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Ralstonia eutropha, containing high poly-β-hydroxybutyrate levels (PHB-A), regulates the immune response in mussel larvae challenged with *Vibrio coralliilyticus*

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Abstract

Marine invertebrates rely maily on innate immune mechanisms that include both humoral and cellular responses. Antimicrobial peptides (AMPs), lysozyme and phenoloxidase activity, are important components of the innate immune defense system in marine invertebrates. They provide an immediate and rapid response to invading microorganisms. The impact of amorphous poly-β-hydroxybutyrate (PHB-A) (1mg PHB-A L^{-1}) on gene expression of the AMPs mytimycin, mytilinB, defensin and the hydrolytic enzyme lysozyme in infected blue mussel larvae was investigated during "*in vivo*" challenge tests with *Vibrio coralliilyticus* (10^5 CFU mL^{-1}). RNAs were isolated from mussel larvae tissue, and AMPs were quantified by q-PCR using the 18SrRNA gene as a housekeeping gene. Our data demonstrated that AMPs genes had a tendency to be upregulated in challenged mussel larvae, and the strongest expression was observed from 24h post-exposure onwards. The presence of both PHB-A and the pathogen stimulated the APMs gene expression however no significant differences were noticed between treatments or between exposure time to the pathogen *V.coralliilyticus*. Looking at the phenoloxidase activity in the infected mussels, it was observed that the addition of PHB-A induced significantly increased activity.

Keywords: *Mytilus edulis* larva, poly-β-hydroxybutyrate, gene expression, antimicrobial peptides, innate immunity, phenoloxidase activity

1. Introduction

In common with other invertebrates, bivalves rely mainly on innate immunity, which by definition lacks adaptive characteristics, to combat against invading pathogens. Antimicrobial peptides (AMPs) appear to be some of the actors in innate immunity that have been conserved during evolution, although their involvement in anti-infectious processes is different according to species, cell type, and tissue [1]. Many antimicrobial peptides are located in epithelia [2] where they prevent invasion by pathogens while others may be especially abundant in circulating cells. They are recognized to be a major component of the innate immune defense system in bivalve mollusks as well [3].

The innate immunity in mollusks is not well understood. Nevertheless, pathogen recognition receptors (PRRs) have been identified in some species [4]. Once the presence of microbes is detected, these intruders are phagocytosed and destroyed by toxic radicals, lysozyme and AMPs, which are involved in bacterial killing by destabilizing their membrane permeability [5]. After engulfment of microorganism, the phagosome undergoes maturation with acidification and sequential fusion with endosomal and lysosomal compartments including granules, which contain diverse families of antimicrobial peptides/proteins [6, 7]. The release into the phagosome of microcidal compounds leads to the rapid neutralization/degradation of the engulfed microorganisms. Among the hydrolytic enzymes that are released into the maturing phagosome, lysozymes are known play an important role in microbial destruction due to their lytic properties on the peptidoglycan of the bacteria cell wall [8].

Jenssen, Hamill and Hancock [9] described in detail the interaction process between the peptide and target cell. It is thought to occur through electrostatic bonding between the cationic peptide and the negatively charged components present on the bacterial outer envelope, such as phosphate groups within the lipopolysaccharides of Gram-negative bacteria or lipoteichoic acids present on the surface of Gram-positive bacteria.

In mussels, four AMPs (defensin, mytilin, myticin and mytimycin) which play a key role in the immune defense were identified and characterized [1]. Different mussel species have various AMPs e.g., myticin appears in *Mytilus galloprovincialis* only, whereas mytimycin

occurs in *M. edulis* only [10]. Some of the AMPs have a wide spectrum of action while others are target-specific. For instance, mytimycin is described as being strictly anti-fungal, mytilin acts extracellularly, whereas myticin can act either intracellularly (during phagocytosis) or extracellularly [1]. Mitta , Vandenbulcke and Roch [1] also reported that defensin producing granulocytes are concentrated in the intestinal epithelia, whereas mytilin and myticin expressing granulocytes are well represented in gills suggesting that the type of AMP may dictate granulocyte allocation in different organs in mussels. *M. galloprovincialis* defensin 1 (MGD1) is an original member of the arthropod defensin family due to the presence of 2 extra-cysteines and one modified amino acid [11]. Defensin, mytilin and myticin were determined *in vitro* to have antimicrobial activity. Differences in expression of these AMPs genes were recorded when mussel *M. galloprovincialis* adults were challenged with various factors such as bacterial infections or by heat shock [3]. To our knowledge, nothing is known about the genes that code for these antimicrobial peptides in blue mussel *M. edulis* larvae or about the regulation of their expression.

Besides the AMP gene expression, another critical component of the immune system of bivalves namely phenoloxidase activity (PO) was examined. As the product of a complex cascade of reactions, PO is generated from proPO through a limited proteolysis by a proPO activating enzyme [12], and is involved in melanization, encapsulation, wound healing, phagocytosis, and pathogen extermination [13, 14]. The soluble form of PO is always involved in humoral immunity while the cellular PO that binds to the surface of hemocytes, is more associated with cell-mediated immunity [15]. Bacterial infection can cause a significant increase in PO in the hemolymph of the bivalves *Crassostrea madrasensis* and *Chlamys farrization* [16].

The compound poly-ß-hydroxybutyrate (PHB), a polymer of the short-chain-fatty acid ß-hydroxybutyrate, was proven to protect experimental animals against a variety of bacterial diseases, including vibriosis in farmed aquatic animals, albeit through undefined mechanisms [17]. Recent research demonstrated that amorphous PHB-A, namely *Ralstonia* cells containing more than 75% PHB on dry weight basis, in particular increases the survival of

mussel larvae (*M. edulis*) [18] and protects them during *in vivo* challenge tests with *V. coralliilyticus* and *V. splendidus* (unpublished).

