Genetic control of the seasonal reproductive cycle in micromammals

Diaa Fawzi Mohamed Mahmoud Massoud

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CIBM

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Dirigida por los Doctores:

Dr. Rafael Jiménez Medina

Dr. Francisco Barrionuevo Jiménez

Doctorando

D. Diaa Fawzi Mohamed Mahmoud Massoud

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To my beloved Mother, for her prayers to me.

To my Wife, for care and support all the time.

And to my Childrens, Jana and Annas with hope for bright future.

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Contents

A. Summary

Seasonal breeding is the process by which species of the temperate zones of the Earth concentrate the reproductive effort in those seasons in which environmental conditions are more favorable. In seasonal breeding mammals, both female and male gonads undergo substantial changes during the transition periods between the breeding and the non-breeding periods, but these processes have been studied in very few species to date, so that the mechanisms of gonad inactivation are not well understood yet. Moreover, most studies are incomplete as only one particular aspect of the entire process was focused in many cases (apoptosis, hormonal variations, ultrastructure, morphological changes, dynamics of cell adhesion molecules). Comprehensive studies including all these features have been performed only in the Iberian mole and the long hairy armadillo. Hence, additional seasonal breeding mammalian species must be investigated in order to elucidate if there is a conserved mechanism of testis regression in mammals or there are several alternative mechanisms that can operate in different species or circumstances. Micro-mammals are ideal species for this kind of studies as multiple captures of wild animals have to be done in order to get statistical significance in the comparisons between different seasons required for many reproductive parameters.

In the present work we investigated for two years four mammalian species, the greater white-toothed shrew, *Crocidura russula*, the Algerian mouse, *Mus spretus*, the wood mouse, *Apodemus sylvaticus* and the Mediterranean pine vole *Microtus duodecimcostatus*, in order to study 1) whether these species experience seasonal breeding, 2) the functional status of the main cell types (Sertoli, Leydig, peritubular myoid and germ cells), structures (seminiferous tubules, lamina propria), and biological processes (spermatogenesis) throughout the seasonal reproductive cycle, 3) the spatio-temporal pattern of expression of several genes involved in testis function, 4) the androgenic function of the testes, 5) the role of both apoptosis and cell proliferation in the process of testis regression, 6) the role of cell junctions in the seasonal dynamics of the germ cells, and 7) the possible existence of associations between altered gene expression patterns and seasonal testis regression in these species, in order to establish possible roles for these genes in the control of seasonal breeding. In *Crocidura russula*, unexpectedly, we found no significant differences between summer and winter males, even though female data confirmed a non-breeding period in the summer, showing that males retain full testis function even when most females are not sexually receptive. This phenomenon was not described before and does not occur in northern populations of the same species. In addition, the reproductive cycle is inverted in the north with respect to south, as the

non-breeding period occurs during the winter in the north and during the summer in the south. Since the non-reproductive period shortens at lower latitude locations, we suggest that in southern populations the non-breeding period is short enough to make testis regression inefficient in terms of energy savings. Two facts support this hypothesis: 1) testes of *C. russula* are very small, a condition derived from their monogamy that implies low investment in spermatogenesis, and 2) the spermatogenic cycle of this species is slow and long. The inverted seasonal breeding cycle and the lack of seasonal testis regression described here are new adaptive processes that deserve further research in this and other species.

In southern Iberian populations of *M. spretus* testes maintain the spermatogenic function during the entire year, but appear slightly reduced in size during the winter if compared with those found in the summer, which could be a mechanism for a lower reproductive activity in this period. Our results indicate that the limited testis reduction that Algerian mice undergo during the winter is mediated by increased apoptosis on meiotic germ cells, a hypothesis consistent with reduced levels of serum testosterone in these males. However, no difference between winter and summer males was detected in our immunohistological study on the expression of several genes. This limited reduction in testicular activity may be, like in the case of the shrew, an adaptive response to a very short period of sexual inactivity. Moreover, we found that the BTB is permeated in the winter testes, even though these gonads look morphologically normal. This is a surprising finding because BTB integrity is required to maintain testicular immune privilege. Nevertheless, our data suggest that Algerian mice experience the testis reduction period without undergoing any drop in their reproductive potential. Reduced virulence of the autoimmune response in the testis micro-environment appears to be a possible explanation for this intriguing phenomenon.

The reproductive timing of the studied populations of *Apodemus sylvaticus* fits very well with a classical model including a continuous reproductive period comprising most of the year, interrupted by a short non-breeding period. Summer testis regression, which is mediated by reduced levels of serum testosterone, implies a dramatic remodeling of the somatic cells of the summer gonad. Leydig cells accommodate to the new smaller space between adjacent seminiferous tubules and Sertoli cells reduce their cytoplasmic volume. Permeation of the BTB during testis regression and altered expression pattern of CLAUDIN11 (a principal component of tight junctions), indicate that the expression of genes encoding cell-adhesion molecules is impaired during testis regression, suggesting that germ cell depletion in this species could occur by direct desquamation as shown in the mole. We found a very significant increase in the frequency of apoptotic cells in the testes of inactive males, suggesting that the role of apoptosis in these testes is to eliminate spermatocytes before they reach more advanced stages of meiosis.

Microtus duodecimcostatus is, together with *A. sylvaticus*, the only species, of the four studied in this work, showing complete testis regression during

the summer. However this only occurs in populations located in wastelands and cereal crops, but neither in natural populations located in poplar groves of the same geographic region nor in the animal house, where reproduction takes place throughout the year. Since all three groups of summer males (wastelands, poplar grove, and animal house) were subjected to the same photoperiod, our observations clearly show that it is the micro-environment in which each vole population is living what determines its reproductive status and the local existence or not of seasonal breeding. When life conditions are favorable, either natural or artificial, voles do not stop breeding, whereas in the poor environment of the wastelands during the summer of south-eastern Iberian Peninsula, testis regression occurs and reproduction is halted.

Gene expression patterns in active and inactive testes from seasonal breeding males were found to be highly conserved, including SOX9, DMTR1, CLAUDIN11, and DMC1. These four genes showed the same expression pattern in all species investigated to date and our data strongly suggest that SOX9 and DMRT1 play important roles in the control of spermatogenesis by Sertoli cells. Other genes, including AR and PCNA showed some expression differences between species, reflecting some species-specific differences in gene regulation.

Summer testis regression was detected in *A. sylvaticus* and *M. duodecimcostatus*, which showed very different levels of apoptosis in the inactive testis, being very high in the former and very low in the latter species. Our results in *A. sylvaticus* and those reported previously for *T. occidentalis* suggest that the role of apoptosis in the inactive testes is to eliminate the germ cells that continue entering meiosis during the non-breeding period. However, the case of *M. duodecimcostatus* is exceptional because in this species the frequency of apoptotic cells decreases during testis regression. Nevertheless, data from this species also support the hypothesis as the number of germ cells entering meiosis in their inactive testis is also very low if compared with that of *A. sylvaticus*.

We found that several features of the regressed testes in seasonal breeding males are quite well conserved from an evolutionary point of view. For instance, the androgenic function of males is diminished during the non-breeding season, suggesting that reduced concentration of androgens is probably the hormonal signal inducing seasonal germ-cell depletion in all species. Another constant feature in the regressed testes of inactive males is the permeation of the BTB, but we found contradictory results in *M. spretus*, as the BTB of this species apparently permeates in winter before any sign of germinative epithelium depletion is visible. Further research is needed to understand the functional connection between androgen concentration, germ cell depletion and BTB permeation in seasonally breeding mammals.

Our results evidence that in the studied populations seasonal breeding is not a species-specific feature. Rather, it depends on the life conditions that every particular individual is facing at each moment. Hence, it appears that these animals show a clear tendency to reproduce continuously at all seasons,

but the environmental cues concomitant with a particular climatic season at a given latitude force then to stop breeding. Two extreme situations can be recognized in mammals regarding the control of circannual reproduction timing: 1) species in which the hypothalamus-pituitary-gonad axis is tightly regulated by the photoperiod and exhibit constant and rigid seasonal breeding patterns; 2) species with opportunistic seasonal breeding, in which photoperiod has little or no influence in their reproduction timing, which mainly depends on micro-environmental cues like water and food availability, temperature, rainfall, etc. European moles, lemmings, hamsters, deers , brown bears, and many other species living in central and northern Europe and similar latitudes in North America and Asia, probably belong to the first group. The greater white-toothed shrew and the Mediterranean pine vole and probably many other species whose area of distribution extends toward lower latitude regions, belong to the second group. All four species analyzed in the present study showed at least one peculiarity in their reproductive biology that makes them different from each other. These multiple species-specific models of circannual testis variation in seasonal breeding species suggest 1) that the mechanisms controlling seasonal reproduction are in fact very plastic and much less rigid than initially considered, and 2) that they appear to be fast evolving. Hence, mammalian populations probably have available multiple ways to get adapted to the unstable environmental conditions that the climate change will probably cause in the future.

B. Resumen

La reproducción estacional es el proceso por el que las especies de las regiones templadas de la Tierra concentran su esfuerzo reproductivo en aquellas estaciones que ofrecen mejores condiciones ambientales. En las especies con reproducción estacional, tanto las hembras como los machos experimentan cambios sustanciales en los periodos de transición entre la época reproductiva y la no reproductiva, pero estos procesos han sido estudiados en muy pocas especies hasta la fecha, de manera que los mecanismos de inactivación gonadal no se conocen bien todavía. Además, la mayor parte de los estudios realizados son incompletos pues sólo enfocan aspectos muy particulares de proceso en muchos casos (apoptosis, variaciones hormonales, ultraestructura, cambios morfológicos, dinámica de la moléculas de adhesión celular). Estudios completos que incluyan todas estas características sólo se han llevado a cabo en el topo ibérico y en el armadillo de pelo largo. Por tanto, es necesario investigar otras especies con reproducción estacional para saber si hay un único mecanismo conservado de regresión testicular, o existen varios mecanismos alternativos que puedan operar en distintas circunstancias. Los micromamíferos son especies ideales para este tipo de estudios, pues permiten la realización de las múltiples capturas de animales silvestres necesarias para alcanzar la potencia estadística requerida para realizar comparaciones entre parámetros reproductivos en distintas estaciones. En este trabajo hemos investigado durante dos años cuatro especies de micromamíferos, la musaraña común, *Crocidura russula*, el ratón moruno, *Mus spretus*, el ratón de campo, *Apodemus sylvaticus* y el topillo mediterráneo, *Microtus duodecimcostatus*, con el fin de estudiar: 1) si estas especies tienen reproducción estacional; 2) el estado funcional de los principales tipos celulares (Sertoli, Leydig, peritubulares y germinales), estructuras (túbulos seminíferos, lamina propria) y procesos biológicos (espermatogénesis) durante el ciclo reproductivo estacional; 3) el patrón espacio-temporal de expresión de varios genes involucrados en la función testicular; 4) la función androgénica de los testículos; 5) el papel de la apoptosis y la proliferación celular en el proceso de regresión testicular; 6) el papel de las uniones intercelulares en la dinámica estacional de las células germinales; 7) la posible existencia de asociaciones entre patrones alterados de expresión génica y la regresión testicular en estas especies, para establecer posibles papeles de estos genes en el control de la reproducción estacional.

Inesperadamente, en *Crocidura russula* no encontramos diferencias significativas entre los machos de verano y de invierno, a pesar de que los datos de las hembras confirmaron un periodo no reproductivo en verano, lo que de-

muestra que los machos retienen todo su potencial espermatogénico incluso cuando las hembras no son sexualmente receptivas. Este fenómeno no ha sido descrito anteriormente y no ocurre en poblaciones de esta especie situadas más al norte. Además, el ciclo reproductivo está invertido en el norte con respecto al sur, ya que periodo no reproductivo ocurre en invierno en el norte y en verano en el sur. Puesto que éste se va acortando conforme avanzamos hacia menores latitudes, sugerimos que en en las poblaciones del sur el periodo reproductivo es lo suficientemente corto como para que la regresión testicular sea ineficiente en términos de ahorro energético. Dos hechos apoyan esta hipótesis: 1) los testículos de *C. russula* son muy pequeños, una condición derivada de su monogamia y que implica un baja inversión en espermatogénesis, y 2) el ciclo espermatogénico de esta especie es lento y largo. El ciclo reproductivo estacional invertido y la ausencia de regresión testicular descritos aquí son nuevos procesos adaptativos que merecen ulterior investigación en esta y en otras especies.

En las poblaciones de *M. spretus* en el sur de la Península Ibérica, los testículos de los machos mantienen la función espermatogénica durante todo el año, pero aparecen ligeramente reducidos en el invierno, si se comparan con los de los machos de verano, lo que podría ser un mecanismo para mantener una menor actividad reproductora en este periodo. Nuestros resultados indican que esta reducción testicular limitada que los ratones morunos sufren durante el invierno está mediada por un incremento de la apoptosis en las células germinales, una hipótesis coherente con los niveles reducidos de testosterona sérica detectada en estos machos. Sin embargo, no detectamos ningunas diferencia entre los machos de invierno y de verano en la expresión de varios genes. Esta reducción limitada en la actividad testicular puede ser, como en el caso de *C. russula*, una respuesta adaptativa a un periodo corto de inactividad sexual. Además, encontramos que la BTB pierde su impermeabilidad durante el invierno a pesar de que las gónadas parecen tener un aspecto normal. Se trata de un hallazgo sorprendente porque la integridad de la BTB es necesaria para mantener el privilegio autoinmune del testículo. Sin embargo, nuestros datos sugieren que estos ratones morunos pasan el invierno sin perder su potencial reproductor. Una posible explicación para este intrigante fenómeno es una reducida virulencia de la respuesta autoinmune en el microambiente testicular.

El programa reproductivo anual de las poblaciones de *Apodemus sylvaticus* estudiadas encaja perfectamente en un modelo clásico que incluya un periodo reproductivo continuo que comprenda casi todo el año, interrumpido por un corto periodo no reproductivo. La regresión testicular del verano, que está mediada por niveles reducidos de testosterona sérica, implica una drástica remodelación de las células somáticas de la gónada. Las células de Leydig se acomodan al nuevo espacio, más reducido, que queda entre los túbulos seminíferos adyacentes, y las células de Sertoli reducen su volumen citoplasmático. La permeabilización de la BTB y la expresión alterada de CLAUDINA11 (un componente principal de las uniones estrechas), indica que la expresión de los

genes que codifican las moléculas de adhesión celular resulta afectada durante la regresión testicular, lo que sugiere que la pérdida de células germinales en esta especie puede ocurrir por descamación directa, tal como se ha demostrado en el topo. Encontramos un incremento muy significativo en la frecuencia de células apoptóticas en los testículos de los machos inactivos, lo que indica que el papel de la apoptosis en estos testículos es eliminar los espermatocitos antes de que alcancen estadios meióticos más avanzados.

Microtus duodecimcostatus es, junto con *A. sylvaticus*, la única especie de las cuatro estudiadas en este trabajo, que muestra regresión testicular completa durante el verano. Sin embargo, esto sólo ocurre en las poblaciones localizadas en páramos y en tierras de cultivos de cereales, pero no en las alamedas de las misma región geográfica ni en el animalario, donde la reproducción tiene lugar durante todo el año. Puesto que los tres grupos de animales (páramos, alamedas y animalario) estuvieron sometidos al mismo foroperiodo de verano, nuestra observaciones muestran claramente que es el microambiente en que vive cada población de topillos lo que determina su estatus reproductivo y la existencia o no de reproducción estacional local. Cuando las condiciones de vida son favorables, ya sea en poblaciones naturales o artificiales, los topillos no cesan la reproducción, mientras que en el pobre ambiente de los páramos en el verano del sudeste de la Península Ibérica ocurre la regresión testicular y la reproducción es detenida. Los patrones de expresión génica en los testículos activos e inactivos de los machos con reproducción estacional están muy conservados, incluidos los genes SOX9, DMTR1, CLAUDIN11, y DMC1. Estos cuatro genes tienen el mismo patrón de expresión en todas las especies investigadas hasta ahora y nuestros datos sugieren claramente que SOX9 y DMRT1 juegan papeles importantes en la regulación de la espermatogenesis por parte de las células de Sertoli. Otros genes, como AR y PCNA mostraron algunas diferencias de expresión entre las especies, lo que refleja la existencia de diferencias específicas en sus mecanismos de regulación.

Detectamos regresión testicular en verano tanto en *A. sylvaticus* como en *M. duodecimcostatus*, que sin embargo mostraron niveles muy distintos de apoptosis en los testículos inactivos, siendo muy altos en la primera y muy bajos en la segunda especie. Nuestros resultados en *A. sylvaticus*, junto con los que publicamos previamente sobre *T. occidentalis*, sugieren que el papel de la apoptosis en el testículo inactivo es eliminar las células germinales que continúan entrando en meiosis durante el periodo no reproductivo. Sin embargo, el caso de *M. duodecimcostatus* es excepcional porque en esta especie la frecuencia de células apoptóticas desciende durante la regresión testicular. Sin embargo, los datos de esta especie también apoyan la hipótesis ya que el número de células germinales que entran en meiosis en sus testículos inactivos es a su vez muy baja si se compara con la de *A. sylvaticus*.

Hemos encontrado que varias características de los testículos involucionados en los machos con reproducción estacional están bastante bien conservados desde un punto de vista evolutivo. Por ejemplo, la función androgénica del ma-

cho está disminuida durante el periodo no reproductivo, lo que sugiere que una menor concentración de andrógenos es probablemente la señal hormonal que induce la pérdida estacional de células germinales en todas las especies. Otra característica constante en los testículos involucionados es la permeabilización de la BTB, aunque encontramos resultados contradictorios en *M. spretus* ya que la BTB de esta especie se permeabiliza aparentemente en invierno, sin que se aprecie ningún signo en desecho del epitelio germinativo. Son necesarios estudios posteriores para comprender mejor la conexión funcional entre la concentración de andrógenos el descarte del epitelio germinativo y la permeabilización de la BTB en mamíferos con reproducción estacional.

Nuestros resultados ponen en evidencia que la reproducción estacional no es una característica específica de especie en las poblaciones analizadas. Mas bien, ello depende de las condiciones de vida que cada individuo afronta en cada momento. Por tanto, parece que estos animales muestran una clara tendencia a reproducirse de forma continua en todas las estacionas, pero los factores ambientales propios de una determinada estación climática en una latitud concreta los fuerzan a cesar la reproducción. Podemos reconocer dos situaciones extremas en los mamíferos en relación con el control del ritmo reproductivo circanual: 1) especies en las que el eje hipotálamo-hipófisis-gónada está estrictamente regulado por el fotoperiodo y muestran patrones rígidos de reproducción estacional; 2) especies con reproducción estacional oportunista en las que el fotoperiodo tiene poca o ninguna influencia en su ritmo reproductivo, que depende principalmente de factores microambientales tales como la disponibilidad de agua y alimento, temperatura, pluviometría, etc... Los topos, lemings, hamsters, ciervos, osos pardos y otras muchas especies que viven en la Europa Central y del Norte y en similares latitudes de América del Norte y Asia, probablemente pertenecen al primer grupo. La musaraña común, el topillo mediterráneo y probablemente muchas otras especies cuyo área de distribución se extiende hasta regiones de menor latitud pertenecen al segundo grupo.

Las cuatro especies analizadas en este estudio mostraron al menos una peculiaridad en su biología reproductiva que la hace distinta de la demás. Estos múltiples modelos de variación testicular estacional específicos de especie sugieren: 1) que los mecanismos que controlan la reproducción estacional son de hecho muy plásticos y mucho menos rígidos de lo que en principio pudimos haber imaginado, y 2) que dichos mecanismos parecen evolucionar muy rápidamente. Por tanto, las poblaciones de mamíferos parecen disponer de múltiples vías para adaptarse a las condiciones ambientales inestables que el cambio climático probablemente cause en el futuro.

C. Introduction

Sexual reproduction is the process in which a new individual is created by combining the genetic material of two individuals of the same species and it is fundamental to the survival and propagation of most living beings. It depends upon the correct development and function of the gonads in both females and males, the organs responsible for the generation of haploid gametes that will fuse during fertilization. The gonad is the only organ that can differentiate into two adult organs, either a testis or an ovary, and the decision to choose one of these fates is taken at the moment of sex determination. In mammals, the sex-determining gene on the Y chromosome, *SRY*, is expressed in the bipotential male gonad and activates a male-specific genetic cascade that triggers testis development. Its absence in the female, leads to the activation of the canonical Wnt signaling pathway in the gonadal anlagen, which results in ovary development. Once the gonad is determined, it undergoes a process of differentiation that results in the formation of functional testes or ovaries that persist in the adult. This is a complex process in which sex-specific cell lineages differentiate and produce a correct cellular environment to generate gametes. When the adult gonad is fully differentiated it is capable of being producing gametes continuously . This is the normal situation in many species including the human, but in nature we also find many species that can halt and resume gamete production in a cyclic fashion: the so called seasonal breeders. In these latter species, this condition is the result of an adaptive process by which breeding takes place when the best environmental conditions occur. In seasonal breeding mammals, both female and male gonads undergo substantial changes during the transition periods between the breeding and the non-breeding periods, but these processes have been studied in very few species to date, so that the mechanisms of gonad inactivation are not well understood yet. In the present study we have tried to gain new insights about the process of testis regression in seasonal breeding mammals. For this, we have studied the seasonal variations occurring in the testes of four species of small mammals living in the province Granada, southern Spain: the greater white-toothed shrew (*Crocidura russula*, Soricidae, Eulipotyphla) the Mediterranean pine vole (*Microtus duodecimcostatus*, Cricetidae, Rodentia),the Algerian mouse or western Mediterranean mouse, (*Mus spretus*, Muridae, Rodentia), the wood mouse (*Apodemus sylvaticus*, Muriade, Rodentia)

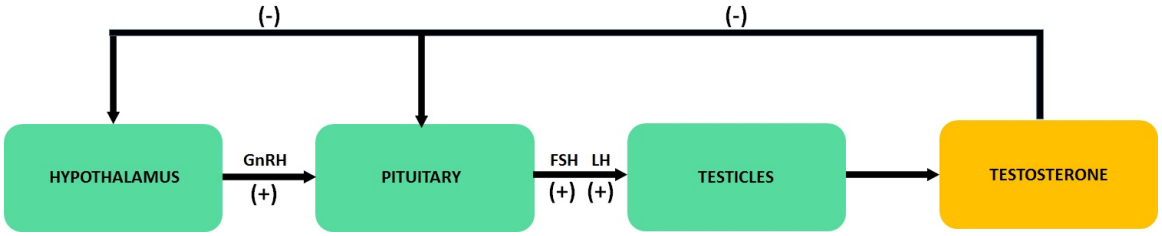


Figure C.1: Schematic representation of the the main actors of the HPG axis

C.1 Seasonal breeding

In females of seasonal breeding species, ovulation takes place during only one period of the year. Similarly, in the male, testis function is abolished during the non-breeding season, a process accompanied of a reduction in the testis size and testosterone release, which implies spermatogenesis arrest and lack of sexual drive. Nevertheless, there is a great variation among species in this respect. In the stallion, for example, fertility may be maintained at a reduced level (Guillaume *et al.*, 1996) during the non-breeding season, whereas in other species spermatogenesis is completely halted and males become azoospermic 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 (Jiménez *et al.*, 1990; Dadhich *et al.*, 2010, 2013), among others. In mammals, reproduction is a complex process hormonally controlled by the hypothalamus-pituitary-gonad axis (HPG). The hypothalamic neurons release the decapeptide GnRH (Gonadotropin-releasing hormone) in the portal venous system, which in turn causes the release of gonadotropic hormones LH (Luteinizing hormone) and FSH (follicle stimulating hormone) in the anterior pituitary (hypophysis). These hormones activate the production of gonadal steroids (testosterone, estradiol and progesterone). Testosterone is the main steroid hormone released by the testis and modulates the production of GnRH in the hypothalamus, and the production of gonadotropins in the pituitary, what implies a feed-back system between the gonad and the pituitary and hypothalamus in the regulation of the HPG axis (see Fig. C.1) (Hayes *et al.*, 2001).

In seasonal breeders, the reproductive status must switch cyclically from active to inactive and vice-versa, what implies that the status of the HPG axis must also change accordingly. The question arises as to which factors are responsible for the variations of the status of the HPG axis between different breeding seasons. Several studies have shown that environmental cues like temperature, stress, availability of food, rainfall and the photoperiod are involved in this process. From them, the most studied to date is photoperiod. This environmental factor provides valuable information about the climatic seasons that remains constant from year to year, but response to photoperiod is species-specific and mainly depends on gestation length. The hamster for

instance, which has a short gestation (3 weeks) and reproduces in the spring an the summer, is a long-day breeder (Sinha Hikim *et al.*, 1988). On the other hand, sheeps, goats, and deers, which have longer gestation lengths (5-6 months) mate in autumn during a period of decreasing daylength and consequently are. 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. 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).

In mammals, the photoperiod is detected by the photoneuroendocrine system that is 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 rhythmic secretion of the hormone melatonin (Simonneaux and Ribelayga, 2003). Pineal and plasma melatonin concentrations are low during the daytime and rise massively during the night-time. Consequently, the profile of the nocturnal melatonin release fluctuates with photoperiod; it is now well established that the duration of melatonin secretion is the critical feature of its signal (Bartness *et al.*, 1993; Pitrosky and Pévet, 1997). Melatonin regulates GnRH secretion via two complementary mechanisms: 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). Although the molecular mechanism controlling these processes are not completely known, one important intermediary component was discovered recently. The complex formed by kisspeptins, a group of peptides of different lengths encoded by the *KiSS-1* gene, together with their receptor GPR54, represent a powerful activator of the HPG axis (Revel *et al.*, 2007). In the syriam hamster, melatonin regulates the expression of *KiSS-1* in the hypothalamic cells responsible of GnRH production, controling this way seasonal reproduction. It is well established that several seasonal breeders use the photoperiod to regulate testicular activity. One example is the Syrian hamster, in which 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 consequences of these changes are the transient atrophy of the gonads and the accessory reproductive organs (Pévet, 1988; Bartke and Steger, 1992).

Photoperiod is thought to be the most common environmental cue controlling seasonal breeding, but several species have been reported in which the role of photoperiod in this process is secondary. In the Californian mouse (*Peromyscus californicus*), for example, water restriction results in a reduction of the size of the reproductive organs and cessation of the reproductive activity,

independently of the photoperiod and the food availability (Nelson *et al.*, 1995). Also, it has been reported that in some ruminants, as the Merino rams (*Ovis aries*), reproduction is not fully photoperiod-dependent (Martin *et al.*, 1990). These animals behave as opportunist breeders with a reproductive strategy depending on short-term responses to environmental cues such as food and social factors. In these species, food availability influences adult testis size, a process that appears to be GnRH-independent . These long-term variations in testis size are not correlated with variations in gonadotropin secretion, and therefore, do not seem to be a consequence of testicular LH variations. These results indicate that other GnRH-independent mechanisms must exist controlling testis activity (Martin *et al.*, 1994). One of these mechanisms has been discovered recently in the musk shrew, *Suncus murinus*, a species in which nutritionally challenged females stop breeding as a consequence of complete loss of sexual receptivity, a response controlled by GnRH-II, another form the the GnRH family (Temple *et al.*, 2003).

C.2 Influence of seasonal breeding in adult testis architecture

In the adult testis of mammals two clearly differentiated compartments are present: the seminiferous tubules and the interstitial cells. The seminiferous tubules house the germinative epithelium which is formed by Sertoli cells and germ cells. Sertoli cells are epithelial somatic cells, with the basal pole oriented toward the outside of the tubule and the apical pole oriented toward the tubular lumen. Their cytological structure is quite complex, with a generally basal nucleus and a very large and morphologically changing cytoplasm that accommodates the spaces between the neighbouring germ cells. Sertoli cells are sustentacular and nurse cells that provide germ cells with the required nutrients and structural support. They also are the main targets of the hormones regulating the spermatogenic function in the seminiferous tubules, such as FSH and testosterone (Skinner *et al.*, 1991), and they establish a regulatory cross-talk with germ cells for the correct timing of spermatogenesis. They also form the blood-testis barrier (BTB) that defines two compartments in the germinative epithelium: adluminal and basal.

Surrounding the seminiferous tubules are the peritubular myoid (PM) cells. In most rodents they form a monolayer of cells, while several layers exist in humans and other species. They are joined by junctional complexes and contain abundant actin filaments which are distributed longitudinally and circularly to the long axis of the seminiferous tubule within one cell. PM cells also contain other cytoskeletal proteins, such as myosin, desmin/vimentin and alpha-smooth muscle actin. They are contractile cells involved in the transport of spermatozoa and testicular fluid in the tubule. PMC secrete a number of extracellular matrix components (fibronectin, type I and IV collagens, proteoglycans) and together with Sertoli cells, which also secrete

extracellular matrix components (laminin, type IV collagen and proteoglycans), cooperate in the formation of the lamina propria. Like Sertoli cells. they also express the androgen receptor and are involved in the hormonal control of spermatogenesis.

The main cellular component of the interstitial compartment of the testis are Leydig cells. They constitute the steroidogenic cell lineage of the testis and secrete androgens including testosterone, dihydrotestosterone, androstenedione and dehydroepiandrosterone.

In males of seasonal breeding species the architecture and the function of the testis change cyclically throughout the year, the fully functional testis of the breeding period generally becoming inactive in the non-reproductive season and then resuming activity again for the next mating season. Despite this general consideration, the mechanisms underlying the transition between the active and inactive periods vary among species, and recent findings indicate that this process is not conserved even in evolutionary related species. Nevertheless, there are several testicular features and processes that have been found to be altered during the reproductive cycle of the species studied to date including, spermatogenesis, testosterone levels, functionality of the blood-testis-barrier, apoptosis and cell proliferation. We provide a state of the art overview of these processes as follows.

C.3 How seasonal breeding influences spermatogenesis and testicular steroidogenesis

Mammalian spermatogenesis is a highly complex process by which spermatogonial "stem cells" are gradually transformed into a highly differentiated haploid cells. This process involves three distinct classes of germinal cells (spermatogonia, spermatocytes, and spermatids), which are usually arranged in concentric layers in the seminiferous tubules. Spermatogenesis can be divided into three distinct phases (mitotic, meiosis, and spermiogenesis) each characterized by specific morphological and biochemical changes of nuclear and cytoplasmic components (Bellve and O'Brien., 1983). In the first phase the spermatogonia stem cells (type A spermatogonia) undergo mitosis, resulting in renewal of germline stem cells as well as type B spermatogonia that continue to undergo differentiation. In the second phase, type B spermatogonia undergo meiosis, generating haploid round spermatids. Then, in the third phase, the spermatids differentiate through complex series of cytological and chemical transformations into spermatozoa; process usually referred to as spermiogenesis. 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 (Bellve and O'Brien., 1983). In seasonal breeding mammals, spermatogenesis is arrested during the non-reproductive season in most of the species studied to

date. This arrest takes place at the level of primary spermatocytes in most of the species studied including hamsters (*Phodopus sungorus* and *Mesocricetus auratus* (Furuta *et al.*, 1994; Sato *et al.*, 2005), squirrels (*Citellus duricus*) (Sheng *et al.*, 2008), armadillos (*Zaedyus pichiy*) (Superina and Jahn, 2009), raccoons (*Procyon lotor*) (Kaneko *et al.*, 2005), monkeys (*Macaca mulatta* and *M. radiata*) (Bansode *et al.*, 2003), mice (*Apodemus speciosus*) (Kuwahara *et al.*, 2000), horses (*Equus caballus*) (Johnson, 1991), and moles (*Talpa europaea* and *T. occidentalis*) (Dadhich *et al.*, 2010), although in the roe deer (*Capreolus capreolus*) (Blottner *et al.*, 1996; Schön *et al.*, 2004) and the red fox (*Vulpes vulpes*) (Andersen Berg *et al.*, 2001) a complete cessation of meiosis onset during the non-breeding season was reported.

As indicated above, spermatogenesis is controlled by the HPG axis. In the male, testosterone is the main feed-back molecule produced by the testis that targets specific neuronal cells in the hypothalamus and pituitary. Most circulating testosterone is produced in the testis by Leydig cells, whose number is regulated by the action of LH and FSH. The amount of testosterone produced is regulated by LH, which controls the expression of the enzymes necessary for testosterone biosynthesis (Payne and O'Shaughnessy, 1996).

Many authors have reported that mammalian testosterone is involved in the control of spermatogenesis, and that it is required for normal spermatogenesis in both quantitative and qualitative terms. Accordingly, in rats, a complete withdrawal of intratesticular testosterone via complete Leydig cell depletion using the Leydig cell-specific cytotoxin ethane-dimethane-sulphonate (EDS), resulted in a complete failure of the spermatogenesis. However, further treatment of the EDS-injected rats with testosterone rescued partially the normal testicular phenotype including maintained testicular weight and increased number of germ cells and diameter of seminiferous tubules at stage VII. In addition to testosterone, FSH also seems to cooperates in maintaining spermatogenesis functionality. Analysis of hypophysectomized and EDS-injected rats, further treated with either FSH or testosterone alone indicated that FSH partially supports spermatogenesis up to the development of round spermatids whereas testosterone is capable of maintaining spermatid development at all 14 stages of the cycle. But when FSH and testosterone were administered together, synergistic effects upon spermatogenesis were observed, being greater than the response expected if their individual effects were simply additive. Thus, it seems that FSH may play a role in normal spermatogenesis and that this role is essential to increase the response of the testis to testosterone (Sharpe *et al.*, 1988).

Testosterone is a signaling molecule that exert its effect by binding to its intracellular receptor, the androgen receptor (AR). In the testis, AR is expressed in peritubular myoid cells, in Leydig cells and in Sertoli cells (Sar *et al.*, 1990). Therefore, it seems that Sertoli cells are the major transducer of testosterone signals required to support germ cell survival and spermatogenesis. When bound by its ligand, the AR translocates into the nucleus where it binds to specific DNA sequences called androgen response elements (AREs), resulting in

the recruitment of co-activators, and the regulation of gene expression (Shang *et al.*, 2002). As it happens in the absence of testosterone, the absence of AR impairs spermatogenesis. In mouse models lacking AR, expression in all tissues (ARKO mice), spermatogenesis is interrupted during meiosis and germ cells beyond the spermatocyte stage are rare (Yeh *et al.*, 2002). In mice in which AR is knocked out selectively in Sertoli cells (SCARKO mice), spermatogenesis rarely progresses beyond the diplotene spermatocyte stage (De Gendt *et al.*, 2004; Tsai *et al.*, 2006). However, with the exception of the *Rhox5* (Pem) homeobox gene (Lindsey and Wilkinson, 1996), little is known about the targets of testosterone signaling in Sertoli cells. In addition to this classic mechanism of action, testosterone may also act via two additional non-classical pathways in Sertoli cells. In the first one, testosterone stimulation depolarizes Sertoli cells and cause calcium influx into them. In the second one, testosterone activates a series of kinases resulting in the activation of the MAP kinase pathway and phosphorylation of the CREB transcription factor (FSH and testosterone signaling in Sertoli cells, (Walker and Cheng, 2005). Thus, in summary testosterone regulates spermatogenesis through two main mechanisms: first, by regulating its level of expression in the feed-back loop that forms with gonadotropins in the HPG axis, and second, this level of testosterone, together with the levels of FSH, regulate spermatogenesis through Sertoli cell signaling (Zhang *et al.*, 2003; Spaliviero *et al.*, 2004; Lindsey and Wilkinson, 1996; Johnston *et al.*, 2004) (Fig. C.2).

In seasonal breeders, spermatogenesis is normally interrupted during the non-breeding season, a process which is normally accompanied of a decrease in the levels of serum-testosterone, as shown in the roe dear, *Capreolus capreolus* (Blottner *et al.*, 1996), rhesus monkeys (Conaway and Sade, 1965), japanese wood mouse, *Apodemus speciosus* (Kuwahara *et al.*, 2000), the beach vole, *Microtus breweri* (Adams *et al.*, 1980), stallions (Johnson, 1985), gray mouse lemur, *Microcebus murinus* (Petter-Rousseaux and Picon, 1981), hedgehog, *Erinaceus europaeus* (El Omari *et al.*, 1989) and moles, *T. occidentalis* (Dadhich *et al.*, 2013). This observation leads to the conclusion that during the non-breeding season particular environmental cues influence the HPG axis function, so that LH and FSH are downregulated. This, in turn, will result in low level of testosterone, no Sertoli cell signaling and spermatogenesis arrest, a situation that would be reverted in the next breeding season.

C.4 Seasonal changes in the blood testis barrier and the expression of testicular cell-adhesion molecules

In the adult testis, Sertoli cells establish the so-called blood testis barrier (BTB), that physically divides the seminiferous epithelium into a basal compartment (outside the barrier) in which spermatogonia, preleptotene, and leptotene spermatocytes reside and an adluminal or apical compartment (inside

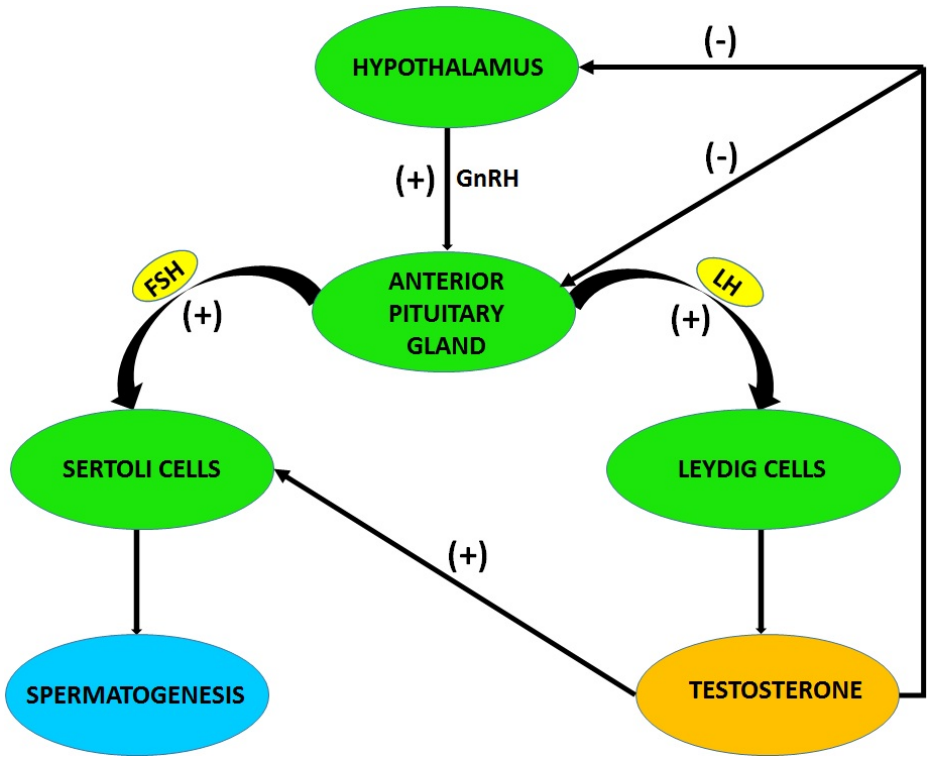


Figure C.2: Schematic representation of the interactions of the HPG axis-molecules in the control of spermatogenesis

the barrier), in which meiotic spermatocytes, spermatids and spermatozoa are located (Russell and Clermont, 1977). The BTB is formed by three morphologically and functionally distinct types of cell junctions (Fig. C.3)

1. **Occluding (tight) junctions**, that contain the following cell-adhesion molecules:

   - Tight junction integral membrane proteins, including occludin, occludin-1B, and various claudins (1, 3, 4, 5, 7, 8, and 11).
   - Tight junctions-associated proteins, including zona ocludins (1-3), cingulin, 7H6 antigen, symplekin, actin, ZAK, ZA-1 TJ, and AF-6.

2. **Anchoring (adhering) junctions**. There are four types (Fig. 3.2):

   - Cell–cell actin-based adherens junctions: N-cadherin, E-cadherin, F-actin, tensin, a-actinin, a- y b-catenin, vinculin, plakoglobin.
   - Cell–matrix actin-based focal contacts: Integrins (at least 20 different integrins are known to exist; but only a1-, a3-, a 5-, a 6-, a 9-, b 1-, and b 4-subunits have been positively identified in the testis), actin, a-actinin, vinculin, talin, nexilin, paxillin, zyxin, tensin, p130cas, Src kinase, FAK, Grb2, collagen, fibronectin, laminin a1 and b 1.

- Cell–cell filament-based desmosomes; desmocollins, desmogleins, desmoplakin, plakoglobin, plakophilin, p120ctn .
- Cell–matrix intermediate filament based hemidesmosomes: Integrin, desmoplakin-like protein, paxillin

3. **Communicating junctions (gap junctions)**, including Connexins 26, 30.3, 31, 31.1, 32, 33, 36, 37, 40, 43, 45, 46, 50, and 57.

During spermatogenesis, spermatocytes must pass through the barrier to gain entry to the adluminal compartment where meiosis can be completed. This process implies Sertoli–Sertoli and Sertoli–Germ cell interactions at the level of cell junctions, and the BTB acts as a dynamic structure that undergoes cyclical changes of "opening" and "closing" to facilitate germ cell migration (Fig. C.3). This event has to be regulated and involves a complex network of signaling cascades and the rapid turnover of junction-associated molecules. The BTB also is of great physiological importance, and selectively permit passage to some molecules that can enter the adluminal compartment. Moreover, it is an immunological barrier to segregate post-meiotic germ cell antigens from the systemic circulation; it creates a unique micro-environment for germ cell development and confers cell polarity. Thus, when the BTB is dysfunctional, germ cell differentiation and development are arrested (Wong and Cheng, 2005).

The molecular pathways that regulate tight junction dynamics in the testis are poorly understood. To date, different signal transduction pathways are implicated in the regulation of this process including protein kinases, protein phosphatases (Balda *et al.*, 1993; Citi, 1992; Li *et al.*, 2001, 2000; Nigam *et al.*, 1991; Nilsson *et al.*, 1996), intracellular Ca_2 (Nigam *et al.*, 1992; Stuart *et al.*, 1996, 1994), G proteins (Balda *et al.*, 1991; Itoh *et al.*, 1993), calmodulin, cAMP, and phospholipase C (Balda *et al.*, 1991; Janecki *et al.*, 1991).

