Adaptation to enemy shifts: rapid resistance evolution to local *Vibrio* spp. in invasive Pacific oysters

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One hypothesis for the success of invasive species is reduced pathogen burden, resulting from a release from infections or high immunological fitness of invaders. Despite strong selection exerted on the host, the evolutionary response of invaders to newly acquired pathogens has rarely been considered. The two independent and genetically distinct invasions of the Pacific oyster *Crassostrea gigas* into the North Sea represent an ideal model system to study fast evolutionary responses of invasive populations. By exposing both invasion sources to ubiquitous and phylogenetically diverse pathogens (*Vibrio* spp.), we demonstrate that within a few generations hosts adapted to newly encountered pathogen communities. However, local adaptation only became apparent in selective environments, i.e. at elevated temperatures reflecting patterns of disease outbreaks in natural populations. Resistance against sympatric and allopatric *Vibrio* spp. strains was dominantly inherited in crosses between both invasion sources, resulting in an overall higher resistance of admixed individuals than pure lines. Therefore, we suggest that a simple genetic resistance mechanism of the host is matched to a common virulence mechanism shared by local *Vibrio* strains. This combination might have facilitated a fast evolutionary response that can explain another dimension of why invasive species can be so successful in newly invaded ranges.

1. Introduction

Species invasions can be considered as evolutionary ‘experiments in nature’ [1] that generate new phenotypes in action [2,3]. Surprisingly, the evolutionary potential of invaders to adapt to altered abiotic and biotic selection pressures in the transition from colonization to expansion has rarely been considered [3,4]. Only recently it has been shown that the adaptation to novel climatic conditions can outweigh or at least match the majority of factors promoting invasion success [5].

One important factor for invasion success is reduced pathogen burden that can result from release from parasite infections i.e. the enemy release hypothesis [6] or immunological superiority [7]. Owing to rapid coevolutionary dynamics host–parasite interactions should also lead to adaptive clines and at least for parasites the rapid adaptation to invasive hosts has been demonstrated [8]. For the host itself an invasion of a new habitat often involves exposure to novel pathogens or parasites [9]. Instead of a release from infection pressure a shift to newly acquired pathogens can be observed. However, the importance of adaptation to new pathogen communities has so far not been investigated.

Here, we present a unique study system, where two independent invasions of Pacific oysters *Crassostrea gigas* (Thunberg, 1793) led to genetically divergent populations [10,11]. Both invasions of the Pacific oyster into the European Wadden Sea occurred throughout the 1990s. While the southern invasion wave can be traced back to the Oosterschelde, the northern invasion stems from British hatchery produced spat farmed on the island of Sylt [12]. Using independent invasion events has a clear-cut advantage over the comparison of adaptive differences between source and invasive populations. For one, adaptations to local selection pressures will be independent and will have
furthermore occurred over a similar time span [2]. Yet, only few studies focused on populations with independent invasion histories [4]. Both invasive populations also differ in their selective history of disease outbreaks. In hot summer months with water temperatures exceeding 19°C, southern oysters were frequently subjected to strong selection by oyster summer mortality with mortality rates exceeding 60% and affecting all size and age classes [13].

A complex interaction of external and internal factors including high temperatures and pathogens like herpes viruses or bacteria of the genus Vibrio spp. (in particular, Vibrio splendidus) are suggested to be involved in mortality events affecting oyster larvae [14], juveniles [15–17] and adults [18,19]. Although herpes viruses (OsHV) were observed during mortality events in the Wadden Sea in 2005 [13], high mortality rates in comparison with low infection rates suggest that also other factors were involved. On top of that screening of oysters from Texel and Sylt for herpes viruses in Pacific oysters by PCR in July 2011 did not give positive results for OsHV-I (M.Y. Engelma, personal communication). Furthermore, resistance towards disease has a genetic basis [20], making it a likely target for selection.

Since the northern population has been spared from disease so far, the aim of this study was to test whether rapid evolution along differential selection gradients within the two invasion waves led to local host adaptation to sympatric disease agents, i.e. Vibrio spp. To answer this question, we conducted two consecutive experiments: in a first reciprocal infection experiment, we determined disease resistance and the underlying efficiency of the cellular immune response towards sympatric and allopatric V. splendidus strains in adult Pacific oysters stemming from both source populations. In order to determine the influence of environmental conditions the experiments were carried out at average summer water temperature (17°C) and elevated temperature (21°C) associated with disease outbreaks. To then generalize our findings, we tested the resistance of laboratory-bred larvae against a broad range of Vibrio strains. Here, we also included crosses between the invasion sources and can therefore not only detect signatures of selection within each invasion wave but are also able to predict whether admixture will put new superior genotypes into action [21–23] that can decisively influence the further spread of biological invasions. We found clear evidence of rapid immunological adaptation to sympatric Vibrio spp. communities and demonstrate that resistance against these is dominantly inherited resulting in increased fitness of admixed populations. Our findings therefore add a new facet to the factors explaining invasion success: rapid adaptation to enemy shifts.