This study focuses on the detection and regulation of the three AMPs mytimycin, mytilinB, defensin and the hydrolytic lysozyme in *M. edulis* larvae. On a second level the impact of PHB-A on the expression of these AMPs genes and on PO activity regulation was investigated during challenge tests with the pathogen *V.coralliilyticus*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Vibrio coralliilyticus is a Gram-negative marine bacterium isolated at the Glen Haven Aquaculture Centre (New Zealand) during 2004 and 2005. This selected strain was reported as a pathogenic *Vibrio* for bivalve larvae culture [19]. *V. coralliilyticus* used in the experiments was mutated to a rifampicin resistant strain. Before use, 10 µl of the stored cultures (in 40% glycerol at -80 °C) were plated on Luria- Bertani plates to which 35g L^{-1} of Instant Ocean® (LB_{35} agar) was added and incubated for 24 h at 17 °C. Single colonies were picked from the plates and cultured overnight in fresh LB_{35} at 17 °C under constant agitation (150 min^{-1}) before each experiment.

2.2. Spawning procedure and larvae handling

Mature blue mussels *(Mytilus edulis)* were transported from the mussel producer Roem van Yerseke, in The Netherlands to the Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Belgium, under cooled conditions. The protocol for mussel spawning and fertilization was followed as described in Hung et al. [18]. The development of the fertilized eggs was regularly monitored. When the morula stage was reached in the majority of the embryos, the embryo solution was sieved through a 30 µm sieve. The remaining sperm was washed away with 0.22 µm filtered and autoclaved seawater (FASW). After this washing step, the embryos were transferred to a 2L glass bottle containing fresh FASW at 18 °C and a mixture of antibiotics rifampicin, kanamycin, and ampicilin (10 mg l^{-1} each) to minimize bacterial interference . After 48h, the straight-hinged D-larvae were washed at least five times with FASW to remove every trace of antibiotics and then re-

suspended in fresh FASW. All manipulations were performed under a laminar flow hood to avoid contamination before the start of the challenge tests.

2.3. Challenge tests

The first experiment was carried out in 48-well tissue culture dishes (TCD), and the second, third and fourth experiment in 1L bottles. Survival was only measured in the small-scale test while the larvae in the bottle experiments were also used for tissue collection. Samples from the second and third experiment were used to measure AMPs gene expression and PO activity, respectively. The fourth experiment was carried out to evaluate a wider time window of gene expression for the different AMPs (till 48 h, based on the results of second experiment).

Each treatment was performed in triplicate. Plate sterility of the control treatments was checked at the end of the challenge by plating 100 µl of the culture water on LB_{35} plates and incubating at 17 °C. If the control was contaminated, the results were not considered, and the experiment was repeated.

2.3.1. Experiment 1

A completely randomized experimental design was followed to evaluate the effect of PHB-A on *V. coralliilyticus* exposure in mussel larvae. The challenge test was performed in 48-well TCDs. Each well contained approximately 100 two-day-old D-larvae. The rearing water contained LB_{35} (0.1% v/v) and rifampicin (10 mg L^{-1}) to suppress growth of ambient bacteria. In treatment 1 (control), larvae were neither PHB-A treated nor challenged, in treatment 2 the mussel larvae were challenged after 6 h with the rifampicin resistant strain *V.coralliilyticus* at a concentration of 10^5 CFU mL^{-1} while in treatment 3 the larvae were supplemented with PHB-A at a concentration of 1 mg L^{-1}. PHB-A consisted of a freeze-dried *Ralstonia eutropha* culture containing 75% PHB on cell dry weight (VITO, Mol, Belgium). In treatment 4, the larvae were supplemented with PHB-A 6 h before being challenged with *V.coralliilyticus*. During the challenge test, larvae were pipetted twice a day to ensure that PHB-A particles were suspended in the water column and available for uptake by the larvae. The survival of the larvae was determined 24h and 48h after the addition of the pathogen.

2.3.2. Experiment 2, 3 and 4

The larvae were submitted to the same treatments as in Experiment 1, PHB-A unfed larvae were compared with PHB-A fed larvae (6hr before exposure to the pathogen) challenged with 10^5 CFU mL^{-1} of *V. coralliilyticus*. Three replicate glass bottles of 1L, containing 50,000 D-larvae each, were sacrificed at each sampling point. Room temperature was 17°C. All the larvae of the three replicate bottles were collected separately on a 60 µm sieve. For experiment 2, samples were collected at the sampling points 0h, 3h, 6h, and 12h (counted just after adding the pathogen). Experiment 3 lasted for 48 hours after the addition of the pathogen during which PO was measured in challenged larvae at 12h, 24h, 36h and 48h while for experiment 4, the larvae were collected at 0h, 3h, 6h, 9h, 18h, 24h and 48h. The larvae were washed with AFSW and transferred to 2 ml centrifuge tubes, weighed, flash-frozen in liquid nitrogen and stored at -80°C for PO measurement and RNA extraction . The procedure for the four experiments is summarized in the Table 1 below:

2.4. Standardization of quantitative real-time qRT-PCR for mussel larvae

2.4.1. Design of gene-specific primers

The sequences of mytimycin, mytilinB, defensin, and lysozyme from two blue mussel species *M. galloprovincialis* and *M. edulis* were selected from the GenBank (http://www.ncbi.nlm.nih.gov). Specific primers for 18srRNA, mytimycin, mytilinB, lysozyme and defensin genes were designed using the Primer3.0 software (http://biotools.umassmed.edu/bioapps/primer3). In this study, the 18srRNA gene was retained as the housekeeping gene.

2.4.2. Gradient PCR

The stock and working solutions of the primers were prepared at 100 µM and 20 µM, respectively. The gradient PCR was performed with Bio-Rad My Cycler™ thermocycler at annealing temperatures between 52 and 66°C. The PCR products from each gene were obtained by separating on a 2% agarose gel in 1X TBE (Tris-borate-EDTA) buffer for 75 minutes at 100 volts.

2.4.3. Bulk PCR and Band elution of PCR product

To have sufficient amounts of PCR product for cloning and sequencing a total of 50 µl of the reaction at each particular annealing temperature was loaded and ran on a 1.5% agarose gel

in 1X TBE (Tris-borate-EDTA) buffer stained with Gel Red. The specific bands of interest were cut and purified from gel slices, following the instructions of the manufacturer (Wizard SV Gel and PCR Clean –up System, Promega, USA).

