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To cite this version:

Fred Jean, Jonathan Flye-Sainte-Marie, Clémence Oudard, Christine Paillard. Handling enhances the development of brown ring disease signs in Ruditapes philippinarum. Journal of Shellfish Research, 2011, in press (xxx). hal-00568169v1

HAL Id: hal-00568169

https://hal.univ-brest.fr/hal-00568169v1

Submitted on 23 Feb 2011 (v1), last revised 23 Feb 2011 (v2)

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Handling induces the development of brown ring disease symptoms in *Ruditapes philippinarum*

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Preprint submitted to Elsevier *May 12, 2010*

Abstract

Brown ring disease (BRD) in the Manila clam is induced by a bacterium *Vibrio tapetis* and is characterized by the formation of a brown deposit of conchiolin on the inner surface of the shell that gives the disease its name. A recent study suggested that *V. tapetis* may benefit from mechanical disruptions of the periostracal lamina and/or the shell margin to enter the extrapallial compartments. Thus, this study also suggested that handling in aquaculture conditions may enhance the development of BRD symptoms. In order to test this hypothesis, we conducted an experiment simulating clam handling. Our results assess that rough handling of *R. philippinarum* in presence of *V. tapetis* significantly increase the prevalence of BRD symptoms compared to undisturbed control clams. As a consequence we recommend to avoid any transfer and manipulation of clams during culture.

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

1 Introduction.

The Manila clam, *Ruditapes philippinarum*, was introduced into France for aquaculture purposes between 1972 and 1975 (Flassch and Leborgne, 1992). In France, this venerid culture became increasingly widespread, and since 1988 natural populations have colonized most embayments along the French Atlantic coast, resulting in important fisheries benefit. Brown ring disease (BRD) in the Manila clam, *Ruditapes philippinarum*, was first observed in North Finistère (France) in 1987 (Paillard et al., 1989). This disease was shown to be caused by *Vibrio tapetis* (Paillard and Maes, 1990; Borrego et al., 1996). Infected clams exhibit a characteristic brown deposit on the inner surface of the valves (Paillard et al., 1989) that gave the disease its name. 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). The effects of BRD on Manila clams have been reviewed by Paillard (2004): the disease causes mass mortalities in cultured clam beds (Paillard et al., 1989; Castro et al., 1992; Paillard, 1992, 2004) and has severely affected venerid culture in northern Brittany. However it has a lower impact in natural beds, where maximum prevalence reaches only 30% (Paillard, 2004).

Althougth post–infection processes (i.e. after penetration of *Vibrio tapetis* into

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extrapallial compartment) have been widely described (Paillard, 2004), mechanisms of entry of *V. tapetis* into the extrapallial fluids remain poorly understood until a recent study: Flye-Sainte-Marie et al. (2008) suggested that the pathogen *V. tapetis* may benefit from mechanical disruption of the periostracal lamina or the valve margins to colonize the Manila clam extrapallial compartment; these disruptions may be induced by the presence of large grains in sediments. This hypothesis also suggests that rough handling of clams in aquaculture conditions, that may disrupt the periostracal lamina and/or valve margins, may enhance BRD development. In order to test this hypothesis an experiment simulating the effect of handling, combined or not with exposure to *V. tapetis*, was conducted to assess the development of BRD.

2 Materials and methods.

2.1 Specimens and experimental plan.

About 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 symptoms was estimated by killing 100 clams randomly chosen.

Clams were randomly subdivided into 12 batches of 50 clams each. During one week of acclimation and 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, which ran for 40 days. 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.

Three batches of 50 clams each were randomly attributed to each of the following experimental conditions :

- Untreated control.
- Handling simulation (hereafter *shaken*): clams were placed in a closed tank without water and manually shaken for 30 seconds before each infection

experiment to simulate shellfish farming handling.

- Exposed to *Vibrio* strain (hereafter *exposed*).
- Handling simulation and exposed to *Vibrio* strain (*shaken* & *exposed*).

The experiment last for about 6 weeks, which is an intermediate duration between experiments by Paillard et al. (2004) and Drummond et al. (2007). On day 40 of the experiment, clams were killed, flesh was removed and valves cleaned under a trickle of water, and were then left to dry.

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 2.5×10^6 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

All shells (including those of moribund individuals sampled throughout the trial) were retained and left 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 appropriate, Tukey's honestly significant difference (HSD) test (Yandell, 1997) was used to assess pairwise differences among groups at the 95% level. Statistical analysis were conducted using R version 2.6.2 statistical software (R Development Core Team, 2006).

3 Results

3.1 Mortality of clams

In total, mortality was low and only 10 clams died during the experiment, 6 of them were derived from "shaken and exposed" clams, 3 from "shaken" group and 1 from control tanks (Tab. 1); ANOVA showed no clear significant difference among treatments ($F = 4.0$; $df = 3$; $p = 0.052$). However, none of the 10 dead clams did present brown ring disease symptoms.

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

		shaken			
		no		yes	
		V. tapetis		*V. tapetis*	
		no	yes	no	yes
brown ring	no	150	149	149	130
disease symptoms	yes	0	1	1	20
mortality		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 symptoms, estimated using 100 clams, was null. On day 40, at the end of the

experiment, there were a total of 22 clams which present brown ring disease symptoms out of a possible 600 clams, of which 20 derived from “shaken 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 "shaken and exposed" group clams were significantly more susceptible to development of brown ring disease symptoms than clams in other groups (Tab. 2). Among the symptomatic clams, 6 presented a CDS superior to 2, and one clam exhibited a CDS equal to 7.

Table 2
Tukey HSD pairwise comparisons of prevalence among experimental groups. ANOVA showed a significant effect of treatment on prevalence of BRD symptoms ($F = 20.83$; $df = 3$; $p < 0.05$)

	prevalence	
	difference	p
exposed *vs* control	0.33	0.986
shaken *vs* control	0.33	0.986
shaken *vs* exposed	0.00	1.0
shaken & exposed *vs* control	6.67	0.001
shaken & exposed *vs* exposed	6.33	0.001
shaken & exposed *vs* shaken	6.33	0.001

4 Discussion and conclusion

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. Previous experimental challenges of Manila clam by *V. tapetis* resulted in mortalities ranging between 2 and 30% (Paillard et al., 2004; Reid et al., 2003; Drummond et al., 2007) after 30 days of experiment. In our trial, cumulative mortality reached an overall total of 1.7% after 40 days, which is **comparable to/more than/less than**/ results obtained by Drummond et al. (2007) who used a similar exposure methodology (immersion rather than inoculation) but did not take handling into account. Moreover, none of the dead clams exhibited brown ring disease symptoms 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.

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 *Vibrio tapetis* benefits from mechanical disruption of the periostracal lamina and shell edge to enter the extrapallial compartment and thus supports the hypothesis emitted by Flye-Sainte-Marie et al. (2008). Furthermore, this strong contrast between ‘"shaken & exposed" clams and other *R. philippinarum* 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 valves 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 important implications for clams culture. Thus we recommend (*i*) to avoid any manipulations, including reseeding practices, in cultured clam beds and (*ii*) to discourage any transfer of clam seed, even for seed coming from non BRD-affected regions, as it implies a increased sensitivity to *V. tapetis*.

5 Acknowledgments

The authors thank Robert Marc for valuable help during field work.

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