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Haemocyte parameters associated with resistance to brown ring disease in *Ruditapes* spp. clams

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Abstract

Brown ring disease (BRD) is a shell disease caused by *Vibrio tapetis*. This pathogen disturbs the periostracal lamina causing the appearance of a brown conchiolin deposit on the inner face of the shell, within the extrapallial space. Although differences in resistance to BRD have been documented, their relationship to possible defense functions has never been investigated. In this study, flow cytometry was used to analyze cellular parameters in asymptomatic and experimentally infected *Ruditapes philippinarum* from France and the west coast of the USA. Parallel analyses were made on *Ruditapes decussatus*, the native European clam, which is highly resistant to BRD. In the haemolymph and extrapallial fluid of animals without BRD, total haemocyte counts, the percentage of granulocytes, and the phagocytic activity against latex beads or *V. tapetis* by the haemocytes were significantly higher in American *R. philippinarum* than in French *R. philippinarum*. In most cases, levels in *R. decussatus* were the highest of all three groups. Four weeks following challenge with *V. tapetis*, BRD prevalence reached 52 in American clams and 100% in French specimens, but only 37% in *R. decussatus*. In symptomatic animals, phagocytosis of *V. tapetis* increased significantly in the resistant species of clam, *R. decussatus*, was unchanged in US clams, and decreased significantly in FR specimens when compared to asymptomatic individuals from each population. Ingestion of *V. tapetis* by haemocytes in the extrapallial fluid, which is in contact with the periostracal lamina, could be the main defense mechanism used to counter the pathogen. Our results suggest that resistance to BRD may well be related to the concentration of granular haemocytes and the phagocytic activity of haemocytes.

Keywords: Bivalve; Bacteria; *Vibrio tapetis*; Resistance; Phagocytosis; Granulocyte

1. Introduction

Cellular defenses in bivalve mollusks rely on haemocytes, the circulating blood cells present in the extrapallial fluids as well as in the haemolymph [1,2]. Measurements of blood cell composition and activity are widely used to evaluate the physiological state of a host and to estimate its potential defense capabilities against disease agents [3–8]. For bivalves, the cellular parameters typically assayed are total and differential haemocyte counts; and the enzymatic, cytotoxic, and phagocytic activities of these cells. It has been suggested that resistance to pathogens could be the result of an efficient defense system, as evidenced by high defense-related haemocyte activities [3,9,10]. Marked changes in all of the above parameters have been associated with infection

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Abbreviations: 90°LS, Right angle light scatter; BRD, Brown Ring Disease; FITC, Fluorescein-isothiocyanate; FLS, Forward light scatter; FR, French; GFL, Green fluorescence; PBS, Phosphate buffered saline; RD, *Ruditapes decussatus*; SSW, Sterile seawater; US, American.

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by pathogens [3–8], but it is rarely clear whether the change is a direct result of defense against the invading pathogen or a consequence of disease-associated physiological debilitation and tissue damage to the host. Although potential roles for putative defense factors have been demonstrated [1,6,7], attempts to correlate high or low levels of one or more of them with successful defense against a pathogen are rare [8]. For example, if high haemocyte counts or phagocytic activity is thought to be important to defense [1,7,8,10], is there a correlation between high numbers of total or phagocytic cells and reduced disease levels or improved survival after challenge by a pathogen?

Over the last decade, a newly described disease has affected an important commercial molluscan species, the Manila clam, *Ruditapes philippinarum*, in western Europe. This species originates from the Indo–Pacific region. It was accidentally introduced to the west coast of the USA in 1936 and was brought to Europe from the USA in the 1970s for aquacultural purposes [11]. Brown Ring Disease (BRD), which affects the inner shell and is caused by the bacterium *Vibrio tapetis*, appeared in cultured clams on the northwest coast of France in 1987, causing high mortalities [12,13]. It later spread to culture beds and wild populations along the western European coast. Interestingly, BRD has never been reported in the USA or the Indo–Pacific region. In a preliminary study, Allam [14] reported that *R. philippinarum* from the western US experienced lower BRD symptoms and better survival than French clams when both were experimentally challenged with *V. tapetis*. The native European species, *R. decussatus*, is also much less susceptible to *V. tapetis* than is the introduced species [14,15]. Thus, the *Ruditapes* spp.– *V. tapetis* relationship provides the biological material for testing the hypothesis that variation in certain supposed cellular defense factors are correlated with measurable variations in disease development.

