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A1–TOXICANTS: RESPIRATORY, ENDOCRINE AND OSMOREGULATORY DISRUPTION

Organised by J.A. Brown, E.W. Taylor and M. Thorndyke for Osmoregulation Respiration and Endocrine Groups

A1.1–Endocrine disruption in UK freshwaters–The story today

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It has been clearly established that effluents from sewage treatment works [STWs] throughout the UK are oestrogenic to fish. In wild indigenous fish, exposure to these effluents disrupts endocrine function and causes intersex (the simultaneous presence of both testicular and ovarian tissue in an individual fish). In intersex fish there are two major changes in gonadal structure namely, disruptions in the gonadal duct and alterations in germ cell development. Altered gonadal ducts in intersex fish occurs as a consequence of effluent exposure in early life. Altered germ cell development probably occurs as a consequence of long-term exposure to STWs effluents; there is a positive correlation between age of wild roach (*Rutilus rutilus*) caught in UK rivers and the severity of the intersex condition. Using a TIE approach on treated effluents, together with laboratory exposures of fish to single chemicals, it has been established that some of the major causative agents of the feminising effects in fish exposed to treated sewage effluents are steroid oestrogens and alkyphenolic chemicals. Effluents, however, are complex mixtures, and other chemicals are likely to contribute to the gender-bending effects. Wild intersex roach have altered sex steroid hormone profiles, altered spawning time and reduced sperm production. Furthermore, these intersex fish (roach) are compromised in their reproductive capacity, producing sperm with poorer mobility and with a lower fertilisation success. This, in turn shows that endocrine disruption (intersex), in wild fish at least, potentially, has population consequences.

A1.2–Mixture effects of endocrine disrupting chemicals

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A number of man-made and natural chemicals present in the aquatic environment have been shown to mimic the action of the natural steroid, oestradiol. The results of both in vivo and in vitro studies imply that many of these xenoestrogens are only weakly active when compared with oestradiol. Furthermore, these chemicals generally exist in the environment in concentrations that appear to be too low to be of concern individually. In the 'real' world, however, aquatic organisms are often exposed to complex mixtures of such chemicals and it is possible that the low concentrations of these chemicals could be additive in producing an effect. This study investigated the effects of binary mixtures of known estrogens in an in vivo fish assay. Juvenile rainbow trout were exposed for 14 days to oestradiol, ethynyloestradiol and 4-nonylphenol, and methoxychlor and to equipotent fixed ratio binary mixtures of each of the individual chemicals with oestradiol. After 14 days, plasma vitellogenin concentrations were measured and dose-response curves constructed for both the individual chemicals and the binary mixtures. Observed effects of the mixtures were compared to predicted effects using the model of Concentration Addition. The results indicate that Concentration Addition provides an accurate prediction for simple mixtures of oestrogenic chemicals. The implication of this concept is that chemicals present at concentrations below their LOEC can contribute to the overall oestrogenic activity of a mixture, which may have serious implications for assessing the hazards of exposure to endocrine disrupting chemicals.

A1.3–Endocrine disruption in a top marine pelagic predator-evidence for endocrine disruption in the Mediterranean swordfish

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Much of the evidence for endocrine disruption resulting from man-made chemicals has come from observations of genital abnormalities in aquatic wildlife. We now report that catches of swordfish (*Xiphia gladius* L.) from

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the Ionian Sea of the Mediterranean contain a high proportion (14%) of males with evidence of intersex gonads. We captured 72 male swordfish in the Central Mediterranean during the 2000 and early 2001 fishing seasons. Ten specimens (13.9%) showed histological evidence of oocytes within the testis, while livers from all of the captured fish showed immunohistochemical staining for vitellogenin. Plasma vitellogenin in all juveniles, males and females was in the range of 0.3–4 mg ml^{-1}, clearly indicative of recent estrogenic exposure. In the neighbouring areas of the Eastern and Western Mediterranean high plasma vitellogenin values were also observed, together with vitellogenin-immunostaining in the liver. Together with overfishing, such reproductive abnormalities as we report predict a catastrophic decline in numbers of these commercially valuable species. The risk to human health of consumption of fish containing high levels of bio-accumulated endocrine disruptors must also be seriously considered especially in the Mediterranean regions where they might form a major component of the diet.

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A1.4–Environmental oestrogens: their role in testicular dysgenesis and hormone-dependent cancers

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Environmental oestrogens are a diverse group of compounds that exert oestrogenic effects in living organisms. They include: (1) industrial pollutants, such as alkylphenols, PCBs, insecticides and herbicides, which are thought to act as endocrine disrupters and have been controversially associated with increases in the incidence of hormone-dependent cancers and defects in reproductive development (testicular dysgenesis, [1]); and (2) phytoestrogens, which are natural constituents of our diets and have been suggested to protect against hormone-dependent breast cancer [2]. Some of the diverse effects of environmental oestrogens may be attributed to ligand-dependent differences in their interaction with oestrogen receptor sub-classes. However, environmental oestrogens are also potent inhibitors of enzymes involved in the generation and removal of endogenous steroid hormones [3, 4]. By inhibiting these enzymes, environmental oestrogens may indirectly influence oestrogenic activity by altering the bioavailability of endogenous oestrone and oestradiol in target tissues. Since dysregulation of oestrogen homeostasis may be an early event in the development of hormone dependent tumours and testicular dysgenesis, compounds which can influence the activity of enzymes involved in endogenous oestrogen metabolism should also be evaluated as potential therapeutic agents for the prevention and treatment of some human cancers.