An interesting observation is that in many cases this perturbation is reversible: when the factor(s) causing the disturbance of of the BTB function disappear, both BTB and spermatogenesis are completely restored. This is the case when one of the key tight junction molecules is perturbed . Occludin is an intercellular adhesion molecule (Schwarze *et al.*, 1999) known to confer direct cell-cell adhesion that likely associates with ZO-1 at a stoichiometric ratio of 1:1 (Furuse *et al.*, 1994). This, in turn, associates with cadherin via fodrin (Cereijido *et al.*, 2000; Provost and Rimm, 1999). Therefore, changes in occludin disrupt anchoring junctions because there are cross-talks between components of the tight junctions and the cadherin-catenin system in the anchoring junctions (Kniesel and Wolburg, 2000; Troxell *et al.*, 2000). Accordingly, intratesticular injection of a purified synthetic occludin peptide caused a reduction in testicular size and weight of 50% in rats. In the occludin-treated testes a massive depletion of germ cells took place and consequently, the seminiferous tubules shrunk significantly, with a significant reduction of tubular diameter 27 days after occludin peptide treatment. In contrast, by

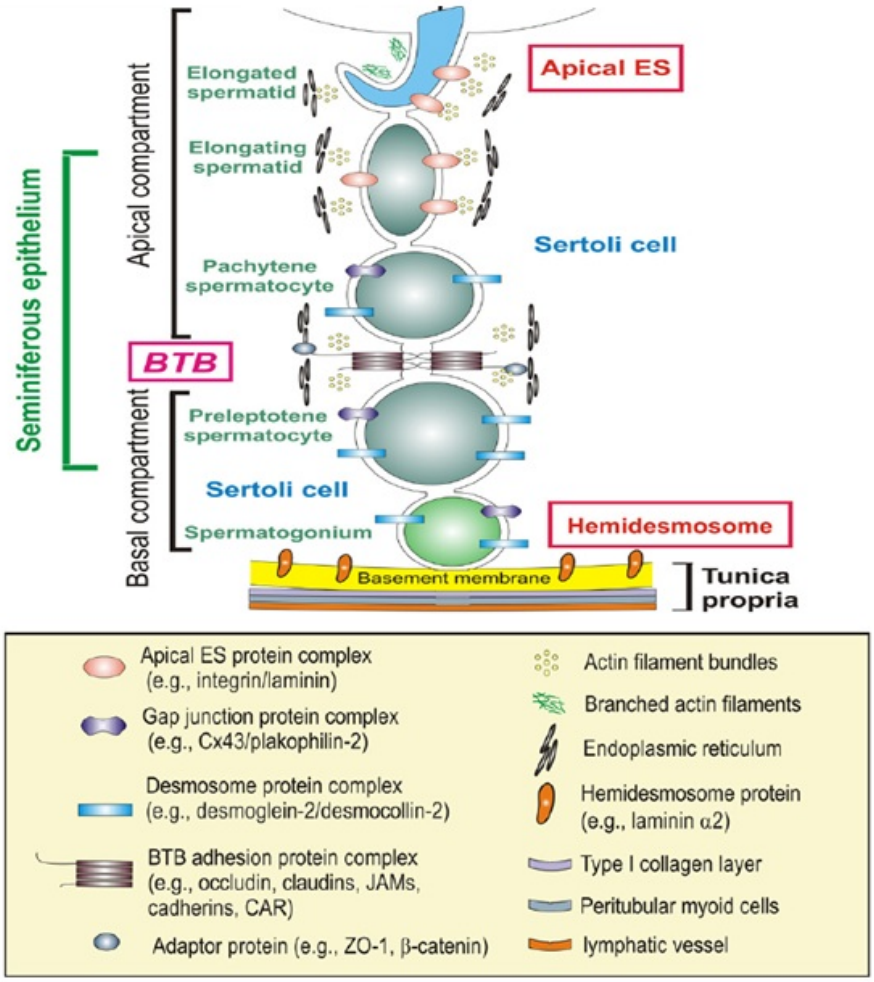


Figure C.3: Schematic drawing illustrating the relative locations of the different types of meiotic germ cells and junctions complexes found in the seminiferous epithelium. The BTB divide the seminiferous epithelium in the basal and the adluminal comparment, and only when germ cells are in the preleptone stage pass through it.

47 days, spermatocytes were visible again, and by 68 days, the morphology of the seminiferous epithelium appeared indistinguishable from controls thus showing full recovery (Chung *et al.*, 2001).

Reversible alterations of the BTB have been also described in the testis of seasonal breeders. In the adult mink, the reproductive cycle comprises two spermatogenic phases, one active lasting 9 months (in turn divided in the first spermatogenic wave, the peak of spermatogenic activity, and the last spermatogenic wave) and an inactive phase lasting 3 months. The BTB cyclically decay during the last wave of the active spermatogenic phase and is reformed during the first wave of the next active phase. The decay and the reformation of the barrier were not coincident with the appearance or disappearance of a particular generation of the germ cell population from the germinative epithelium but were correlated with cyclic cytological changes in Sertoli cells and the rhythmic development and occlusion of the lumen. These observations indicate that during the last spermatogenic wave of the adult mink reproductive cycle, the development of germ cells probably is possible in the absence of a competent, impermeable blood-testis barrier. These results indicate that the transient presence of a permeable epithelial barrier probably does not initiate an autoimmune response of sufficient magnitude to induce the destruction of the germinative epithelium (Pelletier, 1986). Seasonal changes in the BTB have been also described in the Iberian mole, *Talpa occidentalis*. In this species, a lack BTB structures was observed by electron microscopy in the inactive testes. In addition injection of a biotin tracer into the interstitial space of both active and inactive mole testes showed that the tracer was located in the interstitial region and in the basal compartment of testis tubules, but no positive signal was found in the adluminal compartment whereas the biotin tracer was found in the interstitial region and throughout the entire germinative epithelium of the inactive tubules, reflecting a decay of the BTB (Dadhich *et al.*, 2013).

During testicular function, cell-adhesion molecules are not only important for the formation of the BTB, but also play a central role in Sertoli-germ cell interaction and in Sertoli-Sertoli cell adhesion in different locations of the BTB. These interactions are also essential for the migration of the germ cell from the basal to the adluminal compartment (Fig. 3.2). Among these cell-adhesion molecules between Sertoli and germ cells we find desmosome complexes, formed by molecules such as demoglein-2 and desmocollin-2 and, gap junctions proteins, such as connexin 43 and placophilin-2. In the apical region of the germinative epithelium there are apical ectoplasmatic specialization complexes formed by molecules such as integrin and laminin. Since many of these molecules are involved in the formation of the BTB, alteration of the expression of this molecules can affect the functionality of the BTB, and Sertoli-Sertoli and Sertoli-germ cell adhesion. In the Iberian mole, have been shown that the pattern of expression for some of these molecules change between the active and inactive seasons (Dadhich *et al.*, 2013). In this species, the expression patterns of several cell-adhesion molecules appear clearly al-

tered in the inactive testis, including Connexin 43, beta-catenin, N-cadherin, and E-cadherin, which are components of the multi-protein complexes forming ectoplasmatic specialization and other Sertoli-germ cell adhesion complexes, indicating that the control of the expression of these molecules may play an essential role in the mechanisms governing seasonal breeding. The mole data indicate that disturbance of Sertoli-germ cell adhesion structures can enable the massive loss of germ cells of this species by desquamation (sloughing) at the beginning of the non-reproductive season. Similarly, in the long hairy armadillo *Chaetophractus villosus*, a seasonal breeder in which the germinative epithelium of the inactive testis contains only Sertoli and spermatogonial cells, there is a dramatic reduction in the extension of vimentin filaments associated with desmosome-like junctions at the interface between Sertoli and germ cells and the expression pattern of nectin-3, Cadm1, N-cadherin and beta-catenin is altered in the non-breeding season (Luaces *et al.*, 2014). These results suggest a testis regression mechanism similar to that described in the mole.

C.5 The effects of seasonal breeding on apoptosis and cell proliferation

Apoptosis (programmed cell death) is an important cellular process that serves to eliminate damaged, diseased, or superfluous cells from various tissues of the body during organ remodelling and differentiation (Raff, 1998; Cohen, 1999; Lockshin and Zakeri, 2004), and play essential roles in reducing cell numbers during embryogenesis, tissue homoeostasis, and elimination of expendable or potentially harmful cells. Studies that have attempted to inhibit apoptosis (pharmacological or genetic knockouts) during crucial points of development, lead to severe defects, organ dysfunction and/or death of the individual (LeGrand, 1997; Thompson, 1994; Wyllie *et al.*, 1980; Vaux and Strasser, 1996). This highly organized process is initiated by a cascade of signaling events that activate apoptotic pathways to remove target cells without causing any harmful effect to the surrounding cells (Young, 2000). Spermatogenesis is a process characterized by high proliferation rates, and coincidence of spermatogonial proliferation and spontaneous degeneration of spermatogenic cells seems to be a a normal process in the mammalian testis (Allan *et al.*, 1992; Kerr *et al.*, 1992). Thus, up to 75% of potential spermatozoa have been estimated to die in the testis of some 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, (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 occurs regularly (Allan *et al.*, 1988) 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 implies that the seminiferous epithelium generates fewer spermatozoa than would theoretically originate from spermatogonial proliferation. The comprehensive analysis of available data indicate that these spermatogonial apoptosis plays an important role in spermatogonial population 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 maintain 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 chromosomes.

Variation in the number of apoptotic germ cells have been reported in the testis of some seasonal breeders. In these species, the rate of apoptotic cells increase in the inactivating season, the season between the active and inactive periods. In this transitional season the testis undergoes atrophy, which has been associated with increased occurrence of cell death by apoptosis (Furuta *et al.*, 1994; Blottner *et al.*, 1996; Young and Nelson, 2001; Strbenc *et al.*, 2003; Sasso-Cerri *et al.*, 2006). Several reports have demonstrated that apoptosis act as a contributing mechanism to testicular regression in hamsters (Furuta *et al.*, 1994; Morales *et al.*, 2002a), 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 and completely arrested spermatogenesis 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). In the Spanish mole has been reported that apoptosis varies in a season-dependent manner in the testis, affecting mainly late zygotene and pachytene cells during the period of sexual inactivity, but not Sertoli cells. However, apoptosis is not responsible for the massive germ-cell depletion occurring during mole testis regression and a wave of spermatogonial cell proliferation probably restores the number of spermatogonia that were lost during the period of testis inactivity. These findings indicate that mammals are not a homogeneous group regarding the mechanisms by which the cell-content dynamics are regulated in the testes of males from seasonally breeding species (Dadhich *et al.*, 2010) .

C.6 Molecular mechanisms underlying germ cell depletion in the testis of seasonal breeders

Available data suggest that testicular features during seasonal breeding are regulated by the secretion of the HPG axis hormones, which, in turn, are controlled by environmental cues that favours reproduction to take place in the season in which the newborns have more probability of survival. In this work we have focused on the testicular variations during the breeding cycle of mammals, and therefore, it is expected that the change of levels of gonadotropins (FSH and LH) and testosterone between the breeding and the non-breeding season may explain the testis features of either season. Thus, the question arises as to how variations in the level of these hormones can explain the massive germ depletion shown by seasonal breeders during the inactivating season (the transition period between the active and inactive periods). Several studies have shown that testosterone regulate the rate of cell proliferation and apoptosis. Troiano *et al.* (1994) showed that apoptotic cell death was induced in EDS-trated rats, in which leydig cells are selectively destructed resulting in testosterone withdrawal and that this phenomenon was particularly evident in haploid germ cells. In humans, by analysing in vitro cultures of segments of seminiferous tubules from patients with orchidectomy for prostate or testicular cancer, it was shown that apoptosis is induced under serum-free conditions in vitro. It was also reported that this apoptosis was suppressed by testosterone, indicating that testosterone in the human male is a critical germ cell survival factor (Erkkila *et al.*, 1997). There are other reports showing that reduced levels of testosterone induces germ cell death by apoptosis (Kim *et al.*, 2001; Nandi *et al.*, 1999; Sandford *et al.*, 1984). In contrast, organ culture studies have shown that FSH, but not testosterone, is essential for the progression of type A spermatogonia up to the stage of pachytene spermatocytes (Boitani *et al.*, 1993). Taking these results into account, a plausible mechanism to explain the massive germ cell depletion occurring in the inactivating period of seasonal breeders would be that environmental cues reduce the levels of gonadotropins which in turn will reduce the levels of testosterone. This would lead to increased germ cell death by apoptosis and to reduced germ cell proliferation. In this case, the frequency of apoptotic cells must increase and reach a peak only during the period of testis regression and not later, when testis regression has finished and the testis shows a complete spermatogenic inactivity. Of course, the magnitude of the apoptosis increase must be high enough to explain a massive depletion of the germinative epithelium. Later, in the breeding season the hormone levels are restored and spermatogenesis is re-activated. This seems to be the case for a number of non-mammalian species including the Japanese red-bellied newt *Cynops pyrrhogaster* (Yazawa *et al.*, 1999, 2000), the Chinese soft-shelled turtle *Pelodiscus sinensis* (Zhang *et al.*, 2008), the Japanese jungle crow *Corvus macrorhynchos* (Islam *et al.*, 2012), the American crow *Corvus brachyrhynchos* (Jenkins *et al.*, 2007), and the European Starling *Sturnus vulgaris* (Young *et al.*, 2001). However, there

is a number of seasonal breeders in which testis regression was associated with apoptosis, but no conclusive data were reported to assure that it is the cause underlying germ cell depletion. In some instances, an increase in the number of apoptotic cells was observed when the testis was already inactive, after germ-cell depletion had taken place, as in the bullfrog *Rana catesbeiana* (Sasso-Cerri *et al.*, 2006), the white-footed mouse *Peromyscus leucopus* (Young *et al.*, 1999; Li *et al.*, 2009), the hare Lepus europaeus (Strbenc and Bavdek, 2001; Strbenc *et al.*, 2003), and the Guinea pig *Cavia porcellus* (Hingst and Blottner, 1995). In other cases, a slightly increase of apoptosis was detected in the inactive testis, but the transition period was not studied, as in the Syrian hamster *Mesocricetus auratus* (Morales *et al.*, 2002b, 2007). Also in this species, chronic treatment with diethylstilbestrol (DES), a stilbene estrogen acting as an agonist of estradiol-17J3, drastically reduces both FSH and testosterone, leading to a subsequent highly significant surge (10-50-fold) in germ-cell apoptosis, which may explain the germinative-epithelium depletion (Nonclercq *et al.*, 1996). However, this severe experimental situation does not reflect the natural conditions of seasonal testis regression in this species. In the Djungarian hamster *Phodopus sungorus*, the method used to quantify the incidence of apoptosis does not ascertain whether the 5-fold increase observed was high enough to explain testis regression by itself. In addition, there are species in which it has been shown that apoptosis is not the cause underlying testis regression such as the roe deer *Capreolus capreolus* (Blottner *et al.*, 2007) and the Iberian mole *Talpa occidentalis* (Dadhich *et al.*, 2010). Hence, apoptosis cannot explain germ cell depletion during the inactivating period of all the seasonal breeders, and new models are necessary to explain these cases. We have already mentioned that the levels of testosterone affect spermatogenesis, mainly through Sertoli cell signaling. But little is known on how this effect is exerted. Several studies have shown that testosterone promotes Sertoli-Sertoli and Sertoli-germ cell adhesion. Mice with a conditional deletion of the androgen receptor (ARs) in Sertoli cells were infertile and they showed a loss in BTB functionality which was coupled with a reduction in the levels of tight junctions proteins such as occludin and claudins (Strbenc *et al.*, 2003; Blottner *et al.*, 2007). Besides, withdrawal of testosterone resulted in the detachment of developing spermatids from Sertoli cells in the seminiferous epithelium, demonstrating that testosterone is important for cell adhesion (Dadhich *et al.*, 2010; Yan *et al.*, 2008). Moreover, Yan et al., have demonstrated that the integral membrane proteins occludin, JAM-A, and N-cadherin were continuously endocytosed and recycled back to the Sertoli cell surface via the clathrin-mediated pathway, and that testosterone accelerated the kinetics of internalization of BTB proteins from the cell surface and thereby opening the BTB. This indicates that the BTB dynamics is regulated by testosterone via their differential effects on the kinetics of protein endocytosis and recycling in Sertoli cells (Yan *et al.*, 2008). These observations clearly suggest that variations in testosterone levels can alter cell-adhesion molecules expression. Thus, it is conceivable that during seasonal breeding the variations in the levels of testosterone between the active and inactive periods alter

the expression of cell adhesion molecules that, in turn, would control the reproductive status of the male. Studies in the mole *Talpa occidentalis* have shown that this is the case for this species, and a new model to explain testis regression has been proposed. In this species the mechanism of testis regression is a massive desquamation of the germ cells that are eliminated through the epididymis during the inactivating period. In addition, the BTB is not functional. To explain this phenomenon, Dadhich *et al* proposed that the low levels of intra-testicular testosterone alters the expression of cell-adhesion molecules. In this situation Sertoli-Sertoli and Sertoli-germ cell adhesion is relaxed resulting in the desquamation of meiotic and post-meiotic germ cells of the adluminal compartment. During this process the BTB maintain its functionality. Only later, once the adluminal compartment is almost empty and spermatogenesis does not proceed beyond the primary spermatocyte stage, the disassembly of the BTB takes place. Under this situation, mature germ cells are never exposed to the basal environment, thus preventing auto-immunization that would lead to permanent infertility (Dadhich *et al.*, 2013).

C.7 The need of additional studies in new seasonal breeding mammals

Few seasonal breeding mammals have been analysed to date in order to investigate their mechanisms of testis regression. Moreover, most studies are incomplete as in many cases they are only focused on one particular aspect of the entire process (apoptosis, hormonal variations, ultrastructure, morphological changes, dynamics of cell adhesion molecules). Comprehensive studies including all these features have been performed only in the Iberian mole (Dadhich *et al.*, 2010, 2013) and the long hairy armadillo (Luaces *et al.*, 2014). Hence, additional seasonal breeding mammalian species must be investigated in order to elucidate if either there is a conserved mechanism of testis regression in mammals or there are several alternative mechanisms that can operate in different species or circumstances. Micromammals are ideal species for this kind of studies as multiple captures of wild animals have to be done in order to get statistical significance in the comparisons between different seasons required for many reproductive parameters. In the province of Granada, at the South of the Iberian Peninsula, several species of micro-mammals live which are supposed to have seasonal reproduction according to the latitude of this region. Nevertheless, no in depth study of seasonal breeding has been reported in these species to date. Thus, we have performed a detailed morphometric, histological, hormonal and molecular study of the testicular variations of 4 species of micro-mammals living in this region.

C.8 Molecular markers for the study of testis function

Many gene products are currently known to play important roles in the main testicular functions. To gain insight in the genetic control of seasonal breeding, we have studied the expression pattern of some of these genes during the reproductive cycle of the mammalian species analysed. As follows, we will describe them:

C.8.1 *SOX9*

SOX9 expression is an excellent marker for both embryonary and adult Sertoli cells. It is a member of the Group E of *SOX* genes, which also includes *SOX8* and *SOX10*. It is a Sry-box–containing gene that encodes a transcription factor. The human *SOX9* gene encodes a protein of 509 aminoacids consisting of the HMG (high mobolity group) box, which shares 70% amino acid homology to the HMG box of *SRY* (sex determining region on the Y chromosome gene). High resolution mapping showed the cytogenetic location of *SOX9* at 17q24.3-25.1. In addition, the SOX9 protein contains additional protein domains, including two transcriptional activation domains, downstream of the HMG box. It contains a HMG domain, like that of SRY, that acts by binding to and bending specific DNA sites to activate transcription of target genes. The SOX9 protein, unlike SRY, is highly conserved beyond the HMG domain throughout vertebrate evolution. During gonadogenesis, *SOX9* is detected in the bipotential gonad, becoming male-specific after the sex determination stage. From this stage onwards, *SOX9* expression is restricted to the Sertoli cell lineage and persists in the adult (Kobayashi *et al.*, 2005). The up-regulation of *SOX9* in Sertoli cells immediately after the expression of *SRY* suggests a role for *SRY* in the activation of *SOX9* expression and now it is known that *SOX9* is up-regulated by Sry through a 5' enhancer (Sekido and Lovell-Badge, 2008). Conditional inactivation of both *Sox9* alleles in the gonadal anlagen, 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.*, 2006) and 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 (Vidal *et al.*, 2001). The SOX9 protein is known to activate target genes, being the Anti-Mullerian hormone the better characterized (De Santa Barbara *et al.*, 1998). Concerning to the expression and role of SOX9 in the testis of adult mammals, available data are scarce. In the adult rat testis, *SOX9* expression in Sertoli cells is stage-specific expressed, being maximum at the stage VIII of spermatogenesis (Fröjdman *et al.*, 2000). This stage-specific expression pattern of SOX9 in the seminiferous tubules of the adult rats suggests that SOX9 may also have a pivotal role in germ cell differentiation. This pattern of expression was also observed in adult testis of

Talpa occidentalis, which is the only seasonal breeding species in which the expression pattern of this gene has been studied (Dadhich *et al.*, 2011). In this species, we observed that Sertoli cells were more SOX9-immuno-reactive in the testes of moles captured in the season of reproductive inactivity, a fact that was confirmed by RT-Q-PCR analysis.

C.8.2 *DMC1*

DMC1 is a meiosis-specific gene that becomes active during recombination-mediated homology-dependent chromosome pairing and in the strand exchanges that result in chiasmata (Yoshida *et al.*, 1998). It has been shown that *DMC1* gene may also be expressed during mitosis in Arabidopsis (Doutriaux *et al.*, 1998), indicating a conserved role for this gene during these processes. *DMC1* is structurally and evolutionarily closely related to the *Escherichia coli RecA* gene, which is necessary for most of recombinogenic processes in bacteria including conjugation, bacteriophage-mediated transduction, plasmid recombination, and repair of DNA (Bishop *et al.*, 1992). Consistent with these observations, mouse *Dmc1* knockouts are infertile due to defects in chromosome 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 localizes to meiotic chromosomes during early prophase I, when homologous chromosomes synapse. According to these features, DMC1 is an excellent marker for early meiotic cells as it is largely known that recombination takes place in zygotene and pachytene stages of the first meiotic prophase.

In the sexually active mole, *DMC1* shows a normal expression pattern, appearing in the spermatogenic stages containing zygotene and early pachytene meiotic cells. In the inactive testes, the situation was similar as in this species meiosis initiation is not interrupted during the non-breeding season. However, meiotic germ cells die by apoptosis after they reach the pachytene stage (Dadhich *et al.*, 2011)

C.8.3 *PCNA*

Proliferating cell nuclear antigen (PCNA) is a member of the DNA sliding clamp family of proteins, which are structurally and functionally conserved from viruses to humans (Tsurimoto, 1998; Maga and Hübscher, 2003). They are found in eubacteria, archaea, and eukaryotes. Genes which encode the PCNA protein have been isolated from several eukaryotes and archebacteria and the predicted amino acid sequences have been found to be highly conserved (Bult *et al.*, 1996; Kelman and O'Donnell, 1995; Kelman, 1997). They all form ring-shaped complexes with pseudo-hexametric symmetry, encircle DNA, and are capable of sliding around on the DNA in both the 3' and 5' directions

(Dieckman and Washington, 2013). The eukaryotic sliding clamp PCNA was originally identified 35 years ago as an antigen for autoimmune disease in systemic lupus erythematosis patients. After two years, it was found to be differentially expressed during the cell cycle, peaking during S-phase, and its expression was associated with proliferation (Freudenthal *et al.*, 2010). PCNA has been found to play a critical role in several biological processes. It is functioning as an accessory protein for DNA polymerase tethering, nucleic acid metabolism, DNA replication mechanism, chromosomal DNA synthesis and DNA recombination and repair.In addition, PCNA was shown to interact with cellular proteins involved in cell cycle regulation and check point control (Dieckman and Washington, 2013). PCNA is also important for repair of damaged DNA. PCNA functions not only as polymerase co-factor during repair synthesis, but was shown to directly recruit repair factors to sites of DNA lesions and to coordinate repair events. Among the many repair pathways known, mismatch repair (MMR) is one of the best characterized for PCNA involvement. The MMR system repairs misincorporation errors generated during replication through interaction with several MMR proteins, involved at different steps of the process of replication (Moldovan *et al.*, 2006). PCNA is a marker for spermatogonia and zygotene-early pachytene spermatocytes (this gene is transiently down-regulated during leptotene) (Chapman and Wolgemuth, 1994). In the seasonal breeding Iberian mole (Dadhich *et al.*, 2011), PCNA was strongly expressed in the outer layer of cells observed in the seminiferous tubules showing a high density of DMRT1-positive cells (HDD tubules), which corresponds to spermatogonia, and more weakly in an inner layer of cells, corresponding to zygotene-pachytene spermatocytes.

C.8.4 *DMRT1*

DMRT1 (Doublesex- Mab-3-Related Transcriptionfactor 1) was originally located in the band 9p24.3 that is a critical region for sex reversal in humans (Raymond *et al.*, 1998; Shibata *et al.*, 2002). It is a highly conserved transcriptional regulator required for testicular development in vertebrates (Krentz *et al.*, 2009). *DMRT1* gene encodes a protein containing a DM domain that is highly conserved across phyla (Raymond *et al.*, 1998, 2000; Hodgkin, 2002). Genes encoding the DM domain have been described in the fruit fly (*Drosophila melanogaster*) as dsx (doublesex) and in the roundworm (*Caenorhabditis elegans*) as mab-3 (Baker and Wolfner, 1988) (Raymond *et al.*, 1998). The DM domain contains a characteristic double zinc finger motif for DNA binding, that unlike classic zinc fingers binds to the minor groove of DNA rather than the major groove (Zhu *et al.*, 2000; Murphy *et al.*, 2007; Johnsen and Andersen, 2012).

During mouse gonadal development, *DMRT1* is expressed in the genital ridges of both XX and XY embryos. At E12.5 and E13.5 *DMRT1* mRNA is observed in the developing sex cords of the testis as well as in a punctuate pattern in the ovary. At E14.5 and E15.5, *DMRT1* expression is observed

in testis Sertoli cells, but declines in the ovary. Starting at P1, DMRT1 is detectable, in addition to Sertoli cells, in germ cells and reach high levels by P7, just before meiosis begins. 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.*, 1999). In adult testis, DMRT1 is expressed in Sertoli cells and dinamically in spermatogonia with high expression in tubules that are in the initial stages of the spermatogenic cycle (Raymond *et al.*, 2000). This suggests that *DMRT1* may play a role in pre-meiotic germ cells, such as regulating entry to meiosis or controlling the mitotic cell cycle. Knockout studies indicated that murine *Dmrt1* is necessary in the male gonad for survival and differentiation of both somatic and germ-line cells, but has no essential role in sex determination (Raymond *et al.*, 2000). However, male-specific *DMRT1* expression occurs at an earlier developmental stage in the human gonad than in mouse (Moniot *et al.*, 2000) and thus *DMRT1* may play an earlier role in human testis differentiation than in the mouse. Recently it has been showed that the loss of the *DMRT1* transcription factor in mouse Sertoli cells, even in adults, reprograms Sertoli cells into granulosa cells, indicating that *DMRT1* is essential to maintain mammalian testis identity (Matson *et al.*, 2011).

In the testis of adult Iberian moles, *DMRT1* expression varies in a spermatogenic stage- and season-dependent manner. Cells expressing DMRT1 were detected at the periphery of the seminiferous tubules during the active period and the number of DMRT1-positive cells varied widely among different tubules, a situation observed throughout the year, except in the inactive period. Seminiferous tubules showing a high density of DMRT1-positive cells (HDD tubules) were mainly observed at spermatogenic stage VII, whereas tubules with low number of DMRT1-positive cells were more frequent in tubules at stage III. Double immuno-fluorescence with DMRT1-PCNA and DMRT1 and DMC1 showed that DMRT1 expression is limited to Sertoli cells and spermatogonia and that it ceases in the latter coinciding with the onset of meiosis.

C.8.5 *AR*

The androgen receptor gene (*AR*) is located in the human X chromosome (Lubahn *et al.*, 1988) and is a member of the superfamily of nuclear receptors for steroid hormones. The AR protein is located in the cytoplasm forming part of a multiprotein complex (Heemers and Tindall, 2007). When testosterone difusses in the cytoplasm of a target cell, it interacts with the AR protein, which results in phosphorilation and migration to the nucleus, where target genes are regulated through the action of specific androgenic response elements (AREs) present in their promoter region. It is known that AR promotes the expression of several genes including *SRY* (Kallio *et al.*, 1995; Yuan *et al.*, 2001). During fetal life, androgens are responsible for the development of the testes, the Wolffian ducts (that give rise to epididymides, efferent ducts and seminal vesicles), and the male external genitalia (Patrao *et al.*, 2009). Embryonic

Leydig cells produce testosterone from the 8th week of gestation in the human and from 13.5 dpc in the mouse and induces AR expression in the target cells. In the testis, AR is first expressed in the interstitial cells and also in Sertoli cells around birth (You and Sar, 1998; Rey *et al.*, 2009). In adult mice, AR can be detected in several cell types, including Sertoli, peritubular mioid, Leydig, and perivascular muscle cells. In some species it has been reported expression in germ cells (Wang *et al.*, 2009).

In testes of sexually active mole, AR is expressed in Sertoli cells, in peritubular myoid cells and in the interstitial Leydig cells, but not in germ cells. In summer inactive testes, the AR expression pattern was similar to that shown in active testes, but the intensity of the immuno-histochemical signal was weaker, an observation confirmed by RT-Q-PCR, which showed a 2-fold reduction in the levels of AR transcripts with respect to those in active testes.

C.8.6 *P450scc*

Testosterone biosynthesis in Leydig cells is dependent on stimulation by the luteinizing hormone (LH) produced in the anterior pituitary. The first step of steroid hormones synthesis is conversion of cholesterol to pregnenolone in mitochondria, a reaction catalyzed by the cytochrome P450 side chain cleavage (P450scc) enzyme which is rate-limiting, and hormonally regulated (Miller, 1988). Human P450scc is encoded by the *CYP11A1* gene (Nebert *et al.*, 1991), which is expressed in the adrenals, gonads, placenta, and brain (Mellon and Deschepper, 1993). The orphan nuclear receptor steroidogenic factor-1 (SF1), 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). Biochemical and morphological studies suggest that P450scc is located on the matrix side of the inner mitochondrial membrane in the Leydig cells of the male, and in the theca cells of the female gonads (Miller, 1988).

In the testis of the Iberian mole, we found the same expression pattern. Immunohistochemiacal studies showed P450scc expression in the interstitial Leydig cells of both active and inactive testes, but the signal was also weaker in the inactive period. RT-Q-PCR analyses showed a 4.7-fold reduction in the expression levels of CYP11A1 in inactive testes, thus confirming our immuno-histochemical observations.

C.8.7 *Alpha-SMA*

In the seminiferous tubules of mammalian testes, alpha-smooth muscle actin (*alpha-SMA*) is expresed in peritubular myoid (PM) cells, which constitute their external envelop. The main function of these flat cells is to provide structural integrity and contractibility to the tubules (Palombi *et al.*, 1992)).

Also, PM cells are known to cooperate with Sertoli cells in the formation of the basement membrane (Skinner and Fritz, 1985), each cell type contributing different components of this membrane. This suggests that a subset of extracellular matrix molecules can be PM cell-specific. Alpha-SMA is thus a useful marker for PM cells. Although its expression pattern has been analyzed in the Iberian mole throughout the seasonal breeding cycle (Dadhich *et al.*, 2013), no differences were detected between the testes of active and inactive males. Thus, its possible role in seasonal breeding, if any, remains obscure.

D. Objectives

1. To determine whether the species studied experience seasonal breeding and, if so, to mark off the timing of the periods of sexual activity and inactivity.

2. To study the histological and morphometric features of the gonads in each reproductive stage to determine the functional status of the main cell types (Sertoli, Leydig, peritubular myoid and germ cells), structures (seminiferous tubules), and biological processes (spermatogenesis) throughout the seasonal reproductive cycle.

3. To study the spatio-temporal pattern of expression of several genes involved in testis function in active and inactive adult males of the species under study.

4. To study the serum levels of testosterone and the expression pattern of its receptor and biosynthesis enzymes in the testes of active and inactive males.

5. To study the possible role of both apoptosis and cell proliferation in the the process of testis regression in the studied species.

6. To study the role of cell junctions in the seasonal dynamics of the germ cells throughout the seasonal breeding cycle in these species.

7. To look for associations between altered gene expression patterns and seasonal testis regression in these species, in order to establish possible roles for these genes in the control of seasonal breeding.

D. OBJECTIVES

E. Material and methods

E.1 Material analysed

Hola Adult males of four small mammalian species have been analysed in this study: the greater white-toothed shrew, *Crocidura russula* (n=31), the Algerian mouse (also called estern Mediterranean mouse), *Mus spretus* (n=41), the wood mouse, *Apodemus syvaticus* (n=31) and the Mediterranean pine vole, *Microtus duodecimcostatus* (n=58). All females were excluded from the study although their reproductive status was checked and recorded (pregnancy, lactation). Similarly, juvenile males, identified on the basis of low body and testis mass, were also excluded, but the capture date was also recorded. Captures were performed with permission of the Andalusian Environmental Council (*Consejería de Agricultura, Pesca y Medio Ambiente*). This study was conducted following the guidelines and approval of the Ethical Committee for Animal Experimentation of the University of Granada.

E.2 Animal capture methods

All animals were captured alive, using several capture methods depending on the species. Shrews and Algerian mice were captured using small plastic trip trap (NHBS ref. 176705) filled with dry grass and baited with pieces of sardines conserved in oil, grain barley, apple, bread coated with crude olive oil, and live *Tenebrio* larvae. This way, captured animals were provided with warming material and food enough to remain alive until collected. Wood mice were captured using both medium-sized Hipólito type traps and Havahart SHA1020 two door mouse cage Traps, baited with pieces of appel and bread coated with crude olive oil. Mediterranean pine voles were captured using subterranean one-door, cylindric wire-mesh traps designed and made by Dr. Rafael Jiménez (University of Granada), baited with pieces of apple, carrot and/or potato. In the cold months (late autumn, winter and early spring) the traps were set at daytime whereas in the warm months (late spring, summer and early autumn) captures were done at night. Captures were done in several populations of the Granada province (south-estern Spain) located in the localities of La Malaha, Las Gabias y Santa Fe (see Fig.E.1 and table E.1).

Localidad	latitude	longitude	high	number in map
Santa Fe	37° 21' N	3° 78' W	553	5
Las Gabias	37° 13' N	3° 69' W	701	1
Chimeneas	37° 11' N	3° 77' W	765	4
La Malaha	37° 09' N	3° 37' W	730	2
Escúzar	37° 09' N	3° 74' W	755	3

Table E.1: Localities and their geographic coordinates where the animals used in this study were captured

Figure E.1: Map of the zone where the animals used in this study where captured. Numbers indicate the approximate localization

E.3 Tissue collection

Animals were killed by cervical dislocation, weighted, dissected and the testes were collected and weighted aseptically. One of the gonads was frozen in liquid nitrogen for further mRNA purification, whereas the other one was fixed in 50 volumes of Serra's fixative overnight at 4 °C, dehydrated with an ascending series of alcohol, cleared with xylene and embedded in paraffin for routine histology and immuno-histochemistry. Blood was collected immediately after death by cardiac puncture for hormone measurements by radio-immunoassay.

E.4 Paraffin embedding and sectioning

Testes were fixed in serra overnight. 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.

E.5 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.

E.6 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 (for dilution see table E.2) 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.

E.7 Double 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 antibodies (diluted in 1% BSA and 1×PBST, (for dilution see table E.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. The following pair of secondary antibodies were used at a dilution 1:2º00:

    - For PCNA-DMRT1 double IF we used alexa fluor goat anti-mouse 488 and alexa fluor goat anti-rabbit 555
    - For SOX9-DMRT1, DMC1-DMRT1 and CLAUDIN11-DMC1 double IF we used alexa fluor donkey anti-goat 488 and alexa fluor donkey anti-rabbit 555

    They where covered with coverslips and kept in the dark at RT for 1.5–2 hours.

12. Slides were washed tree times with PBST for 10 minutes each and after was added 0.1% Sudan Black for 10 min.

13. Slides were washed tree times with PBST, treated with DAPI (300 nM) for 10 min and washed again tree times with PBST.

14. 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.

Table E.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
SOX9 (Michael Wegner)	SRY related high mobility group box 9	Rabbit polyclonal raised against human protein	1:200
SOX9 (Santa Cruz)	SRY related high mobility group box 9	Goat polyclonal raised against human protein	1:200
DMRT-1	DoubleSex- and Mab3 related transcription factor-1	Rabbit polyclonal raised against human protein	1:1000
PCNA (SantaCruz)	proliferating cell nuclear antigen Pre-meiotic germ	mouse monoclonal raised against human protein	1:400

Table E.2: Primary antibodies used in this study

Gene product (Reference)	Description	Source of antibody	Primary Antibody dilution
	cell marker.		
P450scc (SantaCruz)	Cytochrome P450scc, a steroidogenic enzyme	goat polyclonal, raised against human protein	1:200
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
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
Desmin (Sigma)	Peritubular myoid cell marker	Mouse monoclonal raised against human protein	1:200

E.8 Radioimmunoassy (RIA) for Testosterone level measurement

Blood collected from males was incubated at 4°C for overnight to permit coagulation and then centrifuged at 6 000 rpm for 20 min at 4°C. The supernatant (serum) was collected with a pipette and stored at -80°C.

Radioimmunoassay for *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, 50 μl 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, 500 μl of tracer (I^{125}-labeled testosterone) was added and mixed well. To

obtain total cpm, 500 μl of tracer was added to 2 additional tubes. All the tubes were then incubated for 3 hours at 37°CÀll 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%.

E.9 Terminal deoxynucleotidyl transferase (TdT) dUTP Nick- End Labeling (TUNEL) assay

We performed TUNEL assay on paraffin embedded tissue sections. The protocol is as follows:

1. Heat the slides at 55°C for 20 min.
2. Dewax twice for 10 min. in Xylene on a shaker.
3. Rehydratate in an ethanol series (100% ethanol 5 min, 70% ethanol 5 min, and 50% ethanol 5 min) and then washed twice in PBST.
4. Incubate for 30 min. at 37°C in a moist chamber with Proteinase K [10-20 µg/ml in 10 mM Tris/HCl, pH 7. 4 - 8].
5. Wash twice for 10 min with PBST.
6. Incubate with 50 µl of TUNEL reaction mix a in dark humid chamber for 60 min at 37°C.
7. Wash twice with PBST for 10 min.
8. Add Sudan Black B 0.1% in 70% ethanol in a dark humid chamber for 10 min.
9. Wash twice with PBST for 10 min. in the dark.
10. Counterstain with DAPI for 10 min. in the dark.
11. Wash three times with PBST for 10 min. in the dark.
12. Mount the slides with Vectashield mounting medium, and analyse the slides under the fluorescent microscope.

E.10 BTB Permeability Experiments

We performed the following protocol:

1. Animals were anesthetized with 0.125% avertin (2,2,2-tribromo-ethanol)

2. The testes were exposed, and a total of 50 ul of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co.) freshly diluted in PBS containing 1 mM CaCl2 was placed beneath the tunica albuginea, injecting at several points of the left testis.

3. The right testis was also treated in a similar way, but only PBS with 1 mM $CaCl_2$ was injected in this case as a control.

4. The testes were placed again inside the body of the animals, which were killed 30 min after the tracer injections.

5. The testes were immediately removed and fixed overnight in serra.

6. Treated and control testes were dehydrated, embedded in paraffin (Paraplast plus), and sectioned (7 um thick) according to standard procedures.

7. After deparaffination and rehydration, the tracer was detected by incubating the sections for 30 min with an Alexa Fluor 568-conjugated streptavidin solution (included in the tracer kit) at 25°C.

8. For double detection of the tracer and claudin11, immunoflu- orescence for claudin 11 was performed prior to the tracer-detection technique as described above.

9. In any case, the sections were finally rinsed twice with PBS for 10 min, stained with DAPI, rinsed four additional times with PBS (20 min each), and mounted in Vectashield medium (Vector Labs).

E.11 Solutions

Serra fixative

Mix the following:

60% paraformaldehyde 37%
30% ethanol
10% acetic acid

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

Eosin

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.

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.

proteinasa K solution

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

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

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.

E. MATERIAL AND METHODS

1. First chapter. *Crocidura russula*

Figure 1.1: *Crocidura russula*. Image reproduced with permission of the autor: Mario Cea

1.1 Introduction

True shrews are soricid mammals belonging to the order Eulipotyphla. Shrew species of both Palearctic and Nearctic ecozones have been shown to reproduce seasonally. For instance, the European common shrew, *Sorex araneus* (Brambell, 1935) and the North American short-tailed shrew, *Blarina brevicauda* (Getz *et al.*, 2004), concentrate their reproductive effort during the non-winter seasons. Contrarily, equatorial and tropical shrews reproduce at all times of the year, as observed in the Asian musk shrew, *Suncus murinus* see (Temple, 2004) and in two syntopic white-toothed shrews from Taiwan, *Crocidura attenuata* and *C. kurodai* (Yu *et al.*, 2001), among others.

The greater white-toothed shrew, *Crocidura russula*, which is widely distributed in Western Europe and northwestern Africa, exhibits some exceptional reproductive and population features. It is the only known social shrew species, forming small communally nesting groups in winter and territorial monogamous pairs during the breeding season (Cantoni and Vogel, 1989). The breeding strategy of this species, based on monogamy and male philopatry, together with incomplete female dispersal, permits some degree of coancestry derived from inbreeding, as shown in a population in Switzerland (Balloux *et al.*, 1998). Contrarily, studies based on genetic assignment methods showed that males were less monogamous than expected from previous behavioral studies and that *C. russula* populations exhibit high levels of genetic diversity (Bouteiller and Perrin, 2000). This species is also widely distributed in the Iberian Peninsula (Niethammer and Krap, 1990). Its reproductive features have been investigated in the Ebro Delta region (López-Fuster and Ventura, 1992; López-Fuster *et al.*, 1985), where the breeding season extends from February to September with a clear decrease in sexual activity from August onwards.

According to current knowledge on the reproductive biology of shrews in other European regions (Brambell, 1935; López-Fuster *et al.*, 1985; Jeanmaire-Besancon, 1988; López-Fuster and Ventura, 1992), we hypothesize that southern Iberian populations also reproduce seasonally. No study available to date has described the circannual variations of the testes in males of any shrew species, so that the mechanisms of testis regression operating in Soricidae remain unknown. Similarly, no gene-expression studies in shrew testes have been performed, either. In this chapter, we analyse the testicular histology and gene-expression patterns in males of *C. russula* captured in populations of the south-eastern Iberian Peninsula during warm and cold seasons, the periods in which they are expected to be sexually active and inactive, respectively. The incidence of two major cell processes, i.e. cell proliferation and apoptosis, and the serum testosterone levels have also been studied in these two periods.

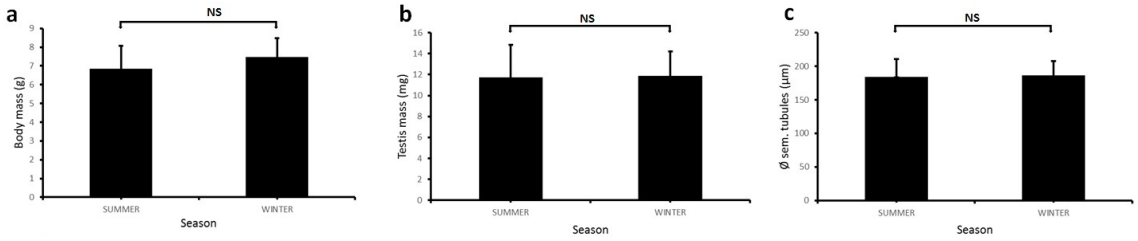


Figure 1.2: Comparison of three morphometric parameters in the winter and the summer groups of *C. russula*: a, body weight; b, testis weight; c, seminiferous tubule diameter.

1.2 Results

1.2.1 Both winter and summer shrews show similar testis morphology

Two study groups were established: 1) group of animals captured in the warmest months (from May to August, N=14), which was called the summer group; and 2) those captured in colder months (from September to February, N=17), called the winter group. Shrews from the winter group were slightly larger (7,48±0.99g) than those from the summer group (6.86±1.21 g) although these differences were not statistically significant (P=0.13; Fig. 1.2 a). Their testis masses were surprisingly similar in the two study groups, showing no significant difference between them: 11.85±2.33 mg in the winter and 11.74±3.09 mg in the summer groups (P=0.91; Fig. 1.2 b). The testis-mass/body-mass ratio was also very similar in the two groups: 0.0016±0.00037 in the winter group and 0.0016±0.00053 in the summer group (P=0.96). Similarly, the diameter of the seminiferous tubules was found to be invariable as well, showing no significant differences between the winter and the summer groups (186.1±21.3 µm and 184.1±26.6 µm, respectively; P=0.710; Fig. 1.2 c)

According to these measurements, the histology of testes and epididymides showed normal features in males of the two groups (Fig. 1.3). Both spermatogenesis and spermiogenesis appeared to be completed as all stages of the spermatogenic cycle were found in the testes of all analyzed males, and their epididymides contained abundant sperm. Signs of degeneration and/or desquamation of the germinative epithelium were absent in all cases as no spermatocytes or other round cells were observed in the lumen of either the seminiferous tubules or the epididymary tube.

