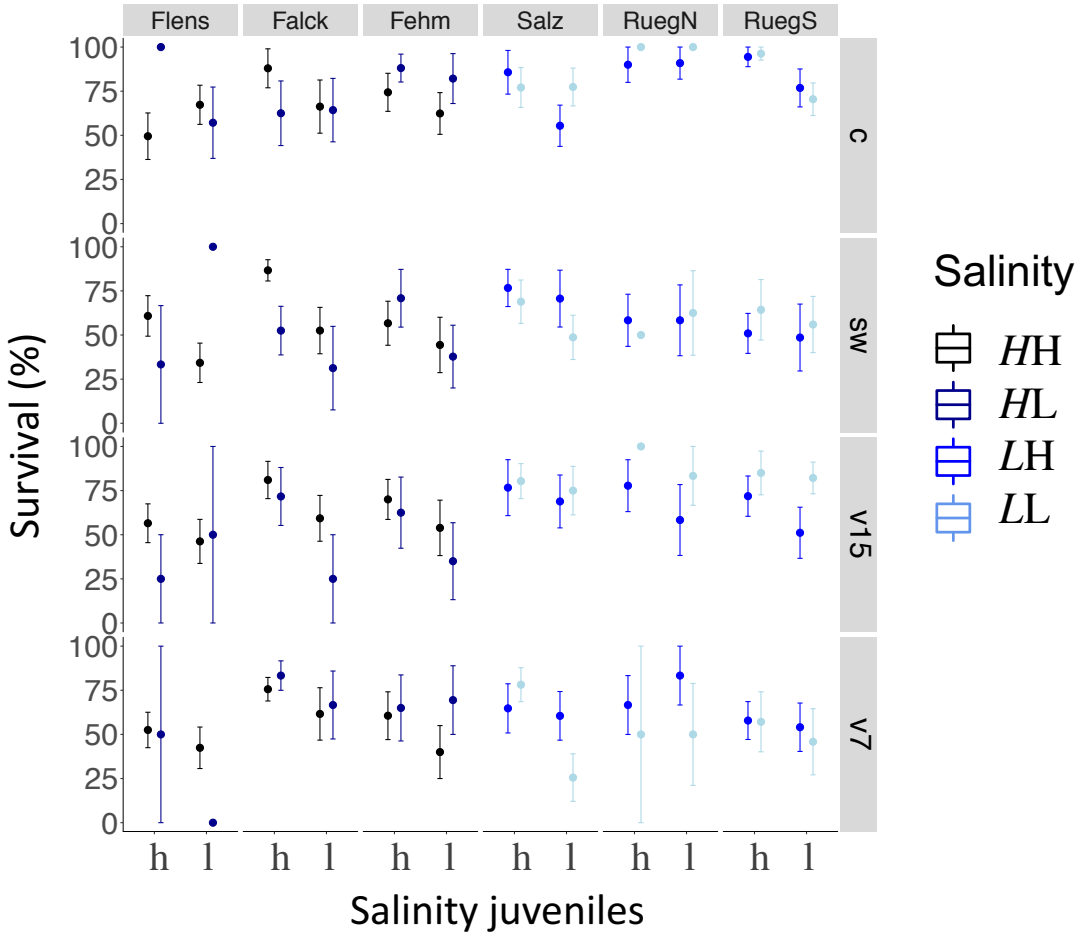


Figure S12: Juvenile survival six days post injection.

