

Effect of temperature on defense parameters in Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*

Christine Paillard*, Bassem Allam, Radouane Oubella

Laboratoire des Sciences de l'Environnement Marin, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Place Copernic, Technopole Brest-Iroise, 29280 Plouzané, France

ABSTRACT: Brown Ring Disease (BRD), a vibriosis affecting the clam *Ruditapes philippinarum*, is present on the Atlantic coasts of Western Europe and is considered to be a cold water disease. The present work investigated the effect of temperature on immune response and its relationships with BRD development. Clams maintained at different temperatures (8, 14 and 21°C) were experimentally challenged with the pathogen *Vibrio tapetis*, the etiologic agent of BRD. Results demonstrated significant effects of temperature on disease development and on hemolymph immune parameters including total and viable hemocyte counts, lysozyme and leucine aminopeptidase activities. Thirty days after challenge, clams maintained at 21°C displayed significantly higher values for all the measured immune parameters in comparison to specimens incubated at 14°C. Improved performance of the immune system was associated with a low BRD prevalence. The recovery process, which occurred mainly at 21°C, was associated with high percentages of viable hemocytes and high activities of leucine amino-peptidase and lysozyme. This laboratory study clearly demonstrates that temperature strongly affects BRD development and clam immune response during infection. Favourable immune status at higher temperature may confer upon the clam a better capacity to fight the disease agent, and therefore to recover more easily.

KEY WORDS: Immunomodulation · Temperature · Hemocyte · Leucine aminopeptidase · Lysozyme · Disease · Clam · *Vibrio*

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INTRODUCTION

Bivalve molluscs possess an open circulatory system and as a result hemocytes are present in sinuses throughout all soft tissues and in extrapallial fluids located between the external epithelium of the mantle and the shell (Cheng 1981, Paillard et al. 1996, Allam & Paillard 1998). In bivalves, immune defense is nonspecific, and based on cellular and humoral factors. Hemocytes constitute the main line of defense against materials recognized as non-self; however, humoral factors, especially circulating enzymes and anti-microbial peptides, also play an important role because they possess various anti-microbial properties (Chu 1988, Cheng 1992, Roch 1999). Lysosomal enzymes such as leucine amino-peptidase (LAP) and lysozyme are particularly crucial because of their ability to hydrolyse protein components of foreign bacterial membranes (Hiroi et al. 1992, Lopez et al. 1997).

Vibrio tapetis is the bacterial agent responsible for Brown Ring Disease (BRD) in the clams *Ruditapes philippinarum* and *Ruditapes decussatus* (Paillard et al. 1989, 1994, Paillard & Maes 1990, Borrego et al. 1996). This disease occurs in wild and reared clam populations of France, England, Ireland, Spain and occasionally Italy, but has never been reported in Japan where *R. philippinarum* originates (Paillard et al. 1994, Allam et al. 2000b). *V. tapetis* colonizes the periostracal lamina, inhibits the normal process of shell biomineralization and thereby provokes an accumulation of melanized organic shell matrix on the inner face of the shell (Paillard & Maes 1995a,b). The pathogen is mainly detected in the extrapallial fluids, on the mantle edge epithelium, and on the periostracal lamina (Paillard 1992, Allam et al. 1996). In severely infected clams, *V. tapetis* can be found within cells on the digestive gland (Plana 1995, Plana et al. 1996). Experimen-

*Email: christine.paillard@univ-brest.fr

tal induction of the disease after *V. tapetis* challenge performed under standard conditions (14°C, 33‰) has shown that physiological functions such as nutrition and the immune defense system are severely affected in diseased clams (Oubella et al. 1994, 1996, Paillard et al. 1994, Plana 1995, Plana et al. 1996). Generally, at 14°C, the immune response in hemolymph, during the development of BRD, displays a 2-phase pattern: an early induction of cellular and humoral response in hemolymph (total hemocyte counts [THC] and enzymatic activities such as leucine aminopeptidase and lysozyme), followed by an inhibition of these defense parameters (Oubella et al. 1993, 1994, Paillard et al. 1994, Allam et al. 2000b). In severely infected clams, a concomitant increase in THC in the extrapallial fluids is measured suggesting their mobilisation to the site of infection (Oubella et al. 1993, 1994, Allam et al. 2000a). Mass mortalities of BRD-infected clams can result from the decline of metabolic activity, sometimes associated with a septicemic invasion of *V. tapetis* in hemolymph and tissues (Plana et al. 1996, Allam et al. 2002).

In marine invertebrates, disease prevalence is controlled by environmental factors (Harvell et al. 1999). In the case of BRD, field studies have suggested the influence of temperature on disease prevalence (Paillard et al. 1997). Along the French Atlantic coast, there is a clear boundary at the Loire river; the observed BRD prevalences ranged between 0 and 3% for the sites located south of the Loire, but ranged between 20 and 45% in sites north of Loire (Paillard et al. 1997). These inter-site prevalence variations could be explained by the pattern of extreme temperatures existing in the south (Paillard et al. 1997). Moreover, *Vibrio tapetis* is very sensitive to extreme temperatures; its *in vitro* growth is inhibited under 4°C and above 25°C (Paillard et al. 1997). Laboratory experiments have also demonstrated the effect of temperature on BRD prevalence, i.e. elevated temperature inhibits the development of BRD and enhances shell repair process (Paillard et al. 1997).

Variations of environmental parameters can also influence defense capacities in invertebrates and vertebrates. Studies have provided evidence for reduced immune function and increased infectious diseases and death rates during the winter for many vertebrates species (Afoke et al. 1993, Lochmiller et al. 1994). During the winter, immune factors may be low in these animals and opportunistic disease agents appear to overwhelm host defenses (Demas & Neslon 1996, 1998, Nelson & Demas 1996). In marine bivalves, environmental temperatures significantly modulate cellular and humoral activities (Feng & Feng 1974, Fisher 1988). Seasonal, extreme and short-term temperature variations affect many parameters related to the immune functions (hemocyte counts and functions

such as spreading, locomotion, and phagocytosis) (Fisher et al. 1987, 1989, Auffret & Oubella 1994, Oubella 1996). In the eastern oyster *Crassostrea virginica* hemocyte counts and phagocytosis are generally less in winter compared to summer (Feng & Canzonier 1970, Fisher et al. 1989). However, other activities, such as locomotion, spreading of hemocytes, and lysozyme levels in the serum display a different pattern with a significant decrease in summer (Feng & Canzonier 1970, Fisher et al. 1989). In the host-pathogen interaction *Crassostrea virginica-Perkinsus marinus*, oysters exhibit greater THC and phagocytic activity at high temperatures when the prevalence and susceptibility to *Perkinsus marinus* are also high (Chu & La Peyre 1993). The relationships between defense parameters modulated by temperature and disease susceptibility to pathogens remain unclear and may vary depending on each host-pathogen model.

Although there is evidence regarding the impact of temperature on BRD prevalence in the field, nothing is known about the relationship between defense-related parameters and BRD susceptibility and recovery at different temperatures. The purpose of this study is to evaluate the effect of temperature on immune response and its relationship to BRD syndrome.

