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Variability of the hemocyte parameters of *Ruditapes philippinarum* in the field during an annual cycle

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ABSTRACT

A field monitoring of hemocyte parameters of the Manila clam *Ruditapes philippinarum* was conducted from July 2004 to September 2005 in Gulf of Morbihan (France), in order to assess (1) the factors controlling the hemocyte parameters of the Manila clam and (2) their relative contribution to the overall variability of these parameters. Monthly, sixty clams were sampled and total hemocyte count (THC), granulocyte and hyalinocyte counts, phagocytosis, phenoloxidase specific activity, length, flesh dry weight, and condition index were measured individually. *Perkinsus* sp. infection and Brown Ring Disease symptoms were also monitored. Temperature and trophic resource were also monitored.

Results indicate that temperature controls granulocyte counts and subsequently THC. Other environmental factors had no direct influence on the measured hemocyte parameters. Almost all measured parameters were significantly affected by size/age and condition index. There were poor relationships between both pathologies and hemocyte parameters presumably because of low infection intensities. Nevertheless, high *Perkinsus* sp. infection intensity significantly increased total and granulocyte counts and decreased phagocytosis.

An interesting result of this study is that the measured biotic and abiotic factors poorly contribute to the explanation of the total variability of hemocyte parameters. Granulocyte concentration was the best explained parameter. However, only 16.4% of its variance was explained by cumulating temperature, length, condition index and *Perkinsus* sp. infection effects. This study emphasizes the need for a better understanding of hemocyte functions and the factors modulating these functions.

1. Introduction

The Manila clam *Ruditapes* (=*Tapes*, =*Venerupis*) *philippinarum* is one of the most extensively cultivated bivalve molluscs. This species was originally endemic to Indo-Pacific waters and its high adaptive capacity to various rearing environments made of it a target species for aquaculture. In Europe, this species was first introduced in France between 1972 and 1975 for aquaculture purposes and later in England, Spain and Italy (Flassch and Leborgne, 1992). In the late 1980s, natural population have developed in Italy (Marin et al., 2003), in England (Jensen et al., 2004; Humphreys et al., 2007) and in most embayments along the French Atlantic coast, resulting in a fishery of ca. 1500 tons in the Gulf of Morbihan at the end of the 1990s. This species is mainly affected by two pathologies: Brown Ring Disease (BRD) and Perkinsosis (see *e.g.* Paillard, 2004a; Villalba et al., 2004). Brown Ring Disease is caused by the bacterium *Vibrio tapetis* (Paillard and Maes, 1990; Borrego et al., 1996) which disrupts the production of periostracal lamina and causes an anomalous deposition of periostracum on the inner shell (Paillard and Maes, 1995a,b). Perkinsosis is induced by the protozoan parasite *Perkinsus* sp. and can affect both *Ruditapes decussatus* and *R. philippinarum* (see Villalba et al., 2004). Both pathologies can interfere with host energy balance (Ngo and Choi, 2004; Park et al., 2006; Leite et al., 2004; Flye-Sainte-Marie et al., 2007a) and can be responsible for mass mortalities (see *e.g.* Paillard et al., 1989; Castro et al., 1992; Paillard, 1992, 2004a; Villalba et al., 2005, 2004). Epidemiological surveys also showed that both pathologies have also been shown to be influenced by environment factors (Paillard et al., 1997; Villalba et al., 2005). Different laboratory experiments have been performed to assess the effect of environmental factors (temperature and salinity) on cellular-defence related parameters (Reid et al., 2003; Paillard et al., 2004) in link with pathologies and few field studies assessed the seasonal variation of these parameters (Matozzo et al., 2003; Soudant et al., 2004). Collection of field data is needed to better understand the relationships between environmental factors, defence-related parameters, physiological status and disease development.

Mainly as a result of aquaculture and fisheries industry and associated disease events, hemocyte systems, thought to be involved

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in immune response of bivalves, were extensively studied during the past 30 years. More recently the interest in using bivalve hemocyte parameters as biomarkers of environmental perturbations appeared. Number of studies allowed to show that hemocyte parameters are controlled by numerous factors such as environmental factors (temperature and salinity), parasites and internal factors (reproduction; see review in Chu, 2000). These factors may contribute to explain the high degree of variability of the hemocyte responses and activities which has been reported only in few studies (see *e.g.* Ashton-Alcox and Ford, 1998; Ford and Paillard, 2007). In the aim of better understanding the linkages between environment, host physiology and disease development, the relative contribution of biotic and abiotic factors to the overall variability of the hemocyte parameters in the field is a key question and remains poorly known.

A multiparametric study was designed to assess the relative effect of environmental and internal factors and diseases on hemocyte parameters in the field. Gulf of Morbihan is one of the largest Manila clam fisheries of Brittany. Meanwhile, information on the physiology and reproduction of the Manila clam of Gulf of Morbihan are available (Laruelle et al., 1994; Laruelle, 1999; Calvez, 2003). Manila clams' populations from this site are known to be moderately affected by both BRD and perkinsosis (Paillard et al., 1997; Paillard, 2004b,a; Lassalle et al., 2007). Seasonal variations were taken into account by monthly sampling over a 1-year period. Flow cytometry methods were applied to determine hemocyte counts, viability and phagocytosis activity.

