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A6–HORMONES AND PHEROMONES: FROM MOLECULES TO ENVIRONMENT

Organised by S.G. Webster and A.J. Mordue for the Endocrinology Group

A6.1 The role of urotensin I in the adaptive physiology of the euryhaline flounder *Platichthys flesus*

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The caudal neurosecretory system (CNSS) is a neuroendocrine gland unique to fish, which secretes urotensin I (UI) and urotensin II (UII). It is located in the last 6 vertebrae of the spinal cord and composed of magnocellular neurones (Dahlgren cells), which terminate in a neuroheamal storage organ, the urophysis. The urophysis drains directly into the caudal vein, that supplies the renal portal system and effects the main osmoregulatory tissues in fish.

The European flounder *Platichthys flesus* is a marine fish but has the ability to survive in fresh water. This euryhaline characteristic makes it an ideal species to investigate the role of the CNSS in osmoregulation. This can be achieved by manipulating the environment of the fish and observing the changes in UI and UII.

Our development of a radioimmunoassay for UI has allowed measurement of urophyseal UI peptide store. Utilisation of this assay has allowed the identification of changes in stored UI when flounder are moved between different salinities. Preliminary data suggests that movement from FW to SW results in an increased storage of UI in the urophysis at 8 hours, followed by a decrease in storage at 24 hrs post transfer.

To understand relative concentrations of UI and UII in osmoregulation the differential secretion of both peptides is of interest. One technique used to investigate this is double immuno gold labelling of urophysal tissue, labelling for UI and UII at the electron microscope level. This has revealed different populations of secretory granules, these include nerve termini which contain

either UI or UII granules alone, termini with separate granules for UI and UII and in a small number of axon endings granules with both peptides colocalised. Taken together with electrophysiological studies (Brierley MJ et al, J Exp Biol. 2003) these results suggest existence of separate subpopulations of Dahlgren cells.

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A6.2 Circulating levels of PTHrP in the European flounder *Platichthys flesus*: so much hormone, but where is it coming from?

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Previously we have described the development of a homologous radioimmunoassay for flounder PTHrP and PTHrP sensitivity to environmental calcium levels, implying a role in calcium homeostasis (Rotllant et al., 2002; Worthington and Balment, 2002). Continuing research has focused on the source of PTHrP, as it exists at levels approximately 10–50 fold higher compared to other circulating hormones (around 100–200 pmol/l in seawater and freshwater fish). Teleosts lack parathyroid glands that secrete PTH in tetrapods and as yet no tissue has been identified as the main secretory source for circulating PTHrP. Tissue homogenisation and extraction from flounder revealed ubiquitously low concentrations of PTHrP at around 100 fmol/g tissue, the pituitary and urophysis in particular having almost no detectable stored content. Removal of the urophysis, however, resulted in a significant drop in circulating PTHrP levels after 48 hours after surgery when compared to control animals, 140 ± 15 (n=6) and 220 ± 36 pmol/l (n=6) respectively (one tailed unpaired t-test, P=0.024). Furthermore, *in vitro* incubation of the urophysis indicated no spontaneous release of PTHrP, whereas primary culture of the pituitary resulted in a release of 1.73 ± 0.31 pmol/g tissue over a 24 hour period, almost 10 fold more than present in homogenised pituitary. We purport that release of PTHrP into circulation may occur con-

stitutively from the pituitary, possibly modulated by urophysiological factors. Research supported by the BBSRC.

A6.3 Role of the hypothalamic-hypophysial-interrenal axis in hypoosmotic regulation in acipenserids

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The components of hypothalamic-hypophysial-interrenal axis in freshwater (FW) and diadromous brackish water (BW) acipenserids during their adaptation to seawater of 12.5‰ salinity were studied. FW-sturgeons are adapted to seawater as osmoconformers, whereas BW-sturgeons have the ability to keep up osmotic and ionic homeostasis under the change of environmental salinity, but their functional possibilities are different.

