

## L1

### **Molecular advances in sperm cryopreservation**

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**Introduction:** Cortisol is inter-converted with cortisone by 11BHSD. We have demonstrated that seminal plasma contains modulators of cortisol metabolism by 11BHSD. The objectives of this study were to establish whether (i) enzyme modulators in bovine and porcine seminal plasma correlate with semen viability following cooling and cryopreservation, (ii) the addition of these compounds to TALP media improves the viability of porcine spermatozoa following cooling.

**Methods:** Bovine and porcine seminal plasma (n=5) was loaded onto a C18 Sep-pak cartridge and sequentially eluted with increasing concentrations of methanol. Each column fraction was tested (at 10% v/v) for effects on 11BHSD activity in rat kidney homogenates over 1h using 100nM [<sup>3</sup>H]-cortisol plus 400uM NADP+. Ejaculates were evaluated for plasma membrane integrity (SYBR-14 positive) and permeability (osmotic resistance test, ORT). Porcine ejaculates were diluted in TALP (2x10<sup>6</sup> sperm/ml) and cooled to 5°C. Bovine ejaculates were diluted in a freezing buffer (3% glycerol; 2x10<sup>6</sup> sperm/ml) and cryopreserved (-5°C at 6°C/min then -196°C at 40°C/min) using a Kryo Save Compact KS1.7/Kryo 10 controlled-rate freezer. Following re-warming, semen viability was correlated with 11BHSD modulators in the seminal plasma of each ejaculate. Porcine ejaculates were subsequently diluted in TALP in the presence/absence of 10% (v/v) fractions of seminal plasma and cooled to 5°C as previously described.

**Results and Discussion:** The %spermatozoa with intact plasma membranes (SYBR-14) following either cooling or cryopreservation positively correlated with the stimulatory potency of the hydrophobic seminal plasma fractions eluted at 55-70% (v/v) methanol (P<0.001). Conversely, bovine spermatozoa membrane permeability (ORT) positively correlated with the inhibitory action of the hydrophilic seminal plasma fraction eluted at 0% (v/v) methanol (P<0.01). Addition of hydrophobic enzyme stimuli extracted from porcine seminal plasma significantly increased membrane integrity following cooling from 58.3±0.3% to 75.0±3.0%. In conclusion, extracts of seminal plasma containing modulators of 11BHSD can significantly improve the survival of cooled and cryopreserved semen.

## L2

### **The oocyte and its role in regulating ovulation rate: a new paradigm in reproductive biology.**

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Ovulation rate in mammals is determined by a complex exchange of hormone signals between the pituitary gland and the ovary and by a localised exchange of hormones within ovarian follicles between the oocyte and its adjacent somatic cells. From examination of inherited patterns of ovulation rate in sheep, several breeds have been identified with mutations in two growth factor genes that are expressed in oocytes. Currently, five different point mutations have been identified in the BMP15 (GDF9B) gene and one in the GDF9 gene. Animals heterozygous for any of the aforementioned mutations have higher ovulation rates (i.e. +0.8-3) than wild-type contemporaries, whereas those homozygous for each of these mutations are sterile with ovarian follicular development disrupted during the preantral stages. The BMP15 mutations result in the production of no or a very truncated form of mature protein or are thought to disrupt dimerisation or receptor binding whereas the GDF9 mutation is thought to disrupt receptor binding. In sheep, GDF9 mRNA and protein are present in germ cells before ovarian follicular formation, and in oocytes of types 1/1a as well as growing follicles, whereas BMP15 is found in oocytes from the type 2 stage of growth. Both GDF9 and BMP15 proteins are present in follicular fluid indicating that they are secreted products. Studies *in vitro* show that granulosa cells are an important target for both GDF9 and BMP15 and that BMPRII is at least one of the receptors mediating their effects. It remains to be

determined which type 1 receptors are involved at the different stages of follicular growth. Immunisation of sheep with BMP15 or GDF9 peptide antigens shows that both growth factors are essential for normal follicular growth and the maturation of preovulatory follicles. Whether GDF9 and BMP15 exert their effects as homodimers or heterodimers remains to be determined. Recently, short-term vaccination of sheep with GDF9 or BMP15 peptide-protein conjugates in water-based adjuvants has been shown to enhance ovulation rate and lamb production. In summary, recent studies of genetic mutations in sheep highlight the importance of oocyte-secreted factors in regulating ovulation rate and these discoveries may help to explain why some mammals have a predisposition to produce two or more offspring rather than one.

## S1

### **Intra-uterine insemination in farm animals and humans**

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Artificial insemination (AI) is the first generation of reproductive biotechnologies, both historically and in terms of numbers around the world. The first reports of AI in animals and humans date from 1322 and 1770 respectively. The introduction of AI in farm animals was forced by sanitary reasons and the first large scale applications with a commercial goal were performed in the late thirties of last century. Before that time, farm animals conceived after natural mating, but venereal diseases spread very fast resulting in serious fertility problems. The discovery of glycerol as cryoprotectant and improvements in the freeze and thaw procedures enabled the use of frozen-thawed semen instead of fresh semen. In this way, the distribution of animal semen was facilitated and AI was mainly performed for economical reasons, especially in dairy cattle industry. In humans however, AI was originally performed in cases of physiological and psychological sexual dysfunction, but later on also in cases of infertility caused by immunological problems. Currently, the most common indications for intra-uterine insemination in human are unexplained infertility and male subfertility. In these cases, intra-uterine insemination (IUI) is considered as a valuable first choice treatment. Intra-uterine insemination is a cheaper, simpler and less invasive treatment option which is more readily available than more invasive techniques of assisted reproduction such as in vitro fertilization (IVF) combined with ovum pick up (OPU) and intra-cytoplasmic sperm injection (ICSI). In contrast with humans, in which the whole ejaculate is needed to achieve pregnancy, the good quality and high number of spermatozoa produced by farm animals permits dilution and production of several insemination doses per ejaculate. However, with the introduction of sex-sorted semen in farm animals, the same problem of lower quality semen as in humans has arisen. Sexing of semen is expensive, time consuming, and harmful for sperm quality. Moreover, to make this procedure profitable, only one tenth of the standard insemination dose is used per insemination. In cattle, pigs and horses it has been shown that conventional insemination with this low number of sex-sorted spermatozoa results in a significant decrease in fertility. To improve the fertility rates with this semen, new insemination techniques have been developed in order to deposit the spermatozoa near the utero-tubal junction. In sows and mares the advantage of deep insemination has already been proven, however in cattle it is still under investigation.

## S2

### **In vitro follicle maturation in humans and domestic livestock**

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The prospects of growing and maturing oocytes *in vitro* holds many attractions for clinical practice, animal production, fertility preservation and research. However, despite much research attention, it has proved remarkably difficult to produce fertile oocytes from cultured primordial and preantral follicles, as we know relatively little about the biology of early folliculogenesis. To date, the greatest successes with follicle culture have been achieved using murine follicles where the normal follicular growth span from the primordial to Graafian follicle stages is relatively short, the follicles are relatively small and the trophic requirements and the interactions between the follicular cells and oocytes have been partially

characterised. Consequently, several authors have demonstrated that it is possible to produce live young from mouse oocytes grown from primordial or, more commonly, preantral stages to maturity *in vitro*. In contrast, the physiological requirements for early follicle growth are less well documented in large animals and humans where follicles are generally much larger and their growth takes months rather than days to complete. Nevertheless, significant advances have been made in the approaches used to harvest and grow ovine, bovine, and porcine follicles *in vitro*. It is now possible to use serum-free culture environments to: initiate and maintain primordial follicle growth over extended periods; induce antral cavity formation in preantral follicles; to manipulate growth rates by inclusion of theca cells and/or culture media additives; to induce steroid biosynthesis after provision of suitable substrate; and in some cases, for oocytes derived from *in vitro* grown follicles to undergo nuclear and cytoplasmic maturation and fertilisation in response to the appropriate stimuli. Importantly, *in vitro* growth can also be achieved with equal efficiency in sheep and mouse follicles harvested from both fresh and cryopreserved tissue. The results of histological evaluation and electron microscopy suggest that the chances of follicle culture succeeding at each developmental stage are increased by preserving the cellular interactions and the phenotype of the follicle cells *in vitro*. The stage is now set to incorporate the methodological advances derived from animal follicle culture into the protocols used to grow human follicles *in vitro*. The immediate goals for human follicle culture include optimisation of the culture environment and improvement of the methods used for isolating and culturing follicles and oocytes at both ends of the size spectrum. Research must also include a full evaluation of the health and genetic normality of oocytes grown for extended periods *in vitro*.

### S3

#### **Derivation of oocytes from mouse embryonic stem cells**

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Continuation of the species requires the formation and development of the sexually dimorphic germ cells. In the early mammalian embryo, the germline and soma are indistinguishable from each other. In the mouse, germ cell competence is induced at embryonic day 6.5 in proximal epiblast cells by signals emanating from the extra-embryonic ectoderm. Even during the specification period, precursor cells give rise to primordial germ cells and certain somatic cells, such as extra-embryonic mesoderm and allantois. The potential of embryonic stem (ES) cells to generate all lineages of the embryo *in vivo* has been widely reported in the literature, in striking contrast to the lack of data describing the derivation of germ cells from ES cells *in vitro*. We attributed the inability to demonstrate the derivation of germ cells from ES cells in culture to the lack of a suitable reporter system allowing for the non-invasive visualization of germ cell formation. We show that mouse embryonic stem cells in culture can develop into oogonia that enter meiosis and recruit adjacent cells to form follicle-like structures and later develop into blastocysts. Oogenesis in culture should contribute to various areas including nuclear transfer, manipulation of the germline, and advance studies on fertility treatment and germ and somatic cell interaction and differentiation.

### S4

#### **Viruses and assisted reproductive technology**

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Interactions between mammalian viruses and cells or tissues of the reproductive tract are relatively common in nature, and the host-animal has developed several mechanisms of protection against viral infection. With the advent of assisted reproductive technologies, some

of these protective actions are circumvented or prohibited. Especially during fertilization and early embryonic development, or during cryopreservation, conditions can prevail which facilitate the entrance of a virus into an embryo. Cattle are an excellent model to study virus-embryo interactions since several well-known bovine viruses are known to have a tropism for the genital tract. Moreover, information on risk assessment can be obtained from large scale studies on bovine embryo transfer and bovine in vitro fertilization and embryo culture. It is the purpose of this review to evaluate possible routes of viral infection, possible sources of contamination and protective measures which can be taken when performing assisted reproductive technology in cattle. Whenever appropriate, the situation in human and in other mammals is discussed. Viral infection can occur by means of vertical transmission of virus via the oocyte. Also the introduction of virus by means of the fertilizing spermatozoon is not inconceivable and needs further investigation. After fertilization, embryos are protected by a glycoprotein coat, the zona pellucida, but recent evidence in bovine, pig and mouse embryos has shown that the pores in the zona pellucida are sufficiently large for mechanical entrapment of certain viruses, while complete penetration and infection of embryonic cells is however considered unlikely. It remains to be determined whether this assumption holds true for human, and for very small viruses. Contamination on the other hand is not related to infection of gametes or embryos but originates from the surrounding media or the manipulations which are performed on the embryos. Dangerous in this respect are gamete micromanipulations which per definition introduce a hole in the zona pellucida. Moreover, recent advances in vitrification with subsequent storage of embryos and semen in open containers directly in liquid nitrogen represent an increased potential for contamination of germ plasm during long term banking. It is obvious that protective measures against viral infection need to be taken, especially when working with embryos from an infected donor or with techniques which compromise the embryo's defence mechanisms. In cattle, the risk for viral infection seems to be rather low when sanitary guidelines are being followed rigorously. Whether this holds also true for human assisted reproduction needs further investigation.

## S5

### **Embryonic gene expression patterns: An analysis of factors with implications for assisted reproductive technologies**

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In vitro maturation of oocytes, in vitro fertilization and extended embryo culture are frequently used in various mammalian species for basic studies in preimplantation development, therapeutic purposes or the production of superior animals. Development is particularly advanced in cattle, where in vitro produced embryos are already an integral part of modern breeding schemes. However, despite significant progress bovine in vitro produced (IVP) and nuclear transfer (NT) derived embryos differ from their in vivo developed counterparts in a number of important characteristics. A pre-eminent observation is the occurrence of the "large offspring syndrome" (LOS) which is correlated with considerable fetal and postnatal losses. A current hypothesis is that early and persistent aberrations in embryonic gene expression patterns are critically involved in the occurrence of LOS. We summarise here results from our extensive studies in which we compared gene expression patterns from IVP- and NT-derived embryos with those from their in vivo produced counterparts, mainly by employing a sensitive semi-quantitative RT-PCR assay. The in vitro maturation period did not affect mRNA expression patterns of several putative marker genes in blastocysts. In IVP-embryos, the relative abundance of transcripts from several developmentally important genes was affected by the basic culture medium and type of supplement (PVA, BSA, serum). In vivo culture in ligated sheep oviducts produced embryos with gene expression patterns nearly similar to the ones observed in their in vivo derived counterparts. In general, numerous aberrations were found in IVP and NT-derived embryos, including a complete lack of expression, an induced de novo expression or a significant up- or down-regulation of a specific gene. The alterations found in our studies may affect a number of physiological functions and are interpreted as a kind of stress response of the embryos to deficient environmental conditions. There is growing evidence that the alterations are caused by epigenetic modifications primarily by changes from the regular DNA-methylation patterns. Unravelling these epigenetic modifications is promising to understand the underlying mechanisms of abnormalities, incl. LOS. In light of reports of an increased proportion of abnormal children born upon use of

ART, the production of embryos with a normal expression profile is of utmost importance. The recently developed cDNA array technology for single embryos will be an essential tool towards this goal. References: Brambrink et al, *BioTechniques* 33,376-385 (2002); Lazzari et al, *Biol. Reprod.* 67,767-775 (2002); Niemann et al, *Cloning and Stem Cells* 4, 23-32 (2002); Wrenzycki and Niemann, in: *Assessment of Mammalian Embryo Quality*, Eds: van Soom and Boerjan, pp. 341-370 (2002).

## S6

### **Fetal androgen excess programs for polycystic ovary syndrome**

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Polycystic ovary syndrome (PCOS) affects 5-10% of reproductive-aged women. Its etiology, however, is unknown. Female rhesus monkeys exposed to fetal male levels of testosterone during discrete periods of gestation exhibit many of the signs and symptoms found in PCOS women. In infancy and adulthood, prenatally androgenized (PA) females exhibit LH hypersecretion, and adult PA females exhibit reduced estradiol negative feedback on LH. During adolescence and adulthood, PA females demonstrate ovulatory dysfunction when compared to untreated controls (C). Adult PA females also exhibit ovarian and adrenal hyperandrogenism, as well as diminished intra-follicular androgenic and estrogenic responses to ovarian hyperstimulation for IVF. Impaired oocyte developmental competency accompanies impaired ovarian responsiveness to recombinant human FSH and is associated with a lack of diminished circulating levels of insulin at oocyte retrieval. Such altered insulin responses reflect a greater dysregulation of metabolic function in PA females. Not surprisingly, PA females are insulin resistant compared to C females of similar age and size, and exhibit impaired pancreatic beta-cell responses to glucose. Possibly as a result, PA females are hyperglycemic and exhibit an increased incidence of type 2 diabetes. Increased abdominal adiposity may contribute to PA females insulin resistance and to their hyperlipidemia. Interestingly, treatment with an insulin sensitizer, pioglitazone, normalizes ovulatory menstrual function in PA females and improves insulin sensitivity, while lowering insulin responses to glucose. The improved reproductive and metabolic responses to pioglitazone causally implicate insulin in the mechanism of ovulatory dysfunction in PA females, similar to findings in PCOS women. Such close mimicry of PCOS characteristics by PA female rhesus monkeys suggests that fetal androgen excess may re-program multiple female organ systems, thus providing a possible etiological origin for this prevalent syndrome impairing women's health. It also suggests that both genetically- and environmentally-determined factors may contribute to PCOS development. Supported, in part, by NIH grants R01-RR13635, R21-RR14093, P50-HD44405, U01-HD44650 and P51-RR00167.

## S7

### **Glucocorticoid metabolism and reproduction: A tale of two enzymes**

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In all reproductive tissues studied to date, the access of glucocorticoids (cortisol and corticosterone) to nuclear corticosteroid receptors is tightly controlled by the 11 $\beta$ -hydroxysteroid dehydrogenase (11BHSD) enzymes. The low affinity, NADPH-dependent type 1 enzyme isoform (11BHSD1) acts predominantly as a reductase to regenerate active glucocorticoids from circulating cortisone/11-dehydrocorticosterone. In contrast, the high affinity, NAD-dependent type 2 enzyme (11BHSD2) catalyses the oxidative inactivation of

glucocorticoids. Hence, the balance in expression and activities of these 2 enzymes can fine-tune the levels of glucocorticoids within a given tissue/cell. With respect to reproductive physiology, these opposing enzymes have been implicated in processes as diverse as the endocrine function of the anterior pituitary gland, testicular and ovarian steroidogenesis, gametogenesis, ovulation, decidualization, placental function, endocrine control of fetal growth, parturition and lactation. Moreover, changes in 11BHS1 activities within reproductive tissues have been linked to intrauterine growth retardation, pre-term labour and cystic ovarian disease, both in women and in domestic livestock. Recently, we have established that ovarian follicular fluid contains a hydrophilic compound that can acutely stimulate cortisol-cortisone inter-conversion by 11BHS1 and a hydrophobic 11BHS1 inhibitor (Thurston et al., 2002, *Reproduction* 124). In the human ovary, levels of the lipid inhibitor of 11BHS1 correlate strongly with the developmental potential of oocytes as reflected by an increased pregnancy rate in women undergoing assisted conception by in vitro fertilisation-embryo transfer (IVF-ET)(Thurston et al., 2003, *Hum.Reprod.*18). In cows and pigs, altered levels of enzyme modulators are observed in spontaneous ovarian cysts (Thurston et al., 2003, *Biol.Reprod.*68), allowing us to advance a new model for the endocrine basis of cystic ovarian disease resulting from endocrine inhibition of glucocorticoid metabolism by the 11BHS1 enzyme. (Supported by grants from Wellcome Trust [05970], BBSRC [48/S15850] & Freemedic plc.)

## S8

### **AMH in the ovary - from receptor signaling to physiological function and clinical impact**

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Anti-müllerian hormone (AMH), is a member of the TGF $\beta$  family of growth and differentiation factors. Also known as müllerian inhibiting substance, AMH plays an essential role during *male* sex differentiation, where it signals the regression of the müllerian ducts. However, in the postnatal *female* AMH plays an important role in growth and development of ovarian follicles. In both rodents and women, AMH is expressed in primary follicles immediately after they have started to grow, i.e. after recruitment, whereas its expression ceases in follicles that have been selected for dominance. This expression pattern is consistent with a role of AMH at two major regulatory checkpoints of follicular development: the entrance of the resting primordial follicles into the growing pool and the exit of follicles either to ovulation or, the majority, to death through atresia. I will discuss several aspects of these roles of AMH in ovarian function as they have been studied in AMH *null* mice, cultures of neonatal ovaries and single follicles. The main conclusion of these studies was that AMH inhibits primordial follicle recruitment and AMH inhibits the growth stimulatory effects of FSH. AMH signaling follows the general pattern as found in the TGF $\beta$  family, i.e. intracellular Smad proteins are activated by AMH through a complex of a specific AMH type II receptor and generic type I receptors. In the Müllerian ducts AMH employs the BMP-like pathway, with the involvement of the type I receptors Alk2 and -3 and activation of Smads-1, -5 or -8. In the ovarian granulosa cells the situation is less clear, although also in a mouse granulosa cell *line* (KK-1) AMH uses the BMP-like pathway. In our further studies of AMH we have addressed the possibility that AMH may act as a marker of the size of the ovarian reserve in women. In a first study, serum AMH levels were compared of volunteer cyclic women at two visits to the gynecologist, which were spaced by 3-5 years. We found that AMH levels dropped during this time interval, consistent with a decrease in the size of the follicle pool. In addition, we observed an excellent correlation between the number of antral follicles as determined by vaginal ultrasonography and serum AMH levels. In a second study, we compared serum AMH levels with number of oocytes picked up during an IVF procedure. Again an excellent correlation was found between AMH levels and success rate of IVF as determined by oocyte pickup. Of course these studies have to be extended to larger numbers, but we may conclude that AMH is an excellent candidate serum marker for the size of the ovarian reserve.

## O1

### **Pre-ovulatory follicle dynamics and subsequent corpus luteum function in the cow**

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**Introduction:** A delayed rise in post-ovulatory progesterone is associated with poor embryo development in the cow though the underlying cause of this aberrant luteal function is poorly understood. The objective of this study was to develop a novel model, in which we can induce a delayed rise in progesterone by manipulating the follicular phase.

**Methods:** Luteolysis was induced in twenty dairy cows in the presence of either a large follicle (LF on day 7-9; n=11) or a small follicle (SF on day 10-12; n=9) to create two groups of cows with differing follicular phases. The cows were ultrasound scanned to determine follicle and corpus luteum (CL) growth and ovulation. Plasma progesterone and oestradiol were analysed 3x daily. Cows were slaughtered on either day 4 (n=4 per group) or day 7 after ovulation and CL weights determined. This study was conducted under the Animal (Scientific Procedures) Act.

**Results and Discussion:** The pre-ovulatory follicle was larger in the LF group than the SF group at luteolysis (13.5 0.4 mm vs 6.7 0.7 mm,  $P<0.001$ ) and ovulation (17.5 0.5 mm vs 13.7 0.7 mm,  $P<0.001$ ) though growth rates did not differ. The LF group experienced a shorter follicular phase and ovulated 36h earlier than the SF group ( $P<0.001$ ). The subsequent CL diameter was not different between the two groups ( $P>0.15$ ). However, CL weight was greater in the LF group on days 4 and 7 post-ovulation ( $P=0.04$ ). Moreover, higher progesterone concentrations were observed in the LF group during the early luteal phase ( $P<0.05$ ). In conclusion, the dynamics of follicle development during the pre-ovulatory period is an important determinant of subsequent CL development and function. The ability to now analyse pre-ovulatory follicles with knowledge of their subsequent luteal potential will finally allow us to elucidate the role of the follicle in determining the adequacy of subsequent luteal function.

## O2

### **Leptin in the bovine corpus luteum - receptor expression and effects on progesterone production**

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**Introduction:** In cattle, leptin has been implicated in the control of ovarian function though a direct effect of leptin on luteal function has not yet been established. This study was conducted to determine if the leptin receptor (Ob-R) is expressed in the bovine corpus luteum (CL) and to examine the effects of leptin on progesterone production by luteinised granulosa cells and dispersed luteal cells *in vitro*.

**Methods:** RT-PCR was used to detect the presence of Ob-R and more specifically the long, biologically active isoform (Ob-Rb) in CL collected on day 2-18 of the oestrous cycle (n=18). The effects of leptin on progesterone production were investigated in granulosa cells induced to luteinise during a 144h culture with serum (n=4) and in dispersed luteal cells prepared from CL (n=8) collected on day 5-8 and cultured for 24h. Luteinised granulosa cells were cultured with recombinant human leptin and/or LR3-IGF-1 and dispersed luteal cells with recombinant human leptin and/or LR3-IGF-1 and/or LH.

