Goat as a Model for Studying R-Spondin Involvement in Ovarian Differentiation in Mammals

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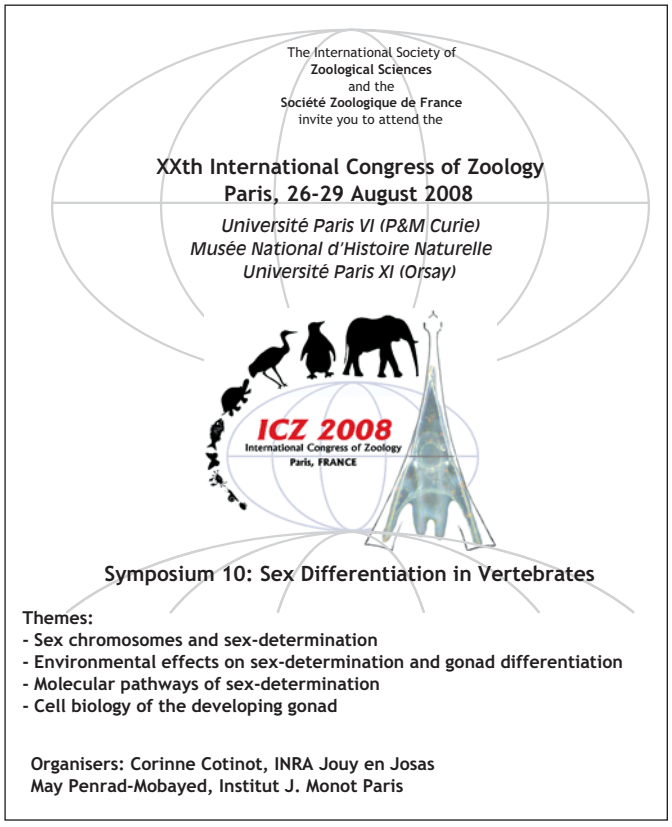

Sex-Specific Differences of the Skeleton in North African Gazelles (Mammalia, Artiodactyla, Bovidae)

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Ruminants frequently display sexual dimorphism in body size and also in horns and upper canines if they are present. Sex-specific differences in size and morphology of bones were analyzed in three captive-bred North African gazelle species *(Nanger dama*, *Gazella dorcas* and *Gazella cuvieri)* from the 'Estación Experimental de Zonas Áridas' (Almería, Spain). Morphologically female skulls are characterized by a more asymmetric basioccipital with less prominent anterior tuberosities, thinner jugular processes, and less depressed supraorbital fosses that are also more separate from each other. Apart from the pelvis, sexual differences are detected in the morphology of atlas and axis. In females, the atlas presents less developed wings with parallel lateral edges and the axis has smaller and less divergent transverse apophysis and a flat superior edge of the spinous process. PCAs run on each of these element databases found that between 93 and 100% of the total variance is explained by the three first principal components. PC1 reflects general size, whereas PC2 and PC3 reveal variations of shape. Males and females are arranged by the three first components confirming important sexual differences in size and morphology of skull, pelvis, atlas and axis. But the biggest species, *N. dama*, shows dimorphism in all skeletal elements, even in the palatal region of skull and in the teeth. Sexual arrangements were checked by means of Discriminant Analysis with a classification rate between 69 and 76% in teeth and between 71 and 100% in skull, 99% in either atlas or axis, and 95% in pelvis.

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In mammals, once the chromosomal sex XY or XX is set up at fertilization, the presence of the *SRY* gene (Sex-determining Region of Y) will determine the fate of the gonad by initiating testis differentiation. In the female counterpart, key genes for ovarian differentiation have been isolated from genetics studies of female-to-male XX sex-reversal: the PIS locus (Polled Intersex Syndrome) in goat, and recently *RSPO1* in human. In goat, the pleiotropic PIS mutation is an 11.7-kb deletion encompassing any genes, but acting on the transcriptional regulation of at least 3 genes, *PISRT1*, *PFOXic* and *FOXL2*, distal from the deletion. Compared to the normal situation ($PIS^{+/-}$ and $PIS^{+/+}$), the expression of these three

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genes is abolished in the ovaries of XX foetuses homozygous for the deletion (PIS$^{-/-}$). So, it seems that one of the PIS-regulated genes has an 'anti-testis' effect. Among the three genes, only *FOXL2* encodes a conserved protein belonging to the forkhead family making it a strong anti-testis candidate gene. As shown in human, an R-Spondin gene *(RSPO1)* is involved in a female-to-male XX sex reversal. Consequently, R-Spondin expression patterns were studied in XX PIS$^{-/-}$ males. *RSPO2* was detected with a female sex-dimorphic expression, and was absent in XX mutants. This leads us to assume that *RSPO2* could be a direct or indirect target of *FOXL2* in ovarian differentiation. To further confirm our presumption, different studies are under progress: (i) cellular localization of RSPO2 in the ovary; (ii) if co-localized with FOXL2, studies of the *RSPO2* promoter.

Identification of New Transcripts Involved in Ovine Ovary Development

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The aim of this study was to isolate by suppressive subtractive hybridization genes differentially expressed between the two main steps of ovarian development in sheep: onset of prophase I meiosis (55 dpc) and follicle formation (82 dpc). Two subtractive libraries (55/82; 82/55) were constructed and 7,296 clones were obtained. Among these, 6,080 clones were sequenced and grouped into 2,101 unique contigs using SIGENAE bioinformatics facilities. Then these contigs were compared with databases from different mammalian species (human, bovine and ovine) and annotated. In both libraries, 99% of contigs possessed an Unigene annotation and 1% were unknown (21/2101). The expression profiles of all unknown contigs were determined by RT-PCR in fetal ovary, testis and in a pool of somatic tissue. Three of them showed an ovary-specific expression that was confirmed by real time RT-PCR. Furthermore, we have found one gene never described in female ovary. This gene showed a high expression level during female meiosis I while its expression remained low in fetal testis and during the other stages of ovary development. Further studies such as 5′ and 3′ RACE analysis to obtained full-length transcripts and ISH to determine cellular localisation are currently in progress for these genes. Moreover, investigations of the folliculogenesis library are under progress. In parallel, these 2,101 contigs will be used to develop a custom-made macroarray, dedicated to ovine ovary differentiation, in order to evaluate ovarian transcriptomes in different physiological and physiopathological conditions.