Neurophysin and arginin-vasotocin-immunoreactive cells of the preoptic nucleus and ATCH-immunoreactive cells of the tuberal nucleus in FW- and BW-acipenserids respond to saline influence by the release of neurosecretory granules into the axons and then in the median eminence. Morphological-functional changes of interrenal gland in BW-sturgeon show that this endocrine organ is included in the osmotic regulatory process. In the interrenal gland of FW-sturgeons, the accumulation of steroid material preponderates after primary release of the hormones during the first hours of saline adaptation.

In FW the contents of cortisol in blood serum of all studied species are equal ($P > 0.05$). Three hours after transfer of FW-sterlet (*Acipenser ruthenus*) to seawater from FW, cortisol level increases by four times and remained high during all experiments (7 days), as well as in FW-Siberian sturgeon (*A. baeri stenorrhynchus*). In anadromous Russian sturgeon (*A. gueldenstaedtii*) cortisol content decreases during the first 12 hours, but it is restored to the end of the experiment. In euryhaline anadromous starry sturgeon (*A. stellatus*) the cortisol level is unchanged.

Dynamics of Na^+/K^+ ATP-ase activity in the gill and the kidney is different in the species studied during their adaptation to seawater. In FW-sterlet the activity of this enzyme was higher (3 times) in the kidney than in the gill not only in fresh water, but also in seawater (6 times). In starry sturgeon, as well as in euryhaline teleosts, Na^+/K^+ ATP-ase activity increases (11 times) in the gill and decrease (10 times) in the kidney during adaptation to seawater. Thus, the distinctions in the function of hypothalamic-hypophysial-interrenal complex give possibility to discuss about the distinction in ion transport ability of different species of acipenserids.

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A6.4 The barnacle settlement factor is a novel protein related to the Alpha macroglobulin superfamily

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The molecular identity of the chemical cues that facilitate gregarious settlement in barnacles has eluded researchers for over fifty years. The determination of these cues is crucial for our understanding of the evolutionary origins of gregariousness and allo-specific associations in cirripedes and for the development of antifouling strategies and aquaculture. In the present study, we demonstrate that the Settlement-Inducing Protein Complex (SIPC), one of the molecules involved in chemical signalling of attraction and settlement in the barnacle *Balanus amphitrite*, is a novel protein related to the alpha-2 macroglobulin (A2M) super family of glycoproteins. The full length of the cDNA, which was obtained by MOPAC and RACE reactions, encodes a complete open reading frame encoding of 1548 amino acids. The deduced amino acid sequence shares the highest homology (31%) with A2M of the soft tick insect. Moreover, the SIPC is likely to have lost the function of the general anti-protease activity characteristic of the A2M since it lacks the canonical thiol ester site that is specific to the family of alpha macroglobulin proteases. The SIPC sequence contains a signal peptide-like hydrophobic N terminal segment of 17 residues suggesting that it is a secreted protein. The tissue distribution was examined by a semi-quantitative RT-PCR and showed that SIPC mRNA is present in all tissues tested and significantly expressed in the cirri. The developmental pattern of expression of the SIPC transcript and protein also revealed that SIPC is expressed in the 7 larval stages and the adult.

A6.5 Phagocytosis by haemocytes of *Manduca sexta*: effects of plasma and of inhibition of signaling pathways

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Phagocytosis is relatively poorly characterised in insect haemocytes compared with that known about this process in vertebrate systems. Furthermore, relatively little has been reported about intracellular signaling pathways that drive phagocytic responses. The work presented aims to explore the effects of plasma and signaling path-

way inhibitors on phagocytosis of bacteria by haemocytes of *Manduca sexta*.

Haemocytes were collected into Grace's medium under sterile conditions and plated onto glass coverslips. The resulting monolayers were washed free of haemolymph and the number of viable haemocytes per coverslip was estimated. Plasma, plasma fractions or inhibitors in Grace's medium were added together with fluorescein-labelled, heat-killed bacteria at different bacteria:haemocyte ratios. Fluorescence of non-internalised bacteria was quenched with trypan blue, the haemocytes were fixed in formaldehyde and numbers of phagocytosed bacteria were counted.

