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A5–COMPARATIVE REPRODUCTIVE AND DEVELOPMENT BIOLOGY

Organised by Kevin Coward (University of Oxford) and Matt Bentley (University of Newcastle) for the Animal Section

A5.1 The reproductive biology and mating behaviour of the salmon louse *Lepeophtheirus salmonis* (Crustacea; Caligidae)

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Lepeophtheirus salmonis is a major pathogen of farmed salmon causing £20 million damage annually in treatments and losses. It has a 10-stage life cycle comprising three planktonic stages and seven stages attached to its salmonid host. There are two preadult mobile stages before the highly mobile adult stage which move between hosts for feeding and mate location. *L. salmonis* is dioecious, the sexes becoming distinct at the preadult 1 stage, and maturing at the adult stage when mating occurs. Adult males take up precopulatory position predominantly with pre-adult II females and copulation occurs between the adult male and recently moulted adult female. Protandry occurs with adult male emergence synchronised with pre-adult II female emergence. Mate recognition occurs by short and long range chemical cues males showing specific behaviours in the presence of pre-adult II and adult virgin females. The role of olfaction and diffusible pheromones in mate location have been assessed with Y-tube behavioural bioassays. Adult male sea lice displayed activation and directional responses to seawater conditioned with preadult II females and also to solid phase extraction (SPE) extracts of the conditioned water. Distillation under vacuum produced volatile semiochemicals that were attractive to males. Our research provides evidence that small lipophilic organic molecules are used by sea lice as sex pheromones to locate a member of the opposite sex.

A5.2 Diatom reproductive toxicology: Implications for marine ecosystems and aquaculture

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Diatoms have traditionally been regarded as the 'grass of the sea', essentially an inert parcel of nutrients in a silica casing. Research within the last decade has begun to challenge this view. It has come to light that diatoms possess a sophisticated chemical defence strategy that revolves around the production of reactive chemical species following lipid peroxidation. The production of defensive chemicals is initiated following mechanical damage to the diatom frustule and cell membranes. The bioactive molecules produced include unsaturated short-chain aldehydes and oxo-acids. These toxins specifically target invertebrate reproductive processes including the oocyte fertilization current, sperm motility and embryonic mitosis. The aldehydes are highly cytotoxic and induce apoptosis and necrosis in exposed cells, embryos and larvae. We have extracted the toxic aldehyde decadienal from the planktonic diatom *Skeletonema costatum* and the benthic diatom *Nitzschia commutata*. We have also investigated the toxicity of diatom-derived aldehydes to benthic marine macroinvertebrates. The polychaete worms *Nereis virens* and *Arenicola marina* exhibit extreme sensitivity to decadienal compared with echinoderm, crustacean and ascidian comparators. We suggest that the aldehydes are functioning as endocrine disruptors in these polychaetes. We argue that diatom toxicity represents a strong selective pressure in benthic systems for the evolution of seasonal spawning strategies. We will also discuss the possible impacts of diatom aldehydes to the aquaculture industry either from the application of diatoms as larval feeds or from direct environmental exposure.

A5.3 Sperm competition and sperm form and function

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Sperm form and function show remarkable diversity, both across and within animal taxa. For example, known sperm lengths range hugely from 28 microns in the porcupine to 58 millimetres in *Drosophila bifurca*. In this talk, I will introduce the extent and nature of this diversity, and consider which evolutionary forces explain such variation in spermatozoa (compared with the female gamete). Sperm competition, when sperm from two or more males are thrown into competition for a female's eggs, is one important and widespread force that spermatozoa are subject to selection from. This form of male:male competition is recognised as a pervasive force in the evolution of male reproduction, but its role at the gamete level remains poorly understood. I will describe studies on insect, fish and mammalian systems, to show how sperm competition influences adaptations in spermatozoa. Specifically, I will use across-species studies to examine comparative evolution of sperm traits. I will then use single-species experimental studies to determine, for that system, the relative influences of sperm size, number and motility for sperm competition and fertilization success.

A5.4 Starting and stopping Ca^{2+} oscillations at fertilization in mammals

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Fertilization in all species triggers an increase in intracellular Ca^{2+} . This increase in Ca^{2+} is responsible for egg activation and co-ordinating the transition from meiosis to mitosis. In mammals, the Ca^{2+} oscillations are initiated within minutes of sperm-oocyte fusion and continue for 3–4 hours. The oscillations stop as the fertilized oocyte enters interphase of the first mitotic division. Surprisingly, some 12 hours later, Ca^{2+} oscillations start again during mitosis of the first mitotic division. These mitotic Ca^{2+} transients are only seen in fertilized embryos suggesting a contribution from the fertilizing sperm. Recent experiments have provided some insight into the temporal organization of these Ca^{2+} oscillations and how they initiate the molecular changes that result in egg activation.