2. Material and methods

(a) Vibrio community structure

Pacific oysters were sampled at six sites covering the entire Wadden Sea (electronic supplementary material, figure S1) in September 2011. Haemolymph samples were taken from each oyster and 4 μl were spread on Vibrio selective thiosulfate citrate bile sucrose agar (TCBS) plates (Fluka Analytica, Sigma-Aldrich, Steinheim, Germany). Plates were incubated at 25°C for 24 h, before we counted the colony forming units (CFU). A random subset of 11–18 single colonies per site were resuspended in 3 ml nutrient solution 1.5% NaCl (1000 ml distilled water, 5.0 g peptone, 3.0 g meat extract) and cultured at 25°C under constant shaking for 24 h. An aliquot of each liquid culture was used for direct amplification of 16s rRNA, GyrB and PyrH. Amplification followed previously established protocols [24] and PCR products were purified and sequenced at the Institute for Clinical Molecular Biology (IKMB), Christian-Albrechts-University Kiel, Germany. Each remaining culture was cryopreserved in medium +50% glycerol at −80°C until further use.

(b) Infection experiments

We performed two sets of infection experiments. One using adult oysters to measure resistance and cellular immune response against two strains of V. splendidus and the other using laboratory-bred larvae to measure resistance against a wide variety of Vibrio strains.

(i) Adult infections

Adult sampling and acclimation. In May 2012, healthy adults that were showing no signs of disease were collected three weeks prior to the experiment from two sites covering the northern (Sylt Island: 55°23.3’ N, 8°26.57’ E) and southern (Texel Island: 53°08.85’ N, 4°54.53’ E) population (electronic supplementary material, figure S1). Oysters were acclimated to the experimental temperatures of 17 and 21°C in constant temperature rooms (temperature shifts during acclimation less than 0.5°C per day). We chose 17°C as it represents contemporary average summer water temperatures, and 21°C as a representative for future predicted water temperatures [25]. Oysters were kept in a flow through system and fed three times a week with 50 000 to 80 000 cells per ml of Isochrysis 1800 Instant Algae (Varicon Aqua Solutions, Worcester, UK). One week prior to the experiment oysters were cleaned of epibionts and were notched on the dorsolateral side of the shell closest to the adductor muscle with a small hand drill.

Adult infections. For the adult infection experiment, we selected two previously described closely related isolates of V. splendidus from each location (O7w_July from Sylt and Tx5.1 from Texel, as described in Thieltges et al. [26]). Bacteria of the Splendidus clade have been shown to be involved in mortality events affecting oyster larvae [14], spats [15] and adult oysters [27,28]. Both strains have been successfully used in past studies [26] and did not show temporal bias in virulence [17] at 17°C. The strains will be referred to as Vibrio north and Vibrio south for geographical reference. To test for patterns of local adaptation across environments, we used a three-factorial design including origin (north, south), temperature (17 and 21°C) and infection (control, Vibrio north and Vibrio south). We used a total of 220 oysters and kept half of the oysters from each site (n = 55) at average summer water temperature (17°C) and the other half at elevated temperature (21°C). Experiments were carried out in one constant climate chamber where oysters were kept individually in single 1 l aerated glass jars placed in temperature controlled water baths. To avoid block effects, jars were randomly distributed over 21 water baths, each containing eight jars at 17°C and eight jars at 21°C. We exchanged the water every second day.

For each experimental group, 20 oysters were infected with Vibrio north or Vibrio south, and the remaining 15 oysters with nutrient solution 1.5% NaCl (as sham control). Treatments followed the infection protocols described in Wendling & Wegner [29]. Briefly, we injected 106 cells of bacterial culture or an equal volume of nutrient solution 1.5% NaCl with a syringe into the adductor muscle through the predrilled hole.

Adult cellular immune response and resistance. Cellular immunological parameters, bacterial load expressed as culturable Vibrio counts and survival were assayed as described in Wendling & Wegner [29]. In short, we monitored survival of all animals daily and additionally collected five random oysters from every treatment group at days 1, 3 and 7 to extract haemolymph (800 μl) from the adductor muscle. In fractions of the haemolymph, we:
(i) measured the total number of circulating haemocytes (THC) using an automated cell counter (Scepter, Merck Millipore, Darmstadt, Germany). (ii) estimated phagocytosis activity per unit of haemocyte protein using neutral red-stained, heat stabilized zymosan as described in Pipe et al. [30], and (iii) determined infection intensity by plating out 4 μl of haemolymph on TCBS plates to count CFU.

