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Effects of the pathogenic *Vibrio tapetis* on defence factors of susceptible and non-susceptible bivalve species: II. Cellular and biochemical changes following in vivo challenge

Bassem Allam [a,*], Christine Paillard [b], Michel Auffret [b], Susan E. Ford [c]

[a] *Marine Sciences Research Center, Stony Brook University, Stony Brook, NY 11794-5000, USA*
[b] *Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest-Iroise, 29280 Plouzané, France*
[c] *Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349-3617, USA*

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Abstract

This work compared the effect of challenge with *Vibrio tapetis*, the etiologic agent of brown ring disease (BRD) in clams, and other bacterial strains on defence-related factors in four bivalve species: *Ruditapes philippinarum* (highly susceptible to BRD), *R. decussatus* (slightly susceptible to BRD), *Mercenaria mercenaria* and *Crassostrea virginica* (both non-susceptible to BRD). Results show that bacterial challenge modulated defence-related factors, namely total and differential haemocyte counts, percentage of viable haemocytes, and lysozyme activity, both in haemolymph and extrapallial fluid. Injection with bacteria induced a response that was dependent upon the bacterial and bivalve species investigated, and upon the site of inoculation: external (pallial cavity), pseudo-internal (extrapallial space), or internal compartment (adductor muscle). The most conspicuous changes were systematically measured in *R. philippinarum* injected with *V. tapetis,* indicating a bacterial pathogenicity particular to the host in which it causes a specific disease syndrome. Alterations of defence-related factors were maximal in haemolymph of clams injected with *V. tapetis* in the muscle, and in the extrapallial fluid when the bacteria were injected into the pallial or the extrapallial cavity. Resistance to the development of the BRD symptom was not related to the extent of the haemocyte reaction measured following in vivo challenge.

Keywords: Bivalve; Brown ring disease; Defence-related factors; *Vibrio tapetis*

* Corresponding author. Fax: +1 631 632 8915.
E-mail address: bassem.allam@stonybrook.edu (B. Allam).

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1. Introduction

In studies of the interactions between the internal defence system of bivalve molluscs and their pathogens, it is often useful to compare known susceptible and non-susceptible host species, and known virulent and non-virulent microorganisms [1–4]. In a companion study [5], we examined in vitro interactions between several bacterial species on circulating haemocytes of three bivalves, including the effect of the pathogen *Vibrio tapetis* on the clam, *Ruditapes philippinarum*, in which it causes brown ring disease (BRD). This bacterium caused decreased haemocyte viability in *R. philippinarum* compared to the hard clam, *Mercenaria mercenaria,* and the oyster, *Crassostrea virginica*; neither of the latter two is susceptible to BRD. *Vibrio tapetis* also decreased phagocytic activity of *R. philippinarum* haemocytes. These results suggest that the deleterious effect of *V. tapetis* on *R. philippinarum* haemocytes may be a major reason for the susceptibility of this host; however, because bivalves possess several layers of defence, both physical and immunological, in vivo challenges may help clarify the relative significance of various defence factors. This is particularly true when a variety of dosing methods is employed, resulting in different barriers being breached [6,7]. While internal defence is provided by humoral and cellular factors of the haemolymph [8,9], defence factors present in pallial [10] and extrapallial fluids [11] constitute the first line of defence after the shell. Mucus produced by pallial organs, the gills and the mantle, agglutinates and lyses bacteria [10,12] and helps the elimination of microorganisms by mechanical processes such as ciliary action [13]. Thus, the internal defence system is generally only exposed to pathogens that have been able to penetrate inside tissues, through breaks or lesions in epithelia.

Since the bivalve internal defence system is largely based on haemocytes, changes in haemocyte counts and activities have been widely used as an in vivo indicator of the pathologic state of animals following natural or experimental contact with pathogens [14–20]. In addition, humoral factors released by haemocytes may help fight invasive microorganisms [21]. These include hydrolytic enzymes, such as lysozyme, which can act directly against bacteria by lysing these microorganisms [8,22–24] or indirectly by increasing the killing activity of other antibacterial substances [25]. In vivo changes in both cellular and humoral factors have already been reported in *R. philippinarum* naturally [19] or experimentally [17,18,20,26] infected with *V. tapetis*. Severely infected *R. philippinarum* exhibited depressed haemocyte counts and phagocytic activity, whereas infections of the more resistant clam species *R. decussatus*, provoked increased haemocyte counts and phagocytic activity [26].

Experimental induction of BRD is typically done by injection of the pathogen into the pallial cavity of the clam where it faces external defence factors described above (agglutinins, lysozyme, etc.). The effect of direct injection of *V. tapetis* into the pseudo-internal (extrapallial - between the mantle and shell) and internal (tissues) compartments on defence parameters have never been studied despite previous observations showing the presence of *V. tapetis* within the extrapallial fluid [19] and inside cells of the digestive diverticula [27] of severely diseased animals.

