

## A4–INTER- AND INTER-ORGANISATIONAL MESSENGERS

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

### A4.1–Localization and physiological effects of RFamides in the corpora allata of *Diploptera punctata* in relation to allatostatins

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The corpora allata of insects receive innervation from neurosecretory cells of the CNS. In cockroaches, some of this innervation carries peptides of the allatostatin (AST) family that inhibit juvenile hormone (JH) biosynthesis.

In the present study, we demonstrate that other nerves to the CA carry members of the FMRFamide (RFa) family of peptides and in some of these, from the lateral cells of the protocerebrum, AST and RFa are colocalized. The quantities of RFa and AST peptides within the CA are similar (~300 fmol/pair CA) and they do not change during the first vitellogenic cycle.

The effect of RFa on JH production is stimulatory only at one stage of the vitellogenic cycle-day 6, the final day of oocyte development, at which time JH production is low. The effect of RFa on the ability of several members of the AST family (Dippu-AST 2, 5, 7) to inhibit production of JH was assessed on CA from mated females of this age. At different concentrations, RFa had an effect only on Dippu-AST 2. This effect was concentration-dependent and bi-modal, i.e. it reduced AST inhibition at 100 nM and increased AST inhibition at 10  $\mu$ M. The lower concentration is more likely physiological and suggests that RFa modulates the effect of AST on JH production. CA from day 6 mated females incubated *in vitro* release about 10% of their content of RFa in 3 hours. Treatment with Dippu-AST 2 (100 nM) results in enhanced release of RFa. This supports the notion that RFa modulates the effect of at least one of the ASTs.

### A4.2–Allatostatin-like peptides: crustacean and vertebrate analogues

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Orcostatins (CST8: AGPYAFGLa, OST-I: SAGPYAFGLa, OST-II: PRVYGFGLa) identified in crayfish *Orconectes limosus* exert inhibitory effects on crayfish hindgut muscle contractility and on stomatogastric pylo-

ric neurons. Orcostatins, 20 similar crab and 45 penaeid peptides belong to a superfamily of allatostatin (AST)-like peptides. Neuronal distributions and haemolymph contents were investigated in *Orconectes* by immunocytochemistry and a competitive EIA using anti-CST8 sera. Crayfish CNS contains interneurons associated with CNS-neuropils and neurosecretory neurons terminating in eyestalk and thoracic neurohaemal organs and appendage muscles. We differentiated CST8-peptides ranging from 20 to 200 fmoles/ml in crayfish haemolymph indicating orcostatins as circulating neurohormones. Furthermore, we provide first evidence for the existence of similar peptides in the Wistar rat brain by identification of peptides in rat brain extracts and by immuno-histochemistry on coronal frozen brains sections. Three-step HPLC- or immunoaffinity purification followed HPLC led to the identification of several CST8-ir fractions analysed by MALDI-TOF, especially two CST8-ir peptides of 3375 Da and 3692 Da which differ considerably from CST8 (793.9 Da) although peptides in the range of 3000–4000 Da have previously been shown in crustacean studies. CST8-ir peptides occur in several rat brain areas, in both fibers and cell bodies. Most intense staining was in the ventral hypothalamus, particularly the arcuate nucleus. Fewer numbers, or less densely labeled cells were present in cortex, thalamus, striatum, and some reticular brainstem areas, a labelling abolished by preabsorption of the antiserum with peptide at  $10^{-9}$  M. Further work on the elucidation of structures and physiological significance of these peptides is in progress.

### A4.3–Olfaction of catecholamines by the goldfish: possible intra-specific functions?

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The current study assessed the olfactory sensitivity of the goldfish (*Carassius auratus* L.) to the catecholamines and their metabolites by recording the electroolfactogram (EOG). Goldfish were found to be sensitive to both adrenaline and dopamine, with thresholds of detection of  $10^{-7.8}$  M and  $10^{-7.9}$  M respectively, but less so to noradrenaline (threshold of detection  $10^{-6.3}$  M). The 3-*O*-methoxy metabolites (metadrenaline, normetadrenaline and 3-*O*-methoxytyramine) evoked larger amplitude EOGs than the non-metabolised form with similar or lower thresholds of detection. However, the olfactory system was less sensitive to the precursors L-tyrosine and L-DOPA, and markedly less so to the  $\alpha$ -

