

**A11/C6–EXTREME ENVIRONMENTS–OSMOREGULATION AND TOXICOLOGY**

Organised by G. Cramb, M.C. Thorndyke and R.D. Handy for the Osmoregulation Group

**A11/C6.1–Osmotic and ionic regulation in European eels during sea-water acclimation and during the yellow- to silver-eel transition**

J.C. Rankin, Huddersfield, UK

Abstract not supplied

**A11/C6.2–Expression of multiple Aquaporin channels in the osmoregulatory tissues of the European eel (*Anguilla anguilla*)**

C.P. Cutler and G. Cramb, School of Biology, University of St Andrews, UK

During acclimation of eels from freshwater (FW) to sea-water (SW), water losses across body surfaces are counteracted by a drinking response, which subsequently involves the uptake of water from the intestine. Duplicate AQP1 aquaporin water channel genes have been isolated from the eel. The first AQP1 isoform was expressed in a wide range of tissues and at high levels in the intestine, where mRNA abundance increased considerably in both yellow and silver developmental stages following SW transfer. Overall higher levels of AQP1 mRNA were found in silver rather than yellow eel intestine. The other eel AQP1 isoform (AQP1 dup) mRNA was found in a smaller range of tissues but its expression was sporadic. AQP 1 mRNA was most abundant in the posterior segment of the intestine with intermediate levels in the middle and lower levels in the anterior region of both yellow and silver eels. Significant up-regulation of intestinal AQP1 mRNA expression was found in all segments and both developmental stages. Both yellow and silver eels had similar intestinal AQP1 expression profiles following SW transfer, with mRNA levels slightly decreased after 6 hours followed by increases after 2 days, 7 days or 3–4 months. However, the data also indicated that AQP1 mRNA expression levels reach maximum faster in silver than yellow eels following SW transfer. The data suggests that AQP1 may play a role in intestinal water uptake following drinking. (Supported by a project grant from NERC).

**A11/C6.3–Cellular distribution of Aquaporin 3 within the gill, kidney and gastrointestinal tract of the European eel (*Anguilla anguilla*)**A.S. Martinez<sup>(1)</sup>, J.H. Lignot<sup>(2)</sup>, C.P. Cutler<sup>(1)</sup> and G. Cramb<sup>(1)</sup>, <sup>1</sup>University of St Andrews, UK; <sup>2</sup>Université Louis Pasteur/CNRS CEPE, Strasbourg, France

In teleost fish such as the European eel, water transport plays a crucial role in osmoregulation in both freshwater (FW) and seawater (SW) environments and a number of tissues work together to maintain body fluid homeostasis. A key water channel protein Aquaporin-3 (AQP3) has been identified in the 'silver' life stage of the European eel, *Anguilla anguilla*. Using a specific polyclonal antibody directed against the C-terminal of the eel protein, AQP3 was found to be expressed in the renal, intestinal, branchial and oesophageal tissues of SW- and FW-acclimated eels and also in the intestinal rectal segment of SW- fish. Western blotting identified a 24 kDa protein in the gills from both FW and SW eels and immunohistochemistry localised the protein to membranes within the chloride cells. SW acclimation induced a three-fold decrease in the branchial protein level. In both FW and SW fish, AQP3 was detected in the brush border membrane of a subset of renal tubules, on the plasma membranes of basal epithelial cells and in epithelial cells within the anterior part of the oesophagus-staining in the posterior segments was restricted to mucous cells. AQP3 also stained macrophages within the intestinal epithelium of FW and SW eels and mucous cells in the rectal epithelium of SW- fish. These results suggest that AQP3 may play an important functional role in water and/or small solute transport in the major osmoregulatory organs; the role of this protein in the physiological processes associated with the acclimation of eels to different environmental salinities will be discussed. (Supported by a project grant from NERC).

**A11/C6.4–Excretion and reabsorption of cations and water in the kidney of different sturgeon species during adaptation to sea water**

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Inulin was used in order to measure the levels of the excretion and the reabsorption of the cations and the

water in the kidney.  $\text{Li}^+$  was used as the marker for the estimation of proximal and distal transport of the water in the nephron. Different Caspian species of acipenserids, that have different ability of osmotic and ionic regulation, were the objects for present investigation: freshwater, potamodromous starlet *Acipenser ruthenus* (that can ability to adapt to brackish water as the com-former), diadromous brackishwater Russian sturgeon *A. gueldenstaedtii* and starred sturgeon *A. stellatus* (that are adapted to brackish water as the regulators). The latter is the most euryhaline species from Caspian acipenserids. In fresh water (FW) these species have similar concentrations of corresponding cations in blood serum. Also Russian sturgeon and starred sturgeon have equal ( $p > 0.05$ ) diuresis (accordingly  $0.82 \pm 0.08$  and  $0.98 \pm 0.09$  ml/h/100 g weight) whereas starlet has higher diuresis ( $1.13 \pm 0.1$  ml/h/100 g weight), than Russian sturgeon has. The excretions of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  by the kidney of three species in FW were equal, whereas the excretion of  $\text{Mg}^{2+}$  by the kidney in starlet was lower than in other species. In FW the kidney fulfils ion sorption function. The kidney of fresh water starlet yields to the kidney of brackish water species in the ability to reabsorb the water significantly. This ability is displayed specially during the adaptation of studied fish to sea water (SW) of 12.5‰ (average salinity of Caspian sea). In FW the reabsorption of water in kidney of the starlet is about 30%, but in SW this level raises only to 50%, whereas in Russian sturgeon and starred sturgeon corresponding values were 50% in FW and increase to 80–90% in SW. Netto of water reabsorption takes place in postproximal segments of the nephron. In SW the diuresis decreases in starred sturgeon in 5 times, in Russian sturgeon in 4 times, in starlet in 2 times, but the latter level is higher in 3 times than the level in starred sturgeon ( $p < 0.05$ ). As a result of high diuresis and high sodium concentration in the urine of the starlet, its kidney excretes  $\text{Na}^+$  more than the kidney of other species. In SW it is observed the secretions of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the kidney of starred sturgeon, only the secretion of  $\text{Mg}^{2+}$  and the reduction of  $\text{Ca}^{2+}$  sorbtion ( $p < 0.001$ ) in the kidney of Russian sturgeon and secretion of  $\text{K}^+$  and  $\text{Ca}^{2+}$  in the kidney of the starlet.