**Results and Discussion:** RT-PCR showed that Ob-R and in particular Ob-Rb, was expressed in the CL at all stages of development. These results were confirmed by sequence analysis. In luteinised granulosa cells, leptin alone (4ng/ml) produced a 20% increase ( $p<0.001$ ) over basal progesterone production, which was augmented by addition of IGF-1 (50ng/ml) to a maximum of 100% of basal ( $P<0.001$ ). Progesterone production by luteal cells was increased ( $P<0.001$ ) by treatment with LH (10ng/ml) but treatment with leptin alone had no effect. In the presence of IGF-1 (100ng/ml) leptin (10ng/ml) caused a small but significant ( $p<0.005$ ) increase in progesterone production, although the response was considerably less than the response to LH. In conclusion, we have shown that the leptin receptor is expressed

in the bovine CL and have demonstrated a modulatory effect of leptin on luteal progesterone production. Work funded by BBSRC

### O3

#### **Fasting stimulates VEGF mRNA expression and steroidogenesis in pig corpora lutea**

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**Introduction:** VEGF is an important regulator of both angiogenesis and vascular permeability and it appears to be involved in the development of corpus luteum as well as in its regression in several species. VEGF production has been shown to be modulated by a variety of stimuli, among which nutrient shortage seems to play an important role. Recently we have demonstrated that short-term fasting in gilts increases VEGF concentrations in follicular fluid and VEGF mRNA levels in theca layer (Galeati et al., *Reproduction*, 126:647-652, 2003). This study was aimed at verifying whether fasting influences VEGF mRNA expression as well as steroidogenesis in pig CL.

**Methods:** Eight prepubertal gilts were treated with 1250 IU eCG followed by 750 IU hCG 60 h later in order to induce follicle growth and ovulation. Five days after hCG treatment, the animals were divided into two groups: group A, normal alimentation with a commercial diet, group B, 72 h of fasting with water available at all times. Ovaries were collected 8 days after hCG treatment and the single CLs (10/sow) were cut in two halves. One half was weighed, homogenized in PBS (0.1 g/ml) and stored until measurement of steroid content (progesterone, P4; testosterone, T; estradiol-17B, E2); the second half was treated with Tri-Pure (50 mg/ml) and stored until VEGF mRNA levels determination.

**Results and Discussion:** Fasting significantly ( $P < 0.01$ ) increased VEGF mRNA content (190.1221.39 vs 108.9610.68 arbitrary units in fasted and normally fed animals, respectively), as well as steroid concentrations (P4: 30.51.47 vs 20.90.87 ng/mg; E2: 0.570.04 vs 0.390.01 pg/mg; T: 23.01.78 vs 14.20.78 pg/mg;  $P < 0.001$ ). The increase in VEGF mRNA induced by fasting likely compensates the nutrients reduction through the formation of new vessels, thus favouring the supply of substrates for steroidogenesis. (This study was supported by a MIUR-COFIN grant)

### O4

#### **Ovarian response, embryo yield and antibody formation after repeated superovulation of pigs**

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**Introduction:** Equine chorionic gonadotropin (eCG) has both a follicle stimulating and luteinizing effect and is, therefore, used for oestrus induction and, in higher dosage, for superovulation of farm animals, in particular pigs. In the present study we investigated ovarian response, embryo recovery and the formation of eCG-neutralising immunoglobulins with repeated superovulation of Goettingen minipigs with eCG/hCG.

**Methods:** Adult sows of the Goettingen Miniature Pig breed ( $n=22$ ) were treated with progestagens (altrenogest, Regumate, Hoechst Roussel Vet., France) for 17 days and, on the following day, with 200 IU eCG and 100 IU hCG (Suigonan, Intervet, Unterschleissheim, Germany). Upon standing oestrus they were mated to a boar once every 24 h. Twelve days after gonadotropin administration embryos were flushed from the uterus via mid-line laparotomy. The procedure was repeated twice with intervals of at least two complete oestrous cycles. Blood samples collected before every eCG/hCG administration and on day of embryo recovery were analysed by ELISA for the presence of immunoglobulins against eCG.

**Results and Discussion:** After the first treatment, 13.4 (SEM 1.9) corpora lutea (Cl) had formed and 9.6 (SEM 1.4) embryos were recovered. With the following treatments ovarian response was not significantly reduced: 9.3 (SEM 1.2) and 8.7 (SEM 1.3) Cl for the second and third treatment, respectively. With the second and third flushings the yield was 6.4 (SEM 0.9) and 5.4 (SEM 1.3) embryos, respectively. Differences between first and third treatment were significant ( $P < 0.05$ ). eCG-immunoglobulin binding was hardly increased after the first

treatment, but significantly after the second and third treatments ( $P < 0.01$  and  $P < 0.05$ ). Between treatments it returned to basal levels. There was, however, considerable individual variation, some animals showing no immune response at all. The study indicates that, in pigs, repeated eCG-administration may cause an immune response, resulting in decreased embryo yield.

## O5

### **Effect of a high fibre diet prior to oestrus on reproductive hormones in gilts**

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**Introduction:** Feeding a high fibre (HF) diet prior to ovulation increases oocyte maturity, prenatal survival (Ferguson et al., 2003, Reproduction, Abstract series 30) and litter size (Ferguson et al, 2003, BSAS Occasional Publication, September 2003) in pigs. This study was designed to gain understanding of the endocrine factors which may be associated with the reproductive benefits of this feeding regime.

**Methods:** LWxLR gilts were fed either a high fibre (50% unmolassed sugar beet pulp inclusion, HF; n=21) or a control (C; n=22) diet during their third post-pubertal oestrous cycle. Oestradiol and progesterone concentrations were measured on days 1, 6, 12, 15, 16, 17 and 18 and days 1-18 respectively after oestrus. For LH, samples were collected every 15 minutes for 8 hours on day 18. LH and oestradiol surges were measured in a selection of the gilts (n=20) and samples were taken every 2 and 4 hours respectively from day 19 of their third post-pubertal oestrous cycle until day 2 of the following cycle. Data were analysed by repeated measures using a REML model and ANOVA with diet as a factor.

**Results and Discussion:** Oestradiol concentrations and the timing of LH and oestradiol peaks were expressed relative to when progesterone concentrations first fell below 10ng/ml. There was no overall effect of treatment on progesterone concentrations. Mean oestradiol concentrations were lower in gilts fed the HF diet, and this difference was significant on the day when progesterone levels first dropped below 10ng/ml (4.69 0.64 pg/ml versus 7.29 0.90 pg/ml  $P = 0.029$ ). Gilts fed the HF diet had more LH pulses (3.30 0.17 versus 2.65 0.19  $P = 0.016$ ). The oestradiol and LH peaks occurred later for gilts on the HF diet (120.3 5.5h (n=6) versus 83.3 4.2h (n=8),  $P = 0.06$ ) and (133.3 3.5h (n=6) versus 106.6 2.4h (n=9),  $P = 0.029$ ), respectively. These data demonstrate that a HF diet decreases plasma oestradiol concentrations and delays the peak in the LH and oestradiol surges. The decrease in circulating oestradiol may be the result of binding of steroids to fibre in the gut. Lower oestradiol concentrations reduce the negative feedback on the hypothalamic pituitary axis which in turn increases the number of LH pulses. It is suggested that these endocrine modifications result in a more mature population of oocytes prior to ovulation leading to improved prenatal survival and increased litter sizes. Supported by Defra, MLC and JSR Farms Ltd in the LINK Sustainable Livestock Production Programme

## O6

### **Are suprabasal progesterone levels involved in the pathogenesis of cystic ovarian disease in dairy cattle?**

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**Introduction:** Recently it was shown that in cows with cystic ovarian disease, the majority of emerging new follicles also became cystic when progesterone levels were within the suprabasal range (Silvia et al., 2001). It is however not known whether this mechanism is also involved in the pathogenesis of primary cysts. Therefore, we compared progesterone concentrations in milk fat during the final 7 days prior to the ovulation of the dominant follicle with those prior to the development of primary cystic follicles in dairy cows early post partum.

**Methods:** Ovarian activity in high yielding dairy cows (n=26) was monitored twice a week by transrectal ultrasound scanning (5 MHz linear probe) from day 7 postpartum until ovulation or formation of a cystic follicle, as approved by the Ethical Committee of the Faculty of Veterinary Medicine. A cystic follicle was defined as being a follicular structure, >2cm

diameter, that persisted for at least 7 days in the absence of a corpus luteum. Daily milk samples were taken after milking and stored frozen ('-20°C') until progesterone determination. Progesterone was determined in milkfat with a RIA, as described earlier (Opsomer et al., 1999). Results were analyzed using a linear mixed effects model, with cow as random factor (S-Plus 2000).

**Results and Discussion:** Four cows were excluded due to anoestrus or irregular progesterone profiles. In 6 animals (23.1%), follicular cysts developed. Progesterone concentrations during the 7 days prior to cyst formation were not significantly different from those prior to ovulation. Two cystic cows did however have suprabasal progesterone concentrations that ranged from 0.3 to 1.4 ng/ml (serum values). These values were significantly higher compared to ovulatory animals ( $P < 0.05$ ). We conclude that suprabasal progesterone can be one of the factors involved in the complex pathogenesis of follicular cysts. (This study was funded by the Ghent University, BOF#011D8501)

## O7

### **Preimplantation development, apoptosis and ultrastructure of in vitro cultured rabbit embryos derived from in vivo fertilized gene-microinjected eggs**

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**Introduction:** Microinjection (Mi) of gene constructs into pronucleus of fertilized eggs is a widely used method to generate transgenic animals. However the efficiency of gene integration and expression is very low because of the low viability of reconstructed embryos resulting from cell fragmentation and cleavage arrest. We aimed to test the hypothesis that the low viability of Mi-derived eggs is caused by a high rate of apoptosis in embryos, as a response to the unfavourable influence of Mi.

**Methods:** Pronuclear stage eggs (19-20 hpc) were microinjected with gene construct into the male pronucleus (gene-Mi); the eggs microinjected with PBS (PBS-Mi) and untreated (non-Mi) eggs were used as controls. Epidermal growth factor (EGF, 0, 20 and 200 ng/ml) was added into the culture medium of non-Mi and Mi-groups and the eggs were cultured up to 96 hpc. Apoptosis was detected using TUNEL assay, ultrastructure was analyzed using electronic microscopy of Durcupan ACM thin sections of the embryo.

**Results and Discussion:** Gene-Mi embryos had significantly lower ( $p < 0.05$ ) blastocyst yields and a higher percentage of cleavage arrested embryos than those in the non-Mi group. Mi-derived blastocysts had a significantly higher TUNEL-index ( $p < 0.001$ ) and lower total cell number ( $p < 0.05$ ) than the non-Mi embryos. 38% of all gene-Mi cleavage-arrested embryos had fragmented blastomeres. Comparison of gene-Mi with PBS-Mi embryos indicated that the deleterious effect of Mi on the embryo is caused by the Mi procedure itself, but not by the gene construct. EGF (at 20 ng/ml) had beneficial effects on the blastocyst development of gene-Mi embryos, completely eliminating the influence of Mi procedure on apoptosis and embryo cell number. Ultrastructural analysis confirmed the occurrence of apoptotic signs (nuclear envelope blebbing, areas with dispersed electron dense material, numerous apoptotic bodies) in Mi-derived cleavage-arrested embryos compared with non-Mi or Mi-derived normal looking embryos. These findings suggest an association between embryo cleavage arrest and apoptosis in Mi-derived embryos. EGF can eliminate any unfavorable influence of Mi on embryo quality. (This study was supported by the Slovakian Science and Technology Assistance Agency under the contract No.APVT-51-002402.)

## O8

### **Permeability of murine zona pellucida to fluorescent particles**

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**Introduction:** The zona pellucida (ZP) of preimplantation embryos forms a barrier against viruses, bacteria and fungi. However, it has been shown that the murine ZP is a porous

structure which makes nesting of pathogens possible. Some viruses of the family *Picornaviridae* are even capable of passing through the murine ZP (Gwatkin and Auerbach, 1969; Heggie and Gaddis, 1979). Because Mouse Hepatitis Virus (MHV) and Minute Virus of Mice (MVM) are important pathogens in mouse colonies, the objective of the experiment was to investigate the permeability of the murine ZP for particles with physical dimensions comparable to MHV (80-160 nm) and MVM (20 nm).

**Methods:** Blastocysts (n=10) were flushed from the uterine horns of 2 superovulated mice on 3.5 dpi. Four ZP-intact blastocysts were exposed for 6 hours to 109.0 or 105.0 fluorescent microspheres of 3 different diameters in 1 ml KSOM. The fluorescent microspheres (Fluorospheres, Molecular Probes) used were: (i) crimson red fluorescent microspheres with a diameter of 200 nm (F-8763), (ii) yellow-green fluorescent microspheres with a diameter of 100 nm (F-8803), (iii) blue fluorescent microspheres with a diameter of 20 nm (F-8781). The localization of the microspheres was visualized by a Bio-Rad Radiance 2100 Blue Laser Diode BLDTM linked to a Nikon Diaphot 300 inverted microscope. Two ZP-intact blastocysts were used as negative controls and cultured in KSOM without fluorescent microspheres.

**Results and Discussion:** In ZP-intact blastocysts cultured in KSOM with 109.0 or 105.0 microspheres per ml, the blue fluorescent microspheres (20 nm in diameter) were able to cross the ZP whereas the other microspheres (100 nm and 200 nm in diameter) were detected in the ZP. Based on these results, the possibility exist that high titres of MVM (>105.0 particles/ml) can cross the murine ZP.

## O9

### The onset of zygotic genome activation measured in single mouse embryos using luciferase reporters and an imaging photon detector

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**Introduction:** One of the most important events of preimplantation development is the timely onset of the zygotic genome activation (ZGA), which is essential for subsequent embryo development. The initial phase of ZGA occurs during the late 1-cell stage in mouse embryos. ZGA is normally assayed by destructive analysis of individual embryos taken from cohorts, which carry either endogenous or injected reporter genes. Here we take advantage of a photon-emitting chemical reaction between the firefly luciferase reporter and its substrate, luciferin to monitor the time course of the initial phase of ZGA in living mouse embryos. Photons were detected using an Imaging Photon Detector (IPD)

**Methods:** Pronuclei of in vivo fertilized and in vitro activated parthenotes (1-cell stage) were injected with firefly luciferase pGL3-control vector DNA and incubated in HEPES-buffered KSOM media containing luciferin (100mM). Real-time photon emission was monitored continuously for up to 25 hours using the IPD system. Integration of luminescent photons for a minimum of 5 minutes was sufficient to detect a photon signal above background from one single embryo.

**Results and Discussion:** Luciferase expression was observed 30 hours following hCG injection in in vivo fertilized embryos and 20 hours after oocyte activation in parthenogenetic embryos. Interestingly, this study has revealed a greater heterogeneity of luminescence pattern within individual embryos than was previously reported. Here each embryo displayed a distinct profile of luciferase expression with respect to timing, rate of increase and peak level of ZGA. The expression profile in each embryo showed a distinct peak followed by a faster decline than the initial transient of expression found during previous studies. Collectively these data show that ZGA can be measured (real-time) in individual living mouse embryos using luminescence reporters and an IPD system. This method will be valuable in understanding the control mechanism(s) responsible for efficient gene expression in mammalian embryos.

## O10

### Effect of cobalt/vitamin B12 status on ovum recovery from superovulated ewes

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**Introduction:** Cobalt deficiency is widespread in Scottish soils and as a consequence, vitamin B12 synthesis may be impaired in grazing ruminants. Vitamin B12 is a co-factor for enzymes involved in energy metabolism (methylmalonyl CoA mutase) and DNA synthesis/methylation (methionine synthase). This study investigated the effect of cobalt/vitamin B12 status on ovum recovery from ewes.

**Methods:** Mature Scottish Blackface ewes from farms of low cobalt status were housed and fed daily 1 kg hay and 0.3 kg whole barley (0.06 mg cobalt/kg DM). Sixteen ewes (Co+) received an intra-ruminal cobalt bolus ~30 days before ovum recovery and 17 ewes (Co-) remained untreated. Oestrus was synchronised (intra-vaginal progestagen, 12 d) and all ewes received 8 i.m. injections of 0.125 mg oFSH at 12 h intervals beginning at 0800 h on d 10 of progestagen treatment. Laparoscopic AI was carried out 46 h after progestagen withdrawal using semen from a single sire. Ova were recovered 6 d after insemination and graded according to their stage of development and morphology (scale of 1-4; 1=excellent, 4=poor). Data were analysed using t-tests and Chi square.

**Results and Discussion:** Mean (+/-SEM) circulating vitamin B12 concentrations in Co+ and Co- ewes on the day of ovum recovery were 1483+/-115.5 and 182+/-2.5 pmol/l respectively (P<0.001). For ewes that had ovulated (Co+, n=16; Co-, n=15), corpora lutea per ewe were 14.4+/-1.28 and 9.9+/-1.55 (P<0.05) respectively. Treatment did not affect the proportion of ova recovered per CL (0.72) or the proportion of recovered ova that contained >32 cells (0.86). For ova containing >32 cells from Co+ and Co- ewes, the median stage of development was in each case late morula and morphological grades were 2.2+/-0.04 and 2.0+/-0.06 (P<0.01) respectively. Results demonstrate that sub-optimal cobalt/vitamin B12 status reduced ovulation rate, but not embryo quality, in ewes undergoing ovum recovery procedures. (Funded by SEERAD).

## O11

### Use of heterologous and homologous radioimmunoassay (RIA) for ovine Pregnancy-Associated Glycoprotein (ovPAG) determination to detect pregnancy in sheep

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**Introduction:** Ovine Pregnancy-Associated Glycoproteins (ovPAGs) are produced by the trophoblastic binucleate cells during gestation and subsequently released in the maternal blood circulation. Determination of ovPAG concentrations in maternal blood can be used for (early) pregnancy diagnosis. In this study, the sensitivity, specificity, and ovPAG concentrations as determined by means of a heterologous and homologous RIA are evaluated.

**Methods:** In a field trial, 95 ewes of the Texel breed were synchronized and inseminated. At the moment of synchronization (D-14) and at 25 (D25), 35 (D35) and 45 (D45) days after insemination, blood samples were taken. Heterologous and homologous RIA were performed on all samples. Samples at D-14 were used as controls for non pregnancy and ewes were considered to be pregnant at D25, D35 and D45, when they lambed 147 ± 5 days after first oestrus (D0). To assess the difference between the two types of tests, SAS version 8 was used to fit a mixed model.

**Results and Discussion:** Time (P<0.01), type of test (P<0.05) and the interaction time\*type of test (P=0.01) had a significant effect on the measured ovPAG concentration. The threshold

level for pregnancy was fixed at 3.0 ng/mL for the heterologous and 6.0 ng/mL for the homologous RIA. The sensitivity of the heterologous RIA was evaluated in all pregnant ewes (n=69) at D25, D35 and D45, and was 99% at D25, but increased to 100% at D35 and D45. Likewise, the homologous RIA had a sensitivity of 100% at each time point. The specificity was assessed in all ewes at D-14 and was 99% for both types of test. In conclusion, both RIA systems were very reliable to detect early pregnancy in ewes. However ovPAG concentrations were significantly higher when measured with the heterologous RIA than with the homologous RIA.

## O12

### **The anti-inflammatory cytokine interleukin-10 protects against LPS-induced miscarriage and preterm labour**

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**Introduction:** Interleukin-10 (IL-10) is an anti-inflammatory and immune-regulating cytokine expressed in the endometrium and placenta. Mice with a null mutation in the IL-10 gene are fertile, albeit with altered fetal and post-natal growth trajectories and placental morphometry (Robertson et al., Biol. Reprod. 2004). IL-10 terminates inflammatory responses and limits inflammation-induced tissue pathology by inhibiting macrophage synthesis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). To investigate the anti-inflammatory role of IL-10 in pregnancy, the susceptibility of IL-10 null mutant mice to low dose LPS-induced miscarriage and preterm labour was evaluated.

**Methods:** IL-10 null mutant C57Bl/6 (IL-10 $^{-/-}$ ) and control (IL-10 $^{+/+}$ ) mice were given 0.25ug LPS (*Escherichia coli*) or PBS ip on d10 of pregnancy and autopsied at d18 to measure fetal resorption. A second group of mice were given 2.0ug LPS on d17 and preterm delivery assessed 24h later. Additional mice were given 20ug LPS on d10 and sacrificed 4h later, when serum, uterine, fetal and placental tissues were assessed for TNF- $\alpha$  content by commercial immunoassay.

**Results and Discussion:** IL-10 deficiency was associated with greater susceptibility to fetal loss after administration of LPS on d10 with fewer mated IL-10 $^{-/-}$  mice carrying viable fetuses at d18 (8% in IL-10 $^{-/-}$  mice, n=12, versus 73% in IL-10 $^{+/+}$  mice, n=15, p<0.001) and increased rate of fetal resorption (89% and 37% in IL-10 $^{-/-}$  and IL-10 $^{+/+}$  mice respectively, p<0.001). After administration of LPS on d17, preterm delivery occurred in 10/12 IL-10 $^{-/-}$  mice versus 5/12 IL-10 $^{+/+}$  mice with 73% and 28% of total fetuses delivered within 24h respectively (p<0.001). Serum and tissues from IL-10 $^{-/-}$  mice (n=9) contained very high levels of TNF- $\alpha$  after acute LPS treatment, with mean levels in serum 330-fold greater, in uterus 38-fold greater and in placenta 7-fold greater (all p<0.005) than in IL-10 $^{+/+}$  mice (n=7). We conclude that IL-10 acts to modulate placental resistance to inflammatory stimuli and to protect against miscarriage and preterm labour by down-regulating expression of the pro-inflammatory cytokine TNF- $\alpha$ .

## O13

### **Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum**

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**Introduction:** Over the past decades there has been a decline in the reproductive performance of high-yielding dairy cows. Characteristics of the intrafollicular environment to which the preovulatory oocyte is exposed may be important factors influencing fertility. Therefore we examined how metabolic serum changes, that occur in early postpartum high-yielding dairy cows, affect the follicular fluid (FF) composition in the dominant follicle (> 8mm).

**Methods:** Nine blood samples were taken per cow from high-yielding dairy cows (n=9) (38.2 +/- 2.7 kg milk per cow per day) between 7 days before and 46 days after parturition, following protocol approval by the Ethical Committee of the Faculty of Veterinary Medicine. From day 14 post partum on and together with blood sampling, FF samples of the largest follicle were

collected by means of transvaginal follicle aspiration. Serum and FF samples were analysed using commercial clinical and photometric chemistry assays for glucose, B-hydroxybutyrate, urea, total protein (TP), triglycerides, non-esterified fatty acids (NEFA) and total cholesterol (TC). Data were analysed using a linear mixed effects model (repeated measurement design).

