Genetic control of Seasonal Reproductive cycle in *Talpa occidentalis*

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Genetic control of Seasonal Reproductive cycle in *Talpa occidentalis*

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Rajesh Kumar Dadhich

Dedicated to my parents
and the whole family

When things go wrong, as they sometimes will,
When the road you're trudging seems all uphill,
When the funds are low and the debts are high,
And you want to smile, but you have to sigh,
When care is pressing you down a bit,
Rest, if you must, but don't you quit.
Life is queer with its twists and turns,
As every one of us sometimes learns,
And many a failure turns about,
When he might have won had he stuck it out;
Don't give up though the pace seems slow–
You may succeed with another blow.
Often the goal is nearer than,
It seems to a faint and faltering man,
Often the struggler has given up,
When he might have captured the victor's cup,
And he learned too late when the night slipped down,
How close he was to the golden crown.
Success is failure turned inside out–
The silver tint of the clouds of doubt,
And you never can tell how close you are,
It may be near when it seems so far,
So stick to the fight when you're hardest hit–
It's when things seem worst that you must not quit.

- Author unknown

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Summary

The production of gametes (gametogenesis) is a requisite for sexual reproduction. Depending on environmental factors, mainly latitude, species may have continual reproduction, which implies uninterrupted gametogenesis, or seasonal reproduction, in which gametogenesis (gonads) activate and inactivate cyclically every year. Testis physiology and its genetic and endocrine control has been studied for many decades in model species like mice, rats and human, which are continual breeding species. However, very little efforts have been made to study seasonal reproduction, which provides us an excellent opportunity to study naturally established mechanisms by which gametogenesis function may be accommodated to the environmental requirements. Most studies carried out on the control of seasonal breeding have been performed in domesticated animals, mainly hamsters, or in semi-wild animals like the roe deer. Studies made in wild animals like the brown hare have been very scarce and focused on particular aspects of seasonal breeding.

Hence, to date no wild animal had been the subject of a comprehensive study in which the different aspects of seasonal breeding control were approached. In the present study we have analised *T. occidentalis*, a species of mole, which shows seasonal reproduction. This species breed during the winter and inactivate during summer. Many morphological changes occur in the gonads of both males and females throughout the annual reproductive cycle. In males, testes are larger during the breeding season and smaller during the non-breading season, so that testicular weight in December may quadruple that in July. Most cells present in the germinative epithelium of fertile testes disappear when they become non-fertile testes, and appear again in the next autumn, when testis function reactivates. In this species, we have studied, for the first time, the main aspects of seasonal variations in the testis, including: 1) the expression patterns of several genes involved in

testis development, like *SOX9*, *DMRT1*, *SF1* and *AMH*, 2) androgen levels and the localization and expression levels of their receptors, 3) seasonal dynamics of somatic and germ cells, 4) apoptosis and cell proliferation, 5) seasonal variations in cell junctions and the status of the blood-testis barrier, and 6) the structural integrity of the *lamina propria* of seminiferous tubules.

This comprehensive study has provided several interesting results. We have shown that the seasonal breeding in the mole is not regulated at the level of meiosis onset, as this is maintained at a constant rhythm throughout the year. Our data suggest that there is a hormonal control exerted by the hypothalamic-pitutary-gonadal axis and that genes like *SOX9* and *DMRT1* may be a part of the testicular mechanism that respond to this hormonal control. We have also shown that inactive adult and pre-pubertal testes are very similar but not identical to each other as genes like *AMH* and *OCT4* which are active in the former, remain permanently repressed in the later. The spermatogonial pool in the mole testis probably fluctuates throughout the breeding cycle due to an imbalance between the cellular processes controlling it: apoptosis, cell proliferation and the rhythm of meiosis onset. In addition, we have shown that apoptosis is not responsible for the massive depletion of germ cells that occurs during testis involution. Rather, it a consequence of cell junction disruption, which may lead to Sertoli-germ cell detachment. The disorganization of the blood-testis barrier in testes of non-breeding moles supports this hypothesis.

In temperate areas of the earth the climatic conditions vary seasonally and lead living organisms to modulate their activity also according to a seasonal rhythm. Reproduction is the biological process in which this influence becomes more evident. The current climate change proceeds inexorably and its negative influence on the fitness of seasonal breeding vertebrates remains uncertain, but may be potentially irreversible in many cases. Hence, having a precise knowledge of the environmental and genetic factors controlling seasonal breeding is an essential tool to face and counteract those probable negative effects. The present study in the mole may increase our understanding of these biological processes.

Resumen

La producción de gametos (gametogénesis) es un requisito para la reproducción sexual. Dependiendo de factores ambientales, principalmente la latitud, las especies puedes presentar reproducción contínua, que implica una gametogénesis ininterrumpida, o reproducción estacional, en la que la gametogénesis, (gónada) se activa e inactiva cícliamente cada año. La fisiología testicular y su control genético y endocrino han sido estudiados durante muchas décadas en especies modelo tales como el ratón, la rata y los propios humanos, que son especies de reproducción contínua. Sin embargo, poco esfuerzo se ha dedicado al estudio de la reproducción estacional, que proporciona una excelente oportunidad para analizar los mecanismos que la naturaleza ha establecido para que la gametogénesis se acomode a los requerimientos ambientales. La mayor parte de los estudios realizados sobre el control de la reproducción estacional se han llevado a cabo en animales domésticos, principalmente hamsters, o en animales semi-salvajes, tales como el rebeco. Los estudios realizados en animales salvajes, como la liebre marrón, han sido muy escasos y solo enfocaban aspectos muy particulares de la reproducción estacional.

Por tanto, ninguna especie salvaje de mamíferos ha sido objeto hasta la fecha de un estudio ambicioso en el que los distintos aspectos del control de la reproducción estacional fuesen abordados. El presente estudio se ha centrado en la especie de topo *Talpa occidentalis*, que se reproduce de manera estacional. Esta especie cría durante el invierno, mientras que inactiva sus gónadas durante el verano. Muchos cambios morfológicos ocurren en dichas gónadas, tanto en los machos como en las hembras, a lo largo del ciclo reproductivo anual. En los machos, los testículos son mucho más grandes durante la época de cría, de manera que su peso en diciembre cuadruplica el de julio. La mayor parte de las células presentes en el epitelio germinativo de los testículos fértiles desaparecen cuando se hacen inactivos, y aparecen de nuevo en el siguiente otoño, cuando se reactiva la función tes-

ticular. En esta especie hemos estudiado, por primera vez, los principales aspectos de las variaciones estacionales que afectan a los testículos. Éstas son las siguientes: 1) Los patrones de expresión de varios genes implicados en el desarrollo testicular, tales como *SOX9*, *DMRT1*, *SF1* y *AMH*, 2) La concentración de andrógenos y la localización y niveles de expresión de sus receptores, 3) la dinámica estacional de las células somáticas y germinales, 4) los procesos de apoptosis y proliferación celular, 5) las variaciones estacionales que afectan a las uniones intercelulares y el estatus de la barrera hemato-testicular y 6) la integridad estructural de la *lámina propia* de los túbulos seminíferos.

Este amplio estudio ha proporcionado varios resultados interesantes. Hemos mostrado que la reproducción estacional en el topo no está regulada a nivel del inicio de la meiosis, ya que ésta se mantiene a un ritmo constante a lo largo de todo el año. Nuestros datos sugieren que hay un control hormonal ejercido por el eje hipotálamo-pituitaria-gónada, y que genes tales como *SOX9* y *DMRT1* pueden ser parte del mecanismo testicular de respuesta a este control hormonal. También hemos mostrado que los testículos adultos inactivos y los prepuberales son muy similares, pero no idénticos, entre sí, ya uqe genes tales como *AMH* y *OCT4*, que están activos en los primeros, quedan permanentemente reprimidos en los segundos. La dotación de células espermatogoniales en el testículo de topo, probablemente fluctúa a lo largo de su ciclo reproductivo debido a un desequilibrio entre los distintos procesos celulares que lo controlan: apoptosis, proliferación celular y ritmo de entrada en meiosis. Además, hemos demostrado que la apoptosis no es responsable de la pérdida masiva de células germinales que tiene lugar durante la involución testicular de *T. occidentalis*. Más bien es una consecuencia de la desorganización de las uniones intercelulares, que puede conducir al desengarce entre las células de Sertoli y dichas células germinales. La desorganización de la barrera hematotesticular, en testiculos inactivos de topo apoya claramente esta hipótesis.

En las áreas templadas de la tierra, las condiciones climáticas varían estacionalmente y llevan a los seres vivos a modular su actividad de acuerdo con ese ritmo estacional. La reproducción es el proceso biológico en el que dicha influencia se hace más evidente. El actual cambio climático parece avanzar inexorablemente, y su influencia negativa sobre la eficacia biológica de los vertebrados con reproducción estacional permanece incierta, aunque podría ser potencialmente irreversible en muchos casos. Por tanto, el conocimiento preciso de los factores ambientales y genéticos que controlan la reproducción estacional es una herramienta esencial para hacer frente, y contrarrestar, esos posibles efectos negativos. Este estudio en el topo puede contribuir a incrementar nuestro entendimiento de tales procesos biológicos.

Introduction

Sexual reproduction in animals depends on correct gonadal development and function in both males and females. Sex determination is the genetic process by which the sex of a particular individual is decided. The direct consequence of sex determination is sex differentiation, which represent the effective development of the individual as a male or a female. In mammals, sex differentiation takes place in two steps. In the first, the undifferentiated gonadal primordia, which are present in both males and females, develop either as testes, or as ovaries. In marsupials and placental mammals, this process depends on the presence of the Y-linked gene *SRY*. This gene acts as the genetic switch for male development by triggering a gene cascade where genes of the male pathway (*SF1*, *SOX9*, *FGF9*, *DMRT1*, *DAX1*, *AMH*, among others) are upregulated, whereas those of the female pathway (*RSPO1*, *WNT4*, *FST*, for instance) are downregulated. Expression of *SRY* at the time of sex determination results in the formation of testes in males (XY). Its absence in females (XX) results in ovarian differentiation at some point later on in development. In the second step, once the gonads are differentiated as testes, the androgens produced by the testis directs the differentiation of the urogenital tract of the male embryo, thus resulting in the formation of the male secondary sex features, including external genitalia. In the absence of testicular androgens, a female phenotype develops. Many of the cited genes are known to be expressed also in the adult gonad, where their functions remain unclear in most cases.

Most wild mammals are seasonal breeders in non-equatorial areas of the Earth. In this animals, gonads undergo circannual variations in their functional condition, so that they are activated and inactivated in a cyclic manner, annually. This process implies the involvement of numerous genetic, endocrine, paracrine and morphogenetic factors which are poorly understood and deserve further research.

The current reproductive biology of seasonal breeding mammals is the result of a long period of adaptation to a seasonally-changing climatic environment. Diverse evidences suggest that drastic climatic changes may influence very negatively the reproductive success in many of these species and hence, the species survival. So, increasing our knowledge about the hormonal and genetic systems controlling seasonal reproduction becomes a priority when a climatic change has been announced to be under way.

In this work we have used the mole *Talpa occidentalis* as a seasonal breeding animal model to study several aspects of gonadal function in both males. These include 1) the possible role that particular genes known to be involved in gonad development, may have in the adult gonadal function and circanual variation, 2) the role of particular hormones in the regulation of the seasonal breeding, 3) the role of some morphogenetic processes (mainly apoptosis and cell proliferation) accompanying these cyclic variations and 4) the possible involvement of cell junctions in the regulation of the processes of testis activation and inactivation.

3.1 Genes involved in gonad development

As indicated above, many genes related to gonad development are also known to be expressed in the adult gonad where their roles, if any, remain obscure. Some of the most relevant genes involved in testis development are reviewed below.

3.1.1 Genes involved in testis development and function

The genetic mechanism of testis determination was the first one to be known, after the discovery of the tesis-determining gene *SRY* in 1990 (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990). This and other genes currently known to be involved in testis determination are reviewed here.

SRY

SRY (Sex related gene on chromosome Y), a transcriptional regulator which control a genetic switch in male development, is necessary and sufficient for initiating male sex determination by directing the development of supporting precursors cells (pre-Sertoli cells) as Sertoli cells, rather than granulosa cells (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990). The open reading frame (ORF) of human *SRY* is contained within a single exon and encodes a 204-amino-acid protein. The protein can be divided into three regions. The central 79 amino acids encode the HMG domain, which functions as a DNA-binding and DNA-bending domain and also contains two nuclear

localization signals. The human SRY C-terminal domain has no obvious or conserved structure, except for the final seven amino acids, which interact *in vitro* with a PDZ domain protein (Poulat *et al.*, 1997). The N-terminal region of the protein also has no obvious structure, but phosphorylation of a sequence within this domain enhances DNA binding activity (Desclozeaux *et al.*, 1998). Comparison of the amino acid sequence of the SRY HMG domains from human, mouse, rabbit, wallaby and sheep reveal 70% identity. In contrast, there is no sequence conservation outside the HMG domain.

The timing of *Sry* expression in the mouse is entirely consistent with a role in sex determination. *Sry* is detected in the genital ridge at 10.5 dpc–12.5 dpc, but by 12.5 dpc, *Sry* is detectable only at low levels (Koopman *et al.*, 1990). Sex-specific differences are apparent at about 11.5 dpc when the male gonad takes on a striped appearance, probably due to cells of the supporting cell lineage differentiating into Sertoli cells and aligning into testis cords, the presumptive seminiferous tubules. In humans, as in mice, the onset of *SRY* mRNA expression defines testis determination. The human gonadal ridge forms around 33 d gestation, and *SRY* is detected at 41 d in XY embryos. *SRY* levels peak at 44 d, when the testis cords are first visible (Hanley *et al.*, 2000). Unlike in the mouse, human *SRY* is not downregulated in the gonads and continues into adulthood. By 52 d gestation in the human the germ cells are surrounded by Sertoli cells, which continue to express *SRY* at a low level (Hanley *et al.*, 2000), suggesting other roles for this gene, possibly in spermatogenesis. DNA sequencing has demonstrated that *SRY* is found in most XX males, and that *SRY* point mutations or deletions are found in only about 15% of XY females (Hawkins *et al.*, 1992). This supports the existence of mutations in sex-determining genes other than *SRY*. A number of genes are expressed soon after the expression of *SRY*, e.g., *SOX9*, *FGF9*, *DHH*, and *VNN1*. Recently, it was reported that *Sry* activates a *Sox9* enhancer thus promoting the upregulation of this gene at the time of sex determination in the mouse (Sekido and Lovell-Badge, 2008). Shortly after the induction of *Sry*, *Sox9* becomes activated, moving from the cytoplasm to the nucleus, and up-regulated in the male gonad. Recent studies have implicated two proteins in nuclear import of Sry: calmodulin (CaM) and β-importin (Argentaro *et al.*, 2003; Schmahl *et al.*, 2000a). In the mouse, the expression of both *Sf1* (Schmahl *et al.*, 2000a) and *Sp1* (Marin *et al.*, 1997) in early embryogenesis is consistent with a role for these proteins in regulating mouse basal *Sry* expression *in vivo*. However the role of *Sry* in seasonal breeders have not been studied till now.

SOX9

The human *SOX9* gene (SRY related High Mobility Group related protein-9) encodes a protein of 509 aa consisting of the HMG box, which shares 70% amino acid homology to the HMG box of SRY. High-resolution mapping refined the location of *Sox9* to 17q24.3-25.1. In addition, the SOX9 protein contains additional protein domains, including two transcriptional activation domains, downstream of the HMG box. The SOX9 protein, unlike SRY, is very highly conserved through vertebrate evolution. The SOX9 HMG domain, like that of SRY, acts by binding (and bending) to specific DNA sites to activate transcription of target genes. A large majority of mutations in *SOX9* are located within the HMG box, suggesting its ability to efficiently bind to target sites is a critical factor. The HMG box of SOX9, like all SOX proteins tested, binds to the SRY high-affinity binding site, AACAAT, and the TCF1 site, AACAAAG (Zurita *et al.*, 2007a). Several studies have shown that the SOX9 protein acts as a potent transcriptional activator both in vitro and in vivo. The SOX9 protein is known to activate transcription of the type II collagen gene and anti-Mullerian hormone (De Santa *et al.*, 1998a). *Sox9* has been implicated in numerous developmental processes including chondrogenesis, formation of cardiac valves, and neural crest, testis and spinal cord development (Barrionuevo *et al.*, 2006b). *Sox9* heterozygous knockout mice die at birth with a syndrome similar to that of human campomelic dysplasia. Conditional inactivation of both *Sox9* alleles in the gonadal anlagen using the CRE/loxP recombination system, leads to complete sex reversal, as shown by expression of the early ovary-specific markers Wnt4 and Foxl2 and by lack of testis cord and Leydig cell formation (Barrionuevo *et al.*, 2006a). The expression profile of *SOX9* was shown to be sexually dimorphic in a number of species including humans, mouse, chicken, turtle, and alligator (Western *et al.*, 1999; Spotila *et al.*, 1998; Wright *et al.*, 1995). Up-regulation of *SOX9* is a key phenomenon in all vertebrates, regardless of the switch mechanism controlling sex determination, i.e., *SRY* in mammals [except for the mole vole (Just *et al.*, 1995), ZW chromosome gene/s in birds (Oreal *et al.*, 1998), and temperature sensitivity of egg incubation in turtles and crocodiles Moreno-Mendoza *et al.* (1999); Western *et al.* (1999). During mouse gonadogenesis, following *Sry* expression, *Sox9* upregulation is the earliest marker of pre-Sertoli cells (Schmitt-Ney *et al.*, 1995; Tho *et al.*, 1998). Sry and Sox9 share a highly conserved HMG box, such that in domain-swapping experiments, the Sox9 HMG box can functionally substitute the Sry HMG box in XX transgenic mice (Mitchell and Harley, 2002). Sox9 co-localizes with Sry in the nucleus of pre- Sertoli cells as early as 11.5 dpc (Lovell-Badge *et al.*, 2002). As the Sox9-expressing population expands between 11.5-12.5 dpc, the number of

cells that co-express Sry decreases until 12.5 dpc, when Sry expression is extinguished and Sox9 expression is confined to Sertoli cells inside cords and persists in adults. Human *SOX9* shows a similar specificity of expression in the developing testis (De Santa *et al.*, 2000). The up-regulation of *SOX9* in Sertoli cells immediately after expression of *SRY* suggests a role for *SRY* in the activation of *SOX9* expression De Santa *et al.* (2000). As indicated above, now it is known that *Sox9* is upregulated by *Sry* through a 5' enhancer (Sekido and Lovell-Badge, 2008). Also, persistent expression of *Sox9* after down-regulation of *Sry* in the mouse supports a role for Sox9 in positively regulating its own expression (Koopman *et al.*, 2001). Ectopic activation of *Sox9* in XX Odsex (Ods) mice, or expression of a *Sox9* transgene (*Wt1*:*Sox9*) in XX embryos produce fully sex-reversed (but sterile) male mice (Scherer *et al.*, 1998).

The constitutive expression of *SOX9* appears to be sufficient to promote testis determination. The exact role of SOX9 in Sertoli cell differentiation, however, is not known. One gene in the Sertoli cell lineage, the expression of which SOX9 was shown to directly regulate, is *AMH*, a marker of Sertoli cells, causing the regression of the female (Mullerian) reproductive tract (Shen *et al.*, 1994a; De Santa *et al.*, 1998b). Deoxyribonuclease 1 footprint analysis showed that SOX9 binds to a *SOX*-like site in the *AMH* proximal promoter, adjacent to the MISRE1 site and, together with SF1 (steroidogenic factor 1), WT1 (Wilms tumor gene 1), and GATA4, directly activates expression of *AMH* (De Santa *et al.*, 1998b; Arango *et al.*, 1999; Tremblay and Viger, 2001a). The SOX9 HMG box, similar to SRY, was found to interact directly with the transport receptor Importin β and CaM mediated pathway (Argentaro *et al.*, 2003).

Regarding the expression and role of SOX9 in the testis of adult mammals, available data are scarce. It is known that SOX9 expression is maximum at the stages of active germ cell mitosis and meiosis i.e. in 15 day old rat testis and also at stage VIII of spermatogenesis in adult testis (Frojdman *et al.*, 2000). The age and stage-specific presence of SOX9 in the testicular cords and in the seminiferous tubules of the adult rats suggests that SOX9 also may have a pivotal role in germ cell differentiation. However, nothing is known about the possible role of SOX9 in the regulation of seasonal breeding.

SF1

Sf1 (Steroidogenic factor-1, also known as *Ad4BP*) is an orphan nuclear receptor that regulates the transcription of an array of genes involved in reproduction, steroidogenesis and male sexual differentiation.

Sf1 is first expressed in the urogenital ridge of the mouse at E9.5 (Ikeda

et al., 1994, 2001) and is one of the earliest markers of the coelomic epithelial cells, the precursors of the somatic lineages of the gonad (Luo *et al.*, 1994). In the male gonad, Sf1-positive cells can be found in a population of coelomic epithelial cells that give rise to both Sertoli and interstitial cell precursors (Schmahl *et al.*, 2000b). SRY is expressed exclusively in these precursor cells, which then show rapid upregulation of *Sox9* (Sekido *et al.*, 2004). Once Sox9 has reached a critical threshold, *Sry* is repressed by means of a Sox9-dependent negative feedback loop (Chaboissier *et al.*, 2004; Sekido *et al.*, 2004). *Sf1* and *Sox9* expression are subsequently maintained at high levels in Sertoli cells (Ikeda *et al.*, 2001). Mutation, co-transfection and sex reversal studies all point to a feed-forward, self-reinforcing pathway in which Sf1 and Sry cooperatively upregulate Sox9 and then, together with Sf1, Sox9 also binds to its enhancer to help maintain its own expression after that of SRY has ceased (Sekido and Lovell-Badge, 2008). Hence, Sf1 is indispensable for the survival and proliferation of progenitor somatic cells of the male gonad. In the Sertoli cell, Sf1 functions within a regulatory complex that also involves Sox9, Wilms' tumor (Wt1) protein, and the GATA4 transcription factor to promote Anti-Müllerian hormone (Amh) expression at 11.5 dpc (Shen *et al.*, 1994b; Nachtigal *et al.*, 1998; de Santa Barbara *et al.*, 1998; Viger *et al.*, 1998).

By 14.5 dpc, Sf1 immunostaining can be detected in the fetal Leydig cells (FLC) (Hatano *et al.*, 1994), which reside in the interstitial compartment between the testis cords. In FLCs, Sf1 controls the expression of steroid biosynthetic enzyme genes including Steroidogenic acute regulatory protein (StAR), Cyp11a1 (cholesterol side-chain cleavage, cytochrome P450scc), 3β-hydroxysteroid dehydrogenase and Cyp17, which encode proteins that mediate the step-wise conversion of cholesterol to testosterone (Morohashi *et al.*, 1992; Leers-Sucheta *et al.*, 1997; Reinhart *et al.*, 1999). Later in testis development, Sf1 expression intensifies in the interstitial Leydig population where it regulates the expression of multiple steroidogenic enzyme genes necessary for testosterone production (Ikeda *et al.*, 1996; Morohashi *et al.*, 1993). Expression peaks in the mouse male gonad at E11.5 and then remains relatively low until E17.5 when it increases significantly (Ikeda *et al.*, 2001).

Homozygous deletion of the gene encoding Sf1 in mice results in adrenal and gonadal agenesis, complete XY sex reversal with persistence of Mullerian structures in males, and abnormalities of the hypothalamus and pituitary gonadotropes. Gonadotropin release was preserved, but testicular development was severely affected. *SF1* mutation causes complete XY sex reversal, including normal female external genitalia and retention of the uterus. This contrasts with disorders of steroid biosynthesis, in which no uterus is present. SF1 regulates the regression of Müllerian structures in

humans, either through direct actions on the *AMH* promoter or secondary to an abnormality of Sertoli-cell development or function.

WT1

WT1 (Wilms' tumor suppressor gene 1) is involved in several human syndromes (Gessler *et al.*, 1990; Baird *et al.*, 1992; Barbaux *et al.*, 1997; Jaubert *et al.*, 2003). It is a complex gene comprising 10 exons and 9 introns. The first six exons encode a transcription regulation domain rich in proline and glutamine, and the four last four domains encode Zinc finger domains (Call *et al.*, 1990; Gessler *et al.*, 1992). A noteworthy feature of this gene is the presence of two regions with alternative splicing located in the exons 5 and 9 (Gessler *et al.*, 1992; Haber *et al.*, 1991). The inclusion of exon 5 during mRNA maturation inserts a 17 amino-acid segment inside the transcription regulation domain, whereas the non-elimination of a region of exon 9, implies the addition of a small sequence of 3 amino acids known as KTS (serine-threonine-lysine) between the zinc fingers 3 and 4. WT1 isoforms are classified as +KTS and -KTS depending on whether the protein carries these three amino acids or not. It has been suggested that the +KTS isoforms, which have RNA-binding sites, may integrate into spliceosomes thus regulating the splicing process of other genes, whereas the -KTS isoforms, which have DNA-binding sites, could act as transcription factors (Bickmore *et al.*, 1992; Hammes *et al.*, 2001).

In mouse, *WT1* is expressed for the first time at 9 dpc in the intermediate mesoderm, in the area where the genital ridge is going to appear shortly later. Subsequently, *WT1* expression is detected in the coelomic epithelium and in the sex cords of the gonadal primordium in both sexes, as well as in the adjacent mesonephros (Armstrong *et al.*, 1993). *WT1* expression is maintained in Sertoli cells in the testis and in follicle cells in the ovary until adulthood. *WT1* is also expressed in uterus (embryonic and adult), oviducts and endometrium of females (Pelletier *et al.*, 1991).

In humans, expression of *WT1* is seen for the first time in the seventh week of gestation, in both the genital ridge and mesonefros (Mundlos *et al.*, 1993; Pritchard-Jones *et al.*, 1990). It is known that problems in the development of patients with testicular WAGR (WT-aniridia-genitourinary tract abnormalities-mental retardation) are due to *WT1* hemicigosis (Baird *et al.*, 1992). Also, the origin in cases of DDS (Denys-Drash syndrome) is the existence of dominant mutations affecting the zinc finger domains encoded by exons 7-10, a fact that can lead to cases of male pseudohermaphroditism (Little *et al.*, 1995). Cases of FS (Frasier syndrome), in turn, are caused by point mutations in intron 9 that affect the sequence of KTS maturation (Barbaux *et al.*, 1997). A reduced fraction +KTS/-KTS produces feminiza-

tion of the testis that may lead to cases of male-to-female sex reversal. This, together with the fact that mice expressing only the -KTS isoform show this type of sex reversal due to a drastic reduction in the expression levels of Sry (Hammes *et al.*, 2001), shows the importance of the role that the +KTS isoforms probably play in male sex differenciation.

All these data suggest that WT1 is involved in the development of the genital ridge and is required for the survival of the gonadal primordium (Hammes *et al.*, 2001). Moreover, WT1 seems to be necessary for 1) the maintenance of the testis cords structure (Gao *et al.*, 2006), 2) the diferenciation of Sertoli cells 3) the survival and proliferaction of embryonic germ cells and 4) the development and function of fetal Leydig cells (Natoli *et al.*, 2004).

FGF9

Fibroblast growth factors (FGF) are a group of proteins with several roles in a variety of biological processes, such as proliferacion, diferenciation, migration and survival of many cell types. To to carry out its function, the FGFs interact with heparin and acts through their receptor FGFR (fibroblast growth factor receptors) in the cell membrane, thus, initiating a cascade of intracellular signals (Ornitz and Itoh, 2001).

A member of this family is *FGF9*, which is located at the 13q11-12 band in the human chromosome 13 (Mattei *et al.*, 1995), and encodes a protein of 208 amino acids (Miyamoto *et al.*, 1993). During mouse embryogenesis, *Fgf9* is expressed in a wide variety of tissues, including the intermediate mesoderm, the myocardium ventricle, the pulmonary pleura, the myoblasts, the motor neurons of the backbone and the olfactory bulb (Colvin *et al.*, 1999). Regarding gonad development, the expression of *Fgf9* is first detected in the undifferentiated gonadal primordium. Later, at 11.5 dpc, *Fgf9* expression disappears in the XX gonad and increases in the XY gonad, shortly after *Sry* expression. Like *Sry*, *Fgf9* expression also occurs in the somatic cells of the testis cords (Kim *et al.*, 2006; Schmahl *et al.*, 2004).

It has been suggested that FGF9 and WNT4 play important roles in sex determination by acting in an antagonistic manner. This hypothesis is based on recent work showing that Wnt4 may repress *Fgf9* in the gonads of XX mice homozygous for a null allele of *Wnt4* (Kim *et al.*, 2006). Transgenic mice lacking Fgf9 die shortly after birth due to defects in the lungs, but they show XY sex reversal (Colvin *et al.*, 2001a,b). In these mice, testicular differentiation is aborted by 12.5 dpc as pre-Sertoli cells fail to proliferate and differentiate. Consequently, no cell migration occurs from the mesonephros and no testis cords are formed (Colvin *et al.*, 2001a; Schmahl *et al.*, 2004). The absence of Fgf9 does not affect *Sry* expression or

its control of *Sox9*, but disturb the maintenance of proper Sox9 levels after sex determination (Kim *et al.*, 2006). Furthermore, *Fgf9* XY mutants experience a massive, apoptosis-mediated loss of germ cells, and the expression of ovary-specific molecular markers at 12.5 dpc (DiNapoli *et al.*, 2006). All these data indicate that *FGF9* gene is essential for testicular development, as it is necessary for Sertoli cell proliferation and differentiation, and for the survival of XY germ cells (DiNapoli *et al.*, 2006; Schmahl *et al.*, 2004). Thus, FGF9 may favour the maintenance of *SOX9* expression in Sertoli cells and repress the expression of genes of the female pathway such as *WNT4* (Kim *et al.*, 2006).

FGF proteins are highly conserved among metazoa. These factors are present in invertebrates and in all groups of vertebrates analyzed (Itoh and Ornitz, 2004). Many orthologs of FGF9 have been identified in the chicken (Garcès *et al.*, 2000) and the amphibious Rana rugosa, where it is involved in gonadal development (Yamamura *et al.*, 2005).

DHH

DHH (Desert hedgehog gene) encodes a homolog of the hedgehog protein of *Drosophila* (Bitgood and McMahon, 1995). In mice it is expressed in Sertoli cells shortly after *Sry* expression (Bitgood *et al.*, 1996). Mice homozygous for a null allele of *Dhh* showed a blockage in the differentiation of fetal Leydig cells. In the adult life, these mutants are defective in spermatogenesis, development of adult Leydig cells and peritubular myoid cells (Bitgood *et al.*, 1996; Pierucci-Alves *et al.*, 2001; Yao *et al.*, 2002). *DHH* mutations cause either partial or total gonadal dysgenesis in XY humans, as a result of defective Leydig cell development (Canto *et al.*, 2004, 2005; Umehara *et al.*, 2000). These data indicate that DHH is a protein necessary for spermatogenesis and of Leydig cell differentiation in both embryonic as well as adult gonads.

DAX1

In humans, duplication of a region of 160 Kb in the short arm of the X-chromosome provokes gonadal dysgenesis and development of ambiguous external genitalia or female individuals XY (Bardoni *et al.*, 1994). This was called the dosage-sensitive sex-reversal locus (*DSS*). Furthermore, deletions or point mutations in this locus cause X-linked congenital adrenal hypoplasia (AHC), a disease that produces insufficient adrenal hypogonadotropism and hypogonadism in XY individuals (Zanaria *et al.*, 1994). A gene named *DAX1* (DSS-AHC critical region on the X chromosome, gene 1) was found in this critical region of the X chromosome (Muscatelli *et al.*, 1994; Zanaria

et al., 1994).

DAX1, also known as *NR0B1* (nuclear receptor type subfamily 0, group B, member 1 gene) is located on the Xp21 band (Bardoni *et al.*, 1994; Zanaria *et al.*, 1994). It contains two exons that encode a 470 amino acids protein which belongs to the family of nuclear receptors (Bae *et al.*, 1996). At the carboxy-terminal end, this protein contains a ligand-binding domain (LBD), similar to that of the rest of members of this family, comprising 3.5 repetitions of a sequence of 65-67 amino acids, which contains two Zinc finger domains (Guo *et al.*, 1996; Lalli *et al.*, 2000).

The expression of *DAX1* is linked to that of *SF1*. Studies in mice show that both genes express in developing and adult tissues like the adrenal gland, gonads, hypothalamus and pituitary gland (Ikeda *et al.*, 1996), indicating that *Dax1* may also be involved in the hypothalamus-gonad axis. During gonadogenesis, *Dax1* is expressed in the gonadal primordium of male and female mice a little later than *Sf1*. In the XY gonad, there is a significant increase in its expression in Sertoli cells up to 12.5 dpc. This expression decreases rapidly but increases again in interstitial cells between 13.5 and 17.5 dpc. In the XX gonad, the expression of *Dax1* increases between 12.5 and 14.5 dpc and thereafter begins to decline (Ikeda *et al.*, 2001).

In humans, there is a subtle expression of *DAX1* in the genital ridge of both XX and XY individuals, which is maintained as the gonad remains undifferentiated. During testis differentiation, this expression is localized mainly in the Sertoli cells of the developing testis cords in 52 days post-ovulation (dpo) embryos. Similarly, *DAX1* is expressed in the somatic cells in the ovary development. These low levels continously expressed in the fetal testis and ovary at 18 and 15 weeks of gestation respectively (Hanley *et al.*, 2000). It has been suggested that the WNT4 protein can stimulate the expression of *DAX1*, either directly or indirectly, as this gene expresses in individuals who carry a duplication of *WNT4* (Mizusaki *et al.*, 2003). Furthermore, some KTS-isoforms of WT1 appear to be involved in the activation of the *DAX1* promoter during early gonadogenesis (Kim *et al.*, 2001).

It has been shown that DAX1 is involved in steroidogenesis. In vitro studies indicate that DAX1 antagonizes the activating function of SF1 on genes involved in the synthesis of steroid hormones (Gurates *et al.*, 2003). In this regard, AHC could be caused by mutations in the LBD, which would cause a decrease of this antagonistic effect (Achermann *et al.*, 2001; Ito *et al.*, 1997). Similarly, the interactions between SF1 and DAX1 in gonadal development could be similar to those described for steroidogenesis. Cell culture studies have shown that DAX1 represses the cooperation between SF1, WT1 and GATA4 in the activation of the *AMH* promoter (Nachtigal *et al.*, 1998; Tremblay and Viger, 2001b). These data indicate that DAX1

antagonized SF1 in the steroidogenesis as in the gonadogenesis. However, more recent experiments contradict this idea and show cooperation between the two proteins to activate genes of somatic cells in the male pathway in the XY gonad (Park *et al.*, 2005).

The possible role of DAX1 in gonadal development has raised great controversy. The initial studies of patients with DSS suggested an anti-testis role for DAX1. Individuals with this syndrome have a 46XY karyotype and do not develop testis. As mentioned above, these patients have a duplicate 160 Kb region on X chromosome. In addition to *DAX1* this region contains genes from the *DAM* family (DSS-AHC critical interval *MAGE*), related to the *MAGE* family (melanoma antigen gene family), whose members encode antigenes associated with tumors (Bardoni *et al.*, 1994). Jimenez et al. (1996b) suggested that DAX1 could antagonize the function of SRY in a dose-dependent manner. This idea was supported by subsequent experiments in mice over-expressing Dax1, which showed delayed testicular development and male to female sex reversal (Swain and Lovell-Badge, 1998; Jiménez and Burgos, 1998). Surprisingly, further studies have shown that DAX1 it is necessary for a proper testicular differentiation. Yu et al, (1998) studied the gonad development of mice with a deletion in the exon 2 of Dax1, which encodes the LBD domain (the $Dax1^{-}$ allele). Adult $Dax1^{-/-}$ males have very small testis, frequently showing poor development of the epithelium and depleted germ cells. In contrast, $Dax1^{-/-}$ females were fertile and showed a normal reproductive system. Subsequently, Meeks et al. (2003a) examined the embryogenesis of these same mice, noting that the testicular development was normal until 13.5 dpc, where some testicular cords were disorganized and incomplete. In another study, Meeks et al. (2003b) analyzed the effects of the $Dax1^{-}$ null allele in mice of the strain *Mus musculus poschiavinus*, which carry a gene wick *SRY* allele (the *YPOS* allele) (Eicher *et al.*, 1995). The authors found that $Dax1^{-}/YPOS$ mice show complete sex reversal. Further studies have also suggested that Dax1 may regulate the expression of *Sox9* in cooperation with Sry and a new protein Tda1.

Finally, Park et al,(2005) used loss-of-function mutations in *Dax1* and *Sf1*, to analyze the impact of these mutations in mice transgenic for a null copy of these two genes. These studies suggests that Dax1 acts synergistically with Sf1 during male gonadogenesis in the mouse. According to these apparently contradictory results, it has been proposed that values in the expression of *Dax1* above and below a certain expression level may alter its proper function during male gonadogenesis (Ludbrook and Harley, 2004). This narrow expression range seems to be very sensitive and might differ between different species. *DAX1* is also highly conserved among different animal taxa. Several orthologs of this gene have been identified in inverte-

brates (Alonso *et al.*, 2001), as well as in birds (Smith *et al.*, 2000), reptiles (Western *et al.*, 1999), amphibians (Sugita *et al.*, 2001), fishes (Wang *et al.*, 2002) and primates (Patel *et al.*, 2001)

DMRT1

A gene termed *DMRT1* (Doublesex-Mab3-Related Transcription factor 1) is located on human 9p24.3 (Veitia *et al.*, 1997) and encodes a transcription factor with a DNA binding domain, the DM domain, which is similar to male sexual regulatory genes *doublesex* of *Drosophila melanogaster* (Burtis and Baker, 1989) and *mab-3* of *Caenorhabditis elegans* (Raymond *et al.*, 1998). Both *doublesex* and *mab-3* play important roles in sex determination and male specific differentiation. The expression of alternative spliced *DMRT1* transcripts is associated with the sex reversal of the teleost swamp eel (Huang *et al.*, 2005). A *Dmrt1* knockout mouse demonstrated that this gene is required for testis differentiation after sex determination (Raymond *et al.*, 1998; Fahrioglu *et al.*, 2007). In mouse, during gonad sexual differentiation *Dmrt1* mRNA expression is maintained only in the XY gonad and becomes restricted to the developing seminiferous tubules of the testis. In mouse gonadal primordium, the genital ridge, first becomes morphologically distinct at about day E10.5 in both XX and XY embryos. Sexual differentiation of the gonad becomes apparent at about E12 and continues for several days. At E10.5 and E11.5, as the genital ridges form from the surrounding intermediate mesoderm, *Dmrt1* mRNA expression is detectable at similar levels in the genital ridges of both XX and XY embryos. At E12.5 and E13.5, as sex-specific structures are becoming apparent in the gonad, *Dmrt1* mRNA is observed in the developing sex cords of the testis as well as in a punctate pattern in the ovary. At E14.5 and E15.5, *Dmrt1* mRNA level declines in the ovary but is maintained in the testis. *Dmrt1* is not expressed in interstitial cells such as Leydig cells. *Dmrt1*, is expressed both in Sertoli cells and Germ cells whereas, *Sox9* expression is confined to pre-Sertoli cells (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996).

In all vertebrates examined, *DMRT1* is expressed in the differentiating male genital ridges and adult testis, including mammals, birds, and reptiles with temperature-dependent sex determination (Raymond *et al.*, 1999b; Smith *et al.*, 1999; Moniot *et al.*, 2000). Testis differentiation is severely defective in *Dmrt1*$^{-/-}$ mutants. *Dmrt1* mRNA is expressed both in germ cells and in Sertoli cells (Raymond *et al.*, 1999a).

Thus, the germ cell death caused by mutation of *Dmrt1* could reflect either a defect in the germ cells themselves or a defect in Sertoli cells, which promote germ cell survival and differentiation (Rassoulzadegan *et al.*, 1993; Bitgood *et al.*, 1996). Immunohistochemistry reveals that in the embryonic

testis DMRT1 protein accumulates primarily in Sertoli cell nuclei, with little or no expression detectable in germ cells. Starting at P1, Dmrt1 levels rise in germ cells and reach high levels by P7, just before meiosis begins. The similar timing of increased Dmrt1 expression in germ cells and the onset of germ cell death in the *Dmrt1*$^{-/-}$ mutant testis suggests a possible cell-autonomous function for Dmrt1 in maintaining the germ line. From P7 through adult stage, DMRT1 protein is present in Sertoli cells and undifferentiated germ cells, but not in differentiating germ cells (Raymond *et al.*, 1999a). In adult testis, *Dmrt1* is expressed in Sertoli cells in all regions of the seminiferous tubules, but is expressed dynamically in premeiotic germ cells (spermatogonia), with high expression only in regions of the seminiferous tubule that are early in the spermatogenic cycle (Raymond *et al.*, 2000a). This further suggests that Dmrt1 may play a role in pre-meiotic germ cells, for example, regulating entry to meiosis or controlling the mitotic cell cycle. Murine Dmrt1 is necessary in the male gonad for survival and differentiation of both somatic and germ-line cells. However, male-specific *DMRT1* mRNA expression occurs at an earlier developmental stage in the human gonad than in mouse (Moniot *et al.*, 2000) and thus *DMRT1* might play an earlier role in human testis differentiation than *Dmrt1* does in mouse. Birds and reptiles also have male-specific *DMRT1* expression prior to gonad differentiation (Raymond *et al.*, 1999a; Smith *et al.*, 1999; Kettlewell *et al.*, 2000), and may be required earlier in gonad development in these species. However, in marsupials like in the eutherian mammals, DMRT1 protein was localized in the germ cells and the Sertoli cells of the testis, but in addition it was detected in the Leydig cells, peri-tubular myoid cells and within the acrosome of the sperm heads. DMRT1 protein was also detected in the fetal and adult ovary pre-granulosa, granulosa and germ cells. Similarly, DMRT1 was also detected in the granulosa cells of all developing follicles in the adult mouse ovary (Pask *et al.*, 2003), as well as in the germ cells of mole ovotestes (Zurita *et al.*, 2007b). Available data suggest a wide role for this gene in the function of both testis and ovaries of mammals.

AMH

Anti-Mullerian hormone (AMH), or Mullerian inhibiting substance (MIS), is responsible for the regression of Mullerian ducts in male fetuses (Jost, 1953). It is a 140 kDa glycoprotein homodimer belonging to the transforming growth factor-b (TGFb) family. The human *AMH* gene maps on chromosome 19 p13.3 (Cohen-Haguenauer *et al.*, 1987) and the bovine, on chromosome 7 (Gao and Womack, 1997). *Amh* gene has also been cloned and/or mapped in mouse (Münsterberg and Lovell-Badge, 1991; King *et al.*, 1991) rat (Haqq *et al.*, 1993) pig (Lahbib-Mansais *et al.*, 1997), chick (Oreal

et al., 1998) and alligator (Western *et al.*, 1999)

AMH is produced as early as the beginning of Sertoli cell differentiation in male fetuses and continues to be synthesized throughout fetal life and after birth until puberty (Picon, 1970; Vigier *et al.*, 1983). This has led to a speculation that AMH plays a local role during gonad differentiation in addition to its function during Mullerian duct regression. As soon as testicular cords begin to assemble in the foetal gonad (Tilmann and Capel, 2002), Amh expression is triggered in differentiating Sertoli cells. In human testis, AMH is secreted from the sixth week of gestational age and provokes irreversible Müllerian duct regression, which is completed by the end of week 9 (Taguchi *et al.*, 1984). In the rat, Müllerian ducts are sensitive to AMH as early as Day 14; regression takes place between Days 16 and 20, then Müllerian ducts are no longer sensitive to AMH (Picon, 1969). In male moles, expression was first detected in s5b embryos, coinciding with the onset of testis differentiation, and it continued until shortly after birth (s9 stage). In s10 males [5 days post partum (dpp)], either little or no amplification was detected in different RT-PCR reactions, suggesting that *AMH* expression slowly declines after birth but never persists in s11 moles. No *AMH* expression was detected throughout ovotestes development in female moles (Zurita *et al.*, 2003).

AMH expression by Sertoli cells remains at high levels through foetal and prepubertal life, which indicates that the end of the critical window of Müllerian duct regression is dependent on the expression pattern of AMH type II receptor (Josso *et al.*, 2006). Owing to its sex-specific and time-restricted requirement during foetal development, *AMH* expression needs to be tightly regulated. DNA 1 footprint analysis showed that SOX9 binds to a SOX-like site in the *AMH* proximal promoter, and, together with SF1 (steroidogenic factor 1), WT1 (Wilms tumor gene 1), and GATA4, directly activates expression of *AMH*, as indicated above (De Santa *et al.*, 1998a; Tremblay and Viger, 2001a).

Except for a transient decline in the peri-natal period (Josso *et al.*, 1993; Schwindt *et al.*, 1997), testicular AMH secretion is maintained at high levels until puberty, when Sertoli cell maturation is characterised by a decreasing AMH activity (Rota *et al.*, 2002). At that time, as the blood-testis barrier develops owing to the establishment of tight junctions between Sertoli cells, directional secretion of AMH seems to switch from the basal to the ad-luminal compartment, which results in higher AMH concentration in seminal plasma than in blood (Fenichel *et al.*, 1999; Fujisawa *et al.*, 2002).

If the whole pubertal development is analysed, a negative correlation between increasing serum testosterone and declining serum AMH levels, can be observed in normal and precocious puberty. Some clinical observations strongly suggesting that androgens are responsible for AMH down-

regulation are further supported by the fact that deficient testosterone production and androgen insensitivity result in abnormally high AMH production (Rey *et al.*, 1994). Finally, experimental evidence for the negative effect of androgens on AMH expression was obtained by stimulating testosterone production in normal and in androgen-insensitive (Tfm) prepubertal mice (Al Attar *et al.*, 1997). The physiological androgen insensitivity of foetal and neonatal Sertoli cells explains, thus, the transient coexistence of high concentrations of androgens and AMH. During pubertal development, *AMH* expression faints in coincidence with the onset of germ cell meiosis (Hirobe *et al.*, 1992; Rajpert-De Meyts *et al.*, 1999). AMH decline and meiotic entry of germ cells are androgen-dependent processes, the temporal and spatial coincidence of AMH down-regulation and initiation of meiosis could be interpreted as two independent phenomena of the elevation of intratesticular testosterone concentration. Similarly, there are indications of regulation of AMH levels by FSH also. In patients with androgen insensitivity or deficient androgen production, serum AMH levels are extremely elevated in the first month of life and after the onset of puberty, but they remain within normal levels during childhood (Rey *et al.*, 1994, 1999). These observations strongly suggested that, in the absence of the inhibitory androgen effect, high FSH levels could increase testicular AMH production.