2.4.4. Cloning

Ten microliters of ligation mixes were prepared in advance, containing 5 µl ligation buffer 2X, 1 µl pGEM-T easy vector, 3 µl insert, and 1 µl T4 enzyme. 50 µl of thawed chemical competent TOP10 *E.coli* was supplemented with 2 µl of a ligation mixture and incubated on ice for 20 minutes. Further, a heat shock of 42°C was applied for 60 seconds after which the *E.coli* were immediately placed back on the ice. 950 µl of SOC medium (Tryptone 2%, Yeast Extract 0.5%, NaCl 0.05%, KCl 0.02%) was added then the mixture was incubated at 37°C at 210 rpm for 1hour. Afterward, a sample of 30, 150, 300 µl was spread on LB supplemented ampicillin (100 mg ml^{-1}) plates and grown in an incubator at 37°C for 16 hours. A single colony was picked up, and colony PCR was performed to see only for positive clones. These clones were picked and grown overnight in LB broth containing ampicillin.

2.4.5. Plasmid extraction and sequencing

The protocol for plasmid extraction is described by the manufacturer of the kit (Wizard Plus Minipreps DNA purification system, Omega). NanoDrop 2000 (Thermo Scientific) measured the concentration of that plasmid. The number of plasmid copies was calculated according to the formula of Whelan, Russell and Whelan [20] and used to build the standard curve for each gene.

The concentration of the primers was between approximately 10 and 20 ηg $µl^{-1}$ and the final volume was 10 µl. Primers T7 and SP6 were used as reverse and forward sequencing primers, respectively. The genomic sequencing company LGC (Germany) conducted the sequencing. The software Vector NTI version 15.0 was used to edit the sequence database. The obtained sequences were used to perform nucleotide BLAST on NCBI to verify their identity for the respective AMPs genes.

2.5. Real-time qPCR standardization of primer concentrations

The concentration of primer from each gene was optimized based on the manufacturer's instructions (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X)). Each primer

pair was prepared at different concentrations varying from 0.1 to 0.35 µM to select the optimal level for defining the standard curve. A standard curve was drawn by plotting the natural log of the threshold cycle (C_T) against the natural of the number of molecules. The plasmids were serially diluted from 10^2 to 10^6 plasmid copy μl^{-1}. The standard curve was run in triplicate per sample in the StepOneTM Real-Time PCR System thermal cycler (Applied Biosystems, Belgium). The concentration of the primer that gave an optimal efficiency of 90 – 110 % with a slope between -3.1 and -3.6 was selected to determine the standard curve for C_T value calculations.

2.6. Total RNA extraction and Reverse Transcription

Before the RNA extraction, the larvae were homogenized using the stomacher homogenizer machine (Minibeabbeater, Biospec products, Branson 1200 Model B1200E-1, USA). Tissue debris of the larvae was removed using the Qiashredder apparatus (Qiagen, Hilden, Germany) to avoid clogging of the RNA extraction columns. Total RNA was extracted using the SV Total RNA Isolation System kit (Promega, USA) following the manufacturer's instructions. Extracts were subsequently treated with DNase I (Fermentas, Germany) to remove the remaining DNA. The RNA concentration was checked with the NanoDrop 2000 (Thermo Scientific) and adjusted to 500 ng μl^{-1} in all samples. The complete DNA degradation within the RNA samples was confirmed by running the DNase-treated RNA sample in the PCR. After confirmation of the RNA quality by electrophoresis, it was stored at – 80^{o}C for further use.

Reverse transcription was performed with a RevertAidTM H minus First strand cDNA synthesis kit (Fermentas GmbH, Baden-Württemberg, Germany) following the manufacturer's instructions with some modifications [21]. Briefly, a mixture of 1 µg RNA and 1 µl random hexamer primer solution was prepared. Then, 8 µl of the reaction mixture containing 4 µl of 5x reaction buffer (0.25 mol^{-1} Tris-HCl pH 8.3, 0.25 mol^{-1} KCl, 0.02 mol^{-1} $MgCl_2$, 0.05 mol^{-1} DTT), 2 µl of 0.01 mol^{-1} dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAidTM H minus M-MuLV Reverse Transcriptase was added. The reaction mixture was incubated for 5 min at 25^{0}C followed by 60 min at 42^{0}C. The reaction was terminated by heating at 70^{0}C for 5 min and then cooled to 4^{0}C. cDNA samples were checked by PCR and stored at −20^{0}C for further use.

2.7. Real-time PCR

Real-time PCR was used to quantify the level of expression of the selected antimicrobial peptide genes. This procedure was performed using Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, Fisher Scientific, Erembodegem, Belgium) as described previously by Yang et al. [22] with some modifications. Briefly, the reaction was performed in a StepOneTM Real-Time PCR System thermal cycler (Applied Biosystems, Belgium) in a total volume of 25 µl, containing 12.5 µl of 2 × SYBR Green master mix, 300 nM of forward and reverse primers and 2 µl of template cDNA. The thermal cycle parameters used for the real-time amplification were an initial activation at 50^0C for 2 minutes, initial denaturation at 95^0C for 10 min followed by 40 cycles of denaturation at 95^0C for 15 s and primer annealing at 60^0C (gene 18srRNA), 63^0C (genes mytimycin, and mytilinB) , and 58^oC (genes lysozyme and defensin) and elongation at 60^oC for 1 min. Dissociation curve analysis in the real-time PCR was performed to check for the amplification of untargeted fragments. Data acquisition was carried out with the StepOneTM Software.

2.8. Real-time PCR analysis ($2^{-\Delta\Delta C}{}_T$ Method)

The real-time PCR was validated by amplifying serial dilutions of cDNA synthesized from 0.5 µg of RNA isolated from mussel larvae samples. Serial dilutions of cDNA were amplified by real-time PCR using gene specific primers. ΔC_T (average C_T value of target – average C_T value of 18srRNA) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 0 for all four target genes. Therefore, the amplification efficiency of reference and the target genes was considered to be equal. Based on this precondition, real-time PCR data were analyzed using the $2^{-\Delta\Delta C}{}_T$ method [23].