This research is supported by Russian Foundation for Basic Research (grants 01-04-49808 and 02-04-48426).

#### **A11/C6.5—Functional restoration of rat intestinal mucosa before and after refeeding**

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Three metabolic adjustments occur during fasting with first, a rapid period marked by increasing mobilisation of fat stores followed by a longer period during which

most of the energy expenditure is derived from lipids while body proteins are efficiently spared. A third phase occurs at an advanced stage, and shows an increase in protein use while fat stores are progressively exhausted. Also, in rat jejunum, crypt cell cycles are stopped following DNA synthesis during phase II, but a marked rise in crypt cell proliferation occurs in phase III, suggesting energy sparing in phase II, and refeeding anticipation in phase III.

In order to study in greater detail the correlations between these metabolic changes and the variations in organ functions, expression and localisation of membrane transporters such as SGLT1 and PepT1 were assessed using rat small intestine as a model, after both phase II and phase III fasting periods and immediately after refeeding. SGLT1 and PepT1 expressions in the brush-border membranes were higher in phase III than in fed animals (controls) and during phase II. Also, after refeeding, morphological and functional restorations of the intestinal mucosa were observed within less than 24 h.

Starved rats in phase II must therefore be in a phase of energy sparing, and could anticipate refeeding in phase III by an early and costly increase in cellular kinetics and protein synthesis. This anticipation in phase III which is concomitant with an increase in food foraging behaviour, could induce optimal absorption of nutrients when food is ingested.

#### **A11/C6.6—The effect of hormones on the osmoregulatory properties of the intestine of the euryhaline European eel, *Anguilla anguilla***

C. Phillips, C.P. Cutler, G. Cramb and N. Hazon, School of Biology, University of St Andrews, UK

The gastrointestinal tract is a key organ involved in maintaining salt and water balance in the euryhaline eel as it migrates between fresh and seawater. In the freshwater environment there is an osmotic gain of water over semi-permeable membranes. However teleost fish maintain osmotic balance by drinking the environmental medium, sodium chloride and other ions are actively absorbed in the intestine and water follows. This is controlled by a number of hormones, including cortisol and angiotensin II, which enhance the ability of fish to survive in these environmental extremes. Hormones such as cortisol and angiotensin II may affect intestinal sodium and water transport and therefore could be essential in the osmoregulatory plasticity of the animal during migration. Using intra-peritoneal implanted mini-osmotic pumps was the effects of singly infused cortisol and angiotensin II was investigated on excised stripped and everted intestines. Investigation of sodium and water uptake was investigated in specific regions previously

identified as having high transport capacity, inhibitable by drug application, and compared between groups of control, and hormonally infused animals. (Supported by a project grant and studentship from NERC).

### **A11/C6.7—Cloning and expression of guanylin-like peptides in teleost fish**

G.D. Wilson, C.P. Cutler and G. Cramb, School of Biology, University of St. Andrews, UK

Euryhaline teleosts migrate between SW environments and FW lakes and rivers at various stages in their life cycle. During such migrations, fish face a number of osmotic challenges. FW is a hypo-osmotic environment which results in an influx of water across permeable body surfaces (predominately the gills). To counteract this effect the fish excrete large volumes of urine and limit their rate of drinking. In contrast when in the hyperosmotic SW environment, fish lose water and cellular dehydration occurs. To overcome these effects fish drink copious amounts of SW and ions and water are taken up across the intestine by regulated ion and water transporters which control the rate and the amount of solute/water absorption. The absorbed monovalent ions are actively secreted back to the environment by gill chloride cells as the water is retained. A number of neuronal and hormonal mechanisms have been identified in teleosts which act together to control fluid absorption and secretion thus maintaining body fluid homeostasis even at the extremes of environmental salinity. We hypothesise that the 15 amino acid peptide hormone guanylin also contributes to this osmotic regulation. Little is known about the physiological roles of this peptide in species other than a few mammals. Using cloning, sequencing and expression techniques our present data suggests that guanylin-like peptides are present in both stenohaline and euryhaline teleosts and in the European eel we have identified two distinct members of the guanylin family. The effect of FW/SW acclimation in the expression of these genes will be discussed. (Supported by a NERC studentship)

### **A11/C6.8—(New presentation) Comparative genomic sequence analysis of CFTR: New Findings**

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The cystic fibrosis transmembrane conductance regulator (*CFTR*) has a highly regulated expression pattern,

but the sequences regulating its transcription remain poorly characterized. We have used a genomics approach (phylogenetic footprinting) to characterize regulatory elements involved in *CFTR* expression. We cloned and sequenced the *CFTR* gene of a euryhaline teleost, the killifish, *Fundulus heteroclitus*, and compared it to teleost and mammalian homologues (*Fugu rubripes*, *Tetradon nigroviridis*, human and mouse). Although the non-coding sequences were generally poorly conserved, several important regulatory elements were identified. A DNase I hypersensitive site in the first intron of *hCFTR*, which correlates with *hCFTR* expression, was conserved in both *mCFTR*, *FuguCFTR* and *kfCFTR*. Four transcriptional elements were common to all the *CFTR* homologues examined: a CCAAT element, cAMP responsive element (CRE), Sp1 element, and glucocorticoid responsive element (GRE). We also cloned and sequenced the *CFTR* promoter from 8 additional *Fundulus* species with varying salinity tolerances. Although the sequences were very similar, a number of important differences emerged, including lack of conservation of many of the elements that were conserved between *F. heteroclitus* and *Fugu rubripes*. We are currently using transient transfection analyses to determine the functional significance of these changes. We conclude that while genomic sequence comparisons can help to identify some highly conserved elements, the pattern of conservation is not always maintained as additional taxa are added. These results point out the advantages and limitations of phylogenetic footprinting as an approach for discovering novel regulatory elements. Supported by NSERC.