Ovarian granulosa cells, the homologous to testicular Sertoli cells, also produce AMH (Vigier *et al.*, 1984) but with several differences: AMH expression only begins at the peri-natal period (Bézard *et al.*, 1987; Ueno *et al.*, 1989; Rajpert-De Meyts *et al.*, 1999), remains low throughout reproductive life and becomes undetectable after menopause (Lee *et al.*, 1996b). Granulosa cells of primary and small cavitary follicles show homogeneous AMH expression, in larger follicles, AMH is mainly produced in cells near the ovocyte and in few cells surrounding the antrum, and no AMH production is observed in atretic follicles and theca cells (Rey *et al.*, 2000). Gonadal AMH secretion shows a clear-cut sexual dimorphism in prepubertal ages, when serum AMH is significantly lower in females; in adults, serum AMH is similarly low in both sexes (Rey *et al.*, 1996; Lee *et al.*, 1996b; Cook *et al.*, 2000). As indicated above, SOX9, SF1 and WT1 cooperate in the activation of *AMH* gene.

3.2 Other genes expressed in the adult gonads and studied in this work

There are many genes, other than those related to sex determination, which are expressed in the adult gonads of mammals. Some of them could show seasonal variation in the expression levels and could then be involved in

seasonal breeding. The genes that we have investigated in this thesis are reviewed below.

P450ssc

Testosterone biosynthesis in Leydig cells is dependent on both acute and chronic stimulation by the anterior pituitary hormone, luteinizing hormone (LH). Conversion of cholesterol to pregnenolone in mitochondria is catalyzed by cytochrome P450scc, the cholesterol side-chain cleavage enzyme, and is the first, rate-limiting, and hormonally regulated enzymatic step in the synthesis of all steroid hormones (Miller *et al.*, 1988). Human P450scc is encoded by a single gene, formally termed *CYP11A* (Nebert *et al.*, 1991), that is located on chromosome 15q23-q24 (Sparkes *et al.*, 1991) and is expressed in the adrenals, gonads, placenta, and brain (Chung *et al.*, 1986; Mellon and Deschepper, 1993).

Chronic stimulation by LH or cAMP is required for optimal synthesis of the P450 enzymes involved in testosterone biosynthesis. Chronic treatment of intact or hypophysectomized rats with LH, or its analog, human chorionic gonadotropin (hCG), results in increased capacity for LH-stimulated testosterone production (Zipf *et al.*, 1978; Chung *et al.*, 1986) and induces enzymes of the steroidogenic pathway (Shaughnessy and Payne, 1982). In contrast, administration of a single high dose of LH or hCG to intact animals results in a decreased capacity for LH-stimulated testosterone production and in a decrease in P450scc activities (Shaughnessy and Payne, 1982) and reduced amounts of testicular mitochondrial P450scc (Luketich *et al.*, 1983) due to negative feedback inhibition of testosterone on anterior pituitary gland. However, treatment of immature porcine testicular cells in culture by hCG increases de novo synthesis of P450scc (Mason *et al.*, 1984).

The orphan nuclear receptor steroidogenic factor-1 (SF1), also known as Ad4-BP, is essential for the expression of all the steroidogenic genes in the adrenals and gonads and also for adrenal and gonadal development (Parker and Schimmer, 1997; Morohashi, 1999). Previous biochemical and morphological studies suggest that P450scc is located on the matrix side of the inner mitochondrial membrane (Miller *et al.*, 1988) in Leydig cells in the male, and in the theca cells in the female gonads.

The mole *Talpa occidentalis*, show high levels of serum testosterone during the breeding season (winter) and low levels during the non-breeding season (summer), suggesting that the expression of *P450ssc* also fluctuates circannually (Jiménez *et al.*, 1993)

SYCP3

SYCP3 (or *SCP3*; Synaptonemal complex protein-3) is a DNA-binding protein and a structural component of the synaptonemal complex, which mediates the synapsis or homologous pairing of chromosomes during meiosis of the germ cells. It is a marker for germ cells which are undergoing meiosis.

In mammals, the synaptonemal complex is composed of proteins (*SYCP-1*, 2, and 3)(Meuwissen *et al.*, 1992; Offenberg *et al.*, 1998; Schalk *et al.*, 1998). In mice, all three genes are expressed only in the testis and ovary and are considered to be germ-cell specific (Wang *et al.*, 2001). Chromosomal defects and specific gene mutations result in male infertility, and many idiopathic cases of infertility are thought to have a genetic basis also (Matzuk and Lamb, 2002). Defects of meiosis during spermatogenesis are a significant cause of Azoospermia, but the causes remain largely unknown. Male mice homozygous for the null mutation of the *Sycp3* (*Scp3*) gene are sterile as a result of massive apoptotic cell death in the testis during meiotic prophase (Yuan *et al.*, 2000). In *Sycp3*-deficient male mice, axial or lateral elements and synaptonemal complexes are not formed. The chromosomes in the mutant spermatocytes do not synapse, and meiosis is arrested. Sycp3-deficient female mice are fertile and produce healthy offspring, although there is an increased risk of intrauterine death owing to the generation of aneuploid oocytes resulting from defective chromosomal segregation (Yuan *et al.*, 2002).

Sycp3 is crucial for male fertility not only in mice but also in humans as there are few reports of specific gene defects resulting in disruption of spermatogenesis and infertility in otherwise healthy men. The human *SYCP3* gene, located on chromosome 12, encodes a DNA-binding protein which is required for completion of spermatogenesis in men.

OCT4

Oct4 belongs to the family of POU (Pit, Oct, Unc) transcription factors that contain a bipartite DNA binding domain, the POU domain (Scholer, 1991; Yeom *et al.*, 1996; Tam and Zhou, 1996). OCT3/4 is a transcriptional regulator, expressed exclusively in pluripotent human embryonic stem cells and premeiotic germ cells (Goto *et al.*, 1999; Hansis *et al.*, 2000). These primordial germ cells (PGCs) are set aside to an extra-embryonic location early during embryonic development, and migrate to the area where the genital ridge will be formed around weeks 5 and 6 of human development (Witschi, 1948). Subsequently, the gonads and sexual differentiation occur in weeks 6 and 7 (Falin, 1969). Between weeks 7 and 9 during ovarian development (referred to as prefollicular stage) the germ cells (oogonia)

and granulosa cells characteristically arrange in cords and sheets without specific organization (Gondos, 1985). The first distinctive change in prenatal germ cell development in the ovary is therefore the onset of meiosis, which starts at 11-12 weeks of gestation and extends into the second trimester (Rabinovici and Jaffe, 1990). Nuclear staining for OCT3/4 was restricted to germ cells. In addition to oogonia, OCT3/4 was occasionally seen in early oocytes, but was never detectable in cells involved in folliculogenesis. The expression of OCT3/4 in fetal ovaries was seen at 24 weeks of gestation. In ovaries of neonates, hardly any OCT4 positive germ cells were detectable.

In fetal human testes, *OCT3/4* has been found to be highly expressed in PGC between weeks 17 and 24 and to a lesser extent at later stages (Looijenga *et al.*, 2003). It is therefore tempting to speculate that both the processes of cell-cell interaction and down-regulation of *OCT3/4* are important for germ cell maturation. It should be noted that this is different from the situation found in mouse follicles, where germ cells after completion of meiotic prophase I express Oct3/4 (Pesce *et al.*, 1998). However, the exact mechanisms of interaction are not yet understood, and deserve further investigation.

Germ cells of male moles behave very similarly to those of other mammals during gonadal development (Zurita *et al.*, 2007a). They are rapidly enclosed into the testis cords at s5b stage, proliferate very actively around birth and migrate to the periphery of the testis cords during the first postnatal stages (s9 and s10). However, moles are seasonal breeders (Jiménez *et al.*, 1990) and they inactivate and activate their gonads during the intermediate seasons, spring and autumn, respectively. These processes of cyclic, seasonal gonad activation and inactivation involve profound changes in many parameters of the reproductive tract of both males and females that cause them to become fertile in the winter and sterile in the summer (Jimenez *et al.*, 1996). One of the most striking features of the gonad development in female moles is that germ cells enter meiosis postnatally at s10 mole infants (about five days old) (Zurita *et al.*, 2007a) as in the case of rabbit (PETERS *et al.*, 1965). In both male and the female moles, germ cells downregulate *OCT4* expression as soon as they enter meiosis. Hence, OCT4 is an excellent marker for mole pre-meiotic germ cells.

α-SMA

In the testis, α-smooth muscle actin (*α-SMA*) is marker for peritubular myoid cells (PMC), which constitute the external portion of the seminiferous tubule and are primarily involved in providing structural integrity and contractibility to the seminiferous tubule (Rodriguez *et al.*, 1997). PMC development is postulated to be dependant on androgens (Bressler and Ross,

1972). For example, PMCs are known to cooperate with Sertoli cells to generate basement membrane (Skinner and Fritz, 1985); Sertoli and peritubular myoid cells have been shown to contribute different components of this membrane, suggesting that a subset of extracellular matrix molecules and the genes that encode them may be PMC-specific. In the adult rat and rabbit, testosterone regulates the contractions of the testicular capsule (Hargrove *et al.*, 1973) which is a result of contractibility in the myoid cells. Evidently, in the fetal testis, rapid growth and differentiation of the tunica albuginea is one of the main reasons for the separation of the testicular cords from the surface epithelium (Paranko, 1987).

In vitro (Tung and Fritz, 1987) and histological (Paranko, 1987; Frojdman *et al.*, 1989) studies suggest that the surrounding mesenchyme plays an important role in the morphogenesis of the gonadal cords. The criteria and markers for mesenchymal cell differentiation during this process have, however, not yet been defined clearly in the early gonadogenesis. In postnatal stages of gonadal development, PMCs develop a smooth muscle-like phenotype, expressing smooth muscle markers α-Sma (Palombi *et al.*, 1992) and Des (Virtanen *et al.*, 1986). In rats, while there was no expression of DES in the XY gonad at 13.5–14.5 days post coitum (dpc), α-SMA expression was seen in a distinct pattern around the edge of the XY gonad, but not in the XX gonad, at both 13.5 dpc and 14.5 dpc and in the mesonephric tubules. This reflected expression in the tunica albuginea, a smooth muscle sheath that covers the testis but not the ovary (Karl and Capel, 1998). The absence of DES and α-SMA expression in PMCs at 13.5 and 14.5 dpc suggests that they do not acquire a smooth muscle-like molecular phenotype until a later stage of testis development. In postnatal stages of gonadal development, PMCs develop a smooth muscle-like phenotype, expressing smooth muscle markers α-Sma (Palombi *et al.*, 1992) and Des (Virtanen *et al.*, 1986), and contracting in vitro after exposure to prostaglandin F2a (Tripiciano *et al.*, 1998). However, it is not known when PM cells start to express these markers. In addition, it is not known the possible role of PMCs in seasonal breeding.

α-SMA is also a useful marker for PMCs in the mole. α-SMA expressisn has been detected in the PMCs surrounding the testicular cords present in both male and female gonads (Barrionuevo *et al.*, 2004).

DMC1

During meiosis, a single round of DNA replication is followed by two rounds of chromosome segregation, meiosis I and meiosis II. Meiosis II is similar to a mitotic division in that sister chromosomes segregate from one another. However, meiosis I is unique in that it involves the segregation of

replicated homologous chromosomes (homologs). A complex series of events occurs between the time of DNA replication and the first meiotic division (Bishop *et al.*, 1992). Prominent among these events is genetic recombination, which occurs at a very high level and is directly required to provide the physical connection between homologs that ensures their proper segregation; other essential events of prophase include chromosome pairing, synaptonemal complex (SC) formation, and chromosome condensation (Roeder, 1990; Kleckner *et al.*, 1991). *DMC1* is a meiosis-specific gene. DMC1 is a structural and evolutionary homolog of the well-characterized bacterial recombination protein, RecA, which is required for nearly all recombinogenic processes in bacteria including conjugation, bacteriophage-mediated transduction, plasmid recombination, and repair of DNA damage (Cox, 1991). DNA sequence analysis of a complementing 3.2 kb genomic subclone and a corresponding *DMCl* cDNA clone revealed a single open reading frame of 1 002 bp (334 codons) interrupted by a 91 bp intron.

The region of strong homology between RecA and Dmcl includes specific portions of the RecA protein known to be important for interaction with ATP, including the two consensus sequences for purine nucleotide binding sites, motifs A and B (Walker *et al.*, 1982). It also includes portions of the protein thought to be important for interactions with DNA and for the ATP-dependent conformational change required for DNA binding (Story and Steitz, 1992). The major differences between Dmcl and RecA occur at the amino and carboxyl termini. The two recombinases colocalise to nuclear foci during meiotic recombination (Bishop, 1994; Chen *et al.*, 1999; Tarsounas *et al.*, 1999). Consistent with these observations, mouse *Dmc1* knockouts are infertile due to defects in chromosome synapsis during meiotic recombination (Pittman *et al.*, 1998; Yoshida *et al.*, 1998). Also Dmc1 interacts with BRCA2 which also appears to play an important role in meiotic recombination in mammalian cells, as it is highly expressed during spermatogenesis in mice (Connor *et al.*, 1997), and localises to meiotic chromosomes during early prophase I when homologous chromosomes undergo pairing (Chen *et al.*, 1999). According to these features, DMC1 is an excellent marker for early meiotic cells as it is largely known that recombination takes place between leptotene and pachytene stages of the first meiotic prophase.

3.3 Gonadal function

Many classical studies have established the main physiological aspects of adult gonad function in relation to both gametogenesis and endocrine activity. More recently, new knowledge is being reported concerning the genetic

control of these processes.

3.3.1 Gametogenesis

Concerning gametogenesis, our study was restricted to males, so here we will refer to spermatogenesis. During this process, spermatogonia, through multiple mitotic and meiotic divisions, develop into mature spermatozoa. In brief, this process may be divided into four stages (de Kretser *et al.*, 2002). First, type A spermatogonia undergo mitosis, resulting in renewal of germline stem cells as well as type B spermatogonia that continue to undergo differentiation. Second, type B spermatogonia undergo meiosis, generating haploid round spermatids. Next, spermiogenesis occurs, wherein round spermatids mature to motile spermatozoa without undergoing further cell division. Spermiogenesis includes formation of the acrosome, nuclear condensation and extensive cellular reorganization including sperm tail development. Finally, interactions with the Sertoli cell mediate the process of spermiation, wherein cytoplasmic material from the spermatid is removed and the mature sperm is released into the lumen of the seminiferous tubule. This entire process is under strict endocrine regulation and there is potential for contraceptives to target all or parts of this process. Although the role of FSH in human spermatogenesis has been debated, it is clear that both FSH and LH are required for quantitatively and qualitatively normal spermatogenesis in men. This has been demonstrated in a series of human studies wherein gonadotropin production and spermatogenesis were suppressed in men using exogenous testosterone and either LH (in the form of hCG) or FSH were given back in a selective fashion (Bremner *et al.*, 1981; Matsumoto and Bremner, 1985; Matsumoto *et al.*, 1983). While either gonadotropin alone can reinitiate spermatogenesis, both LH and FSH are required to achieve normal semen parameters. Matthiesson et al recently expanded upon this work with detailed examination of the relative stages of spermatogenesis affected by selective gonadotropin supplementation using exogenous T + DMPA to suppress endogenous T and spermatogenesis (Matthiesson *et al.*, 2006). Data from both rodents and monkeys suggested that FSH is more important than LH for spermatogonial maturation whereas LH and/or intratesticular androgens are needed for normal completion of meiosis and spermiogenesis/spermiation (McLachlan *et al.*, 2002; Meachem *et al.*, 1999; Moudgal *et al.*, 1997; Shetty *et al.*, 1996). Surprisingly, after 6 weeks of selective LH or FSH withdrawal, only pachytene sperm differed among men lacking FSH (treated with T+ DMPA+hCG) compared to untreated, control individuals; men lacking LH showed no differences compared to untreated men. Although the interpretation of these results is limited by the length of treatment and small numbers of indi-

viduals studied, they suggest that FSH or LH/intratesticular androgens are sufficient to support all stages of spermatogenesis including spermatogonial maturation, meiosis, spermiogenesis and spermiation. These data emphasize the need for hormonally-based male contraceptive strategies to maximally suppress both LH and FSH in order to optimize spermatogenic suppression.

Apart from the hormonal control of spermatogenesis, there are various paracrine factors which regulates this process. Two recent studies (Bowles *et al.*, 2006; Koubova *et al.*, 2006) indicate that exposure to RA controls the entry of mouse fetal germ cells into meiosis. RA is a small, polar molecule that easily diffuses through tissues and acts by binding to nuclear RA receptors (RARs, which include the RARα, β and γ isotypes), which heterodimerize with nuclear retinoid X receptors (RXR α, β and γ) (Mark *et al.*, 2006). RAR-RXR dimers bind to RA response elements (RAREs) and thereby control the expression of RA-responsive genes (Chambon, 1996). *Cyp26b1*, encodes a P450 enzyme, a meiosis inhibitin factor, CYP26B1 (also known as P450RAI2), that acts by catabolizing all-trans RA into inactive metabolites (White *et al.*, 2000; Yashiro *et al.*, 2004). CYP26 enzymes act to regulate local levels of RA in several developmental contexts; such *Cyp26b1* transcripts are detectable in mouse gonads at 11.5 dpc, at higher expression levels in males than females (Bowles *et al.*, 2006). After 11.5 dpc, *Cyp26b1* expression is undetectable in female gonads but is very high in male gonads. Expression of CYP26B1 is associated with Sertoli cells and some interstitial cells, and peaks at around 13.5 dpc (Bowles *et al.*, 2006; Menke and Page, 2002). This profile of expression suggests that CYP26B1 might protect male gonads from the actions of RA between 12.5 and 14.5 dpc (McLaren and Southee, 1997).

It is now well establishes that RA acts to initiate meiosis (Bowles *et al.*, 2006; Koubova *et al.*, 2006). First, RA levels are higher in the female than the male mouse gonad at 13.5 dpc (Bowles *et al.*, 2006). Furthermore, exogenous RA can induce XY germ cells in a cultured mouse fetal testis to enter meiotic prophase (Bowles *et al.*, 2006; Koubova *et al.*, 2006), as judged by the histological detection of condensed meiotic nuclei and the expression of three meiotic markers, named: stimulated by retinoic acid gene 8 (Stra8), synaptonemal complex protein 3 (Sycp3) and dosage suppressor of mck1 homolog (Dmc1) (Chuma and Nakatsuji, 2001; Oulad-Abdelghani *et al.*, 1996).

To prove that CYP26B1 functions as a meiosis inhibitor endogenously, *Cyp26b1*-knockout mice were also examined (Bowles *et al.*, 2006). *Cyp26b1*-null mice die immediately after birth with multiple abnormalities, including limb defects (Yashiro *et al.*, 2004). XY gonads from *Cyp26b1*-null embryos showed upregulation of *Stra8* and *Sycp3* expression at 13.5 dpc, demon-

strating that germ cells in the XY mutant embryos are entering meiotic prophase I at this early stage, like in a normal XX embryo (Bowles *et al.*, 2006). These findings were recently extended by the analysis of a second *Cyp26b1*-null line of mice (MacLean *et al.*, 2007), it was found that, in the absence of CYP26B1, XY germ cells enter meiosis by 13.5 dpc and proceed through meiotic prophase, with some reaching zygotene/pachytene, as judged by histological staining and chromosome spread analysis. In *Cyp26b1*-null ovaries, the wave of *Stra8* expression appears to initiate earlier than is normal (Bowles *et al.*, 2006). Because *Cyp26b1* is expressed initially in the gonads of both sexes (Bowles *et al.*, 2006), we surmise that, in *Cyp26b1* null XX gonads, complete absence of CYP26B1 might allow RA to accumulate to a meiosis-inducing level earlier than in wild-type female gonads.

3.3.2 The hypothalamic-pituitary axis and its regulation

The hypothalamic-pituitary-testicular axis is a classic endocrine loop, with negative feedback of downstream products playing the pivotal regulatory role in maintaining endocrine homeostasis. Testicular production of both hormones and sperm is exquisitely regulated by gonadotropins (Follicle Stimulating Hormone and Leutinizing Hormone) produced by the anterior pituitary while gonadotropin production is under the direct control of pulsatile GnRH secretion from the hypothalamus. In turn, steroid and peptide hormones produced in the testes provide inhibitory signals to the pituitary and hypothalamus. In recent years there have been rapid advances in understanding of the neuroendocrine control of reproduction upstream of GnRH. Initially studies into the genetics of familial hypogonadotropic hypogonadism revealed the critical role of the then orphan G-protein receptor GPR54 in gonadotropin secretion (Veldhuis *et al.*, 1987; de Roux *et al.*, 2003). Previous studies had demonstrated that the 54-amino acid peptide kisspeptin-54 (also called metastin) is the ligand for GPR54 (Kotani *et al.*, 2001; Seminara *et al.*, 2003; de Roux *et al.*, 2003). Subsequent investigations have demonstrated that kisspeptin is expressed in the arcuate, periventricular and anteroventral periventricular nuclei of the forebrain, and kisspeptin appears to have direct inhibitory effects on pituitary gonadotropin release through interaction with GPR54 expressed on pituitary gonadotropes (Dungan *et al.*, 2006). Moreover, the negative feedback of testosterone and estradiol on GnRH secretion is mediated via inhibition of kisspeptin production in the arcuate nucleus of the hypothalamus (Navarro *et al.*, 2004; Smith *et al.*, 2005). Most recently, in contrast to acute administration of kisspeptin which stimulates gonadotropin and testosterone release, chronic administration of kisspeptin lowers serum LH levels in monkeys (Seminara, 2006) and

leads to testicular atrophy in adult male rats similar to that seen with chronic GnRH infusions (Thompson *et al.*, 2006). Together, these studies directly link kisspeptin-GPR54 to male reproduction and demonstrate the importance of this pathway in upstream regulation of testicular function and sex steroid homeostasis (Fig. 3.1).

Testosterone is the main testicular steroid, but significant aromatase activity in the testis and peripheral tissues results in production of estradiol as well. Testosterone clearly inhibits kisspeptin transcription (Navarro *et al.*, 2004) and GnRH and gonadotropin secretion (Matsumoto and Bremner, 1984)). Some of this inhibition is estrogen independent (Kerrigan *et al.*, 1994; Veldhuis *et al.*, 1992) but estradiol seems to play an important role in steroid negative feedback in the male, particularly by decreasing LH production (Finkelstein *et al.*, 1991; Raven *et al.*, 2006; Bagatell *et al.*, 1994). Aromatase inhibition results in significant increases in LH and FSH secretion in normal men (Hauger *et al.*, 1977) which can be restored to normal levels with estradiol supplementation in a non-linear fashion, with higher estradiol levels having increasingly less effect on circulating gonadotropin and T levels (Raven *et al.*, 2006). Moreover, both aromatase inhibition and medical castration result in similar increases in FSH, despite markedly decreased T levels with medical castration compared to aromatase inhibition, suggesting that at least acutely, FSH regulation is more dependent on estradiol than testosterone (Hayes *et al.*, 2001; Schnorr *et al.*, 2001). In contrast, DHT, a non-aromatizable androgen, is a poor inhibitor of gonadotropin production in the male when given at or near physiologic doses (Bagatell *et al.*, 1994; Wang *et al.*, 1998). However, with chronic administration and supraphysiologic dosing, exogenous DHT inhibits both LH and FSH secretion despite concomitant suppression of T and estradiol (Kunelius *et al.*, 2002; Ly *et al.*, 2001) demonstrating that aromatization is not an absolute requirement for negative feedback. Like other sex steroids, progesterone is expressed in the male as well as the female, albeit at lower levels (Bélanger *et al.*, 1994). The precise role of progesterone in normal male physiology is unknown, but progesterone receptors have been demonstrated in the male hypothalamus, pituitary, and male reproductive tract (Luetjens *et al.*, 2006; Heikinheimo *et al.*, 1995; Bethea *et al.*, 1992). However, progestins enhance male hormonal contraceptive efficacy when combined with androgens (Meriggiola *et al.*, 2003), an effect attributed to increased hypothalamic pituitary suppression of gonadotropin secretion either directly or through the androgen receptor.

A non-steroidal Sertoli cell product, inhibin B, also contributes to hormonal feedback in the male. Inhibin B is a dimeric molecule consisting of α and β subunits and a member of the TGF-β family. Inhibin B is predominantly produced by Sertoli cells (O'Connor and De Kretser, 2004), and

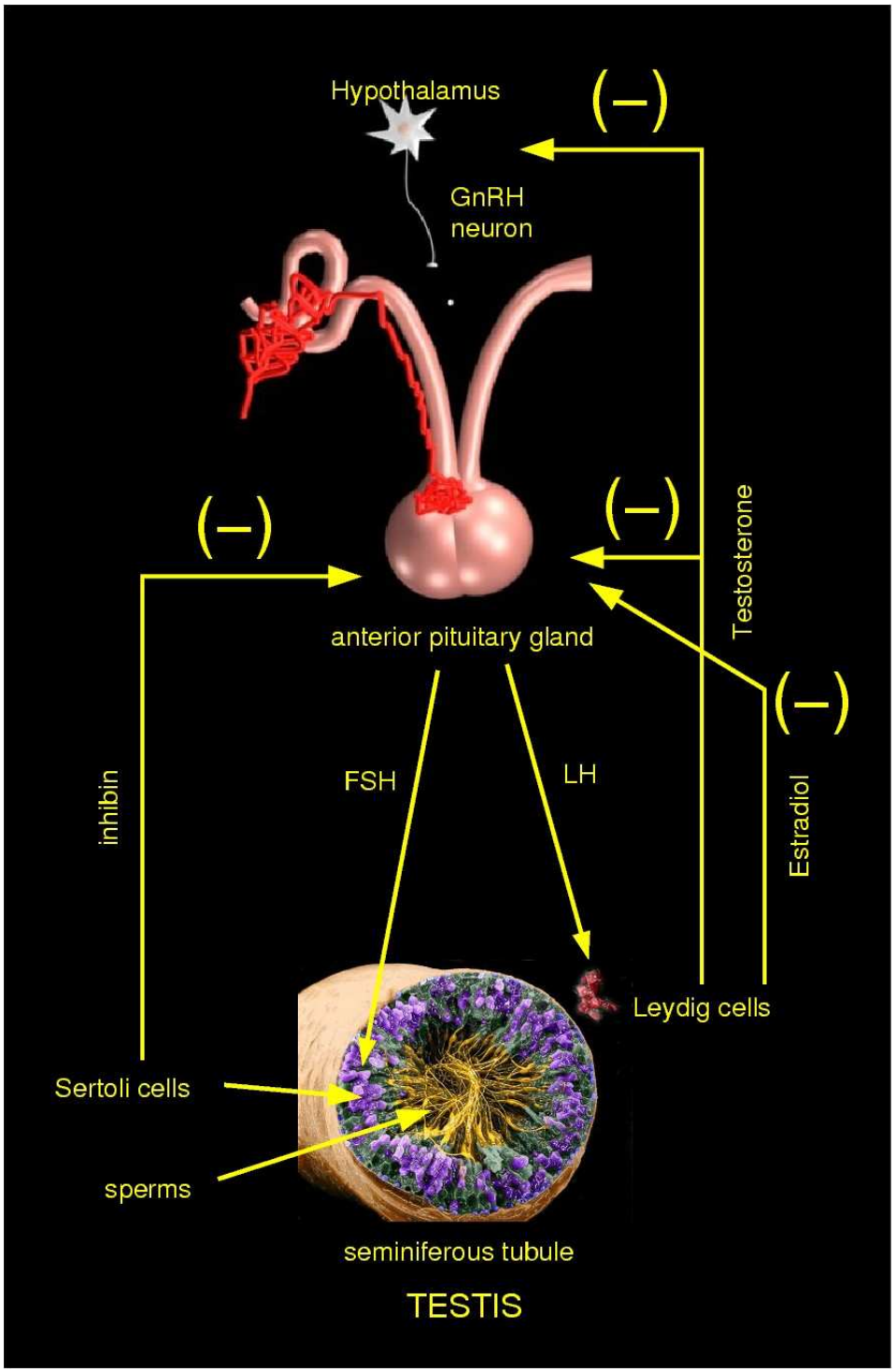


Figure 3.1: Hypogonadal axis and the negative feedback.

inhibin B levels correlate with Sertoli cell number (Ramaswamy *et al.*, 1999). However recent observations in boys with constitutively active LH-receptor mutations resulting in familial male-limited precocious puberty suggest that Leydig cells may produce some inhibin B (Soriano-Guillen *et al.*, 2006). The role for inhibin B as a testicular regulator of FSH, as hypothesized by McCullagh over 75 years ago (McCullagh, 1932) cannot be ruled out. Aside from its role in regulating FSH production and as an indicator of Sertoli cell function, no clear role for inhibin B in testicular physiology or spermatogenesis has been established.

3.3.3 Cell Junctions in the testis

Sertoli cells provide structural support for developing germ cells within the seminiferous epithelium by participating directly in the deposition of extracellular matrix components and permitting the formation of specialized cell junctions. Morphological studies have shown that the Sertoli cell cytoskeleton: 1) maintains shape; 2) positions and transports organelles within the cell; 3) forms and stabilizes the cell membrane at sites of cell-cell and cell-extracellular matrix contact; 4) positions, anchors, and aids in the movement of developing germ cells; and 5) participates in the release of mature spermatids from the seminiferous epithelium at spermiation. The blood-testis barrier (also known as the seminiferous epithelial barrier), a modified occluding junction, is formed by approximately 15–18 d of age in the rat as constituted by Sertoli cell tight junctions located in the basal third of the seminiferous epithelium (Russell and Clermont, 1977; Dym and Fawcett, 1970; Pelletier and Byers, 1992; Pelletier *et al.*, 1997). The blood-testis barrier compartmentalizes the epithelium into two compartments, one is a basal compartment in which spermatogonia, preleptotene, and leptotene spermatocytes reside. The other is an adluminal compartment in which meiotic spermatocytes and spermatids in various stages of spermatogenesis and spermiogenesis exist (Russell and Clermont, 1977) Three main functions are ascribed to the blood-testis barrier: 1) creates a specialized environment; 2) regulates the passage of molecules; and 3) serves as an immunological barrier.

Sertoli cells (nurse cells) are directly involved in the movement of developing germ cells because these cells lack an architecture characteristic of migrating cells, such as fibroblasts (Wilton *et al.*, 1988).

A possible mechanism underlying germ cell movement may involve members of the GTPase superfamily, as shown in a series of recent studies (Lau and Mruk, 2003). Moreover, other studies have shown that the translocation of elongating/ elongate spermatids across the seminiferous epithelium is largely conferred by the ectoplasmic specialization (Vogl *et al.*, 2000;

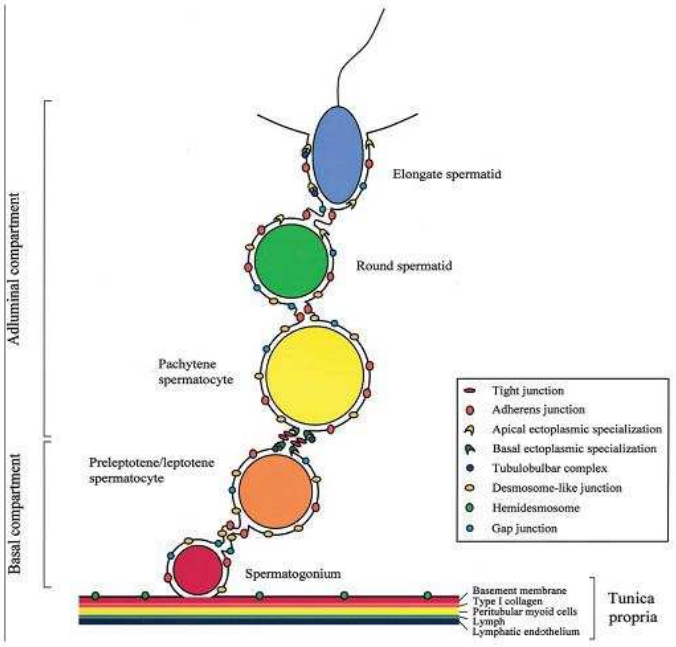


Figure 3.2: Schematic drawing illustrating the relative locations of the different types of junctions found in the testis. Shown are tight, anchoring, and gap junctions. Two testis-specific anchoring junction types are also depicteded: the ectoplasmic specialization and tubulobulbar complex. Tight junctions, basal ectoplasmic specializations, and tubulobulbar complexes that constitute the blood-testis barrier divide the seminiferous epithelium into a basal and adluminal compartment. The proximity of the blood-testis barrier to the basement membrane can be seen. This figure was prepared based on the following original research articles and reviews: (Siu and Cheng, 2004; Dym and Fawcett, 1970; Pelletier and Byers, 1992; Mruk and Cheng, 2004) and was taken from (Mruk and Cheng, 2004)

de Kretser, 1990; Russell and Malone, 1980). It has been shown that microtubules, together with several motor proteins such as dynein (Guttman *et al.*, 2000; Miller *et al.*, 1999) and myosin VIIa (Velichkova *et al.*, 2002), ATPases, and GTPases (Lui *et al.*, 2003) present at the site of the ectoplasmic specialization assist in the migration of elongating/elongate spermatids across the seminiferous epithelium. Sertoli cells also assist in the translocation of early meiotic spermatocytes from the basal to the adluminal compartment during the epithelial cycle in the seminiferous epithelium. This process, which likely requires the disassembly of Sertoli–Sertoli and Sertoli–germ cell adherens junctions, is a highly selective process. For instance, type A spermatogonia cannot be lifted but must remain attached to the basement membrane, suggesting that an intriguing mechanism must exist to determine which specific cell types can be lifted from the basement membrane to the basal compartment.

Sertoli cells are indispensable to the release of mature spermatids from the seminiferous epithelium. This process involves a cascade of events, some of which include: 1) encapsulation of spermatid heads by Sertoli cell cytoplasmic processes; 2) expulsion of spermatids from Sertoli cell crypts; and 3) release of spermatid heads (Vogl *et al.*, 1993).

There are three morphologically and functionally distinct types of cell junctions present in epithelia: 1) occluding junctions (e.g., tight junctions); 2) anchoring (or adhering) junctions (consist of four types: cell–cell actin-based adherens junctions; cell–matrix actin-based focal contacts; cell–cell intermediate filament-based desmosomes; and cell–matrix intermediate filament-based hemidesmosomes); and 3) communicating junctions (e.g., gap junctions). It is known that germ cells, such as pachytene spermatocytes and round spermatids, must remain attached to Sertoli cells throughout the 14 stages of the epithelial cycle in the rat, yet the molecules that confer this adhesiveness are not all known (Chung *et al.*, 1986; De Santa *et al.*, 1998a). Current knowledge shows that Sertoli–Sertoli and Sertoli–germ cell interactions, at the level of cell junctions are of vital importance for the functionality of the testis in human, rats and mice, but little is known about the dynamics of these cell–cell interactions throughout the annual cycle of reproduction in a seasonal breeder. Furthermore, there is no information on the possible role that the cell junctions may have on the regulation of the testis activation and inactivation in the males of seasonally breeding animals.

Out of the many types of cell junctions, only some have been demonstrated to be present in the testis. Figure 3.2 shows schematically the type of junctions and also their main functions.

Because Sertoli-germ cell interactions affect spermatogenesis at the molecular, cellular, and biochemical levels, this continues to be an important area

of research. However, in the past decade attention has largely shifted to the study of cell junction dynamics. This stems from the fact that communication between these cells takes place at the level of cell–cell contact, which in turn affects Sertoli and germ cell function. These demonstrate that a thorough understanding of the biochemical architecture of tight and anchoring junctions and the signaling pathways that regulate them is crucial to the development of new male contraceptives, in addition to those that are currently being investigated

3.4 Seasonal Breeding

In many species, reproductive activity is restricted to a certain period of the year. Reproductive seasonality ensures that in the wild, the young are generally born in spring or summer so that they are more likely reared in an environment that provides optimal conditions for survival. In seasonally breeding females, ovulatory cycles occur only during one part of the year. In the male, testicular size, testosterone release, sperm production and reproductive behaviour are downregulated in nonbreeding season. Outside the breeding season, fertility in the male may be maintained at a reduced level, as in the stallion (Guillaume *et al.*, 1996), or there can be a near or total cessation of spermatogenesis that renders the animal unable to breed for several months. This is the case in a variety of species, including hamsters (Bex and Bartke, 1977), deer (Brown *et al.*, 1979; Clarke *et al.*, 1995), brown bears (Tsubota *et al.*, 1997) and moles (Mathews, 1935; Jiménez *et al.*, 1990), among many others. Marked differences between breeds exist in sheep. In wild or rustic breeds, like the Mouflon or Soay, circannual variations in plasma concentrations of testosterone, gonadotropins and inhibin are significantly more pronounced than in other highly domesticated breeds (Lincoln *et al.*, 1990). Similar differences are found in the degree to which testicular size and spermatogenesis change (Dacheux *et al.*, 1981). In contrast to ewes and most horse mares, which become anovulatory outside the breeding season, stallions and rams are not azoospermic during the nonbreeding season despite a significant reduction in sperm production (Dacheux *et al.*, 1981; Aurich *et al.*, 1996).

Seasonal reproductive activity is regulated by an endogenous rhythm. This rhythm is synchronised with the geophysical year by environmental stimuli, the most important of which is photoperiod. Unlike temperature, availability of food, rainfall or other environmental cues, photoperiod provides information about the season and remains constant from year to year. Photoperiodic stimuli are transmitted via neuroendocrine pathways into hormonal signals that regulate gonadal activity. Reproductive activity is

not a direct function of daylength, but is affected by the photoperiodic history of the animal, the direction of photoperiodic changes and the stage of the circannual rhythm at which a photoperiodic signal is received (Robinson and Karsch, 1987; Gorman and Zucker, 1995). The effects of photoperiod on reproduction can be modified to a certain extent by temperature, nutrition, body condition or age. Animals lacking photoperiodic information e.g., when kept under a constant light regime still show seasonal changes in reproductive activity. However, these are no longer strictly linked to a 12 month period and do not occur in parallel in the animals of a herd (Grubaugh *et al.*, 1982; Jansen and Jackson, 1993).

Responses to photoperiod are species-specific. Species with a long gestation like the horse (320–350 days; (Ginther and Bergfelt, 1992)), as well as the hamster, which gives birth repeatedly during spring and summer (gestation length is 15-16 days in the Syrian hamster and 20–22 days in the Djungarian hamster), are long-day breeders. A gestation length between 5 and 6 months as in sheeps, goats and deers requires that breeding takes place in autumn during a period of decreasing daylength. These species are short-day breeders. Hibernating species, like hamsters, bears, hedgehogs, badgers, squirrels or marmots, are long-day breeders with a breeding season extending from late spring to early or late summer. Spermatogenesis is reactivated shortly before the end of the hibernation phase. In these species, an increase in daylength not only activates testicular function, but also provides the signal leading to the end of the denning period (Fowler, 1988; Tsubota *et al.*, 1997). Although the same photoperiodic stimulus acts differentially on reproductive activity in long and short-day breeders, long days in spring and summer seem to be particularly effective in fine-tuning reproductive activity to a 12-month period in female sheep as well as in stallions (Clay *et al.*, 1987; Woodfill *et al.*, 1994). Reproduction in rams is effectively depressed until autumn, but first signs of the seasonal resumption of reproductive activity can be detected already in spring (Olster and Foster, 1988). Photoperiod may, therefore, synchronize, in part, the endogenous reproductive rhythm over a one year period by comparable mechanisms in long and short day breeders.

In mammals, photoperiodic time measurement is achieved by a photoneuroendocrine system composed of the retina, the suprachiasmatic nucleus (SCN) of the hypothalamus, where the master circadian clock resides, and the pineal gland (Pévet, 1988; Goldman, 2001; Schwartz *et al.*, 2001). The latter transduces daylength into an endocrine signal in the form of the rhythmic secretion of the hormone melatonin (MEL) (Simonneaux and Ribelayga, 2003). Pineal and plasma MEL concentrations are low during the daytime and rise massively during the night-time. Consequently, the profile of the nocturnal MEL release fluctuates with photoperiod; it is now well

established that the duration of MEL secretion is the critical feature of its signal (Bartness *et al.*, 1993; Pitrosky and Pévet, 1997). MEL is thought to regulate GnRH secretion via two complementary mechanisms (Goldman, 1999): a change in the steroid negative feedback on GnRH release (Tamarkin *et al.*, 1976; Goodman *et al.*, 1982; Karsch *et al.*, 1993) and a direct steroid-independent modulation of GnRH secretion (Bittman and Goldman, 1979; Goodman *et al.*, 1982).

Melatonin plays a central role in transmitting daylength information to the whole organism (Fig. 3.3) particularly to the neuroendocrine gonadal axis (Pévet, 1988; Malpaux *et al.*, 2001). In the Syrian hamster, a common model for seasonal breeding, exposure to short days (SD) results in a dramatic inhibition of reproductive activity manifested by a decrease in serum LH, FSH and prolactin, accompanied by a complete suppression of spermatogenesis and a massive reduction of gonadal hormones biosynthesis. The visible consequence of these changes are the atrophy of the gonads and the accessory reproductive organs (Fig. 3.1) (Pévet, 1988; Bartke and Steger, 1992). Melatonin regulates the pulsatile secretion of GnRH from the hypothalamus, thereby influencing LH secretion (Bittman *et al.*, 1985). However, it is not known how the hormone acts to exert this effect. It appears not to act directly on GnRH neurons (Urbanski *et al.*, 1991; Ronchi *et al.*, 1992; Lehman *et al.*, 1997; Brown *et al.*, 2001) and responsiveness to GnRH does not change with photoperiod (Pieper, 1984). Melatonin might regulate GnRH secretion via two complementary mechanisms (Goldman, 1999): a change in the steroid negative feedback on GnRH release (Karsch *et al.*, 1993; Goodman *et al.*, 1982) and a direct steroid-independent modulation of GnRH secretion (Goodman *et al.*, 1982; Bittman and Goldman, 1979).

3.4.1 Photoperiod controls seasonal reproduction via *KiSS1/GPR54*

The *KiSS1* gene encodes a 145 amino acid protein which is processed to generate biologically active peptides of various lengths (10, 13, 14, and 54 amino acids). These peptides belong to the RFamide peptide family and are collectively named kisspeptins, the most abundant type being the 54 amino acid form, kisspeptin-54, also referred to as metastin. GPR54 (also known as AXOR12 and hOT7T175) was initially isolated as an orphan G-protein-coupled receptor (Lee *et al.*, 1999). In 2001, it was found to bind KiSS1 (Muir *et al.*, 2001; Clements *et al.*, 2001), known as the main product from an antimetastasis gene (Lee *et al.*, 1996a; Harms *et al.*, 2003). Binding of KiSS1 to GPR54 triggers a series of intracellular responses via a coupling to G proteins of the Gq/11 subfamily, including calcium mobilization,

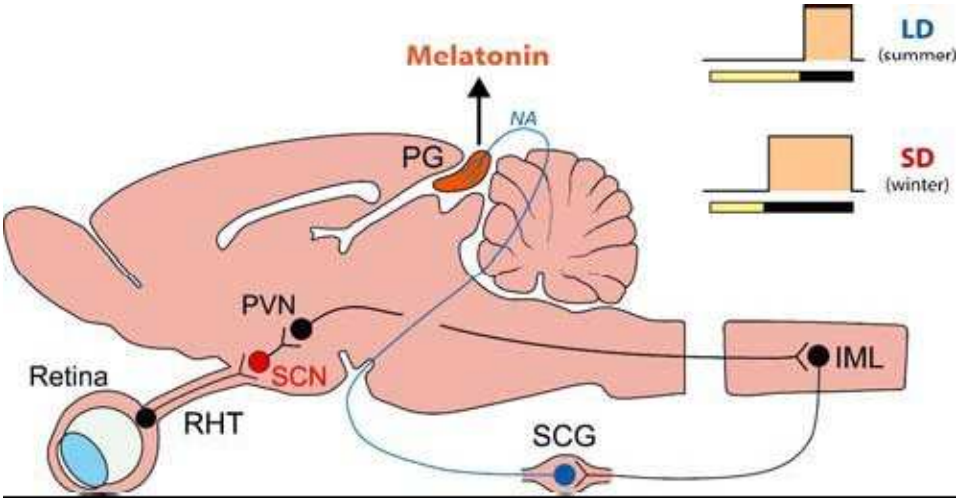


Figure 3.3: The photoneuroendocrine system drives the rhythmic release of melatonin. In mammals, the light/dark information is perceived by the retinal ganglion cells whose axons project, via the retinohypothalamic tract (RHT), to the suprachiasmatic nucleus of the hypothalamus (SCN), seat of the master circadian clock. The SCN controls the rhythm of melatonin synthesis and release by the pineal gland (PG), via a complex multisynaptic pathway. The SCN projects to the paraventricular hypothalamic nucleus (PVN), which in turn projects to the intermediolateral cells (IML) of the upper three segments of the spinal cord. The IML neurons innervate the rostral pole of the superior cervical ganglion (SCG) which projects to the PG where noradrenalin (NA) is released to promote melatonin synthesis and subsequent release into the bloodstream. Because the SCN restricts noradrenalin release to night time, high melatonin plasma level arises during the night only. As a result, the duration of the nocturnal melatonin release changes according to daylength (photoperiod): during summer, long days (LD) are associated with short melatonin peaks, whereas during winter, short days (SD) are associated with longer melatonin peaks (yellow and black bars represent day and night, respectively). The picture has been taken from Revel *et al.* (2007)

phosphatidylinositol-4,5-bisphosphate hydrolysis, arachidonic acid release, MAP Kinase activation (ERK1/2), and probably NFkB induction (Stafford *et al.*, 2002; Becker *et al.*, 2005).

In late 2003, genetic studies showed unexpectedly that the phenotype of humans and mice bearing loss-of-function mutations in GPR54 matched the symptoms of isolated hypothalamic hypogonadism (IHH), with abnormal sexual development and failure to undergo puberty (Seminara *et al.*, 2003; de Roux *et al.*, 2003; Semple *et al.*, 2005). The mutants are unable to drive LH/FSH secretion, although GnRH is synthesized normally in hypothalamic GnRH neurons and gonadotrope cells in the pituitary remain capable of responding to exogenous GnRH.

In a variety of models, central and peripheral administration of kisspeptin potently elicits LH/FSH secretion, which is blocked by GnRH antagonists (Matsui *et al.*, 2004; Irwig *et al.*, 2004; Thompson *et al.*, 2004; Shahab *et al.*, 2005; Kinoshita *et al.*, 2005). However, several lines of evidence indicate that kisspeptin exerts its action on the HPG axis via GPR54, as kisspeptin has no effect in GPR542/2 mice neurons (Irwig *et al.*, 2004), and KiSS1 triggers GnRH release (Messager *et al.*, 2005) without influencing GnRH expression (Navarro *et al.*, 2005a,b). Various groups investigated the regulation of *KiSS1* and *GPR54* expression. Sex steroids influence *KiSS1* expression (Irwig *et al.*, 2004; Navarro *et al.*, 2004) via androgen (AR) and α-type estrogen (ERα) receptors (Navarro *et al.*, 2004; Smith *et al.*, 2005).