MATERIALS AND METHODS

Specimens. Adult clams *Ruditapes philippinarum* (30 to 40 mm) with no clinical signs of BRD were collected from natural stocks in the Bay of Brest (Lanveur, Finistere, France). Two different experiments (Expts 1 and 2) were performed at the same time of year (autumn). Each experiment used 600 clams.

Bacterial strain. For the challenge experiments, the virulent *Vibrio tapetis* strain (P16), previously isolated from diseased *Ruditapes philippinarum* in the Gulf of Morbihan (Tascon, Morbihan, France), was used. Bacteria were grown in marine agar (Difco 2216) at 18°C for 72 h before inoculation experiments.

Animal handling and sampling. After collection, clams were maintained for 3 d in marine aquaria (temperature: 14°C ± 0.1°C; salinity: 34 ± 1‰). The clams were subdivided into 3 batches of 200 individuals. The first batch was maintained at 14°C. For the other 2 batches, the temperature was progressively increased or decreased (1°C d⁻¹) to 21°C or 8°C. Water temperature in the aquaria was controlled by an electronic thermostat system. After being maintained for 2 wk at these 3 different temperatures (8, 14, or 21°C), each batch was divided into 2 subgroups (100 clams each): the first subgroup was challenged with *Vibrio tapetis* as previously described (Paillard & Maes 1990) while

the second one was injected with sterile seawater (SSW). All procedures (emersion, inoculation) were performed in rooms maintained at 8, 14 or 21°C, depending on clam batches, to avoid any change in temperature. Challenged and control clams were incubated in aerated marine aquaria at the above conditions for 4 wk. They were fed daily with cultured algae (*Isochrysis galbana* and *Dunaliella euchlora*). Dead clams were collected daily and counted.

Hemolymph was collected from control and challenged clams as described by Auffret & Oubella (1995) at different time intervals: 3, 14, 24, 30 d for the first experiment (10 clams sample⁻¹), and 0, 14, 30 d for the second one (20 clams sample⁻¹). Time zero clams were sampled before the challenge and corresponded to acclimated clams not submitted to the overnight emersion before the inoculation procedure already described by Paillard & Maes (1990).

Hemolymph immune parameters. Total and dead hemocyte counts: A filtered solution of 0.2% ice-cold SSW-trypan blue was added (v/v) to hemolymph samples to stain dead and damaged cells. Total hemocytes and dead cells were simultaneously counted with a hemacytometer. Results are presented as millions of cells ml⁻¹ for THC and as percentages for dead cells (PDC).

Protein measurement: Hemolymph subsamples were kept in ice cooled vials. Protein determination was made on the hemolymph samples by the method of Bradford (1976) using bovine serum albumin as a standard measured at 595 nm on a spectrophotometer. The values were expressed as mg protein ml⁻¹ of hemolymph.

LAP activity: The LAP activity was determined in hemolymph diluted with deionized water (1:9) by using the method of Oubella et al. (1994). Briefly, after hydrolysis at neutral pH, the substrate (L-leucyl-naphtylamide) (Sigma reagents, ref. 61538) produces L-leucyl-naphtylamine. The absorbance of the dye product is measured by spectrophotometer at 580 nm. The LAP activity was expressed as specific activity (Sigma units ml⁻¹ mg of proteins).

Lysozyme activity measurement: Lysozyme concentration was determined spectrophotometrically according to the method of Shugar (1952) with modifications as previously described by Allam & Paillard (1998). Chicken egg white lysozyme (CEW, Sigma) served as the standard. Lysozyme activity is given as concentration of CEW lysozyme equivalent expressed in µg mg⁻¹ protein.

Characterisation and classification of BRD syndrome. Disease progression was monitored according to the description of Paillard & Maes (1994). According to these authors, conchiolin deposit stages (CDS) range from microscopic brown spots on the inner face of the

shell in the earliest stage (CDS 1) to a complete thick brown ring in the most advanced stage (CDS 7). Three main conchiolin deposit groups (CDG) were considered: CDG I (beginning of infection): CDS 1 and 2; CDG II (intermediate infection): CDS 3; and CDG III (well-developed infection): CDS 4 to 7.

Three stages of shell repair or recovery (SRS) were also described by Paillard & Maes in 1994. Stage 1 corresponds to the apposition of calcified concretions. The partial covering of the deposit by wide calcified plates corresponds to Stage 2. In Stage 3, the brown deposit is completely covered by shell layers. By considering the process of conchiolin deposit formation and the process of shell repair together, 3 phases of the disease are defined. The first phase corresponds to the development of the disease and is characterized only by accumulation of a conchiolin deposit. The second phase is characterized by the 2 processes, conchiolin deposit and shell repair, occurring simultaneously. The third phase corresponds to the end of the recovery process, when the clams have totally covered the conchiolin deposit.

Statistical analysis. Normality fitting was performed using a Kolmogorov-Smirnov test. Where necessary, parameters were expressed as log₁₀ or arc sines. In the tables and figures, data represent non-transformed values.

For both experiments and for each hemolymph parameter, differences between inoculated and control clams and between temperatures were assessed using Student's *t*-test. Significance level was defined at $\alpha = 0.05$.

For the comparison of multiple factors and their interactions (experiment, temperature, date, test), the non-parametric test, the Multiple Range Test (MRT) (confidence level at 95%) and also ANOVA tests (1-way or 4-way interactions at the 95% confidence level, if normality and variance tests allowed parametric tests) were used.

For testing the difference between both experiments, we used only values obtained from the same period of sampling (Day 14 and Day 30 after challenge). Mean deviations were calculated for both experiments to harmonize the data for statistical tests (regression analysis, linear model, confidence level, at 90 or 99%, ANOVA tests at 95% and MRT at the 95% confidence level).

To compare the effect of temperature on each hemolymph parameter of challenged clams a bacterial challenge indicator (BCI) was created. For each immune parameter, this indicator was calculated as

$$BCI = [(I_{1...n}) - C_m]$$

where $I_{1...n}$ corresponds to values from 1 to *n* individuals of *Vibrio tapetis*-inoculated clams (10 < *n* < 21) and

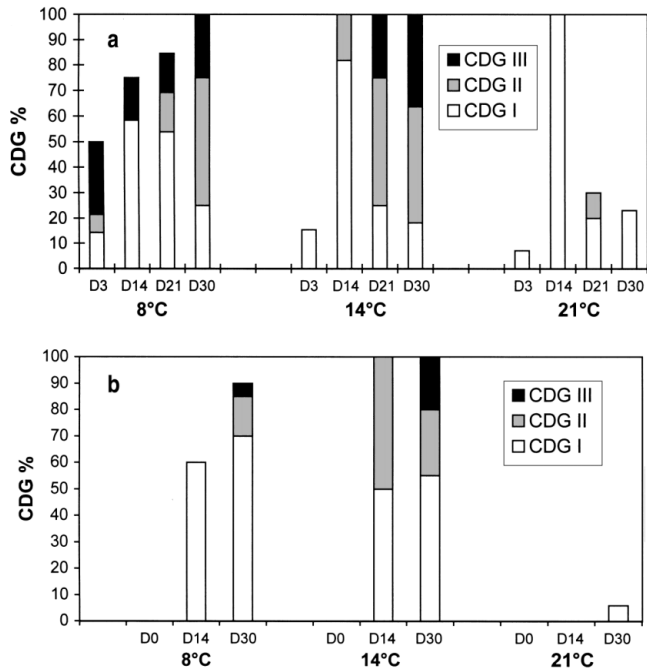


Fig. 1. *Vibrio tapetis* infecting *Ruditapes philippinarum*. Conchiolin deposit group (CDG) prevalence in (a) Expt 1 and (b) Expt 2. At each sampling, $n = 10$ and $n = 20$ for Expts 1 and 2, respectively. D: day

C_m corresponds to the mean of all the data obtained from control individual clams.