### **A11/C6.9—The role of PTHrP in the calcium homeostasis of the European flounder, *Platichthys flesus***

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The recent development of a homologous radioimmunoassay capable of detecting circulating levels of PTHrP in fish (Rotlland et al. unpublished) has allowed preliminary insight into the effect of change in external environment on this hormone. The assay applied to flounder samples is sensitive and accurate with a least detectable limit of 2.5 pg in 25  $\mu$ l of plasma, and intra and inter assay variation coefficients of 4.776% and 10.4% respectively. Initial measurements from animals adapted to seawater (SW) and freshwater (FW) indicate comparable circulating levels at  $0.115 \pm 0.012$  and  $0.182 \pm 0.023$  nmol/l respectively (ANOVA  $p > 0.05$ ). However, in response to an extreme osmoregulatory challenge, de-ionised water (DIW), circulating levels were significantly elevated above those animals adapted

to SW at  $0.186 \pm 0.006$  nmol/l (ANOVA  $p < 0.05$ ). Investigations examining the time course adaptation of animals moving from a high (SW) to a low (FW) calcium environment have revealed that PTHrP levels increase above baseline levels after 24 hours. PTHrP levels return to those of SW adapted controls after 14 days of adaptation to FW. Initial observations also indicate that PTHrP levels fall transiently when fish are moved from FW to SW. These data support the notion that PTHrP may play a role in the calcium homeostasis of the European flounder acting as a hyper-calcitropic factor. This work is supported by the BBSRC.

#### **A11/C6.10—Acclimation of rainbow trout to ion-poor water: cortisol, corticosteroid receptors and ionic regulation**

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The main corticosteroid hormone in fish, cortisol, plays a dual role as a stress hormone (glucocorticoid action) and in the regulation of salt and water balance (mineralocorticoid action). Traditionally, both actions were considered to be mediated by a single receptor of the glucocorticoid subtype (GR). Recently, however, a mineralocorticoid-like receptor (rtMR) was cloned from rainbow trout testis (Colombe et al., 2000, *Steroids* 65:319–328). In the present study, the acclimation of rainbow trout to ion-poor water was used as a tool to investigate the function and regulation of GR and MR in the freshwater fish gill. Juvenile rainbow trout were either maintained in dechlorinated city-of-Ottawa tap water, or were acclimated to artificial soft water (obtained by diluting dechlorinated tap water with reverse osmosis water) for periods of 24 h to 5 days. Plasma cortisol and ion concentrations were essentially unaffected by softwater acclimation. Branchial GR protein content (quantified by Western blots and densitometry) and mRNA expression (quantified using real time PCR) decreased significantly as a result of acclimation to ion-poor water, results that do not conflict with previous findings that a GR antagonist was ineffective in blocking chloride cell proliferation during exposure of rainbow trout to ion-poor water (Sloman et al., 2001, *J. Exp. Biol.* 204:3953–3961). Interestingly, however, while a MR antagonist was found previously to prevent chloride cell proliferation (Sloman et al. 2001), branchial MR mRNA expression (quantified using real time PCR) also decreased significantly during softwater acclimation.

#### **A11/C6.11—Freshwater gills and water-exposed gill cell cultures: physiology and proteomics**

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Rainbow trout gill cells may be cultured, on permeable supports, as intact epithelia. These cultures can then be polarised, by the addition of water to the apical surface. Many of the physiological parameters which have been measured, such as ammonia transport, lipid and protein metabolism, and intracellular sodium content have proven very similar to the fish gill *in vivo*. Measurement of Trans-Epithelial Resistance (TER) provides an assessment of overall epithelial integrity. TER is considerably higher in polarised epithelia than in non-polarised epithelia. Although TER is not related to total cellular protein, it is reduced by inhibiting protein synthesis. Therefore our primary aim was to determine whether gill epithelial polarity is the result of the expression of specific proteins. To achieve this we have made a proteomic comparison of both polarised and non-polarised cultured gill cells and whole gills. This investigation also had a secondary aim. The accepted media supplement, in gill cell culture, is foetal bovine serum (FBS), which clearly contains proteins which are 'foreign' to rainbow trout. Therefore, to refine our primary objective, we also evaluated the substitution of rainbow trout plasma for FBS and thus continued the development of this cellular model as an investigative tool. (supported by NSERC and the European Commission).

#### **A11/C6.12—The 'Cl<sup>-</sup> uptake Metabolon' in freshwater fish – an active mechanism for Cl uptake from freshwater**

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Mitochondria rich cells (MR cells) of the rainbow trout undergo changes in relative distribution and biochemical function during acclimation to one third strength (10 ppt) and full strength seawater (30 ppt). Differential changes in Na<sup>+</sup>/K<sup>+</sup>-ATPase and H<sup>+</sup>-ATPase activity occurred in each of two defined MR cell types during seawater acclimation. Importantly, a high H<sup>+</sup>-ATPase activity in PNA<sup>+</sup> cells was found as was a decrease in activity during seawater acclimation. Re-evaluation of the current model in light of these and other results in the literature suggests that H<sup>+</sup>-ATPase plays a significant role in Cl<sup>-</sup> uptake and acid-base regulation in

freshwater fish. Chloride uptake from very dilute (e.g. 50  $\mu\text{M}$ ) freshwater does not have a favourable driving electrochemical gradient from the water to the fish. Similarly, a strict gradient for  $\text{HCO}_3^-$  from the blood to the water is not sufficient to provide a driving gradient for  $\text{HCO}_3^-$  excretion via an electroneutral anion exchanger under normal conditions. We propose that  $\text{Cl}^-$  uptake is driven by a functional coupling of a basolateral  $\text{H}^+$ -ATPase, an apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (molecular identity as yet undetermined) and an associated carbonic anhydrase. This 'Cl- uptake metabolon', or metabolic unit, functions by actively secreting  $\text{H}^+$  in the basolateral direction, creating a sufficiently high  $\text{HCO}_3^-$  concentration in the local intracellular area of the anion exchanger to drive  $\text{Cl}^-$  uptake via an electroneutral exchanger.