The relative expression was calculated as Relative expression = $2^{-\Delta\Delta C}{}_T$.

$$\text{With } \Delta\Delta C_T = \Delta C_{T,\text{target}} - \Delta C_{T,\text{ control}}$$

$$\Delta C_{T,\text{target}} = C_{T,\text{ target, time x}} - C_{T,\text{ 18srRNA, time x}}$$

$$\Delta C_{T,\text{ control}} = C_{T,\text{ control, time 3}} - C_{T,\text{ 18srRNA, time 3}}$$

RNA extracts from unchallenged mussels taken at 3h were used as a reference: expression in this sample was set at 1 and all other data were normalized accordingly. The 18SrRNA gene was used as a reference gene.

2.9. Phenoloxidase activity

The protocol as reported for *Artemia* by Baruah et al. [24] was slightly modified for use in mussel larvae [25]. Briefly, based on sufficient cellular material (> 0.1 g), the enzymatic transformation of L-3, 4-dihydroxyphenylalanine (L-DOPA) to dopachrome can be used to determine the phenoloxidase (PO) activity of tissues. Before the actual PO analysis, the homogenizing buffer (pH 7.5) was made (0.43% NaCl, 1.25 mM EDTA, 0.5% Triton-X, 5 mM $CaCl_2$). With this buffer, a 0.5 mM stock solution of L-DOPA was prepared (all chemicals purchased from Sigma-Aldrich, Germany). Then, larvae (10% w:w) were homogenized in the buffer using a pestle and stored overnight at 4^0C. Tube filters (0.22 µm) removed cellular debris the following day through centrifuging (10,000 g, 20 min). Triplicate 20 µl aliquots of the resulting larval protein extracts were placed in a 96-well tissue culture plate, and 200 µl of the L-DOPA solution was added to each well. Blank homogenizing buffer samples were included for determining the non-enzymatic dopachrome production. The tissue culture plates were dark-incubated at 30^0C, and absorbance (λ = 490 nm) was measured twice a day for the next 48 hours using the Tecan spectrophotometer (Tecan I-Control). For all treatments, differences in average optical densities at time t and 0 were calculated.

2.10. Statistics and analysis

Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS version 23). Statistical significance of differences in survival between treatment groups was assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

To study the effect of the treatments on expression levels (ΔC_T) of the different genes and on PO, a repeated measures ANOVA taking account of the replicate measurements was performed, with treatment and time as factors. The significance of the interaction between treatment and time was tested. In case the interaction effect was significant, a one-way ANOVA or t-test was performed for each time point followed by post-hoc tests with Bonferroni correction. For all statistical analysis a 5% significant level was used. The changes

in expression between a 0.5 and 2- fold threshold are not considered as biologically relevant (Fig 2, Fig 4, and Table 3).

3. Results

3.1. Survival (Experiment 1)

Survival of mussel larvae in Experiment 1 at 6h, 12h, and 24h was 100% for all treatments (data not shown). However, significant differences were observed between the treatments 48h post-exposure, whereby the addition of PHB-A lowered the mortality for the larvae challenged with *V. coralliitycus* (L+VC+PHB-A) compared to challenged larvae without PHB-A treatment (L+VC). The survival was highest for non-challenged larvae L (CT) and L+PHB-A (Fig 1).

3.2. Design of immune gene primers for blue mussel larvae

Table 2 gives an overview of the primers that were designed to evaluate the gene expression of the 4 selected antimicrobial peptides in mussel larvae by qPCR, using 18srRNA as a housekeeping gene. A standard curve based on the amplification efficiency for each gene was established (Table 3).

3.3. Impact PHB-A and/or pathogen on the expression of the antimicrobial peptides (Experiment 2).

The addition of the pathogen (L+VC) led to a significant down regulation of mytimycin after 12 h in comparison to the control treatment. Remarkably none of the genes were upregulated upon *Vibrio* challenge neither after 6h nor 12 h of exposure. The addition of PHB-A on the other hand led to a significant upregulation of mytimycin and defensin relative to the control after 6 h. This effect disappeared after 12h (Fig 2A & D). After 6 h, in the presence of *V. coralliilyticus* , PHB-A downregulated mytimycin and lysozyme. After 12 h this was only the case for lysozyme. The levels of defensin gene expression were equal for all treatments and time points except for treatment L+PHB-A+VC at 6h post-exposure (Fig 2D). The overall expression pattern, across treatments and time points, is rather similar for mytimycin and defensin. Both *Vibrio* challenge and PHB-A seem to downregulate lysozyme expression at both time points.

3.4. Phenoloxidase activity (Experiment 3)

PHB-A significantly increased the phenoloxidase activity during the 48h of the assay (Fig 3). However, not only PHB-A but also *V.coralliilyticus* triggered a significant increase in larval phenoloxidase activity ($p < 0.05$) as compared to the negative control (unchallenged). Especially, PO activity was significantly increased in the double treatment where the presence both of PHB-A and pathogen resulted in a higher PO activity relative to the control but also to the single treatments.

3.5. Time window of gene expression of AMPs (Experiment 4)

Overall, the expression of the AMPs genes in exposed mussel larvae appeared to be up-regulated during the challenge test with *V.coralliilyticus* and to rapidly increase from 24h onwards following exposure (Fig4A, 4B, and 4D). At 48 hrs, mytimycin, defensin, and mytilinB are expressed 53, 35.7 and 8.3-fold higher respectively, in comparison to the expression at time point 3h. During the 48h challenge test, the expression levels of the lysozyme are down-regulated (Fig 4C). When comparing the expression levels of lysozyme of exposed larvae with the levels in non-challenged larvae, a significant up-regulation was noticed at sampling point 6h (Table 4).

4. Discussion

The pathogenicity of this *V. corallilyticus* strain was again established in this study, confirming the validity of the strain as a pathogen using the described challenge protocol.

Knowledge on the immune system in bivalve larvae is scarce. In order to develop a tool box for studying the immune response in bivalve mollusk, several studies concentrated on designing specific primers for AMPs genes. Adults and larvae of *M. galloprovincialis* have been studied quite extensively: primers for mytilin, myticin, defensin, heat shock protein gene 70 (HSP70), mytilinB, lysozyme and for the house-keeping genes 18srRNA and 28srRNA have been reported [1, 3, 11, 26-28]. The primers developed for *M. galloprovincialis* were tested at several occasions during this research and did not work well for the blue mussel *M. edulis* larvae, challenged with *V. coralliilyticus*. There was also no information on the gene

expression of mytimycin in *M. edulis* species although it is abundantly present in the hemolymph. Therefore it was necessary to first develop the primers for the 3 antimicrobial peptides that were reported to be present in *M. edulis* adults [29] as well as for the hydrolytic enzyme lysozyme.