For testing relationships between BRD development (CDS and CDG, SRS and phases of the disease) and hemolymph parameters, the linear regression test was used. All the data from Expts 1 and 2 were used together for these tests. This was performed on diseased and healthy clams together ($n = 496$ to 502), or only on clams exhibiting shell repair ($n = 88$).

For the factorial correspondence analysis, the calculations were carried out on mean data of squared racine transformed values (Uniwin program).

RESULTS

BRD diagnosis

BRD prevalence, Conchiolin Deposit Groups

At 14°C , all challenged clams collected on Day 14 and Day 30 displayed BRD signs in both experiments. In those incubated at 8°C , BRD prevalence in challenged clams from Day 14 reached 75 and 60%, in Expts 1 and 2, respectively. These percentages increased to 90 and 100% by Day 30 (Fig. 1). Although prevalence was similar on Day 30 in clams incubated

at 14 and 8°C , BRD development was significantly different between these batches. The CDG was lower at 8°C than at 14°C (CDG III = 25% at 8°C and 40% at 14°C in Expt 1; CDG III = 5% at 8°C and 20% at 14°C in Expt 2). The conchiolin deposits were less developed in the second experiment than in the first one (18% and 55% at CDG I, in Expt 1 and 2, respectively).

The development of BRD was relatively low in challenged clams incubated at 21°C . During the first experiment, all clams exhibited BRD signs 15 d following challenge, but at very low CDG (100% at CDG I). One month after challenge, 40% of these diseased clams had recovered, and BRD prevalence decreased to 23%. During the second experiment, only 6% of the challenged clams collected on Day 30 displayed early signs of BRD (CDG I). At 21°C , the clams exhibited significantly lower values of CDG than those incubated at 8 and 14°C (MRT tests).

BRD signs were not detected in control clams (injected with SSW) maintained at 14 and 21°C , for both experiments. However, the control clams collected on Day 30 exhibited 20% BRD prevalence in batches incubated at 8°C , for both experiments.

Shell repair stages and disease phases

At Day 30, the percentage of challenged clams displaying shell repair processes was higher at 21 and 14°C than at 8°C . At 21°C , the SRS reached 65%, for both experiments (Fig. 2). One month following chal-

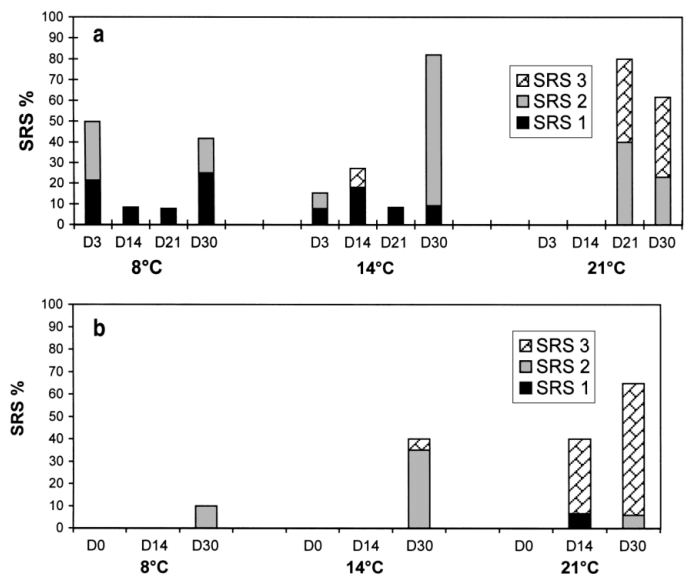


Fig. 2. *Vibrio tapetis* infecting *Ruditapes philippinarum*. Shell repair stage (SRS) prevalence in (a) Expt 1 and (b) Expt 2. At each sampling, $n = 10$ and $n = 20$ for Expts 1 and 2, respectively. D: day

lenge, the percentage of total recovery (SRS 3) was high only in clams incubated at 21°C, reaching 40 and 55% in Expts 1 and 2, respectively. Complete recovery was observed in only 5 to 10% of clams maintained at 14°C, and never in those held at 8°C, for both experiments. In Expt 1, the decrease in BRD prevalence between Day 14 (100%, Fig. 1) and Day 21 (30%) was associated with the process of complete recovery (SRS 3) which reached 40% on Day 21.

The clams conditioned at 21°C showed significantly higher values of SRS and disease phases than those incubated at 8 and 14°C (MRT tests).

Clam mortality

In Expt 1, clam mortality was low in all batches and remained below 10% at the end of the experiment. Similarly, a very low mortality (2 to 5%) occurred during the Expt 2 in control and experimental clams maintained at 8 and 14°C. At 21°C, mortality at Day 30 was low (5%) in challenged clams, but reached 30% in controls.

Temperature effects on hemolymph parameters

Using pooled data from both experiments, temperature significantly modulated all the immune parameters, except protein (ANOVA tests, $p < 0.002$) (Table 1).

Total hemocyte counts

THC level. In control clams, for all samples in both experiments, significantly higher THC were observed in clams incubated at 21°C in comparison with specimens

Table 1. Analysis of variance for the mean deviation of 5 defense parameters (total hemocyte count, THC; percentage of dead cells, PDC; leucine amino-peptidase, LAP; lysosyme, LYSO; protein) with 4 factors without interactions (temperature, test, experiment and date) considering data for both experiments ($n = 180$). Significant p -values are given in **bold**

Factors	THC	PDC	LAP	LYSO	Protein
Temp.	<0.00001	<0.00001	<0.00001	0.00211	0.1952
Test	<0.00001	0.0002	0.0011	0.0039	0.7672
Expt	0.6689	0.0424	0.2654	0.2299	0.7963
Date	0.4998	0.0022	0.5536	0.0456	0.192