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A1.5–Thyroid signalling during fish development

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The thyroid hormones (THs) thyroxine (T4) and triiodothyronine (T3) are believed to mediate the transformation between larva and juvenile (metamorphosis) that occurs during fish development. In addition, maternal THs are present in fish eggs and embryos suggesting that THs may be involved in earlier developmental processes. TH action is mediated by thyroid hormone receptors (TRs). These interact directly with target genes, stimulating transcription when bound to hormone but acting as repressors in the absence of ligand. To examine the role of THs in fish development, thyroid hormone receptors (TRs) have been cloned from several teleost species (sea bream, halibut and salmonids) and their expression during development compared with TH levels and thyroid gland ontogeny. These studies show that small amounts of maternal TR RNA are present and that zygotic expression of TRs begins during early embryogenesis, prior to development of the thyroid gland. TR expression continues through metamorphosis, supporting the proposed roles of THs in this process.

A1.6–Effects of pesticides on pheromonal priming in Atlantic salmon (Salmo salar)

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Previous work has suggested that mature female salmon release at least one sex pheromone, prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$) to the environment. Male salmon detect the pheromone via their olfactory sense and respond with increased blood concentrations of sex steroids and levels of expressible milt. We have studied the effects of low levels of pesticides (diazinon, carbofuran, cypermethrin

and atrazine) found to occur in freshwaters on this pheromonal priming system. In general, when exposed to these pesticides, mature male salmon parr did not respond to $PGF_{2\alpha}$ with increased levels of expressible milt. In addition, blood sex hormone concentrations were not elevated in these pesticide-exposed males after $PGF_{2\alpha}$ stimulation. There is little evidence to suggest that this lack of response is due to altered hormone metabolism. Bile free and conjugated steroid levels in pesticide-exposed males were no different from those of controls. Moreover, there is little evidence to suggest that the testes are unable to respond to pituitary signalling in pesticide-exposed males. However, the results suggest that these pesticides in the water affect the initial detection of the pheromone by the olfactory epithelium. All four pesticides strongly inhibited the olfactory response to the pheromone as judged from the electro-olfactogram (EOG) data. The mechanism(s) by which pesticides inhibit the olfactory detection of pheromones are as yet unknown.

A1.7–Organic pollutants and their effects on the stress response in fish

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Toxicants affect the stress-response of fish, co-ordinated via the hypothalamus-pituitary-interrenal-(HPI)-axis, but the relevant level(s) are unknown. Fresh-water and marine fish were exposed to PCB126 (tilapia and rainbow trout) or Benzo(a)pyrene [B(a)P; turbot], via the diet (50 μg*kg bodyweight^{-1}day^{-1}). After 5 or 7 days, blood samples were taken at-rest, or after net-confinement (superimposed stressor). Plasma ACTH (trout and tilapia), cortisol and glucose (all species) and free-fatty-acids (turbot) were analysed. Resting hormone and metabolic-fuel levels were unaffected. Confinement increased all parameters but cortisol levels were unaffected by either toxicant. ACTH levels were significantly higher in PCB-fed fish and impaired hyperglycaemia occurred after PCB-exposure only. ACTH-injection of turbot (activating the HPI-axis = ACTH-responsiveness) resulted in similarly increased cortisol and metabolic-fuel levels in control and B(a)P-fed fish. Hereafter, trout and tilapia were starved for 3 weeks (mobilising stored PCBs) and sampled at-rest or after confinement. Turbot were fed B(a)P for another 3 weeks and then sampled at-rest, after confinement or after ACTH-injection. Starvation after previous and continuous exposure resulted in lower resting metabolic-fuel levels. Confinement elevated hormone levels but to lesser extents in toxicant-treated than in control fish. ACTH-responsiveness was similar in both groups. We conclude that dietary exposure to B(a)P and PCB126 impairs responsiveness to additional stressors. Depressed corticosteroid responses only become obvious after > 1 week due to initially increased ACTH levels. Absence of such compensation after starvation, in addition to a lack of alteration of ACTH-responsiveness in continuously fed fish, suggests that dietary exposure to organic toxicants interferes with the HPI-axis at the pituitary-level.

A1.8–Individual and species differences in the endocrine responses to toxicants in fish

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Fishes show a great variation in their physiological responses to stressors, as is displayed by differences in the primary, secondary and tertiary stress effects of a standard stressor. These effects include elevation of plasma levels of cortisol, glucose and lactate, changes in plasma ion levels, and reduction in growth, reproductive activity, inhibition of the immune responses and branchial damage. The variation is accounted for by species and strain differences, life history and, in aquaculture, husbandry and feed composition. In addition, marked differences in the stress response can be related to individual differences, in particular connected with rank in hierarchy. Since toxic substances generally evoke a stress response in addition to direct, toxicant-specific effects, the effects of exposure of fish to a standard toxicant vary widely. This holds in particular when fish are experiencing additional stressors, subsequently or concomitantly, independent of whether these are physical (temperature), social (low social rank, isolation, crowding) or chemical (other toxicants): the effects of multiple stressors are normally additive. We have compared the effects to different species exposed to the same standard toxic stressor, the effects of social rank or the sensitivity to toxicants, and the effects of toxicants in combination with physical and social stressors. The results predict that published LC50 values for fish species established under laboratory conditions in general have limited predictive significance for fish under natural or aquaculture conditions.

A1.9–Effects of metals and pesticides on the signalling pathways leading to cortisol synthesis in teleost fish

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Field studies with yellow perch, *Perca flavescens*, diagnosed an impaired cortisol stress response in adult (1) and young of the year fish that accumulated critical tissue burdens of metals in the head kidney and other organs through chronic environmental exposures. A dose-dependant loss of the secretory capacity was also demonstrated in interrenal cells exposed to various metals (Cd, Hg) and pesticides (endosulfan, atrazine, dia-

zinon, mancozeb, *o,p′*-DDD) (2,3) in vitro. Our most recent studies investigated the intracellular targets of selected adrenotoxicants within the interrenal steroidogenic cells of rainbow trout, *Oncorhynchus mykiss*, yellow perch, and two amphibian species (*Xenopus laevis, Rana catesbeiana*). A dose-dependant loss of the capacity to respond to ACTH or dbcAMP was demonstrated in interrenal cells exposed in vitro to Cd and endosulfan while dbcAMP could restore cortisol secretion in cells exposed to *o,p′*-DDD. Substitution with pregnenolone restored cortisol synthesis in cells exposed to Cd, *o,p′*-DDD and endosulfan. The use of agonist and antagonists of specific constituents of the signalling pathways has provided some insight into the steps targeted by specific adrenotoxicants. We have also identified toxicants that are highly cytotoxic and those that disrupt cortisol synthesis without causing cell death. Our data are important for mechanism-based experimental studies in environmental endocrine toxicology and Risk assessment. (Funded by NSERC, CNTC, MITE, Health Canada).