This report describes the results of in vivo challenge with *V. tapetis* on haemolymph factors in four bivalve species with different susceptibilities toward this pathogen. The bivalves studied were *R. philippinarum* (highly susceptible to BRD), *R. decussatus* (slightly susceptible to BRD), *M. mercenaria* and *C. virginica* (both non-susceptible to BRD) [28,29]. A second objective was to compare the effect of *V. tapetis* on certain haemolymph factors with that of other bacteria, including the marine pathogen *Listonella* (=*Vibrio*) *anguillarum*. A final objective was to assess the role of *R. philippinarum* external defence factors in resisting *V. tapetis*. This was accomplished by comparing the effect of injecting *V. tapetis* into (1) the pallial cavity and (2) the pseudo-internal (extrapallial space) and internal (tissue) compartments. Cellular and humoral defence factors were investigated both in haemolymph and extrapallial fluid in order to evaluate the role of each of these two body fluids in fighting bacteria. Because of the multi-objective nature of this paper, we are presenting here a compilation of three different experiments performed in France and in the United States.

2. Material and methods

2.1. Bivalves

Adult clams (*Ruditapes philippinarum* and *Ruditapes decussatus*) without disease symptoms were collected from wild populations in the Bay of Brest (France) except for *R. philippinarum* used in the first experiment (see below) being harvested from Puget Sound (WA, USA). Specimens of the hard clam *Mercenaria mercenaria* were collected near Atlantic City (NJ, USA) and eastern oysters *Crassostrea virginica* were obtained from Delaware Bay (NJ, USA). Animals were maintained in tanks of aerated seawater (32–34 ppt for different clam groups, and 26 ppt for *C. virginica*) at 13 ± 1 °C. They were fed daily using a mixture of cultured algae. All assays were performed on individual specimens.

2.2. Bacteria

Vibrio tapetis (ATCC 4600) was isolated from diseased *R. philippinarum*. The other bacteria employed were *Listonella (=Vibrio) anguillarum* (strain 775), a pathogen of fish and bivalve larvae, and an unidentified non-*Vibrionaceae* bacterial species (R2) frequently encountered in apparently healthy clams (Allam, unpublished). Bacteria were grown on marine agar (1 L distilled water, 15 g agar, 20 g sea salts (Sigma), 4 g peptone, 0.1 g $Fe(PO_4)_2$). Bacterial suspensions used in inoculation experiments were made in sterile seawater (SSW) with bacteria (5×10^8 cfu mL^{-1}) reaching the exponential phase of growth (typically 72 h at 20 °C).

2.3. Inoculation experiments

2.3.1. Effect of *V. tapetis* on total and differential haemocyte counts in clams and oysters

This experiment compared the effect of challenge with *V. tapetis* on circulating haemocytes of *R. philippinarum*, *M. mercenaria* and *C. virginica*. Different batches (n = 15 to 20 per batch) of clams and oysters were separately inoculated into the adductor muscle (posterior muscle for clams) with 0.1 mL (5×10^7 cfu $individual^{-1}$) of *V. tapetis* suspension. Clams were injected by inserting the needle through the hinge ligament. In oysters, the injection was performed through a notch made at the edge of the shell. Control animals were injected with SSW. Prior to injection, 100 μL of haemolymph was collected and analysed to provide Time 0 parameters. Bivalves were then replaced in aerated tanks as described above. Three days following challenge, haemolymph was collected from the adductor muscle (posterior muscle for clams) to measure total and differential haemocyte counts as described below.

2.3.2. Effect of bacterial challenge on haemocyte and plasma parameters in clams

This experiment was designed to study the kinetics of changes in total and live haemocyte counts in clams following injection of different bacterial strains into tissues of two clam species. *Vibrio tapetis*, *L. anguillarum* or R2 were injected (5×10^7 cfu $clam^{-1}$) into the posterior adductor muscle of *R. philippinarum* (120 clams per batch). A fourth batch of clams received heat-killed (60 °C for 2 h) *V. tapetis*, and a fifth batch received SSW (120 clams each). At the same time, two batches of the mildly susceptible clam, *R. decussatus,* received live *V. tapetis* or SSW, also injected into the adductor muscle, to compare its response to that of *R. philippinarum*. Clams were maintained separately (7 tanks total) and haemolymph was collected at different time intervals: 2 h, 6 h, 1, 3 and 7 days following challenge. Total and dead haemocyte counts, protein concentration and lysozyme activity in haemolymph were measured on individual clams.

2.3.3. Role of external defence factors

To investigate the role of external defence factors, present in the pallial and extrapallial spaces, in fighting bacterial pathogens, 500 specimens of *R. philippinarum* were separated into 10 equal batches of 50 clams each. Clams were challenged with bacteria according to three different protocols. Clams from the first batch received 0.1 mL (5×10^7 cfu) of *V. tapetis* suspension into the pallial (mantle) cavity. According to Paillard et al. [30], this method of challenge induces BRD symptoms within days. The same *V. tapetis* inoculum was injected into the posterior adductor muscle or the extrapallial space of two additional batches. Inoculation into the extrapallial space was through a hole made in the central part of the shell using a round dental burr. Care was taken to avoid damaging the mantle. After injection, the hole in the shell was sealed using a piece of sterile glass coverslip and dental paste. Similarly, two sets of control batches were made. The first set (3 batches) was inoculated with live cells of the non-pathogenic strain R2 (5×10^7 cfu in 0.1 mL clam^{-1}) in the pallial cavity, the adductor muscle, or the extrapallial compartment, whereas the second set (3 batches) was injected with 0.1 mL SSW in the same locations. The last batch was untreated in an effort to provide a control material. Each batch was incubated in a separate tank. Total haemocyte counts, protein concentration, and lysozyme activity were measured in clam haemolymph and extrapallial fluid 3 days following challenge.