The activation of the *KiSS-1/GPR54* system is necessary for reproductive activity. The KiSS1 neurons, located in the arcuate nucleus (ARC) and in the anteroventral periventricular nucleus (AVPV) of the hypothalamus, integrate various signals to drive reproductive activity. Gonadal steroids inhibit or stimulate *KiSS1* expression in the ARC and in the AVPV, respectively. It has been shown that photoperiod, via melatonin, can influence *KiSS1* expression in these two structures. This may occur via two complementary mechanisms: 1) a direct action on *KiSS1* expression, and/or 2) a change in the sensitivity of *KiSS1* to the feedback action of sex steroids. It is not known whether melatonin effects on *KiSS1* expression are exerted directly onto KiSS1 neurons or via a relay structure(s). At puberty, activation of the *KiSS1/GPR54* system is presumed to be essential for initiating reproductive activity. In various seasonal species (e.g. the Siberian hamster; but not the Syrian hamster), the rate of development is controlled by photoperiod and melatonin, and KiSS1 neurons could be involved in this mechanism. Finally, the KiSS1 neurons may process a variety of additional signals, including body energy stores, to control reproductive activity

It has been suggested that MEL could act on the KiSS-1 cells, which would be part of the GnRH pulse generator in the MBH and represent interneurons to control activation of GnRH cells (Fig. 3.4). This hypothesis

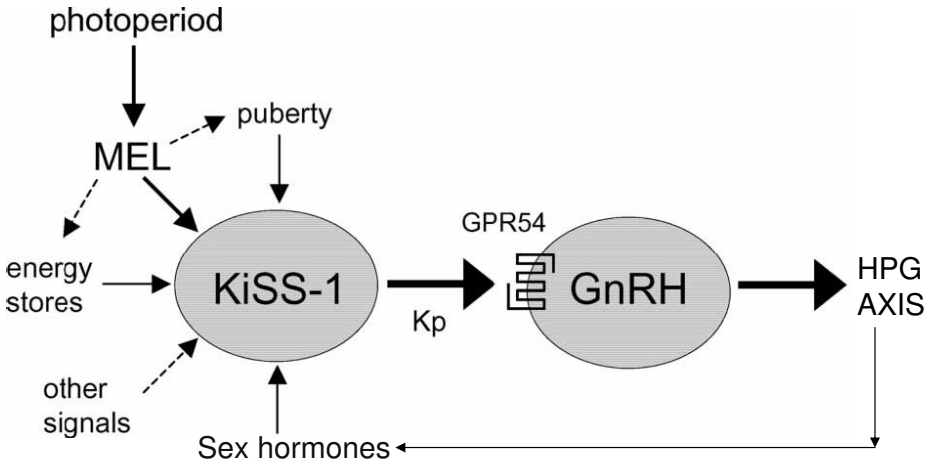


Figure 3.4: Working hypothesis for the photoperiodic control of reproduction. The KiSS1 neurons located in the mediobasal hypothalamus project to GnRH neurons located in the preoptic area. Release of kisspeptin (Kp) activates *GPR54* which in turn induces release of GnRH and activation of the hypothalamo-pituitary-gonadal (HPG). KiSS1 cells receive and integrate signals from diverse origins, including sex hormones that feedback on KiSS1 neurons to downregulate *KiSS1* expression, body energy stores, and possibly other signals (e.g., ambient temperature, stress, and pheromones). We suggest the activity of the KiSS1 neurons is influenced by photoperiod, via the MEL signal, either directly or via intermediate targets. The KiSS1 neurons are primarily activated at puberty, which may be gated by photoperiod through MEL. The piture have been taken from Revel *et al.* (2006)

is supported by preliminary data that indicate KiSS1 expression is down-regulated in Syrian hamsters exposed to short day photoperiod (SD) (Revel *et al.*, 2006) Also interesting is the fact that rat and mouse KiSS1 neurons are affected by sex steroid and coexpress AR and ER α, this may explain, at least partly, the dual steroid-dependent and -independent modulation of GnRH secretion by MEL observed in photoperiodic species. Finally, photoperiod can modulate the rate of development of some seasonal species (Hoffmann *et al.*, 1978; Ebling and Foster, 1989; Shaw and Goldman, 1995). Given the recent data about the role of KiSS1 in the control of puberty, one can then speculate that KiSS1 cells may also play a role in this photoperiodic mechanism.

3.4.2 Role of Apoptosis and Cell Proliferation in Seasonal breading cycle

Spermatogenesis is a complex process consisting of a cascade of mitosis, meiosis and differentiation steps, during which spermatozoa develop from spermatogonia. The whole process is characterized by high proliferation rates. This testicular activity depends on several genetical, seasonal and environmental conditions. For these reasons the production of male germ cells is an important indicator of ecological and anthropogenic factors affecting animal reproduction. The evaluation of spermatogenic efficiency requires a reliable quantification of proliferative and degenerative processes. Modulation of chromatin structure plays an important role in the regulation of transcription in eukaryotes. The nucleosome, made up of four core histone proteins (H2A, H2B, H3 and H4), is the primary building block of chromatin (Workman and Kingston, 1998). The amino-terminal tails of core histones undergo various post-translational modifications, including acetylation, phosphorylation, methylation and ubiquitination (Hansen *et al.*, 1998; Cheung *et al.*, 2000). These modifications occur in response to various stimuli and have a direct effect on the accessibility of chromatin to transcription factors and, therefore, on gene expression (Jaskelioff and Peterson, 2003). Histone H3 is primarily acetylated at lysines 9, 14, 18 and 23 (Hansen *et al.*, 1998; Cheung *et al.*, 2000). Phosphorylation at Ser10, Ser28 and Thr11 of histone H3 is tightly correlated with chromosome condensation during proliferation in both mitosis and meiosis (Hansen *et al.*, 1998; Cheung *et al.*, 2000). Hence we used Phosphohistone H3 (Ser10) used for quantification of proliferation activity.

Apoptosis (programmed cell death) is an active, genetically governed, signal-induced process of selective cell elimination (Schwartzman and Cidlowski, 1993). Coincidence of spermatogonial proliferation and spontaneous degeneration of spermatogenic cells seems to be a general property of normal

testes in mammals (Allan *et al.*, 1992; Kerr *et al.*, 1992). The regulation of testicular cell population size involves apoptosis as a normal, hormonally controlled process in mammals (Blanco-Rodríguez, 1998; Sinha Hikim and Swerdloff, 1999; Print and Loveland, 2000). Corresponding to the high rate of proliferation and the tight control required for achieving the precise homeostasis between Sertoli cells and every germ cell type, cell death is also a prominent phenomenon. Thus, up to 75% of potential spermatozoa have been estimated to die in the testis of adult mammals. Although spermatogonia and spermatocytes have been described in many species as the main cell types that undergo physiological cell death, apoptosis may affect all four classes of male germ cells, viz. spermatogonia, spermatocytes I and II and spermatids (Blanco-Rodríguez and Martínez-García, 1996a, 1998). In the rat testis, spontaneous apoptosis of A2, A3, and A4 type spermatogonia (Allan *et al.*, 1988) occurs regularly while primary and secondary spermatocytes as well as spermatids undergo apoptosis occasionally (Kerr *et al.*, 1992; Brinkworth *et al.*, 1995; Blanco-Rodríguez and Martínez-García, 1996b). However, A1, intermediate, and type B spermatogonia rarely degenerate (Bronson, 1988). Apoptosis results in the seminiferous epithelium generating fewer spermatozoa than would theoretically originate from spermatogonial proliferation. The comprehensive analysis of available data indicate that these spermatogonial apoptosis plays a major role in spermatogonial density regulation as well as in the maintenance of the required homeostasis among the various germ cell types that can be supported by Sertoli cells and fit the seminiferous tubule shape. In addition, germ cell apoptosis seems to play a major role to safeguard the genetic integrity of the male gamete and the synchronization between the spermatogonial and the spermatocyte cycles that originates the mammalian spermatogenic stages, eliminating harmful irreparably damaged cells that are not able to pass checkpoint monitored transitions due to improper synapsis between homologous chromosome.

Seasonal breeders represent a special case in spermatogenesis, because males are subject to annual cycles of testis growth and involution. Regression occurs during decreasing plasma gonadotropin concentrations and reduced testicular testosterone production. Diminished or halted spermatogonial proliferation during the post-breeding period results inherently in reduced spermatogenic cell numbers and testis volume. However, the dramatic reduction by 80%-90% of testis mass during regression strongly suggests the contribution of apoptotic cell elimination with respect to seasonal testicular atrophy. Apoptosis is also induced in response to meiotic arrest in non-seasonally reproducing species (Wolgemuth *et al.*, 2002). Some studies have demonstrated apoptosis as a contributing mechanism to testicular regression in hamsters (Furuta *et al.*, 1994; Morales *et al.*, 2002), white footed

mice (Young *et al.*, 1999) and the European brown hare (Blottner *et al.*, 1995; Strbenc *et al.*, 2003). However, an inverse relationship between proliferation and apoptosis has been found during seasonal changes of testis structure and function in roe deer (Blottner *et al.*, 1995, 1996). The annual cycle in roe deer includes the transition between highly activated (rut) and completely arrested spermatogenesis (winter) and is associated with considerable changes in the mass (by a factor of five) and cellular composition of testis (Blottner *et al.*, 1996; Schön *et al.*, 2004). However, little attention has been directed toward the comparative quantification of proliferation and apoptosis during seasonal periods except in few cases. The considerable changes of involution and recrudescence of testicular activity in seasonal breeders is a result of both proliferation and programmed cell death, acting as antagonists with their maxima and minima at different times during the annual cycle except in the roe deer (Blottner *et al.*, 2007). Due to these contradictory findings, we have checked the the role of proliferation and apoptosis in *T. occidentalis* as we lacked any information about the intra-testicular balance between proliferation, differentiation and death of cells was deficient.

3.5 Moles as Seasonal breaders

Moles of family Talpidae show several singular features concerning their reproductive biology. Female moles of the genus *Talpa* are exceptional among mammals because they develop ovotestes instead of normal ovaries and, nevertheless, they are fertile (Jimenez *et al.*, 1993, 1996). These ovotestes are composed of a small portion of normal ovarian tissue and a generally large portion of dysgenic testicular tissue. In the species *T. occidentalis* the medullary region of the XX gonads develops according to a testis-like pattern which includes cord formation, mesonephric cell migration, profuse vascularization and Leydig and peritubular-myoid cell differentiation (Barrionuevo *et al.*, 2004). Although these features define most of the main events of testis organogenesis (Brennan and Capel, 2004), ovotestes of female moles lack fully differentiated Sertoli cells, as they do not express typical Sertoli cell markers such as AMH or SOX9 . However, Leydig cells in the ovotestes of non-breeding females produce abundant testosterone in postnatal stages of development and during the non-breeding seasons, leading to musculinization of these females. The body of female moles shows additional signs of masculinisation other than gonads. These include a large, penile clitoris where the urethra opens at the tip, an intact perineal region without any vaginal opening during the non-breeding season (the vaginal orifice opens and closes every year) and rudimentary epididymides adjacent

to the ovotestes (Jimenez *et al.*, 1993; Whitworth *et al.*, 1999).

Moreover, moles are strict seasonal breeders. In Southern Iberian Peninsula, moles of the species *T. occidentalis* breed during the winter (Jiménez *et al.*, 1990). Contrarily, moles of the species *T. europaea* living in Central and Northern Europe, breed during the spring. Many morphological changes occur in the gonads of both males and females throughout the annual reproductive cycle. In males, testes are larger during the breeding season and smaller during the non-breading season, so that testicular weight in December may cuadruplicate that in July. The histological features of fertile and non-fertile testes are significantly different. Most cells present in the germinative epithelium of fertile testes disappear when they become non- fertile, and appear again in the next autumn, when testis function reactivates. However, both the mechanism of cell depletion and the identity of the cells remaining inside the testis cords, are currently unknown. In females, the two gonadal components (ovarian and testicular) behave differently throughout the annual breeding cycle. As expected, the ovarian portion of the ovotestis is larger in the winter, due to the presence of growing and mature follicles in non-pregnant females, or large *corpora lutea* in pregnant and lactating individuals. Only primordial follicles are present in the female gonad during the summer. Contrarily, the testicular portion of the ovotestis is much larger during the summer, although little is known concerning which cell types are responsible for these changes in size.

The confluence of several particularities and singularities including seasonal breeding and intersexuality makes *T. occidentalis* to be an animal model of high scietific interest to investigate many aspects of the genetic, endocrine and paracrine control of gonadal function.

Aims and Objectives

1. To study the spatiotemporal pattern of expression of several genes, throughout the seasonal breeding cycle in adult XY gonads of *T. occidentalis* by Immunoflourescence.

2. To look for associations between gene expression patterns and cell functional status, in order to establish possible functions for these genes in controlling seasonal breeding.

3. To performe quantitaive analyses of the expression of these genes by Real Time PCR, in order to determine when functional expression levels are reached.

4. To study the histology of the gonads in each reproductive stage to determine the functional status of the main cell types (Sertoli, Leydig, myoid and germ cells) and structures (seminiferous tubules), throughout the seasonal reproductive cycle.

5. To study the serum and intratesticular levels of testosterone and estradiol and the expression levels of their respective receptors in the testis.

6. To study the role of apoptosis and cell proliferation in the seasonal variations of the mole testis

7. To study the role of cell junctions in the seasonal dynamics of germ cells

Material and Methods

5.1 Material Analysed

A total of 24 males of the mole species *Talpa occidentalis* were captured throughout an entire year, so that a representative sample of specimens in each of the four stages of the reproductive cycle was available. Each individual was asigned to a particular reproductive cycle stage, according to classifying criteria defined as follows:

Active: individuals with a fully functional testes, captured between november and march (december in our case). The weight of the testis, serum testosterone levels and the diameter of the seminiferous tubules are higher than in other stages. All stages of spermatogenesis are presents and sperms can be seen inside the epidydimides.

Inactive: individuals with inactive, sterile testes, captured between june and august (july in our case). The weight and size of the testis is very low, as is the serum testosterone concentration as compared to **active**. The seminiferous tubules are much smaller in diameter and shows only Germ cells at spermatogonia stage. The epidydimides are empty.

Inactivating: individuals with testes reducing in size and loosing spermatogenic function, captured between march and june (april in our case). The serum testosterone concentration is lower than in **active** individuals. The weight of the testis is decresing with respect to **active** males, but still higher than in **inactive** ones. The epidydemis contain cell debris.

Activating: individuals with testes increasing in size and gaining spermatogenic function, captured between august and october (september

in our case). The serum testosterone concentration is much higher than in **inactive** but lower than in **active** males. The weight of the testis is higher as compared to **inactive** testis. The epidydimis may contain less sperms when compared to **active** epidydimis.

According to the above mentioned criterias, the following animals were analyzed in this study: 9 fertiles, 5 inactivating, 7 Infertile and 3 activating.

5.2 Methods

5.2.1 Animal management

Captures

Adults male and female of the insectivorous mole species *Talpa occidentalis* were captured alive in poplar groves around Santa Fe (Granada province, Southern Spain) over the whole breeding season as described previously (Barrionuevo *et al.*, 2004). Moles were captured under permission granted by the Andalusian Environmental Council. The adult moles were handled in accordance with the guidelines and approval of the "Ethical Committee for Animal Experimentation" of the University of Granada. For captures, we used mole traps designed and made by Dr. R. Jiménez at the Departament of Genetics of the University of Granada. Captures were made in December, April, July and early September, thus including all stages of the breeding cycle of *T. occidentalis*. The adult moles were dissected and gonads were removed under sterile conditions. One of the gonads was frozen in liquid nitrogen for further mRNA purification, whereas the other one was fixed in 50 volumes of 4% pareformaldehyde overnight at 4 °C and then embedded in paraffin.

Estimation of the age

The age of each animal was estimated using an index obtained on the basis of dental wear (Jiménez *et al.*, 1988).

The first upper molar from left and right sides were removed from the skull of the animals and measured according to the under a binocular stereomicroscope.

The dental wear index (DWI) was calculated as the arithmetic mean of the index obtained by using the following formula in each of the two molars:

$$DWI = \frac{A}{(B + C)}$$

Table 5.1: Summary of the male moles analysed

Mole code	Dental Wear Index (avg.)	Weight of testis (mg)	Body wight (gm)	Epididymis contents	Reproductive status
TS26	0.93	392	64.2	sperms	Active
TO14	ND	384	61	sperms	Active
TD15	ND	395	64.4	sperms	Active
TD16	ND	308	72.4	sperms	Active
TD19	ND	485	66.2	sperms	Active
TE19	ND	550	58.5	sperms	Active
TE18	ND	440	61.2	sperms	Active
TM22	ND	540	74	sperms	Active
TM23	ND	357	72	sperms	Active
TA26	1.07	204	57.5	some sperms and cell debris	Inactivating
TY27	0.95	151.8	58.3	some sperms and cell debris	Inactivating
TA24	0.83	133.8	60.1	some sperms and cell debris	Inactivating
TY32	1.10	119.6	63	some sperms and cell debris	Inactivating
TY21	0.98	282	68	some sperms and cell debris	Inactivating
TL65	1.44	95	68.1	empty	Inactive
TL59	ND	98	60.1	empty	Inactive
TL76	1.12	100	54.3	empty	Inactive
TL81	1.33	89.8	65	empty	Inactive
TL72	0.98	183	61	empty	Inactive
TL73	1.20	126.4	64.2	empty	Inactive
TL74	0.90	97.9	58.3	empty	Inactive
TS28	1.17	245	63.5	sperms	Activating
TS32	1.65	174	57.7	sperms	Activating

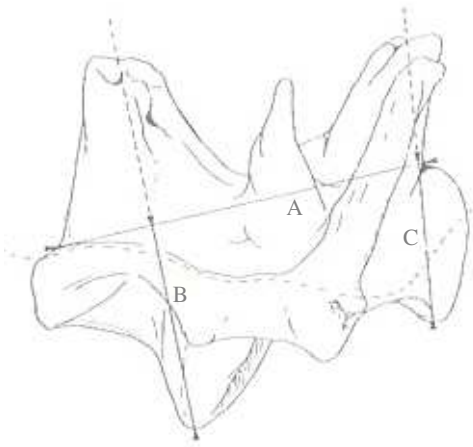


Figure 5.1: Diagramatic representation of a M1 molar piece of *T. occidentalis*, indicating the three parameters, A,B and C, used to calculate the dental wear index (DWI) for estimating the age of animal. The figure is taken from Jimenez et al, 1988

A DWI below 0.80 is indicative of a juvenile individual and a ratio over 0.85 of an adult. Furthermore, before classifying an individual as juvenile or adult, this data was compared with other morphological features like weight of the gonad, weight of the animal or weight of the uterus (in case of females).

5.2.2 Histological methods and immunostaining

Paraffin Embedding and sectioning

After 1 hour of prefixation in 4% paraformaldheyde, testis were sliced to produce 3-5 pieces in order to allow good fixation of the most profound regions overnight. Prefixation was not necessary in the case of ovotestis. Then the gonads were washed twice with 1×PBS for 20 min each and then dehydrated with an ethanol series (50%, 70%, 96%, and 100%) and stored overnight in 100% ethanol at -20 °C. The gonad was then kept in 100% ethanol for 2 hours at 4 °C, washed in ethanol:xylene(1:1) for 1 hour, and then in Xylene for 1 hour, both at room temperature (RT). The gonads were further treated with Xylene at 60 °C, followed by Xylene:Paraffin (1:1) at 60 °C for 1 hour each and incubated in Paraffin at 60 °C overnight.

Next day the paraffin was replaced with fresh paraffin and kept at 60 °C for 4–5 hours and the gonads were oriented as needed (vertically for testis and horizontally for ovotestis), into moulds to produce paraffin blocks.

The paraffin blocks containing the gonads were stored at room temperature for 2-3 hours and then at 4 °C until sectioning.

Haematoxylin–Eosin (HE) staining

HE staining of the sample sections was performed as follows:

1. Dewaxing of the sections was performed by three washes of xylene, 10 minutes each.
2. Samples were rehydrated using an ethanol series (100%, 96%, 70%, 50%), with 10 min. steps.
3. After washing with H_2O for 10 seconds, the sections were stained with haematoxyline for 10 seconds – 3 minutes (The time has to be standardized, for every fresh solution of Haematoxyline).
4. Sections were washed in tap water until the colour of haematoxylene changes from violet to dark violet.
5. Sections were stained with Eosin for 2–3 minutes (The time has to be standardized, with every fresh solution of Eosine).
6. After washing with dH_2O for 10 seconds, the samples were dehydrated using an ethanol series (50%, 70%, 96%, 100%) with 10 seconds in each step. Finally the samples are kept in Xylene and mounted in DPX.

Immunofluorescence

7-9 μm thick sections were cut from paraffin embedded tissue and attached to Tespa coated slides or poly-l-lysine slides (Polysine, VWR International, Belgium).

The protocol for immunoflourescence was as follows:

1. Slides with sample sections were heated at 50 °C for 20 min over a hot plate.
2. Two washes with xylene were performed for 20 min on a shaker.
3. Rehydration Steps (All washings and dehydration steps were performed in Koplin jars on a shaker)

   ETOH 100 % - 5 min.- twice

   ETOH 75 % - 5 min.- once

   ETOH 50 % - 5 min.- once

ETOH 25 % - 5 min.- once

4. Washing: two washes were done with PBST for 10 min. [1×PBST =1×PBS + Tween 20 (0.01%)].

5. Antigen Retrieval: Slides were treated with sodium citrate (0.01 M, pH 6.0 or pH 2.0) for 5 min in a microwave oven at maximum power without allowing the slides to get dry. To prevent drying, extra sodium citrate (0.01 M, pH 6) was kept in the microwave oven and the level of sodium citrate was checked after every 1 min. Sodium citrate at the same temperature was added to the Coplin jars containing the slides with samples, if the level falls below the sample.

6. The slides were left inside the microwave for 30–40 min until they reached RT.

7. They were whased twice with 1×PBST, 10 minutes each.

8. Blocking was done with 10% BSA in 1×PBST for 1 hour at 4 °C. 250-300 μl of blocking agent was added to the slides and covered with coverslip.

9. 200–250 μl of primary antibody (diluted in 1% BSA and 1×PBST, for dilution see table 5.2) was added to the samples on the slides and covered with a coverslip. Some amount of 1×PBST was kept in the staining chamber to maintain the humidity during the O/N incubation at 4 °C.

10. Slides where washed with PBST for 20 min each.

11. Fluorochrome conjugated (FITC or Cy3) secondary antibody diluted in 1% BSA and 1×PBST was added to the samples. They where covered with coverslips and kept in the dark at RT for 1.5–2 hours.

12. Three washes were then performed with PBST, one for 20 minutes and two for 30 minutes each.

13. Vectashield mounting medium (Vector Laboratories, Burlingame, CA) was added and the slides were observed in a flourescence microscope and photographs were taken using Olympus DP70 digital camera installed on a Olympus BX41 microscope.

Immunohistochemistry

1. The sections were cut and deparaffinized with histoclear for 20 minutes (two changes of 10 min)
2. Sections were rehydrated in an ethanol series (100% ethanol 5 min, 70% ethanol 5 min, and 50% ethanol 5 min) and then washed twice in 1×PBST.
3. Antigen retrieval was carried out by Tris-EDTA buffer (pH 9.0) for 10 min (twice) using the microwave method.
4. The section were washed three times in 1×PBST, 5 min each.
5. Blocking was done with 10% normal serum (blocking buffer) of the species in which the secondary antibody is raised (normal goat serum, normal rabbit serum or normal horse serum).
6. The sections were incubated with primary antibody diluted in blocking buffer in a moist chamber for overnight at 4 °C, and washed thrice in 1×PBS for 10 min each.
7. Endogenous peroxidase activity was blocked by treating the sections with 10% hydrogen peroxide (35% w/w) for 1 hour at room temperature, and sections were washed twice in 1×PBST.
8. The endogenous biotin activity was reduced by treating the sections with Avidin for 20 min and followed by treatment with biotin for 20 min.
9. After two washes of 5 minutes each, the sections were incubated with secondary antibody, IgG conjugated with Biotin (1:200 in blocking buffer) for 1 hour in a moist chamber at room temperature.
10. Sections were washed twice in 1×PBST for 10 min each.
11. Sections were incubated with ABC reagent (10 μl of A + 10 μl of BC + 980 μl of 1×PBST , Vector Laboratories, Burlingame, CA) for 1 hour at room temperature in dark.
12. The colour was developed using DAB and counterstained with Haematoxylene
13. Sections dehydrated in an alcohol series, followed by xylene and permanently mounted in DPX.

Table 5.2: Primary antibodies used in this study

Gene product (Reference)	Description	Source of antibody	Primary Antibody dilution
SOX9 (SantaCruz)	SRY related high mobility group box 9	Rabbit polyclonal raised against human protein	1:200
DMRT-1	DoubleSex- and Mab3 related transcription factor-1	Rabbit polyclonal raised against human protein	1:1000
SYCP3 (SantaCruz)	Synaptonemal complex protein-3. Meiotic germ cell marker.	rabbit polyclonal, raised against human protein	1:1000
OCT4 (SantaCruz)	POU family homeodomain protein. Pre-meiotic germ cell marker.	goat polyclonal, raised against human protein	1:400
P450scc (SantaCruz)	Cytochrome P450scc, a steroidogenic enzyme	goat polyclonal, raised against human protein	1:200
SF1 (SantaCruz)	Steroidegenic factor-1		1:500
α-SMA	Smooth muscle protein. Myoid cell marker.	mouse monoclonal, raised against rabbit protein	1:400
Phospho Histone H3 (Upastate cell signaling solutions)	mitosis marker	Rabbit polyclonal, raised against human protein	1:100
DMC1 (SantaCruz)	early meiosis stage marker	goat polyclonal, raised against human protein	1:100
β-catenine (BD Biosciences)	adherent junction marker	mouse monoclonal, raised against rabbit protein	1:100
N-cadherin (BD Biosciences)	adherent junction marker	mouse monoclonal, raised against rabbit protein	1:100
Laminin (BD Biosciences)	basement membrane marker	mouse monoclonal, raised against rabbit protein	1:100
Caspase 3 (RD Systems)	apoptosis marker	Rabbit polyclonal, raised against human protein	1:100
Claudin 11 (SantaCruz)	tight junction marker	Rabbit polyclonal, raised against human protein	1:200
AR (SantaCruz)	androgen receptor	Rabbit polyclonal, raised against human protein	1:200

The secondary antibodies used (at a dilution of 1:200) for Immunohistochemistry were: biotinylated anti-goat IgG(H+L) , biotinylated anti-rabbit IgG(H+L) and biotinylated anti-mouse IgG(H+L)[Vector Laboratories, Burlingame, CA]. The secondary antibodies used (at a dilution of 1:200) for immunoflourescence were: anti-rabbit IgG conjugated with FITC or cya3,anti-goat IgG conjugated with FITC or cya3,anti-mouse IgG conjugated with FITC [Sigma-Aldrich, St. Louis, MO]

5.3 Molecular biology methods

5.3.1 Nucleic acid purification

Isolation of Total RNA

Total RNA was isolated from the testis of *T. occidentalis* using a Qiagen RNeasy kit (QIAGEN, Valencia, CA).Manufacturers protocol was followed with some modifications:

RNAlater stabilized testis was taken out using forceps. The amount of tissue was determined. It was ensured that more than 30 mg of testis was not used.

The appropriate amount of the testis was disrupted and homogenized in 600 μl of Buffer RLT.

The lysate was transferred directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed.

The lysate was centrifuge for 3 min at full speed. The supernatant was carefully removed by pipetting, and transfered to a new microcentrifuge tube.

1 volume of 70% ethanol was added to the supernatant and mixed immediately by pipetting.

700 μl of the sample was transfered to an RNeasy spin column placed in a 2 ml collection tube and centrifuge for 15 s at 8 000×g (10 000řpm). The flowthrough was discarded.

On column DNAse digestion was performed to remove the contaminating genomic DNA.

a) 350 μl Buffer RW1 was added to RNeasy column and centrifuged for 15 s at 8 000× g, and the flow-through was discarded.

b) 10 μl DNase I stock solution was added to 70 μl Buffer RDD gently mixed by inverting tube and was centrifuged briefly.

c) DNase I incubation mix (80 μl) was added directly to RNeasy column membrane and was incubated at room temperature (20-30 °C) for 15 min.

d) 350 μl Buffer RW1 was added to RNeasy column centrifuged for 15 s at 8 000× g and flow-through was discarded.

500 μl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 s at 8 000× g(10 000řpm) to wash the spin column membrane. The flow-through was discarded.

500 μl Buffer RPE was added to the RNeasy spin column. and centrifuged for 15 s at 8 000× g(10 000řpm) to wash the spin column membrane and also to avoid the possible carryover of ethanol.

RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min to eliminate any possible carryover of Buffer RPE.

The RNeasy spin column was placed in a new 1.5 ml collection tube. 50 μl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 8 000× g (10 000 rpm) to elute the RNA.

The RNA was quantified and its quality was checked on 1% agarose gel.

Reverse Transcription

The retrotranscription was performed using iScript cDNA Synthesis Kit (BIO-RAD), which uses a mix of oligodT and random ribonucleotidos allowing the amplificaction of the RNA. For this reaction the following components were mixed:

4 μl 5X iScriptMix Reaction 1 μl iScript Reverso Tansciptase 10 μl Distilled water without nucleases 5 μl RNA (100 fg -1μg Total RNA) 20 μl Total volume

The conditions of Retrotanscription were as follows:

5 min 25 °C

42 °C 30 min

5 min 85 °C

4 °C

Designing of primers

Table 5.3: Primers used in this study

Gene	Description	Primers	bp	Tm
For qRT-PCR				
SOX9	SRY related high mobility group box 9	F: TCATGAAGATGACCGACGAGC R: CTTGTTCTTGCTGGAGC-CGTT	287	62
DMRT1	DoubleSex- and Mab3 related transcription factor1	F: GGAAACCAGTGGCAGATGA R: CAAGCCAGAATCTTGACTGC	195	60
P450scc	Cytochrome P450scc, a steroidogenic enzyme	F: AGGAGGGGTGGACACGAC R: GTCTCCTTGATGCTGGCTTT	177	55
SF1	Steroidegenic factor 1	F: GAGGTGGAGCTGACCACAGT R: TCAAATCCAGGCTGAAGAGG	157	51
CLAUDIN11	tight junction protein	F: TGACTGTTCTTCCCTGCATC R: TAGCCAAAGCTCACGATGG	169	50
ZO2	tight junction protein	F: GGTCTTCCGAGTGGTAGACA R: ACGCATTCTCCAGAAATCTG	181	48
JAM2	tight junction protein	F: GAAGTTAGTGCCCCATCTGA R: GCTGGATTCCCTTCTTTGTC	161	48
NCADHERIN	adherens junction protein	F: ACAATGCCCCTCAAGTGTTA R: CATTAAGCCGAGTGATGGTC	127	49
βCATENIN	adherens junction protein	F: GAAACGGCTTTCAGTTGAGC R: CTGGCCATATCCACCAGAGT	166	52
CONNEXIN43	gap junction protein	F: TGTGATGCGAAAGGAAGAGA R: GATGATGTAGGTTCGCAGCA	178	50
LHβ	beta subunit of Leutinizing hormone	F: GCTGTTGCTGCTGCTGAG R: AGAGCCACAGGGAAGGAGAC	294	50
FSHr	Follicle stimulating hormone receptor	F: TGAGAGCTCACTGGCAAAGA R: CCGGTTCTTATGTGCAACCT	177	51
AR	Androgen receptor	F: ATTTGGATGGCTCCAGATCA R: GCTGAGCAGGATGTGGATTT	177	52
ABP	Androgen binding protein	F: TTGGAGAGCTGTTGGATGG R: GCCAGGAGCATGTCAAAGA	183	51
ERα	Estrogen receptor alpha	F: TCCAGGACTAGGTCCTCTGC R: CCTCTTTGACCGAGAGATCG	189	51
GAPDH	Glyceraldehyde dehydrogenase	F: GATGACATCAAGAAGGTG-GTG R: TCATACCAGGAAATGAGCTTG	179	54
For *in situ* hybridization				
ERα	Estrogen receptor alpha	F: AACGAGTGTGAGATCAC-CAAGC R: CACAGAGTCTGAATTG-GCAAGG	663	62

The primers in table 5.3 were designed by using eprimer3, an interface to the 'primer3' program from the Whitehead Institute. For Real time

PCR reactions primers were designed to amplify 120-200 bp fragment of the mRNA. For *in situ* hybridization primers were designed to amplify 500-700 bp fragment of the mRNA. In case of *ERα*, *FSHr*, *AR*, *CONNEXIN43*, *βCATENIN*, the Real Time primers were designed after amplifying a fragment for *in situ* hybridization. Below is the list of all the primers which were designed and used.

5.3.2 Polymerase chain reaction

PCR was performed using thermal cycler Chromo 4 Real-Time PCR Detection System (BIO-RAD) using the SensiMix dt kit (Quantace R). PCR reaction was set up with total volume of 25 μl:

12.5 μl Sensimix 2X

0.5 μl SYBR Green

0.5 μl forward primer (10 pmol/ μl)

0.5 μl reverse primer (10 pmol/ μl)

2 μl cDNA

9 μl Distilled Water (nuclease free)

The PCR conditions were as follows:

95 °C for 10 minutes

35 cycles of:

- 96 for 15 s
- 57 °C (for different genes, this temperature varies depending on the Tm of the primer)
- 72 °C for 1 minute
- Plate Read
- 72 °C for 3 minutes
- Melting curve from 75 °C to 94 °C read at every 1 °C hold for 1-s

4 °C untill the end

5.3.3 Agarose gel electrophoresis and gel extraction

Agarose gel electrophoresis was performed using submarine gel electrophoresis system (Sharp et al., 1973). Plasmids and DNA fragments were resolved based on size on 1% TAE gels at 5 V/cm. The desired fragment was excised from the agarose gel using QIAquick gel extraction kit (QIAGEN, Germany) following the manufacturer's instructions:

> The desired DNA fragment was excised the from the agarose gel with a clean, sharp scalpel and weighed in a colorless tube.
>
> 3 volumes of Buffer QG were added to to 1 volume of gel and incubated at 50 °C for 10 min (or until the gel slice has completely dissolved).
>
> To help dissolve the gel, the tube was vortexed every 2–3 min during the incubation. After the gel slice has dissolved completely, it was checked that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
>
> One gel volume of isopropanol was added to the sample and mixed.
>
> A QIAquick spin column was placed in 2 ml collection tube, the sample was applied to the column, and centrifuged for 1 min. The maximum volume of the column reservoir is 800 μl. For sample volumes of more than 800 μl, the tubes can be newly loaded and centrifuged again.
>
> The flow-through was discarded and QIAquick column was placed back in the same collection tube.
>
> 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 min.
>
> For washing, 0.75 ml of Buffer PE was added to QIAquick column and 2–5 min later, centrifuged for 1 min.
>
> The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 17 900× g (13 000 rpm).
>
> Now the QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and DNA was eluted by addition of 50 μl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifugating the column for 1 min. Alternatively, for increased DNA concentration, 30 μl of elution buffer was added to the center of the QIAquick membrane, letting the column stand for 1 min, and then centrifuged for 1 min at 17 900 x g (13 000 rpm).

Yield of eluted fragment was quantified by measuring the absorbance at 260 nm spectrophotometrically.

5.3.4 Cloning of the DNA fragments

Preparation of competent bacterial cells by Calcium chloride method

JM109 cells were streaked on LB agar plate and grown overnight at 37 °C.

A single colony was inoculated in 3 ml of LB medium and cultured overnight at 37 °C with shaking at 220 rpm.

The entire 3 ml of primary culture was used as an innoculum for a 100 ml of pre-warmed LB medium (secondary culture). This culture was grown at 37 °C with shaking at 220 rpm till the cell density reached 10^8 cells/ml.

The bacterial culture was then aseptically transferred into sterile, ice-cold, polypropylene GS3 centrifugation bottles and the culture was cooled to 0 °C for 10 min.

The cells were then harvested at 2 700 g (Sorvall HS-4 rotor) for 10 min at 4 °C under sterile conditions. The supernatant LB medium was completely and aseptically removed.

The pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and was incubated on ice for 20 min.

The cells were pelleted and resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ per 50 ml of culture and incubated at 4 °C overnight.

Autoclaved glycerol (80%) was added to a final concentration of 15% and aliquots of 200 μl each were prepared in pre-chilled sterile microfuge tubes.

The aliquots were immediately snap-frozen by immersing them in liquid nitrogen before storing at -70 °C until used.

Ligation

For ligation we used pGEM -T Easy Vector System kit (Promega, Madison, WI USA). The following components were mixed and incubated at 4 °C for overnight:

2× Rapid Ligation Buffer	5 μl
Vector	1 μl
T4 DNA ligase	1 μl
DNA	3 μl

Transformation

An aliquot of competent cells (JM109) were thawed on ice for 5–7 minutes and asseptically mixed with 20–40 ng of the plasmid of interest per 100 μl of competent cells

Cells were incubated on ice for 20 min with flicking at every 5 minutes.

The cells were given a heat shock at 42 °C for 90 s and immediately chilled on an ice bath for 90 s.

For recovery, 150 μl of LB medium was added to the cells and they were incubated at 37 °C with shacking at 220 rpm for 1 hour.

The cells were then plated on LB agar plates supplemented with LB agar containing ampicillin (100 μg/ml), X gal (40 μg/ml) and IPTG (0.1 mM IPTG).

Maintenance of bacterial cultures and plasmid isolation

Single colonies of transformed bacteria were inoculated in 3 ml LB media with ampicillin and cultured at 37 °C with shaking at 220 rpm for 12–14 h.

Plasmid was isolated from the transformed *E. coli* (strain JM109) by QIAprep spin miniprep kit with little modification:

- The transformed *E. Coli* was pelleted at 8 000×g for 5 min at 4 °C.
- 250 μl of buffer P1, containing RNAse A, was added to the pelleted bacterial cells and resuspended nicely by vortexing untill no cell clumps were visible.
- 250 μl of buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times. The lisis was let to stand fot 5 min at room temperature.
- 350 μl of buffer P3 was added and mixed immediately and thoroughly by inverting the tube 4–6 times. The neutralization reaction was carried out for 15 min on ice.

- The lysate was centrifuged for 20 min at 13 000 rpm (≈ 17 900×g) at 4 °C.
- The supernatant was transferred to a new microfuge tubes containing 700 μl isopropanol. The contents were mixed nicely and incubated at -20 °C for 1–2 h.
- The samples were centrifuged at 13 000 rpm for 25 min and isopropanol was removed very nicely.
- the pellete was washed with 70% ethanol at 13 000 rpm for 10 min. The ethanol was removed carefully as its presence may inhibit subsequent enzymatic reactions.
- The plasmid pellet was air dried for 2–3 hours at RT or for 15–20 min at 37 °C.
- The plasmid was resuspended in 40 μl of TE buffer or sterile MilliQ water and stored at -20 °C.
- Yield was quantified by measuring the absorbance at 260 nm.

5.3.5 Screening of colonies for positive insertion of the desired DNA

Restriction digestion of the Plasmid

The restriction digestion of the plasmid was done by mixing the following components

DNA	1 μl
ECoRI	1 μl
10X Buffer	1 μl
H_2O	7 μl

The reaction was incubated for 2 hours at 37 °C

The samples were checked on 1.5% agarose gel

5.3.6 DNA sequencing

DNA sequencing was performed by using Big DYe Terminator V3.1 Cycle Sequencing kit (Applied Biosystems,US). The following reaction was set up-

Sequencing primers (T7 or SP6) or specific primers	1 μl
DMSO	0.5 μl
Big Dye	1.5 μl
DNA	0.5 μl

conditions for sequencing PCR

96 °C for 1 min

24 cycles of:

- Hold on 96 for 10 s
- Hold on 55 °C for 0.05 s (for specific primers this temperature varies depending on the Tm)

60 °C for 3 min

Hold on at 25 °C for ever

Purification of the sequencing PCR reaction

The purification of the sequencing PCR reaction was done using 7% sephadex columns according to the following protocol:

The columns were washed 3 times with 500 μl of dH_2O by centrifugation at 4 000 rpm for 1 minute.

The flow through was discarded and 500 μl of 7% sephadex was added to the columns and centrifuged at 4 000 rpm for 1 min.

The new flow through was discarded and 250 μl of 7% sephadex was added to the columns and centrifuged at 4 000 rpm for 1 min.

The flow through was discarded again and the columns were centrifuged to remove all traces of water.

The collection tube was discarded and the sequencing PCR samples were added to the columns and placed on centrifuge tube containing 15 μl HiDi and centrifuged at 4 000 rpm for 2 min.

The DNA sequence was done using a Sequence analyzer 5.1.1 and the sequence allignment was checked with BioEdit.

5.3.7 *In situ* Hybridization

Preparation of the Riboprobe:

The DNA to be transcribed was cloned into the polylinker site of appropriate transcription vectors (e.g, pGEMT), which contain promoters for SP6 and T7 RNA polymerases. Digestion of the positive clones with relevant Restriction enzymes in order to get an antisense and a sense strand during probe synthesis were performed as follows:

The following components were mixed in a reaction tube:

DNA	25 μg
SalI/SacII	4 μl
10X Buffer	10 μl
H_2O	71 μl

The reaction was incubated for 2 hours at 37 °C

Results were checked on 1.5% agarose gel

Purification of the digestion reaction

Restriction digestion reaction was purified using PCR Purification kit (QIAGEN, Germany) following the manufacturer's instructions:

5× volumes of buffer PB was added to 1 volume of digestion reaction and mixed, and a QIAquick spin column was placed in 2 ml collection tube.

The sample was applied to the QIAquick column, incubated for 3-4 min and and centrifuged for 1 min. The flow-through was discarded and QIAquick column was placed back in the same collection tube.

For washing, 0.75 ml of buffer PE was added to QIAquick column. The column was let to stand 2–5 min after addition of Buffer PE, before centrifugation.

The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 17 900 g (13 200 rpm) to remove the traces of buffer PE.

The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube.

DNA was eluted by addition of 25 μl of Buffer EB (10 mM TrisCl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and the column was centrifuged for 1 min.

Alternatively, for increased DNA concentration, 30 μl of elution buffer was added to the center of the QIAquick membrane, letting the column to stand for 1 min, and then it was centrifuged for 1 min at 17 900 g.

Yield of eluted fragment was quantified spectrophotometrically by measuring the absorbance at 260 nm. The purified digested plasmid was checked on 1.5% agarose gel.

In vitro transcription

In vitro transcription was performed to prepare the sense and antisense riboprobe using Dig-RNA labelling Kit (Roche Applied Sciences).

The following components were mixed:

DNA	25 μg
SalI/SacII	4 μl
10X Buffer	10 μl
water	71 μl

and incubated for 2 hours at 37 °C

The samples were on 1.5% agarose gel

1-4 μg purified template DNA was added to a sterile, RNase-free reaction vial.

Then, enough water (sterile, RNase-free, DMPC-treated,double dist.) was added to the vial to make the total sample volume 13 μl.

The following components were mixed:

purified template DNA (1-4 μg)	13 μl
10× NTP labeling mixture	2 μl
10× Transcription buffer	2 μl
Protector RNase inhibitor	1 μl
RNA Polymerase SP6 or RNA Polymerase T7	2 μl

The components were mixed gently and centrifuged briefly and incubated for 2 h at 37 °C.

2μl DNase I was added to remove template DNA and incubated for 20 min at 37 °C.

The reaction was stopped by adding 2 μl of 0.2 M EDTA (pH 8.0)

Precipitation of the Riboprobe

A mixture was prepared as follows:

Yeast tRNA	1 μl
Tris-EDTA buffer (pH 7.5)	100 μl
4M LiCl	10 μl
absolute ethanol	300 μl

This mixture was added to the riboprobe and incubated at -20 °C for overnight. The mixture was centrifuged at 13 000 rpm for 20 minutes at 4 °C.

The pellet was washed with 70% ethanol and centrifuged again at 13 000 rpm for 20 min at 4 °C The pellet was dried at 37 °C and resuspended in 100 μl of RNAse free water. 1 μl of RNAse inhibitor was added and the probe was stored at -80 °C.

The RNA transcripts was analyzed checking on 1.5% agarose gel electrophoresis (e.g., formaldehyde gels or native gels)

In situ hybridization procedure

In situ hybridization was performed on paraffin sections. We ensured that the sections were fresh and were stored at 4 °C before use. 8-10 μm thick sections were cut from paraffin embedded tissue and attached to tespa coated slides. The detailed protocol is as follows:

1. The slides were incubated at 55 °C in an oven until the paraffin was melted.
2. Two washes with Histoclear were performed for 20 minutes each, on a shaker. All washings and dehydration steps were performed in autoclaved Coplin jars on a shaker.
3. Hydration Steps:

   ETOH 100 % - 5 min.- twice
   ETOH 75 % - 5 min.- once
   ETOH 50 % - 5 min.- once
   ETOH 25 % - 5 min.- once

4. Washing steps: Two washes were done with 1× PBS for 5 min.
5. Prefixation step . Fix the tissue in 4% PFA for 10 min at room temperature.
6. The sections were rinsed in 1× PBS and two washes were done with 1× PBT [1× PBST = 1× PBS + Tween 20 (0.01%)], each for 5 min.
7. Proteinase K treatment: The slides were treated with Proteinase K (1 μg/ml In 0.1 M Tris-HCl, pH 7.5 and 0.05 M EDTA) 37 °C for 2 to 10 min (warm the buffer at 37 °C for 10 min, right before use)

8. The sections were rinsed in 1× PBS and two washes were done with 1× PBT [1× PBST = 1× PBS + Tween 20 (0.01%)], each for 5 minutes.

9. Post-fixation step. The sections were fixed again in 4% PFA for 5 min at room temperature.

10. The sections were rinsed in 1× PBS and two washes were done with 1× PBT, each for 5 min.

11. Acetylation step: Treat the sections with 0.25% Acetic Anhydride / 0.1M Triethanolamine (pH 8.0) for 15 min (For 80 ml volume, 8 ml of 0.1 M Triethanolamine, right before use and quickly add 260 μl Acetic Anhydride. Mix vigorously and quickly put into the jar with slides)

12. The sections were rinsed in 1× PBS and two washes were done with 1× PBT, each for 5 min.

13. The sections were dried at 40 °C and stored at 4 °C, untill used.

14. The probes (sense for the sample and antisense for the control) were mixed with the hybridization solution at a concentration of 1μg/μl and incubated at 85 °C for 3 min.

15. The slides were placed in a humidified chamber and incubated at 55 °C (the incubation temperature depends on the probe) O/N.

16. Washing steps. All washing buffers are prewarmed before washing.

    The coverslips were removed by rinsing the slides in 5× SSC at 55 °C for 10 min.

    One wash was done with 50% Formamide/ 1× SSC at 55 °C for 30 min.

    Another wash was done with TNE buffer at 55 °C for 30 min.

17. RNAse A treatment: The sections were treated with RNAse A (at final concentration at 20 μg/ml in TNE buffer) for 30 min at 37 °C.

18. Washing steps:

    One wash was done with TNE buffer at 37 °C for 10 min.

    Another wash was done with 2X SSC at 65 °C for 20 min.

    Two washes were done with 0.2× SSC at 65 °C for 20 min.

