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Salinity change impairs pipefish immune defence

Simone C. Birrer [a,b], Thorsten B.H. Reusch [a], Olivia Roth [a,*]

[a] Helmholtz Zentrum für Ozeanforschung Kiel (GEOMAR), Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, 24105 Kiel, Germany
[b] Aquatic Ecology, EAWAG, Überlandstrasse 133, 8600 Dübendorf, Switzerland

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ABSTRACT

Global change is associated with fast and severe alterations of environmental conditions. Superimposed onto existing salinity variations in a semi-enclosed brackish water body such as the Baltic Sea, a decrease in salinity is predicted due to increased precipitation and freshwater inflow. Moreover, we predict that heavy precipitation events will accentuate salinity fluctuations near shore.

Here, we investigated how the immune function of the broad-nosed pipefish (*Syngnathus typhle*), an ecologically important teleost with sex-role reversal, is influenced by experimentally altered salinities (control: 18 PSU, lowered: 6 PSU, increased: 30 PSU) upon infection with bacteria of the genus *Vibrio*.

Salinity changes resulted in increased activity and proliferation of immune cells. However, upon *Vibrio* infection, individuals at low salinity were unable to mount specific immune response components, both in terms of monocyte and lymphocyte cell proliferation and immune gene expression compared to pipefish kept at ambient salinities. We interpret this as resource allocation trade-off, implying that resources needed for osmoregulation under salinity stress are lacking for subsequent activation of the immune defence upon infection.

Our data suggest that composition of small coastal fish communities may change due to elevated environmental stress levels and the incorporated consequences thereof.

1. Introduction

Species interactions are likely to be affected by environmental change, in particular when two or more competing species, e.g. parasites and their hosts, or prey and predators differ in their susceptibilities towards environmental variations [1]. This can result in disrupted interaction dynamics and a distorted selection for local adaptation and direction of coevolution [2,3]. Species interactions occur on a continuum from symbiotic (as in Mycorrhiza [4]), over mutualistic (as in plants and its pollinators [5]) to parasitic (as in host–parasite interactions [6]). The two interacting species can, due to different requirements, react adversely upon changing environmental conditions, in particular in the case of two antagonistic species [7]. This affects coevolutionary dynamics, the composition of successful host and parasite genotypes and can ultimately even result in species extinctions [3]. As microparasites usually have a shorter generation time and higher mutation rates, they are supposed to adapt on shorter absolute time scales than their hosts [8]. Microparasites such as bacteria are thus expected to track environmental change with little lag in mean population fitness, and to therefore indirectly profit from global change. This can result in increased parasite virulence and replication rates, and decreased host resistance and higher fitness loss upon parasite exposure [9,10].

Global change has consequences on the mean but also the variation of many environmental parameters [11]. Several global change variables culminate in coastal areas due to their climate dependent functions as sinks for carbon and other atmospheric gases, hence, coastal areas are particularly affected by environmental variation.

Surface ocean salinity has been decreasing in many near-shore areas [12] and is expected to drop locally during the next century due to increased intensity of precipitation [13], freshwater run-off and ice cap melting [11,14]. This will further increase the likelihood of abrupt salinity decreases due to heavy rain events. This impact of global change is predicted to be especially pronounced for the Baltic Sea (Meier et al., 2006). The drop of salinity in the Baltic Sea occurs due to increasing precipitation over Scandinavian land masses and river concomitant freshwater run-off while oceanographic connection to the open Atlantic is limited [15,16]. The Baltic Sea shows a predominant salinity gradient of 3–30 Practical Salinity Units (PSU) with considerable local short-term variations [17]. Due to heavy precipitation predicted in northern Europe, the amplitude of the strong salinity variability near shore and in lagoons is suggested to be enlarged in the future [18].

* Corresponding author. Tel.: +49 431 600 4557.
E-mail address: oroth@geomar.de (O. Roth).

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Empirical data on how global change indirectly alters species interactions, in particular host immune function upon parasite infection, is scarce (but see Refs. [19–21], and the ocean as an important habitat is understudied). Among more mechanistic studies on the effect of salinity stress on animal growth and physiology [22,23], higher background parasite prevalences were observed [23]. In invertebrates, reduced resistance against *Vibrio alginolyticus* and other marine bacteria due to a decreased immune response were reported under changed salinity [24–26]. As yet, only few studies investigated the direct impact of salinity changes on host–parasite interactions in vertebrates. Catadromous fish were in their freshwater phase experimentally exposed to instantaneously different salinities [27,28]. However, the results are conflicting and difficult to interpret [29,30]. Likely reasons for this are treatment conditions that by far exceeded the natural salinity range [31], such that acute stress response cannot be disentangled from a natural salinity stress [32]. Interpretation of data further never exceeds the impact for a single species without bringing data into an evolutionary ecological context.

Hence, we here investigated experimentally how the interaction of a small coastal marine fish, *Syngnathus typhle*, and bacteria of the genus *Vibrio* is influenced by changes and fluctuations in salinity that occur in the natural habitat of pipefish and are predicted for the Baltic Sea in the near future. Decreasing salinity is expected to increase the growth [33] and virulence of *Vibrio* spp. [34] and thus to decrease host fitness due to higher severity of *Vibrio* infections. Our goal was two-fold – to assess how pipefish already now cope with the strong salinity variation that is occurring within the transitional area of the southwestern Baltic, with surface value of 10–24 practical salinity units (PSU) (Supplementary Fig. 1, depicted from Clemmesen-Bockelmann et al., in preparation). Second, this variation is predicted to amplify by increasing frequency of heavy rain events, resulting in salinity values that are likely to be beyond 10 PSU [18]. To the contrary, northern European heat waves (Schär et al Nature 2004) increase precipitation, reduce local river run-off and thus, elevate coastal salinity values. Pipefish of the species *S. typhle* were acclimatized to higher and lower salinity than ambient and artificially infected with a combination of allopatric phylotypes of *Vibrio* spp. Upon infection the activation of the immune defence was measured as a proxy for host resistance and an estimation of its physiological status. Furthermore, the expression of a set of innate and adaptive immune genes was assessed that are involved in the processes of *Vibrio* defence. The results of the present study can have important implications on how future scenarios of global change affect host–parasite interactions.

