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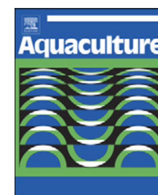

# Osmoregulatory balance in Murray cod, *Maccullochella peelii peelii* (Mitchell), affected with chronic ulcerative dermatopathy

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A B S T R A C T

This study examined the osmoregulatory capability of Murray cod, *Maccullochella peelii peelii*, affected by chronic ulcerative dermatopathy (CUD) in intensive aquaculture. This condition appears to arise only in facilities utilizing groundwater, with the causative agent suggested to be a water-borne factor. Healthy Murray cod (~700 g) were transferred to a CUD-affected farm to monitor the progression of the syndrome and began to show signs of CUD after approximately five months. In order to evaluate possible effects of CUD on osmoregulation; plasma electrolyte concentrations, osmolality, and $Na^+$,$K^+$-ATPase activities were measured, and gill histology and immunohistochemistry were analyzed. Plasma electrolyte concentrations and osmolality of CUD-affected Murray cod were consistent with reference values determined for non CUD-affected fish. A greater number of gill mucous cells were observed in Murray cod cultured at the CUD-affected farm compared to non CUD-affected fish. We also found an un-identified cell type that was present solely in the gills of CUD-affected Murray cod. Gill $Na^+$,$K^+$-ATPase activity was significantly higher in severely CUD-affected Murray cod compared to individuals transferred to the CUD-affected farm. While there appeared to be some minor changes in the gills of CUD-affected fish, this study demonstrated that Murray cod were able to effectively osmoregulate, although, perhaps at an energetic cost.

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## 1. Introduction

In Australia, the culture of native warmwater finfish species has intensified, with many facilities utilizing temperature-controlled indoor tank-based, re-circulating, and flow-through aquaculture systems. Murray cod, *Maccullochella peelii peelii* (Mitchell) are large freshwater teleost fish of the Percichthyidae family that inhabit the Murray–Darling river system of south-eastern Australia. There is a growing interest in Murray cod aquaculture because of its ease of breeding under captive conditions, acceptance of artificial feeds at a young age, high growth rate, and the ability to be cultured at elevated stocking densities (reported in Ingram and De Silva, 2004).

The current aquaculture systems in which Murray cod are farmed, are predominantly supplied by groundwater (Baily et al., 2005). Despite groundwater frequently being used in finfish aquaculture, some water sources have been reported to be unsuitable, including saline groundwater from inland New South Wales (Fielder et al., 2001) and potassium-deficient saline groundwater in Western Australia (Partridge and Creeper, 2004). Groundwater also appears to be associated with the emergence of two new pathologies; skeletal myopathy in juvenile barramundi, *Lates calcarifer* (Partridge and Creeper, 2004), and chronic ulcerative dermatopathy (CUD) in Murray cod and goldfish, *Carassius auratus* (Baily et al., 2005). This condition in Murray cod is also referred to as chronic erosive dermatopathy; however, in this study we will follow the classification of Baily et al. (2005) who states that the appropriate term is ulcerative rather than erosive.

Currently, there is limited information on CUD in Murray cod aquaculture, with the principle cause of the pathology still unknown. The condition results in erosion of the epidermal layer surrounding the cephalic and trunk lateral lines and erosion of the caudal and dorsal fins (Baily et al., 2005). Badly affected fish are severely disfigured, which substantially reduces their marketability. In addition, they display slight increases in mortality and a slower growth rate (reported in Baily et al., 2005). Interestingly, all fish cultured in bore water that precipitates the condition are affected, suggesting that the causative agent is not subject to immunological control.

A similar condition, termed 'hole-in-the-head' disease, has been reported in cichlids such as discus, *Symphysodon discus* (Noga, 2000; Paull and Mathews, 2001) and angelfish, *Pterophyllum scalare* (Paull and Mathews, 2001), as well as Nile tilapia, *Oreochromis niloticus* (Morrison et al., 2007). In all these species the pathology results in lesion formation posterior to the eye and into the lateral line. Paull and Mathews (2001) isolated the flagellate, *Spironucleus vortens*, from head lesions in discus and angelfish and suggested that the severe lesions resulted from flagellate infection. However flagellate infection

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was not apparent in Nile tilapia (Morrison et al., 2007). These authors only isolated bacteria from lesions and not elsewhere, which suggests a secondary infection after the epithelium was damaged. In a study on the development of CUD in Murray cod, Baily et al. (2005) investigated whether viral or bacterial agents were the causative factor; however none were observed. Lesions in affected Murray cod resolved once fish were transferred to Murray river water and the causative agent was suggested to be present in the groundwater.

Given that CUD results in destruction of parts of the epidermal layer, we hypothesized that osmoregulatory homeostasis may be threatened. Ionic gradients in freshwater environments favor the diffusive movement of ions from animals to the environment, and in order to maintain osmotic balance fish must take up ions, such as $Na^+$ and $Cl^-$, from the surrounding water (Marshall and Grosell, 2006). At the same time ion loss must be minimized and a low permeability to ions and water is a feature of the freshwater fish epidermis (Bentley 1962; Fontenot and Neiffer, 2004). Water quality may also have a detrimental effect on fish physiology and biochemistry. For example, changes in gill structure and function and disruptions in osmoregulatory capabilities have been reported in teleost fish after exposure to a wide range of water-borne irritants such as nitrite (Williams and Eddy, 1988; Stormer et al., 1996; Aggergaard and Jensen, 2001), acidic (Ultsch et al., 1981) and alkaline waters (Wilkie et al., 1999) and heavy metals, including mercury (Jagoe et al., 1996), copper (Wang et al., 1998; Monteiro et al., 2005), and aluminum sulphate (Heming and Blumhagen, 1988).

