Developmental and Comparative Immunology 159 (2024) 105221

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Evidence for immune priming specificity and cross-protection against sympatric and allopatric *Vibrio splendidus* strains in the oyster *Magalana (Crassostrea) gigas*

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ARTICLE INFO

Keywords:
Vibrio splendidus
Oyster *Crassostrea gigas*
Immune priming
Cross-protection

ABSTRACT

Infections with pathogenic *Vibrio* strains are associated with high summer mortalities of Pacific oysters *Magalana (Crassostrea) gigas*, affecting production worldwide. This raises the question of how *M. gigas* cultures can be protected against deadly *Vibrio* infection. There is increasing experimental evidence of immune priming in invertebrates, where previous exposure to a low pathogen load boosts the immune response upon secondary exposure. Priming responses, however, appear to vary in their specificity across host and parasite taxa. To test priming specificity in the *Vibrio* – *M. gigas* system, we used two closely related *Vibrio splendidus* strains with differing degrees of virulence towards *M. gigas*. These *V. splendidus* strains were either isolated in the same location as the oysters (sympatric, opening up the potential for co-evolution) or in a different location (allopatric). We extracted cell-free haemolymph plasma from infected and control oysters to test the influence of humoral immune effectors on bacterial growth *in vitro*. While addition of haemolypmph plasma in general promoted growth of both strains, priming by an exposure to a sublethal dose of bacterial cells lead to inhibitory effects against a subsequent challenge with a potentially lethal dose *in vitro*. Inhibitory effects and immune priming was strongest when oysters had been primed with the sympatric *Vibrio* strain, but inhibitory effects were seen both when challenged with the sympatric as well as against allopatric V. splendidus, suggesting some degree of cross protection. The stronger immune priming against the sympatric strain suggests that priming could be more efficient against matching local strains potentially adding a component of local adaptation or co-evolution to immune priming in oysters. These *in vitro* results, however, were not reflected in the *in vivo* infection data, where we saw increased bacterial loads following an initial challenge. This discrepancy might suggests that that it is the humoral part of the oyster immune system that produces the priming effects seen in our *in vitro* experiments.

1. Introduction

Aquaculture of shellfish is a significant contributor to the global food industry (Report of the Joint FAO/WHO 2010) (FAO/2020), however, infectious diseases increasingly cause problems for shellfish and especially oyster aquaculture (Lafferty et al., 2015). In particular, bacteria of the genus *Vibrio* are recognized as important infectious agents impeding aquaculture development (Le Roux et al., 2016; Paillard et al., 2004).

Vibrios are gram-negative bacteria that are widespread in marine and estuarine ecosystems and can be found free-living in the water column, as part of biofilms, or in association with a host (Thompson et al., 2004). *Vibrio* species are causal agents of epizootics, zoonoses, and epidemics, with several *Vibrio* species causing disease in humans (Austin, 2010; Le Roux et al., 2016). *Vibrio* strains from the *Splendidus* clade in particular (e.g. *Vibrio tasmaniensis* and *Vibrio crassostreae*) have been associated with mortality in oysters (Bruto et al., 2017; Lopez-Joven et al., 2018; Rubio et al., 2019). Furthermore, *Vibrio* contributes to Pacific oyster mortality syndrome (POMS) which regularly affects *Magallana gigas* when seawater temperatures reach 16–24 C (Oyanedel et al., 2023; Petton et al., 2021). These frequent mass mortalities made *M. gigas* a model for studying the dynamics of *Vibrio* disease in wild animals (Guo et al., 2015; Le Roux et al., 2016). Oysters, like other invertebrates, have evolved an integrated, highly complex innate immune system to identify and eliminate various invaders through a variety of orchestrated

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https://doi.org/10.1016/j.dci.2024.105221
Received 16 February 2024; Received in revised form 21 June 2024; Accepted 22 June 2024
Available online 24 June 2024

immune responses (He et al., 2011), along with a massive expansion and functional divergence of innate immune genes (Zhang et al., 2012). This innate immune system consists of humoral (e.g. the synthesis of antimicrobial peptides) and cellular (e.g. encapsulation and phagocytosis) mechanisms (He et al., 2011).

In addition to the innate immune responses, the immune systems of invertebrates may also exhibit adaptive-like characteristics, referred to as immune priming. There are multiple examples of immune priming across invertebrate taxa (Kurtz and Franz, 2003; Lemaitre et al., 1997; Little et al., 2003; Pham et al., 2007; Roth et al., 2009; Song et al., 2016; Yue et al., 2013), some of which are supported by molecular data (Lafont et al., 2017; Martins, 2020a). Priming has been demonstrated against viral pathogens in *M. gigas* (Lafont et al., 2017; Martins, 2020b), however, there is only little evidence for priming against bacterial pathogens. It has however been shown that early life environmental microbial exposure can buffer the oyster immune system against immune challenges later in life, and also provide protection against POMS (Fallet et al., 2022). Furthermore, there is evidence of increased cellular and humoral immune responses in *M. gigas* in response to exposure to heat killed *V. splendidus* (Zhang et al., 2014).