The Battle of Sexes: Patterning the Gonad

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Although genetic sex is established at fertilization, Alfred Jost demonstrated that the decision determining whether an embryo develops as male or female occurs later in gestation, in the gonad. The gonad arises as a uniquely bipotential primordium, indistinguishable in XX and XY embryos, and harboring the potential to develop as either a testis or an ovary. Studies suggest that cells in the early gonad are influenced by opposing signals that hold them in a bipotential state. In mammals, transient expression of the Y-linked gene *Sry* in a subset of cells triggers a global shift in this balance toward the testis fate. Although *Sry* normally acts as the genetic switch to initiate male development of the gonad, the sex determination pathway can be manipulated by alterations in the underlying antagonistic signaling pathways. For example, disruption of the Fgf signaling pathway leads to male-to-female sex reversal, while disruption of the Wnt/Rspo signaling pathway leads to partial female-to-male sex reversal. Recent evidence suggests that these opposing pathways may act by influencing the intracellular antagonism between SOX9 and β-catenin. This work leads to the hypothesis that *Sry* is a mammalian invention that is superimposed on an underlying signaling network that may regulate sex determination across vertebrates in species where *Sry* is absent and sex is determined by other genetic elements or by environmental cues.

Rspo1, an Essential Gene for Ovarian Differentiation in Mammals

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The sex of an individual is determined during development through the fate of the gonad. Gonad sex determination is controlled by a balance between two different pathways. While the expression of *Sry* and *Sox9* is sufficient to induce the male developmental program, we have shown that the transcriptional function of β-catenin is activated in XX embryonic gonads, and antagonizes the male determining pathway. Rspo1 is an activator of the Wnt/β-catenin signalling pathway required for ovarian differentiation and ablation of Rspo1 induces female-to-male sex reversal, similar to human XX patients with mutations in *RSPO1*. *Rspo1* is not only involved in the differentiation of somatic cells, but is also required for normal germ cell differentiation and commitment to meiosis. These results demonstrate that *Rspo1* is a crucial gene required for sex determination in mammals.

Reproductive Cycle and Length-Weight Relationship of *Scomber japonicus* Houttuyn, 1782, from the Middle Adriatic Sea

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The chub mackerel, *Scomber japonicus* Houttuyn (1782), is widely distributed in moderate and warm waters of the Atlantic, Pacific and Indian Ocean. This middle-sized pelagic fish species also occurs throughout the Mediterranean and Adriatic Sea. The chub mackerel specimens (n = 790) were collected monthly from the commercial purse seine catches realized during January 1998–November 1999, in the eastern Middle Adriatic Sea. In order to examine some reproductive traits of this commercially important fish species monthly evolution of gonadosomatic index, mass and stage of gonads was used. Overall, fork lengths of chub mackerel specimens varied from 20.1 to 38.0 cm and the mean value was 27.9 ± 3.1 cm. The proportion of males and females was almost equal (♂/♀ = 0.99) but statistically significant differences from expected sex ratio 1:1 ($\chi^2 = 25.7270$; $p < 0.001$) were noticed in favour of females. The period of reproductive activity was from April to September in the Middle Adriatic Sea coinciding with the highest values of gonadosomatic index and weights of gonads as well as with the most developed states of gonads. Length-weight relationship for chub mackerel specimens was: $W = 0.0033\ LF^{3.3457}$ ($r^2 = 0.8479$), thus positive allometry was established.

Temperature-Induced Male Differentiation in the Nile Tilapia: Gonad Gene Expression Using Female Monosex Populations and Divergent Thermo-Sensitive Lines

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Sex in the Nile tilapia, *Oreochromis niloticus*, is governed by the interactions between a complex genetic sex determination system and the influence of temperature. High temperatures applied during a critical period of sex differentiation can induce masculinisation in some progenies originating from both domestic and wild stocks. Differences in thermo-sensitivity were observed between the wild populations and both paternal and maternal effects have been demonstrated, suggesting that it is a heritable trait. This has been confirmed by the development of divergent lines for thermo-sensitivity. Two thermo-sensitive divergent lines were selected with regard to their sex ratios showing high (>90% males) and low responses (only 54%) when treated at 36°C temperatures. At least one of the mechanisms by which temperature exerts its effects on sex differentiation is through a down-regulation of aromatase *Cyp19*. Expression profiles were analysed in monosex XX populations treated at 27°C and 36°C temperatures showing that temperature up-regulates *Sox9s*, *Amh*, *IGFs*, *Dax1* and down-regulates other genes such as aromatase *Cyp19-1a*. Variation in these gene expression levels was associated with the percentage of males obtained by temperature treatments. The divergent thermo-sensitive lines have been used to complement our study done on the monosex populations. Expression of *Cyp19-1a* was also analysed in these two divergent lines.

FOXL2 and SOX9 Show in situ Sex Reversal in Patients with Various Forms of Disorders of Sex Development

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Under normal physiological conditions, the gonadal sex formation of testicular or ovarian tissue is highly separated in mammals and determined by the chromosomal sex constitution. In normal gonads SOX9 protein expression was found to be restricted to the development and maintenance of Sertoli cells. In contrast, FOXL2 was only found in granulosa cells, as well as stromal cells in early development. However, there are a number of pathological exceptions to this rule: both testicular and ovarian tissue can be formed, either in a single gonad, or in two different gonads. This pathological condition is classified as ovo-testicular Disorder of Sex Development (DSD). This study for the first time investigates the presence of both SOX9 and FOXL2 in gonads of patients with various forms of DSD. This study demonstrates the novel finding that in DSD patients the formation of either ovarian or testicular development can only be visualized using immunohistochemistry for FOXL2 and SOX9, respectively. The results demonstrate that SOX9 is a highly informative marker for testicular development and FOXL2 for ovarian development, either present in isolated or mixed constitution. Although SOX9 and FOXL2 could be present within a single histological context, they were never found to be expressed at high levels within the same cell. These observations demonstrate for the first time the additional value of immunohistochemistry for SOX9 and FOXL2, compared to morphology alone, to diagnose the presence of either ovarian or testicular differentiation or both, especially in patients with DSD.