The selection of the housekeeping gene 18srRNA for mussel larvae appeared to be the right choice, based on its expression in both untreated and treated mussel larvae and its stability in expression, independently from the treatment (data not shown). This allowed us to establish the kinetics of expression of the AMPs genes and lysozyme gene in blue mussel larvae in response to a *V.coralliilyticus* challenge.

For a long time, mollusk hemocytes have been reported to be responsible for bactericidal activities mediated by plenty oxic compounds [28], antimicrobial peptides [29] and lysozyme [30], and by phenoloxidase activity [31]. During the last decade, knowledge of immune processes in adult bivalves has significantly improved by the development of genomic tools [32-34]. However, the immune characteristics of larvae remain under-investigated, notably due to the difficulty to isolate hemocytes from larvae. Elston [35] observed phagocytes (described as coelomocytes) containing bacterial fragments in the visceral cavity of *C. virginica* veliger larvae. Dyrynda, Pipe and Ratcliffe [36] have confirmed that some elements of the immune system in adult *M. edulis* are also present in the trochophore and veliger larvae of this species. Recently, several studies reported that hemocytes appear during the gastrular – trochophore developmental stages. At these stages, haemocyte generation/proliferation and induction of immune related genes are concomitant [37]. Phenoloxidase activity is commonly found in invertebrates [38] and is present in hemocytes of the adult mussel and larvae as well [31, 36]. PO activity have been detected in all the disaggregated larval cells from *M. edulis*, showing a strong reaction in the veligers [36] and was also previously observed in cells from the inner mantle fold of the pediveliger of *O. edulis*, [39].. Bacterial infection can cause a significant increase in PO activity in the whole hemolymph of adult bivalves *Crassostrea madrasensis* and *Chlamys farreri* [16, 40]. Recently De Rijcke et al. [25] determined a significant increase in PO activity in blue mussel larvae challenged with the toxin of harmful microalgae. The implications of an elevated larval PO activity are, however, largely unknown as the immunological role of PO is still poorly understood.

In this study, PO activity was detected in homogenates of *M. edulis* larvae and results show that PO enzyme activity was elicited in non-infected mussel larvae in response to larvae fed PHB-A. These findings are consistent with previous studies in other aquaculture species such as *Artemia* [24] and may at least partly explain why mussel larval survival increased when PHB-A was added to the rearing water in this study (Fig 1), and previous experiments [18].

In the mussel *Mytilus galloprovincialis*, the expression of both antimicrobial genes mytilinB and defensin are recorded to be developmentally regulated, and neither gene is expressed in mussels until after larval settlement and metamorphosis according to Mitta et al. [27]. Our findings contradict this statement since defensin was present in the 2-day old D-larvae of *M. edulis* and the gene was clearly upregulated 48hr after Vibrio challenge, suggesting an active role in the immune system.

Our study confirmed the presence of lysozyme in *M. edulis* D-larvae, although its expression was very low. Lysozyme-like and hydrolysis enzyme activities are present in *C. gigas* and *Ruditapes decussatus* larvae.

The existence and diversity of AMP has been revealed both in *M. edulis* [29] and *M. galloprovincialis* [41]. Cellura et al. [3] described how AMPs respond specifically to the challenges, confirming that at least some of the innate immune mechanisms are specifically orientated. Mytimycin for example is linked to fungal infection [29, 42] and also shows very low expression in the velum of *M. galloprovincialis* larvae, when challenged with *Vibrio anguillarum* [43]. However, this is in contradiction to the earlier findings of Mitta et al. [27] who found that both mytilinB and defensin genes in *M. galloprovincialis* are constitutively expressed and not inducible following *Vibrio alginolyticus* (DSMZ 2171) bacterial challenge. Possibly however the host response is strain dependent.

The time window of gene expression of AMPs in the larval phase of *M. edulis* was not determined yet. Measuring gene expression in D-larvae in the presence of both PHB-A and *Vibrio coralliilyticus* at two specific time points namely 6 and 12 h after challenge (experiment 2)revealed that in this short time frame, neither PHB-A nor the bacterial challenge caused upregulation of the measured immune genes (apart from some upregulation after 6h by PHB-A) This might indicate that the infection process had not started yet (in which case the fluctuations are stochastic) or that this particular species is

able to control the host response at the transcriptional level (assuming that infection had started). The PHB-A treatment did not have a major positive effect either and hence does not seem to contribute to the capacity of the host to maintain expression of the tested genes (in this time frame). In view of these observations immune genes expression was monitored over a longer time frame (experiment 4).

The experiment running over 48h demonstrated that mussel larvae (2-day old D-larvae) regulate the tested genes from 24h post-exposure onwards. Mytimycin is the AMPs gene with the highest expression in *M. edulis* larvae in response to the exposure to the pathogenic bacterium *V. coralliilyticus,* followed by defensin and mytilinB. This result indicates that D-larvae are either responding very late to the invading pathogen or that the pathogen is only invading the host after 24 h exposure when an increased expression of the tested genes becomes apparent. This is also in agreement with the results of the first experiment where mortality only occurs after 48 h. The hypothesis of a late defensive response would be in accordance with Balseiro et al. [43] who stated that bivalve larvae are not entirely immune-competent to combat infections by pathogens.

However the PO activity measurements, in larvae 48 hours after exposure, seem to point in the other direction. Here PHB-A stimulates PO, possibly contributing to the capacity of the larvae to handle a Vibrio challenge, in which case PHB-A acts as an immunostimulant. PO activity is also increased upon Vibrio exposure, but that level of PO activity is insufficient for protection against Vibrio. A double exposure, PHB-A, and *Vibrio*, increased PO activity even further. These data seem to suggest that some immunological capacity is available in this developmental stages and that it can be steered, such as by the addition of PHB-A.