The mechanisms of immune priming in molluscs are not yet fully established, however, a body of evidence is growing. There is some evidence of B cell like receptors, CgIgR, with the ability to bind various bacteria, activate downstream signaling pathways, and promote the production of immunoglobulin domain-containing proteins, facilitating haemocytes to eliminate pathogens. (Sun et al., 2020). Futhermore, CgIgR seems to be involved in immune priming in *M. gigas* via histone modification, with Trimethylation of histone H3 lysine 4 (H3K4me3) following priming resulting in enhanced expression of CgIgR (Lian et al., 2024). Furthermore, Total hemocyte counts (THC), number of regenerated hemocytes, and expression levels of hematopoiesis-related genes (e.g. *Cg*Runx1 and *Cg*BMP7) are all significantly increased in oysters following a secondary challenge with *V. splendidus*, suggesting a function for hematopoiesis in immune priming (Zhang et al., 2014). After a second challenge with live *V. splendidus,* the phagocytic activity of haemocytes, involving phagocytic rate and phagocytic index, is specifically enhanced in oysters (Zhang et al., 2014). In addition, the expressions of six putative genes involved in the phagocytosis process (*Cg*Integrin, *Cg*PI3K, *Cg*Rho J, *Cg*MAPKK, *Cg*Rab 32, and *Cg*NADPH oxidase), are significantly up-regulated following secondary challenge (Zhang et al., 2015).

Immune priming can be specific to the bacterial species used for infection (Dhinaut et al., 2018; Sheehan et al., 2020) or even the strain, as demonstrated in the snail *B. glabrata* (Portela et al., 2013). Specificity is thought to be linked to the selection pressure and change of re-encountering a specific pathogen (Dhinaut et al., 2018; Sheehan et al., 2020). In the *V. splendidus* – oyster system there is evidence of local adaptation of oysters and *Vibrio* strains originating from the same location as the oysters (Wendling and Wegner, 2015; Wegner et al., 2019). Closely related strains of *Vibrio splendidus* from opposing ends of the Wadden Sea, about 500 km apart, one sympatric isolated from the Northern Wadden Sea on Sylt (Germany) (O7w_July from Sylt) and allopatric one isolated from the southwestern Wadden Sea on Texel (Netherlands, Tx5.1), had opposing impacts on the immune system activation and survival of oyster isolated at the same locations (sympatric vs. allopatric). Such a pattern can either resemble host-parasite co-evolution by local adaptation (Wendling and Wegner, 2015) or priming by prior contact of oysters to these or closely related strains, but this aspect has not been thoroughly explored.

Against the backdrop of potential co-evolutionary adaptations by priming specificity, we now test the impact of a low dose (priming treatment) on *in vivo* pathogen load in Pacific oysters (cellular and humoral immune defence) and the inhibitory effect of their haemolymph serum on *V. spendidus* growth *in vitro* after primary infection (only humoral immune defence). We then went on to secondarily challenge the oysters with a potentially lethal dose of bacteria. For the secondary challenge we either used the same strain as in the primary challenge or the other strain to evaluate local signatures of specificity in immune priming in relation to cross-protection. Comparing the inhibitory effects of oyster haemolymph between sympatric and allopatric strains in a full factorial design allowed us to test whether low dose primary exposures can function as general immunostimulants that enhance the ability of oysters to resist infectious diseases, or whether specificity of the response towards local co-evolved strains needs to be taken into account on different levels of the immune response.