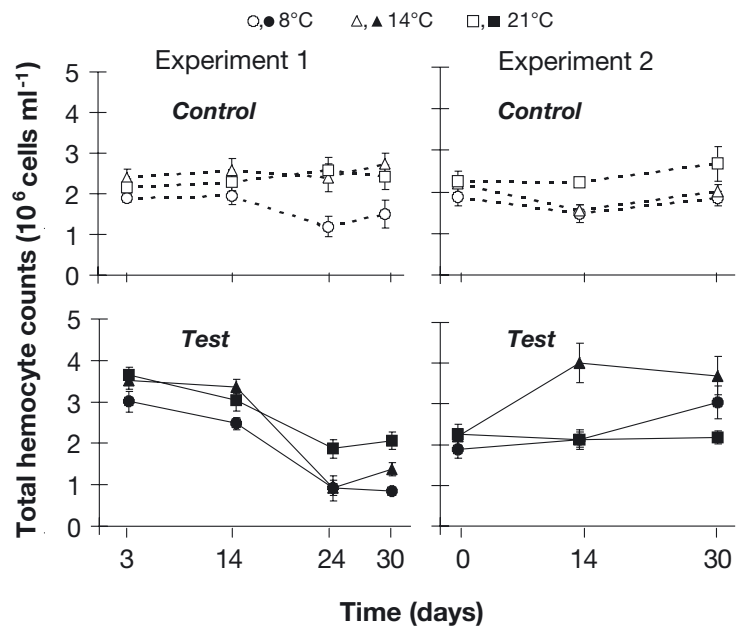


Fig. 3. *Vibrio tapetis* infecting *Ruditapes philippinarum*. Total hemocyte counts (THC) in control and *V. tapetis* challenged (test) clams, maintained at 8, 14 and 21°C. THC is expressed as the number of hemocytes 10^6 ml^{-1} . Vertical bars are standard error of the mean. ($n = 10$ in Expt 1 and $n = 20$ in Expt 2)

held at 8°C (MRT, Student's t -test, $p < 0.05$, ANOVA tests) (Fig. 3). Differences between clams incubated at 21°C and those held at 14°C were significant only in clams from Expt 2 collected on Day 14 and Day 30.

Similar results were observed in *Vibrio tapetis* inoculated clams from Expt 1 with high THC values at higher temperatures (Fig. 3). In contrast, inoculated clams from Expt 2 showed higher THC at 14 than at 8 and 21°C. These differences were significant only on Day 14 (Student's t -test, $p < 0.05$).

THC pattern. On Day 14, using pooled data from clams incubated at all temperatures and both experiments, the mean deviation of THC was significantly higher in inoculated clams than in controls (ANOVA tests, $p < 0.00001$). At 14 d, the THC pattern in challenged clams was different between the first and the second experiments. In Expt 1, for all temperatures, THC on Day 24 were significantly lower than on Day 14 (Student's t -test, $p < 0.05$). This difference was greater at 14 than at 21 and 8°C (on Day 30, the bacterial challenge indicator total hemocyte count [BCI_{THC}] values are all negative and BCI_{THC} is significantly higher at 14°C than at 21 and 8°C, MRT tests) (Table 2). But in Expt 2, for all temperatures, no significant THC decrease in challenged clams between Day 14 and Day 30 was noticed (Student's t -test, $p > 0.05$).

THC and BRD relationship. No significant relationships between hemocyte concentrations and BRD

Table 2. Bacterial challenge indicator (BCI; mean ± SE) in Expt 1 and Expt 2 performed at 3 temperatures: 8, 14 and 21°C. Total hemocyte count, THC; percentage of dead cells, PDC; leucine amino-peptidase, LAP; lysosyme, LYSO; protein. Significant values are given in **bold**. Lettering indicates significant differences between temperature for each parameter and date (using MRT tests)

	THC		PDC		LAP		LYSO	
	Day 14	Day 30	Day 14	Day 30	Day 14	Day 30	Day 14	Day 30
Expt 1								
8°C	401.3 ± 157.91	-636 ± 80.37	0.92^a ± 0.71	2.69^a ± 0.83	1.67^a ± 1.35	-3.15 ± 0.87	7.36 ± 2.1	-7.16^a ± 0.94
14°C	666.72 ± 159.49	-1217.94^a ± 230.34	-0.03^a ± 0.28	1.57^a ± 0.5	2.06^a ± 3.59	-3.94 ± 4.31	9.75^a ± 2.35	-6.39^a ± 1
21°C	768.2 ± 280.84	-343.87^b ± 309.2	2.33^b ± 0.31	1.37^b ± 0.43	17.36^b ± 5.46	1.77 ± 4.63	2.13^b ± 2.24	8.31^b ± 1.82
Expt 2								
8°C	321^a ± 95.91	1197.62^a ± 403.71	1.08^a ± 0.55	0.47 ± 1.09	-0.93^a ± 0.61	1.78 ± 1.27	-0.36^a ± 1.42	5.43^a ± 2.7
14°C	1204.49^b ± 235.52	-133.76^b ± 231.81	4.26^b ± 1.33	2.92 ± 1.01	12.9^b ± 3.95	7.47[*] ± 1.3	11.06^b ± 3.1	-0.61^b ± 3.64
21°C	-58.6^a ± 124.34	-1586.25^c ± 80.74	2.61 ± 0.52	0.7 ± 0.46	1.92^a ± 2.61	-2.31[*] ± 4.91	1.78^a ± 1.61	5.66^a ± 2.47

stages or SRS and disease phases were observed (linear regression tests, Table 3). An increasing tendency of THC with high CDG was noted (Kruskal-Wallis, $p = 0.005$; MRT tests, linear regression test, $p = 0.059$).

Percentage of dead cells

PDC level. The PDCs were significantly higher in clams incubated at 8°C than those held at 14 and 21°C for all clams (challenged and control), all samples and in both experiments (Student's *t*-test, $p = 0.05$, Fig. 4).

PDC pattern. There was no significant effect of temperature on the pattern of PDC (MRT tests and Student's *t*-test, $p > 0.05$). On Day 30, in Expt 1, the bacterial challenge indicator percentage of dead cells (BCI_{PDC}) was significantly higher at 8 and 14 than at 21°C (MRT tests). These latter results were not significant in Expt 2.

PDC and BRD relationship. When all clams were analysed together, the PDC showed a slight but significant increase with development of the disease (Table 3). (Linear regression tests, $p = 0.0002$, and $p = 0.001$, with CDS and CDG, respectively). Asymptomatic clams had a significantly lower percentage of dead cells than clams exhibiting CDG I, II and III of the syndrome (MRT tests, Kruskal-Wallis, $p = 0.00001$).