2. Materials and methods

2.1. Model organisms

Broad-nosed pipefish (*S. typhle*), are native to shallow eelgrass (*Zostera marina*) meadows of the European coastline [35]. This sex-role reversed fish plays a very important role in the seagrass environment as consumer of small mesograzers such as amphipods and isopods. With a body shape designed to mimic a seagrass leaf [36], pipefish are relatively immobile. In shallow waters, they are hence exposed to significant changes of environmental parameters and cannot avoid them by moving to a less affected part of the sea. Recent data from near-shore areas of the southwestern Baltic (Kiel and Eckernförde Bay) suggest that salinity may change daily by 4 PSU (Saderne et al., in preparation), whereas natural variations as a mean from 0 to 5 m water depth range between 11 and 23 PSU (Supplementary Fig. 1, depicted from Clemmesen-Bockelmann et al., in preparation).

Syngnathids serve as a host for *Vibrio* spp. [37], one of the most important and widespread bacterial pathogen group in the ocean, a motile, rod-shaped, gram-negative bacterium which is ubiquitous in the marine environments [38]. Species of the genus *Vibrio* are often generalist pathogens with some phylotypes inducing the disease vibriosis that results in potentially lethal external and internal haemorrhages [37]. *Vibrio* bacteria are opportunistic and many only become virulent upon stress, e.g. a scenario of environmental change [39].

2.2. Fish catching and husbandry

The fish were caught by pulling handnets through the seagrass meadow while snorkeling in water depths of one to three metres. All fish for the experiment were caught at the beginning of June 2011 in Strande, Germany (N 54°43.39′; E 10°16.93′) at a salinity of 18 PSU. Until the start of the experimental phase, the fish were kept in 200 l barrels containing filtered Baltic Sea water, oxygen stones and artificial segrass with light from 5.00 h to 22.00 h. The fish were fed twice a day with frozen and live mysid shrimps.

2.3. Experiment

During the experimental phase the fish were kept in 20 l opaque buckets at 3 different salinities. Seven buckets per salinity were used, each containing eight fish (3 salinities × 6 buckets × 8 fish = 144 fish). Lower salinities were achieved by addition of tap water and higher salinities by addition of a nitrate-free synthetic sea salt (Instant Ocean®). The fish were fed twice a day and half of the water was exchanged every second day. Salinities were adjusted within the first three days of the experiment in steps of 4 PSU (starting salinity: 18 PSU, final low salinity: 6 PSU, final high salinity: 30 PSU). The day the final salinities were reached, fish were infected with a combination of allopatric *Vibrio* strains D11K1, I11E3 (JQ598507), I2K1 (JQ598528), D1K1 (JQ598637), SH54 (JQ598711), D1K3 (JQ598638), D1E3, D12K2 (JQ598420), where the first letter is according to the origin – Italy (I), Denmark (D) or Sweden (S) – and the second letter describes the organ the bacteria was extracted from pipefish gills (K) or eggs (E), or from the surrounding water (H), genebank numbers in brackets [40]. This allopatric bacteria mixture ensured that the infection was novel to the wild caught individuals as suggested due to a strong differentiation pattern of *Vibrio* among pipefish populations [40]. Bacteria were grown according to standard procedures on *Vibrio* selective agar (Thiosulfate Citrate Bile Salts Sucrose (TCBS)) at 25 °C. The following day, a single colony was suspended in 4 ml liquid medium (Medium 1) and grown in an overnight culture at 25 °C. The next morning, strains were mixed and adjusted to a final concentration of 10^7 bacteria cells/ml in phosphate buffered saline (PBS). 24 fish per salinity were injected with 10 μl bacteria solution intraperitoneally. The remaining 24 fish per salinity were injected with the same volume of PBS, as a control for the injection procedure. The low mortality rate (one infected fish from 6 PSU died) showed that the procedure itself did not harm the fish.

Immune measurements were taken one day and eight days post infection (p.i.). On day one, twelve fish per treatment (72 in total) were sacrificed. Upon anesthization, the tail was cut and the blood was collected in RPMI-1640 cell medium (from SIGMA®). The head kidneys were removed and collected upon squashing through a cell sieve for direct immune parameter measurements. Gills were stored in RNAlater™ for subsequent gene expression measurements. The same procedures were done for the second measurement eight days p.i. Since twelve replicates were used per treatment combined with three salinities, two infection states

Table 1
Primer sequences for the examined genes used for the gene expression study. Ubiquitin was used as a reference gene for the calculations of the relative gene expressions. Primer sequences can be found under GenBank accession number HE995536–HE995542 (submission in progress).

Gene name	Symbol	Forw primer sequence 5′–3′	Rev primer sequence 5′–3′	Amplicon length in bp
Complement component 3	*c3*	AGACCCCAACATGAAGCAGT	CTCTCTCAATAGGCTCCATGC	190
Coagulation factor II receptor-like 1	*cf*	TTACAGAGCGGCCTCACC	TCCAGATGCAAAAGCAGGTC	175
Granulocyte colony-stimulating factor precursor	*grcsf*	TTGCAAGGTCTCCAGACAGA	GTTCCTCCTGCTGGCTCTT	178
Interleukin 10	*il*	TTCCTGACTGCACAGTTGCT	TCTTCGATTGTCTGGTCGAG	187
Lymphocyte antigen 75	*la*	GCGCGGATATCCTAACCAT	CATGAGTGCCATCGTACCAC	149
Toll-like receptor 5	*tlr*	CACCTGAAGAAACTCCAGCA	GGAGCGCAAATTGTTAGAGC	218
Ubiquitin	*ubi*	CGTGAAGACATTGACGGGTA	GCAGCACCAGATGAAGAGTG	202

(control and *Vibrio*) and two time points of dissection, we used a total of 144 pipefish for this experiment.

2.4. Immune assays

As first, we assessed direct immune parameters of pipefish individuals by counting the immune cells and by determining the influence of salinity and *Vibrio* infection on the activity of the immune defence. We addressed both components of the innate (monocytes) and adaptive (lymphocytes) immune function in the head kidney (central immune organ), and in the blood (peripheral immune activity). The obtained blood and head kidney cells were cleaned, concentrated and prepared for the fluorescent-assisted cell sorter (FACS) [41–43]. Cell counts (lymphocytes and monocytes) and cell cycle analyses were done using a fluorescent-assisted cell sorter (FACSCalibur by BD Biosciences) and the CellQuest Pro Software (also BD). Cells were counted according to their size and complexity, whereas cells from the innate immune system (monocytes) are larger and of higher complexity than cells from the adaptive immune system (lymphocytes) [41,42]. The cell cycle analysis was performed by determining the amount of DNA in lymphocytes by propidiumiodide staining (cells were killed beforehand in 100% ethanol). Higher DNA accounts for cells in the replicating phase as the set of chromosomes is doubling (G_{01}: resting stage, S: activated cells with doubled chromosomes, G_2M: cells in the stage of myosis and cell division). A higher proportion of cells in the replicating and cell division stage (S and G_2M) indicates an activated adaptive immune system [41,42]. To determine the activity of the innate immune cells (monocytes) in the blood, a respiratory burst assay was performed that gives an estimate of the phagocytotic activity of the cells [44]. For this, the remaining cells were diluted to achieve a concentration of 1.25×10^6 cells/ml and processed according to Ref. [41]. Respiratory burst was measured with a Tecan infinite M200 over three hours every minute. The detected intensity of luminescence reflects the rate of phagocytosis (area under curve), a parameter conserved from invertebrates to vertebrates.