#### **A11/C6.13–Ingestion of water by freshwater fish, effects on osmoregulation and effects of waterborne toxins**

F.B. Eddy, J.H. Best, J. Metcalf and G.A. Codd, University of Dundee

Whilst the control of drinking in freshwater fish has been investigated there is very little information on what happens to the water once it enters the fish. When adult rainbow trout were fed a dry diet, moistening of the gut contents for digestive processes was achieved through increased drinking as well by as fluid secretion from the gut (Ruohonen et al., 1997). Such results indicate that imbibed water is subjected to a complex pattern of absorptive and secretory events in the gut. There is little understanding of the physiological consequences of increased drinking rates when freshwater fish are exposed to waterborne toxins. Lipopolysaccharide (LPS) endotoxins occur at or near the cell surface of Gram negative bacteria, including the cyanobacteria and it can be hypothesised that LPS stimulates the release of NO which in turn stimulates the production of AII, so increasing drinking rates. Greater volumes of water entering the fish would increase exposure of the gut to heterotrophic bacteria and cyanobacteria and increase the opportunity for absorption of the microbial and other water-borne toxins, with the potential for damage to internal organs such as the liver. This idea was tested by designing experiments to determine water uptake in rainbow trout in response to LPS in the presence and absence of whole or disrupted *Microcystis* cells and subsequent indications of liver damage.

#### **A11/C6.14–The importance of socially-mediated individual variation in ionoregulatory ability for toxicological studies in freshwater fish**

K.A. Sloman, G. R. Scott, D.G. McDonald and C.M. Wood, Biology, McMaster University, Hamilton, Canada

We have demonstrated previously that social status of a salmonid fish will affect the uptake rate of sodium from the water and that this has implications for the uptake and accumulation of toxicants that cross the gill epithelia via sodium transport pathways. Subordinate fish will accumulate more copper and silver when compared with dominant fish, due to their higher sodium uptake rates. Here we examined sodium turnover in dominant and subordinate fish in more detail to determine the differences in physiology that are induced by social competition. Branchial and renal sodium loss were both significantly higher in subordinate trout, which resulted in an increased need for sodium uptake from the environment. Branchial sodium loss was by far the largest component of the effect. However, urine flow rate was also elevated, suggesting increased water uptake, but glomerular filtration rate was unaffected. Therefore, subordinate fish, whose ionoregulatory status is already compromised, would likely have much lower thresholds of effect for ionoregulatory toxicants and higher ionoregulatory costs. Subordinate fish may also be at a disadvantage when encountering other ionoregulatory challenges e.g. acclimation to soft water or more acidic water. (Supported by an NSERC Discovery Grant to CMW).

#### **A11/C6.15–Kinetic analysis of Pb-induced ionoregulatory disruption in rainbow trout (*O. mykiss*): effects on $\text{Ca}^{2+}$ , $\text{Na}^+$ , and $\text{Cl}^-$ balance and the role of ion transport enzymes**

J.T. Rogers and C.M. Wood, Biology, McMaster University

Previous studies have shown that exposure to waterborne Pb levels close to the 96 h LC50 of 1 mg Pb  $\text{L}^{-1}$  determined in Hamilton-city dechlorinated tap-water (hardness = 140 mg  $\text{L}^{-1}$  as  $\text{CaCO}_3$ ) has no respiratory impact but rather results in inhibition of branchial  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  influx and subsequent reductions in plasma levels of these ions. These impacts are also observed at Pb concentrations approaching environmental relevance (0.5, 0.25, 0.1 mg Pb  $\text{L}^{-1}$ ). Cir-

cumstantial evidence suggests that Pb-induced hypocalcemia is due to competitive inhibition of calcium uptake. Kinetic analysis revealed typical Michaelis–Menten relationships suggesting competitive inhibition of calcium uptake by Pb. Increases in  $K_m$  values were observed with increasing concentrations of Pb while  $J_{max}$  values did not change significantly. Additionally, an approximate 80% inhibition of high-affinity  $Ca^{2+}$ -ATPase activity occurred after 96 h of acute Pb exposure that could suggest a non-competitive component of uptake inhibition after prolonged exposure to elevated Pb concentrations. Comparably, kinetic analyses of  $Na^+$  and  $Cl^-$  uptake in the presence of Pb suggest that inhibition of  $Na^+/Cl^-$  influx is non-competitive (no change in  $K_m$  values) and is likely due to inhibition of  $Na^+/K^+$  ATPase activity observed upon acute Pb exposure.

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#### **A11/C6.16–Histopathological responses in the gills of the freshwater crab, *Potamonautes warreni* exposed to microbial infestations and cadmium**

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Uptake, transport, storage and defence mechanisms were studied in the freshwater crab, *Potamonautes warreni* harbouring microbial gill infestations and exposed to increasing chronic (0.2, 0.5, 1.0  $mg\ l^{-1}$ ) and acute (2.0  $mg\ l^{-1}$ ) Cd concentrations under controlled laboratory conditions over a period of 21 days. Transmission electron microscopy and X-ray microanalysis showed the loss of microbial gill fauna on exposure to 0.2  $mg\ Cd^{2+}\ l^{-1}$ , Cd becomes incorporated into the crystal deposits and permeates the gill cuticle of *P. warreni*. Degeneration of apical membrane infoldings and vacuolation of epithelial cells occurred together with pinocytosis, endocytosis and pronounced phagocytic activity. Elevated exposures (0.5 and 1.0  $Cd^{2+}\ mg\ l^{-1}$ ) resulted in the swelling and dissociation of mitochondrial outer membranes together with an increase in the transport of Cu, Cl and S by hemocytes to epithelial tissues, depleted of these elements. Cadmium also accumulated in concentric membrane whorls in the hemal canal in aggregates of lysosome-like bodies in the cuticulin-secreting cells of the gill stem. Chronic Cd exposure induced increased fatigue and a mild uncoordinated motor activity in the crabs. At acute exposure of 2.0  $mg\ l^{-1}$  over 48 h, *P. warreni* showed a time-specific rapid loss of motor function, with mild cellular lesions occurring in

gill tissues. The significance of cellular changes in the gill epithelia and the altered motor activity of *P. warreni* with an increase in waterborne Cd concentrations are discussed as potential biomarker responses for monitoring aquatic pollution.