This study was designed to examine the osmoregulatory status of CUD-affected Murray cod in an intensive flow-through groundwater aquaculture system and assess whether CUD and/or the groundwater source had an influence on plasma ion balance, $Na^+,K^+$-ATPase activity and gill histology in Murray cod. These parameters were initially examined in non CUD-affected Murray cod cultured in a re-circulating aquaculture system and used as reference values. Murray cod from this system were transferred to a flow-through system for culture and subsequently developed a mild form of CUD. Comparisons were made between the transferred fish and severely CUD-affected Murray cod that had been cultured in the intensive flow-through system.

## 2. Materials and methods

### *2.1. Animals and experimental protocols*

At Spirit of the Sea (SS) Aquaculture, Warrnambool, Victoria, non CUD-affected Murray cod (~700 g) were housed in a re-circulating aquaculture system (RAS) utilizing groundwater in 10,000 L tanks maintained at 22 °C, pH ~ 7.5. Murray cod were fed Nova Me pellets (Skretting, Tasmania, Australia) continuously on a 12 h cycle by automatic belt feeders. Periodic additions of NaCl (2.0 g $l^{-1}$) were made to the tank for therapeutic reasons. At Brimin Lodge (BL) Murray cod farm, Rutherglen, Victoria, CUD-affected Murray cod (BL-CUD; ~700 g) were maintained in a flow-through groundwater aquaculture system in 5000 L tanks at 18 °C, pH ~ 7.4. All fish cultured in this flow-through system develop the pathology, therefore, 100 unaffected sub-adults were transferred to BL from SS (BL-SS) into 2 × 600 L oxygenated tanks to monitor progression of CUD. These animals were maintained in the flow-through groundwater system in 1500 L tanks in identical conditions to the CUD-affected fish. All fish at BL were fed Barramundi pellets (Ridley Aqua-Feed, Queensland, Australia) once a day to apparent satiation.

### *2.2. Sampling and analytical techniques*

Following transfer to BL, SS fish were allowed to acclimate to their new surroundings for three months prior to the first sampling period in July. The second sampling time occurred five months post-transfer. On the day of sampling, fish were euthanized by an overdose of benzocaine (5 g/L; Sigma, St. Louis, MO, USA), and venous blood samples (4–5 mL) were collected via caudal puncture using 5 mL lithium-heparinised syringes (4 mg/mL; Sigma). Samples were centrifuged for 15 min at 7500 × g and 4 °C (Beckman Allegra™ 21R Centrifuge) to isolate plasma from the cells allowing for the determination of plasma ion concentrations and osmolality. For $Na^+,K^+$-ATPase assay, 3–5 gill filaments were taken from the first gill arch of each animal and frozen in dry ice in SEI buffer (250 mM sucrose, 10 mM EDTA and 50 mM imidazole, pH 7.3). Several replicates were taken to ensure that enough tissue was available for assay. For histology and immunohistochemistry, the first gill arch was removed from each animal, placed in 4% phosphate-buffered formaldehyde for 6 h, rinsed in phosphate buffered saline (PBS) and stored in 70% ethanol until analyzed. All plasma and tissue samples collected were snap frozen in dry ice, transported to Deakin University, Geelong, and stored at −80 °C until required.

During sampling at BL, observations were made and noted regarding the health and appearance of CUD-affected and SS transferred Murray cod, including skin coloration, severity of lesion development, fin erosion and mucus secretion.

#### *2.2.1. Water samples*

Water samples were collected in duplicate from culture tanks at both sample times and analysed for alkalinity, hardness, and ion and trace metal concentrations (Amdel Ltd, Victoria, Australia). Biochemical oxygen demand, pH, total nitrogen, nitrate, nitrite and phosphate concentrations were analyzed at the Deakin University Water Quality Laboratory, Warrnambool.

#### *2.2.2. Plasma ion concentrations and osmolality*

Plasma samples were thawed at room temperature and the concentrations of $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ were determined by atomic absorption spectroscopy (model GBC 933; GBC Scientific, Victoria, Australia). Plasma $Cl^-$ concentrations were measured by a mercuric thiocyanate assay as previously described by Zall et al. (1956), using a LKB Biochrom Novaspec spectrophotometer (model 4049; Biochrom Ltd, Cambridge, UK). Briefly, a standard curve was generated by reading the absorbance of chloride standards (160 mM, 144 mM, 128 mM, and 112 mM in diluent) at 480 nm. These standards were prepared by adding 10, 9, 8, and 7 µL of primary standard (160 mM NaCl), respectively, to 2 mL of chloride color reagent (6.7% methanol, 0.87 mM mercuric thiocyanate, 33 mM nitric acid and 1.35% ferric nitrate, Sigma) and 2 mL of $diH_20$. The absorbance of the unknown plasma samples (10 µL plasma, 2 mL chloride color reagent and 2 mL $diH_20$) were read at 480 nm and chloride concentrations were calculated from the standard curve equation. Plasma osmolality was determined using a VAPRO® vapor pressure osmometer (model 5520; Wescor Inc, Logan, Utah, USA) calibrated with standards.

#### *2.2.3. $Na^+,K^+$-ATPase antibody*

A monoclonal $Na^+,K^+$-ATPase antibody was obtained from the Developmental Studies Hybridoma Bank, which is maintained by the University of Iowa and was developed with assistance from the National Institute of Child Health and Human Development. Dr. Douglas Farmbrough designed the antibody, which is raised against the α-subunit of avian $Na^+,K^+$-ATPase α5 isoform. The $Na^+,K^+$-ATPase α-5 antibody has been used in a number of studies to localize $Na^+,K^+$-ATPase in fish gills (Choe et al., 1999; Dang et al., 2000; Piermarini and Evans, 2000).

