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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 ^{o}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 297.2mmol/L ± 20(SE) and 350.4mmol/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 5.6mmol/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^{o}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.

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41 Keywords: Pacific oyster, salinity, grading, kidney, stomach, digestive gland

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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; Galtsoff 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 in to 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 (DPIPWE Tasmania 2004). 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 (DPIPWE 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).

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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 2004 b; 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

Experimental Design and Setup

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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 sub-lethal 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^{o}C so water temperature was maintained at 8^{o}C in the tanks. For the summer experiment, on the day of collection surface water salinity was 35 and temperature was 18^{o}C on the oyster lease so water temperature was maintained at 18^{o}C in the tanks.

Two independent re-circulating water systems were set up. De-ionised 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

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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 0.25mg/L through daily partial water changes. Salinity was measured daily using a refractometer (Vitalsine, Model SR-6). A basket, which held 13-14 oysters from one 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 0.2-0.4ml of fluid (free water in the closed oyster shell) was collected using a single use disposable plastic 1ml pipette and 0.2-0.4ml of hemolymph, from the pericardial sac, was collected using a 1ml syringe and 21G needle (Becton Dickson). Then the whole oyster was fixed in 10% seawater buffered formalin.

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),

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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 5µm 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 Lola, veterinary pathologist, Animal Health Laboratory, DPIPWE Tasmania) to confirm reproducibility of results and uniform interpretation. For 18 of 20 slides both pathologists agreed in descriptions and grades for all organs. For two slides there was a minor discrepancy between the extent of histopathological changes in renal epithelium (i.e. grade 1 or 2). This was considered a minor discrepancy and did not affect the statistical analysis.

Statistical analyses

Differences in mean hemolymph potassium and sodium concentration, pallial cavity fluid salinity and pH were examined as a function of salinity (normal or low salinity), grading (graded or ungraded), family line, season, and sampling day (day 3 and 10) using a factorial ANOVA. Assumptions of homogeneity of variance were tested using Levene's Test and data were log transformed where necessary. Significant sources of variability were examined with *a posteriori* Tukey's HSD tests.

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Chi–square (χ^2) test of independence was used to determine if the relative frequency of oysters with histopathological changes for each organ or anatomical site differed as a function of salinity (normal or low salinity), grading (graded or ungraded), season (winter or summer water temperature), family line and day sampled (day 3 and 10). The test of independence assumed that the number of individuals in the different histopathological categories were the same for all treatments. If the χ^2 analysis was significant the standardised difference between the expected frequency and the observed frequency was used to identify where differences had occurred. All tests were conducted at significance level $\alpha=0.05$ and all data were analysed using SPSS v18.

In the winter experiment one ungraded oyster at normal salinity sampled on day 3 had insufficient hemolymph in the pericardial sac for testing. During the summer experiment in a low salinity tank one ungraded oyster died at day 5 and results from this animal were not included in the statistical analysis.

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3. Results

At the beginning of winter and summer experiments there was no evidence that any oysters in the experiments had defects or abnormalities in their shells and there was no chipping or break of oyster shell seals in either graded or ungraded oysters. In both experiments on days 3 and 10 there were no gross abnormalities or lesions evident.

There was no evidence that grading and family lines contributed to explaining variability in any of the measured response variables ($P > 0.05$).

Mean pallial cavity salinity differed between groups of oysters that experienced different salinity and was dependent on season and day of sampling ($F_{(season*salinity*day)}=62.670$, df 7,147; $P<0.001$). The mean pallial cavity salinity of oysters held in normal seawater (salinity 35) at day 3 and 10 were 29.4 ± 0.4 (SE) and 28.7 ± 0.4 (SE), reflecting the salinity at the lease. For these reasons, the salinity reading of 35, reported by the commercial farm was probably not representative of the whole lease. In winter (water temperature 8^{o}C) at salinity 9, 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 (Table 1). In contrast, in summer the mean pallial cavity salinity in oysters held at salinity 9 at day 3 was 19.8 ± 1.6 (SE) and day 10 was 22.8 ± 1.6 (SE) lower than oysters at salinity 35.