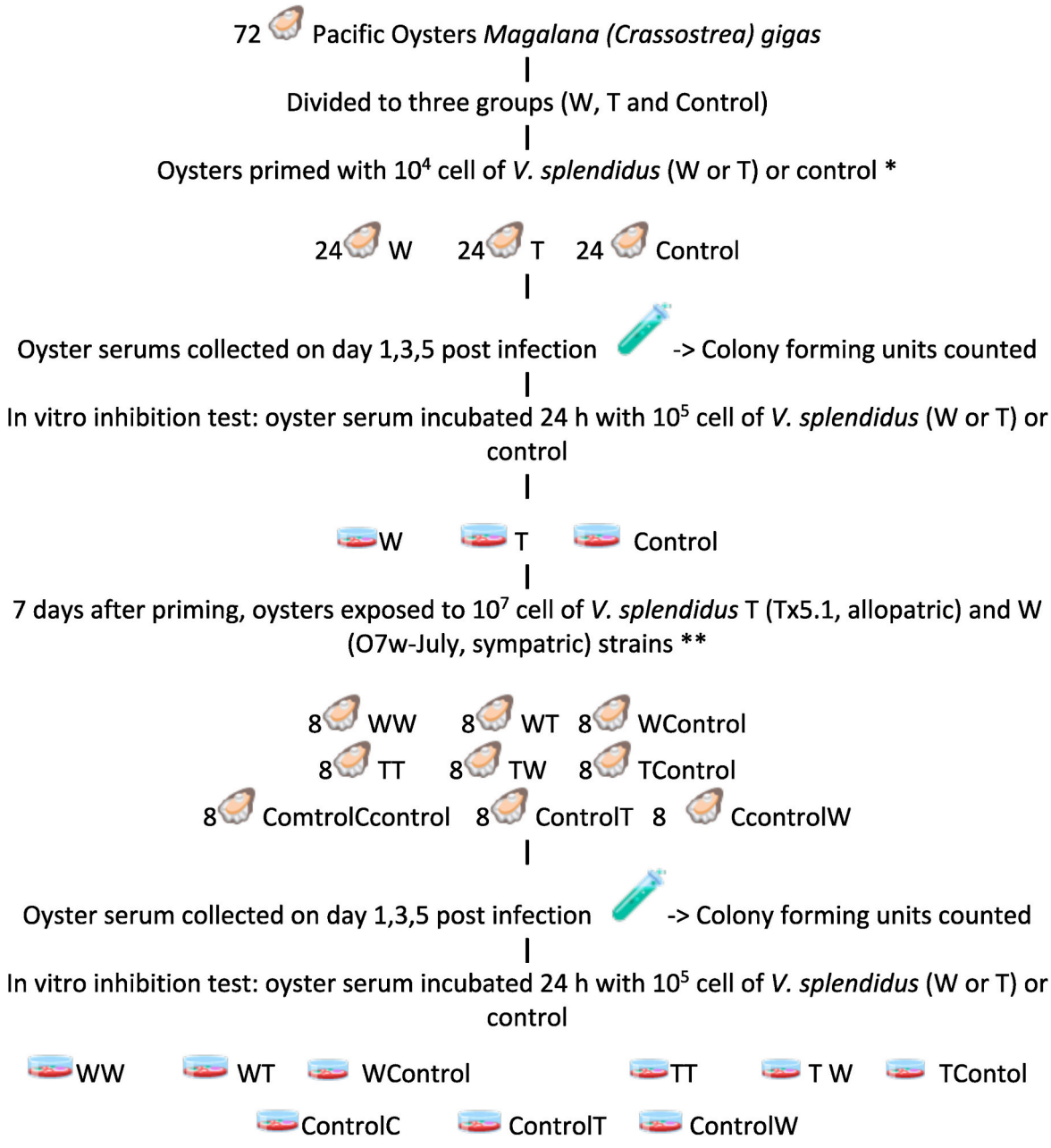
2. Material and methods

2.1. Primary exposure: in vivo experiments

In August 2021, oysters that were showing no signs of disease were collected from a mixed oyster-mussel reef (Oddewatt) on the island of Sylt (5582.330 N, 8826.570 E). Oysters were habituated to the experimental temperatures of 20 C ($\pm$ 0.58 C) in constant temperature rooms. Oysters were kept for three weeks in a flow-through system supplied with fresh filtered sea water. A week prior to the experiment, oysters were cleaned of epibionts and a small hole was drilled on their dorsolateral sides, closest to their adductor muscle. For infection and priming, we selected two previously described closely related isolates of *Vibrio splendidus* from two different locations. Strain O7w_July came from the same site as the oysters Oddewatt (sympatric) while Tx5.1 was isolated from oysters from Texel at south-western end of the Wadden Sea (allopatric) (Thieltges et al., 2013; Wendling and Wegner, 2015). The strains will be referred to as sympatric *Vibrio* vW from Sylt and allopatric *Vibrio* vT from Texel. Both strains elicit a stronger immune response in their sympatric hosts and consequently induce higher mortalities in their allopatric hosts (Wendling and Wegner, 2015). For infection we used a total of 72 oysters kept individually in aerated glass jars placed in temperature-controlled water baths in a constant climate chamber kept at 20 °C. Water was exchanged every two days. Treatments followed the previously established infection protocols (Thieltges et al., 2013; Wendling and Wegner, 2015). Briefly, we injected 100 µl of 10^4 cells/ml of bacterial overnight culture grown in nutrient medium (0.5% peptone, 0.3% meat extract, 1.5% NaCl) or an equal volume of nutrient medium with a syringe into the adductor muscle through the predrilled hole. For the priming challenge, 24 oysters were infected with sympatric *Vibrio north* O7w_July (group W) and 24 oysters were infected with allopatric *Vibrio south* Tx5.1 (group T) and the remaining oysters with nutrient medium (C group as control, Fig. 1). We monitored the survival of all animals daily and additionally collected 24 oysters distributed over all treatment groups on day 1, 3, and 5 post injection to extract haemolymph (100 µl) from the adductor muscle. We used 5 µl of the haemolymph to determine bacterial load expressed as colony forming units (CFU) by plating on vibrio specific Thiosulfate–Citrate–Bile–Sucrose (TCBS) agar plates.

2.2. Primary in vitro experiments

For the primary *in vitro* experiment, we used the haemolymph of the three groups of singly exposed oysters (hW, hT, hC see Fig. 1) isolated on day 1,3, and 5 in triplicate (Fig. 1). Haemolymph was centrifuged at 10,000×*g* at 25 °C for 2 min in order to separate serum from haemocytes. After centrifugation the supernatant serum was filter-sterilized to remove bacteria (0.22 µm pore size) and diluted 1:10 with nutrient medium. Serum from each experimental group (hT, hW, hC) from days 1, 3 and 5 was incubated with the two *V. splendidus* strains, the sympatrically isolated O7w_July (vW) and the allopatrically isolated Tx5.1 (vT) which were cultured in nutrient medium at 25 °C for 24 h, and adjusted to 10^4 cells/ml (Wendling and Wegner, 2015). Ninety-six well plates (Greiner Bio-One, Germany) were filled with 180 µl of diluted haemolymph serum and 20 µl bacteria solution, as well as negative control (culture medium only) and two positive controls *V. splendidus*



represents a Pacific oyster *Magalana* (*Crassostrea gigas*).

represents in vitro sample.

represents oyster serum.