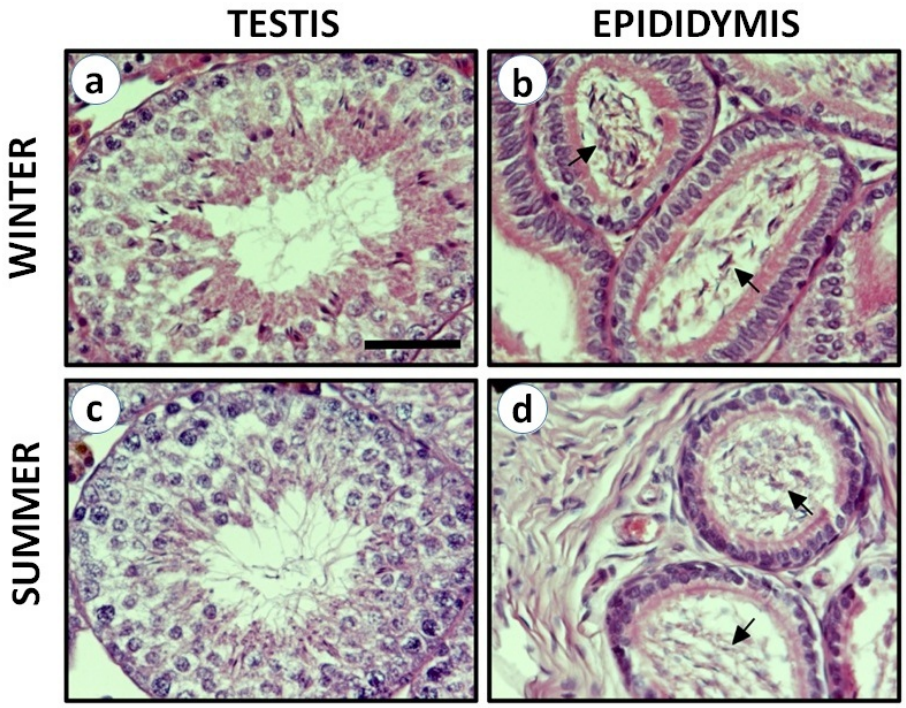


Figure 1.3: Haematoxilin-eosin-stained histological sections of testes (a and c) and epididymides (b and d) from *C. russula* males belonging to the winter (a and b) and summer (c and d) study groups. The morphology of the seminiferous tubules is the expected for sexually active males and the epididymides contain sperm (arrows) in both cases. Scale bar represents 50 µm in all pictures.

1.2.2 Expression of several genes in adult shrew testis is spermatogenic stage- but not season-dependent

We investigated in the testes of *C. russula* the expression pattern of several proteins which have previously been found to show seasonal variations in another species of the order Eulipotyphla, the Iberian mole Talpa occidentalis (Dadhich *et al.*, 2011, 2013). However, again, no differences were detected between the two study groups of *C. russula* (compare left and right columns in Fig. 1.4, although variation was patent for many of these proteins with respect to the stages of the spermatogenic cycle of this species (Parapanov *et al.*, 2007).

The SOX9 gene (SRY-containing box gene 9), a marker for Sertoli cells in the testis (da Silva *et al.*, 1996), showed clear expression variations depending on the spermatogenic cycle, as shown in rats (Fröjdman *et al.*, 2000) as well as in mice and moles (Dadhich *et al.*, 2011) (Fig 1.4 a and b). As described in the latter species, SOX9 is more intensely expressed during the spermatogonial proliferative stages of the cycle (stages I-VI) and expression is restricted to Sertoli cells. The AR protein, was detected in the nucleus of both Sertoli and Leydig cells in males of both winter and summer groups (Fig.1.4 c and d). Contrary to the pattern described in moles (Dadhich *et al.*, 2013), expression of AR in peritubular myoid cells was detectable but not conspicuous in the males of *C. russula*. Positive signal was also detected in the nucleus of some isolated spermatocytes and round spermatids, although it is not clear whether this is a specific expression. In addition, numerous focus-like signals were also observed in cells adjacent to the tubular lumen.

The Claudin11 protein, which is a principal component of the tight junctions forming the blood-testis barrier (BTB), showed a stronger expression in spermatogenic stages IV-XI and was much weaker throughout the rest of the spermatogenic cycle (Figs. 1.4 e and f). The morphology and position of the BTB, as inferred from the expression of Claudin11, was normal in comparison to those described in other species.

The expression of DMC1, a marker for zygotene and early pachytene spermatocytes (Yoshida *et al.*, 1998), was observed in stages VII-IX and absent in all the other cases, as expected from the presence of the aforementioned cell types in these spermatogenic stages. PCNA is a marker for spermatogonia and zygotene-pachytene spermatocytes, but not for the intervening stage leptotene (Chapman and Wolgemuth, 1994). The number of immunoreactive cells was lower in seminiferous tubules in stages I-VI, where only spermatogonia express this protein, and higher in the other stages, where both the spermatogonia and the zygotene-pachytene spermatocytes are simultaneously detected (Figs. 1.4 i and j). In contrast to the situation in other species, the expression in zygotene-pachytene spermatocytes was much more intense than in spermatogonial cells, which was very variable but generally weak in all stages of the spermatogenic cycle of *C. russula*. As in the case of AR, focus-like signals were also abundant in cells of the adluminal compartment, which appear to coincide with the acrosomal region of both round and elongated spermatids.

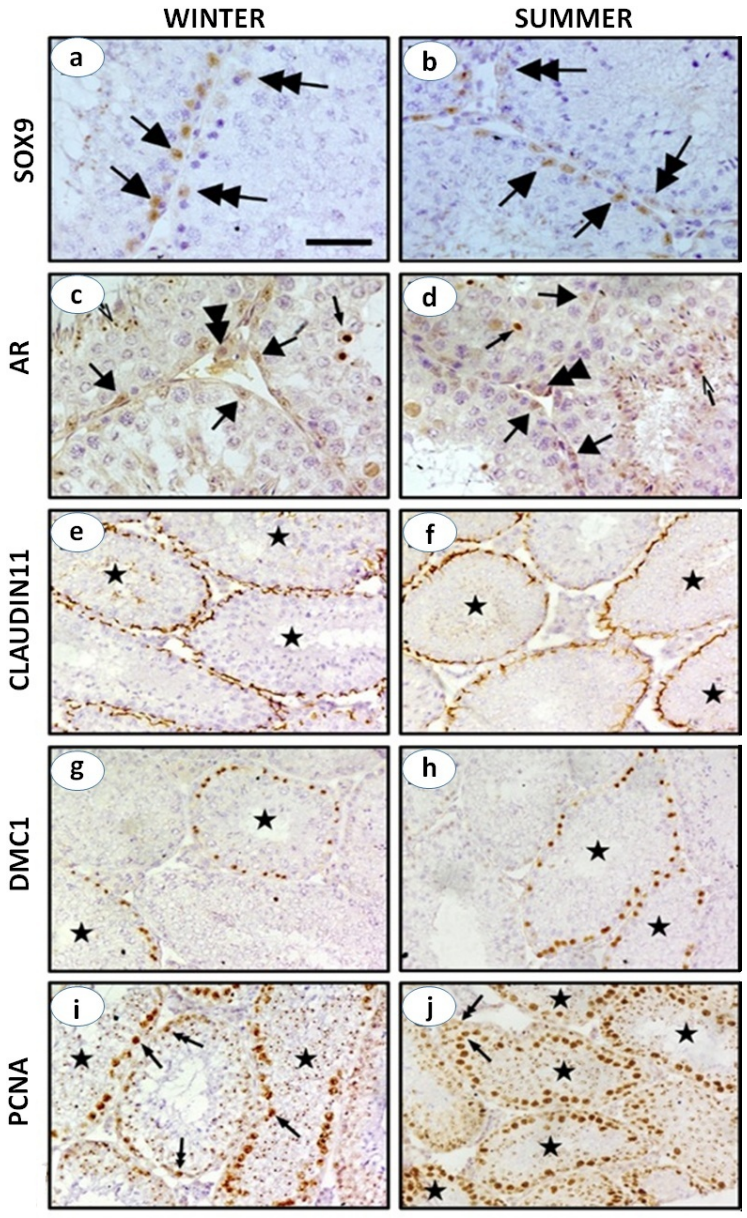


Figure 1.4: Immunohistochemical detection of several proteins in sections of testes from *C. russula* males belonging to the winter (left column) and summer (right column) study groups. No differences between both groups were detected. SOX9 (a and b) expression is strong in the Sertoli cells of the proliferative stages of the spermatogenic cycle (arrows), and very weak in the rest of stages (double arrows). AR (b and c) expression is observed in Sertoli (arrows) and Leydig cells (double arrowheads), as well as in some spermatocytes (small arrows); in addition many small foci-like signals appeared in cells adjacent to the tubular lumen (line arrows). Claudin11 (e and f) is expressed strongly in spermatogenic stages IV-XI (stars) and weakly in the rest of stages. DMC1 (g and h) expression is observed in tubule sections containing zygotene and early pachytene spermatocytes at stages VII-IX (stars). PCNA (i and j) expression was much stronger in zygotene-pachytene spermatocytes (arrows), which appear in stages I-VI (stars), than in spermatogonial cells (double arrows). Scale bar represents 15 μm in a-d and 50 μm in e-j.

Also, we studied the expression pattern of DMRT1, a protein that in T. occidentalis was shown to mark both Sertoli cells and spermatogonia, but not spermatocytes (Dadhich *et al.*, 2011). As described in the mole, two types of seminiferous tubules can be identified in the testes of *C. russula* males according to the number of cells expressing DMRT1. Seminiferous tubules containing a higher number of DMRT1-expressing cells were referred to as HDD (for high density DMRT1) and corresponded to stages VI-VII of the spermatogenic cycle (Fig. 1.5 a and b). These were present in the testes of summer as well as winter males. Similarly, we used LDD to refer to low-density DMRT1 tubules, which corresponded to stages II-IV. To identify the cell types expressing DMRT1 in the shrew seminiferous tubules, we performed double IF using three different antibody pairs: DMRT1-SOX9, DMRT1-DMC1, and DMRT1-PCNA (Fig. 1.5 c-k). DMRT1-SOX9 double IF showed that Sertoli cells expressed both proteins, but there were many other cells that expressed DMRT1 but not SOX9, thus demonstrating that they were germ cells, probably spermatogonia according to their basal position (Figs. 1.5 c-e) and previous reports (Dadhich *et al.*, 2011). This was confirmed with the DMDRT1-DMC1 double IF, as no co-localization of these two proteins was detected in any cell type, showing that meiotic germ cells (spermatocytes expressing DMC1; yellow arrows in Figs. 1.5 f-h) do not express DMRT1, whereas Sertoli and spermatogonial cells do (white arrows). Similar conclusions can be drawn from the results of the double IF using both DMRT1 and PCNA antibodies. Cells expressing only DMRT1 were Sertoli cells (red arrows in Figs. 1.5 i-k), whereas cells expressing only PCNA were spermatocytes (cyan arrows). Spermatogonial cells showed variable staining intensity with both antibodies. Those showing a stronger DMRT1 staining also showed clear PCNA staining (in no case as strong as in spermatocytes) and appeared pinkish-purple colored in the merged image, which included DAPI counter-staining (magenta arrows in Fig. 1.5 k), whereas those with very weak or no DMRT1 staining also showed weak PCNA immuno-reaction and looked blue in the same picture (white arrows).

Having previously analysed the expression of these genes in other mammalian species, we found that the number of DMRT1-positive cells per HDD tubule was clearly lower in the testes of the shrew (35.05±5.24) than in the mouse (46.20±6.07; $P<0.001$) or in the Iberian mole (92.75±20.42; $P<0.001$), as shown in Fig. 1.6. HDD seminiferous tubules contain a single layer of DMRT1-expressing cells in the shrew, whereas in the laboratory mouse or in the mole there were two or even three layers of positive cells in some cases.

1.2.3 Both cell proliferation and apoptosis show no seasonal variation in shrew testes

Since seasonal fluctuations in the germinative epithelium have been shown to be due to circannual variations in the incidence of both cell proliferation and apoptosis, we analyzed these two cellular processes in the testes of shrew males. Fig. 1.7 shows representative photomicrographs of shrew testes

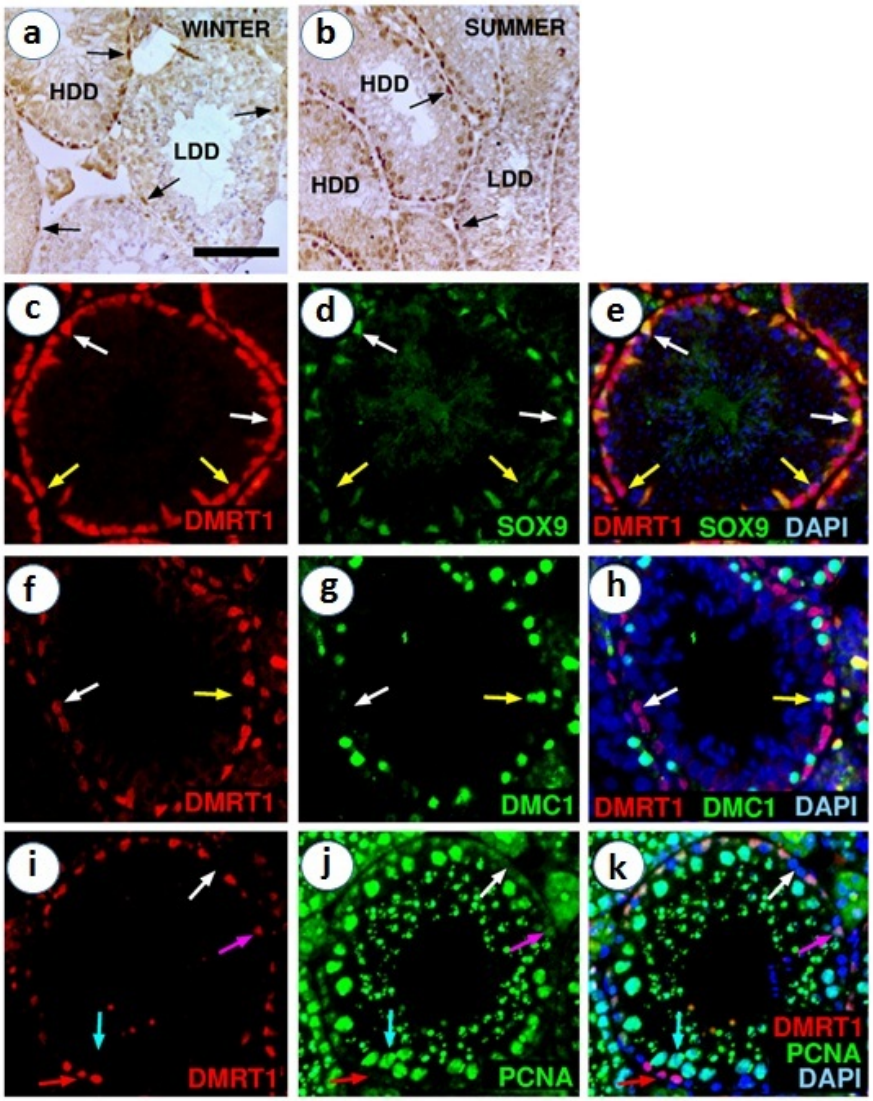


Figure 1.5: mmunostaining of DMRT1 protein in histological sections of testes from *C. russula* males. a-b, immunohistochemical detection of DMRT1 in winter (A) and summer (b) males, showing no relevant difference between the two study groups. Seminiferous tubules containing high density (HDD) and low density (LDD) DMRT1-expressing cells are seen in both cases. c-k, double immunoflurescence of DMRT1 (red) with SOX9 (c-e), DMC1 (f-h), and PCNA (i-k) (green), counter-stained with DAPI (blue). c-d, DMRT1 co-localizes with SOX9 in Sertoli cells (white arrows), showing that the other immuno-reactive cells (yellow arrows) are germ cells. f-h, DMRT1 does not co-localize with DMC1 in any cell type, showing that the germ cells expressing DMRT1 are spermatogonia. i-k, DMRT1 co-localizes with PCNA in spermatogonia but not in both Sertoli cells (red arrows) and spermatocytes (cyan arrows); spermatogonia showing strong (magenta arrows) and weak (white arrows) expression of both DMRT1 and PCNA were observed. Scale bar represents 100 µm in all pictures.

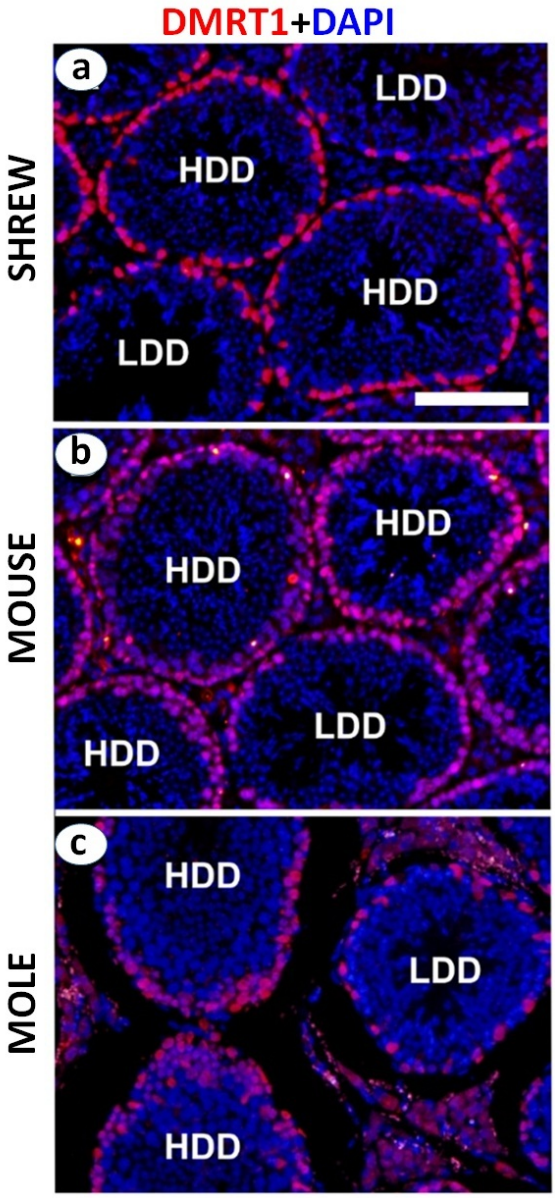


Figure 1.6: Comparison of the abundance of DMRT1-positive cells in testes from shrew (a), mouse (b) and mole (c) males. These cells are more abundant in mice and moles than in shrews, including both HDD and LDD tubules (see text). Scale bar represents 100 µm in all pictures.

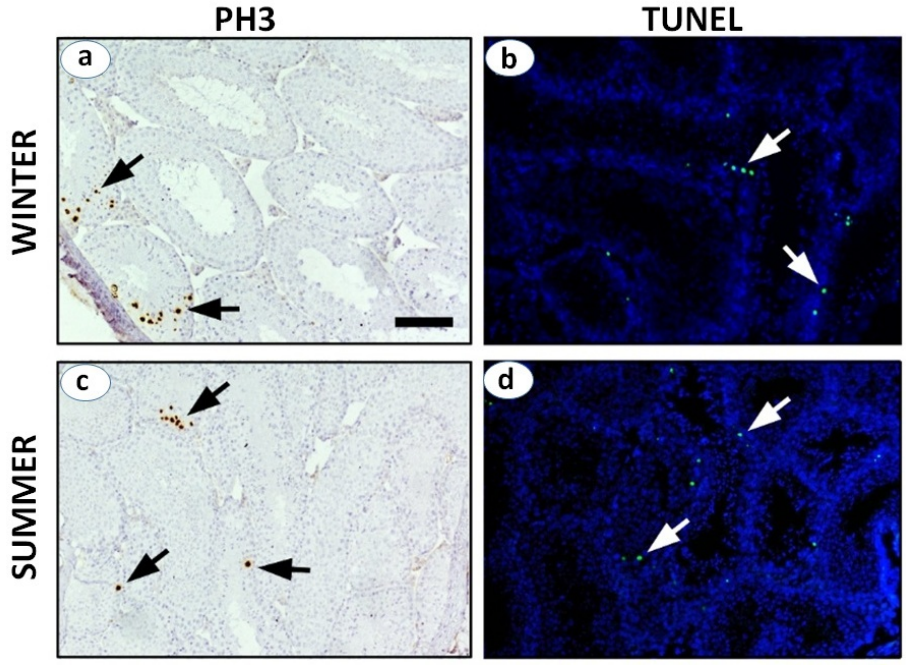


Figure 1.7: Study of proliferation (PH3 immuno-staining; a and c) and apoptosis (TUNEL staining; b and d) in the testes of winter (a and b) and summer (c and d) males of *C. russula*. Similar incidence was observed for the two cellular processes in both study groups. Representative positive cells are marked with arrows. Scale bar represents 100 µm in all pictures.

treated for PH3 immuno-staining, which detects proliferative cells, or TUNEL staining, which identifies apoptotic cells. Wilcox's tests demonstrated that in both cases, the numbers of marked cells per 0.1 mm^2 of testis section were not statistically different in the two study groups. Regarding cell proliferation, the mean values were 3.076 and 3.608 for the winter and the summer groups, respectively (W = 1751.5, P = 0.1942), and for apoptosis the mean values were 2.019 and 1.923 respectively (W= 4274.5, P = 0.8054). These results clearly show that neither cell proliferation nor apoptosis vary seasonally in the testes of shrews from southern Iberian Peninsula.

1.2.4 Winter and summer male shrews show similar levels of serum testosterone

Finally, we also measured the serum concentration of testosterone in males of the two study groups, a feature which has been shown to change seasonally in all seasonal breeding species analysed to date. We detected no significant differences between winter and summer shrew males, although serum concentrations were generally lower in the summer (Fig. 1.8).

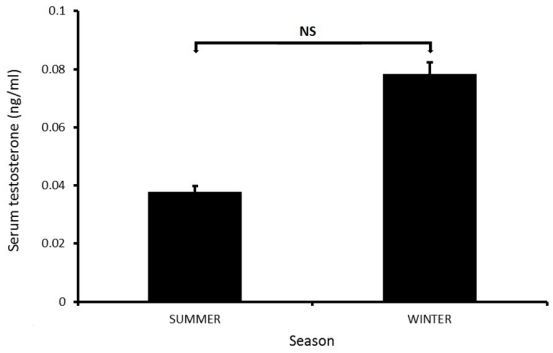


Figure 1.8: Serum testosterone concentrations in winter and summer male shrews. Means and standard deviations are shown.

1.3 Discussion

1.3.1 Adaptive features of the reproductive cycle of *C. russula* in Southern European populations

It is well known that the length of the reproductive period of a particular species is shorter in northern population and increases as latitude decreases, as shown in talpid moles (Jiménez *et al.*, 1990). According to these latitudinal variations, we expected to find a longer breeding season in southern populations of *C. russula* compared with those of Ebro delta (López-Fuster *et al.*, 1985), where the reproductive period is in turn longer than in Switzerland (Jeanmaire-Besancon, 1988). Surprisingly, we found sexually active males of this species throughout the year, suggesting that there is no seasonal breeding in the populations studied, thus preventing us from studying the mechanisms of seasonal testis regression in this species, which was one of the initial purposes of this work. We collected active male shrews in all the coldest months (November, December, January, and February), so it is clear that there is no testis regression in winter. However, regarding summer months, we collected males in June, July and September, but were unable to capture any male in August after several trapping days. Since Luaces *et al.* (Luaces *et al.*, 2013, 2014) have reported recently that in the large hairy armadillo testis regression occurs in just 15 days and then germinative epithelium recovery is completed within 45 days, one might envisage a very short period of testis regression in August also for *C. russula*. However, two facts rule out this possibility: 1) the total duration of spermatogenesis in this species is very long, lasting 54.7 days (Parapanov *et al.*, 2007, 2008), which makes it very improbable for shrew males to reach complete recovery of the germinative epithelium by mid September, if they have to initiate testis regression from mid July on, two dates in which we captured fully active males (see supplementary table 1); 2) we collected two very young juvenile individuals (1 male and 1 female) on October 5th, 2012, which implies that mating should occur in August (consider 28-30 days for ges-

tation plus 15-20 days for weaning), thus showing that male shrews are sexual active in this month.

This lack of testis regression in shrew males from southern Spain contrasts with the situation described for the same species in populations in Ebro delta (only 400 Km to the north of the populations studied here) where López-Fuster and collaborators found old adult males with extremely reduced testis size during the winter (the non-breeding season there), a fact that strongly suggests that males from Ebro delta do undergo seasonal testis regression, like those from more northern populations in Europe presumably also do (López-Fuster *et al.*, 1985). However, data from males are probably not sufficient to state that there is really no seasonal breeding in southern populations of *C. russula.* Data from captured females clearly show that pregnant and lactating females were found mainly in autumn and winter (15 out of 21 captured females), but very infrequently in the summer months (1 out of 10), showing that most, but not all, females stop breeding in the summer. Consistently, large, adult females captured in the summer were found to have inactive ovaries lacking maturing follicles. We lack data from spring females, but the presence of juvenile animals in the summer indicates that reproduction also occurs in the spring. These data indicate that there is in fact a non-breeding season which is determined exclusively by females. This is consistent with the data reported by Rogers Brambell, who established that the onset, and probably also the end, of the breeding season of the common shrew, *Sorex araneus*, is determined by the female and not by the male (Brambell, 1935). It is noteworthy that the seasonal breeding cycle in the southern populations appears to be inverted with respect to that of northern ones, where reproductive inactivity occurs in winter. This is not surprising as the same phenomenon also occurs in populations of other mammalian species living in the same region near the city of Granada, including the Iberian mole (Jiménez *et al.*, 1990; Dadhich *et al.*, 2010, 2011, 2013) and two rodents, *Microtus duodecimcostatus* and *Apodemus sylvaticus* (present study; see below). The extreme drought that characterizes summer in the south-eastern Iberian peninsula probably determines this reversed cycle as it is at this time when small mammals face the harshest life conditions in this region.

The existence of a female-driven seasonal breeding cycle in southern populations of *C. russula* raises two questions: 1) how do females control the circannual variations of their reproductive activity? and 2) why do testes in males not undergo a seasonal regression as in northern populations? Little is known about the mechanisms controlling sexual activity in females. In species with conspicuous seasonal breeding that undergo long periods of sexual inactivity, it is presumed that the photoperiod-dependent activity of the hypothalamic-pituitary-gonadal axis, ultimately determines gonad activity both in males and in females, as shown in the hamster (Clarke and Caraty, 2013). On the other hand, there are also species with a natural tendency to reproduce continuously throughout the year but in which females may undergo a transient receptivity drop when they face survival difficulties such as food

restriction. This is the case of the musk shrew, *Suncus murinus*, which has become an animal model for studying nutritional regulation of reproduction (Temple, 2004). This tropical mammal shows continual reproduction although it is nevertheless sensitive to photoperiod (Rissman *et al.*, 1987). However, nutritionally challenged females stop breeding as a consequence of complete loss of sexual receptivity. This response is controlled by the GnRH-II hormone, the best evolutionarily conserved neuropeptide of the GnRH family (Kauffman, 2004). GnRH-II reactivates the mating behavior of *S. murinus* females subjected to food restriction within a few minutes after administration (Temple *et al.*, 2003). In the case of *C. russula*, the fact that the breeding season is longer in regions with less extreme climate conditions suggest that this species could eventually have continual reproduction in regions providing a stable and favorable environment. Thus, it is possible that the non-breeding periods observed in our study and in other populations were controlled by GnRH-II, as described in *S. murinus*, considering that it is in these periods when shrew females may find more difficulties to fulfil their nutritional requirements (summer droughts in the south, and icy winters in the north). The fact that a few females exceptionally reproduce in the summer is consistent with this hypothesis, as all individuals are not exposed to the same micro-environment and some of them could enjoy more benign life conditions. Further study comparing the expression of GnRH-II and other neuropeptids in the brain of summer and winter female shrews will permit us to test this hypothesis.

Regarding male shrews, the lack of testis regression during the non-breeding period is unique among mammals as all species with seasonal reproduction studied to date show a testis involution that implies almost complete ablation of the meiotic and post-meiotic components of the germinative epithelium. The existence of some receptive females in the summer seems not to be the cause of the peculiar situation we have shown in *C. russula,* as they appear to be quite exceptional. Rather, it could be related to the fact that males are relatively monogamous, a condition generally associated with the presence of small testes (Hosken *et al.*, 2001; Parapanov *et al.*, 2009), and a reduced spermatogenesis investment would not represent an excessive energy cost if maintained during the non-breeding season. However, this cannot be the only cause, as data reported by López-Fuster *et al.* indicate that males from populations of northern Spain undergo testis regression (López-Fuster *et al.*, 1985). Hence, it is under the conditions of southern populations that the phenomenon occurs. One possibility is that the non-breeding period in the populations studied here is very short, inducing a process of testis regression that would become disadvantageous in energetic and reproductive terms as the required immediate germinative epithelium renewal would be onerous and slow. The long duration of spermatogenesis in *C. russula* (54.7 days) (Parapanov *et al.*, 2007, 2008) compared with that of two other shrew species (37.6 days in *S. araneus* and 39.1 days in *N. fodiens*), supports our hypothesis. Our finding that summer males show a clear tendency to have lower levels of serum testosterone (mean differences were only near the 5% statistical significance, probably due to small sample size), is also consistent with this hypothesis, as a moderate

reduction in the serum testosterone levels would permit reduced sexual drive but not a depletion of the germinative epithelium. For this, we conclude that the lack of testis regression in a mammalian population with seasonal breeding represents a new adaptive mechanism that has not been previously described. Further studies defining better the reproductive cycle of the females of this species may help to shed light on this intriguing issue.

1.3.2 The role of DMRT1 expression in the shrew testis

Finally, the spermatogenic stage-specific expression of DMRT1 observed in the shrew, showing a pattern very similar to that described in the mole, supports our hypothesis (Dadhich *et al.*, 2011) that this protein is involved in the control of spermatogenesis, probably by regulating spermatogonial proliferation. However, the number of cells expressing DMRT1 inside the seminiferous tubules was significantly lower in the shrew than in mice or moles, showing that the number of germ cells entering meiosis in every consecutive spermatogenic round is lower in the shrew than in the other two species. This could result in smaller seminiferous tubules, a possibility consistent with the smaller relative size of the testes in *C. russula* (around 3 mg per gram of body mass in our winter study group) if compared with mice (around 8 mg/g) or moles (around 14 mg/g). However, measurements of the seminiferous tubule diameter in all three species showed that they are very similar (185.9±22.5 μm in the winter shrew, 180.31±18.84 μm in the mouse, and 178.44±17.39 μm in the winter (sexually active) mole; $P>0.05$ in all three comparisons). Alternatively, these results could also be explained if the spermatogenic cycle in the shrew were slower than in other species. Parapanov *et al.* analysed the testes of three shrew species including two showing polygamy, high metabolic rate, and large testes (*Sorex araneus* and *Neomy fodiens*), and one exhibiting monogamy, low metabolic rate, and small testes (*C. russula*), and demonstrated that the length of the spermatogenic cycle in the later (12.1±0.60 days) is longer than those of the two former species (8.4±0.26 and 8.7±0.14 days, respectively) (Parapanov *et al.*, 2007, 2008). These data are clearly consistent with our finding of a comparatively low number of proliferating spermatogonial cells in *C. russula*, as a slower meiosis implies that meiotic cells occupy their space in the germinative epithelium during a longer time and can thereby maintain a constant volume of the seminiferous tubule even if the total number is reduced.

2. Second chapter *Mus spretus*

Figure 2.1: *Mus spretus*. Image reproduced with permission of the autor: Igor Evstafiev

2.1 Introduction

The Algerian mouse, also called the western Mediterranean mouse, *Mus spretus*, is a miomorphic rodent species belonging to the family Muridae. Its taxonomy was a subject of debate for many years, but recent molecular studies have established that this is a species clearly separated from the house mouse, *Mus musculus* (Wilson and Reeder, 2005). It is a small murid with short snout and small eyes and ears, and differentiates from the house mouse in the length of the tail, which is shorter in the Algerian mouse. This mammal inhabits south-western Europe and north-western Africa. In Europe, it is present in the Iberian peninsula, except in the north, and throughout the Balearic Islands. In Africa, it is found in the Maghreb regions (Amori *et al.*, 2008). It prefers open terrain, avoiding dense forests. It is primarily nocturnal and an opportunistic omnivore, feeding on grass seeds, fruits, and insects. In the province of Granada, this species is particularly abundant in the banks of water streams, including those with non-permanent water supply, where we captured most animals used in this study.

The annual reproductive cycle of *M. spretus* in southern Iberian Peninsula was studied by Vargas et al. (Vargas *et al.*, 1991). Two well-marked phases are defined for this species: a period of sexual inactivity in winter extending from November to January, with a reduction in the size of testes and seminal vesicles, and a period of sexual activity during the rest of the year. In addition, two phases of maximum activity was recorded in the active period, occurring in April–May and August–September. A photoperiod-depending seasonal reproductive cycle is suggested for this species. *M. spretus* has an average life expectancy lower than 4 months, with a longevity of 14–15 months, and the animals do not usually survive their second winter (Palomo *et al.*, 2009; Cassaing, 1982; Cassaing and Croset, 1985).

2.2 Results

2.2.1 Testes of *Mus spretus* remain active throughout the year but are smaller in winter

Four study groups, named winter, spring, summer and autumn, were established according to the season of capture (2.1).We compared the body mass of the males from each of the four study groups with each other and found no statistically significant differences between them (P>0.05 in all possible comparisons using student's-t tests; table 1). In contrast, analysis of the testis mass showed significant differences between the winter group and the other three groups. Testes of winter males were significantly smaller than those from males in the rest of the year (tables 2.1 and 2.2; Fig. 2.2).

Histological analysis revealed that both winter and summer testes contained seminiferous tubules covering all the stages of the spermatogenic cycle,

	Winter	Spring	Summer	Autumn
Body mass (g)	12.19±1.87	12±0.89	14±1.73	11.9±1.1
Testis mass (mg)	63.06±17.31	87.5±22.54	85.89±18.99	84.82±9.53
n	16	16	9	10

Table 2.1: Body and testis mass values and number of adult males of *M. spretus* analyzed in each season. Data are expressed in mean ± standard deviation.

P value	Winter	Spring	Summer	Autumn
Winter		0.04606*	0.00964**	0.00046***
Spring			0.88833	0.79025
Summer				0.88031

Table 2.2: Matrix of *P* values corresponding to the statistical Student's t-tests performed to compare the testis mass of every study group with each other, as shown in table 1. Asterisks indicate the level of statistical significance.

with a very well developed seminiferous epithelium and mature spermatozoa in the lumen. Consistently, the epididymides were full of spermatozoa, although the summer epididymides appeared to contain more spermatozoa than the winter ones (Fig. 2.3). However, the size of the winter tubules appeared reduced when compared to summer ones. Accordingly, the differences between the diameter of the seminiferous tubules in both study groups (183.29 ±16.01 µm for the summer group and 146.41± 20.06 µm for the winter group) were shown to be highly significant ($P < 0.001$; Fig. 2.4). Nevertheless, this reduction in the width of the seminiferous tubules of the winter males is not as important as in other species, representing a decrease of just 20% with respect to that of the summer males.

2.2.2 Expression of both Sertoli and germ cell marker genes is maintained in the testes of winter males of *M. spretus*

We compared the expression pattern of several Sertoli cell markers between winter and summer adult testes of *M. spretus*. Previous studies have shown that in the adult testis of both mice and moles, *SOX9* expression is spermatogenic stage-dependent in Sertoli cells, being stronger in stages I-IV and weaker in stages VII-X (Dadhich *et al.*, 2011). Inmunohistochemistry for SOX9 revealed the same expression pattern in the testes of adult Algerian mice (Fig. 2.5 a and b). However, unlike other species, this expression pattern was observed in both summer and winter testes, showing no seasonal variation. We also performed DMRT1 immunohistochemistry and, like in both the mole (Dadhich *et al.*, 2011) and the shrew (see above), we also found two types of semi-

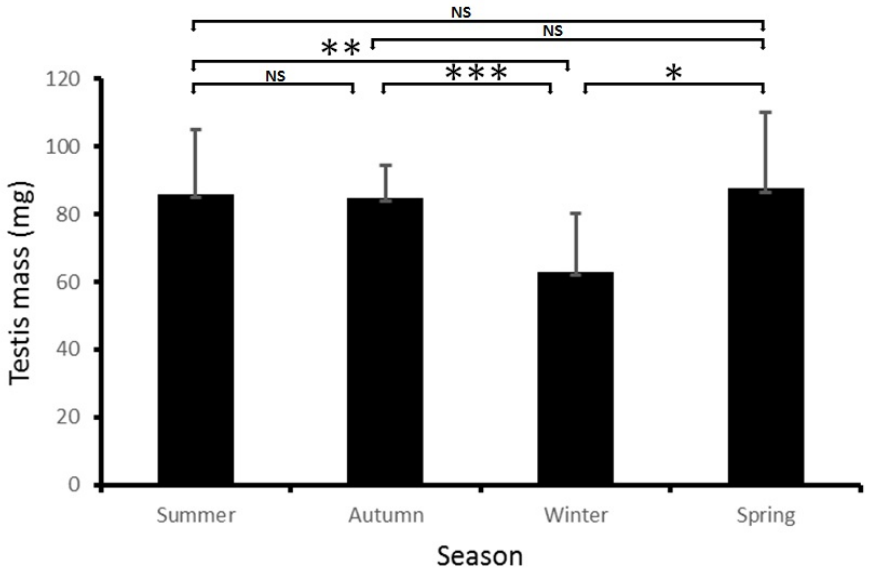


Figure 2.2: Seasonal variations in the testis mass of adult males of *M. spretus* and significance of the differences detected according to Student's t-tests. Asterisks indicate the level of statistical significance. Ns, non signifficant differences.

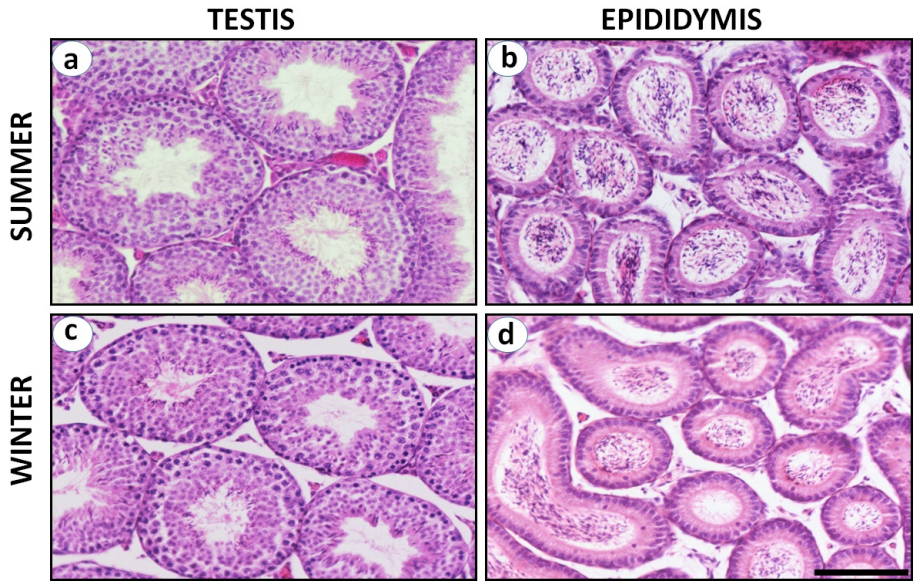


Figure 2.3: Haematoxylin-eosin-stained tansverse sections of testes (a and c) and epididymides (b and d) from summer (a and b) and winter (c and d) males of *M. spretus*. In both cases the seminiferous tubules show active spermatogenesis and the epididymides contain abundant sperm. Scale bar represents 100 µm for all pictures.

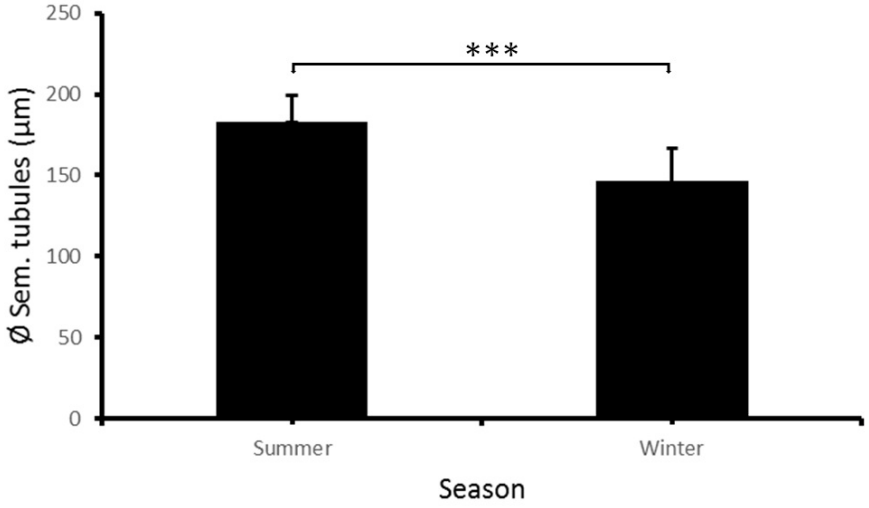


Figure 2.4: Seasonal variations in the seminiferous tubules diameter of testes from adult males of *M. spretus* and significance of the differences detected according to Student's t-tests. Asterisks indicate the level of statistical significance ($P < 0.001$)

niferous tubules depending on the number of DMRT1-positive cells: LDD and HDD (Fig. 2.5 c and d). As described in these species, both LDD and HDD seminiferous tubules are spermatogenic cycle-specificic, being LDD tubules observed in stages I-IV, whereas HDD appeared in stages VI-VII.

SOX9-DMRT1 double immunofluorescence revealed that DMRT1 expression in LDD tubules is almost exclusively restricted to Sertoli cells, whereas in HDD tubules the DMRT1 signal is also found in other cells located in the basal compartment of the seminiferous tubules, which probably corresponds to spermatogonia (Fig. 2.6). Nevertheless, there were no seasonal variation in this expression pattern, which was similar in both winter and summer testes of *Mus spretus*.