**Results and Discussion:** All cows lost body condition (0.94 +/- 0.09 points) during the experimental period. In FF, glucose concentrations were consistently higher (+/- 10%) and the TP, triglycerides, NEFA and TC concentrations were significantly lower (+/- 17%, 40%, 37% and 45% respectively) than in serum ( $P < 0.05$ ). The glucose, B-hydroxybutyrate, urea and TC concentrations in serum and in FF changed similarly over time ( $P < 0.05$ ). Especially glucose, B-hydroxybutyrate and urea concentrations were well correlated. The results from the present study confirm that the typical metabolic adaptations in serum of high-yielding dairy cows shortly post partum, are reflected in follicular fluid and, therefore, may affect the quality of both the oocyte and the granulosa cells. This study was supported by IWT-Vlaanderen (grant 13236).

## O14

### **Localization of estrogen receptor beta (ERB) mRNA within various bovine ovarian cell types using *in situ* hybridisation**

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**Introduction:** The B subtype of the estrogen receptor is largely distributed in the ovary of many species but data on the bovine ovary are scarce. The objective of the current study is to describe cellular expression of ERB mRNA in all different cell types of the bovine ovary using *in situ* hybridisation.

**Methods:** Paraffin-embedded ovarian tissue sections of two cows with different plasma progesterone concentrations were used (cow 1: 3.5 ng/ml; cow 2: 1.0 ng/ml). A 602 bp fragment of ERB mRNA was cloned, sequenced and digoxigenin (DIG)-labelled. Subsequently, *in situ* hybridisation was executed by incubating the sections with the DIG-labelled cRNA antisense probe and the sense probe as a control. For the semi-quantitative evaluation of ERB mRNA expression, the ERB mRNA score (SERB mRNA) was expressed using the formula:  $SERB\ mRNA = 0 \times n\ (SI0) + 1 \times n\ (SI1) + 2 \times n\ (SI2) + 3 \times n\ (SI3)$  with n the percentage of cells exhibiting a staining intensity 0 (absent), 1 (weak), 2 (moderate) or 3 (strong).

**Results and Discussion:** These data show that the ERB mRNA expression was cell specific and present in all follicular stages, in the corpora lutea and corpora albicantia, in the superficial and deep stroma, in the tunica albuginea and in the surface epithelium of the ovaries of both cows. These findings suggest a role of ERB in the development of those structures. ERB mRNA was present in the cytoplasm of these cell types, but could also be found in the nucleus of the oocyte of primordial, primary and secondary follicles. The SERB mRNA was nearly similar in all ovarian structures of both cows, except for the tertiary follicles in which expression was slightly higher in the cow with the lower plasma progesterone concentration which might suggest a negative dose-response. This study was supported by project grants #01100501 and #011B4101 from the BOF (Ghent University).

## O15

### **Angiogenic activity of swine granulosa cells**

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**Introduction:** Angiogenesis, the formation of new vessels from pre-existing ones, is a process of critical importance. Since the ovary is a site of cyclic angiogenesis it represents a valuable model for this study. Granulosa layer, though avascular, is involved in the control of follicular angiogenesis as it represents the main component that produces Vascular Growth Endothelial Factor (VEGF). In previous studies we have demonstrated that an hypoxic environment is established during follicular growth; at the same time we have observed an increase of VEGF production by granulosa cells during follicular maturation, pointing out a possible relationship between hypoxia and VEGF production. The aim of this study is to investigate the role of swine granulosa cells in follicular angiogenesis, in particular the

modulatory effects of hypoxia on their angiogenic activity. To this purpose we set up a reliable bioassay which allows the study of porcine Aortic Endothelial Cells (AOC) growth on a three dimensional fibrin gel matrix.

**Methods:** AOC were cultured on microcarries (MC) beads and then pipetted into a solution of fibrinogen (1 mg/ml) and thrombine (250 ul). 106 granulosa cells from follicles > 5 mm, were seeded in 24 wells plates and incubated for 24 h, then subjected for 18 h to normoxia (19% O<sub>2</sub>), partial (5% O<sub>2</sub>) or total (1% O<sub>2</sub>) hypoxia. After incubation culture media were collected and used to stimulate AOC included in the fibrin matrix. Endothelial cell proliferation was measured at 48, 96, 144, 192 h by means of Scion Image Beta (Scion Corporation, MA, USA).

**Results and Discussion:** A significant ( $p < 0.01$ ) increase of AOC proliferation at each time of measurement was induced by culture media from granulosa cells subjected to partial and total hypoxia. These data suggest that hypoxia stimulates angiogenic factors production by granulosa cells promoting endothelial cell proliferation. This work was supported by a MIUR COFIN grant

## O16

### **Expression of Kit Ligand and c-Kit receptor in goat ovaries**

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**Introduction:** Relative little information is available on the local factors that regulate folliculogenesis in goats. We studied the presence and distribution of a Kit Ligand (KL) / c-Kit receptor system in goat ovaries to find evidence for its possible role in folliculogenesis.

**Methods:** Ovaries of cyclic goats were collected and fixed in paraformaldehyde for immunohistochemical localisation of KL and c-Kit proteins or used to collect samples for RT-PCR. Primordial, primary and secondary follicles were isolated mechanically and, after repeated washings to remove the stromal cells, 15 follicles from each of three different categories were used for PCR. Cumulus cells, mural granulosa and theca cells from small (< 3mm) and large (> 3mm) antral follicles as well as luteal cells, surface epithelium and medullary samples were collected to study the mRNA expression.

**Results and Discussion:** KL protein was found in granulosa cells of follicles at all stages (primordial, primary, secondary, small and large antral follicles) as well as in corpora lutea, in surface epithelium and in the medulla (in vascular walls). Correspondingly, the mRNAs for both types of KL (KL-1 and KL-2) could be detected at similar ovarian sites, except in primordial, primary and secondary follicles that only expressed KL-1. Positive immunoreactivity for c-Kit was observed in oocytes from the primordial follicle stage onwards, in granulosa and theca cells of antral follicles as well as in corpora lutea, surface epithelium and blood vessels. The mRNA for c-Kit was detected at the same sites as the protein. It is concluded, that the presence of KL and c-Kit in goat ovaries is widespread, being present in follicles at all stages of development, corpora lutea, surface epithelium and medullary vascular tissue. This distribution pattern points to a crucial role of granulosa-derived KL in follicle growth and differentiation in goats. (This work was supported by CAPES Foundation, Brazil)

## O17

### **Does the pre-ovulatory LH surge regulate the expression of mRNAs for transforming growth factor alpha (TGF $\alpha$ ) and its receptor (EGF-R) in the post-LH surge ovine follicle?**

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**Introduction:** Neovascularization of the post-ovulatory follicle is obligatory for its transformation into a corpus luteum but its physiological control by angiogenic factors remains speculative. This study tests the hypothesis that the pre-ovulatory LH surge stimulates neovascularization in the postovulatory follicle by regulating mRNA expression of the angiogenic factor, TGF $\alpha$  and/or its receptor (EGF-R) in the follicle.

**Methods:** Sixteen adult ewes were divided into four equal groups and made hypogonadotrophic using a GnRH-agonist. Once confirmed hypogonadotrophic, folliculogenesis was induced by the continuous infusion of FSH (0-72h; 5ug/h) plus pulsatile LH injections (0-72h; 2.5ug/4h, 73-84h; 2.5ug/3h, 85-120h; 1.25ug/h), followed by a 4-hour infusion of 0, 50, 100 and 200ug of LH. The animals were killed 8h after the end of the LH infusion and one ovary per ewe was collected and stored at -80°C. The expression of TGF $\alpha$  and EGF-R was determined on frozen sections using *in-situ* hybridisation with digoxigenin-11-UTP labelled riboprobes.

**Results and Discussion:** The LH infusion did not affect the total number of follicles (>0.5mm) per ovary. The mRNA for TGF $\alpha$  was widely expressed in granulosa and theca but expression of EGF-R mRNA was limited to granulosa in antral follicles. The infusion of LH did not affect the number of follicles expressing mRNA for TGF $\alpha$ . However, there was a dose related increase in follicles expressing mRNA for both TGF $\alpha$  and EGF-R. Compared to controls, there were increases of 64% (50ug), 73% (100ug) and 136% (200ug) in the number of follicles expressing both genes. These data demonstrate that a simulated LH surge induced the expression of EGF-R in granulosa cells of antral follicles. We suggest that the induction of the TGF $\alpha$  receptor by LH facilitates the action of TGF $\alpha$  by inhibiting aromatase and stimulating neo-vascularization in the post-LH surge follicle. This work was supported by grants from the Wellcome Trust.

## O18

### **Dependence of primordial follicle formation on FIGLA may be mediated by regulation of MCL1 expression**

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**Introduction:** The process of primordial follicle formation occurs towards the end of mid-gestation in humans and is a central determinant of a woman's reproductive lifespan. The oocyte-specific transcription factor Figla is essential for primordial follicle formation but the relevant target genes are unknown. We have demonstrated that expression of *MCL1*, an anti-apoptotic factor present in oocytes, increases between 16 and 19 weeks gestation in the human fetal ovary. *FIGLA* mRNA expression also increases 40-fold over the same gestational range. We have therefore investigated whether expression of *MCL1* is regulated by FIGLA.

**Methods:** Electrophoretic mobility shift assays (EMSA) were performed to determine whether FIGLA complexes can bind to a putative site in the human *MCL1* promoter, and transient transfection assays used to demonstrate whether FIGLA complexes could up-regulate a luciferase marker gene downstream of a *MCL1* promoter fragment.

**Results and Discussion:** Human FIGLA protein and its co-factor E12 were generated in vitro and shown to bind specifically to *MCL1* promoter probes containing the putative E-box binding site by EMSA. The function of such complexes was demonstrated by the co-operative 7-fold up-regulation of *MCL1* promoter driven luciferase by co-expression of FIGLA and E12 in transient transfection assays. *MCL1* may therefore be a target gene of FIGLA, and increased expression of this anti-apoptotic factor part of the mechanism whereby FIGLA is essential for oocyte survival at the time of primordial follicle formation.

## O19

### **Heat stress *in vitro* affects steroid production by theca and granulosa cells from bovine preovulatory follicles**

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**Introduction:** High environmental temperatures markedly reduce the fertility of cattle, but reports on the effects of heat stress on bovine follicular steroidogenesis are inconsistent. We examined the steroidogenic responses of theca interna (TI) and granulosa cells (GC) to heat stress *in vitro*, thus avoiding the confounding indirect effects of elevated temperature observed *in vivo*.

**Methods:** Preovulatory bovine follicles (n=5) were obtained on Day 8 of the oestrous cycle, 36 h after a luteolytic injection of PGF2 $\alpha$ . GC were isolated and cultured (200,000 cells/well) with testosterone (0.5 uM) and 0, 2 or 100 ng/ml LH or FSH. Pieces of TI (3/well) were

cultured with 0, 2 or 100 ng/ml LH. Treatments were applied in duplicate to follicle cells cultured at each of three temperatures: 37°C, 39°C or 41°C. Medium was collected and replaced every 24 h for 4 days and steroid concentrations were determined by RIA.

**Results and Discussion:** Secretion of estradiol (E2) by GC during 24-96 h of culture was lower at 41°C than at 37°C or 39°C in the presence and absence of gonadotrophins ( $P < 0.05$ ). Secretion of androstenedione (A) by TI was lower at 41°C than at 37°C in both control (24-96 h) and LH (2 ng/ml)-stimulated (0-96 h) cultures ( $P < 0.05$ ). In contrast, secretion of progesterone (P4) by both GC and TI was higher at 41°C than at 37°C throughout the culture period for all treatments ( $P < 0.05$ ). These results suggest that heat stress causes GC and TI to luteinize prematurely (i.e. decreased E2 and A and increased P4). Premature luteinization has been associated with reduced fertility in cattle with prolonged dominant follicles and may be partially responsible for the lower fertility observed in heat-stressed cattle. (Supported by USDA Multistate Project NE-1007.)

## O20

### **Allografting of mouse hemi-ovaries under the kidney capsule versus back muscle**

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**Introduction:** The aim of this study was to compare follicular development after allografting mouse hemi-ovaries fragments under the kidney capsule versus an intra-muscular site.

**Methods:** Ovaries of 12 day-old (C57Blx6)F1 female hybrids containing primordial follicles were allografted to three ovariectomized 6 week-old recipients. A hemi-ovary was allotransplanted under the kidney capsule and the other hemi-ovary was injected inside the back muscle. One week after grafting, ovarian stimulation with intraperitoneal injection of 1IU FSH (Puregon, Organon, The Netherlands) was given every second days for two weeks. Finally two doses of 5IU FSH were given followed by 5IU hCG (Chorulon, Intervet, The Netherlands) 48 hrs later. Another 14 hours later all animals were autopsied and grafts were fixed in neutral formalin and processed for hematoxylin-eosin staining. Histological examination was done after serial sectioning.

**Results and Discussion:** All grafts were recovered. Antral follicle development was observed in both kidney and muscle grafts. In the musclegrafts three ovulating follicles were observed. The released oocytes were found inside a big cavity formed around the ovulation site. Revascularisation was more prominent in muscle grafts than in kidney capsule grafts. These preliminary results suggest that graft position may influence follicular development in allografted mouse hemi-ovaries. Follicular development seems to be more advanced in back muscle grafts than in kidney capsule grafts. Back muscle grafting may have advantages in terms of the route of grafting by injection, the size of the graft, the tissue compartment allowing large follicles to develop and of follicle monitoring. Additional data will be collected.

## O21

### **Endogenous modulators of 11B-hydroxysteroid dehydrogenase (11BHSD) activities in bovine and porcine seminal plasma**

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**Introduction:** In semen, cortisol is inter-converted with cortisone by 11B-hydroxysteroid dehydrogenase (11BHSD). Previously, we have demonstrated that human follicular fluid contains compounds that can modulate cortisol metabolism by 11BHSD. The objectives of the present study were to establish whether bovine and porcine seminal plasma contain similar endogenous modulators of 11BHSD activities.

**Methods:** Bovine (n=5) and porcine (n=5) seminal plasma samples were collected from animals of proven fertility undergoing regular semen collection for artificial insemination. Each seminal plasma sample was loaded onto a C18 Sep-pak cartridge, sequentially eluted with increasing concentrations of methanol and each column fraction was tested (at 10% v/v) for effects on oxidative 11BHSD activity in rat kidney homogenates. 11BHSD activity was assayed over 1h using 100nM [<sup>3</sup>H]-cortisol plus 400uM NADP<sup>+</sup>.

**Results and Discussion:** Porcine whole seminal plasma stimulated NADP<sup>+</sup>-dependent oxidation of cortisol by 11BHS1 by up to 1.85-fold ( $P < 0.01$ ). When fractions of porcine seminal plasma samples were tested, the hydrophobic components eluted at methanol concentrations of between 55-70% (v/v) methanol stimulated NADP<sup>+</sup>-dependent 11BHS1 activities by up to 2.7-fold ( $P < 0.01$ ). The hydrophilic fractions of porcine seminal plasma had no significant effect on 11BHS1 activity ( $P > 0.05$ ). Conversely, whole bovine seminal plasma inhibited 11BHS1 activity by 86.8% ( $P < 0.01$ ). As in the pig, fractions of bovine seminal plasma eluted at 55-70% (v/v) methanol stimulated NADP<sup>+</sup>-dependent oxidation of cortisol by up to 1.4-fold ( $P < 0.01$ ). However contrary to observations in pig seminal plasma, the hydrophilic fractions of bovine seminal plasma eluted at low methanol concentrations (<10% v/v) inhibited NADP<sup>+</sup>-dependent 11BHS1 activity by up to 43.8% ( $P < 0.01$ ). In conclusion, we have established that both porcine and bovine seminal plasma samples contain hydrophobic stimuli of NADP<sup>+</sup>-dependent oxidation of cortisol by 11BHS1. Moreover, bovine seminal plasma contains additional hydrophilic compounds that inhibit 11B-dehydrogenase activities.

## O22

### **Evidence for continued maturation of spermatozoa after entry into the vas deferens of the spinifex hopping mouse, *Notomys alexis***

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**Introduction:** In most mammals, spermatozoa complete post-testicular maturation in the caput and corpus epididymides, with storage of sperm occurring in the cauda epididymides. Normally, sperm nuclear chromatin condensation is completed during epididymal migration, when thiols are oxidized to disulphide bonds. In the spinifex hopping mouse, *Notomys alexis*, sperm pass relatively quickly through the epididymis, with some storage occurring in the distal region of the vas deferens. The aim of this study was to determine whether in *N. alexis*, the vas deferens contains a more mature sperm population than the cauda epididymidis.

**Methods:** Sperm were collected from the cauda epididymides and vasa deferentia. The amount of thiols in sperm nuclei was determined by staining with 0.5mM monobromobimane (mBBr), with or without pre-treatment with 1mM dithiothreitol (DTT). Fluorescence intensity was measured in grey scale values (gsv) ( $n=3$ , ~100 sperm/animal). To determine the effect of detergent on chromatin integrity, sperm were incubated with 1% sodium-dodecyl-sulphate (SDS) in borate buffer, pH9.0, for 30min, stained with DAPI, and viewed by fluorescence. Chromatin decondensation was determined by amount of nuclear swelling ( $n=3$ , ~100 sperm/animal). Results are given as mean $\pm$ -SD.

**Results and Discussion:** After staining with mBBr there were significantly brighter sperm in the cauda epididymides (60.8 $\pm$ -23.7gsv) than in the vasa deferentia (34.5 $\pm$ -17.9gsv) (unpaired t-test,  $p < 0.001$ ). Following DTT treatment, much brighter fluorescence occurred in both populations (cauda; 155.7 $\pm$ -26.2gsv, vas; 155.7 $\pm$ -26.2gsv). After SDS incubation sperm nuclei in the cauda epididymides (13.8 $\pm$ -3.4 $\mu$ m<sup>2</sup>) were slightly larger than in the vasa deferentia (13.0 $\pm$ -2.9 $\mu$ m<sup>2</sup>) (unpaired t-test,  $p < 0.05$ ), with both populations being larger than controls (cauda; 10.5 $\pm$ -2.0 $\mu$ m<sup>2</sup>, vas; 10.7 $\pm$ -2.1 $\mu$ m<sup>2</sup>). In conclusion, sperm nuclei in the vas deferens are more resistant to SDS decondensation and contain fewer thiols than those in the cauda epididymidis. This suggests increased disulphide bonding in sperm nuclei during sperm passage from the cauda epididymidis to the vas deferens.

## O23

### **Sperm distribution in the genital tract of the bitch following artificial insemination**

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**Introduction:** Canine spermatozoa may be stored for several days within the reproductive tract of the bitch since natural matings 9 days before ovulation can still be fertile. Since there is scant information about the localization of this sperm reservoir, this study was performed to investigate the sperm distribution in the genital tract of the bitch following artificial insemination (AI) in relation to the time of ovulation.

**Methods:** Eight beagle dogs were inseminated intravaginally with 500 x 10<sup>6</sup> spermatozoa: 2 dogs before ovulation, 3 dogs during ovulation and 3 dogs 2 to 3 days after ovulation.

Ovariohysterectomy was performed 24h after AI. Half of the genital tract was divided into 8 segments (corpus uteri, caudal, middle and cranial uterine horn, uterotubal junction (UTJ), isthmus, ampulla and infundibulum) which were processed for histology (HIS) and scanning electron microscopy (SEM). From each segment, the number of spermatozoa and the percentage of uterine crypts containing spermatozoa were assessed on 30 histological sections and 20 electron microscopic areas. The contralateral uterine horn and oviduct were flushed with physiological saline. The data were analyzed using univariate analysis of variance.

**Results and Discussion:** HIS and SEM revealed that the spermatozoa were mainly located in the uterine crypts and at the UTJ, while few spermatozoa were detected in the oviducts. No differences ( $P>0.05$ ) were found among the different segments of the uterus for the percentage of crypts with spermatozoa. AI before or during ovulation resulted in higher percentages of uterine crypts with spermatozoa ( $P<0.05$ ) and a higher number of spermatozoa at the UTJ ( $P<0.05$ ). The number of spermatozoa obtained from the flushing was low. In conclusion, our data suggest that the uterine crypts and the UTJ act as sperm reservoirs in the bitch, and that sperm distribution in the genital tract is affected by the time of AI.

## O24

### **Expression of progesterone receptor(s) during capacitation and incidence of acrosome reaction induced by progesterone and zona proteins in boar spermatozoa**

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**Introduction:** Sperm acrosome reaction (AR) is a prerequisite step for in vivo fertilization. In the vicinity of oocyte, zona protein(s) (ZP) and progesterone (P4), a component of follicular fluid, are proven to be responsible for physiological AR induction. Questions are arisen to how the P4 receptor(s) express during the process of sperm capacitation and how the AR incidence is linked to these ligand induction.

**Methods:** Following capacitation (Melendrez et al., 1994) for 0, 2, 4 and 6 h, pooled fertile boar semen ( $n=2-3$ ) with 80% sperm motility were labelled with P4-BSA-FITC (100 ug/mL) to detect the presence of P4 receptor(s). Parallel sperm samples were treated with P4 (10 ug/mL) for 20 min to test AR inducing efficiency at different time points. To compare the ability of ZP and P4 to induce AR, spermatozoa capacitated in a modified medium supplemented with 1mg/mL heparin for 4 h, were then treated with heat solubilized ZP (150 ug/mL), P4 (10 ug/mL) or ZP+P4 for 20 min. FITC-peanut agglutinin staining was applied to observe the disrupt acrosomal morphology.

**Results and Discussion:** Expression of P4 receptor(s) enhanced with time (onset: 27 $\pm$ 5%, 2h: 41 $\pm$ 4%, 4h: 49 $\pm$ 4% and 6h: 52 $\pm$ 4%, mean $\pm$ se  $n=6$ ) as evidenced by increasing percentage of spermatozoa with completed cap fluorescent staining. In parallel sperm samples, percentages of AR induced by P4 were 9 $\pm$ 2%, 14 $\pm$ 2%, 18 $\pm$ 2% and 24 $\pm$ 2%, respectively. In solvent control at all time points, less than 10% spermatozoa had undergone AR. Capacitation for 4 h onward showed optimal receptor expression and AR induction. After sperm incubation in heparin-medium, P4, ZP and P4+ZP treatments induced higher AR incidence than control (39 $\pm$ 8%, 41 $\pm$ 8% and 45 $\pm$ 7% vs 32 $\pm$ 8%). P4+ZP exerted more inducing action than P4 alone ( $P<0.01$ ). Inducing capacity of P4 was comparable to that of ZP. The expression of P4 receptor(s) is associated with capacitating process and that appears to be required for P4-induced AR. P4 and ZP seem equally capable of autonomously carrying the AR induction but lacking synergetic or additive effect. Both might represent alternative pathways thus resulting in a backing system for this prerequisite acrosomal exocytosis. (Supported by NSC 90-2313-B-005-114; 91-2313-B-005-131)

## O25

### **Modulation of the mouse testis transcriptome during postnatal development and in selected models of male infertility**

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**Introduction:** Spermatogenesis is a highly co-ordinated process involving complex cell interactions and changing patterns of gene expression. Up to 4% of the mouse transcriptome is specific to spermatogenesis (Schulz N et al. 2003). Despite detailed knowledge of testicular structure and cell biology, our understanding of the molecular pathways that define different testicular cell types and their interactions remains rudimentary. In turn, this has hindered understanding of the causes of male infertility. The objective of this study was to develop a cDNA array-based platform for analysis of spermatogenesis and use it to investigate key models of infertility.

