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ScienceDirect
Aquaculture 275 (2008) 342 – 346
Aquaculture
www.elsevier.com/locate/aqua-online

Effects of the muscle relaxant, magnesium chloride, on the Sydney rock oyster (*Saccostrea glomerata*)

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Received 23 May 2007; received in revised form 3 December 2007; accepted 8 December 2007

Abstract

The effects of the muscle relaxant magnesium chloride ($MgCl_2$) were monitored in the Sydney rock oyster, *Saccostrea glomerata*, under laboratory conditions. The relaxant was first tested for efficiency and then for potential deleterious effects on immunological and spawning activity. A range of immunological parameters were tested including, total haemocyte frequencies, acid phosphatase, superoxide and phenoloxidase activities in the haemolymph, as well as total haemolymph protein levels. All of the oysters exposed to $MgCl_2$ demonstrated total relaxation within 6h of the initial exposure. Total haemocyte frequencies, acid phosphatase activity and superoxide activity all increased significantly during the first 48h after exposure to the relaxant but declined later. Phenoloxidase activity increased immediately after relaxation but then decreased consistently until 48h after exposure. Overall, the data indicate that $MgCl_2$ is an effective relaxant for use on *S. glomerata*, and that any potentially deleterious effects of the relaxant do not persist for more than 96h.

Keywords: *Saccostrea glomerata*; Immunological activity; Magnesium chloride; Oysters; Relaxant; Stress

1. Introduction

Modern oyster culture is becoming increasingly invasive due to new hatchery techniques for assessing gonad maturation and inducing spawning, and tissue sampling for research. This has created a need for muscle relaxants that allow non-lethal invasive procedures to be performed on commercial oyster species (Culloty and Mulcahy, 1992; Norton et al., 1996). Using these relaxants minimises sampling stress and reduces research-related mortalities. To be effectively applied to research and commercial aquaculture, an oyster relaxant must be easily administered and have high relaxant efficiency. It must also be non-toxic to both the oyster and the operator, and pose no downstream threats to human health (Acosta-Salmon and Davis, 2007).

Muscle relaxants have been used in the pearl oyster industry to reduce stress during the operative process. Anaesthetics commonly used during these operations include benzocaine and propylene phenoxetol (Norton et al., 1996; Mills et al., 1997; O'Connor and Lawler, 2002). However, the Sydney rock oysters' ability to remain closed for long periods of time (up to 2weeks) means that these chemical relaxants have proven unsuitable for use in this species (W. O'Connor, pers. comm.). The salt, magnesium chloride ($MgCl_2$), has been shown to depress cilia movement in the American oyster, *Crassostrea virginica* (Galtsoff, 1964) and has been used to induce gaping in the Pacific oyster, *Crassostrea gigas* (Whyte and Carswell, 1983). $MgCl_2$ is also a successful muscle relaxant for several other bivalves, including the flat oyster, *Ostrea edulis* (Culloty and Mulcahy, 1992) and the scallop, *Pecten fumatus* (Heasman et al., 1995).

The current study investigates the response of the Sydney rock oyster, *Saccostrea glomerata,* to $MgCl_2$. The Sydney rock oyster industry is currently the fourth largest producer of edible oysters

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doi:10.1016/j.aquaculture.2007.12.004

in the world, with production valued at over US$27million at the farm gate. The relaxant was initially assessed for its general effectiveness and rate of action. The physiological stress induced by exposure to the relaxant was also assessed by monitoring spawning events and immunological activity of oysters during recovery after relaxation. Numerous external stressors have been shown to affect immunological activity in oysters (Lacoste et al., 2002; Hegaret et al., 2003; Butt & Raftos, 2007; Butt et al., 2007), and so certain immunological parameters appear to be good gauges of physiological stress. We monitored a number of these immunological parameters, including, total haemocyte numbers, phenoloxidase, acid phosphatase and superoxide activities, as well as the total protein concentration of haemolymph.

2. Materials and methods

2.1. Oysters

Sydney rock oysters (*S. glomerata*) came from Port Stephens, New South Wales, Australia. The individuals used during trials for relaxant efficacy and spawning responses were first acclimated in a recirculating conditioning system at 24°C for 2weeks prior to relaxant exposures. Experiments were undertaken at the New South Wales Department of Primary Industries (NSW DPI) Fisheries Research Centre, Port Stephens, or at Macquarie University, Sydney.

2.2. Relaxant exposures

To determine the efficacy of $MgCl_2$ as a muscle relaxant for *S. glomerata*, 80 oysters were placed in a solution containing 50g L^{-1} $MgCl_2$ prepared in distilled water. Preliminary experimentation found this to be the best concentration for effective relaxation (unpublished data). Salinity was adjusted to between 28 and 30ppt using common sea salt (Aquasonic). After immersion in the relaxant solution, oysters were monitored for a period of 3h so that the time to complete relaxation could be recorded. Oysters were considered relaxed when gentle irritation of the mantle induced no response (O'Connor and Lawler, 2002). Once relaxed, oysters were removed from the immersion bath and allowed to recover in aquaria containing recirculating filtered seawater.

