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HOSTS ARE AHEAD IN A MARINE HOST–PARASITE COEVOLUTIONARY ARMS RACE: INNATE IMMUNE SYSTEM ADAPTATION IN PIPEFISH *SYNGNATHUS TYPHIE* AGAINST *VIBRIO* PHYLOTYPES

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Microparasites have a higher evolutionary potential than their hosts due to an increased mutation rate and a shorter generation time that usually results in parasites being locally adapted to their sympatric hosts. This pattern may not apply to generalist pathogens as adaptation to sympatric host genotypes is disadvantageous due to a narrowing of the host range, in particular under strong gene flow among host populations. Under this scenario, we predict that the immune defense of hosts reveals adaptation to locally common pathogen phylotypes. This was tested in four host populations of the pipefish *Syngnathus typhie* and associated bacteria of the genus *Vibrio*. We investigated the population divergence among host and bacteria populations and verified that gene flow is higher among host populations than among parasite populations. Next, we experimentally assessed the strength of innate immune defense of pipefish hosts using in vitro assays that measured antimicrobial activity of blood plasma against sympatric and allopatric *Vibrio* phylotypes. Pipefish plasma displays stronger antimicrobial activity against sympatric *Vibrio* phylotypes compared to allopatric ones. This suggests that host defense is genetically adapted against local bacteria with a broad and unspecialized host spectrum, a situation that is typical for marine systems with weak host population structure.

KEY WORDS: **Gene flow, genetic diversity, host–parasite interaction, immune defense, local adaptation.**

Parasitism is one of the strongest evolutionary forces across the living world with the potential to rapidly change the genotypic composition of hosts, as well as their distribution and abundance (Thompson 1988; Ebert and Hamilton 1996; Keesing et al. 2010). By exploiting host resources and reducing their fitness, parasites impose selection on hosts to evolve an efficient immune defense (Sorci et al. 1997). Such adaptation and counteradaptation is the hallmark of host–parasite coevolution, that is, the reciprocal evolution of the parasite's virulence and the host's resistance (Hamilton 1980). Adaptation to common host genotypes leads to an increased fitness of a parasite genotype (Kaltz and Shykoff 1998; Lively and Dybdahl 2000; Kawecki and Ebert 2004) and to a rise of its frequency in the population (Clay and Kover 1996). Selection is thus suggested to work against allopatric and to benefit sympatric host–parasite combinations (Kawecki and Ebert 2004). However, due to the generally smaller genomes and higher mutation rates of parasites and their often shorter generation time, a time lag between adaptation and counteradaptation of the host

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and the parasite is expected (Hamilton et al. 1990). Local parasite adaptation has been shown in various systems. Outstanding examples are the snail-trematode system *Potamopyrgus antipodarum* and *Microphallus* sp. (Lively and Dybdahl 2000; Lively et al. 2004) and *Daphnia magna* and its microparasites (Ebert 1994; Refardt and Ebert 2007) where the host lags behind the adaptation of the parasite (Kaltz and Shykoff 1998). However, this scenario is strongly affected by the genetic structure of host and parasite populations (Gandon et al. 1996; Abida et al. 2010). Empirical studies, which used phage-bacteria or invertebrate models, suggested parasite migration (gene flow among parasite populations) to increase parasite local adaptation (Lively and Dybdahl 2000; Morgan et al. 2005; Vogwill et al. 2010). On the other hand, reduced selection for parasite local adaptation is expected when gene flow and migration is lower among parasite than among host populations (Gandon 2002; Hoeksema and Forde 2008), favoring a stronger selection pressure for host local adaptation (Oppliger et al. 1999; Gandon et al. 2008), in particular when population sizes are relatively small (Gandon and Nuismer 2009).

Parasite local adaptation can also be viewed as increasing specialization of parasites/pathogens to a narrow spectrum of host species or even genotypes (Little et al. 2006; Magalhaes et al. 2009). The benefits of more efficient resource utilization under such specialization are traded-off against fitness costs of a reduced infection range, for example, transmission cost (Eizaguirre and Lenz 2010). This can ultimately curb the evolution of more specialized parasites (Kawecki 1998) and, for a generalist parasite, implies a reduced selection for local adaptation. This should particularly apply if the host species is rarely encountered (Gandon and Michalakis 2002; Lajeunesse and Forbes 2002; Kniskern et al. 2010). Hence, both infections with multihost (generalist) parasites and higher gene flow among host populations should be conducive for host local adaptation, and in combination, the effects may even be additive (Delmotte et al. 1999; Kaltz et al. 1999). The potential for local adaptation to a generalist parasite has only rarely been addressed (Kniskern et al. 2010), and we are not aware of any study in the ocean, although here host populations are genetically often highly connected (Palumbi 1992). Further, the most abundant and diverse marine group of pathogenic bacteria of the genus *Vibrio* is opportunistic and has a broad host range (Thompson et al. 2004). In particular for vertebrates in the marine realm, where above conditions apply, host local adaptation can thus be expected.

Host–parasite interactions can be assessed by measuring infectivity patterns on the parasite side or by assessing attributes of the immune defense on the host side (Dybdahl and Storfer 2003). As immune defense is costly (Sheldon and Verhulst 1996), the host needs to adjust its optimal defense strategy to the pervailing parasite genotypes (Boots and Bowers 2004). In vertebrates, parasite-induced selection typically results in a combination of the innate and the adaptive immune pathways that most effectively prevent infection. The innate immune system of vertebrates consists of a fast and unspecific self to nonself recognition without known establishment of memory (Tort and Mackenzie 2003). The adaptive immune system, on the other hand, is highly specific and reveals stronger and faster defense upon secondary exposure to the same parasite genotype (immune memory) (Zinkernagel et al. 1996).