Gender-Bending Chemicals and the Mammalian Fetal Gonad

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Most mammals adhere to a general pattern of gonadal development and then subsequent sexual differentiation along the lines outlined by Alfred Jost over 40 years ago. For development of a reproductively competent male, establishment of testes leads to

androgen action, in-utero and post-natally, which is critical for the development of a urogenital system based on Wolffian ducts. In contrast, females develop ovaries as a result of a different cascade of genes and establish a urogenital system based on Mullerian ducts. It is well recognised from experimental studies that excess androgen masculinises the female fetus while excess oestrogen interferes with male fetal masculinisation, in both instances impairing fertility. While there are mammals, including the spotted hyena and several mole species, which deviate from this basic pathway, in the majority of mammals disturbance of endocrine signalling adversely affects reproductive development. However, over the last 20 years there has been increasing concern about the potential detrimental effects of environmental chemicals, including heavy metals and endocrine-disrupting compounds, on reproductive development and health in humans, domestic species and wildlife. These chemicals have complex mechanisms of action and additive effects in complex mixtures and at low doses. The levels of exposure that developing mammals receive and the effects of the exposures remain poorly understood. In addition the data concerning the effects of exposure are sometimes contradictory. However, there is sufficient evidence for so-called 'gender-bending' actions of fetal exposure to these environmental chemicals. In this presentation the basic processes in male and female reproductive development and the evidence for their disturbance by exposure to environmental chemicals will be discussed.

Collaborators: Sharpe RM, Evans NP, Rhind SM, Cotinot C, Fischer B, Pocar P, Sinclair K, Lea RG, O'Shaughnessy PJ.

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The Cres/Testatin Subgroup, a Reproductive Tract Specific Subgroup of Genes within the Cystatin Family 2 Protease Inhibitors in Sexual Development

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CMM L8:02, Stockholm, Sweden

Testis differentiation is initiated with the expression of the Y-linked factor Sry in XY gonads. Factors, such as Sox9, Fgf9 and Dhh, have been identified downstream of Sry and proven to cause gonad development failures or sex reversals in humans or in mouse models when disrupted. Characterization of novel genes will help to clarify basic mechanisms behind gonad development as well as facilitate diagnostics of gonad dysgenesis. Testatin was previously isolated by our group in a screen searching for novel genes expressed in early mouse sex differentiation. Testatin is specifically up-regulated in the developing testis just after expression of Sry. Testatin belongs to the Cres/testatin subgroup of cystatin family 2 protease inhibitors that show a reproductive tract restricted expression (testis, epididymis, ovary, pituitary) in contrast to the broad expression profile of family 2 cystatins, which implies a specialized function within the reproductive tract. We generated testatin knockout mice to evaluate the role of testatin in male sexual development. We observed normal fetal testis development and fertility in male testatin knockout animals (1). An explanation could be redundancy between the subgroup members. We evaluated the expression profile of the Cres/testatin subgroup in fetal testis with real-time PCR and in situ hybridization and we show that three of the subgroup members together with testatin (Cres, cystatin SC, Cystatin TE-1) are expressed in mouse fetal testis (2). In conclusion, future generation of conditional triple knockout mice would further evaluate the role of the Cres/testatin subgroup in male sexual development.

Gene Expression Profiling during Gonadal Differentiation in Chicken

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In birds sex is determined by a ZZ/ZW chromosome system in which the female is heterogametic. Several genes involved in mammalian gonadal differentiation have been identified in the chicken and their expression patterns investigated, but their function(s) as well as the molecular pathways in which they are involved are still poorly understood. In the light of current knowledge, this study aimed at identifying new actors of chicken gonadal differentiation in order to improve our understanding of the female and male molecular pathways in birds. Based on real-time RT-PCR, we studied the expression profiles of over 100 candidate genes during gonadal differentiation. Our set of candidates included ligands, receptors, signaling molecules, enzymes and transcription factors, known for their roles in sex differentiation, reproduction and embryogenesis in other species. The hierarchical clustering of genes based on the similarity of their temporal expression patterns allowed the statistical identification of gene clusters with sex or/and stage, or/and side specific signatures. For instance, the analysis of the distribution of genes in clusters suggested a differential involvement of the members of the TGFβ system in chicken testicular and ovarian differentiation along with, for some of them, their implication in the mechanisms underlying gonadal left-right asymmetry which features the ovarian development in birds. In addition, we also identified new Z-linked actors of chicken testicular differentiation. To conclude, the findings of this study gave new insight on the molecular mechanisms of chicken gonadal differentiation and provided good candidate genes for further functional analysis.

New Mechanisms Involved in Meiosis Prevention by Fetal Mouse Testes

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Fetal testes and ovaries differentiate from morphologically identical, bipotential gonads. In mice, the proliferative germ cells (GC) remain very similar in male and female gonads until about

13.5 days post-conception (dpc). At this age, female GC initiate meiosis whereas in the testis, GC undergo mitotic arrest with all GC having entered the quiescence phase by 15.5 dpc. Recent findings indicate that retinoic acid (RA) is the key factor in committing GC toward the female pathway as it induces meiosis in mouse fetal germ cells. GC in the fetal testis are protected against the effects of RA by CYP26b1, a male-specific enzyme that degrades RA. In this study, we evidenced distinct testicular pathways involved in the prevention of the fetal meiosis. Using a co-culture model in which an undifferentiated XX gonad is cultured next to a fetal or neonatal testis, we demonstrated that the testis prevented the meiosis initiation in the XX gonad. This testicular effect was function of the stage of the testis and was not correlated with the expression of Cyp26b1. Moreover, addition of RA or ketoconazole, an inhibitor of Cyp26b1, to the medium did not prevent the testicular meiotic inhibitory effect on the GC of the co-cultured ovary. We evidenced that this testicular effect was due to secreted factor(s) as conditioned medium of fetal testes also inhibits meiosis in the XX gonad. Lastly, molecular weight cut-off experiments indicated that the factor's weight is higher than 10 kDa. In conclusion, our results demonstrate that diffusible testicular factors specifically produced during fetal and neonatal lifes have the potentiality to prevent meiosis independently of the activity of Cyp26b1.