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A1.10–Multi-level analyses of endocrine disruption in shore crabs from UK estuarine and coastal environments

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The shore crab, *Carcinus maenas,* is potentially an indicator of endocrine disruption in the marine environment. Shore crabs from three 'un-polluted' sites (Oban and Arisaig, W. Scotland; Lindisfarne, Northumberland) were compared with crabs collected from three 'polluted' sites (Tyne and Tees estuaries, NE England; Tamar estuary, Plymouth). A multi-level approach, which combines morphometric analyses with behavioural assays of responses to sex pheromone, is being employed to assess endocrine disruption. Morphometric analyses indicate that crabs from the polluted sites show some evidence of intersex and superfemale characteristics, based on abdominal area, chela depth and pleopod length. Intersex males show a degree of feminisation, whereas superfemales have exaggerated female characteristics. Crabs from un-polluted sites show little or none of these atypical features. A stone assay was used, in which male crabs were presented with a stone treated with pheromone-conditioned water obtained from freshly moulted females. Positive responses, in which males approach the stone and initiate pre-copula behaviour, were observed in randomly selected male crabs from Oban and Arisaig. Response of Lindisfarne male crabs (both normal and potentially intersex) were variable; ranging from attempting to form pre-copula pairs to ignoring the pheromone treated stone, and further study is required. The responses of potentially intersex and normal male crabs from the polluted sites to stone assays is again varied. The majority of assay crabs generally interact with the pheromone treated stone without exhibiting pre-copula behaviour, or they show no response. Furthermore, work will relate behavioural and morphometric observations to moulting hormone (20HE) titres.

A1.11–Endocrine disruption in invertebrates: examples of masculinisation?

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Endocrine disruption (ED) poses a serious threat to life on earth and evidence shows that effects may be expected in all species as a result of the early development in evolution of hormone-regulated processes in plants and animals. Invertebrates play key roles in ecosystem functioning, as decomposers and pollinators and as the food source for many species of vertebrates, including humans. In environmental management and risk assessment invertebrates have traditionally served as effective indicators of the impact on ecosystems caused by pollution. However, despite their importance, ED has hardly received any attention in this group of species. The endocrine systems of invertebrates tend to regulate the same processes as in vertebrates, namely development, growth, and reproduction. Some of the endocrine-regulated processes associated with development, growth, and reproduction are unique to specific groups of invertebrates. All invertebrates extensively use neuropeptides to transduce neuroendocrine signals, but steroid hormone usage appears to have undergone significant divergence between deuterostome and protostome invertebrates. Compared to vertebrates, few studies have examined the effects of environmental endocrine disruptors on the reproductive biology of these invertebrate phyla. Nevertheless, several indications exist that chemical pollutants act as endocrine disruptors in invertebrates. One of the best known phenomena linked to endocrine disruption is the occurrence of imposex in female shoreline whelks. There is also considerable evidence for the interference of contaminants with steroid metabolism of the sea star, *Asterias rubens*. In addition, effects of t-octylphenol were found on the sex characteristics of female daphnids, *Daphnia magna*. These observations indicate that endocrine disruption may in some invertebrate species lead to masculinisation of females.

A1.12–Nitrite, a combined respiratory, ion regulatory and endocrine disrupter

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Nitrite is a potential problem in aquatic environments. Freshwater fish actively take up nitrite across the gills, leading to high internal concentrations. Seawater fish are less susceptible but do take up nitrite across intestine and gills. Nitrite has multiple physiological effects. Its uptake is at the expense of chloride, leading to chloride depletion. Nitrite also activates efflux of potassium from skeletal muscle and erythrocytes, disturbing intracellular and extracellular K^+ levels. Its passage of the erythrocytic membrane leads to oxidation of haemoglobin to methaemoglobin, compromising blood O_2 transport. Hyperventilation is observed, and eventually tissue O_2 shortage becomes reflected in elevated lactate concentrations. Heart rate increases rapidly, before any significant elevations in methaemoglobin or extracellular potassium occur. This suggests nitrite-induced vasodilation (possibly via nitric oxide generated from nitrite) that is countered by increased cardiac pumping to re-establish blood pressure. Nitrite may form or mimic nitric oxide and thereby interfere with a number of processes regulated by this local hormone. This also relates to a recently reported inhibition of steroid hormone synthesis by nitrite. Detoxification of nitrite includes its oxidation to nitrate, and the elimination of nitrite occurs both via gills and urine. The susceptibility to nitrite varies between species and in some cases also within species. Rainbow trout fall into two groups with regard to susceptibility and physiological response. These two groups are not related to sex but show different nitrite uptake rates.