deaminated metabolites (3,4-dihydroxyphenyl glycol, 3,4-dihydroxy mandelic acid and dihydroxyphenylacetic acid). Sensitivity to metabolites both  $\alpha$ -deaminated and 3-*O*-methoxylated was similar to the  $\alpha$ -deaminated form. Cross-adaptation studies suggested that, whilst there is some degree of commonality of the receptor mechanisms with L-tyrosine and L-serine, a proportion of the response to the catecholamines is due to distinct receptors. Similarly, the 3-*O*-methoxy metabolites also had (a) separate receptor mechanism(s) although, again, there was overlap with the adrenaline/dopamine receptor site(s). The  $\alpha$ -adrenoreceptor antagonist prazosin or the peripheral DA<sub>2</sub> dopamine receptor antagonist domperidone caused partial attenuation of the responses to adrenaline and dopamine but had much less effect on those of the 3-*O*-methoxy metabolites. The  $\beta$ -adrenoreceptor antagonist sotalol was ineffective. This suggests that the olfactory catecholamine receptors are structurally and functionally different from systemic adreno- and dopamine receptors. It is possible that release of catecholamines or their 3-*O*-methoxy metabolites to the water plays a role in chemical communication.

#### **A4.4—Chemical communication in the tilapia (*Oreochromis mossambicus*): the story so far**

E.N. Barata, P.C. Hubbard, P. Frade, O.G. Almeida, A. Miranda and A.V.M. Canário, CCMar, Universidade do Algarve

Despite their high degree of social organisation, chemical communication has received little attention in the cichlids. The current study investigated the possibility for chemical communication between individuals of the Mozambique tilapia. Males are territorial aggregating in breeding leks, and their reproductive success appears to be dependent on female choice. During their courtship display, males markedly increase their rate of urination only in the presence of pre-ovulatory females. Recording of the electro-olfactogram showed that females have a high olfactory sensitivity to substances released in male urine, and that urine from territorial males is more potent than urine from non-territorial males. Thin layer chromatographic fractions of male urine showing olfactory activity may contain sulphated steroids and/or bile acids. In addition, males are able to discriminate between females of different reproductive status, modulating their reproductive behaviour accordingly. This discrimination is likely to be based on olfactory input regarding substances released by females via their urine and/or faeces; both body fluids are more potent odorants from pre-ovulatory females than post-ovulatory. These results suggest that male urine contains a putative pheromone affecting female actions, the male being able to regulate the timing and intensity of signal release. Final-

ly, males also increase urination rate on encountering an intruding male, and have high olfactory sensitivity to male urine. In conclusion, these results strongly suggest that chemical communication is important to this species, not only in reproduction, but also in male–male interactions. The chemical identity of the substances involved and their exact biological functions must now be established.

#### **A4.5—Sea lice Olfaction: behavioural responses of infective larvae to host odours**

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The sea louse, *Lepeophtheirus salmonis* Krøyer 1838, is an important, highly specific ectoparasite of salmonid hosts, causing substantial economic losses to global production of Atlantic salmon and sea trout. The infective stage of the life cycle is the larval copepodid, its role being to successfully find and settle on a suitable host fish to ensure development to the adult stage. The mechanisms of host location and identification are believed in part to involve the use of host-derived chemical cues. Evidence is presented to show that the copepodid stage shows behavioural responses to such cues. Copepodid behavioural responses were observed in simple flow chambers and Y-tubes on addition of seawater conditioned with host-derived chemical cues. Copepodids were monitored for the presence of directional (taxis) and non-directional (kinesis) responses. Taxis was quantified using horizontal Y-tube assays which allowed lice to exhibit preference for one stimulus over another. Kinesis was quantified using a digital tracking system to compare copepodid behaviour and movement between potential stimuli. *L. salmonis* copepodids modified their behaviour in response to host odours, with both directional and non-directional responses being observed. The implications of these findings are discussed in terms of the host location process, behavioural search patterns and the potential use of such chemicals in integrated pest management strategies.

#### **A4.6—Acanthocephalan parasite infection exerts a controlling influence on osmoregulation in the freshwater amphipod *Gammarus pulex***

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The acanthocephalan endoparasite *Polymorphus minutus* requires 2 hosts to complete its life cycle. *Gammarus pulex* acts as an intermediate host, and the definitive host is a bird. Following ingestion of a parasite egg, the larval

stage of *P. minutus* bores into the haemocoel of *G. pulex* where it develops into a cystacanth. When the cystacanth is fully developed it alters the behaviour of *G. pulex* so that its risk of predation by the definitive host increases. The amphipod *Gammarus pulex* is widely distributed in standing and running fresh water habitats in Britain. When acclimated to a range of salinities up to 30‰ sea-water it is hyperosmotic to the medium. However haemolymph ion concentrations and whole body sodium fluxes change with salinity of the medium. The aim of this investigation was to determine if the agent that alters the behaviour of infected *G. pulex* would also disturb osmoregulation.