2. Materials and methods

2.1. Clam sampling

From July 2004 to September 2005, 60 *R. philippinarum* ranging from 20 mm to 50 mm were monthly sampled at low tide from the natural clam bed of Bailleron island in Gulf of Morbihan, southern Brittany, France (Fig. 1). Clams were stored in an icebox until being processed in the laboratory. A total of 1020 individuals was sampled from 17 sampling dates. At each sampling date, 60 clams were processed individually according to the protocol that follows.

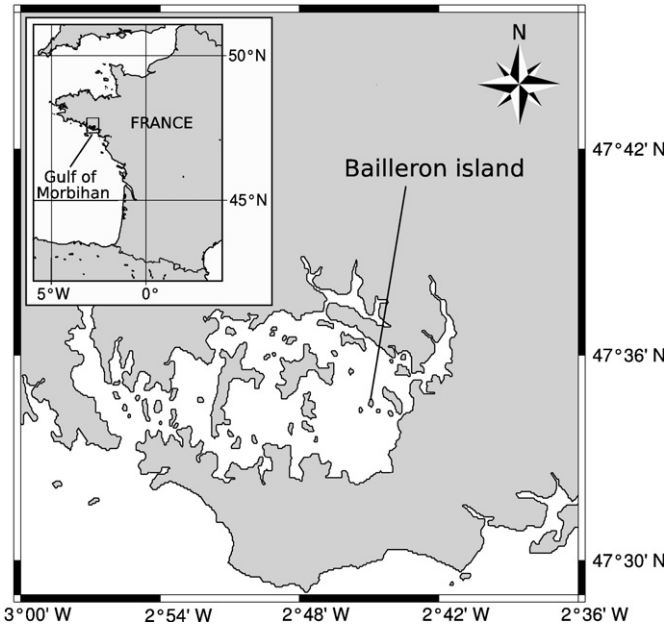

Fig. 1. Location of Bailleron island in Gulf of Morbihan, Southern Brittany, France.

2.2. Analysis of hemocyte parameters by flow cytometry

2.2.1. Hemolymph sampling

A minimum of 450 µL of hemolymph was withdrawn from the adductor muscle of individual clams using a 1 mL plastic syringe fitted with a 25-gauge needle and observed under microscope to control the sample and sampling qualities. Hemolymph samples were filtered through a 80 µm mesh in order to eliminate large debris and were stored individually in 1.5 mL micro-tubes held on ice.

2.2.2. Instrumentation

Analysis of hemocyte parameters was performed using a FACScalibur flow cytometer (Becton-Dickinson, San Diego, CA, USA) equipped with a 488 nm argon laser. The light scattered by particles indicated (1) their size through the FSC detector (Forward SCatter height) and (2) their internal complexity through the SSC detector (Side SCatter height). The flow cytometer is equipped with three specific fluorescence sensors: FL1 (green, 500–530 nm), FL2 (orange, 550–600 nm) and FL3 (red, >630 nm) allowing the detection of autofluorescence or fluorescent dyes.

2.2.3. Hemocyte viability, total and differential hemocyte counts (THC and DHC)

These parameters were measured following the protocol developed by Delaporte et al. (2003). Briefly, 100 µL of hemolymph from each individual was added in a tube containing 200 µL of anti-aggregant solution for bivalve hemocytes (AASH; Auffret and Oubella, 1994) and 100 µL of filtered sterile seawater (FSSW). Samples were incubated 2 h at 18 °C in dark conditions with 4 µL of SYBR Green working solution (obtained by diluting 10× the commercial solution; Molecular probes, Oregon, USA) and propidium iodide (PI, Sigma) at a final concentration of 10 µg mL^{-1}. Live and dead cells containing DNA are stained by SYBR Green; whereas, dead cells are only stained by PI. SYBR Green fluorescence is detected by the FL1 detector of the flow cytometer, and PI fluorescence is detected by the FL3 detector. By using a density plot visualisation of FL1 vs FL3, it was possible to estimate precisely the percentage of dead cells in each sample.

A density plot visualisation of SSC vs FL1 allowed differentiation and gating of hemocytes stained by SYBR green from other particles in the hemolymph. This allowed to calculate THC by taking into account the flow rate of the cytometer calculated according to the method of Marie et al. (1999).

Similarly to Allam et al. (2002a), two distinct sub-populations could be identified on a FSC vs SSC density plot: granulocytes (high SSC and high FSC), hyalinocytes (low SSC and high FSC). Results of THC, granulocyte and hyalinocyte counts are expressed as number of cells per mL of hemolymph.

2.2.4. Phagocytosis assays

Phagocytic activity of hemocytes was measured following the protocol described in Delaporte et al. (2003) and Labreuche et al. (2006) using 2 µm diameter latex fluorescent beads (fluoresbrite microspheres YG 2.0 mm, polysciences, Eppelheim, Germany). A 150 µL sub-sample of hemolymph, primarily diluted with 150 µL of FSSW, was brought in contact with 30 µL of the working solution of fluorescent beads (obtained by diluting 50× the commercial solution) in micro-tubes. Tubes were incubated for 2 h at 18 °C in dark condition. Analysis by flow cytometry allowed to detect hemocytes containing fluorescent beads on the FL1 detector. The phagocytic activity of hemocytes was calculated as the percentage of hemocytes that have ingested three fluorescent beads or more.

2.2.5. Phenoloxidase activity

Ninety-six-well plates containing 100 µL hemolymph samples were thawed and phenoloxidase activity measured as described by Reid et al. (2003). Briefly, 50 µL of Tris–HCl buffer (0.2 M, pH = 8) with