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Abstract

Brown ring disease (BRD) in the Manila clam is characterized by the formation of a brown deposit of conchiolin on the inner surface of the shell that gives the disease its name. The development of the signs of BRD may be favored by the entry of bacteria in the extrapallial compartments *via* mechanical disruptions of the periostracal lamina and/or chipping of the shell margin. In order to test this hypothesis, we conducted an experiment simulating clam handling under aquaculture conditions and we checked for prevalence of BRD signs. Our results assess that rough handling of *R. philippinarum* in presence of the bacterium *V. tapetis* significantly increase the prevalence of BRD signs. As a consequence our results show that minimizing manipulations and transfers of clams during culture is beneficial to avoid the development of BRD signs.

Key words: *Ruditapes philippinarum*, brown ring disease, handling, shellfish farming

1. Introduction

The Manila clam, *Ruditapes philippinarum*, has been introduced during the 70's in a number of european countries for aquaculture purposes (Flassch and Leborgne, 1992) and is now a widespread species. Brown ring disease (BRD) in the Manila clam, *Ruditapes philippinarum*, was first reported in North Finistère (France) in 1987 (Paillard et al., 1989). The disease is characterized by a brown deposit on the inner surface of the valves (Paillard et al., 1989) that gave the disease its name ; those signs go in hand with the proliferation of the aetiological agent of BRD, the bacterium *Vibrio tapetis* (Paillard and Maes, 1990; Borrego et al., 1996). BRD has been responsible for mortalities of Manila clam in several European countries such as France, Italy, Spain, Portugal and England (Paillard et al., 1989; Paillard and Maes, 1990; Castro et al., 1992; Paillard et al., 1994; Figueras et al., 1996; Allam et al., 2000). Infection disrupts the production of the periostracal lamina and causes an anomalous deposition of periostracum on the inner shell of infected clams (Paillard et al., 1994; Paillard and Maes, 1995a,b). Althougth post–colonization processes (i.e. after penetration of *Vibrio tapetis* into extrapallial compartment) have been widely described (Paillard, 2004), mechanisms of entry of *V. tapetis* into the extrapallial fluids remain poorly understood. A previous study (Flye-Sainte-Marie et al., 2008) suggested that the pathogen, *V. tapetis*, may benefit from mechanical disruption of the periostracal lamina or chipping of the valve margins to colonize the Manila clam extrapallial compartment. These results suggest that rough handling of clams in aquaculture conditions may enhance BRD development.

The present experiment was designed to test this hypothesis. For this purpose we simulated rough handling of clams, as it may occur for clam cultivation purpose during transport and manipulations (*e.g.* between nursery and growing plot, or when

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small individuals are harvested and reseeded after harvest of commercially mature clams). We combined or not this treatment with exposure to *V. tapetis*, to assess its effect on the development of BRD signs.

2. Materials and methods.

2.1. *Specimens and experimental plan.*

More than 700 Manila clams, larger than 25 mm, were collected at low tide by hand on the 30th of January 2008, on the Lanveur mudflat, Bay of Brest, France. Particular care was taken to avoid any effect of handling on clams : during collection in the field clams were stocked cautiously, one after the other, in boxes containing rags and transfered to the Laboratoire des Sciences de l'Environnement Marin (Brest, France). At the laboratory clams were gently rinsed individually in seawater. Initial prevalence of the brown ring disease signs was estimated by sacrificing and evaluating 100 clams randomly chosen.

Six hundred clams were randomly subdivided into 12 batches of 50 clams each. Throughout the experiment, each batch was maintained in 20 liters tanks of aerated filtered (0.5μm) seawater at 16°C, which is near optimal temperature for brown ring disease development (Paillard, 2004); a pump generated a smooth current in each tank. Filtered seawater was renewed every fifth day throughout the trial. Clams were fed with one liter of cultured *Isochrysis aff. galabana* (concentration of 50 cells / μL) per week and per tank. Tanks were checked on a daily basis for mortalities and moribund clams. Any gaping individuals were presumed moribund and removed.

After one week of acclimation, the 12 batches were randomly distributed into 4 triplicates; each triplicate was then attributed to one of the following experimental conditions:
– Untreated control.
– Handling simulation (hereafter *handled*)
– Exposed to *Vibrio* strain (hereafter *exposed*).
– Handling simulation and exposed to *Vibrio* strain (*handled and exposed*).

In order to simulate shellfish farming rough handling, *handled* clams were placed in a closed tank without water and manually roughly shaken for 30 seconds; the tanks were then filled with 20 liters of fresh filtered seawater. *Handled and exposed* clams were submitted to experimental infection as described below immediately after simulation of handling.

2.2. *Experimental infections*

V. tapetis strain CECT 4600 was grown in marine agar (Difco 2216) at 18°C for 48 to 72 hours. Bacterial colonies were resuspended in filtered seawater. Bacterial suspension was added in *V. tapetis* exposed tanks to reach a final concentration of 10^6 cells ml^{-1}, which is the same order of magnitude as in Drummond et al. (2007). A first exposition was performed on the 8th of February 2008 (day 8) and water was renewed after 24 hours. A second exposure experiment was conducted on the 22th of February 2008 (day 22) and water was renewed after 5 days. During exposures, clams were regularly monitored to verify that their shells were opened and that they were actively filter-feeding. After each infection experiment, the water was drained and the clams remained out of water for 1 hour, in order to induce the closure of the valves and the incorporation of *V. tapetis* in the pallial cavity. The tanks were then filled with 20 liters of fresh filtered seawater.