Parasite infection was found to profoundly alter cation regulation. At an acclimation salinity of 15‰ sea water there was a marked hyperregulation of haemolymph sodium, potassium, magnesium and calcium concentration in infected *G. pulex* with respect to control *G. pulex* acclimated to the same salinity. However, no difference in whole animal water permeability was caused by parasite infection. Surprisingly parasite infection reduced sodium influx despite increasing haemolymph sodium concentration. This apparent discrepancy was resolved when experiments demonstrated parasite infection lowered sodium efflux. Experiments currently in progress will determine the inter organismal messengers involved.

#### **A4.7—Sex pheromone in nereidid polychaetes and shorecrabs—from maturation to reproduction**

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Spawning events such as the mass swarming in nereidid polychaetes require environmental as well as endocrine biochemical 'timing' to ensure synchronised maturity and reproduction within a population. Sex pheromone production in *Nereis succinea* is a late event in the lifespan of these monotelic polychaetes, with significant amounts of the pheromone 'Nereithione' (Cysteinyl-Glutathione, CSSG) produced only once the individuals have reached the final stages of metamorphosis into the reproductive form. Pheromone production in females is directly correlated to oocyte growth and can be used as a marker for maturation. Upon leaving the sediment to undertake the spawning event culminating in gamete release into the water, female heteronereids release substantial amounts of pheromone (mg) using glutathione and Cysteine as precursors. This depletes GSH levels in the coelom significantly and is seen as a 'cost of signalling'. Only actively swimming females release labelled pheromone when injected with labelled GSH, suggesting a significant role in the spawning process itself in the production of the cue. In shore crabs, *Carcinus maenas* sex pheromone production in females only

occurs around the time of moulting, and is maximal 3–5 days after the moult, when hardening out of the new cuticle would make further matings physically difficult.

#### **A4.8—A novel action of the endocannabinoid anandamide in mammalian brain**

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It is well established that endocannabinoids such as anandamide reduce neuronal excitability and transmitter release by modulating presynaptic  $Ca^{++}$  and  $K^+$  channels, either through cannabinoid (CB1) receptor stimulation or direct action. We investigated the ability of anandamide and other cannibimimetics (AM 404 and WIN 55,212–2) to interact with voltage-gated sodium channels using biochemical and electrophysiological techniques. We found that low micromolar concentrations of anandamide, AM 404 and WIN 55,212–2 inhibit veratridine-dependent depolarization of synaptoneurosomes, veratridine-dependent release of L-glutamic acid and GABA from purified synaptosomes and [ $^3H$ ]batrachotoxinin A 20- $\alpha$ -benzoate binding to voltage-sensitive sodium channels. Moreover, in cortical neurons anandamide, AM 404 and WIN 55,212–2 fully suppress TTX-sensitive sustained repetitive firing without influencing primary spikes. Anandamide's action is reversible and its inhibitory potency at sodium channels is increased by inhibition of fatty acid amidohydrolase. Significantly, the potent CB1 antagonist AM 251 is unable to reduce the inhibitory effects of anandamide and the cannibimimetics on voltage-sensitive sodium channels. We propose that voltage-sensitive sodium channels participate in a novel signaling pathway involving anandamide. This mechanism depresses synaptic transmission in brain by damping neuronal capacity to support action potentials and reducing evoked release of both excitatory and inhibitory transmitters. In addition to depressant actions of AM 404 (as an anandamide uptake inhibitor) and WIN 55,212–2 (as a CB1 receptor agonist) at synapses, these cannibimimetics clearly produce similar effects through sodium channel blockade.