For clams displaying SRP, the PDC decreased significantly with the process of recovery. There was a negative relationship between PDC and SRS and disease phases (regression linear tests, $p = 0.0047$ and $p = 0.0025$, respectively). With analysis of all clams, the healthy individuals (asymptomatic and recovered clams, SRS 3)

exhibited a significantly lower percentage of dead cells than diseased clams with no shell repair (SRS 0) or weak shell repair (SRS 1) (MRT tests). Similarly, asymptomatic clams (phase 0) and recovered clams (phase 3) exhibited

Table 3. Linear regression tests between defense parameters (total hemocyte count, THC; percentage of dead cells, PDC; leucine amino-peptidase, LAP; lysosyme, LYSO; protein) and Brown Ring Disease (BRD) susceptibility (conchiolin deposit stages, CDS; conchiolin deposit groups, CDG; shell repair stages, SRS; disease phases)

	CDS	CDG	SRS	Disease phases
THC	No	+ $r^2 = 0.71$ $p = 0.059$ $n = 499$	No	No
PDC	+ $r^2 = 2.81$ $p = 0.0002$ $n = 499$	+ $r^2 = 3.1$ $p = 0.001$ $n = 499$	- $r^2 = 8.9$ $p = 0.0047$ $n = 88$	No
LAP	- $r^2 = 1.5$ $p = 0.006$ $n = 499$	- $r^2 = 1.18$ $p = 0.0149$ $n = 499$	+ $r^2 = 10.04$ $p = 0.0025$ $n = 88$	No
Lysozyme	+ $r^2 = 1$ $p = 0.0379$ $n = 496$	+ $r^2 = 1.09$ $p = 0.02$ $n = 496$	+ $r^2 = 4.11$ $p = 0.0629$ $n = 88$	+ $r^2 = 1.99$ $p = 0.0016$ $n = 499$
Protein	No	No	No	- $r^2 = 2.2$ $p = 0.0008$ $n = 502$

lower concentrations of dead cells than those in Phase 1 and 2 of the disease (MRT tests, Kruskal-Wallis, $p < 0.00001$).

Leucine amino peptidase

LAP level. In both experiments and all clams, the LAP activity was higher in clams incubated at 21°C than those held at 8°C. Significant differences were noted for all sampling intervals in both control and challenged clams from the first experiment, and in both control and challenged clams collected on Day 30 during Expt 2 (Student's *t*-test, $p < 0.05$) (Fig. 5).

Data from both experiments show that the mean deviations for LAP were significantly higher at 21 and 14°C than at 8°C (on Day 30, they were also significantly higher at 21°C than at 14°C) (Student's *t*-test, $p < 0.05$, MRT tests, ANOVA tests).

LAP pattern. A significant effect of temperature on the LAP pattern was measured in challenged clams in comparison to controls collected at the end of the experiments. On Day 30, for both experiments, the absolute value of BCI_{LAP} was higher at 14°C than at 8°C and 21°C (significant only in Expt 2) (Table 2).

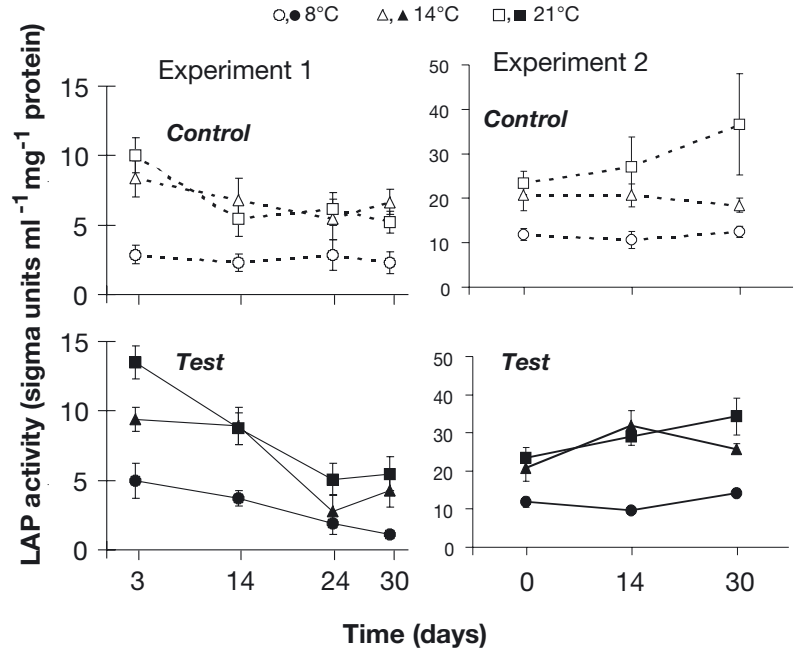


Fig. 5. *Vibrio tapetis* infecting *Ruditapes philippinarum*. Leucine amino-peptidase (LAP) activities in control and *V. tapetis* challenged (test) clams, maintained at 8, 14 and 21°C. Vertical bars are standard error of the mean ($n = 10$ in Expt 1 and $n = 20$ in Expt 2)

LAP and BRD relationship. When diseased and healthy clams are analyzed together, regression tests show that the LAP activity was negatively correlated with CDS and CDG (linear regression tests, $p = 0.006$ and $p = 0.0149$, respectively) (Table 3).

Further analyses of clams displaying a shell repair process have shown that the LAP activity increased significantly with the process of recovery. There is a statistically positive relationship between LAP activity and SRS, and disease phases at the 99% confidence level (regression linear tests, $p = 0.0025$ and $p = 0.0010$, respectively). Recovered clams (SRS 3, Phase 3) showed significantly higher LAP activity than clams in SRS and disease Phases 0, 1 and 2 (MRT tests, Kruskal-Wallis, $p = 0.0013$).

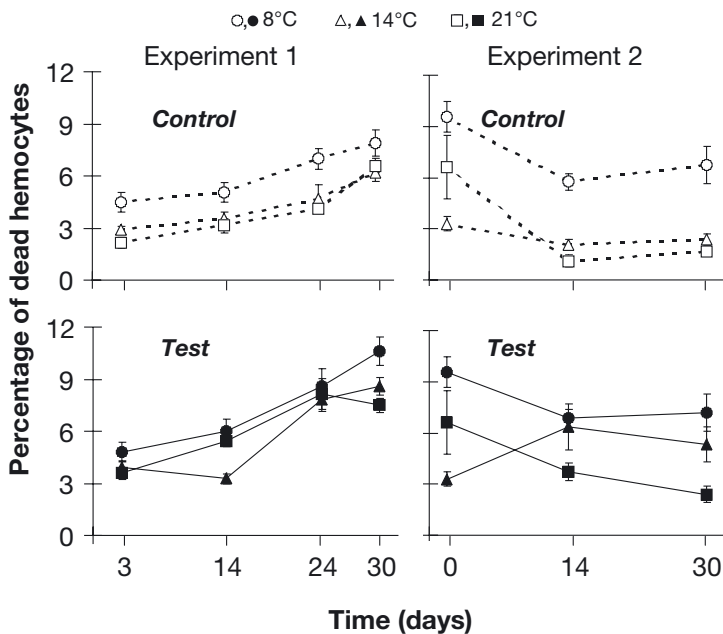


Fig. 4. *Vibrio tapetis* infecting *Ruditapes philippinarum*. Percentage of dead cells (PDC) in control and *V. tapetis* challenged (test) clams, maintained at 8, 14 and 21°C. Vertical bars are standard error of the mean ($n = 10$ in Expt 1 and $n = 20$ in Expt 2)

Lysozyme

LYSO level. In control clams from both experiments, the specific lysozyme activities were always higher in clams incubated at 8°C compared to clams held at 21°C (significant differences detected only in Expt 1, Student's *t*-test, $p < 0.05$) (Fig. 6). Similarly, the mean deviation for lysozyme activity was sig-