In order to check whether spermatogenesis is affected in the reduced testes of winter males of *M. spretus*, we analyzed the expression of several germ cell markers. As mentioned above, the first molecular marker analysed was DMRT1, which is expressed in spermatogonial cells. PCNA immunohistochemistry showed that all positive cells were close to the basal surface of the seminiferous tubules (Fig. 2.7 a and b). We could distinguish two types of spermatogenic cycle-dependent PCNA-positive tubules: those with a monolayer of PCNA-positive cells, corresponding to spermatogenic stages I-IV, and those with a multilayer of PCNA-positive cells, which are seen in stages VI-VII. Furthermore, DMRT1-PCNA double immnuofluorescence (Fig. 2.8) revealed that the former kind of tubules corresponded to the LDD tubules detected with DMRT1 staining, the expression of this gene being almost exclusively restricted to Sertoli cells, whereas PCNA expression was observed in

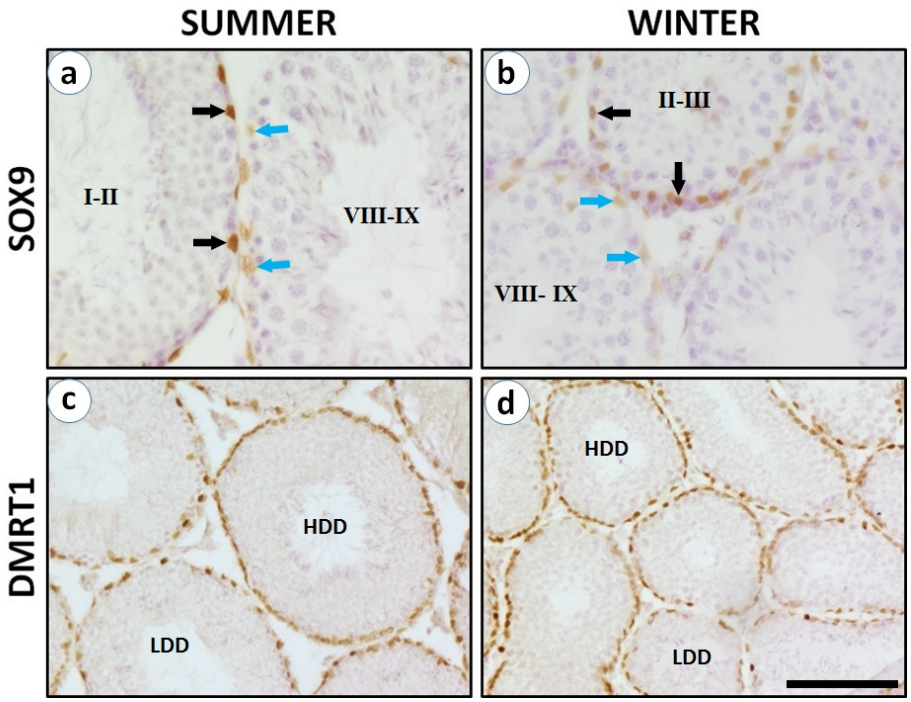


Figure 2.5: Immunohistochemical detection of both SOX9 (a and b) and DMRT1 (c and d) proteins in the testes of summer (left column) and winter (right column) males of *M. spretus*. SOX9 expression is stronger in I-IV (black arrows), and a weaker in stages VII-X (cyan arrows). No relevant difference was observed in DMRT1 expression between the two groups (c and d). Two types of testis tubules can be distinguished regarding the content of DMRT1-positive cells: low-density DMRT1-expressing (LDD) tubules, in which expression is almost restricted to Sertoli cells and appear in stages I-IV, and high-density (HDD) ones, in which the expression is observed in both Sertoli and spermatogonial cells, which are observed in stages VI-VII. Scale bar represents 100 µm in all pictures.

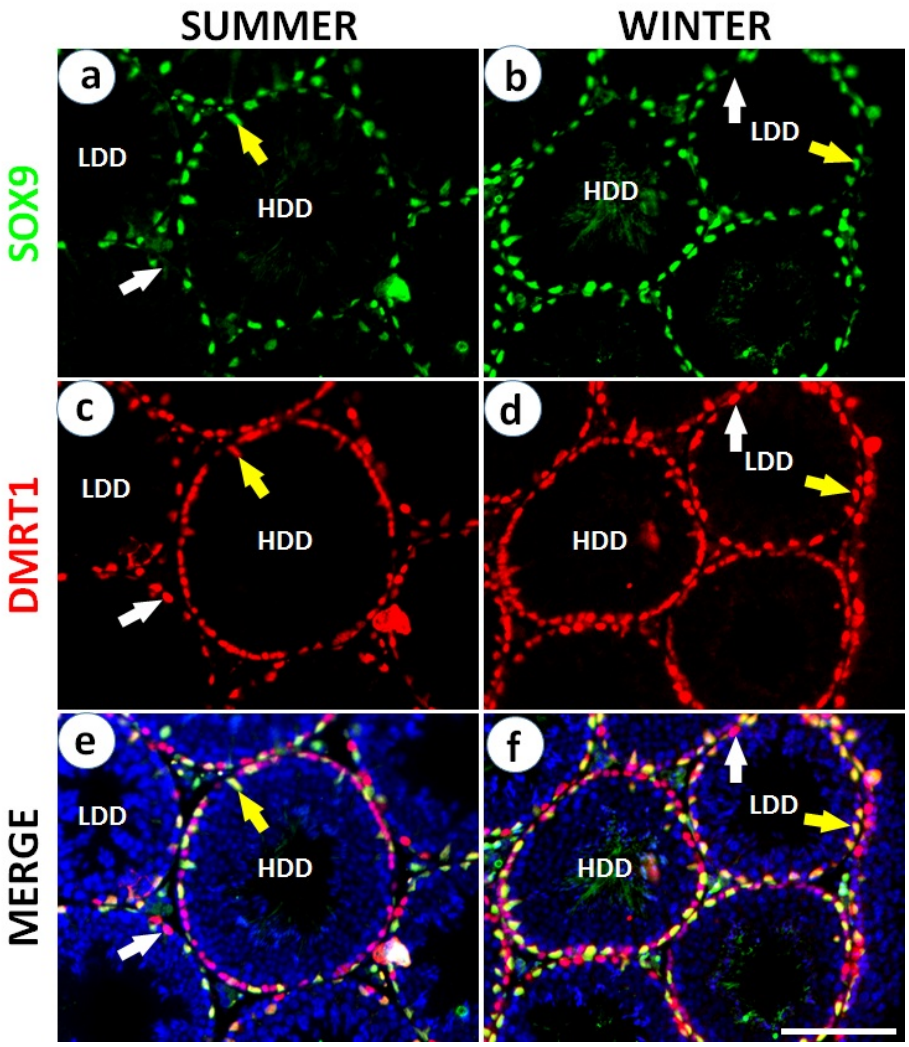


Figure 2.6: Double immuno-fluorescence for SOX9 (green; a and b) and DMRT1 (red; c and d) proteins in testes from summer (left column) and winter (right column) males of *M. spretus*. Pictures in e and f show merged images with DAPI counterstain (blue). DMRT1 co-localizes with SOX9 in Sertoli cells (yellow arrows), indicating that the other immuno-reactive cells (white arrows) are spermatogonia. Scale bar represents 100 µm in all pictures.

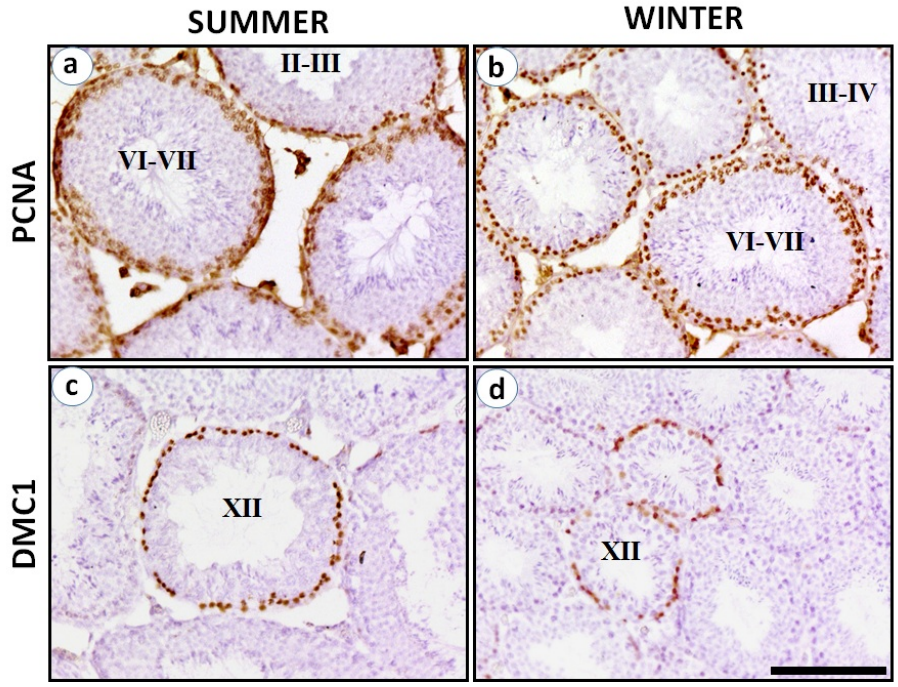


Figure 2.7: Immunohistochemical detection of both PCNA (a and b) and DMC1 (c and d) proteins in testes from summer (left column) and winter (right column) males of *M. spretus*. Scale bar represents 100 µm in all pictures.

spermatogonia. However, in tubules showing several layers of PCNA-positive cells, DMRT1 and PCNA proteins co-localized in spermatogonial cells, but there were in addition PCNA-positive, DMRT1-negative cells located in the inner layers which corresponding to zygotene-pachytene spermatocytes. The same expression pattern was observed in the testes of winter males of *M. spretus*. Finally, we analysed DMC1 expression by immunohistochemistry (Fig. 2.7 c and d) and found that only seminifeous tubules corresponding to spermatogenic stages II-IV contained DMC1-positive cells. Double immunofluorescence for both DMRT1 and DMC1 proteins showed no co-localization of both markers in any cells, indicating that DMC1 was expressed only in tubules containing zygotene-early pachytene spermatocytes, but not in spermatoiconia (Fig. 2.9).

2.2.3 The blood testis barrier is transiently permeated in the testes of winter males of *Mus spretus*

To check the status of the blood testis barrier in summer and winter testes of *M. spretus*, we performed immuno-histochemistry for CLAUDIN11. We found a dynamic CLAUDIN11 expression pattern with stronger immunoreactivity in tubules in stages IV-XI and a weaker expression in tubules of the rest of the spermatogenic cycle stages (Fig. 2.10).

We also checked the integrity of the BTB by injecting a biotin tracer into

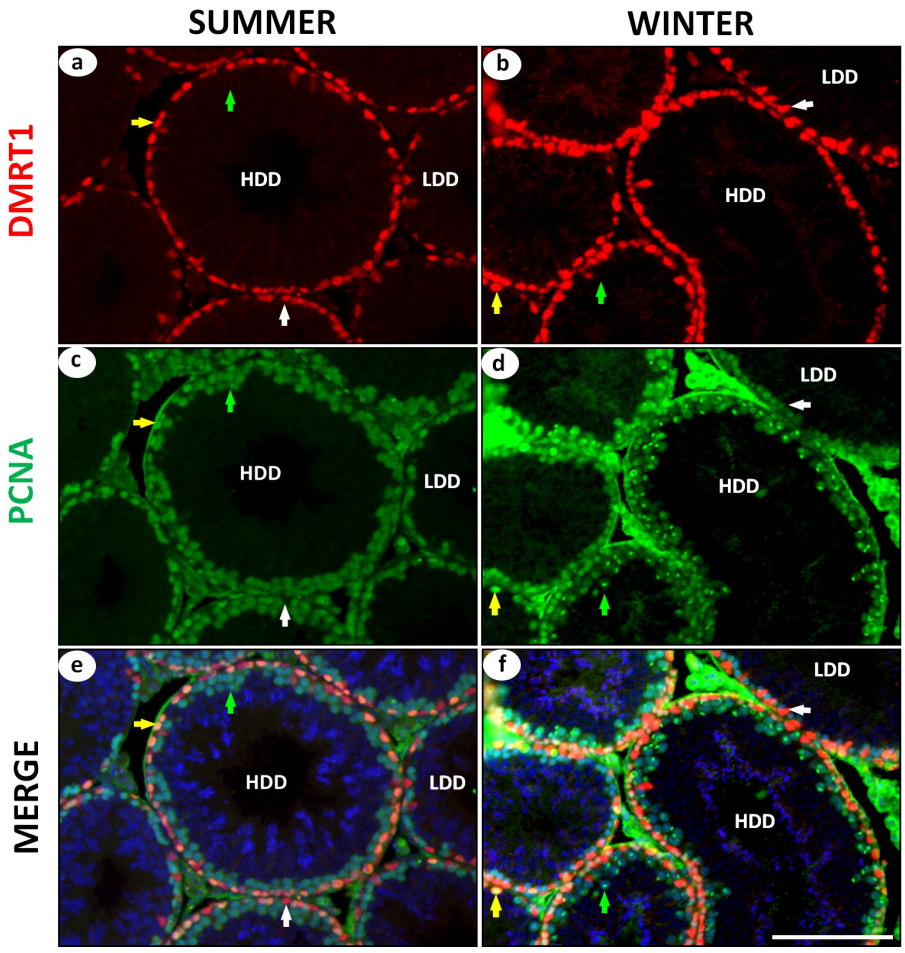


Figure 2.8: Double immunofluorescence for both DMRT1 (red; a and b) and PCNA (green; c and d) proteins in testes of summer (left column) and winter (right column) males of *M. spretus*. Pictures in e and f show merged images with DAPI counterstain (blue). PCNA co-localizes with DMRT1 in spermatogonial cells (yellow arrows), but not in Sertoli cells (white arrows) and in zygotene-early pachytene spermatocytes (green arrows). Scale bar represents 100 µm in all pictures.

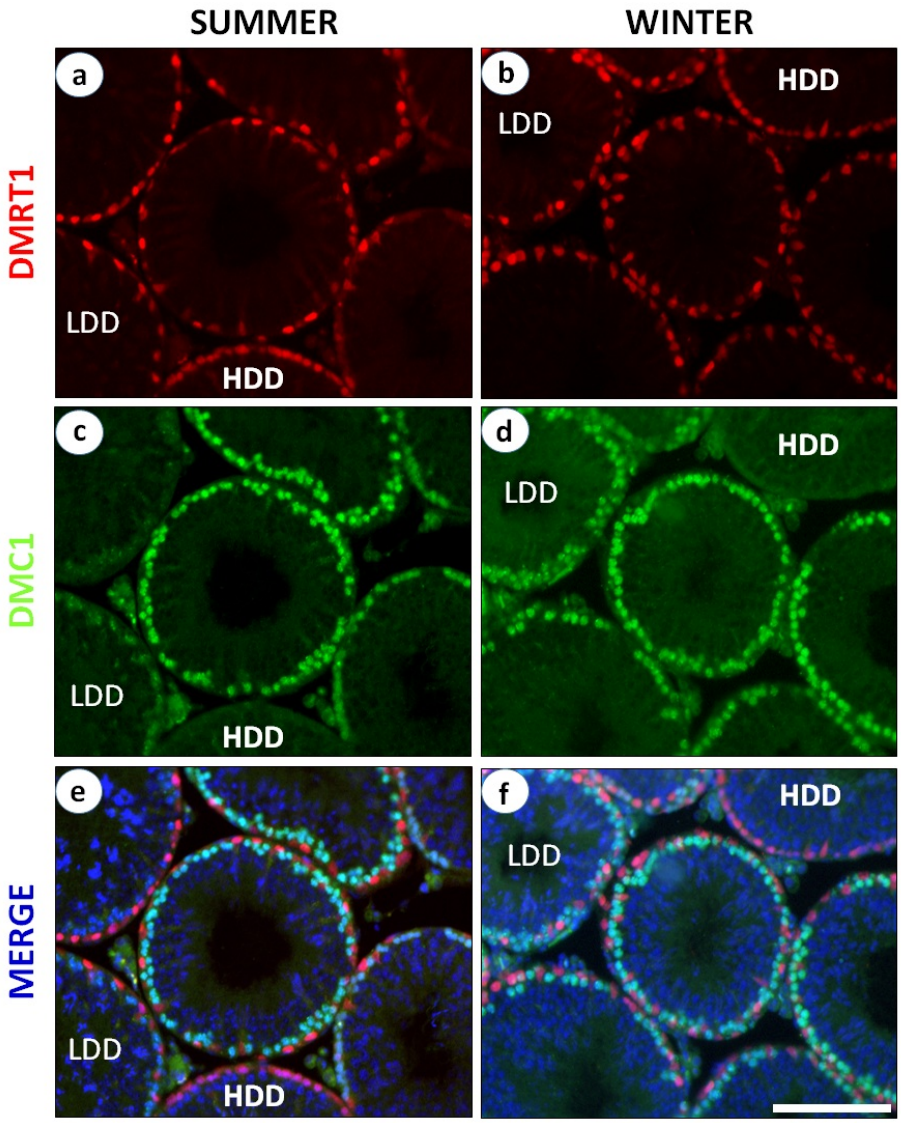


Figure 2.9: Double immunofluorescence for both DMRT1 (red; a and b) and DMC1 (green; c and d) in testes of summer (left column) and winter (right column) males of *M. spretus*. Pictures in e and f show merged images with DAPI counterstain (blue). DMRT1 and DMC1 do not co-localize in any cell type in both summer and winter testes. Scale bar represents 100 µm in all pictures.

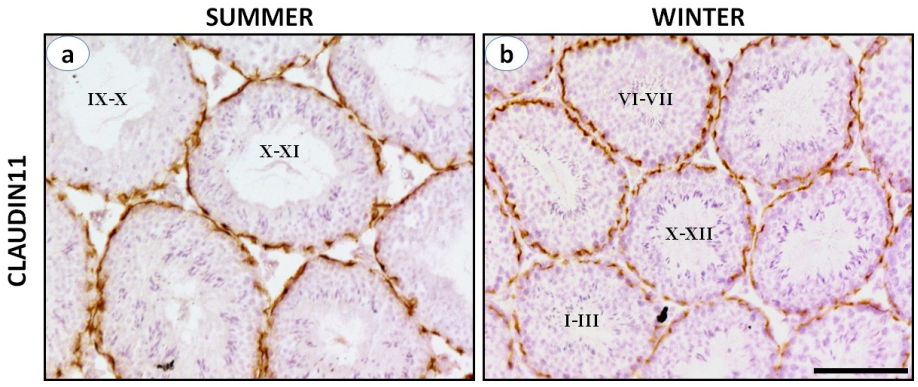


Figure 2.10: mmunohistochemical detection of CLAUDIN11 in the testes of summer (a) and winter (b) males of *M. spretus*. Stronger expression is detected in seminiferous tubules of the stages IV-XI of the spermatogenic cycle in both study groups. Scale bar represents 100 µm in all pictures.

the interstitial space of the testes of summer and winter males. In the summer testes we observed that the biotin tracer was detected in the interstitial tissue as well as in the basal compartment of the seminiferous tubules but not in the adluminal compartment (Fig. 2.11 a and c). Surprisingly, biotin immunoreaction was clearly observed in both basal and adluminal compartments in the testes of the winter group, reflecting impaired BTB impermeability in these apparently fertile animals. (Fig. 2.11 b and d).

To confirm this observation, we performed two additional experiments. Firstly, we made double immuno-fluorescence for both CLAUDIN11 and the biotin tracer (Fig. 2.12), which confirmed that the tracer either co-expressed or was located basal to CLAUDIN11 signal in the seminiferous tubules of summer testes. In contrast, the biotin signal was observed at both sides of CLAUDIN11 domain in the winter testes. Secondly, we checked the location of meiotic germ cells relative to the BTB in both winter and summer testes of *M. spretus*, by performing CLAUDIN11-DMC1 double immuno-fluorescence (Fig. 2.13). In both cases all DMC1-positive cells were located inner to the CLAUDIN11 domain, indicating that when germ cells pass through the BTB they have already entered meiosis.

Knowing this dysfunction of the BTB in the testes of winter males, we wanted to check the status of the lamina propria in order to look for possible differences between the summer and the winter testes. For this, we studied the localization of several proteins involved in the formation of this structure. Immunohistochemistry for alpha–smooth muscle actin (alpha-SMA) and for DESMIN, two markers of the peritubular myoid cells, and for LAMININ, a major component of the basement membrane (Fig. 2.14), showed a positive signal around the seminiferous tubules that remained unaltered in both winter and summer testes, indicating that the lamina propria conserves its integrity throughout the year.

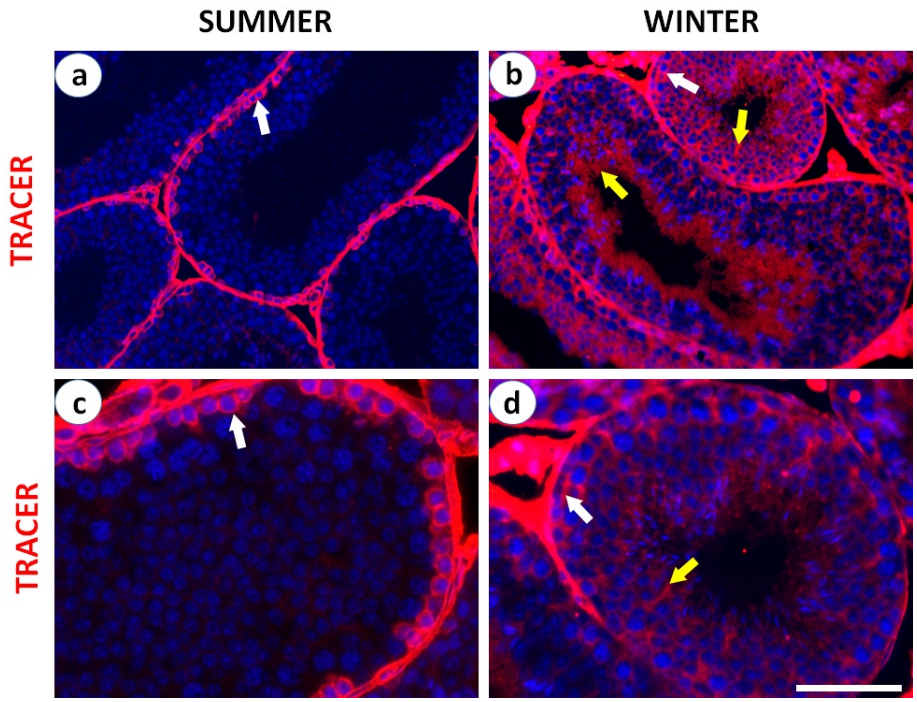
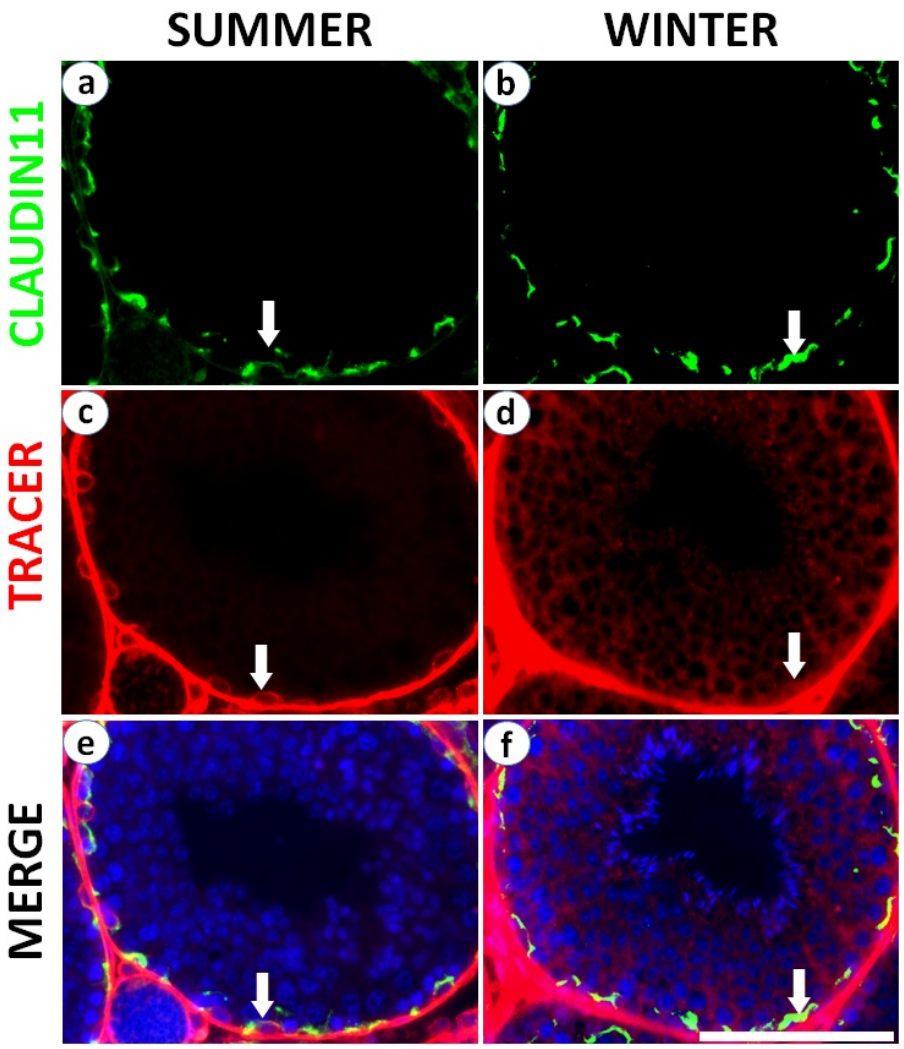


Figure 2.11: Biotin tracer immuno-detection after injection in the interstitial space of the testis from summer and winter males of *M. spretus*. The tracer (red fluorescence) is located in the basal compartment of the seminiferous epithelium in both summer and winter testes (white arrows in all pictures), but it also appears in the adluminal compartment of the winter testes (yellow arrows in b and d). Nuclei were counterstained with DAPI (blue fluorescence in all pictures). Scale bar represents 100 µm in a and b, and 50 µm in c and d.



White arrows

Figure 2.12: Double immuno-fluorescence for both CLAUDIN 11 (green; a and b) and the biotin tracer (red; c and d) in testes from summer (left column) and winter (right column) males of *M. spretus*. Pictures in e and f show merged images with DAPI counterstain (blue). Biotin is located in the basal compartment of summer testes and at both sides of the CLAUDIN11 domain in the winter ones. Scale bar represents 50 µm in all pictures.

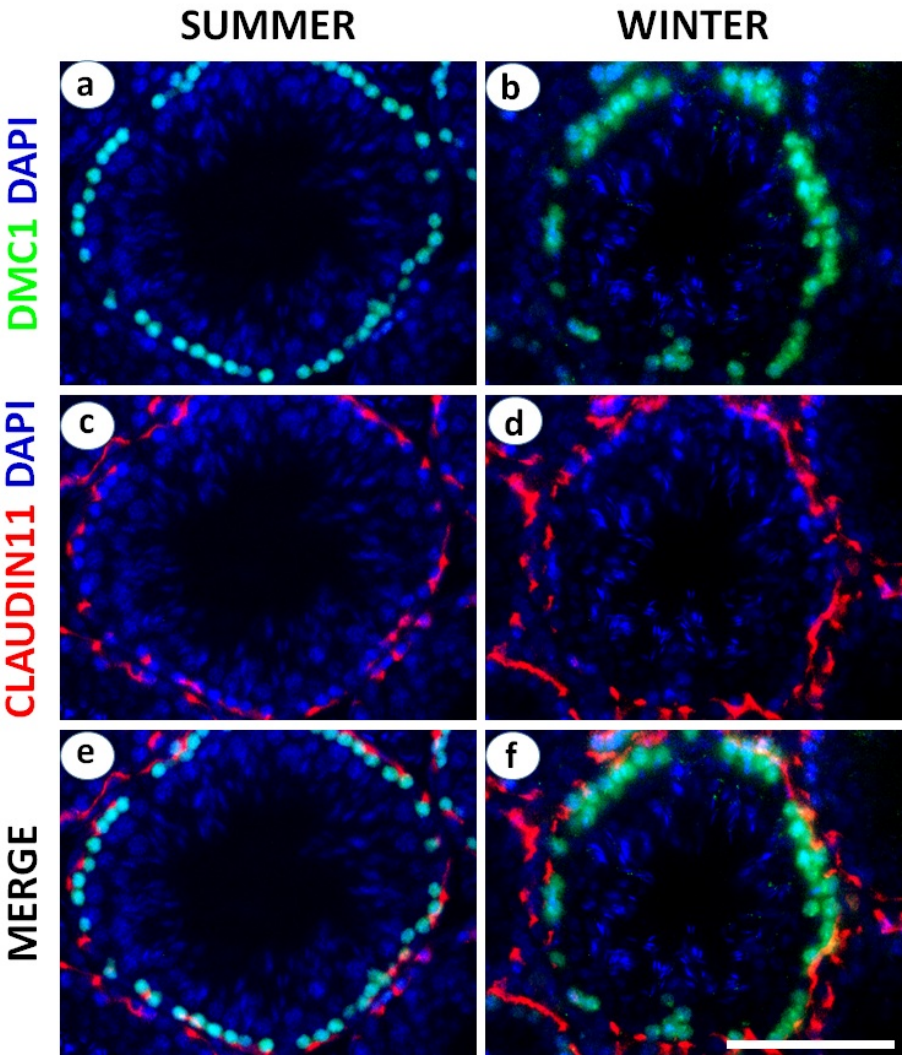


Figure 2.13: Double immune-fluorescence for both DMC1 (green; a and b), and CLAUDIN 11 (red; c and d), and DAPI counterstaining (blue; a-f) in testes of summer (left column) and winter (right column) males of *M. sprestus*. All DMC1-positive meiotic cells are located inner to the CLAUDIN11 domain in the two study groups. Scale bar represents 50 µm in all pictures.

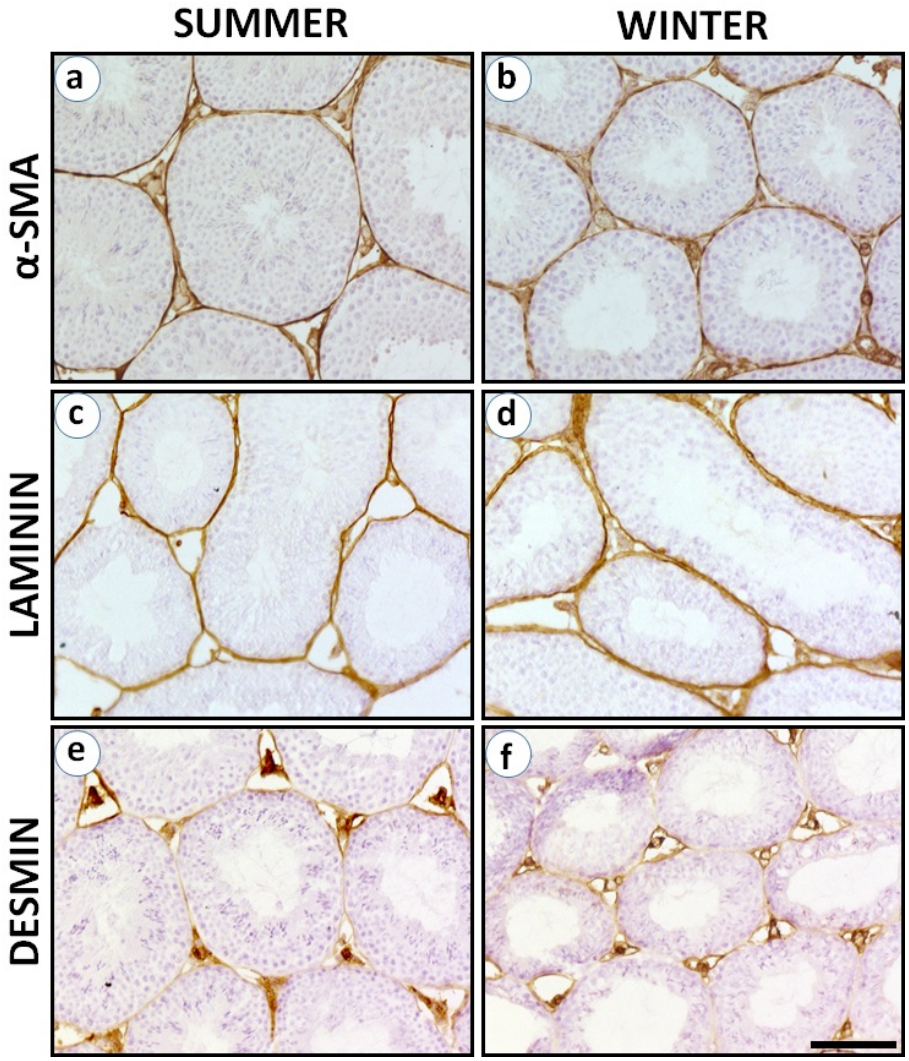


Figure 2.14: Immunohistochemical detection of alpha-SMA (a and b), LAMININ (c and d), and DESMIN (e and f) in the testes of summer (left column) and winter (right colum) males of *M. spretus*. The same expression pattern is observed in the two study groups for all three proteins. Scale bar represents 100 µm in all pictures.

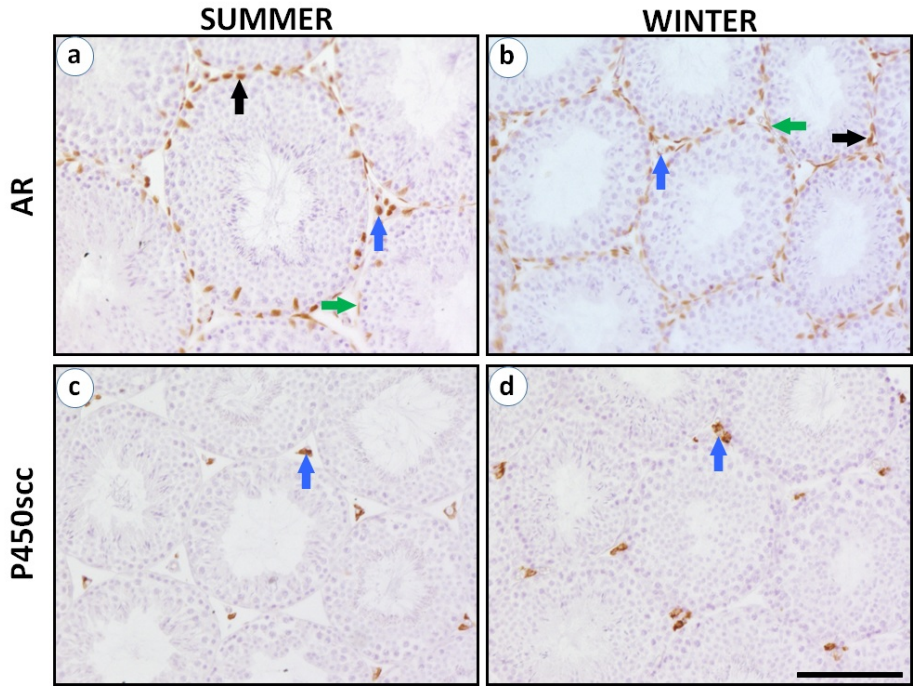


Figure 2.15: Immunohistochemistry for both AR (a and b) and P450scc (c and d) in transverse sections of testes from summer (left column) and winter (right column) males of *M. spretus*. Nuclei were counterstained with haematoxylin. AR expression is detected in Sertoli cells (black arrows), Leydig cells (blue arrows), and peritubular myoid cells (green arrows), and P450scc is expressed in Leydig cells (blue arrows), in both summer and winter testes. Scale bar represents 100 µm in all pictutes.

2.2.4 Steroidogenesis is reduced during the winter in males of *Mus sprestus*

The expression of several genes responsible for the production of steroid hormones and signaling were also studied in summer and winter *M. spretus* testes by immunohistochemistry. As reported for the Iberian mole (Dadhich *et al.*, 2013) we found that the androgen receptor (AR) was expressed in Sertoli, Leydig, and peritubular myoid cells in both summer and winter testes, although AR-immunoreactivity appeared weaker in the later (Fig. 2.15). We also observed that the cholesterol side-chain cleavage enzyme (P450scc) was strongly expressed in Leydig cells, with no difference in the signal intensity detected between summer and winter testes. Finally, we also determined the serum concentration of testosterone by radioimmunoassay (RIA) and found a significant reduction in androgen levels in the winter animals with respect to those of the summer ones (Fig. 2.16).

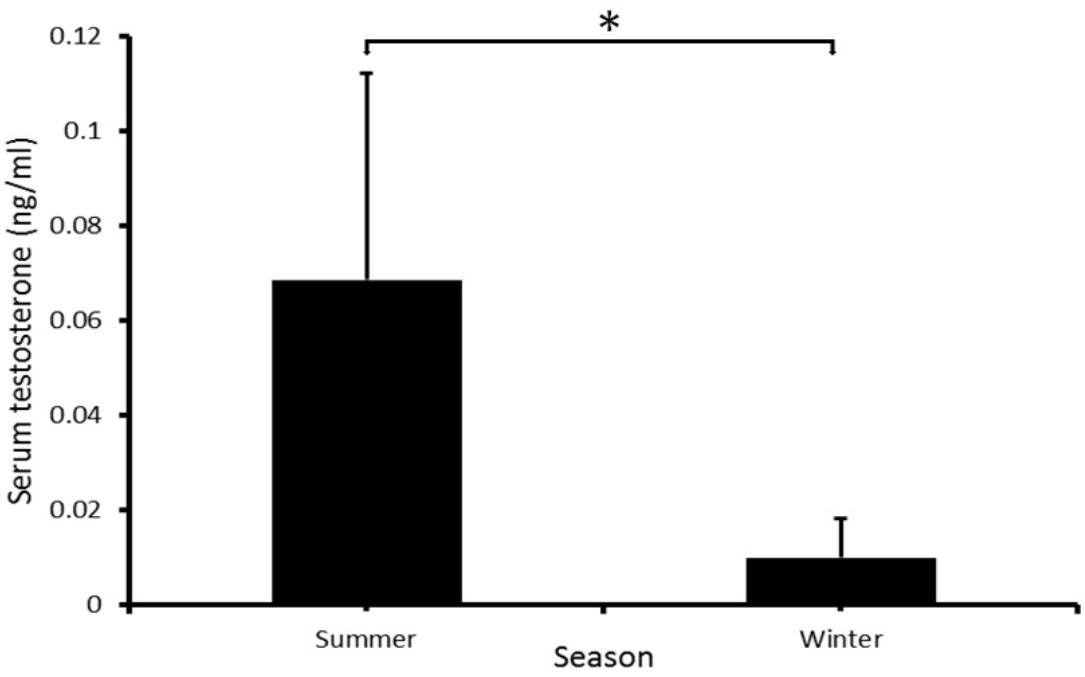


Figure 2.16: Levels of testosterone measured by radio-immunoassay in the serum of both summmer and winter males of *M. spretus*. A clear reduction is observed in the winter with respect to the summer group.

2.2.5 Study of apoptosis and cell proliferation

Several studies have reported differences in the number of cells that undergo apoptosis or proliferate between testes in the different breeding seasons. Based on these observations, the hypothesis that this is the cause for testis regression in the males of many animal species reproducing seasonally has been proposed. To check whether these processes are also altered in the testes of either summer or winter males of *M. spretus*, we performed TUNEL assays to detect apoptotic cells (Fig. 2.17 a and b) and PHOSPHO-HISTONE 3 (PH3) immunohistochemistry (Fig. 2.17 c and d) to detect proliferating cells. We found that both processes were slightly reduced in summer testes when compared to winter ones. Thus, the number of apoptotic cells was 1.63 ± 1.81 cells/0.1 mm^2 in the summer and 2.12 ± 2.29 cells/0.1 mm^2 in the winter. Comparison using a Wilcox's test revealed the existence of significant differences between the two study groups (W = 11699, P = 0.04172; Fig. 2.18). Similarly, the number of proliferating cells was 9.33 ± 10.04 cells/0.1 mm^2 in the summer and 13.69 ± 15.09 cells/0.1 mm^2 in the winter, although in this case differences were only near the statistical significance (Wilcox's test: W =14864, P = 0.057; Fig. 2.19).

2.3 Discussion

2.3.1 The causes of winter size reduction in the testes of *Mus spretus*

The situation we have just described in the Algerian mouse is relatively new if compared with other species investigated for seasonal changes in testis function. In strict seasonal breeders, males undergo complete testis regression, as occurs in hamsters (Bex and Bartke, 1977), deer (Brown *et al.*, 1979; Clarke *et al.*, 1995), brown bears (Tsubota *et al.*, 1997) and moles (Jiménez *et al.*, 1990; Dadhich *et al.*, 2010, 2013), among others. Contrarily, in the southern Iberian populations of *M. spretus* testes maintain the spermatogenetic function during the winter but appear slightly reduced in size if compared with those found in the summer. This case could be considered similar to that of the stallion, in which fertility may be maintained at a reduced level during the non-breeding season (Guillaume *et al.*, 1996), although we do not know whether this functional reduction is also accompanied of a size reduction of the testes in this species. Perhaps, testis reduction in the Algerian mouse during the winter is a mechanism for a lower reproductive activity in this period. The lower testis mass of the winter *M. spretus* is clearly a consequence of reduced diameter of the seminiferous tubules, which even representing only 20% of the summer ones, it is very consistent from a statistical point of view. Hence, unlike the shrew, the reduced spermatogenetic function in the winter males of this species is not achieved through a slower

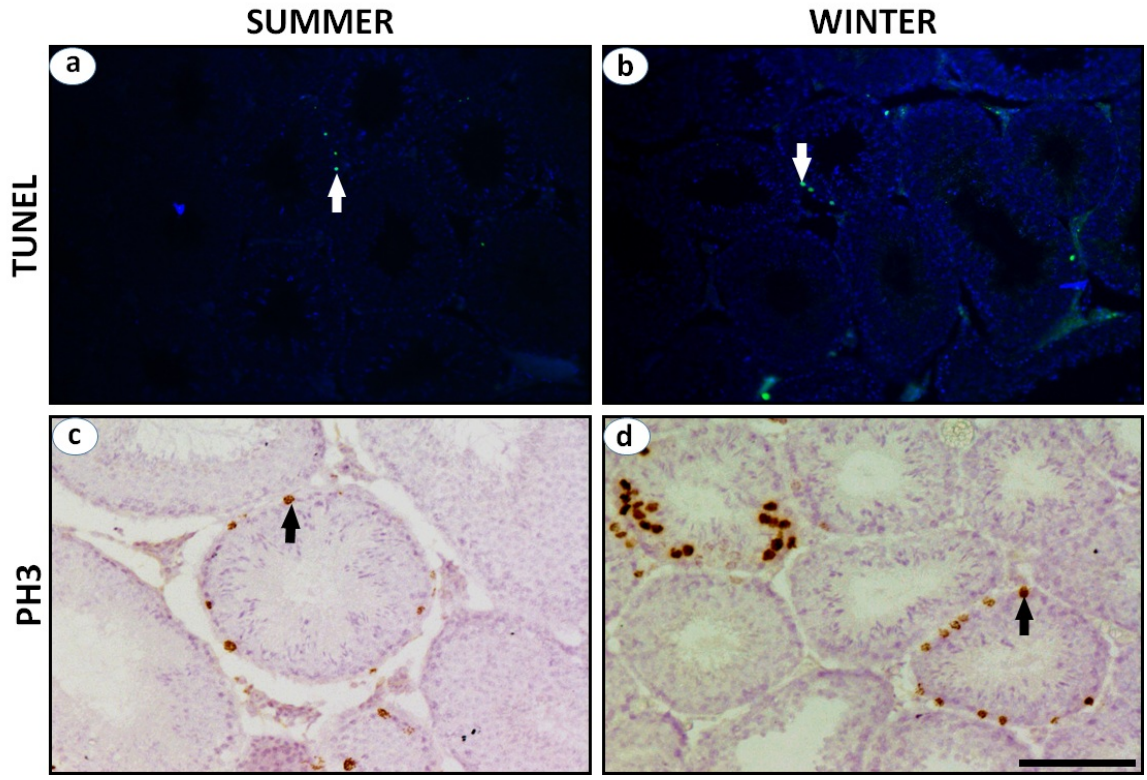


Figure 2.17: TUNEL staining (a and b) to detect apoptotic cells, and PH3 immunohistochemistry (c and d) to detect proliferating cells in transversal sections of testes from summer (left column) and winter (right column) males of *M. spretus*. Arrows point to positive cells. Scale bar represents 100 µm in a and b and 100 µm in c and d.