#### *2.2.4. Histological and immunohistochemical analysis*

Gills were dehydrated through a graded series of ethanol and xylene washes in an automated tissue processor (Leica TP 1010) before being embedded in Paraplast® (Tyco Healthcare, NSW, Australia). Serial sections were cut and placed on silane subbed slides and left to

**Table 1**
Chemical characteristics of Brimin Lodge (BL) groundwater, Warrnambool (SS) groundwater and water in the culture tanks from both aquaculture facilities

| Water source | Sample time | pH | Alkalinity ($mg\ l^{-1}$) as $CaCO_3$ | Hardness ($mg\ l^{-1}$) as $CaCO_3$ | Total Nitrogen ($mg\ l^{-1}$) | Nitrate ($mg\ l^{-1}$) | Nitrite ($mg\ l^{-1}$) | Phosphate ($mg\ l^{-1}$) | BOD ($mg\ l^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| *Supply* | | | | | | | | | |
| BL Bore | – | 6.40 | 120 | 50 | 0.67 | 0.67 | <0.01 | 0.03 | <2.0 |
| SS Bore | – | 7.43 | 350 | 630 | 6.9 | 6.9 | <0.01 | 0.26 | <2.0 |
| *Culture tank* | | | | | | | | | |
| BL-FL | 1 | – | 110 | 150 | 0.04 | 0.04 | <0.01 | 0.07 | 2.0 |
| | 2 | 7.74 | 110 | 140 | 1.20 | 0.53 | 0.02 | 0.09 | 4.0 |
| SS-RAS | 1 | 7.30 | 150 | 410 | 22.0 | 20.0 | 0.46 | 2.70 | 10. |
| | 2* | 7.35 | 210 | 590 | 40.0 | 39.0 | 0.12 | 3.20 | 2.0 |

Sample times represent the month in which sampling occurred; 1: July and 2: September. Asterisk indicates the sample time that occurred 24 h after salt addition to the culture tank. BL-FL: Brimin Lodge flow-through system; SS-RAS: Spirit of the Sea Aquaculture re-circulating aquaculture system. BOD: Biological oxygen demand.

dry overnight. Slides were dewaxed in three changes of xylene and rehydrated through a graded series of ethanol to water. For histological analysis of the gills, several slides from each animal were stained with haematoxylin and eosin, Masson's trichrome, or PAS-alcian blue. To determine the localization of $Na^+,K^+$-ATPase activity, sections were washed 3 × 10 min in 0.01 M PBS, pH 7.4, and endogenous peroxidase activity was blocked by incubating sections in a 3% hydrogen peroxide solution for 25 min. After blocking for 1 h with antibody diluent (10% normal goat serum, 0.1% bovine serum albumin, 0.01% sodium azide in PBS, pH 7.4), sections were incubated with $Na^+,K^+$-ATPase primary antibody (1:500) for 48 h at 4 °C. Sections were then washed in 0.01 M PBS (3 × 10 min) and a Vector stain ABC kit (Vector laboratories, Burlingame, CA, USA) was used to detect primary antibody. This involved incubating sections with a biotinylated HRP-conjugated goat anti-mouse secondary antibody for 30 min, washing in 0.01 M PBS, followed by a 30 min incubation with avidin-biotin complex. Slides were washed in 0.01 M PBS, rinsed briefly in 0.1 M Tris (pH 7.4), before colour development in 3% 3'3 diaminobenzidine tetrahydrochloride in 0.1 M Tris. The trailing edge (afferent side of the filament) showed the greatest density of mitochondrial-rich cells (MRC), therefore, sections from this region were used to quantify MRC on secondary lamellae. Fifty secondary lamellae were chosen at random to be counted and the mean of these values were taken as the count for that animal. In an attempt to ensure an even plane of section, only lamellae that were of equal height on both sides of the filament were counted. For histological examination, serial sections were counter-stained with haematoxylin and eosin or stained with alcian blue. Mucous cell proliferation was determined by visual grading of the cells to be either high (>10 mucous cells visible per lamellae present on virtually all lamellae), medium (3–10 mucous cells visible on virtually all lamellae) or low (1–3 mucous cells visible per lamellae, many lamellae with no visible mucous cells). To eliminate experimenter bias, the examination of mucous cell proliferation and MRC cell counts was conducted in a randomized, deidentified manner, with the identification key being held by a third party.

### *2.2.5. $Na^+,K^+$-ATPase activity analysis*

$Na^+,K^+$-ATPase activity was assessed by a kinetic assay that measured oxidation of NADH following the method developed by McCormick (1993). Immediately prior to assay, gill samples were thawed and extra SEI buffer, with the addition of Na deoxycholic acid (0.1%), was added. Tissue was homogenized on ice using a Kontes pellet motor pestle for 10–15 s and centrifuged in a Beckmann Allegra™ 21R centrifuge at 5000 × g for 1 min to pellet any remaining solid. Homogenate (10 µL) was assayed in quadruplicate reactions in assay mixture (50 mmol $l^{-1}$ imidazole, 2.8 mmol $l^{-1}$ phosphoenolpyruvate, 0.22 mmol $l^{-1}$ NADH, 0.7 mmol $l^{-1}$ ATP, 4 U/mL lactic dehydrogenase, 5 U/mL pyruvate kinase, 47 mmol $l^{-1}$ NaCl, 5.25 mmol $l^{-1}$ MgCl and 10.5 mmol $l^{-1}$ KCl) with two replicates containing ouabain (0.5 mM; Sigma), a $Na^+,K^+$-ATPase inhibitor. The optical density of the reaction was read at 340 nm at 10 s intervals for a period of 10 min on a SpectraMax 340PC$^{384}$ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Known concentrations of ADP were incubated with assay mixture to form a standard curve of ΔmOD vs. nmoles ADP and a standard curve of known concentrations of NADH in imidazole buffer vs. mOD was also run. Homogenates were assayed for protein concentration/10 µL using a Pierce BCA protein assay kit against a series of albumin standards. Values for $Na^+,K^+$-ATPase activity were reported as µmoles ADP $mg^{-1}$ protein $h^{-1}$.