Similarly mean hemolymph sodium ($F_{(season*salinity*day)}=170.373$, df 7,147; $P<0.001$) and hemolymph potassium ($F_{(season*salinity*day)}=62.670$, df 7,147; $P<0.001$) differed between groups of oysters that experienced different salinity and was

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dependent on the season and day of sampling. In the winter, by day 10, mean hemolymph sodium and potassium in experimental oysters held at salinity 9 were 80.3mmol/L ± 6.5 (SE) and 2.3mmol/L ± 0.5 (SE) lower (respectively) than oysters held at salinity 35 (Table 1). In summer, mean hemolymph sodium (mean ± SE mmol/L) for oysters at salinity 9 on day 3 and 10 were 297.2mmol/L ± 20(SE) and 350.4mmol/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 5.6mmol/L ± 0.6(SE) and 7.9 mmol/L ± 0.6 (SE) lower than oysters at salinity 35.

In summer and winter hemolymph pH was on average 7.3 with a range of 7.2 to 7.4 and there was no evidence that the variability in pH among the oysters was explained by any of the experimental factors (all terms in ANOVA $P > 0.05$).

There were microscopic changes in the stomach and intestines (χ^2=80.896, df 7; P<0.001), digestive gland tubules (χ^2=94.883, df 7; P<0.001) and kidney (χ^2=20.741, df 7; P=0.004) in oysters and the frequency of these changes was associated with salinity, season and day of sampling (Table 2). These microscopic changes in oysters (which were either grade 1 or 2; no grade 3 changes were seen) were more common in low salinity compared to normal salinity and this was particularly the case in summer. Expanded intercellular spaces, intracytoplasmic vacuoles in epithelial cells (taking up greater than 70-80% of the cytoplasm and moderately expanding the cell) and hemocyte infiltrate (diapedesis) in the walls of the stomach (Fig. 2), digestive gland tubules (Fig. 4), and intestines (all grade 1 to 2) were observed. In the kidney there were expanded intercellular spaces in tubules and intracytoplasmic vacuoles in epithelial cells (taking up greater than 70-80% of the cytoplasm and moderately expanding the cell) lining the renal tubules (grade 1 to 2) (Fig. 6).Oysters in winter at low salinity had only limited microscopic changes in the kidney. These changes were mild (grade 1) intracytoplasmic vacuoles in renal cells and intercellular expanded spaces in the kidney and other kidneys, from oysters at normal salinity showed no microscopic findings (Fig. 5). There were no severe (grade 3) changes in any organs. As the frequency of grades 1 and 2 for histopathological changes did not differ between treatment groups based on salinity, season,

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day, family or grading ($P > 0.05$), grades 1 and 2 were pooled for each histopathological description (Table 2).Oysters in winter at normal salinity showed no microscopic changes in the alimentary tract (normal stomach, Fig 1 and normal digestive gland, Fig. 3). Family line and grading did not affect the frequency of histopathological changes due to low salinity in either summer or winter.

The most significant histopathological findings from the oyster which died in low salinity in the summer experiment were mild (grade 1) diffuse infiltrate of hemocytes into the wall of the stomach, intestines and digestive gland tubules (grade 2) and atrophy of digestive gland tubules (grade 1). Hemolymph potassium was 13.8mmol/L and sodium was 136mmol/L. These were within the range for other oysters at salinity 9.

4. Discussion:

This experiment demonstrated that elevated summer temperature and low salinity working together were associated with renal and alimentary changes in osmoconforming oysters. This is consistent with the influence of temperature and salinity on physiological processes in oysters, such as Eastern oyster *(C. virginica)* (Shumway 1996).

