Summer immune depression associated with increased susceptibility of the European abalone, *Haliotis tuberculata* to *Vibrio harveyi* infection

Marie-Agnès Travers\textsuperscript{a,b}, Nelly Le Goic\textsuperscript{b}, Sylvain Huchette\textsuperscript{a}, Marcel Koken\textsuperscript{b,1}, Christine Paillard\textsuperscript{b,1,*}

\textsuperscript{a} France Haliotis, Kerazan, Lilia, 29880 Plouguerneau, France

\textsuperscript{b} Laboratoire des Sciences de l’Environnement Marin, CNRS UMR 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, 29280 Plouvzané, France

**Abstract**

*Haliotis tuberculata* mortality outbreaks have occurred in France since 1998 and were attributed to a pathogenic *Vibrio harveyi*. These mortalities were recorded in September, a month with abalone reproduction and characterised by high seawater temperatures. The importance of gonadal maturation and temperature increase on abalone immunity and susceptibility to *V. harveyi* infection needed to be clarified. Therefore, an immune survey analyzing a large panel of parameters was performed from June to September 2007 on abalone from the Bay of Brest. The data obtained were put in relation with abalone reproductive status and its susceptibility to *V. harveyi*.

Most parameters showed clear patterns from early to late summer and during gametogenesis, phagocytosis and phenoloxidase activity were reduced, whereas basal reactive oxygen species production and agglutination titres were significantly increased. Total haemocyte counts went up after the partial spawning event at the end of June, and cell complexity diminished. Using a Principal Component Analysis, the “haemolymph profile” was shown to decrease in parallel with spawning and gonadal maturation processes, and reached a minimum just after total spawning. A significant correlation between this “haemolymph profile” and disease susceptibility allowed us to establish for the first time in abalone, a clear concordance between maturation and spawning processes, immune status and abalone susceptibility to *V. harveyi*.

© 2008 Elsevier Ltd. All rights reserved.

**1. Introduction**

Important mortality outbreaks have been reported in *Haliotis tuberculata* populations since 1998 in Brittany and Normandy, France [1]. During these mortality outbreaks, *Vibrio harveyi* was isolated and identified as the pathogenic agent [2]. *V. harveyi* is a gram-negative bacterium, which was previously described as fish, mollusc and crustacean pathogen in warm seas [3]. The mortalities occurred mainly in September, and killed up to 80% of the estimated stock [1,2]. The importance of seawater temperature and sexual maturation for field mortalities associated with *V. harveyi* was suggested [1] but the precise impact of sexual maturation on abalone physiology and particularly on its immune system remained unclear. In oysters and mussels, it was shown that some haemocyte activities (phagocytosis, adhesion) decreased during gametogenesis, suggesting that in these molluscs the immune system is modulated by the reproduction [4–8]. So even though there is some evidence of immune depression in molluscs during their spawning period, a proven link with disease susceptibility is in general lacking [4,5].

Mollusc cellular and humoral immunity is thought to depend mainly on the haemocytes present in the haemolymph and in the animal’s tissues. These cells are responsible for recognition, phagocytosis and elimination of non-self particles via direct hydrolysis [9], production of reactive oxygen species (ROS) [10], of antimicrobial peptides [11–13] and of non-self recognition molecules and immune effectors (lectins, opsonins and components of the prophenoloxidase system) [14].

Recently, a first detailed analysis of the cellular component of the *H. tuberculata* immune system was undertaken to start unravelling the functioning of its immune system. It was shown that in *H. tuberculata* only two haemocyte types, large hyalinocytes and blast-like cells, exist in contrast to the situation in bivalves where three or four cell types can be distinguished [15]. However, the abalone humoral system received very little attention, and only the phenoloxidase, lysozyme and acid phosphatase activities were shown to be modulated by environmental factors [ammonia, nitrite...
and temperature) [16–20]. So far no information exists on the modulation of abalone immune during physiological modifications like those encountered during spawning [21].

In the present study, the “haemolymph profile” was assessed by measuring a large panel of haemocyte and serum parameters (haemocyte counts, size, internal complexity and mortality, phagocytosis index, basal ROS production, plasma agglutination titres, superoxide dismutase, phenoloxidase and leucine amino peptidase activities). For the first time it was tried to correlate immune status to the reproductive status of the abalone and to their susceptibility for their pathogen V. harveyi. By studying a large panel of immune parameters linked to a Principal Component Analysis, we were able to prove that abalone develop disease only when high temperatures coincide in time with an immune depression, with the stressful spawning periods and the presence of a pathogenic bacterium.