## *2.3. Statistical analysis*

Data are reported as the mean ± SE. Murray cod plasma electrolytes, osmolality and gill $Na^+,K^+$-ATPase activity were tested for normality via a normal probability plot using Minitab v12.0. Following this, data sets were tested for homogeneity of variance and compared via a one-

**Table 2**
Concentrations of major and minor ions in the Brimin Lodge (BL) groundwater, Warrnambool (SS) groundwater, and water in culture tanks from both aquaculture facilities

| Water source | Sample time | $Na^+$ | $Ca^{2+}$ | $K^+$ | $Mg^{2+}$ | $Cl^-$ | $Fe^{2+}$ | $P^{3-}$ | $Al^{3+}$ | $Si^{4+}$ | $Sr^{2+}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ | $mg\ l^{-1}$ |
| *Supply* | | | | | | | | | | | |
| BL Bore | – | 47 (2.04) | 7.0 (0.17) | 1.5 (0.04) | 8.0 (0.33) | 56 (1.63) | <0.1 (<0.01) | <0.1 (<0.01) | <0.005 | 19 | 0.11 (<0.01) |
| SS Bore | – | 310 (13.48) | 180 (4.49) | 2.4 (0.06) | 41 (1.69) | 550 (15.96) | <0.1 (<0.01) | <0.1 (<0.01) | <0.005 | 11 | 0.84 (<0.01) |
| *Culture tank* | | | | | | | | | | | |
| BL-FL | 1 | 160 (6.96) | 19 (0.47) | 3.4 (0.09) | 24 (0.99) | 360 (10.45) | <0.1 (<0.01) | 0.2 (<0.01) | <0.005 | 13 (0.46) | 0.280 (<0.01) |
| | 2 | 110 (4.78) | 18 (0.45) | 2.0 (0.05) | 23 (0.95) | 260 (7.55) | <0.1 (<0.01) | <0.1 (<0.01) | <0.005 | 13 (0.46) | 0.280 (<0.01) |
| SS-RAS | 1 | 220 (9.57) | 120 (2.99) | 4.3 (0.11) | 29 (1.19) | 370 (10.74) | <0.1 (<0.01) | 2.3 (0.07) | <0.005 | 6.0 (0.21) | 0.630 (<0.01) |
| | 2* | 450 (19.57) | 170 (4.24) | 9.0 (0.23) | 39 (1.60) | 690 (20.03) | <0.1 (<0.01) | 3.5 (0.11) | <0.005 | 9.6 (0.34) | 0.830 (<0.01) |

Sample times represent the month in which sampling occurred; 1: July and 2: September. Data presented in ( ) are water concentrations as mM. Asterisk indicates the sample time that occurred 24 h after salt addition to the culture tank. BL-FL: Brimin Lodge flow-through system; SS-RAS: Spirit of the Sea re-circulating aquaculture system.

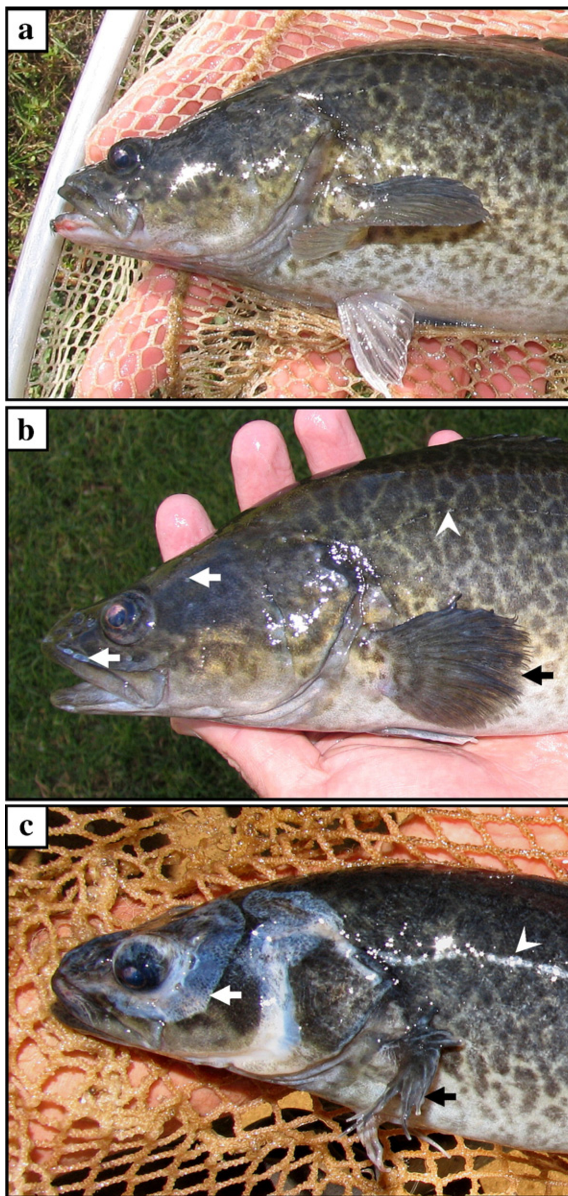


**Fig. 1.** (a) Healthy Murray cod showing no signs of CUD, (b) pit formation in sensory canals on the head (white arrows) and lateral line (white arrowhead) and early fin erosion (black arrow) of fish five months after transfer from Spirit of the Sea Aquaculture to Brimin Lodge, and (c) erosion of epidermis surrounding sensory canals on the head (white arrow), lateral line (white arrowhead) and fin erosion (black arrow) of a severely CUD-affected Murray cod. Note lesions on Murray cod transferred from Spirit of the Sea Aquaculture were minor in comparison to severely CUD-affected fish.

way ANOVA using SPSS v11.5. If significant differences ($p<0.05$) were found, a post-hoc multiple comparisons test (Tukey) was applied to determine these differences.