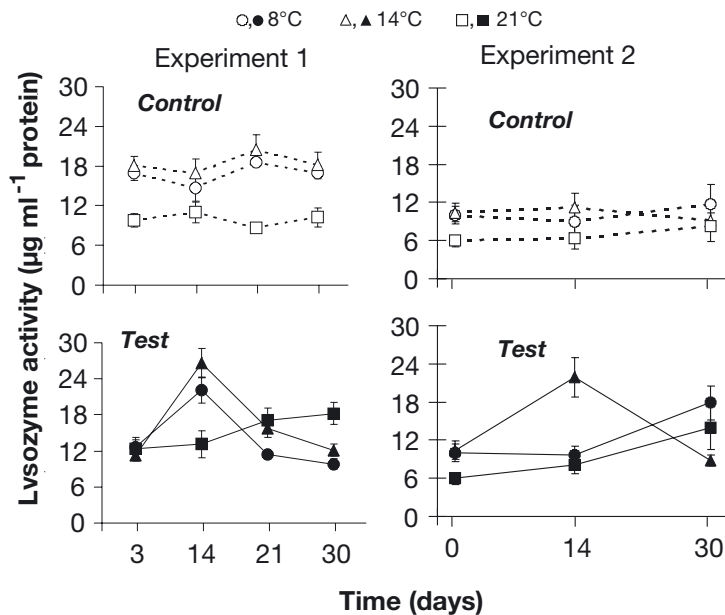


Fig. 6. *Vibrio tapetis* infecting *Ruditapes philippinarum*. Lysozyme activities in control and *V. tapetis* challenged (test) clams, maintained at 8, 14 and 21°C. Vertical bars are standard error of the mean (n = 10 in Expt 1 and n = 20 in Expt 2)

nificantly higher at 8 and 14°C than activity measured at 21°C (MRT and ANOVA tests). In contrast, challenged clams from both experiments collected on Day 30 displayed a higher lysozyme level at 21°C than at 14°C. In Expt 1, the specific lysozyme activity was also significantly higher at 21 than at 8°C (MRT tests).

LYSO pattern. For both experiments, the lysozyme pattern between Day 14 and Day 30 was dependent upon the incubation temperatures. At 14°C, the lysozyme activity decreased in both experiments between Day 14 and Day 30 (Fig. 6). In contrast, at 21°C, the lysozyme activity increased progressively between Day 14 and Day 30 and reached significantly higher values than controls incubated at the same temperature by the end of the experiment. The temperature effect on bacterial challenge indicator lysozyme (BCI_{LYSO}) confirms this tendency for both experiments (Table 2). At Day 30, BCI_{LYSO} was significantly higher at 21°C than at 14°C (MRT tests).

LYSO and BRD relationship. Lysozyme activity increased with the development of the disease (Table 3). A statistically positive relationship between lysozyme activity and CDS and CDG was noted (regression linear tests, $p = 0.0379$ and $p = 0.02$, respectively).

For clams displaying a shell repair, lysozyme activity increased significantly with the process of recovery (SRS, $p = 0.0629$). In fact, healthy clams exhibited a significantly lower lysozyme activity compared to BRD clams that had not begun the shell repair pro-

cess (SRS 0) and those which completed the shell repair process (i.e. recovered clams, SRS 3) (MRT tests). Similarly, the lysozyme activity increased significantly with the disease phases (regression linear test, $p = 0.0016$).

Protein

Protein level. No significant effect of temperature was noted on protein concentrations in control or challenged clams in either experiment.

Protein pattern. No significant effect of temperature on the pattern of protein content was noticed (MRT tests).

Protein and BRD relationship. No relationships were found between protein activity and BRS (CDS and CDG). In fact, recovered clams exhibited significantly lower protein concentrations than healthy or CDG 1 and CDG 2 clams (MRT tests).

No relationship was found between protein concentration and SRS. Healthy clams and diseased clams with no shell repair showed significant higher protein concentrations in hemolymph compared to recovered clams (MRT tests).

Similarly, asymptomatic clams (Phase 0) had significantly higher protein concentrations than individuals in Phase 3 of the disease (MRT tests).

Experiment effects on hemolymph parameters

Significant differences were observed between the experiments for all measured parameters, except protein content, both in control and challenged clams. However, no differences were observed between experiments in mean deviation data for all studied hemolymph parameters, except the percentage of dead hemocytes, (Table 1). The mean deviation for dead cells was higher in Expt 1 than in Expt 2 (ANOVA, $p = 0.04$, and MRT test).

Date effects on immune parameters

Date effect was only significant for 2 immune parameters, the PDC and lysozyme activity (Table 1).

Challenge effects on immune parameters

Challenge with *Vibrio tapetis* caused significant changes in all studied parameters, except protein contents (Table 1).

Interaction factors on immune parameters

Multifactor ANOVA analysis

There were 2 main significant interactions showing the effect of temperature combined with other factors on the measured immune parameters. The interaction of temperature and inoculation had a significant effect on the mean deviation of THC ($p = 0.0246$, ANOVA). The interaction of temperature and date also showed a significant effect on the mean deviation of lysozyme ($p = 0.0001$, ANOVA).

Factorial correspondence analysis

A factorial correspondence analysis was performed on the mean values of all immune parameters measured in Expt 1 (Fig. 7). The first 2 axes were the principal contributors to the total inertia (76.7% for Axis 1; 14.7% for Axis 2). The other axes were under 10%. Relative and absolute contributions to these 2

first axes allowed the identification of 2 gradients, a temperature and an inoculation gradient. For the temperature gradient, mean data corresponding to low temperature (8°C) and lysozyme were isolated together in the positive values of the Axis I. Axis 2 isolated all the data obtained at the higher temperature (21°C) and the LAP immune parameter in the negative values. Factorial correspondence analysis confirmed that temperature had a modulating effect, particularly on lysozyme and LAP activities. The contribution of LAP and lysozyme were high and represented on average 41 and 27% of the total, respectively, for Axis 1. Mean data corresponding to control data and THC and lysozyme were isolated together in the positive values of Axis 1 for the inoculation gradient. The positive values in Axis 2 isolated almost all the *Vibrio tapetis*-inoculated clam data and PDC. This factorial correspondence analysis study showed that *V. tapetis* inoculation had a strong effect on the PDC parameter. The contribution of PDC was very high for Axes 1 and 2, representing 23 and 51% of the total, respectively.