In summer oysters at low salinity 9 opened their shells and began to osmoconformed to low salinity sooner than those in winter. In estuarine environments following sudden exposure to low salinity, bivalves molluscs, such as *Mytilus edulis*, initially close their shells in response to low salinity (Hoyaux et al. 1976; Davenport 1981). Shell closure is initiated by sudden fall in intracellular sodium concentration (Natochin et al. 1979). Bivalves cannot feed or take in oxygen, while their shells are shut (Dame 2012). Warmer water temperatures increase respiration rate and oxygen demand in oysters, like other bivalves, (Shumway and Koehn 1982) and limit how long oysters can remain closed (Loosanoff 1953; Galtsoff 1964). In the summer experiment it is possible that increased respiratory demand at the higher temperature (Shumway and Koehn 1982) over-rode the intracellular signals from ion

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receptors to maintain the shell closed (Natochin et al 1979). After bivalves open their shells, the extracellular fluid osmoconforms to the surrounding water (Berger and Kharazova 1997). In the low salinity tanks in summer hemolymph sodium and potassium fell by day 3, as oysters opened their shells and began to osmoconform to low salinity water, 9 (Loosanoff 1953; Galtsoff 1964).

Accumulation of anaerobic metabolites is another reason why oysters cannot keep their shells closed after persistent stressful environmental challenges (Zubkoff and Ho 1982), such as low salinity. When bivalve molluscs are closed they accumulate anaerobic metabolic by-products such as succinate and fatty acids (Wijsman 1976; Zubkoff and Ho 1982) from the incomplete oxidation of glycogen for adenosine triphosphate generation through the tricarboxylic acid pathway (de Zwaan 1977). To extend the period they can remain shut under anaerobic conditions (closed shell) bivalves decrease their rate of metabolism (de Zwaan and Wijsman 1976; Hawkins and Bayne 1992; Hochachka and Somero 2002). In winter oysters at day 3 in low salinity demonstrated this metabolic adaptability by remaining closed but with no significant decrease in pallial cavity fluid pH, suggesting decreased metabolic rate with minimal production of acidic metabolic by-products (de Zwaan 1977). The cool water (8^{o}C) of the winter experiment may have slowed oyster metabolic rates (Loosanoff 1953), allowing a longer period of anaerobic metabolism before animals had to open their valves.

One of the key microscopic changes, associated with low salinity and elevated summer temperature, in osmoconforming oysters was expanded intercellular spaces and significantly vacuolated cells in the kidney. Similar changes have been reported in *Mytilus* sp. when challenged by abrupt low salinity (Khan and Saleuddin 1986) and when the remaining mussels were returned to seawater the renal changes resolved. Based on this evidence and the fact there was no necrosis of renal cells or irreversible damage to renal cell integrity or intercellular structures (Myers and McGavin 2007), the expanded intercellular spaces and vacuolated renal cells in the Pacific oysters, during the experiment, may also be reversible.

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247 Significant intracellular vacuolation distending epithelial cells was common in both renal and alimentary epithelial cells in oysters at
248 low salinity. The roles of renal cells include low grade partial osmoregulation (along with the gills) of hemolymph, excretion of
249 nitrogenous waste and phagocytosis and pinocytosis of excreted product through lysosomal intracytoplasmic vacuoles (Florey
250 1966; Grizel 2003). As low salinity and elevated temperature increased, osmotic stress and respiratory demand may have
251 increased not only the osmoregulatory role of the kidney but also the demand and production of phagocytic lysosomal vacuoles.
252 Similarly the significant intracytoplasmic vacuoles in the digestive gland may have related to the increased metabolic demand due
253 to low salinity and increased temperature. Intracytoplasmic lysosomes are one of the key processes for digestion in bivalve
254 molluscs (Owen 1972; Pal et al. 1990; Weinstein 1995). Stomach and intestines do not play a primary role in intracellular digestion.
255 However, vacuolation in the stomach and intestines in osmoconforming oysters at summer may have reflected the accumulation of
256 breakdown metabolic products retained within lysosomal vacuoles for release into the lumens (Galtsoff 1964).
257
258 Pollution can also cause intracytoplasmic vacuolation. Intracytoplasmic vacuolation of digestive glands were found in *Mytilus edulis*
259 exposed to polluted waters in Tvarminne area Gulf of Finland (Baltic sea) (Sunila 1987), *Crenomytilus grayanus* exposed to
260 polluted waters in Peter the Great Bay, Sea of Japan (Usheva et al. 2006) and *Mytilus edulis* exposed to metals such as copper
261 and cadmium (Sarasquete et al. 1992). Pollutants or heavy metals were unlikely to have contributed to the vacuolation in the
262 experimental oysters in this trial because these oysters were from leases which tested negative for heavy metals (including copper,
263 zinc, cadmium, lead, aluminium) and pesticides (including Endosulfan, Tributyltin, Malathion). This testing was overseen by the
264 Tasmanian Shellfish Quality Assurance Program (TSQAP) (A. Turnbull, TSQAP, pers. comm.). In addition, distilled water, which
265 was not the source of pollutants, was used in the trials to dilute the sea water.
266
267 Along with intracytoplasmic vacuolation of alimentary epithelial cells, there was mural transmigration of hemocytes, known as
268 diapedesis (Onstad et al. 2006; Jones 2010) between the expanded intercellular spaces in oysters at low salinity in summer.