## 3. Results

### 3.1. Water quality

Chemical characteristics of the groundwater utilized at both aquaculture facilities are shown in Table 1. Groundwater at SS contained higher concentrations of $CaCO_3$, nitrate, nitrite, and phosphate. This groundwater was also more saline and electrolyte enriched compared to that at BL, containing higher concentrations of $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $K^+$, $Cl^-$ and $P^{3-}$ (Table 2). Elevated $Na^+$ and $Cl^-$ concentrations were observed in culture tanks at SS in September (sample time 2) and were attributed to the addition of salt to culture tanks 24 h prior to sampling. Both groundwater sources contained levels below detection limit (<0.005 mg $l^{-1}$) of major trace metals including, arsenic, beryllium, cadmium, chromium, cobalt, copper, lead, nickel, selenium, tin and zinc (results not shown).

### 3.2. CUD development

Severely CUD-affected Murray cod cultured at BL (BL-CUD) displayed necrosis of the dermal layer overlaying sensory canals on the head and lateral line when compared to non-affected fish from SS (Fig. 1a, c). The condition also resulted in erosion of the dermis on the fins, with severe cases occasionally resulting in eye loss (results not shown). Fish affected by CUD were observed to be darker in color and appeared to secrete excess mucus. Murray cod transferred to BL from SS (BL-SS) began showing visual signs of the syndrome approximately five months post-transfer. These included dark coloration and pigmentation of the skin, formation of distinct pits, albeit small, surrounding sensory canals on the head and lateral line, and slight fin erosion (Fig. 1b).

### 3.3. Plasma ion concentrations and osmolality

Murray cod plasma $Na^+$ concentrations slightly increased over time, with BL-CUD having significantly higher plasma $Na^+$ concentrations in September compared to values obtained in July (Table 3). Plasma $Ca^{2+}$ and $Mg^{2+}$ concentrations varied between stocks and sample times and there were no significant differences in plasma $K^+$ and $Cl^-$ concentrations or osmolality between BL-CUD and BL-SS.

### 3.4. Histological analysis

All three groups (SS, BL-SS, and BL-CUD) had individuals with varying degrees of mucous cell proliferation, ranging from high to low (Fig. 2a–c). However, qualitatively, animals held in groundwater at BL showed greater mucous cell proliferation than animals from SS, and BL-CUD showed greater mucous cell proliferation than BL-SS animals (Fig. 2d). The only other observable difference noted between animals affected by CUD compared to those unaffected, was the presence of a small unidentifiable cell type (UCT) (Fig. 3). These cells were small and ovoid with a highly prominent cell membrane, a dense nucleus and cytoplasm condensed into small, rod-shaped, highly eosinophillic particles. The UCT was present in all animals at BL irrespective of whether there was mucous cell proliferation or not, and were not observed in any of the animals at SS. Sections examined for positive $Na^+$, $K^+$-ATPase immunoreactivity and counterstained with haematoxylin and eosin demonstrated that $Na^+$,$K^+$-ATPase immunoreactivity was not observed in these cells (Fig. 3).

**Table 3**
Plasma ion concentrations and osmolality of non CUD-affected Murray cod at Spirit of the Sea (SS) Aquaculture, SS stock transferred to Brimin Lodge (BL-SS) and CUD-affected Murray cod at Brimin Lodge (BL-CUD)

| System | Sample time | $[Na^+]$ (mM) | $[Ca^{2+}]$ (mM) | $[K^+]$ (mM) | $[Mg^{2+}]$ (mM) | $[Cl^-]$ (mM) | Osmolality (mmol $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| SS | 1 | 133.7±1.5 | 3.08±0.04 | 3.36±0.18 | 0.67±0.03 | 116.2±2.0 | 269.7±2.0 |
| | 2 | 147.0±3.3 | 2.82±0.04 | 3.42±0.10 | 0.56±0.02 | 118.1±2.0 | 293.6±1.7 |
| BL-SS | 1 | 145.1±2.6$^A$ | 2.50±0.07$^A$ | 3.17±0.16 | 0.76±0.03$^A$ | 103.3±7.3 | 293.6±5.1 |
| | 2 | 148.9±3.4$^A$ | 2.89±0.05$^B$ | 2.29±0.23 | 0.71±0.01$^{AB}$ | 106.0±3.0 | 289.7±2.4 |
| BL-CUD | 1 | 131.7±2.7$^B$ | 2.75±0.10$^{AB}$ | 3.09±0.23 | 0.95±0.06$^C$ | 107.3±5.7 | 294.5±6.6 |
| | 2 | 154.4±2.0$^A$ | 2.90±0.08$^B$ | 2.73±0.22 | 0.58±0.04$^B$ | 108.1±3.2 | 293.6±2.5 |

Sample times represent the month in which sampling occurred; 1: July and 2: September. Values represented as mean±SE, $n=8$. Mean values in columns with different superscripts are significantly different (ANOVA; $p<0.05$).