We show that Ca^{2+} transients at fertilization can continue for many hours if the formation of the pronuclei is

prevented using inhibitors of nuclear transport. Furthermore, the mitotic Ca^{2+} transients are initiated after the nuclear envelope has become permeable to 70kDa fluorescent dextrans and stop with the initiation of the formation of the nuclear membranes in the 2-cell embryo. These observations suggest a model whereby the temporal organization of Ca^{2+} transients is controlled by nuclear sequestration and release of a paternally-derived Ca^{2+} releasing factor. Further experiments suggest the molecular identity of the factor is the novel sperm-specific phospholipase C (PLC), PLC ζ . Ca^{2+} oscillations induced by PLC ζ show the same temporal organization as found at fertilization. Furthermore, immunofluorescence reveals that PLC ζ localises to the pronuclei, consistent with it being regulated by nuclear sequestration.

A5.5 Physiological ecology of larval settlement of the barnacle *Balanus amphitrite*

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The settlement process of barnacles encompasses attachment of the cypris larva to the substratum and metamorphosis to the juvenile barnacle. It is well known that chemical and physical cues can influence the likelihood that encounters with the substratum will translate into settlement. The aim of the present study was to examine competence to settle in terms of physiological status, vis-à-vis, hormone, lipid and cyprid major protein (CMP) levels, in cyprids of *Balanus amphitrite* 'stored' at two temperatures (6 and 23 °C). The major ecdysteroids present in the cyprid stage were determined by RIA and LC-MS as 20-hydroxyecdysone (20E), ecdysone (E) and ponasterone A (PoA). Levels of methyl farnesoate (MF) were determined after sample purification by HPLC and GC-MS-SIM analysis. When hormone profiles were related to propensity to settle, MF and PoA were found to be useful markers of physiological age; E and 20E profiles proved too complex for this purpose. Lipid and CMP measures further supported the interpretation of the hormone titre results. The utility of the biochemical markers of physiological age was assessed in laboratory and field studies. First, the effect of two known chemical inducers of gregarious settlement, the settlement-inducing protein complex (SIPC) and waterborne cue, on hormone titres was investigated. Both cues were found to cause a significant drop in the titres of

20E, E and MF and a significant rise in the titre of PoA. Remarkably, the hormonal status of day 0 cyprids (23 °C) that had been exposed for a few hours to these cues was comparable to that of day 3 cyprids. Secondly, the markers were tested in the field using wild *Semibalanus balanoides* cyprids. ‘Late season’ cyprids, which showed enhanced settlement rates over their ‘early season’ counterparts, were also found to have hormone titres and energy reserve levels indicative of physiologically older cyprids. Energy reserve levels (e.g. lipid) have been used previously to indicate physiological age. This is the first study to examine the utility of hormone titres for this purpose.

A5.6 Physiological interactions between sperm and oviduct epithelium

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The oviduct has been considered a passive conduit conveying gametes to the site of fertilisation and the products of conception to the uterus. Recent studies have begun to reveal an important modulating role in the maturation of spermatozoa and in controlling their approach to fertilisation. In our studies of the pig oviduct epithelium, we have shown that epithelial cells in culture will support sperm viability and reduce motility. Spermatozoa attach to these oviduct cells by the rostral plasma membrane. Epithelial cells from prepubertal gilts were used since these performed as well as those from adult sows. Apical plasma membranes (APM) of epithelial cells from adult sows were prepared and then incubated with spermatozoa. These preparations showed a dose-response in their ability to support sperm viability *in vitro*. Furthermore, they supported sperm viability irrespective of the stage of the cycle (follicular or luteal) from which they were prepared, or the anatomical position in the oviduct; isthmus APM was no more effective than ampullary APM. Heat treatment of APM at 100 °C diminished the capacity to support sperm viability. A solubilised extract of APM was found to be active and would support sperm viability and motility over a 7-day period. Spermatozoa became relatively immotile during this period but could be revived in fresh medium. Current studies are exploring the characteristics of the active components. In this presentation, our own work will be reviewed in the context of other literature demonstrating that the oviduct is instrumental in fine-tuning sperm development and progress towards fertilisation.