2.5. Gene expression assay

In addition to the cellular measurements, we assessed the expression of several immune relevant genes to evaluate the activity of the immune system on the genetic level. Gene expression was measured with quantitative real-time polymerase chain reaction (rt-QPCR). Immune relevant genes were identified based on an expressed sequence tag (EST) library of *S. typhle* genes and comprised major immune functions: *complement component 3* (*c3*), *coagulation factor II receptor-like 1* (*cf*), *interleukin 10* (*il*), *lymphocyte antigen 75* (*la*), *toll-like receptor 5* (*tlr*) and *granulocyte colony-stimulating factor precursor* (*grcsf*). Primers flanking amplicons of a target size of 150–200 bp were designed using Primer3 v. 0.4.0 (Table 1). In addition, primers for three housekeeping genes (HKGs) – *ubiquitin* (*ubi*), *cytochrome b* (*cytb*) and *beta-actin* (*b-act*) – were designed and tested. To do so, the expression of HKG candidates was measured in samples from different salinity and *Vibrio* treatments. As gene with the most stable expression across all samples, *ubi* was chosen as baseline for further measurements using the ΔCT method. The immune functions of the single genes are explained in Table 2. In order to ensure that no genomic DNA (gDNA) would be amplified during the procedure, a gDNA digestion was performed previous to the reverse transcription of the RNA. Non-reverse transcription controls were later included in order to test for a complete removal of all gDNA. Primer efficiencies were tested using cDNA dilution series. Only primers with a slope between 0.9 and 1 and correlations $0.9 < r^2 < 1$ were considered further.

For the quantification of mRNA as a measure of gene expression we randomly chose five replicates per treatment from the first dissection date (one day after infection) and extracted the RNA from the gills with an InviTrap®Spin Tissue RNA Mini Kit from Invitek. Extraction yields were measured using a spectrophotometer (NanoDrop®ND-1000 from peQLab). For reverse transcription (RT), using the QuantiTect®Reverse Transcription Kit from Qiagen, the RNA concentration was 5 ng RNA/μl. Triplicate reaction mix (20 μl total volume) contained 0.2 μl of forward and reverse primers (500 μM), 10 μl of Fast SYBR®Green Master Mix (Applied Biosystems) and 4 μl template. For every sample, non-reverse transcribed RNA after the gDNA wipeout step was run with the primers for *ubi* to test for complete gDNA digestion and non-template controls (NTC) were used for every primer mix. The thermo cycling protocol was 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s, followed by a dissociation phase to verify the melting curves, where the samples were slowly heated from 65 °C to 90 °C. In the StepOne Plus Real-Time PCR System (Applied Biosystems) we manually set the CT threshold to 0.5. Samples that

Table 2
Names and functions of immune genes assessed during gene expression measurements.

Immune gene	Function	Innate or adaptive?	Reference
c3	Activation of the complement system, opsonisation of bacteria	Innate	NCBI
cf	Involved in thrombotic response: blood is clotted to prevent blood loss	Innate	Wikipedia
grcsf	Support of proliferation and differentiation of haematopoietic cells	Innate	Tabbara, 1993
il	Anti-inflammatory cytokine	Mainly innate	NCBI
la	Endocytic receptor, reduces proliferation of B lymphocytes	Adaptive	UniProtKB
tlr	Recognition of PAMPs and mediation of production of necessary cytokines	Innate	NCBI
ubi	Labels regulatory proteins for degradation	None	Hershko et al., 1998

were not amplified (one sample) or samples of which the RNA negative control was amplified (seven samples) were excluded from the analyses.

For all measurements, the standard deviations (SD) and means were calculated. Further, the coefficient of variation (CV) was determined as $CV = SD_{CT}/CT_{mean}$. Samples for which $CV > 0.04$, were excluded from the analysis (Bookout & Mangelsdorf, 2003).

The relative gene expression of every gene, $-\Delta CT$, was calculated using the formula $-\Delta CT = CT_{ubi\ mean} - CT$. With these values all plots and all statistical analyses were conducted. Furthermore, a value for $-\Delta\Delta CT$ (formula: $-\Delta\Delta CT = -\Delta CT_{Vibrio} - -\Delta CT_{control}$) was calculated for every gene in every individual. Applying the formula $RQ = 2^{-\Delta\Delta CT}$ to these values yielded the relative quantity (RQ) of every gene, i.e. the amount of the gene under treatment conditions (here *Vibrio* infection) is RQ times the level of the gene in the control treatment [45].

2.6. Statistics

Data were analysed using univariate and multivariate analysis of variance (ANOVA/MANOVA). Data were tested for normality using the Shapiro–Wilk test and transformed if appropriate. In all analyses, salinity (6, 18 or 30 PSU), infection status (control or *Vibrio*) and timepoint of dissection were included as fixed factors (using R version 2.11.1). All significant ANOVAs were followed by post-hoc Tukey's HSD tests. For the SIMPER tests that were conducted with the gene expression data (using Primer v6), all negative values had to be eliminated from the data set, because a fourth root transformation was done to enable the comparison of very high and very low values [46]. The negative $-\Delta CT$ values were eliminated by the addition of a constant to all the values of the target gene. Missing values were replaced by the mean expression value of the affected gene (dummy variables).

For analysis of the lymphocyte activity of individuals that were dissected eight days p.i., the results of plate three were excluded due to a measurement problem of the FACS. This reduced the sample size from $N = 72$ to 48 for the second measurement date.

2.7. Correlation of gene expression and immune function

We correlated the direct immune measurements with the gene expression data in order to determine whether or not particular immune genes can be used as indicators for direct immune performance. For these Pearson correlations, the mean $-\Delta CT$ values of the different genes for every treatment were used. No experiment-wise error correction was performed, because these correlation tests were considered to be explorative.