(ii) Larval infections

**Larval crossing and rearing.** Over a 2 day period in July 2012, we created 40 full-sibling families from randomly collected northern (Sylt) and southern (Texel) oysters (electronic supplementary material, figure S1). We bred four crossing groups with 10 families each: NN (Northfemale × Northmale), NS (Northfemale × Southmale), SN (Southfemale × Northmale) and SS (Southfemale × Southmale) as well as all interactions as independent variables. Experimental challenges were carried out using northern (NN, SS, hybrids) as well as all interactions as independent variables. We grouped Vibrio strains to contrast effects between both larvae sources (Sylt and Texel) and other sites (Husum, Büsum, WilhemsHAVEN, Norden). To further identify differences in oyster survival depending on geographical origins of Vibrio isolates, we used a linear weighted regression with survival rate averaged over all Vibrio strains per site (weighted by 1/σ²) as the dependent variable and geographical distance of to the Vibrio community to the northem site as the independent variable for all crossing groups. Since the relationship between survival rate and geographical distance in the hybrid group was not linear, we chose a quadratic polynornial regression to determine the relationship between survival rate and geographical distance of the Vibrio community.

We used a heuristic approach to identify Vibrio strains causing patterns of local adaptation. To do so, we calculated the deviance explained by the interaction term of a generalized linear model predicting survival by cross type and Vibrio strain. We repeated this analysis after removal of each Vibrio strain and recorded the resulting deviance change of the interaction term when compared with the model containing all strains. We then ordered all 76 strains based on their contribution to the interaction deviance term and subsequently removed strains in descending order until the crossing group × Vibrio strain interaction term was not significant any more. Strains that were removed from the model this way can be considered to show patterns of local adaptation. We conducted a Fisher’s exact test to identify whether Vibrio origin and genetic affiliation were responsible for the observed pattern of local adaptation.

3. Results

(a) Vibrio community structure

We successfully genotyped 76 different Vibrio strains for all three genes (16s, PyrH, CytB), of which we could unambiguously assign 75 strains to nine different Vibrio species from three distinct clades (figure 1), i.e. the *Splendidus* clade (92%), the *V. anguillarum* clade (7%) and the *V. splendidus* clade (1%). Within the *Splendidus* clade, we found seven different species (V. chagassi, V. crassostrenae, V. cromaticolor, V. gigantis, V. kanalae, V. splendidus, V. tasmaniensis), while all members of the Vibrio core belonged to the species *V. aegilopticus*, and the member of the *Anguillarum* clade was identified as *V. aestuarius*. The Vibrio community composition did not significantly differ between all sampling sites (Unifrac significant test, p = 0.07) and overall was dominated by the *Splendidus* clade (electronic supplementary material, figure S1).

(b) Infection experiments

We infected two different life stages to address two different scientific questions. First, we were interested in differences of...
immune response between sympatric and allopatric infections, for which we had to use adults. To extrapolate our results obtained from adult infection experiments to a wider range of Vibrio isolates, we conducted a larvae infection experiment.

(i) Adult infections

Survival. Upon infection, mortality started at day 1 and continued until day 5 with no more deaths observed until day 7. Therefore, experiments were terminated at day 7. Infection and temperature significantly increased mortality (logistic regression: infection $\chi^2 = 20.82, p < 0.001$; temperature: $\chi^2 = 16.86, p < 0.001$). At 17°C mortality did not differ with respect to oyster and Vibrio origin, $\chi^2 = 2.37, p = 0.5$. However, at 21°C, oysters from north and south showed significantly lower mortality rates when infected with their sympatric strain compared with the allopatric infection: significant oyster origin × Vibrio origin interaction: $\chi^2 = 14.3, p = 0.003$ (figure 2).