To investigate population divergence and local adaptation in a host–parasite system, we throughout used the broad-nosed pipefish *Syngnathus typhie* and associated bacteria of the genus *Vibrio*. We tested whether hosts reveal a stronger innate immune response against sympatric compared to allopatric pathogen genotypes. Pipefish and *Vibrio* phylotypes (during the manuscript isolates are named phylotypes as species determination is unclear in bacteria) were sampled along a North-South gradient at four locations (Sweden, Denmark, Germany, and Italy). First, as a prerequisite for a scenario of host local adaptation, we assessed the population structure of *S. typhie* with 13 microsatellite markers and the community structure of the matching *Vibrio* phylotypes. We then designed an in vitro experiment to test whether strength of plasma antimicrobial activity, as essential function of the innate immune response, differs among allopatric and sympatric *Vibrio* phylotypes measured in inhibition zone assays.

Material and Methods

MODEL SYSTEM

The parasite

Vibrio are the most abundant and diverse opportunistic pathogens in the marine world (Thompson et al. 2004), and thus on the globe, as 73% is covered by oceans. Bacteria of the genus *Vibrio* occur in a continuum from pathogenic, over opportunistic to symbiotic and free living (Wilson and Hastings 1998; Thompson et al. 2004). Their pathogenic potential is tightly coupled to environmental conditions, in particular temperature and salinity, with higher temperature and lower salinity promoting infection and virulence (Martin et al. 2002; Rosenberg and Ben-Hain 2002). Beyond a critical bacterial cell density virulence genes are expressed (quorum sensing) (Thompson et al. 2004). Quorum sensing makes it highly unpredictable for a host whether associated *Vibrio* will turn pathogenic, which makes a clear-cut assignment of *Vibrio* spp. to a-pathogenic and pathogenic phylotypes difficult (Belkin and Colwell 2006). In its virulent form, *Vibrio* has a strong impact on its widespread invertebrate and vertebrate hosts and is further a common disease agent in aquaculture and aquaria fish (Hjeltnes and Roberts 1993; Austin and Austin 2007).

The host

The broad-nosed pipefish, *S. typhie*, is distributed along a North-South gradient, from Northern Norway to Southern Portugal

(Wilson and Veragut 2010), and can only be found in shallow seagrass meadows (Froese and Pauly 2008). The majority of bacteria isolated on Syngnathids belong to Vibrionacea (Balcazar et al. 2010b). *Vibrio harveyi* species cause mass mortalities in captive bread sea horses with almost 90% mortality (Alcaide et al. 2001). Phylotypes of *V. anginolyticus* and *V. splendidus* have recently been isolated from seahorses with signs of infection (Balcazar et al. 2010a).

SAMPLING

Pipefish *S. typhie* individuals and *Vibrio* phylotypes were sampled at four European locations along a North-South gradient (Swedish population: Fiskebäckskil (Sweden, 58°24′80″N; 11°44′62″E); Danish population: Lemvig (Denmark, 56°56′30″N; 8°29′61″E); German Population: Wackerballig (Germany, 54°75′57″N; 9°87′66″E); Italian Population: Venice Lagoon (Italy, 45°25′20″N; 12°27′85″E). Water temperatures at all locations ranged from 11 to 13°C. Pipefish were sampled by snorkelling in a water depth from 0 to 3 m and caught with hand nets. Fish were brought to the laboratory within 24 h in coolers, fin clips for DNA extraction were taken upon anestisation with MS-222, and the fish were slowly adapted to summer conditions (18°C and 16:8 h light regime). Salinities were kept constant and according to the site pipefish were caught (15–29 psu). The pipefish were acclimatized to laboratory conditions between 10 and 16 days before the use for the experiment.

MICROSATELLITE MARKER DEVELOPMENT

RNA was extracted from gills and head kidneys of five pipefish with a RNeasy (Qiagen, Germany) kit and pooled to a total of 25 μg RNA. A normalized cDNA library was constructed and cDNA was used for 454 sequencing on a Roche GS FLX. About 500,000 reads were obtained (David Haase, Olivia Roth, Thorsten B.H. Reusch, unpubl. data). Microsatellite regions were identified using the software Tandem repeat finder (http://tandem.bu.edu/). Primers were designed for regions with di-, tri- and tetranucleotide repeats using Primer 3 (Rozen and Skaletsky 2000). Microsatellite sequences and associated primer pairs are given in Table S1, and were deposited in GenBank under accession numbers JQ598279–JQ598290.

GENOTYPING OF HOST POPULATION STRUCTURE

To assess the population genetic structure of the hosts, we genotyped 13 microsatellite loci in 26 pipefish individuals per population. Three microsatellite loci were taken from an earlier study (Jones et al. 1999), and 10 microsatellites were newly developed (deposited under Genbank accession numbers JQ598279–JQ598290).

DNA from finclips was extracted using a 96-well DNA extraction kit (Invitek) according to the manufacturer's protocol. Multiplex-polymerase chain reaction (PCR) was performed for a combination of the primer sets Typh 4/16 and for Typh 12 (Jones et al. 1999). The newly developed microsatellite primers were multiplexed as described in Genbank where also the PCR protocols can be found.

All microsatellite data were size called and scored using the software GeneMarker ® (http://www.softgenetics.com/GeneMarker.html). Based on the obtained multilocus genotypes, Wright's F_{ST}-values (Weir and Cockerham 1984) were calculated by using 1000 permutations with the software Genetix (Belkhir et al. 1998), to test for population structure. As a second test for population structure, we employed a Bayesian cluster analysis implemented in the software STRUCTURE (Pritchard et al. 2000). The number of most likely clusters was inferred using the method of Evanno et al. (2000). Deviations from Hardy–Weinberg equilibrium was assessed using the software Genetix (Belkhir et al. 1998), whereas the presence of null alleles was tested with MICROCHECKER (Van Oosterhout et al. 2004).