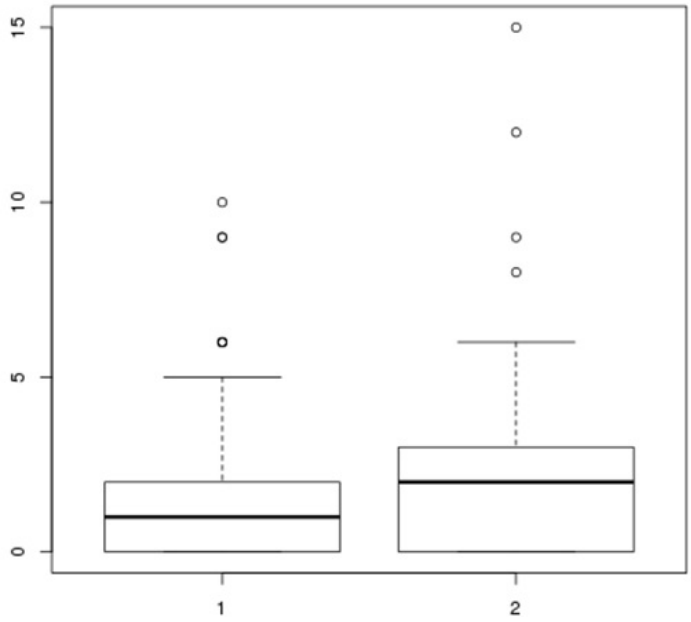


Figure 2.18: Comparison of the number of apoptotic cells per 0.1 mm^2 in testes from summer and winter males of *M. spretus*, analysed using a Box plot. Apoptotic cells are significantly more frequent in the winter testes as demosntrated by a Wilcox test.

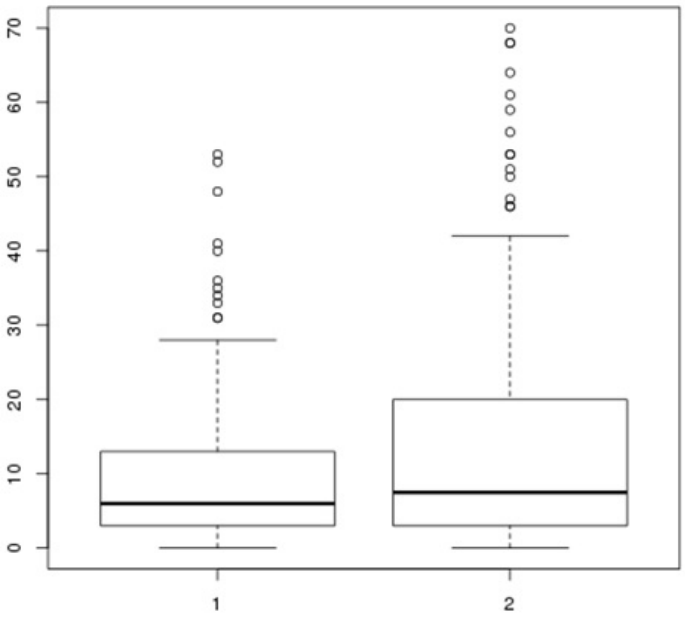


Figure 2.19: Comparison of the number of proliferating cells per 0.1 mm^2 in testes from summer and winter males of *M. spretus*, analyzed using a Box plot. Proliferating cells are more frequent in the winter testes, but statistical significance of a Wilcox test is slightly below the conventional 5% value.

spermatogenic cycle but by a reduction of the number of germ cells contained in the seminiferous tubules. This could be possible by either reducing the number of cells entering meiosis by decreasing the levels of spermatogonial proliferation, or increasing the level of spermatocyte depletion by apoptosis. Our results clearly support the latter hypothesis. Firstly, cell proliferation, which is known to involve mainly spermatogonial cells, is not reduced but slightly increased in the winter males (see Fig. 2.19). Secondly, apoptosis, which is known to affect mainly primary spermatocytes and spermatids, was shown to be significantly increased in the winter testes with respect to the summer ones (see Fig. 2.18). Hence, our findings suggest that the limited testis reduction that Algerian mouse testes undergo during the winter in the south-eastern populations if the Iberian Peninsula is mediated by increased apoptosis on meiotic germ cells. Elevated apoptosis levels in winter males is consistent with reduced serum testosterone in these animals (see Fig. 2.16), as it is well known that intra-testicular androgens negatively regulate apoptosis in the testis (Erkkila *et al.*, 1997; Nandi *et al.*, 1999; Troiano *et al.*, 1994), and low levels of testosterone in the serum reflect a reduction in the intra-testicular concentration of this hormone. However, unlike the summer Iberian mole, in which reduced intra-testicular testosterone induced also a massive desquamation of the germinative epithelium (Dadhich *et al.*, 2013), no evidence of germ cell sloughing was detected in the winter *M. spretus*. Several hypotheses can explain these differences: 1) the effects of testosterone on these processes may not be exactly the same in all species, 2) the intra-testicular levels of testosterone may be finely controlled, existing different effects induced at different testosterone reductions, and 3) additional control elements, other than androgens, are likely to exist that may differ between the Iberian mole and the Algerian mouse, inducing the observed differences. The expression patterns of several genes expressing in Sertoli cells, mainly SOX9 and DMRT1, suggest that they may play important roles in the control of the spermatogenetic cycle and seasonal reproduction. However, no difference between winter and summer males was detected in our immuno-histological study on the expression of these genes in the *M. spretus* testes. These showed the same expression pattern than that described for the active testes of other species, including Talpa occidentalis and Mus musculus (Dadhich *et al.*, 2011), as well as *C. russula*, *A. sylvaticus* and *M. duodecimcostatus* (studied here). It is well known that immuno-histochemistry and immuno-fluorescence are not quantitative techniques and it is possible that the expression levels of these genes could be altered in the winter testes of *M. spretus*, as shown in Talpa (Dadhich *et al.*, 2011). Hence, further studies are required to quantify the expression of SOX9 and DMRT1 by q-RT-PCR. The precise reasons why Algerian mice undergo a limited and transient testis size reduction in the winter, instead of a full testis regression, as observed in other species, remain unknown for the moment. However, we know that the testis reduction period in the populations investigated here is very short, occurring only in December and early January, as males from November and late January showed normal large testes. Hence, this limited reduction in testicular activity may be, like

in the case of the shrew, an adaptive response to a very short period of sexual inactivity. In these circumstances, complete testis regression could be disadvantageous in energetic terms as the required immediate germinative epithelium renewal would be relatively costly. Considering that the testes of *M. spretus* are larger than those of *C. russula*, this argument makes even more sense in the former species as there would be much more testicular tissue to be depleted and then regenerated again in so short time. The possibility also exists that there is not a period of sexual inactivity but a period of reduced but not null reproductive activity. In this case, males could retain some, but not full, spermatogenic production because some females could be receptive in that period. Like in the shrew again, further studies comparing the expression of GnRH-II in the brain of summer and winter female Algerian mice and defining better the reproductive cycle of the females of this species will help to shed light on this issue.

2.3.2 The surprising BTB permeation in the functional testes of winter*M. spretus*

The most striking finding in our study of *M. spretus* is the demonstration that the BTB is permeated in the winter testes, even though these gonads look morphologically normal, apart from a light size reduction, and are functional producing abundant sperm. This is surprising because it is assumed that BTB integrity is required to maintain testicular function in spermatogenically active testes. The main task of the BTB appears to be the protection of meiotic and postmeiotic germ cells from the immune system, as these cells do not exist when the establishment of self-tolerance takes place. Permeation of the BTB has been associated with diverse pathologic processes, including inflammation, infection, and trauma resulting in germ cell depletion (Comhaire *et al.*, 1999; Johnson, 1970; Lewis-jones *et al.*, 1987). Autoimmune orchitis is a common cause of human male sterility (Silva *et al.*, 2014). Hence, the situation in the Algerian mouse suggest different scenarios to explain the lack of a functional BTB. Firstly, we could admit that BTB permeation leads to autoimmunization of these animals against their own germ cells and, ultimately, to irreversible sterility. Considering that the average life expectancy of Algerian mice is very short and that these animals rarely survive their second winter (Palomo *et al.*, 2009; Cassaing, 1982; Cassaing and Croset, 1985) , this hypothesis could appear plausible if most animals were born in the spring, as autoimmunization would occur at the end of their active reproductive life. However, this is not the case as some births were found to occur in October and it does not make sense that these young males become sterile just after reaching the puberty. Furthermore, we never captured the putative autoimmunized, azoospermic males, probably exhibiting testicular abnormalities, that would be expected to exist in the months immediately after the testis reduction period. Accordingly, it is more likely that Algerian mice experience the testis reduction period without undergoing any drop in

their reproductive potential. But how is this possible if the BTB is transiently permeable and these animals continue producing meiotic cells and sperm? One possibility is that the BTB impairement is only partial, so that testes of winter males become permeable for very small molecules, as the biotin tracer used in our experiment (339 Daltons), but remains impermeable for larger molecules like antibodies (150.000 Daltons for the IgG, which are the smallest immunoglobulins). Furthermore, to induce autoimmunization, the cells of the immune system must enter into direct contact with the meiotic cells and, even though macrophages and other blood cells may infiltrate into tissues passing between endothelial cells by dispedesis, they could not run through the BTB to reach the adluminal compartment if this complex junctional structure is not completely abolished. The normal disposition of early primary spermatocytes (DMC1-positive) with respect to the CLAUDIN11 domain in the winter testes supports this hypothesis, but further electron microscopy and permeation studies using antibodies as tracers may help to elucidate this point. Even if BTB permeation is complete, the possibility still exists that winter males may scape from autoimmunization. It is currently accepted that the BTB alone does not account for all the aspects of the testicular immune privilege (Fijak *et al.*, 2011). It is known that many autoantigens are shared by both meiotic and spermatogonial cells, which are outside the BTB, and that sperm are weakly protected in the rete testis region, where the BTB is incomplete. These findings suggest that other mechanisms in addition to the physical separation provided by the BTB, probably exist to ensure testicular immune privilege. Reduced virulence of the autoimmune response in the testis micro-environment appears to be one of these additional mechanisms (Fijak *et al.*, 2011).

3. Third chapter *Apodemus sylvaticus*

Figure 3.1: *Apodemus sylvaticus*. Free reproduction image taken from Wikipedia

3.1 Introduction

The wood mouse, *Apdemus sylvaticus*, is a small rodent of the family Muridae present in Europe and north-western Africa. It has been reported that this species frequently inhabits mainly forest habitats but also steppes, crop fields, rocky and mountain environments (Hooper, 1968), (Jamon, 1982), (Schenk, 1987; Zima, 2004). Wood mice of genus *Apodemus* have become an important mammalian model for studying the evolutionary dynamics of mammals (Zima, 2004).

(Lee *et al.*, 2001) studied the annual reproductive cycle of adult males of the Formosan wood mice (*Apodemus semotus*) which inhabit the Alishan area in Taiwan. Testes, epididymides, and seminal vesicles were lightest and spermatogenesis was virtually arrested in December-January. During the remainder of year, all animals examined showed active spermatogenesis. A reduction in the mass of the reproductive organs and diameter of seminiferous tubules took place in May–July, but spermatogenesis was not affected. Levels of testicular and plasma testosterone were elevated in March–April and August–September and were significantly correlated with the mass of testes, epididymides, and seminal vesicles, and with the diameter of seminiferous tubules. Whereas day length and temperature were correlated with mass of testes, epididymides, and seminal vesicles, precipitation was not.

It has been suggested that the reproductive cycle of *A. sylvaticus*, in central and north Europe starts early in the spring and ends by late summer or early autumn (Rood, 1965; Moreno and Kufner, 1988). However, the annual breeding and population density variation patterns of wood mice in the Mediterranean scrubland of southwestern Spain extends from August to April (Moreno and Kufner, 1988) . In the coastal zone of Algeria (and probably in the entire north-western Africa) reproduction of *A. sylvaticus* seems to be restricted to autumn and winter (Kowalski, 1985), probably because of the mildness of climate during this period and the extreme heat of the summer which might be inhibitory (Klaa, 1999). The meiotic behaviour of the sex chromosomes of *A. sylvaticus* has been studied in populations of Granada province in south-eastern Iberian Peninsula (Stitou *et al.*, 2001), but the seasonal reproductive cycle of this species in this region has not been reported to date.

3.2 Results

3.2.1 Males of *Apdemus sylvaticus* undergo summer testis regression in south-eastern Iberian Peninsula

Four study groups, named winter, spring, summer and autumn, were established according to the season of capture. Data on body mass, testis mass, and testis/body mass ratio of the males of *A. sylvaticus* analyzed are summarized

in table 3.1. Comparison between the body mass of individuals captured in all four seasons showed that autumn animals were significantly larger than those of the other three seasons, the smaller individuals being captured in the spring (table 3.2; Fig. 3.2). Regarding testis mass, statistical analysis revealed a highly significant reduction in the summer group when compared with the other groups (table 3.3; Fig. 3.3). In addition, significant difference was also detected in the comparison between spring and autumn males. However, since the spring and the autumn mice were the smaller and the larger animals analyzed, respectively, we suspected that these differences in testis mass could be a consequence of the different body size. Hence, we also calculated the testis/body mass ratio for the animals of all four study groups and compared them as previously (table 3.4; Fig: 3.4). In this case, only summer males showed a ratio significantly reduced, the other three groups showing no difference to each other. These data indicate that testis regression in the populations of *A. sylvaticus* analyzed here exclusively occurs in the summer months but not in the rest of the year. Consistently, we considered that the winter and the summer males of these populations are the most representative of the breeding and the non-breeding periods, respectively, and we used a sample of animals from these seasons for further histological, morphometric, immuno-histochemical and hormonal comparative studies.

	Winter	Spring	Summer	Autumn
Body mass ± SD (g)	25.25± 2.87	23.1±3.17	27±1.63	33.33± 2.5
Testis mass ± SD (mg)	439.5±114	431.9±114	88.125±36.079	576±62.38
Gonad/Body mass (mg/g)	18±5	19±5	3±1.5	17±0.7
n	4	10	4	3

Table 3.1: Values of body mass, testis mass, and testis/body mass ratio and number of males of *A. sylvaticus* analyzed in each season in south-eastern populations of the Iberian Peninsula.

P (body mass)	Winter	Spring	Summer	Autumn
Winter		0.26476	0.34027	0.0118*
Spring			0.01211*	0.00391**
Summer				0.02785*

Table 3.2: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the body mass of every study group with each other, as shown in table 3.1. Asterisks indicate the level of statistical significance.

Consistent with their different size, winter testes showed a normal histology, with seminiferous tubules covering all the stages of the spermatogenic cycle, well developed seminiferous epithelium and mature spermatozoa in the lumen, whereas summer testes exhibited a very thin germinative epithelium

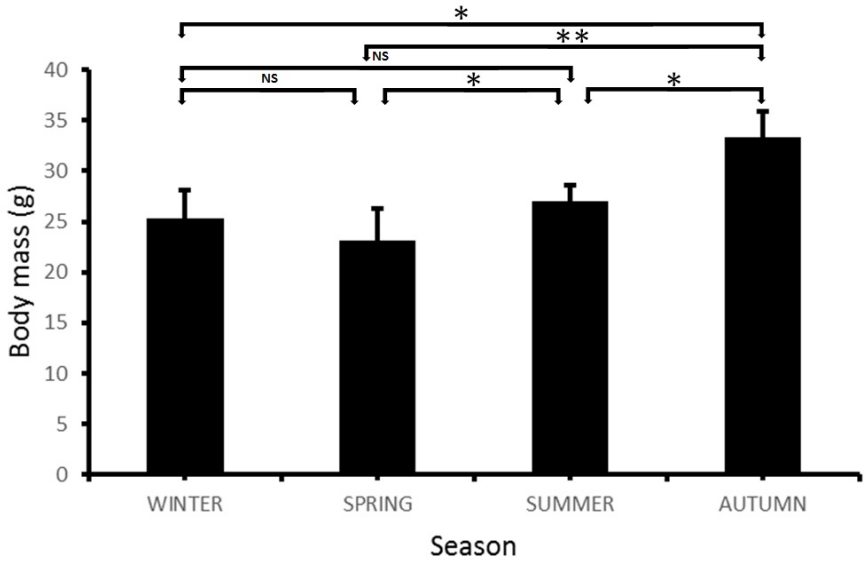


Figure 3.2: Seasonal variations in the body mass of adult males of *A. sylvaticus*. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

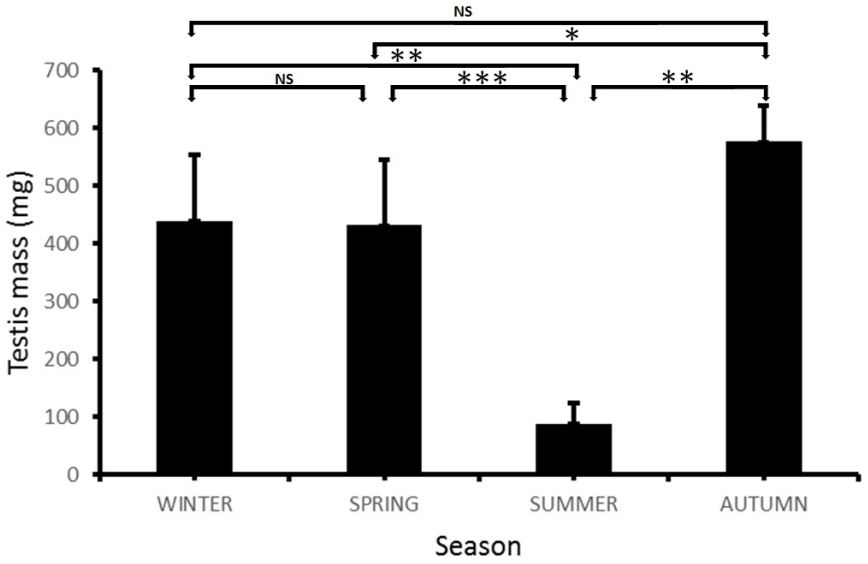


Figure 3.3: Seasonal variations in the testis mass of adult males of *A. sylvaticus*. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

P (Testis mass)	Winter	Spring	Summer	Autumn
Winter		0.9143	0.00583**	0.1021
Spring			2.1E-6***	0.02735*
Summer				0.00121**

Table 3.3: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the testis mass of every study group with each other, as shown in table 3.1. Asterisks indicate the level of statistical significance.

P (Testis mass/Body Mass)	Winter	Spring	Summer	Autumn
Winter		0.67653	0.00611**	0.90935
Spring			7.02E-07***	0.33557
Summer				5.12E-05***

Table 3.4: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the testis/body mass ratio of every study group with each other, as shown in table 3.1. Asterisks indicate the level of statistical significance.

and no mature spermatozoa in the lumen (Fig. 3.5 a and c). Statistical analysis confirmed a significant reduction in the diameter of summer seminferous tubules (195.85 ± 22.96 µm for the winter group and 75.11 ± 12.18 µm for the summer group; Student-t test, $p < 0.001$, Fig. 3.6). Consistent with these observations, the epididymides in the winter group were full of spermatozoa, whereas the summer epididymides were completely empty (Fig. 3.5 b and d). Thus, in the province of Granada *A. sylvaticus* do not reproduce in the summer season and during this period the testes undergoes a process of regression.

3.2.2 Spermatogenesis is arrested at the spermatocyte stage in *Apdemus sylvaticus* summer testes

We also compared the expression pattern of Sertoli cell markers between winter and summer adult testes of *A. sylvaticus*. Immuno-histochemistry revealed a pattern of expression for SOX9 in the testes of winter males of this species similar to that described before for *C. russula*, *M. musculus* (see above) and *T. occidentails* (Dadhich *et al.*, 2011). SOX9 expression is spermatogenic cycle-dependent with strong expression in the stages I-IV and a weaker expression in the stages VII-X. However, the summer seminiferous tubules showed an uniform SOX9 staining intensity and the distance between neighbouring SOX9-positive cells was very reduced. This latter observation is probably a consequence of the shrinkage that seminiferous tubules undergo in this season. (Fig. 3.7 a and b). Again, as described in the mole (Dadhich et al., 2011) as well as in the shrew and the Algerian mouse (see above),

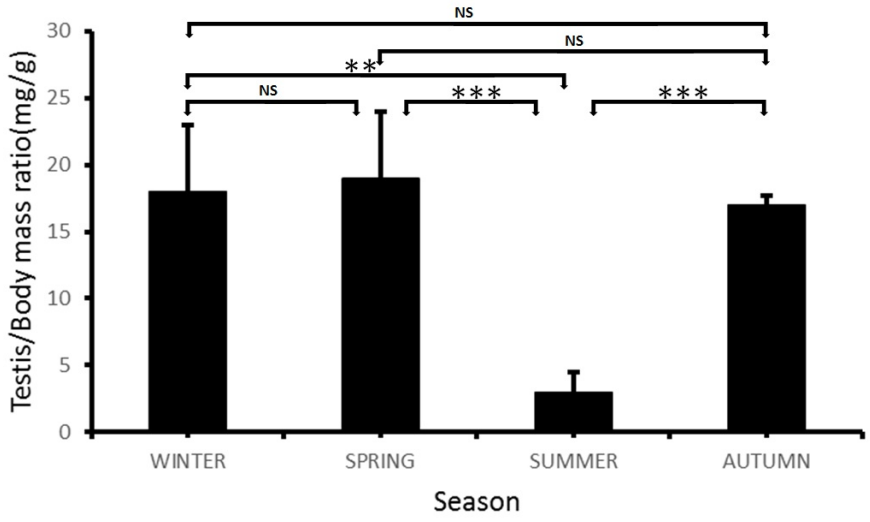


Figure 3.4: Seasonal variations in the testis/body mass ratio of adult males of *A. sylvaticus*. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

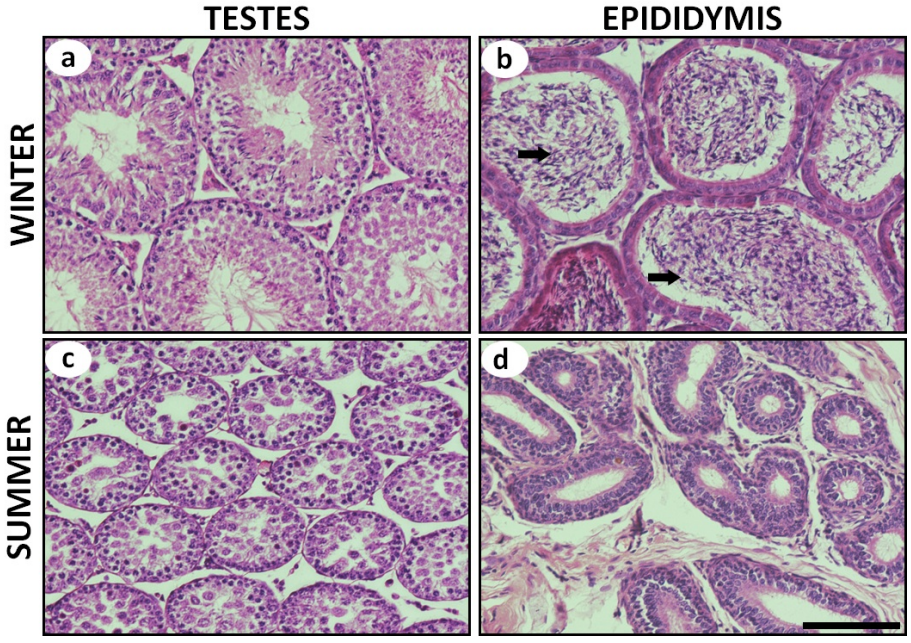


Figure 3.5: Haematoxylin-eosin-stained tansverse sections of testes (a and c) and epididymides (b and d) from winter (a and b) and summer (c and d) males of *A. sylvaticus*. In the winter testis the seminiferous tubules show active spermatogenesis and the epididymides contain abundant sperm. Scale bar represents 100 µm for all pictures.

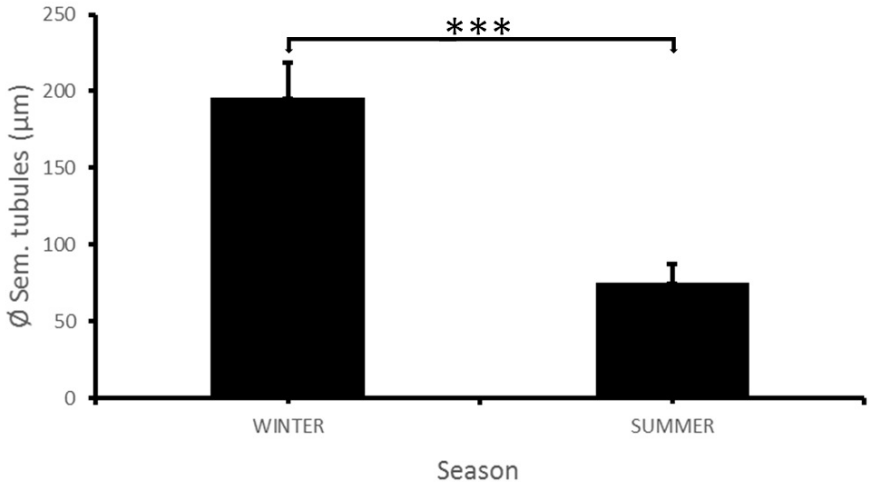


Figure 3.6: Comparison of the testis tubule diameter between summer and winter testes of adult males of *A. sylvaticus*. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

studies of DMRT1 expression in the testes of winter males of *A. sylvaticus* showed two types of seminiferous tubules depending on the number of DMRT1-positive cells: LDD and HDD (Fig. 3.7 c and d). Both types of tubules were spermatogenic stage-specific, with LDD tubules corresponding to stages I-IV and HDD tubules to stages VI-VII. In the summer testes, we also found two types of tubules depending on the number of DMRT1 cells, LDD-like and HDD-like seminferous tubules. SOX9-DMRT1 double immuno-fluorescence showed that in the winter testes DMRT1 expression is almost exclusively restricted to Sertoli cells in LDD tubules, whereas in HDD tubules the DMRT1 signal is also found in other cells located in the basal compartment of the germinative epithelium, which correspond to spermatogonia (Fig. 3.8). In the LDD-like tubules of summer testes most of the DMRT1-positive cells were also SOX9-positive, indicating that most cells in these tubules are Sertoli cells and the few SOX9-negative DMRT1-positive cells are spermatogonia. In general, the latter cells maintain a basal position, whereas Sertoli cell nuclei move to more internal locations, forming something similar to a pseudo-stratified epithelium. In contrast, in summer HDD-like testis tubules about half of DMRT1-positive cells were also SOX9-negative, indicating an increase in the number of spermatogonial cells in these tubules. These observations show that the summer testes of *A. sylvaticus* actively experience the spermatogonial proliferative phases of the spermatogenic cycle. Furthermore, we always detected cells negative for both SOX9 and DMRT1 in the innermost region of summer testes tubules suggesting that these cells are meiotic spermatocytes and showing that spermatogenesis must be arrested beyond the spematogonial proliferative stage.

The expression pattern of PCNA in the testes of winter males of *A. sylvati-*

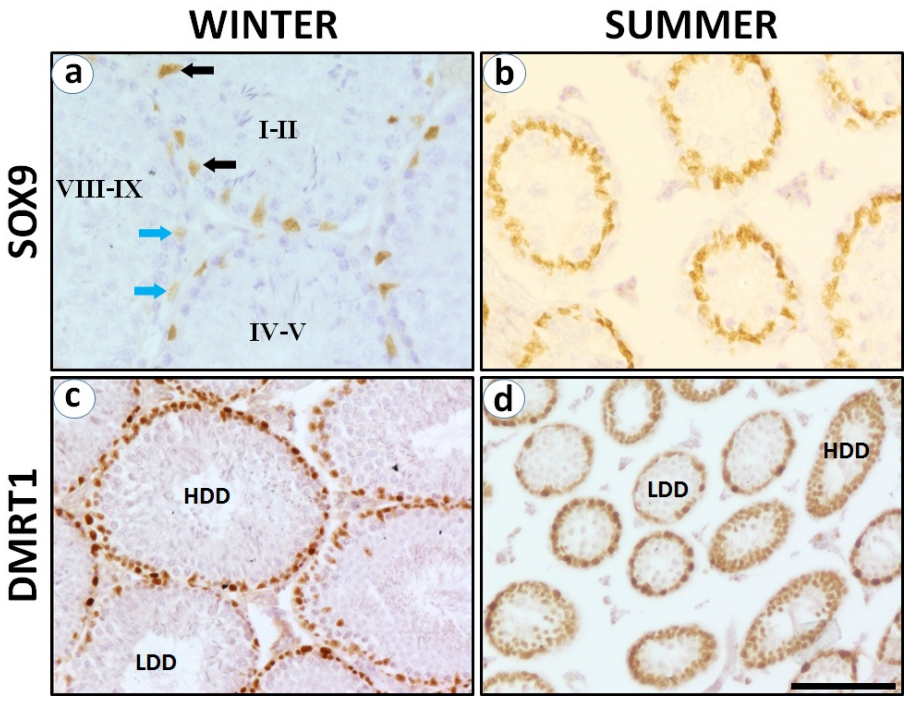


Figure 3.7: Immunohistochemical detection of both SOX9 (a and b) and DMRT1 (c and d) proteins in the testes of winter (left column) and summer (right column) males of *A. sylvaticus*. SOX9 expression is stronger in stages I-IV (black arrows), and a weaker in stages VII-X (cyan arrows). No relevant difference was observed in DMRT1 expression between the two groups (c and d). Two types of testis tubules can be distinguished regarding the content of DMRT1-positive cells: low-density DMRT1-expressing (LDD) tubules, in which expression is almost restricted to Sertoli cells and appear in stages I-IV, and high-density (HDD) ones, in which the expression is observed in both Sertoli and spermatogonial cells, which are observed in stages VI-VII. Scale bar represents 100 µm in all pictures.

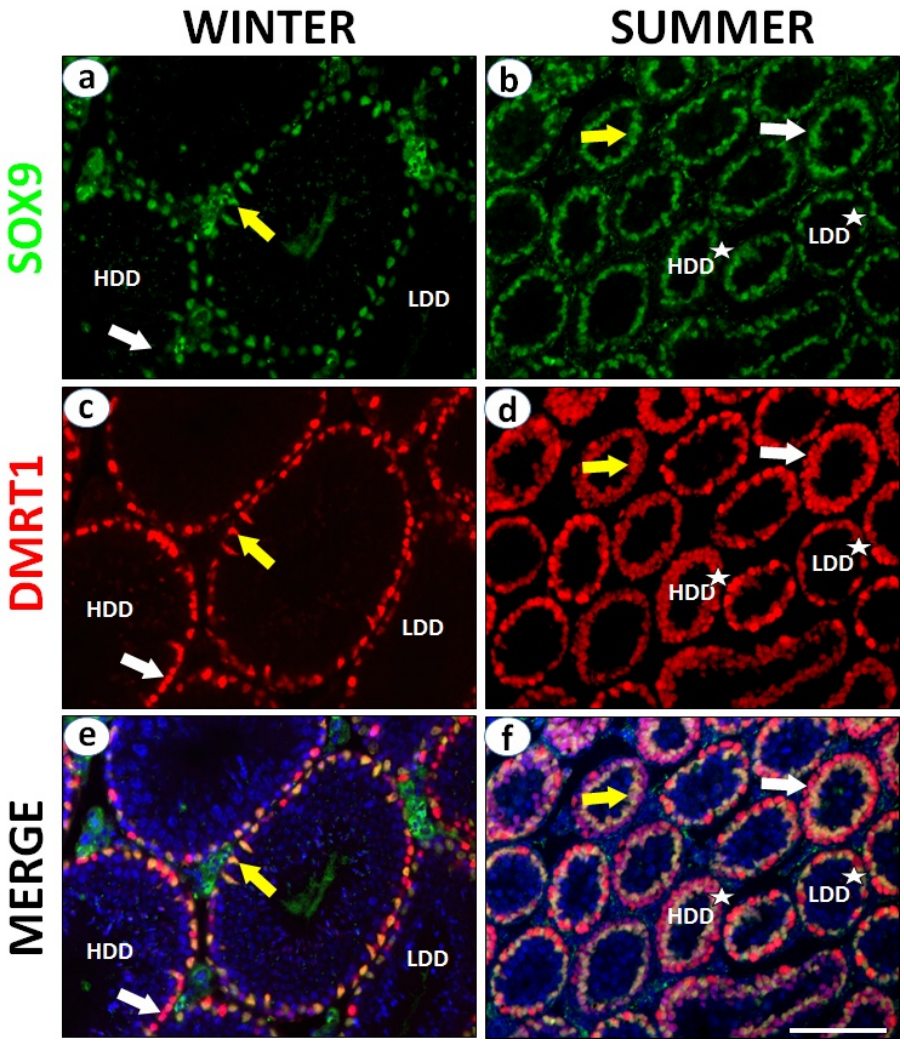


Figure 3.8: Double immuno-fluorescence for SOX9 (green; a and b) and DMRT1 (red; c and d) proteins in testes from winter (left column) and summer (right column) males of *A. sylvaticus*. Pictures in e and f show merged images with DAPI counterstain (blue). DMRT1 co-localizes with SOX9 in Sertoli cells (yellow arrows), indicating that the other immuno-reactive cells (white arrows) are spermatogonia. Scale bar represents 100 µm in all pictures.

cus was similar to that of the species studied above (*C. russula* and *M. spretus*), being spermatogenic-cycle dependent in both spermatogia and zygotene-pachytene spermatocytes (Fig. 3.9 a and b). In the summer testis, many tubules expressed PCNA in most cells, although with varying intensity, and only a few tubules were observed with PCNA-negative cells in the innermost zone (Fig. 3.9 b). As we have shown in the previous chapters for *C. russula* and *M. spretus*, DMRT1-PCNA double immnuo-fluorescence in winter testes of *A. sylvaticus* also permited the identification of Sertoli cells (DMRT1-positive, PCNA-negative), spermatogonial cells (DMRT1-positive, PCNA-positive) and zygotene-pachytene spermatocytes (DMRT1-negative, PCNA-positive) (Fig. 3.10). In the summer testes, DMRT1 and PCNA also co-expressed in spermatogonial cells of both LDD-like and HDD-like testis tubules. But while in HDD-like tubules most of the cells were spermatogonia and only a few zygotene-pachytene spermatocytes were observed in the innermost region of the tubules, in LDD-like tubules only one layer of spermatogonial cells was located near the basal surface and most of the inner cells were zygotene-pachytene spermatocytes. In both cases, a very reduced number of DMRT1-negative PCNA-negative cells were observed in the innermost zone of the tubules. Finally, as observed in the other species studied in this work, we detected a spermatogenic cycle-dependent expression of DMC1 in zygotene-early pachytene spermatocytes (Fig. 3.9 c and d). In both winter and summer testes, expression of DMC1 was observed in some testis tubules but not in others. DMRT1-DMC1 double immunofluorescence showed no co-localization of both proteins in any cell of either winter or summer testes. In the latter, DMC1 was only expressed in LDD-like seminiferous tubules, in which almost all the cells located in the innermost part of the tubules were DMC1-positive (Fig. 3.11), indicating that in summer testes of *A. sylvaticus*, spermatogenesis do not progress beyond the zygotene-pachytene stage.

3.2.3 The blood testis barrier is impaired in the summer testis of *Apdemus sylvaticus*.

We performed in *A. sylvaticus* the biotin tracer experiment as described previously for *M. spretus* to investigate the status of the blood-testis barrier in the two study groups. In the winter testes, biotin (red color) was detected in both the interstitial tissue and the basal compartment of the seminiferous tubules but not in the adluminal compartment (Fig. 3.12 a and c). However, it was detected in both basal and adluminal compartments in the testes of the summer group, showing that the BTB of inactive testes can be permeated by the tracer. (Fig. 3.12 b and d). Moreover, immuno-histochemistry for CLAUDIN11 revealed a dynamic CLAUDIN11 expression pattern showing stronger signal in tubules in stages IV-XI and a weaker expression in tubules of the reminder stages (Fig. 3.13). Double immuno-fluorescence for both CLAUDIN11 and the biotin tracer (Fig. 3.14), showed that the tracer either coexpressed or was located basal to the CLAUDIN11 signal in the seminiferous

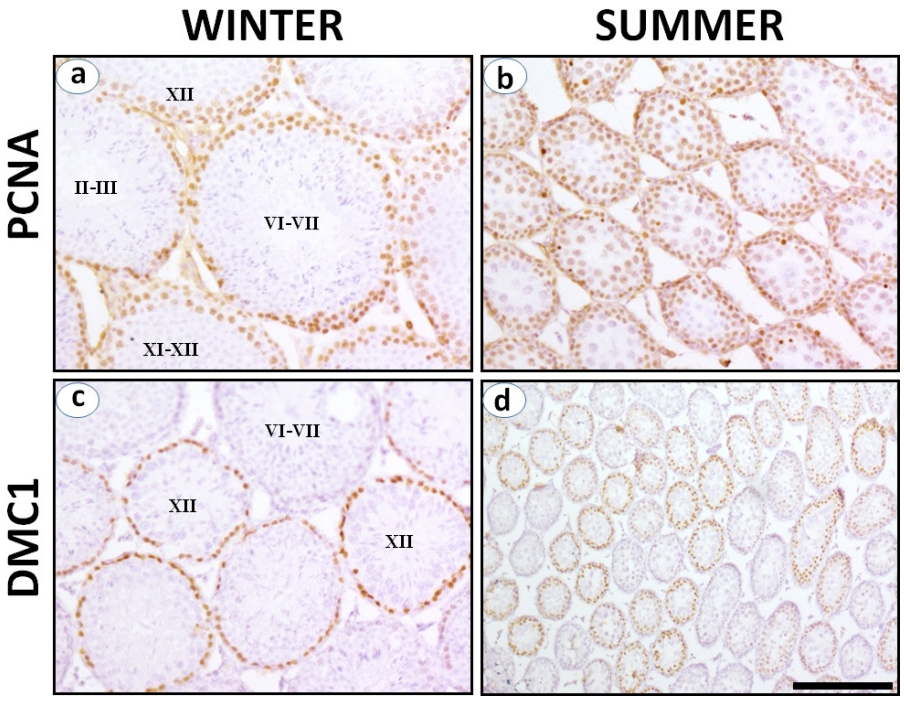


Figure 3.9: Immuno-histochemical detection of both PCNA (a and b) and DMC1 (c and d) proteins in testes from winter (left column) and summer (right column) males of *A. sylvaticus*. The spermatogenic cycle stages of some seminiferous tubules are indicated in Roman numerals. Scale bar represents 100 µm in all pictures.

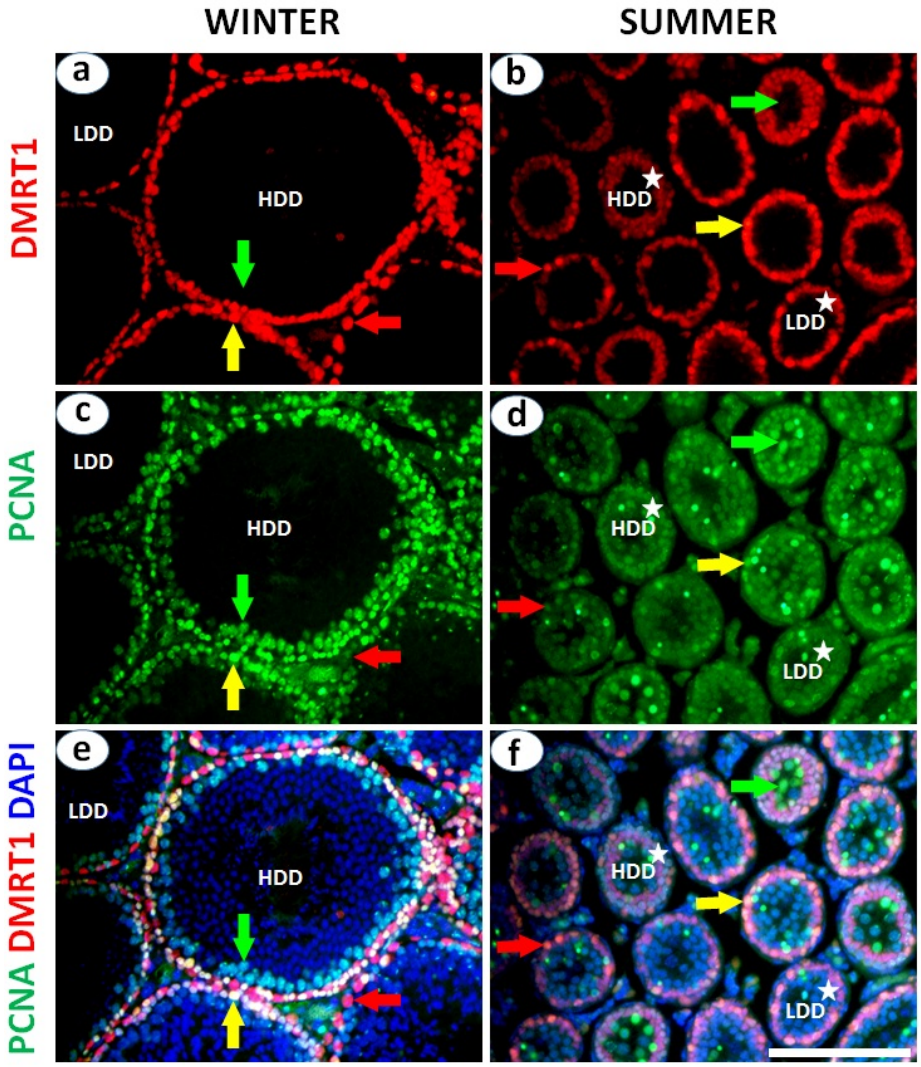


Figure 3.10: Double immuno-fluorescence for both DMRT1 (red; a and b) and PCNA (green; c and d) proteins in testes of winter (left column) and summer (right column) males of *A. sylvaticus*. Pictures in e and f show merged images with DAPI counterstain (blue). PCNA co-localizes with DMRT1 in spermatogonial cells (yellow arrows), but neither in Sertoli cells (red arrows) nor in zygotene-early pachytene spermatocytes (green arrows). Scale bar represents 100 µm in all pictures.

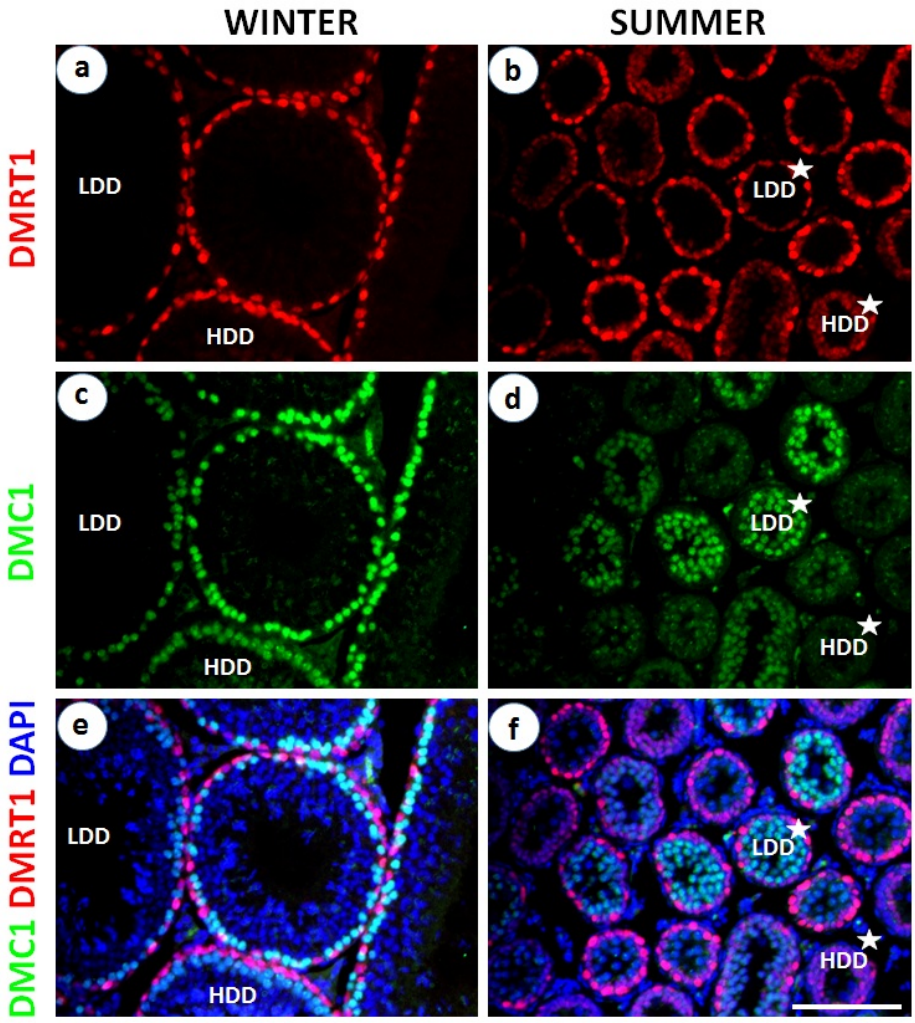


Figure 3.11: Double immuno-fluorescence for both DMRT1 (red; a and b) and DMC1 (green; c and d) in testes of winter (left column) and summer (right column) males of *A. sylvaticus*. Pictures in e and f show merged images with DAPI counterstain (blue). DMRT1 and DMC1 do not co-localize in any cell type in both summer and winter testes. Scale bar represents 100 µm in all pictures.