Taken together it is apparent that the immunological response in larvae as monitored by gene expression can only be interpreted at its best if simultaneously the infection process is visualized (by e.g. immunohistochemistry). Such data would allow a better understanding of the dynamics of the immune response.

In conclusion, this study reports the successful development of 5 primers (3 AMPs, 1 hydrolytic enzyme, 1 house-keeping gene) to monitor the immune response of blue mussel (*M. edulis)* larvae. PHB-A regulated immune gene expression was found in challenged mussel

larvae, as revealed by PO activity in unchallenged and challenged (48h after exposure) larvae. This may at least partly explain why larval survival increases in the presence of PHB-A.

It was demonstrated that 2 day-old blue mussel larvae activate the expression of the three AMPs, mytimycin, mytilinB and defensin upon invasion by the pathogen *V. coralliilyticus*. The level of expression is up- and down-regulated in the first 12hours after exposure in an unclear pattern, but rapidly increases from 24 to 48 h post-exposure. The expression of lysozyme, however, remains very low and stable during the first 48 h after challenge. Mytimycin gene is one of the genes with the strongest expression in blue mussel larvae.

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Table 1: Summary of the challenge tests with *V. coralliilyticus* (10^5CFU mL^{-1}) in the presence of PHB-A (1 mg L^{-1}) added 6 h before the pathogen

Challenge experiment (Exp.)	Vessel	Sampling point	Evaluation
Exp.1	Tissue culture Dishes (TCD)	48h	Survival of larvae
Exp.2	Glass bottle of 1 L	0, 3, 6 and 12 h	AMPs gene expression
Exp.3	Glass bottle of 1 L	12,24,26 and 48h 48h	Phenoloxidase activity
Exp.4	Glass bottle of 1 L	0, 3, 6, 9, 12, 24, 48	AMPs gene expression

Table 2: Characteristics of the primers used for expression analysis with RT-qPCR

No	Gene	GenBank accession no.	Primer	Amplicon length (bp)	Annealing temperature
1	18srRNA	L33448.1	F: TTAAGAGGGACTGACGGGGG R: TTGGCAAATGCTTTCGCAGT	93	60 °C
2	Mytimicin	JN825739.1	F: CCATTGTTGGGACTGCACTG R: CGGTCCCCACGTTCATAACA	123	63 °C
3	MytilinB	AF177540.1	F: CAGAGGCAAGTTGTGCTTCC R: GGAATGCTCACTGGAACAACG	125	63 °C
4	Lysozyme	AF334662.1	F: CCAACGACTATTCATGTGCCT R: TCCCCTTGGACCTCCATTGT	122	58 °C
5	Defensin	JN935271.1	F: CCCAGCACCGATTCTAGGAC R: AAGCGCCATATGCTGCTACT	140	58 °C

Table 3: RT-qPCR amplification efficiencies for all immune genes

No.	Gene	Funtion	Slope	R^2	Amplification efficiency (%)
1	18SrRNA	Housekeeping gene	-3.5	0.99	93
2	Mytimycin	Target gene	-3.2	0.99	103
3	MytilinB	Target gene	-3.1	0.98	109
4	Lysozyme	Target gene	-3.3	0.98	100
5	Defensin	Target gene	-3.3	0.98	102

Table 4: Significant differences (*) in gene expression regulation (↑up or ↓down) of 4 AMPs between challenged (V. *coralliilyticus* 10^5 CFU mL^{-1}) and unchallenged blue mussel larvae (negative control) in function of exposure time (Experiment 4).

Gene	Post exposure (h)				
	6	9	12	18	24
Mytimycin	*↑				*↑
MytilinB	*↓	*↓			*↑
Lysozyme	*↑	*↓	*↓		
Defensin	*↑		*↓		*↑

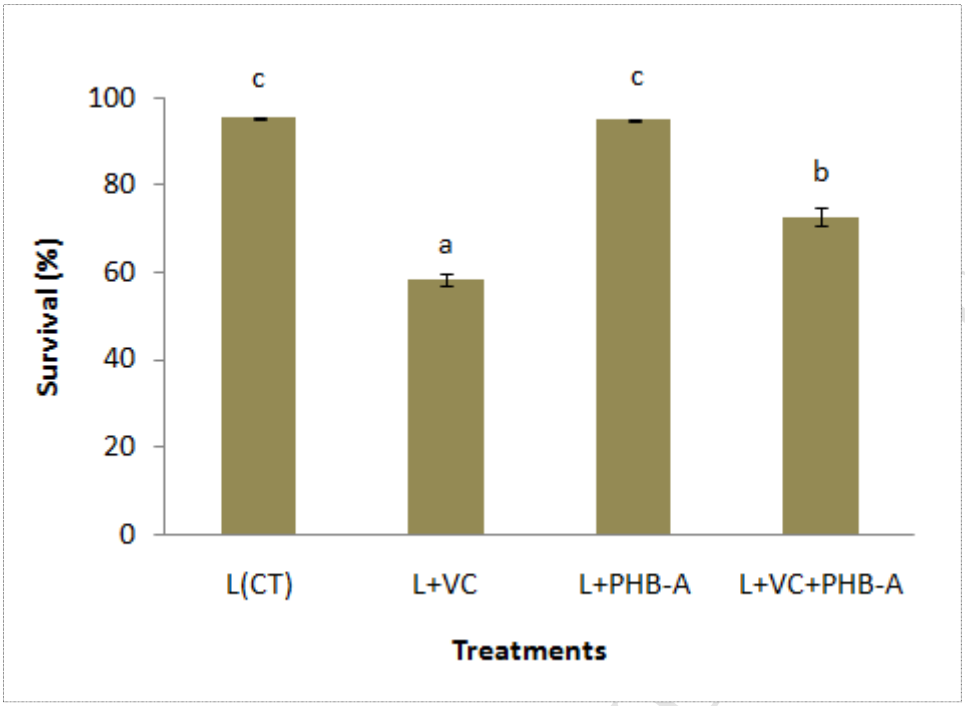

Fig 1: Experiment 1. Survival (%) of mussel larvae after 48h exposure to *V. coralliilyticus* (10^5 CFU mL^{-1}) in the presence or not of PHB-A (1 mg L^{-1}). Data are expressed as mean ± Standard Error (SE) of three replicates. Different letters indicate significant differences ($p < 0.05$).