    Two additional washes were done with Maleic buffer (MABT) at room temperature, each for 5 min.

19. Blocking: Blocking was done with 10% HISS (heat inactivated sheep serum)/Maleic buffer for 1 h at room temperature.

20. Primary Antibody incubation: Slides were incubated with Sheep anti-DIG Fab Ab (1:1 000 in Blocking buffer, 10% HISS/Maleic buffer) at 4 °C for O/N in a humidified chamber.

21. Washings:

    The sections were rinsed in Maleic Buffer and washed thrice in the same Buffer, for 5 min each.

    The sections were washed with NTMT buffer (0.1 M Tris-HCl, pH 9.5 (20 °C), 0.1 M NaCl, 0.05 M $MgCl_2$, 0.01% Tween 20) for 10 min.

    The substrate, NBT/BCIP (200 μl of the stock solution were added to 10 ml 0.1 M Tris-HCl, pH 9.5 (20 °C), 0.1 M NaCl) was applied on the slides till the colours developed.

    Slides were rinsed in NTMT buffer (pH 9.0) and checked under the microscope.

    Slides were then washed twice with 1× PBS, each for 5 min.

22. The sections were mounted in Glycerol and stored at room temperature.

5.3.8 RealTime PCR

Retrotranscription of RNA

The retrotranscription was performed using iScript cDNA Synthesis Kit (BIO-RAD), which uses a mix of oligodT and random ribonucleotidos allowing the amplificaction of the mRNA. For this reaction, the following components were mixed:

4 μl	5X iScriptMix Reaction
1 μl	iScript Reverso Tansciptase
10 μl	Distilled water without nucleases
5 μl	RNA (100fg -1μg Total RNA)
20 μl	Total volume

The conditions of Retrotanscription were as follows:

25 °C	5 min
42 °C	30 min
85 °C	5 min
4 °C	Forever

Amplification and quantification by RT-qPCR

After synthesis of the first strand of each of the four different stages, the quantification of the expression of genes of interest using a thermal cycler Chromo 4 Real-Time PCR Detection System (BIO-RAD) using the SensiMix dt kit (Quantace R).
PCR reaction total volume of 25 μl:

12.5 μl	Sensimix 2X
0.5 μl	SYBR Green
0.5 μl	forward primer (10 pmol/ μl)
0.5 μl	reverse primer (10 pmol/ μl)
2 μl	cDNA
9 μl	Distilled Water (nuclease free)

The PCR conditions were as follows:

95 °C for 10 minutes

35 cycles of:

- 96 for 15 s
- 57 °C (for different genes, this temperature varies depending on the Tm of the primer)
- 72 °C for 1 minute
- Plate Read
- 72 °C for 3 minutes
- Melting curve from 75 °C to 94 °C read at every 1 °C hold for 1-s

4 °C till the end

The identity of all amplified fragments were confirmed by DNA sequencing. The efficiency of amplification was confirmed by quantifying serial dilutions of products amplified by PCR. Amplification efficiency value, E was calculated from the slope of the standard curve using the following formula:

$$\Delta E = 1 - 10^{-1/m}$$

where E is the efficiency of reaction and m is the slope.
The %efficiency was calculated using the formula:

$$\%Efficiency = (E - 1) \times 100$$

It was ensured that an amplification efficiency of 90%-105% was achieved for all the genes at their amplification temperature.

Gene expression levels were calculated by ΔCT method: In our studies GAPDH was used as a reference gene. The following formula was used to calculate the relative expresssion of the gene of interest with respect to GAPDH:

$$\Delta CT = 2^{CT_{(GAPDH)} - CT_{(target)}}$$

where target is target gene and GAPDH was reference gene. The values obtained were again standardized by dividing the lowest expression in one the stages. This was necessary for better comparison of the gene expression in various stages. The products of RT-qPCR reaction were analyzed on 1% agarose gel in order to verify the amplification of the gene

5.3.9 Hormone measurement by RadioImmunoassay

Testosterone and estradiol were measured from the serum of the males and females moles from various stages of their annual breeding cycle. All the samples were measured in triplicates.

Sample preparation

Serum separation

Blood collected from various male and female moles were incubated at 4 °C for overnight. In the morning the coagulated blood was centriguged at 6 000 rpm for 20 min at 4 °C . The supernatant (serum) was stored at -80 °C .

Testicular fluid separation for measuring intratesticular testosterone and estradiol

The testis was homogenized in 100μl of 1× PBS. The homogenate was centriguged at 10 000 rpm for 20 min at 4 °C . The supernatant (testicular fluid) was stored at -80 °C .

Procedure for Radioimmunoassay

Procedure for estradiol measurement using Radioimmunoassay

Radioimmunoassay for the in vitro determination of estradiol in serum was carried out using a commercial kit (ESTR-CTK-4 kit, from DiaSorin, Saluggia, Italy) following manufacturer's instructions. Briefly, 100μl of standard, control or samples were added to individual anti-estradiol antibody coated

tubes. The concentrations of the standards used were as follows: 0, 12, 38, 160, 540, 1500, 5000 pg/ml. To each tube, 500μl of tracer (I^{125} -labeled estradiol, concentration) was added and mixed well. To obtain total cpm, 500μl of tracer was added to 2 additional tubes. All the tubes were then incubated for 3 hours at 25 °C with shaking (350 rpm). All the contents of these tubes of were aspirated carefully except the 2 tubes labeled for total cpm. Total radioactivity (T) and bound radioactivity (B) were measured for 1min. (Counter) The standard curve for the determination of estradiol concentrations in samples were measured at the same time as the standards. Standard curve was plotted using a semi-logarithmic curve fit with B/T (%) on vertical axis and the estradiol concentration of the standards on the horizontal axis (pg/ml). Results were obtained from the standard curve by interpolation. The analytical sensitivity of kit was $<$ 11 pg/ml. Inter-assay coefficient of variation was found to be below or equal to 11.2% and Intra-assay coefficient of variation was found to be below or equal to 12.1%

Procedure for Testosterone measurement using Radioimmunoassay

Radioimmunoassay for the in vitro determination of testosterone in serum was carried out using a commercially available kit (TESTO-CTK kit, from DiaSorin, Saluggia, Italy) following manufacturer's instructions. Briefly, 50μl of standard, control or samples were added to individual tubes coated with testosterone antiserum raised in rabbit. The concentrations of the standards used were as follows: 0, 12, 38, 160, 540, 1500, 5000 pg/ml. To each tube, 500μl of tracer (I^{125}-labeled testosterone) was added and mixed well. To obtain total cpm, 500μl of tracer was added to 2 additional tubes. All the tubes were then incubated for 3 hours at 37 °C Ȧll the contents of these tubes of were aspirated carefully except the 2 tubes labeled for total cpm. Total radioactivity (B0) and bound radioactivity (B) were measured. The standard curve for the determination of testosterone concentrations in samples were measured at the same time as the standards. Standard curve was plotted using a semi-logarithmic curve fit with B/ B0 (%) on vertical axis and the testosterone concentration of the standards on the horizontal axis (ng/ml). Results were obtained from the standard curve by interpolation.

The analytical sensitivity of RIA kit was 0.02 ng/mL (0.07 nmol/L) at 95% confidence limit. Inter-assay coefficient of variation was found to be below or equal to 7.2% and Intra-assay coefficient of variation was found to be below or equal to 8.1%

5.3.10 Statistical Analysis

All statistics were done using students T test using the graphpad software (GraphPad Software Inc, CA). The datas were considered statistically significant, if $P < 0.05$.

Chemicals

Ethidium bromide, bromophenol blue, xylene cyanol, calcium chloride, sodium acetate, chloroform, trypan blue, agarose for running samples for Southern hybridization, ethylenediaminetetraacetic acid (EDTA), glycerol, sodium dodecyl sulfate (SDS), ampicillin (sodium salt), kanamycin monosulphate, salmon sperm DNA, Ficoll 400000, Polyvinylpyrrolidone 40000, BSA, Sodium chloride, Sodium citrate, DEPC, Formamide, Dextran sulphate, Yeast tRNA, Tween 20, Maleic acid, Tris base, EDTA (disodium salt), Magnesium chloride. and TRI reagent were procured from SIGMA Chemical Co. (St. Louis, USA). Methanol, acetic acid, formaldehyde, diethyl ether, isopropyl alcohol and isoamyl alcohol were purchased from Merck (Spain). Agarose for routine use was purchased from Amresco (Solon, Ohio, USA). All other chemicals were procured from SIGMA Chemical Co. (St. Louis, USA)unless otherwise stated. The 100 bp DNA ladder and 1 kb DNA ladder were purchased from Biotools laboratories, Madrid.

5.3.11 Kits

Plasmid isolation kits (both mini and maxi-scale) and gel extraction kit were purchased from QIAGEN (Hilden, Germany). Reverse transcription kit was obtained from Promega (Madison,WI, USA) and Dig RNA Labelling Kit (SP6/T7) from Roche Diagnostics (Mannheim, Germany). High prime DNA labeling kit was purchased from Roche Diagnostics (Mannheim, Germany), Big DYe Terminator V3.1 Cycle Sequencing kit (Applied Biosystems,CA,US).

5.4 Solutions

4% paraformaldehyde

For 100 ml of a 4% paraformaldehyde stock solution:

4 g of paraformaldehyde was taken and added to 80 ml of ddH_2O. The solution was heated to 60 °C–70 °C. 1 N NaOH was added and mixed until complete solubilization. The solution was cooled at RT. 10 ml of PBS 10×

was added and mixed. The pH was adjusted to 7.2–7.4. The volume was adjusted to 100 ml with ddH_2O. The solution filtered using a 0.2 μm 25 mm nylon syringe filter.

10X Phosphate Buffer Saline (PBS)

Dissolve the following in 800 ml distilled H_2O.

80 g	NaCl
2.0 g	KCl
14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4

pH was adjusted to 7.4. The volume was adjusted to 1 l with additional distilled H_2O. Sterilization was done by autoclaving. pH was adjusted to 7.2-7.4, if required.

Haematoxylin solution

2 g haematoxyline and 40 g of Potas alum was added to 20 ml of Ethanol and 400 ml of ddH_2O, respectively and mixed. After 24 hours, the solutions were mixed and 1 g of mercuric oxide was added. The resulting solution was heated to form a clear solution and then cooled and filtered and stored at RT.

Eosin solution

4.5 g of eosine was dissolved in 450 ml of ddH_2O. 4 drops of acetic acid glacial was added and stirred nicely. The solution was filtered and stored at RT.

Karnowsky

To make a volume of 5 ml:

The following components were added to a 10 ml tube:

- 4% Paraformaldehyde: 1.25 ml
- sodium Cacodylate 0.4M pH 7.3-7.4: 1.25 ml
- 25% Glutaraldehyde : 0.5 ml
- 0.25% calcium chloride : 1 ml
- distilled H_2O: 1 ml

Mix by stirring.

carbonate buffer 0.1M

To make a 500 ml carbonate buffer add the following components:

Na_2CO_3: 5.30 g

$NaHCO_3$: 4.24 g

The pH 9.4 was adjusted

Peroxidase Substrate solution

For a volume of 25 ml:

Add the following components:

- Sodium citrate buffer 0.15M pH 5: 12.5 ml
- distilled H_2O : 12.5 ml
- 1 tablet of OPD[1] (O–fenil diaminobenzidine)

Cover with aluminium foil and store at 4 °C for maximum 1 week.

Extraction buffer

The following components were added:

Tris–HCl 10 mM pH 7.5

NaCl 10 mM

EDTA 2 mM

proteinasa K solution

100 mg of lyophilized proteinasa K was dissolved in 10 ml of sterile H_2O.

Lysis buffer

The buffer contained the following components:

[1]OPD: O–Phenylendiamine dihydrochloride tablet (10 mg/tablet), ref. SIGMA P8287–50TAB.

Tris–HCl 10 mM pH 7.5

NaCl 10 mM

EDTA 2 mM

5% SDS

DNAse free RNAse solution

The following components were added:

- Tris.HCl 10 mM pH 7.5
- NaCl 15 mM

RNAse A was dissolved at a concentration of 10 mg/ml in the above buffer.

The solution was heated to 100°C for 15 min for inactivating the DNAse.

Cool the solution to room temperature.

50X Denhardt's reagent

For 100 ml of 50X Denhardt's Reagent, following components were added:

Ficoll: 1 g

Polyvinylpyrrolidone: 1 g

BSA: 1 g

The solution was filtered and stored at -20 C.

20X SSC

For 100 ml of 20X SSC, following components were added:

Sodium chloride: 17.53 g

Sodium citrate: 8.82 g

DEPC-treated water 80 ml

The pH was adjusted to 7.0 with concentrated HCl. The volume was adjusted to 100 ml. the solution was autoclaved and stored at RT.

Hybridization Buffer

For preparing 10ml of hybridization buffer, following components were added:

1 M Tris pH 7.5: 1 ml

5 M Nacl: 12 ml

0.5 M EDTA pH 8: 200 μl

20% SDS: 1.25 ml

Dextran sulphate: 10 g

Yeast tRNA (50 mg/ml): 400 μl

50 X Denhardt's reagent: 2 ml

Formamide: 50 ml

The volume was adjusted to 100 ml using DEPC-treated water.

Formamide/SSC/Tween washing buffer

For 600 ml of this buffer, following components were added:

Formamide: 300 ml

20× SSC: 30 ml

Tween20: 600 μl

The volume was adjusted using DEPC-treated water.

5× MAB, pH7.5

For 500 ml, following components were added:

Maleic acid: 29 g

Sodium chloride: 20.5 g

The pH was adjusted to 7.5 with 10 N NaOH. The volume was adjusted using DEPC-treated water.

1× MABT, pH 7.5

For 500 ml, following components were added:

5× MAB: 100 ml

Tween 20: 5 ml

The volume was adjusted using DEPC-treated water.

Blocking Solution

For 10ml, following components were added:

1× MABT: 5 ml

Heat inactivated sheep serum: 5 ml

5 M Sodium chloride

73.125 g of Sodium chloride was dissolved in DEPC-treated water and volume was adjusted to 250 ml. The solution was autoclaved and stored at RT.

1 M Tris-HCl, pH 7.5

For, 200 ml, 24.228 g of Tris base was dissolved in 180 ml of DEPC-treated water. The desired pH was adjusted using concentrated HCl. The volume of the solution was adjusted to 200 ml using DEPC-treated water.

2 M Tris-HCl, pH 9.5

For 200 ml, 48.456 g of Tris base was dissolved in 180 ml of DEPC-treated water. The desired pH was adjusted using concentrated HCl. The volume of the solution was adjusted to 200 ml using DEPC-treated water.

0.5 M EDTA, pH 8.0

18.612 g of EDTA (disodium salt) was added to 80 ml water and pH was adjusted to 8.0 with 10 N NaOH. The volume was adjusted to 100 ml as final volume with water. The solution was autoclaved and stored at RT.

1 M Magnesium chloride

For 250 ml, 50.82 g magnesium chloride was added to 220 ml water. The volume was adjusted to 250 ml with water.

NTMT buffer, pH 9.5

For 1000 ml, following components were added:

5 M NaCl: 20 ml

2 M Tris-HCl pH 9.5: 50 ml

1 M $MgCl_2$: 50 ml

The pH was adjusted to 9.5 and 2 ml of Tween-20 (50%) was added. The volume was adjusted to 1000 ml with DEPC-treated water.

Luria Bertani (LB) medium

10 g of tryptone, 5 g of yeast extract and 10 g of sodium chloride were dissolved in MilliQ water and volume was made upto 1 l. This medium was sterilized by autoclaving at 15 lbs/square inch (121 °C) for 20 min (Bertani, 1951). For LB agar preparation 15 g of Bacto Agar was added per litre of the above medium prior to autoclaving.

Results

6.1 Expression of genes involved in gonad development during seasonal breeding cycle of *Talpa occidentalis*

Many of the genes involved in mammalian gonad development are known to be expressed also in the adult gonads of males and/or females, although their function here remains unclear in most cases. In this study we have analysed the expression patterns of three of the most relevant genes in this context, *SOX9*, *DMRT1* and *AMH*, throughout the circannual seasonal breeding cycle in testes of *T. occidentalis* males, to investigate whether expression variations may be associated with the cyclic changes observed in these gonads. The present study, may certainly lead to better understanding the role of these genes in the regulation and maintenance of spermatogenesis.

SOX9

Using a specific anti-SOX9 antibody, the localisation of this protein was studied in transversal sections of testes from moles captured in the four stages established throughout the seasonal breeding cycle (Fig.6.1). The immunohistochemical analysis showed that the SOX9 protein is present in the Sertoli cells of the testes in all four stages, thus sugegsting the absence of any seasonal variation in its expression. However, some variation was observed concerning the intensity of the immunostaining. SOX9 postive cells varied in a spermatogenic stage-dependent manner between different seminiferous tubules in the active testis. Also, Sc in the inactive testis were in general more immunoreactive than those in inacive gonads.

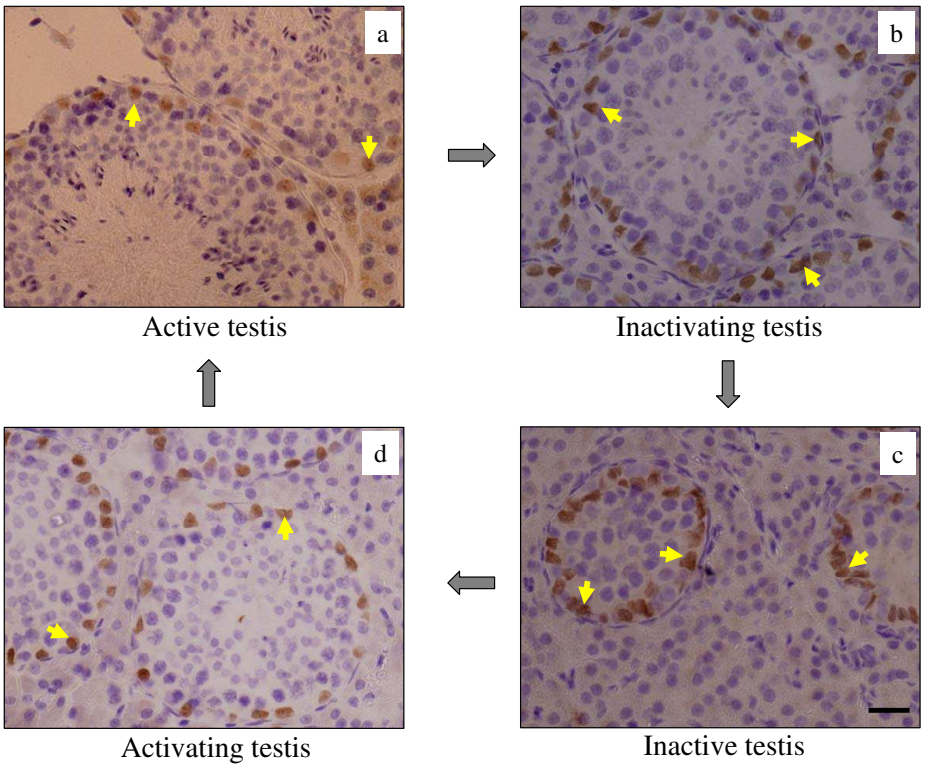


Figure 6.1: SOX9 detection in transverse sections of testes from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). SOX9 is expressed in the Sertoli cells (yellow arrows) in all the stages analyzed although the intensity of the immunostaining depends on the stage of the spermatogenic cycle. Scale bar = 20 μm

Since we knew the nucleotide sequence of a fragment of the mole *SOX9* (our unpublished data), specific primers were designed to perform a quantitative real time PCR analysis of the expression of this gene (Fig.6.2, Fig.6.3). This study showed that the levels of SOX9 mRNA are relatively low in all stages except in the inactive testis, where these levels are about 5-fold higher. In order to confirm that there is no variation in the number of Sc in the active and the inactive stage, we calculated the number of Sertoli cells per tubule in both of these stages. However, there was no significant difference in the number of Sc during the seasonal breeding cycle (Fig.6.5).Hence, since SOX9 is exclusively expressed by Sertoli cells, in our opinion, realtime expression level data could probably be distorted by the possibility that the variations that testis mass undergoes throughout the year (mainly caused by wide variations in the number germ cells), were much higher than those affecting Sertoli cells. Accordingly, we have calculated the sectional area Sertoli cell density (number or Sertoli cells per unit of section area; in our case the section area unit selected was the area of photomicrographs made with a Olympus DP70 digital camera installed on a Olympus BX41 microscope, using a 40X objective). This study showed that Sertoli cell density in the inactive testis is 2.55 times higher than that of the active one. Hence, if we correct the SOX9 expression levels data accordingly (by dividing the inactive expression value by 2.55), then the new data show that the levels of *SOX9* expression are infact higher in the inactive stage, although the difference with those observed in the active testis is not as large as that measured before any correction (Fig.6.4).

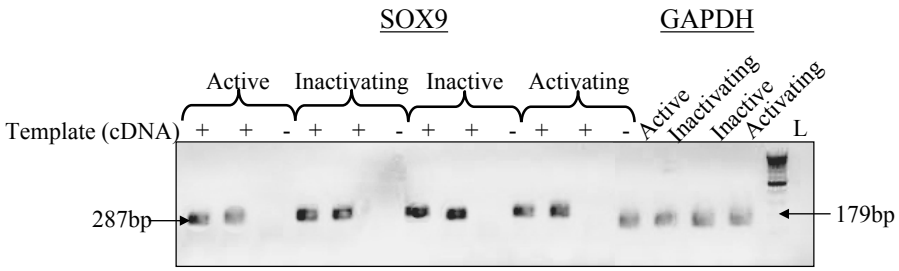


Figure 6.2: *SOX9* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *SOX9* expression levels were checked using quantitative real time PCR. Amplification of 287 bp was observed for *SOX9* in all stages analyzed. Expression of *GAPDH*(179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA. (-) reactions performed without cDNA. L:100 bp ladder

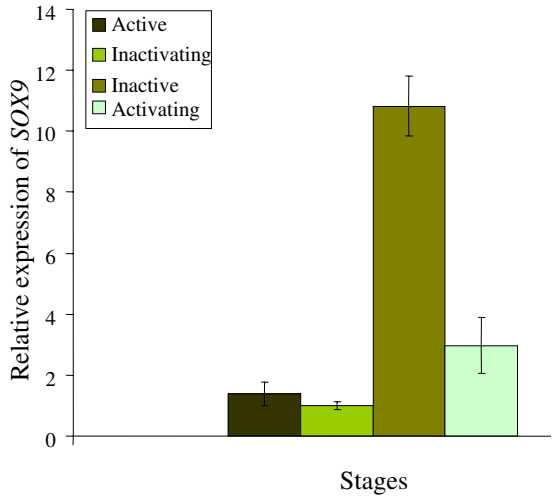


Figure 6.3: Measured expression levels of *SOX9* in the testis from *T. occidentalis*, throughout the seasonal breeding cycle. Expression was found to be very high in the inactive stage whereas it was very low in the active stage. Real time PCR reactions were performed, using *GAPDH* as a reference gene for quantification.

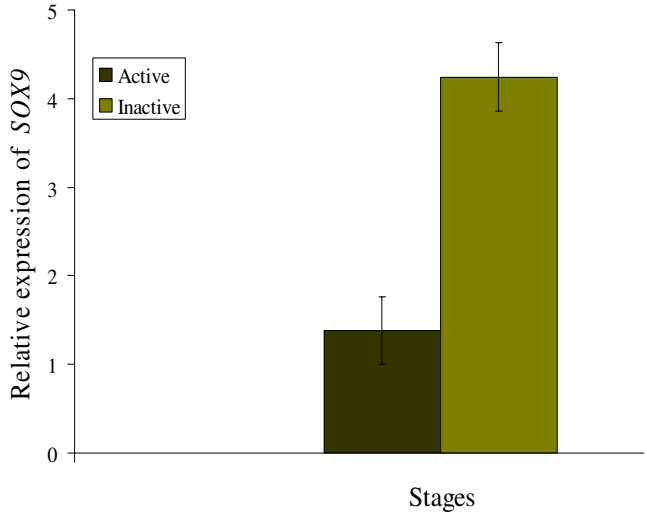


Figure 6.4: Corrected expression levels of *SOX9* in the testis from *T. occidentalis*, during the active and the inactive stages.

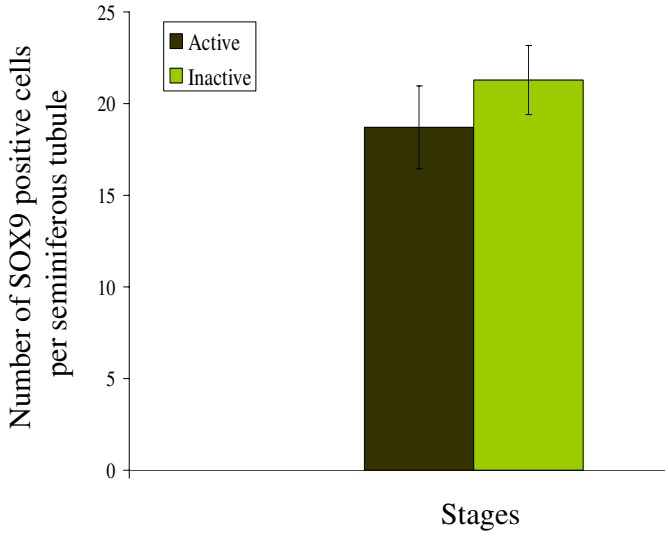


Figure 6.5: Comparison of the number of SOX9 immunoreactive cells per seminiferous tubule in the testis from *T. occidentalis*, during the active and the inactive stages.

DMRT1

The immunohistochemical analysis of DMRT1 showed that this protein is present in the nucleus of peripheral cells in seminiferous tubules in all stages of the reproductive cycle of *Talpa occidentalis* (Fig.6.6). Two types of seminiferous tubules were found in these peparations, regarding the density of DMRT1-positive cells in them: low density (LDD) and high density (HDD) tubules (see Fig.6.6a) Like in the case of of SOX9, we knew the nucleotide sequence of a fragment of DMRT1 in this species (our unpublished data), so that it was possible to measure the expression levels of this gene by real time PCR (Fig.6.7, Fig.6.8). In this case, expression was much higher in the active testis than in the rest of the cycle stages. Since DMRT1 is produced by Sertoli cells, we could apply here the same correction used in the case of SOX9. This reduced even more the expression value corresponding to the inactive testis, thus making the differences between active and inactive gonads more evident (Fig.6.9).

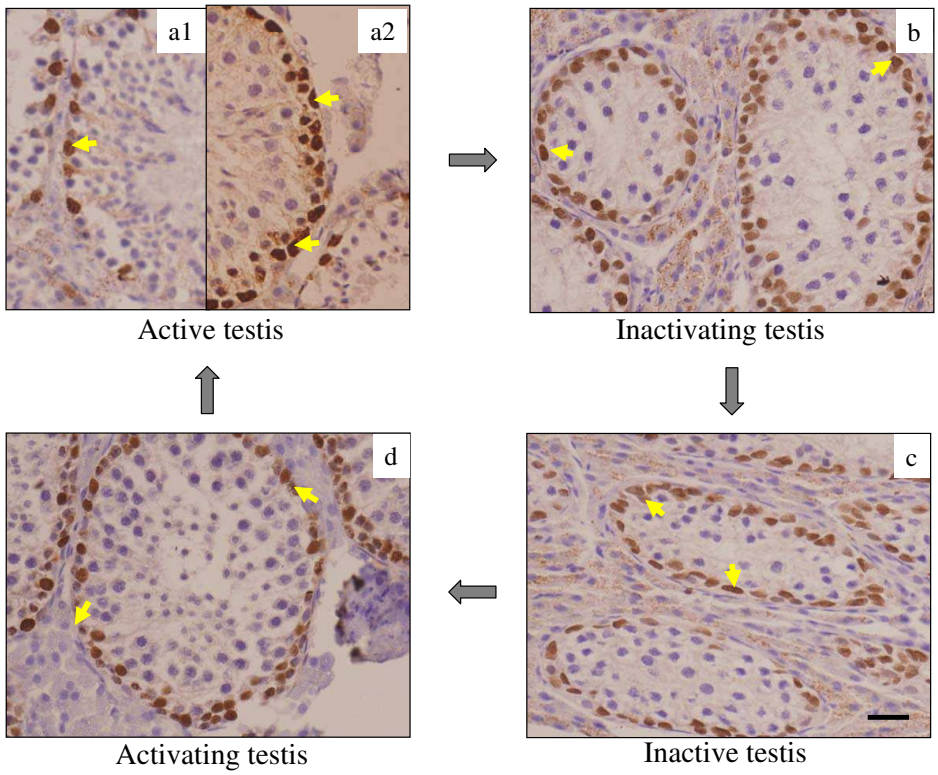


Figure 6.6: DMRT1 detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). DMRT1 is expressed in the Sertoli cells in all the four stages analyzed (yellow arrows), and in the spermatogonia in a stage-specific manner. This is evident in the active stage (**a**), where low density (**a1**) and high density (**a2**)seminferous tubules were observed regarding the abundance of DMRT1- positive cells. These are Sertoli cells in (**a1**) and Sertoli cells and germ cells in (**a2**) Scale bar = 20 μm

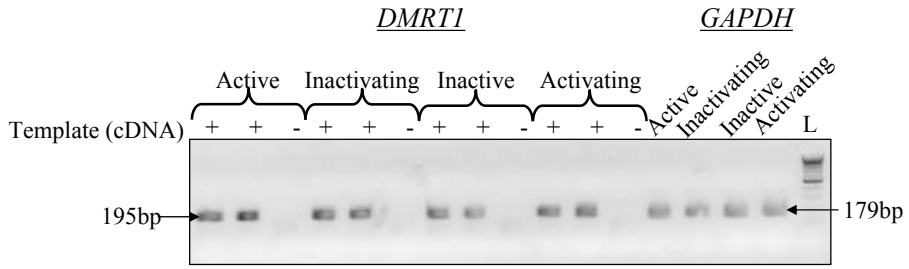


Figure 6.7: *DMRT1* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *DMRT1* expression levels were checked using quantitative real time PCR. Amplification of 195 bp was observed for *DMRT1* in all stages analyzed. Expression of *GAPDH* (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA. (-) reactions performed without cDNA. L:100 bp ladder

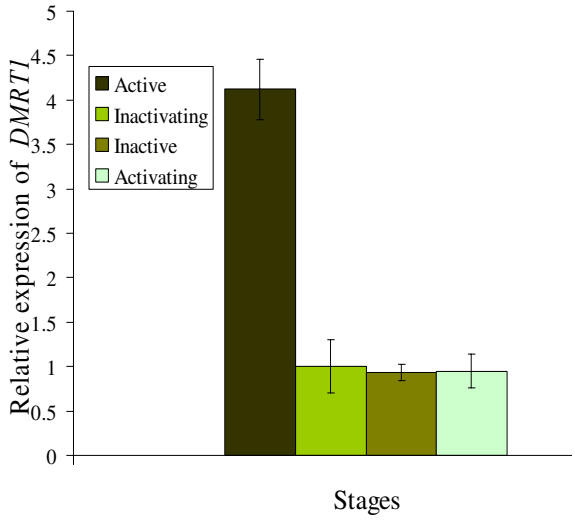


Figure 6.8: Measured expression levels of *DMRT1* in the testis from *T. occidentalis*, throughout the seasonal breeding cycle. Expression was found to be very high in the active stage whereas it was low in rest of the stages. Real time PCR reactions were performed, using *GAPDH* was used as a reference gene for quantification

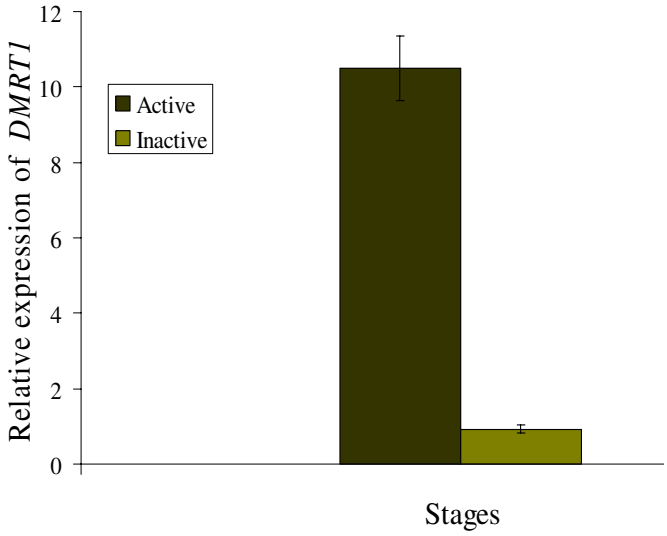


Figure 6.9: Corrected expression levels of *DMRT1* in the testis from *T. occidentalis*, during the active and the inactive stages.

AMH

As expected, AMH is not expressed in testes of adult male moles of *T. occidentalis*, in any of the four stages of the seasonal breeding cycle (Fig.6.10).

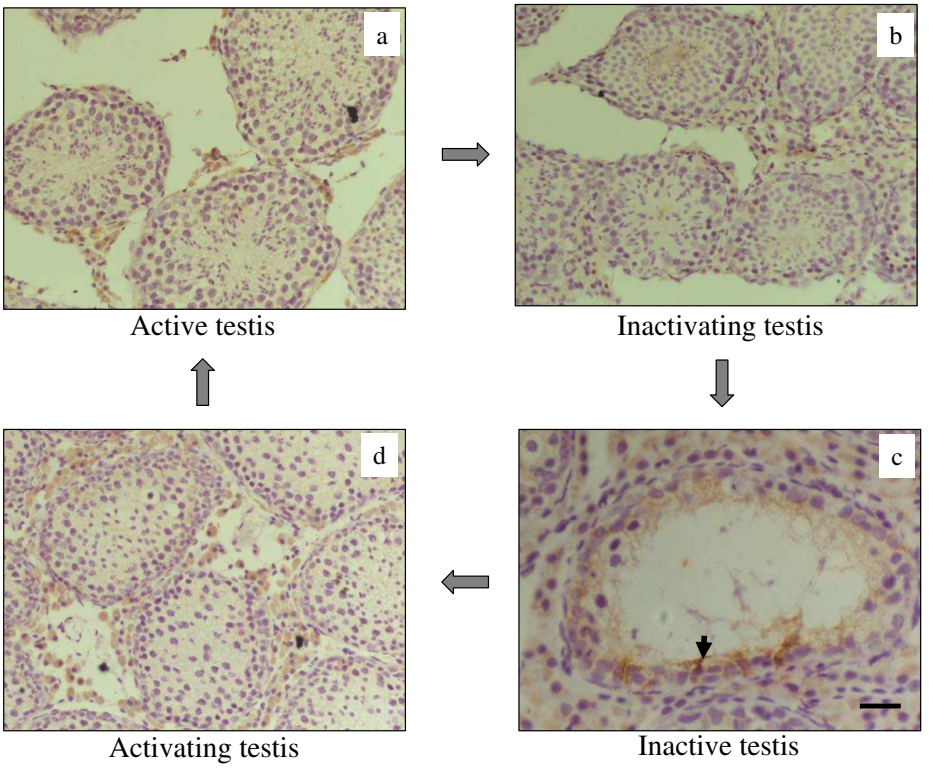


Figure 6.10: Immunohistochemical analysis in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*, using an antibody specific for anti-mullerian hormone. Active (**a**) Inactivating (**b**) Inactive (**c**) Activating (**d**). AMH was not detected in Sertoli cells of any of the analyzed stages. Scale bar = 20 μm

SF1

The SF1 protein is present in two cell types of the mole testis. Leydig cells appear strongly immunoreactive to SF1 in all stages of the reproductive cycle. A similar expression pattern was observed in Sertoli cells, although in this case, immunoreactivity was not as intense as that detected in Leydig cells (Fig.6.11).

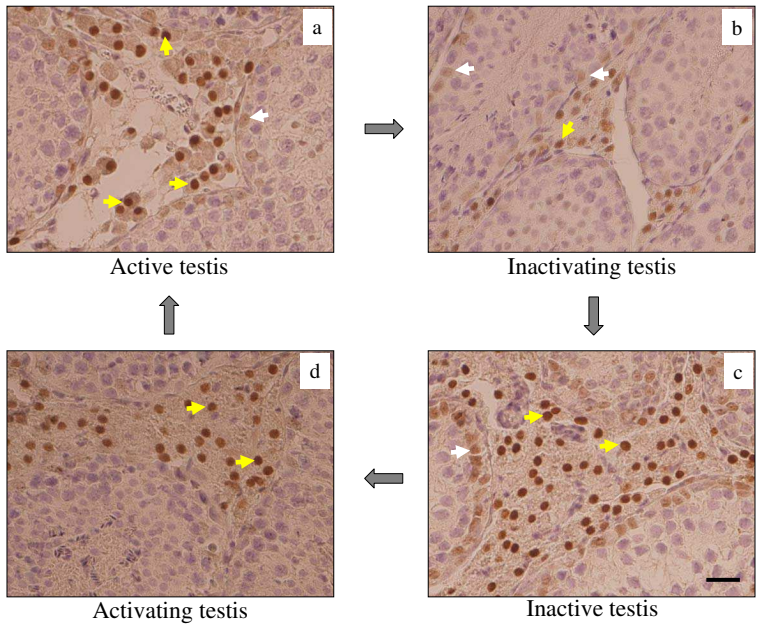


Figure 6.11: SF1 detection in transverse sections of testes from four representative stages during the seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). SF1 is strongly expressed in the Leydig cells (yellow arows) and weakly in Sertoli cells (white arrows) in all the four stages analyzed. Scale bar = 20 μm.

In order to quantify the expression of this gene, a 157 bp fragment of the mole SF1 was amplified, using oligonucleotide primers designed according to the consensus sequence of this gene from several mammalian species, and sequenced as described above (Fig.6.12). Once the identity of the amplified fragment was confirmed by the sequence, these primers were used in real time PCR reactions, performed on cDNA obtained from testes of males in the four stages of the reproductive cycle, to quantify the expression levels of SF1 in these gonads (Fig.6.13, Fig.6.14). The levels of SF1 mRNA are

maximum in the active testis, and very low in the inactive one, the two transition stages showing intermediate expression levels.

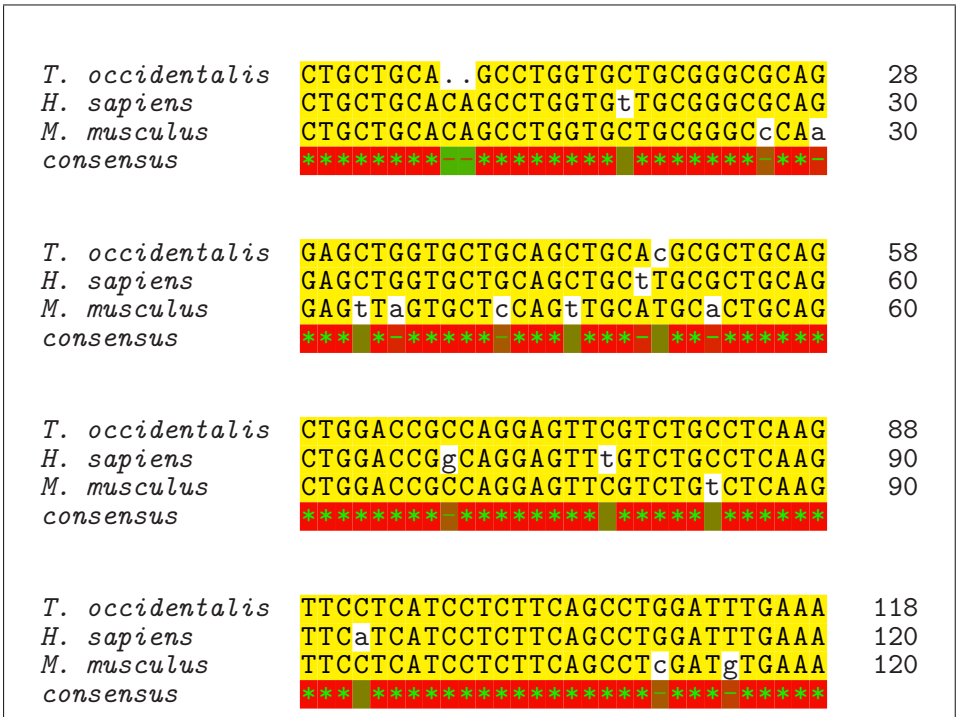


Figure 6.12: Comparison of nucleotide sequences of *Steroidogenic Factor 1* (*SF1*)fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. The mole fragment showed 89% homology with that of *Homo sapiens* and 88% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

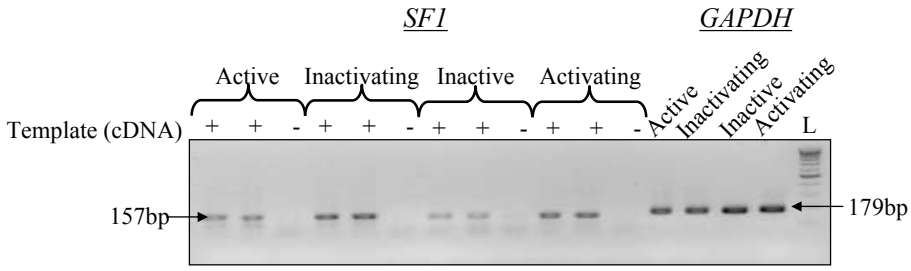


Figure 6.13: *SF1* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *SF1* expression levels were checked using quantitative real time PCR. Amplification of 157 bp was observed for *SF1* in all stages analyzed. Expression of GAPDH (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA. (-) reactions performed without cDNA. L:100 bp ladder

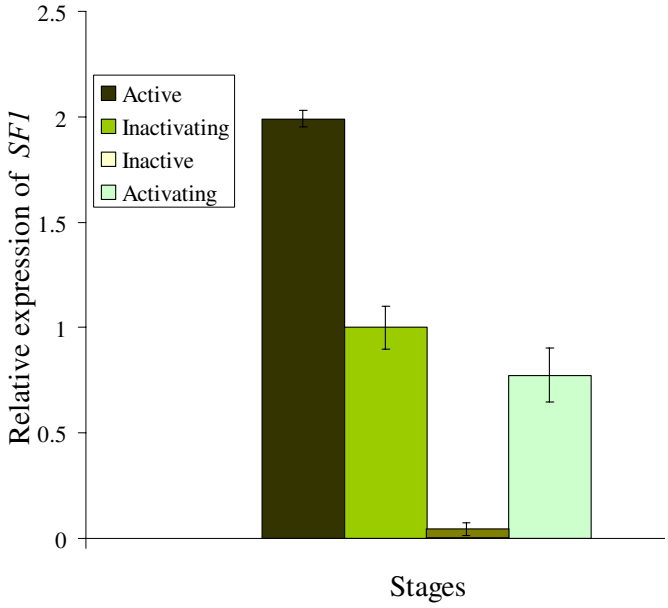


Figure 6.14: Expression levels of *SF1* in the testis from *T. occidentalis*, throughout the seasonal breeding cycle. Expression was found to be very high in the active stage whereas it was very low in the inactive stage. Real time PCR reactions were performed, using *GAPDH* was used as a reference gene for quantification

6.2 Steroidogenesis in the gonads of *Talpa occidentalis* during the annual seasonal breeding cycle

In addition to SF1, which is involved in both testis differentiation and steroidogenesis, we have investigated the expression of several genes responsible of controlling steroid hormones production and reception. These are P450scc, AR and ERα. The levels of testosterone and estradiol were also measured in both serum and testicular fluid obtained from male moles in the four stages of the reproductive cycle.

6.2.1 P450scc

Immunohistochemistry analysis showed that this protein is present in the cytoplasm of the Leydig cells in the four stages analysed (Fig. 6.15). Leydig cells in active and activating testes were found to be strongly immunoreactive to P450scc, whereas those in inactivating and inactive gonads were less immunoreactive.

On the basis of the known sequence of this gene from the mole (our unpublished data), oligonucleotide primers were designed to amplify a 177bp fragment, which was used to quantify the expression of P450scc in testes of moles in the four stages of the reproductive cycle (Fig.6.15, Fig.6.16, Fig.6.17). The quantitative real time PCR analysis showed that this gene is expressed in all stages with a similar intensity, as the differences observed were not statistically significant (Fig.6.17; P=0.9439). However, as indicated above for SOX9, these data could be distorted by the presumably higher relative abundance of Leydig cells in inactive than in active testes, as seminiferous tubules are much bigger in later stage. So, we have calculated the sectional area Leydig cell density (number or Leydig cells per unit of section area) in active and inactive testes. In this case, as Leydig cells organize into large and compact patches, mainly in the inactive stage (see Fig.6.15), we have measured the sectional area occupied by Leydig cells in the selected photographs. This study showed that Leydig cell density in the inactive testis is 7.23 times higher than that of the active one. Hence, if we correct the P450scc expression levels data accordingly (by dividing the inactive expression value by 7.23), then the new data show that Leydig cells produce much more P450scc protein during the active than during the inactive stages (Fig.6.18). A statistic analysis (student t) shows that these defferences are highly signifficant (P=0.0013)

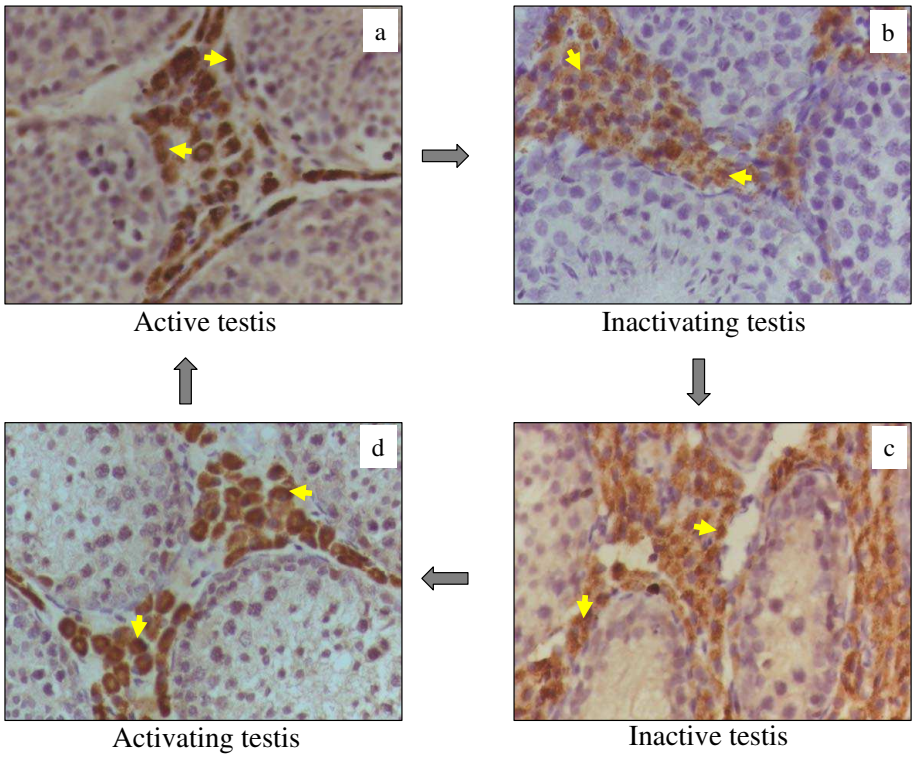


Figure 6.15: P450scc detection in transverse sections of testis from 4 representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). Strong immunoreactivity was observed in the cytoplasm of activating(**a**)and Active (**a**) testes. Leydig cells of Inactivating (**b**) and Inactive(**c**) were less immunoreactive(yellow arrows). Scale bar = 20 μm.