A1.13–Respiratory, endocrine, and osmoregulatory effects of copper exposure in fish

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Micromolar concentrations of aqueous copper causes rapid oedema of the gill epithelium, increased costs of respiration, and disturbance to branchial Na regulation. However, disturbances to body systems during chronic dietary exposure to Cu are less well known. We have shown that chronic exposure to 500 mg Cu/kg food for three months increases lamellar length, which we interpret as an adaptive change to maintain oxygen uptake efficiency. These changes occur in the absence of major haematological or ionoregulatory disturbances (Handy et al. 1999, Aquatic Toxicology, 47, 23–41). We also demonstrate adjustments to circadian rhythms in Cu exposed trout (Campbell et al, CJFAS, in press), that may relate to a loss of circulating serotonin. The pathophysiology of endocrine disturbances are explored using histochemical methods and a perfused gut bioassay, on tissues from rainbow trout exposed to 1000 mg Cu/kg food over 6 weeks. The gut morphology was mainly normal, but brain tissue showed foci of necrosis and oedema in some fish. However, preliminary histochemical investigations suggest normal melatonin/serotonin secretion in the pineal organ. Melanomacrophage centres were also prominent in the kidney of exposed fish. Gut contractility bioassays reveal a loss of gut circadian rhythm in vitro, and some loss of responsiveness to additions of serotonin. Overall, these data suggest that Cu injury to the brain and damage to peripheral hormone receptors may play a role in the chronic toxicology of excess dietary copper.

A1.14–Interactive effects of ammonia and hypoxia on the swimming performance of brown trout (Salmo trutta)

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Exposure of adult brown trout for 24 h to concentrations of 100 μmol l^{-1} or 200 μmol l^{-1} NH_4Cl caused an increase in plasma total ammonia to 386 ± 42 μmol l^{-1} or 771 ± 92 μmol l^{-1}, respectively, compared with 133 ± 29 μmol l^{-1} in control fish (mean $\pm$ SE, $n = 6$, T = 15 °C). This ammonia accumulation was associated with a significant decline in maximum sustainable swimming speed (U_{crit}) from 2.24 ± 0.15 body lengths s^{-1} (BL s^{-1}) in control trout to 1.46 ± 0.09 BL s^{-1} or 1.08 ± 0.16 BL s^{-1} in trout exposed to 100 μmol l^{-1} or 200 μmol l^{-1} NH_4Cl, revealing a direct negative relationship between plasma ammonia concentration and U_{crit}. Impaired exercise performance in ammonia-exposed fish was associated with increased costs of swimming (measured as O_2 uptake), reduced maximum tailbeat frequency, and a partial depolarisation of white muscle and brain. Exposure to hypoxia (11 kPa, 55% air-saturation) caused a 45% decline in U_{crit} in control animals, down to 1.23 ± 0.09 BL s^{-1}, as a consequence of reduced aerobic scope. Hypoxia did not, however, cause the same proportional decline in performance in the ammonia-exposed fish; both groups had a U_{crit} of approximately 1 BL s^{-1}. Therefore, hypoxia had no further effect on the reduced performance of animals exposed to 200 μmol^{-1} NH_4Cl. Elevated water ammonia and hypoxia do not have additive effects on trout swimming performance.

A1.15–Biological effects of paralytic shellfish toxins on Atlantic salmon and bivalve molluscs

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Paralytic shellfish toxins (PSTs), a group of potent neurotoxins produced by marine dinoflagellates, are known to cause fish kills and accumulate in filter feeding bivalves. Little is known about the sub-lethal effects of exposure from this group of toxins on marine organisms. Laboratory based exposure experiments on Atlantic salmon (*Salmo salar*) indicate that intra-peritoneal exposure to low doses (2–4 μg/kg) of saxitoxin causes an induction of hepatic glutathione *S*-transferase (GST) activity within four days. Doses approximating the LD_{50} for this compound (4 μg/kg) had little effect on blood plasma ionic concentration of surviving fish. Mussels (*Mytilus edulis*), like other invertebrates, appear insensitive to the paralytic effects of PSTs. Exposure to high doses (intra-muscular, >100 μg/100 g soft tissue) of saxitoxin, however, causes an induction of digestive gland GST activity. This is in contrast to scallops (*Pecten maximus*) which showed no induction of GST activity after acquiring high digestive gland toxicities from feeding on cultures of toxic dinoflagellates. After toxic events, scallops retain PSTs considerably longer than mussels. It is suggested that the induction response of GST in mussels may be partly responsible for this discrepancy in toxicokinetics between the two species.

A1.16–Effects on chub (Leuciscus cephalus) of chronic exposure to sub-lethal levels of complex pollutant mixtures in an urban river in Italy

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This study has compared the physiological and biochemical status of resident fish species at two sites on the River Lambro (Lombardy, Italy) which were similar in their hydrological characteristics but which differed in chemical and ecological quality. Levels of bioavailable polyaromatic hydrocarbons and heavy metals, measured by passive samplers, were higher at an urban site (Brugherio) than at an upstream site (Merone). Brugherio had a lower extended biotic index and reduced diversity of fish species relative to Merone. Wild chub (mass approx. 150 g) were collected at Merone and exposed in cages at each site for 3 weeks, in May and September 2001. Exercise performance was then assessed, as an indicator of their physiological status. In both June and September, chub at Brugherio exhibited impaired performance, their capacity to repeat a swim test was significantly less than that of fish at Merone. Performance of fish at Merone was similar to that of chub maintained for 3 weeks in clean biofiltered water. Impaired performance of fish from Brugherio was a consequence of reduced aerobic scope. In September 2001, chub caged at Brugherio had significantly higher liver EROD activity and metallothionein levels than chub from Merone. An absence of such differences in June may have been because the fish were in their breeding season. The results indicate that links can be drawn between the chemical quality of a water body and the species richness, physiological status, and ecotoxicological biomarker expression, of its resident fish populations.