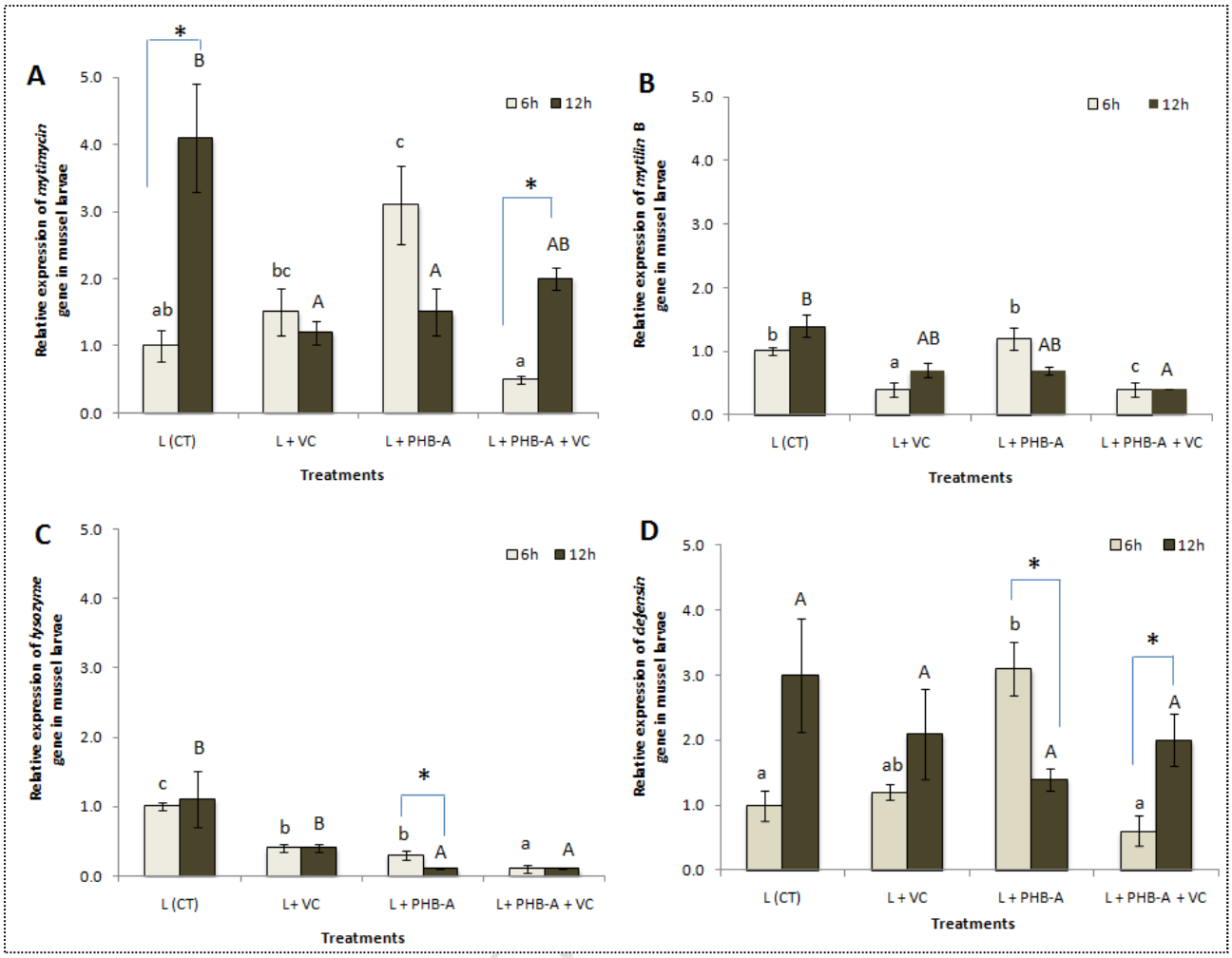

Fig 2: Experiment 2. Expression of immune genes (A) mytimycin, (B) mytilinB (C) lysozyme, and (D) defensin in unchallenged (L) and challenged (L+VC) mussel larvae with *V.coralliilyticus* at 10^5 CFU mL^{-1} (n=3, mean ± standard error). PHB-A (1 mg L^{-1}) was added to the mussel larvae culture water 6 h before the pathogen. The expression of AMP genes in the control treatment (CT) was regarded as 1.0. Different small and capital letters indicate significant differences between the treatments for the sampling points 6 h and 12 h post challenge respectively. Asterisks denote a significant difference between sampling point 6 h and 12 h post challenge for a particular treatment (T-test, $P < 0.05$).