2. Material and methods

2.1. Animal sampling and temperature survey

From June to September 2007 (June 12th, July 12th, August 2nd, September 4th and 26th) abalone were caught by scuba diving in the Bay of Brest near Trébérón Island. At each dive 34 animals (between 60 and 80 mm) were collected. For each sampling, haemolymph from 10 animals was withdrawn and kept on ice before analysis. The animals were sized, weighed and dissected. The other 24 abalone were separated into three groups of eight individuals before bacterial immersion challenges: (a) one group was placed at 19°C and inoculated after 48 h with pathogenic V. harveyi (b) one (control 1) was placed at 19°C and (c) one (control 2) was placed at 17°C. Temperature probes were placed at the Trébérón Island sampling sites in proximity of a group of abalone, and temperature was registered every hour over a period of four months. Daily means were calculated.

2.2. Bacterial challenges

2.2.1. Bacterial strain

For the challenge experiment, the virulent V. harveyi strain, ORM4, was used. This strain was isolated from diseased H. tuberculata in Normandy (France) in 1999 [2]. The strain was GFP-tagged for easy identification [22]. Bacteria were grown in Luria–Bertani broth (LB, Sigma) supplemented with extra salt (20 g L⁻¹, f.c) (LBS) in a temperature controlled Infor® shaker at 28°C for 18 h before inoculation.

2.2.2. Immersion challenges and susceptibility

Abalone were challenged at 19°C, 48 h after capture, in 0.5 μm filtered seawater containing 10⁵ CFU mL⁻¹ of freshly grown and washed GFP-tagged bacteria. Two controls were used: abalone placed without bacteria at 17°C and 19°C. Seawater was renewed each day and tanks were checked twice a day; moribund or dead animals were removed. We voluntarily choose to limit the acclimation period as much as possible (in the limit of 2°C per day) to not influence the immune status that was measured the day of fishing on the 10 animals before challenging the other 24 animals of the same group.

2.2.3. Bacterial identification

Detection of V. harveyi was performed on moribund abalone according to the following method. Haemolymph was withdrawn from the cephalic sinus, and muscle tissue was homogenized in filtered and sterilized seawater (FSSW). Tenfold serial dilutions of these extracts were prepared and plated on two different media: V. harveyi-specific medium (Vha) and thiosulfate-citrate-bile salts-sucrose TCBS (Vibrio-selective medium), and grown for 24–48 h at 28°C. Predominant strains were isolated from TCBS and Vha, and three times streak plated.

Bacterial DNA (liquid culture-propagated) from pure cultures was extracted by the proteinase K/phenol-method [23], and about 10 ng of DNA was used for PCR reactions. A 1500 bp fragment from 16S rDNA was amplified using eubacterial universal primers (forward primer – 5’AGACGTGTGATCCCTTCGACTAG3’ and reverse primer – 5’CGGTTACCTTGTAACG3’ [24]. A 390 bp fragment of tosR DNA was amplified with the V. harveyi-specific primers (Vh-tosR-F 5’-TTCTGAGACGACCTAC-3’ and Vh-tosR-R 5’-TGCAGACTGGTAAAGAC-3’) [25]. The fragments were gel-purified and underwent direct sequencing.

2.3. Haemolymph parameter measurements

2.3.1. Haemolymph collection

Haemolymph was collected from the cephalic arterial sinus with a 5-ml hypodermic syringe (25 G needle) according to Travers et al. [15] and transferred into a 1.5 ml tube maintained on ice until flow cytometry analysis. All measurements were done on each individual. Total haemocyte count (THC), haemocyte mortality percentages, phagocytosis index, and basal haemocyte reactive oxygen species (ROS) production were assessed.

For each sample, the remaining haemolymph was used for determining: (1) phenoloxidase (100 μL) and superoxide dismutase (100 μL) activities as well as total protein content (10 μL); (2) 1 mL was centrifuged at 600 × g for 10 min at 4°C. The supernatant was divided into 150 μL for determining the agglutination titre and 10 μL for total protein content. The cell pellet was resuspended in 1 mL of FSSW and divided into 100 μL for the leucine amino peptide assay and 10 μL for total protein content determination. All samples were quick frozen and stored at −80°C.

