

A12–COMPARATIVE REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY

Organised by K. Coward and M. Bentley for the Animal Section. The Animal Section gratefully acknowledge the sponsorship of Seabait Ltd and the Marine Biological Association

A12.1–The spark of life – how calcium kickstarts development of the embryo

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Changes in intracellular calcium constitute one of the central signalling mechanisms utilized by living cells. One important physiological process where calcium signalling plays a central role is the activation of the egg by the sperm at fertilization. In all species studied, egg activation is triggered by a rise in calcium levels within the egg. In sea urchins, frogs and fish, a single, explosive wave of calcium crossing the egg is observed. In contrast, in mammals, nemertean worms and ascidians, the sperm triggers a series of periodic increases in intracellular calcium that have been termed calcium oscillations.

Despite the fact that interest in the mechanism of egg activation goes back over a century, the mechanism by which the sperm causes intracellular calcium release remains to be resolved. Here we present evidence that the physiological agent of egg activation in mammals is a novel sperm-specific phospholipase C isoform – PLCzeta. A recombinant version of PLCzeta triggered calcium oscillations identical to those seen at fertilization. Immuno-depletion of PLCzeta from sperm extracts removed the ability to cause calcium release. The mechanism of egg activation in non-mammals remains unclear. We provide evidence that sperm from teleost fish (tilapia) contain a soluble sperm ‘factor’ or calcium releasing agent, similar to that found in mammals. In sea urchins, we have recently identified a novel phospholipase C of the delta subclass that is present in both sperm and eggs. We present evidence suggesting a role for this protein at fertilization in sea urchins. Research supported by MRC.

A12.2–NAADP-mediated calcium signalling in the sea urchin egg

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NAADP is a highly potent mobilizer of calcium ions from intracellular stores. It has recently been shown that the NAADP-sensitive stores are acidic lysosomal-related compartments (Churchill et al., 2002). Local calcium release by NAADP in turn triggers calcium-induced calcium release pathways by recruiting additional calcium release channels based in the endoplasmic reticulum.

We recently reported a dramatic increase in NAADP during sea urchin egg fertilization that was largely due to production in sperm upon contacting egg jelly (Churchill et al., 2003). The NAADP bolus plays a physiological role upon delivery to the egg based on its ability to induce a cortical flash, a depolarization-induced activation of L-type calcium channels. The sperm-induced cortical flash was not apparent in eggs desensitized to NAADP. It is likely that the NAADP increase plays a physiologically relevant role during fertilization and provides the first demonstration that NAADP is a genuine second messenger.

Churchill, G. C. et al. (2003). ‘Sperm deliver a new second messenger: NAADP.’ *Curr. Biol.* In press.

Churchill, G. C. et al (2002). ‘NAADP mobilizes Ca²⁺ from reserve granules, a lysosome-related organelle, in sea urchin eggs.’ *Cell* 111: 703–708.

A12.3–Studies on hepoxilins and trioxilins as barnacle hatching factors

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Barnacles are a constituent of the hard fouling organisms that encase man-made structures submerged in the marine environment. As a result, there is much interest in controlling factors of developmental processes in these animals with a view to developing novel antifoulants. Following fertilisation, barnacle eggs (embryos) are brooded in the mantle cavity. The hatching of these is thought to be under the control of ‘barnacle hatching factor’ (BHF) to give rise to dispersive larvae. BHF activity is thought to reside in a variety of eicosanoids including 8-hydroxyeicosapentaenoic acid and trihydroxy fatty acid derivatives similar to trioxilins. In mammals, the biologically inactive, trioxilins are the breakdown products of active hepoxilins. Therefore, the current study examines whether trioxilins and hepoxilins are produced by barnacles (*Elminius modestus*) and whether they have BHF activity. Using a combination of mass spectrometry and high-performance liquid chromatography, it was shown that barnacles produce both trioxilin A₄ and trioxilin A₃ from eicosapentaenoic and arachidonic acids respectively. Authentic trioxilin A₃ was used to determine if these compounds had hatching activity. At all concentrations tested (10⁻⁹–10⁻⁶ M) it was inactive. Hepoxilin A₃, however, significantly stimulated hatching at 10⁻⁶ M. The stable hepoxilin ana-

logue, PBT-3, also had BHF activity at 10^{-7} M. Overall, these studies show that only hepoxilins have BHF activity.