* Primary challenge

**Seondary challenge

(caption on next page)

Fig. 1. The depicted figure delineates primary and secondary challenges within a sequence of both *in vivo* and *in vitro* experiments conducted on Pacific oysters *Magalana (Crassostrea) gigas*. In the primary *in vivo* phase, oysters were subjected to infection with *Vibrio splendidus* strains sympatric or W (O7w_July) and allopatric or T(Tx5.1). During the primary *in vitro* experiments, haemolymph obtained from individually exposed oysters served as a pivotal component for evaluating the immune response. The haemolymph serum underwent filter sterilization and subsequent incubation with *V. splendidus* strains with a microplate reader, followed by the measurement of resulting growth curves over 24 h. Subsequently, in the secondary challenge, oysters were divided into three subsets. These subsets were subjected to infection with either sympatric or allopatric *Vibrio* strains within the treatment groups, while the control group received a nutrient medium (0.5% peptone, 0.3% meat extract). Haemolymph was systematically collected once more to ascertain bacterial load. The secondary *in vitro* challenge was designed to study cross-protection. Haemolymph was categorized into nine distinct groups and placed within a 96-well plate. Bacterial strains were introduced, and ensuing growth curves were plotted.

strains: O7w_July (W) and Tx5.1(T) with only medium. In total we assayed 6 experimental groups (hT.vT, hW.vT, hC.vT, hT.vW, hW.vW, hC.vW) from three days (1,3, and 5 dpi) to give 18 experimental groups that we assayed in triplicates resulting in 54 samples. Growth curves were measured at 25 °C with shaking in a microplate reader (Synergy 2, Biotek) by taking measurements at OD_{550} nm every 15 min for 24 h.

2.3. Secondary challenge: bacteria preparation for infection and in vivo to cross-protection assay

Each of the primary challenge group (T, W, C see above) was divided into three groups with 8 oysters per group (Fig. 1). After seven days they were infected with sympatrically isolated *Vibrio* O7w_July, allopatrically isolated *Vibrio* Tx5 or nutrient solution as control in a fully factorial design (Fig. 1). The timing between priming and reinfection was to provide sufficient time for the acute immune response to clear the infection with these strains (Wendling and Wegner, 2015). By allowing this interval between exposures, we aimed to mimic a realistic scenario where the immune system has been primed by a prior encounter with the pathogen before facing a secondary challenge. We injected 100 μl of 10^7 cells/ml solution of *V. splendidus* strains T, W and equal volume of nutrient solution (1.5% NaCl) with a syringe into the adductor muscle through the predrilled hole. This resulted in nine experimental groups reflecting the combinations of primary and secondary challenges (primed with T: TT,TW,TC; primed with W: WT,WW,WC and control in primary challenge: CT, CW, CC). We collected 24 oysters distributed over all treatment group on day 1, 3, and 5 post injection to extract haemolymph (100 μl) from the adductor muscle. We used 5 μl of the haemolymph to determine bacterial load expressed as colony forming units (CFU) by plating on TCBS agar plates.

2.4. Secondary in vitro challenge to study cross-protection

The secondary *in vitro* cross-protection experiment followed a similar setup to the primary experiment. For the secondary *in vitro* cross protection experiment, the oyster haemolymph was divided into 9 groups for days 1,3 and 5 and kept in triplicates (Fig. 3) resulting in nine groups of haemolymph (hTT: primed with T and exposed to T, hTW: primed with T and exposed to W, hTC: primed with T and exposed to control medium and accordingly for the other groups primed with W (hWT, hWW, hWC) or without priming (hCT, hCW, hCC), and 180 μl of 1:10 diluted haemolymph were added to a 96 well plate in triplicates. Cultures of both bacterial strains (W,T) were grown in nutrient medium at 25 °C for 24 h, and 10^4 cells were added to each well, and OD_{550} nm was measured every 15 min for 24 h to generate growth curves in a Synergy 2 plate reader (Biotek, Germany).

2.5. Statistical analysis

All data were analyzed with R version 4.1.3. We analyzed *in vivo* CFU counts using the GLM function with both a Poisson, quasi-Poisson and a negative binomial error distribution and used AIC model comparison to find the best fitting model with "day", "bacterial strain" and their interaction as fixed factors. For bacterial growth Gompertz curves were fitted to the growth rate data using the *growthcurve* package and carrying capacities (K) were calculated from these curves as response variable for the *in vitro* analyses. The best-normalize package was used to transform data from the *in vitro* experiments for use in linear models using the Ordered Quantile transformation. For *in vitro* data AIC model comparison was carried out to find the best fitting combination of fixed factors and interactions (Tables S1 and S8). Differences were deemed significant at $p < 0.05$, If significant differences were indicated at the 0.05 level, then post hoc multiple-comparison (Tukey's) tests were carried out using the 'multcomp' and 'esmeans' packages in R to examine significant differences among treatments.

3. Results

3.1. Primary challenge

3.1.1. Bacterial load In vivo

Bacterial loads were measured from haemolymph of Oysters which had been primed with a sublethal dose of either sympatric (vW) bacteria, allopatric (vW) bacteria or a control solution. Priming with both allopatric and sympatric bacteria resulted in increased bacterial loads *in vivo* following a single challenge. The highest loads were found in oysters inoculated with sympatric (vW) bacteria.

