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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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1 **Abstract**

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

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

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15 **1 Introduction.**

16 The Manila clam, *Ruditapes philippinarum*, was introduced into France for  
17 aquaculture purposes between 1972 and 1975 (Flassch and Leborgne, 1992).  
18 In France, this venerid culture became increasingly widespread, and since 1988  
19 natural populations have colonized most embayments along the French At-  
20 lantic coast, resulting in important fisheries benefit. Brown ring disease (BRD)  
21 in the Manila clam, *Ruditapes philippinarum*, was first observed in North Fin-  
22 istère (France) in 1987 (Paillard et al., 1989). This disease was shown to be  
23 caused by *Vibrio tapetis* (Paillard and Maes, 1990; Borrego et al., 1996). In-  
24 fected clams exhibit a characteristic brown deposit on the inner surface of the  
25 valves (Paillard et al., 1989) that gave the disease its name. Infection disrupts  
26 the production of the periostracal lamina and causes an anomalous deposi-  
27 tion of periostracum on the inner shell of infected clams (Paillard et al., 1994;  
28 Paillard and Maes, 1995a,b). The effects of BRD on Manila clams have been  
29 reviewed by Paillard (2004): the disease causes mass mortalities in cultured  
30 clam beds (Paillard et al., 1989; Castro et al., 1992; Paillard, 1992, 2004) and  
31 has severely affected venerid culture in northern Brittany. However it has a  
32 lower impact in natural beds, where maximum prevalence reaches only 30%  
33 (Paillard, 2004).

34 Although post-infection processes (i.e. after penetration of *Vibrio tapetis* into

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

## 46 2 Materials and methods.

### 47 2.1 Specimens and experimental plan.

48 About 700 Manila clams, larger than 25 mm, were collected at low tide by  
49 hand on the 30<sup>th</sup> of January 2008, on the Lanveur mudflat, Bay of Brest,  
50 France. Particular care was taken to avoid any effect of handling on clams :  
51 during collection in the field clams were stocked cautiously, one after the other,  
52 in boxes containing rags and transferred to the Laboratoire des Sciences de  
53 l'Environnement Marin (Brest, France). At the laboratory clams were gently  
54 rinsed individually in seawater. Initial prevalence of the brown ring disease  
55 symptoms was estimated by killing 100 clams randomly chosen.

56 Clams were randomly subdivided into 12 batches of 50 clams each. During one  
57 week of acclimation and throughout the experiment, each batch was main-  
58 tained in 20 liters tanks of aerated filtered (0.5 $\mu$ m) seawater at 16°C, which is  
59 near optimal temperature for brown ring disease development (Paillard, 2004);  
60 a pump generated a smooth current in each tank. Filtered seawater was re-  
61 newed every fifth day throughout the trial, which ran for 40 days. Clams were  
62 fed with one liter of cultured *Isochrysis aff. galabana* (concentration of 50 cells  
63 /  $\mu$ L) per week and per tank. Tanks were checked on a daily basis for mortal-  
64 ities and moribund clams. Any gaping individuals were presumed moribund  
65 and removed.

66 Three batches of 50 clams each were randomly attributed to each of the fol-  
67 lowing experimental conditions :

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

- 71 experiment to simulate shellfish farming handling.  
72 • Exposed to *Vibrio* strain (hereafter *exposed*).  
73 • Handling simulation and exposed to *Vibrio* strain (*shaken & exposed*).

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

## 78 2.2 *Experimental infections*

79 *V. tapetis* strain CECT 4600 was grown in marine agar (Difco 2216) at 18°C  
80 for 48 to 72 hours. Bacterial colonies were resuspended in filtered seawater.  
81 Bacterial suspension was added in *V. tapetis* exposed tanks to reach a final  
82 concentration of  $2.5 \times 10^6$  cells ml<sup>-1</sup>, which is the same order of magnitude  
83 as in Drummond et al. (2007). A first exposition was performed on the 8<sup>th</sup>  
84 of February 2008 (day 8) and water was renewed after 24 hours. A second  
85 exposure experiment was conducted on the 22<sup>th</sup> of February 2008 (day 22)  
86 and water was renewed after 5 days. During exposures, clams were regularly  
87 monitored to verify that their shells were opened and that they were actively  
88 filter-feeding. After each infection experiment, the water was drained and the  
89 clams remained out of water for 1 hour, in order to induce the closure of the  
90 valves and the incorporation of *V. tapetis* in the pallial cavity. The tanks were  
91 then filled with 20 liters of fresh filtered seawater.