2.3.1.1. Haemocyte parameter measurements. Morphological and functional analyses were performed on a FACS-Calibur flow cytometer (Becton Dickinson, France) equipped with a 488 nm laser.

2.3.1.1.1. Total haemocyte count (THC), morphology and mortality. A total of 200 μL of haemolymph were diluted into 200 μL of anti-aggregant solution AASH (1.5% EDTA, 6.25 g L⁻¹ NaCl, in 0.1 M phosphate buffer, pH 7.4) [26], and samples were filtered through an 80 μm nylon mesh to eliminate aggregates and debris before flow cytometry analyses. Samples were incubated for 30 min with SYBR Green I fluorescent dye (Molecular Probes, 10⁻³ dilution of the commercial stock solution) and propidium iodide fluorescent dye (10 μg mL⁻¹, f.c). Only dead cells which lost membrane integrity incorporated propidium iodide and the resulting fluorescence was measured by the FL3 detector. Total haemocyte count (THC) was assessed by counting at least 10,000 particles in 30 s. Cells were identified by their characteristic DNA peak (FL1), as previously described [15]. Results were expressed as cells mL⁻¹. Haemocyte mortality was presented by giving the percentage of dead cells present in each sample.

2.3.1.1.2. Phagocytosis index and phagocytosis capacity. The phagocytosis protocol was adapted from Allam et al. [27]. A sub-sample of haemolymph was diluted (1:1) in FSSW and 400 μL of this solution was added to a 24-wells plate (Cellstar, Greiner Bio- one). Haemocytes were allowed to adhere for 15 min at 18°C and 100 μL of fluorescent beads (Fluoresbrite YG Microspheres, 2.00 mm, Polysciences, 1:200 in distilled water) were added. After 3 h at 18°C, supernatants were removed and trypsin (2.5 mg mL⁻¹ in AASH) was added to detach the adherent cells. Plates were shaken for 10 min and AASH was used to stop the reaction by dilution. By flow cytometry, beads are identified through their...
green fluorescence. Non-ingested particles were easily distinguished from engulfed ones by their size on a FSC (forward scatter, representing particle size) vs SSC (side scatter, representing internal complexity of particles) and a FSC vs FL1 plot. The phagocytosis index is defined, as previously described [28], as the percentage of haemocytes phagocytosing three or more beads.

2.3.1.1.3. Reactive oxygen species (ROS) production. The ROS protocol was adapted from Lambert et al. [10]. To avoid inhibitory effects of the anti-adherent and of high cell adhesion in FSSW, ROS production was measured in FSSW exactly 10 min after addition of 2',7'-dichlorofluorescein diacetate (DCFH-DA, 0.1 mM f.c.). Results are given as the mean intra-haemocytic fluorescence level of the DCF end product expressed in arbitrary units (U.A.) representing the basal ROS production.

2.3.1.2. Enzymatic activity measurements

2.3.1.2.1. Phenoloxidase assay. PO activity was measured according to Ford and Paillard [29]. Briefly, 100 μL of 2 mM l-DOPA in 0.2 M Tris–HCl pH 8 was added to 100 μL of total haemolymph in 96-well flat-bottomed micortitre plates and the absorbance at 490 nm was read continuously for 15 min. One unit of PO activity was defined as the absorbance change of 0.001 in 1 min. Results were expressed as unit activity per mg of protein contained in the sample (U mg protein⁻¹).

2.3.1.2.2. Superoxide dismutase assay. The SOD activity assay was adapted from Ahmed et al. [30]. Briefly, the activity of 100 μL of total haemolymph was measured in presence of 100 μL of deoxy- genated 0.2 mM pyrogallol diluted, just before use, in an air-equilibrated Tris–cocodylate buffer (0.05 M, pH 8.2) containing 1 mM diethylenetriamine pentaacetic acid (DTPA). Inhibition of normal auto-oxidation of pyrogallol in the presence of DTPA by the SOD protein was measured in a continuous reading at 405 nm during 1 h. A standard of purified Escherichia coli SOD allowed to express results as Units equivalents of E. coli SOD per mg of protein (U eq mg protein⁻¹).