CFU counts differed significantly by the day of haemolymph extraction post injection (Negative binomial GLM: Chisq = 13.2, d.f. = 2, p = 0.001, Table S4), the strain of bacteria the host was inoculated with (Negative binomial GLM: Chisq = 38.3, d.f. = 2, $p < 0.0001$) and the interaction of the two (Chisq = 12, d.f. = 4, p = 0.017). We selected a negative binomial model for this analysis as there were high Cook's distances in the Poisson and quasipoisson models and AIC comparison of the Poisson and negative binomial model showed that the negative binomial model was the better fit (Poisson 782, negative binomial 246). The posthoc multiple comparison tests revealed that injection with sympatric *Vibro* W resulted in higher bacterial loads than either allopatric bacteria or control inoculation and injection with allopatric bacteria resulted in higher bacteria loads than control treatment only on day 5 (comparision of means, $p < 0.05$, Fig. 2, Table S5). Over time, bacterial

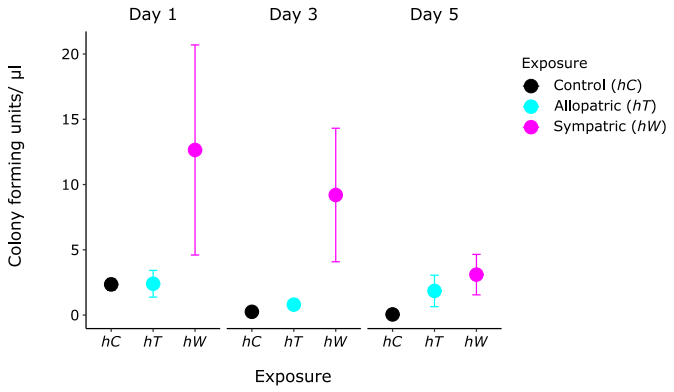


Fig. 2. *In vivo* bacterial load of haemolymph after primary challenge of oysters infected by the sympatric *Vibrio* O7w_July and the allopatric *Vibrio* Tx5.1 as well as the control group of oysters on day 1,3 and 5 post infection. Challenge with sympatric *Vibrio vW* resulted in higher bacterial loads than allopatric *Vibrio* or control inoculation and injection with allopatric bacteria resulted in higher bacteria loads than the control treatment only on day 5.

load significantly decreased only in the control treatment (pairwise comparison of means, $p < 0.05$), albeit on a comparatively low level (Fig. 2). However, there was also a trend towards a decrease in bacterial load in the sympatric treatment (pairwise comparison of means, $p = 0.09$), where interindividual variation was very high on day 1 and 3 (Fig. 2).

3.1.2. *Bacterial growth with haemolymph serum in vitro*

A subsample of haemolymph extracted from the primed oyster described above was used in an inhibition assay test, to test whether it influenced the growth of the bacterial strains *in vitro*. In all cases bacterial growth in the presence of the primed haemolymph was either the same or higher than in the non-primed control. The highest growth was found with sympatric bacteria exposed to haemolymph of oysters primed with the same sympatric bacteria.

Carrying capacities of haemolymph from the oysters used for the priming exposure were determined for both OW7_july (W) and Tx5.1 (T) bacteria. For the primary exposure the data were transformed using an ordered quantile normalizing transformation to achieve the most accurate normal distribution (Peterson and Cavanaugh, 2020). We used AIC model selection to compare a range of models including day, the bacteria the oysters were injected with (W or T) and the bacteria grown with exposed haemolymph *in vitro* (W or T) to find the best fitting model (Table S1). The model that best fit the data contained all three terms and the interaction between all three with an Akaike weight representing a relative likelihood of 84% (Table S1). There was a significant difference in bacterial growth rate *in vitro* among injection treatments (linear model: $F = 13.57$, d.f. $= 2$, $p < 0.0001$, Fig. 3, Table S2). The growth in the two *Vibrio* treatments did not differ significantly from one another, however, bacteria reached higher carrying capacities in both in comparison to the control (Tukey multiple comparison tests $p < 0.05$, Fig. 3, Table S3), suggesting that here may have been substances in the haemolymph that promote growth. Both vW and vT grew best in the haemolymph challenged with the matching strain (vW in hW and vT in hT), indicating that there was some strain specific serum alteration to favor growth of the matching strain. This effect was strongest for sympatric vW indicating that vW might have evolved mechanisms to maximise habitat manipulation in the oyster tissue. Interestingly, however, bacterial carrying capacity *in vitro* is significantly reduced over time. Again, this effect was strongest when oysters were injected with sympatric *vW* (Tukey multiple comparison test, difference between days one and five, $p < 0.05$, Fig. 3, Table S3). This is the opposite to the pattern seen in control injected oysters, where the carrying capacity on day one is lower but increases significantly with allopatric bacteria (Tukey test $p < 0.05$, Fig. 3, Table S3) and with sympatric bacteria (Tukey test $p = 0.065$, Fig. 3, Table S3). This might indicate that the most efficient inhibition response is mounted against sympatric vW, and that the dynamics of the balance between growth enhancement and inhibition is dependent on shared evolutionary or environmental history.