Correlation between Ovarian Steroidogenesis and β-Endorphin in the Lizard *Uromastyx acanthinura*: Immunohistochemical Approach

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In mammals, opioid peptides are involved in various physiological processes including reproductive function. The major sites of biosynthesis are hypothalamus, solitary bundle nucleus and hypophysis intermediary lobe. β-Endorphin, one of the opioid peptides is also synthesized in the ovary. In *Uromastyx acanthinura*, the localization of this peptide and sex steroid was investigated by an immunohistochemical approach. β-Endorphin is strongly distributed in the granulosa cells and oocyte cytoplasm of the previtellogenic follicles of sexually quiescent lizards (winter) when steroidogenesis is interrupted. In spring, the signal becomes low, or event absent, in the vitellogenic and previtellogenic follicles. The granulosa cells of the previtellogenic follicles show an important synthesis of 17β-estradiol. Females that do not undergo vitellogenesis in spring show the same profile as winter quiescent females. These findings represent the first evidence of the presence of β-endorphin in the ovary of *Uromastyx acanthinura*. The seasonal variations observed in the reproductive cycle suggest that this opioid peptide is involved in the modulation of seasonal steroidogenesis.

How Different Are Sex Determination Mechanisms in Reptiles?

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The distribution of sex determination modes among reptile species is still enigmatic from an evolutionary point of view. Some species exhibit genotypic sex determination (GSD), but some others show environmentally dependent sex determination (ESD). One theory, widely accepted, proposes that ESD and GSD are mutually exclusive. Another hypothesis considers that sex could be determined both by genotypic and environmental factors, the co-occurrence of ESD and GSD being so possible. Under this theory, in some cases the sex determination mode would be a mixed form between ESD and GSD. In other cases, according to the relative importance of these factors, sex determination could be mainly genotypic or environmentally dependent. Our study focuses on the evolution of sex determination modes in reptiles. To explain the observed distribution of ESD and GSD in reptiles, we discuss these two theories and the different hypotheses on the evolution of sex determination modes they imply. We searched the literature for data about turtle species which show temperature-dependent sex determination (TSD), GSD or mixed modes. We also included data about the described TSD patterns for concerned species. Based on these data, we propose and test hypotheses on the evolution of ESD and GSD in turtles in particular, and in reptiles in general.

Adrenal Cortex Contents of Androstenedione in Rabbit *(Orynctolagus cuniculis)*

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Exploration of the adrenal androgen activity with the aim of knowing the endocrine physiology of the domestic rabbit in local populations of Algeria was carried out. Histology and morphometric measurements of the glands and evaluation of their contents of androstenedione hormone were done. Male rabbits, 37 days (n = 14), 60 days (n = 5) and 6 months old (n = 2), from the experimental farm (ITELV) were sacrificed. Adrenal glands were quickly taken, streamlined, the left (awkward) gland was fixed in Bouin Hollande for the histological study and the right adrenal gland was crushed in phosphate buffer pH 7.4 for the dosage of androstenedione by RIA kit. The histo-morphometric study shows that the zonation of the adrenal cerebral cortex is net in the animals of 37 days, the thicknesses of glomerulosa, fasciculata and reticularis zones expressed in percents are respectively 9.36%, 62.99%, and 27.63% of the total thickness of the cerebral cortex, and these values are not different from those observed in rabbits of 60 days and 6 months. The adrenal contents of androstenedione in 100 mg of adrenal weight are 2.90 ng at 37 days, 4.54 ng at 60 days and 1.34 ng in 6-month-old rabbits. Even though, in the rabbit, the adrenal cerebral cortex, according to its production of androstenedione (which is the metabolite of the DHEA and a potential direct precursor of testosterone) might participate in the installation of puberty.

Gonadal Development of Freshwater Turtle *(Malayemys macrocephala)* Embryos Exposed to Environmentally Relevant Doses of Cadmium

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Cadmium contaminated sediment plumes have formed on the Mae Tao River Basin, Tak Province, Thailand, possibly as a result of zinc mining activities. We are using the freshwater turtle *(Malayemys macrocephala)* as a sentinel to monitor the reproductive effects of exposure and, by inference, the potential for human health impacts. Since contaminant may affect turtle by exposing through eggshell as well as maternal transfer via yolk, we thus examined the effect of cadmium on turtle embryos. Freshly laid eggs of *M. macrocephala* were collected from a reference site with no history of cadmium contamination. Representative eggs were analyzed for cadmium contamination by an ICP/ES to verify that the cadmium content in egg yolk is non-detectable. The effect of cadmium was determined using eggs subjected to in ovo exposure to cadmium chloride. The doses used in this study (0, 3, 30 and 300 μg of total cadmium/g egg weight) were based on the concentrations in soil at Mae Tao area. Eggs were kept in an incubator at a constant temperature that yields 1:1 sex ratio until hatching. It was found that low doses of cadmium significantly prolonged the hatching time compared to that of the control. However, the hatching success and hatching weight are not significantly different among groups. Since cadmium may interfere with processes of gonadal development, results on sex ratio and gonadal development of these hatchlings will be presented. The potential of the environmentally relevant dose of cadmium on turtle development and its implication for sentinel system will be discussed.