2.3.1.2.3. Agglutination titre. Agglutination titre was performed as previously described [31]. Horse erythrocytes were purchased from AES laboratories, washed three times in PBS, and the final pellet was resuspended to give a 2% erythrocyte suspension in TBS (50 mM Tris–HCl, 150 mM NaCl, 10 mM CaCl₂, pH 7.2). A twofold serial dilution was prepared with 100 μL of cell-free haemolymph supernatants in 96-well U-shaped bottom microplates, using TBS as diluent. This was mixed with 100 μL of the 2% erythrocyte suspension, and plates were incubated at room temperature for 1 h. Agglutination titres were determined by the pattern of erythrocyte sedimentation. Results are expressed as the reciprocal of the highest dilution which showed a positive pattern of agglutination [31].

2.3.1.2.4. Leucine amino peptidase assay. LAP activity in haemocytes was measured as described by Ford and Paillard [29]. Just after addition of 75 μL of 0.2 M Tris–HCl pH 8 to 100 μL haemolymph, 25 μL of the substrate (10 mM leucine p-nitroanilide in Tris–HCl pH8) was added into a 96-microplate. After mixing, optical density (OD) at 405 nm was measured for each 5 min during 30 min. Results are expressed as OD change per minute per mg of protein (ΔOD min⁻¹ mg protein⁻¹). Controls, without haemolymph, but containing leucine p-nitroanilide, Tris–HCl and distilled water, were run in parallel and their values were subtracted from the test values.

2.3.1.2.5. Total protein content. Protein analysis was carried out on 10 μL samples using the BCA Protein Assay (Bio-Rad) according to the manufacturer’s description. Briefly, 200 μL of dye reagent was added to 10 μL of sample, incubated at 37 °C for 1 h and the absorbance was measured at 595 nm. Sample ODs were compared to a standard curve of Bovine Serum Albumin (BSA) and results were expressed as mg of protein mL⁻¹.

2.4. Biometry, condition indexes and histology

In the laboratory, abalone were removed from their shell and organs were separated and weighed. Wet and dry (100 °C for 48 h) weights of the viscera were recorded for each individual and visceral condition indices (VCI) were calculated as VCI = visceral dry weight × 100/(Shell length)³ [32].

A small fragment of the gonad was weighted, fixed in Bouin’s solution for 48 h and embedded in paraffin using standard methods. Histological sections of 5 μm were cut, mounted on glass slides and stained with the Masson’s trichrome technique [33].

Each histological section of gonad tissue was examined under the microscope, and for each female, in at least 10 randomly selected fields the individual surfaces of about 150 randomly chosen mature oocytes (with clearly visible nucleoli to ensure that each section passed through the centre of the gamete) was measured by Image-Pro Express v6.0. Theoretical diameters were then calculated as: dtheoretical = √(4 × surface/π).

2.5. Statistical analyses

Statistical analysis was performed using an ANOVA, based on variance analysis. THC and agglutination titre values were log transformed and the percentage of haemocyte mortality values were (arcsin of the square root) transformed prior to statistical analysis. Note, however, that in the figures the original (non-transformed) data are presented.

Multivariate analyses were used to provide a reduced description of a large data set, to permit analysis of relationships between variables. Data were evaluated using discriminant analysis (DA) and Principal Component Analysis (PCA). These analyses were followed by linear correlations and ANOVA. The analyses were performed with the Statgraphics Plus 5.1 statistics software.

3. Results

3.1. Spawning date and temperature

Spawning of abalone was estimated by comparing histological data of the gonads with the visceral condition index. The observed decrease of the mean diameter of mature ovocyes and of the visceral condition index between June 12th and July 12th (from 116 to 101 μm and from 7.6 × 10⁻⁶ to 5.6 × 10⁻⁶, respectively) was indicative for a partial spawning event between these two dates (Fig. 1A, B, Table 1). A second decrease of these parameters was observed when total spawning occurred between September 4th and September 26th. Mature ovocytes had disappeared at the end of September, and the visceral index had decreased from 6.3 × 10⁻⁶ to 5.4 × 10⁻⁶ (Fig. 1A, B, Table 1).

During the survey period, the mean seawater temperature in the Bay of Brest was 16.4 °C ± 1.1 °C, s.d., with an extreme minimum of 13.7 °C and a maximum of 18.1 °C. The temperature never exceeded 18 °C for more than one day, and the mean temperature during the complete spawning period (September 4th and September 26th) was 16.7 °C. No mortalities were observed in the field during this survey.

