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Hemolymph chemistry and histopathological changes in Pacific oysters (*Crassostrea gigas*) in response to low salinity stress

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ABSTRACT

This study described seasonal differences in the histopathological and hemolymph chemistry changes in different family lines of Pacific oysters, *Crassostrea gigas*, in response to the stress of an abrupt change to low salinity, and mechanical grading. The most significant changes in pallial cavity salinity, hemolymph chemistry and histopathological findings occurred in summer at low salinity. In summer (water temperature 18 °C) at low salinity, 9 (25.7% of full salinity), the mean pallial cavity salinity in oysters at day 3 was 19.8 ± 1.6 (SE) and day 10 was 22.8 ± 1.6 (SE) lower than oysters at salinity 35. Associated with this fall in pallial cavity salinity, mean hemolymph sodium for oysters at salinity 9 on day 3 and 10 were 297.2 mmol/L ± 20(SE) and 350.4 mmol/L ± 21.3(SE) lower than oysters at salinity 35. Similarly mean hemolymph potassium in oysters held at salinity 9 at day 3 and 10 were 5.6 mmol/L ± 0.6(SE) and 7.9 mmol/L ± 0.6 (SE) lower than oysters at salinity 35. These oysters at low salinity had expanded intercellular spaces and significant intracytoplasmic vacuolation distending the cytoplasm of epithelial cells in the alimentary tract and kidney and hemocyte infiltrate (diapedesis) within the alimentary tract wall. In contrast, in winter (water temperature 8 °C) oyster mean pallial cavity salinity only fell at day 10 and this was by 6.0 ± 0.6 (SE) compared to that of oysters at salinity 35. There were limited histopathological changes (expanded intercellular spaces and moderate intracytoplasmic vacuolation of renal epithelial cells) in these oysters at day 10 in low salinity. Mechanical grading and family line did not influence the oyster response to sudden low salinity. These findings provide additional information for interpretation of non-lethal, histopathological changes associated with temperature and salinity variation.

1. Introduction

Varying temperature and salinity in inter-tidal and estuarine environments influence oyster feeding, growth, respiration, oxygen consumption, and excretion rates (Galtsoff, 1964; Chavez-Villalba et al., 2005; DiGialleonardo et al., 2005; Dunphy et al., 2006; Dame, 2012). Oysters, which are euryhyaline, osmoconform to gradual salinity changes, over a range of temperatures (Kinne, 1964; Gullian and Aguirre-Macedo, 2010). However, in response to abrupt freshwater flooding, oysters will shut their shells (Galtsoff, 1964, 1972; Davenport, 1981; Shumway, 1996). If the fall in salinity is marked and persistent, feeding and respiration will cease and mortalities can occur, particularly in spring and summer when oysters have a fast metabolic rate (Galtsoff, 1964; Shumway, 1996). Significant oyster mortalities were recorded in Georges and Moulting Bays, north eastern Tasmania, Australia, after severe freshwater flooding (1 in 50 year event) in February 2004 (summer), with up to 90% oyster stock losses in leases closest to the river mouth as it flowed into Georges Bay (DPIWE Tasmania, 2004a).

During the significant oyster mortality in Georges and Moulting Bays, there was an abrupt fall in surface water salinity (2 was the lowest recorded salinity) which persisted for 9–10 days across leases in Georges and Moulting Bay (DPIWE Tasmania, 2004a). Regardless of mortality rates, oysters showed digestive tubule atrophy and leydig necrosis (DPIWE Tasmania, 2004a). In leases closer to George River Mouth, which had high oyster mortality rates, oysters showed microscopic osmotic changes such as expanded intercellular spaces of the alimentary tract (stomach, intestines, and digestive gland) and kidneys and dilated renal tubules, consistent with exposure to low salinity flood waters

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(DPIWE Tasmania, 2004a). However, some microscopic changes could not be related to the oysters' response to freshwater stress, for example, multifocal erosion of the mantle, which was also seen predominantly in high mortality leases (DPIWE Tasmania, 2004a).

Alongside elevated temperature, genetic oyster traits and mechanical grading are potential predisposing risk factors associated with freshwater oyster mortality events(Lacoste et al., 2001; Li and Vanderpeer, 2002; Percival and Ellard, 2004; Zhang and Li, 2006). Survival traits, such as the ability to withstand freshwater flooding, are more heritable than commercially selected traits such as growth rate and condition (Evans and Langdon, 2006). For these reasons family lines may respond differently to freshwater stress, in the same way as some family lines had better survival rates than others during summer mortality events in France (Huvet et al., 2010). The process of grading, which includes automated size sorting, facilitates faster oyster growth as similar sized animals are grown together in baskets or trays (Zhang and Li, 2006). In Tasmania, oyster losses have been reported following grading, particularly after high rainfall events (Batley et al., 2010).

Histopathology is commonly used as part of oyster health surveillance programs, disease and mortality investigations (Ellis et al., 1998; DPIWE Tasmania, 2004b; Kim and Powell, 2006) and bio-monitoring programs (Yevich and Yevich, 1994; Kim and Powell, 2007) because it has potential to explore the interaction of oysters with pathogens or the environment (Yevich and Yevich, 1994; Grizel, 2003; Myers and McGavin, 2007; Berthe, 2008; Kim and Powell, 2009). In the absence of infectious pathogens, water data and physiological changes are important for interpreting microscopic changes (Bignell et al., 2008). For these reasons, the aim of this study was to explore the haemolymph chemistry and histopathological responses of Pacific oysters (*Crassostrea gigas*) to an abrupt fall to low salinity, in controlled tank trials and assess if the interaction of grading, season, or family line with salinity influenced the oysters' response.