**Methods:** Two subtracted cDNA libraries enriched for testis specific and germ cell specific genes were constructed, sequenced and used to generate cDNA microarrays. Using these arrays, we examined the expression profiles of mouse testes during prepubertal development, in selected germ cell depleted models, and in a series of teratozoospermic models with deletions on the Y chromosome long arm. All animals were housed and killed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

**Results and Discussion:** Library sequencing showed that the combined gene set contains over 1905 different genes, of which over 1000 are uncharacterised. 1790 genes showed significant change during testis development, demonstrating successful targeting of our gene set to this process. Cluster analysis of the data has enabled us to deduce the probable cellular pattern of expression of the genes on the array, highlighting genes likely to be important at different stages of germ cell development. Data from the Y-deleted models has allowed discovery of a new gene on the Y chromosome, and also implicated a number of X-linked genes in the loss of fertility exhibited by these models. These findings suggest a competitive interaction between the gonosomes during spermiogenesis, akin to the Stellate locus in *Drosophila* (Aravin AA et al.2001).

## O26

### **Interchromosomal effect in infertile men with Robertsonian or reciprocal translocation**

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**Introduction:** Somatic chromosomal abnormalities are frequently found in infertile men, particularly in those with low sperm count and/or seeking ICSI. These abnormalities mostly consist in numerical sex chromosome abnormalities and translocations (Robertsonian or reciprocal). In this study, we searched for the occurrence of non-disjunction of chromosomes not involved in translocations during meiosis, phenomenon called interchromosomal effect and first described by Lejeune et al (1965).

**Methods:** We evaluated aneuploidy for chromosomes 7, 9, 13, 18, 21, X, and Y in spermatozoa of five infertile men referred for ICSI: two patients carrying a Robertsonian translocation and three a reciprocal translocation. Ejaculate samples of these five patients and four controls (men with a 46,XY karyotype and normal sperm parameters) were studied in dual FISH 7-9, dual FISH 13-21 and triple FISH X-Y-18.

**Results and Discussion:** A statistically significant increase of disomy X, Y and XY ( $p=0.009$ ,  $p=0.004$ ,  $p<0.001$ ) was found in the Robertsonian der(13;14)(q10;q10) carrier but not in the der(14;21)(q10;q10) carrier compared to controls. Among reciprocal translocation carriers, a significant increase of disomy 21 ( $p=0.033$ ) was observed in a sole patient with a t(9;22)(q21;q11.2). The increase of meiotic non-disjunction for chromosome 21 and sex

chromosomes is a recurrent event found in other studies. According to our results and some published data, the interchromosomal effect of translocations on some specific chromosomes is likely in infertile men. Moreover, this phenomenon shows inter-individual variations which cannot be predicted. The risk of aneuploidy in sperm of males used for ICSI should be evaluated. It has to be superadded to that of meiotic segregation of the translocation to give a more precise and personalized risk assessment of aneuploidy in the offspring of those men.

## O27

### **Contraceptive effect of M1 equatorial segment protein in male hamster (Mesocricetus auratus)**

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**Introduction:** M1 is an antigenic protein, localized specifically at the equatorial segment of hamster spermatozoa. Previous study revealed that the M1 monoclonal antibody (mab) blocked hamster *in vitro* fertilization (Mat Noor and Moore, 1999) and also resulted contraceptive effect on female hamsters (Mahanem and Dzulsuhaimi, 2003). In this study, an active immunization of male hamsters was conducted to evaluate the *in vivo* effect of M1 antigen on male hamsters fertility.

**Methods:** Mature male hamsters (n = 6/group) received injection intraperitoneally at doses of 10 µg and 30 µg M1 antigen respectively. Nine days after successful mating with proven fertile female hamsters, the females were sacrificed for implantation site count. Histological analyses on immunized males testes were carried out to determine any abnormality due to M1 antigen immunization. Data on the number of implantation sites are expressed as mean +/- SEM and analyzed using the student t-test. Values with a confidence level of P < 0.05 were considered significant. Handling and treatment of animals were conducted in accordance with the guidelines for the care and use of laboratory animals established by the Animal Research Committee of the university.

**Results and Discussion:** Active immunization of 10 µg and 30 µg of M1 antigen respectively showed a significant decrease in number of implantation sites (P < 0.05). Histological analyses on testes of immunized males clearly showed a sign of aspermatogenesis particularly at 30µg of M1 antigen injections. These results suggest a possible basis for the development of M1 antigen as a potential contraceptive antigen. (This study was supported by project grants from IRPA 09-0202-0060 and TORAY foundation).

## O28

### **Oocyte-cumulus communication: Impact of gap junctional communication and GDF-9 on peroxiredoxin 6 expression**

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**Introduction:** Peroxiredoxins (PRDX1 to PRDX6) form a new family of peroxidases involved in cell signalling and antioxidant protection. We showed earlier that bovine oocytes and cumulus cells present an up-regulation of PRDX6 transcripts and protein after *in vitro* maturation. Our objective was to study the effect of cumulus-oocyte communication and GDF-9 on the expression of PRDX6.

**Methods:** Experiment 1: Clumps of cumulus cells were matured in presence or absence of denuded oocytes, or as cumulus-oocyte complexes. Clumps of cumulus were obtained by aspiration through a 75 µm pipette. These samples were matured *in vitro* for 24 hours in TCM-199 supplemented with EGF. Experiment 2: Clumps of cumulus cells were matured in TCM-199 supplemented with medium conditioned either by mouse recombinant GDF-9 transfected cells, or by mock transfected cells. In the two experiments, PRDX6 expression was studied using semi-quantitative RT-PCR with normalisation to histone H2a expression.

**Results and Discussion:** An up-regulation of PRDX6 was observed in oocytes only when they were matured as cumulus-oocyte complexes. On the contrary, cumulus-oocyte junctions were not needed for the up-regulation in cumulus cells, even though the presence of oocytes was required. It therefore seems that cumulus cells regulate PRDX6 expression in oocytes

through cell junctions, whereas oocytes regulate PRDX6 expression in cumulus cells via paracrine signalling. Next, we tested whether GDF-9 could be the paracrine factor. Our results showed that GDF-9 increased PRDX6 expression by two folds in cumulus cells cultured alone ( $p < 0.001$  at 100 ng/ml GDF-9 versus control). Together, our results demonstrated that interaction between oocytes and cumulus cells are necessary for the regulation of PRDX6 expression in both cell types. GDF-9 might be the oocyte factor responsible for the increase of PRDX6 expression in cumulus cells. (Gregory Leyens is a Research Fellow of the Fonds National de la Recherche Scientifique, Belgium.)

## O29

### **Novel functional characterization of expression of prostaglandin F<sub>2a</sub> synthase (PGFS) and microsomal prostaglandin E<sub>2</sub> synthase (mPGES) in porcine oestrous cycle endometrium and in early pregnancy**

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**Introduction:** Prostaglandins produced in the uterine endometrium and the PGF<sub>2a</sub>/PGE<sub>2</sub> ratio play important role in the regulation of the oestrous cycle and establishment of pregnancy. PGFS and mPGES are the two distinct downstream catalyzing enzymes, which regulate the production of both prostaglandins. We hereby investigated the functional appearance of the PGFS and mPGES expression along with the porcine oestrous cycle and early pregnancy.

**Methods:** Endometrium samples were analyzed from cyclic (n=27) and pregnant gilts (n=7). PGFS expression was examined by RT-PCR, quantitative RT-PCR and Western blot, whereas mPGES expression was investigated by RT-PCR and quantitative RT-PCR.

**Results and Discussion:** A 978-bp RT-PCR product which corresponds to the positive control (lung) mRNA, was identified both in endometrium collected from the oestrous cycle (4-21 days) and pregnant (15-25 days) gilts. PGFS mRNA expression levels (arbitrary units) were highest on days 12-13 and 14-15 of the oestrous cycle (mean $\pm$ SEM, 0.037 $\pm$ 0.0005, 0.031 $\pm$ 0.006;  $p < 0.05$  and  $p < 0.01$ , compared to midluteal and follicular phase), medium during midluteal 6-9 days (0.013 $\pm$ 0.002;  $p < 0.05$  and  $p < 0.01$ , compared to 12-13, 13-14 days and follicular phase), and lowest during follicular phase (0.004 $\pm$ 0.001;  $p < 0.01$ , compared to both 12-13, 14-15 days and midluteal days) and high in 15-, but low in the 20-25 days of pregnancy ( $p < 0.05$ , compared to all groups). A 37-38 kDa protein band of PGFS was significantly increased on days 13-14 of the oestrous cycle and also showed similar mRNA expression patterns in pregnancy, compared to follicular phase. A 333-bp RT-PCR product of mPGES was detected in endometrium and mPGES mRNA quantitation demonstrated no significant upregulation across the oestrous cycle comparing to PGFS but similar increase on 15-25 of the pregnancy was observed. Taken together, this is a novel report characterizing the functional expression and changes of PGFS and mPGES in porcine endometrium during the oestrous cycle and early pregnancy.

## O30

### **Role for prostaglandins in the autocrine/paracrine regulation of 11B-hydroxysteroid dehydrogenase (11BHSD) activity in human granulosa-lutein (hGL) cells**

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**Introduction:** In the ovary, cortisol is inactivated by isoforms of 11BHSD. In placenta and uterus, glucocorticoid metabolism by 11BHSD can be increased by prostaglandins (PGs). Since PGs are paracrine regulators of ovarian function, the objective of this study was to establish whether endogenous PGs can regulate the activity and expression of 11BHSD in hGL cells.

**Methods:** hGL cells were isolated from follicular aspirates of women undergoing assisted conception (n=6). After isolation on 60% Percoll, cells were cultured in serum-supplemented medium. On day 3 of culture, cells were transferred to serum-free medium and treated for 4h or 24h with the preferential PGH synthase inhibitor meclofenamic acid (MA: 0, 0.01, 0.1, 1,

10, 100uM). 11BHS activities were assessed by radiometric conversion assays using either 100nM 3H-cortisol or 3H-cortisone to assess 11B-dehydrogenase (11B-DH) or 11-ketosteroid reductase (11-KSR) activities, respectively. Steroids were extracted, resolved by TLC, and quantified using a radiochromatogramme scanner. Parallel experiments were performed using 0uM and 100uM MA and Western blots conducted to determine effects of MA on 11BHS protein expression (n=3).

**Results and Discussion:** Suppression of local PG synthesis in hGL cells with MA resulted in concentration-dependent decreases in both 11B-DH and 11-KSR activities. Over 4h, 100uM MA decreased 11B-DH and 11-KSR activities by 31.9+/- 4.8% (p<0.05) and 50.6+/- 4.9% (p<0.01) respectively. Although treatment with MA for 24h had no further effect on 11B-DH activity (25.9+/-4.8% inhibition, p<0.01), the effects on 11-KSR activity was more marked: maximal suppression of 11-KSR activity was achieved at 0.1uM MA (56.2 +/- 9.3% decrease, p<0.01). Since 100uM MA had no effect on 11BHS protein expression (p>0.05) over both 4h and 24h, we conclude that endogenous PGs are necessary to maintain 11BHS activities within hGL cells and are implicated in the post-translational regulation of 11BHS within the human ovary. (Supported by MRC studentship G69/1756)

### O31

#### **Glycoconjugates of the zona pellucida of murid rodents: Is there evidence for species-specificity?**

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**Introduction:** Oligosaccharides account for approximately half the zona pellucida (ZP) mass. They are important for structural support and provide adhesive ligands for sperm receptors that may bind species-selectively. Lectins, sugar-binding proteins, may be used to probe sugar composition. If the sperm-adhesion property of the ZP is species-selective then it may be that the ZP of closely related species have different sugar compositions. To test this, we compare lectin-binding patterns, and hence sugar composition, of ZP from three murid rodent species: *Mus musculus* (laboratory mouse) and two Australian hydromyine species, *Notomys alexis* (spinifex hopping mouse) and *Pseudomys australis* (plains mouse).

**Methods:** Ovaries from young *M. musculus*, *N. alexis* and *P. australis* were fixed for 24h in Rossman's Fluid, washed in 95% alcohol, routinely processed and embedded in paraffin wax. 7 um sections were incubated with various FITC-labelled lectins and viewed by epifluorescent and phase contrast microscopy. Intensity of the lectin staining of the ZP around oocytes of antral follicles was determined both qualitatively and quantitatively with digital image analysis.

**Results and Discussion:** The ZP of all three species stained with lectins specific for N-acetylglucosamine (DSA, LEA, S-WGA and WGA), B-gal(1-4)N-acetylglucosamine (ECA) and B-gal(1-3)N-acetylgalactosamine (PNA), albeit at varying intensities; however, there was no staining with lectins specific for  $\alpha$ -mannose (ConA),  $\alpha$ -glucose (ConA), or  $\alpha$ -fucose (JEA-I). The ZP of *M. musculus*, but not those of *N. alexis* or *P. australis*, stained with DBA and SBA, indicating presence of  $\alpha$ -N-acetylgalactosamines. These results suggest that the ZP of the hydromyine rodents have more similar sugar composition to each other than either does to that of the more distantly related *M. musculus*. They do not support the hypothesis that closely related species have different ZP sugar composition; the findings thus question species differences in oligosaccharides and hence perhaps species-specificity of zona-sperm adhesion.

### O32

#### **Ploidy and development of artificial mouse oocytes and zygotes**

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**Introduction:** For patients without gametes, there are currently no treatment options leading to genetically own children. These sterile patients have to rely on sperm or oocyte donation. Artificial production of gametes through haploidization may offer an alternative strategy. The artificial creation of a gamete involves the transfer of a diploid somatic nucleus into an enucleated oocyte followed by induction of polar body extrusion to reduce artificially the diploid chromosome number to haploid status. The aim of this study was to evaluate the efficiency of producing artificial oocytes and zygotes with correct ploidy. We also analysed the developmental capacity of the artificial zygotes and artificial oocytes fertilised by IVF or ICSI.

**Methods:** Somatic cumulus cell nuclei were injected into non-enucleated oocytes to produce artificial zygotes and into enucleated mature mouse oocytes to produce artificial oocytes. The expected ploidy of artificial zygotes and oocytes is 40 and 20 chromosomes, respectively. Further we verified whether different time intervals (3, 5, 8h) between injection and activation influenced the number of artificial oocytes and zygotes showing correct chromosome number. Finally fertilisation and developmental potential was investigated.

**Results and Discussion:** The expected chromosome numbers were found in 12% of artificial zygotes and 15% of artificial oocytes. Varying the time interval between injection of the somatic nucleus and activation (3, 5, 8hrs) tended to increase the efficiency up to 18% and 23%, respectively in the 5h time interval. Fertilization rate was 90% for artificial zygotes, 37% for artificial oocytes after IVF and 53% after ICSI. Blastocyst formation rates were 14, 8 and 9%, respectively. Ploidy analysis shows that the efficiency of obtaining artificial zygotes and oocytes with correct ploidy was low and that developmental potential was severely hampered. These observations question the possibility of obtaining chromosomally normal embryos from artificial oocytes or zygotes.

### O33

#### **PKA localises to mitochondria in mouse oocytes**

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**Introduction:** It has recently been shown that both isoforms of the regulatory subunit of Protein Kinase A (PKA) RI and RII are present in mouse oocytes. PKA-RII is known to bind to A Kinase Anchoring Proteins (AKAPs) that are present on many intracellular structures, including the endoplasmic reticulum, mitochondria, golgi and the cytoskeleton. In oocytes the RII subunit shows a punctate distribution in the cytoplasm with an aggregation around the germinal vesicle (GV). The aim of this study is to identify the structure which binds the RII subunit in mouse oocytes.

**Methods:** Oocytes were obtained from PMSG primed and 14 day old MF1 mice. mRNA for the catalytic and regulatory subunits of PKA, fused with eCFP and YFP respectively, was microinjected and left for 2 hours to express prior to imaging. Mitochondria were labelled by incubation in tetramethylrhodamine ethyl (TMRE; 100 nm, 10 min). The cells were then scanned using a confocal microscope fitted with a metahead to allow the separation of the 2 closely emitting fluorophores YFP and TMRE.

**Results and Discussion:** In GV stage oocytes both PKA and mitochondria show a punctate distribution in the cytoplasm with perinuclear localisation around the GV, overlay of these two signals demonstrated that PKA was co-localised with the mitochondria (n=8). However, in meiotically incompetent oocytes obtained from pre-antral follicles, different patterns of distribution for PKA and mitochondria were observed. PKA was diffuse throughout the cytoplasm (n=7), while the mitochondria showed a punctate pattern throughout the oocyte. These results demonstrate a structure for localised PKA signalling is formed during the acquisition of meiotic competence and that this is localised to the mitochondria. (This study was supported by a project grant from the Wellcome Trust.)

### O34

#### **The effects of caffeine on MPF and MAPK kinase activities and parthenogenetic development of ageing ovine oocytes**

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**Introduction:** Maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) are key regulators of both meiotic and mitotic cycles. MII oocytes contain high levels of both kinases, however, these activities decline with age. Caffeine (an inhibitor of Myt1/Wee1 activity can increase MPF and MAPK activities in ovine oocytes (Lee and Campbell, 2004 Reprod Fertil Dev), however the effects of caffeine treatment on the activation and developmental potential of ovine oocytes is unknown. The aims of this study were to examine the effects of ageing and caffeine treatment on MPF and MAPK activities, activation rates and development.

**Methods:** Ovine oocytes were matured in vitro. Control and enucleated (16-18h post onset of maturation (hpm)) oocytes were cultured until 24 hpm and then treated with caffeine 10mM for 6 hr. 10 oocytes were sampled and analysed for MPF and MAPK as previously described (Ye JP et al., 2003 *Reprod.* 125, 645-656). Oocytes were activated in medium containing 5ug/ml A23187, and then cultured in SOF, 7.5ug/ml cytochalasin B, with or without 10ug/ml of cycloheximide (CHXM) for 5 hrs. Cleavage was assessed on day 2 and development to blastocyst on day 7. Statistical analysis was performed using the Chi-square test.

**Results and Discussion:** Both kinases reached maximum activities 24 hpm and then decreased. Enucleation did not affect activities but caffeine treatment significantly increased both. The decline of MPF and MAPK on activation was not affected by caffeine treatment. A significant difference was observed in activation rates between A23187 alone or A23187 + CHXM in 24hpm oocytes (23.7% vs 83.6%) and 30hpm caffeine treated oocytes (42.8% vs 88.5%), but not in 30hpm control oocytes (92.2% vs 94.2%). Developmental to blastocyst was higher in 30hpm oocytes activated with A23187 + CHXM (30.2%) than 24hpm oocytes (22.2%), and in 30hpm caffeine treated oocytes a significant difference in development was observed (3.1% vs 30.2%).

### O35

#### **Connexin 43 as a parameter of cumulus functionality in meiotic maturation of bovine oocytes in vitro**

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**Introduction:** It was shown previously that mechanical disruption of cumulus-oocyte gap junctional communication by removal of cumulus cells before maturation did not prevent oocytes from resuming the meiotic maturation in vitro but resulted in suppressed developmental competence of oocytes and decreased number of blastocysts. The current study was aimed a) to study the effect of cdk kinases inhibitors roscovitine and butyrolactone-I (BL-I) on connexin 43 (Cx43) expression in bovine cumulus-oocyte complexes (COCs) and b) the effect of gap junction inhibition on Cx43 expression in cumulus cells and maturation of bovine oocytes in vitro.

**Methods:** Bovine COCs were obtained from slaughterhouse ovaries and matured in M199 medium supplemented or not with specific cdk or gap junction inhibitors. Expression of Cx43 was assayed by immunocytochemistry and immunoblotting. Histone H1 kinase activity was assayed as a parameter of meiotic resumption and nuclear maturation was checked by Hoechst staining.

**Results and Discussion:** At the GV stage, the cumulus cells are coupled by large gap junctions (GJ), which are characterized by ring-shaped pattern of expression of Cx43. During 24h of maturation in vitro, the pattern of Cx 43 expression changed to punctuate labelling, which is typical for small GJ. This change was not inhibited by the addition of roscovitine or butyrolactone-I to the culture medium. Immunoblots showed that both non-phosphorylated (43 kDa) and phosphorylated (45 kDa) forms of Cx43 were expressed in bovine COCs and that during in vitro culture the amount of phosphorylated form increased irrespective of culture conditions. This suggests that the process of GJ restructuring in cumulus cells may be independent of meiotic resumption in the oocyte. On the other hand, blocking specifically the GJ communication in bovine cumulus cells by affecting the membrane fluidity (heptanol) or Cx43 phosphorylation (AGA) interfered with generation or transmission of signals participating in the control of meiotic maturation and inhibited restructuring of GJ and the resumption of meiosis and nuclear maturation. This observation indicates that signals generated and/or transmitted by cumulus cells and transmitted to oocyte through gap junctions may be involved in meiotic regulation and acquisition of oocyte developmental competence. (Supported by EU grant QLK3-CT1999-00104 and GACR, grant nr.523-01-0136).

### O36

#### **FSH delays nuclear maturation but enhances development of pig oocytes *in vitro***

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**Introduction:** FSH is often added to oocyte maturation media, but its function is unclear. Using synchronised pig oocytes, we have detected a distinct modulatory role for FSH in maturation.

**Methods:** Experiments were performed to define the effects of gonadotrophins and EGF on meiotic progression. The basic medium (B) was M199 containing BSA (0.1%) and cysteine (0.57mM). Treatments were supplemented with FSH (50 ng/ml; F), LH (0.2 ug/ml; L), EGF (10 ng/ml; E) or combinations (FL, FE and LE). Oocytes were synchronised with cycloheximide (5 ug/ml, 12h; Ye et al, 2002, Rep.Fert.Dev. 14:433-442), before culture (12 or 24h) in treatment media. Three to seven batches were studied for each treatment; maturation rates were compared by non-parametric ANOVA. One batch was cultured in F for 36h to observe further maturation. To examine developmental potential, synchronised oocytes were matured in LE-medium (up to 36h), with or without FSH, and fertilised *in vitro*. Putative embryos were cultured in NCSU23 for 6d.