2.4. Fluid sampling

Body fluids were sampled as previously described [11]. Briefly, haemolymph was collected from the adductor muscle (posterior adductor muscle in clams). In control clams, a hole was made at the central part of the shell to allow collection of extrapallial fluid. The extrapallial fluid of clams previously inoculated in their extrapallial space was harvested through the hole already made during the injection by taking off the glass coverslip. In oysters, the sampling was made through the notch previously made for the injection procedure.

2.5. Total, viable and differential haemocyte counts

Total haemocyte counts were assessed microscopically using a haemocytometer (Experiments 1 and 2) or using a Coulter Counter, Model Z particle counter (Experiment 3) on aliquots of each body fluid. Aliquots were first fixed using iso-osmotic formalin-based (3% final concentration, Experiment 1) or glutaraldehyde-based (2.5% final concentration, Experiment 3) solutions. During Experiment 2, total and viable haemocytes were counted simultaneously in mixtures (v/v) of samples with 0.2% trypan blue in seawater.

Differential haemocyte counts were performed by flow cytometry. The percentage of granular and agranular cells was determined using a Coulter EPICS flow cytometer as previously described [31].

2.6. Protein and lysozyme measurements

Haemolymph and extrapallial fluid samples were immediately centrifuged to separate supernatant and cell pellet. Resulting fractions were frozen (−80 °C) until subsequent processing, typically within the next week. Protein contents and lysozyme activity were measured in supernatants as described in [11], by adapting the original methods described by [32] for protein, and by [33] for lysozyme. Bovine serum albumin and chicken egg white (CEW) lysozyme served as standards, respectively. Results were expressed as mg protein mL^{-1} and μg chicken egg white lysozyme equivalent mg protein^{-1}.

A summary of experimental conditions and measured parameters is given in Table 1.

Table 1
Experimental conditions and measured parameters

Experiment	Bivalve species	Inoculum	Injection site	Analysed fluid	Parameters measured	Sample times[a]
1. Effect of *V. tapetis* on circulating haemocytes	*R. philippinarum* *M. mercenaria* *C. virginica*	*V. tapetis* SSW	Adductor muscle	Haemolymph	Total haemocyte counts Differential haemocyte counts Bivalve mortality BRD development	3 days
2. Kinetics of changes in haemocyte counts	*R. philippinarum* *R. decussates*	*V. tapetis* *L. anguillarum* R2 SSW	Adductor muscle	Haemolymph	Total haemocyte counts Percentage of dead haemocytes Protein Lysozyme Clam mortality BRD development	2 & 6 h 1, 3 & 7 days
3. Effect of injection site on circulating haemocytes	*R. philippinarum*	*V. tapetis* R2 SSW	Adductor muscle Extrapallial space Pallial cavity	Haemolymph Extrapallial fluid	Total haemocyte counts Protein Lysozyme Clam mortality BRD development	3 days

SSW, sterile seawater.
See text and following tables for more details.

2.7. Statistics

Arcsine transformations were applied to the percentage granulocytes before the use of statistical tests. Total haemocyte counts were Log_{10}-converted before comparisons. However, tables and figures show means and standard errors of non-transformed values. For each experiment and for each species, differences among treatments were tested using a one-way general linear model ANOVA followed by Fisher's PLSD post hoc test. Differences were considered significant at $\alpha = 0.05$.

3. Results

3.1. Development of BRD and mortality

Signs of BRD (macroscopic and/or microscopic organic spot on the inner face of the shell) appeared only in *R. philippinarum* inoculated with *V. tapetis* in the pallial cavity (Experiment 2), reaching 42% on Day 3. None of the other bivalves, including *R. philippinarum*, injected with *V. tapetis* within the adductor muscle or the extrapallial space, showed any disease symptoms. Similarly, inoculation with bacteria other than *V. tapetis* did not induce the development of BRD in *R. philippinarum*. The percentage of dead animals ranged from 0% to 3%, and was not significantly different from 0, in all batches of all species in all experiments on Day 3. Mortality reached 14% on Day 7 in *R. philippinarum* injected within the adductor muscle with live *V. tapetis* (Experiment 2). During this experiment, the injection of *V. tapetis* induced no mortality in *R. decussatus*.

3.2. Haemocyte changes in clams and oysters following challenge with V. tapetis

3.2.1. Total haemocyte counts

The inoculation of live *V. tapetis* within the adductor muscle caused a significant increase in total haemocyte counts (THC) in haemolymph of both the French (5.3×10^6 cells mL^{-1}) and the American (5.9×10^6 cells mL^{-1}) *R. philippinarum* in comparison to SSW-injected controls (2.5 and 2.6×10^6 cells mL^{-1}, respectively; Table 2). A similar increase was noted in *M. mercenaria* (Table 2). Conversely, challenge with *V. tapetis* induced no significant change in THC, compared to SSW injection, in *R. decussatus* or *C. virginica*.