A1.17–Gill metal handling as a determinant of metal induced osmoregulatory disruption in freshwater fish

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An increase in aquatic metal concentrations results in a disruption to gill osmoregulatory function of freshwater fish. To avert potentially fatal perturbations to ionic homeostasis an integrative response is required that increases the capacity for cation sequestration, reduces diffusional cation loss and reduces the concentration of bioactive metal in the gill. Identified mechanisms to avert cellular metal toxicity are: (1) reduce metal uptake; (2) increase intracellular metal-binding protein concentrations and (3) increase metal export. Assessment of gill branchial Ag(I) and Zn(II) metal handling on exposure to sublethal levels of the metals has shown that one of the main cellular defence mechanisms appears to be a downregulation of the apical metal entry step in freshwater fish. In the case of Zn(I), there is also an increase in basolateral extrusion probably via a putative zinc transporter, Znt-1. There is no known Ag(I) specific transporter, however a basolateral ATPase capable of transporting Ag(I) has been identified. Furthermore, characterisation of this transporter shows that Cu(II) inhibits Ag(I) transport suggesting that it may belong to the Cu-ATPase family of proteins. Whether this transporter is up-regulated in fish exposed to Ag(I) is unclear. By the use of subtractive cDNA hybridisation and screening of heterologous cDNA macroarray libraries, the genomic response of gills of rainbow trout exposed to Zn(II) and Ag(I) was assessed. Searches of gene databases provide putative information regarding the function of a number of these up-regulated genes. These suggest that upon metal exposure the gill increases expression of proteins involved in paracellular integrity, protein synthesis, energy synthesis, and immunity.

A1.18–Development of a Gill cell culture system to assess aquatic metal pollution

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Speciation (i.e., complexation by naturally occurring ligands) strongly influences toxicity of metals to fresh water fish. Cell culture systems previously have tried to replicate metal ligand binding and the toxicological interaction, however, this has proven to be unsuccessful as the cells must be bathed in cell culture media. A cell culture system is being developed using cell culture inserts, where primary gill cells are grown on polyethylene terephthalate (PET) track-etched membrane supports. This method allows test water samples to be placed on the apical side, while the cells are bathed in media on the basolateral side, mimicking a fresh water gill. This system will allow the elucidation of interactions between waterborne metals, water chemistry, physiological response systems (e.g. stress response, metallothionein induction, and metal uptake regulation), and toxicologically relevant effects (e.g. ion transport disruption). The physiological and toxicological responses can then be utilised as a bioindicator of water contamination by toxic metals. The cell culture system is now being characterised with comparison to live fish studies and physiological response systems are being calibrated for the sensitivity as a bioindicator.

A1.19–Branchial ion exchange in freshwater stingrays of the Rio Negro: effects of low pH and dissolved organic carbon

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Potamotrygonidae, the only stenohaline freshwater elasmobranchs, are endemic to the dilute, often acidic blackwaters of the Rio Negro, Brazil, so-called because of their high dissolved organic carbon (DOC: humic, fulvic and other acids). Potomotrygon tested in ion-poor blackwaters (Na^+, Cl^-, and Ca^{2+} = 10–30 mmol.l^{-1}; pH = 6.1; DOC = 8.35 mg C.l^{-1}) were ammoniotelic and exhibited blood chemistry typical of freshwater teleosts. Unidirectional Na^+ and Cl^- influx rates, measured with radiotracers,displayed saturation kinetics, with similar low affinity K_m (300–500 umol l^{-1}) and moderate J_{max} values (400–700 umol kg^{-1} h^{-1}). In order to understand the possible protective role of the high level of natural DOC (8.35 mg C l^{-1}), stingrays acclimated to Rio Negro water were compared to stingrays acclimated to laboratory water with similar ion concentrations but low in DOC (0.59 mg l^{-1}). In both media, Na^+ and Cl^- influxes were strongly inhibited by acute exposure to pH 4.0, but efflux rates increased only in laboratory water. Experimental ten-fold elevation of water calcium levels protected against the increase in efflux rates upon pH 4.0 exposure in laboratory water, but had no effect in Rio Negro water. However, addition of Aldrich humic acid to laboratory water (with or without added calcium) to achieve the same DOC concentration as Rio Negro water did not protect against the increase in Na^+ and Cl^- efflux rates upon exposure to pH = 4.0, and indeed greatly exacerbated them. We conclude that natural DOC in blackwaters may offer a protective action at the gills similar tothat of calcium, and this is not duplicated by Aldrich humic acid.

A1.20–Why does social status influence uptake of waterborne trace metals?

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We have demonstrated that the social status of a rainbow trout will affect the rate of uptake of copper from the water. Sub-ordinate fish will take up more copper from the water than dominant fish, resulting in higher liver and gill tissue accumulation. Similarly, we have shown that sub-ordinate fish have a higher uptake rate of sodium from the water when compared with dominant fish. The mechanisms behind the observation that social competition mediates individual variation in copper uptake are currently being investigated. From previous studies sub-ordinate fish are known to have higher standard metabolic rates and circulating concentrations of plasma cortisol than dominant fish. Increased metabolic rate has the potential to increase copper uptake across the gills by increasing ventilation volume and gill permeability, while elevated concentrations of cortisol are known to alter the ion transport mechanisms across the gills. Recent work investigating the transport of copper across the gill epithelia has suggested that copper may be transported via the sodium pathway thus the higher uptake rates of sodium in sub-ordinate fish may explain higher copper accumulation. By altering metabolic rate, cortisol concentrations and sodium uptake rates independently of social interaction we aim to address the question—why does social status influence uptake of waterborne trace metals? (Supported by an NSERC Strategic Grant, ICA, ILZRO, NiPERA, Cominco, Noranda and Falconbridge).

A1.21–The effects of copper on heart rate, pleopod beat and osmoregulation in the freshwater amphipod Gammarus pulex

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Heart rate and pleopod beat were used as indicators of physiological stress in the freshwater amphipod *Gammarus pulex* when exposed to varying concentrations of copper. A continuous flow-through chamber allowed

measurements of heart rate and pleopod beat. Significant reductions in both heart rate and pleopod beat were found when animals were exposed to 1 and 10 mg/l $CuCl_2$ over a 1 h period. The effect of copper on osmoregulation in *G. pulex* was determined from the analysis of blood sodium concentrations, apparent water permeability and gill Na^+, K^+-ATPase activity. Significant reductions in blood sodium concentration were found in animals exposed to 100 μg/l copper or greater. These changes in blood sodium concentration were rapid and occurred within 2 h of copper exposure for both 100 and 1000 μg/l copper. The effects of copper exposure on the apparent water permeability of *G. pulex*, expressed as half time for exchange of body water ($t_{1/2}$), was investigated. Copper concentrations of 100 and 1000 μg/l copper had no significant effect on $t_{1/2}$ values after 24 and 102 h exposure, suggesting no structural gill damage caused by copper toxicity. Gill Na^+, K^+-ATPase has previously been shown to be an important osmoregulatory enzyme in crustacean gills. This work found copper concentrations greater than 10 μg/l copper significantly reduced Na^+, K^+-ATPase activity. These findings will be discussed with respect to mechanisms of copper toxicity.