**Results and Discussion:** At 14h, media B, L and E produced similar rates of maturation to MI (81.0%, 93.8%, 76.6%; NS). By 24h, fewer oocytes had reached MII in B (56.0%;  $P < 0.05$ ) than in L (80.2%) or E (87.0%). In medium F, a significant proportion remained at GV until 14h (44.0%,  $P < 0.001$ ) with 42.1% ( $P < 0.01$ ) reaching MI; by 24h, only 45.5% ( $P < 0.001$ ) had reached MII. Extending culture in F to 36h appeared to allow maturation to MII (73.3%). Culture in FL and FE produced similar maturation rates to that in F alone. The proportion of oocytes cleaved 2d after IVF was higher for those matured with FSH (70.7%) than without (37.9%;  $P < 0.01$ ). The number of blastocysts (day 6) was also higher (32.8% vs. 16.9%;  $P < 0.01$ ). In conclusion, FSH slows meiosis in pig oocytes but increases developmental potential. (Supported by BBSRC)

### O37

#### **Immunohistochemical detection of MMP-1 and TIMP-1 expression in the cyclic canine endometrium**

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**Introduction:** Matrix metalloproteinases (MMPs) play a pivotal role in human endometrial remodelling. Recently, MMP-2, MMP-7 and MMP-9 activities have also been assessed in extracts of the canine endometrium during different cycle stages by means of zymography. However, this study offered no information on the localisation of the investigated MMPs nor data on tissue inhibitors of MMPs (TIMPs) or collagenases (MMP-1, MMP-8), which play a crucial role in extracellular matrix degradation in human endometrium. Therefore, the present study was framed to verify expression and localisation of collagenase-1 (MMP-1) and its inhibitor TIMP-1 in the cyclic canine endometrium by means of immunohistochemistry.

**Methods:** Paraffin-embedded uterine samples of several dogs that were ovariectomised in five veterinary clinics during pro-oestrus (n = 8), oestrus (n = 10), early metoestrus (n = 9), late metoestrus (n = 15) and anoestrus (n = 16) were cut into 5 µm sections. For MMP-1 and TIMP-1 detection, purified mouse antibodies directed against both latent and active human MMP-1 (1:25) and human TIMP-1 (1:500) (BD Biosciences, San Diego, USA) were used, respectively. Both MMP-1 and TIMP-1 expression were evaluated in the surface epithelium, the superficial stroma, the deep stroma, the crypts and the basal glands by means of immunohistochemical scores. Morphometric analyses and collagen percentage were assessed using a computerized image analysis system (Olympus BX61 light microscope). The data for MMP-1 and TIMP-1 expression and collagen percentage were analysed using the non-parametric Kruskal-Wallis test and the Tukey's test, whereas the morphometric data were analysed using ANOVA and the Tukey's test.

**Results and Discussion:** The present study showed no cyclic changes of TIMP-1, whereas MMP-1 was significantly more expressed in the superficial stroma during pro-oestrus than in late metoestrus ( $P < 0.05$ ) and in the basal glands during oestrus than in anoestrus ( $P < 0.05$ ). Most prominent changes for MMP-1 were noticed in the deep stroma, in which MMP-1

expression was significantly higher during pro-oestrus and oestrus than during late metoestrus and anoestrus ( $P < 0.05$ ). Endometrial thickness increased during oestrus and early metoestrus mainly due to glandular proliferation whereas collagen content of the deep stroma was significantly lower in early metoestrus than in late metoestrus. These findings suggest a role for MMP-1 in canine endometrial proliferation rather than in endometrial degradation as has been suggested in humans. (This study was supported by the BOF (#011B4101) of Ghent University)

### O38

#### **Exploring the extracellular matrix of the bovine oviduct - modulations of hyaluronan associated receptors and synthases *in vivo* and *in vitro***

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**Introduction:** The oviduct contributes to reproductive success through providing an optimal environment for the final maturation of gametes and the early embryonic development. The aim of this study was to investigate a proposed role of the extracellular matrix component hyaluronan (HA) in bovine oviducts. The transcript regulation of two HA synthases (HAS2, HAS3) as well as two HA receptors (CD44, RHAMM) were approached in the bovine oviduct *in vivo* during the oestrous cycle. Furthermore, the influence of exogenous HA was investigated on oviduct epithelial cells (BOEC) *in vitro*.

**Methods:** Bovine oviducts collected at the slaughterhouse were grouped depending on the stage of the oestrous cycle and dissected into ampulla and isthmus. In addition, an epithelial cell suspension culture (BOEC) was established and stimulated with HA time-dependently and with respect to high (1580 kDa) or low (100 kDa) molecular weight. Total oviductal and BOEC RNA was extracted and specific transcripts were quantified using real-time RT-PCR. In addition, HA and immunoreactive CD44 protein were localised on serial oviductal cryosections.

**Results and Discussion:** HA and CD44 protein were detected in the lamina propria of the oviductal villi, indicating a functional HA-system in the bovine oviduct. Remarkable local and cyclic changes of CD44 mRNA occurred *in vivo*, most pronounced in the isthmus around estrus. CD44 transcripts were significantly up-regulated by the ligand HA *in vitro* irrespectively of the molecular weight. In contrast, RHAMM mRNA was found down-regulated after HA supplementation. Due to an absolute higher expression level responding to exogenous HA, HAS3 seemed to be of greater importance than HAS2 in BOEC. As reported earlier the expanding cumulus-oocyte-complex produces large amounts of HA, therefore this study places the HA components present in the oviduct into peculiar interest for studying local oocyte-oviductal-communication during early reproduction. (Supported by DFG Ei 296/10-1 and Evangelisches Studienwerk eV.)

### O39

#### **Oestrogen receptor alpha inhibits the NF-kappaB pathway in a bovine endometrial stromal cell line**

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**Introduction:** NF-kappaB, acting through the NF-kappaB DNA binding site, is critical in cellular signalling in endometrial cells. However, several steroid hormone receptors, including the oestrogen receptor (ER), have been shown to inhibit the activation of the NF-kappaB pathway in specific cell lines. The aim of this study was to develop a transfection model in which to study the interaction between ER, oestradiol 17-beta and NF-kappaB in endometrial cells.

**Methods:** Studies were carried out using a bovine stromal (BST) cell line generated from day 16 cyclic cow endometrium. A CMV IE-1 promoter containing three NF-kappaB DNA binding sites was inserted into pBLSeAP forming pBLCMVSeAP to investigate the enhancement of transcription induced by NF-kappaB. A Xenopus Vitellogenin A2 oestrogen responsive element (ERE) was inserted in pBLCMVSeAP to form pBLCMVSeAP+ERE. BST cells were co-transfected by electroporation with 5ug pBLCMVSeAP or pBLCMVSeAP+ERE plus 5ug pCMVhER to investigate the inhibition of NF-kappaB by ER-alpha. The transfected cells were

treated with oestradiol 17-beta (1 or 10 nM) with or without the anti-oestrogen ICI 162,780 (500nM) to examine the role of oestradiol 17-beta in NF-kappaB function.

**Results and Discussion:** ER-alpha decreased the expression of SeAP by 30% ( $P < 0.05$ ) in transfected BST cells. Oestradiol had no effect on this response. This indicates that ER-alpha inhibits NF-kappaB transactivation and represses downstream transcription. Replacement of the CMV promoter in pBLCMVSeAP+ERE with a H. simplex tyrosine kinase promoter (which does not contain NF-kappaB sites) reversed these effects. In this construct co-transfection with pCMVhER and addition of oestradiol 17-beta both stimulated expression ( $P < 0.05$ ). The effect of oestradiol 17-beta was blocked by addition of anti-oestrogen. In conclusion, this transfection model can be used to demonstrate NF-kappaB inhibition by ER-alpha and to study the specific cellular signalling pathways involved.

## O40

### **Immune response in mice following in utero infection with Mouse Hepatitis Virus and Mouse Minute Virus**

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**Introduction:** Viral infections of laboratory mice are eliminated by embryo transfer. However, use of this technique also harbours the risk of re-introducing pathogenic viruses. The aim of the present study was to determine the minimum doses sufficient to cause infection with two of the most prevalent mouse pathogens, Mouse Hepatitis Virus (MHV) and Mouse Minute Virus (MMV), during the embryo transfer process.

**Methods:** Day 2.5 seronegative Swiss pseudopregnant mice, 8-12 weeks of age, were inoculated in utero (i.u.) with 1.5 ul of varying doses of MHV-A59 and MMVp according to procedures used for the transfer of embryos into the uterus. For each dose, 4 mice were treated. Control mice were sham-inoculated with either 0.9% NaCl or M2 medium. Mice were kept in individually ventilated cages for the duration of the experiment. Sera from day 14, 21 and 28 were tested for the presence of antiviral antibodies using enzyme-linked immunosorbent assay (ELISA).

**Results and Discussion:** Seroconversion in both MHV-A59 and MMVp groups occurred within two to three weeks after inoculation. The present data show that, under embryo transfer conditions, the lowest dose required to cause seroconversion in pseudopregnant Swiss mice corresponds to 300 TCID<sub>50</sub> MHV-A59 and 0.03 TCID<sub>50</sub> MMVp. During embryo transfer, a maximum of 1.5 ul medium together with embryos are transferred. This means that in order to cause infection and seroconversion during embryo transfer media must be contaminated with at least 105 TCID<sub>50</sub>/ml MHV-A59 or 101 TCID<sub>50</sub>/ml MMVp, the dose of MHV-A59 being 104 times higher than that of MMVp. (This study was supported by the GV-SOLAS).

## O41

### **Substrate induction of COX-2 expression by polyunsaturated fatty acids (PUFAs)**

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**Introduction:** Prostaglandin endoperoxide H synthases-1 and -2 (cyclooxygenases (COX) -1 and -2) catalyse the rate limiting steps of prostanoid synthesis from C20 n-6 polyunsaturated fatty acids (PUFAs). COX-2 activity is affected by changes in enzyme concentration as well as by substrate availability. We tested the hypothesis that COX-2 expression is induced by the PUFAs which are the enzymes' substrates.

**Methods:** COX-2 levels were measured by Western blotting (Santa Cruz; antibody SC 1745) in bovine endometrial stromal (BST) and bovine endometrial epithelial (BEND) cell lysates. To investigate COX-2 promoter elements responsible for PUFA induction, a bovine COX-2 promoter fragment containing 1356 bp 5' to the transcription start site cloned by PCR and inserted into pCAT promoter 3 vector (Promega) was transiently transfected by electroporation into BST and BEND cells. pCAT basic and pCAT control vectors were controls. To test effects of PUFAs on COX-2 promoter function, the fatty acids were added to

culture medium for up to 48 hr after transfection, and the cells lysed for chloramphenicol acetyl transferase (CAT) assay.

**Results and Discussion:** Arachidonic acid (0.05mM) induced COX-2 level 9-fold in BST cells and 2.9-fold in BEND cells 6 and 12 hr respectively after addition to culture media. Consistent with rates of endogenous prostanoid production, the level of COX-2 was higher in BST than BEND cells. Arachidonic acid increased CAT expression 3.8- and 3.2-fold in BST and BEND cells respectively after 48 hr. 8,11,14-Eicosatrienoic acid (0.05mM, 48 hr) also increased CAT expression and COX-2 level up to 11-fold in BST and BEND cells. In BST cells, linoleic acid (0.002mM) increased both COX-2 level (9.1-fold, 48 hr) and CAT expression (2.3-fold, 36 hr), but with a slower time course than observed with the 20-carbon fatty acids; it was ineffective in BEND cells. These data suggest COX-2 expression may be controlled by substrate induction.

## O42

### **The pattern of COX-2 and EP<sub>4</sub> mRNA expression in the ovine cervix during the oestrous cycle**

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**Introduction:** The anatomy of the ovine cervix prevents the routine use of transcervical artificial insemination, limiting its' use in the sheep industry. There is a degree of cervical relaxation at oestrus, enabling cervical penetration in 10-15% of ewes. This may be attributable to the actions of gonadal steroid hormones and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) on the ovine cervix. This study aims to determine the localisation and pattern of mRNA expression of the enzyme cyclooxygenase-2 (COX-2), involved in PGE<sub>2</sub> synthesis, and the PGE<sub>2</sub> receptor EP<sub>4</sub> in the cervix during the oestrous cycle.

**Methods:** Oestrus was synchronised in ten ewes and their cervixes collected under Home Office approval during the mid-luteal phase of the cycle (n = 4), and during (n = 3) or following (n = 3) the LH surge. The expression of COX-2 and EP<sub>4</sub> mRNA was analysed by *in-situ* hybridisation using digoxigenin-11-UTP labelled ovine riboprobes.

**Results and Discussion:** Blood hormone analyses confirmed that cervixes were collected at the pre-designated stages of the oestrous cycle. *In-situ* hybridisation showed that EP<sub>4</sub> and COX-2 mRNAs are located in cervical luminal epithelium (LE) and stroma. The expression of EP<sub>4</sub> was not significantly different during the oestrous cycle. However, COX-2 expression in the LE was significantly higher during the LH surge compared to post-LH surge (p = 0.006) and luteal phase (p = 0.001) expression, although there was no difference between post-LH and luteal phase expression (p = 0.34). In stroma the expression of COX-2 differed significantly (p < 0.001) between different stages of the cycle (during LH > post-LH > luteal). These results illustrate that cervical COX-2 mRNA expression is regulated throughout the sheep oestrous cycle, and that EP<sub>4</sub> mRNA expression is not. We suggest that the increase in COX-2 observed during the LH surge may be a result of the peri-ovulatory changes in oestradiol concentrations.

## O43

### **FSH and IGF promote both the secretion of 'total' inhibin-A and activin-A by bovine granulosa cells and modify the relative abundance of different molecular mass (Mr) variants**

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**Introduction:** Western-blotting of bFF using antibodies against inhibin/activin subunits reveals multiple immunoreactive species corresponding to different Mr-variants generated by differential post-translational processing and/or dimerization of precursor molecules synthesized by granulosa cells. Here we have used isolated bovine granulosa cells to investigate whether treatment with FSH or IGF modifies the processing pattern of inhibin/activin subunit precursors detected in cell-conditioned media.

**Methods:** Three batches of granulosa cells were cultured (12-well plates; 0.5 million cells/well) for 6 days with/without FSH, LR3-IGF-1 (IGF), or both (see Glister et al 2001 BOR 65:120-128). Conditioned media (day 4-6) were analysed for inhibin-A and activin-A by ELISA

and then desalted/concentrated by 10K-cut-off ultrafiltration and analysed by SDS-PAGE/western blotting using monoclonal antibodies against inhibin-a and inhibin/activin-BA-subunit. Membranes were probed with <sup>125</sup>I-labelled-anti-mouse IgG and images captured/quantified using a phosphorimager.

**Results and Discussion:** FSH and IGF enhanced secretion of 'total' inhibin-A, 'total' activin-A and raised activin-A/inhibin-A ratio ( $P<0.005$ ); co-treatment promoted an even greater response ( $P<0.001$ ). Western-blotting revealed activin-A (24kDa), pro-BA (58kDa), five Mr inhibin-A forms (34, 56, 65, 82, 108 kDa) and two a-monomer forms (27, 50kDa). Moreover, the abundance of each ir-a and BA band increased with FSH/IGF treatment. When each ir-a band intensity was expressed as a proportion of total ir-a per lane, a similar proportion contributed to each inhibin-A and a-monomer band, irrespective of treatment. However, in the case of BA-subunit, the proportion contributing to 34 and 56kDa inhibin-A decreased with FSH/IGF treatment ( $P<0.01$ ); conversely the proportion contributing to higher Mr inhibin-A forms and to activin-A increased ( $P<0.001$ ). These findings indicate that: (i) availability of BA-precursor rather than a-subunit-precursor is the limiting factor for inhibin-A/activin-A formation; (ii) under FSH/IGF-stimulated conditions relatively less high Mr inhibin-A is processed to low Mr forms whilst relatively more activin-A is synthesized. (supported by BBSRC grant 45/S14995)

#### O44

##### **BMPs 2, 6 and 15 are regulators of porcine theca cell function *in vitro***

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**Introduction:** Bone morphogenetic proteins (BMPs) are emerging as a family of proteins crucial in the regulation of fertility and ovulation rate. Previous work has shown that these factors are capable of modulating porcine granulosa cell steroidogenesis and proliferation *in vitro*. However, little information currently exists regarding the effect(s) of BMPs on theca cell function. The aim of this study was to determine the effects of BMPs -2, -6 and -15 on porcine theca cells cultured under serum-free conditions.

**Methods:** Theca cells were isolated from healthy 2-6mm follicles of mature porcine ovaries and cultured under serum-free conditions. Culture medium was supplemented with: 10ng/ml insulin, 0 or 100ng/ml LR3 IGF-1, 0 or 0.01ng/ml LH and 0, 3, 30 or 100ng/ml recombinant human BMP-2, -6 or -15 per well of a 96-well plate. Medium was replaced every 48h and cells were cultured for 144h, after which viable cell number was determined.

**Results and Discussion:** BMP-2 ( $p<0.001$ ) and BMP-6 ( $p<0.006$ ) suppressed progesterone synthesis. BMP-15 stimulated progesterone production ( $p<0.001$ ). There were significant interactions between BMP-6 and both LH ( $p<0.001$ ) and IGF-1 ( $p=0.001$ ), and also between BMP-15 and both LH ( $p=0.006$ ) and IGF-1 ( $p=0.002$ ), with regard to progesterone production. Oestradiol synthesis was suppressed by BMP-2 ( $p<0.004$ ) and BMP-6 ( $p<0.04$ ), but was promoted by BMP-2 in the absence of LH ( $p=0.021$ ). BMP-15 stimulated oestradiol production at 48h ( $p=0.003$ ) but inhibited it at 144h ( $p<0.001$ ). Interactions between BMP-15 and LH ( $p=0.024$ ) and IGF-1 ( $p<0.001$ ) were observed with regard to stimulation of oestradiol production. BMPs-2 and -15 increased viable cell number ( $p<0.001$ ). These results provide further evidence of a functional BMP system in the porcine ovary. Each BMP appears to modulate steroidogenesis and proliferation of theca cells differently suggesting that each BMP plays a specific role in the control of follicle development. *Funded by BBSRC*

#### O45

##### **The function of co-cultured porcine granulosa and theca cells is modulated by BMPs 2, 6 and 15 in conjunction with oocytes in a serum free system**

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**Introduction:** BMPs belong to the transforming growth factor B family and play a key role in ovarian physiology and female fertility. We have previously shown that a functional BMP system exists in the porcine ovary. The aim of this study was to examine the effect of BMPs 2, 6 and 15 on granulosa cells co-cultured with theca cells and/or oocytes.

**Methods:** Granulosa and theca cells were isolated from 2-6mm healthy follicles of mature porcine ovaries and cultured under serum-free conditions supplemented with: 100ng/ml LR3 IGF-1, 10ng/ml insulin, recombinant human BMP-2 (100ng/ml), BMP-6 (30ng/ml) or BMP-15 (100ng/ml) and 1ng/ml FSH + 100ng/ml testosterone (granulosa) or 1ng/ml FSH + 0.01ng/ml LH (co-culture), with and without 1 or 5 denuded oocytes per well of a 96-well plate. Medium was replaced every 48h and cells were cultured for 144h, when viable cell numbers were determined.

**Results and Discussion:** All BMPs studied suppressed progesterone by granulosa and/or by co-cultured granulosa/theca cells (with/without oocytes). Granulosa: there were significant interactions ( $P < 0.049$ ) between oocytes and BMP-6 or BMP-15. Granulosa/theca co-culture: BMP-2 ( $P < 0.001$ ) and BMP-6 ( $P < 0.001$ ). There were significant interactions between BMPs 2 ( $P < 0.001$ ), 6 ( $P = 0.028$ ) or 15 ( $P < 0.012$ ) and oocytes. BMPs 2 and 6 stimulated oestradiol synthesis by granulosa cells (+/- oocytes). Granulosa/theca co-culture: BMPs 2 and 6 inhibited oestradiol synthesis. BMP-2 ( $P < 0.001$ ) and BMP-6 ( $P < 0.001$ ). BMP-15 increased oestradiol synthesis ( $P = 0.056$ ). Only BMP-2 interacted with oocytes ( $P = 0.011$ ). BMP-6 and oocytes interacted ( $P = 0.028$ ) to decrease viable granulosa cell numbers. Granulosa/theca co-culture: BMP-6 stimulated cell proliferation ( $P = 0.04$ ). There was also an interaction between BMP-2 and oocyte addition ( $P < 0.001$ ). These results provide evidence that BMPs play an important role in co-ordinating the paracrine responses between granulosa and theca cells and the oocyte to prevent premature luteinization. Specifically, BMPs 2, 6 and 15 attenuate oocyte-derived signals in the regulation of somatic cell function. *Funded by BBSRC*

#### O46

##### **Expression of bone morphogenetic protein-6 (BMP-6) in the bovine foetal ovary at different stages of gestation**

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**Introduction:** The transforming growth factor-b (TGF-b) superfamily is made up of a number of peptide growth factors. These regulate cell proliferation and differentiation in a number of different organs. Three of these proteins are expressed by the oocyte, BMP-6 being one of these oocyte-secreted factors. BMP-6 is known to be expressed by oocytes of primary follicles in rodents and primordial follicles in adult ruminants. However, presence of the TGF-b superfamily has not previously been investigated in bovine foetal ovaries.

**Methods:** Ovaries were obtained from slaughterhouse bovine fetuses (n=13) ranging from 5.8-55.6cm crown-rump length, which equates approximately to 2-6.5 months of gestation. Ovaries were also obtained from fetuses (n=5) at known dates of pregnancy (d109 and d123). The ovaries were fixed in 4% paraformaldehyde, dehydrated through a series of alcohols and embedded in paraffin wax. Expression and localisation of BMP-6 was determined by immunohistochemistry, using DAB as a detection system.

**Results and Discussion:** At 2 months gestation, some light staining was observed around the nests of oogonia. At 3.5months, primordial follicles were observed and expression was localised to the oocyte of these follicles. At 6.5 months, expression was seen in the oocytes of both primordial and primary follicles. In conclusion, for the first time, expression of BMP-6 has been demonstrated to be present in bovine foetal ovaries. As in the adult ovary, expression was localised to the oocyte. These results indicate that expression is present in the first trimester (2 months) as well as the second and third (6.5 months). Although quantitative analysis is required, expression of BMP-6 appears to be stronger during the later stages of gestation. These results support a possible role of BMP-6 in follicular cell differentiation. (Funded by BBSRC and The University of Nottingham)

#### O47

##### **Mitochondrial transfer between porcine oocytes improves fertilisation outcome**

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**Introduction:** The use of assisted reproduction technology has become more wide ranging for the treatment of infertility. However, the treatment available for women with either poor

oocyte quality or repeated embryo development failure is limited. Recently, cytoplasmic transfer was introduced though considerable concerns have been raised regarding safety and the transmission of donor oocyte mtDNA. Using a porcine model, our aim was to determine whether mitochondria isolated from competent oocytes and introduced into incompetent oocytes would improve fertilisation rates and embryo development.

**Methods:** Oocytes were aspirated from 20 porcine ovaries and stained with Brilliant Cresyl Blue (BCB), a marker of oocyte developmental competence, to provide populations of BCB+ (competent) and BCB- (incompetent) oocytes. These oocytes were then cultured in vitro for 24h. The BCB- oocytes were then divided into 2 groups. Mitochondrial populations were isolated and purified from BCB+ oocytes and then injected into BCB- oocytes from another ovarian source. The supplemented oocytes were then divided into two groups and treated by: 1) traditional IVF, where oocytes were inseminated after 24h of mitochondrial transfer; 2) ICSI, where the mitochondria and sperm were injected together after 48h of in vitro maturation. The control groups consisted of sham injected oocytes and parthenogenetically activated oocytes.