3. Results

3.1. Salinity effect

Salinity treatments affected immune activity in pipefish. Individuals kept under low salinity revealed the highest monocyte counts in the head kidney (Table 3 (monocytes): salinity, $p = 0.011$; posthoc: Supplementary Table S1; Figs. 1 and 2). The monocyte counts in the blood were lower at ambient salinity (18 PSU) compared to the low (6 PSU) and the high (30 PSU) salinity treatment (Table 3 (monocytes): salinity, $p < 0.001$; posthoc: Supplementary Table S1; Figs. 1 and 2). The amount of monocytes correlated negatively with the amount of lymphocytes, hence, the opposite of the above discussed pattern was found for lymphocytes, the main cells of the adaptive immune system.

The proportion of lymphocytes in the head kidney that were in the mitosis and cell division phase (S & G_2M) was highest in the ambient salinity (18 PSU) one day post infection (Table 3 (proliferating lymphocytes): salinity × timepoint, $p = 0.002$; posthoc: Supplementary Table S1; Figs. 1 and 2). Eight days post infection (p.i.) all three salinity treatments attained similar values. In the blood, fish from the low salinity treatment (6 PSU) had an upregulated lymphocyte activity compared to the fish from the ambient salinity. The high salinity resulted in an intermediate lymphocyte activity between the low and intermediate salinity (Table 3 (proliferating lymphocytes): salinity, $p = 0.026$; posthoc: Supplementary Table S1; Figs. 1 and 2).

Table 3
The results of the ANOVA to assess the effect of *Vibrio*, salinity, timepoint and the interactions on monocyte counts and lymphocyte proliferation in the head kidney and in the blood. Timepoint 1 corresponds to measurements taken one day after infection, whereas timepoint 2 represents the data from eight days after infection. Posthoc tests (Tukey HSD) can be found in Supplementary Table S1.

ANOVA immune parameters					
	df	Head kidney		Blood	
		F	*p*	*F*	*p*
Monocytes					
Salinity	2	4.667	0.011	9.845	<0.001
Vibrio	1	1.044	0.309	0.132	0.717
Timepoint	1	26.230	<0.001	20.055	<0.001
Salinity × Vibrio	2	0.018	0.982	0.390	0.678
Salinity × timepoint	2	2.746	0.068	0.463	0.630
Vibrio × timepoint	1	4.212	0.042	0.232	0.631
Salinity × Vibrio × timepoint	2	0.703	0.497	0.489	0.615
Proliferating lymphocytes					
Salinity	2	2.834	0.063	3.763	0.026
Vibrio	1	18.298	<0.001	4.587	0.035
Timepoint	1	109.919	<0.001	0.019	0.892
Salinity × Vibrio	2	0.341	0.712	0.662	0.518
Salinity × timepoint	2	6.797	0.002	1.340	0.251
Vibrio × timepoint	1	0.685	0.410	2.018	0.158
Salinity × Vibrio × timepoint	2	0.071	0.931	0.792	0.456
Respiratory burst					
Salinity	2			6.276	0.003
Vibrio	1			1.136	0.290
Timepoint	1			17.817	<0.001
Salinity × Vibrio	2			0.001	0.999
Salinity × timepoint	2			13.391	<0.001
Vibrio × timepoint	1			0.761	0.386
Salinity × Vibrio × timepoint	2			0.262	0.771

Respiratory monocyte burst was significantly affected by salinity, timepoint and by an interaction among salinity and timepoint. One day p.i. the pipefish in the high salinity treatment upregulated their phagocytosis rate such that fish from high salinity had significantly higher phagocytosis values than both fish from low and ambient salinity (Table 3 (respiratory burst): salinity, $p = 0.003$; timepoint, $p < 0.001$; salinity × timepoint, $p < 0.001$; posthoc: Supplementary Table S1; Figs. 1 and 2). Salinity affected immune gene expression significantly (Table 4, MANOVA, salinity, $p = 0.003$). In detail, the expression of the genes *grcsf* and *il* significantly decreased with increasing salinity (Table 4, ANOVA, *grcsf*, $p < 0001$; *il*, $p = 0.038$, posthoc tests underneath; Fig. 3). In the two genes that contributed to the salinity effect (*grcsf* and *il*) differences between the low and high salinities were found. Fish from the high salinity on average had the lowest expression of the three genes. *grcsf* expression in the high salinity treatment was even lower than in the ambient salinity.

3.2. Effects of Vibrio infection

Individuals kept at ambient and high salinity displayed higher monocyte counts one day after *Vibrio* infection in the head kidney (Table 3 (monocytes): Vibrio × timepoint, $p = 0.042$; posthoc: Supplementary Table S1; Figs. 1 and 2), suggesting an activation of the innate immune system upon *Vibrio* exposure. The fish from low