A1.22–Sublethal effects of copper on respiration and osmoregulation of the freshwater crab, Potamonautes warreni

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Whole animal rates of oxygen consumption, carbon dioxide production and nitrogen excretion were measured in freshwater crabs exposed to a sublethal copper concentration of 1 mg copper ions per liter (at 25 °C). Lipid peroxidation is initiated within 3 h after start of exposure and is maintained for (at least) 21 days. This is an uncontrolled breakdown with hepatopancreas free fatty acid levels (quantified and identified by GC-MS) five times above control levels. Glycolytic action is reversed for the duration of a 7 day experiment, and ^{14}C-glucose and ^{14}C-glycogen is anabolized from injected ^{14}C-lactate in an animal that is predominantly reliant on carbohydrates for energy. As the importance of carbohydrate metabolism fluctuates during the molting cycle, impacts on different molting stages needs to be assessed. Chloride concentrations and osmotic pressure in haemolymph and urine are unchanged over a 7 day exposure period. The simplistic deduction would be that copper causes no perturbation of osmoregulatory mechanisms, but more detailed investigation gives us an idea of the strain placed on osmoregulatory mechanisms to maintain salt balance. In copper-exposed animals the kidney's salt-concentrating capability, as measured by ^{51}Cr-EDTA experiments, is impaired, leading to an increase in urine filtration rate to prevent excess salt loss. It is unclear how ion uptake mechanisms on the gills or gill membrane permeabilities are impaired. Mortality as a measurable end-point is not feasible in monitoring pollution impacts. We argue that integrated measures of animal health and long-term effects on reproductive success are required in order to better quantify the long-term survival of species.

A1.23–Mechanism of osmoregulatory disturbance in marine teleost fish during copper exposure

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A concentration dependant disturbance of plasma ions revealed that copper is an osmoregulatory toxicant in the marine gulf toadfish. The osmoregulatory disturbance resulted from at least three different events. Copper (50 μM) caused initial inhibition of drinking rate, which subsequently was stimulated to levels greatly exceeding control values. Branchial Cl^- and Na^+ extrusion was inhibited by 90% after two and five days of exposure, respectively, with no signs of recovery within 16 days of exposure. Isolated intestinal segments revealed that HCO_3^- secretion was the most sensitive intestinal transport process exhibiting marked inhibition after 5 and 16 days of copper exposure, which explains reduced HCO_3^- concentrations in the intestinal fluids of exposed fish. In addition to osmoregulatory disturbance, mild disturbances in nitrogen metabolism were seen. Copper at 10 μM did not cause osmoregulatory disturbance. Physiological parameters during exposure to 50 μM copper stabilized after 16 days and remained constant, though different from controls, for up to 30 days of exposure. Consistent with effects of 50 μM copper both on gill and intestinal function, substantial copper accumulation was seen in both tissues. In addition, the kidney and the liver accumulated copper, while all other tissues exhibited normal concentrations. Copper at 10 μM did not lead to significant accumulation of copper in internal organs, presumably due to an observed elevated biliary excretion. Elevated biliary excretion during exposure to 50 μM copper was also evident but not sufficient to prevent internal copper accumulation. (Supported by a Pilot Project Grant from UM NIEHS Center ES05705).

A1.24–Acute nickel toxicity occurs by a respiratory rather than an ionoregulatory mechanism in the rainbow trout

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Over 120 h, mean arterial oxygen tension in cannulated adult rainbow trout (200–340 g) exposed to 11.6 mg Ni/L (~40% of the 96 h LC50) drops gradually to ~ 35% of control values. This drop in arterial oxygen tension is accompanied by a respiratory acidosis. By 96 h, arterial carbon dioxide tension rises to more than double control values with a concomitant drop in arterial pH of 0.15 units.

Additionally, increases in ventilation rate, plasma total ammonia, and plasma lactate suggest an increasing diffusion limitation at the gills over time. Analysis of plasma ions obtained by cannulation shows no significant decreases in plasma Na, Cl, Ca or Mg, with Ni exposure.Additionally, unidirectional flux measurements on juvenile (2 g) rainbow trout using radiolabelled Na, Cl and Ca show no impact on uptake of these physiological ions with exposure to 15 mg Ni/L (the 96 h LC50 for 2 g fish in Lake Ontario tap water), further suggesting that acute Ni toxicity is primarily of respiratory origin and not ionoregulatory.

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A1.25–Toxicant effects on exercise performance and oxygen uptake in salmonids

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The shift in the field of aquatic toxicology from lethal responses to sublethal mechanisms is welcomed for two reasons. First, better environmental management is likely to emerge, and second, toxicology experiments can now be used as probes to discover how animals function. Our work has examined the effects of aquatic toxicants that target the cardiorespiratory system of fishes. Dose-response studies show that toxicants such as 2-(thiocyanomethylthio)benzothiazole (TCMTB), pentachorophenol and rotenone have sublethal cardiorespiratory effects only at doses that approach the LC_{50} value. We found this surprising given existing paradigms concerning the predicted impacts of toxicants that load or limit the metabolic capacities of fish. For example, rotenone, a known inhibitor of mitochondrial electron transport, had little effect on routine metabolic rate. Pentachlorophenol, a known mitochondrial uncoupling agent, while increasing routine metabolic rate, had little effect on maximum metabolic rate. In fact, exposure to petacholorophenol appeared to improve the ability of sockeye salmon to recover for exercise under mild hypoxia! Lastly, TCMTB could produce rather severe lesions on gill lamellae before appreciable effects were seen on critical swimming speeds. Collectively, these observations provide important insights into fish cardiorespiratory physiology during exercise that challenge current concepts of how toxicant-induced changes to oxygen uptake and metabolic capacity relate to exercise performance in salmonids and may have important applications to whether or not a particular sublethal effect should be considered 'adverse'.