2. Materials and methods

2.1. Experimental design and setup

A three factor orthogonal experimental design was used to examine differences in histopathological changes of Pacific oysters (*Crassostrea gigas*) in response to water salinity (normal salinity 35 and low salinity 9), grading (graded and ungraded oysters), and breeding (two family lines). Salinity and grading were fixed factors, while family line was a random factor and three replicate tanks were used for every combination of the three factors. A salinity of 9 was used as this is the lower end of the mesohyaline range for oysters (Galtsoff, 1964) and we wished to examine the sublethal effects of salinity reduction on oysters and not the lethal effects.

The experiment was run twice, once in summer (February 2010) and once in winter (July 2009), to determine if the response depended on water temperatures. Pacific oysters were collected from a commercial oyster farm in north-western Tasmania and sent, on ice, by overnight courier to the Animal Health Laboratory DPIPWE Tasmania, Launceston. The two family lines used in the winter experiment were lines YC06-22E and YC06-4A, while the summer experiment used lines PI 1 and PI 3. Different family lines were used because the family lines used in the winter experiment were all harvested and unavailable for the summer experiment. The mean length of Pacific oysters for the winter experiment was 97 ± 8 mm (mean ± SD, $n = 80$) and for the summer experiment 75 ± 6 mm (mean ± SD $n = 77$). Half the oysters from each family line were graded at the farm before being sent to the laboratory.

At the oyster lease in north-western Tasmania the water temperature and salinity were recorded by the farmer using an alcohol thermometer and refractometer (Vitalsine, Model SR-6) on the day oysters were collected. On the day of collection for oysters for the winter experiment surface water salinity was 35 and temperature was 8 °C so water temperature was maintained at 8 °C in the tanks. For the summer experiment, on the day of collection surface water salinity was 35 and temperature was 18 °C on the oyster lease so water temperature was maintained at 18 °C in the tanks.

Two independent re-circulating water systems were set up. Deionised water was used to dilute seawater, at salinity 35, to salinity 9 for the treated tanks. Each water system had three 60L tanks and a biofilter with aeration provided by movement of water through the system and a water stone in each tank. Daily water ammonia/ammonium was tested with an NH_3/NH_4 API test kit and water temperature was measured three times daily with an alcohol thermometer. Ammonium/ammonia levels were maintained at or below 0.25 mg/L through daily partial water changes. Salinity was measured daily using a refractometer (Vitalsine, Model SR-6). A basket, which held 13–14 oysters consisting of the four combinations of family line and grading, was suspended in each tank. The oyster farm provided three fewer oysters for the summer experiment than for the winter experiment.

The experiment ran for 10 days and 40 oysters were randomly sampled on day 3 and another 40 on day 10, from each treatment group. It was decided not to feed oysters during the 10 day experiment as the microalgae usually used to feed Pacific oysters in culture would have lysed at salinity of 9. Oyster shells were examined for abnormal conformation, shape and defects (e.g. fluting) at the beginning of the experiment and when sampled on days 3 and 10. Each oyster was opened by removing the flat shell valve and examined for the presence of gross lesions in the oyster meat. The colour, distribution, pattern, shape, contour, size, organ or site and change in texture of any lesions were recorded. From each oyster pallial cavity 0.2–0.4 ml of fluid (free water in the closed oyster shell) was collected using a single use disposable plastic 1 ml pipette and 0.2–0.4 ml of hemolymph, from the pericardial sac, was collected using a 1 ml syringe and 21G needle (Becton Dickson). Then the whole oyster was fixed in 10% seawater buffered formalin.

2.2. Analytical methods and histopathology

Concentrations of sodium and potassium in the oyster hemolymph were determined using a Konelab automated biochemical analyser. Samples were diluted 1 in 2 or 1 in 3 with distilled water so concentrations were not above the limit of detection of the Konelab automated biochemical analyser. Pallial cavity fluid salinity was measured using a refractometer (Vitalsine, Model SR-6), and pH was measured with a pH meter (MiniLab pH meter with ISFET solid state sensor, model IQ125 manufactured by IQ Instruments, Carlsbad, California, USA).

Formalin fixed tissues (10% buffered seawater formalin) were embedded in paraffin, cut at 5 μm thickness, one slide per animal (including cross sections of all organs), mounted and stained with haematoxylin and eosin, using standard techniques. All 157 slides were read by one pathologist (GK) and each organ or anatomical site (kidney, heart, mantle, interstitium, gonad, ganglia, gill, stomach, intestine, digestive gland) of the oyster was examined. Histopathological changes were recorded and graded using a four point grading scale; 0 = normal tissue and no microscopic changes, 1 = mild changes with minor alteration to organ architecture, 2 = moderate changes that affected and/or disrupted >½ of the organ architecture, 3 = severe changes with marked disruption or effacement of the majority of the organ architecture. A subset of 20 slides was independently read by a second pathologist (Susan