PHYLOTYPE IDENTIFICATION OF *VIBRIO* ISOLATES

To assess the population genetic structure of the parasite, we measured diversity in *Vibrio* obtained from a subsample of 10–16 pipefish per location (Denmark: 16 pipefish, Italy: 16 pipefish, Germany: 10 pipefish, Sweden: 10 pipefish). To this end, swabs from the whole intestines, liver, kidney, gills and, if present, eggs were placed on *Vibrio* selective Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) plates (Fluka Analytica, Buchs, CH) immediately after capture (< 3 h). In addition, we took five water samples per location. All plates were incubated at 15°C for 48 h. Separate organs of single fish individuals were assigned to be infected with *Vibrio* when at least one colony appeared within three days. Five colonies of each positive plate (one plate per organ and fish) were picked and grown in Nutrient agar 1.5% NaCl (1000-mL distilled water, 5.0 g peptone, 3.0 g meat extract) at 25°C over night under constant shaking for molecular characterization of *Vibrio* phylotypes.

The genetic affiliation (phylotype) of the *Vibrio*-cultures was determined using the DNA sequence of three genes, *recA*, *pyrH*, and *16S rDNA* (Table S1). *Vibrio* cells were lysed (3 min, 100°C) prior to PCR-amplification after Thompson et al. (2005). PCR products were purified wit GeneJet™ Gel extraction Kit (Fermentas GmbH, St. Leon-Rot, Germany). Sequences were obtained using standard Sanger sequencing with BigDye version 3.1 chemistry (Applied Biosystems) on an ABI capillary sequencer according to standard protocols.

PHYLOGENETIC ANALYSES

The taxonomic and phylogenetic affiliation of local *Vibrio* communities was guided by phylogenetic analyses based on the DNA sequences obtained for three genes individually and on a

Table 1. The total number of distinct *Vibrio* phylotypes isolated from pipefish *Syngnathus typhie* from four different locations (Sweden, Denmark, Germany, and Italy) that were used for the phylogeny are displayed here according to the gene sequences (*rec A*, *pyr H*, 16S *rDNA*). In addition also the differing sequences are mentioned (different) and the number of reference strains used in the phylogeny.

	Total number of bacteria phylotypes		
	♯sequenced	♯different	♯reference
rec A	401	102	35
pyr H	318	94	36
16S r DNA	148	75	10

concatenated alignment (Table 1; see also Table S2 for GenBank accession numbers). Contigs between forward and reverse sequenced reads (> 300 bp) were assembled with CodonCode Aligner (version 3.6.1) and then aligned by eye together with reference sequences obtained from GenBank (Table S3). Phylogenetic analyses were performed with PAUP* (version 4.0b10 for Unix; (Swofford 2003) and GARLI (version 0.951 (Zwickl 2006). The best fitting model of sequence evolution for the maximum likelihood searches was determined with jModeltest (version 12.2.0) and applied in heuristic searches with PAUP*. A maximumlikelihood bootstrap analysis with 100 pseudoreplicates was done with GARLI. Haplotype genealogies for all genes and 16S rDNA have been drawn with Adobe© Illustrator© CS3 (version 13.0.0) according to the maximum likelihood trees generated as described above and following the procedure described by Salzburger et al. (2011). Recombination search was done with Genetic Algorithms for Recombination Detection (GARD) (Pond et al. 2006a,b).

BACTERIAL COMMUNITY ANALYSIS

To test for differences in the structure of *Vibrio* phylotypes from the four different locations (Denmark, Sweden, Italy, and Germany), as multivariate statistics an analysis of similarity (ANOSIM) has been computed using Primer 6 (Clarke and Warwick 1994). The ANOSIM has been run for each gene separately and for 999 permutations.

EXPERIMENTAL DESIGN FOR IN VITRO LOCAL ADAPTATION TEST

To assess adaptation of the host immune response to sympatric versus allopatric *Vibrio* phylotypes, we performed in vitro experiments with the response variable antimicrobial activity measured as size of an inhibition zone invoked by fish plasma. We picked 12 *Vibrio* phylotypes from four locations at random (Italy $n = 4$; Denmark $n = 3$; Sweden $n = 3$; Germany $n = 2$) and tested host immune response against them. To this end, blood plasma from all four populations of pipefish was collected and its potential to inhibit bacterial growth was assessed in an inhibition zone assay (fish used for assays: Italy $n = 11$; Denmark $n = 45$; Sweden $n = 30$; Germany $n = 45$). Pipefish were killed with MS222. Blood was collected in a heparinized capillary (Na-heparinized, Brand GMBH + Co. KG, Wertheim, Germany) and centrifuged at full speed for 2 min to separate the plasma from cellular blood compartments. Plasma was stored at −20°C until further processing within less than seven days. From each fish between 15 and 20 μl blood was collected resulting in plasma volumes between 6 and 10 μl. For one inhibition zone assay, 2-μl plasma is needed implying that not every pipefish could be tested against each bacterium phylotype in an inhibition zone assay. Bacteria phylotypes were thus randomly chosen for every fish, resulting in three to five bacteria phylotypes tested for every individual. Pipefish were sampled regardless of their sex or reproduction status.

INHIBITION ZONE ASSAY TO DETERMINE ANTIMICROBIAL ACTIVITY

Vibrio phylotypes were taken from a frozen glycerol stock (40% glycerol) and grown in nutrient 1, 1.5% NaCl medium at 25°C over night. Inhibition zone plates were prepared with nutrient 1, 1.5% NaCl agar. Liquid agar was cooled to 40°C and liquid cultures of the *Vibrio* phylotypes were added to a final concentration of 10^7 cells/mL. Petri dishes were cast with 4 mL of bacteria medium. Using a Pasteur pipette, eight holes were stamped per agar plate, in each of the holes 2 μl of thawed plasma was added. Each petri dish included one tetracycline sample (concentration: 1 mg/mL) as positive control, along with distilled water as a negative control, petri dishes were incubated at 25°C for 16–20 h. Diameters of inhibition zones were measured to the nearest 0.1 mm and standardized to the size of the antibiotic inhibition within plates.