Plasma markedly reduced phagocytosis: for example, when 50% v/v plasma was included and 500 bacteria were added per viable haemocyte, the mean number of phagocytosed bacteria was 0.05 ± 0.02 per haemocyte compared with 2.19 ± 0.15 per haemocyte for Grace's medium ($n=6$). Even at 10% v/v plasma, a small inhibition of phagocytosis was observed. This reduction in mean number of phagocytosed bacteria was partially reversed by the addition of heat-treated plasma at 50% v/v. Both genistein, a tyrosine kinase inhibitor, and LY294002, a specific phosphatidylinositol 3-kinase inhibitor, at concentrations of 10–100 micromolar, reduced the mean number of phagocytosed bacteria (e.g. for genistein 100 at micromolar, mean number of phagocytosed bacteria was 0.03 ± 0.003). The implications of these, and other results will be presented.

A6.6 An antidiuretic with diuretic activity: *Tenebrio* ADFb stimulates secretion by cricket Malpighian tubules

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Spring *et al.* (1988) reported the presence of an antidiuretic hormone (ADH) in the house cricket (*Acheta domestica*) that reduced Malpighian tubule (MT) secretion, but it was not further characterised. Two antidiuretic factors (Tenmo-ADFa,b) have been identified in the mealworm (*Tenebrio molitor*) and shown to reduce MT secretion by activating a cGMP-dependent cAMP phosphodiesterase which lowers the level of intracellular cAMP (Eigenheer *et al.*, 2002, 2003). The ADH activity described by Spring *et al.* (1988) suggests related peptides could be present in *Acheta*. At 1 μ M, Tenmo-ADFa had no effect on secretion by cricket MTs, whereas Tenmo-ADFB had diuretic rather than antidiuretic activity. The maximum response to Tenmo-ADFB is 70% that of acetakinin-II (Achdo-KII) and the EC_{50} is 0.2 μ M. Like Achdo-KII, the activity of Tenmo-ADFB is Cl⁻-dependent and it has no effect on the urine $[Na^+]:[K^+]$ ratio. In addition, both peptides depolarise V_{tep} , but have little effect on V_{bl} . The second messenger pathway activated by Tenmo-ADFB in *Acheta* MTs is unlikely to be cGMP, which hyperpolarises V_{tep} . Cyclic AMP can also

be excluded, because it too hyperpolarises V_{tep} and the actions of Tenmo-ADFB and 8Br-cAMP are synergistic. Taken together, the results suggest that Tenmo-ADFB, in common with Achdo-Ks, opens a chloride conductance pathway across the epithelium, thereby increasing NaCl/KCl transport into the lumen with water following osmotically. Cricket MTs lack stellate cells and the chloride conductance pathway is most likely paracellular, because there is no evidence for Cl channels in the basal membrane of principal cells.

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A6.7 Endocrine cascades and signalling during ecdysis and postmolt in crustaceans

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The brief period leading up to ecdysis can be loosely described as an endocrine melee! Very dramatic declines in circulating ecdysteroids precede massive but transient release of hyperglycaemic hormone (CHH) from gut endocrine cells, followed (within a few minutes) by a similar surge in crustacean cardioactive peptide (CCAP). These hormones control water uptake during moulting and subsequent stereotyped behaviours during ecdysis. We were interested in further investigating these (and new) endocrine phenomena, to develop models of moult control during the brief period prior to, and following ecdysis in the shore crab *Carcinus maenas*.

By using quantitative RT-PCR we have described expression patterns of the ecdysteroid receptors EcR and USP during premoult, and have shown that administration of the ecdysteroid agonist Tebufenozide increased gut CHH expression. Furthermore, we have shown that CHH is involved in an autocrine positive short-feedback loop involving cGMP. A very surprising finding, at odds with accepted models of moult control was that a brief but massive surge of moult-inhibiting hormone (far larger than any seen during intermoult) could be reproducibly observed, just prior to epimeral line rupture. Whilst it has long been known that circulating calcium levels rise during premoult and fall during postmoult, we found that there is a brief, but dramatic fall in Ca^{2+} , just prior to epimeral line rupture. Subsequent investigation of possible neurohormonal control of calcium, implicates involvement of novel calcitonin and PTHrP signalling system which may be important in de- and re-calcification events during pre- and post-moult.