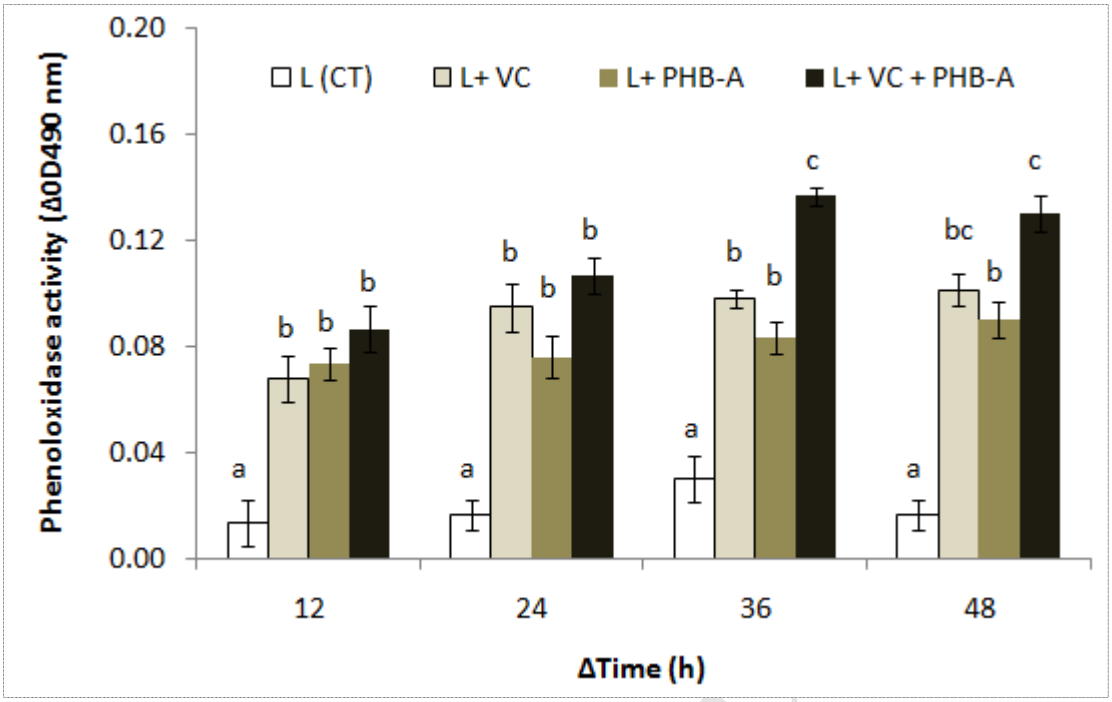

Fig 3: Phenoloxidase activity (PO) in the mussel larvae collected after 48 h exposure to the different treatments (mean ΔOD±SE, n=3). Data represent the increase in dopachrome over a 48 hours time frame in which PO activity in the 4 samples was measured. Data are expressed as mean ± Standard Error (SE) of three replicates. Different letters indicate significant differences for a given time point ($P < 0.05$).

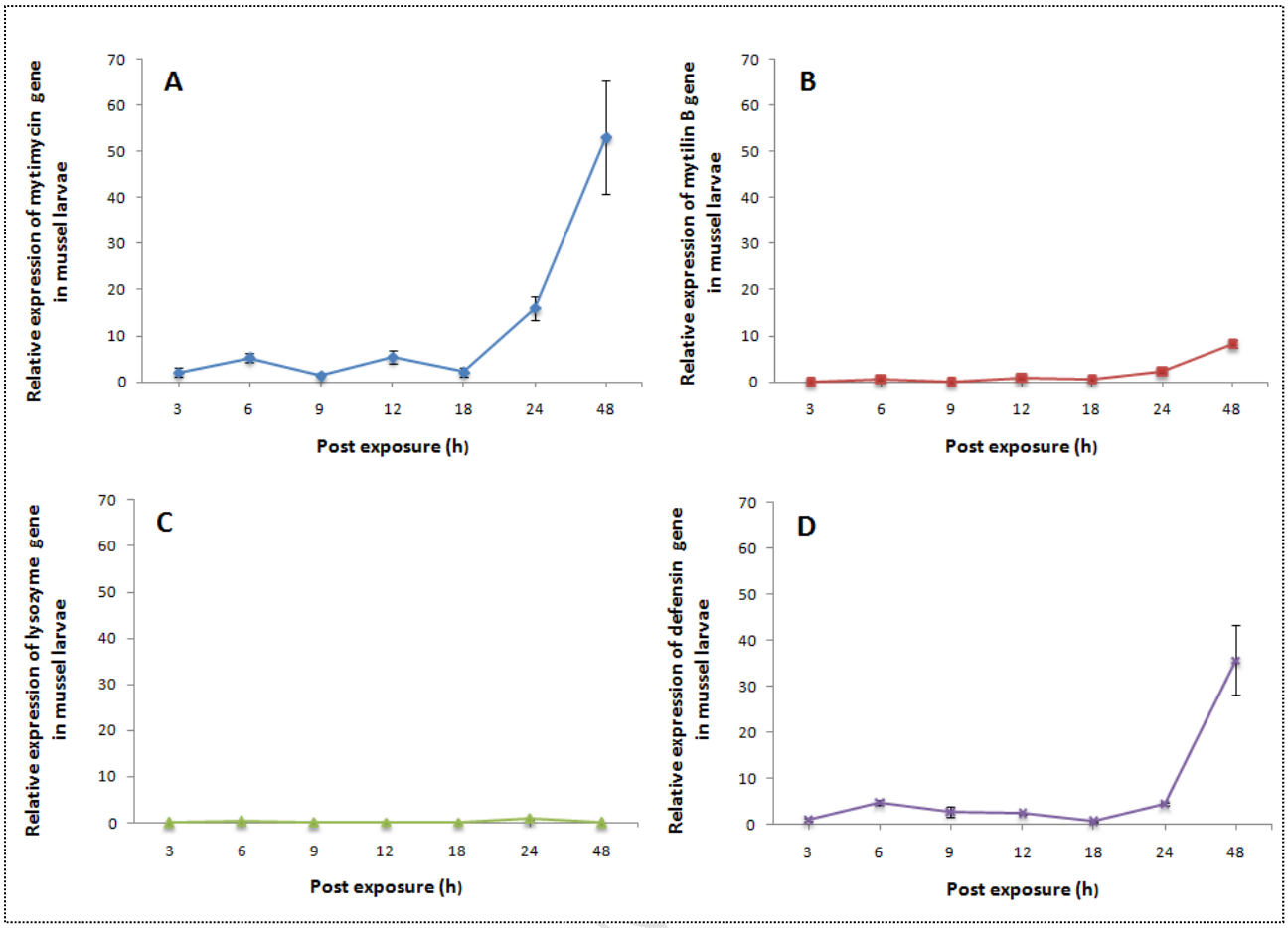

Fig 4: Expression of immune genes (A) mytimicin, (B) mytilinB, (C) lysozyme and (D) defensin in challenged mussel larvae with *V. coralliilyticus* at 10^5 CFU mL^{-1} (n=3, mean ± standard error). The expression of AMPs gene in the negative control was regarded as 1.0 at 3h.

Highlights

Our study is innovative because for the first time

*Specific primers are developed for 4 antimicrobial peptides in *Mytilus edulis* larvae .

*The effect of PHB in blue mussel larvae has been related to gene expression .

*The antimicrobial peptide defensin was identified in *Mytilus edulis* larvae