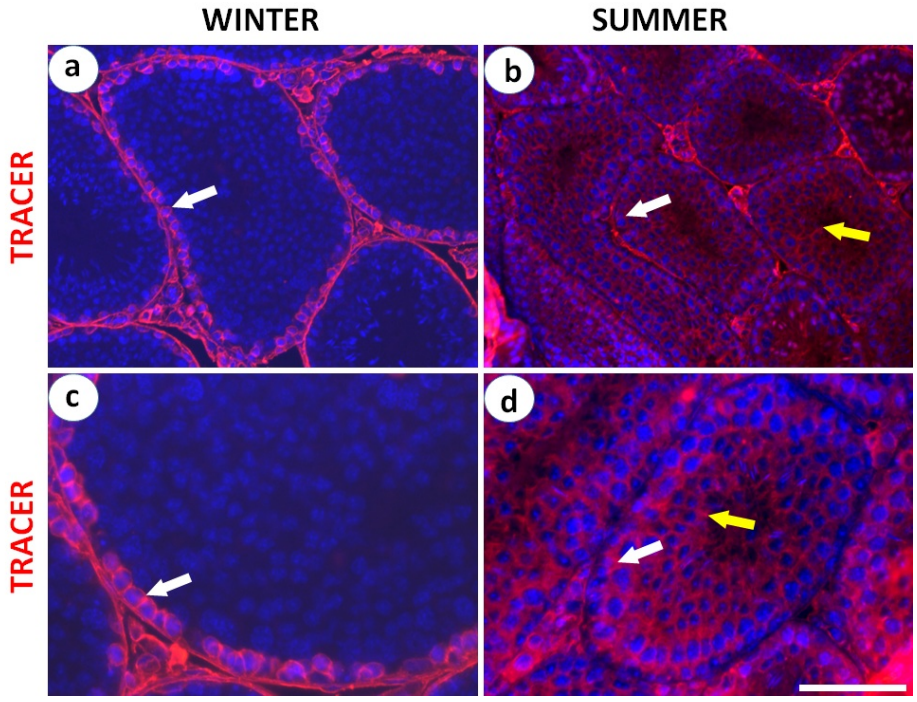


Figure 3.12: Immuno-detection of biotin tracer after injection in the interstitial space of the testis from winter and summer males of *A. sylvaticus*. The tracer (red fluorescence) is located in the basal compartment of the seminiferous epithelium in both winter and summer testes (white arrows in all pictures), but it also appears in the adluminal compartment of the summer testes (yellow arrows in b and d). Nuclei were counterstained with DAPI (blue fluorescence in all pictures). Scale bar represents 100 µm in a and b, and 50 µm in c and d.

tubules of winter testes, whereas it was observed at both sides of CLAUDIN11 domain in the summer testes. Similarly, CLAUDIN11-DMC1 double immunofluorescence showed that all DMC1-positive cells were located inner to the CLAUDIN11 domain in the winter testis, showing that all cells passing through the BTB are meiotic cells, whereas meiotic cells can be seen located at the other side of the BTB in the summer group, evidencing impaired function of this structure in the regressed testis of *A. sylvaticus* (Fig. 3.15).

As we did before for the case of *M. musculus*, we checked the status of the lamina propria to compare it between winter and summer testes of *A. sylvaticus* using immuno-histochemistry for alpha-SMA, DESMIN, and LAMININ (Fig. 3.16). All three proteins were located around the seminiferous tubules, showing no differences between winter and summer testes, which suggests that the lamina propria is not altered during testis regression in this species.

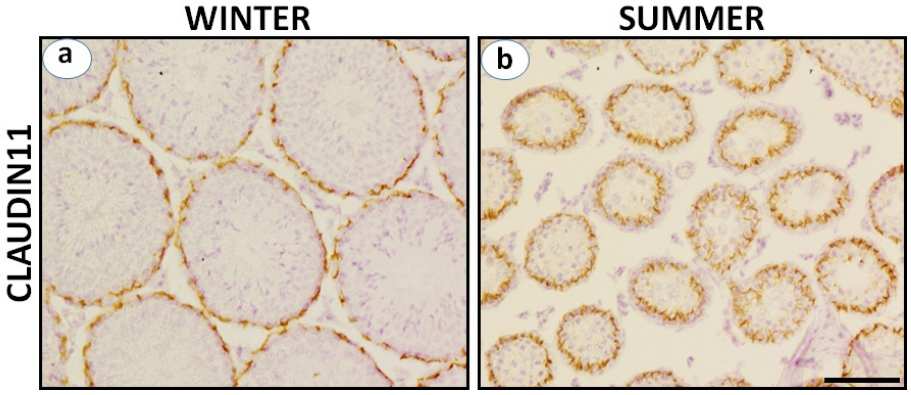


Figure 3.13: Immuno-histochemical detection of CLAUDIN11 in the testes of winter (a) and summer (b) males of *A. sylvaticus*. Scale bar represents 100 µm in all pictures.

3.2.4 Reduced steroidogenesis during the summer in males of *A. sylvaticus*

To study the androgenic function in winter and summer males of *A. sylvaticus*, we analyzed by immuno-histochemistry the expression of AR and P450scc. We observed an expression pattern identical to that described for *M. spretus*. AR was expressed in Sertoli, Leydig, and peritubular myoid cells in both winter and summer testes, although in PM cells AR-immunoreactivity was clearly stronger in the summer testes (Fig. 3.17 a and b). Similarly, P450scc was strongly expressed in Leydig cells in both summer and winter testes (Fig. 3.17 c and d). Finally, RIA analysis evidenced a clear reduction in testosterone levels in the summer animals compared with those of the winter ones (Fig. 3.18).

3.2.5 Increased levels of both apoptosis and cell proliferation in the inactive testis of *A. sylvaticus*

Using TUNEL assays to detect apoptotic cells (Fig. 3.19 a and b) and PH3 immuno-histochemistry to detect proliferating cells (Fig. 3.19 c and d), we found that both processes were clearly increased in summer testes when compared to winter ones. The number of apoptotic cells was 1.08 ± 3.13 cells/0.1 mm^2 in the winter and 33,89 ± 15.29 cells/0.1 mm^2 in the summer. Comparison using a Wilcox's test showed highly significant differences between the two study groups ($W = 121$, $P<0.001$; Fig. 3.20). Similarly, the number of proliferating cells was 3.83 ± 6.35 cells/0.1 mm^2 in the winter and 11.71 ± 9.75 cells/0.1 mm^2 in the summer, differences being again highly significant (Wilcox's test: $W =6292$, $P<0.001$; Fig. 3.21).

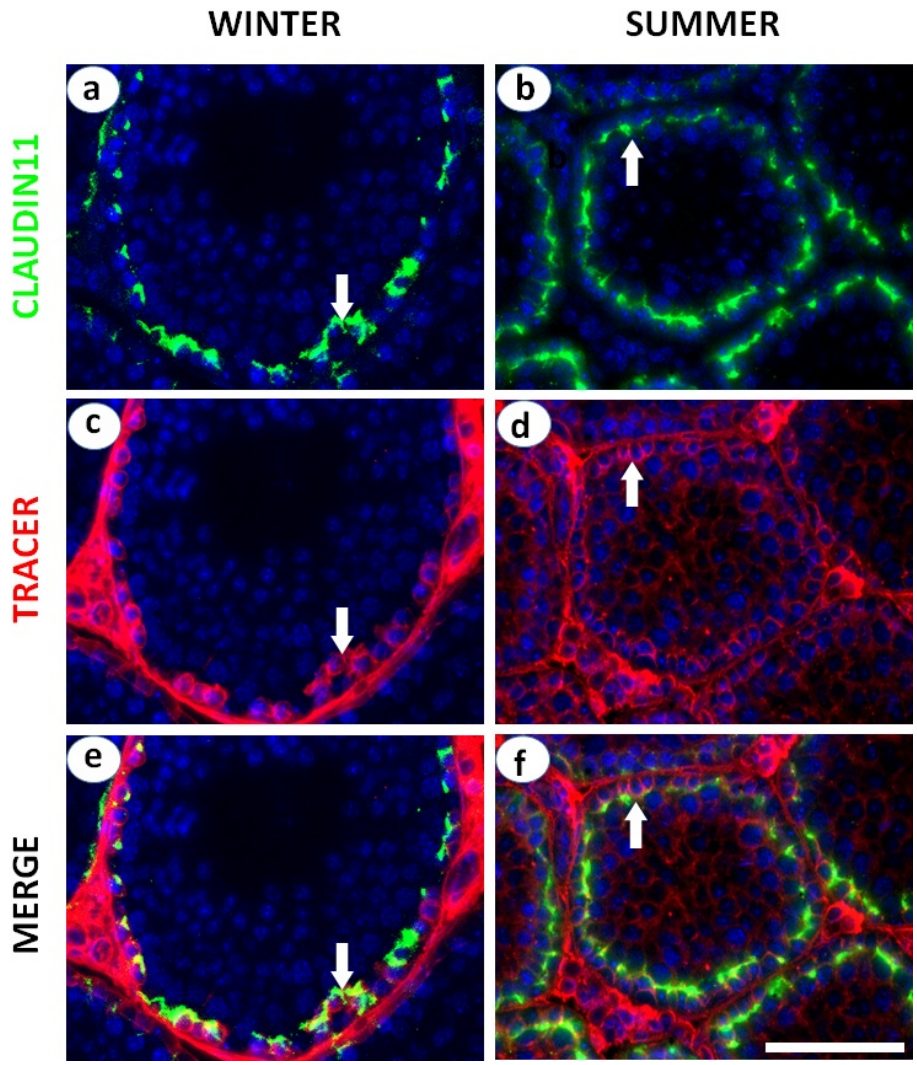


Figure 3.14: Double immuno-fluorescence for both CLAUDIN 11 (green; a and b) and the biotin tracer (red; c and d) in testes from winter (left column) and summer (right column) males of *A. sylvaticus*. Pictures in e and f show merged images with DAPI counterstain (blue). Biotin is located in the basal compartment of winter testes and at both sides of the CLAUDIN11 domain in the summer ones. Scale bar represents 50 µm in all pictures.

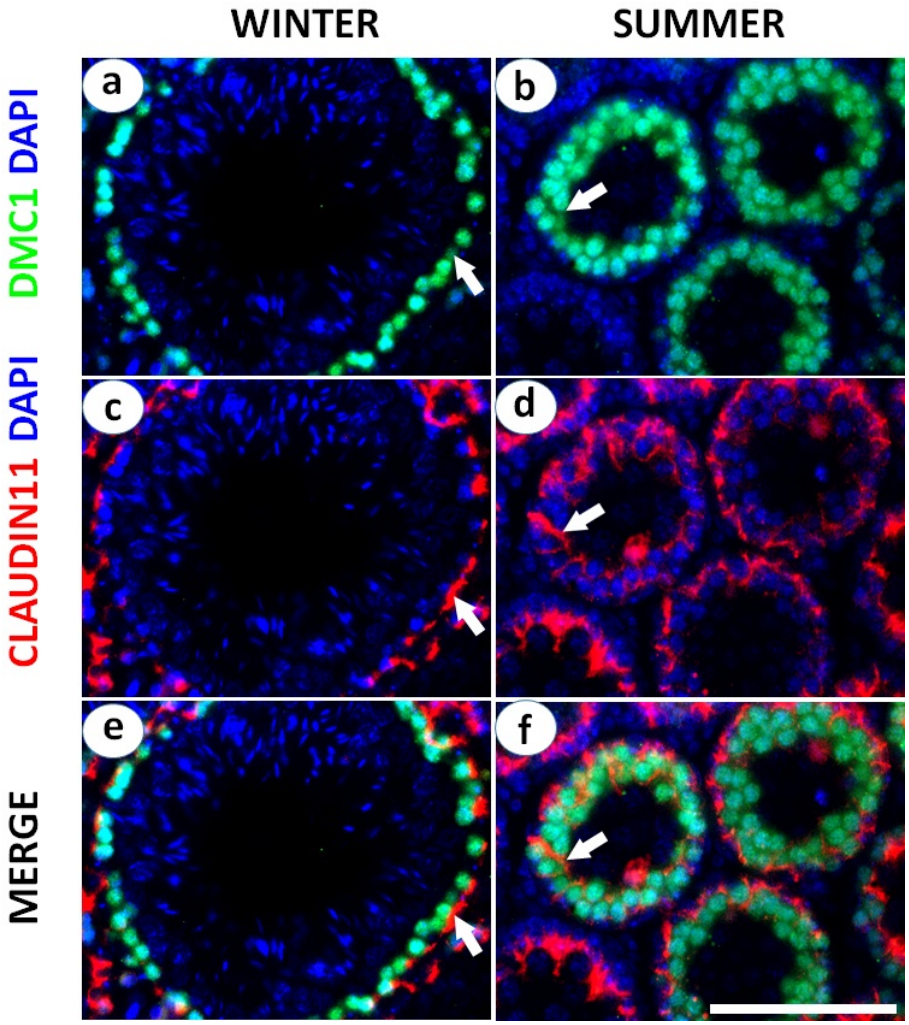


Figure 3.15: Double immune-fluorescence for both DMC1 (green; a and b), and CLAUDIN 11 (red; c and d), and DAPI counterstaining (blue; a-f) in testes of winter (left column) and summer (right column) males of *A. sylvaticus*. All DMC1-positive meiotic cells are located inner to the CLAUDIN11 domain in the winter group, but some meioic cells are seen basal to the BTB domain in the summer group (arrows). Scale bar represents 50 µm in all pictures.

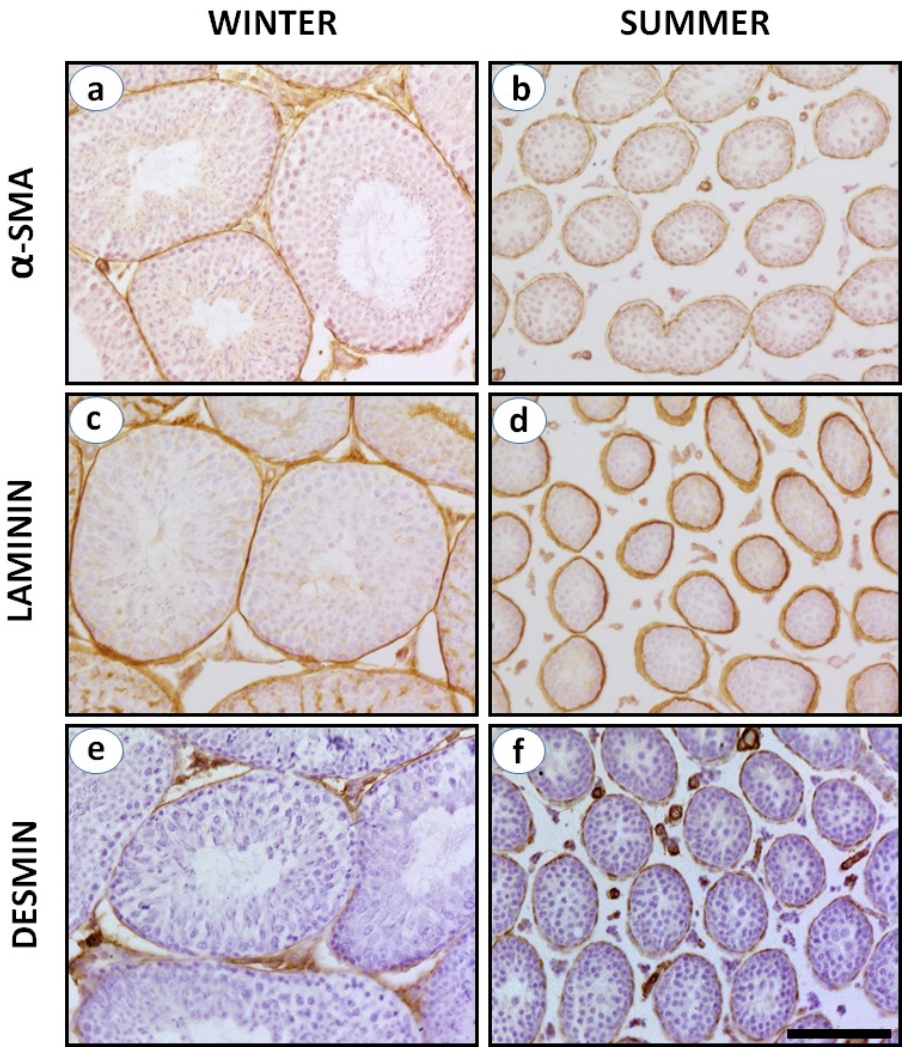


Figure 3.16: Immunohistochemical detection of alpha-SMA (a and b), LAMININ (c and d), and DESMIN (e and f) in the testes of winter (left column) and summer (right column) males of *A. sylvaticus*. The same expression pattern is observed in the two study groups for all three proteins. Scale bar represents 100 µm in all pictures.

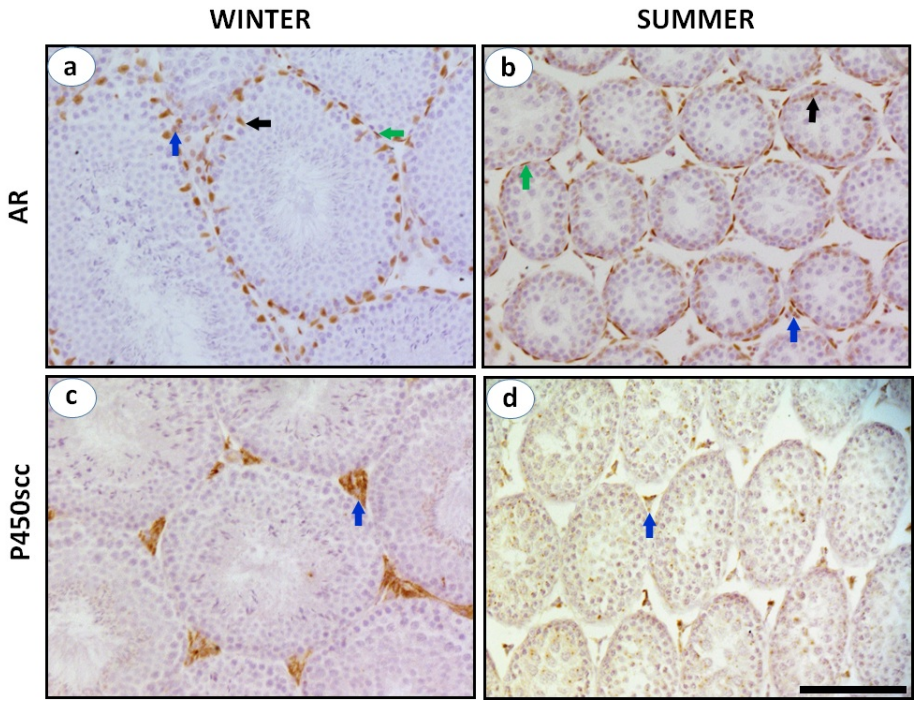


Figure 3.17: Immuno-histochemistry for both AR (a and b) and P450scc (c and d) in transverse sections of testes from winter (left column) and summer (right column) males of *A. sylvaticus*. Nuclei were lightly counterstained with haematoxylin. AR expression is detected in Sertoli cells (black arrows), Leydig cells (blue arrows), and peritubular myoid cells (green arrows), and P450scc is expressed in Leydig cells (blue arrows), in the two study groups. Scale bar represents 100 µm in all pictutes.

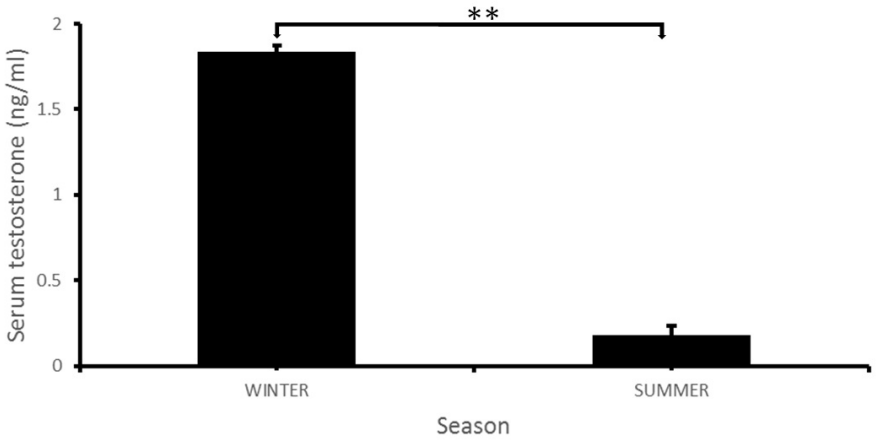


Figure 3.18: Levels of testosterone measured by radio-immunoassay in the serum of both winter and summer males of *A. sylvaticus*. A clear reduction is observed in the summer with respect to the winter group.

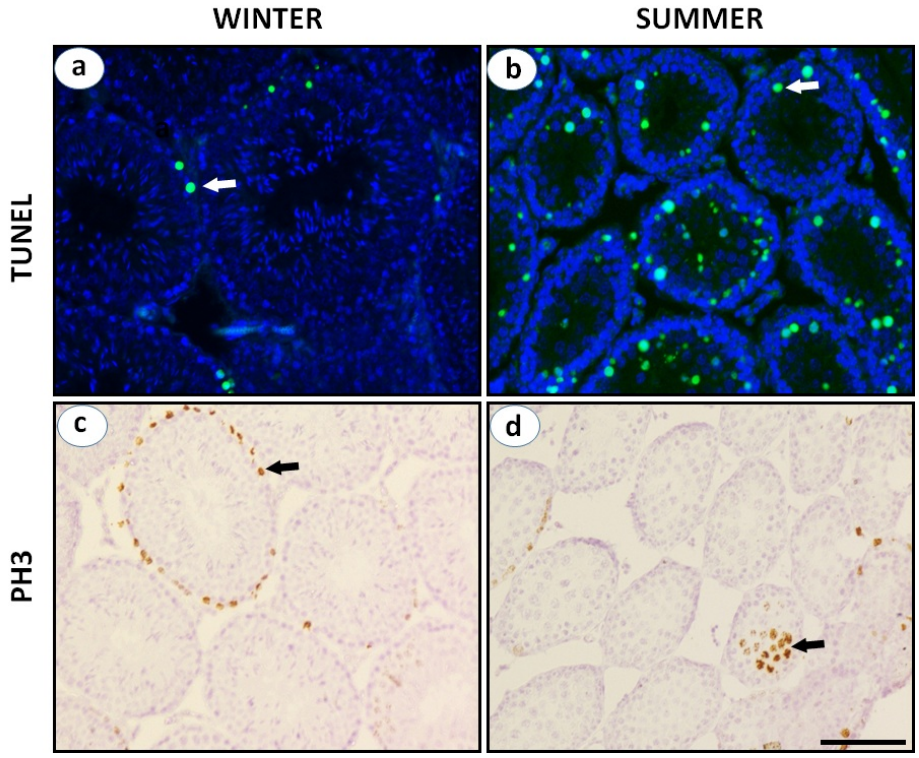


Figure 3.19: TUNEL staining (a and b) to detect apoptotic cells, and PH3 immunohistochemistry (c and d) to detect proliferating cells in transversal sections of testes from winter (left column) and summer (right column) males of *A. sylvaticus*. Arrows point to positive cells. Scale bar represents 100 µm in a and b and 200 µm in c and d.

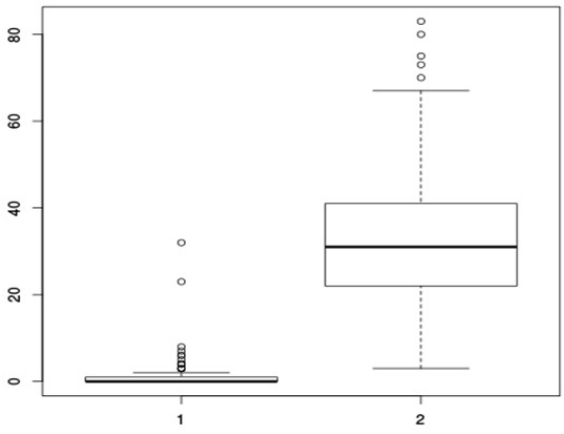


Figure 3.20: Comparison of the number of apoptotic cells per 0.1 mm^2 in testes from winter and summer males of *A. sylvaticus*, analyzed using a Box plot. According to a Wilcox test, apoptotic cells are significantly more frequent in the summer testes.

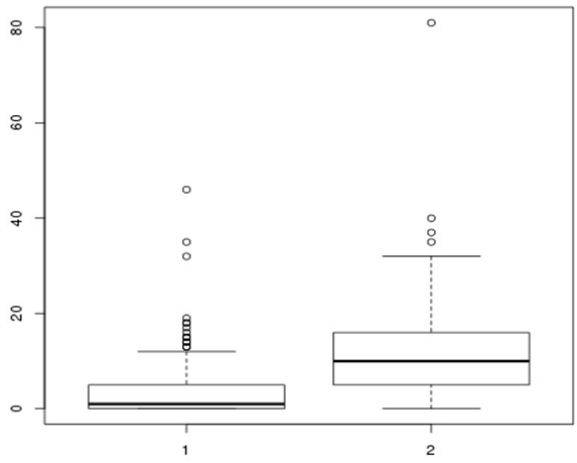


Figure 3.21: Comparison of the number of proliferating cells per 0.1 mm^2 in testes from winter and summer males of *A. sylvaticus*, analyzed using a Box plot. A wilcox text demonstrated that proliferating cells are significantly more frequent in the summer testes.

3.3 Discussion

3.3.1 Short period of summer testis inactivity in *A. sylvaticus*

The reproductive timing of the populations of *Apodemus sylvaticus* we have studied in this work fits very well with a classical model including a continuous reproductive period comprising most of the year, interrupted by a non-breeding period, which in this case takes place during the summer. Populations of the Iberian mole, *Talpa occidentalis*, in the south-eastern Iberian Peninsula, located in the same region than those of *A. sylvaticus* we have studied here, showed a very similar pattern, although in this case the non-breeding season is quite longer, extending from March to October (Jiménez *et al.*, 1990; Dadhich *et al.*, 2010, 2011, 2013). Although the number of individuals captured do not permit us to establish accurately the temporal limits of the non-breeding period of *A. sylvaticus*, available data indicate that it is relatively short, because we captured fully active males in mid June and in early October. In addition, it seems that testis regression in this species is probably quite fast, because we captured by late June adult males with quite reduced testes. Hence, considering the time required for completing the recovery of the germinative epithelium after the period of spermatogenic rest, which may take more than one month (the precise duration of the spermatogenic cycle of *A. sylvaticus* is unknown), the testes of this species are probably inactive for less than two months in the populations analyzed in the present study. There must be in addition some degree of interindividual variation in the length of the period of spermatogenic inactivity, as we captured in July both males with testes

suggesting to be in a process of spermatogenesis reactivation and males with completely inactive testes (not shown).

3.3.2 Seasonal Sertoli and Leydig cell remodeling in the testes of *A. sylvaticus*

The considerable depletion of most of the adluminal portion of the germinative epithelium during wood mouse testis regression implies a dramatic remodeling of the somatic cells of the gonad. Reduction in the seminiferous-tubule diameter forces Leydig cells to accommodate to the new available inter-tubular space, but a continuous matrix of interstitial tissue similar to that described in the Iberian mole in which thin tubules are embedded (Dadhich *et al.*, 2013) is not organized in *A. sylvaticus*. Since most apoptosis and cell proliferation was detected in germ cells, but not in the cells located outside the seminiferous tubules (Leydig cells), as reported for the roe deer (Blottner and Schoen, 2005), this remodeling is probably mediated by active cell movement. The cytologic alterations of Sertoli cells during testis regression appears to be even more severe. In the Iberian mole, we previously reported that the number of Sertoli cells remains invariable throughout the breeding cycle, suggesting that these cells are either not affected by apoptosis or affected to a similar magnitude at all the stages of the cycle (Dadhich *et al.*, 2010), and that the numeric density of Sertoli cells was higher in inactive than in active testes, showing that these cells reduce their volume considerably during testis regression (Dadhich *et al.*, 2013). In *A. sylvaticus* the situations is very similar as Sertoli cell nuclei are much more closely packed in the seminiferous tubules of inactive summer testes, forming a structure resembling a pseudostratified epithelium, than in the active testes, in which a considerable distance of several dozens of microns may separate the nuclei of adjacent Sertoli cells (see Fig. 3.7 a and b). These data suggest that, like in the mole, in the inactive testis of the wood mouse Sertoli cells undergo a drastic reduction of the cytoplasmic volume that permit the same number of cells to accommodate to the severely reduced space of the summer seminiferous tubule. On the other hand, in the viscacha (*Lagostomus maximus maximus*) Sertoli cells, no variation in the cell volume was reported in a very similar situation of seasonal testis regression (Muñoz *et al.*, 2001). Consistently, no variation was detected in the viscacha Sertoli cell membrane, whereas in the mole these cells retain most, if not all, of the cell membrane during testis regression (Dadhich *et al.*, 2013) by forming superposed membrane infoldings. Our current data on the inactive testes of *A. sylvaticus* suggest that a similar process may take place in this species. The summer testes of the wood mouse show very thin seminiferous tubules in which the germinative epithelium is almost completely lost. In the Iberian mole, we previously showed that this depletion takes place by desquamation of living germ cells due to disorganization of the cell junctions maintaining the architecture of the germinative epithelium. The most important junctional structure organized between Sertoli cells is the BTB, which contains several

types of cell junctions and additional junction types are also established between Sertoli and germ cells (Cheng and Mruk, 2009; Mruk and Cheng, 2004; Vogl *et al.*, 2009; Cheng and Mruk, 2010; Mruk *et al.*, 2008; Yan *et al.*, 2008; Kopera *et al.*, 2010). A desquamation-based germ cell depletion implies that the composition and organization of the cell-adhesion molecules making up these cell junctions must undergo important alterations. In the wood mouse, two pieces of evidence including 1) the permeation of the BTB during testis regression, and 2) the altered expression pattern of CLAUDIN11 (a principal component of tight junctions), indicate that the expression of genes encoding cell-adhesion molecules is impaired during testis regression, suggesting that germ cell depletion in this species could occur by direct desquamation as demonstrated in the mole. Further study of the testes and epididymides of wood mouse males captured at the precise time of testis regression will help to elucidate this question.

3.3.3 Testis regression in the wood mouse is probably mediated by intra-testicular androgen suppression

It is known that testicular cell-junctions are regulated by hormones, although the mechanisms of this process is not completely understood. FSH has a key role in the regulation of Sertoli-Sertoli tight junctions forming the BTB in the Djungarian hamster (Tarulli *et al.*, 2008) and gonadotropins regulate rat testicular tight junctions in vivo (McCabe *et al.*, 2010), whereas Sertoli cell-specific ablation of the androgen receptor gene (AR) showed that testosterone regulates BTB permeability by controlling the expression of CLDNN3 (the gene for CLAUDIN3) and that the subsequent BTB permeation leads to the loss of the testicular immune privilege (Meng *et al.*, 2011). Furthermore, it was reported that testosterone up-regulates CLAUDIN 11 expression in primary cultures of mouse and rat Sertoli cells (Gye, 2003; Florin *et al.*, 2005), whereas both FSH and testosterone regulate the expression and localization of CLAUDIN11 and the subsequent formation of tight junctions between rat Sertoli cells cultured in vitro (Tu'uhevaha *et al.*, 2007). Also, it was found that suppression of intra-testicular androgen, induced by administration of testosterone implants, induces massive germ-cell depletion affecting only the adluminal compartment of rat seminiferous tubules without perturbing the BTB. In the present study, we show that the serum testosterone levels in males of *A. sylvaticus* are highly reduced in the non-breeding season, suggesting a parallel reduction of the intra-testicular androgen levels and thus, as in the rat and the mole, this reduction might be the hormonal signal inducing seasonal germ-cell depletion in the wood mouse.

3.3.4 The role of apoptosis and cell proliferation in the inactive testis of *A. sylvaticus*

Cell proliferation and apoptosis are two fundamental cellular processes known to have important roles in the normal functioning of the testis, but their circannual variations in the testes of males reproducing seasonally have been subject to diverse interpretations (discussed in (Dadhich *et al.*, 2010). In the testes of adult males of *A. sylvaticus* we found that apoptosis varies in a season-dependent manner, affecting mainly late zygotene and pachytene spermatocytes during the period of sexual inactivity, as no further meiotic stages were found in these gonads. Currently, we lack data from males captured at the precise time in which testis regression takes place and hence we cannot define yet the role of apoptosis during this process in this species. However, like in the mole (Dadhich *et al.*, 2010), we found a significant increase in the frequency of apoptotic cells in the testes of inactive males of the wood mouse, when compared with those of active ones. Since apoptotic cells are mostly located in the innermost areas of the regressed seminiferous tubules, it is very likely that these cells are in fact primary spermatocytes in zygotene-pachytene stages and that the role of apoptosis in the inactive testes is to eliminate these cells before they reach more advanced stages of differentiation. The increased levels of cell proliferation in these testes is more difficult to interpret as there is no apparent reason why spermatogonia (the testicular cell type dividing more frequently) should require higher rates of proliferation during the non-breeding period. In the mole, this increase was detected in the period of spermatogenesis recovery just after the non-breeding season, and it was hypothesized that it could serve to restore the loss of germ cells derived from the apoptotic elimination of spermatocytes in the inactive period (Dadhich *et al.*, 2010). In the wood mouse, the increase levels of cell proliferation concomitant with the highest levels of apoptosis during the non-breeding season could play the same role. In both species, testes could have restored their pools of germ cells just before the initiation of a new breeding season.

4. Fourth chapter *Microtus duodecimcostatus*

Figure 4.1: *Microtus duodecimcostatus*. Image reproduced with permission of the autor: Jorge Espier.

4.1 Introduction

Voles constitute a group of rodents belonging to the family Cricetidae, subfamily Arvicolinae, with 20 genera (von Schrank, 1798). Voles of genus *Microtus* are predominantly herbivorous small mammals present in the northern hemisphere, and form an ecologically diverse group. Most species prefer open habitats, such as grasslands, meadows or pastures, although some species are also associated to forested areas (Getz, 1985). The Mediterranean pine vole, *Microtus duodecimcostatus* (Sélys-Lonchamps, 1839), is distributed across most of the Iberian Peninsula, except in the north-western region and some scattered areas in Spain, occurring also in southern France (Shenbrot and Krasnov, 2005; Cotilla and Palomo, 2007). They are characterized by a burrowing behavior as they spend most of their time in underground tunnels and burrows. These voles make extensive network of shallow tunnels, throwing up small piles of earth as they excavate them. They do not show any typical morphological adaptation to a fossorial life like those noticed in true moles (genus *Talpa*) or other "typical" subterranean rodents (Madureira, 1984; Mathias, 1990; Giannoni *et al.*, 1993; Santos *et al.*, 2009).

As mentioned above this vole species occupies open Mediterranean type habitats, including both natural and agricultural lands (Cotilla and Palomo, 2007). It consumes mostly subterranean plant parts (e.g. bulbs, roots, rhizomes, etc.), although aerial parts are sometimes also eaten (Borghi and Giannoni, 1997; Cotilla and Palomo, 2007). *M. duodecimcostatus* is a common rodent pest in vegetable crops and orchards (Cotilla and Palomo, 2007; Mira and Mathias, 2007). In the studied areas, this vole can be considered sexually active throughout the year, with a peak of sexual activity in winter and spring, from November to May with maximum in February/March (Mira, 1999.; Santos *et al.*, 2009). Ventura *et al.* (2010) studied the breeding characteristics of the Lusitanian pine vole, *Microtus lusitanicus*, a similar species common in south-western Europe. The authors revealed that reproduction in a population of the north-west of the Iberian peninsula (El Bierzo) probably occurs all year round, although sexual activity could be slightly lower in winter. They also noticed that in males and females monthly mean of adult body mass did not vary significantly during the year. In the province of Granada, this species is relatively abundant in various habitats including cereal crops, aldmon tree plantations, wastelands, poplar groves and irrigated vegetable gardens, where it causes occasional pest damages with a pluriannual frequency (our personal observations). The reproduction timing of this species in this geographic area has not been studied to date.

4.2 Results

4.2.1 Males of *Microtus duodecimcistatus* living in wastelands undergo summer testis regression in the south-eastern Iberian Peninsula

Like in previous species, four study groups, named winter, spring, summer, and autumn, were established according to the season of capture in the wastelands and cereal crops, which were the habitats with more dense populations of this species in the area of study. Data on body mass, testis mass, and testis/body mass ratio are summarized in table 4.1. When we compared the body mass of individuals captured in all four seasons we found no significant differences between the four study groups (table 4.2; Fig. 4.2). Contrarily, statistical analysis revealed a highly significant reduction of the testis mass in the summer group when compared with the other three groups, denoting the existence of summer testis regression in this species (table 4.3; Fig. 4.3). It is noteworthy that winter males, even exhibiting the lowest body mass, had the largest testes, even existing significant differences when compared with those of the autumn, suggesting a slow testis recovery after summer and a pick of reproductive activity in the winter. Consistently with these observations, the testis/body mass ratio of summer males was highly significantly lower than that of the other three study groups, whereas the ratio of winter males was significantly higher than that of the others as well. Only the comparison between spring and autumn males revealed no difference in this parameter (table 4.4; Fig. 4.4. The histological analysis of the testes from winter and summer males of *M. duodecimcostatus* clearly supported the hypothesis that males from wastelands and cereal crops of south-eastern Iberian Peninsula undergo summer testis regression. Whereas winter males had large, spermatogenically active testes and epididymides full of spermatozoa (Fig. 4.5 a and b), summer ones had regressed testes with reduced seminiferous tubules and empty epididymides (Fig. 4.5 c and d). According to these results, we considered that the winter and the summer males of these populations are the most representative of the breeding and the non-breeding periods, respectively, and we used a sample of animals from these seasons for further histological, morphometric, immuno-histochemical and hormonal comparative studies.

4.2.2 Testis regression does not occur in other habitats

From previous studies carried out in our laboratory with this species(Carnero *et al.*, 1991), we new that Mediterranean pine voles are relatively easy to maintain and reproduce in captivity. Taking advantage of this knowledge we planned to check whether males maintained in captivity under optimal living conditions and summer photoperiod, would undergo testis regression

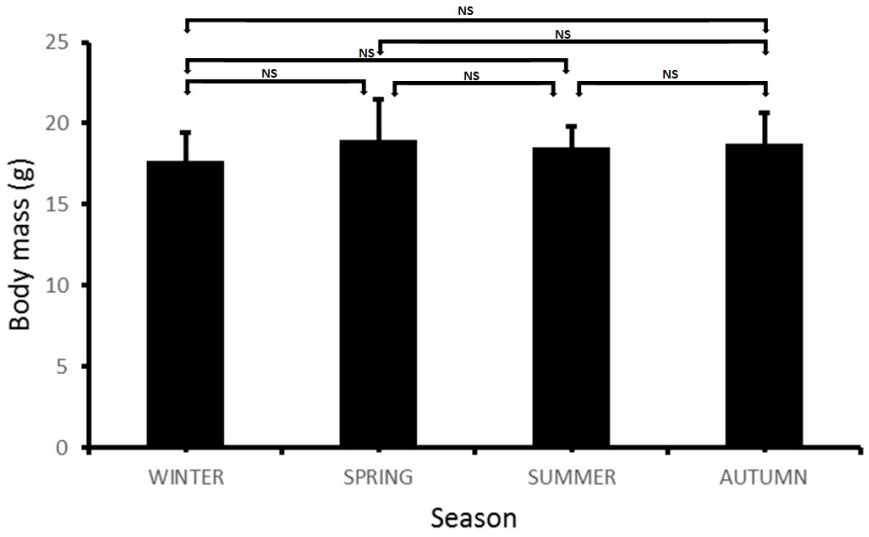


Figure 4.2: Seasonal variations in the body mass of adult males of *M. duodecimcostatus*. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

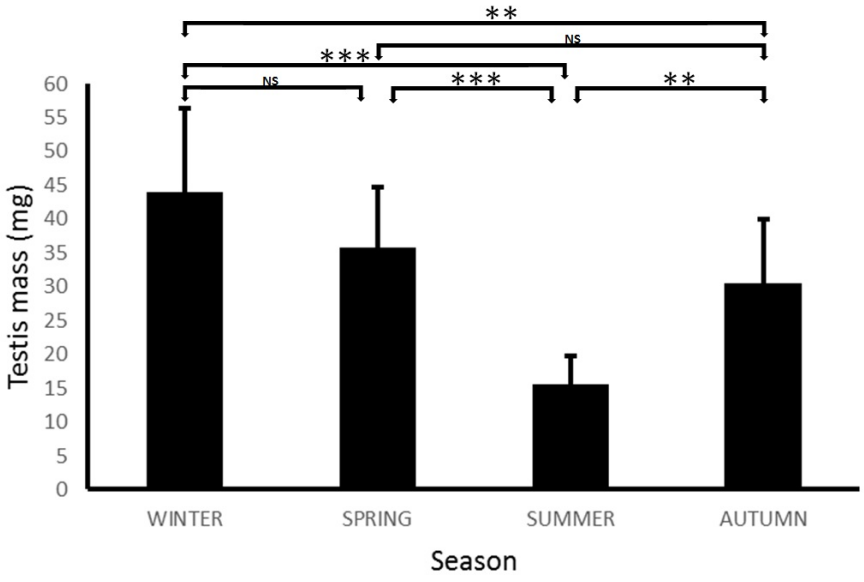


Figure 4.3: Seasonal variations in the testis mass of adult males of *M. duodecimcostatus*. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

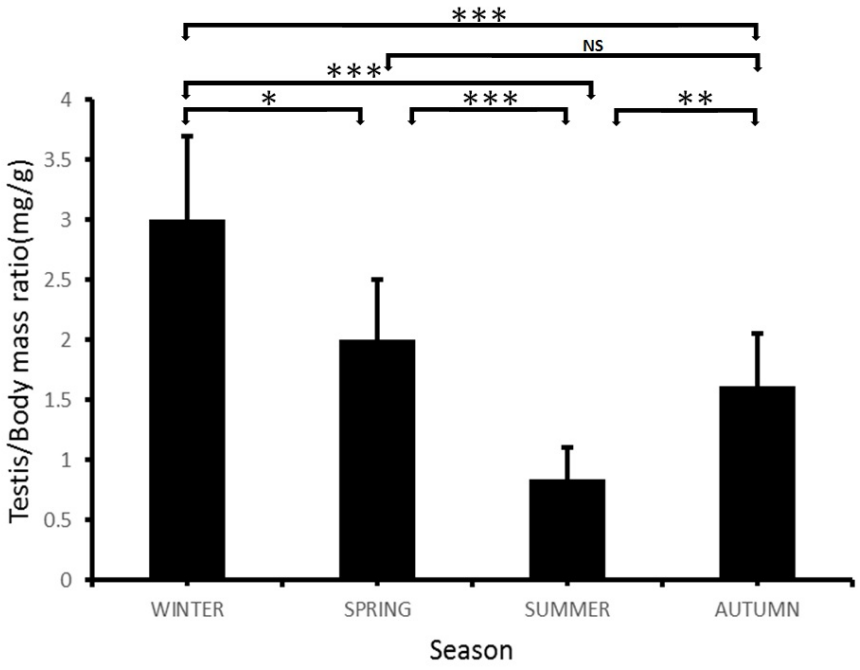


Figure 4.4: Seasonal variations in the testis/body mass ratio of adult males of *M. duodecimcostatus*. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

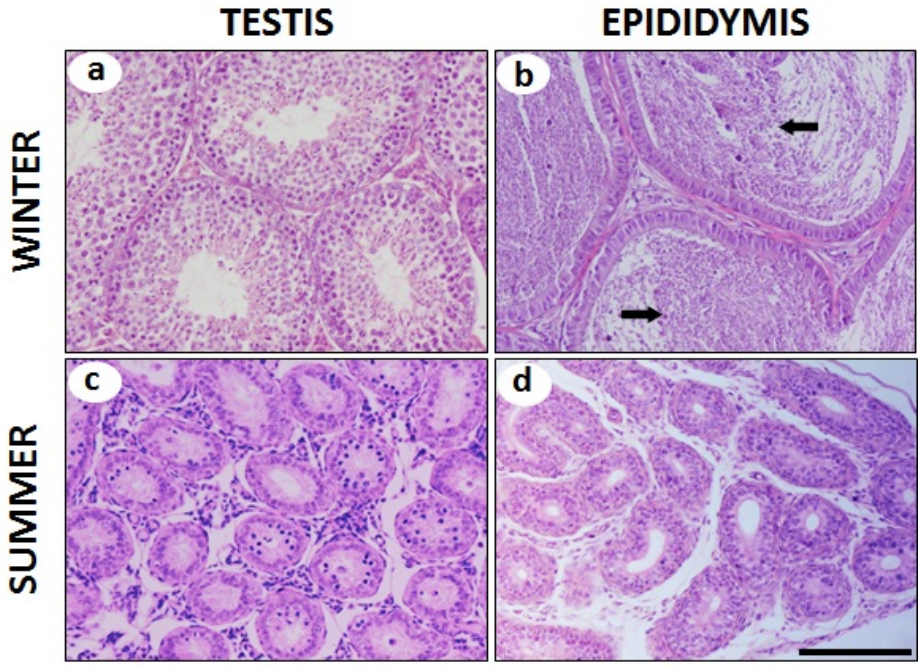


Figure 4.5: Haematoxylin-eosin-stained tansverse sections of testes (a and c) and epididymides (b and d) from winter (a and b) and summer (c and d) males of *M. duodecimcostatus*. In the winter testis, the seminiferous tubules show active spermatogenesis and the epididymides contain abundant sperm (black arrows). Scale bar represents 100 µm for all pictures.