Supported by NERC (GR3/12765)

A12.4—Developmental expression of genes controlling sexual development in the zebrafish (*Danio rerio*)

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Sexual differentiation and development in fish are mediated by sex steroids. Exposure to these hormones and their chemical mimics (endocrine disrupting chemicals—EDCs), can disrupt the normal pattern of development in these processes. Gonad, cell and tissue responses to these substances are well characterised in fish, but the molecular mechanisms mediating these effects are poorly understood. Even less is known about the molecular mechanisms of chemical sexual disruption at the level of the hypothalamic–pituitary axis. To understand the effects of EDCs on genes controlling sexual development requires information on the normal pattern and levels (and variation therein) of their expression, this information has not been forthcoming for any fish species.

In this study, expression of a suite of genes known to play central roles in sexual development was measured in the gonad and brain in the zebrafish (*Danio rerio*) during normal development at 30, 60, 90 and 120 (sexually mature fish) days post-fertilisation using PCR techniques. The target genes studied included oestrogen receptors—(ER alpha and gamma), P450 aromatase (gonadal [alpha], neural [beta]) and 20 beta-hydroxysteroid dehydrogenase (20 Beta-HSD). Both oestrogen receptors were expressed in the gonad and brain; with greater levels of expression for ER alpha in neural tissue and ER gamma in gonadal tissue. P450 aromatases were expressed in both gonad and neural tissue, with highest expression in the female neural tissue. 20 Beta-HSD was predominately in the gonad and expressed at the highest levels in the ovary. There were life stage differences in the expression of these genes which will be presented.

A12.5—Experimental reproductive biology in the deep sea

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The deep sea is the largest single environment on earth containing the longest single environmental gradient: pressure. In addition to pressure, the deep sea for the

most part is cold ($<4^{\circ}\text{C}$), with the exception of the Mediterranean and Red Seas, or close to hydrothermal vents. Rather than being a tranquil environment, the deep sea is very dynamic with high biodiversity, benthic storms, seasonal processes and environments independent of solar radiation for their primary production. Experimental reproductive biology in the deep sea is still in the relatively early phases of development. Experiments fall into two main categories. Those experiments carried out at the seabed with the aid of submersibles or ROVs, and those where the deep-sea animals are brought to the surface and maintained at pressure. In addition, there have been a series of experiments that address the questions of how invertebrates have penetrated the deep sea. In this short review I am going to describe a series of experiments that have contributed to our understanding of biological processes in the deep sea. The first experiment examines the fertilization success of deep-sea echinoids. The theme of echinoid biology is considered in the second set of experiments that examined how larvae of the genus *Echinus* may have invaded the deep sea from high latitudes using the sinking of cold dense waters as a pathway. The third set of experiments examines the potential distribution of larvae of the hydrothermal vent shrimp *Rimicaris exoculata*.

A12.6—Aquatic mating by dispersal and collection of sperm

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A diverse range of marine animals release dispersing aquatic sperm which fertilise eggs that have been retained by the female; the resulting progeny are often brooded. This may be termed 'spermcast' mating, and has apparent parallels with reproductive processes in some algae and with angiosperm mating by pollen dispersal, but differs from copulatory mating or broadcast spawning for external fertilization. Studies on laboratory cultures of two sessile, modular, marine invertebrates with spermcast mating have revealed extensive similarities between the two species, despite their belonging to different major clades of the Metazoa. Both are self-sterile hermaphrodites. Sperm are long-lived following release (in comparison with external fertilizers), and may be utilised at very low external concentrations. Fertilization is truly internal, and follows extensive contact between sperm and maternal tissue. Mating is governed by compatibility systems. Compatible allosperm may undergo prolonged storage before syngamy occurs, allowing the gradual accumulation of male gametes by

the female, potentially from different sources. Sperm precedence effects are present. In both species, the receipt of compatible allosperm triggers vitellogenic egg growth, which is otherwise deferred. In one species, the budding of entire female modules is also triggered by uptake of sperm by non-reproductive (feeding) modules. In the other, the size to which eggs are grown by a particular female depends on the identity of the sperm source. The triggering of female investment by sperm appears a recipe for sexual conflict in these remotely mating hermaphrodites.