#### **A4.9—Effects of $CAP_{2b}$ , ion replacement, and 2,4-DNP on *Musca domestica* Malpighian tubules**

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*Musca domestica* principal cell basolateral membrane potential ( $V_{b1}$ ) was determined to be  $-41.96 \pm 0.47$  mV

( $n=100$ ), cell negative and the transepithelial potential ( $V_{\text{tep}}$ ) was  $48.06 \pm 0.89$  mV ( $n=82$ ), lumen positive, yielding a mean apical potential of 90.02 mV. Chloride-free saline leads to an increase in  $V_{\text{bl}}$  and a slow increase in  $V_{\text{tep}}$ . Phosphate-free saline leads to a slow, steady decrease in both  $V_{\text{tep}}$  and  $V_{\text{bl}}$ . Furthermore, 2,4 Dinitrophenol, an uncoupler of mitochondrial oxidative phosphorylation leads to a rapid collapse of  $V_{\text{tep}}$  and  $V_{\text{bl}}$ . All effects were reversible upon return to *Musca* saline. Application of CAP<sub>2b</sub> (100 nM) leads to a rapid and sustained collapse of the luminal membrane potential. Concurrently, CAP<sub>2b</sub> causes a small increase in  $V_{\text{bl}}$ . Significantly, CAP<sub>2b</sub> has no effect on  $V_{\text{tep}}$  or  $V_{\text{bl}}$  if applied in chloride-free saline. This indicates that the effects of CAP<sub>2b</sub> are dependent on the presence of extracellular chloride. Interestingly, in *D. melanogaster*, physiological levels of CAP<sub>2b</sub> cause hyperpolarisation of the transepithelial potential. However, as discussed above, we find that CAP<sub>2b</sub> causes a rapid, sustained depolarization of the transepithelial potential with a concurrent hyperpolarization of the basolateral membrane potential. This suggests that in *Musca*, in terms of stimulation of fluid secretion, CAP<sub>2b</sub> acts in a similar manner to the kinin family of peptides. Specifically they open the shunt pathway for the passage of anions (predominantly chloride) from the bathing saline into the lumen. Among other conclusions, our results suggest caution must be shown with regards to the use of *D. melanogaster* as a model insect.

#### A4.10—Calcium signalling in *Manduca sexta* haemocytes

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Although the responses of haemocytes to immunostimulants have been studied extensively, relatively little is known about the intracellular signaling pathways that are evoked during these responses. The work presented aimed to explore the role of calcium in plasmatocytes of *Manduca sexta* in response to substances that have been implicated in playing a role in insect immunity. Plasmatocytes were isolated from haemolymph under sterile conditions using a differential cell fractionation method with *p*-nitrophenol guanidinobenzidine and plated onto glass coverslips for imaging. Calcium imaging was performed using Fura-2 as the calcium reporter. Octopamine, a biogenic amine which has been implicated in enhancing phagocytosis and the nodulation response in other insects, induced increases in intracellular calcium in a dose-dependent manner. These were dependent on the presence of extracellular calcium and did not require mobilization of calcium from intracellular stores. Caffeine, a pharmacological agonist of the ryanodine receptor, also induced an increase in intracellular calcium, which suggests the presence of this recep-

tor in the endoplasmic reticulum. Adipokinetic hormones I, II and III from *Locusta migratoria* failed to produce a calcium response in *Manduca* plasmatocytes.

#### A4.11—Changes in apolipoprotein-III in response to injection of immunogens are related to the activation of prophenoloxidase in the haemolymph of *Locusta migratoria*

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Prophenoloxidase in the haemolymph of adult *Locusta* is activated in response to injection of the immunogen laminarin, and co-injection of adipokinetic hormone (AKH) prolongs the period of activation of the enzyme. However, these responses are absent in 5<sup>th</sup> instar hoppers and newly emerged adults, and only become evident four days after the final moult. Intriguingly, this pattern of change in prophenoloxidase activation correlates with the patterns of change in the concentration of apolipoprotein-III (apoLp-III) in the haemolymph, and of AKH-induced lipid mobilisation. Thus, injection of *Lom*-AKH-I has no effect on the phenoloxidase response to laminarin in larvae or newly emerged adults, but in adults older than four days co-injection of the hormone prolongs the maintenance of high levels of phenoloxidase in the haemolymph for several hours [Goldsworthy, G., K. Opoku-Ware, and L. Mullen (2002). *Journal of Insect Physiology* 48(6): 601–608]. After injection of laminarin or another immunogen, bacterial lipopolysaccharide (LPS), the concentration of apoLp-III decreases, and (lipid-loaded) LDLp forms in the haemolymph: changes that are remarkably similar to those occurring after injection of AKH-I. However, measurement by immunoassay of the titres of AKH-I in the haemolymph does not indicate release of endogenous AKH-I by these immunogens. Furthermore, while passive immunisation of locusts against AKH-I prevents lipid mobilisation in response to injected AKH-I, it does not alter metabolic or immune responses to injection of laminarin or LPS. The awards of a University of London Jubber Research Studentship to LM and a Leverhulme Trust Research Fellowship to GG are gratefully acknowledged.