3.2. Susceptibility

Abalone susceptibility, indicated by the percentage of survival after immersion with V. harveyi ORM4 bacteria at 19 °C, was evaluated in the laboratory from June to September. Survival decreased progressively from 90% in June to 10% in September (Fig. 1C). In both September samples, several control abalone placed at 19 °C in 0.5 μm filtered seawater without bacteria also died (Fig. 1C). The other control of abalone, placed at lower temperature, 17 °C...
without bacteria, did not suffer any mortality. From the moribund 19 °C control animals, in both September samples, a culture of non-GFP expressing bacteria could be isolated from haemolymph and muscle which was directly pure. By PCR with *V. harveyi*-specific toxR primers and after 16S sequencing, the isolated bacteria were shown to correspond to non-GFP expressing *V. harveyi*, suggesting that these abalone may have been disease-free carriers.

3.3. Immunity

3.3.1. Collecting date effect on haemolymph immune parameters

In August, total haemocyte counts peaked \(7.8 \times 10^6\) cell mL\(^{-1}\) \(p < 0.01\), Fig. 2A, Table 1) and at the same time, the very low \(< 3\%\) haemocyte mortality decreased and reached a minimum \(1.5\%\, p < 0.05\), Fig. 2A, Table 1). A decrease of cells size (not significant) and complexity was also observed in August and at the end of September (Table 1).

Phagocytosis, represented by the phagocytosis index, showed also a significant decrease from July to September (Fig. 2B, Table 1, \(p < 0.001\)). Basal ROS production presented two peaks in July and September (180 and 191 UA, respectively, Fig. 2B, Table 1, \(p < 0.001\)).

Significant differences in mean phenoloxidase activity (PO, \(p < 0.001\), Fig. 2B, Table 1), agglutination titre \((p < 0.05\), Fig. 2C, Table 1) and total protein content \((p < 0.05\), Fig. 2C, Table 1) were also observed according to the collecting date. No differences were found in LAP and SOD activity throughout the survey (Table 1).

It is noticeable that cell complexity and PO activity significantly decreased with both spawning events, whereas total protein content and phagocytosis significantly decreased between spawning events, i.e., simultaneously with the gonadal maturation process (Table 1)

3.3.2. Differences between collecting date and gonad classes

Discriminant analysis was performed on all immune data to validate sampling groups (i.e., the physiologically significant groups), as statistically significant groups. Therefore, five groups (each sampling) were postulated. Classification functions permitted to correctly classify 93% of individuals for sampling
groups (Wilks’s lambda = 0.003 and p-value < 0.001) and could thus be used for further statistical analyses (Fig. 3).

Similarly, with all immune data, discriminant analysis was performed to validate gonadal development states (i.e., the biologically significant groups based on histology), as statistically significant groups. Therefore, three groups were postulated: before spawning (June 12th), between partial and total spawning (July 12th, August 2nd and September 4th) and after total spawning (July 26th), leading to functions that were 100% of individuals for gonad maturation groups (ANOVA).

We also tested the variation of immune parameters by these three histologically significant groups: similar tendency was found, as all parameters (except total protein content and cell mortality) significantly influenced by dates were also significantly influenced by gonadal development states. Concordance between immune parameter variations and reproduction events was thus observed.

### 3.3.3. Collecting date effect on global haemolymph profile

In the Principal Component Analysis performed on all measured immune parameters (Fig. 5), principal components PC1 and PC2 explained more than 53% of the total variance. The PC1 explained 34% of the total variance and was characterised by high loadings of several variables (haemocyte complexity, size, PO activity and phagocytosis index). These highly differentiating variables are directly associated with low basal ROS production and agglutination titre (or vice versa).

The first component of this PCA, corresponding to the so-called “haemolymph profile” (HP), was extracted. The effect of the collecting date on the HP was significant as it significantly decreased both in July and at the end of September, i.e., during both spawning events (p < 0.05, Fig. 6A). Similarly, between July and August, i.e., during gonadal maturation, a significant decrease of HP was observed (Fig. 6A).

### 3.3.4. Immunity and susceptibility

A statistically significant regression explaining 62% of susceptibility variance was observed between the HP and animal susceptibility (survival rate after infection at 19 °C, p < 0.001, Fig. 6B). When the HP was high (high haemocyte complexity, size, DOPA oxidase activity and phagocytosis index associated with low basal ROS production and agglutination titre), abalone survival rates were also high.