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Diapedesis through the alimentary tract (and other organs) can be a normal finding in aquatic bivalve molluscs, such a *Mytilus edulis* (Onstad et al. 2006; Jones 2010). However, diapedesis was observed more commonly at low salinity in summer along with expanded extracellular spaces in the alimentary tract. Mural diapedesis through the alimentary tract wall can be due to pathogenic or benign environmental bacterial chemotactic factors (Cheng and Howland 1979) or pollution stress when heavy metals are transferred to the alimentary tract lumen by hemocytes within intracytoplasmic tertiary lysosomes (George 1983). Heavy metal stress was unlikely a cause for diapedesis in the alimentary tract of these oysters.

Histopathological changes in osmoconforming oysters in the summer experiment were similar but not as severe as the histopathological findings from live oysters sampled during the summer freshwater flood and oyster mortality event in Georges Bay and Moulting Bay 2004 (DPIWE Tasmania 2004a). Numerous oyster mortalities are often associated with the combined effect of freshwater flooding and elevated ambient temperatures (Gunter 1950; Butler 1952; Gunter 1953; Owen 1953; Andrews et al. 1959; Burrell 1977). Common histopathological changes seen in both oysters from the Georges Bay and Moulting Bay mortality event and the experimental oysters at salinity 9 in summer, included expanded intercellular spaces in the walls of the stomach, intestines and digestive glands, with diffuse hemocyte infiltrate in these organs and expanded intercellular spaces in the kidneys. Additional microscopic changes seen in oysters from Georges Bay 2004 and not in the experimental oysters at low salinity in summer were epithelial necrosis in the alimentary tract, gonad necrosis, leydig tissue necrosis and mantle erosion. There was no significant necrosis evident in the alimentary tract of oysters in the summer experiment at salinity 9 and this was consistent with the lack of rise in potassium hemolymph levels at salinity 9 in summer. Elevated hemolymph potassium reflects cell rupture and death in molluscs (Burrell 1977; Natochin et al. 1979). Other microscopic findings only seen in oysters from the flood event in 2004 included myositis of the adductor muscle and expanded extracellular spaces and hemolymph vessels in the mantle. The histopathological changes in oysters collected from the Georges and Moulting Bay mortality event in 2004 were not consistent with Iridovirus, *Marteilia* spp, *Bonamia* spp, *Haplosporidium* spp, *Perkinsus* spp, *Nocardia* spp infection (DPIWE Tasmania 2004a). No

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recognisable pathogens were seen in the histopathological sections of animals in this experiment. In particular, the microscopic findings associated with low salinity were not consistent with the range of microscopic changes associated with Ostreid Herpesvirus-1 infection (Friedman et al. 2005; Jenkins et al. 2013).

In this experiment there was no evidence that differences among the variables measured were attributable to the oysters' breeding history or their exposure to grading stress just prior to arriving at the laboratory for the experiment. Grading would directly affect the response to an abrupt change in low salinity if the shell seal was broken during grading (Loosanoff 1953). Because there were no significant chips or breaks in the shell seals or valves after grading, it appeared that the oysters had a complete seal when exposed to low salinity water and as a result grading had no effect. The oysters of all four families had a similar response to abrupt change to low salinity suggesting there is little significant genetic variation in this trait between the four family lines.