A1.26–Ionoregulation and nitrogen excretion in fish inhabiting a eutrophic, periodically alkaline lake

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This study has focused on four fish species; roach (*Rutilus rutilus*), rudd (*Scardinius erythrophthalamus*), perch (*Perca fluviatilis*) and pike (*Esox lucius*), native to a shallow lake (Slapton Ley) in Devon, UK that has been eutrophic for several decades. These fish regularly experience alkaline water for 3 summer months (mean = pH 9.5, peaks of pH 10), with little spatial or diurnal variation and hence no obvious refuges. The deleterious effects of pH's > 8.5 on ionoregulation and nitrogen excretion have been well documented in salmonids. If coarse fish show similar susceptibility, then fish inhabiting this lake are seasonally exposed to potentially harmful conditions. The relative tolerance of these four Slapton species to alkaline water (pH 9.5) was investigated in the laboratory with respect to the disruption of ammonia excretion and Na^+ balance. Further analyses of the Na^+ uptake kinetics are being made, together with a comparison of the same four species obtained from a non-eutrophic source to explore whether any adaptation to high pH has occurred. Results indicate that all four species experience a similar inhibition of ammonia excretion (39–58%) upon acute exposure to pH 9.5 water (cf. ~90% in salmonids), but have markedly different tolerances with respect to Na^+ balance (e.g. unaffected in rudd, but a large net loss in perch ~ -750 nmol g^{-1} h^{-1}). The relative tolerance of the four Slapton species and salmonids to high pH will be discussed in relation to their different lifestyles and ionoregulatory strategies (e.g. Na^+ uptake kinetics and inherent ion losses).

The Na^+ uptake kinetics has revealed that perch display a K_m of 46.9 μM and V_{max} of 474.4 nmol g^{-1} h^{-1}, indicating a relatively high affinity Na^+ transporter, while rudd do not show a saturable Na^+ uptake curve up to 360 μM Na^+.

A1.27–Effects of 17α-ethinyloestradiol (EE_2) on gill structure and ionic regulation in the shore crab, Carcinus maenas

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EE_2 is a compound released into the environment via sewage treatment works, as a result of human consump-

tion of the contraceptive pill and post-menopausal hormone-replacement therapy drugs. The detrimental effects of various components of sewage effluent have been well established in birds, reptiles and fish. However, very little is known about the effects on crustaceans and other aquatic invertebrates. Crab gills are used for gas exchange, salt regulation, and nitrogenous waste excretion. Therefore, if a pollutant, such as EE_2, is in direct contact with the gill it has the potential to have a dramatic effect on a number of vital life processes. An initial experiment was carried out exposing female shore crabs (*Carcinus maenas*) to 10 ng/l EE_2 and 100 ng/l EE_2 for 2 weeks in 50% seawater. No statistically significant data was obtained from this experiment. A more detailed investigation was then carried out exposing crabs to EE_2 for 1 week or 4 weeks in 100% and 50% seawater. Samples of haemolymph and gill tissue were taken for analysis. It was found that EE_2 did not significantly affect blood glucose levels in crabs adapted to 50% or 100% seawater. However, crabs exposed to EE_2 in 50% seawater showed a significantly higher blood osmolarity compared to the controls. Blood monovalent ions (Na^+, K^+ and Cl^-) have also been measured along with gill lamellar structure. The significance of these results to the ionic regulatory ability of EE_2-exposed crabs will be discussed.

A1.28–Effect of seawater desalination waste products on turbot (Scophthalmus maximus) embryos and larvae

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Seawater desalination is vital in many countries. In this process salt is removed from seawater using several techniques. The waste product is a hot, hypersaline brine poured back to the sea. This contains several substances such as corrosion products, antiscaling additives (polycarbonic acids, polyphosphates), antifouling additives (chlorine and hypochlorite), halogenated organic compounds formed after chlorine addition, antifoaming additives, anticorrosion additives, oxygen scavengers (sodium sulfite), acid, heat and the concentrate which has excess salts. We studied the effect of bromoform, a halogenated organic compound detected at the outlet of several desalination plants in Kuwait in the Arabian Gulf. Turbot embryos and larvae were exposed to normal and hypersaline seawater mixed with bromoform ($CHBr_3$) at 735.8 ng/l. The results showed increased mortality in the embryos and larvae exposed to bromoform and hypersalinity and significant effects on egg diameter, oil globule diameter, larval length, and yolk sac volume between treated and non- treated animals. Histological sections showed significant difference in skin thickness and notochord diameter also. Whole body chloride concentrations have also been measured.

A1.29–The role of stress proteins in endocrine disruption of rainbow trout (Oncorhynchus mykiss)

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The surfactant and anthropogenic pollutant, 4-nonylphenol (4-NP) is now ubiquitous in industrial and sewage treatment processes. This compound is noted because of its toxicity, estrogenic effects and ability to bioaccumulate in aquatic organisms. Indeed, 4-NP exerts an estrogenic effect on fish and has been shown to affect growth in salmonids. To date however, the cellular and molecular processes underpinning these reproductive and growth effects are not well understood. In this regard, stress or heat shock proteins (hsps) have a critical role in endocrine signal transduction. Hsps, notably hsp90, complex with steroid hormone receptors and appear to significantly contribute to the receptor's hormone binding ability. In our study, we do not observe any significant growth effects on juvenile rainbow trout 6 weeks following a low, but environmentally relevant exposure (2 24 h pulses of 20 μg/l) to 4-NP. This dose, however, was sufficient to induce endocrine disruption in these fish as indicated by a significant increase in liver vitellogenin (yolk) protein, a well-known marker of endocrine disruption. The stress inducible form of hsp90 protein appeared to significantly decrease following treatment with 4-NP, while the constitutive form of the hsp was not affected. This may not be surprising if you consider that 4-NP is less specific for the estrogen receptor compared to estrogen itself, and perhaps activates fewer receptors. The aim of this study is to understand the physiological effects and mechanisms of action of 4-NP in fish by exploring the role of hsps in sex steroid signal transduction.