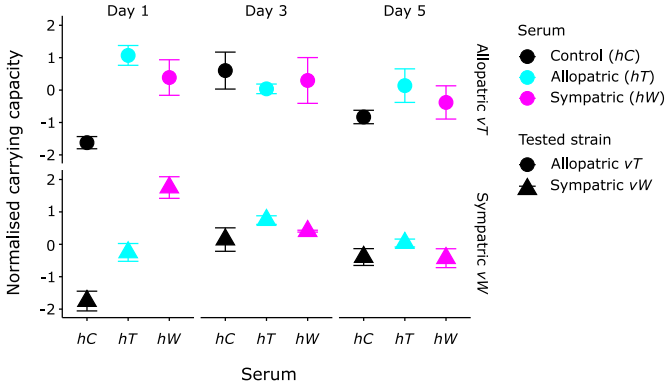


Fig. 3. Carrying capacities of haemolymph serum primed with different exposures (hW, hC and hT) for *Vibrio* strains vT and vW after 1, 3 and 5 days post infection. Primary exposure with either strain increased carrying capacity, but was strongest for matching combination of Vibrio strain and haemolymph on day 1 (vW – hW, vT-hT). Highest carrying capacity was reached for the vW-hW combination of day 1, but decreased significantly over time, whereas low carrying capacities were only found in hC on day one, but also increased significantly with allopatric bacteria (Tukey test<0.05), and showed a trend with sympatric bacteria (Tukey test $p = 0.065$) throughout the course of the experiment.

3.2. *Secondary challenge*

3.2.1. *Bacterial load in vivo*

Following priming with sublethal doses of bacteria, a group of oysters were exposed to a second, higher dosage of bacteria in a fully factorial design. Contrary to our expectations bacterial growth following secondary challenged *in vitro* was increased by priming treatment. The highest growth was in matching combinations, where oysters were exposed to the same bacteria twice.

The objective was to determine whether initial priming with a low dose of either sympatric or allopatric bacteria, resulted in a specific response to a second higher dose (10^7 cells) of either the sympatric or allopatric strain. Here, the negative binomial model was the best fit with a lower AIC than the Poisson model (Poisson: 8617, negative binomial 911) and a lower deviance than the quasipoisson model. Primary strain (Negative binomial GLM: Chisq $= 6.01$, d.f. $= 2$, $p = 0.05$, Table S6) significantly influenced the number of bacterial cells accumulating *in vivo*, with CFUs being significantly higher when primed with allopatrically isolated *Vibro* (vT) than in the control treatment (comparison of means, $p < 0.05$, Fig. 4, Table S7). Secondary strain also significantly influenced the outcome (Negative binomial GLM: Chisq $= 8.36$, d.f. $= 2$, $p = 0.015$, Table S6), which was again a result of individuals infected with allopatrically isolated bacteria (vT) having higher bacterial loads than the control (comparison of means $p < 0.05$, Fig. 3, Table S7). The interaction between primary and secondary exposure was also significant (negative binomial GLM: Chisq $= 11.2$, d.f. $= 4$, $p = 0.024$, Table S6), indicating that priming influences the response to a later exposure. Especially, during later stages of the infection this interaction term manifested in homologous combinations of primary and secondary challenge showing the highest bacterial loads for each priming group (vW in hW and vT in hT, Fig. 4). Interestingly, bacterial load was significantly higher in oysters exposed twice to allopatric bacteria than when primed with either sympatric or no bacteria. Load was also higher when exposed twice to sympatric bacteria than a control infection but

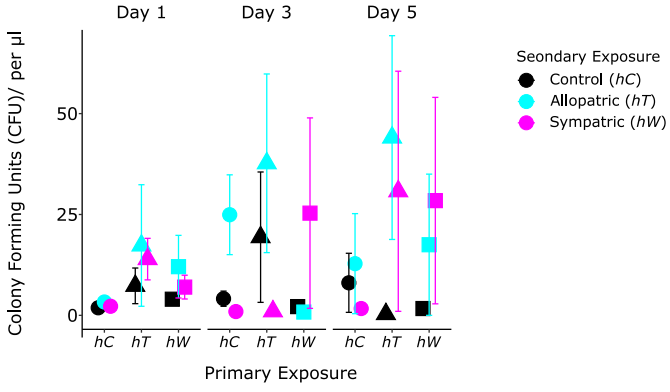


Fig. 4. ***In vivo*** Bacterial load after the secondary challenge of oysters primed with sympatric (vW), allopatric (vT) *V. splendidus* or without priming (C). A significant interaction between the primary and secondary exposures in the *in vivo* experiment (Negative binomial GLM: Chisq $= 11.2$, d.f. $= 4$, $p = 0.024$) indicates that priming influences the response to subsequent exposures. Oysters primed with sympatric or allopatric bacteria showed significantly higher bacterial loads when exposed twice, suggesting priming increases *in vivo* growth rather than inhibiting it.

not higher than in allopatric priming (pairwise comparisons of means, $p < 0.05$, Fig. 4, Table S7). These data suggest that priming leads to increased *in vivo* growth, rather than inhibition and mirror the first set of *in vitro* results where we saw higher carrying capacities with homologous combinations of primed haemolymph and infecting strain.