92 In control tanks, clams were treated as above except that the bacterial sus-  
93 pension was not added.

## 94 2.3 *Characterisation and classification of brown ring disease syndrome*

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

103 Variations of prevalence and mortality among treatments were tested using  
 104 analysis of variance (ANOVA). Tank effect was always neglected because it was  
 105 never significant when we tried to take it into account. When appropriate,  
 106 Tukey’s honestly significant difference (HSD) test (Yandell, 1997) was used to  
 107 assess pairwise differences among groups at the 95% level. Statistical analysis  
 108 were conducted using R version 2.6.2 statistical software (R Development Core  
 109 Team, 2006).

### 110 3 Results

#### 111 3.1 Mortality of clams

112 In total, mortality was low and only 10 clams died during the experiment,  
 113 6 of them were derived from “shaken and exposed” clams, 3 from “shaken”  
 114 group and 1 from control tanks (Tab. 1); ANOVA showed no clear significant  
 115 difference among treatments ( $F = 4.0$ ;  $df = 3$ ;  $p = 0.052$ ). However, none of  
 116 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	
		<i>V. tapetis</i>		<i>V. tapetis</i>	
		no	yes	no	yes
brown ring disease symptoms	no	150	149	149	130
	yes	0	1	1	20
mortality		1	0	3	6

#### 117 3.2 Prevalence and intensity of brown ring disease

118 At the beginning of the experiment, the initial prevalence of brown ring disease  
 119 symptoms, estimated using 100 clams, was null. On day 40, at the end of the

120 experiment, there were a total of 22 clams which present brown ring disease  
 121 symptoms out of a possible 600 clams, of which 20 derived from “shaken and  
 122 exposed” tanks (Tab. 1); ANOVA showed a significant effect of treatment on  
 123 prevalence ( $F = 20.83$ ;  $df = 3$ ;  $p < 0.05$ ). According to the Tukey HSD pair-  
 124 wise comparisons, the "shaken and exposed" group clams were significantly  
 125 more susceptible to development of brown ring disease symptoms than clams  
 126 in other groups (Tab. 2). Among the symptomatic clams, 6 presented a CDS  
 127 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 <i>vs</i> control	0.33	0.986
shaken <i>vs</i> control	0.33	0.986
shaken <i>vs</i> exposed	0.00	1.0
shaken & exposed <i>vs</i> control	6.67	0.001
shaken & exposed <i>vs</i> exposed	6.33	0.001
shaken & exposed <i>vs</i> shaken	6.33	0.001

## 128 4 Discussion and conclusion

129 Repeated mechanical disturbance is known to stress small sized *Ruditapes*  
 130 *philippinarum* (Marin et al., 2005). However, our experimental handling of  
 131 adult *R. philippinarum*, even associated with exposure to *Vibrio tapetis*, did  
 132 not result in significant increased mortality. Previous experimental challenges  
 133 of Manila clam by *V. tapetis* resulted in mortalities ranging between 2 and 30%  
 134 (Paillard et al., 2004; Reid et al., 2003; Drummond et al., 2007) after 30 days  
 135 of experiment. In our trial, cumulative mortality reached an overall total of  
 136 1.7% after 40 days, which is **comparable to/more than/less than/** results  
 137 obtained by Drummond et al. (2007) who used a similar exposure methodology  
 138 (immersion rather than inoculation) but did not take handling into account.  
 139 Moreover, none of the dead clams exhibited brown ring disease symptoms in  
 140 the current trial; it can thus be hypothesized that the observed mortality is  
 141 independent of the infection and handling challenge during this 40 days trial.

142 Our results show that the sole exposure to *V. tapetis* does not lead to a

143 higher prevalence of brown ring disease than for control clams. The influence  
144 of handling associated with *V. tapetis* exposure is obvious after a 40 days trial  
145 (Tab. 2). This result confirms that *Vibrio tapetis* benefits from mechanical  
146 disruption of the periostracal lamina and shell edge to enter the extrapallial  
147 compartment and thus supports the hypothesis emitted by Flye-Sainte-Marie  
148 et al. (2008). Furthermore, this strong contrast between "shaken & exposed"  
149 clams and other *R. philippinarum* should thus be taken into account for future  
150 experiments, especially during growth season: as mentioned by Flye-Sainte-  
151 Marie et al. (2008), disruptions of the periostracal lamina and valves margins  
152 may occur more easily during this period because of the fragility of the newly  
153 calcified layers on valve margins.

154 The increased vulnerability of handled *R. philippinarum* exposed to *V. tapetis*  
155 may have important implications for clams culture. Thus we recommend (i) to  
156 avoid any manipulations, including reseeded practices, in cultured clam beds  
157 and (ii) to discourage any transfer of clam seed, even for seed coming from  
158 non BRD-affected regions, as it implies a increased sensitivity to *V. tapetis*.

## 159 5 Acknowledgments

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

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