#### A4.12—Investigating the relationship between lipid status and bacterial lipopolysaccharide-induced activation of the immune system in locusts

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Injection of bacterial lipopolysaccharide (LPS) induces nodule formation in mature fed adult locusts, but does

not increase phenoloxidase activity unless adipokinetic hormone (AKH) is co-injected. When different components of LPS from the *E. coli* Rd mutant are tested, the mono- and the di-phosphoryl Lipid A components behave in a similar way to the intact LPS but, remarkably, detoxified (de-lipidated) LPS activates phenoloxidase in the absence of AKH, although co-injection with hormone enhances this response. Interestingly, short periods of starvation make adult male *Locusta* sensitive to injection of intact LPS, even without co-injection of AKH. How does starvation enable the activation of the prophenoloxidase cascade by LPS, mimicking the effect produced when LPS is co-injected with AKH? Evidence is presented to show that increased mobilisation of lipid (such as occurs during starvation, or after injection of AKH) is a key feature of the phenoloxidase response to immunogens like LPS or laminarin (a  $\beta$ -1,3 glucan). Furthermore, the lipid mobilised after injection of LPS is shown to be diacylglycerol, which is the class of lipid mobilised in the haemolymph during flight or starvation. The haemolymph of the LPS-injected locusts has high antimicrobial activity in comparison with naïve controls, and the influence of AKH-I on the appearance of antimicrobial activity in the haemolymph will be discussed. The award of a Leverhulme Trust Research Fellowship to GG is gratefully acknowledged.

#### **A4.13—Novel roles for adipokinetic hormones in the immune responses of locusts and beetles**

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Nodule formation and the activation of the prophenoloxidase cascade have been studied in *Locusta migratoria* and *Tenebrio molitor*. Injection of bacterial lipopolysaccharide (LPS) induces nodule formation in mature adult locusts but does not increase phenoloxidase activity. Nodules form mainly associated with the dorsal diaphragm on either side of the dorsal blood vessel, but sometimes with smaller numbers on the ventral diaphragm either side of the nerve cord. Co-injection of adipokinetic hormone-I (*Lom*-AKH-I) and LPS increases phenoloxidase activity and brings about dose-dependent increases in nodule formation and. In locusts the activation of the prophenoloxidase is associated with increased lipid mobilisation, whereas stimulation of nodule formation occurs independently of this effect. In larvae of *Tenebrio*, injection of *Tem*-HrTH also affects nodule formation and phenoloxidase activity but, this time, the effects are inhibitory. Compared with locusts, smaller numbers of nodules are formed in *Tenebrio* in response to injection of LPS, and they are widely distributed within the haemocoel. Further, injection of LPS reduces the activity of haemolymph phenoloxidase. Co-

injection of *Tem*-HrTH with LPS reduces the numbers of nodules formed, and inhibits phenoloxidase activity further. It is argued that these differences in the effects of AKH on the immune responses of *Locusta* and *Tenebrio* to LPS injection are reflections of differences in the energy metabolism of the two insects.

The award of a Leverhulme Trust Research Fellowship to GG is gratefully acknowledged.

#### **A4.14—Genes, transcription and translation (or not) the tangled web of the hyperglycaemic hormones in crustaceans**

J.S. Chung and S.G. Webster, Biological Sciences, Bangor

Multiple forms of CHH neuropeptides present in various tissues (i.e. X-organ, pericardial organ, and gut) of the green shore crab, *Carcinus maenas* might result from either different gene products or alternative splice variants. With regard to peptide levels, CHHs in both X-organ-sinus gland (XO-SG) and pericardial organs (PO) were invariant throughout moult cycle, with exception of a slight increase in the level of PO CHH during premoult. A similar situation was seen for mRNA levels (measured by quantitative RT-PCR) but in this case much greater increases in PO CHH transcript numbers occur during premoult. In contrast, there were large changes in type and quantity of CHH transcripts in the gut tissues during the moult cycle. For example, intermoult gut contained a CHH transcript variant, which was never translated, whilst the presence of a gut CHH transcript and neuropeptide, identical to XO-SG CHH, was stage-specific to premoult. The mechanism behind the different patterns of CHH expression during premoult is not yet understood. However, we noticed that the sudden change in the expression patterns (from untranslated to translated CHH mRNAs) was coincident with a high titre of haemolymph ecdysteroids. This suggests that the expression of CHHs of X-organ and of PO may be quite differently regulated from that of gut (possibly having different upstream promoter regions). We are currently measuring the quantitative expression of ecdysteroid receptors (EcRs) and ultraspiracle (Usp) to see whether transcription levels of these correlate with rising ecdysteroid titre and CHH transcript expression patterns in these CHH producing tissues.