### 4. Discussion

The objective of this study was to compare abalone immune status in correlation with gonadal development state, and to study the relationship between reproduction and susceptibility to *V. harveyi*.

#### 4.1. Increased susceptibility to bacterial infection after spawning

As suggested by previous work [1], there is a clear relationship between spawning period and abalone disease susceptibility. Such a relationship has also been shown in the Pacific oyster, *Crassostrea gigas*, as spawning effort has been correlated to increased mortalities [34] in both triploid oysters, considered as sterile [5], and oysters in low trophic condition, due to “summer mortalities” [35].

Abalone susceptibility to *V. harveyi*, estimated by bacterial immersion challenges at 19 °C, was maximum in September coinciding with the period of total spawning. Bacterial susceptibility increased from 10% mortality during the partial summer spawning period to reach 90% mortality just after the major autumn spawning event. Myrand et al. reported in mussels also the importance of a second, and major mussel spawning on the appearance of summer mortalities [35].

In September, abalone were found to be potentially free carriers of *V. harveyi*, which was revealed when 90%, apparently healthy animals died when placed in laboratory conditions adequate for disease development, i.e., at a temperature of 19 °C. Similar observations were done after susceptibility experiments performed in September on field abalone in Normandy (Basuyaux, personal communication). It is important to note that in this study, the control abalone placed at 17 °C did not develop any
mortalities although they came from the same field-positions as the 19 °C animals. This result underlines the major role of temperature as a trigger for vibriosis development in *H. tuberculata*. Temperature during spawning is one of the keys for the *V. harveyi* infection to succeed, as it requires at least 18 °C to obtain disease in experimental infections [36]. Seawater temperature measured in the Bay of Brest during the summer of 2007 never exceeded 18 °C for more than a day, and only reached a mean of 

Fig. 2. Immune parameters follow-up. (A) Total haemocyte counts (THC, cell mL⁻¹) and haemocyte mortality rates (%). (B) Phagocytosis index (%) and PO activity (U mg protein⁻¹). (C) Basal oxidative production (ROS, A.U. Arbitrary Units), agglutination titre (log) and total protein content (PROT, mg mL⁻¹).

Fig. 3. Discriminant analysis (DA) of immune parameters classed by dates. Sampling (1) corresponds to June 12th, (2) to July 12th, (3) to August 2nd, (4) and (5) to September 4th and 26th.

Fig. 4. Discriminant analysis (DA) of immune parameters classed by histological gonad maturation groups. Group (1) ‘before spawning’ corresponds to June 12th, group (2) ‘between partial and total spawning’ to July 12th, August 2nd, and September 4th and group (3) ‘after total spawning’ to September 26th.
16.7 °C during the major spawning period. *V. harveyi* is known to have a high optimal growth temperature of about 30 °C, and its growth capacities around 16 °C are rather limited (Travers, unpublished results). Disease development in Brittany waters is probably depending on very little temperature differences. The temperatures of less than 18 °C in the Bay of Brest could, therefore, explain the absence of natural mortalities, as well as the free carrier status of abalone, and disease development at 19 °C in the laboratory.

However, proving a free carrier status in a natural population implies detecting a very small number of bacteria somewhere in or on an abalone. This can only be done by classical bacterial plating of a completely “crushed” animal (as PCR will never be able to detect these small numbers due to the limited amount of bacterial DNA over abalone DNA). The other problem that could limit such detection is the existence of *V. harveyi* VBNC stages (viable but non-cultivable) that remain virulent in the environment [37]. Only laboratory experiments using a controlled environment, bacterial plating and GFP-tagged pathogens will be able to clarify this interesting issue.

4.2. Relationship between reproduction and immune depression

Many studies reported the effect of specific pathogens on mollusc an immunity. These are often pathogen-specific, and show only how a pathogen undermines the animal’s immune system [38]. The results cannot be generalised to other models or conditions. The approach here is more general and asks whether seasons or maturation can affect immunity, and eventually lead to an increased susceptibility to pathogens.