In control tanks, clams were treated as above except that the bacterial suspension was not added.

2.3. *Characterisation and classification of brown ring disease syndrome*

The experiment last for about 6 weeks, which is an intermediate duration between experiments by Paillard et al. (2004) and Drummond et al. (2007) that allows the development of visible signs of BRD at the chosen experimental temperature. On day 40 of the experiment, clams were sacrified, flesh was removed and valves cleaned under a trickle of water, and were then left to dry until further analysis.

All shells (including those of moribund individuals sampled throughout the trial) were retained and left to dry. The disease intensity was estimated by the extent of the symptomatic deposit according to the criteria of Paillard and Maes (1994) in which conchiolin deposit stages (CDS) range from microscopic brown spot on the inner face of the shell in the earliest stages (CDS 1), to a thick brown deposit covering most of the inner shell in the most advanced stage (CDS 7).

2.4. *Statistical analyses*

Variations of prevalence and mortality among treatments were tested using analysis of variance (ANOVA). Tank effect was always neglected because it was never significant when we tried to take it into account. When ANOVA was significant, Tukey's HSD test (Yandell, 1997) was used to assess pairwise differences among groups. Statistical analysis were conducted using R statistical software version 2.6.2 (R Development Core Team, 2006).

3. Results and discussion

3.1. *Mortality of clams*

Repeated mechanical disturbance is known to stress small sized *Ruditapes philippinarum* (Marin et al., 2005). However, our experimental handling of adult *R. philippinarum*, even associated with exposure to *Vibrio tapetis*, did not result in significant increased mortality. In total, mortality was low and only 10 clams died during the experiment: 6 of them were derived from "handled and exposed" clams, 3 from "handled" group and 1 from "control" tanks (Tab. 1); ANOVA showed no clear significant difference among treatments ($F = 4.0$; $df = 3$; $p = 0.052$). Moreover, none of the dead clams exhibited brown ring disease signs in the current trial; it can thus be hypothesized that the observed mortality is independent of the infection and handling challenge during this 40 days trial.

Table 1
Distribution of Manila clams *R. philippinarum* presenting or not presenting brown ring disease signs amongst different treatments and distribution of dead clams amongst treatments. Numbers are sum of individuals amongst triplicates in each treatment.

	control	handled	exposed	handled and exposed
nb. asymptomatic clams	150	149	149	130
nb. symptomatic clams	0	1	1	20
nb. dead clams	1	0	3	6

3.2. *Prevalence and intensity of brown ring disease*

At the beginning of the experiment, the initial prevalence of brown ring disease signs, estimated using 100 clams, was null. On day 40, at the end of the experiment, there were a total of 22 clams presenting brown ring disease signs out of a possible 600 clams, of which 20 derived from "handled and exposed" tanks (Tab. 1); ANOVA showed a significant effect of treatment on prevalence ($F = 20.83$; $df = 3$; $p < 0.05$). According to the Tukey HSD pairwise comparisons, the "handled and exposed" group clams were significantly more susceptible to development of brown ring disease signs than clams in any other group (Tab. 2).

Table 2
Tukey HSD pairwise comparisons of prevalence of the brown ring disease among the experimental groups (D, value of the difference; p, associated *p-value*) . ANOVA showed a significant effect of treatment on prevalence of BRD signs ($F = 20.83$; $df = 3$; $p < 0.05$)

	exposed	handled	handled and exposed
control	$D = 0.33$ $p = 0.986$	$D = 0.33$ $p = 0.986$	$D = 6.67$ $p = 0.001$
exposed		$D = 0.00$ $p = 1$	$D = 6.33$ $p = 0.001$
handled			$D = 6.33$ $p = 0.001$

Our results show that the sole exposure to *V. tapetis* does not lead to a higher prevalence of brown ring disease than for control clams. The influence of handling associated with *V. tapetis* exposure is obvious after a 40 days trial (Tab. 2). This result confirms that mechanical disruption of the periostracal lamina and shell edge enhances the development of BRD signs; our results support the hypothesis that *Vibrio tapetis* may benefit from mechanical disruptions to enter extrapallial compartment (Flye-Sainte-Marie et al., 2008). Furthermore, this strong contrast between "handled & exposed" clams and other treatments should thus be taken into account for future experiments, especially during growth season: as mentioned by Flye-Sainte-Marie et al. (2008), disruptions of the periostracal lamina and chipping of valve margins may occur more easily during this period because of the fragility of the newly calcified layers on valve margins.

The increased vulnerability of handled *R. philippinarum* exposed to *V. tapetis* may have implications for clams culture. Thus our results suggest (*i*) to limit manipulations, including reseeding practices, in cul-

tured clam beds and (*ii*) to minimize transfers of clam seed, even for seed coming from non BRD-affected regions, as those manipulations imply an increased sensitivity to brown ring disease.

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