#### **A11/C6.17–(Note New Title) – A teleost fish endocrinological paradox – three functionally distinct corticosteroid receptors and only one ligand, cortisol**

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In teleost fish the corticosteroid cortisol regulates both glucocorticoid and mineralocorticoid actions, unlike mammals where these functions are regulated by cortisol and aldosterone, respectively. Initially the actions of cortisol were believed to be controlled by a single glucocorticoid receptor (GR), but we have identified a cDNA clone (rtGR2) in rainbow trout showing high sequence homology with other GRs, and different from the initial trout GR (rtGR1). The discovery of a mineralocorticoid receptor in trout suggests a far more complex regulation of corticosteroid induced gene expression in fish. Phylogenetic analysis indicates GR gene duplication is common to fish. Real-time PCR reveals that the two GRs are ubiquitous, and the dexamethasone-binding affinities ( $K_d$ ) of rtGR1 or rtGR2 are similar. Differences exist between the two receptors in their transactivational activity. Co-transfection of rtGR1 or rtGR2 expression vectors in Chinese hamster ovary (CHO-K1) cells along with a reporter plasmid (MMTV-LUC) containing multiple glucocorticoid response elements (GRE) shows that both receptors initiate transactivation in the presence of cortisol and the synthetic glucocorticoid dexamethasone. But, there is a pronounced difference in the sensitivity, rtGR2 being over an order of magnitude more sensitive than rtGR1. This is confirmed by:

1. similar sensitivity patterns being observed in COS-7 (derived from green monkey kidney) cells, and with different reporter plasmids;
2. inhibition of transactivation by the antagonist RU486 being more effective with rtGR1.
3. 1  $\mu M$  aldosterone and deoxycortisol only inducing rtGR2 transactivational activity.

Experiments to determine whether (and how) these receptors regulate different physiological pathways are underway.

### A11/C6.18—Genomic exploration of the metal response in fish

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The metal-responsive transcription factor-1 (MTF-1) is a positive transcriptional regulator that mediates gene expression in response to an increased concentration of loosely ligated zinc in the cytosol. Upon activation by zinc, MTF-1 binds to metal responsive elements (MRE) of target genes where it stimulates transcription. MRE also confer transcription in response to cadmium and silver, but the mechanism involved is less clear. Genes, such as metallothionein, that are directly involved in metal regulation typically have several MRE copies that act synergistically. Conversely, orphan MRE have little influence on gene transcription. Through animal and cell experiments, we have observed surprisingly many genes in teleost fish that are induced by zinc and cadmium, have no obvious direct role in zinc regulation. To examine how widespread MRE mediated gene expression is in teleost fish, we interrogated the *Fugu rubripes* genome for putative MRE regulated genes. A search was constructed to detect predicted genes with at least two conservative MRE core consensus sequences, CGCRCNCG, within  $-2$  kb of the first predicted ORF. This exercise returned 1029 genes, of which 365 had no Ensembl annotation and 664 were possible orthologues to genes with known function. Many of the recognised genes encoded proteins with roles in cell structure, solute transport and signal transduction. A substantial number of brain-specific genes were also found. It seems likely that the widespread occurrence of MRE activated genes reflects the emerging role of zinc in cell signalling processes rather than a stress response to metals.

### A11/C6.19—The nutritional physiology and molecular biology of trace metal homeostasis during dietary iron deficiency and excess in rainbow trout (*Oncorhynchus mykiss*)

P. Carriquiriborde, Antorchas Foundation and National Research Council of Argentina; R.D. Handy, S.J. Davies, R. Serwata, Biological Sciences, University of Plymouth; and N. Bury, Health and Life Sciences, King's College London

The effect of dietary iron levels on the health of rainbow trout was studied. Fish weighting  $79.1 \pm 12.2$  g were fed a semi-purified diet supplemented with 0 (Low), 100 (Normal) and 1500 (High) mg/kg of iron during eight weeks. Survival, growth rate, condition factor and hepa-

tosomatic index were not affected by diet-iron content. Hb and Hct were significantly higher in high-iron fed fish (HIFF) at week-6 but became normal at week-8, low-iron fed fish (LIFF) showed a significant decrease in Hb (from  $7.7 \pm 0.6$  to  $6.6 \pm 0.6$  mg/dL) and MEH (from  $68.9 \pm 6.8$  to  $57.3 \pm 6.2$   $\mu$ g/cell) since week-6 and 8 respectively. Iron increased significantly with time in serum, stomach, intestine and liver of HIFF. There was an inverse relationship between tissue iron status and Mn levels, with Mn levels decreasing in HIFF. Cu content was always lower in the intestine of HIFF and was significantly increased in LIFF at week-8. Serum unsaturated iron-binding capacity was significantly reduced from  $396.0 \pm 40.1$  to  $354.0 \pm 49.3$   $\mu$ g/dL in HIFF since week-4, total iron-binding capacity was significantly lower in LIFF ( $482.0 \pm 55.03$   $\mu$ g/dL) than in HIFF ( $575.6 \pm 107.07$   $\mu$ g/dL) after week-8 and percentage of transferrin saturation was increased 12.6% in HIFF since week-4 and reduced 11.8% in LIFF since week eight. Ferrireductase activity was increased 3.7 fold in the intestine of LIFF and in the liver of HIFF since week-4 and 6 respectively. Lipid peroxidation was significantly increased in intestine and liver of HIFF since week-4 and 8 respectively. Results indicate that rainbow trout is able to regulate iron metabolism in a wide range of dietary iron concentrations and during relative long periods of exposure without detriment in growth and survival.

### A11/C6.20—Acute and chronic effects of waterborne nickel exposure on the rainbow trout (*Oncorhynchus mykiss*)

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Exposure to acutely lethal levels of waterborne Ni (10 mg/L) causes respiratory toxicity in the rainbow trout. Ni acts exclusively at the gill, causing a diffusive limitation of gas exchange, leading to a decrease in arterial oxygen tension, an increase in carbon dioxide tension and subsequent respiratory acidosis. Predictably, ventilatory parameters of adult rainbow trout were significantly affected by 48 h. Ventilation rate, volume, and stroke volume were increased by 11, 95, and 65%, respectively, while oxygen utilization decreased by 28%. Chronic exposure to far lower levels of waterborne Ni (245–395  $\mu$ g Ni/L) also affected respiration in rainbow trout, though effects were seen only in exercised fish. After 35 d of exposure, no differences were seen between control and treated fish in any of 16 respiratory, hematological, metabolic, and ventilatory parameters measured in fish at rest. However, when fish were exercised, experimental animals had a significantly lower maximal oxygen consumption rate (36%) and aerobic scope for activity (37%) than control fish. Following 35

days of recovery in clean water, maximal oxygen consumption (21%) and aerobic scope for activity (26%) were still depressed. Adverse respiratory effects persisted in experimental fish despite almost complete clearance of gill Ni during the 35 day depuration period, suggesting that Ni exposure causes irreversible ultrastructural damage to the gill. This hypothesis is being tested by morphometric analysis of gills from fish exposed to Ni both acutely and chronically. (Supported by NSERC Strategic, NiPERA, ICA, ILZRO, Falconbridge, Noranda, Cominco).