In summary the stress effects of elevated summer temperature and abrupt change to persistent low salinity caused microscopic changes in the kidney and alimentary tract of osmoconforming oysters. Describing these microscopic changes will aid diagnosticians in their interpretation of molluscan histopathology. These results will also enable better management of stress events experienced by oysters in culture conditions and ultimately inform industry of the nature of mortality events due to environmental challenges.

Further research is required to compare these microscopic changes in oysters challenged at the lower end of mesohyaline salinity 9 to oysters challenged by freshwater in summer.

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HIGHLIGHTS

- We subjected Pacific oysters to abrupt low salinity and normal salinity
- At low salinity, microscopic changes in the alimentary tract and kidney were seen
- These changes were more common in summer than winter
- These findings help interpret microscopic changes related to temperature and salinity

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Figure 1: The normal stomach wall consists of tall epithelial cells with elongated nucleus set in the median part of the cell.

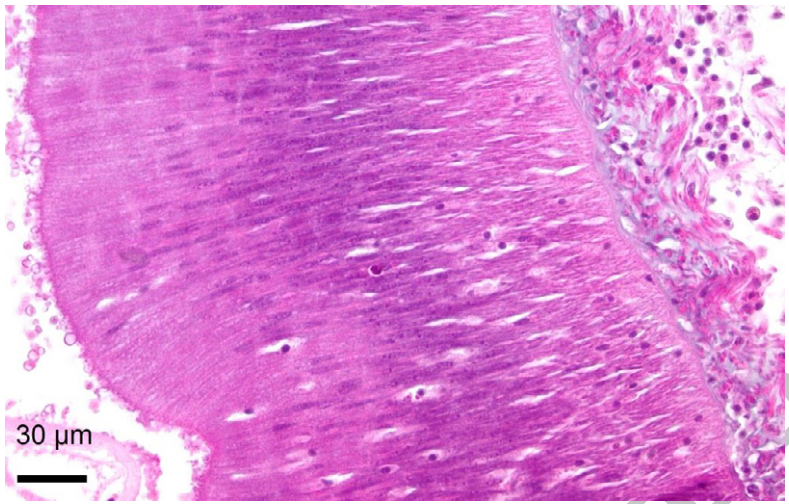

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Figure 2: The stomach wall is moderately, diffusely expanded by intercellular spaces and haemocyte infiltrate (arrows) in the stomach wall, in an oyster at low salinity

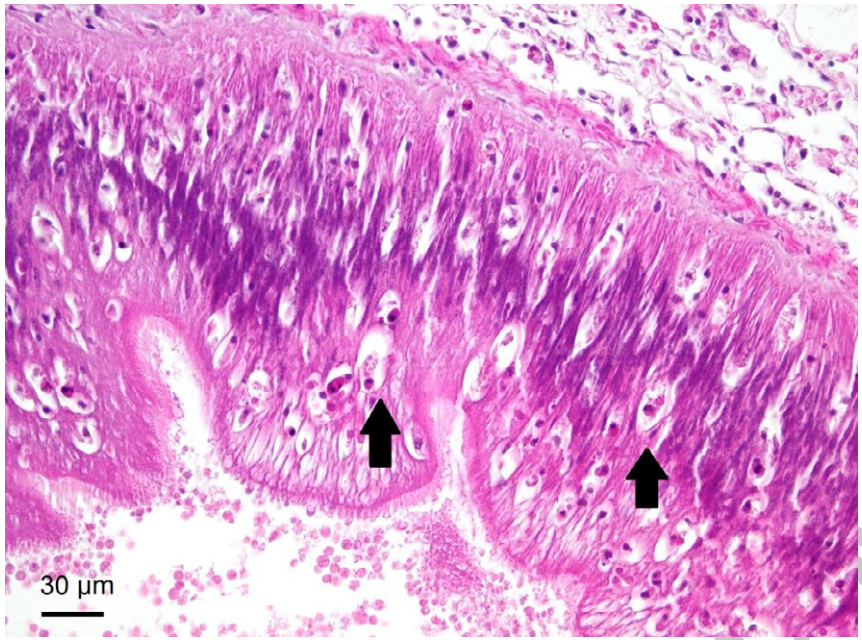

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Figure 3: Normal digestive glands consist of digestive ducts (DD) and digestive tubules (DT).