Table 2
Total haemocyte counts (10^6 cells mL^{-1}, mean ± SEM) and percentage of granulocytes (mean ± SEM) in haemolymph 3 days following injection of *V. tapetis* into the adductor muscle of four bivalve species

	Bivalve species/measured parameter				
	RP-FR ($n=10$)	RP-US ($n=20$)	*R. decussatus* ($n=6$)	*M. mercenaria* ($n=18$)	*C. virginica* ($n=15$)
Total haemocyte counts					
V. tapetis	5.3 ± 0.9^b	5.9 ± 0.7^b	2.5 ± 0.4^a	3.7 ± 0.4^c	4.1 ± 0.3^b
SSW	2.5 ± 0.2^a	2.6 ± 0.4^a	2.5 ± 0.3^a	2.4 ± 0.3^b	3.9 ± 0.8^b
Day 0	2.3 ± 0.2^a	2.0 ± 0.2^a	2.5 ± 0.2^a	1.7 ± 0.2^a	2.3 ± 0.4^a
Percentage granulocytes					
V. tapetis	NA	76.3 ± 3.0^b	NA	82.0 ± 2.2^b	45.2 ± 2.7^b
SSW	NA	62.5 ± 3.4^a	NA	74.7 ± 2.9^a	39.2 ± 2.7^b
Day 0	NA	59.3 ± 4.3^a	NA	73.2 ± 3.4^a	24.6 ± 2.3^a

RP-FR and RP-US, French and American *R. philippinarum*, respectively; NA, not-assayed. For each parameter, letters (a, b and c) represent differences between experimental and SSW-injected control individuals within each species (Fisher's PLSD post hoc test, $P < 0.05$).

3.2.2. Percentage granulocytes

The percentage of granular haemocytes followed trends similar to THC (Table 2). The percentage of granulocytes increased significantly in *V. tapetis* challenged *R. philippinarum* and *M. mercenaria* in comparison with SSW-injected controls. In *C. virginica*, the increase was observed in both batches in comparison with values measured on Day 0. Small granulocytes, a distinct subpopulation of granular cells present in *C. virginica* [31,34], also increased in oysters regardless of injection with *V. tapetis* (3.5±0.4%, mean±SEM) or SSW (2.9±0.3%), in comparison with respective values measured on Day 0 (1.3±0.2).

3.3. Kinetics of haemolymph responses in clams challenged with different bacterial strains

3.3.1. Total haemocyte counts

A rapid increase (within hours) in THC was observed in the haemolymph following injection of bacteria (live and heat-killed *V. tapetis*, and live *L. anguillarum* and R2) into the adductor muscle of *R. philippinarum* (Fig. 1a). Differences between bacteria-injected and SSW-injected clams became significant 6 h following challenge, at which time the highest counts were measured in clams injected with *L. anguillarum* (4.2×10^6 cells mL^{-1}). Haemocyte counts regained normal values in clams injected with heat-killed *V. tapetis* and R2 on Day 1 and Day 3, respectively. On Day 3, the highest haemocyte counts were observed in clams injected with live *V. tapetis* (5.3×10^6 cells mL^{-1}, more than twice control values; Table 3), followed by animals

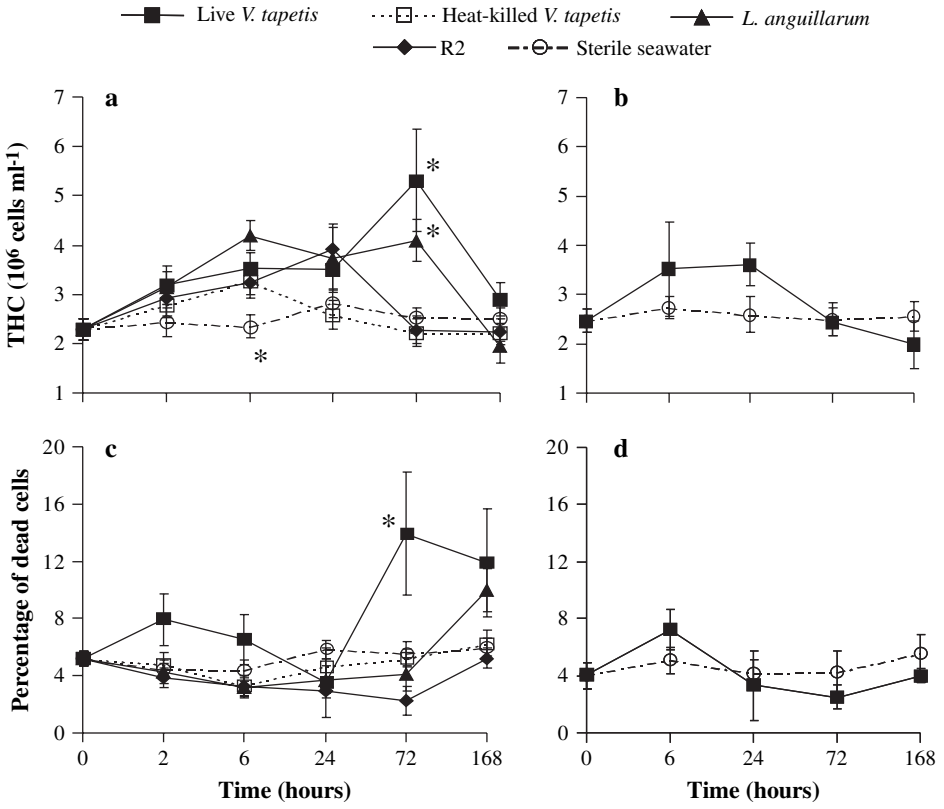

Fig. 1. Effect of bacterial challenge on total haemocyte counts (a,b) and percentage of dead cells (c,d) in *Ruditapes philippinarum* (a,c) and *Ruditapes decussatus* (b,d). *Significant differences with other data points measured at the same time interval ($P<0.05$, Fisher's PLSD post hoc test). $n=10$ clams except for *R. decussatus* where $n=6$. Mean±SEM.