**A11/C6.21—Regulation of squirrelfish metallothionein by zinc and estradiol: Implications in zinc homeostasis and sequestration**

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Squirrelfish of the family, *Holocentridae*, contravene normal zinc accumulation criterion. The females of this family accumulate up to 100 times the amount of Zn in liver tissue than do other known vertebrates. Additionally, female squirrelfish show a significant elevation of Zn in the gonads as compared with other species. Metallothionein (MT) is a small, cysteine-rich, metal binding protein that avidly binds metal ions in the IB and IIB groups of the periodic table. A strong, positive correlation between squirrelfish hepatic MT concentrations and hepatic zinc concentrations has been previously indicated. Recent findings indicate that MT mRNA transcription is up-regulated in response not only to zinc but also to estradiol. Additionally zinc influx rates are higher in cells pre-treated with estradiol. High MT levels and high zinc levels in female squirrelfish are seasonal, and related to reproductive cycles. This presentation illustrates the mechanisms employed by female squirrelfish to enable Zn accumulation in the liver and ovaries while protecting against Zn toxicity.

**A11/C6.22—An in vitro biotic ligand model (BLM) for silver binding to cultured gill epithelia from rainbow trout (*Oncorhynchus mykiss*)**

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A novel double-seeded insert preparation using reconstructed gill epithelia (pavement cells and mitochondria-rich cells) of the rainbow trout in primary culture on filter membrane supports in vitro was employed to develop a possible in vitro BLM for silver. Radio-

labelled silver (<sup>110m</sup>Ag as AgNO<sub>3</sub>) was placed in the apical media (fresh water), and the appearance of <sup>110m</sup>Ag in the cells themselves (binding) and in the basolateral culture media (transport) was monitored. Apical silver exposure up to 100 µg L<sup>-1</sup> had no adverse effects on TER and TEP and there was no effect on Na<sup>+</sup> flux rates or Na<sup>+</sup>-K<sup>+</sup>-ATPase activity. Silver-binding was concentration-dependent and saturable with Km ≈ 10 µg L<sup>-1</sup> (10<sup>-7</sup> M). Na<sup>+</sup> competitively inhibited silver binding to the cells in soft water, whereas Ca<sup>2+</sup> showed no competitive effect. More silver was bound to the cells at higher Cl<sup>-</sup> concentration, whereas elevations in dissolved organic carbon (DOC) significantly reduced silver binding. Greater silver binding to the cultured epithelium was observed at lower pH. These results suggest that silver, possibly Ag<sup>+</sup>, can enter the cells through Na<sup>+</sup> channels, but not through Ca<sup>2+</sup> channels, while AgCl may enter passively via diffusion across the apical surface. Similarities and differences from the in vivo BLM for silver (1) will be discussed (supported by Kodak Canada & NSERC CRD Program).

(1). McGeer, J.C., Playle, R.C., Wood, C.M., and Galvez, F. (2000). A physiologically based BLM for predicting the acute toxicity of waterborne silver to rainbow trout in fresh waters. *Env. Sci. Technol.* 34: 4199–4207.

**A11/C6.23—Plasma clearance of cadmium and renal ionoregulation in chronically cadmium pre-exposed trout**

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Chronic effects of metals in fish are not yet well defined. Our study examines the plasma clearance of cadmium (Cd) and renal ionoregulation in rainbow trout (*Oncorhynchus mykiss*; ~0.3 kg) after exposure to waterborne Cd (3 µg/L = 26.7 nmol/L) or dietborne Cd (500 mg/kg = 4.45 mmol/kg diet) for 30 days. Following pre-exposure, the clearance of a single bolus of <sup>109</sup>Cd (7 µg/kg = 64.4 nmol/kg) injected into the bloodstream through an indwelling arterial catheter (waterborne group) or a single bolus of <sup>109</sup>Cd (290 µg/kg or 2580 nmol/kg) infused via a stomach catheter (dietborne group) were determined. Plasma clearance of Cd in waterborne Cd-acclimated fish was faster than that in non-acclimated trout. Pre-exposure to dietary Cd increased the uptake rate of Cd from the gut into the blood plasma, but did not affect the clearance kinetics of Cd from the plasma. Plasma clearance of Cd in the dietary acclimation group was lower than Cd clearance in waterborne acclimation group. Trout pre-exposed to Cd are presently being examined for ionoregulatory functions of the kidney by collecting urine samples via surgically implanted urinary bladder catheters. Glomerular filtration rate (GFR), Urine flow rate (UFR),

ammonia, urea, pH, glucose, and urinary excretion of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cl}^-$  and  $\text{Cd}^{2+}$  are being determined. We conclude that metal-acclimation plays an important role in internal metal handling for detoxification and homeostasis in trout, though responses differ between waterborne and dietary acclimation to Cd (Supported by NSERC Strategic, ILZRO, NiPERA, ICA, Falconbridge, Cominco, and Noranda).

### **A11/C6.24–Apoptosis, cell proliferation and migration in rat small intestine during the Metabolic adjustments occurring through fasting and after refeeding**

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The effects of fasting and refeeding on apoptosis, cell proliferation and migration were studied in rat jejunal mucosa in order to relate the phenotypic flexibility of this organ to the three metabolic changes occurring during fasting. These metabolic adjustments are 1/a rapid increase in fat store mobilisation, 2/a longer period during which most of the energy expenditure is derived from lipids while body proteins are efficiently spared and 3/an increase in protein use while fat stores are progressively exhausted.