	Winter	Spring	Summer	Autumn
Body mass ± SD (g)	17.70± 1.69	19± 2.5	18.5± 1.29	18.75±1.91
Testis mass ± SD (mg)	43.99± 12.27	35.72±9.03	15.47±4.35	30.43±9.56
Testis/Body mass (mg/g)	3±0.76	1.9±0.5	0.84±0.26	1.61±0.44
n	16	9	4	12

Table 4.1: Values of body mass, testis mass, and body/testis mass ratio and number of males of *M. duodecimcostatus* initially analyzed in each season in south-eastern populations of the Iberian Peninsula.

P Body mass (g	Winter	Spring	Summer	Autumn
Winter		0.18977	0.34178	0.14616
Spring			0.64501	0.80603
Summer				0.81316

Table 4.2: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the body mass of every study group of *M. duodecimcostatus* males with each other, as shown in table 4.1. Asterisks indicate the level of statistical significance.

as free wild males do at the same time in the wastelands. For this, we established a small colony of voles (around 30 individuals) which was checked periodically for the presence of pregnant females and new offspring, to monitor the reproductive status of the adult animals. We used laboratory rat cages (30x50cm) and one male and one female vole were placed in each cage and feed with mouse pellets and fresh vegetables (potato and carrot). Abundant dry hay and wood shaving substrate were also provided to permit the animals to hide and dig.

Adult voles born in captivity were also coupled trying to avoid endogamous crosses. Table 4.5 summarizes the body and testis mass, and testis/body mass ratio of captive males analyzed in the summer. The testes of these captive males were significantly heavier than those of the summer wastelands males and similar in mass to those of spring and autumn, but lighter than those of winter wild males (Table 4.6; Fig. 4.6. Similar results were obtained in the analysis of the testis/body mass ratios (Table 4.7; Fig. 4.7). In this laboratory colony, which was set in the winter and maintained until the end of the next summer, voles reproduced continuously, even during the summer, coinciding in time with the period in which wild males living in wastelands only 11 Km apart from the animal house, had fully regressed testes. This is consistent with the normal histology observed in both the active testes and the epididymides of the animal house summer males (Fig. 4.8 a and b).

In addition, we knew that scattered small populations of Mediterranean pine voles are found in the extensive poplar cultivations near the locality of

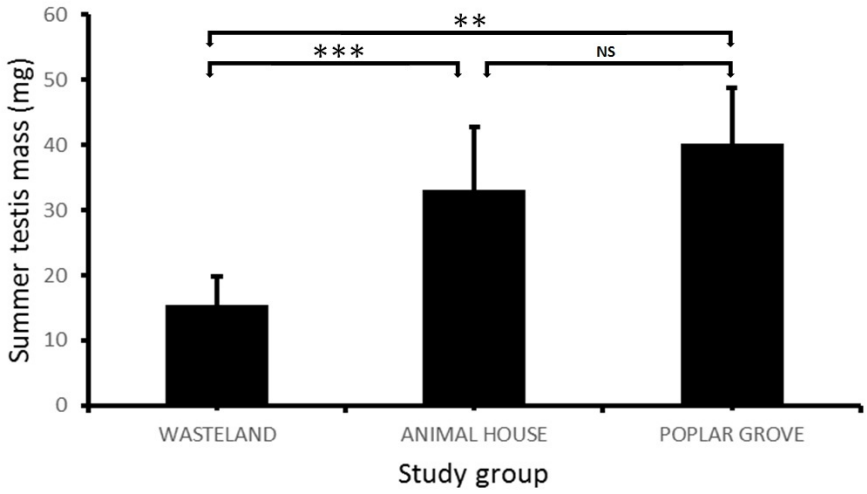


Figure 4.6: Seasonal variations in the summer testis mass of adult males of *M. duodecimcostatus* from three different origines: wastelands, animal house, and poplar grove. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

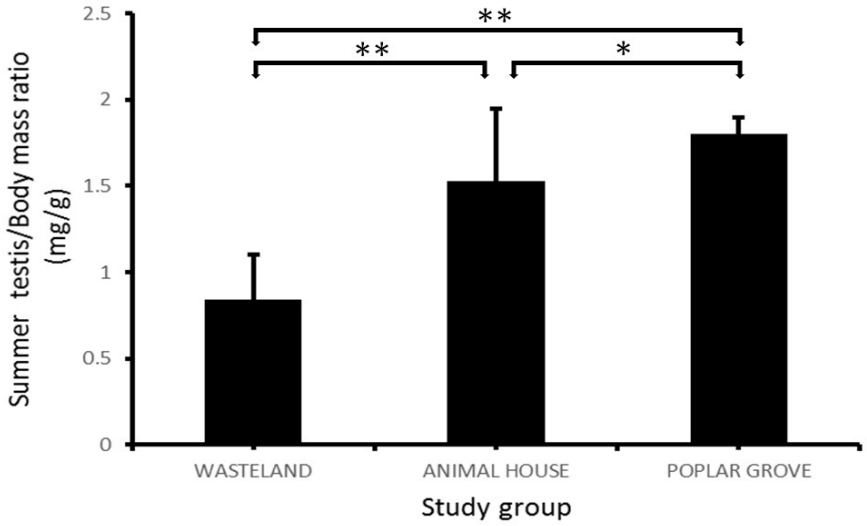


Figure 4.7: Seasonal variations in the summer testis/body mass ratio of adult males of *M. duodecimcostatus* from three different origines: wastelands, animal house, and poplar grove. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

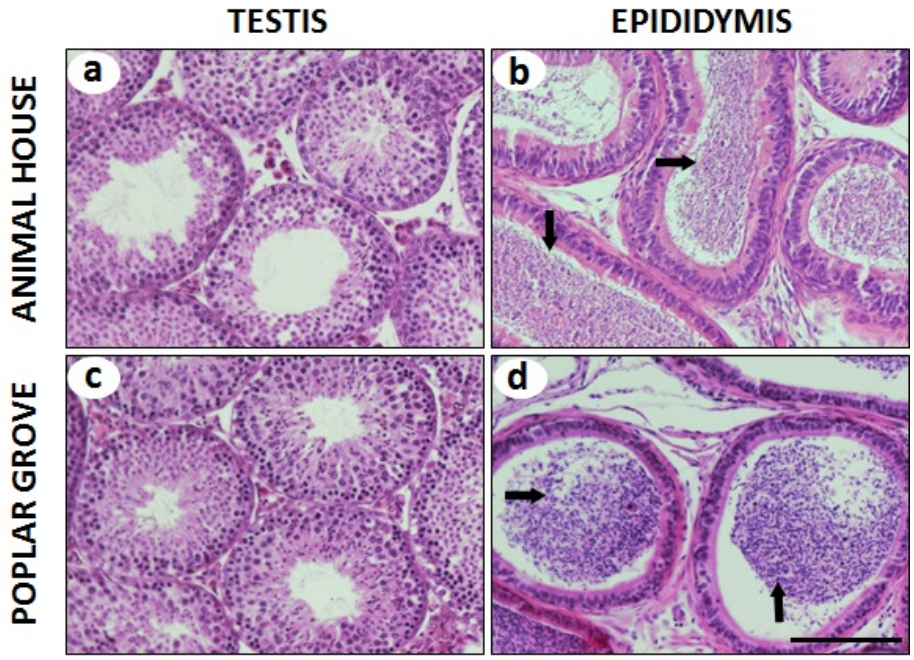


Figure 4.8: Haematoxylin-eosin-stained tansverse sections of testes (a and c) and epididymides (b and d) from animal house (a and b) and poplar grove (c and d) males of *M. duodecimcostatus*. In both cases, the seminiferous tubules show active spermatogenesis and the epididymides contain abundant sperm (black arrows). Scale bar represents 100 µm for all pictures.

P (Testis mass)	Winter	Spring	Summer	Autumn
Winter		0.06803	1.7E-06***	0.00291**
Spring			0.00022***	0.21139
Summer				0.00113**

Table 4.3: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the testis mass of every study group of *M. duodecimcostatus* males with each other, as shown in table 4.1. Asterisks indicate the level of statistical significance.

P (Testis mass/Body mass)	Winter	Spring	Summer	Autumn
Winter		0.02481*	2.07E-06***	0.00059***
Spring			0.0004***	0.18033
Summer				0.00192**

Table 4.4: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the testis mass of every study group of *M. duodecimcostatus* males with each other, as shown in table 4.1. Asterisks indicate the level of statistical significance.

Santa Fe, where members of our laboratory have repeatedly captured individuals of the Iberian mole species *Talpa occidetanlis* for diverse developmental and seasonal reproduction studies (see Jiménez et al. 2013, for a review). The living conditions in the poplar groves during the summer are absolutely different from those that voles can find in the wastelands. Here, temperature may exceed 45ºC in July and August, the land becoming completely dry and hard, which oblige the voles to remain underground most of the time and preclude them from digging new tunnels. Contrarily, during the summer in poplar groves sunshine never reach the ground, humidity is high (due to frequent irrigation), temperature is moderate (never higher than 30-35ºC), food is abundant and the land is soft, permitting voles to renew their tunnel network also in this season. Hence, these populations represent a natural alternative to the animal house colony experiment, so we decided to captured some few voles in the summer to check whether they were reproductively active or not. Table 4.5 summarizes the body and testis mass, and the testis/body mass ratio of wild male voles captured in a poplar grove during the summer. The testes of males captured in a poplar grove were significantly heavier than those of the summer wastelands males and similar in mass to those of the other study groups, including both wild and captive males (Table 4.6; Fig. 4.6). Similar results were obtained in the analysis of the testis/body mass ratios, although in this case, unlike for the testis mass, significant differences were found between captive and poplar grove males, the latter showing higher values (Table 4.7; Fig. 4.7). The body mass of these animals was very similar to that of captive males and heavier than wild males living in the wastelands at any season, although

	Animal house	Poplar grove
Body mass ± SD (g)	22.07±3.72	22.25±3.94
Testis mass ± SD (mg)	33.11±9.63	40.25±8.5
Gonad/Body mass (mg/g)	1.53±0.42	1.8±0.1
n	13	4

Table 4.5: Values of summer body mass, testis mass, and body/testis mass ratio and number of males of *M. duodecimcostatus* analyzed from a laboratory colony maintained for several months in an animal house and from a poplar grove population in south-eastern Iberian Peninsula.

P (Testis mass)	Animal house	Poplar grove
Winter	0.01253*	0.49964
Spring	0.52434	0.41722
Summer	0.00025***	0.00481**
Autumn	0.49212	0.10243
Animal house		0.21

Table 4.6: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the summer testis mass of every study group of *M. duodecimcostatus* males captured in wastelands with those captured in a poplar grove and those maintained in an animal house, as shown in tables 4.1 and 4.5. Asterisks indicate the level of statistical significance.

these differences did not reach the 5% statistical significance level, probably due to low sample size (Table 4.8; Fig. 4.9). The four males captured during the summer in a poplar grove population, which is only 9 Km apart from the studied wastelands, had fully active spermatogenesis, as confirmed by the corresponding histological analysis of both testes and epididymides (Fig. 4.8 c and d).

Since all groups of males studied here were subjected to the same summer photoperiod, these observations clearly show that it is the micro-environment in which each vole population is living what determines its reproductive status and the local existence of seasonal breeding or not. When life conditions are favorable, either natural or artificial, voles do not stop breeding, whereas in the poor environment of the wastelands during the summer of south-eastern Iberian Peninsula, testis regression occurs and reproduction is halted.

P (Gonad mass/Body Mass)	Animal house	Poplar grove
Winter	0.00018***	0.00216**
Spring	0.08257	0.5668
Summer	0.00366**	0.00256**
Autumn	0.62944	0.18304
Animal house		0.04584*

Table 4.7: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the summer testis/body mass ratio of every study group of *M. duodecimcostatus* males captured in wastelands with those captured in a poplar grove and those maintained in an animal house, as shown in tables 4.1 and 4.5. Asterisks indicate the level of statistical significance.

P (body mass)	Animal house	Poplar grove
Winter	0.00124**	0.10205
Spring	0.03127*	0.20192
Summer	0.01062*	0.15243
Autumn	0.01084*	0.17343
Animal house		0.94124

Table 4.8: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the summer body mass of every study group of *M. duodecimcostatus* males captured in wastelands with those captured in a poplar grove and those maintained in an animal house, as shown in tables 4.1 and 4.5. Asterisks indicate the level of statistical significance.

4.2.3 Spermatogenesis is arrested at the spermatocyte stage in the testes of reproductively inactive males of *M. duodecimcostatus*

Immuno-histochemistry for SOX9 revealed that in winter males of *M. duodecimcostatus* the expression pattern was similar to those described above for the other studied species, being spermatogenic cycle-dependent with stronger expression in the stages I-IV and a weaker expression in the stages VII-X. Contrarily, the summer seminiferous tubules showed an uniform SOX9 staining and a reduced distance between neighbouring SOX9-positive cells (Fig. 4.10 a and b). Also as described previously for other species, studies of DMRT1 expression in the testes of winter males of *M. duodecimcostatus* identified two types of seminiferous tubules depending on the number of DMRT1-positive cells: LDD and HDD (Fig. 4.10 c and d). LDD tubules appeared in stages I-IV of the spermatogenic cycle whereas HDD tubules were more frequent in stages VI-VII. Two types of tubules were also found in the summer testes with different amount of DMRT1-positive cells. Double immuno-fluorescence for both SOX9 and DMRT1 in testes of winter males of *M. duodecimcostatus* showed

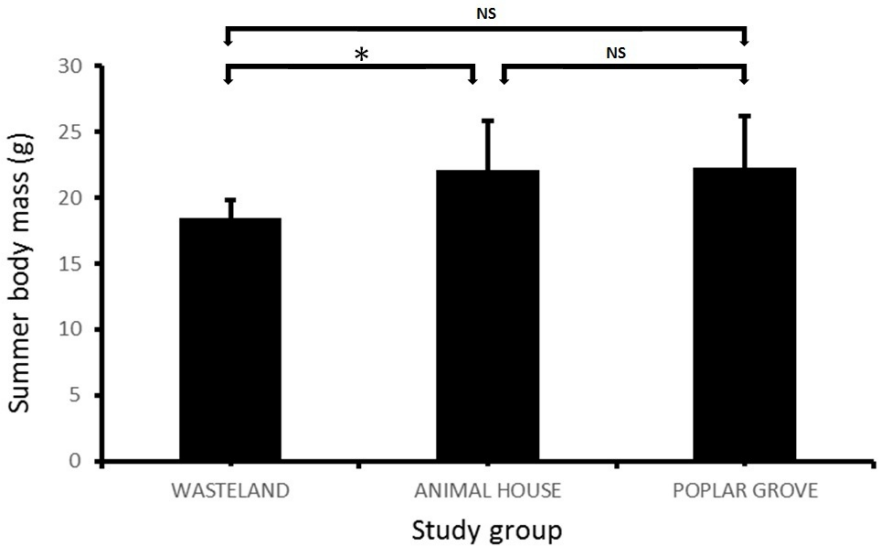


Figure 4.9: Seasonal variations in the summer body mass of adult males of *M. duodecimcostatus* from three different origines: wastelands, animal house, and poplar grove. Asterisks represent the levels of statistical sigificance of the differences detected in Student's t-tests.

that DMRT1 expression is almost exclusively restricted to Sertoli cells in LDD tubules, whereas it is also found in spermatogonial cells in the HDD tubules (Fig. 4.11). In the testis of inactive males, LDD-like tubules contained mainly cells positive for both DMRT1 and SOX9-positive, indicating that most cells in these tubules are Sertoli cells, and only some few SOX9-negative DMRT1-positive spermatogonial cells. As observed in the inactive testis of *A. sylvaticus*, in these tubules Sertoli cell nuclei are displaced to internal locations and formed what appeared to be a pseudo-stratified epithelium, whereas spermatogonial cells maintain a basal position. However, summer HDD-like contain more spermatogonial cells. Hence, like in *A. sylvaticus*, these observations suggest that the summer testes of *M. duodecimcostatus* undergo cyclic spermatogonial proliferative phases. Tubules in these inactive testes also contain some few meiotic spermatocytes which were negative for both SOX9 and DMRT1, but no spermatids, showing that spermatogenesis is arrested at the spermatocyte stage in the inactive testes of this species.

In winter males of *M. duodecimcostatus*, the expression pattern of PCNA in both spermatogia and zygotene-pachytene spermatocytes was spermatogenic cycle-dependent, as observed in other species analysed in this work (Fig. 4.12 a). In the summer testis, different tubules showing varying intensity of PCNA-staining could be observed (Fig. 4.12 b). As we described in the chapters for *C. russula*, *M. spretus*, and *A. sylvaticus*, we used double DMRT1-PCNA immnuo-fluorescence in the winter testes of *M. duodecimcostatus* to identify Sertoli cells (DMRT1-positive, PCNA-negative), spermatogonial cells (DMRT1-positive, PCNA-positive) and zygotene-pachytene spermatocytes

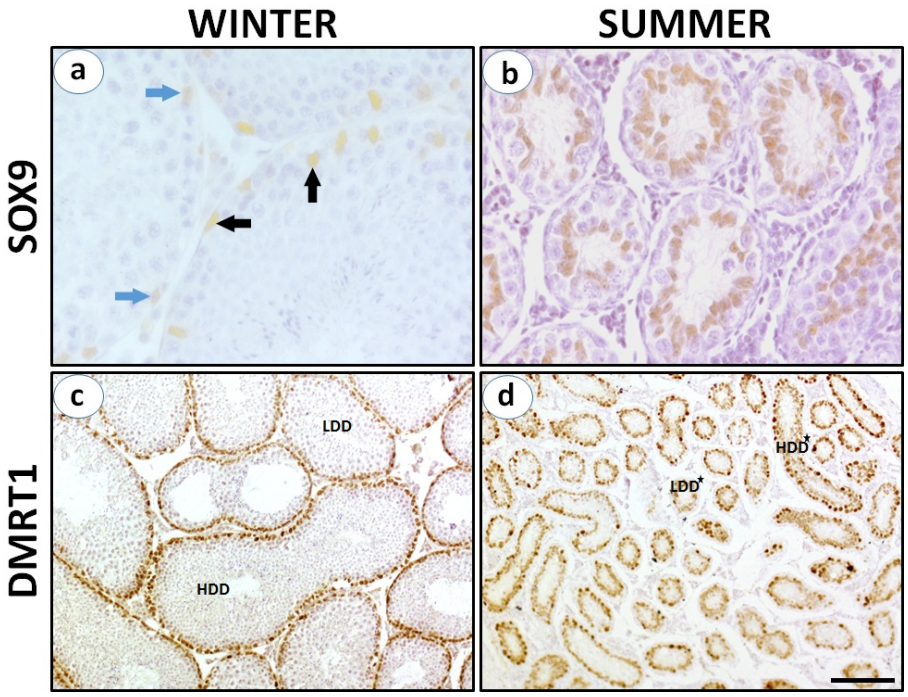


Figure 4.10: Immunohistochemical detection of both SOX9 (a and b) and DMRT1 (c and d) proteins in the testes of winter (left column) and summer (right column) males of *M. duodecimcostatus*. SOX9 expression is stronger in stages I-IV (black arrows), and weaker in stages VII-X (cyan arrows). No relevant difference was observed in DMRT1 expression between the two groups (c and d). Two types of testis tubules can be distinguished regarding the content of DMRT1-positive cells: low-density DMRT1-expressing (LDD) tubules, in which expression is almost restricted to Sertoli cells and appear in stages I-IV, and high-density (HDD) ones, in which expression is observed in both Sertoli and spermatogonial cells, which are observed in stages VI-VII. Scale bar represents 20 µm in in a and b, and 100 µm in c and d.

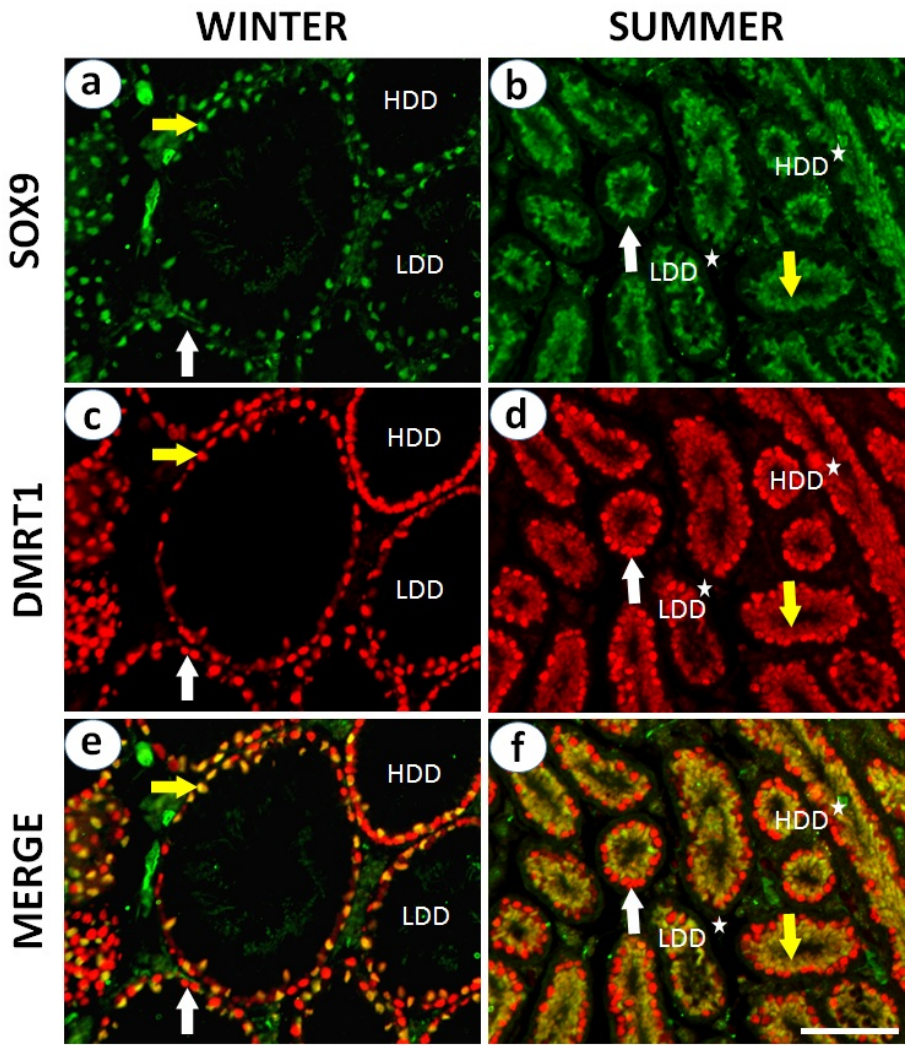


Figure 4.11: Double immuno-fluorescence for SOX9 (green; a and b) and DMRT1 (red; c and d) proteins in testes from winter (left column) and summer (right column) males of *M. duodecimcostatus*. Pictures in e and f show merged images. DMRT1 co-localizes with SOX9 in Sertoli cells (yellow arrows), indicating that the other immuno-reactive cells (white arrows) are spermatogonia. Scale bar represents 100 µm in all pictures.

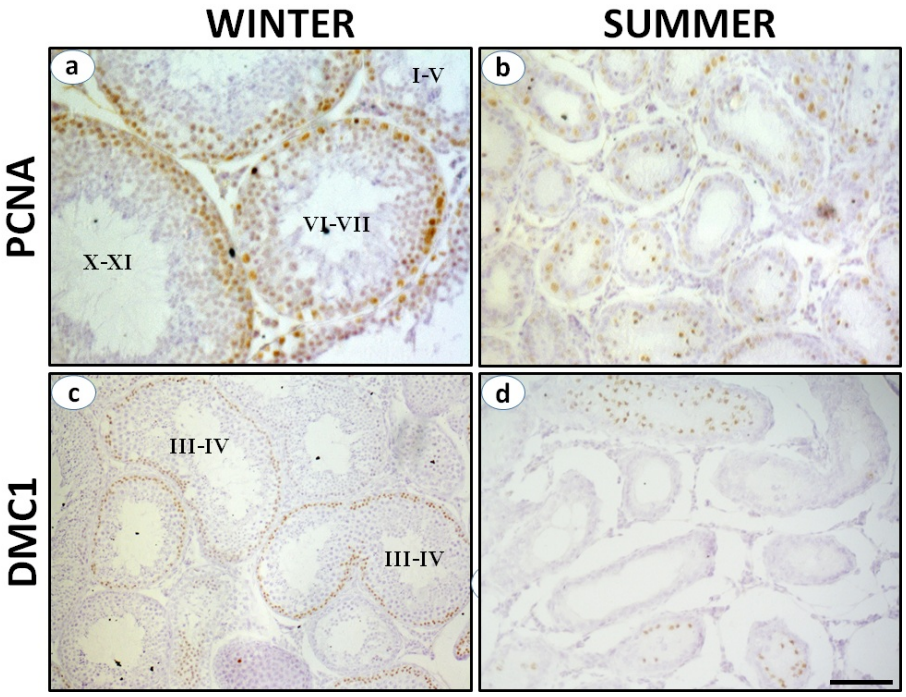


Figure 4.12: Immuno-histochemical detection of both PCNA (a and b) and DMC1 (c and d) proteins in testes from winter (left column) and summer (right column) males of *M. duodecimcostatus*. The spermatogenic cycle stages of some seminiferous tubules are indicated in Roman numerals. Scale bar represents 25 µm in a and b, and 100 µm in c and d.

(DMRT1-negative, PCNA-positive) (Fig. 4.13). The three cell types could also be identified in the tubules of the inactive testis, but in this species, distinguishing between LDD- and HDD-like tubules was not as easy as in *A. sylvaticus*. The expression of DMC1 in zygotene-early pachytene spermatocytes of *M. duodecimcostatus* was spermatogenic cycle-dependent, as observed in the other species studied in this work, and detected in some testis tubules but not in others in both winter and summer testes (Fig. 4.12 c and d). DMC1 showed no co-localization with DMRT1 in any cell of either winter or summer, as demonstrated using double immuno-fluorescence (Fig. 4.14). The absence of cells types other than the DMC1-positive zygotene-pachytene spermatocytes in the innermost region of some tubules in inactive summer testes of *M. duodecimcostatus*, shows that spermatogenesis do not progress beyond this meiotic stages in these gonads.

4.2.4 The blood testis barrier is impaired in the summer testis of *M. duodecimcostatus*.

We also performed in *M. duodecimcostatus* the biotin tracer experiment to study the status of the blood-testis barrier in active and inactive males. In

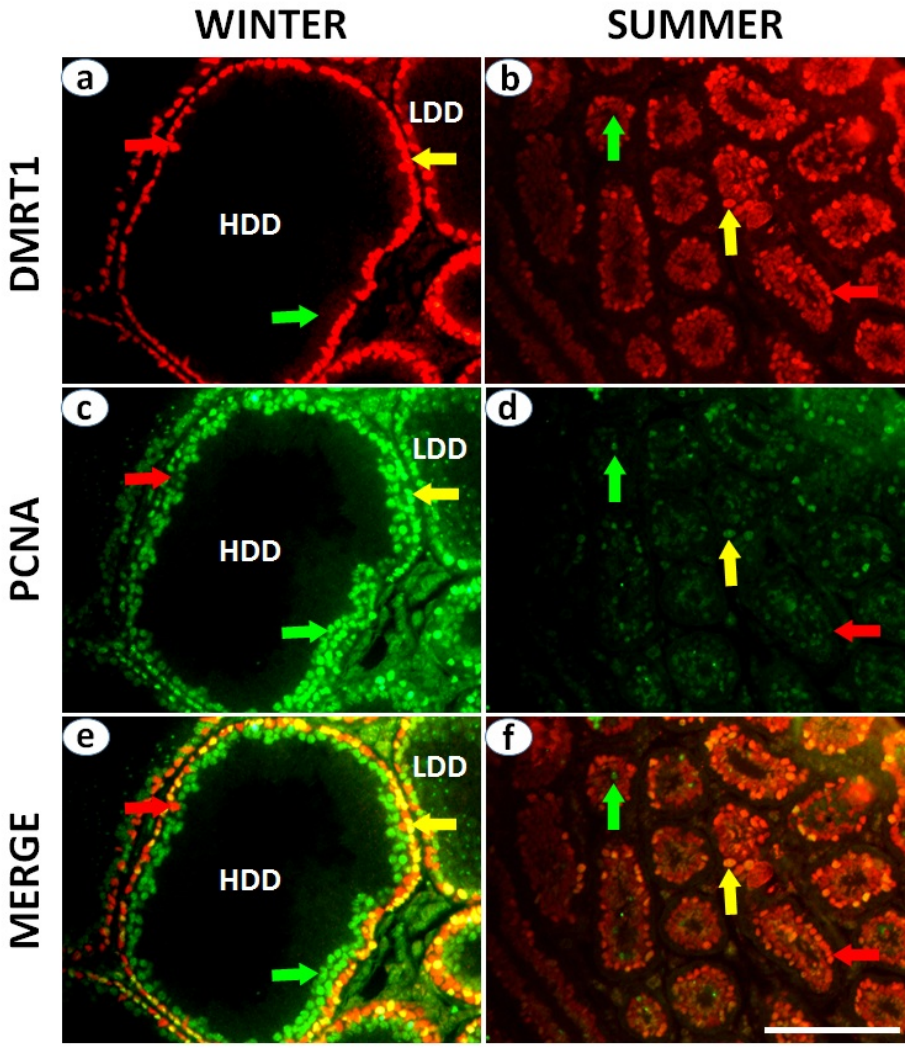


Figure 4.13: Double immuno-fluorescence for both DMRT1 (red; a and b) and PCNA (green; c and d) proteins in testes of winter (left column) and summer (right column) males of *M. duodecimcostatus*. Pictures in e and f show merged images. PCNA co-localizes with DMRT1 in spermatogonial cells (yellow arrows), but not in Sertoli cells (red arrows) and in zygotene-early pachytene spermatocytes (green arrows). Scale bar represents 100 µm in all pictures.

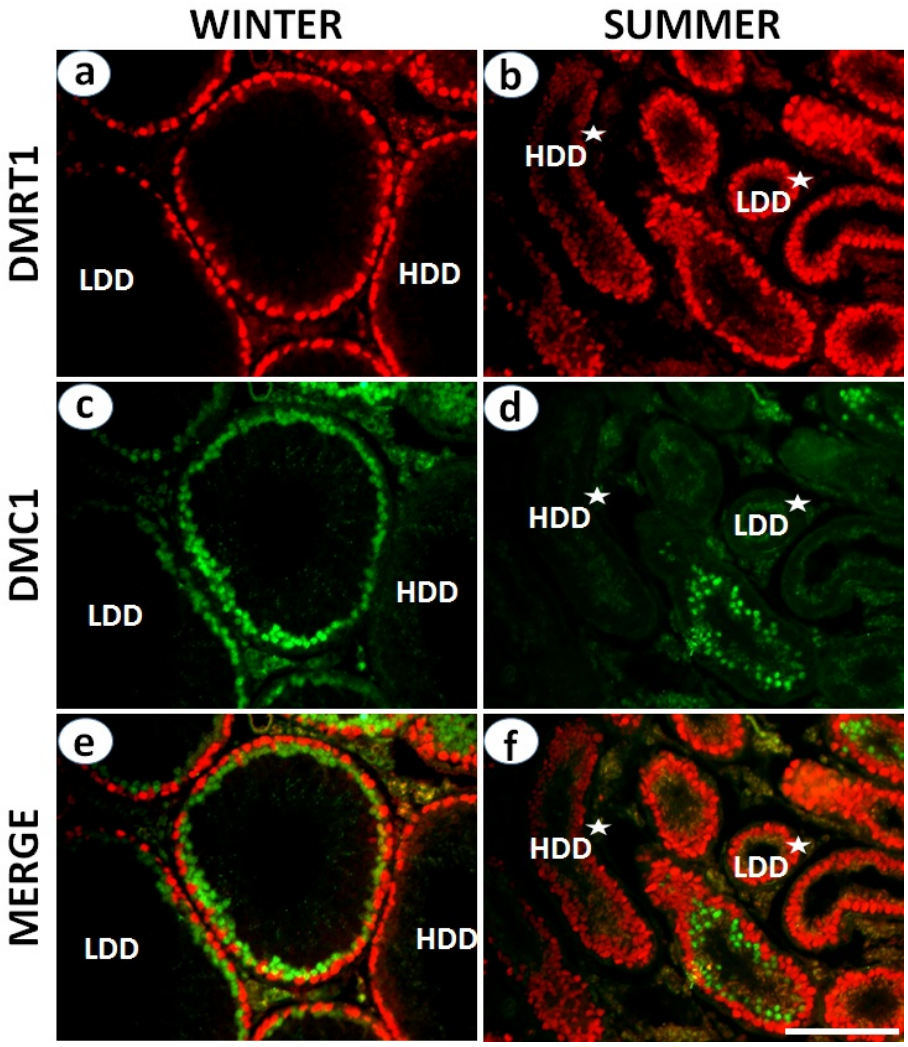


Figure 4.14: Double immuno-fluorescence for both DMRT1 (red; a and b) and DMC1 (green; c and d) in testes of winter (left column) and summer (right column) males of *M. duodecimcostatus*. Pictures in e and f show merged images. DMRT1 and DMC1 do not co-localize in any cell type in both summer and winter testes. Scale bar represents 100 µm in all pictures.

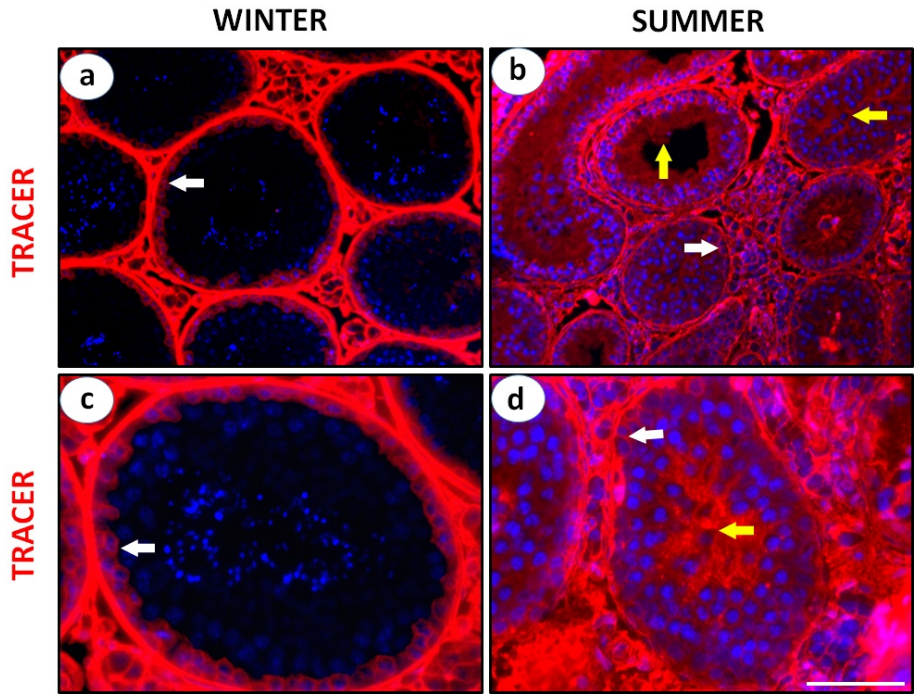


Figure 4.15: Immuno-detection of biotin tracer after injection in the interstitial space of the testis from winter (a and c) and summer (b and d) males of *M. duodecimcostatus*. The tracer (red fluorescence) is located in the basal compartment of the seminiferous epithelium in both winter and summer testes (white arrows in all pictures), but it also appeared in the adluminal compartment of the summer testes (yellow arrows in b and d). Nuclei were counterstained with DAPI (blue fluorescence in all pictures). Scale bar represents 100 µm in a and b, and 50 µm in c and d.

the winter, the tracer (red color) was seen in both the interstitial tissue and the basal compartment of the seminiferous tubules but not in the adluminal compartment (Fig. 4.15 a and c). However, like in *Mus* and *Apodemus*, it could pass to the adluminal compartment as well in the testes of summer males, showing the impaired condition of the BTB in the testes of inactive voles. (Fig. 4.15 b and d). Immuno-histochemistry for CLAUDIN11 revealed a stronger signal in tubules in stages IV-XI and a weaker expression in tubules of the rest of stages (Fig. 4.16 a). Claudin11 expression was completely disorganized in the inactive testes of summer males (Fig. 4.16 b).

Double immuno-fluorescence for both CLAUDIN11 and the biotin tracer (Fig. 4.17), showed the tracer located basal to the CLAUDIN11 signal in the seminiferous tubules of winter testes. However, the CLAUDIN11 signal was intermingled with the tracer one in the summer testes, forming no defined barrier domain. Moreover, DMC1-CLAUDIN11 double immuno-fluorescence showed that all cells located inner to the CLAUDIN11 domain were DMC1-positive in the winter testis, showing that all cells passing through the BTB have already entered meiosis (Fig. 4.18). Contrarily, meiotic cells can be seen

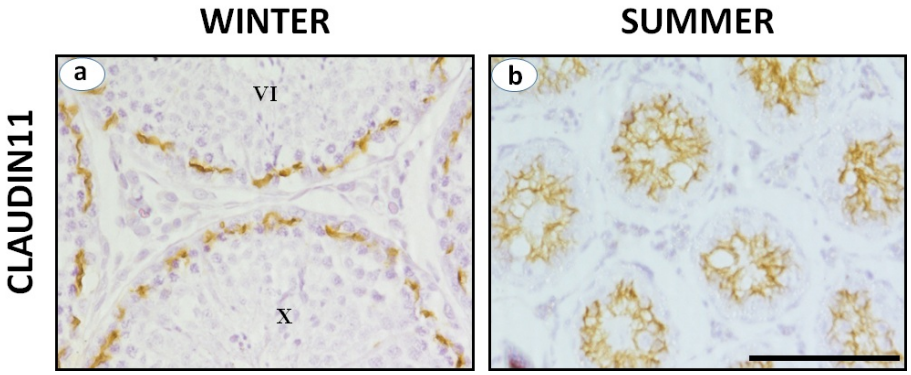


Figure 4.16: Immuno-histochemical detection of CLAUDIN11 in the testes of winter (a) and summer (b) males of *M. duodecimcostatus*. Roman numerals indicate spermatogenic cycle stages. Scale bar represents 50 µm in all pictures.

located in basal positions at the other side of the BTB in the summer group, evidencing that the function of this structure is impaired in the inactive testes of summer males of *M. duodecimcostatus*.

We also checked the status of the lamina propria in winter and summer testes of *M. duodecimcostatus* using immunohistochemistry for alpha-SMA, DESMIN, and LAMININ (Fig. 4.19). All three proteins were detected around the seminiferous tubules, in both winter and summer testes, suggesting that the lamina propria is not altered during testis regression in this species.

4.2.5 Summer males of *M. duodecimcostatus* show reduced steroidogenesis

The androgenic function of winter and summer males of *M. duodecimcostatus* was analyzed using both immuno-histochemistry for AR and P450scc. The expression pattern observed was not different from that described for other species. AR was detected in Sertoli, Leydig, and peritubular myoid cells in both winter and summer testes (Fig. 4.20), whereas P450scc was strongly expressed in Leydig cells in both summer and winter testes. The RIA analysis performed to measure the levels of serum testosterone was also extended to voles from both the animal house and the poplar grove. Results showed a clear reduction in testosterone levels in the summer, compared with those of the other study groups, the higher levels being detected in the poplar grove population (tables 4.9 and 4.10; Fig. 4.21). Testosterone levels of summer males were significantly lower than those of both the animals captured in wasteland during the winter, and those captured in the poplar grove in the summer, but differences were not significant when compared with the animal house animals analyzed in the summer.