Many immune parameters measured in this study showed variations depending on the collecting date. THC, cell complexity, haemocyte mortality, phagocytosis capacity, basal ROS production, phenoloxidase activity and agglutination titre significantly changed over time. After a first spawning in August, an increase in THC, associated with a decrease in cell size and cell complexity, could indicate haemocyte release from tissues or active haemocyte division, as it was previously suggested in bivalves [39,40].

Two spawning events were recorded for abalone in the Bay of Brest in 2007, in June–July and in September. Phagocytosis and PO activity, which are two main haemocyte immune capacities, were significantly decreased during the maturation process or spawning events, respectively. “Haemolymph profile” (HP) extracted from the PCA analysis varied also in concordance with the maturation and spawning processes, as HP decreased after spawning events and during gonadal maturation (i.e., less cells, smaller and less complex, low phagocytosis and PO activity associated with high basal ROS production and agglutination). Reduced phagocytosis activity was previously reported during the reproductive effort for clams [41], oysters [4], blue mussel [42] and shrimp [43]. Some authors suggested a potential control of the immune system by steroid hormones that could explain the phagocytosis suppression during the reproductive effort [42].

However, it should be noted that many other factors can also cause seasonal fluctuations in oysters, clams and mussels, such as PAH (Polycyclic Aromatic Hydrocarbons), PCB (Polychlorinated Biphenyls), metals, TBT (Tributyltin) [44–46], toxic algae [47,48], high or low temperatures [19,28,49,50], salinity [51], dissolved oxygen [52] or “stress” [21,53]. Therefore, due to our approach with a random sample of a field population, we cannot exclude that some variation (particularly haemocyte concentrations) in our data...
may be attributed to other abiotic factors. Besides this reservation, our data clearly show a sound concordance between the evolution of immune parameters or HP and the maturation status of the animals.

Because of the poorly developed tools in this disease model, the haemocyte parameters presented in our study are mainly related to the immune function. As the haemocyte is not only an immune cell, it is appropriate to develop tools to allow studying the implication of haemocyte in their other functions (calcification, metabolite transport, etc.).

4.3. Immunity suppression: link with abalone susceptibility to infection by V. harveyi

Two main periods of immune depression are in general reported: winter, principally because of low food availability leading to a decrease in condition index [54,55]; and late summer, because of the spawning effort [56]. These periods are generally associated with either cold- or warm-water diseases, as they also correspond to parasite or bacteria proliferation.

For example, reduced phenoloxidase activity in some populations was shown to facilitate lethal infections in Sydney rock oyster by Martellia sydneyi [57], and immune depression caused by stress was shown to increase bacterial susceptibility in vitro and in vivo in oysters [58,59] or abalone [53]. But all these studies did not provide a link of the changes in immune status with the animals’ natural physiology state.

The only example to our knowledge where a more global and follow-up approach was used is the MOREST program on the Pacific oyster, C. gigas. They show that well-fed normal oyster, diploid oysters, or families selected as more susceptible to “summer mortality” were all highly vulnerable to pathogens, and presented an immune-depressed phenotype. This, in comparison with food-limited normal oysters, triploid oysters, or genetically selected “summer mortality-resistant” oysters. The susceptibility for “summer mortalities” was partly explained with the depressed immune-phenotype and the highly active spawning processes in the first group of animals [60,61].

Similarly, in the present work, a clear positive correlation between a natural abalone immune status in a specific period and survival after bacterial challenge was found. This suggests that immune depression associated with the spawning period (low phagocytosis, low phenoloxidase activity, low THC with small and poorly complex cells, and high basal ROS production) increases abalone susceptibility to V. harveyi.

In conclusion, a concordance between maturation and spawning processes, immune status and abalone susceptibility to V. harveyi was demonstrated. However, since, as well the gonadal development, as the pathogen’s proliferation are shown to be directly influenced by temperature, their respective contribution in disease development should be dissected to understand their relative importance.

Acknowledgement

We thank Robert Marc,Jonathan Fly-Sainte Marie, Frédéric Jean, Joëlle Richard, Laurent Chauvaud and Erwan Amice for all the scuba dive sampling. We thank Dr. Hélène Hégaret for her careful revision of the manuscript, and Dr. Philippe Soudant and Dr. Christophe Lambert for stimulating discussions and suggestions. This study was supported by ‘France Halitiotis’ SCEA, and the “Région Bretagne”. This is contribution No. 1087 of the European Institute for Marine Studies (Brest, France).

References