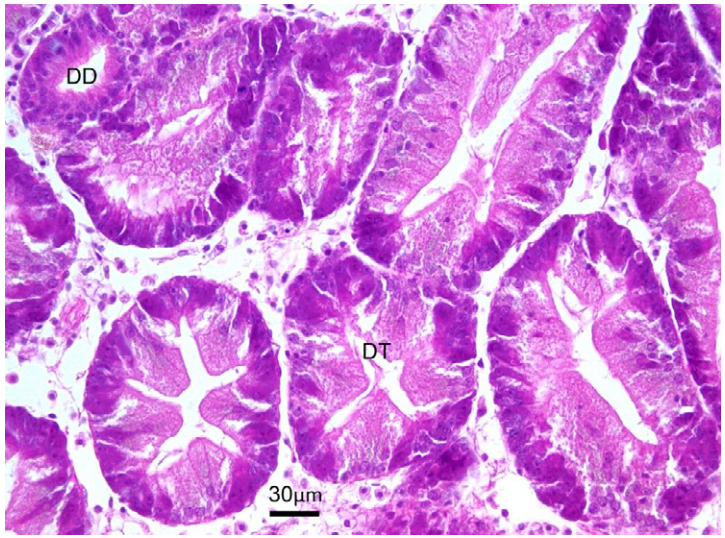

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Figure 4: Expanded intercellular spaces (arrow) and haemocyte infiltrate within the digestive tubular walls in an oyster at low salinity

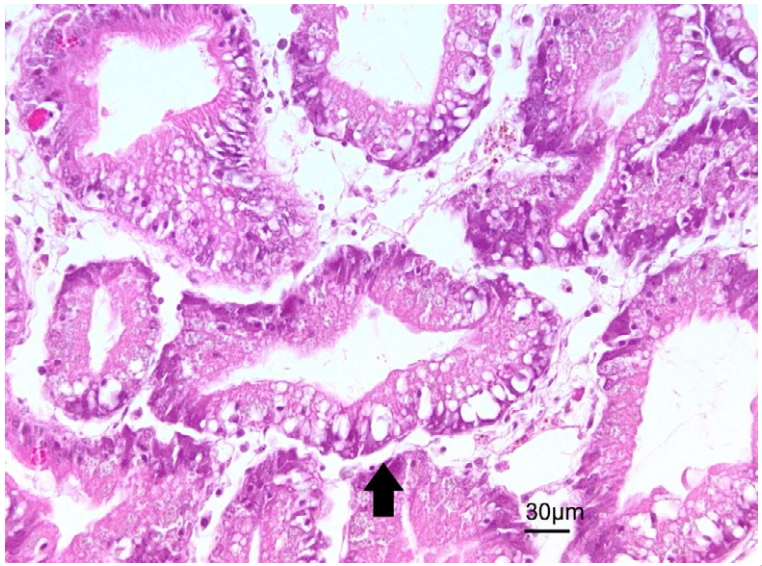

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Figure 5: Normal renal tubule (RT) lined by closely packed columnar epithelium (E), with apical poles.