Considerable experimental data on changes in putative defense factors in *R. philippinarum* after experimental injection of *V. tapetis* are available [8,12,14,16,17]; however, they do not include information on the effectiveness of the various factors in combating BRD. In addition, phagocytosis was not measured. In the present study, we performed several common cellular assays on three groups of clams that had previously demonstrated different susceptibility to BRD [14]. Clams were challenged with *V. tapetis*, after which disease development and cellular parameters were compared among the three groups. The recently described occurrence of functional haemocytes in clam extrapallial fluid [2,18,19] was of major interest since it is in this compartment that the BRD symptom, a perturbation of the periostracal lamina caused by *V. tapetis*, is found and where haemocytes would come into initial contact with the pathogen. We hypothesized that if a particular cellular factor is important in combating the pathogen, it should be correlated with the development of disease symptoms and with survival of the host.

2. Materials and methods

2.1. Experimental clams

Specimens of *R. philippinarum* were obtained from two locations: the Bay of Brest (Brittany, France; length = 41.1 ± 0.58 mm, mean ± SEM) and southern Puget Sound, Washington (USA; 41.8 ± 0.32 mm) in April 1998 (13 and 14°C at the two collection sites, respectively). Specimens of *R. decussatus* (39.7 ± 0.22 mm) were collected from the same location, and at the same time, as French *R. philippinarum*. On arrival in the laboratory, the clams were immediately placed in quarantined 35-litre aerated, standing-water tanks (about 33 clams per tank), in which they were kept during the remainder of the study. Water was maintained at 13°C and 34 ppt, and clams were fed daily throughout the experiments using a mixture of cultured algae.

2.2. Bacteria

V. tapetis (*ATCC 4600*) strain (P16) was previously isolated from symptomatic clams in Brittany (France) by Paillard [20]. Two bacterial species not known to be pathogenic to adult *Ruditapes* spp., *Vibrio anguillarum* (strain 775, [21]) and *Vibrio splendidus* (ATCC 33125) were graciously provided by Dr Catherine Boettcher at the University of Maine (USA). All bacterial strains were grown on marine agar and used during their exponential phase of growth (typically 72 h after subculture).

2.3. Inoculation with V. tapetis

After an acclimation period of 12–14 days, clams from each of the three groups were challenged with *V. tapetis* using the method of Allam [22] in order to induce BRD. Briefly, a *V. tapetis* suspension of 10^8 cells ml^{-1} in sterile seawater (SSW) was made from a 72-h culture grown on marine agar. Each experimental clam was inoculated via the pallial cavity with 5×10^7 *V. tapetis* in 0.5 ml. Control clams were inoculated with 0.5 ml of SSW. After inoculation, clams were maintained as described above for 4 weeks, a period shown by Paillard [12] to be sufficient to induce BRD symptoms in most susceptible clams. At that time, haemolymph and extrapallial fluid were collected (see below). The clams were then shucked and the percentage of clams showing the organic deposit, characteristic of BRD, on the inner face of the shell (symptomatic individuals) was determined in each group. The stage of BRD development was classified according to the standard rating system developed by Paillard [23]. In this classification system, the BRD index is calculated according to the extent and thickness of the brown deposit, and ranges theoretically between 1 (a very light, localised deposit) and 18 (very heavy, extensive deposit). A mean BRD index was calculated for each group. The percentage of clams displaying the repair process, which consists of covering the brown deposit with new calcified layers [23], was also recorded.

2.4. Body fluid sampling

Haemolymph and extrapallial fluid samples were collected as previously described [2]. For each clam, the sample volume varied with compartment: 500–800 μl for haemolymph, and about 700–1200 μl for extrapallial fluid (both valves). Samples were immediately divided into two subsamples: the first subsample (300 μl) was diluted with ice-cold SSW (1:3, v/v) and processed for the phagocytosis assay; the second (200 μl) was fixed with formalin (3% final concentration) and used for total and differential haemocyte counts. All assays were performed on individual clams.

2.4.1. Total haemocyte counts

Total haemocyte counts were assessed microscopically using a haemacytometer. Results are presented as the number of cells ml^{-1} of haemolymph or extrapallial fluid.