**Results and Discussion:** The fertilisation rates for BCB- oocytes supplemented with mitochondria (IVF=31%, n=90; ICSI=33.1%, n=89) was significantly greater than ( $P<0.001$ ) for BCB- oocytes (IVF=17.6%, n=105; ICSI=20.5%, n=81). There was no significant difference in the cleavage rates for sham injected BCB- inseminated oocytes against BCB- inseminated oocytes or for parthenogenodes ( $P<0.001$ ). The transfer of mitochondria from competent oocytes to incompetent oocytes demonstrates the importance of this organelle role during fertilisation.

## O48

### **Carbohydrates and glycoproteins involved in bovine fertilization in vitro**

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**Introduction:** Whereas the importance of numerous carbohydrates and glycoproteins in sperm-zona binding, acrosome reaction and sperm-oolemma interactions has been well documented in human and mouse, few studies have investigated the role of glycoconjugates in bovine fertilization. Therefore, the objective of the present study was to identify carbohydrates and glycoproteins that are involved in bovine in vitro fertilization.

**Methods:** In vitro matured cumulus-oocyte complexes were inseminated in the presence of a variety of carbohydrates and glycoproteins to determine which molecules act as competitive inhibitors of bovine oocyte penetration. To exclude a possible toxic influence on spermatozoa, sperm motility was evaluated over time by means of computer-assisted sperm analysis in the presence of carbohydrates and glycoproteins that inhibited the penetration rate with 40% or more.

**Results and Discussion:** Among the glycoconjugates tested, D-mannose, fucoidan, dextran sulphate and fibronectin were the most potent inhibitors of oocyte penetration (90% or more inhibition), while L-fucose and vitronectin inhibited the penetration rate to a lesser extent (around 50% inhibition). Other carbohydrates caused less than 40% inhibition (i.e. D-galactose, N-acetyl-D-galactosamine, D-fucose and sialic acid) or were not effective as inhibitors of oocyte penetration (i.e. mannan, N-acetyl-D-glucosamine, dextran and heparan sulphate). Heparin was the only carbohydrate that significantly increased the penetration rate. L-fucose, dextran sulphate and vitronectin did not significantly influence total and progressive sperm motility, whereas D-mannose, fucoidan and fibronectin caused a significant, but slight reduction in both motility parameters. In conclusion, these results are indicative for the involvement of D-mannose, L-fucose, fucoidan, dextran sulphate, fibronectin and vitronectin in bovine in vitro fertilization. (This research was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders and the Fund for Scientific Research - Flanders (Belgium).)

## O49

### Effect of replacement of follicular fluid on pregnancy rates in the horse

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**Introduction:** Intrafollicular insemination (IFI) has been used to obtain pregnancies in humans (Zbella et al., 1992; Nuojua-Huttunen et al., 1995), however in horses recent attempts using IFI have been unsuccessful at obtaining a pregnancy, even when mares were inseminated concomitantly using artificial insemination with 500 million motile spermatozoa (AI-500)(Eilts et al., 2002; Meintjes et al., 2000). Successful IFI would circumvent uterine inflammation in mares susceptible to post-breeding endometritis. Poor survival of spermatozoa in follicular fluid (unpublished data, S. Eilts); the unknown effects of artificial media on ovulation, fertilisation, and sperm storage; and the unknown IFI sperm number may contribute to the disappointing results. The objective of this study was to determine if a pregnancy could be obtained by replacing the follicular fluid with a culture medium when performing IFI.

**Methods:** The study was performed with approval of the LSU Animal Care and Use Committee. Ten reproductively sound mares were used during a total of 24 oestrous cycles. At 36 hours after hCG or GnRH administration mares had IFI performed with either 50 million (n=4) or 500 million (n=5) motile spermatozoa in embryo culture medium, or had follicular fluid replaced with culture medium concomitant with AI-500 (n=10), or had only AI-500 performed (controls n=5)

**Results and Discussion:** All 5 control mares inseminated by AI-500 became pregnant. Only one of ten became pregnant after follicular fluid replacement concomitant with AI-500 and none became pregnant after IFI. These data suggest that the intrafollicular environment in the preovulatory follicle might be unfavourable for fertilisation or sperm viability and that follicular fluid manipulation may be detrimental to ovulation or fertilisation. Further studies are under way to examine the sperm cell viability after IFI.

## O50

### Requirement for mitochondrial ATP production during sperm-triggered Ca<sup>2+</sup> oscillations in the mouse egg

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**Introduction:** At fertilization, repetitive increases in the intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>c</sub>) drive the completion of meiosis and initiate the development of the quiescent egg into an embryo. While the requirement for an ATP supply is evident, the relative roles of potential ATP sources remains unclear in the mammalian egg, and the specific role of mitochondria in [Ca<sup>2+</sup>]<sub>c</sub> regulation as well in the sperm-triggered Ca<sup>2+</sup> oscillations is unknown.

**Methods:** We used fluorescence and luminescence imaging of mitochondrial redox state, [Ca<sup>2+</sup>]<sub>c</sub> and [ATP]<sub>c</sub> to investigate mitochondrial activity in single mouse eggs. We used pharmacological inhibitors of mitochondria to assess their role during the activation of development

**Results and Discussion:** Simultaneous imaging of mitochondrial redox state (NADH and flavoprotein autofluorescence) and [Ca<sup>2+</sup>]<sub>c</sub> revealed that sperm-triggered Ca<sup>2+</sup> oscillations are transmitted to the mitochondria where they directly stimulate mitochondrial activity. Inhibition of mitochondrial oxidative phosphorylation caused release of ER Ca<sup>2+</sup> due to local ATP depletion. Mitochondrial ATP production is an absolute requirement to maintain a low resting [Ca<sup>2+</sup>]<sub>c</sub> and to sustain sperm-triggered [Ca<sup>2+</sup>]<sub>c</sub> oscillations. Luminescence measurements of intracellular [ATP]<sub>c</sub> from single eggs confirmed that mitochondrial oxidative phosphorylation is the major source of ATP synthesis in the dormant unfertilised egg. These observations show that a high local ATP consumption is balanced by mitochondrial ATP production, and that balance is critically poised. Mitochondrial ATP supply and demand are thus closely coupled in mouse eggs. As mitochondrial ATP generation is essential to sustain the [Ca<sup>2+</sup>]<sub>c</sub> signals that are critical to initiate development, mitochondrial integrity is clearly fundamental in sustaining fertility in mammalian eggs.

## P1

### **Developmental potential of tetraploid pig embryos generated by electrically stimulated fusion of two-cell embryos**

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**Introduction:** Tetraploid embryos can be used as supporting cells for creation of genetically modified mammalian embryos, tissues or even individuals. They have been produced using several methods, of which electrically stimulated fusion of two-cell embryos appears to be the most efficient one. The aim of this study was to assess development and ploidy of presumably tetraploid pig embryos prepared under two different protocols.

**Methods:** Embryos in the 1- or 2-cell stage were flushed from oviducts of hormonally stimulated and mated sows at 48 and 66 h after hCG injection, respectively, and cultured in a modified medium NCSU 37 at 38.5°C under an atmosphere of 5 % CO<sub>2</sub>, 6 % O<sub>2</sub> and 89 % N<sub>2</sub>. In Experiment I, the embryos were flushed at 1-cell stage (Day 0), cultured in vitro overnight to 2-cell stage (Day 1) and then fused. In Experiment II, freshly recovered embryos at 2-cell stage (Day 1) were fused. Fused embryos were cultured until Day 7 and scored for blastocyst formation and number of nuclei. The ploidy of embryos was assessed by fluorescence in situ hybridization of chromosomes 1 and 10.

**Results and Discussion:** Development to blastocyst stage of tetraploid embryos, generated from the cultured 2-cell embryos was significantly inferior to the development of control diploid 1-cell embryos (29.1±9.7 versus 66.8±9.7 %). However, development of tetraploid embryos produced from the freshly recovered 2-cell embryos and control 2-cell embryos was very similar (89.9±6.1 versus 81.3±3.4 %). Detection of chromosomes 1 and 10 by in situ hybridization showed that more than 85 % of the cultured control embryos were diploid while 15 % of the embryos were mosaic. Among the fused embryos 50% were tetraploid, 29 % mosaic and 21 % diploid. These results show that cleavage- and blastocyst stage tetraploid embryos can be efficiently produced by electrofusion of the freshly recovered 2-cell embryos.

## P2

### **Secretion of interferon-tau (IFN-t) by ovine pre-implantation embryos during in vitro culture (IVC) in the absence or presence of granulocyte-macrophage colony stimulating factor (GM-CSF)**

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**Introduction:** Previous studies have shown that inclusion of GM-CSF during IVC stimulates IFN-t secretion by ovine blastocysts. Here, underlying mechanisms were explored by including GM-CSF in IVC at different stages of zygote development.

**Methods:** Using abattoir-derived oocytes and after standard in vitro maturation and fertilisation procedures, IVC was performed in modified synthetic oviductal fluid containing 0.3% w/v albumin (SOFA). Zygotes (day 1) were cultured in groups of 10 in 0.05ml drops. Media were changed at 48h intervals and spent (conditioned) media analysed for IFN-t. Recombinant ovine GM-CSF (5 ng/ml) was absent (A) or present (P) during period 1 (days 1-3), period 2 (days 3-5) and period 3 (days 5-7) in 6 combinations: AAA, AAP, APP, PPP, PPA and PAA. Blastocyst yield, grade, diameter and cell numbers were assessed up to day 7 of culture. Data were analysed either by the Kruskal-Wallis test (period 1) or using General Linear Model after log transformation (periods 2 and 3).

**Results and Discussion:** There were no differences between treatments in terms of blastocyst yield or quality. IFN-t (ng/ml) was first detected after period 1 when concentrations (means with s.e.) were greater in the presence of GM-CSF (A v P, 2.9+/-1.38 v 13.5+/-2.63, P<0.001). Production of IFN-t during period 2 was greater than period 1, but was influenced more by the presence of GM-CSF during period 1 (A v P, 190+/-24.7 v 518+/-63.3, P<0.001) than period 2 (A v P, 301+/-55.3 v 408+/-55.3, NS). Similarly IFN-t production during period 3 (AAA, 154+/-22.4, AAP, 131+/-18.8, APP, 474+/-130, PPP, 585+/-126, PPA, 421+/-82.0, PAA, 645+/-223) was determined by the presence of GM-CSF during period 1 (P<0.001) rather than period 3 (NS). Thus stimulation of IFN-t production by GM-CSF appears to be mediated by events prior to activation of the embryonic genome and may be related to oocyte characteristics. (SAC receives financial assistance from SEERAD).

### P3

#### **Inclusion of bovine serum from different dietary backgrounds influences embryo viability in vitro**

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**Introduction:** Dietary carbohydrates and lipids affect bovine oocyte quality in a manner dependent on oocyte donor body composition (Adamiak *et al.*, 2004; *Reprod. Fertil. Dev.*, in press). Here we cultured bovine zygotes to the blastocyst stage in vitro in the presence of serum harvested from oocyte donors from that study.

**Methods:** Sera from Thin and Fat heifers offered a high fibre (F) or starch (S) diet alone (0) or with 6% w/w (6) protected fat (Ca soaps of fatty acids) were added to 20 ul drops of SOF at 10% v/v. Bovine zygotes (n = 1,480), from oocytes matured and fertilised using standard procedures, were cultured in these drops at 38.8°C under oil in a humidified atmosphere of 5% CO<sub>2</sub> and 5% O<sub>2</sub>. Fatty acid (FA) analysis, pyruvate metabolism, total cell counts and TUNEL analysis were conducted on Day 8 blastocysts. Data were analysed by ANOVA.

**Results and Discussion:** Plasma FA for F0, F6, S0 and S6 were 0.75, 1.82, 0.50 and 1.39 ug/ml; SED=0.07; P<0.001. Cleavage rates averaged 70.8% and were unaffected by serum source. Blastocyst yields averaged 24.0+/-2.1, 21.0+/-1.9, 18.2+/-2.4 and 23.1+/-3.0% for F0, F6, S0 and S6 (P=0.09), and were higher (P<0.05) with serum from Thin than from Fat animals (24.2+/-1.9 vs 18.9+/-1.2%). FA (ng/embryo) were 106.7+/-3.5, 121.7+/-0.62, 94.3+/-3.27 and 108.2+/-0.59 for F0, F6, S0 and S6 (P<0.001). Total cell number/embryo (109.0+/-5.0) did not differ between treatments. Pyruvate metabolism (pmol/embryo/3h; 16.5+/-6.5, 26.1+/-2.9, 26.1+/-3.1 and 19.2+/-2.5; P<0.05) and TUNEL positive cells (3.8+/-0.4, 6.9+/-0.9, 6.4+/-0.7 and 5.3+/-0.5% for F0, F6, S0 and S6, respectively; P<0.01) were greater for F6 and S0 diets. In conclusion, serum source influences FA uptake by embryos in vitro and can alter early embryo viability by modifying energy metabolism and the incidence of apoptosis. (SJA supported by The Perry Foundation, Nottingham University)

### P4

#### **Mineral profile of the infertile buffaloes in Multan, Pakistan**

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**Introduction:** Population of Pakistan is increasing at a rate of 2.2% annually whereas the population of livestock is increasing marginally. So, there is shortage of milk and meat for the low-income group. The solution of this problem is to enhance the reproductive and productive potential of the livestock. Apart from macro-elements, some microelements play very important role in the reproductive performance of buffaloes. Minerals are necessary for oestrus, maintenance of developing foetus in uterus and pregnancy. Deficiency of minerals has been shown to result in low fertility rate due to irregular oestrus, silent heat and delayed ovulation. The aim of the present investigation is to compare the mineral profile in infertile (acyclic) and fertile (cyclic) buffaloes.

**Methods:** Blood samples from fifty buffaloes aged 3-5 years (acyclic=25; cyclic=25) were collected, serum was harvested and stored until analysed for sodium, potassium, magnesium and copper by atomic absorption spectrophotometer. The protein was removed from the serum by taking 1.5 ml of serum in a tube, mixed with one ml of 1N HCl and kept in for 10 min. Then 8 ml of 10% trichloroacetic acid (TCA) was added, and mixed thoroughly. This mixture was centrifuged at 3000 rpm for 10 min. Supernatant was separated and used for mineral analysis. The results are expressed as Mean SEM. The means were compared by t test.

**Results and Discussion:** Mean SEM for sodium, potassium, magnesium and copper in cyclic group (fertile) were 142.0 6.4 (mEq/l), 4.7 1.3 (mEq/l), 24.3 3.9 (mg/l) and 92.5 5.1 respectively whereas the mean values for sodium, potassium, magnesium and copper in acyclic group (infertile) were 144.8 5.3 (mEq/l), 4.1 0.9 (mEq/l), 27.4 4.2 (mg/l) and 64.3 3.9 respectively. The comparison of these means in infertile and fertile groups revealed that sodium, potassium and magnesium were not different (P > 0.05) in both groups whereas copper was significantly higher (P < 0.05) in fertile group when compared with infertile group. The results of this study indicate that the low level of copper in infertile group may be

responsible of acyclic behaviour in buffaloes and consequently affecting reproductive performance in these animals and could suppress the milk and meat production.

## P5

### **Blood progesterone and 17-B estradiol concentration in non-pregnant Caspian miniature mares**

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**Introduction:** The Caspian miniature horse is an ancient breed of horse, which was thought to be distinct for many years. This breed is a charismatic versatile little horse, existing only in small numbers, has now been recognized as holding an ancestral position in the history of the modern horse breeds, in all probability preceding that of the Arab horse. This article is performed for identifying reproductive hormone secretion in the Caspian miniature mares during oestrous cycle.

**Methods:** Growth and regression of follicle and corpus luteum and variation of plasma progesterone and 17-B estradiol were assessed during two successive years. The evaluation was performed during the spring and summer seasons on two completely healthy groups of mares with 12 heads respectively. Follicular waves were determined by use of clinical observation and ultrasonography and were evaluated after statistical analysis along with daily blood samples and hormonal assessment by RIA and usage of gamma counter method. Using 5 MHz liner scanners did sonographic assessment. The time intervals between start up to separation, ovulation and separation were 7 and 8.7±0.68 days respectively.

**Results and Discussion:** The plasma concentration of progesterone was 1 ng/ml from the day 14 of the cycle, and a significant increase to 8 ng/ml was observed 24 hours after ovulation. The plasma concentration of 17-B estradiol had an increase amount from 6 days before ovulation and its maximum amount to 44.5±65.6 was obtained 2 days before ovulation and decreased rapidly afterwards. The results showed the significant increase in plasma progesterone 24 hours after ovulation while in other breeds of horse and ponies between 12-24 hours. The duration of high-level progesterone secretion (more than 8 ng/ml) was 4 days. In other breeds this time is 5-7 days. The significant increase in plasma estradiol was 6 days and the duration of high-level secretion was 2 days. In this study the differences and similarities between this breed and the ponies were determined.

## P6

### **Expression, storage and secretion of LH and associated proteins in adult male and female mice: a cautionary tale with respect to the LHBT2 gonadotroph cell line**

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**Introduction:** The LBT2 gonadotroph cell line derived from a male mouse pituitary tumour is used extensively to study gonadotroph function. Because of differences between male and female mice in LH storage and secretion we examined the role of GnRH using wild type and hypogonadal (hpg: no GnRH) male and female mice.

**Methods:** Study 1. Plasma and pituitaries were collected from adult male (n=20) and female mice (n=25). Plasma and pituitary LH was measured by RIA, pituitary LHb, secretogranin II (SgII) and chromogranin A (CgA) mRNA by Taqman PCR and gonadotroph numbers by ICC for LH. Study 2. The same parameters were measured in male and female hpg mice together with control siblings (n=6 in all groups). Results were analysed using ANOVA and unpaired t-tests.

**Results and Discussion:** There were no differences in gonadotroph numbers between male and female adult wildtype pituitaries, but a 40%(male) and a 30%(female) reduction in hpg mice. Normal male mice had significantly lower plasma LH (1.52 ± 0.09 v 2.43 ± 0.16ng/ml), but significantly higher pituitary LH (7.3 ± 1.4 v 2.3 ± 0.13mg/ml) than females with significantly higher LHb (23.5 ± 0.5 v 4.3 ± 1.7), SgII (x5) and CgA (x7) mRNA levels. In hpg mice plasma LH was significantly lower in males than females (0.31±0.01 v 0.63±0.02) and pituitary LH and LHb, SgII and CgA mRNAs were similar to, or lower than, females suggesting a potential role for GnRH +/- neonatal but not fetal testosterone in masculinizing gonadotroph function. Thus normal male pituitary function differs from females but the

absence of GnRH results in levels equivalent to females. Whether this sexually dimorphic activity occurs in the fetal pituitary when the clonal tumour from which the LbT2 cells were derived was forming, is not known. It is possible that results obtained with LbT2 cells reflect male more than female gonadotroph function.

## P7

### **Growth repression in diethylstilbestrol/dimethylbenz[a]anthracene (DES/DMBA)-induced rat mammary gland tumour using Hecate-CGB conjugate**

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**Introduction:** In this present study, we induced mammary gland tumours in Wistar rats using combined prenatal-exposure to synthetic oestrogen DES and postnatal-exposure to DMBA, and investigated the susceptibility of Hecate-CGB conjugate (a fusion of 23-amino acid lytic peptide Hecate and a 15-amino acid fragment of the B-chain of chorionic gonadotropin, CG), to treat DES/DMBA-induced mammary gland tumours.

**Methods:** Rats with tumours were equally randomised (n=10/group) and treated with either sham (control) or Hecate or Hecate-CGB (12 mg/kg body wt/once/week, for 3 weeks) by tail vein injections and sacrificed one week after the last injection. Ethic approval was obtained from the local Research Ethics Commission.

**Results and Discussion:** RT-nested-PCR/Southern blotting analyses revealed alternatively spliced mRNA for luteinizing hormone receptors (LHR) in tumour tissues of 5 (out of 30) females, further confirmed by Western blot analysis. The percent of tumour volume increase was lowest in the group treated by Hecate-CGB (45.3±27.6), in comparison with Hecate-treated and sham-treated control group (324.8±78.1 and 309.9±51.2, respectively; P<0.001). Hecate-CGB induced a significant decrease in tumour burden compared with control (9.5±2.1 vs 21.6±2.9 mg/g body wt; P<0.01). A smaller reduction of tumour burden was also observed in Hecate-treated females (17.6±1.6 vs 21.6±2.9 mg/g body wt; P<0.05). The mechanism of Hecate-CGB conjugate action in repression of DES/DMBA-induced tumour growth needs to be further analysed, as the regression appeared also in tumour tissues with very low or undetectable levels of LHR expression. Our results proved the principle that targeted ablation of mammary gland tumour by Hecate-CGB conjugate is possible. Lytic peptides are known to disrupt preferentially prokaryotic and cancer cell membranes maintaining large membrane potentials than healthy eukaryotic cells with low membrane potential, thus this known phenomenon could have been the additional mechanism involved in this tumour repression mechanism.

## P8

### **Changes in plasma leptin concentrations over parturition in primiparous and multiparous dairy cows and the effect on subsequent fertility**

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**Introduction:** In dairy cows leptin is thought to play a role in the repartitioning of nutrients following parturition. The post parturient fall in leptin may encourage an increase in dry matter intake whereas sustained low leptin concentrations post partum have been associated with poor energy status.

**Methods:** This trial assessed the effects of dry period metabolic status on subsequent fertility. Blood samples for leptin radioimmunoassay were collected from 168 first calving heifers and 141 multiparous cows precalving and at weeks 2, 5 and 7 post partum. Milk samples were taken twice weekly for progesterone (P4) analysis and P4 profiles were classified as: (1) normal, (2) delayed start (P4 <3 ng/ml for >40 days), (3) ceased to cycle or (4) prolonged luteal activity. Insemination records and P4 profiles were used to determine the interval to conception.

**Results and Discussion:** Cows exhibited significantly lower leptin concentrations than heifers at all four times measured (P< 0.001). In both age groups, the pre-partum sample was significantly higher (P< 0.001) than those taken after calving, but values did not change from

weeks 2-7 post partum. In cows, there were significantly higher leptin concentrations at precalving and week 2 in animals with a delayed resumption of ovarian cyclicity in comparison with normal profile cows. This difference was not observed in heifers. However, heifers taking >150 days to conceive had higher leptin values pre-calving than those conceiving in <150 days ( $P < 0.01$ ). In conclusion, the higher leptin values in heifers may be due to their greater adiposity. Higher leptin concentrations before calving were associated with a delayed return to ovarian cyclicity in cows and a longer calving to conception interval in heifers. Overfeeding in late pregnancy may thus have a negative effect on subsequent fertility, emphasizing the importance of calving animals in an appropriate body condition. Supported by DEFRA and the MDC.

## P9

### **Establishment of an ELISA to assess PAG-concentrations in blood and milk of dairy cows**

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**Introduction:** Availability of a RIA for measuring plasma PAG permits the detection of pregnancy in cows (Zoli et al., 1992). The present investigation addresses the establishment of a PAG-ELISA for blood and milk.