#### **A4.15—cDNA cloning of a vasa-like gene of the green shore crab, *Carcinus maenas***

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The origin of germ cells and the molecular mechanisms of primordial germ cell (PGC) determination in crusta-

ceans is unclear. Vasa is a member of the DEAD (Asp–Glu–Ala–Asp) protein family that plays an indispensable role in the germ cell determination both in vertebrates and in invertebrates. In the present study, we isolated and characterised a vasa cDNA from the green shore crab, *C. maenas*. This is a first step towards understanding the molecular mechanisms of PGC determination and gonad development, which subsequently might be useful as a molecular marker for identifying PGCs during development. Additionally, an understanding of PGCs determination will be useful in determining the nature and mode of action of androgenic hormone in decapod crustaceans. Cloning of vasa cDNA was performed by degenerate- and RACE-PCR. The cDNA (2905 bp) contained an open reading frame (ORF) conceptually translating a 695 amino acid residue protein. Overall amino acid similarity to *Drosophila* Vasa was 50%. The predicted amino acid sequence of *Carcinus* Vasa contained eight consensus sequences for the DEAD protein family and three arginine–glycine–glycine (RGG) repeats, a common characteristic of known Vasa homologues. The predicted vasa contains one retroviral-type zinc finger motif. Vasa-related proteins containing the zinc finger motif are known only in a nematode, an ascidian and a hydra. The RNA expression patterns are currently being analysed.

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#### **A4.16—Ontogeny of peptidergic neurones during embryonic development of crabs**

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Development of neurosecretory neurones containing moult-inhibiting hormone (MIH), crustacean hyperglycaemic hormone (cHH) and pigment-dispersing hormone (PDH) was studied during embryonic development of the shore crab, *Carcinus maenas*, using wholemount confocal microscopy. For the 'simplest' system (MIH), immunopositive perikarya (2 pairs) were first observed in the dorsal eyestalk anlage, during early eye development. In later stages of embryogenesis, immunopositive material was observed in the sinus gland, but no further MIH immunopositive structures were seen during development. For CHH, a similar situation occurs, but 4 pairs of perikarya expressed cHH during eye anlage formation. However, during later embryogenesis, a complex pattern of neurones in the thoracic ganglia begin to express cHH, which project axons to the pericardial organs (PO). These probably express the PO-cHH variant. A few days before eclosion, a novel set of segmentally arranged peripheral neurones start to express cHH (XO-cHH variant). These are particularly prominent in the abdomen, where each seg-

ment contains a pair of dorsal and median neurones. These appear to be involved in a massive surge in cHH release immediately prior to larval eclosion. For PDH, an extremely complicated pattern of immunoreactive neurones develop. These are first seen in the brain, before eye anlage formation. Subsequently, neuronal architecture reminiscent of that seen in the adult eye develops, and finally, immunopositive axons and dendrites appear throughout the entire ventral nerve cord. This work is supported by BBSRC, ref. 5/S13837

#### **A4.17—Temptation, scent, and dance— An experimental analysis of chemically and visually mediated anemonefish and grouper behavior**

H.-D. Huang and D. Rittschof, Marine Laboratory, Duke University; M.-S. Jeng, Inst. Zoology, Academia Sinica

Anemonefish display specific swimming behaviors when chemically prompted by their hosts (Murata et al., 1986), and these movements are similar to anemonefish behavior elicited by an approaching predator. The present study tests the hypothesis that anemonefish function to lure predators to become anemone prey. Within 60 seconds of exposure to anemone *Entacmaea quadricolor* odors, naïve clownfish *Amphiprion ocellaris* responded by performing relatively low time proportion of head-up swimming. Both individual and simultaneous, visual and chemical factors from a predator, grouper *Epinephelus melanostigma*, significantly enhanced clownfish seesawing behavior. When tested with visual cues, life size anemone and clownfish decoys, naïve grouper showed no aggression. The grouper became alert and aggressive when exposed to odors from the assemblage. When tested with combined chemical and visual cues from the live symbiotic assemblage, the grouper displayed aggressive behavior and attacked. These data support the lure hypothesis.