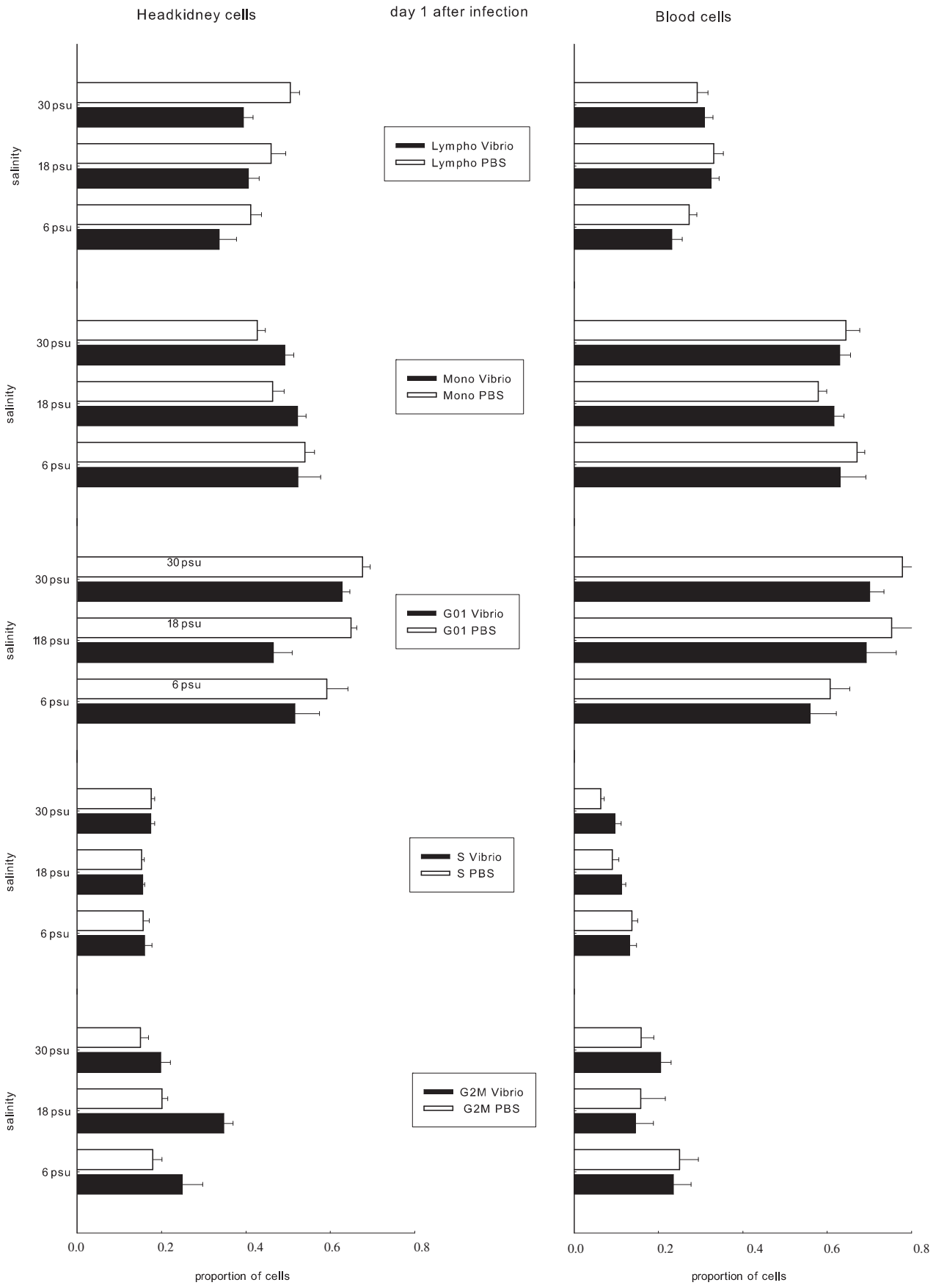

Fig. 1. Evaluation of influences of salinity (6 PSU, 18 PSU, 30 PSU) and *Vibrio* (*Vibrio*: black; PBS: white) effects on direct immune traits such as proportion of lymphocytes and monocytes, and lymphocyte proliferation (G_{01}, S, G_2M phase) one day after infection. Bars show mean plus standard error.

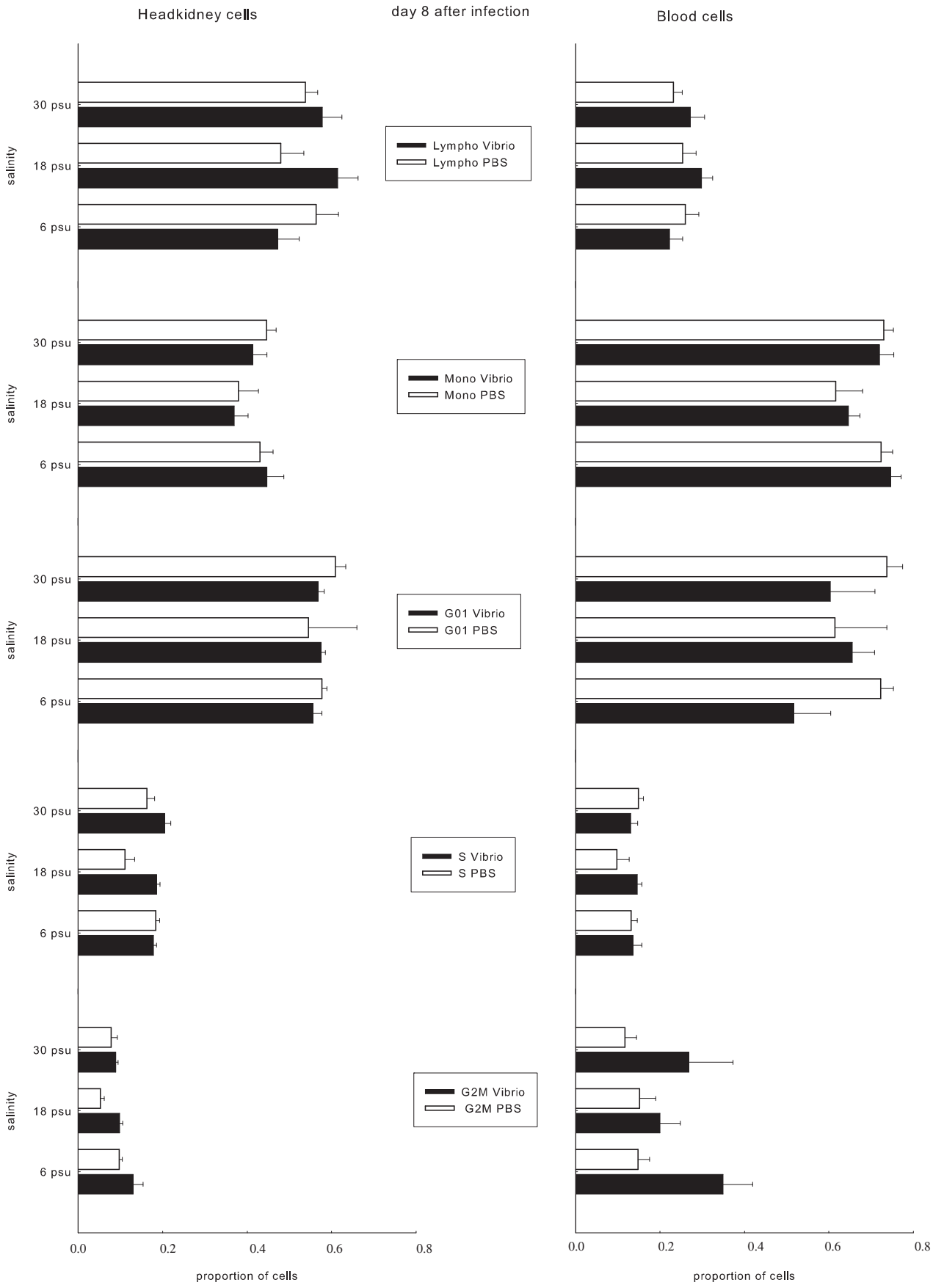

Fig. 2. Evaluation of influences of salinity (6 PSU, 18 PSU, 30 PSU) and *Vibrio* (*Vibrio*: black; PBS: white) effects on direct immune traits such as proportion of lymphocytes and monocytes, and lymphocyte proliferation (G_{01}, S, G_2M phase) eight days after infection. Bars show mean plus standard error.

Table 4
Gene expression data of seven immune genes assessed overall in a MANOVA and in single ANOVAs.