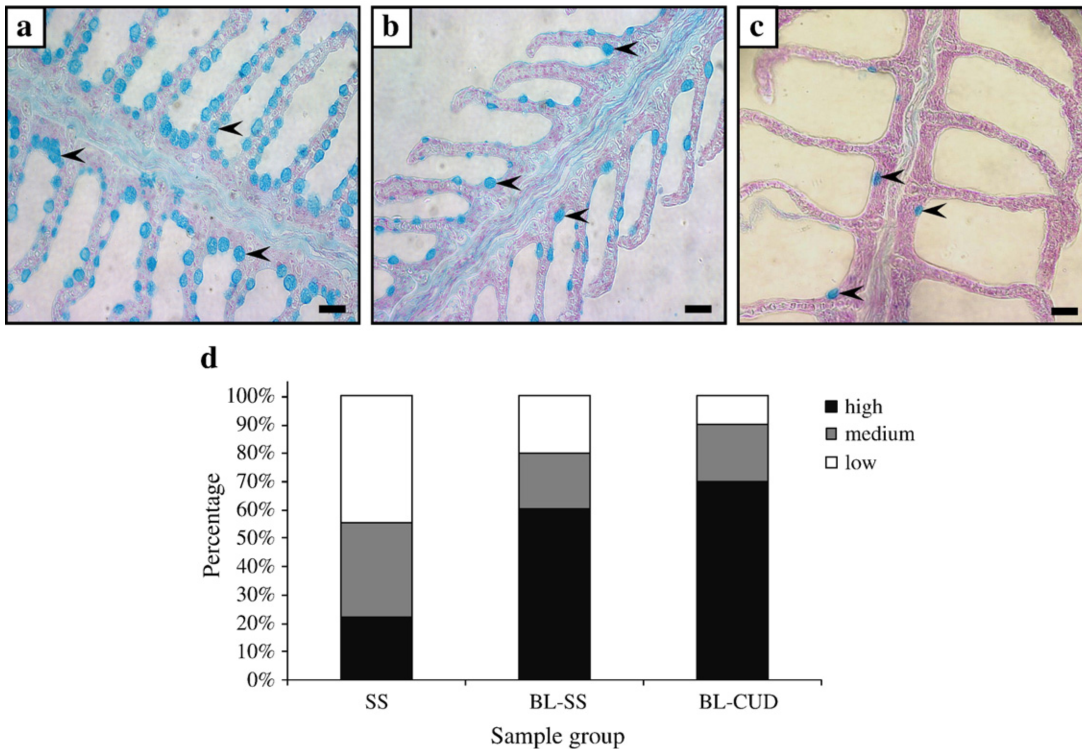


**Fig. 2.** Alcian blue and nuclear fast red stained sections showing mucous cell proliferation (arrow heads) on the gill epithelium in Murray cod, (a) gill lamellae with high mucous cell proliferation, (b) gill lamellae with medium mucous cell proliferation, (c) gill lamella with low mucous cell proliferation and, (d) percentage of animals with high, medium or low mucous cell proliferation in Murray cod cultured at Spirit of the Sea Aquaculture (SS; $n=7$), fish transferred to Brimin Lodge from the Spirit of the Sea Aquaculture (BL-SS; $n=9$), and severely CUD-affected Murray cod at Brimin lodge (BL-CUD; $n=9$). Scale bars = 25 µm.

### 3.5. $Na^+$,$K^+$-ATPase in MRC

$Na^+$,$K^+$-ATPase immunoreactivity occurred in cells located in the interlamellar regions predominantly at the base of lamellae. $Na^+$,$K^+$-ATPase immunoreactivity was also observed on cells extending up the length of the lamellae (Fig. 4). Immunoreactive cells were concentrated on the trailing edge of the filament and were absent or rare on the leading edge in fish from SS and BL. There was no significant difference between the number of immunopositive cells in BL-SS fish (5.50 ± 0.99) when compared to BL-CUD fish (6.07 ± 0.49; results not shown). BL-CUD and BL-SS showed large aggregates of MRC, whereas MRC occurred singly in unaffected SS fish (Fig. 4a–c). Immunoreactivity was not observed in negative controls (results not shown). $Na^+$, $K^+$-ATPase activity in the gills was significantly higher in BL-CUD Murray cod (1.04 ± 0.10 and 0.97 ± 0.11 µmoles ADP $mg^{-1}$ protein $h^{-1}$) compared to BL-SS fish (0.77 ± 0.08 and 0.67 ± 0.06 µmoles ADP $mg^{-1}$ protein $h^{-1}$) in July and September samples (Fig. 5). Murray cod cultured at SS had relatively low gill $Na^+$, $K^+$-ATPase activity at both sample periods (July: 0.52 ± 0.12 µmoles ADP $mg^{-1}$ protein $h^{-1}$; and September: 0.47 ± 0.11 µmoles ADP $mg^{-1}$ protein $h^{-1}$).

## 4. Discussion

This study was the first to investigate the impact of CUD on the osmoregulatory capability of Murray cod. We clearly demonstrated via determination of blood plasma electrolytes, osmolality and gill $Na^+$,$K^+$-ATPase activities that CUD does not negatively impact osmoregulation, and that severely affected fish may compensate a compromised epithelium by producing more iono-regulatory cells with increased $Na^+$, $K^+$-ATPase activity.

SS stock cultured in the BL groundwater began showing visual signs of CUD approximately five months post transfer, including dark skin coloration, small lesion development, and slight fin erosion. Baily et al. (2005) previously demonstrated that 6-week old juvenile Murray cod showed lesion development after one month in groundwater and fin erosion after approximately two months. Therefore, the condition appears to have a more rapid onset in juvenile Murray cod compared to adult fish. The higher surface area to volume ratio of the smaller animals suggests that they will be exposed to a relatively larger dose of the causative agent, if it is water-borne. Alternatively, susceptibility to CUD may be influenced by the stage of development of fish.

Many factors have been identified that impede efficient osmoregulation in freshwater teleost fish, including disease (Byrne et al., 1995; Chen et al., 2003), water quality (Ultsch et al., 1981) and

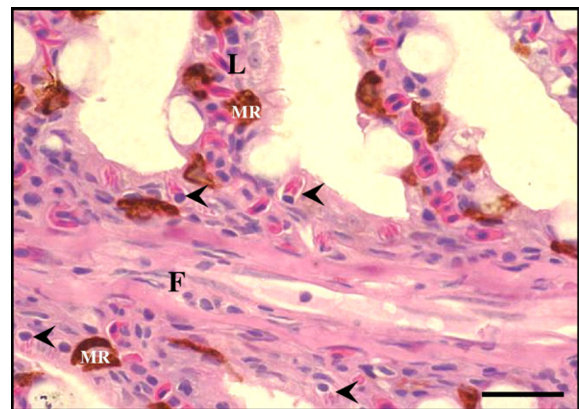


**Fig. 3.** $Na^+$,$K^+$-ATPase immunoreactivity counterstained with haematoxylin and eosin to determine if un-identified cell type might be mitochondria-rich cells. Cross-section of gill filament (F) showing lamellae (L), mitochondria-rich cells (MR) and un-identified cell type (arrow heads). Scale bar = 10 µm.