Table 3
Haemolymph parameters (mean ± SEM) measured 3 days following injection of different bacterial strains into the adductor muscle of *R. philippinarum*

Measured parameter	Bacterial strain				
	SSW	*V. tapetis*	H-K *V. tapetis*	*L. anguillarum*	R2
THC	2.5 ± 0.2^{a}	5.3 ± 0.9^{b}	2.2 ± 0.2^{a}	4.1 ± 0.4^{b}	2.3 ± 0.3^{a}
PDC	5.2 ± 0.8^{a}	13.9 ± 4.3^{b}	5.2 ± 0.6^{a}	4.1 ± 1.1^{a}	2.2 ± 1.0^{a}
Protein	3.2 ± 0.2^{a}	5.8 ± 0.7^{b}	3.1 ± 0.3^{a}	4.2 ± 0.4^{a}	3.8 ± 0.3^{a}
Lysozyme	5.4 ± 0.8^{a}	14.8 ± 2.3^{b}	6.3 ± 1.0^{a}	9.0 ± 1.4^{a}	5.8 ± 0.9^{a}

THC, total haemocyte counts (10^6 cells mL^{-1}); PDC, percentage of dead haemocytes; Protein, $\times 10^{-1}$ mg mL^{-1}; Lysozyme, μg equivalent chicken egg white lysozyme mg $protein^{-1}$; H-K *V. tapetis*, heat-killed *V. tapetis*; R2, non-Vibrionaceae bacterial species. Letters (a and b) represent differences between treatments (Fisher's PLSD post hoc test, $P < 0.05$). $n = 10$ clams $sample^{-1}$.

injected with *L. anguillarum*. Afterwards, haemocyte counts decreased in these batches, eventually reaching values measured in control clams. In *R. decussatus,* results showed an early non-significant increase in haemocyte counts following challenge with live *V. tapetis*, however, these counts regained values observed in SSW-injected animals on Day 3 (Fig. 1b).

3.3.2. Dead haemocyte counts

In *R. philippinarum*, an early increase in percentage of dead cells (PDC) was observed in clams 2 h following injection with live *V. tapetis* into the muscle (Fig. 1c). Thereafter, PDC decreased until Day 1, after which a new increase was observed. The only statistically significant difference was noted on Day 3, between clams injected with live *V. tapetis* and the other groups (Fig. 1c, Table 3). A slight decrease was noted in this batch at the end of the experiment, while a non-significant increase was measured in clams injected with *L. anguillarum*. The injection of live *V. tapetis* did not affect haemocyte viability in *R. decussatus* (Fig. 1d).

3.3.3. Protein concentration

Protein concentration increased rapidly in all *R. philippinarum* clams inoculated with bacteria; however, these changes were not significant relative to SSW-injected clams (Fig. 2a). On Day 3, protein concentrations were significantly higher in the haemolymph of clams injected with live *V. tapetis* (0.58 mg mL^{-1}, Table 3) when compared to all the other batches. A decrease was noted subsequently. The injection of live *V. tapetis* did not affect protein contents in the haemolymph of *R. decussatus*, in which similar values were measured in SSW- and *V. tapetis*-injected clams (0.28 and 0.31 mg mL^{-1} on Day 3, respectively; Fig. 2b).

3.3.4. Lysozyme activity

The injection of bacteria into the muscle of *R. philippinarum* caused a rapid increase in lysozyme activity in haemolymph (Fig. 2c). At 6 h, lysozyme activity was significantly higher in all challenged clams in comparison with SSW-injected animals. On Day 1, lysozyme activity regained control values in clams injected with heat-killed *V. tapetis* and R2, but increased in clams injected with *L. anguillarum* (14 μg mg $protein^{-1}$) and particularly in those challenged with live *V. tapetis* (22 μg mg $protein^{-1}$, maximal value). The activity then gradually decreased until the end of the experiment, although higher values were systematically measured in clams injected with live *V. tapetis* (Fig. 2c, Table 3). Lysozyme activity was not significantly modified in the haemolymph of *R. decussatus* following challenge with live *V. tapetis* (Fig. 2d).