A12.7—Exposure of gametogenic *Nereis virens* (Polychaeta: Annelida) to copper-spiked sediment and its sub-lethal effects on reproduction

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The infaunal polychaete *Nereis virens* (King ragworm) is often exposed to elevated concentrations of pollutants during its adult stages. Copper is present in many coastal sediments and analysis has shown that concentrations can vary between 24 and 44 mg kg⁻¹ dry weight (DW) around Portsmouth, up to 2000 mg kg⁻¹ DW in some UK estuaries (Bryan & Langston, 1992).

To investigate the effects of copper on reproduction, gametogenic adults were incubated for 3 months prior to spawning in control sediment (background concentration: 24 mg kg⁻¹ DW) and copper-spiked sediment (nominal concentrations: 50, 500, 1000 mg kg⁻¹ DW). Results show that cumulative adult mortality is correlated with increasing concentrations of copper, with males being more susceptible than females. Data also indicate that males spawn prematurely (up to 3 weeks before the predicted time) in the higher concentrations. To investigate the effect on gametes, oocytes were stripped from females at monthly intervals and diameters measured. Results show that oocyte growth is significantly reduced at the 500 and 1000 mg kg⁻¹ DW concentrations compared to the control. At the time of natural spawning, oocytes were fertilized in vitro with pooled sperm from males collected from the natural population. These higher concentrations of copper at which the females were incubated had a significant effect on fertilization success and on subsequent embryo development to the blastula stage.

Bryan, G.W. and Langston, W.J. (1992). Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution*, 76: 89–131.

A12.8—Effects of exposure to treated sewage effluent during early life on sexual development in roach (*Rutilus rutilus*)

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Surveys of UK rivers have shown a high incidence of sexual disruption in roach (*Rutilus rutilus*) living downstream from some sewage treatment works (STW). It has been proven that STWs effluents are oestrogenic to fish, resulting in the induction of vitellogenin. Field data also strongly support the contention that disruption in gonad development in fish is caused by exposure to STW effluents. A previous study showed that exposure of juvenile male roach from 50–150 days post-hatch (dph) to a single STWs effluent induced feminisation of the reproductive ducts but did not induce intersex (the simultaneous presence of both male and female germ cells in the gonad). In this study we exposed fertilised roach embryos/fry to two STWs effluents, from fertilisation up to 300 days post hatch (dph; to cover the full period of gonadal sex differentiation in this species) to determine if effluent exposure during this period induces the intersex condition. Both effluents were oestrogenic to roach inducing vitellogenin. The magnitude of the vitellogenic responses paralleled the effluent content of steroid oestrogens. Both effluents also contained low µg/L concentrations of alkylphenols. Feminisation of the reproductive ducts occurred in all male fish in concentrations of effluent of 80% and higher, and depuration studies confirmed that this effect was permanent. There was, however, no evidence of major germ cell disruption in fish from either site. It still remains to be proven that STWs effluent induced germ cell disruption and if so what the critical life phase for this effect is.

