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Combined effects of temperature and salinity on functional responses of haemocytes and survival in air of the clam *Ruditapes philippinarum*

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ABSTRACT

The combined effects of temperature and salinity on both immune responses and survival in air of the clam, *Ruditapes philippinarum*, were evaluated for the first time. The animals were kept for 7 days at three differing temperature (5 °C, 15 °C, 30 °C) and salinity values (18 psu, 28 psu, 38 psu), and effects of the resulting 9 experimental conditions on total haemocyte count (THC), Neutral Red uptake (NRU), haemolymph protein concentration, and lysozyme activity in both haemocyte lysate (HL) and cell-free haemolymph (CFH) were evaluated. The survival-in-air test was also performed. Two-way ANOVA analysis revealed that temperature influenced significantly THC and NRU, whereas salinity and temperature/salinity interaction affected NRU only. Temperature and salinity did not influence significantly HL and CFH lysozyme activity, as well as haemolymph total protein content. Survival-in-air test is widely used to evaluate general stress conditions in clams. In the present study, temperature and salinity were shown to influence the resistance to air exposure of *R. philippinarum*. The highest LT_{50} (air exposure time resulting in 50% mortality) value was recorded in clams kept at 18 psu and 15 °C, whereas the lowest value was observed in clams kept at 28 psu and 30 °C. Overall, results obtained demonstrated that temperature and salinity can affect some functional responses of haemocytes from *R. philippinarum*, and suggested a better physiological condition for animals kept at 15 °C temperature and 18 psu salinity.

1. Introduction

In the Mediterranean area, two clam species belonging to the genus *Ruditapes* (Mollusca, Bivalvia) are widespread: *Ruditapes decussatus* and *Ruditapes philippinarum*. The two Venerid species are phenotypically similar and very important for fishery and aquaculture practises. *R. decussatus* is an Atlantic-Mediterranean species and the clam fishery based on this species is considered as one of the oldest in Europe [1]. *R. philippinarum*, native to Japan, Korea, and the Philippines, was introduced in 1983 in the Lagoon of Venice (where *R. decussatus* is the native species) for breeding purposes, and subsequently spread rapidly. This is mainly due to the fact that *R. philippinarum* is hardier and faster-growing than *R. decussatus* [2]. At the present, *R. philippinarum* contributes 91% to European yields of the two species [2].

Environmental factors including temperature, salinity, oxygen, nutrients, and contaminants can affect marine bivalves. For example, salinity values lower than 20 psu caused reductions in filtration and respiration rates in the mussel *Mytilus edulis* [3]. However, after an acclimation phase, *M. edulis* can tolerate lower salinity, till to 15 psu [3]. Scope for growth (a quantitative measurement of animal energy status) of the Chilean scallop, *Argopecten purpuratus*, was negatively affected by low salinity (24 to 18 psu) compared with 27 and 30 psu [4]. Anoxic incubations with seawater diluted with demineralised water induced at the lowest salinity (50% seawater) a significant increase in the capacity of *M. edulis* to survive anoxia, as compared to both 75% seawater and control (100% seawater, corresponding to 32 psu) [5]. In the Indo-Pacific mytilid *Brachidontes pharaonis*, it has recently been observed a reduction in clearance rate with a decrease in temperature (from 20 °C to 11 °C) and salinity (from 37 to 15 psu). In the same study, scope for growth showed negative values at 15 psu, at all tested temperatures [6]. The combined effects of temperature and salinity on body condition index (an overall indicator of bioenergetic status) and the RNA/DNA ratio (a biochemical indicator of cellular stress) have been evaluated in the eastern oyster, *Crassostrea virginica*: the results showed that there was a greater ability of animals to withstand extreme salinity conditions at lower temperatures [7].

Haemocyte-mediated immune parameters are suggested to be particularly sensitive to variations in environmental factors [8]. It

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has been demonstrated that lysosomal and cell membranes of haemocytes from the mussel, *M. edulis*, were destabilised at 0 °C compared with those of haemocytes from mussels acclimated at 10 °C [9]. Reduced lysosomal membrane stability was also recorded in haemocytes from the oyster, *Ostrea edulis*, maintained at 15 °C [10]. In a recent study, Monari et al. [11] have demonstrated that the highest temperature (30 °C) significantly increased total haemocyte count (THC), whereas it decreased the phagocytic activity of haemocytes from the clam *Chamelea gallina.* In that study, total superoxide dismutase (SOD) activity also decreased significantly in haemocytes from clams held at 30 °C, whereas it increased in cell-free haemolymph from clams held at 25 °C and 30 °C [11]. Salinity can also influence immune responses in bivalves. In oysters (*O. edulis*) kept for 7 days at 32, 25 and 16 psu, the highest salinity caused the growth of the opportunistic bacterial pathogen *Listonella anguillarum*, increased the number of circulating large granulocytes, and decreased hydrogen peroxide concentration in the haemolymph [12]. In the same oyster species, animals acclimated at 32, 28 and 25 psu showed that the Neutral Red dye (used to monitor cell membrane stability) was confined to the lysosomal compartment for approximately 80 min, whereas in oysters acclimated at 16 and 19 psu, the dye was released from the lysosomes rapidly, suggesting that the lipid membranes within the cell were less stable [13]. In clams (*C. gallina*) kept at 28 psu, THC significantly increased compared with animals kept at 34 and 40 psu, whereas higher phagocytic activity was recorded in haemocytes from clams kept at 34 psu, compared with those kept at 28 and 40 psu [14].