2.4.2. Differential haemocyte counts

Fixed haemolymph and extrapallial fluid samples, each containing about 2.5×10^5 cells, were analyzed as previously described [24] using a Coulter EPICS C flow cytometer equipped with an argon laser and operated at a wavelength of 488 nm. Log 90° (Log90°LS) and forward light scatter (FLS) data were collected and stored as list-mode files. Two subpopulations were discernible on two-parameter histograms (Log90°LS × FLS). Each subpopulation was sorted onto glass slides using the EPICS C and its identity was confirmed microscopically. Hyalinocytes were located in the lower channels of both light scatter axes, whereas granulocytes appeared in the higher channels. The percentage of each subpopulation was calculated by bitmapping (electronic outlining) that cell group and comparing the number of cells in the bitmap with the total number of analyzed cells.

2.5. In vitro phagocytosis measurements

2.5.1. Test particles

Both fluorescent latex beads (2.02 μm in diameter, Fluoresbrite Calibration grade, Polysciences, USA) and fluorescein-isothiocyanate (FITC)-labeled bacteria (*V. tapetis*, *V. anguillarum* and *V. splendidus*) were used to measure the phagocytic activity of haemocytes. Each bacterial strain was labeled and used individually. Bacteria (10^9 CFU) were fixed in 2% formalin, washed three times with PBS, and suspended in 1 ml PBS (pH 7.4) containing 1 mg FITC (Sigma). The mixture was incubated for 30 min at room temperature, washed twice in PBS, and finally resuspended in SSW.

2.5.2. Phagocytosis assay

The phagocytosis assay was modified from the procedure of Alvarez [25] as described in Allam [14]. Test particles (beads or labeled bacteria) were dispersed by expelling them through a 26-gauge needle and diluting them with SSW to give a final concentration of 6×10^6 particles ml^{-1}. Two hundred microliters of this suspension were placed in each well of a 24 well microplate, which was then centrifuged (200***g*** for 10 min for beads, and 400***g*** for 30 min for

bacteria) at 20°C to form a uniform monolayer of particles on the bottom of each well. Aliquots of haemolymph or extrapallial fluid diluted in SSW were added to each well (200 μl containing about 1.2×10^5 haemocytes) to give approximately a 1:10, cell:bead or cell:bacteria ratio. Control wells, in which phagocytosis was prevented, were established by adding Cytochalasin B at 10 μg ml^{-1} final concentration to wells containing fluorescent particles before the addition of haemocytes [25]. For each clam, two test and one control well were assayed for each fluid compartment. Following incubation (30 min at 20°C), Cytochalasin B was added (10 μg ml^{-1} final concentration) to each experimental well to stop haemocyte activity. Attached cells were released by trypsinization (0.4% trypsin in 1% EDTA–saline solution for 10 min) and gentle sonication for 1 min at room temperature. Formalin was then added to a final concentration of 3% to fix the sample, which was transferred to a microfuge tube where it was held on ice until processed for flow cytometry (within an hour).

2.5.3. Flow cytometry

Gains and photomultiplier high voltage settings were adjusted to include phagocytosing and non-phagocytosing cells on the two-parameter display plot. The settings were based on forward light scatter (FLS) and green fluorescence (GFL) signals [14]. List-mode data were collected for a total of 10,000 particles in each sample. Bitmaps were used to outline cells that were or were not associated with fluorescent particles in each control and test sample. The percent phagocytosis was computed as the ratio of bead-associated haemocytes to total haemocytes x100. For each clam, the percent phagocytosis was calculated as the difference between the arithmetic mean of percent phagocytosis in both test wells and percent phagocytosis in control well (added with Cytochalasin B, percent phagocytosis was always below 0.5%).

2.6. Statistics

Arcsine transformations were applied to the percentage phagocytosis and the percentage granulocytes before the use of statistical tests. Differences between *V. tapetis*-injected clams that developed BRD (symptomatic) and clams that did not (asymptomatic) were assessed using a Student's *t*-test. In the initial analysis, two groups of symptomatic and asymptomatic clams were recognized: those injected with the pathogen and those injected with SSW. There were no statistical differences, however, between the two groups of symptomatic clams or the two groups of asymptomatic clams in the haemocyte parameters measured, so all symptomatic clams and all asymptomatic clams were pooled within each of the three origin/species groupings. Within each disease category, differences among the three groups were tested using a one-way general linear model ANOVA followed by a Fisher's PLSD post-hoc test. Differences were considered significant at $\alpha = 0.05$.