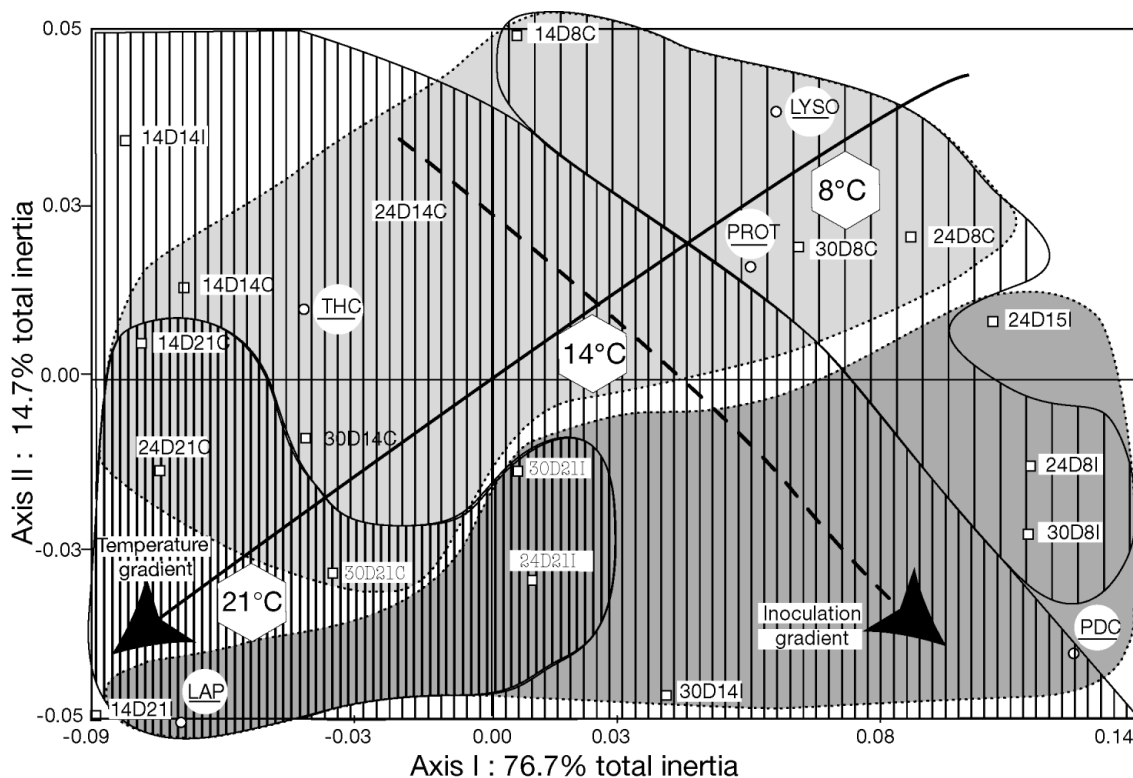


Fig. 7. *Vibrio tapetis* infecting *Ruditapes philippinarum*. Factorial correspondence analysis on mean data of immune parameters of Expt 1. The temperature gradient from 8 to 21°C is noted (areas surrounded by continuous line and vertically hatched; arrow with solid line). Inoculation gradient from control to *V. tapetis* challenged clams (areas surrounded by dotted line and grey-shaded; arrow with dashed line). THC: total hemocyte counts; PDC: percentage of dead cells; LAP: leucine amino-peptidase; LYSO: lysozyme; PROT: protein. The mean of each data set was expressed with an abbreviation: XD (14D, 24D or 30D) corresponds to the day of sampling, the number (8, 14 or 21) to the temperature (°C) of clam incubation and the letter (C or I) to the clam status (Control or Inoculated)

DISCUSSION

In marine invertebrate diseases, prevalence is often controlled by environmental factors. Temperature is a major factor that can explain spatial and temporal variations of disease prevalence (Harvell et al. 1999). In the present study, experimental *Vibrio tapetis* contaminations carried out at different temperatures demonstrated relatively low prevalence (6 to 20%) at 21°C in comparison to 100% prevalence at both 14 and 8°C after 4 wk challenge. The high temperature (21°C) not only retarded development of the disease, but enhanced recovery processes. Temperature has been repeatedly reported to be a key factor in bacterial diseases of poikilotherms (Lauckner 1983, Sindermann 1990). Temperature influences the severity of disease in several bivalve species, particularly during larval stages (see review of Sindermann 1990). Summer mortalities attributed to *Vibrio* sp. infections and high water temperature are frequently described, particularly in *Crassostrea gigas* (Lipovsky & Chew 1972, Lacoste et al. 2001, Waechter et al. 2002). Generally, major shellfish diseases become more severe with higher temperatures, and experimental challenge with pathogens has shown that high temperatures enhance disease prevalence (Elston et al. 1982, Chu & La Peyre 1993). However, a few cold water diseases have also been reported in bivalves. For instance, *Mikrocytos mackini*, the agent of the Denman Island disease in the oyster *C. gigas*, did not develop in oysters held at temperatures above 14°C. Temperatures below 10°C are required for the development of the disease and associated mortalities (Hervio et al. 1996, Bower et al. 1997). In the case of BRD, the present results are in accordance with the field survey along the French Atlantic coast and with epidemiological studies performed in Europe; this bacterial disease is reported mainly in areas where seawater temperature is relatively low in summer (in northern Europe, i.e. England, Ireland, Brittany, and in southern Europe, i.e. Galicia, Portugal) (Paillard et al. 1994, 1997). In Italy, sporadic cases of BRD have been reported in lagoons in Venice during winter, but these infections have not spread along the Adriatic coast. The very low level of BRD in Italy may be primarily due to the fact that higher temperatures (up to 21°C) in the sediment during the warm seasons limit *V. tapetis* proliferation.

Temperature is also a key factor modulating the host immune defense in invertebrates (Thompson et al. 1978, Fisher 1988). Laboratory experiments reported here clearly demonstrate that temperature significantly modulates the host immune status and also the response of clams after challenge with *Vibrio tapetis*. The impact of temperature on hemolymph characteris-

tics was analysed in 2 ways: (1) whether it produced an effect on the level of defense parameters in control and challenged clams, and (2) whether it modifies the kinetics of the immune response of challenged clams during disease development. Temperature effect combined with BRD development and immune parameters will be discussed subsequently.

In the present study, temperature predominantly affected the total hemocyte counts, the percentage of viable cells, and the activity of LAP, whether the clams were challenged or not. For control and challenged clams from both experiments, these parameters were generally higher at 21°C than at 8 and 14°C. This result can be explained by the assumed higher metabolism of *Ruditapes philippinarum* which originated in the Philippines where water temperatures are higher (Gouletquer 1989, Laruelle 1999). Heartbeat rate in *Crassostrea virginica* and O₂ consumption, respiration rate and filtration rate in *R. philippinarum* are highest at elevated temperatures (Feng 1965, Mann 1979). Therefore, an elevated metabolism at higher temperatures in clams could explain the higher concentration of circulating hemocytes due either to a higher production rate of hemocytes at 21°C or mobilisation of hemocytes from tissues towards the hemolymph. The THC in many marine bivalves, such as *Mytilus galloprovincialis* and *C. gigas*, follow a seasonal pattern, and are positively correlated with water temperature (Oubella 1996, Carballal et al. 1998). Similar increases in circulating hemocytes at high temperatures have also been observed during experimental studies in *C. virginica* (Chu & La Peyre 1993) and in the gastropod *Biomphalaria glabrata* (Stumpf & Gilbertson 1978). As in controls, challenged clams incubated at 21°C displayed higher THC than specimens incubated at 8 and 14°C. Similar results have also been demonstrated in *C. virginica* challenged with the protozoan *Perkinsus marinus* (Chu & La Peyre 1993). The PDCs in hemolymph can be used as an indicator of the physiological state of bivalves. In laboratory and field experiments, high percentages of dead hemocytes (up to 20%) have been measured in hemolymph and extrapallial fluids of oysters and clams and were often followed by mortalities (Paillard et al. 1996, Paillard, unpubl. data). Thus, the high percentages of dead hemocytes measured here at 8°C either in control or inoculated clams, could reflect poor physiological condition for this exotic species at lower temperatures. Autophagocytosis is one of the principle ways to eliminate dead hemocytes from circulation (Scro & Ford 1990). In *R. philippinarum*, phagocytosis activity is significantly lower at 8 and 14°C than at 21°C (Allam 1998). Thus, the high percentages of dead hemocytes at 8°C could be the result of low autophagocytic activity at lower temperatures. In the case of BRD, the

