
Society for Experimental Biology Annual Main Meeting 31st March – 4th April 2007, Glasgow, Scotland

A1/C6–IN VITRO TECHNIQUES FOR INVERTEBRATE AND PISCINE PHYSIOLOGICAL AND ECOTOXICOLOGICAL STUDIES

A1.1

Cultured branchial epithelia from freshwater rainbow trout: Tools for understanding gill function

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Cultured gill epithelia from dispersed gill cells of freshwater rainbow trout grown on semipermeable supports are a unique cell culture preparation inasmuch they can maintain polarity and integrity for an extended period during apical freshwater exposure. Evolving from an original preparation comprising only pavement cells without hormonal supplementation (Wood and Pärt, 1997), several techniques are now available for growing epithelia with specialized properties, including incorporation of mitochondrial rich cells and endocrine support (Wood et al., 2003). We will first review how these preparations have expanded our understanding of gill physiology – e.g. hormonal effects on ion permeability and transport, ammonia permeation, metal toxicology (Zhou et al., 2005), lipid metabolism, proteomics (Smith et al., 2005) – and then move to studies using differential seeding of various cell types. Finally we will present very recent experiments using both cultured epithelia and intact trout to understand the actions of DOC from natural waters (“natural organic matter”) on the electrical properties of the gills. (NSERC, Kodak Canada).

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Wood, C.M., Pärt, P., 1997. Cultured branchial epithelia from freshwater fish gills. *J. Exp. Biol.* 200: 1047–1059.

Wood, C.M., Eletti, B., Pärt, P., 2003. New methods for the primary culture of gill epithelia from freshwater rainbow trout. *Fish Physiol. Biochem.* 26: 329–344.

Zhou, B., Nichols, J., Playle, R.C., Wood, C.M., 2005. An in vitro biotic ligand model (BLM) for silver binding to cultured gill epithelia. *Toxicol. Appl. Pharmacol.* 202: 25–37.

doi:10.1016/j.cbpa.2007.01.069

A1.2

Trout gill cells in primary culture on solid and permeable support

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Trout gill cells in primary culture on solid and permeable support were compared. Primary cultures were carried out by seeded gill cell, after trypsin dissociation, at $0.4\text{--}0.5 \times 10^6$ and $1.5\text{--}2 \times 10^6$ cells/cm² on solid and permeable support respectively. Whereas most of cell types present in primary culture were similar whatever culture support (pavement cells, mucus cell but no mitochondria rich cell), epithelium structure was different. Gill cells on permeable support stratified to 2–7 cell layers compared to 1–3 on solid support. On solid support, most of mucus cells were in contact with apical side and inserted among pavement cells whereas on permeable support mucus cells were covered by pavement cells with a small opening to apical side. Gene expression of gill ion transporters and hormonal receptors were analyzed in cultured gill cells and this study indicated rather similar expression levels in both systems. Cortisol, added in culture medium 24 h after seeded, had no effect on cell and epithelium morphology after 9–11 days of culture. Interestingly, cortisol treatment increased total cell number but only on cultured cells on permeable membrane. Furthermore, cortisol maintained transepithelial resistance ($25\text{--}30 \text{ k}\Omega \text{ cm}^2$) after 11 days in culture whereas this TER decreased in control condition. In conclusion, gill cells in primary culture on permeable support present (i) a closer

morphology to in situ epithelium and (ii) specific response to cortisol treatment.

doi:10.1016/j.cbpa.2007.01.070

A1.3

Evidence for sodium-linked ammonia transport in cultured gill epithelia from freshwater trout

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Ammonia is continuously produced by fish and must be eliminated across the gills. However, the mechanisms by which ammonia moves through the branchial epithelium remain unsettled. For example, the relative importance of simple NH_3 diffusion versus carrier-mediated processes is unclear, and the potential involvements of Na^+/K^+ -ATPase, Na^+/H^+ exchangers and K^+ channels are not certain. Part of the obstacles in answering these questions are the technical difficulties in controlling various parameters (e.g. internal ammonia concentration, pH) when using the whole fish as an experimental model. However ammonia transport can be studied across cultured gill epithelia containing both pavement and mitochondria-rich cells grown on a porous membrane (Kelly and Wood, 2001). The cells develop polarity and the apical side can be exposed to freshwater. We have revisited this approach with a focus on concentration-dependent kinetics and pharmacology. Ammonia flux across the cultured epithelium showed some evidence of saturation and rectification, and increased with chloride-cell enrichment. Ouabain inhibited ammonia flux in a dose-dependent manner. Amiloride also inhibited ammonia flux, whereas phenamil, quinidine and bumetanide had no effect. These results suggest that Na^+/K^+ -ATPase and Na^+/H^+ exchanger are involved in branchial ammonia transport. The cultured branchial epithelium appears to be a useful model to elucidate the mechanisms of ammonia transport across fish gills.