#### **A4.18—Urination rate of male tilapia (*Oreochromis mossambicus*) is highly dependent on social context**

O.G. Almeida, E.N. Barata, P.C. Hubbard and A.V.M. Canário, CCMar, Universidade do Algarve

The Mozambique tilapia (*Oreochromis mossambicus*) has high olfactory sensitivity to male conspecific urine. To establish whether this could have a communicative function, the rate of urination by males was measured in different social contexts; in the presence of non-reproductively receptive (post-ovulatory) females, reproductively receptive females (pre-ovulatory), intruder males and resident males. Urine was visualised by i.m. injection of a dye (isosulfan blue), and the frequency and

duration of urination recorded during a control period (isolated) followed by an experimental period (in the presence of one conspecific). In the presence of pre-ovulatory females, the males' urination rate increased dramatically, and remained elevated throughout the recording period (45 minutes). This was associated with other, typical, courtship behaviours. In the presence of post-ovulatory females, urination also increased, but not as markedly. Similarly, the males showed some courtship behaviours, but not with the frequency or intensity as in the presence of pre-ovulatory females. In the presence of an intruding male, the resident's rate of urination increased dramatically. However, this was then followed by a fight, during which urination dropped to near-zero. Conversely, the urination rate of the intruding male did not change throughout the experiment. These results not only suggest that male tilapia are able to tell both sex and reproductive status of conspecifics, but also that males modulate their rate of urination depending on the social context. Given the high olfactory sensitivity to males' urine, this is strongly indicative that chemical communication, via substances released in the urine, is occurring in this species.

#### **A4.19—Olfactory detection of a putative reproductive pheromone in tilapia (*Oreochromis mossambicus*)**

P. Frade, E.N. Barata, P.C. Hubbard and A.V.M. Canário, CCMar, Universidade do Algarve

Female tilapia (*Oreochromis mossambicus*) have high olfactory sensitivity to the urine of conspecific males. The current study was designed to assess whether the strength of this putative pheromonal message is dependent on the social status of the male, and to investigate the chemical nature of the compound(s) involved. Urine was collected from two groups of males; 'territorial' and 'non-territorial' (differentiated by coloration and behaviour) and pooled. The two pools were then subjected to solid-phase extraction and reverse-phase thin-layer chromatography (TLC). Fractions were then recovered and tested for olfactory potency by recording electro-olfactograms (EOG) from females. Other TLC plates were stained with Zimmerman's reagent or phosphomolybdic acid. Females had high olfactory sensitivity to untreated urine of both pools but the territorial urine evoked EOGs of significantly higher amplitude than non-territorial. This was also true of the solid-phase extracts. Staining with Zimmerman's reagent revealed a diffuse zone staining heavily in fractions 12–14. These fractions also evoked large amplitude EOGs: the fractions from territorial urine again evoked larger amplitude EOGs than non-territorial urine. Sulphated bile acids and steroids

also migrated to this zone. Their non-sulphated equivalents do not migrate so far (fractions 7–9) and these fractions of either pool did not evoke large amplitude EOGs. Given that males dramatically increase their urination rate in the presence of sexually mature females, the current study strongly implicates (a) sulphated steroid(s) released in the males' urine as playing a role in chemical communication during reproduction. The exact chemical identity and biological function are currently under investigation.