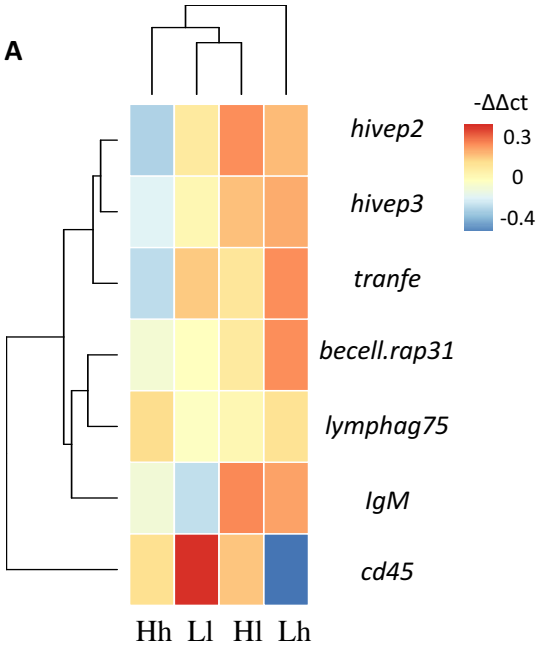
Juvenile survival six days post infection is plotted on the y axis for different *treatments*: control (c), sham injection with seawater (sw), injection with *V. alginolyticus* evolved at 15 PSU (v15) or at 7 PSU (v7). Survival is plotted for each sampling site separately (Flensburg, Falckenstein, Fehmarn, Salzhaff, Ruegen North and Ruegen South). The x-axis indicates the *developmental salinity* after hatching (h – high/15 PSU, l – low/7PSU). Italic letters and colors specify *origin salinity* of the parental generation (*H*: 15 PSU, black & dark blue; *L*: 7 PSU blue & light blue). The 2nd letter indicates *acclimation salinity* in the lab (H: 15 PSU, black and blue; L: 7 PSU, dark & light blue).

Table S12A: Analysis of Deviance Table (Type II tests) for endpoint mortality of juvenile survival after infection. To test for differences between treatments we used a generalized linear model (glm). Survival was measured as ratio of dead and alive fish. Fixed factors are comprised of *origin salinity*, *acclimation salinity*, *Offspring salinity* and *Origin salinity:Sampling site*. Table factors and interactions of the reduced model.

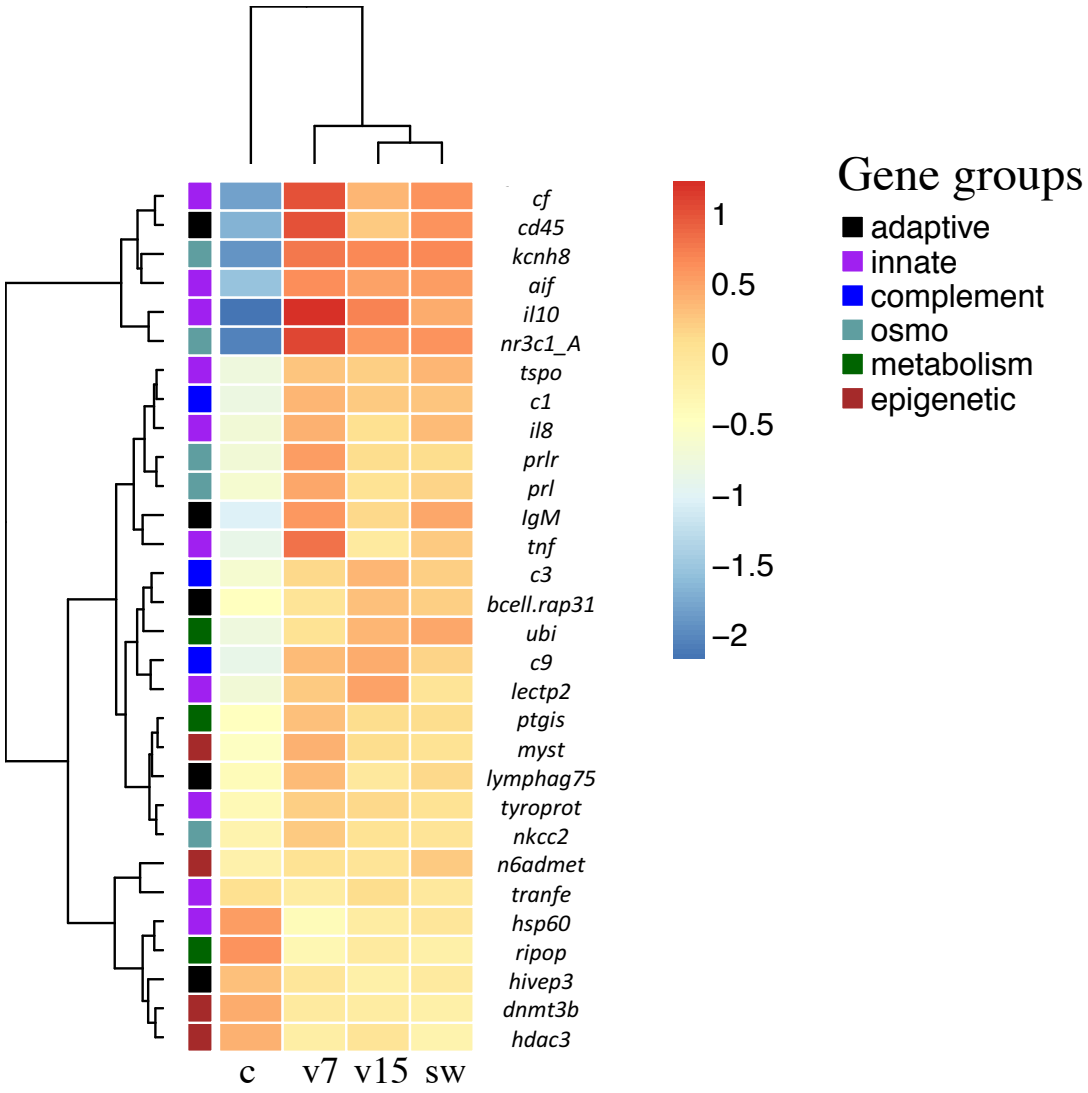
	df	Chisq	p
Origin salinity	1	9.1	**0.003***
Acclimation salinity	1	0.0	0.910
Developmental salinity	1	40.2	**< 0.001***
Treatment	3	50.9	**< 0.001***
Origin salinity:Acclimation salinity	1	1.0	0.312
Origin salinity:Treatment	1	6.6	0.084
Acclimation salinity:Treatment	1	0.3	0.959
Origin salinity:Developmental salinity	4	0.3	0.616
Parental acclimation salinity:Developmental salinity	1	0.1	0.704
Treatment:Origin salinity	3	2.3	0.514
Origin salinity:Sampling site	4	39.5	**< 0.001***
Origin salinity:Acclimation salinity:Treatment	3	13.0	**0.005***