Kelly, S.P., and Wood, C.M., 2001. The cultured branchial epithelium of the rainbow trout as a model for diffusive fluxes of ammonia across the fish gill. *J. Exp. Biol.* 204: 4115–4124.

doi:10.1016/j.cbpa.2007.01.071

A1.4

Transcriptomic analysis of metal responses in cultured fish gill cells

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Fish use gills to take up nutritional metals from their surrounding water and the gill is also a primary site of toxicity for a range of essential and nonessential metals. The response of the gill epithelium to excess metal is complex and

involves regulation of numerous genes and proteins. Some of the pathways mediating metal responses are local to the gill, but there is also a systemic component involving endocrine signals. We have used gill cell cultures to investigate the local pathways leading to transcriptional metal responses in gills of rainbow trout and zebrafish. The responses in primary rainbow trout cells to metals were found to be highly dependent on culture condition and influenced by factors, such as seeding density and addition of antibiotics and serum to the culture medium. In optimised cell culture conditions it was possible to generate transcript profiles, which were unique to zinc, cadmium, copper and silver. A zebrafish gill cell line, CF4, was used to genetically interrogate the involvement of metal-responsive transcription factor-1 (MTF-1) in the transcriptional response to zinc at the genomic level. A RNAi knock-down system for MTF-1 was generated in CF4 cells and the transcriptomic response to zinc with or without MTF-1 knockdown established with oligonucleotide microarrays. Using this data linked to sequence analysis, an inventory of MTF-1 regulated genes in CF4 cells is being generated.

doi:10.1016/j.cbpa.2007.01.072

A1.5

The use of *Xenopus laevis* oocytes for characterising metal transport proteins

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Xenopus laevis oocytes are routinely used as a tool for heterologous expression and functional characterisation of ion transporters and channels. With respect to metal transport proteins the use of this system was integral in the identifying the ferrous ion importer, Divalent Metal Transporter 1 (DMT1) and has been used to characterise other component of the iron uptake pathway (IREG1, DcytB and recently a Haem transporter) essential for iron uptake in humans. We have also used this system to characterise the properties of fish iron transport proteins and have identified a novel splice variant of DMT present in rainbow trout that lacks the last 2 trans membrane domains but is still functional and widely expressed in tissues. However, there is a need to combine both radiolabelled tracer experiments with electrophysiological studies in *Xenopus* oocytes expressing transport proteins to truly understand their properties and function. The *Xenopus* oocyte also contains a number of endogenous membrane transporters that are important during fertilisation and development. Recent studies have shown that silver elicits a membrane current in defolliculated *Xenopus* oocytes, which implies that caution is needed when designing electrophysiological experiments using oocytes expressing metal transport proteins.

doi:10.1016/j.cbpa.2007.01.073

A1.6

***In vitro* and *in vivo* effects of polycyclic aromatic hydrocarbons on Pacific Oyster, *Crassostrea gigas* (Thunberg), hemocyte parameters**

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In vitro and *in vivo* on Pacific oyster, *Crassostrea gigas*, hemocyte parameters. For the *in vitro* experimentation, effects of selected PAHs were monitored after contact with hemocytes. In a first step, based on the use of pool or individual hemolymph protocols were compared. Similar results were obtained for pool and individual analysis. In a second step, hemolymphs from 45 oysters were pooled and *in vitro* contacts with selected PAHs were carried out during 24 h. After 24 h, seven pollutants (fluorene, pyrene, anthracene, phenanthrene, chrysene, indeno [1,2,3-*c,d*]pyrene and heavy fuel oil (HFO)) induced a decrease of hemocyte mortality. Fluorene, pyrene and HFO decreased significantly phagocytosis activity. Esterase and lysosomal activities were increased by naphthalene and dibenz[*a,h*]anthracene respectively. The *in vivo* effects of the soluble fraction of HFO (730 ng L⁻¹) were also investigated on two hemocyte parameters: cell mortality and phagocytosis activity. After nine days of contact between the soluble fraction of HFO, oysters were transferred and maintained into non contaminated seawater for one month. The effects on hemocyte parameters were assessed. The soluble fraction of HFO induced a significant decrease of phagocytosis activity. This work is one of the first which allowed a real comparison of the effects of different PAHs on hemocyte parameters *in vitro* and *in vivo*.