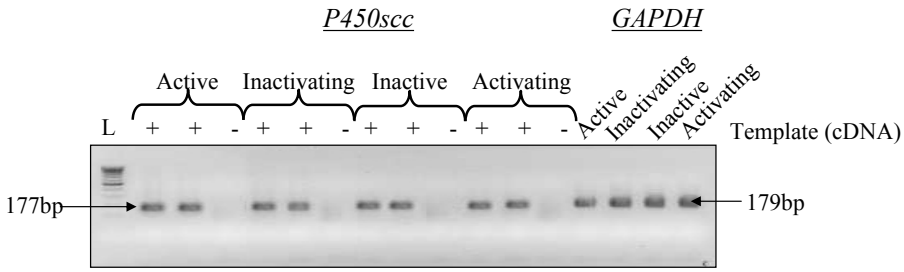


Figure 6.16: *P450scc* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *P450scc*expression levels were checked using quantitative real time PCR. Amplification of a 177 bp fragment was observed for P450scc at all the stages analyzed. Expression of *GAPDH* (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA. (-) reactions performed without cDNA. L:100 bp ladder

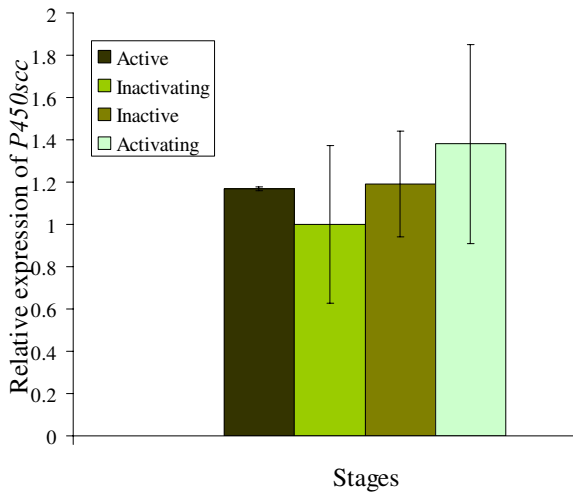


Figure 6.17: Expression levels of *P450scc* in the testes from *T. occidentalis*, throughout the seasonal breeding cycle. Expression seems to be similar in all the stages analyzed. Real time PCR reactions were performed, using *GAPDH* as a reference gene for quantification

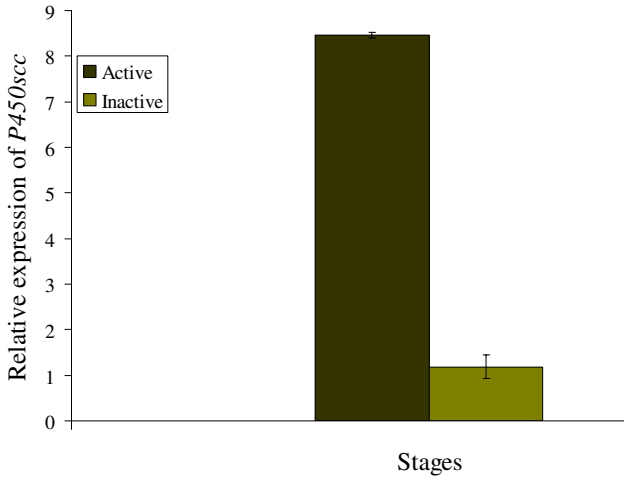


Figure 6.18: Corrected expression levels of *P450scc* in the testis from *T. occidentalis*, during the active and the inactive stages.

AR

The immunohistochemical study of the AR protein showed that it is present in a variety of cell types in the testis of T. occidentalis (Fig. 6.19). AR was detected in Sertoli cells inside the seminiferous tubules, and in Leydig and peritubular myoid cells, outside of them. This expression pattern was common to all stages of the seasonal reproductive cycle of moles, although AR was also detected in round spermatids of the active testis, in a spermatogenic stage-dependent manner.

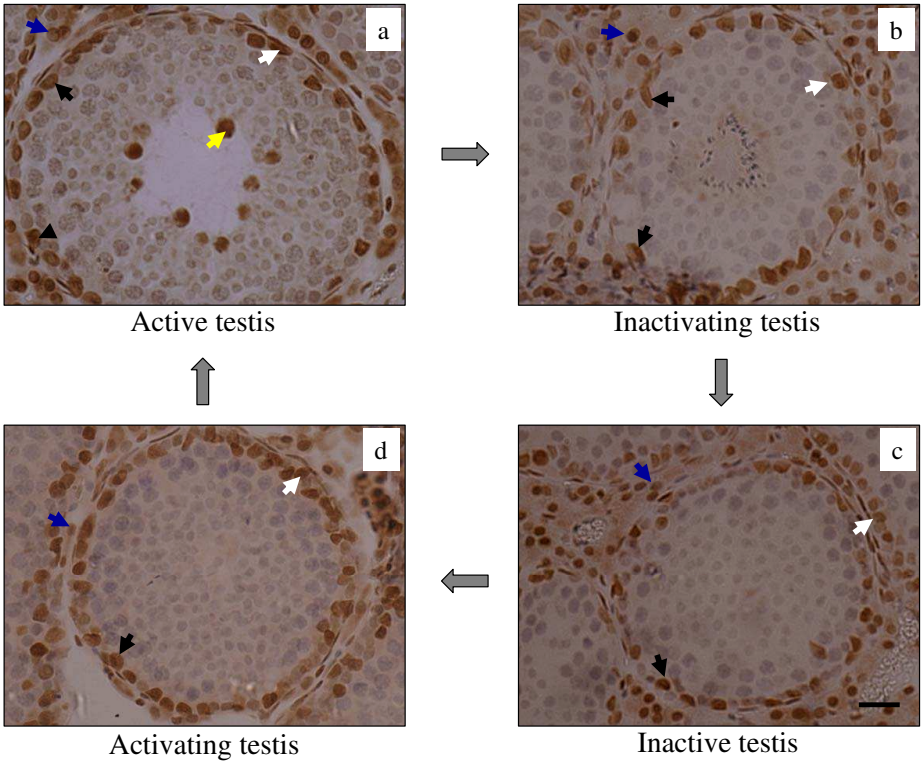
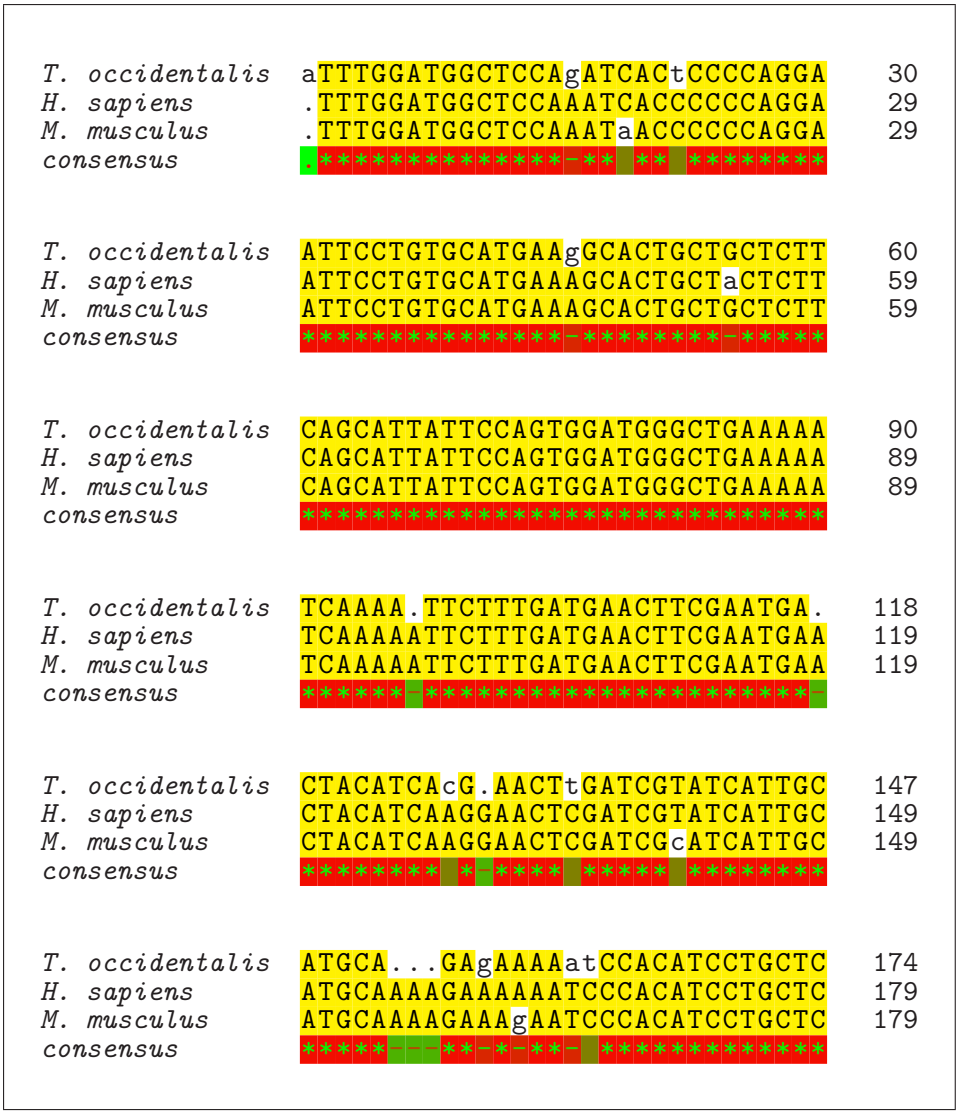


Figure 6.19: AR detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**) Inactivating (**b**) Inactive (**c**) Activating (**d**). AR is detected in the Sertoli cells (white arrows), Leydig cells (blue arrows), peritubular myoid cells (black arrows) Germ cells (yellow arrows) in active stage(**a**), whereas in rest of the stages AR is localized to Sertoli cells (white arrows), Leydig cells (blue arrows) and peritubular myoid cells (black arrows). AR was also detected in the elongating and round spermatids in the active testis, in a stage dependent manner (yellow arrows). Scale bar = 20 μm

As described above for *SF1*, we amplified and sequenced a 177 bp fragment of the mole *AR* gene (Fig.6.20, Fig.6.21), and used this fragment to

quantify the expression of this gene in the four stages of the reproductive cycle (Fig. 6.22). This study indicated that AR mRNA in activating and active testes doubles in abundance that of inactivating and inactive gonads.



```
T. occidentalis   Agc   177
H. sapiens        A..   180
M. musculus       A..   180
consensus         *..
```

Figure 6.20: Comparison of the nucleotide sequences of *Androgen Receptor*(*AR*)fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. The mole fragment showed 91% homology with that of *Homo sapiens* and 93% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

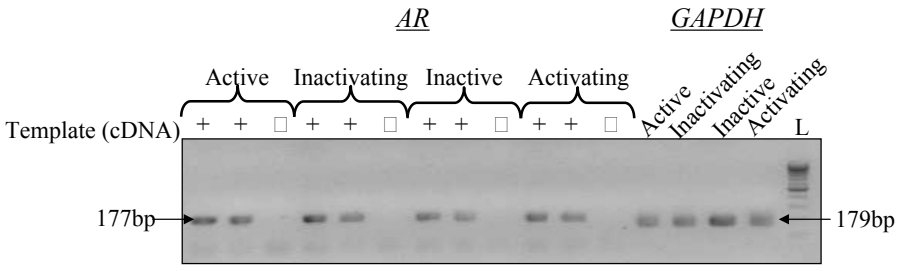


Figure 6.21: *AR* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *AR* expression levels were checked using quantitative real time PCR. Amplification of a 177 bp was observed for *AR* at all the stages analyzed. Expression of *GAPDH* (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA and (-) reactions performed without cDNA. L:100 bp ladder

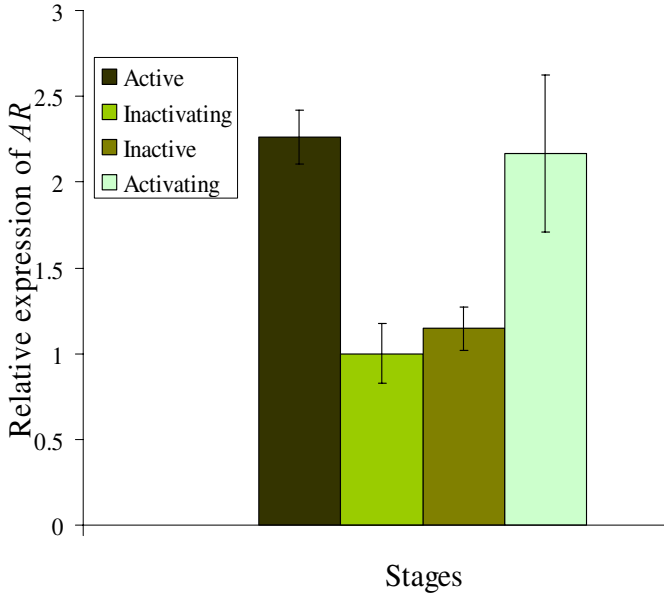


Figure 6.22: Expression levels of *AR* in the testis from *T. occidentalis*, throughout the seasonal breeding cycle. *AR* expression level was found to be higher in active and activating than in inactivating and inactive gonads. Real time PCR reactions were performed, using *GAPDH* as a reference gene for quantification

ERα

No commercial antibody was found to be immunoreactive to this protein from *T. occidentalis*. Alternatively, we investigated its location by in situ hybridisation. For this, a 663 bp fragment of the gene was amplified and sequenced (Fig.6.24), in order to produce the necessary Digoxigenin-labelled riboprobes. In situ hybridisation showed that *ERα* mRNA is present in the Leydig cells in all stages of the reproductive cycle, except in the inactive testis (Fig.6.23).

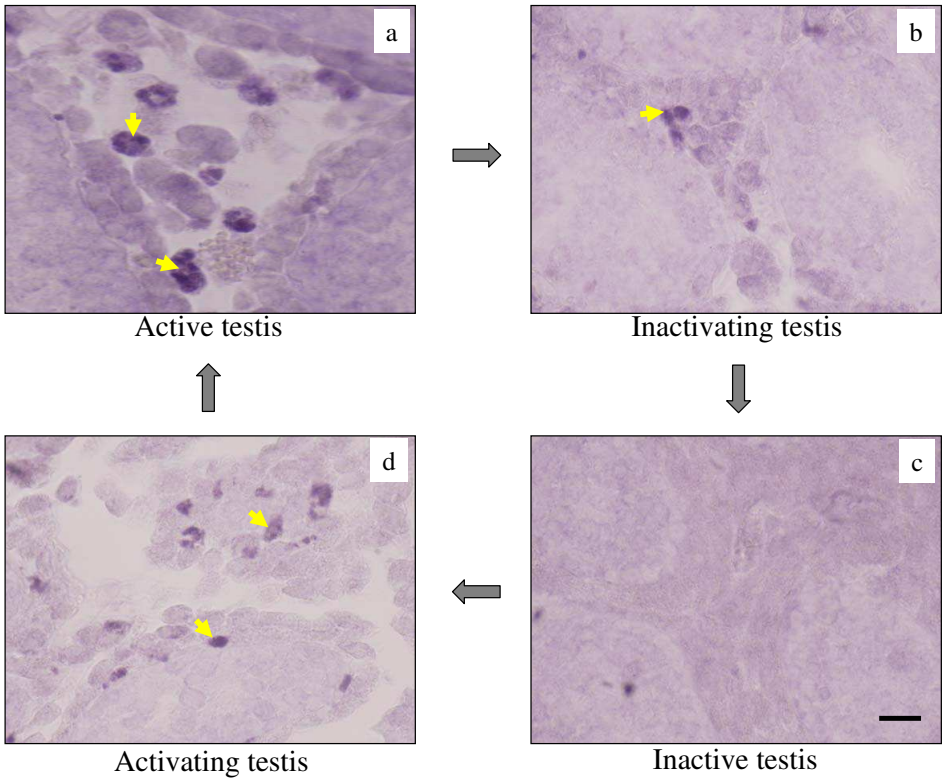
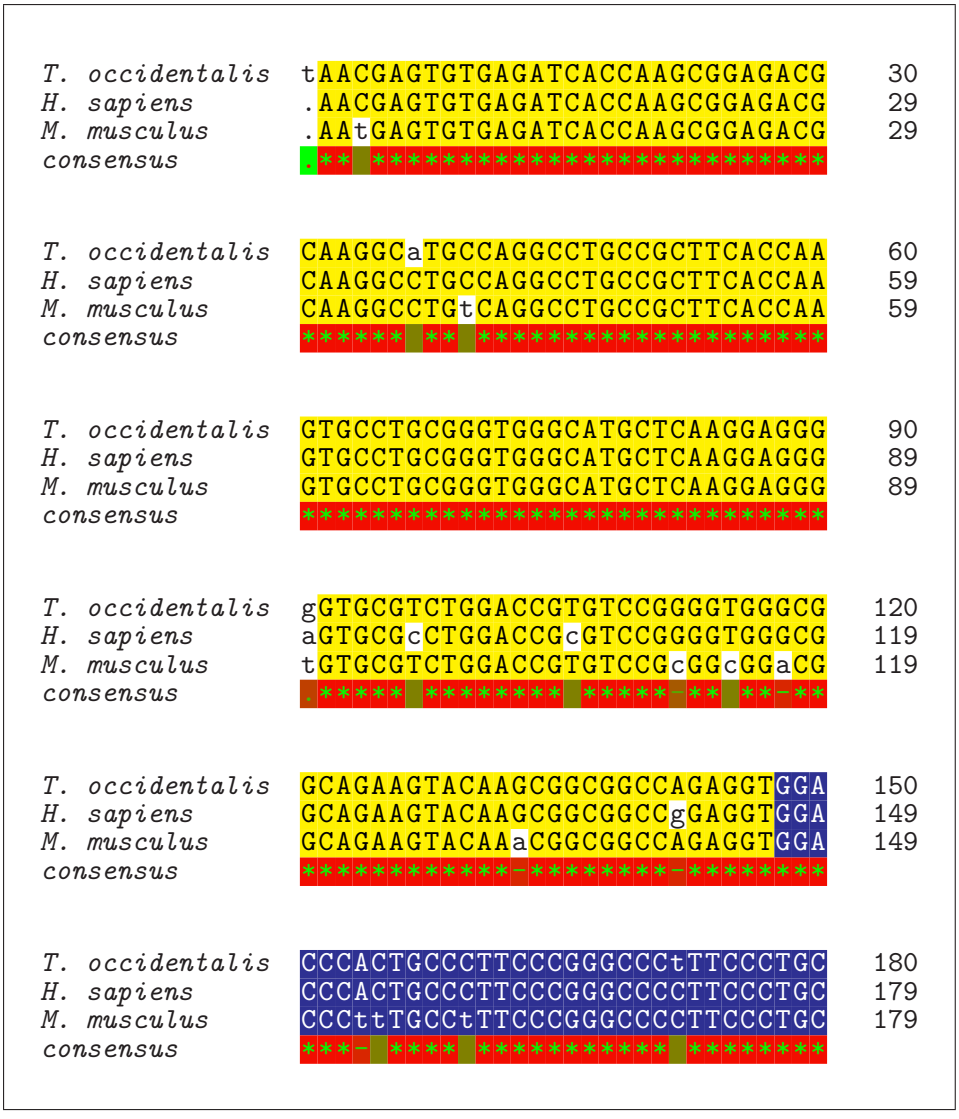
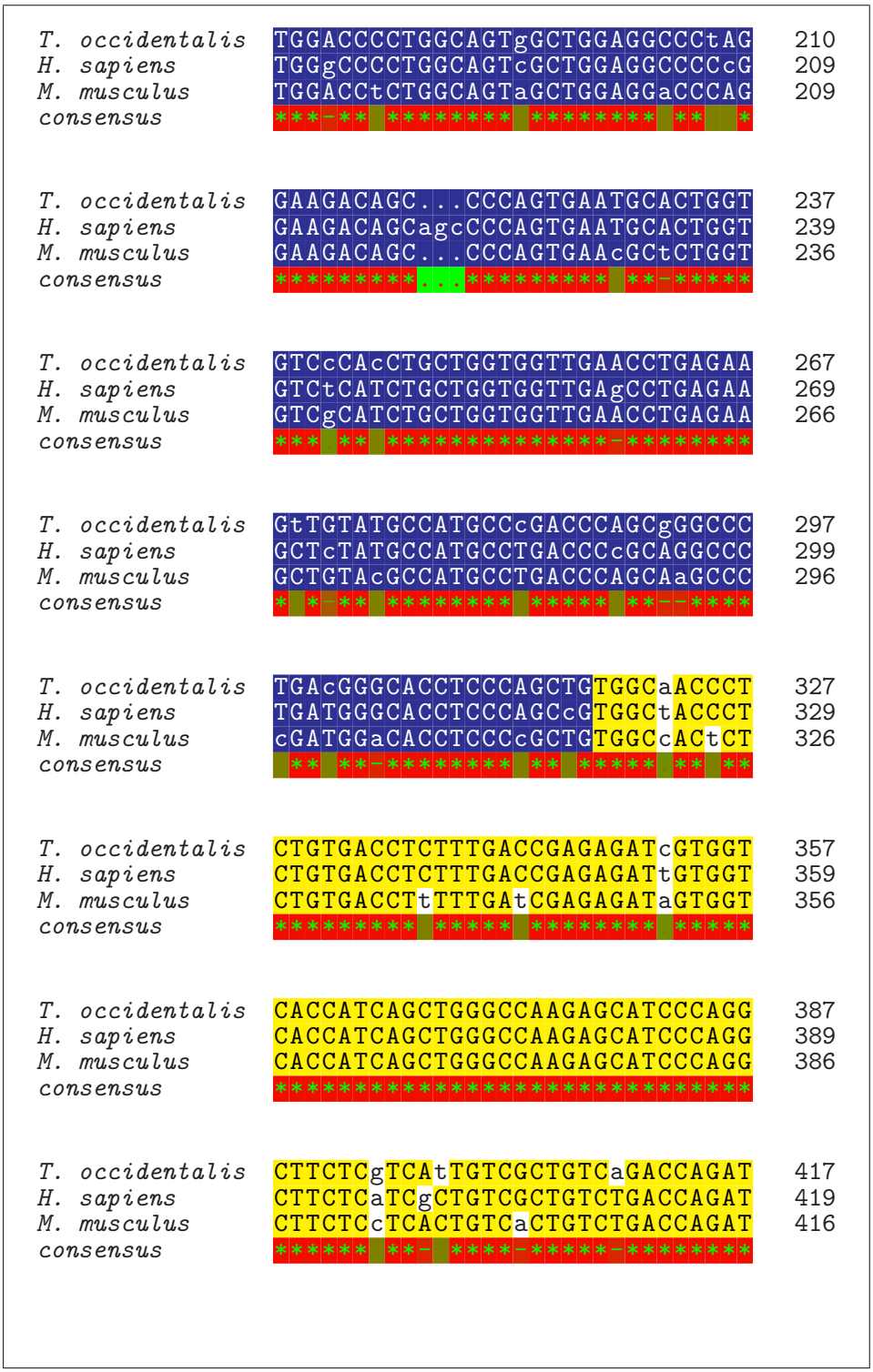


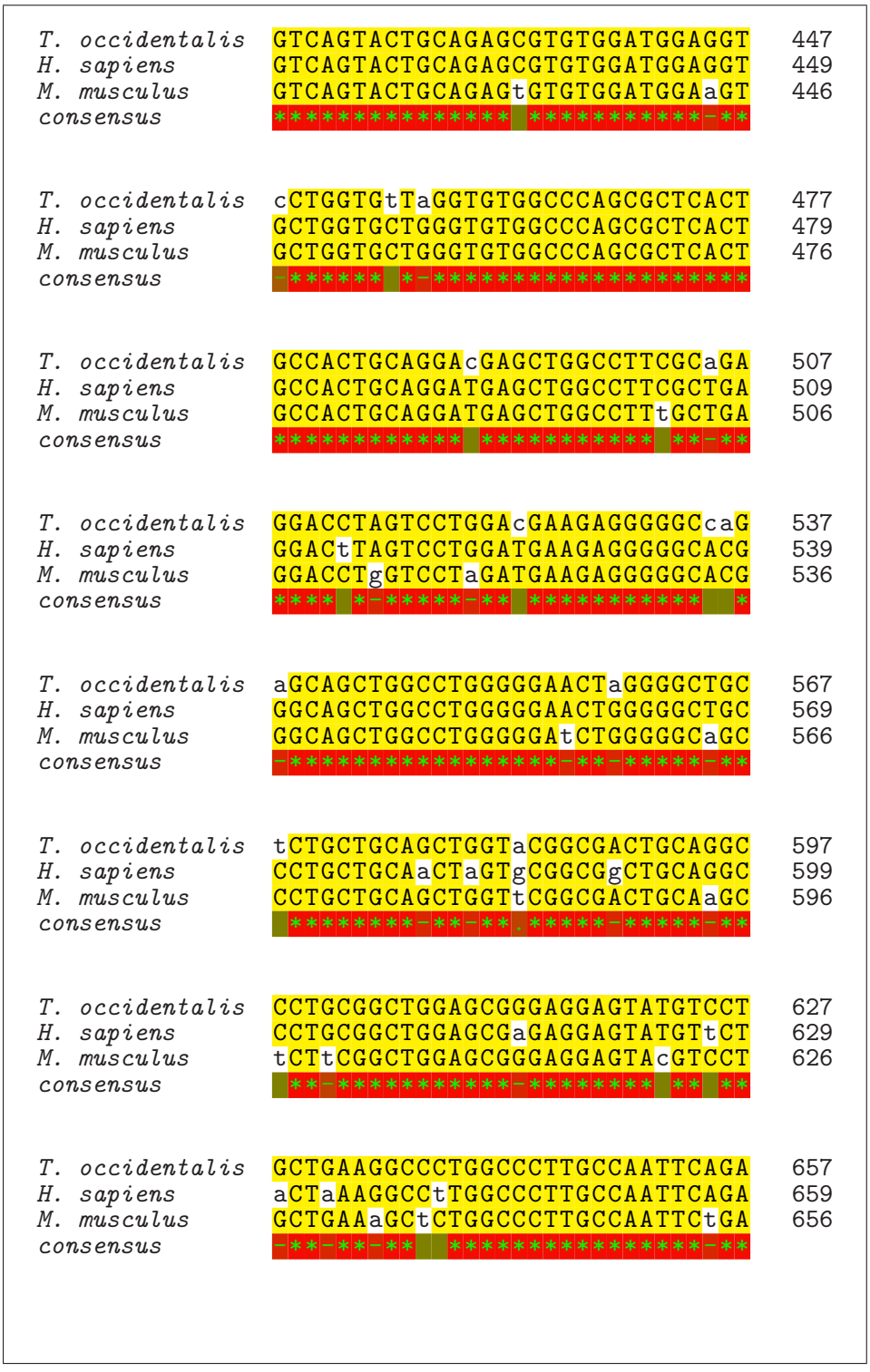
Figure 6.23: *ERα* mRNA expression (*in situ* hybridization) in transverse sections of testis from four representative stages during seasonal breeding cycle of *Talpa occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). *ERα* is expressed in the Leydig cells in all the stages analyzed except in the inactive stage (yellow and black arrows). Scale bar= 20 μm

For quantification purposes, a 189 bp fragment of this gene was also amplified and sequenced from the larger fragment used for in situ studies (Fig. 6.24). This shorter fragment is located between nucleotides 148 and 317 of the larger one (Fig.6.24)The real time PCR quantification showed

that *ERα* is highly expressed in active testes, and much less in the remaining stages (Fig.6.25, Fig.6.26).




T. occidentalis TGGACCCCTGGCAGTgGCTGGAGGCCCtAG 210
H. sapiens TGGgCCCCTGGCAGTcGCTGGAGGCCCCcG 209
M. musculus TGGACCtCTGGCAGTaGCTGGAGGaCCCAG 209
consensus
T. occidentalis GAAGACAGC...CCCAGTGAATGCACTGGT 237
H. sapiens GAAGACAGCagcCCCAGTGAATGCACTGGT 239
M. musculus GAAGACAGC...CCCAGTGAAcGCtCTGGT 236
consensus
T. occidentalis GTCcCAcCTGCTGGTGGTTGAACCTGAGAA 267
H. sapiens GTCtCATCTGCTGGTGGTTGAgCCTGAGAA 269
M. musculus GTCgCATCTGCTGGTGGTTGAACCTGAGAA 266
consensus
T. occidentalis GtTGTATGCCATGCCcGACCCAGCgGGCCC 297
H. sapiens GCTcTATGCCATGCCTGACCCcGCAGGCCC 299
M. musculus GCTGTAcGCCATGCCTGACCCAGCAaGCCC 296
consensus
T. occidentalis TGAcGGGCACCTCCCAGCTGTGGCaACCCT 327
H. sapiens TGATGGGCACCTCCCAGCcGTGGCtACCCT 329
M. musculus cGATGGaCACCTCCCcGCTGTGGCcACtCT 326
consensus
T. occidentalis CTGTGACCTCTTTGACCGAGAGATcGTGGT 357
H. sapiens CTGTGACCTCTTTGACCGAGAGATtGTGGT 359
M. musculus CTGTGACCTtTTTGAtCGAGAGATaGTGGT 356
consensus
T. occidentalis CACCATCAGCTGGGCCAAGAGCATCCCAGG 387
H. sapiens CACCATCAGCTGGGCCAAGAGCATCCCAGG 389
M. musculus CACCATCAGCTGGGCCAAGAGCATCCCAGG 386
consensus
T. occidentalis CTTCTCgTCAtTGTCGCTGTCaGACCAGAT 417
H. sapiens CTTCTCaTCgCTGTCGCTGTCTGACCAGAT 419
M. musculus CTTCTCcTCACTGTCaCTGTCTGACCAGAT 416
consensus


T. occidentalis GTCAGTACTGCAGAGCGTGTGGATGGAGGT 447
H. sapiens GTCAGTACTGCAGAGCGTGTGGATGGAGGT 449
M. musculus GTCAGTACTGCAGAGtGTGTGGATGGAaGT 446
consensus
T. occidentalis cCTGGTGtTaGGTGTGGCCCAGCGCTCACT 477
H. sapiens GCTGGTGCTGGGTGTGGCCCAGCGCTCACT 479
M. musculus GCTGGTGCTGGGTGTGGCCCAGCGCTCACT 476
consensus
T. occidentalis GCCACTGCAGGAcGAGCTGGCCTTCGCaGA 507
H. sapiens GCCACTGCAGGATGAGCTGGCCTTCGCTGA 509
M. musculus GCCACTGCAGGATGAGCTGGCCTTtGCTGA 506
consensus
T. occidentalis GGACCTAGTCCTGGAcGAAGAGGGGGCcaG 537
H. sapiens GGACtTAGTCCTGGATGAAGAGGGGGCACG 539
M. musculus GGACCTgGTCCTaGATGAAGAGGGGGCACG 536
consensus
T. occidentalis aGCAGCTGGCCTGGGGGAACTaGGGGCTGC 567
H. sapiens GGCAGCTGGCCTGGGGGAACTGGGGGCTGC 569
M. musculus GGCAGCTGGCCTGGGGGAtCTGGGGGCaGC 566
consensus
T. occidentalis tCTGCTGCAGCTGGTaCGGCGACTGCAGGC 597
H. sapiens CCTGCTGCAaCTaGTgCGGCGgCTGCAGGC 599
M. musculus CCTGCTGCAGCTGGTtCGGCGACTGCAaGC 596
consensus
T. occidentalis CCTGCGGCTGGAGCGGGAGGAGTATGTCCT 627
H. sapiens CCTGCGGCTGGAGCGaGAGGAGTATGTtCT 629
M. musculus tCTtCGGCTGGAGCGGGAGGAGTAcGTCCT 626
consensus
T. occidentalis GCTGAAGGCCCTGGCCCTTGCCAATTCAGA 657
H. sapiens aCTaAAGGCCtTGGCCCTTGCCAATTCAGA 659
M. musculus GCTGAAaGCtCTGGCCCTTGCCAATTCtGA 656
consensus

```
T. occidentalis   CTCTGTGaatcg   669
H. sapiens        CTCTGTG.....   666
M. musculus       CTCTGTG.....   663
consensus         *******.....
```

Figure 6.24: Comparison of nucleotide sequences of *ERα* fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 94% homology with that of *Homo sapiens* and 90% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green. Nucleotides in the blue block correspond to the fragment used in real time PCR reactions

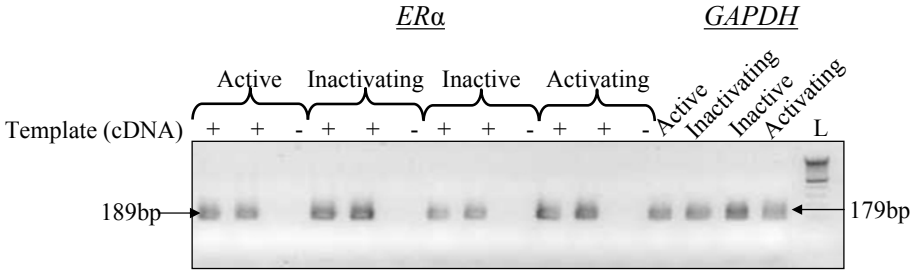


Figure 6.25: *ERα* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *ERα* expression levels were checked using quantitative real time PCR. Amplification of a 189 bp was observed for *ERα* at all the stages analyzed. Expression of *GAPDH* (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA. (-) reactions performed without cDNA. L:100 bp ladder

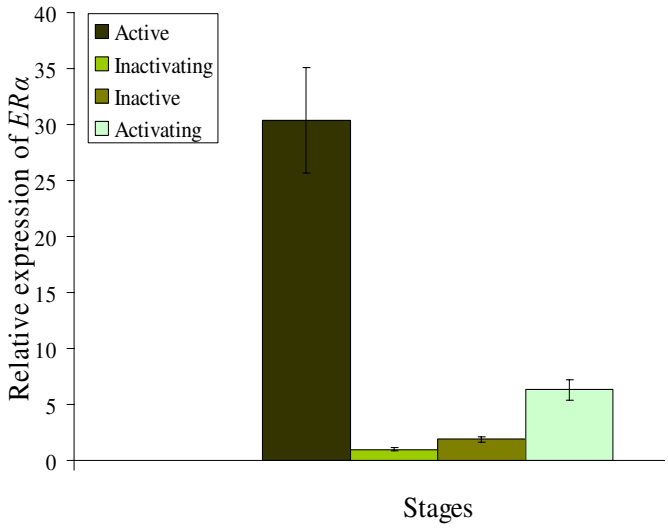


Figure 6.26: Expression levels of *ERα* in the testis from *T. occidentalis*, throughout the seasonal breeding cycle. Higher expression levels were detected in the active (**a**) testes than in the rest of the stages. Real time PCR reactions were performed, using *GAPDH* was used as a reference gene for quantification.

6.2.2 Hormone measurements

Testosterone and estradiol measurements were performed in males (serum and intra-testicular fluid) and females (serum) of *Talpa occidentalis*, by the radio-immunoassay technique, in different stages of the reproductive cycle.

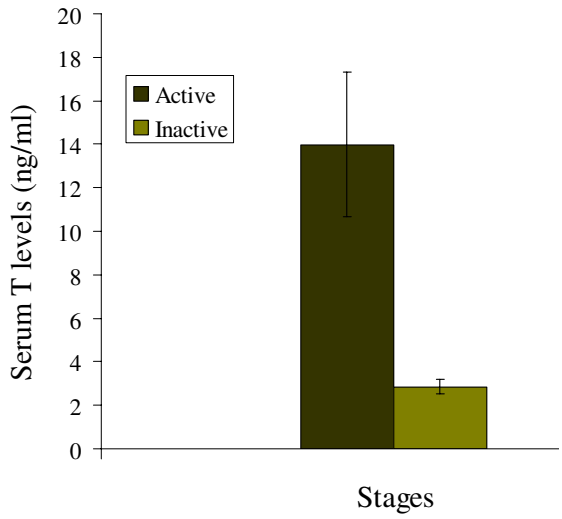


Figure 6.27: Serum testosterone levels in males of *T. occidentalis*. Serum was extraxted from blood of active and inactive moles and T levels were measured by RIA. Testosterone production was clearly higher in the breeding (active) stage.

Testosterone

In male moles, testosterone levels were much higher in the active than in the inactive stage in both the serum (Fig.6.27) and the intra-testicular fluid (Fig.6.28), although the concentration measured was about seven times higher in the intra-testicular fluid than in serum.

Estradiol

In males serum estradiol levels were about five times higher in the inactive than in the active testis (Fig.6.29), whereas no significant hormone concentration differences were observed between active and inactive testes in the intra-testicular fluid (Fig.6.28).

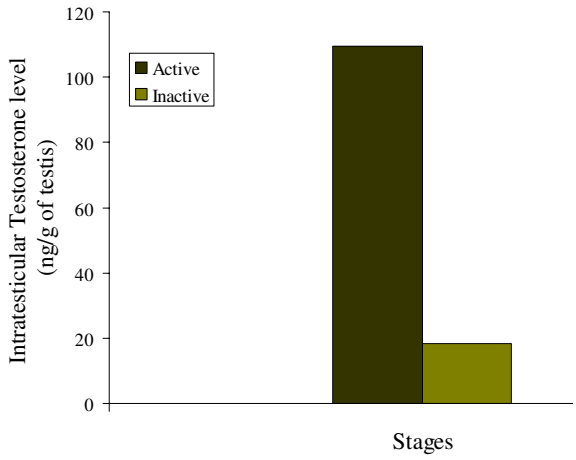


Figure 6.28: Intratesticular testosterone levels in males of *T. occidentalis*. Testes homogenate, of active and inactive moles, was prepared and T levels were measured by RIA. Intratesticular testosterone production was clearly higher in the breeding (active) stage.

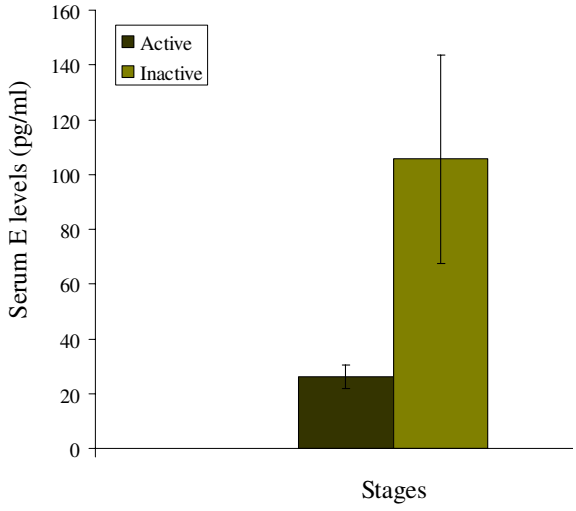


Figure 6.29: Serum estradiol levels in males of *T. occidentalis*. Serum was extraxted from blood of active and inactive mole and estradiol levels were measured by Radioimmunoassay. Estradiol production was clearly higher in the non-breeding (inactive) stage

6.3 Control of meiosis onset during seasonal breeding cycle of *Talpa occidentalis*

6.3.1 Initiation of meiosis

Male moles become temporally sterile (azoospermic) every year during the non-breeding season. This raises the hypothesis that meiosis is completely stopped every year during the non breeding season. In this context it was interesting to investigate whether germ cells de-differentiate to pre-pubertal stages during the period of inactivity. In this case it was expected that they would express some primordial germ cell markers like OCT4. Accordingly, we performed immunoflouresence studies using an antibody specific for OCT4 as shown in (Fig.6.30). Contrarily to this hypothesis, we found that OCT4 protein is absent in the testes of moles in any of the four stages of the seasonal reproductive cycle.

To mark early spermatogenic cells, we have used antibodies specific for DMC1 and γH2AX, expressed by recombining early prophase spermatocytes at zygotene and pachytene, as well as SYCP3, a component of the synaptonemal complex, present in zygotene-diplotene spermatocytes. Immunostaining performed with these antibodies clearly evidenced that immunoreactive cells appear in basal positions of the seminiferous tubules in testes from males captured in all four stages of the reproductive cycle of this species (Fig.6.31, Fig.6.32, Fig.6.33).

Late spermatogenic cells were detected with CRM1, a nucleo-cytoplasmic transporter expressed in round spermatids. This protein is present in activating and active testes in a stage-dependent manner, but it is absent in the gonads of inactivating and inactive male moles (Fig.6.34).

In order to quantify the relative abundance of these cell types, we first counted the total number of spermatocytes per tubule in testes belonging to the four representative stages of the reproductive cycle (Fig.6.35, Fig.6.36). For preletotene/leptotene spermatocytes (DMC1 positive) the average values were 40.5 ± 5.79 and 22.7 ± 2.11 for active and inactive testes, respectively. In the case of zygotene/pachytene cells (SYCP3 positive), the counts were 48.8 ± 4.96 and 15.6 ± 2.72 for active and inactive testes, respectively. According to this quantification method, the difference between active and inactive gonads was highly significant ($P<0.0001$) for both cell types. However, in our opinion, this method may lead to misleading results because seminferous tubules are in fact much bigger in the active than in the inactive testis. However, a quantification based on the percentage of primary spermatocytes in the seminiferous tubule would also be misleading because these cells are not homogeneously distributed in it. Instead, primary spermatocytes always peripherally located in the tubule, so that their density

does not vary according to its area (which is directly proportional to its volume), but according to their perimetric length. Hence, we calculated the perimetric length spematocytes density (Fig.6.35), by counting their number every 100 μm of the perimetric length in the same seminiferous tubules previously analyzed. In our opinion, this parameter reflects more reliably seasonal variations in the abundance of these cell types in the testes of *T. occidentalis* (Fig.6.35). By using this method we obtained very different but realistic results. The cell density values corresponding to preleptone/leptotene were 6.7±0.96 and 5.9±0.55 for active and inactive testes, respectively (Fig.6.35; P=0.0434). For the case of zygotene/pachytene, these values were 8.1±0.82 and 4.9±0.85 for active and inactive testes, respectively (Fig.6.36; P=0.0001).

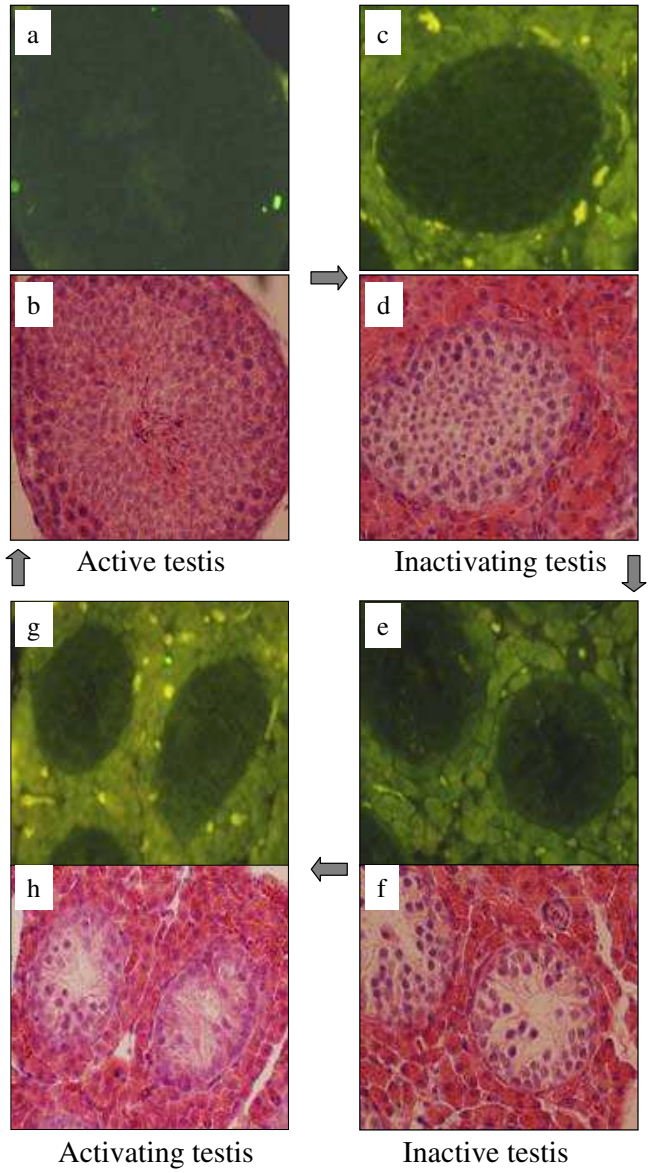


Figure 6.30: OCT4 detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**c**), Inactive (**e**)and Activating (**g**). *OCT4* is not expressed in any of the stages analyzed. Haematoxylin-eosin staining is shown for the different stages in **b**, **d**, **f**, **h**. Scale bar = 20 μm

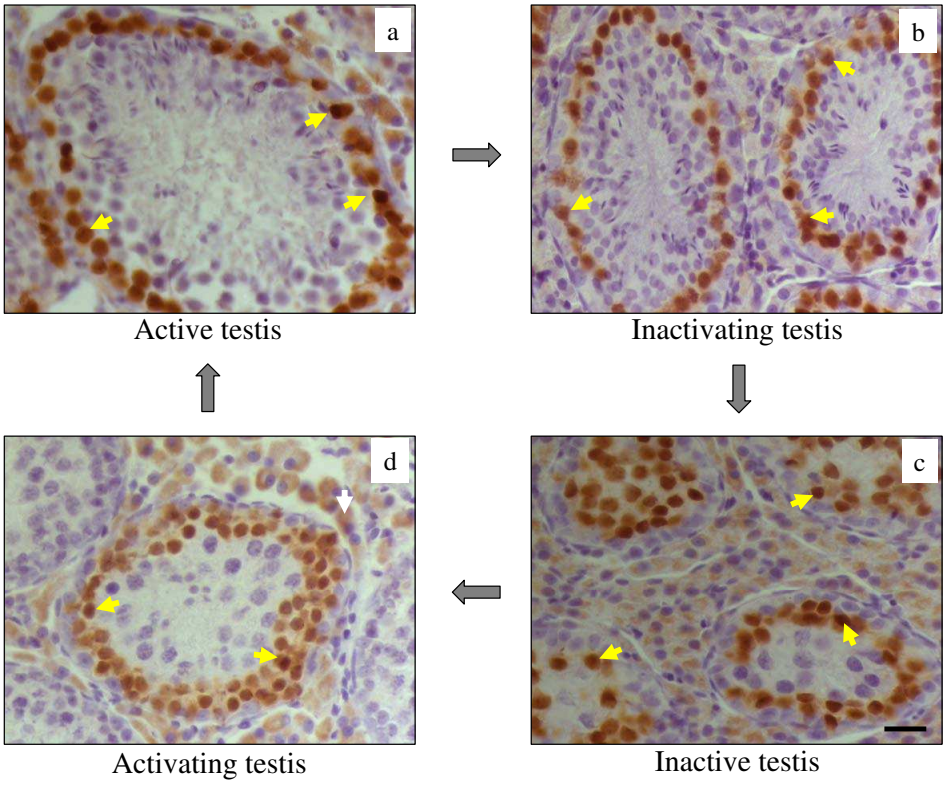


Figure 6.31: DMC1 detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). Early meiotic germ cells are immunoreactive to DMC1 in all the four stages (yellow arrows). Scale bar = 20 μm.