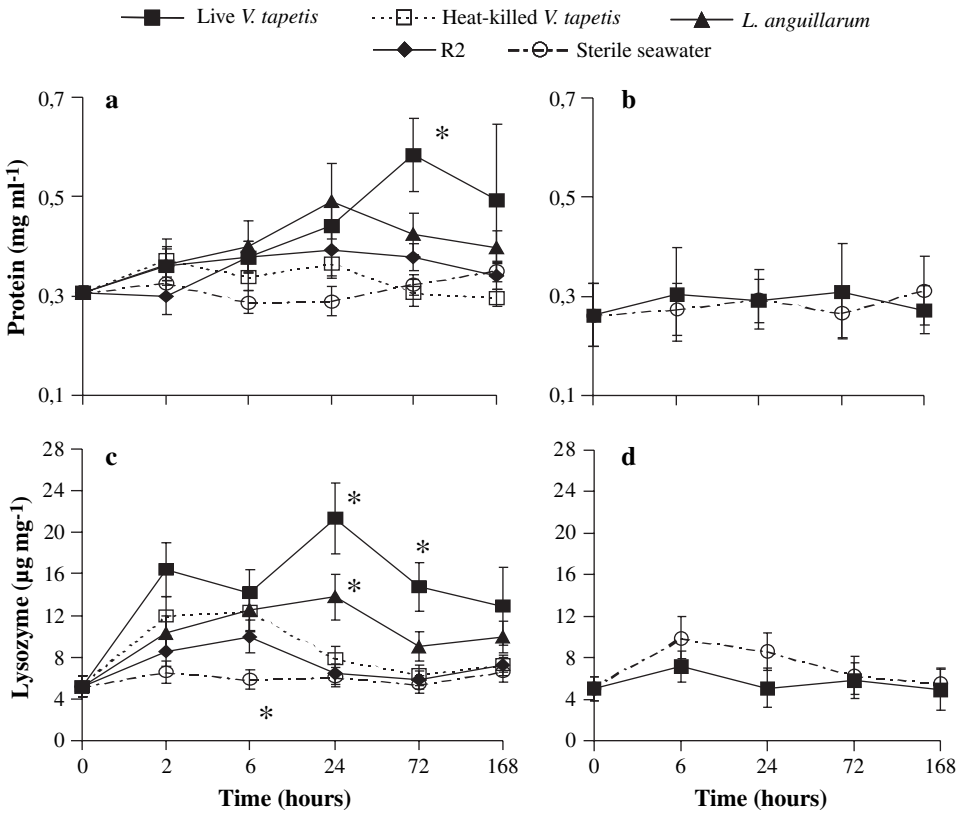

Fig. 2. Effect of bacterial challenge on protein contents (a,b) and lysozyme activity (c,d) in *Ruditapes philippinarum* (a,c) and *Ruditapes decussatus* (b,d). *Significant differences with other data points measured at the same time interval ($P<0.05$, Fisher's PLSD post hoc test). $n=10$ clams except for *R. decussatus* where $n=6$. Mean ± SEM.

3.4. Responses of R. philippinarum following injection of V. tapetis into different compartments

3.4.1. Total haemocyte counts

In agreement with previous experiments, haemocyte counts increased markedly in haemolymph of clams injected with *V. tapetis* within the adductor muscle (6.3×10^6 cells mL^{-1}), in comparison with non-treated clams and those injected with SSW (2.1 to 2.3×10^6 cells mL^{-1}, Table 4). Injection of the non-pathogenic strain R2 into the muscle also caused a slight increase when compared with SSW-injected clams. Furthermore, high circulating haemocyte counts were observed in all treated clams, regardless of the inoculum (including clams injected with SSW), when compared with non-treated controls. The inoculation within the pallial cavity caused an increase only when *V. tapetis* was introduced.

Significant increases in haemocyte counts were observed in the extrapallial fluid of all treated clams in comparison with non-treated controls (Table 4). This increase was most marked in clams inoculated in their extrapallial cavity with bacteria, particularly with *V. tapetis*. The injection of *V. tapetis* in the pallial cavity also caused an increase in haemocyte counts in the extrapallial fluid.

3.4.2. Protein contents

In haemolymph, protein concentration increased significantly in clams inoculated with *V. tapetis* in the adductor muscle in comparison with all the other treatments (Table 5).

Table 4
Total haemocyte counts (10^6 cells mL^{-1}, mean±SEM) in haemolymph and extrapallial fluid of *R. philippinarum* 3 days following injection of bacteria into the adductor muscle (AM), the extrapallial space (ES) or the pallial cavity (PC)

Inoculum	Site of inoculation/analysed fluid		
	AM	ES	PC
Haemolymph (Untreated controls: 2.1 ± 0.3^{a})			
V. tapetis	6.3 ± 1.2^{c}	3.4 ± 0.3^{b}	3.4 ± 0.4^{b}
R2	3.6 ± 0.3^{b}	3.4 ± 0.3^{b}	2.0 ± 0.2^{a}
SSW	2.3 ± 0.3^{a}	3.8 ± 0.3^{b}	2.3 ± 0.3^{a}
Extrapallial fluid (Untreated controls: 2.5 ± 0.3^{a})			
V. tapetis	$3.7\pm0.6^{a,b}$	5.1 ± 0.7^{b}	5.2 ± 0.6^{b}
R2	2.9 ± 0.5^{a}	4.4 ± 0.4^{b}	2.4 ± 0.3^{a}
SSW	2.4 ± 0.2^{a}	$3.6\pm0.6^{a,b}$	2.3 ± 0.3^{a}

R2, non-Vibrionaceae bacterial species. Letters (a, b and c) represent differences between treatments (bacterial strain, site of inoculation) within each body fluid (Fisher's PLSD post hoc test, $P<0.05$). $n=10$ clams $sample^{-1}$.