Although the above-mentioned studies have demonstrated effects of temperature and salinity on immune responses in bivalves, no information – to our knowledge at least – are available in the literature about the combined effects of temperature and salinity on bivalve immunosurveillance. As a consequence, in the present study, combined effects of differing temperature (5, 15, and 30 °C) and salinity values (18, 28, and 38 psu) on some important immune parameters of the clam *R. philippinarum* were evaluated for the first time, in order to elucidate clam capability in modulating haemocyte functional responses. The survival-in-air test was also performed in order to highlight a possible relationship between decreased resistance to air exposure and reduced immunocompetence in stressed clams. Indeed, the survival-in-air test is generally recognised as a rapid, simple and low-budget method for examining whether environmental parameters may have perturbing effects leading to reduced fitness of bivalves [15]. The experimental temperatures and salinities were chosen taking into account that values close to those tested in this study can be recorded in the Lagoon of Venice. In particular, both the extreme temperatures (5 and 30 °C) and salinities (18 and 38 psu) tested can be experienced by clams in shallow waters (<1 m depth) which are present in most part of the Lagoon.

2. Materials and methods

2.1. Animals and experimental setup

Specimens of *R. philippinarum* (3.5–3.8 cm shell length) were collected in a reference site located inside a licensed area for clam culture in the Lagoon of Venice, and acclimatised in the laboratory for 5 days before experiments. Clams were maintained in 75-l aquaria with a sandy bottom and aerated seawater (salinity of 35 ± 1 psu, temperature of 17 ± 0.5 °C, pH 8.1) and fed with microalgae (*Isochrysis galbana*). Clams were then transferred to three isothermal rooms and acclimatised to experimental conditions by increasing or decreasing progressively seawater temperature (2 °C day^{-1}, till to 5, 15 and 30 °C) and salinity (1–2 psu day^{-1}, till to 18, 28, and 38 psu). Salinity was adjusted by addition either of artificial hyperaline seawater (Instant Ocean® salt, Aquarium Systems, France) or distilled water. Clams (60 for each experimental condition) were kept for 7 days at the nine temperature/salinity combinations, in large aquaria provided with a sandy bottom and well-aerated seawater, and fed with microalgae (initial concentration of about 100,000 cells l^{-1}). The seawater was changed every 48 h. Both seawater parameters (temperature, salinity and pH) and clam mortality were checked daily.

2.2. Haemolymph sampling

Haemolymph (about 350 μl per clam) was collected from the anterior adductor muscle with a 1-ml plastic syringe and stored in ice. For each experimental condition, 3 pools of haemolymph from 5 clams each were prepared. Pooling was necessary to obtain enough haemolymph for analyses. After pooling, 250 μl of haemolymph were immediately used to determine THC, while 1 ml was used for Neutral Red uptake (NRU) and total protein assays. Lastly, 250 μl were used to measure lysozyme activity in both haemocyte lysate (HL) and cell-free haemolymph (CFH).

2.3. Total haemocyte count (THC)

THC was determined by a Model Z2 Coulter Counter electronic particle counter/size analyser. Pooled haemolymph (250 μL) was added to 19.75 ml of filtered sea water (FSW). THC results were expressed as number of haemocytes ($\times 10^6$) ml $haemolymph^{-1}$.

2.4. Pinocytotic activity assay

The cationic probe Neutral Red (NR) was used to evaluate the capability of haemocytes to carry out pinocytosis [16,17]. This assay is faster than the phagocytosis assay, but equally responsive [17]. Pooled haemolymph (1 ml) was centrifuged at 780 *g* for 10 min, haemocytes (at final concentrations of 10^6 cells ml^{-1}) were resuspended in an equal volume of 8 mg l^{-1} NR dye (Merck) solution in FSW, and incubated at room temperature (about 18–20 °C) for 30 min. Haemocytes were then centrifuged at 780 *g* for 10 min, resuspended in distilled water, sonicated at 0 °C for 30 s with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12,000 *g* for 15 min at 4 °C. Supernatant, corresponding to haemocyte lysate (HL) was collected for the NRU assay. Absorbance at 550 nm was recorded with a Beckman 730 spectrophotometer. Results were expressed as optical density per ml haemolymph (OD ml $haemolymph^{-1}$).

2.5. Haemolymph total protein concentration

The Quick Start Bradford Protein Assay Kit (Bio-Rad) was used to measure haemolymph total protein concentration. Briefly, 1 ml of reagent (Quick Start Bradford Dye Reagent) was added to 20 μl of haemolymph and incubated for 5 min at room temperature. A standard curve was drawn using bovine serum albumin (BSA) as standard. Absorbance was read at 595 nm, and results were expressed as mg proteins ml $haemolymph^{-1}$.

2.6. Lysozyme activity assay

Lysozyme activity was quantified in both haemocyte lysate and cell-free haemolymph. Pooled haemolymph was centrifuged at 780 *g* for 10 min. The supernatant, corresponding to cell-free haemolymph (CFH), was collected, whereas the haemocytes were resuspended in distilled water, sonicated at 0 °C for 1 min, and then centrifuged at 780 *g* for 30 min to obtain HL. CFH and HL were frozen and stored at −80 °C before analyses. Fifty μl of CFH and HL