Cellular immune parameters and infection intensity. After 7 days, we lost all southern oysters that had been infected with Vibrio north at 21°C and therefore excluded day 7 from the analysis. Infection with either Vibrio isolate significantly increased the amount of total culturable Vibrio counts, i.e. CFU and cellular immune parameters (figure 3). To specifically examine local adaptation between oysters and Vibrio, we excluded the control group from further analysis. Cellular immune parameters and infection intensity significantly increased with warmer temperatures (table 1 and figure 3). CFU was highest after 12 h and decreased
(ii) Larval infections

Three days after artificial fertilization, we lost four families (1 NN, 1 SS and 2 SN). Resistance of cross types varied significantly between Vibrio strains \(F_{1,391} = 6.47, p = 0.01\), but did not depend on phylogenetic distance between strains within each cross type (Mantel test: northern oysters: \(p = 0.91\), southern oysters: \(p = 0.63\), artificial hybrids: \(p = 0.87\)) nor for the whole dataset (Mantel test: \(p = 0.77\)). On the other hand, we observed a significant main effect for oyster origin (binomial GLM: \(\chi^2 = 55.04, p < 0.001\)), Vibrio group (binomial GLM: \(\chi^2 = 31.32, p < 0.001\)) with the interaction between Vibrio group and oyster origin explaining most of the variation (binomial GLM: \(\chi^2 = 183.64, p < 0.001\)). For both northern and southern cross types \(R^2 = 0.84\) and \(R^2 = 0.62\) respectively, figure 4a, we observed a linear relationship with distance to the infecting Vibrio community, i.e. both cross types displayed highest survival rates when infected with sympatric Vibrio spp. strains. By contrast, hybrids were equally resistant to northern and southern Vibrio spp. strains as the pure cross types (NN and SS) and showed lowest survival at intermediate distance from both origins (quadratic distance \(R^2 = 0.81\); figure 4a) indicating that resistance to infection with sympatric Vibrio strains is dominantly inherited. Averaged over all Vibrio sampling sites, hybrids had a higher mean survival than pure lines (ANOVA: \(F_{2, 280} = 5.38, p = 0.006\); figure 4b).

Out of the tested 76 Vibrio strains, our heuristic approach identified 10 strains from several groups within the V. splendidus clade that showed typical local adaptation patterns (figure 4c). Out of these 10 strains the major proportion (60%, Fisher’s exact test: \(p = 0.05\)) was isolated from the sites where brood stocks were collected (i.e. Sylt and Texel, figure 4c) indicating that the evolution of specific resistance resulted from a local process over short period of time.

4. Discussion

Rapid evolution of invasive species in response to natural selection in the novel environment is a key feature in determining
invasion success [5]. Based on controlled infection experiments with two genetically divergent invasive populations of the Pacific oyster C. gigas, we could show that rapid evolution can also occur to adapt to a broad range of newly acquired opportunistic pathogens encountered in the new habitat. Oysters showed increased cellular immune efficiency giving them higher resistance when infected with sympatric Vibrio spp., suggesting that a common feature shared by otherwise only distantly related Vibrio spp. strains can serve as a target for local immunological adaptation. The dominant inheritance of resistance in hybrids between the two invasions further suggested that the genetic mechanism of resistance relies on only few loci, thus facilitating a fast and independent evolutionary response over only 20 years since the introduction of Pacific oysters into the Wadden Sea. This corresponds to six to seven generations at most when considering that Pacific oysters populations have been established in the mid-1990s (K Reise 2012, personal communication; [35]) and that they need approximately 3 years to reach maturity.

(a) Local adaptation to Vibrio communities is environment dependent

For all oyster origins, we detected no significant difference in disease resistance to either Vibrio strain at ambient water temperature (17°C). However, at higher water temperatures we detected higher resistance to sympatric Vibrio mediated by a stronger cellular immune response (figure 3b,c). High water

![Figure 4](http://rspb.royalsocietypublishing.org/)

**Figure 4.** (a) Survival rate (%) of artificially bred oyster populations: NN (red), SS (black) and hybrids (blue), infected with Vibrio spp. from six different sampling sites along the Wadden Sea coast from north, i.e. Sylt to south, i.e. Texel. Weighted linear regression: NN ($t_r = -4.69, p = 0.009$), SS ($t_r = 2.57, p = 0.06$), polynomial regression: Hybrids ($t_r = -3.59, p = 0.04$). (b) Mean survival rate (mean ± s.e.m., n = 688) per host population. (c) Strains (%) identified to promote local adaptation displayed per origin (left) and Vibrio species (right).

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Table 1. Infection intensity (i.e. total culturable Vibrio counts) and cellular immune parameters (MANOVA, Pillai’s trace statistic). (Significant factors ($\alpha = 0.05$) are highlighted in bold.)
temperatures on the one side led to increased proliferation rate of the pathogen (figure 3a), while it also led to an increased immune response compared with 17°C (figure 3b,c). Our experimental results thus match the natural condition under which oyster mass mortalities occurred, i.e. when water temperatures exceed a critical value of 19°C [36] leading to more favourable conditions for Vibrio growth. This may suggest that immune surveillance may be costly [7] and is enhanced when conditions are favourable for bacterial disease. With the risk of disease being increased with rising water temperature [37], the environment becomes more selective and allowed us to detect otherwise cryptic patterns of local adaptation.