A5.7 The *ets* family of transcription factors in the sea urchin embryonic mesoderm

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The ease of *cis*-regulatory analysis in the sea urchin embryo facilitates the study of the transcriptional events that control development. Mesoderm in the sea urchin embryo is entirely constituted by two general types of mesenchyme cells, primary (PMCs) and secondary mesenchyme cells (SMCs). Although different for several developmental aspects, these two populations of cells share one important feature: at a certain point of their developmental history they undergo epithelio-mesenchyme transition (EMT). These cells and the sea urchin embryo therefore represent a relatively simple model for studying transcriptional regulation during EMT. In previous studies, we identified a *cis*-regulatory device capable of specifically driving expression in both PMCs and SMCs at the time of their EMT, thus supporting the existence of a pan-mesodermal regulatory program in the sea urchin embryo. Interestingly, this *cis*-regulatory element contains an essential consensus sequence for the *ets* family of transcription factors. To study the function of these factors in mesenchyme formation, we isolated from different stage sea urchin cDNA libraries various members of the *ets* family and characterized among them three genes, belonging to the ETS1/2, ERG and ELK subgroups, respectively: *SpEts*, which is expressed only in PMCs early in development and in both PMCs and SMCs during gastrulation; *SpErg*, which is expressed in both PMCs and SMCs; *SpElk*, which is present in SMC precursors and some regions of the endoderm. We are currently analyzing the role of these transcription factors during sea urchin development by knocking out the downstream function of these genes using injection in zygotes of morpholino antisense oligonucleotides or dominant repressor mRNAs.

A5.8 Glowing gonads: Use of an *in vivo* approach to investigate the novel signalling protein PLC ζ and its role at fertilisation

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In all species studied, egg activation and embryo development is triggered by a rise in intracellular calcium. It is thought that a sperm specific soluble protein factor is responsible for the calcium rise. Recent work has revealed that the sperm factor is a novel protein, a sperm-specific member of the phospholipase C (PLC) class of proteins, called PLC ζ . We have been investi-

gating how PLC ζ is generated in the testes and sperm using a novel transgenic technique, *in vivo* gene transfer. *In vivo* gene transfer is a new technique whereby a transgene is micro-injected directly into the seminiferous tubules of the testis. An electrical current is applied, opening pores in the spermatogenic cell membranes, introducing the transgenic DNA into the cell, to be expressed using the cells transcriptional machinery. We are using mammalian expression vectors to link PLC ζ to a fluorescent marker, namely green and yellow fluorescent protein, allowing us to visualise and track the expression pattern of PLC ζ within a living tissue. The ability to assess the function of PLC ζ in a living system is vital to our understanding of its activity during development, how it is regulated and how it behaves at fertilisation. Without the luxury of a working *in vitro* spermatogenic cell culture system the use of *in vivo* gene transfer should allow us to assess the function of PLC ζ while bypassing traditional time-consuming and complicated transgenic techniques.

A5.9 Interactions between Eph and FGF signalling revealed by somitogenesis in zebrafish

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Somites are the segmental units of the vertebrate mesoderm and give rise to all the skeletal muscles and the vertebral column. They are formed periodically during vertebrate segmentation by the process of somitogenesis. It has been suggested that somitogenesis involves a molecular segmentation clock: gene expression oscillates on and off in the cells of the presomitic mesoderm (PSM). A wavefront of maturation then sweeps back through this tissue, arresting oscillation and initiating somite differentiation. Cells arrested in different phases of their cycle express different genes and give rise to the anterior or posterior half of a somite (1).

Eph receptors are cell surface receptor tyrosine kinases and, together with their membrane bound ephrin ligands, they have been shown to have important roles in somite boundary formation (2). Disruption of Eph signalling by injection of a dominant negative ephrin leads to disruption of somite boundary formation (2). Timelapse analysis showed that later in development irregular shaped and bigger somites are formed on the injected side. According to the clock and wavefront model (1), larger somites can be formed by either a change in the period of oscillation of the clock, or a delay in the operation of the wavefront. We investigated these possibilities using *in situ* hybridisation analysis of dominant negative ephrin injected embryos.

(1) Cooke and Zeeman. *J Theor Biol.* 1976 May 21;58(2):455-76.

(2) Durbin et al. *Genes Dev.* 1998 Oct.1;12(19):3096-109.

5.10 Photoperiodic manipulation and its effect upon the reproductive performance of the Nile tilapia, (*Oreochromis niloticus*)

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Tilapias are now a major aquaculture species with production levels of over a million tonnes annually. The hatchery production of fry is still very inefficient due to the relatively low fecundity and lack of spawning synchrony. Any methodology that enables farmers to synchronise the reproductive cycles of their broodstock would have immense practical advantages. Light is already known to play an important role in the initiation of gonad maturation in other fish species. In this investigation, the reproductive performance of 32 siblings of Nile tilapia was evaluated under four different photoperiods: short day (6L:18D), normal day (12L:12D), long day (18L:6D), and continuous illumination (24L:0D). Significantly larger eggs ($P < 0.05$) were produced under normal daylength (12L:12D) compared to other treatment groups. Fish reared under long daylength (18L:6D) exhibited significantly higher ($P < 0.05$) total fecundity (2408 ± 70 eggs spawn⁻¹) and relative fecundity (7.2 ± 0.2 eggs g⁻¹ body weight) concomitant with a significant reduction in inter-spawn-interval (ISI, 15 ± 1 days) in comparison with the rest of the trials. This investigation shows that long daylength (18L:6D) helps improve some important reproductive traits in Nile tilapia, and suggests that such methodology may be used to alleviate the production problems caused by low fecundity and poor spawning synchrony, and thus play a valuable future role in tilapia culture.