TEST FOR IMMUNE MEMORY UPON BACTERIA EXPOSURE

Wild-caught pipefish used in the in vitro experiment are likely to already have been exposed to *Vibrio* phylotypes in the field that may be similar to those used in our assays. To exclude that such immune priming influenced our experiment, we verified whether potential differences in inhibition zone between sympatric and allopatric phylotypes revealed immune memory upon a secondary exposure, which would be a prerequisite for such priming. Pipefish from one location (Wackerballig, Germany) were exposed to an allopatric phylotype of *Vibrio* to induce an immune response against a novel bacterium. To do so, *S. typhie* individuals ($n = 10$ per treatment) were either injected with 50 μl of a bacteria solution of 10^6 cells/mL dissolved in PBS of the Italian *Vibrio* phylotype I11Ma2, or with 50 μl of PBS as control. Individuals were kept singly for two weeks in buckets of 2.5 L and fed daily with live and frozen mysids. After two weeks,

they were killed with MS222 and their plasma was collected and frozen as described above. We performed in vitro inhibition zone assays against the same *Vibrio* phylotype I11Ma2. Larger inhibition zones of *Vibrio* exposed pipefish would indicate confounding effects of immune memory.

SPECIFICITY INDEX AND STATISTICAL ANALYSIS

In order to test for host immunological local adaptation in the in vitro inhibition zone assays, we calculated specificity indices after Schulte et al. (2011). For each bacterium phylotype, a "bacterium phylotype specificity index" was calculated to estimate the strength of bacterium inhibition within a bacterium strain and between host populations. This was done by subtracting the mean of all allopatric inhibition measurements from the mean of all sympatric inhibition measurements per phylotype. A positive index suggests that the plasma of the sympatric pipefish inhibited growth of the bacterium more than the allopatric pipefish plasma, although a negative index indicates the opposite. In a similar way, "host population specificity indices" were calculated to assess the inhibition of bacteria growth within the same host population that differ between bacteria phylotypes, this time subtracting allopatric inhibition measurements from the mean sympatric values. As statistical test for the "bacterium phylotype specificity index" analyses of variance (ANOVAs) (with location as fixed factor, size of inhibition zone as response variable) were performed for each of the 12 bacteria phylotypes and alpha-values were corrected using the false discovery rate (FDR) approach (Benjamini and Hochberg 1995). A general linear model could not be computed because every pipefish individual carried only enough plasma to be tested against three to four bacterial strains (i.e., 6–10 μl). The "host population specificity index" was analysed with four single one sided *t*-tests, alpha-values were FDR corrected.

Results

PIPEFISH POPULATION STRUCTURE

Allele frequencies obtained at 13 microsatellite loci revealed that pipefish populations were genetically differentiated into a Southern (Italy) and a Northern genetic cluster (Germany, Denmark, Sweden). This applied to both the calculation of pairwise F_{ST} values and a Bayesian population clustering method implemented in the software STRUCTURE that revealed $k = 2$ clusters as likely number of subgroups (Pritchard et al. 2000). We did not detect any null-alleles and all populations were in Hardy–Weinberg equilibrium (Belkhir et al. 1998).

VIBRIO COMMUNITY

The *Vibrio* community composition differed strongly among all four sites. An ANOSIM of the *Vibrio* phylotypes showed among all three genes (*rec A*, *pyr H*, and *16S rDNA*) that locations differed significantly in their phylotype composition (Table 2). The similarity percentage analysis (SIMPER) within locations suggested the degree of uniformity of a *Vibrio* phylotype community (Table S4). *Vibrio* sequences were tested for recombination using GARD (Pond et al. 2006a,b). We did not detect any recombination in *16S rDNA* and *pyr H*. However, we detected one recombination breakpoint at base 364 in the gene *rec A* at base pair 364 ($P = 0.0002$). This did not affect our analysis because we only used the phylogeny to guide our taxonomy.

Table 2. **An analysis of similarity (ANOSIM) for all sequences used in the *Vibrio* phylogeny either for *rec A* (global $R = 0.042$, global $P = 0.001$) *pyr H* (global $R = 0.105$ global $P = 0.001$) or *16S rDNA* (global $R = 0.207$ global $P = 0.001$). ANOSIM was done with 999 permutations. Bold numbers display values with $R > 0.2$.**

rec A Location	Sweden	Italy	Germany
Denmark	**0.001**	**0.001**	0.001
Sweden		**0.001**	0.001
Italy			**0.001**
pyr H Location	Sweden	Italy	Germany
Denmark	0.001	**0.001**	0.001
Sweden		**0.001**	0.001
Italy			**0.001**
16S rDNA Location	Sweden	Italy	Germany
Denmark	0.009	**0.002**	0.002
Sweden		**0.001**	0.001
Italy			0.001

PHYLOGENETIC ANALYSES IN *VIBRIO*

The haplotype genealogies on the basis of the three *Vibrio* genes (*rec A*, *pyr H*, and *16S rDNA*) confirmed that there is only little overlap between the sampling populations in terms of shared haplotypes (Figs. S1–S3). The Italian *Vibrio* population appeared to be the most diverse; both in terms of number of haplotypes, and the average pairwise distances between haplotypes (cf. network depictions in Figs. S1–S3).