humoral response has been focused on lysosomal enzymes such as LAP, an exopeptidase which is particularly important because of its ability to hydrolyse protein components of foreign bacterial membranes (Hiroi et al. 1992). The present study has shown that LAP activity was positively correlated with temperature in challenged and control clams. In both cases, this positive correlation suggests that enzyme levels are enhanced in hemolymph with increased temperature. Hypersynthesis of hemolymph enzymes is a phenomenon which plays a protective role by acting against invading micro-organisms including bacteria (Mohandas & Cheng 1985). However, the activity of LAP remained lower following exposure to low temperature (8°C). The observed lower level of LAP in cold temperature exposed-clams may reveal a weakened physiological state. These results suggest that lower temperature is a stressor that may cause immunosuppression. Decrease of this humoral defense mechanism with low temperature may interfere with the immunocompetent functions of the immune system, and may increase the susceptibility of populations to *Vibrio tapetis* in the field. Numerous publications have described the effect of salinity on LAP activity, but to date, no one has reported a temperature effect.

Temperature also affects the pattern of the immune response of clams following challenge with *Vibrio tapetis* (Table 2). Clams maintained at 14°C, which corresponds to standard conditions used during experimental infection since 1990, exhibited immune response patterns in accordance with kinetics described in previous publications (Oubella et al. 1993, 1994, Paillard et al. 1994, Allam et al. 2000b). The kinetics of the immune response in clams incubated at 8°C was relatively close to the one in clams held at 14°C, showing an early increase in THC, LAP and lysozyme activities that reach maximal values about 2 wk after challenge. These 3 parameters decreased thereafter and relatively low values were maintained up to 4 wk following contamination. Decreases in these hemolymph parameters in the late phase could be a consequence of the continuous migration of hemocytes from hemolymph to the site of infection (i.e. extrapallial fluids) (Paillard et al. 1994). In the present study, in both experiments on Day 30, the BCI was generally higher at 14°C than at 8 and 21°C for most of the immune parameters (THC, PDC and LAP). This confirms that 14°C is the best temperature to detect changes of these parameters following challenge with *V. tapetis*. On the other hand, the incubation of clams at 21°C had a greater effect than 14°C on the pattern of lysozyme activity during the development of BRD. In control clams, lysozyme activity was negatively correlated with temperature. This result is in accordance with data reported in *Crassostrea virginica* by Chu & La

Peyre (1993). In contrast, challenged clams displayed an increase of lysozyme activity at 21°C, reaching values comparable to those measured in control clams maintained at 8°C. This increase in lysozyme activity could be associated with the recovery process occurring at 21°C 3 wk post-challenge. Lysozyme may be one of the immune parameters playing a major role against the proliferation or invasion of micro-organisms such as gram-positive and -negative bacteria (Cheng et al. 1975, 1977, Cheng 1983, Lopez et al. 1997). In addition to direct bactericidal activity, lysozyme could enhance the activity of other anti-bacterial molecules which could be directly active against *V. tapetis*, a process already described for other gram-negative bacteria by Smith et al. (1995).

In the present study, protein levels in clam hemolymph were not dependent upon the temperature at which the clams were incubated. This result is unlike the findings of Chu & La Peyre (1993), who described a decrease in protein levels in the hemolymph of *Crassostrea virginica* maintained at high temperatures, associated with a decrease in condition index. In Manila clams, recent experiments performed in similar laboratory conditions but during another period of the year (January and February) compared to the present study have shown a decrease of protein and condition index in individuals maintained at 21°C compared to those at 13 and 8°C (Paillard unpubl. data). This lower protein level and condition index at high temperature could be due to high metabolism and nutritional stress compared to clams maintained at 8 and 14°C (Chu & La Peyre 1993).

The present study also demonstrated good reproducibility between experiments and underlines the strong effect of temperature on host immune response in clams. To explain minor differences in immune parameters between both experiments, infection severity must be considered, i.e. a delay in BRD group development in Expt 2 compared to the Expt 1.

Temperature has a strong effect on BRD development, which means that the elevated temperature not only inhibits the development of the BRD syndrome but also enhances the recovery processes. In the present study, BRD development was related to a significant decrease in hemocyte viability and LAP activity in hemolymph. In contrast, the recovery process was associated with high levels of these 2 immune parameters and also with an increase in lysozyme activity. During the different phases of the disease, the decrease in protein concentration in the hemolymph can be seen and associated with the 2 processes occurring during BRD, the conchiolin deposit and the shell repair. These 2 external defense mechanisms may require higher protein concentrations compared to normal shell growth.

In conclusion, temperature can be considered a key factor controlling BRD development and defense parameters. The factorial correspondence analysis confirms the modulating effect of temperature on hemolymph defense parameters, particularly on lysozyme and LAP parameters. In our opinion, in the case of the BRD, the favourable immune status at 21°C may confer on the clam a better capacity to fight the disease agent and therefore enable it to recover more easily. This hypothesis is in accordance with other experiments in our laboratory which have shown that the pathogen was still present at lower concentrations (near the detection limit of ELISA, 10^4 CFU ml⁻¹) in the host maintained at 21°C, but it is not capable of inducing BRD symptoms or mortalities (Paillard unpubl. data). Transfer of clams from 21 to 14°C has shown that these individuals were able to develop BRD (Paillard unpubl. data). In the present study, high hydrolytic enzyme activities in the hemolymph, associated with high numbers of viable and phagocytic hemocytes at high temperatures, probably help fight the disease agent and maintain low levels of pathogenic bacteria. Low BRD prevalence (14 %) by pallial challenge, takes at least 10^3 UFC ml⁻¹ of *Vibrio tapetis*, so if the clam can maintain the *V. tapetis* level in the extrapallial fluids under this critical concentration, it could be considered asymptomatic. To better understand the effect of temperature on host-pathogen interactions, further experiments are in progress to investigate how temperature and other environmental factors such as salinity or trophic resources modulate the immune competency of hemocytes against bacterial pathogens, as well as the virulence factors of *V. tapetis*.

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