Fifty oysters, to be tested for immunological activity, were exposed to 50g L^{-1} $MgCl_2$ for 3h. They were then returned to aquaria containing seawater without $MgCl_2$ to recover.

Controls were established for all experiments by holding fifty oysters in aquaria containing normal seawater without $MgCl_2$.

2.3. Haemolymph collection

Haemolymph for immunological assays was extracted from the pericardial cavity of shucked oysters. Approximately 700μL of haemolymph was withdrawn from each oyster using a 1mL syringe and 22-gauge needle. Samples were immediately transferred to polypropylene tubes and held on ice.

2.4. Total haemocyte counts

Fifty μL of fresh haemolymph were added to an equal volume of marine anticoagulant (MAC, 0.1M glucose, 15mM trisodium citrate, 13mM citric acid, 50mM EDTA, 0.45M sodium chloride, pH 7.5). This haemolymph suspension was then fixed with 50μL paraformaldehyde (4% w/v) and the number of haemocytes per mL was estimated using an improved Neubauer haemocytometer.

2.5. Total protein content of haemolymph

Whole haemolymph from individual samples was centrifuged for 5min (5000 × g, 4°C) to generate a cell free supernatant. The total protein concentrations of the supernatants were determined spectrophotometrically using a Bio-Rad Protein Assay kit (Bio-Rad, Regents Park, NSW). Absorbance was measured at 595nm and protein concentrations were interpolated from a standard curve generated with bovine serum albumen.

2.6. Phenoloxidase assays

Whole haemolymph from individual oysters was collected without MAC and frozen and thawed (− 80°C to room temperature) to rupture haemocytes. The lysates were then centrifuged for 1min (5000 × g, 4°C) to remove cellular debris. Phenoloxidase assays were carried out according to the protocol of Butt et al. (2006). Briefly, assays were undertaken in 96-well microtitre plates (Sarstadt, Germany) using the substrate, hydroquinine monomethyl ether (Fluka, Switzerland; 5mM), and the chromogenic nucleophile, 3-methly-2-benzothiazoline hydrazone (Sigma-Aldrich, Castle Hill, NSW; 1mM) prepared in phosphate buffered saline (PBS; Amresco, Ohio; pH 7.4) (Dicko, 2002). Absorbance was measured at 492.0nm immediately after the addition of substrates and again after a 1h incubation at 22°C. The change in absorbance per minute was calculated with the data adjusted to account for absorbance in wells containing phenoloxidase substrate in the absence of oyster haemolymph (negative controls) and the total protein content of haemolymph. Phenoloxidase activities are presented as the change in absorbance per minute per microgram of protein ($\Delta OD_{490} min^{-1} \mu g^{-1}$ protein).

2.7. Acid phosphatase assays

Assays used to measure acid phosphatase activity were modified from the methods of Xia et al. (2000). Eighty microlitres of haemocyte lysates (prepared as above) were added in triplicate to 96-well flat bottom microtitre plates. One hundred and twenty microlitres of *p*-nitrophenol phosphate (Sigma-Aldrich, 2.5mg mL^{-1}) were then added to each well. Plates were incubated at 37°C for 90min before the reaction was terminated by the addition of 50μL sodium hydroxide (Fluka, Switzerland, 0.1M). Absorbance was measured at 405nm and enzyme activity was interpolated from a standard curve generated using a *p*-nitrophenol standard (Sigma-Aldrich).

2.8. Superoxide assays

Superoxide anion generation was measured by the reduction of nitroblue tetrazolium to insoluble blue formazan (Pipe, 1992). One hundred microlitres of fresh oyster haemolymph was added to flat bottom 96-well microtitre plates. Plates were left at room temperature for 30min to allow haemocytes to settle and adhere to the wells. Twenty microlitres of nitroblue tetrazolium (Sigma-Aldrich, 5mg mL^{-1}) was then added to each well and the plates were incubated at room temperature for a further 2h. Supernatants were then extracted leaving the stained haemocytes to air dry. One hundred microlitres of dimethyl sulphoxide (Sigma-Aldrich) were finally added to each well to dissolve the cytoplasmic formazan formed during the reaction. Absorbance was measured at 620nm.

2.9. Data analysis

Data were analysed using SPSS v.15 software (SPSS Inc. 2006). One-way analyses of variance (ANOVA) were performed to determine whether statistically significant differences existed in the immunological parameters tested at different times after relaxation. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Magnesium chloride is an effective muscle relaxant for Sydney rock oysters

Oysters began to actively siphon $MgCl_2$ soon after being immersed in the relaxant solution. More than 60% of oysters were completely relaxed after 3h exposure to $MgCl_2$. Six hours after initial exposure, the relaxant was 100% effective. Fig. 1 shows the cumulative percentage of oysters opening within 3h after initial exposure to $MgCl_2$. The proportion of oysters to relax in the period 0–30min was significantly less than in the periods from 60–90min ($\chi^2 = 5.041$, $df = 1$, $p < 0.05$) and 150–180min ($\chi^2 = 10.801$, $df = 1$, $p < 0.05$). Similarly, significantly fewer oysters relaxed in the period from 90–120min compared to