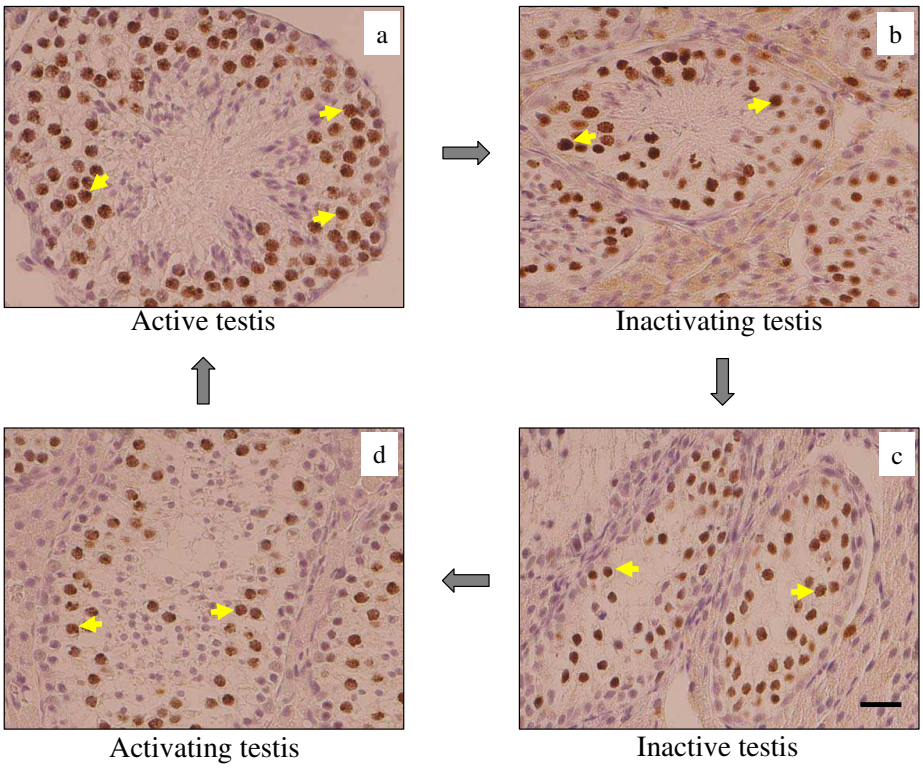


Figure 6.32: γH2AX detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). Zygotene and pachytene spermatocytes are marked with anti-γH2AX in all the four stages (yellow arrows). Scale bar = 20 μm

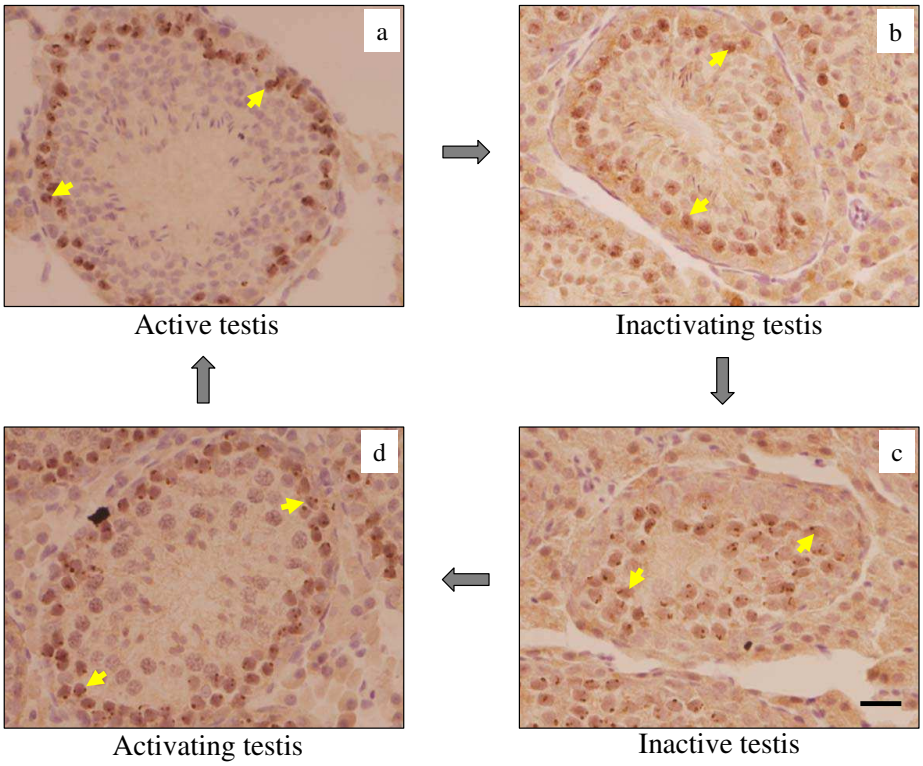


Figure 6.33: SYCP3 detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). Early prophase I spermatocytes are marked with anti-SYCP3 in all the four stages (yellow arrows). Scale bar = 20 μm

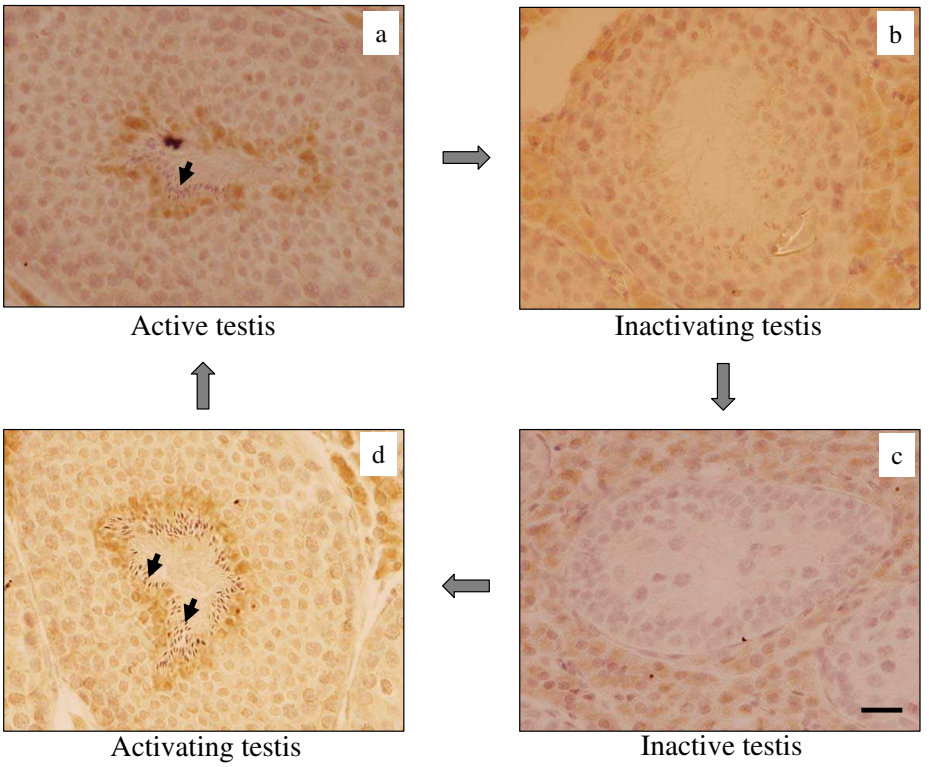


Figure 6.34: CRM1 detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). CRM1 is detected in the round spermatids in activating (**d**) and active testes (**a**) in a stage dependent manner, but not in the inactivating (**b**) and the inactive gonads(**c**) (black arrows). Scale bar = 20 μm

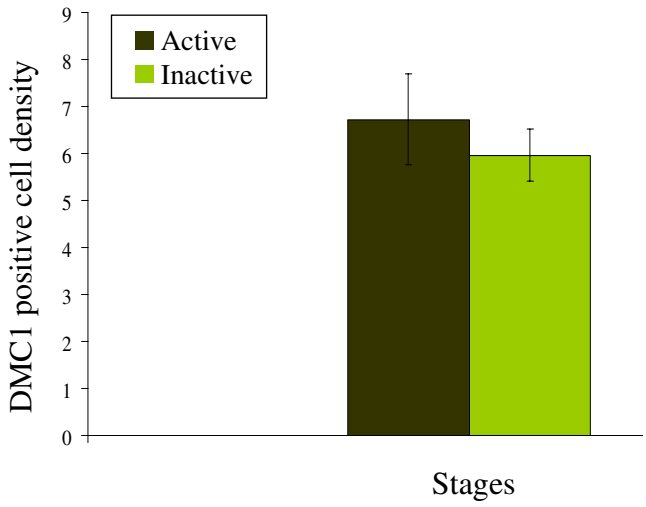


Figure 6.35: DMC1 positive cell (preleptotene/leptotene stage) density during the active and the inactive stages of seasonal breeding cycle in *T. occidentalis*. No significant differences were found between active and inactive stages.

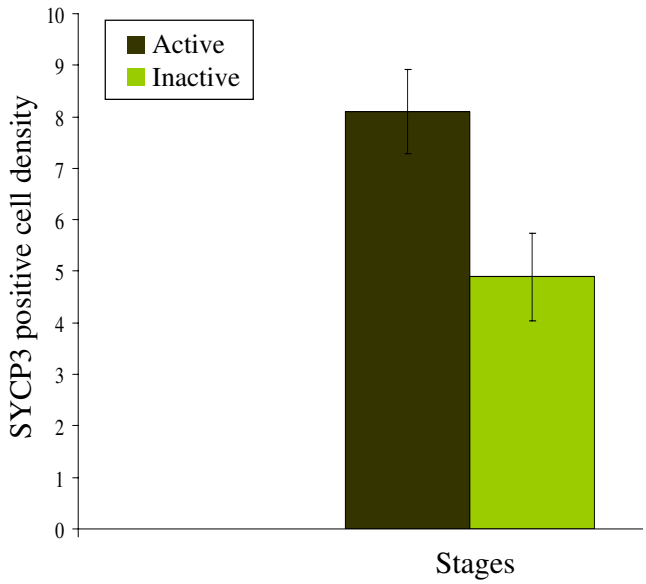


Figure 6.36: SYCP3 positive cell (pachytene/zygotene stage) density during the active and inactive stages of the seasonal breeding cycle in *T. occidentalis*. No significant differences were found between active and the inactive stage.

6.4 Role of apoptosis and cell proliferation in the testis during seasonal breeding cycle of *Talpa occidentalis*

6.4.1 Apoptosis

It is well known that in *Talpa occidentalis*, as well as in other species with seasonal reproduction , testes experiences considerable size and weight variations throughout the breeding cycle, and that these changes are mainly due to variation in the diameter of the seminiferous tubules. Ultimately this is a consequence of the fact that seminiferous tubules in the active testes are full of germ cells in different stages of meiosis, which are completely absent in the inactive gonad. This implies that during the transition stage of testis inactivation, a massive depletion of germ cells takes place resulting into a completely azoospermic testis. Therefore it was interesting to investigate the possible role that apoptosis could play in this process of testis inactivation.

Fig.6.37 shows the result that we have obtained in the immunohistochemical study we performed using an antibody specific for CASPASE3, a typical apoptosis marker. This protein was found to be present in peripheral cells of the seminiferous tubules of the testes in all the four stages of the reproductive cycle of *T. occidentalis*. In no case inner cells were found to be immunoreactive to this antibody. Surprisingly all Leydig cells also appeared to be caspase positive. But this was not specific staining as the same pattern was also present in the negative control.

In order to quantify the influence of apoptosis in the process of testis inactivation, a first approach was to count the total number of apoptotic cells present per tubule in testes belonging to the four representative stages of the reproductive cycle (Fig.6.38). This analysis showed that this number is relatively high in all stages except in the inactive testes where it is significantly lower (P=0.0001, between active and inactive). Differences between the other stages were not significant (P=0.064, between active and inactivating, for example). However, these data are not very informative because seminferous tubules are in fact much bigger in the active than in the inactive testis. Consequently, we also counted the total number of cells in the same seminiferous tubules where the previous counting was done in order to obtain the percentage of apoptotic cells (Fig.6.39). The percentage of CASPASE3 positive cells were found to be significantly lower in the active than in the other three stages (P=0.0001, between active and inactivating). However, as in the case of primary spermatocytes, apoptotic cells always show a peripheral location in the seminiferous tubules, so we

calculated the perimetric length apoptotic cell density ((Fig.6.40). According to this quantification method, no significant differences were observed between inactivating, inactive and activating stages (P>0.05). Significant differences were found between active and inactivating (P=0.0005), and between inactivating and inactive stages (P=0.0001). Similarly, differences were also significant between active and inactive stages (P=0.0001). Contrarily, no significant differences were found between activating and active or inactivating stages (P>0.05).

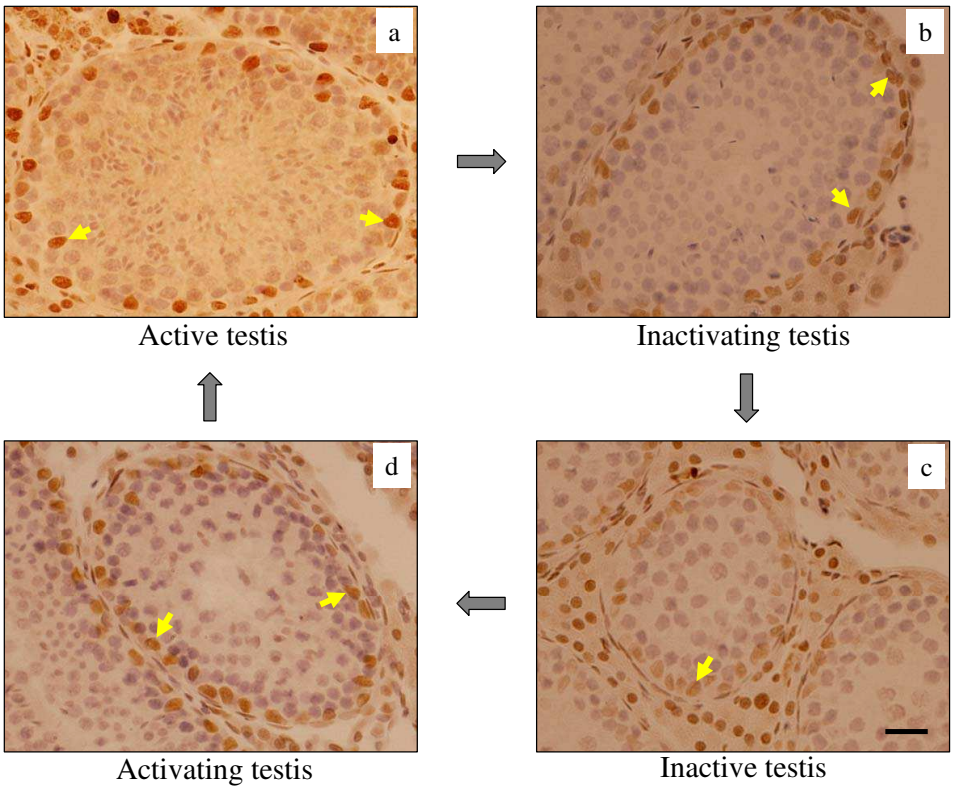


Figure 6.37: CASPASE3 detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**)and Activating (**d**). Apoptotic cells (shown by yellow arrows) are relatively abundant in the seminferous tubule in all the stages but were lesser in the inactive stage. CASPASE3 positive cells (apoptotic cells) were located near to the periphery. All Leydig cells appear to be immunoreactive but showed less staining intensity. Scale bar = 40 μm

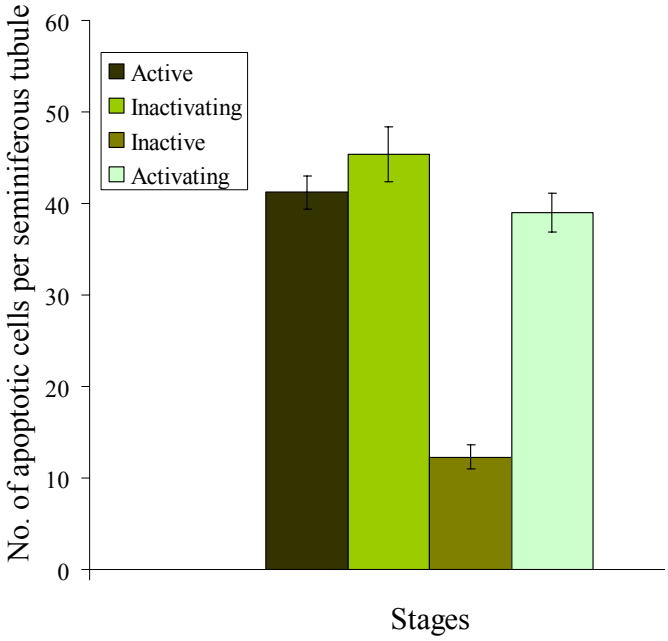


Figure 6.38: Quantitative analysis of the number of apoptotic cell per seminiferous tubule in various stages of the seasonal breeding cycle in *T. occidentalis*. There were more *CASPASE3* positive cells in the active, inactivating and activating stages than in the inactive stage. Countings were made in 10 randomly selected seminiferous tubules from each of the four stages

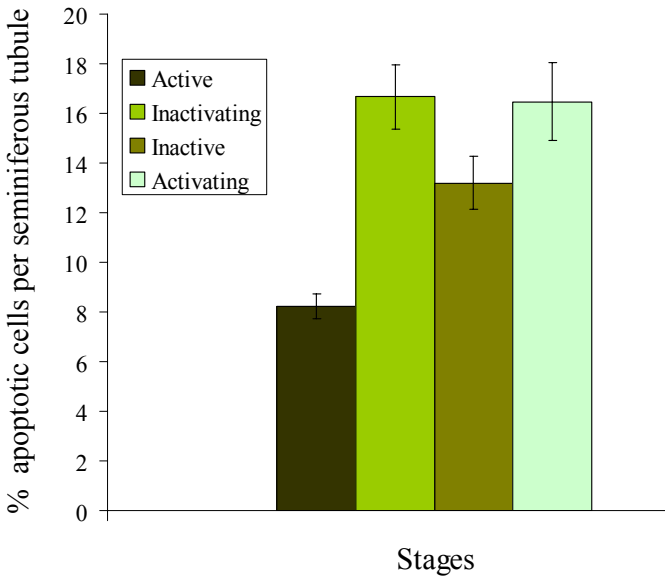


Figure 6.39: Percentage of apoptotic cells per seminiferous tubule in various stages of seasonal breeding cycle in *T. occidentalis*. This percentage was significantly lower in the active stage than in other stages. Countings were done in the same seminiferous tubules analyzed in Fig.6.38, Fig.6.39

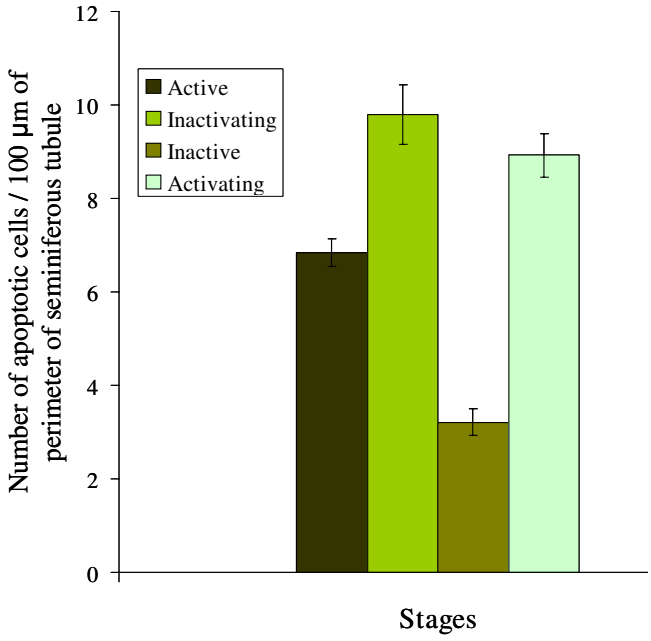


Figure 6.40: Quantification of the number of apoptotic cell per 100 μm of perimetric length in seminiferous tubule of testes in various stages of the seasonal breeding cycle in *T. occidentalis*. Significant differences were found between active and inactivating, and between inactivating and inactive stages, as well as between active and inactive stages. No significant differences were found between activating and active or inactivating stages. Perimetric lenghts were measured in the same seminiferous tubule analyzed in Fig.6.38

6.4.2 Cell Proliferation

We performed immunohistochemical stainings with an antibody specific for PHOSPHO HISTONE 3, a protein present in all proliferating cells, to study the role of cell proliferation during the seasonal breeding cycle of *T. occidentalis* (Fig.6.41). To quantify the density of proliferating cells, we counted their number per section area as described above for the case of SOX9-positive cells.

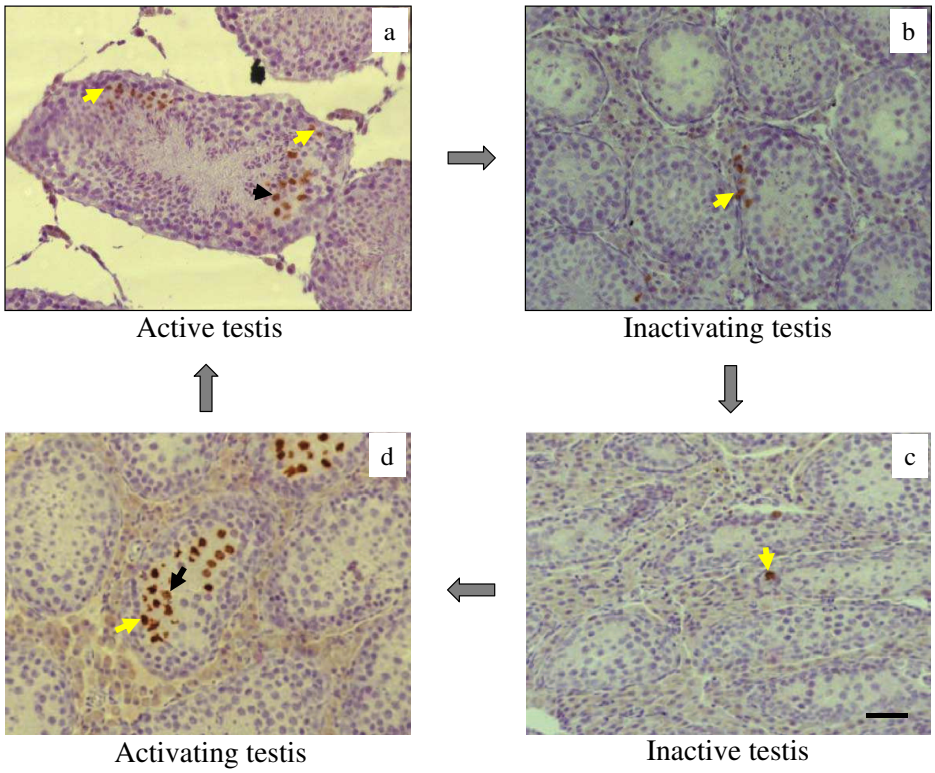


Figure 6.41: PHOSPHO HISTONE 3 detection in transverse sections of testis from four representative stages during the seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**)and Activating (**d**). Basal proliferating cells were minimum in the active, inactivating and the inactive testes. The basal PHOSPHO HISTONE 3 positive cells (yellow arrow) increases dramatically in the activating and decreases during the active stage. Black arrows point to inner, non-basal cells. Scale bar = 40 μm

We found that proliferating cells were present in all four stages of this cycle, although they are clearly more frequent in activating and active stages than in the inactivating and the inactive testes, as demonstrated in the

corresponding quantitative analysis (Fig.6.42). However, according to the position of the PHOSPHO HISTONE 3-positive cells observed in our preparations, we suspected that not all of them were mitotic spematogonia. Many positive cells occupied inner, non-basal positions, suggesting that they were in fact meiotic cells (Fig.6.41). This is consistent with recent reports showing that phosphorylation of HISTONE H3 is also coupled to chromatin condensation in meiosis (Wei *et al.*, 1998; Houben *et al.*, 2005; Swain *et al.*, 2007). Hence, as we mainly wanted to quantify the density of proliferating spermatogonia, we made a new count in which only basal positive cells were considered. In this case, we calculated the sectional density of PHOSPHO HISTONE 3-positive cells, by counting their number in complete testis sections and dividing these numbers by the area (in square mm) occupied by those sections. This new quantifications provided quite different results (Fig.6.43). The lower density of mitotic spematogonia was found in the active testes. This density increases gradually through inactivating and inactive stages and undergoes a statistically significant increase (P=0.0359) during the activation of the gonad. Consequently, there is a highly significant decrease (P=0.0037) of mitotic activity once the spermatogenesis is completely restored (active stage).

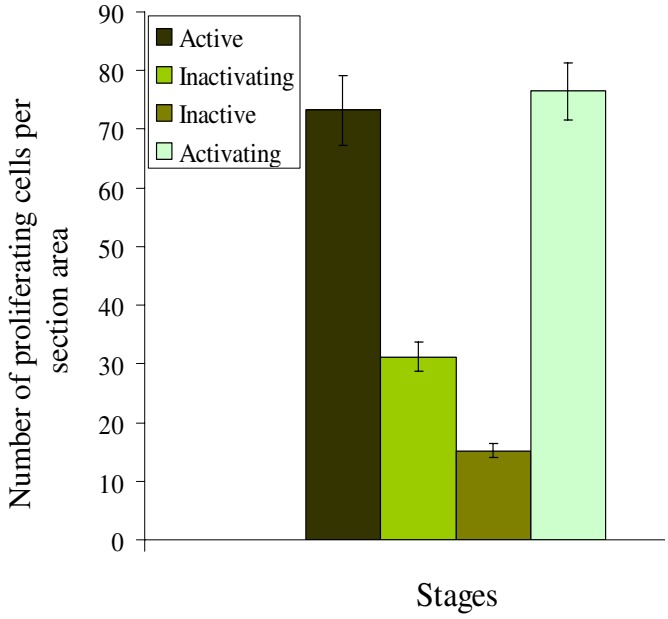


Figure 6.42: Number of proliferating cells per sectional area in various stages of seasonal breeding cycle in *T. occidentalis*. Significant differences were found between active and inactivating stages and between the later and the inactive stage. No significant difference was obsereved between activating and active stages

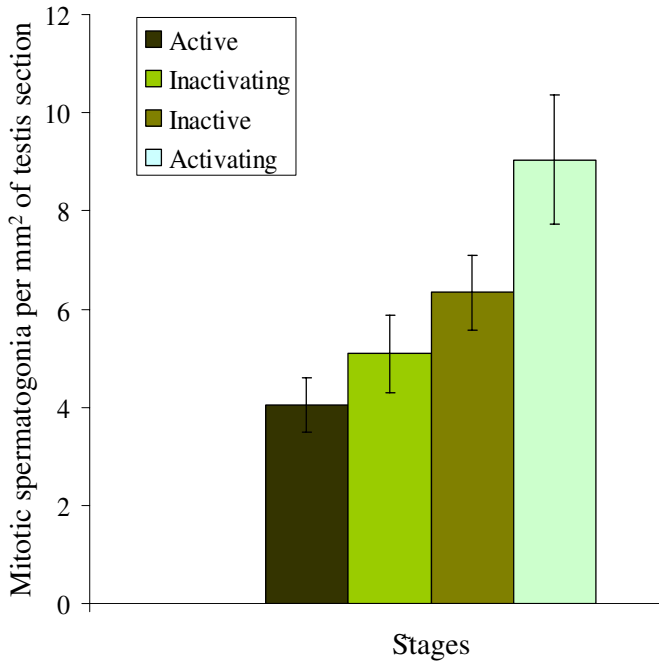


Figure 6.43: Proliferating spermatogonial cells per square mm of testis section in various stages of seasonal breeding cycle in *T. occidentalis*. There was gradual increase in the basal proliferating from the active stage (with minimum) to the inactive stage and a peak was observed in the activating stage. Significant differences were found between activating stages and rest of the stages

6.5 Regulation of cell junctions in the testis during seasonal breeding cycle of *Talpa occidentalis*

Although apoptosis could have a role in the massive cell depletion that takes place in the testis during the inactivation process, there are two facts suggesting that additional factors may contribute to this phenomenon: 1) apoptotic cells always occupy a basal position near the periphery of the seminiferous tubules, and 2) most inner cells are lost without showing any evidence of undergoing apoptosis. Hence, there is also possible that many live germ cells were depleted simply by loosing their junctional linkage to either Sertoli cells or other germ cells, thus falling into the tubular lumen.

According to this hypothesis it was interesting to investigate the status of the cell junctions in the four stages of the reproductive cycle of *T. occidentalis*. The cell junctions could be studied by different methods including electron microscopy and immunohistochemistry or immunofloures-cence. In the present study we have emphasized on the immunohistochemical and quantitative real time PCR. Hence, we have studied the localization of several proteins which are structural components of different types of cell junctions- CLAUDIN11, CONNEXIN43, N-CADHERIN, B-CATENIN and ZONA OCCLUDIN-2. All of these proteins are specially abundant in the inter-Sertolian specializations forming the blood testis barrier (BTB).

6.5.1 CLAUDIN11 (CLDN11)

We performed immunohistochemical studies using an antibody against CLDN11. We observed two distinct patterns of localization of CLDN11 in the seminiferous tubules during the seasonal breeding cycle of *T. occidentalis*. It was found that CLDN11 localization was basal relative to germ cells in the active and the activating testes. In these stages the staining was very sharp and continuous around the Sertoli cells. However, the staining showed a diffuse pattern with wide gaps in the inactivating and the inactive testes (Fig.6.44). We also amplified a fragment of 169 bp of the mole CLDN11 (Fig.6.45, Fig.6.46) to study the expression level of the *CLDN11* gene. This paralleled quite well the gonad activity curve of the mole, i.e. fertile testes had significantly higher levels of CLDN11 than inactive testes (P=0.0001). The transition stages, inactivating and activating, had intermediate levels of *CLDN11* mRNA ((Fig.6.47).

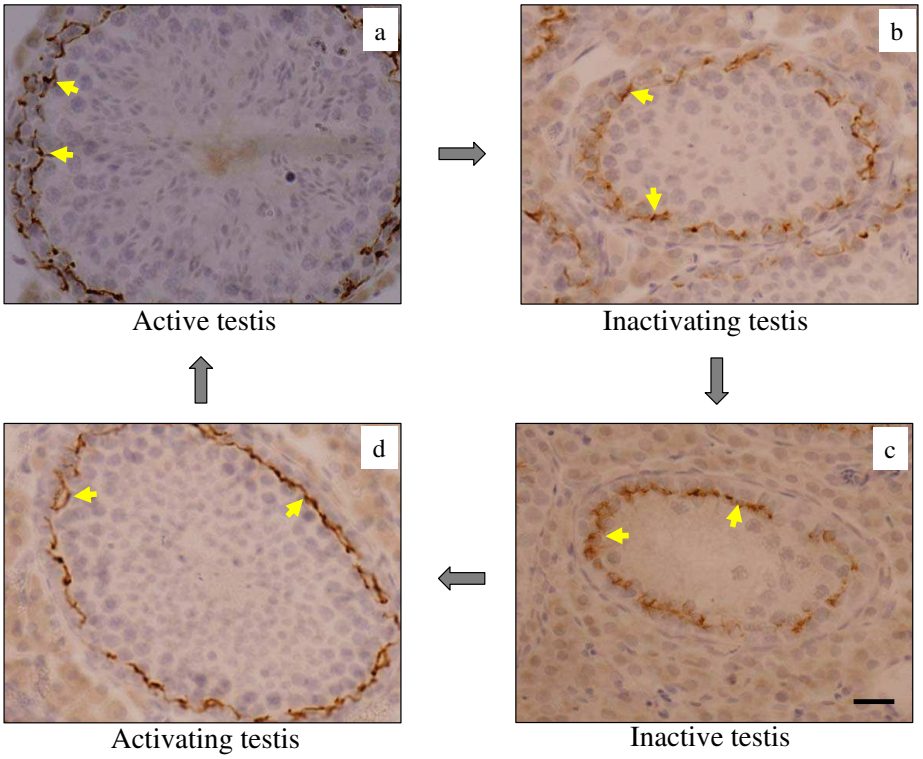


Figure 6.44: CLAUDIN11 (CLDN11) detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). Intense expression around the Sertoli cell was clearly visible in activating (**d**) and active (**a**) testes, whereas only a diffused immunoreactivity was observed in the same area of inactivating (**b**) and inactive (**c**) testes (arrows). Scale bar = 20 μm

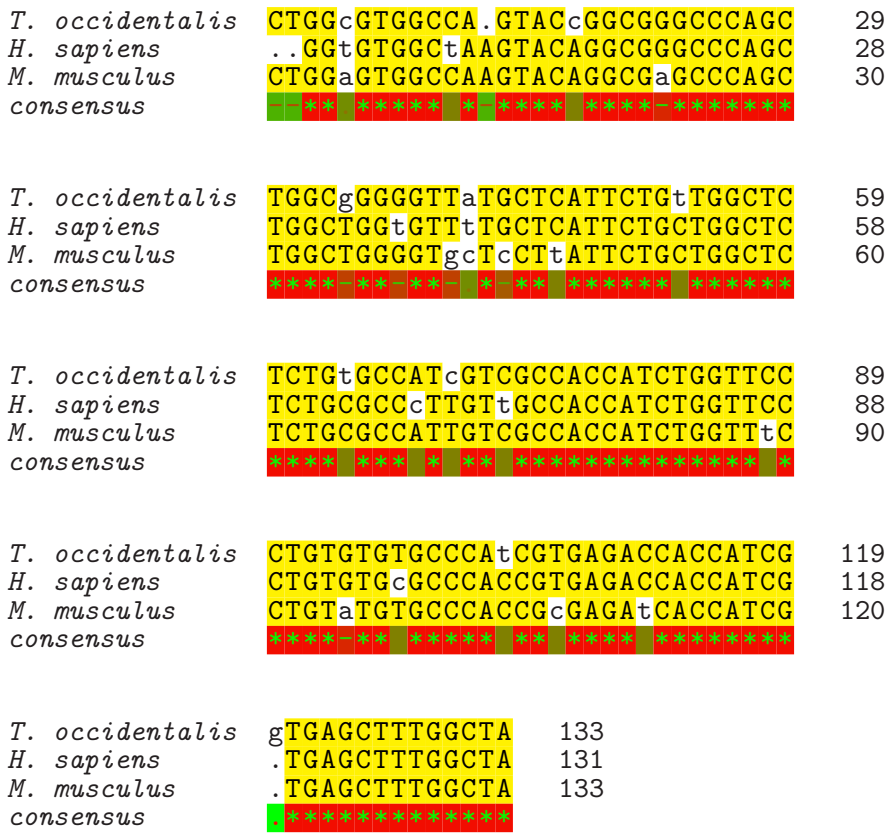


Figure 6.45: Comparison of the nucleotide sequences of *CLAUDIN11 (CLDN11)* fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 88% homology with that of *Homo sapiens* and 86% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

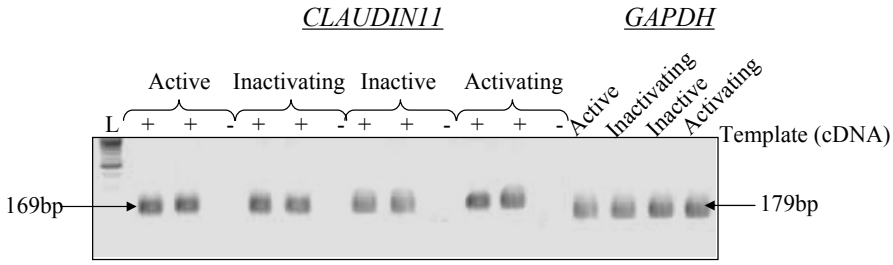


Figure 6.46: *CLAUDIN11* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* from four stages of their annual breeding cycle and expression levels were checked using quantitative real time PCR. Amplification of a 169 bp was observed for *CLDN11* at all the stages analyzed. Expression of *GAPDH* (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA. (-) reactions performed without cDNA. L:100 bp ladder

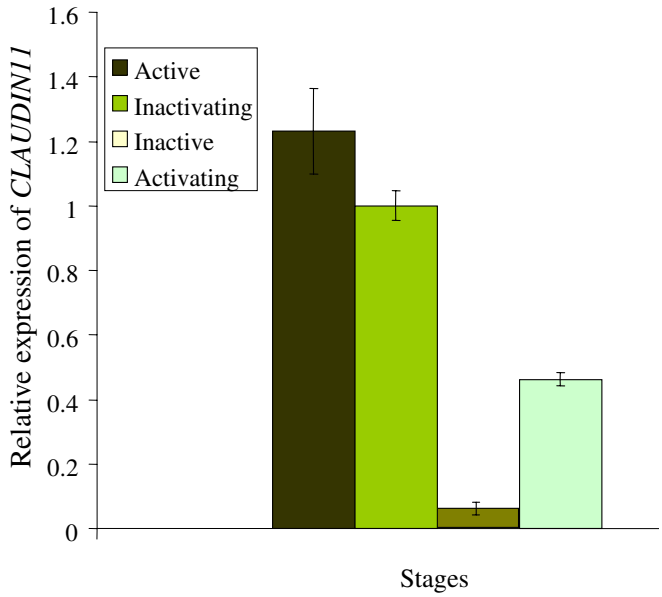
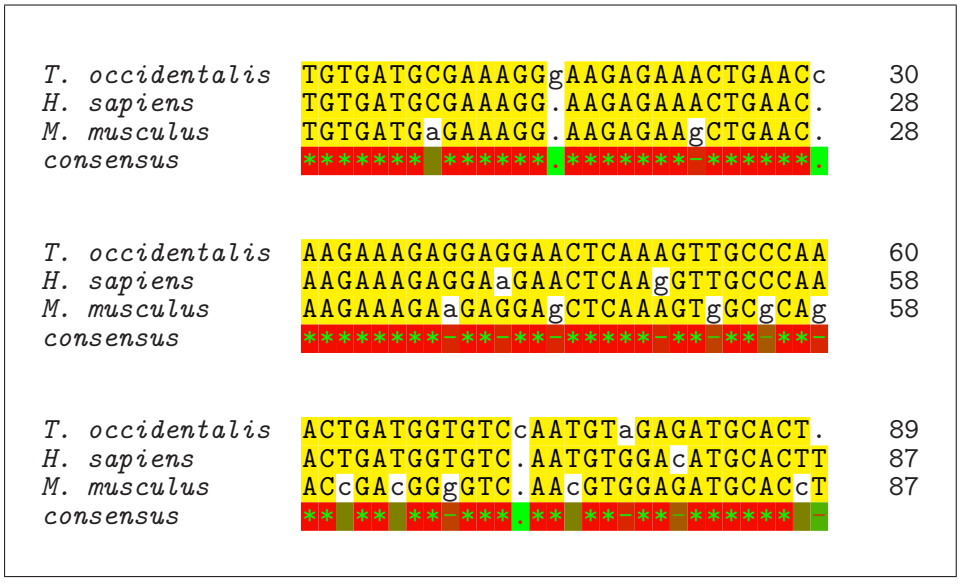


Figure 6.47: Expression levels of *CLAUDIN11* in the testis from *T. occidentalis*, throughout the seasonal breeding cycle. High expression levels were detected only in active (**a**) and inactivating testes (**b**) and very low expression in inactive (**c**) and activating (**d**) gonads. Real time PCR reactions were performed, using *GAPDH* as a reference gene for quantification.

6.5.2 CONNEXIN43 (CON43)

In general, testicular cells of moles appear less immunoreactive to the CON43 antibody we used when compared with the CLDN11 antibody. Nevertheless, we could find a well defined localization pattern for this protein. Leydig cells showed the strongest expression of this protein with sharp foci located between adjacent cells in all stages except the activating stage. Inside the seminiferous tubules a faint staining appeared between inner cells of the germinative epithelium and a little stronger between peripheral cells. This pattern was observed in active and the inactivating stages but was more evident in the later. In the inactive testes only the inner cells of seminiferous tubules appeared to be immunoreactive. In the activating testes, CON43 was not detectable inside the seminiferous tubule (Fig.6.48). Using a 178 bp fragment of the mole CON43 gene (Fig.6.49, Fig.6.50), we performed a quantitative analysis of this gene. From low levels of *CON43* mRNA in the active testes, a strong upregulation of the gene is observed during the inactivating stage to reach the maximum in the inactive testes. Beyond this point, the gene is downregulated again so that a minimum expression level was observed in the activating stage (Fig.6.51). Like in the the case of *P450scc*, correction was also required to be done in the expression level of CON43 mRNA. After the corrections, this gene seemed to maintain a constant level throughout the sesaonal breeding cycle as the expression levels between the active and the inactive stages were not statistically significantly (Fig.6.52; P>0.05).



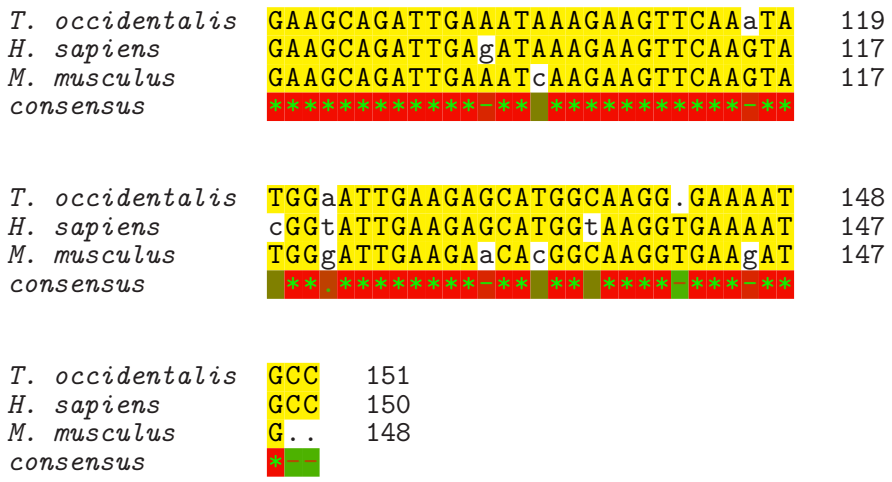


Figure 6.49: Comparison of the nucleotide sequences of *CONNEXIN43* (*CON43*) fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 90% homology with that of *Homo sapiens* and 84% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

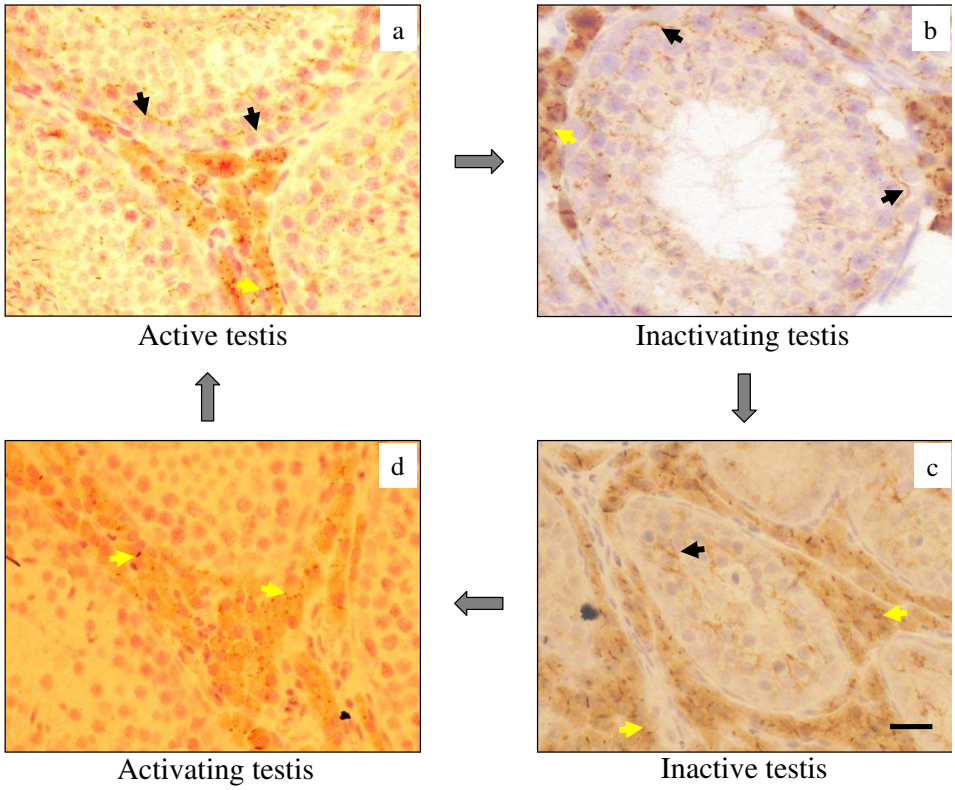


Figure 6.48: CONNEXIN43 detection in transverse sections of testes from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**)and Activating (**d**). Leydig cells appeared strongly immunoreactive in all the stages except in activating testes (yellow arrows). CON43 was also present, although very weakly, around Sertoli cells in active and inactivating testes but in a very disorganized manner in the inactive stage (black arrows). Scale bar = 20 μm

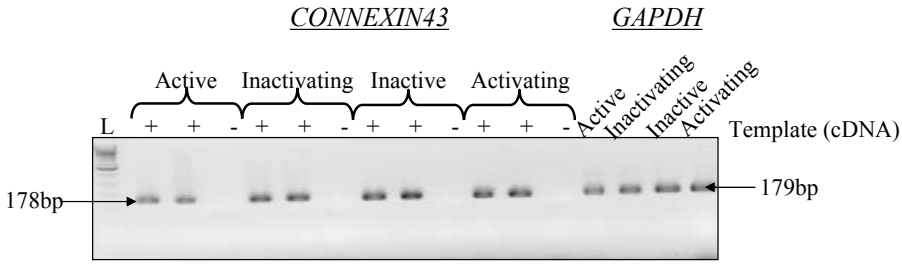


Figure 6.50: *CONNEXIN43* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *CON43* expression levels were checked using quantitative real time PCR. Amplification of a 178 bp was observed for *CON43* at all the stages analyzed. Expression of *GAPDH* (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA. (-) reactions performed without cDNA. L:100 bp ladder

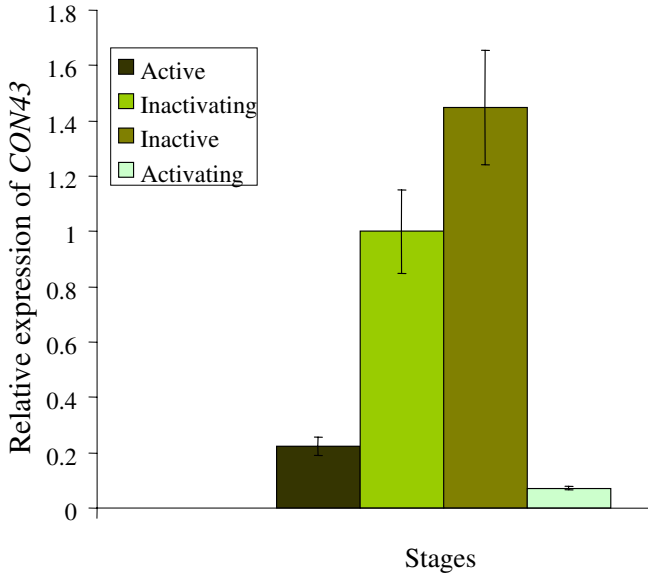


Figure 6.51: Expression levels of *CONNEXIN43* in testes from *T. occidentalis*, throughout the seasonal breeding cycle. Expression increased gradually from active to inactive stages, where a maximum was observed, and then it rapidly declined during the activating stage. Real time PCR reactions were performed, using *GAPDH* as a reference gene for quantification

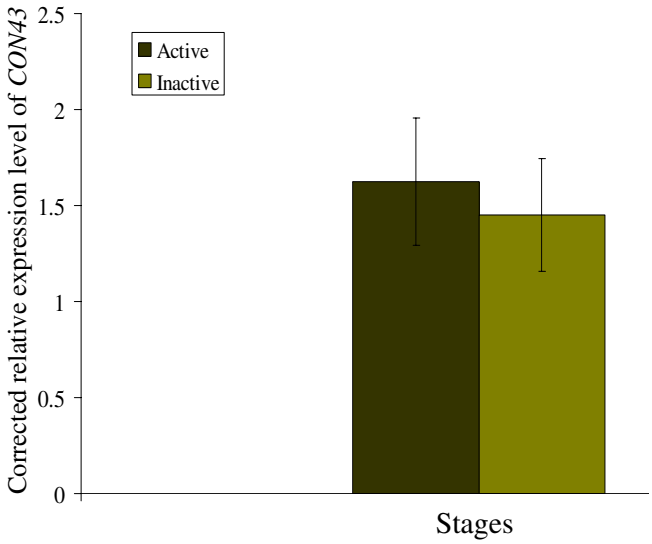


Figure 6.52: Corrected expression levels of *CON43* in the testis from *T. occidentalis*, during the active and the inactive stages.