In the extrapallial fluid, protein contents increased significantly in treated clams when compared with the non-treated control, regardless of the nature of the inoculum (Table 5).

3.4.3. Lysozyme activity

In haemolymph, the highest lysozyme activity (22 μg mg $protein^{-1}$) occurred in clams injected in their muscle with *V. tapetis* (Table 6). Inoculation of this pathogen within the extrapallial space and in the pallial cavity also caused an increase in lysozyme activity in haemolymph.

In the extrapallial fluid, lysozyme activity was maximal in clams inoculated with *V. tapetis* into the extrapallial space (19.5 μg mg $protein^{-1}$), followed by those injected with the pathogen into the adductor muscle (16.5 μg mg $protein^{-1}$, Table 6). Changes caused by injection of SSW or by inoculation with R2 were not significant in haemolymph or in the extrapallial fluid.

4. Discussion

By applying a set of various pathogenic stimuli to several bivalve species, this study demonstrated that defence-related factors could be selectively modulated in vivo as a function of the bacterial challenge.

Table 5
Protein contents ($\times10^{-1}$ mg mL^{-1}, mean±SEM) in haemolymph and extrapallial fluid of *R. philippinarum* 3 days following injection of bacteria and SSW into the adductor muscle (AM), the extrapallial space (ES) or the pallial cavity (PC)

Inoculum	Site of inoculation/analysed fluid		
	AM	ES	PC
Haemolymph (Untreated controls: 2.7 ± 0.2^{a})			
V. tapetis	5.1 ± 0.7^{c}	$3.4\pm0.4^{a,b}$	3.8 ± 0.2^{b}
R2	$3.7\pm0.2^{a,b}$	$3.3\pm0.1^{a,b}$	$3.3\pm0.2^{a,b}$
SSW	$2.9\pm0.3^{a,b}$	$2.9\pm0.3^{a,b}$	$3.5\pm0.4^{a,b}$
Extrapallial fluid (Untreated controls: 2.0 ± 0.1^{a})			
V. tapetis	2.1 ± 0.2^{a}	3.0 ± 0.2^{c}	2.1 ± 0.2^{a}
R2	1.9 ± 0.2^{a}	$2.8\pm0.2^{b,c}$	2.1 ± 0.2^{a}
SSW	1.9 ± 0.1^{a}	2.9 ± 0.2^{c}	$2.3\pm0.2^{a,b}$

R2, non-Vibrionaceae bacterial species. Letters (a, b and c) represent differences between treatments (bacterial strain, site of inoculation) within each body fluid (Fisher's PLSD post hoc test, $P<0.05$). $n=10$ clams $sample^{-1}$.

Table 6
Lysozyme activity (μg equivalent chicken egg white lysozyme mg protein^{-1}, mean±SEM) in haemolymph and extrapallial fluid of *R. philippinarum* 3 days following injection of bacteria into the adductor muscle (AM), the extrapallial space (ES) or the pallial cavity (PC)

Bacterial strain	Site of inoculation/analysed fluid		
	AM	ES	PC
Haemolymph (Untreated controls: 5.2±1.0[a])			
V. tapetis	22.0±2.6[e]	13.8±2.4[d]	10.5±1.3[c,d]
R2	7.9±1.5[a,b,c]	9.0±2.0[b,c]	4.3±1.0[a]
SSW	5.5±1.0[a,b]	7.6±1.5[a,b,c]	4.8±1.0[a]
Extrapallial fluid (Untreated controls: 7.0±1.3[a])			
V. tapetis	16.5±1.8[b,c]	19.5±3.8[c]	11.6±1.7[a,b]
R2	8.5±1.4[a]	13.3±1.7[a,b]	8.9±2.4[a]
SSW	7.9±1.3[a]	12.8±2.2[a,b]	9.1±1.9[a]

R2, non-Vibrionaceae bacterial strain. Letters (a, b, c, d and e) represent differences between treatments (bacterial strain, site of inoculation) within each analysed fluid (Fisher's PLSD post hoc test, $P<0.05$, $n=12$ clams sample^{-1}).