3. Results

Four weeks after inoculation, conchiolin deposits on the inner face of the shell that are characteristic of Brown Ring Disease were visible in all clam groups challenged with *V. tapetis*. The prevalence of BRD, however, varied according to both origin and species: 100 and 52% in *R. philippinarum* from France (FR, $n = 75$) and the United States (US, $n = 120$), respectively, and 37% in *R. decussatus* (RD, $n = 38$). Moreover, among animals that developed BRD, the average disease index was low in US *R. philippinarum* and *R. decussatus* (mean BRD indices of 2.4 and 1.8, respectively) compared with FR *R. philippinarum* (8.9). Among symptomatic clams, the recovery process (recalcification) was observed in 89% of *R. decussatus*, 62% of US *R. philippinarum*, and only 12% of FR *R. philippinarum*. No BRD symptoms were observed in SSW-injected control clams in the *R. decussatus* (n = 24) or US *R. philippinarum* ($n = 120$) groups, but a few (17%) light conchiolin deposits were found in the FR *R. philippinarum* ($n = 74$), in which *V. tapetis* were apparently present at the time of collection. Mortality was $< 4\%$ in all control and experimental clams.

Among asymptomatic clams, the total number of haemocytes in the haemolymph of US *R. philippinarum* was significantly higher than that found in either FR *R. philippinarum* or *R. decussatus* (Table 1). In all comparisons, clams with BRD symptoms had significantly greater total haemocytes than did asymptomatic animals from the same location/

Table 1
Haemocyte parameters in American (US) and French (FR) *Ruditapes philippinarum*, and *Ruditapes decussates* (mean ± SEM). Number of clams is indicated within parentheses. For each parameter, double bars represent significant differences (Student's *t*-test, $P < 0.05$) between asymptomatic and symptomatic animals from the same population, and letters (a and b; or x, y and z) represent differences among asymptomatic or symptomatic clams from different populations, respectively (ANOVA, $P < 0.05$)

	R. philippinarum—US		*R. philippinarum*—FR		*R. decussatus*	
	Asymptomatic	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic	Symptomatic
Haemolymph						
Total haemocyte counts (10^6 cells ml^{-1})	3.1 ± 0.1 (59) a	3.8 ± 0.2 (43) x	2.5 ± 0.2 (20) b	3.6 ± 0.1 (54) x	2.4 ± 0.2 (33) b	3.4 ± 0.3 (9) x
Granulocytes (%)	67.2 ± 2.9 (59) a	73.2 ± 3.0 (43) x	55.2 ± 4.1 (20) b	51.3 ± 3.6 (54) y	64.2 ± 4.1 (33) a	83.7 ± 3.7 (9) z
Phagocytosis of beads (%)	17.0 ± 0.6 (56) a	17.4 ± 1.1 (45) x	12.9 ± 0.7 (24) b	8.9 ± 0.6 (51) y	16.7 ± 0.9 (32) a	23.4 ± 0.8 (14) z
Extrapallial fluid						
Total haemocyte counts (10^6 cells ml^{-1})	3.9 ± 0.2 (59) a	4.9 ± 0.2 (43) x	3.2 ± 0.2 (20) b	4.6 ± 0.2 (54) x	3.6 ± 0.2 (33) a,b	5.1 ± 0.6 (9) x
Granulocytes (%)	79.4 ± 2.0 (59) a	91.2 ± 1.7 (43) x	70.3 ± 3.1 (20) b	84.6 ± 1.6 (54) y	80.6 ± 3.2 (33) a	95.1 ± 1.0 (9) x
Phagocytosis of beads (%)	17.6 ± 1.2 (56) a,b	14.9 ± 1.2 (45) x	14.8 ± 0.7 (24) a	10.7 ± 0.9 (51) y	19.3 ± 1.0 (32) b	22.2 ± 1.2 (14) z

species, however, there were no statistical differences among the three groups of symptomatic clams. In asymptomatic individuals, haemocyte counts were significantly higher in the extrapallial fluid of US compared to FR *R. philippinarum* (Table 1); *R. decussatus* was intermediate and not statistically different from the others. The development of BRD was associated with a significant increase in haemocyte densities in all clam groups, but as in the haemolymph, without significant differences among the groups. In each population, haemocyte densities were significantly higher in the extrapallial fluid than in the haemolymph of either symptomatic or asymptomatic clams ($P < 0.05$).