6.5.3 *NCADHERIN* (*CDHN*) and *β-CATENIN* (*CTNNB1*)

The CDHN-CTNNB1 complex form one of the functional units of the tight junction in the testis. According to this, the expression pattern of these two proteins was similar in the mole testes. Like CLDN11 and unlike CON43, CDHN and CTNNB1 were not detectable between Leydig cells. Contrarily, these proteins appeared throughout the seminiferous tubules in all stages, but showing different staining intensities in different tubular areas, being higher towards the basement membrane (in between Sertoli cells) and lower in the inner areas ((Fig.6.53 and (Fig.6.54). However, this peripheral protein localization was not observed in the inactive stage.

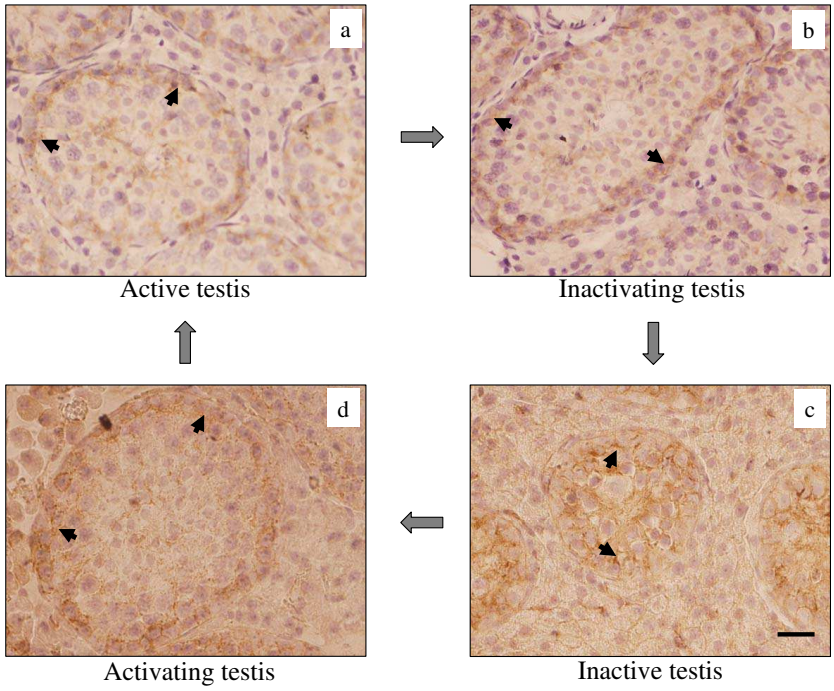


Figure 6.53: NCADHERIN (CDHN) detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**) and Activating (**d**). Although the localization of CDHN was not as precise as that of CLDN11, it clearly appeared in the peripheral area of seminiferous tubules occupied by Sertoli cells in all the stages, except the inactive testis, where the expression was more diffused and extended to the inner regions. Scale bar = 20 μm

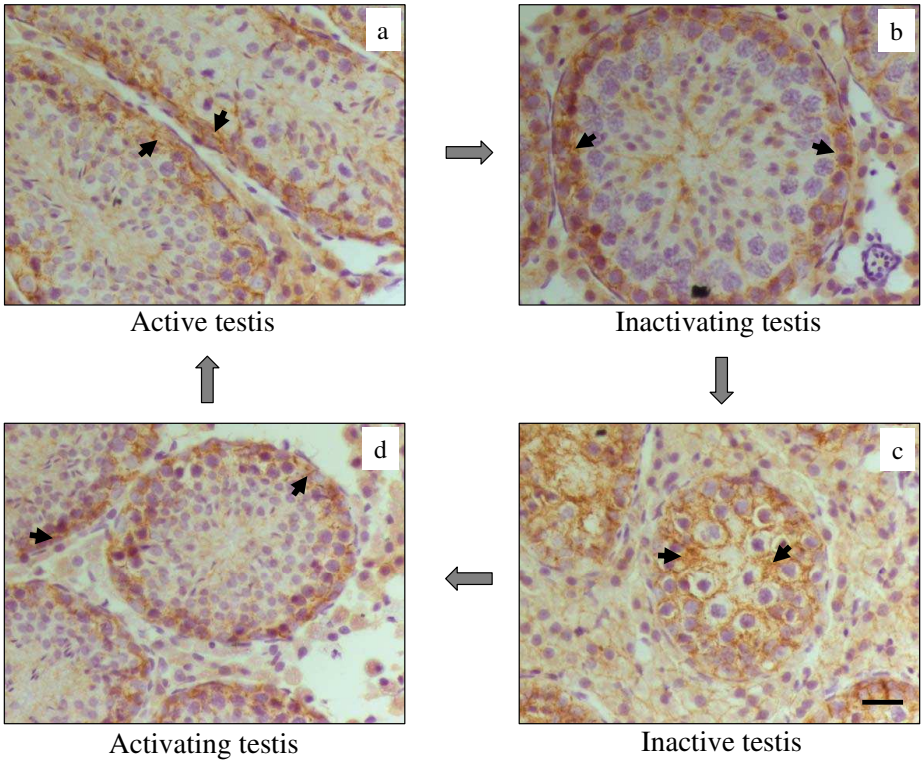


Figure 6.54: β-CATENIN (CTNNB1) detection in transverse sections of testes from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**)and Activating (**d**). Like CDHN, this protein appeared in the peripheral area of the seminiferous tubule occupied by the Sertoli cells in all the stages except the inactive testis where the expression was more diffused and extended to the inner regions. Scale bar = 20 μm