Modulation and Immunohistochemical Localization of P450 Aromatase on Algerian *Psammomys*

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Epididymal function is androgen dependent, but recent evidence indicates that estrogens are also important. They have a major importance in fertility. Estrogen receptors invalidation in the mouse induces an abnormal epididymal phenotype and infertility. The aim of this study is analysis of aromatase in sand rat epididymis and to highlight its modulation during castration, castration then treatment and efferent ducts ligation. Three groups were constituted: castrated groups for one month, castrated groups during 36 days then treated during 15 days by testosterone, and animals having undergone efferent ducts ligation. The organs were taken in Hollande Bouin for immunohistochemistry. In order to locate P450 aromatase responsible for the estrogen production, an indirect method was applied by using anti-mouse aromatase antibodies against human aromatase. The research of P450 aromatase on proximal epididymis does not show positive reactions on lumen, epithelium and conjunctive tissue in the control. Castration, castration then testosterone treatment and efferent ducts ligation does not show positive reaction on all compartments of proximal epididymis. In distal epididymis, P450 aromatase shows proximal cauda epididymis localization. This staining increases and becomes more intense towards the distal cauda principal cells cytoplasm and not the clear cells. Castration experiments show enzyme disappearance and testosterone treatment induces its reappearance. In the animals having undergone efferent ducts ligation, the positive reaction persists only in the distal part of the cauda epididymis; it always remains in the cytoplasm of the principal cells. The absence of P450 aromatase in proximal epididymis excludes its expression in this segment or its testicular origin. Its immunolocalization on principal cells cytoplasm of the distal epididymis and its androgen-dependence lindicates a modulating role of T/E balance other than T/DHT balance and does not exclude estrogens impact on epididymal function.

Development of a Goat-Specific anti-SRY Antibody: Preliminary Results on Goat SRY Protein Expression

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In mammals, the *SRY* gene (Sex-determining Region of Y chromosome) is the master regulator of male sex determination. Despite 15 years of studies, the molecular and cellular mechanisms operating downstream of SRY remain undefined. The *SRY* gene is not well-conserved among mammals both in terms of protein structure and gene expression. In mouse, *Sry* is first expressed around 10.5 days post-coïtum (dpc), reaches a peak of expression at 11.5 dpc, and is extinguished shortly after 12.5 dpc. In sheep, *SRY* expression is first detected in male gonads at 23 dpc, remains highly expressed during 18 days between 27 and 44 dpc and decreases but is not totally absent from 49 dpc until a few days after birth. In the post-partum (pp) sheep testis, *SRY* is expressed again from 12 dpp until adulthood. By contrast, in goat *SRY* mRNAs are detectable from the earliest studied stage (25 dpc) until adulthood. In order to determine the cellular and sub-cellular localization of SRY protein at different developmental stages in goat, we have developed polyclonal antibodies against the C-terminal part of goat SRY protein, excluding the HMG-box. Our objective is to confirm the specificity and effectiveness of our antibodies against goat SRY protein and to compare the developmental expression profiles of SRY proteins between goat and sheep.

Impact of Phtalates on Human Fetal Testis in vitro

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During the last decades, a decrease in sperm production and an increase in genital abnormalities have been described in human. They are suspected to belong to the same Testicular Dysgenesis Syndrome. This syndrome may result from a defect in testis development during fetal life, due to an increasing exposure to endocrine disruptors [Sharpe and Skakkebaek, 1993] widespread in the environment, among which we found the phthalates. One of the most important phthalates is di(2-ethylhexyl)-phthalate (DEHP) which is metabolized in its major active compound: MEHP. Exposure of rodents to phthalates impairs testis functions. Moreover, recent clinical investigations have reinforced this hypothesis [Swan et al., 2005; Main et al., 2006]. We have studied the effects of MEHP, with doses from 10^{-6} to 10^{-4} M, on the development of human fetal testis. The testes have been obtained from legal pregnancy abortion (7 to 12 weeks). This study has been performed in vitro in an organotypic culture system, previously developed in our laboratory [Lambrot et al., 2006]. With the higher dose, we show for the first time that MEHP has a negative effect on the number of human gonocytes by increasing their apoptosis without any changes in their proliferation rate. But contrary to the rat and whatever the dose used, MEHP does not affect testosterone production of the human fetal testis.

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Fat Reserves and Moisture Content in Relation to Sexual Cycle of Sardine, *Sardina pilchardus* (Walbaum, 1792), in the Eastern Middle Adriatic Fishery Grounds

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The fat reserves, moisture and sexual cycle of sardine, *Sardina pilchardus* [Walbaum, 1792], were studied from monthly random samples of purse seine catches from March 2004 to February 2005. Catches were performed in offshore (Dugi Otok) and inshore waters (Virsko more) of the Mid Adriatic Sea. A total of 1,219 sardine specimens were collected, out of which 668 were males and 541 females. Fish were measured, weighed and sexed. The sexual cycle analysis was based on the temporal evolution of gonadosomatic index and gonad mass. Gonadosomatic index (GSI) was calculated by expressing the monthly gonad weight as a proportion of the total body weight. The fat content was examined on the basis of monthly analyses of mesenteric fat in the visceral cavity and by determining the amount of lipid content in sardine tissues using Soxhlet's s method. The total length of sardine ranged from 13.0 to 19.0 cm and the mass ranged from 16.72 to 51.45 g. The reproductive period was from October to May, coinciding with the highest gonad weights and gonadosomatic indices. The mean percentage of mesenteric fat grades in visceral cavity points to the greatest fat quantities in August, when grade 4 (fattest fish) presence amounted to 72%. The value is the result of an increase of mesenteric fat started in June and proceeding in the successive months until October. During autumn, a decrease trend is evident and it becomes pronounced in winter and spring when the lowest mesenteric fat quantities are recorded. Thereafter an increase in mesenteric fat for sardine was recorded, indicated by the records of grades 3 and 4. Due to such variations in mesenteric fat and tissue lipid during the year, i.e. its greatest levels in summer (out of spawning) and lowest levels in the colder part of the year (during sardine spawning), it was assumed that quantity of sardine fat was affected by its sexual cycle. Lipid content in terms of sardine tissue dry weight showed the same trends as mesenteric fat – highest amounts during resting phase of reproduction (41.1%) and lowest during the peak of spawning (1.0–2.2%). Amounts of total lipids and moisture in sardine tissues showed that females had more fat content and less moisture than males. An inverse correlation between fat content and sexual cycle on one side and lipid content and moisture on the other has been noted.