3.2.2. Inhibition after secondary challenge in vitro

Interestingly the *in vitro* data showed a different pattern to the *in vivo* data. By far the highest levels of inhibition were seen following priming and exposure to matching sympatric bacteria, indicating that humoral priming occurs, but mainly in response to sympatric bacteria.

The *Vibrio* growth data suggested a degree of inhibition when bacteria were grown in serum from doubly exposed oysters *in vitro*. Data were transformed using an ordered quantile normalizing transformation (Peterson and Cavanaugh, 2020), and the best fitting model contained the fixed factors primary and secondary exposure and day and the interaction of all three (100% Akaike weight, Table S8). The effects of primary treatment alone (Linear model: $F = 6.33$, d.f. $= 2$, $p = 0.07$, Fig. 5, Table S9), secondary treatment alone (Linear model: $F = 123.93$, d.f. $= 2$, $p < 0.0001$, Fig. 5, Table S9) and day alone (Linear model: $F = 139.61$, d.f. $= 2$, $p < 0.0001$, Fig. 5, Table S9), with a significant reduction in carrying capacity over time. All possible interactions of primary treatment, secondary treatments were highly significant (Linear model $p < 0.0001$, Table S9). Treatments with both bacterial strains resulting in significantly lower carrying capacities than the control treatment (Tukey multiple comparison tests $p < 0.05$, Table S10). Within the allopatric secondary exposure, both primary treatment with allopatric and sympatric bacteria resulted in significantly higher carrying capacities than the control (Tukey multiple comparison tests $p < 0.05$, Fig. 5, Table S10) but did not differ from one another. Within the sympatric secondary exposure, however, carrying capacities were significantly lower in the sympatric primary exposure than in either the control or allopatric treatments of the secondary exposure (Tukey multiple comparison tests $p < 0.05$, Fig. 5, Table S10), whereas the control and allopatric treatments did not differ from one another. In contrast to the primary exposure, the haemolymph exposed to sympatric vW twice showed the biggest inhibitory effect, suggesting that some specific humoral immune memory against the locally encountered sympatric strain could have evolved.

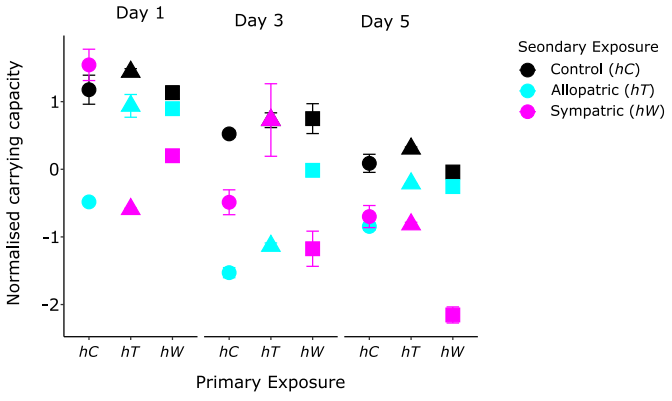


Fig. 5. Pooled data of carrying capacities of vT and vW cultured with haemolymph serum after priming and secondary exposure with *Vibrio* strains vT and vW after 1,3 and 5 days post infection data from growth with vT and vW were pooled for simplification as the strain being grown hat no significant impact on the outcome and was excluded in the best fitting model. **T**he interaction between primary and secondary infection was significant (Nested linear model: Chisq $= 67.4$, d.f. $= 4$, $p < 0.0001$). Within the control secondary treatment there were no significant differences among primary treatments. Within the allopatric secondary treatment, both primary treatment with allopatric and sympatric bacteria resulted in significantly higher carrying capacities than the control (Tukey multiple comparison tests $p < 0.05$) but did not differ from one another.

4. Discussion

We investigated the effects of humoral immune priming with two sympatric and allopatric strains of *V. splendidus* in Pacific oysters by comparing growth dynamics *in vivo*, where bacteria were exposed to the cellular and humoral immune defence, to growth dynamics *in vitro* where only humoral components affected *Vibrio* growth. We specifically asked whether priming with sympatric bacteria, sharing evolutionary and environmental history with their host, differs from priming with allopatric bacteria. We found that sympatric *Vibrios* seem to be better adapted to colonizing oysters from the same location than allopatric *Vibrios*. Yet, oyster haemolymph primed with the sympatric *Vibrios* shows the highest inhibitory effects *in vitro*. The latter lends strong support to the notion that immune priming against bacterial infections can occur on the humoral level in *M. gigas*, although *in vivo* where humoral and cellular immune responses are intertwined, we do not see a similar effect. Further experiments investigating cellular immunity against these strains *in vitro* could help to reveal mechanistic components of the *in vivo* response and elucidate the role of cellular immunity in immune priming.

As the bacterial load in primed host individuals was higher than in the controls, this might suggest that colonization was mainly driven by the primary exposure. We show here that cell free haemolymph serum of oysters primed with allopatrically isolated *Vibrio* can have inhibitory properties against the growth of *Vibrio* bacteria in subsequent challenges. This extends previous immune expression data (Zhang et al., 2014) demonstrating priming in the *M. gigas* – *Vibrio* system on the cellular level to priming effects on humoral immune components.