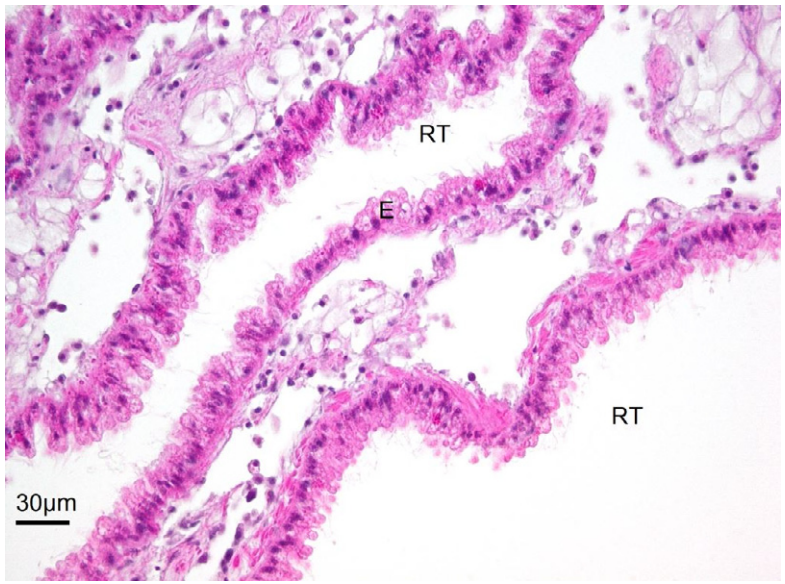

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Figure 6: There are expanded intercellular spaces (arrow) in the kidney tubule in an oyster at low salinity.

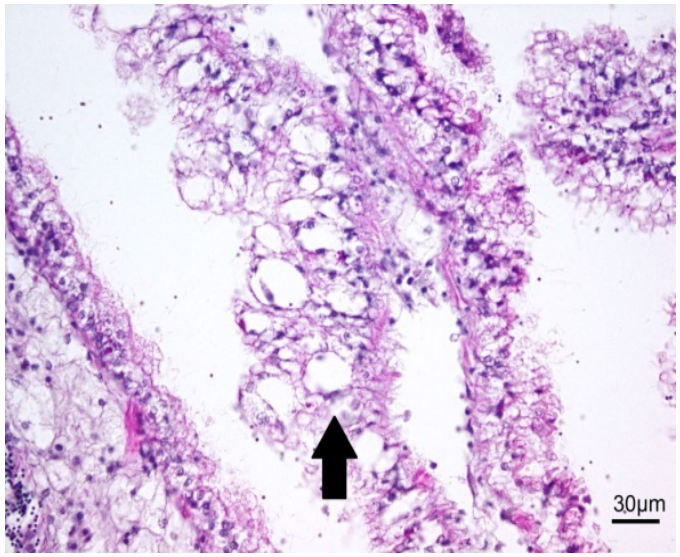

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Table 1 Oyster pallial cavity salinity and haemolymph potassium and sodium results due to season, salinity and day of sampling. For each of the three variables, treatment means with different letters are significantly different from one another

Variable	Winter				Summer			
	Water salinity 9 ppt		Water salinity 35 ppt		Water salinity 9 ppt		Water salinity 35 ppt	
	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=20)	Day 10 (n=20)	Day 3 (n=19)	Day 10 (n=17)	Day 3 (n=20)	Day 10 (n=20)
Pallial cavity salinity (ppt) mean ± SE	29.0 ± 0.4^{a}	22.7 ± 0.4^{b}	29.4 ± 0.4^{a}	28.7 ±0.4^{a}	15.4 ± 0.7^{d}	13.1 ± 0.7^{d}	35.2 ± 0.5^{c}	38.0 ±0.7^{c}
Haemolymph potassium (mmol/L) mean ± SE	10.0 ± 0.4^{e}	8.5 ± 0.4^{f}	9.5 ± 0.4^{e}	10.7 ± 0.4^{e}	6.3 ± 0.4^{h}	4.5 ± 0.4^{h}	12.0 ± 0.4^{g}	12.4 ± 0.4^{g}
Haemolymph sodium (mmol/L) mean ± SE	347.1 ± 4.6^{i}	263.7 ± 4.6^{j}	350.5 ± 4.7^{i}	344.0 ± 4.6^{i}	170.0± 15^{l}	134.0 ± 15^{l}	468.1 ± 15^{k}	484.4 ± 15^{k}