As in the case of total haemocyte densities, the percentage of granulocytes in the haemolymph of asymptomatic animals was significantly higher in US than in FR *R. philippinarum* and intermediate in *R. decussatus* (Table 1). The presence of BRD symptoms did not significantly alter the proportion of granulocytes in either *R. philippinarum* group, but increased it in *R. decussatus*. Among symptomatic animals, the highest percentage of granulocytes was observed in *R. decussatus* (83.7%) followed by US *R. philippinarum* (73.2%) and finally FR *R. philippinarum* (51.3%). Extrapallial fluid of *R. decussatus* and US *R. philippinarum*, both symptomatic and asymptomatic, contained significantly higher percentages of granulocytes when compared with FR *R. philippinarum* (Table 1). In all three groups, symptomatic clams had significantly greater percentages of granulocytes (84.6–95.1%) than did asymptomatic clams (70.3–80.6%). The percentage of granulocytes was significantly higher in the extrapallial fluid than in haemolymph.

Haemocytes from the haemolymph of asymptomatic *R. decussatus* and US *R. philippinarum* displayed a significantly higher phagocytic activity (17.0 and 16.7%, respectively) relative to those obtained from FR *R. philippinarum* (12.9%) (Table 1). Phagocytic activity by haemocytes from the extrapallial fluid of the same individuals was statistically similar to, and fell in the same order as, the haemolymph haemocytes: *R. decussatus* displayed the highest activity (19.3%) followed by US *R. philippinarum* (17.6%) and finally by FR *R. philippinarum* (14.8%) (Table 1). In both compartments, the presence of BRD symptoms was associated with a significant increase in phagocytic activity in *R. decussatus*, no change in US *R. philippinarum* and a significant decrease in FR

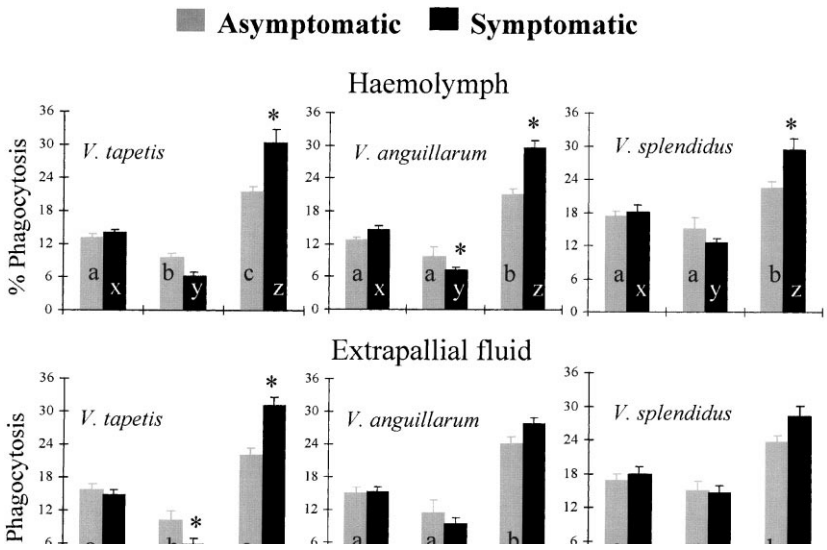

Fig. 1. Phagocytosis of FITC-labeled bacteria by haemocytes from haemolymph and extrapallial fluid of asymptomatic and symptomatic clams (mean ± SEM). RP-US and RP-FR: American and French *R. philippinarum*, respectively. RD: *R. decussatus*. *: significant difference between asymptomatic and symptomatic animals within each population (Student's *t*-test, $P < 0.05$). Letters (a, b and c; or x, y and z) represent differences among asymptomatic or symptomatic clams from different populations, respectively (ANOVA, $P < 0.05$).

R. philippinarum. The fraction of phagocytic cells was about 50–100% higher in US than in FR *R. philippinarum* (extrapallial fluid and haemolymph respectively), and 100–160% more in *R. decussatus* than in FR *R. philippinarum*.