#### **A4.20—Olfactory discrimination of female reproductive status by male tilapia (*Oreochromis mossambicus*)**

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Male tilapia (*Oreochromis mossambicus*) modulate their behaviour differentially in the presence of pre-ovulatory and post-ovulatory females, respectively. In the absence of marked visual or behavioural differences in the females, the current study assessed whether this discrimination could be based on odors released by females in different reproductive condition. Post-ovulatory (one to three days after the last ovulation) and pre-ovulatory females (one day prior to predicted ovulation) were kept in isolation for two hours and solid-phase extracts were made of water samples. Samples of body-fluids (urine, faeces, bile and plasma) were also taken and pooled. The olfactory sensitivity of males to these samples was assessed by recording of the electro-olfactogram (EOG). Solid-phase extracts of the water samples from pre-ovulatory females consistently evoked EOGs of greater amplitude than those of post-ovulatory females ( $P < 0.01$ ). Similarly, the urine and faeces of pre-ovulatory females elicited larger responses in males than those of post-ovulatory females ( $P < 0.001$  and  $P < 0.05$ , respectively). No differences were noted in the responses to dilutions of bile fluid or plasma from the two groups of females ( $P = 0.92$  and  $P = 0.85$ , respectively). These results suggest that male tilapia could use olfactory information to discriminate the reproductive status of females. Furthermore, the odorants involved are likely to be released mainly via the urine and faeces but are unlikely to be circulating factors or present in bile fluid. Whether males use only olfactory information, or a mixture of visual and olfactory input, is currently under investigation, as is the chemical identity of the odorants.

#### A4.21–The role of monoamines in regulation of salt balance in terrestrial crustaceans

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Unregulated urinary salt loss is problematic for animals drinking only freshwater and would require compensatory dietary uptake. Land crabs produce isosmotic urine but reduce salt loss by reabsorbing salt via the gills to produce a dilute excretory fluid (P); a kidney analogue. Branchial salt reclamation is regulated in response to changes in dietary salt availability. The regulation of branchial salt reabsorption was investigated in the terrestrial brachyuran crab *Gecarcoidea natalis* and anuran crab *Birgus latro* on Christmas Island. Both species adjusted the volume of urine and extent of salt reclamation in response to the amount of ingested salt. In *B. latro* infusion of dopamine or dibutyl-cAMP ( $8.7 \times 10^{-7}$  mol.l<sup>-1</sup> haemolymph) inhibited Na and Cl uptake in animals acclimated to freshwater and markedly reduced the gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. Dopamine stimulated the production of cAMP within the branchial epithelial cells. In *G. natalis* during the dry season, serotonin but not dopamine or cAMP increased urine production by at least 16%. In *G. natalis* drinking freshwater, serotonin (but not dopamine or cAMP) increased net Na influx by stimulating branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase. Red crabs drinking saltwater reduced net uptake through increased diffusive loss while Na<sup>+</sup>/K<sup>+</sup>-ATPase were unchanged. Serotonin and dopamine abolished the increased diffusive loss and re-established net influx. Up-regulation of net flux in red crabs was reminiscent of marine brachyuran crabs, and in contrast to ion transport in *Birgus*, in which regulation is inhibitory. Both species show aspects of regulation specific to life on land.

#### A4.22–Host Settlement of the Salmon Louse, *Lepeophtheirus salmonis*

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The salmon louse, *Lepeophtheirus salmonis* (Copepoda: Caligidae) shows strong host specificity and needs to recognise and identify its host prior to settling. Current

research at Aberdeen is investigating the long distance cues used in host location but little is known of the mechanisms by which the infective copepodid selects its host at close range. Results were expressed as the mean number of lice per species and statistically analysed using generalized linear modelling or chi-squared tests. Surface area was found not to have an effect on lice burden and so, was removed from the analysis. Sea trout had significantly more lice than salmon. In addition, the presence of one fish species did not affect the numbers of lice on the other species. Salmon had more lice than both non-salmonid species. The fins were preferentially settled and attached to by fish. Results described here will provide the basis for identification of the close range chemical cues used for settlement.

#### A4.23–Behavioural Responses of Larval Sea Lice to Host Odours

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The copepodid of the sea louse *Lepeophtheirus salmonis* Krøyer 1838 is the infective stage of this economically important fish ectoparasite. Its role is to successfully find and settle on a suitable host fish to allow development to the adult stage. The mechanisms of host location and identification are believed to involve the use of host-derived chemical cues. We present evidence to show that the copepodid stage shows behavioural responses to such cues. Copepodid behavioural responses were observed in simple flow chambers and Y-tubes on addition of seawater conditioned with host-derived chemical cues. Copepodids were monitored for the presence of directional (taxis) and non-directional (kinesis) responses. Taxis was quantified using horizontal and vertical Y-tube assays which allowed lice to exhibit preference for one stimulus over another. Kinesis was quantified using a digital tracking system to compare copepodid behaviour and movement between potential stimuli.

The copepodid of the sea louse modified its behaviour in response to host odours, with both directional and non-directional responses being observed. The implications of these findings are discussed in terms of the host location process and the potential use of such chemicals in integrated pest management strategies.