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Table 2. The number of oysters with histopathological changes in the kidney, digestive gland, stomach and intestines (combining grade 1 and 2) on day 3 and 10, in either low, 9, or normal, 35, salinity, for each season. Arrows indicate if observed values are greater or less than expected values on the assumption that salinity did not influence the number of oysters with histopathological changes

	Winter				Summer			
	Water salinity 9		Water salinity 35		Water salinity 9		Water salinity 35	
Histopathological finding	Day 3 n=20	Day 10 n=20	Day 3 n=20	Day 10 n=20	Day 3 n=19	Day 10 n=17	Day 3 n=20	Day 10 n=20
kidney – expanded intercellular spaces and intracytoplasmic vacuolation	5↓	7↑	6	3↓	13↑	8↑	4↓	3↓
Digestive gland - expanded intercellular spaces intracytoplasmic vacuolation and haemocyte infiltrate	0↓	0↓	0↓	0↓	14↑	13↑	1↓	2↓
Stomach and intestines - expanded intercellular spaces, intracytoplasmic vacuolation and haemocyte infiltrate	0↓	0↓	0↓	0↓	11↑	10↑	0↓	0↓

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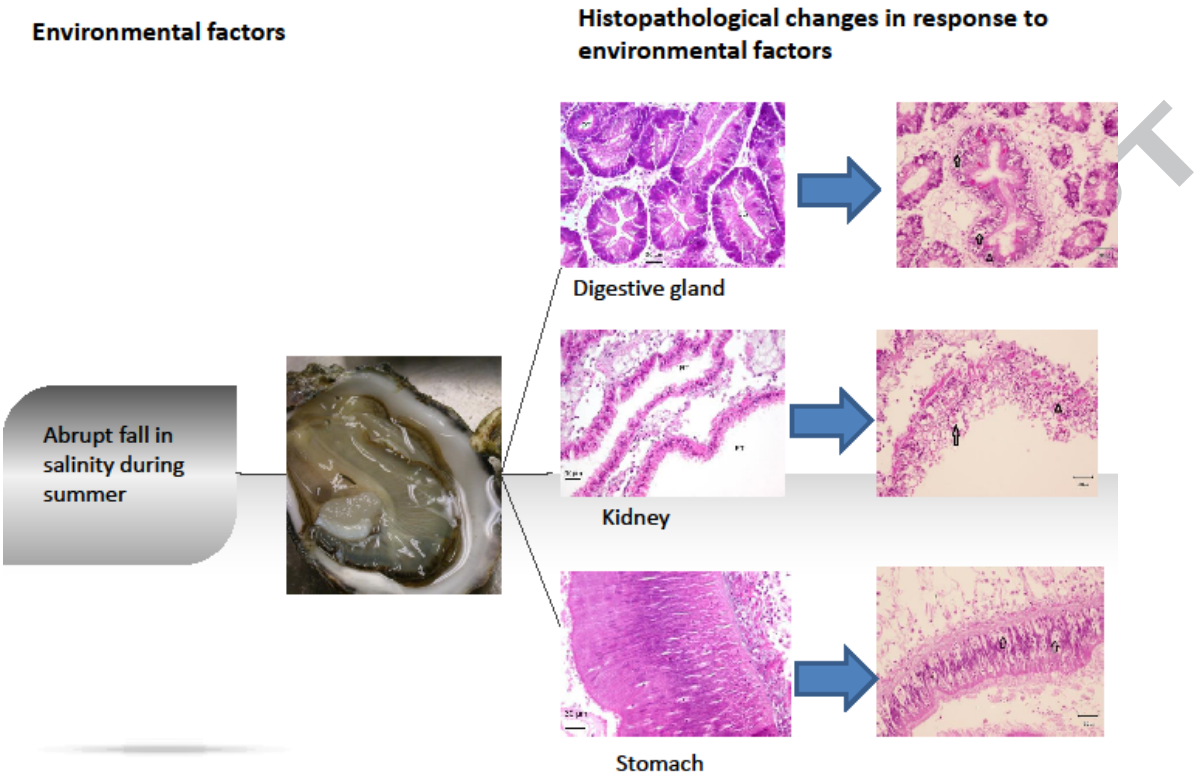

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