In a maximum likelihood tree of the concatenated data (Fig. 1), the majority of haplotypes were not shared among populations (for details Figs. S1–S3). The phylotypes clustered into four separate clades that largely corresponded to sampling locations in Italy, Germany, Denmark, and Sweden. Sequences of common *Vibrio* phylotypes obtained from GENBANK were located in different clades, and only marginally overlapped with the new

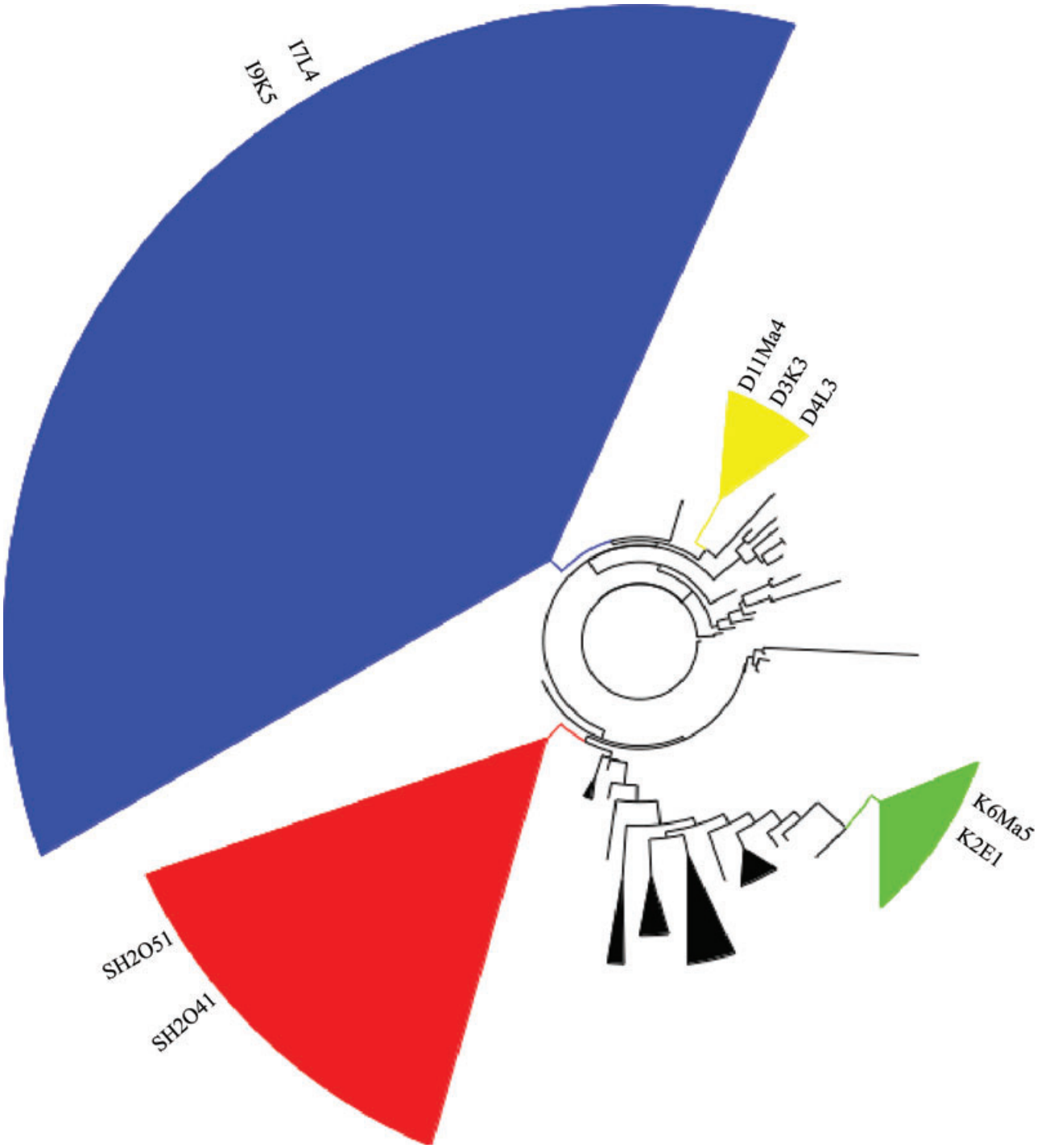

Figure 1. Maximum likelihood tree of concatenated *Vibrio* phylotype gene sequences (*pyrH*, *recA*, and *16S rDNA*). Phylotypes used for inhibition zone assays are mentioned, all other are just displayed as colors respective to the sample location (yellow for Denmark: D3K3, D11Ma4, D4L3; blue for Italy: I7L4, I9K5; red for Sweden: SH41, SH51; green for Germany: K2E1, K6Ma5). All black lines correspond to reference phylotypes mentioned in Table S2. In total 82 Danish phylotypes, 370 Italian phylotypes, 75 German phylotypes, and 312 Swedish phylotypes were used for this maximum likelihood tree. For a more detailed picture see Table S3 and Figures S1–S3.

sequences rendering an exact phylotype-identification impossible. All *Vibrio* phylotypes used for the inhibition zone assays were taken from within the cluster of their respective spatial origin (Fig. 1).

PREVALENCE DATA IN PIPEFISH HOSTS

Vibrio prevalence varied between 81% for Danish pipefish, 87% for Italian pipefish, 90% for German pipefish, and 40% for Swedish pipefish. Counting organs singly, organs from Danish