**Methods:** Purified bPAG-1, extracted from bovine placental tissue, and bPAG-1-antibodies raised in rabbits were supplied by J.F. Beckers (Liege, Belgium). bPAG-1 was biotinylated to serve as tracer. Nunc Microtiter plates were coated with sheep-anti-rabbit-IgG and stored dry at  $-20^{\circ}\text{C}$  or with 300  $\mu\text{l}$ /well of a N-azide buffer at  $4^{\circ}\text{C}$ . Plates were coated with 100  $\mu\text{l}$ /well of anti-bPAG-1-IgG and pre-incubated for 0, 10 or 20h. Buffers used for PAG measurement in blood were two standard ELISA buffers (TP-1, TP-2), steroid-free bull serum (TP-3) or serum from non-pregnant cows (TP-4). For measurement in milk, TP-1, TP-2, milk from non-pregnant cows (TP-M1) and fat-free milk (TP-M2) were used. Measurements were conducted by ELISA and RIA (J.F. Beckers) on blood and milk from 12 cows between 180 to 250 d pregnant. PAG-concentrations were calculated by linear regression in the logit-log transformation of the standard values. Recovery rates (addition of 100  $\mu\text{g}/\text{ml}$  PAG) and intra- and inter-assay variance were determined.

**Results and Discussion:** It turned out that the method of storage of coated plates had no effect. The least intra-assay variability was encountered with 20h of pre-incubation (vs. 0 or 10h). The most suitable buffer system was a combination of TP-1 and TP-3 for blood and TP-1 and TP-M2 for milk. Intra- and inter-assay variability was 10 and 14% for blood and 7 and 9% for milk. Recoveries were 94 to 107% for blood and 96 to 108% for milk. The correlation between RIA and ELISA measurements in blood was  $r=0.96$  ( $P < 0.01$ ). It may be concluded that the PAG-ELISA is a suitable alternative for measuring plasma PAG and may also be applied to milk.

## P10

### **Measurement of progesterone concentration in plasma samples: Comparison of three radioimmunoassay procedures**

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**Introduction:** Plasma progesterone (P4) measurement is an established method of early pregnancy detection in many animal species. Radioimmunoassay (RIA) is a simple and rapid procedure to measure concentration of hormones in various biological material as blood and milk. In the plasma, P4 is bound to binding proteins, like transcortin, which could interfere with the RIA. To displace P4 from these binding proteins, we propose to compare the conventional extraction method with two direct assays including the use of a displacing agent, the ANS (8-Anilinoaphtalene-1-sulfonic acid Ammonium Salt)(Fluka).

**Methods:** For a specific extraction procedure of progesterone from blood or milk sample (200 $\mu\text{l}$ ), petroleum ether (BDH)(60-80 degreesC) was used as specific organic solvent. After evaporation of the organic phase containing P4 and solubilization in a phosphate gelatin buffer, an incubation step was performed during 4 hours at 4 degreesC in presence of tracer and antiserum. In each case, the derivative 11-a-hemisuccinate-P4 was used either to produce specific antibodies after coupling with BSA and immunization of rabbits or to prepare the tracer with high specific activity after coupling with 2-iodo125-histamine and HPLC

purification. In the direct assays, 300µg of ANS per 50µl of sample were added and incubated in the same conditions of time and temperature. We compared a direct assay performed in liquid phase and conclude by a classical second- antibody method to another in solid-phase including the preparation of coated tubes by an indirect method. For this last one, a purified preparation of goat anti rabbit -IgG serum is first coated on the wall of the tube before to bind the first anti-P4 serum in order to present the antigen binding sites of the molecule.

**Results and Discussion:** The three methods are sensitive (0,2nanogramms/ml). and well correlated. In a cow followed by daily blood samples, the P4 profiles were identical for the concentrations ranging from 0.2 to 10 nanogramms/ml. All the three methods present excellent agreement between ovarian function and oestrus detection. They can serve as an indicator of early foetal death and a control of insemination success. Practically, the solid phase RIA was more rapid and easier to realize a routine laboratory.

## P11

### **Identification of volatile compounds in buffaloes (*Bubalus bubalis*) with special reference to oestrous cycle**

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**Introduction:** Chemical cues (pheromone) reportedly play a major role in mammalian reproduction and behaviour and are found to be significant in the induction of ovulation, as well as identifying the receptivity of female. The sources of chemical signals are urine, faeces, vaginal mucus and specialized scent glands. Urine is known to contain a large array of compounds that may confound the isolation of pheromones. The present investigation was carried out to examine the buffalo urinary profiles across oestrous cycle so as to clarify the urine contains chemical information that is potentially useful in communication.

**Methods:** The urine samples were collected in all the three stages proestrus, estrus and postestrus at the District Livestock Farm, Orathanadu, India. The solvent dichloromethane was used to extract the compounds from urine and the same was filtered through a silica-gel column (60-120 mesh) for fractionation and chemical identification by gas chromatography-mass spectrometry (GC-MS; QP 5000, Shimadzu, Japan).

**Results and Discussion:** Nearly 16 compounds were identified in all the three stages which include alkanes, alkenes, ketones, alcohol, aldehyde, and acid. The proestrus urine contained i) 4, methyl-1-3 cyclohexene- 1 ol, ii) 2,4- dimethyl-2-decene, iii) 1- iodo decane and 2-methyl-2- undecanethiol; estrus urine contained i) 9-octa decenoic acid, ii) acetic acid, iii) 4-methyl phenol iv)1-chloro octane, v) 2-octanone, vi) 1-iodo undecane, vii) 9-octa decenal; postestrus urine had i) 2,6-bis phenol, ii) 9-octadecenal, iii)9-decene-1-ol iv) 1-chloro octane, v)1-iodo decane. The results show that the alkane compounds appear to be present in all the three stages of estrous cycle, for instance, 1- iodo decane identified in pre estrus and post estrus stages whereas 1- chloro octane identified in estrus and post estrus. The compounds, 9-octa decenoic acid and 4-methyl phenol are repeatedly identified only in the estrus urine sample. The present study concludes that the excretion of volatile compounds in the urine of female buffaloes differs from one phase to other, and findings provide additional support to the possibility of identifying the estrus phase of buffalo by detecting urinary pheromonal compounds.

## P12

### **Expression of *Nrip1* during development of the male reproductive tract of mice**

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**Introduction:** The transcriptional activity of nuclear receptors is mediated by cofactor proteins (coactivators or corepressors) that are implicated in chromatin remodelling and the recruitment of the transcription machinery. Adult male mice devoid of *Nrip1* gene (encoding a nuclear receptor corepressor) have a high percentage of 'hairpin-looped' epididymal sperm. In the adult mouse, *Nrip1* is present in Leydig cells, some seminiferous tubules, the efferent ducts and parts of the epididymis suggesting that *Nrip1* plays a role in both endocrine and spermatogenic functions in the adult male. The present study focussed on determining the pattern of *Nrip11* expression during early development in the testis and male tract.

**Methods:** *Nrip1* expression can be identified in *Nrip1* -- mice in which *Nrip1* has been replaced by a lacZ-neo fusion gene expressing beta-galactosidase. Wildtype, heterozygote and knockout male mice were killed at 2-day intervals from the day of birth to postnatal day 20 and the testes and reproductive tract were stained using X gal.

**Results and Discussion:** There was no endogenous beta-galactosidase activity in the testis or efferent ducts of wildtype animals at any age, but became evident in the vas deferens and cauda epididymis by day 12 and in the corpus epididymis by day 14. Endogenous activity was present in the initial segment of the caput epididymis by day 16 and in the rest of the epididymis by day 20. In heterozygote and *Nrip1* -- mice, beta-galactosidase activity (reflecting *Nrip1* expression) was evident from day 0 and persisted until beyond Day 20 in the interstitial tissue and was present throughout the seminiferous tubules from day 12. There was also strong expression throughout development in the efferent ducts and along the whole length of the epididymis. These results are consistent with a regulatory role of *Nrip1* in nuclear receptor mediated responses of the male reproductive tract development.

### P13

#### **RNAi evidence that GDF9 mediates oocyte regulation of cumulus cell expansion in mice**

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**Introduction:** In mice, growth differentiation factor-9 (GDF9) is an oocyte-specific secreted protein that plays an essential role in early follicular development. However, the role of GDF9 at later stages of follicle development is uncertain. In this study, RNAi was used to knockdown GDF9 levels in oocytes in order to investigate the possible role of this protein in mediating oocyte regulation of cumulus expansion.

**Methods:** Fully-grown oocytes from mice maintained in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 were injected with either GDF9 dsRNA; BMP15 (a closely related gene also expressed by oocytes) dsRNA or injection buffer alone and cultured for 24 h. To determine the efficacy of the RNAi procedure, oocytes were then used for measurement of GDF9 and BMP15 mRNA levels using real-time RT-PCR, and for measurement of GDF9 protein levels using Western blotting and immunofluorescence. To investigate the role of GDF9 in cumulus expansion, 24 h after injection oocytes in the three treatment groups were cocultured with oocyctectomised cumulus cell complexes in the presence of 0.5 IU / ml rFSH for a further 24 h.

**Results and Discussion:** GDF9 dsRNA but not BMP15 dsRNA or injection buffer knocked down GDF9 mRNA levels in oocytes within 24 h of injection. Similarly, GDF9 protein levels were lower in the GDF9 dsRNA-injected oocytes. During a further 24 h culture period, oocytes injected with either BMP15 dsRNA or injection buffer alone, but not oocytes injected with GDF9 dsRNA, enabled FSH-stimulated cumulus cell expansion. This study strongly supports the idea that GDF9, but not BMP15, is a key mediator of oocyte-enabled cumulus cell expansion in mice. (This work was supported by project grants from the Royal Society and the BBSRC)

### P14

#### **Evolution of the putative sperm adhesion region of zona pellucida C glycoprotein in murid rodents: Is there evidence for species-selectivity?**

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**Introduction:** The mammalian egg coat, the zona pellucida (ZP), is an extracellular matrix of 3 to 5 glycoproteins. Studies on the laboratory mouse suggest that ZPC O-linked oligosaccharides attached to the serine/threonine residues of exon 7 provide adhesive ligands for sperm receptors. Furthermore, exon 7 amino acid residues may be under positive Darwinian selection. Changes to the amino acid sequence within this region may alter glycosylation, and hence sugars available for sperm adhesion. If this adhesion is species-selective then the amino acid sequence of closely related species might differ. To test this we determined the amino acid sequence of the putative sperm adhesion region of ZPC from more than 40 species of Australian hydromyine rodents.

**Methods:** DNA was extracted using either the salt extraction method or PureGene DNA Isolation Kit (Gentra, Minneapolis, MN). Primers, designed from *Notomys alexis* cDNA, were used to PCR amplify, and sequence, the ZPC region of exon 6 through to exon 7, the sequences visually aligned, then compared.

**Results and Discussion:** Within the sperm adhesion region of exon 7 (Cys-328 to Gln-343), more than three-quarters of species show 100% amino acid identity. Unlike *Mus* and *Rattus*, all have an additional serine residue, at position 336 and all, except two *Melomys* species, have a second serine residue at position 341. Five species of pebble-mound mice have a serine to proline substitution at position 334 and two other species of *Pseudomys* have a serine to leucine substitution at position 331. This study shows that, in the Australian hydromyine rodent radiation, there was full conservation of the putative sperm adhesion region between closely-related species; it argues against this region, in at least this group of rodents, being under positive Darwinian selection.

## P15

### **Centrosome functions in first cell cycle organisation of horse oocytes following parthenogenesis and nuclear transfer**

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**Introduction:** In animal cells, the microtubules of the cytoskeleton typically associate with the centrosome to assemble the complex microtubule-organizing centre (MTOC). Some animal cells lack centrosomes entirely yet they are still capable of forming complex microtubular structures such as the mitotic spindle. In this study, we monitored the functions of the centrosome on first cell cycle organisation in the horse oocytes following parthenogenesis and nuclear transfer.

**Methods:** MII oocytes generated by in vitro culture system were subjected to parthenogenetic stimulation or nuclear transfer. The oocytes were cultured for 12-14 h after each treatment and then fixed in a glycerol-based microtubule-stabilising solution followed by 2.5% paraformaldehyde in PBS (Simerly and Schatten, 1993). First cell cycle organisation was detected by indirect immuno-fluorescent staining, utilising a mouse anti-alpha-tubulin antibody which stained the microtubules green and a rabbit anti-gamma-tubulin antibody which stained the centrosome red. The stained oocytes were mounted under a coverslip in anti-fade mounting medium containing TOT3 (blue chromatin), and examined by confocal microscopy.

**Results and Discussion:** In parthenogenetically activated oocytes centrosomes were not found at opposite poles of the microtubular spindles at the MII stage, or after their further development following chromosomes separation. However, red staining gamma-tubulin did remain associated with the microtubules. On the other hand, 2-4 red-stained centrosome-like structures were observed in oocytes reconstructed by nuclear transfer, and these appeared to be associated with the introduced donor chromatin. This finding suggests that centrosome structures are not an essential component in the formation of the metaphase spindle in horse oocytes, but they are introduced from the donor cells into the reconstructed oocytes following nuclear transfer.

## P16

### **Cytoplasmic polyadenylation during *in vitro* maturation of bovine oocytes**

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**Introduction:** Cytoplasmic polyadenylation of mRNAs plays a key role during oocyte cytoplasmic maturation and for the subsequent control of early embryonic development. We have studied the polyadenylation status of developmentally important genes during in vitro maturation of bovine oocytes.

**Methods:** Cumulus-oocyte complexes (COCs) were aspirated from follicles ranging from 3 to 8mm in diameter. They were then matured 24 hours under 20% O<sub>2</sub> in TCM-199 supplemented with 10 ng/ml EGF and 0.4mM pyruvate, without serum. COCs were denuded, and pools of 20 oocytes and of cumulus granulosa cells were made before and at different stages of maturation, and stored at - 80°C until use. Changes in the poly(A) tail length were

studied using RACE-PAT, a PCR-based technique, where the length of the smear obtained after PCR gives an estimate of the length of the poly(A) tail. Transcripts analysed were cyclin A and B. Cordycepin (a strong inhibitor of polyadenylation) was also used during maturation. Another purine, 3-deoxyguanosine (3-dG) was used as a control.

**Results and Discussion:** A longer poly(A) tail was found after maturation for the Cyclin A and B transcripts (suggesting a probable translational activation). Kinetics studies performed during maturation showed clearly that the lengthening of the poly(A) tail for cyclin B takes place between 6 and 12h from the onset of the maturation process. Cyclin A seems to follow the same time pattern, but the increase in length is not as important as in cyclin B. When COCs were matured during 24h in the presence of cordycepin polyadenylation was completely abolished for the studied transcripts. If the inhibitor was added at 12h from the onset of maturation, polyadenylation was no longer prevented, showing that cytoplasmic polyadenylation of cyclin A and B effectively occurs in the first 12h from the onset of *in vitro* maturation in bovine oocytes.

## P17

### **Low developmental competence of bovine oocytes after *in vitro* maturation is unrelated to apoptosis**

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**Introduction:** Only a small proportion of oocytes recovered from abattoir ovaries may develop to the transferable stage, which could be partially due to the use of oocytes of inferior quality. Aim of this study was to clarify whether apoptosis takes place in bovine oocytes, and if so, whether it is correlated to oocyte developmental competence.

**Methods:** Immature cumulus-oocyte complexes (COC) were grouped into 3 categories: G 1, oocytes coated by many layers of compact cumulus cells (n = 133); G 2, oocytes with only 1 - 5 layers of cumulus cells (n = 102); and G 3, oocytes with expanded cumulus cells (n = 108). They were matured, fertilized and cultured in SOFaa medium up to 168 hpi *in vitro*. Cleavage rate and blastocyst formation were recorded at 24 and 168 hours post insemination (hpi) respectively. The experiments were repeated three times. To investigate apoptosis, fresh, matured and fertilized oocytes of various groups were randomly picked up after puncturing, 24 hours post-maturation and 24 hpi for tests. Oocyte morphology was evaluated by inverted microscopy, DNA fragmentation and caspase activity were investigated by using TUNEL assay and a carboxyfluorescein labelled caspase inhibitor FAM-YVAD-FMK.

**Results and Discussion:** Morphological apoptotic features (such as membrane budding and cell fragmentation), DNA fragmentation and caspase activity were not observed in any oocytes examined at the three points of time. There was no difference in cleavage rate between groups whereas the blastocyst formation differed significantly (G 1(28.6%) vs G 2(9.8%) vs G 3(5.6%)) (p = 0.01) (Chi-square analysis, SPSS for windows). Our results implies that inferior developmental competence of oocytes is not caused by DNA fragmentation, caspase activity or cell fragmentation and membrane budding of the oocyte itself, while the amount and morphology of the surrounding cumulus cells is a strong predictor of oocyte developmental competence.

## P18

### **The cellular origin of basal lamina proteins in the bovine ovarian follicle**

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**Introduction:** The mechanism by which the follicular basal lamina is remodelled during growth is unknown. Its elucidation requires knowledge of the cellular origin of basal lamina proteins. Available Northern hybridisation and immunocytochemical data on collagen IV and laminin provide only circumstantial evidence of their sites of production. Here we have used *in situ* hybridisation to identify specific groups of cells in the granulosa and theca layers where these proteins are expressed.

**Methods:** Bovine ovarian tissue was obtained from the abattoir, fixed in 4% paraformaldehyde, embedded in wax, and sectioned to 5 microns. Digoxigenin-labelled antisense riboprobes (with sense controls) to collagen IVa2 and laminin a1 chains were constructed from published cDNA sequences and used to detect mRNA by *in situ* hybridisation. Hybridisation conditions for both probes were optimised at 37°C and 40% formamide, with washing at 47°C and 40% formamide. Stringency was precisely controlled by varying the concentration of SSC. Alkaline phosphatase-conjugated anti-DIG provided a localised blue precipitate, on Fast Green FCF-counterstained sections. Only healthy antral follicles were examined.

**Results and Discussion:** mRNAs for collagen IVa2 and laminin a1 were each expressed in the theca interna and granulosa layers. For each probe, assessment at tissue and cell levels showed similar intensities of expression in each layer. Signal in sense controls was negligible. Expression in the theca was widely distributed, including adjacent to the basal lamina, and was not confined to endothelium. Expression in the granulosa was uniform from mural to antral layers. This study demonstrates, for the first time, that theca and granulosa cells both express proteins found in the basal lamina. Future models of tissue remodelling during follicle growth should take account of protein subunits contributed from both sides of the basal lamina. Supported by BBSRC

## P19

### **The effect of oxytocin on the porcine ovarian follicle functions in vitro: the role of protein kinase A and ERK-related MAP-kinase**

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**Introduction:** The aim of our *in vitro* experiments was to study the role of oxytocin (OT), cAMP/protein kinase A (PKA) and mitogen-activated protein kinase (ERKs MAP-kinase) in the control of ovarian cell functions as well as the role of PKA and MAPK on mediating OT effects on these processes.

**Methods:** The whole porcine ovarian follicles were cultured in the presence or absence of OT (1, 10, 100 ng/ml), PKA inhibitor Rp-cAMPS (10 nM), MAP-kinase inhibitor PD98059 (1 µg/ml) or their combination. The release of prostaglandins F (PGF) and E (PGE) were determined by RIA. PKA (α-Cat subunit), the proliferation-associated peptide PCNA and ERK-1,2 content in cell lysates were analyzed by Western-blotting.

**Results and Discussion:** OT stimulated the release of PGF and PGE, accumulation of PKA, ERK-1/2 and PCNA in cell lysate. A PD98059 decreased the basal PGF and PGE output, as well as reduced both ERK-1 and ERK-2 accumulation in cell lysates. Rp-cAMPS decreased PKA accumulation in cell lysates. Both PD98059 and Rp-cAMPS prevented the OT-induced stimulation of PKA, ERK-1, ERK-2, PGF and PGE, whilst OT-stimulated PCNA accumulation was only slightly modified by these blockers. These observations suggest that OT, PKA and ERKs MAPK can be involved in the control of PGs release and proliferation of ovarian cells. Effects of OT on PGs release can be mediated by both PKA and MAPK, whilst their role in mediating the proliferative effects of OT seems to be minor, assuming the involvement of other intracellular messengers.

## P20

### **Effects of body condition at calving, postpartum nutrition and suckling restriction from day 45 postpartum on follicular dynamics in Brown Swiss cows**

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**Introduction:** The interval from calving to first ovulation is mainly affected by nutrition and suckling. Previous studies have reported that complete restoration of pituitary LH occurs by 5-6 weeks after calving (Nett, 1987) and that restricted suckling effect on LH pulsatile release and postpartum interval (PPI) appears to be exerted in a short term. The aim of this study was to determine the effect of suckling restriction from day 45 postpartum on follicular dynamics and PPI of suckler cows fed to different nutrition levels.

**Methods:** The experiment was a 2x2x2 factorial design, in which the factors were body condition at calving (BC) (2 (Low, n=18) vs 3 (High, n=24), in a scale of 5), postpartum nutrition (75 (Low) vs 175 (High) MJ ME/d) and suckling frequency (once-a-day (from day 45 postpartum to weaning) vs ad libitum suckling). Follicular development was monitored daily by transrectal ultrasonography from calving to detection of second ovulation.

**Results and Discussion:** Each follicular wave was characterised by the simultaneous emergence of 6.5 follicles, with 4.7 mm in diameter, independently of BC, postpartum nutrition and number of follicular wave. Maximum diameter and growth rate of dominant follicles were smaller in the low BC and nutrition levels. There were gradual increases in maximum diameter and growth rate of dominant follicles related to the proximity to ovulation ( $P < 0.001$ ). PPI was markedly affected by the interaction between BC and postpartum nutrition (194.4, 69.4, 33.7 and 27.4 days, in LL, LH, HL and HH, respectively,  $P < 0.001$ ), being the intensity of postpartum nutrition effect modulated by BC. In the current conditions of nutrition, suckling frequency did not affect follicular dynamics. It is concluded that in extreme BC and postpartum nutrition levels, calf restriction from day 45 postpartum was a useless practice to induce ovulation. (Study supported by INIA94-072 (Spain MST) and EC-AIR3-CT94-1124).

## P21

### The effects of IVF aspiration on the temperature, dissolved oxygen levels and pH of follicular fluid

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**Introduction:** A considerable amount of research has been carried out which seeks to quantify the relationship between various physiological parameters in follicular fluid and oocyte developmental capacity. The work reported here investigated the effect of aspiration through a standard IVF follicle aspiration kit on the temperature, dissolved oxygen levels, and pH of follicular fluid.

**Methods:** The temperature profile of follicular fluid was monitored using thermocouples placed at various positions in an aspiration kit. Dissolved oxygen was measured before and after aspiration using a miniature Clark style electrode. pH was determined before and after aspiration using a needle glass membrane electrode.

**Results and Discussion:** The results indicated that the temperature of follicular fluid drops an average of 8 degrees C upon aspiration. Dissolved oxygen levels rose by as much as 8 vol%. The fluid pH was unchanged over the course of aspiration. The cause of the temperature and dissolved oxygen changes was investigated. The temperature change was attributed mainly to evaporation of fluid in the bulk collection tube. The major source of oxygen contamination was air in the bulk collection tube. This result implies that determination of oxygen in follicular fluid should be carried out on samples taken before the fluid has reached the collection tube. This work also highlights the importance of giving pre-analytical sample collection due consideration when attempting to describe the relationship between physiological fluid parameters and oocyte quality.