A12.9—Biorhythmicity and photoperiodism in the seasonal reproduction of *Nereis*: a case study for environmental genomics

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The nereid prostomium receives environmental inputs, and their transduction affects behavioural rhythms (e.g. foraging behaviour), somatic growth (e.g. segment proliferation) and the key life cycle transition that culminates in life terminating reproductive activity. The inputs include photoperiodic, diel and tidal signals entraining

endogenous circadian and circa-tidal rhythmicity. In long photophase conditions segment proliferation and caudal regeneration occur under a permissive neuroendocrine signal but the rate of segment formation is determined locally. Transition to short photophase days initiates a physiological state change in which foraging behaviour and regeneration capability is suppressed and sexual development may occur. This is followed by completion of sexual maturation or a spontaneous state reversal to a feeding non-reproductive state. Special interest relates to a ganglionic region of the brain that includes both mechanoreceptors and ciliary photoreceptors closely linked to neurosecretory output tracts. Analysis of the molecular mechanism involved in signal input and transduction is being investigated using a cDNA/micro-array approach, coupled to conventional gene screening, for the identification of the clock and signal transducing genes involved.

A12.10–Growth factors in fish: IGFs and myostatin expression

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Growth of teleost fish is largely genetically controlled but it is also influenced by environmental factors. Many fish species continue to grow throughout life, and therefore the adult body size is not fixed. In particular, these fish grow through a mixture of hyperplastic and hypertrophic mechanisms that continue even after sexual maturation.

Growth-promoting factors and hormones form a complex regulatory network in which a pivotal role is played by the growth hormone (GH) and insulin-like growth factor axis. In all fish studied so far, the IGF system (IGFs) seems to be correlated with cell proliferation although the expression pattern in tissues differs between species. Other growth factors involved in development mechanisms belong to the TGF- β superfamily and our group is particularly interested in myostatin, which is a negative regulator of muscle development in higher vertebrates, but may have other functions in fish. Our methodological approaches include cellular and molecular techniques applied to larval and post larval development of species of interest for aquaculture, with the aim of characterising these growth-regulating genes and their expression as a means to understanding their structure, function and evolution.

A12.11–Regeneration and Budding in Echinoderms and Tunicates: An adult developmental phenomenon?

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Echinoderms and tunicates are marine invertebrate deuterostomes that have the unique ability to undergo extensive regeneration as adults. This can take place following trauma as during arm loss in seastars and brittlestars or may be part of a normal asexual reproductive process such as budding in tunicates. We have used a combination of cellular and molecular techniques to study regeneration in tunicates and echinoderms. In tunicate neural complex regeneration there is good evidence for the delamination of 'neuroblasts' from putative neural placode-like structures that are retained in the adult. In budding tunicates the phenomenon is even more remarkable with complete adults generated from a variety of pre-existing and apparently fully differentiated adult tissues including endoderm, mesoderm or ectoderm. We are exploring the role of BMPs and putative adult 'stem cells' in this phenomenon using cell proliferation markers, cDNA library screens and ISH. Initial evidence suggests a general role for haemoblasts with lineage plasticity and totipotency.

In echinoderms we have also focused on the role(s) of BMPs and cloned a number of BMP2/4 homologues from both crinoids and ophiuroids (see Bannister et al. this volume). Expression studies suggest that these genes, hitherto considered to be expressed early in embryonic development, are recruited in the regenerating adult system to re-iterate their embryonic role in the regulation of neural non-neural identity.

Supported by BBSRC, NERC, KVA and VR.

A12.12–Genomics of the HOX gene cluster

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The Hox family of homeobox genes encodes transcription factors that control different aspects of metazoan development. They appear clustered in the genomes of those animals in which their relative positions have been mapped. Although clustering is assumed to be a general property of Hox genes in all bilaterians, just a few species have been studied in sufficient detail to support the claim. Linear duplication of genes inside the cluster, as

well as full-cluster duplications account for the actual complexity of HOX clusters in the different animal groups that have been studied (mainly vertebrates). Understanding how the Hox genes are regulated during development will depend, ultimately, on the generation of more powerful tools for cloning intact HOX clusters and for elucidating their cis-regulatory components. Echinoderms are providing us with tools to analyze both. Moreover, and in order to clarify the roles of the Hox genes themselves, we will need to characterize in detail their downstream targets, and some progress in this direction is coming, mainly, from the recent use of arrayed libraries. Hence, a comprehensive study of Hox target genes in tissues and organisms promises, in the long term, to give us a clear idea of the role of that Hox genes play during development and how they have changed over evolutionary time.