While the *in vitro* results are consistent with the hypothesis of immune priming, our *in vivo* results indicate that priming, contrary to our initial expectations, leads to an increase in *in vivo* bacterial growth rather than inhibition. This suggests that the initial exposure to the bacteria enhances their growth within the host organism. However, in contrast to the *in vivo* findings, the *in vitro* experiments revealed a degree of inhibition upon a second exposure. Specifically, when the bacteria were examined in haemolymph obtained from doubly exposed oysters, their growth was somewhat suppressed. There are several reasons that might potentially contribute to this incongruity. The changes in immune response upon priming with *Vibrio* reported here could be influenced by both cellular and humeral immune components (Wang et al., 2018). Here, our *in vivo* data encompass the potential for both factors to be at play. On the other hand, in our *in vitro* experiments we use cell-free filtered haemolymph or serum. This means that priming expressed in the form of cellular immunity, for example, phagocytosis would not influence the experimental outcomes. Not only does this filtering of haemolymph remove the vibrio it also removes other microbes that might be present in the haemolymph limiting potential inhibitory interactions they might have with the injected *Vibrio*. In effect, in the *in vitro* experiments we purely test the impact of priming via humeral immune defenses. In the context of *in vivo* conditions, oysters may experience additional regulatory variables or cellular interactions that influence their immune responses in a manner distinct from what is observed in isolated *in vitro* environments.

It is interesting to note that treatments involving both bacterial strains (allopatric and sympatric) showed a significant increase in carrying capacities compared to the control treatment. This suggests that haemolymph serum contains, next to humoral immune effectors, also substances that promote growth of specific microbes. These substances could simply be additional nutrients, but we also observed a significant interaction between the primary and secondary treatments, indicating that their combined effects on growth are not merely additive but rather exhibit an interactive relationship. The primary-secondary strain specific effect suggests these growth promoting substances must exert specific effects. Such specificity could result from downregulation of strain specific immune effectors or strain specific signals enhancing growth (Milton, 2006), and can be viewed as a beneficial habitat

modification increasing population growth. Intriguingly, no significant differences were observed between the primary treatments within the secondary control treatment. This implies that the specific type of primary treatment did not have a significant impact on growth when no secondary exposure occurs, further indicating that signals from the primary exposure pertain throughout the secondary exposure.

When exposed to sublethal doses or heat killed *Vibrio M. gigas* modifies its immune response to prepare for future exposures. Upon initial contact, oysters rapidly identify *Vibrio* bacteria by recognizing certain molecular patterns associated with these bacteria (Huang et al., 2018). This initiates a series of immunological responses, which involve the activation of immune cells and the synthesis of antimicrobial substances that can differ between populations (Schmitt et al., 2013). The immune system of oysters acquires the ability to distinguish several strains of *Vibrio*, resulting in enhanced efficiency and accuracy of immune responses (Wendling and Wegner, 2015), providing the potential for specificity in primed responses. Multiple exposures to *Vibrio* bacteria result in an augmented and specific immune response (Fallet et al., 2022; Lafont et al., 2017), which confers durable immunity against subsequent *Vibrio* infections. The acquired immunity demonstrated by oysters may persist for an extended duration, hence reducing their susceptibility to subsequent infections. In this study, secondary challenge *in vitro* experiment demonstrated a sharp decrease in caring capacity in both strains when combined with sympatric-sympatric *hW-hW* haemolymph. This suggests a specific immune activation by the sympatric strain that elicits broad effectors working against both closely related strains and balances out the positive effects on growth.

Invertebrates in general have been the subject of a long discussion regarding their immunological memory (Schmid-Hempel, 2005), and several reports have suggested that some forms of immune memory may exist in invertebrates, generally referred to as specific immune priming (Lafont et al., 2017; Schmid-Hempel, 2005). Most studies investigating immune priming focus on enhanced survival due to changes in immune responses (Rowley and Powell, 2007), following previous encounters with pathogens or their products and providing protection against reinfection (Contreras-Garduño et al., 2016). For example, insects, which were previously believed to lack immunological memory owing to the fact that they do not posses specific memory cells, are now known to illicit immune priming (Prakash and Khan, 2022), also in a strain specific manner (Roth et al., 2009).For molluscs, examples of strain specific priming are, however, much more limited. This study underscores the multifaceted dynamics of bacterial priming on oyster hosts, with implications for understanding the intricate interplay between bacterial strains, priming sequence, and their impact on humeral and cellular immune response ultimately determining oyster health and bacterial growth.

We demonstrated that humoral immune priming stimulates oyster immune responses against both sympatric and allopatric strains of *V. splendidus,* enhancing specific memory and acquired immunity in oysters. This adds to a growing body of evidence in oysters that immune priming occurs in response to both viral and bacterial threats. Furthermore, we demonstrate that both the bacterial strain and its history with its host impact priming responses. This knowledge could be used for vaccine programs to prevent disease outbreaks in aquaculture.

Funding statement

Noushin Afatahery was supported by Scholars at Risk, the Ernst-Reuter Gesellschaft and the FU Berlin.

CRediT authorship contribution statement

Noushin Arfatahery: Writing – original draft, Investigation, Formal analysis, Data curation. **Charlotte Rafaluk:** Writing – review & editing, Visualization, Supervision, Formal analysis. **Jens Rolff:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **K. Mathias Wegner:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank Christin Manthey, Dirk Mikolajewski and Caroline Zanchi for helpful discussions and Afshin Motavaly for statistical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2024.105221.

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