6.5.4 ZONA OCCLUDIN2 (ZO2)

The localization of ZO2 in the mole testes was not feasible because in this species this protein was not immunoreactive to the commercially available anti-ZO 2 antibodies. However, we were able to study its expression level by quantitative real time PCR analysis. For that, we amplified a 181 bp fragment of the mole ZO2 gene (Fig.6.55 and Fig.6.56). The quantitative analysis (Fig.6.57) showed that this gene was highly expressed in active testes, but it gradually downregulated during the inactivating stage and reached a minimum expression level in the inactive gonad. Expression was still very low during the activating stage which meant that a very strong upregulation takes place just before the active stage.

```
T. occidentalis   AGCTGGGCC.CTGGCTGGCCGTGAGGATCG   29
H. sapiens        AGCTGGGCaACTGGCTGGCtGTGAGGATtG   30
M. musculus       AGCTGGGCCACTGGCTGGCCGTGAGGATCG   30
consensus         ********* *********** ********* *

T. occidentalis   GaAACGAGTTGGAaAAAGGCTTAATCCCCA   59
H. sapiens        GGAACGAGTTGGAGAAAGGCTTAATCCCCA   60
M. musculus       GGAAtGAGcTGGAGAAAGGCTTgATCCCtA   60
consensus         *-**  *** ***-********-******  *

T. occidentalis   ACAAAAGCAGAGCTGAGCAAATGGCCAGTG   89
H. sapiens        ACAAgAGCAGAGCTGAaCAAATGGCCAGTG   90
M. musculus       ACAAAAGCAGAGCcGAGCAAATGGCCAGTG   90
consensus         ****-********** **-************

T. occidentalis   TTCAGAATGCtCAGCGgGACA.tGCTGGGG  118
H. sapiens        TTCAaAATGCCCAGaGAGACAACGCTGGGG  120
M. musculus       TcCAGAATGCCCAGCGAGAgAACGCcGGGG  120
consensus         * **-******* *** *-**-*-* ** *****
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T. occidentalis   ACAGGGCAGATTTCTGGAGA.TGCGT   143
H. sapiens        ACcGGGCAGATTTCTGGAGAATGCGT   146
M. musculus       ACAGaGCAGAcTTCTGGcGgATGCGT   146
consensus         ** * *-****** ****** *-*****
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Figure 6.55: Comparison of the nucleotide sequences of *ZONA OCCLUDIN 2* (*ZO2*) fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 90% homology with that of *Homo sapiens* and 87% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

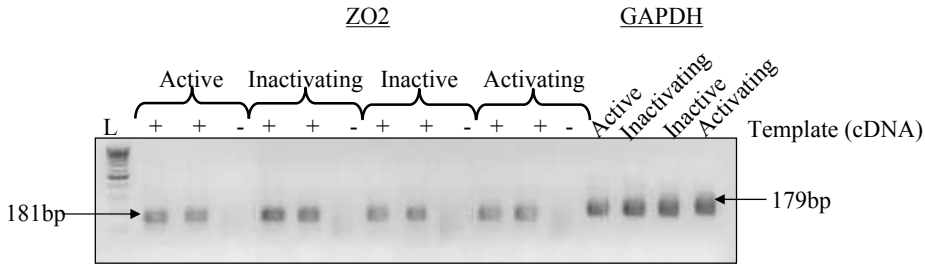


Figure 6.56: *ZO2* amplification in *T. occidentalis*. Total RNA was isolated from testes of *T. occidentalis* at various stages of their annual breeding cycle and *ZO2* expression levels were checked using quantitative real time PCR. Amplification of a 181 bp was observed for *ZO2* at all the stages analyzed. Expression of *GAPDH* (179 bp) from each stage (shown on the right) was used as reference for quantification. (+) reactions performed with cDNA.(-) reactions performed without cDNA. L:100 bp ladder

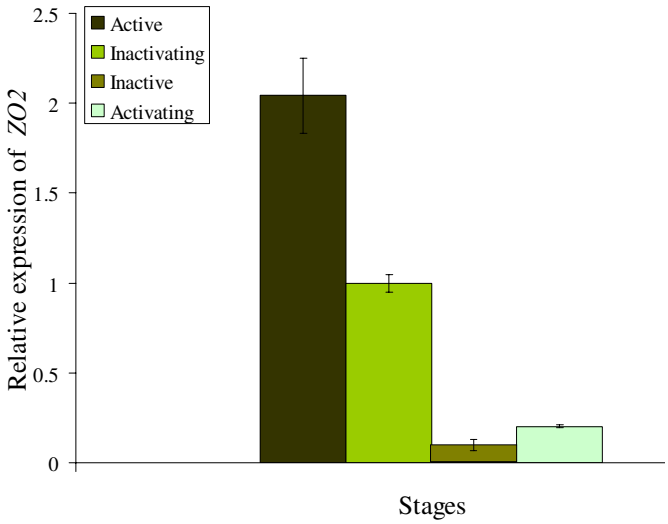


Figure 6.57: Expression levels of *ZO2* in the testis from *T. occidentalis*, throughout the seasonal breeding cycle. Expression decreased gradually from active (where it was maximum) to the inactive stage (where it was minimum). Then it again reactivated in the activating testes. Real time PCR reactions were performed, using *GAPDH* as a reference gene for quantification.

6.6 Integrity of the *lamina propria* of the seminiferous tubules throughout the seasonal breeding cycle of *Talpa occidentalis*

There were two reasons that prompted us to investigate whether the *lamina propria* of the seminiferous tubules maintain their integrity in the mole testes throughout its seasonal breeding cycle: 1) in the above results we have shown that the BTB becomes disorganised during the inactive stage, so that it could be possible that something similar could happen to the *lamina propria*; 2) previous studies carried out in our lab demonstrated that the *lamina propria* of the testis cord-like structures present in the testicular portion of female mole ovotestis, undergo some degree of disorganization during the estrous (active) stage, which raises the hypothesis that a similar phenomenon might occur in the male.

To study the integrity of the *lamina propria*, we performed immunostaining using antibodies specific for LAMININ, a major component of the basement membrane, and α SMOOTH MUSCLE ACTIN, a marker for the peritubular myoid cells (Fig.6.58 and Fig.6.59). Our results clearly show that presence of both markers around the seminiferous tubules remains unaltered throughout the seasonal breeding cycle, indicating that the *lamina propria* conserves its integrity in all the stages of the cycle.

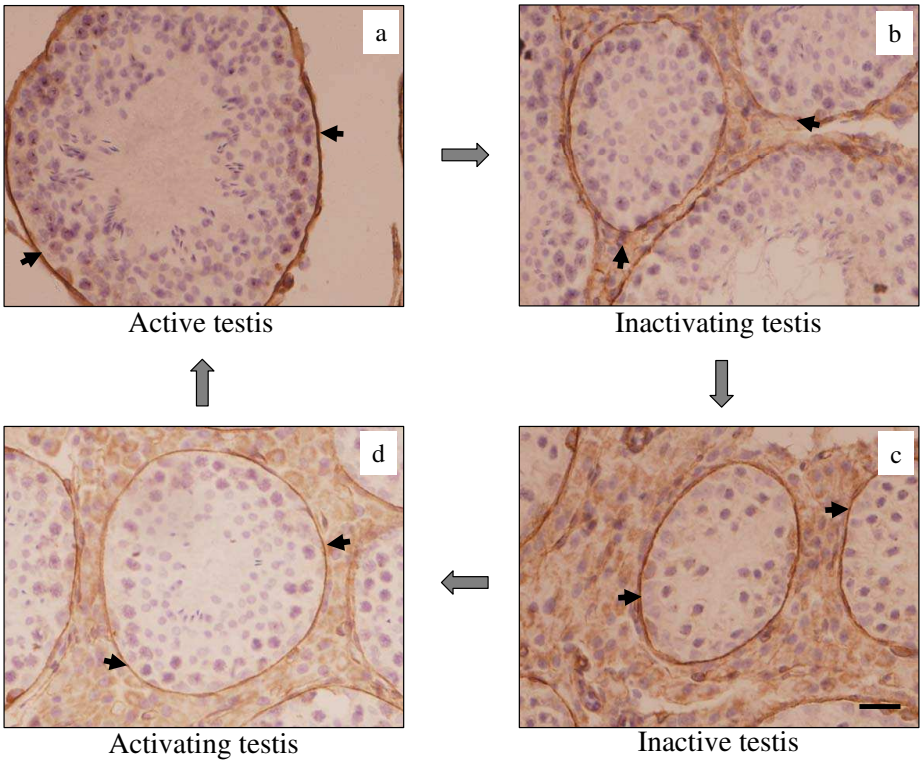


Figure 6.58: LAMININ detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**)and Activating (**d**). The basement membrane of the seminiferous tubules maintained the integrity throughout the seasonal breeding cycle in *T. occidentalis*. Laminin was detected in all stages therby, indicating that the emphlamina propria remains intact. Scale bar = 20 μm

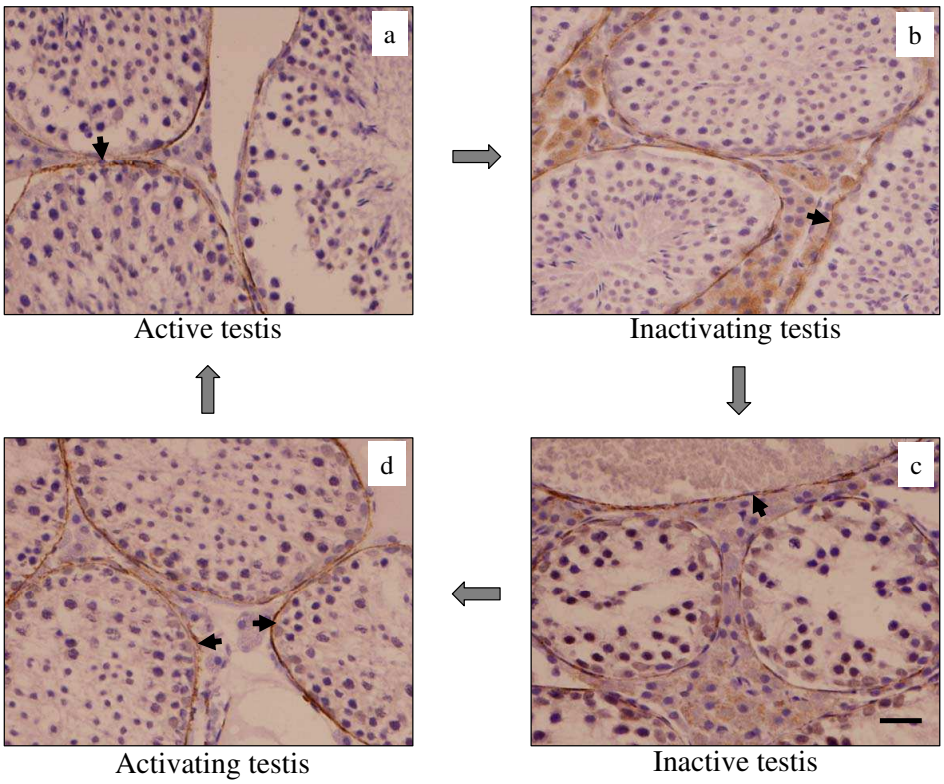


Figure 6.59: α SMOOTH MUSCLE ACTIN (αSMA) detection in transverse sections of testis from four representative stages during seasonal breeding cycle of *T. occidentalis*: Active (**a**), Inactivating (**b**), Inactive (**c**)and Activating (**d**). Peritubular myoid cells maintain the integrity of the tubular envelope throughout the seasonal cycle as they completely surround the tubule in all the stage. Scale bar = 20 μm

6.7 Preliminary data for future studies

As indicated above in the ¨Aims and Objectives¨ section, a general objective of our laboratory is to understand how seasonal reproduction in mammals is regulated from the genetic, hormonal and environmental points of view, using *T. occidentalis* as an animal model. Clearly, this is a very ambitious project that extends largely beyond the scope a single doctoral thesis. Consequently in this work we have focussed on several aspects of seasonal reproduction in male moles, but research is being continued in several others, some of which are already under way. As a consequence, we have some interesting preliminary results, which may increase our understanding of the genetic control of the seasonal breeding cycle of *T. occidentalis*. In many cases the aim is to investigate the expression of particular genes throughout the seasonal breeding cycle of this species for which these genes must be identified and cloned. In this context, we have identified and amplified fragments of genes like *LUTEINIZING HORMONE-β subunit* (*LHβ*), *ANDROGEN BINDING PROTEIN*(*ABP*), *FOLLICLE STMULATING HORMONE RECEPTOR*, *CDHN*, *CTNNB1*, *JUNCTIONAL ADHESION MOLECULE-2* (*JAM2*).

We can basically consider our preliminary results in two broad categories, 1) genes which encodes for gonadotropins and their receptors, which play important roles during reproduction in both seasonal and non-seasonal breeding species, and 2) the cloning and sequencing of tight junction protein genes, the localizations of which we have already studied, here.

1. Seasonal reproduction is under the control of GnRH which acts through the gonadotropins- LH and FSH. The alpha subunit of these two hormones is common for both of them whereas they have unique beta subunits. LH is one of the gonadotropins controlling the production of testosterone. Since all gonadotropins are protein hormones, they are species-specific and hence the commercial antibodies could not be used to study their levels in species, like the mole, which are not standard animal models. From our results, it is clear that we have substantial knowledge about the levels of testosterone in serum as well as in testis. We also have data about the localization and expression levels of AR and, P450scc and SF1 - two of the many important genes involved in steroidogenesis. It would be interesting to study the expression level of LHβ in the testis and also could be used for measuring the serum LHß levels by using RIA. This study will be soon possible as we have already amplified and sequenced a fragment of the mole LHβ (Fig.6.60).

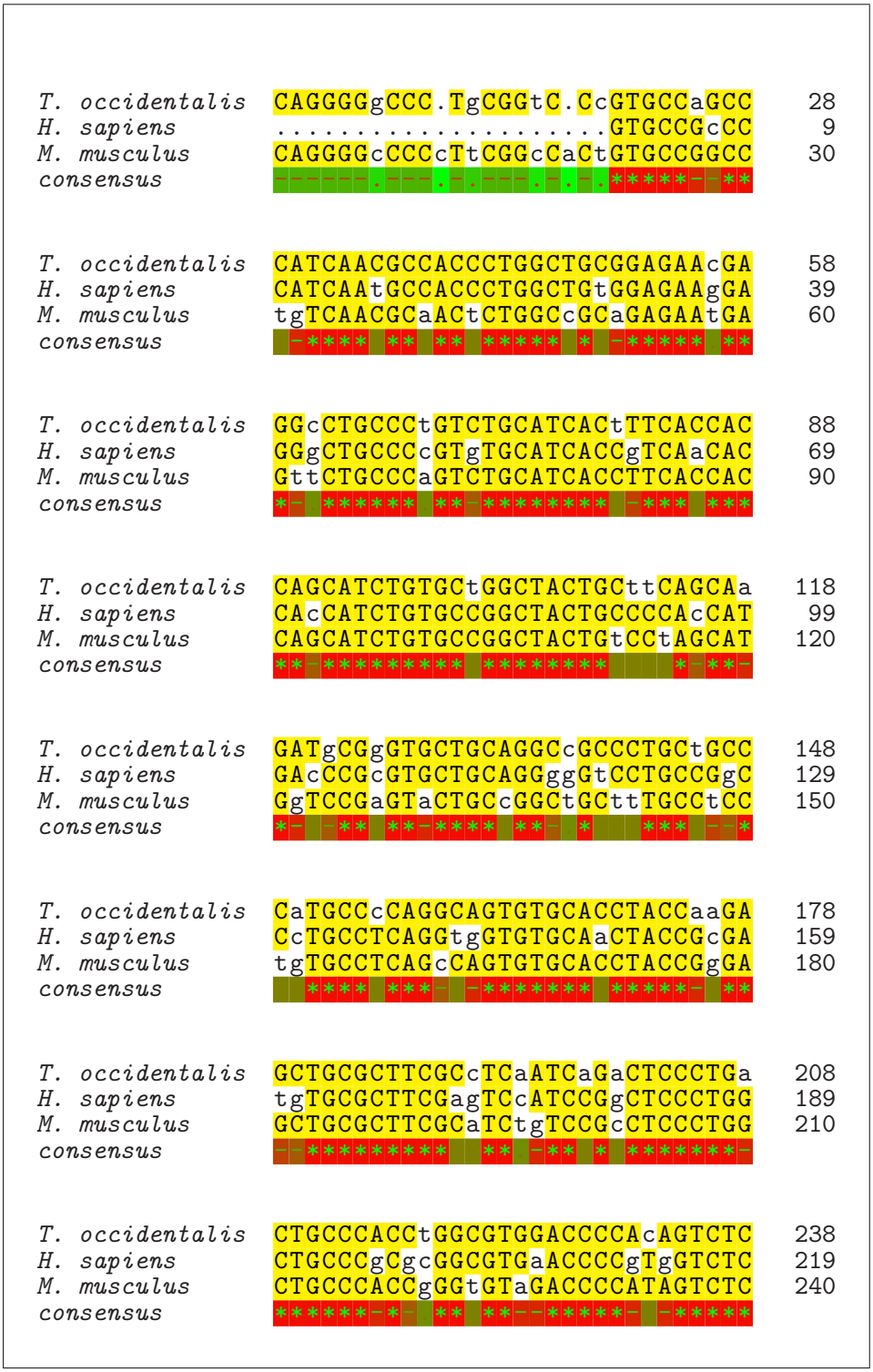
   The other gonadotropin, FSH, is also under study, but despite of our

repeated efforts we were not yet able to amplify a fragment of the mole FSHβ gene, which could be used for quantitative real time PCR analysis. Nevertheless, we could amplify a 178 bp fragment of FSHR gene, the receptor of FSH (Fig.6.61).

ANDROGEN BINDING PROTEIN (ABP), is produced specifically by Sertoli cells in the testes and permits the accumulation of androgens in the luminal fluid of the seminiferous tubule. Since its regulation is under the influence of FSH on Sertoli cells, it would be of interest to study how the levels of this protein would vary during the seasonal breeding cycle of *T. occidentalis*. We have already amplified and sequenced a 183 bp fragment of this gene in the mole which will be used in future quantification of the expression of this gene. (Fig.6.62)

2. The complex CDHN-CTNNB1 constitutes one of the functional units of tight junctions and their expression patterns were constant in the testes of male moles throughout their seasonal breeding cycle. Hence, it will be interesting to study the expression level of these two genes. Accordingly, we have obtained the sequences of fragments of these genes from the mole to be used in these studies (Fig.6.64 and Fig.6.63).

   It would be also interesting to study the localization of the JAM2 protein which is also a very important tight junction component. Preliminary analysis carried out with a commercial antibody specific for the human protein did not yield results of the desired quality. So, further experiments will be performed, either with this one or with other antibodies, in order to obtain proper results. This includes the possibility of preparing our own mole-specific antibody. For expression quantification, or for eventual in situ hybridization, it is also necessary to clone and sequence a fragment of this gene in the mole, a work which is already done (Fig.6.65).


T. occidentalis CAGGGGgCCC.TgCGGtC.CcGTGCCaGCC 28
H. sapiens .....GTGCCGcCC 9
M. musculus CAGGGGcCCCcTtCGGcCaCtGTGCCGGCC 30
consensus
T. occidentalis CATCAACGCCACCCTGGCTGCGGAGAAcGA 58
H. sapiens CATCAAtGCCACCCTGGCTGtGGAGAAgGA 39
M. musculus tgTCAACGCaACtCTGGCcGCaGAGAAtGA 60
consensus
T. occidentalis GGcCTGCCCtGTCTGCATCACtTTCACCAC 88
H. sapiens GGgCTGCCCcGTgTGCATCACCgTCAaCAC 69
M. musculus GttCTGCCCaGTCTGCATCACCTTCACCAC 90
consensus
T. occidentalis CAGCATCTGTGCtGGCTACTGCttCAGCAa 118
H. sapiens CAcCATCTGTGCCGGCTACTGCCCCAcCAT 99
M. musculus CAGCATCTGTGCCGGCTACTGtCCtAGCAT 120
consensus
T. occidentalis GATgCGgGTGCTGCAGGCcGCCCTGCtGCC 148
H. sapiens GAcCCGcGTGCTGCAGGggGtCCTGCCGgC 129
M. musculus GgTCCGaGTaCTGCcGGCtGCttTGCCtCC 150
consensus
T. occidentalis CaTGCCcCAGGCAGTGTGCACCTACCaaGA 178
H. sapiens CcTGCCTCAGGtgGTGTGCAaCTACCGcGA 159
M. musculus tgTGCCTCAGcCAGTGTGCACCTACCGgGA 180
consensus
T. occidentalis GCTGCGCTTCGCcTCaATCaGaCTCCCTGa 208
H. sapiens tgTGCGCTTCGagTCcATCCGgCTCCCTGG 189
M. musculus GCTGCGCTTCGCaTCtgTCCGcCTCCCTGG 210
consensus
T. occidentalis CTGCCCACCtGGCGTGGACCCCAcAGTCTC 238
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consensus

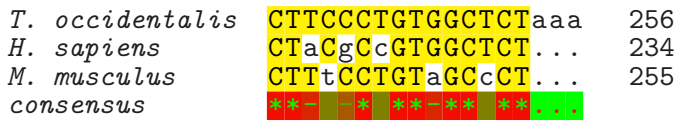

Figure 6.60: Comparison of the nucleotide sequences of *LHβ* fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 78% homology with that of *Homo sapiens* and 80% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the the species, while differences are represented with diffent colour intensities between red and green.

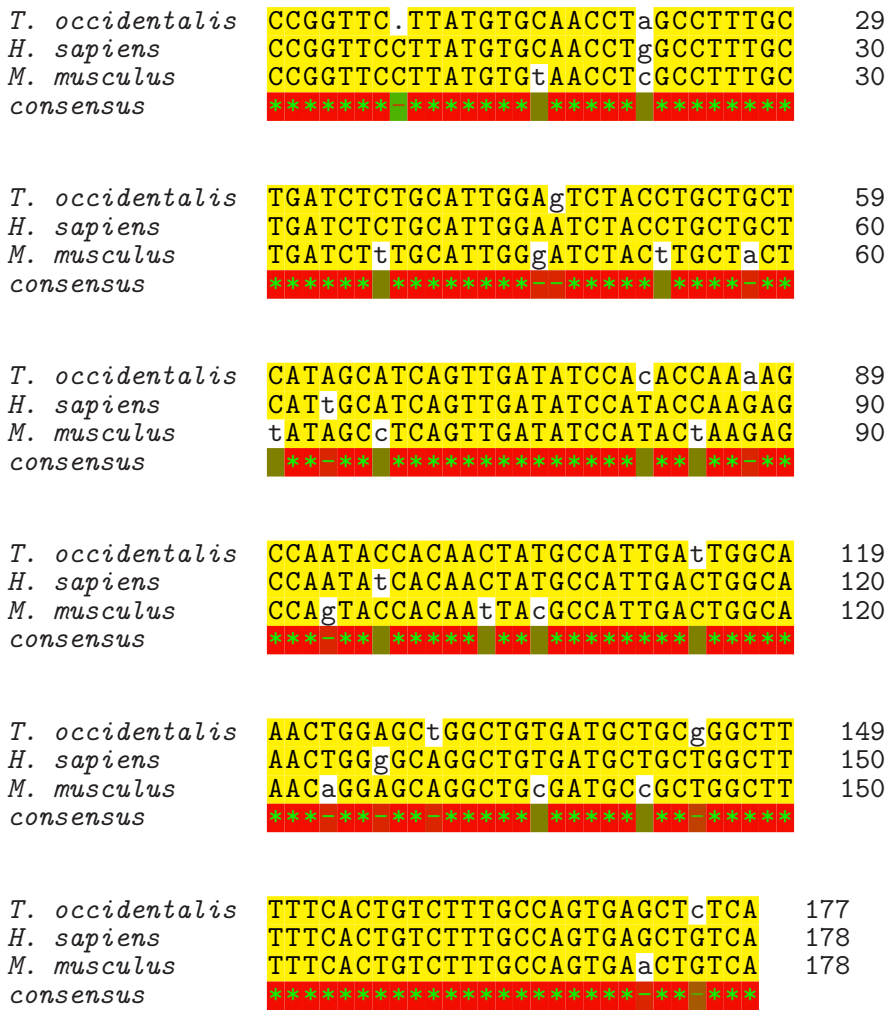

Figure 6.61: Comparison of the nucleotide sequences of *FSHr* fragments from *T. occidentalis*, *Homo sapiens*and *Mus musculus*. Mole fragment showed 89% homology with that of *Homo sapiens*and 84% homology with that of *Mus musculus*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

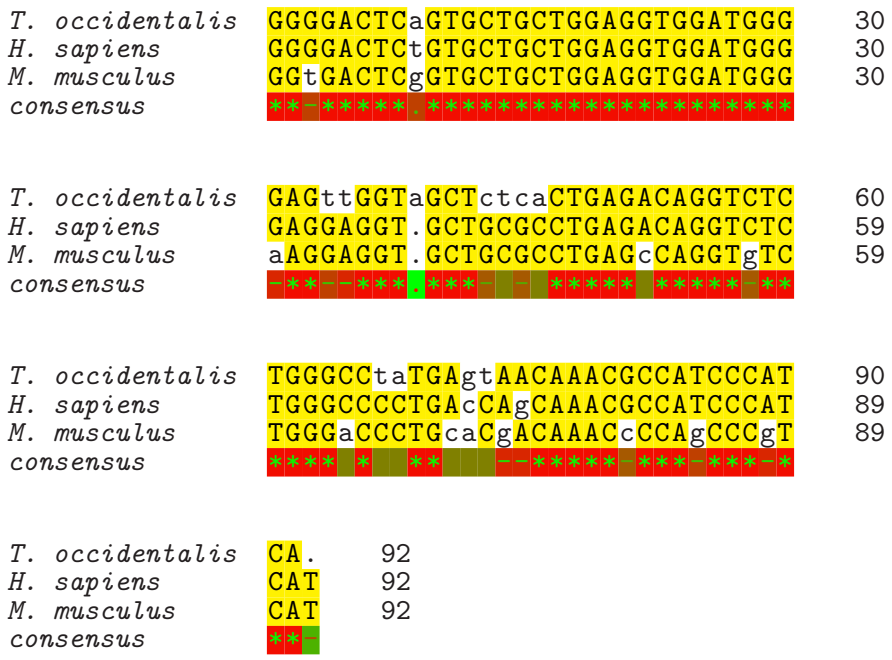

Figure 6.62: Comparison of the nucleotide sequences of *Androgen binding protein*(*ABP*) fragments from *T. occidentalis*, *Homo sapiens*, *Mus musculus*. Mole fragment showed 86% homology with that of *Homo sapiens* and 76% homology with that of *Mus musculus ABP*. Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

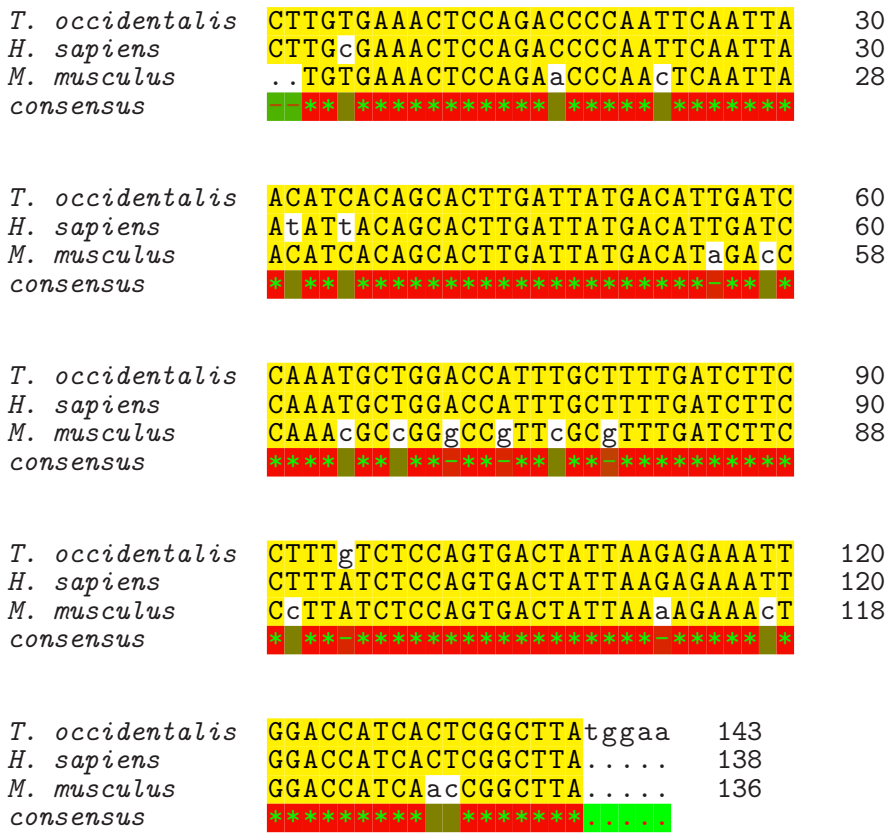

Figure 6.63: Comparison of the nucleotide sequences of *NCADHERIN*(*CDHN*) fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 97% homology with that of *Homo sapiens CDHN* and 88% homology with that of *Mus musculus* Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

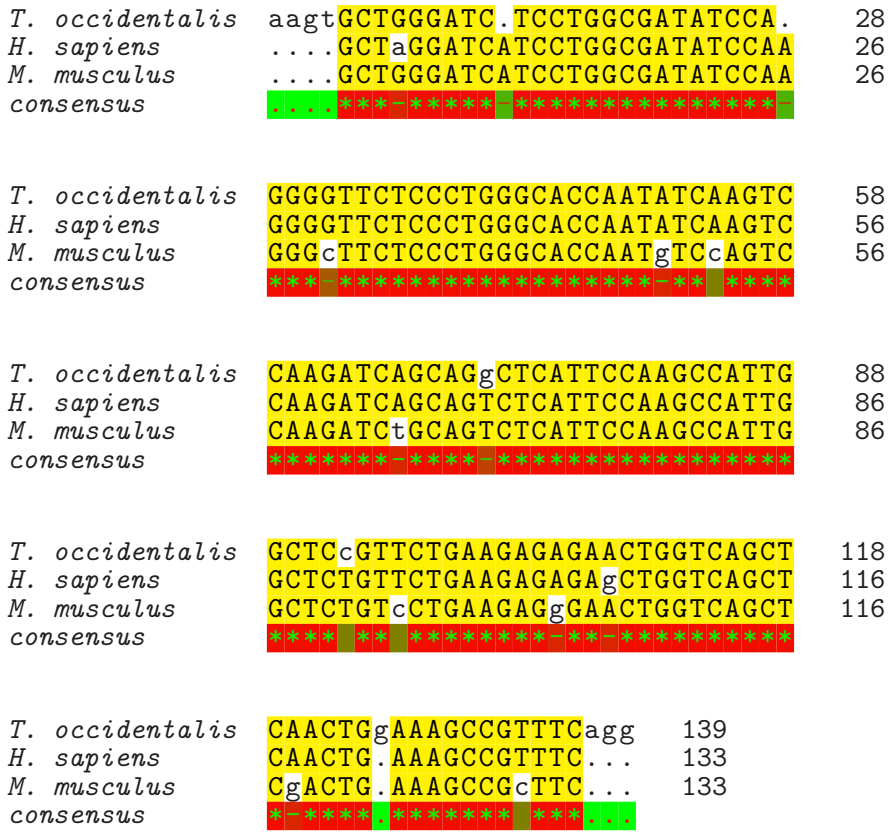

Figure 6.64: Comparison of the nucleotide sequences of *β-CATENIN*(*CTNNB1*) fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 95% homology with that of *Homo sapiens CTNNB1* and 90% homology with that of *Mus musculus* . Red blocks with * indicate the presence of coincidence with the fragments from the three species, while differences are represented with different colour intensities between red and green.

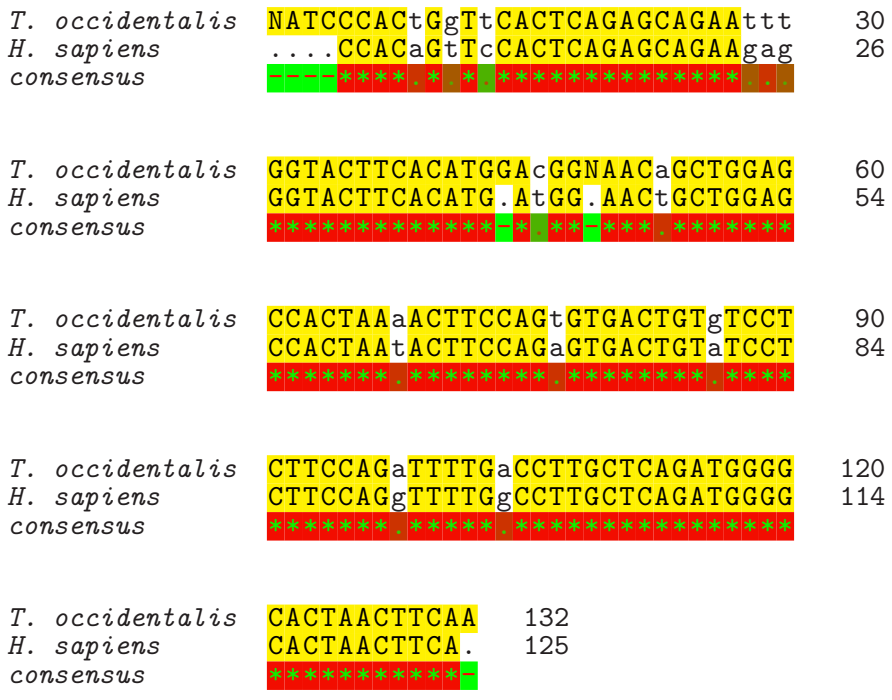

Figure 6.65: Comparison of the nucleotide sequences of *JAM2* fragments from *T. occidentalis*, *Homo sapiens* and *Mus musculus*. Mole fragment showed 84% homology with that of *Homo sapiens JAM2*. Red blocks with * indicate the presence of coincidence with the fragments from the the species, while differences are represented with diffent colour intensities between red and green.

Discussion

There are two major mechanisms that may be envisaged by which testes can be cyclically inactivated in seasonal breeding mammals: 1) by acting at the hormonal level, reducing for example the production of gonadotropic hormones, and 2) by acting at the level of meiosis control, possibly inhibiting the meiosis onset. To elucidate these questions is one of the major aims of the present work.

Here we have analyzed, both qualitatively and quantitatively, the expression of four genes, SOX9, SF1, DMRT1 and AMH, whose expression was already analyzed in our laboratory during gonad development (Carmona *et al.*, 2008, in press). The fact that moles are seasonal breeding species where gonads undergo cyclic periods of activity and inactivity, made it interesting to investigate whether the expression of these genes also follow a seasonal pattern, which would also be an indication of having some role in testis function. In the following sections, we will discuss the expression pattern of these genes and their possible causes. The control of meiosis onset, the role of apoptosis and cell proliferation, the hormonal control and the involvement of testis cell junctions will also be discussed in relation to the seasonal variation in the testes of adult male moles of the species *T. occidentalis*.

7.1 *The season-dependent expression of SOX9 in the adult mole testis suggests a role in gonad function*

It is known since 1996 that SOX9 expression is maintained in the somatic cells of the adult testis (Kent *et al.*, 1996). More recently, it has also been reported that SOX9 is transiently expressed during follicular development

in the adult ovary (Notarnicola *et al.*, 2006). However, to our knowledge, only one paper has been reported to date addressing the functional implications of SOX9 expression in the adult testis (Frojdman *et al.*, 2000). In the rat, SOX9 expression is weak after birth, a situation which is maintained until day 15, when the presence of the SOX9 protein significantly increases into the testis cords. In adult Sertoli cells SOX9 expressed in a spermatogenic stage-specific manner. Based on theses data, Fröjdman *et al.* (2000) suggested that this age and stage-dependent expression of SOX9 suggests a pivotal role in germ cell differentiation.

The expression of SOX9 had not been previously studied in the adult testis of any seasonal breeding mammal. Our study is thus the first attempt to investigate the possible involvement of this gene in seasonal breeding. Concerning the expression of SOX9 in the active testis of the mole, our study is equivalent to that carried out in adult rats by Notarnicola *et al.* (2006), and our results are also similar to those of these authors: Sertoli cells express SOX9 in a stage-dependent manner. However, we report for the first time the expression status of SOX9 in inactive testes from a seasonal breding mammal, showing that, surprisingly, expression levels here are higher than those observed in active testes. In the mouse developing testis, Sox9 is upregulated by a synergistic action of SRY and SF1 proteins on a Sox9-specific enhancer (Sekido and Lovell-Badge, 2008), and FGF9 signalling is also needed to establish its expression (Colvin *et al.*, 2001a; Schmahl *et al.*, 2004). Our current data do not permit to establish the molecular mechanism controlling SOX9 expression in the mole, but the extremely low expression levels of SF1 observed in inactive moles (see bellow), does not suggests that this gene may participate in the upregulation of SOX9 during this stage of the seasonal cycle.

A higher expression of SOX9 in the inactive testis suggests an inhibitory role for this gene on particular aspect of the spermatogenic function. Alternatively, this fact could reflect that SOX9 expression may be negatively controlled, either directly or indirectly, by a factor produced by some spermatogenic cell type, which disappears once the gonad becomes inactive. These two hypotheses are consistent with the spermatogenic stage-dependent expression pattern of this gene in the active testis. The seasonal-dependent expression of SOX9 is also consistent with a possible role for this gene in the control of seasonal breeding.

7.2 *Continued presence of primary spermatocytes and intratesticular testosterone may explain the lack of AMH expression in the adult mole testes*

As indicated above, *AMH* is kept repressed during the whole adult life of the male mole as known for humans, mice and rats which are not seasonal breeders (Al Attar *et al.*, 1997). This expression pattern in the mole is not surprising regarding the period of testis activity, when mole testes are functionally equivalent to those of mice and human. However, some additional explanation is necessary to understand why *AMH* remain repressed during the phase of testis inactivity, especially when both human with Tfm syndrome (testicular feminization due to androgen insensitivity caused by mutation in the AR gene) and AR mutant mice, maintained high levels of serum AMH (Josso, 1995; Rey *et al.*, 1994, 1993, 1996). It is known that SOX9, WT1, SF1 and GATA4 regulate the expression of the *AMH* gene in the embryonic Sertoli cells during testis development (Shen *et al.*, 1994b; Nachtigal *et al.*, 1998; de Santa Barbara *et al.*, 1998; Vigier *et al.*, 1984), and that FSH also stimulates AMH production by increasing Sertoli cell proliferation and *AMH* gene transcription in postnatal testes (Lukas-Croisier *et al.*, 2003).

As discussed below, inactive testes in adult moles are not functionally equivalent to a prepubertal testis as far as the expression of *AMH* is considered. We do not know currently the expression pattern of all the factors involved in the activation of the mole AMH gene as we were not able to study the expression of WT1, GATA4 and FSH due to the lack of antibodies immunoreactive to the mole proteins. However, we do know the status in the mole testis of two factors known to synergistically inhibit *AMH* expression in mice, i.e. primary spermatocytes and intratesticular testosterone (Al Attar *et al.*, 1997). In the active testis of the mole, there is no doubt that these two factors may be exerting this inhibitory action. We will also clearly show in this work (see below), that in the inactive testes of adult moles meiotic onset is not interrupted, so that primary spermatocytes are always present. Concerning the role of testosterone during this stage, our results indicate that although testosterone levels decrease significantly during the testis inactivation process, a basal level of testosterone is maintained, mainly in the intratesticular environment (20 ng/ml), that could retain some functional activity. This is particularly relevant if we consider that the expression of AR (the testosterone receptor) in the inactive testis, only decreases to a half of its level in the active stage. Hence, it is possible that testosterone-mediated inhibition of the *AMH* gene can be maintained throughout the

seasonal breeding cycle of *T. occidentalis*.

According to the above insights, we conclude that the continued presence of primary spermatocytes and/or the effect of intratesticular testosterone, may be responsible for the permanent inhibition of the AMH gene in the testis of *Talpa occidentalis*.

7.3 *Localization and expression levels of the mole DMRT1 gene suggest a role in the control of both spermatogenesis and seasonal breeding*

DMRT1, a gene involved in the sex determination process of many zoological groups, has recently been reported to be expressed in the adult testis of the eutherian mammals (Pask *et al.*, 2003), where DMRT1 protein is localized in Sertoli, Leydig and peritubular myoid cells, as well as in the sperm. In the mole, we found two types of seminiferous tubule with distinct staining patterns, which were identified as low density (LDD) and high density (HDD) DMRT1- positive seminiferous tubules. Concerning the identity of the positive cells, the interphase morphology of their nuclei indicate that these cells are not spermatocytes. However, since both Sertoli cells and spermatogonia occupy basal positions in the semniniferous tubules, a morphological identification of these cells was not possible. Similarily, it was not possible to study the colocalization of SOX9 (a marker for Sertoli cells) and DMRT1, due to the fact that the antibodies which we found useful to be used in the mole for these two proteins had been raised in the same species (rabbit).

In the adult testis of chicken, DMRT1 expression was observed in the Sertoli cells in all regions of seminiferous tubules whereas spermatogonia showed a spermatogenic stage-specific expression pattern for this gene (Raymond *et al.*, 2000b). According to this data from the chicken, we hypothesized that the DMRT1 positive cells in LDD tubules were Sertoli cells and that the additional positive cells observed in the HDD ones were spematogonia. To test this hypothesis, we compared the number of SOX9 positive cells in the seminferous tubule of active mole testes (18.7±2.26), with that of DMRT1 positive cells in LDD tubules (20.1±3.31). We found that these numbers were not significantly different (P=0.567; Fig.7.1), suggesting that the DMRT1 positive cells in LDD tubules were in fact Sertoli cells. On the other hand, the number of DMRT1 positive cells in the HDD tubules (40.7±5.07), was significantly different from that in the LDD tubules (P=0.0001; Fig.7.2). These data strongly suggest that DMRT1 is expressed in the premeiotic spermatogonia of the mole in a spermatogenic stage-specific manner, suggesting a role for this gene in the functions of these

cells. Zarkower and colleagues reported almost identical results in the adult testis of both chicken (Raymond *et al.*, 1999c) and mouse (Raymond *et al.*, 2000b), and suggested that DMRT1 could be involved in the regulation of meiosis onset or in the control of the mitotic cell cycle, a hypothesis which could also be valid for the case of moles. These data clearly suggest that DMRT1 is probably involved in the control of testis function in vertebrates and that this role is well conserved at least in mammals and birds.

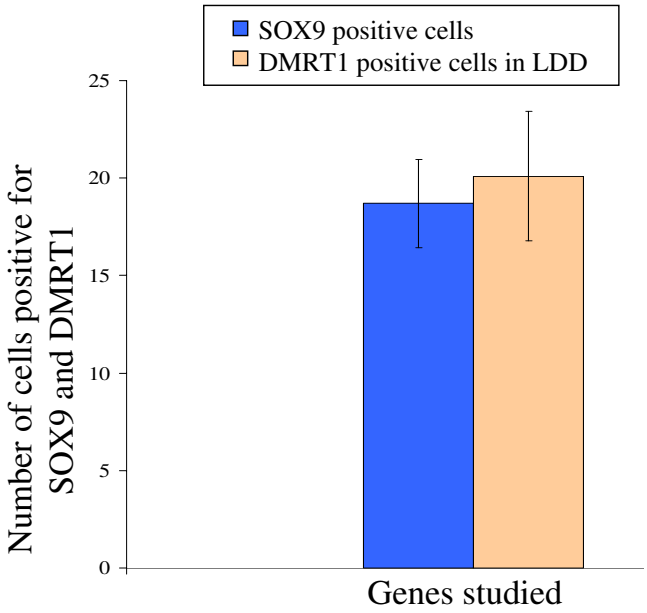

Figure 7.1: Comparison of the number of SOX9 positive cells per seminiferous tubule in the active testis and DMRT1 immunorecative cells in the LDD seminiferous tubules of the active testis

We have also shown that DMRT1 is expressed in the peripheral cells of the seminferous tubules of inactive mole testes. As the number of DMRT1 positive cells in these tubules is not significantly different from that of SOX9 positive cells in the same tubules (P=0.1408; Fig.7.3), we deduced that these are Sertoli cells. This means that spermatogonia do not express DMRT1 during the period of testis inactivity in the mole seasonal breeding cycle. This is consistent with our real time expression studies that show higher expression levels in the active than in the inactive mole testis (see Fig.6.8). We report here for the first time the expression profile of this gene in seasonal breeder, showing a clear dependence on the seasonal stage. This fact prompt us to hypothesize that DMRT1 could be involved in the control of

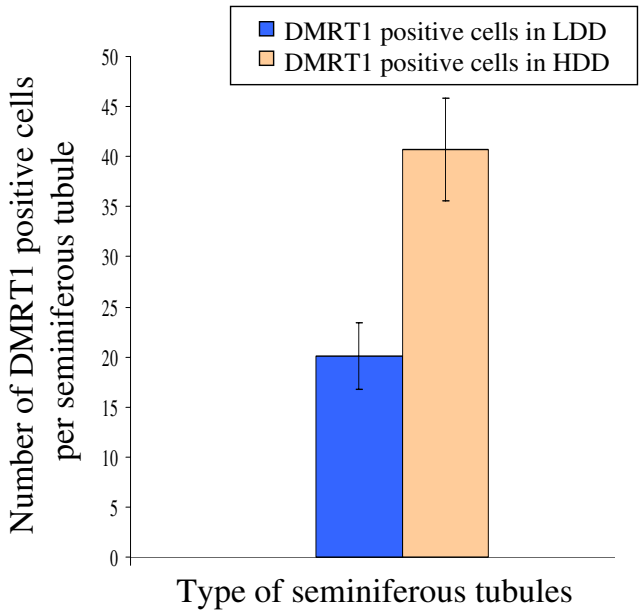

Figure 7.2: Comparison of the number of DMRT1 immunorecative cells in the LDD and HDD seminiferous tubules of the active testis

seasonal breeding in mammals and perhaps also in birds. It has been reported that DMRT1 expression is regulated by FSH and phorbol esters in postnatal Sertoli cells in vitro (Chen and Heckert, 2001). Hence, this opens the possibility that seasonal breeding may be controlled by gonadotropins by regulating DMRT1 expression in spermatogonia either, directly or indirectly.

7.4 *During the inactive stage of seasonal breeding, mole germ cells do not regress to pre-pubertal stages of differentiation*

As stated above, the regulation of cyclic sperm production in seasonal breeding species could be exerted at the level of meiosis onset. According to this idea, testis inactivation could be produced by inhibiting the entry of new spermatogonia into meiosis at the end of each breeding season, so that testes would become azoospermic, once the last round of spermatogenesis is completed. Then it could be possible that the remaining spermatogonia would regress to pre-pubertal stages of differentiation. To check this hypothesis we performed immunoflourescence analyses using an antibody specific

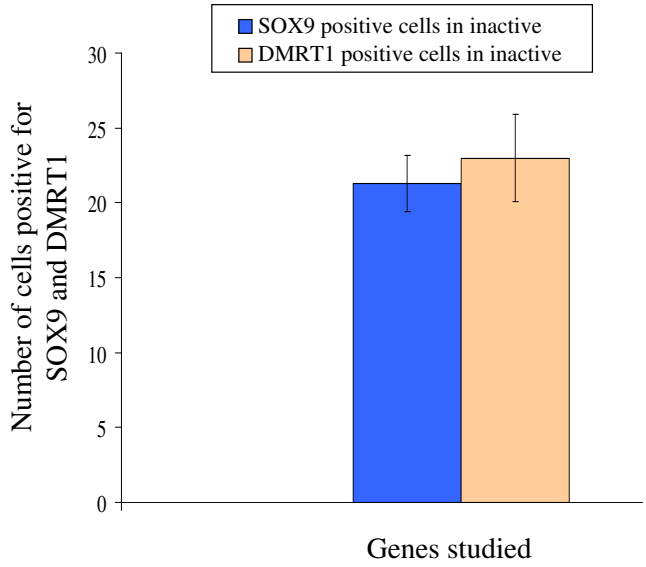

Figure 7.3: Comparison of the number of SOX9 and DMRT1 immunoreactive cells in the inactive testis of the mole

for OCT4, a typical pluripotent germ cell marker (Fig.6.30). However, we found that germ cells in adult mole testes lack this protein at any stage of the seasonal breeding cycle. This fact clearly indicates that after puberty germ cells do not undergo any de-differentiation.

7.5 *Meiosis onset is never interrupted during the seasonal breeding cycle of the mole*

To investigate the status of meiosis regulation in mole testes throughout the seasonal breeding cycle, several early (DMC1, γH2AX and SYCP3) and late meiotic markers (CRM1) were used in immunohistochemical analyses. We found that zygotene and pachytene spermatocytes are continuously observed in the inactive testis, showing that meiosis onset is not interrupted during this period. Contrarily, round spermatids were also present in some stages of the active and activating testes, but never in inactive gonads, showing that late spermatogenic as well as spermiogenic cells disappear during the inactivation of the testes and remain completely absent during the entire stage of testis inactivity. Consequently, a massive depletion of these cell types take place during testis inactivation. Then, once the testis is inactivated, new cells entering into meiosis are probably lost by apoptosis shortly

after or around pachytene stage (see below).

In order to study the germ cell dynamics throughout the seasonal breeding cycle of the mole, we quantified the number of pre-pachytene spermatocytes using the perimetric length cell density parameters described above. This permited us to compare the mole with other seasonal breeding mammals. In the roe deer (*Capreolus capreolus*), it has been established that regressed testes lack meiotic cells indicating that a complete cesation of meiosis onset takes place (Hartung and Schoppmeyer, 1986; Marchlewska-Koj and Kruczek, 1988; Blottner *et al.*, 1996; Schön *et al.*, 2004). In photoinhibited Djungarian (Siberian) hamsters (*Phodopus sungorus*), Meachem et al (2005) found that the number of spematocyes during the short day photoperiod (SD) were reduced to 10% of that observed in sexually active males (subjected to a long day photoperiod, LD). Similarly in photoinhibited (SD) golden (Syrian) hamsters (*Mesocricetus auratus*), Sinha Hikkim et al, (1988) reported 42% of preleptotene and 8% of pachytene spermatocytes with respect to LD animals. According to these references, moles are clearly different from roe deers as onset of meiosis is not compromised during the testis regression. In fact, in the mole there is only a small decrease in the density of pre-leptotene/leptotene cells when we compared active with inactive testis indicating that the rhythm of entry into meiosis is kept almost constant throughout the seasonal breeding cycle. Hamsters seem to be more similar to moles in the fact that meiosis onset is not completely stopped in SD males. However, since these studies provided the number of cells per tubule, and did not use the quantification method that we proposed here, quantitative comparisons between hamsters and moles cannot be performed for the moment.

7.6 *Apoptosis is only partially responsible for the massive depletion of germ cells occurring during inactivation of the mole testis*

Apoptosis is a hormonally controlled process, known to operate during normal testicular function, regulating the testicular cell population (Blanco-Rodríguez and Martínez-García, 1998; Sinha Hikim and Swerdloff, 1999; Print and Loveland, 2000). Its role has previously been investigated in mammalian species with both seasonal and non-seasonal reproduction (Blottner *et al.*, 2006).

Several authors have reported that spermatogonia and primary spermatocytes are the main cell types that undergo apoptosis, although apoptotic secondary spermatocytes and spermatids have also been described (Blanco-Rodríguez and Martínez-García, 1996b, 1998). Our studies during the sea-

sonal breeding cycle in the mole showed that apoptotic cells are always located in the peripheral region (mostly basal) of the seminiferous tubules, a fact which is consistent with the identification of these cells as either spermatogonia or primary spermatocytes, as indicated by these authors. On the other hand, we never found apoptotic cells located in more inner, non-basal positions of the tubule, indicating that both secondary spermatocytes and spermatids undergo apoptosis very rarely, or may be never, in the mole.

The role of apoptosis in testis function has been studied in several mammals. However, there was no consensus regarding the quantification method used to study the incidence of apoptosis. Blottner *et al.* (1996) used ELISA for evaluating apoptosis. Furuta *et al.* (1994); Morales *et al.* (2002) and Strbenc *et al.* (2003) quantified the number of apoptotic cells per unit of testis area or volume. This method implicated to include different numbers of tubules due to their different diameter in different reproductive stages. A more recent study (Blottner *et al.*, 2006) quantified the number of apoptotic cells per tubule cross section which, in the authors´ opinion, provides a better standardization method. Our study in the mole showed that the number of apoptotic cells per seminiferous tubule was significantly higher in the active than in the inactive testis (Fig.6.38; P=0.0001), as Blottner *et al.* (2006) found in the roe deer. However, like these authors, we think that this difference may correspond to the presence the different number of germ cells present in both samples. Accordingly, we calculated the percentage of apoptotic cells with respect to the total number of cells inside the same seminferous tubules, finding that the process of apoptosis is now higher in the inactive than in the active testis. Blottner *et al.* (2006) found a lack of inter-stage differences when applied a similar correction in their study of the roe deer. Nevertheless, in our opinion, this is neither a good method to quantify apoptosis, because apoptotic cells are not homogeneously distributed inside the seminiferous tubule. As indicated above they are always located in the periphery of the tubule, at least in the mole (Fig.6.37). So, apoptosis quantification would be better measured in terms of density of apoptosis cells in the peripheral area, instead of density of apoptotic cells in the whole area of the seminiferous tubule. According to this idea, we defined the perimetric length apoptotic cell density parameter, as the number of apoptotic cells per every 100 μm of perimetric length of the seminiferous tubule, which in our opinion is a more suitable method to quantify apoptotic cells in this type of tissue.

According to this quantification method, perimetric length apoptotic cell density was statistically not different in active and activating stages. However, apoptotic cells in inactive testes were 46.9% of those in active ones, which was a statistically significant difference (P=0.0001). Nevertheless, the number of apoptotic cells in active testes was 69.9% of that found in the

inactivating stage, where the maximum density of these cells was observed, which was also significantly different (P=0.0002) to each other. This implied that apoptotic cell density of the active testis increases by 43.9% when the transition to the inactivating stage takes place.

Before discussing about the role that apoptosis may play in the seasonal breeding cycle of the mole, we should identify which cell types are being affected by apoptosis in the mole testis. Since we have shown that the number of Sertoli cells do not vary significantly (Fig.6.5) throughout this cycle, our data clearly indicate that Sertoli cells are either not affected by apoptosis or affected in a similar magnitude at the different stages of the seasonal breeding cycle. This is in clear agreement with the data reported by Blottner and Schoen (2005) and Blottner *et al.* (2006) in the roe deer. If apoptosis does not affect differentially the Sertoli cells, then differences between stages in the density of apoptotic cells must necessarily be attributed to the germ cells. A valid criterion that may help in the identification of which germ cells are affected by apoptosis, is the location of apoptotic cells in the seminiferous tubule. We have seen (Fig.6.37) that apoptotic cells in the mole testis are always located in a basal position. Since this is the normal location of spermatogonia and primary spermatocytes, these two cell types are very probably the only germ cells that undergo apoptosis in these testes.

To investigate the role of that apoptosis may have on seasonal breeding, we have analyzed the dynamics of different germ cell types during the breeding cycle in *T. occidentalis*. To identify the different cell types present in the seminiferous tubule, we have used very precise methods based on the detection of specific genetic markers (DMC1 for early primary spermatocytes,γ H2AX and SYCP3 for later primary spermatocytes and CRM1 for round spermatids), unlike the previous studies (Hartung and Schoppmeyer, 1986; Sinha Hikim *et al.*, 1988; Marchlewska-Koj and Kruczek, 1988; Blottner *et al.*, 1996; Schön *et al.*, 2004; Meachem *et al.*, 2005) in which only morphological criteria were used. Like for apoptosis, we have calculated the perimetric length density of cells in early and late stages of the first meiotic prophase and compared them between active and inactive mole testes. Our results indicate that there is a small decrease in the density of DMC1 positive cells (leptotene) in inactive testes with respect to that in active ones (Fig.6.35; P=0.0434). On the other hand, a highly significant (Fig.6.36; P=0.0001) decrease was observed in the case of late primary spermatocytes (SYCP3 positive cells). Considering that we had found a significant increase in the density of apoptotic cells during the inactivating stage, our data clearly suggest that apoptosis has very little or no effect on early primary spermatocytes, whereas it probably has a strong influence on later primary spermatocytes (zygotene and pachytene). This view is also sup-

ported by the fact that both the density of apoptotic cells and that of zygotene/pachytene cells decrease to a half when we compare active with inactive mole testes (compare Fig.6.40 and Fig.6.36).

Our results also indicate clearly that apoptosis is not responsible for massive germ cell depletion taking place in the mole testis during the inactivating stage as no CASPASE3-positive cell was observed in inner regions of the seminiferous tubules in none of the reproductive stages. Hence, further explanation was needed for the loss all these cells, which represents 73.7% of the active testis weight. Taken together, these findings indicate that apoptosis is responsible for the depletion of just a small proportion of the germ cells that takes place during the seasonal testicular regression in the mole.

7.7 *A wave of cell proliferation probably restores the number of spematogonia lost during the inactivity period*

It is well known that spermatogonia proliferate by mitosis to maintain the pool of germ cells in the testis. This is necessary because spermatogonia are lost either by apoptosis or by entry into meiosis. In mammals with non-seasonal reproduction the rates of spermatogonia loss and proliferation must be balanced so that their number can be maintained constant (Kimura *et al.*, 2003; Blottner *et al.*, 2007). However, seasonal breeding mammals need spermatogonia during the breeding stage only, so that it is interesting to know whether their number is also maintained at a constant level throughout the year. In the mole we observed that proliferation increases gradually from active to inactive stages, but a proliferation peak takes place during the activation stage. This suggest that at the beginning of the active stage, the number of spermatogonia is most probably maximum, which is consistent with the fact that during this period the levels of spermatogonial proliferation are minimum (Fig.6.43). The fact that the rhythm of meiosis onset seems to be constant throughout the year is also consistent with this hypothesis. According to this idea, it is likely that the number of spermatogonia is maximum at the beginning of each breeding season, decreases gradually during the inactivation and the inactive stages and must be restored again before the new breeding season (activation stage). Future studies using spermatogonial-specific markers (when available) may permit us to test this hypothesis.

The role of cell proliferation has also been studied in other seasonal breeding mammals. In both the brown hare (Strbenc *et al.*, 2003) and the roe deer (Blottner *et al.*, 1995) a peak of cell proliferation was detected

in the months previous to the breeding season, thus coinciding with our results in the mole. Similarly, in the Syrian hamster, Morales *et al.* (2002) studied photoinhibited and non-photoinhibited animals and found that SD hamsters showed higher rates of cell proliferation than LD animals.

7.8 *Low levels of intratesticular testosterone may explain seasonal testis inactivation*

Although it is well established that testosterone (T) is required for spermatogenesis, the biological mechanism(s) that underlies the androgen dependency of spermatogenesis is poorly understood. Sertoli cells are considered to be the target cells for androgen action. Testosterone action is mediated by the androgen receptor (AR), which after binding to testosterone, translocates into the nucleus, and initiates androgen-dependent gene transcription (Blok *et al.*, 1992; Vornberger *et al.*, 1994; Bremner *et al.*, 1994; Zhu *et al.*, 2000). In rats, AR nuclear location is stage-specific, occurring more frequently during stages VII-VIII (Vornberger *et al.*, 1994; Bremner *et al.*, 1994; Zhu *et al.*, 2000), the so-called androgen-dependent stages of the spermatogenic cycle (Sharpe *et al.*, 1992; O'Donnell *et al.*, 1996).

In the present study, we have measured serum and intratesticular T levels and have analyzed the expression pattern of AR in moles at different stages of their seasonal breeding cycle. The relationships between germ cell dynamics, AR expression and the intratesticular levels (ITT) levels in mammals are not very well established yet. A number of studies on the effects of T levels on AR expression have been made, but different, and sometimes contradictory, conclusions were reported. Some of them established a clear relationship between AR expression and androgen levels (Bremner *et al.*, 1994; Tan *et al.*, 2005; Turner *et al.*, 2001). However, others have suggested that androgens may influence the levels of AR protein but not those of AR mRNA (Hill *et al.*, 2004). A possible explanation for this phenomenon is that androgens regulate in fact the intracellular localization of AR, thus resulting in a decrease of the intra-nuclear levels of this protein, instead of its transcription levels (Blok *et al.*, 1992). In the mole, we found a clear half-fold decrease in the amount of mRNA in the inactive stage of the seasonal breeding cycle, with respect to that in the active one, a finding which is in clear contradiction with the hypothesis proposed by Hill *et al.* (2004). This AR transcription decrease in the inactive mole testis can be associated with the coinciding decrease in the serum and intratesticular levels of testosterone. However, since FSH is also known to directly influence AR expression (Blok *et al.*, 1989), and we currently lack any data on FSH levels and FSHR expression in the mole, no cause-effect relationship can yet

be established for these parallel variations of T and AR in this animal.

The ITT concentration in the mole was around 7 times higher than the physiological T level in serum in both the active and the inactive stages. The lowest ITT concentration (20 ng/ml) coincides in time with complete disruption of spermatogenesis. This abrupt decrease in the concentration of testosterone is clear consequence of the downregulation of genes like P450scc and SF1, necessary for testosterone production, that we have verified in this work (Fig.6.11 and Fig.6.15). The presence of low ITT levels in the inactive mole testis is especially relevant in the light of previous studies in the rat, indicating that quantitatively normal spermatogenesis can be maintained only when ITT levels are above 20 ng/ml, which is a concentration about 10-fold higher than that of the rat serum (Awoniyi *et al.*, 1990; Zirkin, 1998; Kim *et al.*, 2001). It was reported that when ITT levels fall below the threshold of 20 ng/ml, step 19 spermatids fail to be released, pachytene spermatocytes undergo apoptosis, and round spermatids are sloughed from the seminiferous epithelium (Sharpe *et al.*, 1992; O'Donnell *et al.*, 1996; Awoniyi *et al.*, 1990; Zirkin, 1998; Kim *et al.*, 2001). These events are initiated specifically at stages VII-VIII and presumably occur due to reduced AR-mediated, androgen-dependent gene transcription in the Sertoli cells present in these stages of the spermatogenic cycle (Vornberger *et al.*, 1994; Bremner *et al.*, 1994; Zhu *et al.*, 2000; Sharpe *et al.*, 1992; O'Donnell *et al.*, 1996). In the mole, germ cells that were eventually lost as a consequence of reduced ITT could then be restored again in the next activating stage of the breeding cycle, when ITT levels rise. This is consistent with other findings in the rat, indicating that the administration of high doses of exogenous T directly into the testis (not in the peripheral blood), thus rising the ITT levels above the critical threshold of 20 ng/ml, are sufficient to re-establish the spermatogenic activity of the testis (Awoniyi *et al.*, 1989, 1990; Zirkin, 1998).

7.9 *High levels of serum estradiol in the inactive mole*

Our study in the mole showed that serum estradiol levels were siginificantly higher in the inactive than in the active stages of the seasonal breeding cycle (P=0.0086; Fig.6.29). Based on the well known roles of gonadotropic hormones in other mammals, we presume that in the mole FSH and LH levels should be higher during the breeding season. Accordingly, the high levels of serum estradiol present during the inactive stage suggest that it might be involved in the inhibition of the LH and FSH secretion during the inactive stage (by negative feedback mechanism) as reported in previous

studies (Finkelstein *et al.*, 1991; Bagatell *et al.*, 1994; Raven *et al.*, 2006). Estrogens exert their cellular effects through estrogen receptor (ER) that exist at least in two subtypes, ERα and ERβ (Green *et al.*, 1986; Mosselman *et al.*, 1996). In spite of high levels of estradiol in the serum of moles, ERα mRNA expression showed very low expression in the inactive stage when compared with the active stage ((Fig.6.26;P=0.0001), which is consistent with data in the rat showing that high estradiol levels results into down-regulation of estrogen receptors (Lee and Gorski, 1998).

Studies of ERα-deficient mice have provided direct evidence about the physiological role of estradiol in male reproductive organs. The ERαKO males were infertile (Luconi *et al.*, 1999). Their testes appeared normal until puberty after which it began to degenerate, with disruption of spermatogenesis (Hess *et al.*, 1997). The infertility was supposed to be caused by impaired fluid re-absorption in the efferent ductules, resulting in dilated sperms, increased back-pressure in the seminiferous tubules and related atrophy of the seminiferous epithelium (Hess *et al.*, 1997). Similarily, ER *ERβ*KO (estrogen receptor β knock out) and ArKO (aeromatase knockout) mice were also sterile (Hess *et al.*, 1997). These data clearly show that estrogen receptors are necessary for testis functionality, which is consitent with our observations in the mole that the higher ERα expression levels were observed in moles with active testes. This situation in active moles coincides with the lower levels of serum estradiol (Fig.6.23), indicating that this low levels are sufficient to exert its physiological role. Hence, current knowledge suggests that intra-testicular estrogen levels should play an important role in the mole testis physiology. So, investigating the concentration of intratesticular estradiol and the expression levels of ERβ and aeromatase in the mole is one of our next objective in our research on mole seasonal breeding.

7.10 *Disruption of blood testis barrier during testis inactivation suggest that cell junctions may be involved in the massive cell depletion occurring in this stage*

Regarding the process of testis regression in the breeding cycle of *T. occidentalis*, there are several facts that have to be considered: 1) this process implies a massive depletion of germ cells from inner regions of the seminiferous epithelium (all germ cells beyond pachytene stage); 2) this cell depletion takes place in a relatively short time, in which the epididymides appear filled with germ cells and cell debris (our unpublished results); 3) as indicated above, apoptosis is only partially responsible for this massive cell

depletion so that most of these cells were probably live when eliminated. Any explaination for this phenomenon has to take into account all these features. A plausible explaination is that germ cells detach from the germinative epithelium thus falling inside the tubular lumen and being finally eliminated through the epididymis and the urethra. This implies that either elimination or disorganization of cell junctions could be responsible for this massive cell depletion.

Many studies have shown that cell junctions have pivotal roles in several aspects of testis function. This is mainly evident in the formation of the so-called blood-testis barrier (BTB), that compartmentalizes the germinative epithelium into two compartments. One is the basal compartment which contains spermatogonia, preleptotene and leptotene spermatocytes, the other is the adluminal compartment which contains all the rest of meiotic and post-meiotic germ cells (Dym and Fawcett, 1970; Cavicchia and Sacerdote, 1988). The BTB is mainly composed of tight junctions, although adherens junctions and gap junctions also participates in its structure (Dym and Fawcett, 1970; Pelletier and Byers, 1992). A very important aspect of testis function is that preleptotene and leptotene spermatocytes must translocate from the basal to the adluminal compartment of the seminiferous epithelium to continue its differentiation. This implies the opening and closing of the inter-Sertoli tight juctions at the level of BTB. Apart from the BTB, Sertoli cells and germ cells are intimately attached to each other by adherens junctions and tubulobulbar complexes (Mruk and Cheng, 2004; Lui *et al.*, 2002). Hence, germ cell migration through the seminiferous epithelium involves a restructuring of these cell junction types.

In our studies in the mole, we have analyzed molecular markers specific for the three main junction types present in the testis. CLAUDIN11 and ZONA OCCLUDIN 2 for tight junctions, CONNEXIN 43 for gap junctions and N-CADHERIN and β-CATENIN for tight and adherens junctions. Based on the existence of a well defined pattern of CLAUDIN11, N-CADHERIN and β-CATENIN immunostaining located at the peripheral regions of the seminiferous tubules in the mole, our results strongly suggest that a well formed and probably functional BTB exist during the activating and active stages of the seasonal breeding cycle in this species (Fig.6.44, Fig.6.53 and Fig.6.54). This is clearly consitent with the finding that the presence of CLDN11 in non-photoinhibited Djungarian hamsters, is well correlated with BTB function (Tarulli *et al.*, 2008). On the other hand, the well defined pattern of tight junction proteins disappear in the inactive mole testis, indicating that the BTB barrier disorganizes during this stage. This situation also coincides with that of the Djungarian hamster, where photoinhibited animals have been shown to loose BTB functionality. FSH has a key role in the regulation of this process (Tarulli *et al.*, 2008). However,

Meng *et al.* (2005) have shown that androgens regulate the permeability of the BTB. Different results were reported by Xia et al (2005) in the rat where disruption of Sertoli-germ cell adhesion function effected adherens junctions without perturbing BTB integrity. It is very interesting to note that the effects in the Djungarian hamster testis were caused by gonadotopin suppression, whereas those observed in the rat, were a consequence of androgen suppression. Moreover, it is also noteworthy that both hamsters and moles are seasonal breeders whereas rats are not. According to these considerations, we would hypothesise that seasonal changes in the BTB integrity are regulated by gonadodropins, either directly or indirectly through androgens. This hypothesis is also consistent with the fact that BTB disruption caused by gonadotropin suppression is reversible, a condition necessary for seasonal breeders. A similar phenomenon could also affect the adherens junctions, which are mainly responsible for linkages between Sertoli and germ cells. This is consistent with our observations on the localization pattern of N-CADHERIN and β-CATENIN, which are structural components of adherens junctions, show that a reorganization of these proteins occurs during the seasonal breeding cycle (Fig. 6.53 and Fig. 6.54). Hence, like in the case of the BTB functionality, linkages between Sertoli and germ cells could disappear, thus permitting a massive germ cell depletion that takes place during the inactivation of the mole testis.

There are three main functions of the BTB. 1) to provide a specialized environment 2) to regulate transit of molecules and 3) to provide an immunological barrier (Mruk and Cheng, 2004). It has been largely assumed that the integrity of the BTB must be maintained at all times so that post-meiotic germ cell-specific antigens remain sequestered from the systemic blood circulation (see Xia *et al.*, 2005). Although this may be true in the case of continual breeders, this seems not be the case in seasonal breeders. Both the Djungarian hamsters, where testis regression was artificially induced by photoinhibition (Tarulli *et al.*, 2008), and the Iberian mole, in which wild animals were directly taken from nature (the present study), show that the adluminal compartment of the seminiferous tubule is also exposed to the immunological system during testis inactivity, a possible generalized normal situation in seasonal breeders. Disruption of the BTB in regressed testes of seasonal breeders may be a situation derived from the lack of meiotic products inside the seminiferous tubules, so that there is no need to protect them from the immunological system. In this context, an inactive adult testis would be similar to a pre-pubertal testis, as the BTB is formed at puberty (Dym and Fawcett, 1970; Steger *et al.*, 1999).

7.11 *Integrity of the* lamina propria *is not compromised during the seasonal breeding cycle*

In the adult testis, seminiferous tubules are enveloped by a series of non-cellular and cellular layers forming the so called *lamina propria* (Leeson and Leeson, 1963; Dym and Fawcett, 1970). The outer cells of the seminiferous epithelium (basal Sertoli cells and spermatogonia), are in close contact with the basement membrane (a testis specific form of extracellular matrix) (Dym, 1994). The peritubular myoid cells are the other major component of the *lamina propria* and are located outside the basement membrane. Several studies have established that the basement membrane is involved in BTB dynamics and spermatogenesis (Salomon and Hedinger, 1982; Lehmann *et al.*, 1987). Laminins and collagens, which are the two major components of the basement membrane, have been shown to play a key role in the regulation of Sertoli cell tight junction dynamics (Tung *et al.*, 1993; Siu *et al.*, 2003). All this information indicates that integrity of the basement membrane is critical to maintain the functional status of both BTB and spermatogenesis.

Taking into account that there is disruption of the BTB in the inactive mole testis, it was interesting to investigate whether the *lamina propria* also undergoes some seasonal variations during the breeding cycle of this species. We studied the localization of laminin, a component of the basement membrane, and αSMA, a typical marker of peritubular myoid cells (Fig.6.58 and Fig.6.59). Our results indicates that unlike BTB, neither the basement membrane nor peritubular myoid cells appear to change throughout the seasonal breeding cycle, indicating that the *lamina propria* maintains its integrity during the entire adult life in moles. No similar studies have been performed to date in other seasonal breeding mammals.

7.12 *Inactive adult testes vs prepubertal testes. Differences and similarities*

In species with continual reproduction, the process of spermatogenesis activation takes place just once in life, during a period called puberty. On the other hand, seasonal breeders activate and inactivate their gonads cyclically, so that testes are activated once every year. This raises the question as to whether inactive adult testes of seasonal breeders are functionally equivalent to the pre-pubertal testis of continual breeders. In this regard, we report here for the first time, a comprehensive study addressing several aspects of testis function in a seasonal breeding mammal that provides sufficient elements to compare these two physiological stages.

Table 7.1 summarizes the main differences and similarities between pre-pubertal and adult inactive testes in *T. occidentalis*. These data indicate that pre-pubertal and adult inactive testes are similar in most aspects but they are not identical as some features are clearly different. In the case of *T. occidentalis*, we found differences concerning the expression of AMH and the presence of cells undergoing meiosis onset. We have already discussed in detail about the reasons why AMH may be permanently repressed in adult testes. However, regarding meiosis onset it is interesting to note that seasonal breeding species may show differences in this respect. So meiosis onset represents a difference between pre-pubertal and adult inactive testes in the mole but not in the roe deer, where meiosis stops completely during the stage of reproductive inactivity (Blottner *et al.*, 1996)

Table 7.1: The main differences and similarities between pre-pubertal and adult inactive testes

Event/Process	Pre-pubertal testis[a]	Inactive adult testis
SOX9 expression in Sc	+	+
DMRT1 expression in Sc	+	+
DMRT1 expression in Gc	-	-
OCT4 expression in Gc	-	-
SOX9 expression in Sc	+	+
SF1 expression in Sc	-	-
SF1 expression in Lc	-	-
BTB integrity	-[b]	-
Basement membrane integrity	+	+
Serum testosterone levels	-	-
Meiosis onset	-	+
AMH expression	+	-

[a] Data from the pre-pubertal mole testis were obtained from previous published and non-publishes studies performed in our laboratory.

[b] for the moment we lack direct data about the BTB integrity in prepubertal moles but it is known that in other species BTB is formed at puberty, so that we assume that pre-pubertal mole testis lack it. Sc, Sertoli cells; Gc, Germ cells; Lc, Leydig cells

7.13 A hypothesis on the control of seasonal breeding in moles

Our study of several aspects of seasonal breeding in the mole testis indicate that this process is not regulated at the level of meiosis onset, as the rhythm of entry into meiosis by spermatogonia seems to be constant throughout the adult life of this animal. In our opinion, there is an endocrine mechanism that controls this process in which gonadotropins and androgens are the main components. According to this hypothesis, in a breeding mole, the anterior pituitary produces gonadotropic hormones (FSH and LH), stimulated by the presence of GnRH, which is in turn produced by the hypothalamus as an indirect consequence of stimulating seasonal factors. Although it is well established that most mammals are sensitive to the photoperiod as the main seasonal factor controlling reproduction, the fossorial lifestyle of the mole makes it unclear whether it is the photoperiod or a different element (temperature, rainfall, etc.) what exerts this control. LH stimulates Leydig cells to produce testosterone and other androgens, and FSH acts directly on Sertoli cells promoting different functions (see Fig.7.4).

The process of testis regression could be produced as a consequence of GnRH-mediated decrease in the production of gonadotropins, when seasonal factors mark the end of the breeding season. The absence of LH leads to a decrease in the production of androgens. A direct consequence is that the AR gene is down regulated in Sertoli cells, resulting into two detrimental effects: 1) apoptosis affecting zygotene/pachytene primary spermatocytes is activated thus preventing the differentiation of any germ cells entering into meiosis; 2) tight and adherens junctions are disrupted, resulting in both loss of functionality of the BTB and detachment of the germ cells present in the adluminal compartment of the seminferous epithelium. The later phenomenon leads to a massive and rapid depletion of meiotic and post-meiotic germ cells. Moreover, the absence of FSH results in 1) disruption of the nursing functions of the Sertoli cells, and 2) disengagement of the tight junction structure (see Fig.7.5).

When the environmental conditions announce a new breeding season, the testis initiates an activation process which is stimulated by a rise in the concentration of serum gonadotropins. This reverses the inhibitory effects described above: 1) rise in the serum and intratesticular androgen concentrations, 2) reorganization of the BTB and Sertoli-germ cell adherens junctions, and 3) reduction of apoptosis to basal levels. The consequence is that spermatogenesis is again fully functional, a situation which will persist until the next quiescence season.

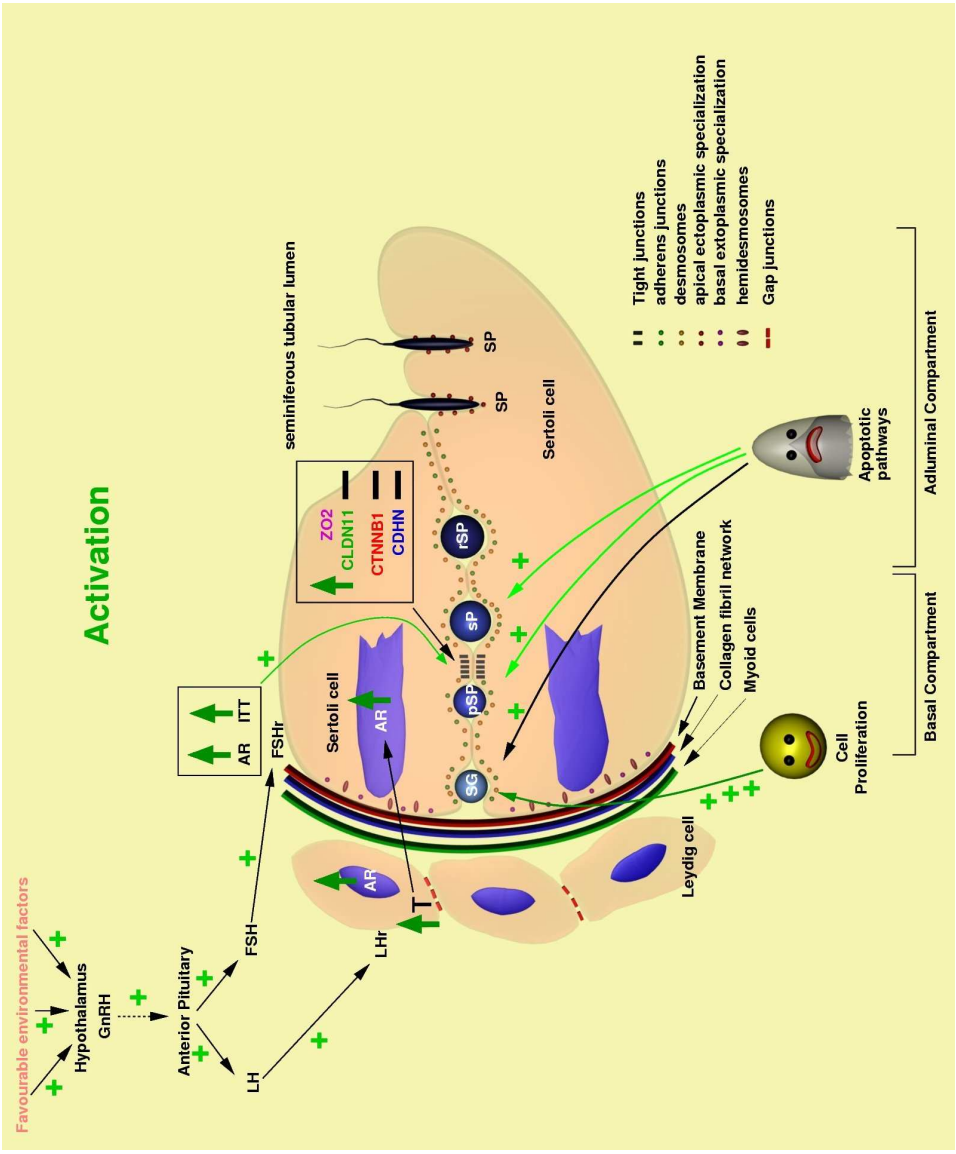

Figure 7.4: Activation

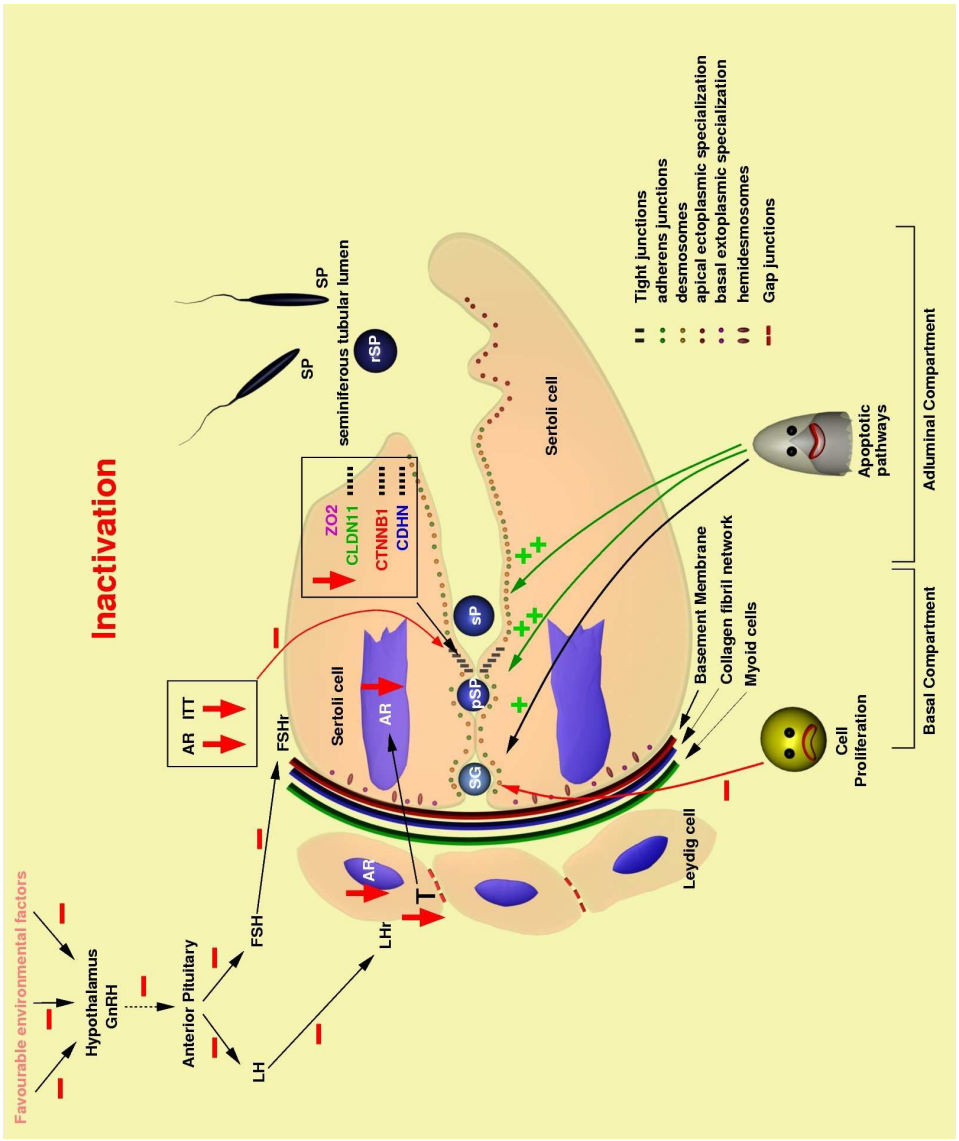

Figure 7.5: Inactivation

Conclusions

1. The expression patterns of *SOX9* and *DMRT1* in the adult mole testis suggests a role for these genes in seasonal breeding.

2. The permanent repression of the *AMH* gene in the adult mole testis may be a consequence of the continued presence of primary spermatocytes and intratesticular testosterone.

3. Mole germ cells never regress to pre-pubertal stages of differentiation in adult testes.

4. The constant rhythm of meiosis onset throughout the seasonal breeding cycle of *T. occidentalis* indicates that the control of seasonal reproduction is not established at this level, at least in this species.

5. Apoptosis is not responsible for the massive depletion of germ cells occurring during regression of the mole testis.

6. Imbalance between apoptosis, cell proliferation and meiosis onset rate may lead to seasonal variations in the spermatogonial pool in *T. occidentalis* testes.

7. Testosterone and estradiol probably play important roles in the control of seasonal breeding in moles.

8. Disruption of blood testis barrier during testis inactivation suggest that loss of cell junctions is primarily responsible for the massive cell depletion occurring in this stage.

9. The integrity of the *lamina propria* is not compromised during the seasonal breeding cycle of *T. occidentalis*.

Conclusiones

1. Los patrones de expresión de *SOX9* y *DMRT1* en el testículo de topo adulto sugieren que estos genes tiene algun papel importante en el control de la reproducción estacional.

2. La represión permanente del gen *AMH* en el testículo de topo adulto es posiblemente una consecuencia de la presencia continuada de espermatocitos primarios y testosterona intratesticular.

3. Las células germinales del topo adulto inactivo nunca regresan a estadios de diferenciación pre-puberales.

4. El ritmo contante de entrada en meiosis a lo largo del ciclo reproductivo estacional de *T. occidentalis* demuestra que el control de dicho ciclo no se establece a este nivel, al menos en esta especie.

5. La apoptosis no es responsable de la perdida masiva de células germinales que ocurre durante la inactivación del testículo de topo.

6. Un desequilibrio entre apoptosis, proliferación celular y ritmo de entrada en meiosis puede producir variaciones estacionales en la población de espermatogonias del testículo de topo adulto.

7. La testosterona y el estradiol probablemente desempeñan papeles importantes en el control del ciclo reproductivo estacional de *T. occidentalis*.

8. La desorganización de la barrera hemato-testicular durante la etapa de inactivación, sugiere que la pérdida de uniones intercelulares es ser responsable directa de la eliminación masiva de células germinales que tiene lugar durante dicha etapa.

9. La integridad de la *lamina propia* no se ve afectada durante el ciclo reproductivo estacional de *T. occidentalis*.

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