The cellular mechanisms of this phenotypic plasticity in the small intestine were assessed using classical histology, immunohistochemistry and Environmental Scanning Electron Microscopy (ESEM).

ESEM observations using fresh and unfixed tissues confirmed the importance of gut resorption through fasting. Crypt cell cycles were stopped following DNA synthesis during phase II, but a marked rise in crypt cell proliferation (DNA synthesis and mitosis) was observed in phase III. Also, apoptosis at the tips of the villi ceased in phase III. After refeeding, morphological restorations of the intestinal mucosa occurred in less than 24 h. Cell renewal was partially restored after 24 h of refeeding and totally after 3 days of refeeding following phase III. While the cellular activity decrease in phase II could save energy, the lack of apoptosis along with the increased cell proliferation and migration in phase III could, however, indicate refeeding anticipation. These different cellular events are thus, key elements in order to understand the phenotypic plasticity of the digestive system in response to environmental pressure such as fasting.

### **A11/C6.25–Peroxidation of protein in blood serum of freshwater and brackishwater sturgeons during adaptation to hyperosmotic medium**

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Spontaneous (Sp.P) and stimulated by  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  (St.P.) peroxidation of protein in blood serum (E/mg protein,  $\lambda=363$  nm) of freshwater sturgeons (starlet-, Siberian sturgeon – *A. baeri stenorrhynchus*) and brackishwater sturgeons (Russian sturgeon – *A. gueldenstaedtii*, great sturgeon – *Huso huso*) during their adaptation to sea water of 12.5‰ salinity were studied. In fresh water, starlet a Siberian sturgeon, have higher levels of Sp.P and St.P. than brackishwater sturgeons have. The levels of these parameters in starlet (obligatory freshwater species) are higher than in Siberian sturgeon that has temporary migration to the estuary for the feeding. In fresh water great sturgeon and Russian sturgeon have similar levels of Sp.P as well as St.P After the adaptation of starlet to sea water during 168 hours significant decrease of Sp.P and the tendency to the decrease of St.P are observed comparatively with these parameters in fish from control (in fresh water). This fact shows that starlet has decrease of the level of the metabolism and cellular breathing. During analogous experiment with Siberian sturgeon, the change in Sp.P. level was absent, but St.P. level increases. Thus pathological effort of adaptive system in Siberian sturgeon is not discovered for this situation, but functional effort is observed. In great sturgeon and Russian sturgeon in fresh water Sp.P. levels were similar. During the adaptation of both species to sea water, significant increase of this parameter is observed only after 12 hours of the experiment. For next 12 hours this parameter decrease to original level that is support without change to the end of the experiment. Thus, during the adaptation of brackishwater sturgeons to sea water, functional effort of the systems of the adaptation is happened only for the transfer of fish to hypoosmotic regulation. In great sturgeon St.P. decreases after 12 hours of the experiment, then is restored to original consequence (in fresh water). This fact suggests the fall of functional activity of adaptive systems during the transfer of great sturgeon to hypoosmotic regulation. In Russian sturgeon St.P. increases significantly after 72 hours of fish adaptation to sea water, but this level decrease and after 168 hours of the experiment reaches control range. It is means that functional effort of this species reaches maximum level after 72 hours. It is possible to conclude that the adaptation to sea water

is happened with higher physiological effort in freshwater sturgeons, than in brackishwater sturgeons, and the systems of the peroxidation and the antioxidants take place in this adaptation. This research is supported by Russian Foundation for Basic Research (grant 01-04-49808).

#### **A11/C6.26—Putative involvement of CHH isoforms on osmoregulation in the crayfish *Astacus leptodactylus***

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Effects of eyestalk neuroendocrine factors on osmoregulatory parameters were studied in the crayfish *Astacus leptodactylus*. Bilateral eyestalk removal in animals maintained in freshwater was followed by a significant decrease in hemolymph osmolality and Na<sup>+</sup> concentration and by a 50% increase in weight after one molting cycle, while the Cl<sup>-</sup> concentration did not change. These effects suggest that factors are probably released from the eyestalk to compensate the water influx and/or the loss of ions at ecdysis. CHH isoforms from the sinus gland have been identified and characterized by high performance liquid chromatography (HPLC) associated with direct enzyme-linked immunosorbent assay (ELISA) on collected fractions and by glucose quantification bioassay. The X-organ/sinus gland complex of *A. leptodactylus* synthesizes several neurohormones and different CHH-related peptides including stereoisomers (CHH and D-Phe<sup>3</sup>-CHH). Injection of the CHH-immunoreactive chromatographic fractions induced a strong hyperglycemic activity, the D-Phe<sup>3</sup>-CHH being the most efficient isoform. Destalked crayfish were injected with purified CHH HPLC fractions. The D-Phe<sup>3</sup>-CHH fraction significantly increased the hemolymph osmolality and Na<sup>+</sup> content 24 hours after the injection. Two other CHH-related peptides did not induce a significant effect on osmolality but caused a small increase in Na<sup>+</sup> concentration (lower than the increase observed after injection of the D-Phe<sup>3</sup>-CHH). No variation was observed in Cl<sup>-</sup> concentration following injection of all CHH isoforms.

This study shows for the first time in a freshwater crustacean the involvement of CHH isoforms in the neuroendocrine mediation of osmoregulation, and more especially the higher effect of the D-Phe<sup>3</sup>-CHH on hemolymph osmolality and Na<sup>+</sup> concentration.

#### **A11/C6.27—Study of osmotic pressure and ion regulation of an invasive Comb-jelly, *Mnemiopsis leidyi*, in the Caspian Sea**

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*Mnemiopsis leidyi* is one of the species of comb-jelly. The main habitat of this ctenophore is the east coast of North and South Americas, which covers the Atlantic coastal zone from latitude 40° North to 40° South (Agassiz, 1865). The existence of *M. leidyi* in Caspian Sea was first reported in Dec. 1999; the density of this ctenophore at that time was so little that access to some samples was a difficult task (Esmaeili et al., 1999). After five months, however, the biomass increased considerably and the preliminary evidence imply that *M. leidyi* have found the Caspian Sea a suitable environment for their growth (Khodabandeh et al., 2002). Study of ion regulation and osmotic pressure in *Mnemiopsis leidyi* in Southern Caspian Sea showed that this species lives in salinities of 12/5–16 ppt. Body fluid osmolarities were 237–312 mOsm/lit over the habitat range of 278–354 mOsm/lit.