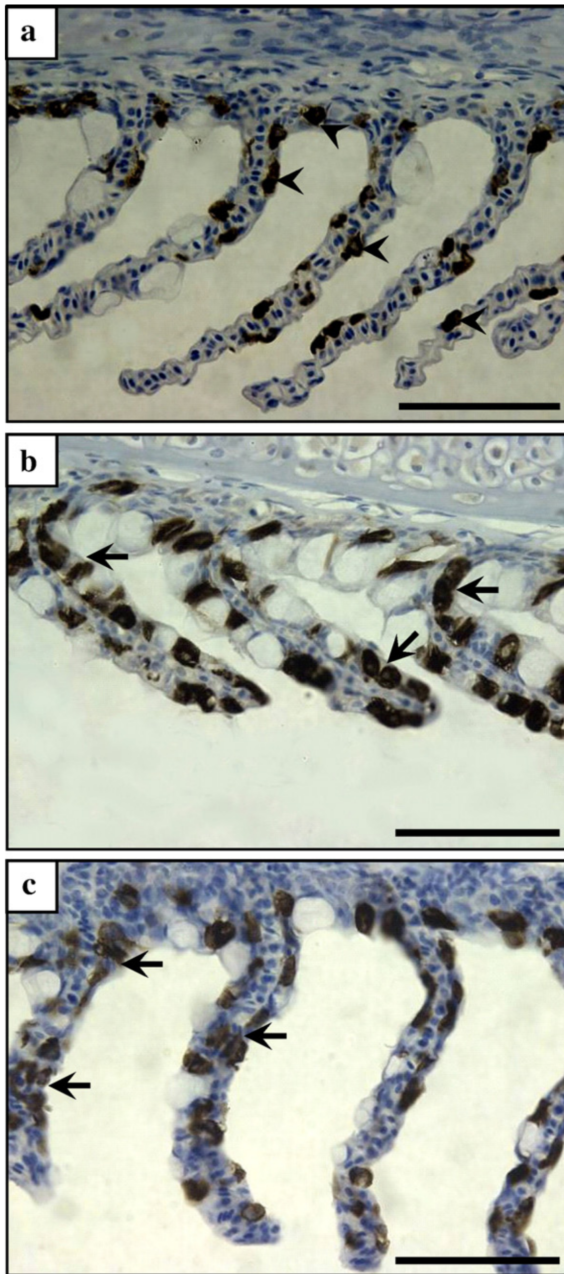


**Fig. 4.** $Na^+,K^+$-ATPase immunoreactivity in MRCs located in the lamellae of Murray cod, (a) SS fish, (b) BL-SS fish, (c) BL-CUD fish. Cross sections of gill filament taken from the trailing edge of the filament and sections counter stained with haematoxylin. Note MRCs occur singly (arrow heads) in non CUD-affected fish (a), and aggregates of MRCs (arrows) are present in fish affected with CUD (b and c). Scale bar = 100 µm.

exposure to toxicants (Wang et al., 1998; Monteiro et al., 2005). To date, there have been no studies conducted on the osmoregulatory capabilities of Murray cod, with baseline blood electrolyte concentrations and osmolality values unknown. Analysis of plasma electrolyte concentrations and osmolality of non CUD-affected Murray cod in a RAS were used as reference values and were consistent with values previously reported for other freshwater teleost fishes, such as rainbow trout, *Oncorhynchus mykiss* (Heming and Blumhagen, 1988; Greco et al., 1995; Wang et al., 1998), carp, *Cyprinus carpio* (Evans 1993), and tilapia, *Oreochromis hybrid* (Wright et al., 1990; Hrubec et al., 2000). Plasma $Na^+$ and $Cl^-$ concentrations and osmolality of BL-SS fish were consistent with values determined for BL-CUD Murray cod. Observed significant differences in plasma $Ca^{2+}$ and $Mg^{2+}$ concentrations between these stocks were consistent with values determined for non CUD-affected fish and were still within the normal ranges previously identified in other freshwater teleost fish (Heming and Blumhagen, 1988; Wright et al., 1990; Evans 1993; Greco et al., 1995; Wang et al., 1998; Hrubec et al., 2000).

Plasma osmolality and $Na^+$ and $Cl^-$ concentrations in the severe CUD-affected fish were not dramatically lower than transferred fish; therefore the compensatory ion uptake must be sufficient for these animals to maintain homeostasis. However, the slower growth rates reported for CUD-affected fish by Baily et al. (2005) may, in part, be attributable to the energetic cost of effectively iono-regulating in the face of a damaged epidermis. Therefore, CUD appears to have no affect on the osmoregulatory capabilities of Murray cod. The suggested ion losses, if present, across the eroded epidermis were not sufficient enough to cause any visible osmotic stress in Murray cod. It would be interesting in future investigations to determine the rate of $Na^+$ and $Cl^-$ loss across the damaged epidermis in CUD-affected Murray cod.