Haemocytes from the haemolymph of both symptomatic and asymptomatic *R. decussatus* phagocytosed FITC-labeled bacteria at significantly higher rates (22–31%) than did haemocytes from the other groups (Fig. 1). Phagocytic rates of US *R. philippinarum* were intermediate (13–18%) and those for French clams, the lowest (6–15%). Differences were significant for both symptomatic and asymptomatic clams challenged with *V. tapetis*, but only for symptomatic clams exposed to the other bacterial species. Differences between clams with and without BRD symptoms displayed an identical pattern for all bacterial species: an increase (always significant) in *R. decussatus*; a decrease (significant for *V. tapetis*) in FR *R. philippinarum*; and no change in US *R. philippinarum*. The uptake of *V. tapetis* by haemolymph haemocytes of symptomatic clams was more than 120% higher in US *R. philippinarum* and about 400% higher in *R. decussatus* than in FR *R. philippinarum*. Haemocytes from both US and FR *R. philippinarum* populations, symptomatic or not, phagocytosed *V. splendidus* at significantly greater rates than they did *V. anguillarum* or *V. tapetis*. Phagocytic rates measured for haemocytes from the extrapallial fluid were nearly identical to those from the haemolymph (Fig. 1). Cells from *R. decussatus* showed the highest phagocytic activity against each bacterial species, US *R. philippinarum* were intermediate, and FR *R. philippinarum* had the lowest rates, although differences between *the R. philippinarum* groups were not always significant for *V. anguillarum* and *V. splendidus*. For the most part, changes associated with BRD symptoms fell in the same order as they did for haemolymph haemocytes, although the differences were smaller, and significant in two comparisons only: the increase for *R. decussatus* and the decrease for *R. philippinarum*, both exposed to *V. tapetis*. Compared to FR *R. philippinarum*, uptake of *V. tapetis* in the extrapallial fluid of symptomatic clams was about 150% higher in US clams, and about 400% higher in *R. decussatus* (Fig. 1).

Changes in the percentage of phagocytic haemocytes do not reflect the true changes in the subpopulation. To estimate changes in absolute numbers of phagocytic cells, the density of total haemocytes was multiplied by the percentage of cells that phagocytosed *V. tapetis* in each clam. This analysis showed

that although the proportion of phagocytic cells declined in FR *R. philippinarum* with BRD, the absolute number remained unchanged ($0.25 \pm 0.05 \times 10^6$ vs $0.24 \pm 0.06 \times 10^6$ cells ml^{-1} in haemolymph, and $0.31 \pm 0.07 \times 10^6$ vs $0.30 \pm 0.09 \times 10^6$ cells ml^{-1} in extrapallial fluid of asymptomatic and symptomatic clams, respectively) because the total haemocyte densities increased in the symptomatic clams. By the same calculation, the phagocytic haemocyte numbers increased significantly in US clams with BRD ($0.43 \pm 0.03 \times 10^6$ vs $0.57 \pm 0.04 \times 10^6$ cells ml^{-1} in haemolymph, and $0.60 \pm 0.06 \times 10^6$ vs $0.81 \pm 0.08 \times 10^6$ cells ml^{-1} in extrapallial fluid of asymptomatic and symptomatic clams, respectively) even though the proportion of phagocytic cells did not change, because of the increase in total haemocytes. Finally, the concentration of phagocytic haemocytes rose by 78% in the haemolymph of symptomatic *R. decussatus* ($0.54 \pm 0.04 \times 10^6$ vs $0.96 \pm 0.08 \times 10^6$ cells ml^{-1}), and more than doubled in the extrapallial fluid ($0.78 \pm 0.07 \times 10^6$ vs $1.65 \pm 0.14 \times 10^6$ cells ml^{-1}), because both the total haemocyte density and the proportion of phagocytes increased.

4. Discussion

The results of our study demonstrated clear differences in Brown Ring Disease (BRD) prevalence, intensity, and recovery between two species of the clam, *Ruditapes* (*philippinarum* and *decussatus*), and between US and French populations of *R. philippinarum*, all experimentally challenged with the same dose of the causative agent, *V. tapetis*. Concurrent measurement of three cellular components of the internal defense system showed that variations in the percentage of granulocytes and phagocytic cells were consistently and positively correlated with variations in resistance to Brown Ring Disease, whereas total haemocyte densities showed no consistent associations.

All three components of the molluscan internal defense system that we measured are commonly used to assess overall health of an individual or population, and are considered as indices of potential resistance against pathogens [3,7,9,26,27]. Our study compared cellular parameters in clams with obvious symptoms of the disease and those without, which we termed 'asymptomatic'. The former consisted of animals with a brown deposit assayed at the end of the study. The latter included control clams, which represent the prechallenge condition because previous studies have consistently demonstrated no change over time in haemolymph and extrapallial fluid parameters of *R. philippinarum* injected with SSW [8,14,17,18]. It also includes clams injected with *V. tapetis* that successfully fought the challenge and did not develop BRD (US *R. philippinarum* and *R. decussatus*, only) because there were no statistical differences between these and nonchallenged controls.