Immune genes	MANOVA																
ANOVA	df	Overall		*c3*		*cf*		*grcsf*		*il*		*la*		*tlr*		*tnf*	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Salinity	2	7.731	0.003	1.751	0.228	0.510	0.617	22.064	<0.001	4.822	0.038	1.061	0.386	0.256	0.780	6.806	0.016
Vibrio	1	85.044	0.002	0.013	0.913	6.220	0.034	90.545	<0.001	5.405	0.045	9.108	0.015	1.614	0.236	0.839	0.384
Salinity × Vibrio	2	5.868	0.008	0.065	0.937	0.129	0.881	11.683	0.003	0.092	0.003	2.467	0.140	0.131	0.878	1.052	0.389
Posthoc (Tukey)																	
18 − 6									0.145		0.094						0.339
30 − 6									<0.001		<0.001						0.182
30 − 18									0.014		0.005						0.926
Vibrio − control							0.010		<0.001		0.002		0.310				
18, ctrl − 6, ctrl									0.949								
30, ctrl − 6, ctrl									0.193								
6, Vibrio − 6, ctrl									0.014								
18, Vibrio − 6, ctrl									0.107								
30, Vibrio − 6, ctrl									0.999								
30, ctrl − 18, ctrl									0.564								
6, Vibrio − 18, ctrl									0.001								
18, Vibrio − 18, ctrl									0.013								
30, Vibrio − 18, ctrl									0.966								
6, Vibrio − 30, ctrl									<0.001								
18, Vibrio − 30, ctrl									<0.001								
30, Vibrio − 30, ctrl									0.145								
18, Vibrio − 6, Vibrio									0.961								
30, Vibrio − 6, Vibrio									0.003								
30, Vibrio − 18, Vibrio									0.040								

salinity, however, did not up-regulate their innate immune system further under *Vibrio* exposure.

In the head kidney, all infected fish showed a higher lymphocyte activity (Table 3 (proliferating lymphocytes): Vibrio, $p = 0.001$; posthoc: Supplementary Table S1; Figs. 1 and 2). This upregulation was particularly pronounced in the ambient salinity treatment one day p.i. In the blood, *Vibrio* infection resulted in a higher lymphocyte activity in the salinity stress treatments, only eight days p.i. However, *Vibrio* infection neither affected the monocyte activity in the head kidney nor in the blood (Table 3 (respiratory burst): Vibrio, $p = 0.290$; Figs. 1 and 2).

The genes *cf*, *grcsf*, *il* and *la* expression were upregulated upon *Vibrio* infection (Table 4, MANOVA: Vibrio, $p = 0.002$; ANOVA: *cf*, $p = 0.034$; *grcsf*, $p < 0.001$; *il*, $p = 0.045$; *la*, $p = 0.015$; Fig. 3). The significant interaction between salinity and *Vibrio* was solely due to the gene *grcsf* (Table 4, MANOVA: Vibrio × salinity, $p = 0.008$; ANOVA: *grcsf*, Vibrio × salinity, $p = 0.003$; Fig. 3). Whereas the levels of gene expressions were not significantly different in the uninfected fish among the three salinities, the overall immune gene upregulation upon *Vibrio* infection decreased with increasing salinity, i.e. fish from low and ambient salinity had a higher *grcsf* expression than fish from high salinity.

The relative quantity (RQ) values for the tested genes in the different salinities are shown in Table 5. In the low salinity the infected fish downregulated the *c3* expression, whereas in the other salinities the expression was upregulated upon *Vibrio* challenge. The genes *grcsf* and *il* were considerably upregulated in the low and ambient salinity treatments, *il* was additionally upregulated in fish from the high salinity treatment. The gene *tlr* was also substantially influenced by the bacterial infection, but only in the intermediate and high salinities.

3.3. Correlation between cellular measurements and gene expression

To connect the biological relevance of gene expression patterns and the immune parameters, correlation analyses were done for all individuals that were used in both assays (−ΔCT values were correlated with direct immune parameters). The gene *c3* was positively correlated with the number of lymphocytes in the blood $r_{c3} = 0.4824$, $p_{c3} = 0.0126$. Furthermore, *grcsf* and *il* expression was negatively correlated with the number of lymphocytes in the head kidney ($r_{grcsf} = 0.5931$, $p_{grcsf} = 0.0023$ and $r_{il} = -0.4732$, $p_{il} = 0.00127$). Additionally, they had a positive correlation with the activity of the lymphocytes ($r_{grcsf} = 0.5644$, $p_{grcsf} = 0.0041$ and $r_{il} = 0.5434$, $p_{il} = 0.0034$). We further found a positive correlation between the gene expression of *cf* and the activity of the lymphocytes in the head kidney ($r_{cf} = 0.4639$, $p_{cf} = 0.0297$) (Table 6, Fig. 4).

4. Discussion

This experiment investigated immune reactions of the pipefish *S typhle* to a realistic scenario of salinity change and increasing variability predicted for the Baltic Sea in the coming decades [15]. As challenge, we used infections with a cocktail of allopatric *Vibrio* phylotypes. Under salinity change, uninfected pipefish induced their innate immune defence (monocyte counts & phagocytosis activity) and decreased the activity of their adaptive immune defence (reduced lymphocyte proliferation). Pipefish kept at high salinity upregulated innate immunity only on the short scale, e.g. monocyte counts were only higher in the periphery (blood monocytes), and phagocytosis activity was only increased at the first measurement day. In contrast, pipefish at low salinity induced their monocyte production both in the head kidney and in the periphery, which shows that the activation took place on a longer time scale.

This rather unspecific stress response apparently prevented further activation of the adaptive immune defence upon *Vibrio* infection. One day p.i., the lymphocyte activity in the head kidney was only induced in fish that were kept at ambient salinity. Fish from ambient salinity already ceased their lymphocyte activity eight days post infection, implying a successful clearance of *Vibrio*. In contrast, only by that time, the lymphocytes in fish from low and high salinity proliferated. Such a delayed activation of adaptive immune defence under salinity stress may be due to a resource allocation trade-off. Costly investment into immune defence is