Immunohistochemistry for $Na^+,K^+$-ATPase in the gills of Murray cod localized the enzyme to cells predominantly located on the trailing edge of the gill filaments. Specifically, positive immunoreactivity occurred on cells that were located on the lamellae and the interlamellar region. The location and intensity of the $Na^+,K^+$-ATPase immunoreactivity is consistent with these cells being mitochondria-rich cells (MRC) also referred to as chloride cells (Choe et al., 1999; Katoh et al., 2003). These cells are the iono-regulatory cells of the gills, and therefore express large amounts of $Na^+,K^+$-ATPase protein. Proliferation of MRC is a common pathology to water-borne toxicants in fish (Takashima et al., 1995), but there was no apparent increase in cell number between BL-SS and BL-CUD groups. Murray cod from BL, BL-SS and BL-CUD, showed aggregates of MRC cells, whereas, MRC occurred singly in SS fish. It is uncertain whether the aggregates of MRC are a result of CUD or the difference in ion concentration of the water at BL compared to SS. Nevertheless, the increase in $Na^+,K^+$-ATPase activity is reflective of increased ion uptake in CUD-affected Murray cod, presumably to offset an increase in ion loss across the damaged epithelium. Notwithstanding this, the low $Na^+,K^+$-ATPase activity in the gills of unaffected fish from SS may have been attributed to the higher salt concentration in the SS water source. Scott et al. (2005) found that $Na^+,K^+$-ATPase activity in the gills of the common killifish, *Fundulus heteroclitus*, was lower when animals were acclimated to brackish water than when they were acclimated to freshwater. It is possible that the increased ions in the external media could lower the transepithelial potential, reducing the workload of the $Na^+,K^+$-ATPase. Future studies should attempt to discern the relative influence of CUD and culture water ion composition on $Na^+,K^+$-ATPase activity and iono-regulatory cell number.

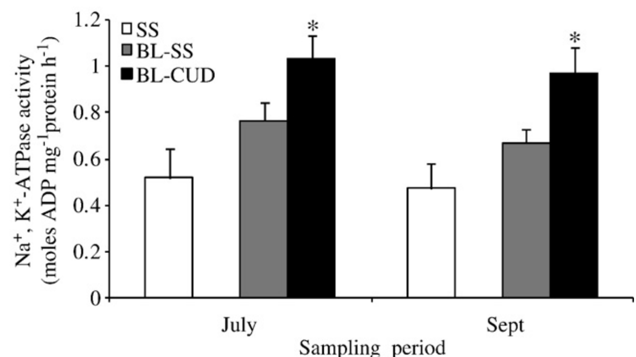


**Fig. 5.** Activity of branchial $Na^+,K^+$-ATPases (mean ± SE, $n = 8$) in non CUD-affected Murray cod at Spirit of the Sea Aquaculture (SS), SS Murray cod transferred to Brimin Lodge (BL-SS) and CUD-affected Murray cod at Brimin Lodge (BL-CUD). Asterisk (*) denotes any significant differences observed between BL-SS and BL-CUD (ANOVA, $p < 0.05$).

Histological analysis of Murray cod gills revealed a proliferation of mucous cells in CUD-affected fish, and although it was not uniform across all fish, it was far more likely to occur in CUD fish than non CUD-affected fish. Mucous cell proliferation is a non-specific response to a stressor observed in a variety of situations, including disease (Roberts and Powell, 2003), metal toxicity (Lock and van Overbeeke, 1981; Julliard et al., 1993; Anderson et al., 1995; Tao et al., 2000) and acid toxicity (Miller and Mackay, 1982). Mucus has an important role in fish homeostasis, providing a protective function as a physical barrier between the epithelium and the environment. In addition, immunoglobulins have been detected in the mucus of plaice, *Pleuronectes platessa* (Fletcher and Grant, 1969), and the armored catfish, *Ictalurus punctatus* (Zilberg and Klesius, 1997). These results suggest mucus also has a role in specific defenses against pathogens. The secretion of mucus creates an unstirred layer directly next to the gill epithelia, resulting in the movement of ions which are solely dependent on diffusion in this layer (Coombs et al., 1972; Schlichter 1982). An increased number of active mucous cells in the gills of CUD-affected Murray cod could result from a need to increase the unstirred layer in the gills, possibly to prevent an irritant from coming into contact with the gill, to assist in ion uptake, and/or to replace mucus being sloughed off or broken down.

An unknown cell type was observed in the gills of all Murray cod cultured in groundwater at BL, irrespective of the severity of the symptoms displayed. The unknown cells were not immunopositive for $Na^+$,$K^+$-ATPase and were similar in appearance to rodlet cells, which are usually found in epithelial tissues. The function of rodlet cells is not entirely clear, but they are most likely holocrine immune cells and increases in rodlet cell numbers have been observed in incidences of parasite infection (Dezfuli et al., 2003; Dezfuli et al., 2004; Bosi et al., 2005; Mazon et al., 2006), osmotic shock (Dezfuli et al., 2006), $Ca^{2+}$ deficient water (Balabanova 2000), and exposure to pesticides (Dezfuli et al., 2006). The appearance of rodlet cells can therefore occur in the absence of lesions, pathogens or without any other inflammatory response (Manera and Dezfuli, 2004), which appears to be the case in CUD-affected Murray cod. Since no pathogens have ever been associated with CUD, the appearance of the presumed rodlet cells are more likely in response to the currently unknown disturbing agent in bore water. Further study using electron microscopy would be needed to confirm the identity of the unknown cell type.

Despite the compromised epidermal layer, CUD-affected Murray cod were able to effectively maintain their plasma ion concentrations and osmolality within ranges consistent with non CUD-affected fish and other freshwater teleost species. Whilst osmoregulation appeared to be unaffected, gill histology demonstrated that CUD-affected Murray cod had increased mucous cells and an unknown cell type in the gills, indicating that a water-borne irritant may be present in the BL groundwater that resulted in the development of CUD. This theory is consistent with the observation by Baily et al. (2005), who showed that lesions of CUD-affected Murray cod resolved when transferred into water that was sourced from the Murray river. Studies are currently underway to identify the unknown cell type in the gills of CUD-affected fish and to establish the component of the groundwater responsible for the CUD syndrome.

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