Sex Differentiation in Mammals: What about Polled Goats?

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In goats, the PIS (Polled Intersex Syndrome) mutation is responsible for both the absence of horns in males and females and sex-reversal affecting exclusively XX individuals. The mode of inheritance is dominant for polled trait and recessive for sex-reversal. In XX PIS$^{-/-}$ mutants, expression of testis-specific genes is observed very precociously during gonad development. Nevertheless, a delay of 4–5 days is observed in comparison with normal testis differentiation in XY males. By positional cloning, we have demonstrated that the PIS mutation is an 11.7-kb regulatory deletion affecting the transcriptional expression of 3 genes, *PISRT1, PFOXic* and *FOXL2* which should act synergistically to promote ovarian differentiation. The transcriptional extinction of these 3 genes leads, very early, to testis-formation in XX homozygous PIS$^{-/-}$ mutants. Since the discovery of this mutation in 2001, progress has been made in order to understand the molecular functioning of this complex locus and the role of the different PIS-regulated genes. Our current understanding of this locus will be presented in regards to our main results obtained these last years, demonstrating that for some aspects goat gonad differentiation seems more closely related to non-mammalian vertebrates than to mouse.

A Diversified Role for *SOX9* Gene in Amphibian Gonads

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Our purpose is to identify common mechanisms implied in the acquisition of gonad identity in vertebrates. *SOX9* is one of the highly conserved genes, which seems to be a key factor in gonad differentiation. Its role in the male determining pathway was clearly confirmed in mammals. Accumulating evidences showed that this gene is also expressed in male specific manner in reptiles and birds. However, its function in gonad differentiation in fishes and amphibians remains to be elucidated. Indeed, RT-PCR studies showed that *SOX9* m*RNA* is expressed during gonad development of both sexes. To approach the role of the *SOX9* gene in amphibians, we carried out in situ hybridization and immunostaining studies in one urodelan and two anurans species: *Pleurodeles waltl*, *Xenopus laevis* and *Xenopus tropicalis*. Results show that *SOX9* mRNA is expressed at the later stage of gonad differentiation in both sexes. However, SOX9 protein expression greatly differs between male and female gonads. In the testis, SOX9 protein expression is restricted to the nuclei of Sertoli-like cells whereas in the ovary, SOX9 protein is initially detected in the cytoplasm of previtellogenic oocytes and then translocated into the nucleus at the vitellogenic stage. These data suggest that SOX9 has a diversified role in gonad differentiation throughout vertebrate evolution.

Microdissection and DOP-PCR as a Way to Study the Evolution of an Old Sex Chromosome Pair Using Two Sister-Species of Tilapia with Different Sex Determination Systems, *O. aureus* and *O. niloticus*

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Within the tilapia group, the sex determining locus has been located on linkage group 1 (LG1) in *Oreochromis niloticus*, whereas in *O. aureus* both LG1 and LG3 are sex-linked. Using specific BAC clones as probes for FISH, LG3 and LG1 have been located on the largest and on a smaller chromosome pair, respectively. The largest pair presents various traits of a relatively old sex chromosome, whereas LG1 seems to be at an early stage of sex chromosome evolution. Evidence for interactions between the two sex linked loci has been demonstrated at least in *O. aureus*, with minor factors (genetic and environmental factors) also modulating sex ratios. We have taken advantage of the >3-fold larger size of one of the chromosome pairs to microdissect it in order to search for genes specific to this large pair. This chromosome was microdissected from metaphase preparations of homogametic genotypes (XX and YY for *O. niloticus*, and ZZ for *O. aureus*). Microdissected chromosomes were amplified by DOP-PCR and the DOP-products were then used to screen a gonadal cDNA library. Positive clones were hybridized by FISH on metaphase spreads of different genotypes from both species. We evidenced positive signals located on the large chromosomes of both strands indicating specificity of the probes, despite the presence of large amounts of repetitive sequences on these large sex chromosomes. The conservation of the structure of the large pair between the two species is discussed.

Sexual Dimorphism in Persian Long-Tailed Desert Lizard, *Mesalina watsonana* (Sauria: Lacertidae), with Especial View on the Rensch'es Rule

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A total of 174 specimens (84 females, 90 males) of adult Persian long-tailed desert lizard *(Mesalina watsonana)* were either collected from various regions of the Iranian Plateau during 2005–2007 or examined using museum material. These specimens were studied based on 24 metric and 12 meristic characters. The T-test and Discriminate Function Analysis (DFA) were employed to analyze the data for sexual size dimorphism. In ten metric and three meristic characters, significant differences ($p < 0.05$) were found between the two sexes of adult lizards. Geographic variation in nine, out of 12, meristic characters was found in males, but interestingly these characters in various populations of females were invariable. According to the Rensch'es rule Sexual Size Dimorphism (SSD) characteristically increases with size when males are the larger sex and decreases with size when females are the larger sex, such that logarithmic plots of males against females size across the species having a slope greater than one. This is the case with *Mesalina watsonana* so that the males are larger than the females especially in those characters pertaining to head region. The possible importance of sexual dimorphism in taxonomic and ecologic criteria is discussed.