A5.11 Studies on the biosynthesis and reproductive functions of novel eicosanoids in barnacles

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Barnacles are key hard fouling organisms that encase man-made structures submerged in the marine environment. Hence, there is much interest in determining the

controlling mechanisms in the reproduction and settlement of such organisms with a view to developing new antifoulants. In the current study we report on the generation of a novel eicosanoid in the sub-tidal barnacle, *Balanus balanus*, and its role in fertilisation. Eicosanoids are C20 polyunsaturated fatty acid derivatives with a diverse range of activities. Previous studies have examined the nature of the products generated in barnacles and shown their potential involvement in larval settlement and hatching.

The testis of *B. balanus* was found to produce a wide range of eicosanoid-like compounds. The main compound had a λ_{\max} of 240 nm. Under acidic conditions this compound was converted to a range of conjugated triene and conjugated pentaene-containing compounds. Using a combination of high performance liquid chromatography and mass spectrometry, the structure of the parent compound was elucidated as 8,13-dihydroxy-eicosapentaenoic acid (8,13-diHEPE). This appears to be generated as a result of the action of an 8-lipoxygenase and cytP_{450} activities. Incubation of spermatozoa from *B. balanus* with this 8,13-diHEPE had no obvious effect on sperm motility. Placing barnacles in sea water containing the 8,13-diHEPE, however, caused a significant increase in muscular contractions, which as reported previously are believed to be linked to post-copulatory behaviour. The mechanism of this action remains to be elucidated.

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A5.12 The effect of dietary arachidonic acid concentration on Atlantic halibut (*Hippoglossus hippoglossus*) broodstock performance. Assessment of egg, milt and larval quality

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In order to evaluate the impact of four different diets on halibut broodstock, eight tanks were set up. Each tank contained 15 females and 5 males. The four broodstock diet formulations contained 0.4% arachidonic acid (ARA), (0.4g ARA/100g dry feed), 0.6% ARA, a control feed not supplemented with ARA and a control feed in which a proportion of the fish meal was replaced with squid meal, to improve palatability. The experimental diets have now been fed for a period of 3 years. The spawning period is regulated by photoperiod. Four tanks spawn in May and the four others spawn in August. A

PIT tag identifies each fish. This allows individual length and weight data to be collected every 2 months. During the spawning season, milt, eggs, at different developmental stages, and yolk sac larvae are sampled for subsequent biochemical analyses (lipids, fatty acids and $\text{PGF}_{2\alpha}$). Fish fed the enriched ARA diets have shown significantly better growth compared to the other two treatments. During the second spawning season, fish fed on 0.4% ARA significantly improved their whole production. Fatty acid analyses on eggs, yolk sac larvae and milt show significant ARA uptake and deposition relative to the diet.

A5.13 Reproduction and embryonic development of the comb-jelly, *Mnemiopsis leidyi*, in the south Caspian Sea conditions

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The invasion of *Mnemiopsis leidyi* in the south Caspian Sea was reported by Esmaeili et al. at the end of 1999. *M. leidyi* is a simultaneous hermaphrodite and possesses gonads (ovary and spermatophore) in their rows. In this study we tried to figure out fecundity rate and early life stages of *M. leidyi* in south Caspian conditions. Our results show that we could observe several categories and a range of variability of hatching time, fecundity rates, reproduction size and temperature dependent mortality. Two categories of hatching have been observed. The eggs were hatched after 20–24 hours, which seems to be the same as what has been reported from Black Sea strain. The eggs were hatched after about 12–15 hours, the new category first seen in south Caspian Sea. After fertilization of eggs into water, an ovoid larvae develops (Cydippid). This free swimming larvae grows naturally into a new Comb-jelly that is similar to the results of previous studies. Fecundity investigations show that, each *Mnemiopsis* carries about 43–87 eggs along each meridional canal and about 24–51 of them in each auricular canal. The fecundity was in average 264 and 553 eggs/day for specimens 18 and 29 mm length, respectively. The average fecundity of *M. leidyi* in the north Caspian Sea is measured 1174 and in the Black Sea 3348 eggs/day (Shiganova, unpublished). Our results show that, the fecundity seems to be much lower than that reported from north Caspian and the Black Sea, and depends on different size, temperature, salinity and food concentration. Also individuals placed in more than 30 °C have had high mortality rate and low fecundity.