Table S12B: Tukey HSD for juvenile survival after injection. Table shows significant and discussed post hoc pairwise comparisons of the significant interaction between *origin salinity*, *acclimation salinity* and *treatment* on juvenile mortality. Abbreviations: *L/H – origin salinity;* H/L – *acclimation salinity;* v15/v7/sw/c - *treatment (*v15: *Vibrio* evolved at 15 PSU; v7: *Vibrio* evolved at 7 PSU; sw: injection with autoclaved seawater; c: control, i.e. no injection)

	Estimate	Std. Error	z value	Pr (>|z|)
*L*Hc - *H*Hsw	-1.25	0.27	-4.7	< 0.01*
*H*Lc - *H*Hv15	-1.07	0.30	-3.6	0.030*
*L*Hc - *H*Hv15	-1.34	0.27	-5.0	< 0.01*
*L*Lc - *H*Hv15	-0.90	0.25	-3.6	0.026*
*L*Lv15 - *H*Hv15	-1.04	0.30	-3.4	0.046*
*L*Hc - *H*Hv7	-1.27	0.27	-4.7	< 0.01*
*H*Lsw - *H*Lc	1.31	0.38	3.5	0.039*
*H*Lv15 - *H*Lc	1.36	0.38	3.6	0.028*
*L*Hc - *H*Lsw	-1.59	0.35	-4.5	< 0.01*
*L*Hc - *H*Lv15	-1.64	0.36	-4.6	< 0.01*
*L*Lc - *H*Lv15	-1.19	0.34	-3.4	0.040*
*L*Lv15 - *H*Lv15	-1.33	0.38	-3.5	0.038*
*L*Hsw - *L*Hc	1.31	0.30	4.3	< 0.01*
*L*Hv7 - *L*Hc	1.16	0.30	3.9	< 0.01*
*L*Lv7 - *L*Hc	1.30	0.31	3.2	< 0.01*
*L*Hv15 - *H*Hv15	-0.39	0.25	-1.5	0.971



Supplement figure S 13: Hierarchical heat map of juvenile gene expression (-ΔΔCt) for adaptive immune genes. Capital letters indicate parental acclimation salinity (H - high and L - low). Small letters indicate *developmental salinity* (h - high, l – low).



Supplement Figure S 14: Hierarchical heat map of juvenile gene expression (-ΔΔCt) for genes differentially expressed 10 days post hatch treatment: untreated control (c), injection with *Vibrio alginolyticus* evolved at 7 PSU (v7) or *V. alginolyticus* evolved at 15 PSU (v15) and sham injection with seawater (sw).

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