Sex Determination and Gonadal Development in the Common Toad *(Bufo bufo)*

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Amphibians have been traditionally used as animal models in embryology. Nevertheless, the knowledge about the mechanisms involved in sex determination and differentiation are very scarce

in this group. In the species *Bufo bufo* there are no sex chromosomes morphologically distinguishable. However, by analysing the sex ratio of the offspring of sex reversed animals it is possible to conclude that the females are the heterogametic sex in this species. That is, the species *Bufo bufo* has a ZZ/ZW chromosome system for sex determination. Regarding gonadal development, the species of the genus *Bufo* have the particularity of developing undifferentiated ovaries (Bidder's organs) in the cranial portion of male and female gonads. To study gonadal development in *B. bufo*, we are cloning in this species orthologous genes to those involved in gonadal development in other vertebrate groups. The study of the expression pattern of these genes during gonadal differentiation, and its relation with the morphological changes that take place, will enable us to establish their role in this process. The *SOX9* gene, involved in Sertoli cell differentiation, is among the genes we are studying. This gene codes for a transcription factor closely related to the *SRY* gene, is male-specific and necessary for testis differentiation. In *B. bufo* this gene is expressed only in developing male gonads, starting at the stage where morphological differences start to be observed between developing testes and ovaries.

The Effect of Dexamethasone on Testicular Structure during Neonatal Life

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The effect of dexamethasone on gonadal function during neonatal life has been studied in Wistar rats. The experimental approach consisted of treating newly-born rats with dexamethasone (a synthetic glucocorticoid) at the rate of 1 μg/5 g/day for 2–3 weeks. The treatment was effected by intra-peritoneal injection. Batches of control animals received the same volume of 0.9% NaCl. The weight of the rats was determined each day and they were killed at the end of each treatment. The organs were removed, weighed and fixed in Bouin-Holland fluid for structural analysis. A decrease in body ($p < 0.001$) and testicular weight is observed in treated animals in comparison with controls. Histological slices of the testes treated over 21 days show an increase in mean diameter of the seminal tubes. Spermatogenesis appears to be blocked at the spermatogonial stage for the majority of the seminal tubes. In the control rats, the cells of the germinal line are at spermatocyte stage 1. No significant variation in the average diameter of Leydig cells, of their nuclei and of the nuclei of Sertoli cells is observed by comparison to controls. Spermatogenesis of rats at 15 days is blocked at the spermatogonial stage; without significant difference between the treated animals and the controls. In conclusion, it seems that dexamethasone administered during neonatal life influences weight and spermatogenesis.

Identification of Novel Sex Determining Genes by Copy Number Analysis of Patients with Disorders of Sexual Development

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Disorders of sexual development, ranging in severity from genital abnormalities to complete sex reversal, are surprisingly common. The cause of these problems is most often the failure of the complex network of genes that regulate development of testes or ovaries. Mutations in the critical testis-determining genes *SRY* and *SOX9* account for approximately 20% of XY females with complete gonadal dysgenesis. We have little idea about what other genes do account for the remaining 80% of patients. In contrast, 90% of XX males with gonadal dysgenesis are due to Y translocations that include *SRY*. We have collected DNA from 34 patients with gonadal dysgenesis (XX males lacking *SRY* and XY females without mutations in *SRY*), which have been hybridized to Affymetrix Genome-Wide Human SNP Array 6.0. Copy number analysis has been performed with a custom designed algorithm. So far two causative rearrangements have been identified in known genes (a duplication of *DAX1* and a deletion of ~1 Mb in the upstream regulatory region of *SOX9*). Other rearrangements have been found encompassing candidate sex determination genes identified in mouse models, and there have been several genes deleted or duplicated that are not listed in the database of genomic variants. These are currently being confirmed and de novo status is being checked in parental DNA. A set of the most interesting candidate genes is being sequenced to identify small mutations. The combination of these powerful approaches is helping us to identify new genes and their regulatory regions involved in sex determination.

Histological and Immunohistochemical Analysis of the Vagina during Estrous Cycle of the Algerian Wild Lybian Jird *(Meriones libycus)*

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The female reproductive cycle of *Meriones libycus*, a nocturnal gerbillidae rodent, living in Algerian Sahara (Beni-Abbes, 30°7′ N, 2°10′ W) is characterized by a short breeding period at spring and beginning of summer, and a long quiescent sexual period from summer until the end of winter. In order to evaluate the

physiological variations and hormonal control affecting the genital tract, some histological and immunohistological methods were used to understand the cyclic variations in vaginal tissues. During the breeding cycle, vaginal epithelial cells showed cyclic variations. More particularly, keratinisation and exfoliation of epithelial cells were observed during the estrus phase; a proliferative activity of basal cells was also observed at this moment. Immunohistochemical methods revealed that the distribution of estrogen and progesterone receptors performed cyclic patterns. The highest labelling of all the hormone receptors was observed on the basal cells layer, showing that these ones can be implicated in vaginal regeneration.

A Conserved Role for *R-SPONDIN1* in Vertebrate Ovary Development

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R-SPONDIN1 (RSPO1) is a novel regulator of the Wnt/β-catenin signalling pathway. Loss-of-function mutations in human *RSPO1* cause testicular differentiation in 46,XX females, pointing to a role in ovarian development. Here we report the cloning and comparative expression analysis of *R-SPONDIN1* orthologues in the mouse, chicken and red-eared slider turtle, three species with different sex-determining mechanisms. Gonadal *RSPO1* gene expression is female up-regulated in the embryonic gonads in each species at the onset of sexual differentiation. In the embryonic mouse gonad, *Rspo1* mRNA is expressed in the somatic cell lineage of females, with little or no expression in germ cells. In the chicken embryo, *RSPO1* expression becomes elevated in females at the time of ovarian differentiation, coinciding with female-specific activation of the *FOXL2* gene and estrogen synthesis. RSPO1 protein in chicken is localised in the outer cortical zone of the developing ovary, the site of folliculogenesis and oocyte differentiation. Chicken *RSPO1* expression is down-regulated in embryos treated with an aromatase enzyme inhibitor, indicating that it is influenced by oestrogen. In the red-eared slider turtle, which exhibits temperature-dependent sex determination, female up-regulation of *RSPO1* occurs during the temperature-sensitive period, when gonadal development is responsive to temperature. Accordingly, *RSPO1* expression is temperature-responsive, and is down-regulated in embryos shifted from female- to male-producing incubation temperatures. These results indicate that *RSPO1* is up-regulated in the embryonic gonads of female vertebrates with different sex-determining mechanisms. Taken together, the findings indicate that *R-SPONDIN1* is an ancient, conserved part of the vertebrate ovary-determining pathway.