A1.30–Endocrine disruption: metabolic and proteomic responses

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This study addresses growth and proteomic responses, to endocrine disruption, in rainbow trout (*Oncorhynchus mykiss*). Whilst, in this species, differential feeding causes differences in overall protein synthesis there is little information regarding differences in specific protein expression. Also, whilst numerous examples exist of particular proteins induced as specific stress responses, no attempt has been made to resolve those resulting from growth differences and from endocrine disruption. Proteomic analysis may provide an appropriate tool. Therefore the aim here is twofold; (1) to

investigate the relationship between endocrine disruption and growth, and (2) to evaluate proteomic changes as potential biomarkers to endocrine disruption. Individual growth rates were recorded for 3 weeks prior to and after injection with either a 0.5 mg kg^{-1} (high), 0.5 μg kg^{-1} (medium) or 0.005 μg kg^{-1} (low) β-oestradiol dose, or corn oil (β-oestradiol solvent) only. Non-injected fish growth was also monitored over the same period. Liver samples were then taken and the proteome analysed by 2D-PAGE, followed by image analysis using Phoretix gel analysis software. The effects on growth appear to be concentration dependent. In non- and corn oil injected fish and fish injected with high or medium dose β-oestradiol individual pre- and post-injection growth rates are maintained following β-oestradiol treatment. However, following low dose β-oestradiol injection growth rates are not reflective of pre-injection growth. The retention of growth rate appears to be associated with an increase in hypertrophy. In addition proteomic changes are detected which are unique to differential growth rates and/or endocrine disruption.

A1.31–Effects of metabolic inhibition on cellular volume in hepatocytes from goldfish (Carassius auratus)

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We studied the effects of cyanide (CN), a blocker of oxidative phosphorylation and iodoacetic acid (IAA), a blocker of glycolysis, on relative cell volume (Vc) and potassium fluxes of goldfish hepatocytes. Changes in Vc were monitored by calcein fluorescence microscopy. Influx and efflux of K^+ were assessed by radioactive measurements using $^{86}Rb^+$. In anisotonic media, cells displayed a reversible osmometric change of Vc. In contrast, Vc remained unchanged in the presence of IAA, CN or both. Whereas metabolic blockers had no effect on K^+ efflux, the influx of K^+ decreased acutely to approximately 50% in the presence of CN or IAA + CN, but showed not significant change in the presence of IAA alone. Net K^+ loss observed in the presence of CN or IAA + CN amounted to 0.11 nmol K^+/million cells/min, yielding a steady decrease of intracellular Ki which, however, did not significantly change intracellular osmolarity. These results provide evidence for a lack of any regulatory role of intracellular K^+ in volume regulation of anaerobic goldfish hepatocytes.With grants from UBA, CONICET and ANPCyT of Argentina, Austrian Science Foundation and the Austrian Academy of Sciences.

A1.32–Fish liver (Chondrostoma polylepis and Barbus bocagei) as bioindicator of aquatic pollution in Mondego basin

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Fish liver performs an important role in pollutant metabolism being susceptible to lesions induced by their action. Since the liver structure is related with its physiological and biochemical activities, histological changes can be used as bioindicators for the evaluation of the impact caused by the increasing level of rivers contamination. The aim of this study is to evaluate the changes observed in *Chondrostoma polylepis* and *Barbus bocagei* liver and use them in a program for the biomonitoring of aquatic environment in Mondego Basin. There have been chosen 4 different sampling regions along the river, namely M1 to M4. The first one was used as control, since the region did not show significant changes induced by man action. The principal lesions observed in the hepatic tissue were focal vacuolization, lymphocytic foci, necrosis, increased pool of macrophages aggregates and changes in hepatocytes structure and distribution. This work explores the possibilities for including biological tools in pollutant exposure assessment.

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A1.33–Copper effect on gill epithelium ultrastructure of tilapia, Oreochromis niloticus

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Copper is an essential micronutrient but is toxic at elevated concentrations. Copper sulfate ($CuSO_4$) used in agriculture as fertilizers and pesticides leaches into the aquatic environment resulting in toxicity in aquatic organisms. Toxic levels of $CuSO_4$ are known alter the ultrastructure of gill epithelium of teleost fish. Mor-

phology of fish gills may also be used as an indicator of sublethal effects of heavy metals. The purpose of this study was to investigate the effects of sublethal and lethal $CuSO_4$ concentrations on gill histology and ultrastructure of the tilapia (*Oreochomis niloticus*). Three days exposure to sublethal concentration (2.5 mg l^{-1}) resulted in epithelial lesions including interstitial edema, vasodilatation and total loss of the columnar cells in superficial filament epithelium (sFE). Electron microscopic analysis revealed an enhanced number of cells characterized by dense core vesicles, likely immunological in origin, and a differentiation of macrophage-like cells deep within the FE. In the lamellae, effects of 3-day exposure included disappearance of pericytes, activation of covering cells and rupture of pillar cells. Lamellar fusion, atrophy of the vascular axis, and even to necrosis of the FE and lamellae were also noted in some samples. After increased exposure time, regeneration of the FE and adaptation to the pollutant were found. Higher concentrations (5.0 mg l^{-1}) of $CuSO_4$ were found to increase the number, size and extent of lesions recorded in gill epithelium.