In invertebrates, phagocytosis provides an important component of cellular immunity [38]. Upon pathogen infection, haemocytes migrate actively towards the site of infection, locally increasing the concentration of immune effectors attacking the pathogen [39]. At high temperatures, a higher heartbeat rate can result in enhanced production and circulation of haemocytes [40]. Consistently, at higher temperatures, we observed increased haemocyte counts (THC) and a higher phagocytosis rate. And while we are aware that several other molecular as well as cellular immune parameters may have contributed to local immunological adaptation, it is nevertheless tempting to speculate that the increased number of THC and the enhanced phagocytosis rate are involved in limiting the pathogen load of the faster growing Vibrio population in the case of sympatric combinations at elevated temperatures (figure 3b).

By using 76 different Vibrio strains isolated from haemolymph of six different oyster populations covering 535 km of Wadden Sea coastline, we could further show that resistance against sympatric Vibrio strains is valid for a broad range of strains. Overall, Vibrio spp. communities associated with oyster haemolymph showed similar species distributions throughout the entire Wadden Sea (electronic supplementary material, figure S1). This indicates that the taxonomic composition of strains used here was independent of the invasion source of the host. While oyster associated microbiota can assemble according to host genotype [41], Vibrio spp. in oyster haemolymph are most probably taken up from the environment and vary seasonally with environmental temperature [24,42]. Vibrio spp. are virtually absent from oyster tissue during winter months [41], and their abundance and diversity increases from spring to summer, reaches a peak during spawning season before decreasing towards autumn [24].

Thus, Pacific oysters do not have a constant spawning season before decreasing towards autumn [24]. Summer mortality syndrome (SMS) varied with the intensity of selection with narrow-sense heritability being higher in sites where only a small fraction of adults, i.e. the most resistant individuals, were removed [20]. In addition, resistance to V. parahaemolyticus and V. harveyi in the clam Meretrix meretrix is associated with single nucleotide polymorphisms in the I-type lysozyme gene [52]. If resistance of Pacific oysters to Vibrio spp. has a similar genetic basis, rapid evolution of disease resistance may result from a ‘regime shift’, where extensive mortalities remove susceptible individuals [53], supported by sweepstake reproductive success, where only a small fraction of adults, i.e. the most resistant ones, will reproduce successfully [53,54]. Hence abiotic and biotic environmental threats can rapidly alter the genetic composition of a single oyster population, thereby creating spatially and temporally varying populations that possess higher fitness in their native habitat.
5. Conclusion

We could show that oysters can rapidly adapt to widespread communities of pathogenic *Vibrio* spp. While invasion success has partly been attributed to a release from parasites encountered in the native habitat (i.e. enemy release [6]), we can now add a new facet for explaining invasion success: rapid adaptation to enemy shifts. Conditions supporting rapid adaptation were the likely generalism of *Vibrio* spp. in terms of host choice [43], a genetic mechanism shared by local *Vibrio* strains and the dominant inheritance of resistance. Immunological superiority in terms of reduced self-harm has been implicated in invasion success [7], but it is unclear to which extent evolutionary potential of resistance/tolerance contributes to population growth in new environments. Since any pattern of local adaptation is constrained to a given environment with all its abiotic and biotic features, evolutionary potential of any host (invasive and native) seems to be an important aspect of species persistence in the light of fast changing environments like coastal oceans. For *Vibrio* spp., a group containing many widespread opportunistic pathogens, it is well known, that virulence depends on environmental parameters, such as temperature or salinity [55,56]. If temperature rises, as predicted by current climate change models, an increase in virulence has to be expected [37] that potentially disrupts patterns of local host adaptation of all potential hosts.

Invasive species can have high evolutionary potential to adapt to changing abiotic conditions [5]. If they also have a higher potential to rapidly adapt to altered virulence of generalist pathogens in general, far reaching consequences for future development of marine ecosystems can be expected. Therefore, knowledge of underlying genetic mechanisms of rapid local adaptation and their interactions with the biotic and abiotic environment will be a crucial component in predicting evolutionary change in response to increasing virulence of generalist pathogens resulting from rising temperatures.

Ethics statement. Approval for collecting oysters was given by the Nationalparkamt Schleswig-Holstein http://www.nationalpark-wattenmeer.de/.

Data accessibility. Data are available at http://doi.pangaea.de/10.1594/PANGAEA.833020. All DNA sequences are available at GenBank with Accession Numbers KMS56003 through to KMS56074 and KMS579635 through to KMS579705.

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