Of the three clam populations studied, *R. decussatus* developed the lowest BRD prevalence and intensity followed by American *R. philippinarum*, and finally the French population of *R. philippinarum* which developed the heaviest disease symptoms. These findings are consistent with previous reports showing lower BRD development in *R. decussatus* wild populations and in those experimentally challenged with *V. tapetis* compared to French *R. philippinarum* [12,14,15]. Our haemocyte assays showed that *R. decussatus* or US *R. philippinarum* (mostly the former) always had the highest proportion of granulocytes and phagocytic haemocytes and FR *R. philippinarum* always had the lowest. Further, this ranking was observed whether or not the clams had BRD symptoms. In contrast, there was no consistent pattern to the ranking of total haemocyte densities among the clams. Because of the consistent differences observed in asymptomatic clams, we conclude that the more resistant *R. decussatus* and US *R. philippinarum* had higher baseline proportions of granulocytes and phagocytic cells compared to the very susceptible FR *R. philippinarum*. On the other hand, there was no consistent evidence that higher resting haemocyte densities were associated with reduced BRD development.

It must be emphasized that these differences occurred not only in the haemolymph, but also in the extrapallial fluid. The extrapallial space is important in BRD because it is where the potential defense elements first encounter *V. tapetis* [2,18]. Within hours of experimental exposure, the bacteria colonize the periostracal lamina, disorganizing it causing the formation of the brown organic deposit, which becomes heavily colonized with *V. tapetis* [22,28,29]. In this study, total haemocyte densities and the percentage of granulocytes were always higher in the extrapallial fluid than in the haemolymph. They were inversely correlated with BRD

development and positively correlated with recovery rates.

Mean haemocyte densities increased in all clams with BRD, as has been previously reported [8,14,18,19], but changes in the percentage of granulocytes and phagocytic haemocytes associated with disease symptoms varied considerably among groups. The percentage increased in *R. decussatus*, decreased in FR *R. philippinarum*, and was unchanged in US *R. philippinarum*. In assessing this finding, it is important to recall that symptomatic *R. decussatus* had only very light deposits and most were recovering, as evidenced by recalcification of the BRD deposit. In contrast, nearly all of the FR *R. philippinarum* had heavy deposits with almost no signs of repair. Thus, although they were classified as symptomatic, most of the *R. decussatus* appeared to be successfully overcoming the *V. tapetis* challenge. For the symptomatic *R. decussatus*, therefore, it can be argued that the increased proportion of phagocytic haemocytes was correlated with a successful defense rather than a consequence of the disease itself. In contrast, the depressed phagocytic activity measured in FR *R. philippinarum* was probably the result of diminished physiological capacities in sick clams [30,31] or the inhibitory effects of *V. tapetis* on haemocytes of this host (unpublished data). In fact, high percentages of dead haemocytes found in *R. philippinarum* with BRD in a previous study is an indication of the debilitating effect of BRD on the clam's cellular defense system [18,19]. The average disease index in US *R. philippinarum* was relatively light and about half the symptomatic clams showed evidence of repair. The intermediate phagocytic activity in this group may indicate a mixture of responses—most evidencing a successful outcome, a few reflecting disease—caused debilitation. Finally, it is important to point out that the absolute density of phagocytic haemocytes increased in *R. decussatus* and US *R. philippinarum* with BRD symptoms. These two groups had, by far, the most effective response to the *V. tapetis* challenge, as measured by low disease stage and high recovery.

In addition to changes associated with increases in total haemocyte numbers, there was evidence of changes in activity of individual haemocytes. Granulocytes produce a wide array of hydrolytic enzymes and antibacterial substances and are considered to be the most phagocytic haemocytes in *Ruditapes* spp. [32,33]. However, increases in the percentage of phagocytic cells cannot be explained simply by increases in the fraction of granulocytes. In this study, the percentage of granulocytes increased in the extrapallial fluid of all symptomatic clams, but the percentage of phagocytic cells increased in *R. decussatus* only. This indication of an enhanced phagocytic capacity during the Brown Ring Disease process suggests that *V. tapetis* cells, or their products, may induce the uptake of foreign particles by granulocytes of *R. decussatus* to a greater extent than in *R. philippinarum*. However, until the effects of *V. tapetis* on granulocytes are tested, this point is highly speculative.