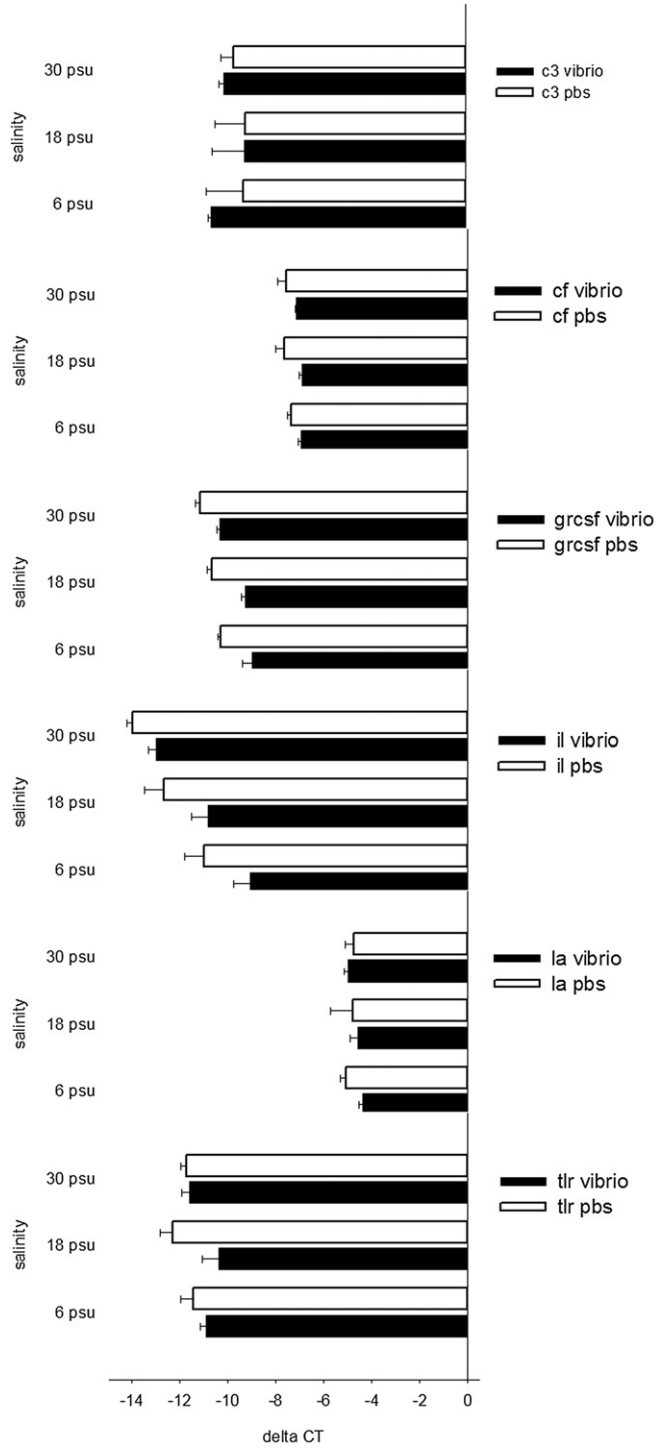

Fig. 3. Gene expression profiles upon *Vibrio* (black) or sham control exposure (white) and salinity change (6 PSU, 18 PSU, 30 PSU) for all measured immune genes. Gene expression data are standardized to the housekeeping gene *ubiquitin* and are depicted as −ΔCT. Bars show mean plus standard error.

traded-off against the investment into other life-history traits for example related to growth and reproduction [47,48]. Whereas pipefish exposed to high salinity experienced a low stress level and were still able to slightly up-regulate their immune defence upon infection, pipefish exposed to low salinity invested maximal energy into salinity stress handling and were only able to activate their specific immune defence eight days p.i.

Table 5
Relative quantity (RQ) values of the tested genes for the mature fish in three different salinities. Values compare the extent of *Vibrio* expression over the control expression.

RQ values			
Gene	6 PSU	18 PSU	30 PSU
c3	0.401	1000	269
cf	1.344	1.485	1.528
grcsf	2.751	2.510	1.678
il	4.870	7.651	2.158
la	1.517	1.523	1.031
tlr	1.512	7.557	2.134
tnf	0.761	1.782	1.046

The higher lymphocyte proliferation upon *Vibrio* exposure coincided with an immediate upregulation of the monocyte counts, whereas higher lymphocyte counts were only identified eight days after infection. This illustrates the complex interaction of the innate and adaptive vertebrate immune system. The innate immune system as a first line of defence is immediately available as opposed to the adaptive immune system that needs time before reaching full efficiency, visible here by delayed lymphocyte maturation and proliferation.

Our results suggest that pipefish activate their innate immune system upon salinity change as a general stress response. This possibly resulted in a decreased energy budget available for an activation of the specific immune responses upon subsequent bacterial infections, followed by a delayed adaptive immune response under salinity stress. This could imply a negative effect on host fitness and could cause host mortality due to increased time available for bacterial replication. Our data are in accordance with earlier studies in Ref. [49] that suggested an increased disease prevalence at lower salinity due to salinity stress and a decreased immune response.

The immune dynamics detected in this study were further supported by the gene expression patterns that were, however, only measured one day p.i. for logistic constraints (sample size restrictions). Pipefish from the ambient salinity treatment upregulated immune gene expression (*cf*, *grcsf*, *il* and *la*) upon a bacterial infection which presumably enabled them to cope with the infection more efficiently than fish kept at low and high salinity. Upregulation of *cf* expression increases blood clotting in order to inhibit blood loss and to decrease the flow speed and thus to counteract a distribution of the bacteria in the body. *cf* positively correlated with the activity of the lymphocytes in the head kidney. Hence, when the lymphocytes were active in the head kidney and prepared to fight the pathogen, *cf* was expressed and active to prevent meanwhile spreading of the pathogen in the host until the lymphocytes are transported into the blood. *tlr* expression was only induced after infection at ambient and high salinity, similar to the temporal patterns in *c3* expression that was, however, downregulated at low salinity. This suggests that the activation of the complement system which initiates the opsonization of bacteria is decreased under low salinity. *c3* links the innate to the adaptive immune system and its expression is positively correlated with lymphocyte counts in the blood what supports the decreased early adaptive immune activity if infected pipefish were kept at low salinity. The expression of *grcsf* that is responsible for the stimulation of monocyte proliferation was in general upregulated upon a *Vibrio* infection. It was only overall downregulated at high salinity compared to the ambient salinity, similar to *il* that was also downregulated at high salinity which indicates a high risk of inflammation at high salinity. *grcsf* and *il* correlated negatively with the amount of adaptive immune cells (lymphocytes) in the head

Table 6
Results of the Pearson's correlation calculations that correlate immune gene expression values with direct cellular immune parameters. Bold numbers indicate significant correlations (upper line: *p*-value, lower line: correlation coefficient *r*). *N* gives number of fish used for the measurements.