A12.13—An orthologue of vertebrate cannabinoid receptors in the urochordate *Ciona intestinalis*, a model organism for post-genomic developmental biology and physiology

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The vertebrate receptors CB₁ and CB₂ are activated by endocannabinoids and Δ⁹-tetrahydrocannabinol, the psychoactive ingredient of cannabis. We recently discovered CiCBR, a G-protein coupled receptor in the urochordate *Ciona intestinalis* that is orthologous to vertebrate cannabinoid receptors and the first putative cannabinoid receptor gene to be identified in an invertebrate (Elphick et al., 2003; Gene 302, 95–101). Phylogenetic analysis reveals that although CiCBR forms a clade with vertebrate cannabinoid receptors, it is positioned outside the CB₁ and CB₂ clades, indicating that the common ancestor of CiCBR and vertebrate cannabinoid receptors predates a gene (genome) duplication event that gave rise to CB₁ and CB₂ in vertebrates. The discovery of CiCBR and the absence of orthologues of CiCBR in protostomian invertebrates (e.g. *Drosophila*) indicate that the ancestor of vertebrate CB₁ and CB₂ cannabinoid receptors originated in a deuterostomian invertebrate. Moreover, analysis of CiCBR function in *Ciona* may reveal the “ancestral” physiological roles of the cannabinoid receptor before the emergence of separate CB₁ and CB₂ receptors in vertebrates. We are currently investigating the effects of cannabinoids on CiCBR-expressing CHO cells. In addition, preliminary analysis of *Ciona* using CiCBR-antibodies reveals a pattern of immunostaining strikingly similar to CB₁-expression in the mammalian brain, with CiCBR-like immunoreactivity

localised on fibres and varicosities in the neuropile of the cerebral ganglion. This suggests that presynaptic targeting of cannabinoid receptors in neurons may be an evolutionary ancient phenomenon that is conserved from *Ciona* to man. Therefore, *Ciona* could be exploited as a model ‘simple system’ for analysis of neuronal endocannabinoid signalling.

A12.14—Polyembryony: a twinned response to sperm limitation and restricted gene flow in the sea?

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Polyembryony, the splitting of a single sexually produced embryo into many clonal copies, seems to get the balance of sexual and asexual reproduction wrong, yet it persists in a diverse range of organisms. This project investigates cyclostome bryozoans, an entire order with this mode of reproduction, where microsatellite markers and spatial autocorrelation have been used to investigate whether sperm limitation and inbred local population structure may be important for the evolution and maintenance of this life history.

A12.15—Role of Eph signalling in Zebrafish somitogenesis

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The hindbrain, mesoderm and limbs of all vertebrates from xenopus to zebrafish and to chick contain repetitive segmental structures. Somites are mesoderm blocks that will give rise to all the skeletal muscles and the vertebral column. Somitogenesis is characterised by 3 main events:

1. Oscillation of gene expression in the cells of the presomitic mesoderm (PSM) under the control of the ‘segmentation clock’
2. Stoppage of the clock by a wavefront of maturation, initiation of differentiation and establishment of the anterior/posterior (A/P) identity
3. Epithelialisation and formation of a boundary between somites

Eph receptors are cell surface receptor tyrosine kinases and together with their membrane bound ephrin ligands, they have been shown to be key regulators of cell adhesion having important roles in somite boundary forma-

tion (1). Two members of this family, EphA4 and ephrinB2a are expressed in the anterior and posterior half of developing and formed somites respectively. Disruption of Eph signalling by injection of a dominant negative ephrin lead to disruption of somite boundary formation (1) and this persists later in development. Lack of Eph signalling might be affecting the differentiation of the PSM, the A/P identity of cells or the tim-

ing and the operation of the wavefront. These possibilities were investigated using markers of A/P identity and maturation of somites. Furthermore, it is known that FGF signalling is a component of the wavefront; effects of Eph signalling on the FGF pathway were also addressed.

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