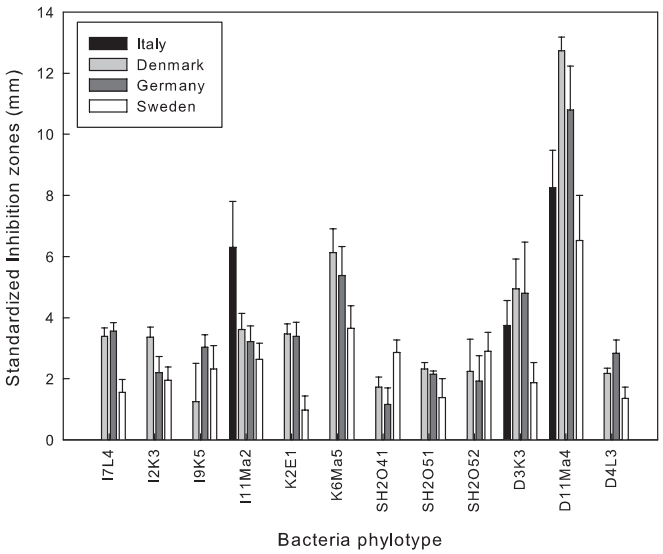

Figure 2. **Plasma antimicrobial activity from pipefish of different locations (black: Italy, dark gray: Germany, light gray: Denmark, white: Sweden) against different phylotypes of *Vibrio* spp isolated at different locations (I7L4, I2K3, I9K5, I11Ma2: Italy; K2E1, K6Ma5: Germany; SH2O41, SH2O51; SH2O52: Sweden; D11Ma4, D4L3; Denmark). Results are shown as standardized (against antibiotic inhibition) inhibition zone diameter in millimeter. Error bars show standard error.**

and Italian fish both had the highest *Vibrio* prevalence with 65% of the organs harboring cultivable *Vibrio* bacteria, followed by pipefish from Germany with a prevalence of 45% and lowest prevalence was found in Swedish pipefish with a *Vibrio* prevalence of 20%.

IMMUNE RESPONSE OF PIPEFISH HOSTS VIA INHIBITION ZONE ASSAY

Specificity indices calculated according to the inhibition zone measurements suggested host immunological adaptation to sympatric *Vibrio* (Fig. 2). The "bacteria phylotype specificity index" that reflects the potential of local adaptation within one bacterium phylotype but between pipefish populations was calculated for nine of 12 bacteria phylotypes (Table 3 and Fig. 3). Against the other three phylotypes (I7L4, I2K3, I9K5), the Italian (sympatric) plasma was not tested. These strains were exclusively used to display general differences in strength of *Vibrio* growth inhibition between the Northern populations to be able to disentangle potential effects of local adaptation and specificity from a general higher capacity to inhibit growth of *Vibrio*. From the nine "bacteria phylotype specificity indices" calculated eightwere positive, only one index was negative (D4L3), however, statistically not different from zero. From the eight positive indices, six (I11Ma2, K2E1, K6Ma5, SH2O41, D3K3, D11Ma4) were significantly different from zero and thus indicate host local adaptation. The

Table 3. **Specificity indices of bacterial phylotype specificity (bacterium specificity) and host population specificity (host specificity). Positive values suggest host local adaptation, negative values parasite local adaptation. Italian pipefish plasma was only tested against I11Ma2, K6Ma5, D3K3, and D11Ma4, hence against I7L4, I2K3, and I9K5 no sympatric values could be calculated and specificity indices are thus missing. Results of statistical tests using ANOVA are given for bacterial specificity; results of *t*-tests are given for host population specificity. x = missing values. Significant differences from zero are indicated (*$P \leq 0.05$).**

	Italy I7L4	Italy I2K3	Italy I9K5	Italy I11Ma2	Germany K2E1	Germany K6Ma5	Sweden SH2041	Sweden SH2051	Sweden SH2052	Denmark D3K3	Denmark D11Ma4	Denmark D4L3	Host specificity	*t*-tests	FDR
Italy	x	x	x		x		x	x	x			x	**0.036**	0.393	0.038
Germany													**0.067**	0.316	0.050
Sweden													**1.380**	<0.001*	0.025
Denmark													**1.651**	<0.001*	0.013
Bacterium specificity				**3.171**	**0.630**	**0.102**	**1.300**	**1.010**	**0.850**	**1.450**	**4.289**	**−0.080**			
ANOVAs	>0.001*	0.030*	0.373	0.018*	0.016*	0.004*	0.030*	0.829	0.631	0.020*	0.003*	0.009*			
FDR	0.004	0.038	0.042	0.025	0.021	0.008	0.033	0.050	0.046	0.029	0.013	0.017			

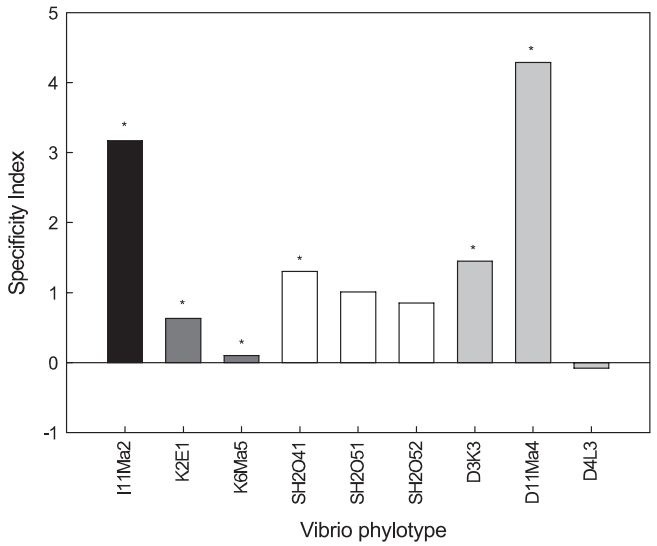

Figure 3. "Bacterium phylotype specificity indices" calculated for every *Vibrio* phylotype used to in vitro measure differences in immune response between sympatric and allopatric host populations. The mean of the inhibition zones (Fig. 2) of allopatric combinations was subtracted from the mean of the sympatric combinations. Significant differences from zero are indicated (*$P \leq 0.05$; Table 3).