Defence factors were differentially modulated according to the bacterial strain and bivalve species used, and were further confined to a particular body fluid (haemolymph or extrapallial fluid) depending on the site of inoculation. The injection of *V. tapetis* into the muscle induced a significant increase in haemocyte counts in *R. philippinarum* and *M. mercenaria*, but not in *R. decussatus* or *C. virginica*. Similarly, the most significant changes in defence-related factors were systematically measured in *R. philippinarum* injected with *V. tapetis*, which is highly pathogenic to that particular clam species. Earlier work described specific induction of defence-related response in bivalves by particular microorganisms [23,29,35,36]. This specificity is generally considered a result of specific interaction between microorganism and the challenged bivalve at the molecular level involving both host's [37,38] and pathogen's [39] recognition factors. Our results denote an increased alteration with increased bacterial toxicity. For instance, challenge of *R. philippinarum* with the non-pathogenic bacteria R2 caused relatively slight changes in haemocyte numbers and lysozyme activity, followed with the slightly pathogenic *L. anguillarum* and finally *V. tapetis* which induced the greatest alterations in measured parameters. These results are in accordance with an earlier report on in vivo toxicity of these bacterial strains to *R. philippinarum*, denoting that injection of *V. tapetis* inside the adductor muscle caused heavy mortality in this clam, while injection of *L. anguillarum* and R2 caused low and no mortality, respectively [6]. Similarly, the percentage of dead haemocytes increased in *R. philippinarum* injected with live *V. tapetis* into their muscle, but not in *R. decussatus* (Fig. 1). A recent study demonstrated that *V. tapetis* possesses thermosensitive cytotoxic factors that are able, in vitro, to kill haemocytes from *R. philippinarum* and, to a far lesser degree, those from *M. mercenaria* and *C. virginica* [5]. Thus, it emphasizes the greater sensitivity of *R. philippinarum* haemocytes toward *V. tapetis*. Overall, it seems obvious that the triggering of change observed in haemocyte and plasma components does not necessarily represent onset of efficient neutralization of a pathogen, as the most resistant bivalve species had relatively stable immune parameters after challenge. However, it should be noted that, except for 'percentage of dead haemocytes' that clearly reflects the toxic effect of bacteria (mainly *V. tapetis*), the changes measured in haemolymph might be helping bivalves overcome the stresses due to bacterial challenge and shell damage. Percentage granulocytes increased significantly in haemolymph of clams (*R. philippinarum* and *M. mercenaria*) injected with live *V. tapetis* into muscle in comparison with SSW-injected animals; this observation suggests that mobilized haemocytes following bacterial challenge are mainly granulocytes. Granulocytes are considered to be the major effector cells for internal defence in bivalve molluscs because of their high phagocytic activity [40,41] and their ability to produce a wide array of enzymes and antimicrobial substances [42,43], including lysozyme which increased significantly in bacteria-challenged *R. philippinarum*. Additionally, the percentage of granulocytes was previously correlated with

phagocytic activity of haemocytes and with resistance of clams to BRD [26]. Thus, increased numbers of haemocytes, particularly granulocytes, observed in the present work following injection of bacteria into tissues, are probably beneficial to the bivalve because these cells are able to phagocytose *V. tapetis*, both in vivo and in vitro [41,26,6,44]. Similarly, an increase in soluble protein concentration was previously reported in bivalves following challenge with pathogenic bacteria [45,38] and protozoans [3], and is believed to be related to the synthesis and/or release of compounds that play a role in the elimination of pathogens from tissues, as agglutinins (glycoproteins), which increased in oysters challenged with bacteria [45].

Unlike *V. tapetis*, the R2 strain did not induce any perceptible change in defence factors when introduced into the pallial cavity of *R. philippinarum*, whereas it did when directly injected into tissues. This result underlines the efficiency of external (physical and immune) defence factors present at interfaces in limiting the damage caused by high numbers of pathogenic and non-pathogenic microorganisms. It should be noted that changes in defence factors might be limited to haemolymph or extrapallial fluid according to the site of injection. For instance, injection of bacteria (*V. tapetis* or R2) into the muscle induced a significant increase in haemocyte counts only in haemolymph, in comparison with SSW-injected animals (Table 3). On the other hand, the injection of bacteria into the extrapallial space caused an increase in haemocyte counts only in the extrapallial fluid (Table 3: significant difference between clams injected with *V. tapetis* and SSW-injected clams, Student *t*-test, $P<0.05$). Similar trends were also noted for the other measured parameters (Tables 4 and 5). These results suggest that haemocyte numbers are separately controlled within each body fluid and that haemocytes can be mobilized toward haemolymph or extrapallial fluid, according to the origin of detected stimuli. These findings suggest that the measurement of defence factors in haemolymph may not always be suitable for studying the immune response in bivalves affected by 'peripheral' infections such as shell diseases.

Haemocyte counts and protein concentrations increased in haemolymph and extrapallial fluid of all batches injected in the extrapallial space, whether the clams were injected with bacteria or with sterile seawater (Table 3). These changes were probably related to shell damage rather than to the introduction of seawater into the extrapallial space, since the injection of sterile seawater into the adductor muscle did not induce any significant changes in haemocyte counts during the same time frame. An increased number of haemocytes was observed microscopically in the extrapallial space of several gastropod and bivalve molluscs during shell repair [46–50]. This increase is probably the result of the mobilization of haemocytes from tissues toward the mantle-extrapallial space during the repair process. These cells play a role in the transportation of calcium and metabolites to damaged sites [46,47,51,52]. By means of their ability to phagocytose foreign materials [11], haemocytes present in the extrapallial fluid provide a cellular defence barrier during the repair of the damaged physical barrier. On the other hand, the increased protein concentration in the extrapallial fluid following shell damage may be related to the repair process that requires deposition of an organic matrix predominantly composed of protein compounds [50].

In conclusion, this work suggests a high specificity of *V. tapetis* to *R. philippinarum*, in accordance with in vitro studies [5], thus reflecting specific host-pathogen interactions. In the case of BRD, disease resistance seems to be a function of the extent to which host cells are damaged by the pathogen rather than the magnitude of the defence reaction following challenge with *V. tapetis*. The strategy used by each bivalve to counter bacterial challenge may differ according to the characteristics of their system of defence and to the pathogenicity/immunogenicity of the bacteria. The molecular basis of this disparity, particularly between *R. philippinarum* and *R. decussatus*, which can be both affected by BRD, should be addressed by developing appropriate challenge experiments.

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