Effects of Laboratory Exposure to Cadmium-Contaminated Field Environments on Gonadal Development of Guppy *(Poecilia reticulata)*

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Exposure to low levels of cadmium, a known endocrine disruptor, may induce changes in gonadal development and differentiation resulting in adverse effects on animal reproduction. In Mae Sot District of Tak Province, Thailand, concerns have been raised over the contaminated sediment plume resulting from runoff and natural irrigation systems that leach cadmium from zinc mining areas into agricultural areas. Although there is no report of acute toxicity on aquatic animals in this area, the impact of long-term environmental exposure to cadmium on their reproduction is of attention. In this study, *Poecilia reticulata* was used to investigate effects of laboratory exposure to cadmium-contaminated field environments on the neonate fish. Experimental aquaria were set up using water and sediment samples collected from contaminated and reference sites in Tak Province. Guppies at the age of 1 day post partum were raised in each aquarium for 8 weeks. After exposure, all guppies were sampled for histological study of the gonads. Growth of the fish in term of mean standard length is not significantly different between guppies raised in the reference site or contaminated site conditions. The difference in sex ratio and gonadal development and differentiation will be compared. The implications of the effects of exposure to environmentally relevant doses of cadmium on sex differentiation and gonadal development of the neonate guppies will be discussed.

Retinoic Acid Triggers Meiosis Entry in the Urodele Amphibian *Pleurodeles waltl*

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Pleurodeles waltl is a urodele amphibian that displays a genetic mode of sex determination. Sex differentiation can later be modulated either by temperature or hormonal treatment. Like in mammals, germ cell lineage is specified during embryonic development through cell-cell interactions. Recently, retinoic acid (RA) was demonstrated to promote meiosis entry in female mouse foetuses while this event is delayed until puberty due to the degradation of RA in male gonads. To further investigate the time-course of germ cell specification and differentiation in *Pleurodeles waltl*, we analysed the expression of the germ cell marker VASA and the meiosis marker DMC1 during normal development. We show that germ cells specifically express VASA protein at hatching, enter meiosis in late larval life in females while this event occurs two months after metamorphosis in males. We set up a protocol for organotypic cultures of gonad-mesonephros

(GM) complexes and demonstrate that RA is necessary and sufficient to induce meiosis entry in *P. waltl*. Furthermore, we analysed Raldh2 and Cyp26b1, the enzymes required for RA synthesis and degradation respectively, in steroid-induced sex reversal conditions. The data indicate that meiosis entry depends on Cyp26b1 repression in the gonad. Taken together, these results confirm with molecular data the late specification of germline in urodeles, indicate for the first time that RA-dependent meiosis entry could be a conserved mechanism of germ cells differentiation between urodeles and mammals and provide evidence for complex cross-talks between steroid production and RA biosynthesis in the course of sex differentiation.

Sex Determination and Sex Chromosome Evolution in the Platyfish *Xiphophorus maculatus*

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In contrast to the situation observed in mammals and birds, fish display an amazing diversity of sex determination systems. Almost nothing is known about the molecular and evolutionary basis of this phenomenon. Bacterial artificial chromosome (BAC) contigs including the sex-determining region of the platyfish *Xiphophorus maculatus* and covering 2.5–3.5 megabases of the X and Y chromosomes have been constructed and sequenced. Thirty-nine BAC clones have been sequenced so far to completion, leading to the identification of 63 sex chromosomal genes. Interestingly, eleven of these genes are also located on human sex chromosomes, but no synteny was found with other fish gonosomes. Through comparison between X and Y sequences, the sex-determining region has been delimited. Gene candidates have been identified for all loci involved in pigmentation, cancer formation and sexual development that are linked to the master sex-determining gene of the platyfish. Numerous genes, including the melanocortin hormone receptor gene *mc4r*, are amplified in the sex-determining region of *X. maculatus*. Gonad-specific genes were identified, including two genes with so far unknown function organized in tandem and exclusively expressed in oocytes. One gene, called *swimy*, is present on the Y but not on the X chromosome and represents an excellent candidate for the master sex-determining gene. This gene encodes a ubiquitously expressed protein, with different predicted domains involved in nucleic acid binding and protein-protein interactions. Interestingly, through the use of an alternative exon in spermatogonia, a testis-specific longer isoform is produced, with different domains involved in protein modification.

Sex Differentiation of the Urodele Amphibian *Pleurodeles waltl*

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In the sexually dimorphic urodele amphibian *Pleurodeles waltl*, sex determination obeys female heterogamety. Female (ZW) and male (ZZ) genotypes can be deduced from tail biopsy samples submitted to a biochemical analysis of the sex linked marker peptidase-1. The first histological changes in the differentiating gonad are observed at the end of larval life (stage 53): germ cells are localized in the cortex of the ovary but in the medulla of the testis. Meiosis entry occurs before metamorphosis in females (stage 54 + 2 months) whereas it occurs only two months post-metamorphosis in males. Experiments using organotypic cultures suggest that retinoic acid is involved in this event. The differentiation of the testis is very astonishing: the caudal extremity initiates spermatogenesis whereas the cephalic part remains in an undifferentiated status and contains primordial germ cells whose meiosis entry is delayed. Besides, the differentiation of the testis takes place lifelong and leads to several lobes named 'multiple testis'. This model displays a few original features that can be helpful for the understanding of sex differentiation. First, when genetically female larvae (ZW) are reared at 32°C (instead of 20°C) during a thermo-sensitive period (stage 42 to stage 54), they develop as phenotypic fertile males. Several studies have demonstrated that estrogen synthesis is inhibited in case of temperature-induced sex reversal and have pointed out steroids as key factors in the sex differentiation pathway. Second, parabiosis experiments can be performed. Ovarian development is impaired but testis differentiation is normal suggesting a role of other hormones, maybe AMH.