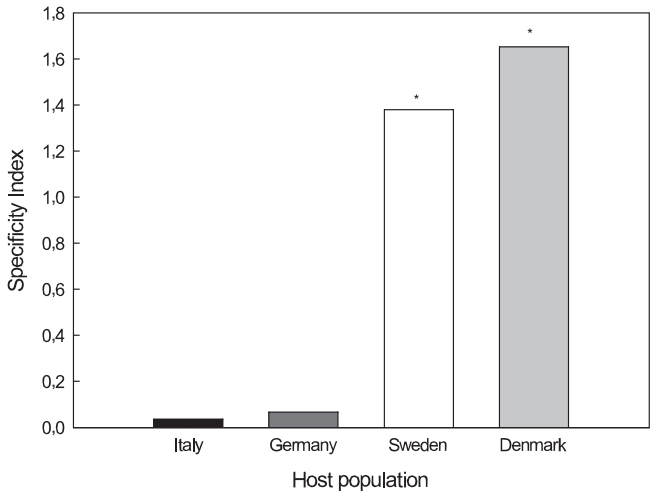

Figure 4. "Host population specificity indices" calculated for four *Syngnathus typhie* populations. Differences in immune response between sympatric and allopatric *Vibrio* phylotypes were assessed in vitro using an inhibition zone assay. The mean of the inhibition zones (Fig. 2) of allopatric combinations was subtracted from the mean of the sympatric combinations. Significant differences from zero are indicated (*$P \leq 0.05$; Table 3).

two positive but not significantly from zero different specificity indices (SH2O51 and SH2O52) belong to two phylotypes from Sweden. Swedish plasma in general had a decreased potential to inhibit the growth of *Vibrio* bacteria, according to the three additional Italian phylotypes that were used to assess the strength of general humoral immune activity against *Vibrio* measured as inhibition zone. Furthermore, the capacity to inhibit *Vibrio* from German and Danish plasma was not differentiable against some *Vibrio* phylotypes, even if sympatric *Vibrio* phylotypes were included (more details in Table S5).

The calculations of the "host population specificity index" to estimate local adaptation between bacteria phylotypes within the same population revealed positive values for all four locations (Table 3 and Fig. 4). Only pipefish plasma from Sweden and Denmark inhibited the sympatric bacteria phylotypes to a larger extent than the allopatric phylotypes, the strength of inhibition from Italian and German pipefish plasma was not stronger in inhibiting sympatric than allopatric *Vibrio* phylotypes. This suggests host local adaptation in two of four populations.

IMMUNE MEMORY UPON BACTERIA EXPOSURE

To differentiate between a true measurement of local adaptation and immune memory, German pipefish were exposed to injections with the Italian *Vibrio* phylotype I11Ma2 or as a control with PBS. We could not detect a difference in the size of the inhibition zones due to prior *Vibrio* infection of the pipefish individuals (*t*-test, two-sided, $P = 0.1199$). Inhibition zones of PBS injected pipefish had, after correction for differences among plates with the antibiotics positive control standard measurement, a mean diameter of 3.03 ± 0.589 (mean $\pm$ standard error), the inhibition zones of *Vibrio* injected pipefish a diameter of 1.65 ± 0.594.

Discussion

This study provides evidence that in generalist pathogens such as *Vibrio* bacteria, hosts are immunologically adapted to their counterpart. Although such a pattern is expected from theory (Gandon et al. 1996; Gandon and Michalakis 2002; Gandon et al. 2008; Gandon and Nuismer 2009), empirical data are rare (Oppliger et al. 1999). Hence, our data provide the first assessment of host local adaptation in widespread fish and their associated bacteria in marine coastal systems. Two prerequisites for host local adaptation are met in the *S. typhie*—*Vibrio* host–pathogen system, (1) *Vibrio* bacteria are generalist pathogens that would risk reducing their broad infection range upon local adaptation, and (2) the population structure of the host revealed essentially no genetic divergence among three northern locations, whereas bacterial communities associated with reservoirs (water) and fish hosts were remarkably differentiated. Our study design does not permit to identify the different contributions of processes driving host local adaptation. Probably, both selection pressures inter population exchange among hosts and generalism on the bacteria side, promote the evolution of host immunological local adaptation observed here.

Accordingly, in six of nine *Vibrio* phylotypes used for *in vitro* inhibition zone assays, the pipefish plasma inhibited the growth of their sympatric *Vibrio* phylotypes to a larger extent than that of allopatric phylotypes. Conversely, in no case were allopatric *Vibrio* phylotypes inhibited more strongly than the sympatric one. Immune memory could potentially explain this result. However, our results suggest that animals exposed to an allopatric *Vibrio* phylotype did not enhance their antimicrobial activity two weeks after exposure. This time should be sufficient for an adaptive immune response, as in a previous study, the upregulation of components of the adaptive immune system was identified within the same time frame (Roth et al. 2011). Because we can thus rule out that our inhibition assay is confounded with immune memory against sympatric *Vibrio* phylotypes due to previous exposure, the most likely explanation for the pattern observed is local host adaptation.

The potential of local adaptation is optimally analyzed with a fully crossed general linear model considering host and parasite main effects and their interaction (Thrall et al. 2002). The experimental design applied here deviated from the ideal because the amount of plasma in a single pipefish is only sufficient to conduct inhibition zone measurements against three to four different bacteria phylotypes, which leads to an incomplete design. One possible solution is the calculation of specificity indices that was recently proposed by Schulte et al. (2011). Applying this index, we successfully demonstrated host immunological adaptation against local bacteria phylotypes, which also extended to the level of host populations. Even though most host–pathogen combinations suggested host local adaptation, the sympatric plasma was not always differentiable in the strength of inhibition from all two to three allopatric combinations. In particular, the extent to which bacterial growth was inhibited by Danish and German pipefish plasma could not be differentiated for several bacterial phylotypes, although *Vibrio* communities between Germany and Denmark were taxonomically highly distinct. Among Italian pipefish plasma, only a limited number of bacteria phylotypes was tested, which has lowered the statistical power and thus the likelyhood to find host local adaptation. Swedish pipefish plasma generally showed a decreased antimicrobial activity against *Vibrio* compared to the plasma of the other three locations possibly because *Vibrio* is less abundant in Sweden. This corresponds with recent data on differences in immunocompetence among pipefish populations (Roth et al. 2011). Against one Swedish phylotype, the sympatric plasma had the strongest antimicrobial activity compared to plasma from the other locations, the antimicrobial activity of Swedish plasma against the other two Swedish phylotypes could further not be differentiated from the activity of allopatric pipefish plasma. This suggests an upregulation of antimicrobial activity in Swedish fish when sympatric bacteria phylotypes are met, as against all reference phylotypes antimicrobial activity of Swedish plasma was decreased compared to plasma from fish from all other locations.