p-value	Head kidney			Blood			
r	Lymphocytes ($N = 29$)	Monocytes ($N = 29$)	G_2M ($N = 29$)	Lymphocytes ($N = 29$)	Monocytes ($N = 29$)	G_2M ($N = 29$)	Luminescence ($N = 29$)
c3 ($N = 26$)	0.3903	0.1246	0.7804	**0.0126**	**0.0371**	0.4946	0.6920
	0.1758	−0.3090	0.0575	**0.4824**	**−0.4109**	−0.1402	−0.0816
cf ($N = 22$)	0.5411	0.4602	**0.0297**	0.8614	0.5804	0.8208	0.6993
	−0.1377	0.1660	**0.4639**	−0.0395	0.1247	0.0513	0.0873
grcsf ($N = 24$)	**0.0023**	**6.38E-04**	**0.0041**	0.1841	0.0738	0.3728	0.0803
	−0.5931	**0.6467**	**0.5644**	−0.2806	0.3716	0.1904	−0.3641
il ($N = 27$)	**0.0127**	**0.0038**	**0.0034**	0.2052	0.0571	0.5130	0.0561
	−0.4732	**0.5377**	**0.5434**	−0.2518	0.3705	0.1316	−0.3719
la ($N = 12$)	0.8018	0.7223	0.4580	0.3283	0.4823	0.4884	0.8365
	0.0583	−0.0825	0.1712	0.2243	−0.1622	0.1600	−0.0480
tlr ($N = 27$)	0.0758	0.3083	0.0779	0.6515	0.8577	0.9835	0.5295
	−0.3474	0.2036	0.3451	0.0911	0.0362	−0.0042	−0.1265
tnf ($N = 27$)	0.3168	0.5521	0.7289	0.8893	0.4816	0.6633	0.6290
	−0.2001	0.1197	0.0699	−0.0281	0.1414	−0.0878	−0.0816

kidney, and at the same time positively with an increased activity of these cells. This implies that a decrease in amount of lymphocytes acts as a trigger for the expression of *grcsf* and *il*, which then in turn activate the proliferation of cells.

Summarized, the results of this study suggest that pipefish activate their immune system upon salinity stress and that this in turn can reduce the potential of parasite defence. Environmental stress can, however, result in different immune activation patterns

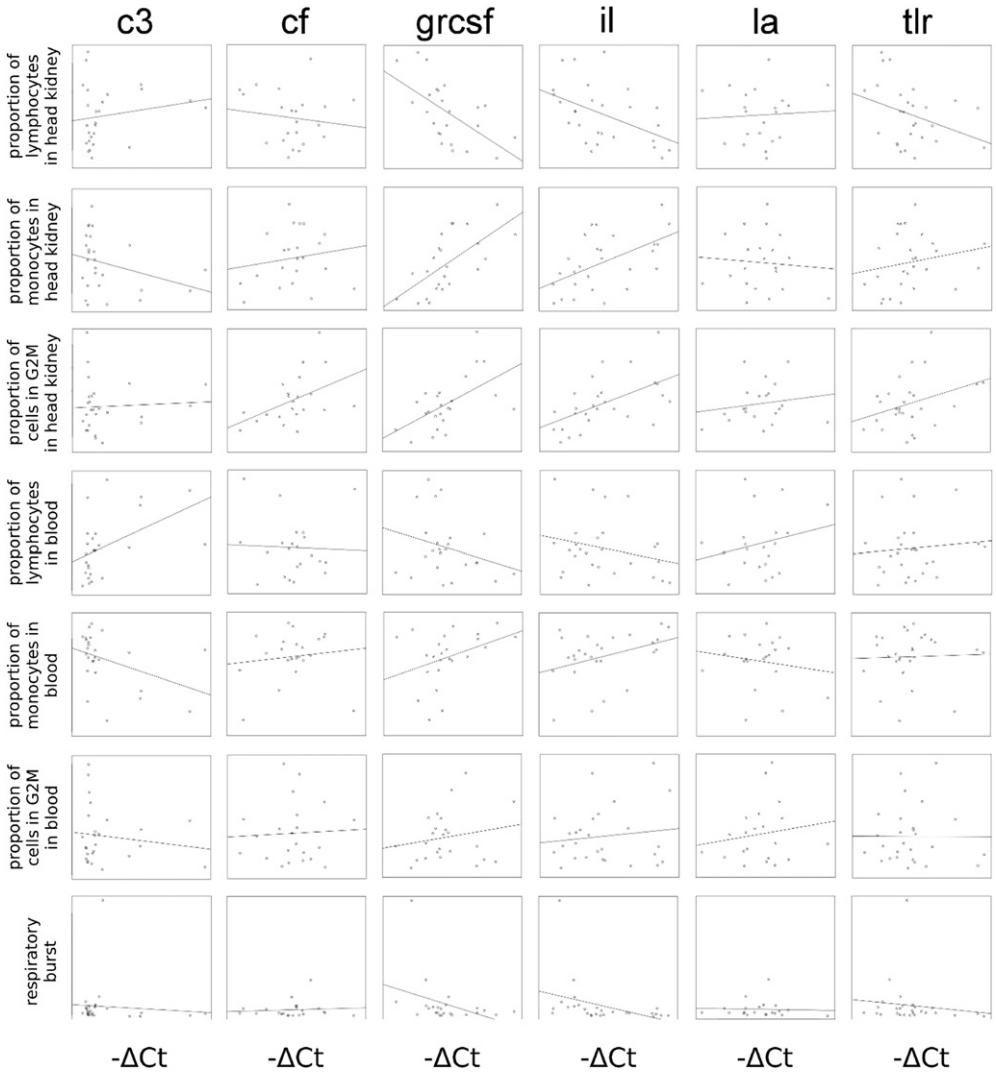

Fig. 4. Correlation plots of immune genes with direct cellular immune traits assessed. Immune gene values are given as −ΔCT.

and dynamics. Whereas exposure to low salinity triggered a long lasting activation of the innate immune system, the high salinity treatment activated the immune system only on the short run but yielded more active cells that were fighting the infection in the periphery. A long lasting immune reaction is costly and potentially results in a decreased defence against the bacterial infection.

For the next decades a drop of salinity in the Baltic Sea is predicted [15,16]. In addition, the pronounced salinity variation in the southwestern Baltic Sea is predicted to be amplified during the next decades [18]. According to our data this may compromise the immune reaction of fish hosts against widespread bacterial infections. We here detected immune defence dynamics to be slower and less efficient if fish experience a change in salinity. This can ultimately result in reduced parasite resistance and negatively affect fitness.

Finally, we have to consider that salinity change is only one part of a future global change scenario in the Baltic Sea. To understand the full impact of the changing environment on pipefish, empirical data must be collected that investigate the effects of a combination of environmental changes. Only this will enable us to predict the impact of the interactive effects of a future global change scenario on parasite defence, life-history and biodiversity in the ocean.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2012.08.028.

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