doi:10.1016/j.cbpa.2007.01.074

A1.7

3-Dimensional culture of invertebrate ganglia for studies of neural repair

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doi:10.1016/j.cbpa.2007.01.075

A1.8

ECVAM key area ecotoxicology: Summary of activities and future perspectives

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The European Centre for Validation of Alternative Methods (ECVAM) is part of the European Commission and is

responsible for the development and validation of methods which would reduce, refine and replace (3Rs) the use of animals for the safety testing of chemicals and other products. ECVAM's activities in ecotoxicology started 2001 with a workshop on "The use of fish cells in ecotoxicology" (Castaño et al. (2003). ATLA 31, 317–351) followed by the establishment of a ECVAM Taskforce Ecotoxicology composed by Commission and Non-commission experts giving scientific advice on 3Rs methods and testing strategies.

The approaches focus on acute aquatic toxicity and bioaccumulation being the two endpoints in the regulatory framework (e.g. REACH, agrochemicals, pharmaceuticals), which use large numbers of fish. Thus, the threshold approach, a reduction strategy for acute aquatic toxicity testing recently approved by the ECVAM Scientific Advisory Committee (ESAC) has the potential to reduce the number of fish by 50%. Its implementation is discussed at European Union and OECD level. In partnership with regulatory bodies, industry and academia ECVAM is involved in various projects aiming at the complete replacement of the acute fish test using a testing strategy based on fish cells and fish embryos.

ECVAM collaborates with ILSI HESI on reducing the number of fish in bioaccumulation testing and will launch a study evaluating the use of *in vitro* methods for this purpose. More information on ECVAM: Marlies.Halder@jrc.it.

doi:10.1016/j.cbpa.2007.01.076

A1.9

Fish cell lines in physiology and ecotoxicology of fish

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Fish cell lines can be derived from primary cultures of cells, tissues or organs taken directly from organisms. As all vertebrate cell lines, they can be propagated for a limited number of times, in which case they are finite, or indefinitely, in which case they become immortal. Compared to other *in vitro* models, cell lines are easy to handle and maintain. They generally comprise a continuous source of experimental material and because of their homogeneity, produce well reproducible results. Yet, based on available methods of derivation and culture, they tend to lose some of the characteristics of their origin. The ability of cell lines to express specific functions therefore needs careful consideration prior to application to the research question at hand. Initially, fish cell lines were derived to study piscine pathogens. By now, however, they are widely used in fish physiology and ecotoxicology and many new cell lines from different fish have been initiated in recent years. Research relying on fish cell lines ranges from studies on nutritional requirements to the development of methods to replace fish in the ecotoxicological risk assessment of chemicals and effluents. The presentation will outline specifics of fish cell line derivation and culture, illustrate their potential in physiology and ecotoxicology as well

as highlight research needs for their continued and even broader use.

doi:10.1016/j.cbpa.2007.01.077

A1.10

Fish hepatocyte cultures as an alternative to *in vivo* tests for screening oestrogen receptor active chemicals

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A wide variety of chemicals in the environment have the potential to disrupt the endocrine systems of wildlife. Of particular concern are environmental oestrogens, which can adversely affect reproduction and development. At OECD and US EPA there has been a focus on the development of *in vivo* approaches for screening chemicals with oestrogenic activity. However, the number of fish used for regulatory purposes is rising and there is increasing desire to reduce animal testing. In turn this is driving the need for the development of alternative, *in vitro* techniques to screen chemicals for adverse effects.

In the current study, stimulation and inhibition of vitellogenin (VTG) synthesis in primary cultures of carp (*Cyprinus carpio*) hepatocytes was adopted to assess the relative oestrogenic potency of a range of steroidal oestrogens that have environmental relevance. Compounds screened included the natural oestrogen, 17 β -oestradiol (E2), and oestrogens included in the contraceptive pill and hormone replacement therapy, specifically, ethinylloestradiol (EE2), the equine oestrogen, equilenin (EQL), and one of its metabolites, dihydroequilenin (DHQ). These steroid oestrogens induced time- and dose-related inductions of VTG, both at the level of protein and gene expression. EE2 was significantly more potent than E2, while EQL and DHQ were considerably less potent. The responses of these oestrogen receptor agonists *in vitro* reflected their relative potencies *in vivo* using VTG induction.