Our study covered four host populations and several *Vibrio* phylotypes. We possibly underestimated the *Vibrio* diversity associated with *S. typhie*, as only phylotypes that were directly isolated in pipefish organs and cultivable on TCBS agar were analyzed. However, our aim was to identify *Vibrio* phylotypes that occur in the organs of *S. typhie* and are potentially attacked by antimicrobial activity within the host. *Vibrio* is a very diverse bacteria genus in which only some species are obligate pathogens (Thompson et al. 2004). In opportunistic *Vibrio* phylotypes, virulence can be triggered by environmental effects and quorum sensing (Pujalte et al. 2003; Thompson et al. 2004), an interspecific bacterial signaling mechanism that leads to expression of virulence (Fuqua et al. 1994). This makes the potential pathogenicity of associated *Vibrio* unpredictable to the host. Hosts are thus likely to induce immune defense upon most *Vibrio* infections. With the in vitro assay applied in this study, we exclusively measured host immune defense, whereas deliberately excluding confounding effects of parasite virulence or prevalence, as the number of bacteria cells used was standardized for every in vitro assay. Measuring local adaptation as infection prevalence and not immune function as in our study, has the advantage that host and parasite effects are simultaneously incorporated, which makes it possible to assess interaction effects. Therefore, we cannot rule out that in direct infection experiments, sympatric bacteria would find a way to evade host immune response and thus overcome the stronger immune reaction of the local hosts. We can also not address whether both host and parasite are locally adapted, as during host–parasite coevolution, host and parasite may simultaneously attain reciprocal adaptation. If during that state of host–parasite coevolution, host and parasite main effects are seperately investigated, a pattern of both host and parasite local adaptation can be found. The pattern of *Vibrio* diversity presented here reflects a snapshot for spring 2010. It is known that the structure of the *Vibrio* community underlies temporal variation (Montes et al. 2006) and shows certain temperature dependence (Pujalte et al. 2003). The clear pattern of differentiation among location implies selection for diversification to occur in this host–pathogen system. Our observation of strong bacterial community divergence seems at odds with the "everything-is-everywhere but the environment selects" hypothesis (Baas-Becking 1934), where all spatial differentiation in very small organisms is erased by their enormous dispersal potential. Our dataset, however, is not exhaustive enough to rule out that all phylotypes occur at every sampled location, albeit at much lower abundance. Deep amplicon next-generation sequencing of phylogenetic markers such as *16S rDNA* would probably lead to the identification of many more phylotypes (Wegner et al., in press), which could, in principle, be more similar between the populations than the ones we identified. We further detected

recombination at one locus in the *rec A* sequences. However, as we here used the phylogeny exclusively as guidance for the taxon determination and not to rely on the direct phylogenetic relationship, recombination is only of minor relevance.

Patterns of host local adaptation have so far mainly been demonstrated in non-vertebrate systems (Kaltz et al. 1999; Abida et al. 2010). The existence of immunological adaptation of the vertebrate host suggests that in several situations selection on the host is strong to adapt to its parasite (Greischar and Koskella 2007; Hoeksema and Forde 2008). This may particularly be true in marine systems that harbor a great phylogenetic diversity of facultative pathogenic prokaryotes (Riemann et al. 2008) where pathogenicity is triggered by environmental cues (Thompson et al. 2004), and where hosts, on the other hand, form large interconnected metapopulations (Palumbi 1992; Bohonak 1999). Future work will aim at disentangling different selection pressures for host and parasite local adaptation in more detail including the direction of selection on innate versus adaptive immunity.

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Supporting Information

The following supporting information is available for this article:

Table S1. Sequences of all primers used during this study, either for microsatellite loci (Sy_ty_1 – Sy_ty_24) or for genotyping the bacteria of the genus *Vibrio* spp. (*pyrH*, *recA*, 16S rDNA).

Table S2. All isolated *Vibrio* phylotypes from the four different sampling locations (D: Denmark, I: Italy, K: Germany, S: Sweden) are displayed here.

Table S3. Reference *Vibrio* sequences used for the phylogeny. The references sequences were aligned to the sequences of the particular gene (*16S rDNA*, *pyrH*, *recA*).

Table S4. SIMPER results: Numbers show similarities in percentages of *Vibrio* phylotypes within and between locations.

Table S5. Displays results of single ANOVAs testing for differences in the antimicrobial activity against several *Vibrio* phylotypes for plasma from the different pipefish locations.

Figure S1. The haplotype network for *rec A* shows the proportion of sequences from Sweden in red, from Italy in blue, from Denmark in yellow, and the proportion of German sequences is displayed in green.

Figure S2. The haplotype network for *pyr H* shows the proportion of sequences from Sweden in red, from Italy in blue, from Denmark in yellow, and the proportion of German sequences is displayed in green.

Figure S3. The haplotype network for *16S rDNA* shows the proportion of sequences from Sweden in red, from Italy in blue, from Denmark in yellow, and the proportion of German sequences is displayed in green.

Supporting Information may be found in the online version of this article.

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