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A6.8 Human-derived host masking compounds in Scottish biting midges

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Midges in Scotland have an enormous economic impact affecting outdoor industries (forestry, aquaculture) recreation, tourism and the wellbeing of rural communities. Previous work has shown that individual humans vary in their attractiveness to biting insects. Host-masking chemicals produced by non-attractive persons can be identified and used as natural human repellents for individuals who are attractive to midges. This lack of attraction in non-target individuals is due to specific components in the several hundred skin emanations. Such chemicals may prevent insects from recognising and locating a potential host. The current project aims to identify these specific chemicals, and to develop a new class of safe, natural insect repellents.

New behavioural bioassays were developed for use in the field. Individual human volunteers were ranked, using these bioassays, according to how attractive they were to midges. Skin emanations were collected from two volunteers and the extracts of these tested in the field bioassay. The extracts were found to be attractive to midges in a dose-dependant manner. This bioassay will be used in further laboratory and field tests to screen more volunteers, and the resultant extracts will be analysed and identified using gas chromatography and mass spectrometry.

A6.9 The search for natural repellents from human beings against mosquitoes

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Blood sucking insects are differentially attracted to human beings. This is likely to be caused by differences in the semiochemical profiles of individual human hosts. Despite substantial research into insect-human interactions, the responsible semiochemicals have not yet been identified. An explanation for this difference is that unattractive individuals either lack attractant chemical compounds or may produce compounds that 'mask' the activity of attractants. A special air entrainment technique was developed and used to collect volatile chemicals from human volunteers, and the extracts analysed

by gas chromatography (GC), coupled GC-electroantennography (GC-EAG) and GC-mass spectrometry (GC-MS). For the first time electrophysiological responses of female yellow fever mosquitoes, *Aedes aegypti* to volatiles from air entrainment extracts were recorded, and a number of electrophysiologically active compounds were identified. Previous behavioural studies have confirmed the differential attractiveness of volunteers to biting insects. A quantitative and qualitative analysis of compounds within the extracts, from individuals known to be differentially attractive to biting insects, has revealed significant differences in chemical profiles. The identification of behaviourally active compounds could lead to new and improved control technologies, whereby 'masking' compounds could potentially be incorporated into new, safe and natural repellents against biting insect pests.

A6.10 Melatonin binding sites in gill, gut and kidney of flounder and rainbow trout

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The pineal neurohormone melatonin (N-acetyl-5-methoxytryptamine) is a potent regulator of circadian and seasonal rhythms in vertebrates, including fish. In all species examined, plasma melatonin concentration shows a diurnal rhythm, with the higher levels during the dark period. In fact, melatonin is a multifaceted hormone engaged in many physiological processes. Many of its well-established physiological effects are mediated via high-affinity cell membrane receptors belonging to the superfamily of G-protein – coupled receptors. In this study, specific binding of ligand 2-[¹²⁵I] iodiomelatonin to homogenates from selected tissue membranes were investigated in flounder and rainbow trout. Flounder and rainbow trout were kept in sea- and freshwater, respectively, at artificial light-dark cycle 12L:12D. The kidney, gill and gut samples were collected during the day and at night. The kidney tubules and dispersed gill cells were prepared. The binding affinities (K_d) and maximal binding densities (B_{max}) were calculated for each tissue at the two time points. The binding sites with K_d values in the picomolar range appeared of high affinity. K_d and B_{max} values were different for both species, for each tissue and time point. This study provides the first evidence for the presence of melatonin binding sites (Mel

membrane receptors) in fish gut tissue, kidney tubules and dispersed gill cells and supports the earlier findings of Mel binding in kidney and gill tissue (Kulczykowska, 2002). Thus, the data strongly suggest new potential tar-

gets for melatonin action at these sites and consequently an influence of melatonin on water/ion balance in fish. Work supported by the Royal Society and Committee for Scientific Research