These data show that primary cultures of carp hepatocytes offer potential as a sensitive screen to detect chemicals that interact with oestrogen receptor(s) and thus could potentially be applied at the screening stage of suspected oestrogen receptor active chemicals. Our work, however, did find considerable inter-animal variability in the responsiveness of hepatocytes and the causes and biological significance of this are being investigated.

doi:10.1016/j.cbpa.2007.01.078

A1.11

Organ perfusion methods for *in vitro* fish physiology and toxicology: An overview with example data from gill and intestine

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Organ perfusion methods are available for a range of tissues including gill, gut, liver and heart of fish. The advantages of the perfused organ approach include the ability to identify the role of a particular organ in a physiological or toxicological process; whilst retaining control over physiological parameters such as blood flow, dosing regimes, hormones, and other factors in the blood. Organ perfusions can be used to explore accumulation or metabolism. Critically, the methods require some training prior to use and physiological or biochemical integrity may be hours rather than days. Viability criteria and histological integrity of preparations are discussed, and should reflect the normal physiological structure/function of the organ. The utility of the preparations are illustrated with reference to salt and trace metal uptake across the perfused trout gill preparation, as well as pharmacological investigations of solute transport in the intestine. The latter include the effects of cyanide on copper and mercury transport, and the effect of sodium and chloride transport inhibitors on trace metal uptake across the catfish intestine.

doi:10.1016/j.cbpa.2007.01.079

A1.12

Measuring intestinal water transport *in vitro*: Gravimetric method vs. non-absorbable marker

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Measuring ion and water transport rates using the gut sac technique requires accurate determinations of volume change, which are usually estimated gravimetrically. However, inconsistencies can result while blotting the sac and from fluid accumulation in the sub-epithelial layers, leading to consideration of alternative methods. Polyethylene glycol (PEG) is one of the most commonly used volume markers *in vivo*, but has yet to be comprehensively tested *in vitro*. Using European flounder (*Platichthys flesus*), we investigated whether [¹⁴C] PEG in the mucosal fluid represented an improvement. Our results show that using [¹⁴C] PEG yields a water transport rate (J_v) of $1.33 \pm 1.49 \mu\text{l cm}^{-2} \text{h}^{-1}$ compared to $4.64 \pm 0.98 \mu\text{l cm}^{-2} \text{h}^{-1}$ gravimetrically ($P=0.011$). On average, $99.7 \pm 0.04\%$ of the original [¹⁴C] PEG activity could be recovered from the sac at the end of a flux (only $0.2 \pm 0.02\%$ leaking to the serosa). However, serial sampling of the mucosal saline over a 6 h period suggested PEG may not distribute homogeneously within the sac, thus influencing the calculation of J_v . [¹⁴C] PEG activity in the mucus was found to be strongly correlated with the amount of mucus present, and over the course of a 2–3 h incubation activity in the mucus increased from $1.1 \pm 0.1\%$, after taking the initial sample, to $2.1 \pm 0.4\%$ upon removal of the final sample. In conclusion, using PEG as a non-absorbable marker has some drawbacks, for the intestine of *P. flesus* at least, and the precise causes are under further investigation.

doi:10.1016/j.cbpa.2007.01.080

A1.13**Overview of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)**

A. Cook, V. Robinson, (NC3Rs, United Kingdom)

The National Centre for the Replacement, Refinement and Reduction of the use of Animals in Research (NC3Rs) provides the UK focus for funding and promotion of research into the 3Rs. The Centre was established in September 2004 to translate the UK government's commitment to the 3Rs both strategically and operationally through the funding of high quality 3Rs science. The Centre is an independent, scientific organisation reporting into the DTI. It has a non-executive board and a wide range of founders (largely from the Government) and stakeholders, including industry, academia, Government and regulators, animal welfare organisations, the media and the public. Since its launch, the

NC3Rs has become a catalyst and focus for 3Rs research in the UK. This has been achieved by:

- Providing dedicated research funding for the 3Rs;
- Improving access to 3Rs information and resources, eg. the NC3Rs website (www.nc3rs.org.uk);
- Raising the kudos of the 3Rs in the scientific community;
- Identifying and fostering specific 3Rs initiatives and activities;
- Working with regulators on the acceptance of alternative methods;
- Maximising efforts and resources through partnerships and collaboration.

In summary, the NC3Rs takes a scientific approach to minimising the use and improving the lives of animals in research and this is achieved by championing a range of initiatives to increase investment, effort and energy in the 3Rs in the UK.

doi:10.1016/j.cbpa.2007.01.081