## P22

### The effect of ascorbic acid on ovine preantral follicle development *in vitro*

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**Introduction:** Basement membrane remodelling occurs during early follicle growth at the time of antral cavity formation. However, the mechanism of this process has yet to be established. This study used preantral follicle culture technology to address this question.

**Methods:** Two methods were used to harvest preantral follicles of 170-230um diameter from abattoir derived sheep ovaries. Theca-free follicles (TFFs, n=72) were harvested from minced ovarian cortex by needle dissection following a 1 hr digestion in collagenase 1A and DNase. In contrast, theca-intact follicles (TIFs, n= 92) were isolated from thin strips of ovarian cortex using needles. Individual follicles were cultured at 37°C in 5% CO<sub>2</sub> for 30 days in 200 ul of serum-free media with (+) or without (-) 50ug/ml of the basement membrane component L-Ascorbic Acid (AA). Culture media was replenished on alternate days. Follicle morphology, diameter and antrum formation were measured twice weekly. After 30 days follicles were

fixed for either transmission electron microscopy or immunohistochemical analysis of Proliferating Cell Nuclear Antigen (PCNA).

**Results and Discussion:** The data show that TFFs grew faster ( $P < 0.05$ ) than TIFs. Antral cavities formed in 45.5% of TFFs. In contrast 23.3% of TIFs cultured in AA(-) media formed antral cavities by extrusion of the granulosa-oocyte complex through the basement membrane. This phenomenon was reduced ( $P < 0.05$ ) for TIFs grown in AA(+) media, where 52.6% formed antral cavities and retained an intact theca. Terminal follicle diameter was similar within follicle type (TFF AA(+): 547 +/- 41  $\mu$ m; TFF AA(-): 602 +/- 61  $\mu$ m) but was significantly different ( $P < 0.05$ ) between the TFF and TIF follicles (TIF AA(+): 428 +/- 25  $\mu$ m; TIF AA(-): 441 +/- 29  $\mu$ m). After 30 days the majority of the growing follicles were PCNA positive. These data indicate that inclusion of AA in culture medium facilitated basement membrane modelling and antral growth in theca intact preantral follicles *in vitro*.

## P23

### **BMPs -2, -6 and -15 interact with oocytes to affect porcine theca cell function *in vitro***

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**Introduction:** Bone morphogenetic proteins are intra-ovarian peptides involved in regulating ovarian follicle growth and development. BMPs-2, -6 and -15 have been shown to modulate theca cell growth and function *in vitro*. The aim of this study was to ascertain if BMPs -2, -6 and -15, in conjunction with oocytes, are capable of affecting porcine theca cell behaviour when cultured under serum-free conditions.

**Methods:** Theca cells and oocytes were isolated from healthy 2-6mm follicles of mature porcine ovaries. Theca cells were cultured under serum-free conditions in medium supplemented with: 10ng/ml insulin, 100ng/ml LR3 IGF-1, 0.01ng/ml LH, either 30ng/ml recombinant human (rh)BMP-2, 30ng/ml rhBMP-6 or 100ng/ml rhBMP-15 and either 0, 1 or 5 oocytes per well of a 96-well plate. Medium was replaced every 48h and cells were cultured for 144h, after which viable cell number was determined.

**Results and Discussion:** Oocytes stimulated oestradiol synthesis in the presence of BMP-2 ( $p = 0.001$ ) and BMP-6 ( $p = 0.003$ ) despite an overall inhibitory effect of BMPs-2 and -6 on oestradiol production ( $p < 0.001$ ). Oocytes were also observed to increase BMP-15-stimulated oestradiol production ( $p < 0.022$ ). Oocytes stimulated progesterone synthesis in the presence of BMP-6 ( $p = 0.003$ ) despite an overall inhibitory effect of BMP-6 on progesterone production ( $p < 0.016$ ). However, no interactions between oocytes and either BMP-2 or BMP-15 were observed with regard to progesterone production. There was a significant interaction between BMP-2 and a single oocyte in terms of increased viable cell number ( $p = 0.034$ ). The results of this study provide evidence of communication between oocytes and theca cells. This communication is altered in response to treatment with BMPs-2, -6 and -15, in that oocytes act to stimulate BMP-modulated steroidogenesis. This study provides further support for BMPs having specific roles within the porcine ovary. *Funded by BBSRC*

## P24

### **Evidence that fetal programming during pregnancy affects postnatal development of Holstein/Friesian calves**

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**Introduction:** Dairy cows normally calve for the first time at about 2 years, when not yet fully grown. Subsequent pregnancies occur during lactation. Therefore in primiparous (PP) cows the fetus competes for nutrients with the mother's demand for her own growth while in multiparous (MP) cows they compete with milk production. Human epidemiological studies have shown that low birth weight can predispose offspring to a variety of metabolic diseases in adult life. This study investigated the hypothesis that competition with maternal growth or lactation limits nutrient availability to the fetus thus compromising calf development.

**Methods:** 123 female Holstein-Friesian calves were monitored from birth to conception. All animals were measured at birth, 3, 6, 9 and 15 months and 55 were selected for metabolic

profiling at 6 months of age and just before first service. Subsequent insemination and fertility data were collected.

**Results and Discussion:** PP cows produced smaller calves than MP cows ( $P < 0.05$ ) but these offspring demonstrated catch-up growth by 6 months of age. This was associated with significantly higher concentrations of IGF-I ( $P < 0.009$ ). However smaller, low birth weight offspring of MP cows remained smaller than their high birth weight peers. Within the MP cows there was a significant trend towards the low birth weight calves coming from older dams (3 or more lactations) with higher peak milk yields ( $> 42$  kg/day). Calves of PP dams required slightly fewer services to conceive successfully than MP offspring, and conceived at a faster rate. High and low birth weight offspring of MP cows conceived at similar rates. The evidence supports the hypothesis that the size of the calf at birth is influenced by the age and yield of the dam and this in turn influences the metabolic characteristics of the growing calf. (Sponsored by DEFRA).

## P25

### **Towards in utero gene therapy: adenovirally-mediated transduction of first trimester human placental fibroblasts with sense or antisense IGFs influences growth, migration and survival**

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**Introduction:** Fetal growth disorders lead to neonatal morbidity and mortality and an increased risk of developing cardiovascular disease and diabetes in later life. The insulin-like growth factors (IGFs) are key regulators of growth during pregnancy and their levels are reduced in intrauterine growth restriction (IUGR). Recent in vivo studies suggest IGFs control fetal growth in part through their effects on placental development. Overexpression of IGFs in placental cells offers a possible approach to improving growth in utero without genetic modification of the fetus.

**Methods:** A series of recombinant adenoviruses was generated containing IGF-I or IGF-II cDNA inserted bicistronically with sequence encoding the green fluorescent protein (GFP). Sense and antisense constructs were created for each gene. Placental fibroblasts (PF; vimentin-positive, cytokeratin 17-negative) were isolated from explants of first trimester placenta and could be passaged and frozen for storage. Transduced cells were characterised by GFP fluorescence, western blotting, 3H-thymidine uptake, survival and migration. Transwell coculture assays were used to examine the effects of IGFs produced by the virally transduced cells on either PFs or PL-4 trophoblasts as target.

**Results and Discussion:** Expression of GFP and IGF-I or IGF-II could be detected 72h post-infection by immunocytochemistry, and Western blotting demonstrated the presence of a 7kDa protein in the medium. Thymidine incorporation by PF increased 7-fold ( $p < 0.03$ ) after infection with an adenovirus containing the IGF-I gene and 4-fold ( $p < 0.05$ ) with the IGF-II gene. Incorporation was attenuated relative to controls after cells were transduced with either IGF gene in the antisense orientation ( $p < 0.05$ ). PF apoptosis increased 7-fold when cells were switched to serum-free medium; this effect was fully abrogated by transduction with either IGF-I or IGF-II. 2.5-fold (IGF-I) and 1.8-fold (IGF-II) increases in apoptosis relative to the serum-free control were observed in cells transduced with antisense virus. The results suggest that increased IGF production could influence the mesenchymal compartment of the placenta as well as adjacent trophoblast and vascular cells during development, resulting in effects on placental growth and function. Antisense inhibition of the survival effect of endogenous gene product in placental cells suggests a role for both IGFs in normal placental development. Thus a small placenta may arise because of increased apoptosis or the loss of proliferative or migratory capacity in either trophoblast or mesenchymal compartments. The ability of PF-derived IGF to stimulate responses in paracrine assays also suggests the possibility of placental-to-fetal delivery of transgenes.

## P26

### **Radioimmunoassay of bovine placental lactogen (bPL) using an antiserum raised in guinea pigs: measurement in foetal plasma**

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**Introduction:** As the bovine placental lactogen (bPL) was suspected as a main regulator in foetal growth, six foetuses were catheterised *in utero* and blood samples were removed for bPL, IGF-I and IGF-II measurements after anti-bPL purified immunoglobulins (IgG) injections. The aim of this study was to develop a new radioimmunoassay system using an antiserum raised in guinea pig in order to measure the circulating bPL concentrations in presence of bPL antibodies originated from rabbits.

**Methods:** Plasma samples (50ul, in duplicate) and appropriately diluted working standards (0.8 to 100 ng/ml) were incubated overnight at room temperature after the addition of primary antibody (100 ul guinea pig anti-bPL, diluted to 1:256,000). Recombinant bPL (rbPL, Lot#AFP9152C; NHPP, NIDDK & Dr. Parlow, USA), was radiolabelled by lactoperoxidase method. The following day, 100 ul of [125I]-rbPL was added to all the tubes and a further incubation at room temperature (4 h) was realised. Free and bound [125I]-rbPL were separated after the addition of second antibody (rabbit anti-guinea pig IgG antiserum), incubation (1 h) and centrifugation (1,500 xg). The supernatant was discarded and the pellet was washed and counted in a gamma counter.

**Results and Discussion:** The standard dilution curves for both the rabbit (Beckers, 1982) and guinea pig systems were very similar. The two systems were used to measure bPL in 8 different foetal sera giving 5.3, 12.2, 9.1, 9.5, 6.7, 4.5, 15.3 and 0.6 ng/ml in the rabbit RIA system and 5.4, 10.9, 7.3, 9.7, 6.3, 4.9, 14.7, 1.0 in the guinea pig RIA system. In conclusion our guinea pig RIA is useful to determine bPL concentrations in foetal sera. It remains to be established how the interference of the administration of IgG anti-bPL of rabbit origin can be discarded in the assay. (This study was supported by grants from the Belgian Ministry of Agriculture and Ministry of the Wallonne Region-DGA).

## P27

### **Investigation on prenatal endocrinology: preliminary results on long term catheterisation of bovine foetuses**

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**Introduction:** Foetal catheterisation is a efficient tool allowing to access the foetal peripheral circulation. The aim of this study was to develop a surgical technique in order to realise a long term foetal blood sampling in bovine species.

**Methods:** Eleven Holstein pregnant cows were used for this study (ULg Ethics Committee number 216). Gestational ages on the day of surgery varied from 6 till 8 months. Before surgery diet was observed. Antibiotics and selenium/vitamin E were given. The catheterisation of foetuses was based on the technique described by Taverne et al. (1988) with some modifications. After a 2-day flush of the catheter with a heparin solution (200-250 IU/ml in 0.9% saline), foetal blood samples were taken 1-2 times per day.

**Results and Discussion:** The duration of surgery varied from 2 to 8 h (surgery including a maternal catheter), with a mean of 4 h 30 min for the first 5 cows (C1 to C5, Group 1) and 2 h 30 min for the last six cows (C6 to C11, Group 2). In cows from Group 1, some problems

were found within 7 days after surgery: peritonitis followed by abortion, myopathy, premature birth, foetal septicaemia and intra-catheter coagulation (for C1 to C5, respectively). In this group, foetal catheters were not functional for more than 3 consecutive days. In the Group 2, five cows calved at 259-277 days of pregnancy (27 to 95 days after surgery). The last one, which was surgered at about the 6th month of pregnancy, calved at term. Foetal blood could be taken during a period from 10 to 92 days, with a mean of 20 days. In conclusion, our technique makes available foetus blood for long term endocrine and physiological investigations before birth. (This study was supported by grants from the Belgian Ministry of Agriculture and Ministry of the Wallonne Region-DGA).

## P28

### **Cryopreservation of chimpanzee spermatozoa**

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**Introduction:** The chimpanzee is one of the endangered animals in the world. Gamete cryopreservation is a useful tool for maintaining their genetic resources. Spermatozoa, especially ejaculated spermatozoa, can be collected easily from individuals compared to oocytes. We cryopreserved ejaculated spermatozoa collected by non-surgical technique and assessed their fertilization ability after thawing.

**Methods:** Semen was collected by artificial vagina or masturbation from 12 males maintained at Kumamoto Primate Research Park, Sanwa Kagaku Kenkyusho Co., Ltd. Collected semen was washed in HTF medium. After centrifugation, spermatozoa were frozen using the method described by Gabriel et al. (2000). Frozen spermatozoa were kept in liquid nitrogen. After thawing at 37°C for 10 min, spermatozoa were washed in HTF medium, and then their concentration and motility was measured by the C-IMAGING SYSTEM (Compix, Inc.). Spermatozoa were also fertilized with hamster zona-free oocytes *in vitro*. Oocytes were fixed and stained, and the spermatozoa in the oocytes were assessed at 24h after insemination.

**Results and Discussion:** Although sperm concentration was different in each male, motile spermatozoa were observed in all males (37.5-73.0%). Spermatozoa were fertilized with hamster zona-free oocytes in all males and a high fertilization rate was obtained (68.8-100%). In this study, chimpanzee ejaculated spermatozoa could be frozen and stored while retaining their fertilization ability.

## P29

### **Maximum yield of spermatozoa at 30 minutes in conventional and Micro-Swim Up**

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**Introduction:** The Swim Up method is widely applied during in human and bovine *in vitro* fertilization. The aim of the present study was to develop a simple and inexpensive method to observe the concentration changes over time during Swim Up and to define the time point of maximum concentration of spermatozoa in the upper layer.

**Methods:** Frozen semen from six Holstein Friesian bulls were used. The Swim Up was carried out according to Parrish et al. (1986), with slight modifications: the top 750  $\mu$ l of media was harvested and centrifuged at 300 g for 10 min. In case of Micro Swim Up 25  $\mu$ l sperm was layered under 147  $\mu$ l Sperm-TALP, the top 110  $\mu$ l of the media was removed, and centrifuged for 10 minutes at 300 g. The concentration of spermatozoa in the pellets was measured by counting in Makler chamber. We compared sperm concentrations by the two methods at 5, 15, 30, 45 and 60 minutes. The experiment was repeated three times. Paired t-tests and one-way ANOVA were done by Microsoft Excel.

**Results and Discussion:** The concentration of spermatozoa at 30 minutes was significantly higher than in other time points in both methods ( $p < 0.05$ ). Significantly higher concentration was observed in Micro Swim Up in the case of every bull in every time point ( $p < 0.05$ ). We

found significant individual differences between bulls ( $p < 0.05$ ), but not between replicates ( $p > 0.05$ ). In conclusion, the maximum yield of both Swim Up procedures was at 30 minutes in spite of the experiences of the conventionally used 1 hr-long incubation. The Micro-Swim Up seems to be a reliable and economical tool to test the kinetic changes of sperm concentration. Supported by Ministry of Agriculture and Region Development 56805/2001, National Fund of Research and Development. 4/031/2001.

### P30

#### **Evaluation of prototype protein-free extenders and cryo-extendors for bovine spermatozoa**

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**Introduction:** Traditionally sperm cryo extendors have contained egg yolk and glycerol as cryoprotectants. However, extendors with egg yolk are difficult to standardize and might contain contaminants, while glycerol can be toxic to sperm. Therefore an extender without protein or glycerol (PGFE) and a cryo extender without protein (CWP) have been developed for bovine sperm (Nidacon, Sweden). However, since semen extendors with egg yolk are widely used, a further cryoextender was developed for the use with egg yolk (CEY). The study compares two commercially available extendors (Triladyl and Andromed, Minitube, Germany) with Nidacon's prototype extendors.

**Methods:** Semen was obtained from 9 bulls from Svensk Avel, Skara, Sweden. Semen samples were extended (1:8) in PGFE, Triladyl and Andromed. Sperm concentrations were assessed and samples diluted with the corresponding extender to 80 million sperm/mL. Samples were extended 1:1 in cryomedium and stored in the refrigerator for 2 hours before freezing for 7 minutes in liquid nitrogen vapour. Sperm extended in PGFE were frozen with both Triladyl and Andromed. Sperm extended in Triladyl and Andromed were frozen with the corresponding cryomedium. After a minimum of 48 hrs in liquid nitrogen, samples were thawed at 37°C for 30 sec. Sperm motilities were assessed subjectively.

**Results and Discussion:** Sperm in CEY show better post thaw motilities (50%) compared with Triladyl (40%) when initially extended in PGFE. When sperm were extended in Triladyl, post thaw motilities were significantly lower (20%, 5% for Triladyl and CEY respectively). CWP compared with Andromed also gives better post thaw motilities (50% and 43% respectively  $n=4$ ). As in the case of Triladyl, Andromed gave better post thaw motilities when sperm were initially extended in PGFE. Preliminary results on the protein-free cryo extender look very promising, and will be investigated further.

### P31

#### **Comparison of controlled-rate freezing and vapour freezing of turkey spermatozoa**

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**Introduction:** Cryo-preserved sperm have been widely used for artificial insemination in many different species but not in turkeys, possibly because of low post-thaw viability. This study will focus on a comparison of a controlled freezing and a vapour freezing technique for turkey sperm, to improve post-thaw sperm viability.

**Methods:** Turkey ejaculates were collected at a commercial turkey farm into Turkey Semen Extend (TSE) in a 1:1 dilution. TSE is an extender specially formulated for extending turkey sperm prior to freezing (Nidacon, Gothenburg, Sweden). A turkey sperm cryoprotectant containing glycerol (Nidacon, Gothenburg, Sweden) was added in a 1:1 ratio. Payett straws were filled with 100  $\mu$ L of the mixture. Half of the straws were frozen using liquid nitrogen vapour and half were frozen with a cryochamber and freeze control unit (CL-8000;Cryologic PL). Freezing with vapour: The straws were placed in a refrigerator for 60 min to equilibrate. The straws were placed horizontally on a cushion 2 cm above the liquid nitrogen surface. After 30 min the straws were plunged into liquid nitrogen for storage. Freezing with cryochamber: The straws were placed inside the cryochamber and the freezing programme executed. The cooling rates used were: 20°C to 4°C at 1°C/min; Hold for 10 min; 4°C to -30°C at 5°C/min; Freefall When the temperature reached -80°C the straws were plunged into liquid nitrogen for storage. After 48 h storage the straws were thawed at room temperature, and sperm motility was assessed subjectively.

**Results and Discussion:** Post-thaw motility was better for sperm frozen in the cryochamber (51.1%) compared with sperm frozen in liquid nitrogen vapour (19.2%) Future experiments will investigate the programmed cooling rates to improve post-thaw survival and the fertilising capacity of thawed sperm.

### P32

#### **Volume regulation in boar spermatozoa: involvement of a volume-sensitive osmolyte and anion channel (VSOAC), possibly CLC-3**

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**Introduction:** The ability to maintain cellular volume is an important general physiological function. Swelling induced by hypotonic stress results in the opening of channels, through which ions exit with accompanying water loss (regulatory volume decrease, RVD). RVD has been shown to occur in mammalian spermatozoa, primarily through the opening of quinine-sensitive potassium channels. However, as yet, direct evidence for the participation of anion channels in sperm RVD has been lacking.

**Methods:** Hypotonically induced swelling in boar spermatozoa was investigated essentially in a HEPES-buffered NaCl-based medium using electronic cell sizing; further investigations involved recording cell volume in sperm suspensions treated with gramicidin in media in which sodium chloride was replaced by sodium sulphate. Membrane integrity was evaluated by flow cytometry. SDS-PAGE and Western blotting were used to detect chloride channels proteins.

**Results and Discussion:** The following results were obtained: (1) The anion channel blockers NPPB, tamoxifen and DIDS increased hypotonic swelling in concentration-dependent fashion, whereas verapamil (P-glycoprotein inhibitor) had little effect. The most potent, NPPB and DIDS, blocked RVD without affecting cell membrane integrity (propidium iodide exclusion) at effective concentrations. Such an inhibitor profile is similar to that reported for VSOACs in other cell types. (2) When gramicidin was included to dissipate Na<sup>+</sup>/K<sup>+</sup> gradients, a biphasic volume increase was observed only under hypotonic conditions. We hypothesised this finding as follows: During RVD, separate channels for potassium and chloride are opened. Exit of potassium down its concentration gradient drives a tandem exit of chloride against a concentration gradient. Once the potassium gradient is dissipated by gramicidin, chloride ions enter into the cell through the chloride channel, accompanied by sodium ions. In support of this hypothesis, gramicidin swelling could be reduced by NPPB, and suppressed completely by replacing chloride in the medium with sulphate, an ion which does not pass through chloride channels. (3) The chloride channel type CLC-3 may be the VSOAC in other cell types. Following SDS-PAGE and Western blotting under non-reducing conditions, a commercial anti-CLC-3 antibody detected specific protein bands of about 85 kDa in boar sperm extracts. Our observations provide direct evidence that a chloride channel (possibly CLC-3) is involved in the process of volume regulation in boar spermatozoa.

### P33

#### **The vaginal microflora and canine herpes virus 1 titres throughout the oestrous cycle of breeding bitches**

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**Introduction:** The purpose of this study was to make a qualitative and quantitative assessment of the vaginal microflora, combined with a serological follow-up of CHV1 titres in relation to the oestrous cycle to relate changes in the aerobic vaginal bacterial flora and canine herpes virus 1 (CHV1) (re)infection with fertility problems in dogs.

**Methods:** This study was performed on 34 breeding bitches from 2 kennels with and 2 kennels without fertility problems. Blood samples and vaginal swabs were taken at the four stages of the oestrous cycle. Blood samples were collected for the determination of CHV1 seroneutralizing (SN) antibody titres and for the examination of plasma progesterone

concentrations. Vaginal swabs were taken for the staging of the oestrous cycle and for bacteriological examination. The results were evaluated using univariate logistic regression and ordinal logistic regression (SPSS 11.0).

**Results and Discussion:** The number of bacteria isolated was significantly influenced by the stage of the oestrous cycle. Bacterial counts were higher during pro-oestrus and oestrus. In the individual bitch the flora changed during the oestrous cycle but when all samples were compared, no specific bacterial species could be associated with a certain stage of the oestrous cycle. No significant differences in the vaginal flora were found between bitches from kennels with and without reproductive disorders. In kennels with fertility problems significantly more bitches were seropositive for CHV1. In all but one bitch, serum-neutralizing (SN) antibody titres to CHV1 did not change during the oestrous cycle. While this bitch seroconverted, no effect on the fertility status was found. It was concluded that no correlation between the aerobic vaginal bacterial flora and/or CHV1 and fertility could be demonstrated.