Our comparison of phagocytic rates for various bacterial species suggested that some differences among the clam groups tested are independent of the target particle, whereas others show selective uptake of some particles. For instance, *R. decussatus* had superior phagocytic rates for beads and all *Vibrio* spp. alike. In contrast, US *R. philippinarum* showed higher uptake of both beads and *V. tapetis* compared to *FR R. philippinarum*, while for the nonpathogenic *Vibrio* spp., this difference was observed only in symptomatic clams. Selective uptake of particles by *R. decussatus* haemocytes was reported by Lopez [32], who noted that haemocytes phagocytosed yeast cells and *V. tapetis* more actively than they did *Perkinsus atlanticus* zoospores. These differences could be related to recognition factors (lectins) present on bivalve haemocytes [34] or in plasma [35,36,37]. In our study, haemolymph and extrapallial fluid were diluted (1:3, v:v) using SSW without any cross-dilution of plasma from other individuals or groups (i.e., without suspending haemocytes from FR animals in plasma from US or RD specimens) thus, it is not known if the differences in percent phagocytosis between US and FR clams and between asymptomatic and symptomatic animals could be the result of differences or changes in such 'opsonizing' factors in the soluble fraction of the body fluids. However, in a recent work, Lopez-Cortes [33] noted that *R. philippinarum* and *R. decussatus* haemolymph proteins are not required for identification and internalization of non-self particles, including *V. tapetis*.

The response of the US population of *R. philippinarum* was clearly different from that of the FR population. US clams developed fewer and lighter

BRD symptoms, and showed better recovery than the French population. These differences were similar to those of an earlier study [14] in which BRD developed in 57% of *V. tapetis*-injected US *R. philippinarum*, contrasted with 93% BRD development in a comparably treated French population. The finding of the same results in two separate experiments suggests a true difference in resistance between the two groups, at least under experimental challenge. Without rearing the two groups in a common environment before challenging them with the pathogen, it is impossible to determine whether the differences in either parameter are the result of environmental factors to which the clams were exposed before the experiments, or whether they are genetic. It is interesting, however, to note that the more resistant group came from the west coast of the United States where BRD has never been reported and the more susceptible, from an area in France, where BRD has been observed since 1992 and where one would expect that some resistance might have developed. It is more typical that a population with no history of exposure to a pathogen would be the more susceptible.

The total haemocyte count is probably the most commonly measured cellular index, primarily because it is an easy parameter to measure. However, high numbers of haemocytes are beneficial only if they can effectively eliminate the pathogen or perform some other role in the disease process, such as wound plugging or tissue repair [7,38]. In some instances, circulating hemocyte densities do appear to be correlated with successful defence, as in the case of the freshwater crayfish, *Pacifastacus leniusculus*, in which experimental reduction of circulating haemocyte numbers resulted in proliferation of *Aphanomyces astaci* (cause of crayfish plague) from latent infections in the normally 'resistant' host species [26]. In others, the evidence is equivocal. For instance, Chu, La Peyre and colleagues [3,7,39] found a lack of consistent correlation between the survival of two oyster species (susceptible *Crassostrea virginica* and resistant *C. gigas*) exposed to the pathogen *Perkinsus marinus* and various hemocyte parameters, including total density, either before or after challenge. In fact, these authors concluded that haemocytes may not play a direct role in defense against *P. marinus*.

Although circulating haemocyte densities increased in resistant strains of *C. virginica* exposed to another oyster pathogen, *Haplosporidium nelsoni* [6], a similar conclusion was reached about their role based on the very low in vitro phagocytic rates for the pathogen, and the lack of difference in these rates between *C. virginica* strains resistant and susceptible to the pathogen, and between *C. virginica* and the highly resistant *C. gigas* [40]. Interestingly, these authors reported no differences in the densities of circulating haemocytes between uninfected resistant or susceptible *C. virginica* strains. Thus, in the case of two major oyster diseases, total haemocyte densities or even the numbers of potential phagocytes may be irrelevant because these cells do not appear to be very effective either in phagocytosing or in killing the parasites, although they may perform other functions.

In the case of BRD, on the other hand, both in vivo and in vitro phagocytosis, and in vivo elimination of the pathogenic agent *V. tapetis* have been clearly demonstrated (present study; [14] and unpublished data, [33]). Although total haemocyte counts alone provided little information about the potential 'resistance status' of the clams, the consistent, positive correlation found between baseline numbers of potential (granulocytes) and active phagocytic haemocytes, and reduced disease development after experimental challenge is strong indication that these particular cells play a role in combating *V. tapetis* in vivo. The continued high concentrations of granular and phagocytic cells during successful recovery from the disease is further evidence of their role. Of particular importance in assessing our results is the fact that low BRD development and recovery were associated with high numbers of phagocytic cells in the extrapallial fluid, the site of initial encounter of the clam with the bacterial pathogen.

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