Determinations of the concentrations of Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>++</sup>, Ca<sup>++</sup>, So<sub>4</sub> and Cl<sup>-</sup> ions in the body fluid of *M. leidyi* were also carried out. Comparative studies between body fluid and seawater revealed that the concentrations of Na<sup>+</sup>, Mg<sup>++</sup>, Ca<sup>++</sup>, So<sub>4</sub><sup>-</sup> and Cl<sup>-</sup> ions were higher in seawater, except the K<sup>+</sup> ion concentration which was lower.

#### **A11/C6.28—Structure and development of the antennal glands in *Astacus leptodactylus***

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The ontogeny of the excretory antennal glands was studied during the embryonic and post-embryonic development of *Astacus leptodactylus*. The different stages investigated were: -embryos at EI (eye index) 190, 220, 250, 350, 385, 410, and 440 μm (hatching occurs at EI 440–450 μm); -juveniles stages I, II, and 6 months old; -adults. The tubule has been observed from the metanauplius stage EI 190 μm, the labyrinth and coelomosac from EI 320 μm, and the bladder from EI 385 μm. An ultrastructural study has shown that the coelomosac cells

present distinctive pedicels and numerous intracellular dense bodies, vesicles and vacuoles at all stages. The labyrinth is lined by cuboidal cells which possess a brush border (microvilli); the microvilli present the characteristic bleb formations. The organization of the brush border is different in embryos and in adults. The epithelial cells of the tubule region are smaller and devoid of brush border; they present basal infoldings associated with elongated mitochondria and numerous vacuoles.

The enzyme  $\text{Na}^+ - \text{K}^+$  ATPase has been detected through immunocytochemistry in the antennal glands of embryonic stages, from EI 220  $\mu\text{m}$  in the distal tubule, from EI 385  $\mu\text{m}$  in the proximal tubule, and from 385/410  $\mu\text{m}$  in the bladder. The ontogeny of the antennal glands therefore starts early during the embryonic development. The development of the antennal glands before hatching is one of the major adaptations that allow crayfish to spend their entire life cycle in freshwater.

#### **A11/C6.29 – Effects of storage on amnesic shellfish poisons toxins (AST) in king scallops (*Pecten maximus*)**

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Since 1998, king scallops obtained from Scottish offshore sites have been monitored for domoic acid (DA) and epi-DA, the amnesic shellfish toxins (AST) (Gallacher et al., 2000). However, there is limited published information on the effects of storage conditions (e.g. freezing) on concentrations of AST within scallop tissues. The stability of AST under different storage conditions should impact on how samples are managed/stored for both routine monitoring programmes and prior to scientific investigations.

The objective of this study was to assess the effect of freezing whole king scallops (i.e. in their shell) and dissected scallop organs on AST concentrations in different scallop organs and tissue. Scallops were stored frozen for up to 10 days either whole or dissected into the adductor muscle (AM), gonad (GO), and all remaining tissue (RD) – including the hepatopancreas, the outer mantle, and the gills (see Fig. 1). Subsequently these tissue types from both the whole and dissected animals were analysed for AST content using Liquid Chromatography with UV diode array detection (LC-UVDAD; Quilliam et al., 1995).

- AST concentrations showed a significant increase in the AM and a significant decrease in RD following frozen storage of whole king scallops.
- Frozen storage of whole scallops may lead to contamination of the AM with AST during defrosting. This

may be caused by toxic hepatopancreas (HP) fluid coming into contact with the AM.

- AST concentrations in tissues that were dissected prior to freezing change significantly, but this is probably due to high inter-animal variation.

#### **A11/C6.30–Molecular basis for the aphid response to an osmotically-challenging diet**

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The sucrose-rich phloem sap of plants has a higher osmotic pressure than aphid body tissues, presenting these phloem-feeding insects with an osmoregulation challenge. Aphids avoid osmotic collapse, at least in part, by reducing the osmotic pressure of the gut. This is achieved by the conversion of ingested sucrose to longer-chain oligosaccharides, which are voided in the honeydew.

The sucrose-transglucosidase activity in the mid-gut of aphids mediates the sugar transformations and is a key component of aphid osmoregulation. Our aim is to establish the molecular basis of sucrose-transglucosidase activity and the underlying regulatory network, particularly in terms of the aphid response to changes in dietary sucrose concentration and osmotic pressure. We describe the first element of the programme, to establish the molecular and protein identity of the sucrose-transglucosidase activity in the gut of the pea aphid, *Acyrtosiphon pisum*. The molecular approach, to construct and probe a pea aphid cDNA library for candidate enzymes, is being informed by our work at the protein level. Protein with high sucrose activity has been purified from isolated aphid guts and is being analysed by mass spectrometry to obtain amino acid sequence data.

#### **A11/C6.31–Identification and sequence of the Na, K-ATPase alpha sub-unit of the bull shark, *Carcharhinus leucas***

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The bull shark, *Carcharhinus leucas*, is rare amongst the elasmobranchs in that it is able to tolerate both seawater and freshwater. Elasmobranch body fluids are hyperosmotic ( $\sim 600\text{--}1100 \text{ mOsm l}^{-1}$ ) in both seawater ( $\sim 900\text{--}1000 \text{ mOsm l}^{-1}$ ) and freshwater ( $\sim 100 \text{ mOsm l}^{-1}$ ) environments. Despite this high osmotic concentration, the plasma salt concentrations are maintained at a

level similar to that of other marine fish. This is achieved by efficient transport and regulation of water and ions which requires the expression and function of a number of membrane transporters which are fundamental to osmoregulation, including the Na, K-ATPase (sodium pump). Using RT-PCR we have amplified,

cloned and sequenced a number of cDNAs encoding the partial sequence of the bull shark Na, K-ATPase alpha subunit. The nucleotide and putative amino acid sequences will be analysed and compared to those published for other species. (Supported by a project grant and studentship from NERC).