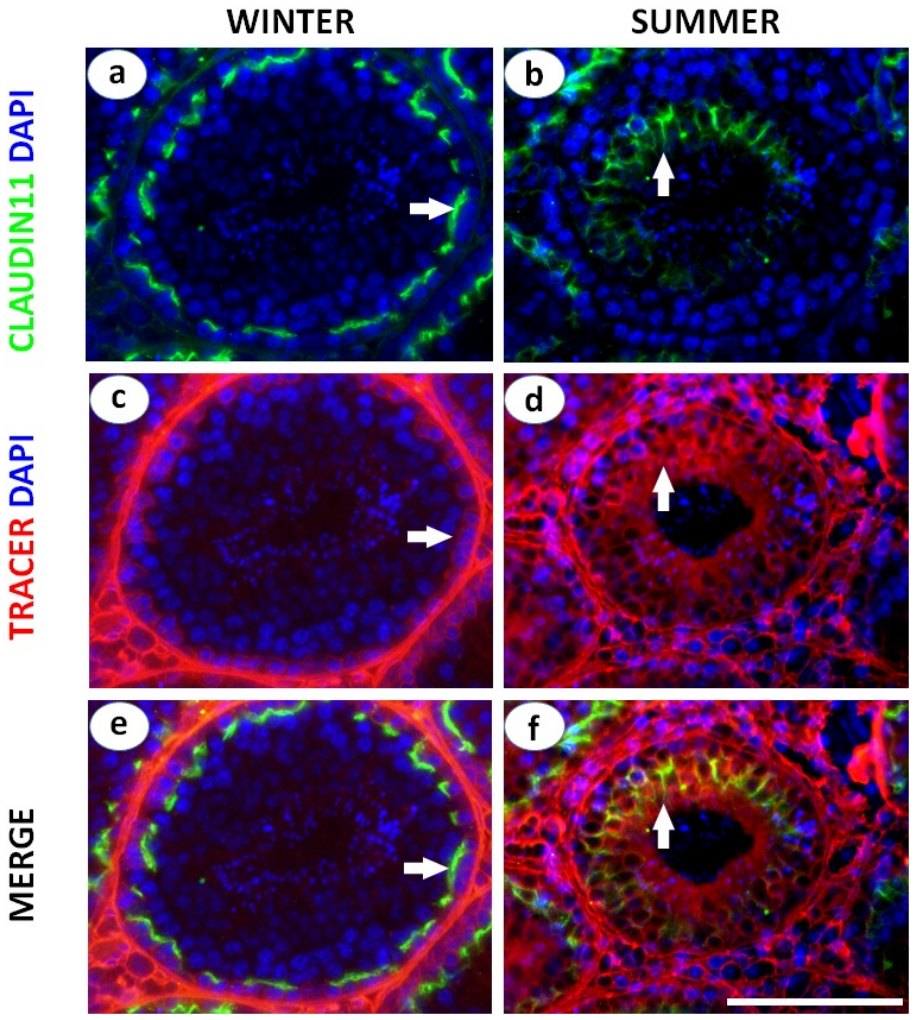


Figure 4.17: Double immuno-fluorescence for both CLAUDIN 11 (green; a and b) and the biotin tracer (red; c and d) in testes from winter (left column) and summer (right column) males of *M. duodecimcostatus*. Pictures in e and f show merged images with DAPI counterstain (blue). Biotin is located in the basal compartment of winter testes and at both sides of the CLAUDIN11 domain in the summer ones. Scale bar represents 25 µm in all pictures.

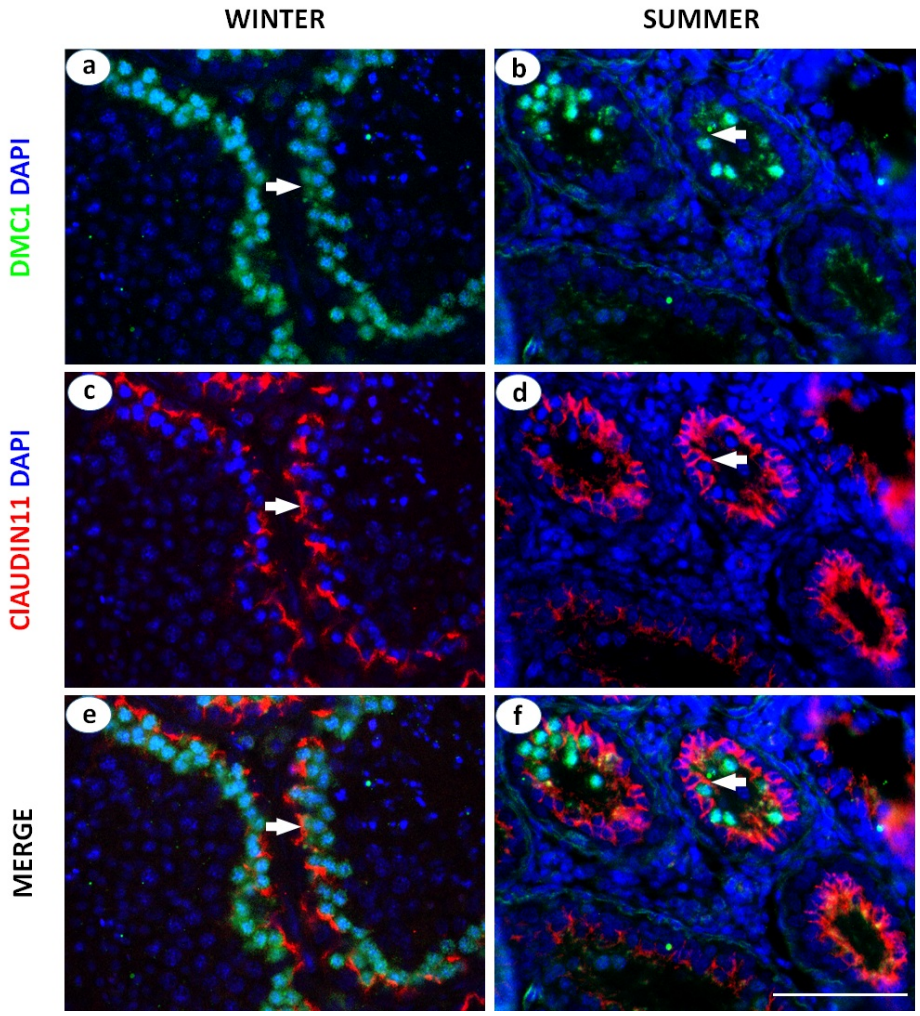


Figure 4.18: Double immune-fluorescence for both DMC1 (green; a and b), and CLAUDIN 11 (red; c and d), and DAPI counterstaining (blue; a-f) in testes of winter (left column) and summer (right column) males of *M. duodecimcostatus*. All DMC1-positive meiotic cells are located inner to the CLAUDIN11 domain in the winter samples, but some meiotic cells are seen basal to the BTB domain in the summer testes (arrows). Scale bar represents 25 µm in all pictures.

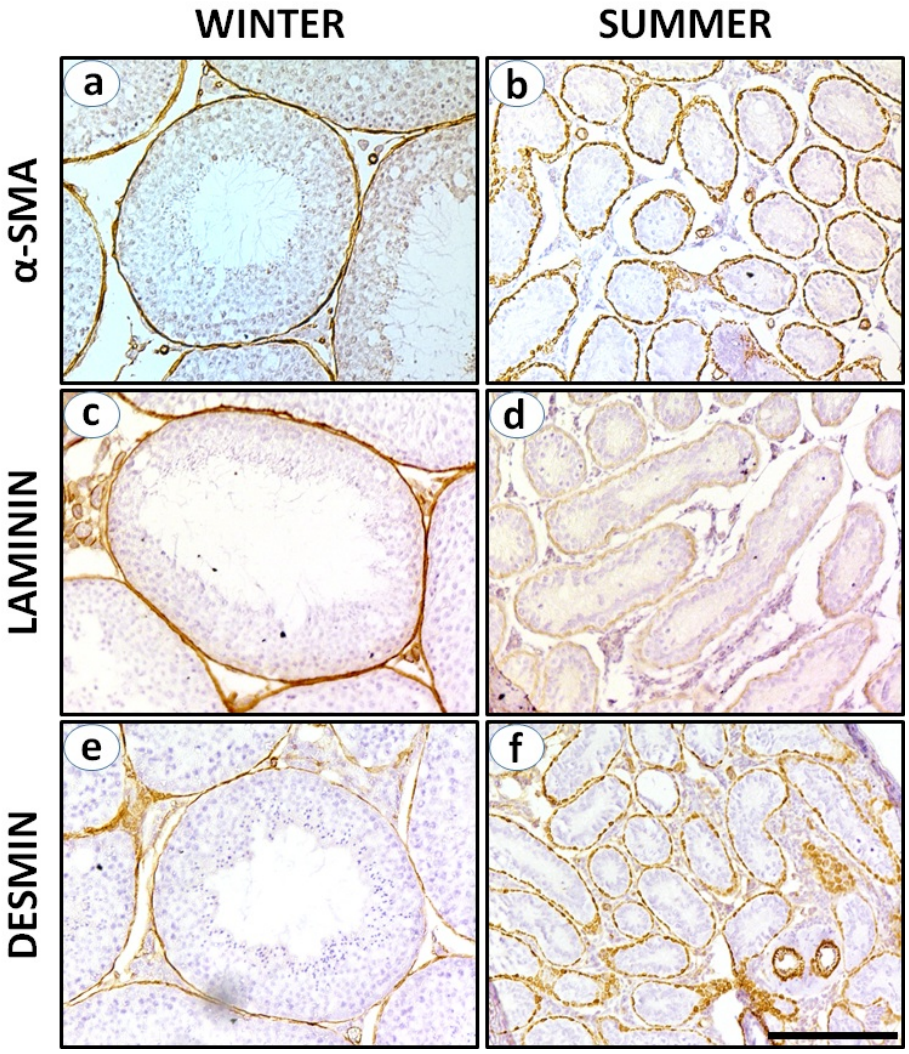


Figure 4.19: Immuno-histochemical detection of alpha-SMA (a and b), LAMININ (c and d), and DESMIN (e and f) in the testes of winter (left column) and summer (right column) males of *M. duodecimcostatus*. The same expression pattern is observed in the two study groups for all three proteins. Scale bar represents 50 µm in all pictures.

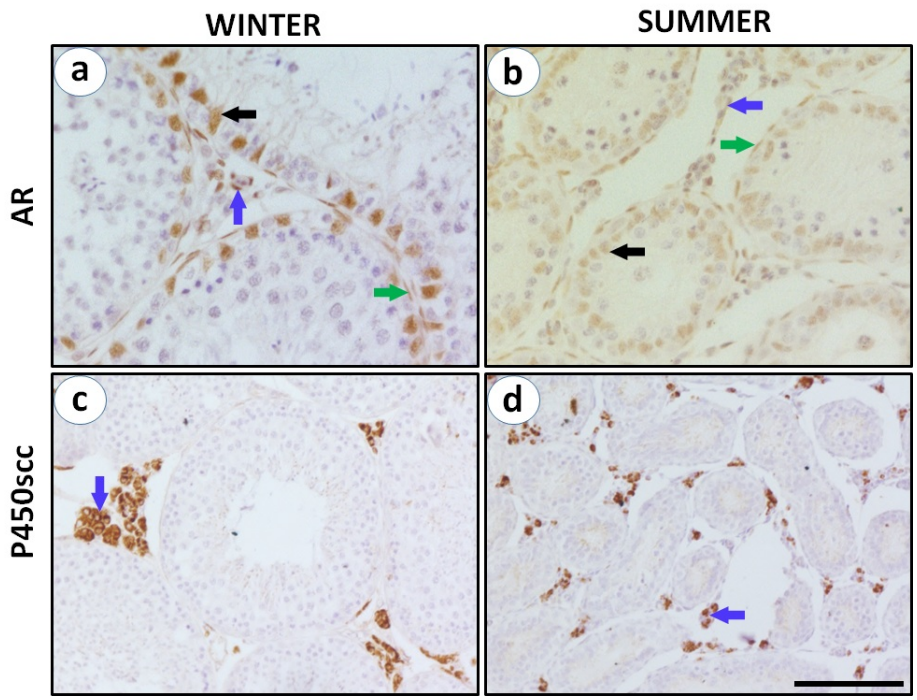


Figure 4.20: Immuno-histochemistry for both AR (a and b) and P450scc (c and d) in transverse sections of testes from winter (left column) and summer (right column) males of *M. duodecimcostatus*. Nuclei were lightly counterstained with haematoxylin. AR expression is observed in Sertoli cells (black arrows), Leydig cells (blue arrows), and peritubular myoid cells (green arrows). P450scc is expressed in Leydig cells (blue arrows), in the two study groups. Scale bar represents 50 µm in all pictutes.

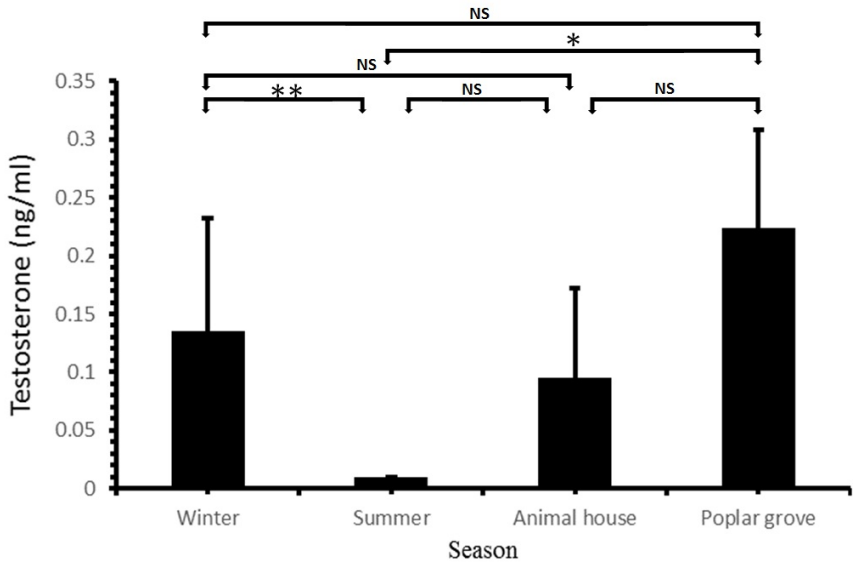


Figure 4.21: Levels of testosterone measured by radio-immunoassay in the serum of males of *M. duodecimcostatus* from wastelands in winter and summer, from the animal house and from the poplar grove. The summer males have lower levels than the other groups. Asterisks indicate the significance level of the differences detected using Student-t tests.

Testosterone (ng/ml)	Winter	Summer	Animal house	Poplar grove
Mean±SD	0.13±0.097	0.009±0.007	0.095±0.076	0.22±0.084

Table 4.9: Mean and standard deviation values of the concentrations of serum testosterone measured by RIA in males of *M. duodecimcostatus* from wastelands in winter and summer, animal house and poplar grove populations.

4.2.6 Reduced levels of apoptosis in the inactive testis of *M. duodecimcostatus*

As usual, apoptotic cells were detected using TUNEL assays (Fig.4.22 a and b) and proliferating cells were revealed with PH3 immuno-histochemistry (Fig. 4.22 c and d). The number of apoptotic cells was 9.50 ± 6.14 cells/0.1 mm^2 in the winter and 4.00 ± 2.43 cells/0.1 mm^2 in the summer. Comparison using a Wilcox's test showed highly significant differences between the two study groups (W = 2492, P<0.001; Fig. 4.23). Similarly, the number of proliferating cells was 5.77 ± 6.37 cells/0.1 mm^2 in the winter and 2.90 ± 2.38 cells/0.1 mm^2 in the summer, differences being again highly significant (Wilcox's test: W = 1924.5, P<0.099; Fig. 4.24). In summary, contrarily to the observed in *A. sylvaticus* and in *M. spretus*, apoptosis is more frequent in the winter testes. However, cell proliferation showed no significant differences between the two study groups of *M. duodecimcostatus*.

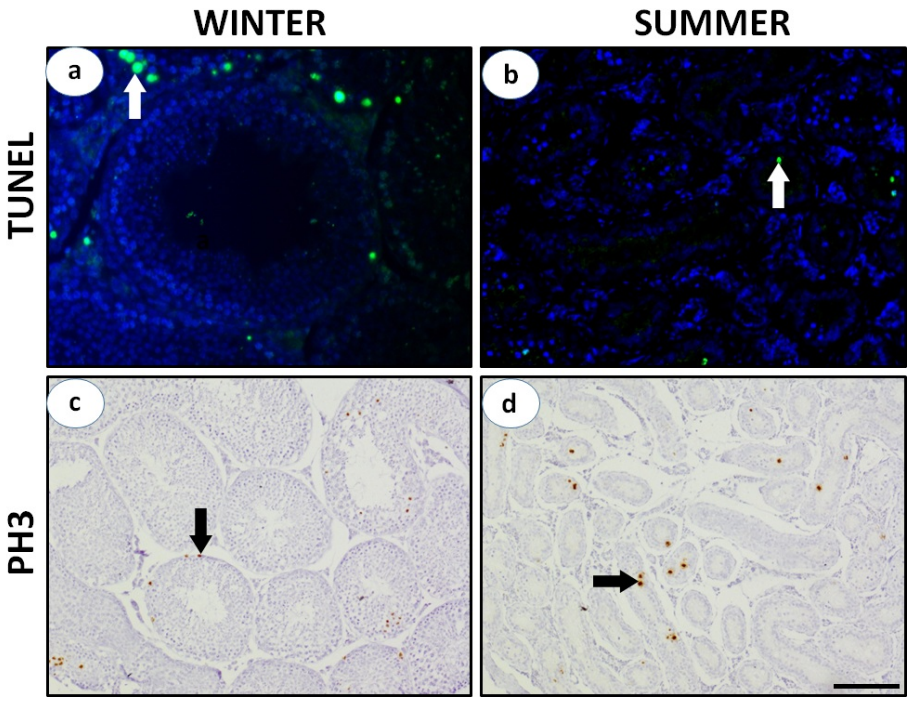


Figure 4.22: TUNEL staining (a and b) to detect apoptotic cells, and PH3 immunohistochemistry (c and d) to detect proliferating cells in transversal sections of testes from winter (left column) and summer (right column) males of *M. duodecimcostatus*. Arrows point to positive cells. Scale bar represents 50 µm in a and b and 100 µm in c and d.

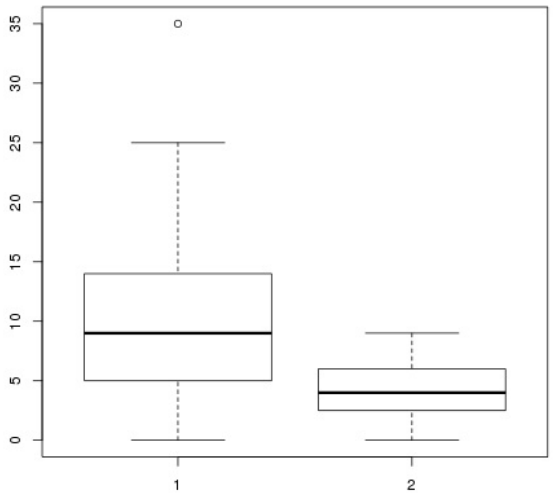


Figure 4.23: Comparison of the number of apoptotic cells per 0.1 mm^2 in testes from winter and summer males of *M. duodecimcostatus*, analyzed using a Box plot. Apoptotic cells are significantly more frequent in the winter testes according to a Wilcox test.

P value	Winter	Summer	Animal house	Poplar grove
Winter		0.00269**	0.44495	0.20364
Summer			0.11189	0.0478*
Animal house				0.10478

Table 4.10: Matrix of P values corresponding to the statistical Student's t-tests performed to compare the serum testosterone levels of every study group of *M. duodecimcostatus* males with each other, as shown in table 1. Asterisks indicate the level of statistical significance.

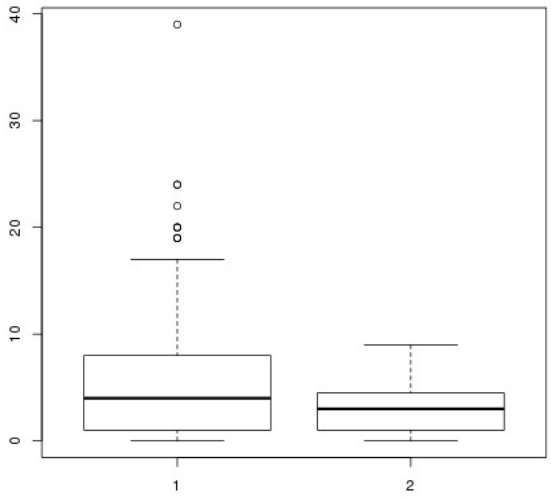


Figure 4.24: Comparison of the number of proliferating cells per 0.1 mm^2 in testes from winter and summer males of *M. duodecimcostatus*, analyzed using a Box plot. a Wilcox test shows no significant differences between the two study groups.

4.3 Discussion

4.3.1 Seasonal breeding in *M. duodecimcostatus* does not depend on photoperiod

Microtus duodecimcostatus is, together with *A. sylvaticus*, the only species, of the four studied in this work, showing complete testis regression during the non-breeding period, which in the south-eastern Iberian populations analyzed here, takes place during the summer months in both cases. However, unlike the wood mouse, the period of reproductive inactivity seems to be much longer in the Mediterranean pine vole. The testicular mass of voles living in wastelands was not maintained at stable values during the non-summer months, existing a clear peak of higher weight in the winter, which may reflect a period of maximal spermatogenic activity and probably an increased reproduction rate. Data of testis/body mass ratio paralleled very well those

of testis mass because the body mass of these animals showed no significant variations throughout the year. Alternatively, the intermediate values of testis mass in spring and autumn might reflect that the processes of testis inactivation and activation, respectively, could be slow, existing reproduction only during the winter months. The lack of pregnant and lactating females in these intermediate seasons supports this hypothesis. Nevertheless, this is true regarding the populations analyzed in wastelands and cereal crops, that undergo severe drought and very high temperatures during the summer. Animals of the same species living in less extreme conditions, either natural (poplar groves) or artificial (animal house), do not experience testis regression and maintain breeding activity throughout the year. The better life conditions in both poplar grove and animal house lead to a higher body mass, mainly in the latter, when compared with that of animals captured in the wastelands at any season.

Since all three groups of males studied here during the summer (wastelands, poplar grove, and animal house) were subjected to the same photoperiod, our observations clearly show that it is the micro-environment in which each vole population is living what determines its reproductive status and the local existence or not of seasonal breeding. When life conditions are favorable, either natural or artificial, voles do not stop breeding, whereas in the poor environment of the wastelands during the summer of south-eastern Iberian Peninsula, testis regression occurs and reproduction is halted. It is well known that, apart from the photoperiod, other environmental cues exist, including food availability, rainfall, temperature, among others, that may influence the reproduction timing of seasonal breeding species. Unlike the photoperiod, the mechanism of action of these environmental cues remains unknown, although numerous investigations are being done, mainly in canary birds (Leitner *et al.*, 2003) and hamters (Paul *et al.*, 2009). The later authors have shown that a non-photic environmental cue, food availability, may influence the expression of Kisspeptin and RFRP, two hypothalamic neuropeptides known to be involved in the control of the Hypothalamus-pituitary-gonad axis, if photoperiod is maintained at an intermediate rhythm (13.5 hours light), but not at stimulating long-day pattern (16 h. light). Hence, non-photic cues are thought to modulate the timing of reproduction in photoperiod-dependent species, but little is know about their function in species en which photoperiod appears to play a less important role. This is the case of the Mediterranean pine vole populations we have investigated here, which could become a useful animal model for the study of the genetic and functional response to the environmental cues influencing seasonal reproduction.

4.3.2 The regressed testis of the Mediterranean pine vole

As discussed previously for other mammalian species, including the wood mouse (see above) and the Iberian mole (Dadhich *et al.*, 2013), depletion of most of the adluminal portion of the germinative epithelium during the vole testis

regression involves a dramatic remodeling of both Sertoli and Leydig cells which is probably mediated by cell movement, as these cells rarely undergo apoptosis or cell proliferation in the adult testis (Blottner and Schoen, 2005; Dadhich *et al.*, 2010). Regarding Leydig cells, the situation in the vole is similar to that described above for the wood mouse as continuous matrix of these cells is formed embedding the reduced seminiferous tubules of the inactive testis, as described in the Iberian mole (Dadhich *et al.*, 2010). Remodelling in Sertoli cells appears to be much better conserved, as most species show an increased numeric density of these cells in the inactive testis, showing that these cells reduce their volume considerably during testis regression (Dadhich *et al.*, 2013). The exception may be the viscacha, where (Muñoz *et al.*, 2001) reported no reduction in the volume of Sertoli cells from inactive testes. Unlike the Iberian mole, in which Sertoli cells retain most of the cell membrane during testis regression (Dadhich *et al.*, 2013) exhibiting superposed membrane infoldings, no variation was detected in the viscacha Sertoli cell membrane. Our results on the inactive testes of *M. duodecimcostatus* suggest a situation similar to that of the Iberian mole.

Currently, we lack data on the process of testis inactivation in the Mediterranean pine vole, but we know that it results in seminiferous tubules with almost completely depleted germinative epithelium, as described for other mammalian species. Nevertheless, like in the wood mouse, two features of the vole inactive testis, including the permeation of the BTB during testis regression and the altered expression pattern of CLAUDIN11, suggest that the cell-adhesion molecules are impaired during testis regression in this species. Accordingly, the hypothesis of a desquamation-based germ cell depletion similar to that described in the Iberian mole (Dadhich *et al.*, 2013), is also favored as the testis-regression mechanism in the Mediterranean pine vole. Testes and epididymides from males captured at the precise time of testis regression will be studied to test this hypothesis. We have also discussed previously the role of both gonadotropin hormones and intra-testicular testosterone on the expression of cell-adhesion molecules in the testis, which are fundamental for the maintenance of the germinative epithelium architecture and the BTB (Tarulli *et al.*, 2008; McCabe *et al.*, 2010; Gye, 2003; Florin *et al.*, 2005; Tu'uhevaha *et al.*, 2007). In *M. duodecimcostatus*, like in the wood mouse (see above) and the Iberain mole (Dadhich *et al.*, 2013), serum testosterone levels decrease significantly during testis regression, another fact supporting the hypothesis that testis regression in this species is mediated by germ-cell sloughing.

4. FOURTH CHAPTER *MICROTUS DUODECIMCOSTATUS*

5. General discussion

5.1 Conserved gene expression patterns in active and inactive testes from seasonal breeding males

Even though the testis features and the mechanism of testis regression in the four species studied here, *C. russula*, *M. spretus*, *A. sylvaticus*, and *M. duodecimcostatus* were very variable – in fact we did not find two identical cases – the winter and summer expression patterns of several testicular cell markers were found to be very well conserved. Several proteins, including SOX9, CLAUDIN11, and DMC1, showed no expression differences between the four species and with respect to those of the mouse and the Iberian mole (Dadhich *et al.*, 2011, 2013), confirming a high degree of evolutionary conservation of their respective functions in the testis. Spermatogenic stage-depending expression of SOX9 in all species studied to date, including rats (Fröjdman *et al.*, 2000), mice, moles (Dadhich *et al.*, 2011) and those analyzed here, suggests an important role for this protein in the control of spermatogenesis, and probably also in seasonal testis involution as expression is even stronger in seasonally regressed testes of moles (Dadhich *et al.*, 2011). Furthermore, ablation of both Sox8 and Sox9 genes just after testis differentiation stages induces spermatogenic failure in mice at the age of 5 months (Barrionuevo *et al.*, 2009), suggesting that spermatogenic function requires a tight regulation of SOX9 in Sertoli cells. DMC1 and CLAUDIN11 have well-known roles in meiotic recombination and blood-testis barrier formation, respectively, and their expression patterns in the shrew testis indicate that both functions occur normally in these species. Other genes analyzed in in this study showed expression differences between species. Apart from normal expression in Sertoli and Leydig cells, in the shrew AR showed very weak or no expression in peritubular myoid cells and consistent foci-like expression in spermatid cells near the tubular lumen, a pattern not previously observed. Similarly, PCNA is expressed in the Sertoli and spermatogonial cells of the four species studied here, as well as in early spermatocytes but, contrary to that described in the mole (Dadhich *et al.*, 2011) and the mouse (our unpublished data), expression in spermatocytes is much stronger than in spermatogonia. In addition, there is also a consistent focus-like expression in the acrosomal region of round spermatids of the shrew. Since PCNA is involved in DNA repair (Shivji *et al.*, 1992), this expression may denote the presence of a species-specific acute DNA damage in this cellular region. Although the origin of these inter-species differences remain unknown

for the moment, our data unravel some peculiar features of the shrew spermatids that deserve further study. The spermatogenic stage-specific expression of DMRT1observed in the four studied species, showing a pattern very similar to that described in the mole, supports our hypothesis (Dadhich *et al.*, 2011)that this protein is involved in the control of spermatogenesis, probably by regulating spermatogonial proliferation. Apart from a difference between the shrew and the other species regarding the number of DMRT1-expressing cells, the expression pattern of this gene was shown to be equal in all species studied to date, indicating a very well conserved function in the adult testis of mammals.

5.2 The role of apoptosis in the germ cell dynamics of seasonal breeding mammals

The causes of seasonal testis regression has been investigated in a number of vertebrate species, including reptiles, amphibians, birds, and mammals. The generally accepted hypothesis derived from these studies was that apoptosis is the main cell process mediating seasonal testis involution (reviewed by (Young and Nelson, 2001; Pastor *et al.*, 2011). However, the discovery of species in which apoptosis is clearly not the cause of testis regression challenges this paradigm. (Dadhich *et al.*, 2013) classified the studied vertebrate species into three main groups: 1) cases in which the involvement of apoptosis in testis regression has been unambiguously demonstrated, including one salamander and four bird species (Yazawa *et al.*, 1999, 2000; Zhang *et al.*, 2008; Islam *et al.*, 2012; Jenkins *et al.*, 2007; Young and Nelson, 2001) 2) cases in which apoptosis was associated but not conclusively demonstrated to be the cause of testis regression, including one frog and five mammalian species (Sasso-Cerri *et al.*, 2006; Young *et al.*, 1999; Young and Nelson, 2001; Strbenc *et al.*, 2003; Strbenc and Bavdek, 2001; Hingst and Blottner, 1995; Morales *et al.*, 2002a, 2007; Nonclercq *et al.*, 1996) ; and 3) cases in which conclusive evidence was reported excluding apoptosis as the cause of testis regression, including two mammals, the roe deer Capreolus capreolus (Blottner et al., 2007) and the Iberian mole *Talpa occidentalis* (Dadhich *et al.*, 2010). The conclusion was that apoptosis could cause testis-regression in birds and maybe also in amphibians but not in mammals, where the apoptosis paradigm is not sufficiently supported. Based on our studies in the Iberian mole (Dadhich *et al.*, 2013) we recently described a new mechanism of testis regression based on germ-cell desquamation, that has also been further reported in the long hairy armadillo (Luaces *et al.*, 2014).

In the present study, we showed the existence of summer testis regression in two rodents, the wood mouse, *A. sylvaticus* and the Mediterranean pine vole, *M. duodecimcostatus*. In the former species, like in the Iberian mole, we observed increased levels of apoptosis in the inactive testis. Since in both species apoptotic cells are mainly located in the innermost area of the seminiferous tubules, indicating that affected cells are primary spermatocytes (pachytene

cells), and taking into account that no further meiotic cells are observed in these tubules, our interpretation of these results is that the role of apoptosis in the inactive testes of *T. occidentalis* and *A. sylvaticus* is to eliminate the germ cells that continue entering meiosis during the non-breeding period. However, the case of *M. duodecimcostatus* is exceptional because in this species the frequency of apoptotic cells decreases during testis regression, becoming lower in the testes of inactive males. Hence, if our hypothesis on the role of apoptosis in the inactive testis of seasonal breeding males is correct, then the rate of meiosis onset should be lower in *M. duodecimcostatus* than in species showing increased apoptosis in the non-breeding period. To test this, we compared the areal cell density of DMC1-positive cells (zygotene and early pachytene spermatocytes) in active and inactive testes of *A. sylvaticus* and *M. duodecimcostatus* and found no significant differences between the winter (active) testes of both species, which implies a similar rate of meiosis initiation in both cases (Table 5.1; Fig. 5.1 a; P=0.075). On the other hand, the areal density of meiotic cells in *M. duodecimcostatus* was much lower than that of *A. sylvaticus* in the summer (inactive) testis (Table 5.1; Fig. 5.1 b; $P < 0.001$). This results clearly show that meiosis onset is extremely reduced during the vole inactive season, existing only some few meiotic cells that have to be depleted by apoptosis in this period. In conclusion, our results in both *A. sylvaticus* and *M. duodecimcostatus* also support our hypothesis that the main role of apoptosis in the inactive season of mammals is to eliminate the germ cells that enter meiosis.

DMC1$^+$ cells per 0.15 mm^2	Summer testes		Winter testes	
	Microtus duodecim.	*Apodemus sylvaticus*	***Microtus*** *duodecim.*	*Apodemus sylvaticus*
Mean ± SD	35.6±21.7	227.4±67.7	175.6±48.1	139.2±16.3
P value	4.65E-05		0.075	

Table 5.1: Areal density values of DMC1-positive (meiotic) cells in the testes of summer and winter males of both *M. duodecimcostatus* and *A. sylvaticus*. Data are expressed in mean ± standard deviation. P values correspond to mean comparisons made using Student's t-tests.

5.3 Conserved features in the regressed testes of seasonal breeding species

Circannual testis changes have been studied in a few mammalian species with seasonal reproduction. These include the white-footed mice Peromyscus leucopus (Young *et al.*, 1999; Young and Nelson, 2001), the hare Lepus europaeus (Strbenc *et al.*, 2003; Strbenc and Bavdek, 2001), the Syrian hamster

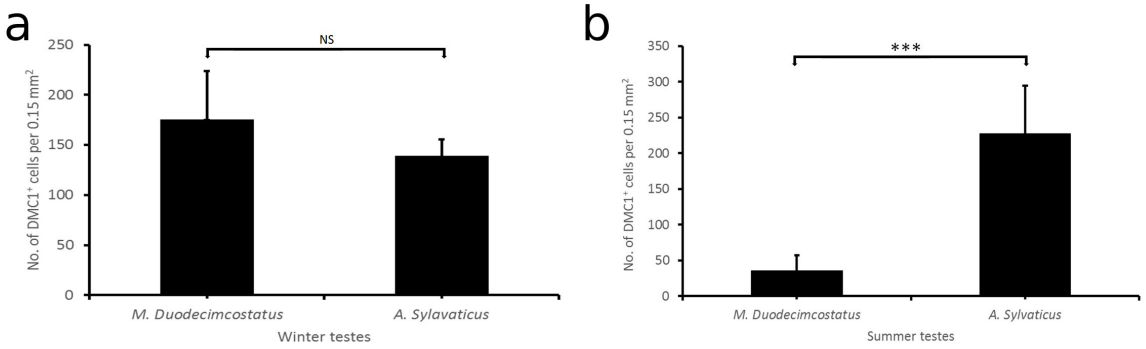


Figure 5.1: Comparisons of the areal density of DMC1-positive (meiotic) cells in the testes of winter (a) and summer (b) males of both *M. duodecimcostatus* and *A. sylvaticus*, and significance of the differences detected according to Student's t-tests. Asterisks indicate the level of statistical significance

Mesocricetus auratus (Morales *et al.*, 2002a, 2007), the Djungarian hamster Phodopus sungorus (Hoffmann, 1979), the roe deer Capreolus capreolus (Blottner *et al.*, 2007), the European mole *Talpa europaea* (Suzuki and Racey, 1978), the Iberian mole *Talpa occidentalis* , the long hairy armadillo Chaetophractus villosus(Luaces *et al.*, 2014), among others, including the four species studied in this work, *Crocidura russula*, *Mus spretus*, *Apodemus sylvaticus*, and *Microtus duodecimcostatus*. From these, only six species have been the subject of multidisciplinary studies including morphological, morphometric, histological, cytological, gene-expression, and hormonal analyses in which comparison between active and inactive males was performed. These are the Iberian mole, *T. occidentalis* (Dadhich *et al.*, 2013), the long haired armadillo, C. villosus (Luaces *et al.*, 2013, 2014), and the four species studied in the present work, although only in two of them, *A. sylvaticus* and *M. duodecimcostatus*, males undergo complete testis regression during the non-breeding period, like mole and armadillo males also do. When we compared the biological features of sexually inactive males in these four species, which belong to three different mammalian orders (Eulipotyphla, Cingulata and Rodentia), several of them were found to be quite well conserved. For instance, in all species inactive males exhibit reduced levels of circulating testosterone, which appear to be a condition necessary for testis regression. Compelling evidence exists that testicular cell-junction dynamics is regulated by hormones and that impairment of Sertoli-germ cell junctions is the main testis regression effector in both moles and armadillos (Dadhich *et al.*, 2013; Luaces *et al.*, 2014). It is known that both testosterone and gonadotropins regulate the expression of testicular cell-adhesion molecules (Tarulli *et al.*, 2008; McCabe *et al.*, 2010; Meng *et al.*, 2011; Gye, 2003; Florin *et al.*, 2005; Tu'uhevaha *et al.*, 2007; Xia *et al.*, 2005). In the seasonal breeding species analyzed to date, the androgenic function of males is diminished during the non-breeding season, suggesting that reducing the production of androgens is probably the hormonal signal inducing seasonal germ-cell depletion. Another constant feature in the regressed

testes of inactive males is the permeation of the BTB, a process also subject to hormonal control, although it is controversial whether the control is exerted by FSH or testosterone or both (Xia *et al.*, 2005; Tarulli *et al.*, 2008; Meng *et al.*, 2005, 2011; Tu'uhevaha *et al.*, 2007). According to our observations in the testis of *T. occidentalis*, (Dadhich *et al.*, 2013) suggest that a reduction of the intra-testicular testosterone levels beyond a given threshold would lead to the disassembly of the Sertoli cell apical ectoplasmic specializations and thus to a massive germ-cell desquamation, whereas a further reduction to an even lower threshold would be necessary to induce BTB permeation. However, we have found now contradictory results in *M. spretus*, as the BTB of this species apparently permeates in winter before any sign of germinative epithelium depletion is visible. The levels of serum testosterone in the inactive males of *M. spretus* are lower than those measured in active ones, but it is clear that both cases cannot be explained in the same way, as germ cell depletion only occurs in the mole. Further research is needed to understand the functional connection between androgen concentration, germ cell depletion and BTB permeation in mammals.

Profound remodeling of testicular somatic cells is also a well conserved feature during testis regression in seasonally breeding males. The loss of most of the germinative epithelium implies a drastic reduction of the seminiferous tubule diameter, a fact that force Sertoli cells to reduce the cytoplasmic volume, becoming tightly packed in the tubular periphery. In some cases, as observed in *A. sylvaticus* and in *M. duodecimcostatus*, but not in *T. occidentalis*, the nuclei of these cells is pushed towards inner locations in the seminiferous tubule because those of spermatogonia remain adjacent to the basal membrane and there is no room for all of them. In the mole, it was shown that Sertoli cells in the regressed testis retain most of their cytoplasmic membrane by forming deep, superposed infoldings. Regarding Leydig cells, only the two mole species mentioned above, T. europaea and *T. occidentalis*, exhibit a particular remodeling pattern in the inactive testis. A high number of Leydig cells are packed together within the inter-tubular space existing no lymphatic sinusoidal space observed between them. This way, a continuous, dense matrix interstitial cells is formed in the inactive testis in which the seminiferous tubules are embedded loosing contact to each other (Suzuki and Racey, 1978; Dadhich *et al.*, 2013). This unique feature has not been described in any other mammalian species. The most frequent situation in the rest of species, including rodents, hamsters, deers, and rabbits is the presence of clusters of Leydig cells occupying the interstitial spaces between adjacent, spermatogenically inactive, seminiferous tubules. The testes of inactive males of *A. sylvaticus* or *M. duodecimcostatus* (see Figs. 3.5 c and 4.5 c) may exemplify the aspect of testes in the non-breeding males of most mammalian species.

5.4 Multiple species-specific models of circannual testis variation in seasonal breeding species

Most of the species cited above, including the Iberian mole, show strict seasonal breeding with a non-breeding period taking place every year in which testes of males become spermatogenically inactive and reduced in size. By contrast, all four species analyzed in the present study showed at least one peculiarity in their reproductive biology that makes them different from each other. In *C. russula*, males scape from undergoing testis regression even though most females become non-receptive in the summer and stop breeding for a couple of months. In *M. spretus*, males conserve spermatogenically active testes throughout the year, although a slight size reduction accompanied of BTB permeation takes place in winter. *A. sylvaticus* shows a classical seasonal breeding pattern with testes becoming fully inactive during the summer. And finally, in *M. duodecimcostatus*, testis regression occurs in some natural habitats (wastelands and cereal crops) but not in others, either natural (poplar groves) or artificial (animal house). In our opinion, it is not by chance that this high inter-species variability is detected in populations located in southern Iberian Peninsula, which represent in most cases the meridional border of the distribution areas of many European mammals (see below). The most noteworthy example of these differences is found if we compare the situation in *M. spretus* and in *A. sylvaticus*. These are two rodent species belonging not only to the same family (Muridae), but also to the same subfamily (Murinae) and are not only sympatric, but also syntopic, occupying the same habitat. In fact, we captured animals of both species in the same locations, setting their respective traps mixed and separated by just a few meters from each other. Despite these similarities, which denote a quite close phylogenetic relationship and probably very similar lifestyles, males of M. espretus undergo a moderate although significant testis reduction in the winter, whereas those of *A. sylvaticus* experience complete testis regression in the summer. The lack of evident causes that may explain this surprising divergence implies that either very subtle species- and/or population-specific characteristics or non-conspicuous environmental cues, or both, may serve to define a particular circannual reproduction timing. The genetic and physiological mechanisms by which apparently irrelevant environmental cues are transduced to induce a reproductive response remain obscure and the study of examples like this of *M. spretus* and *A. sylvaticus* may help to unravel them. Nevertheless, two conclusions can be drawn from this knowledge regarding the mechanisms controlling seasonal reproduction: 1) that they are in fact very plastic and much less rigid than initially considered, and 2) that they appear to be fast evolving. Hence, mammalian populations probably have available multiple ways to get adapted to the unstable environmental conditions that the climate change will probably cause in the future.

5.5 Breaking paradigms on seasonal breeding

Seasonal breeding is generally considered a species-specific feature, existing mammals reproducing seasonally, those inhabiting the temperate zones of the Earth, and others exhibiting continual reproduction, the tropical and equatorial ones. However, this paradigm is now challenged by our findings in the greater white-toothed shrew, *Crocidura russula*. We clearly show that the reproductive pattern of this species in southern European populations is very different from those reported in northern ones. Whereas in Switzerland (Jeanmaire-Besancon, 1988) and in Ebro delta (López-Fuster *et al.*, 1985) shrews experience a non breeding season in the winter, which is longer in more northerly populations and involves testis regression in males, in the populations we have investigated the non-breeding period is very short and males retain fully testis function throughout the year, a fact that can be considered a new adaptive feature. Moreover, some few females can remain receptive and reproduce during the resting period. These results evidence that in these populations seasonal breeding is not a species-specific feature. Rather, it depends on the life conditions that every particular individual is facing at each moment. Hence, it appears these animals show a clear tendency to reproduce continuously at all seasons, but the environmental cues concomitant with particular climatic season at different latitudes force then to stop breeding. Similarly, little attention is generally paid to the fact that the reproductive period is not a constant feature for each particular species, which are frequently classified as winter or summer breeders. According to our knowledge, the less favorable conditions for the reproduction of *C. russula* are found during the winter in the north and during the summer in the south, and this is the reason why the non-breeding period is inverted in northern with respect to southern populations. The inflexion latitude for this species appears to be located somewhere between northern and southern Iberian Peninsula, as shrews from Ebro delta stop breeding in winter (López-Fuster *et al.*, 1985) whereas those from Granada do it in summer. A similar reproductive pattern is observed the European populations of *A. sylvaticus*. In southern Great Britain this species reproduces in the spring-summer but not in the winter (Randolph, 1977), although winter breeding can take place occasionally if food availability (mainly acorns) is exceptionally good (Smyth, 1966). However, our present study shows that the non-breeding season of the wood mouse in southern Spain is the summer, coinciding with most species living in this geographic region. The case of *M. duodecimcostatus* adds more diversity. The distribution area of this species includes southern France and most of the Iberian Peninsula. (Paradis and Guedon, 1993) found that in northern populations of this species (Montpellier), reproduction takes place throughout the year, existing no resting period. In contrast, we have shown that in the wastelands near Granada city, reproduction is halted during the summer months, whereas in a poplar grove located in the same geographic region reproduction continues at that time, as it occurs with animals maintained in captivity. Again, like *C. russula*, *M. duodecimcostatus* provides clear evidences that seasonal breeding

is not an intrinsic reproductive feature for this species and that micro-environmental cues interacting with each particular animal in each particular moment condition whether reproduction must be halted or not. According to these considerations, two extreme situations can be recognized in mammals regarding the control of circannual reproduction timing: 1) species in which the HPG axis is tightly regulated by the photoperiod and exhibit constant and rigid seasonal breeding patterns; 2) species with opportunistic seasonal breeding, in which photoperiod has little or no influence in their reproduction timing, which mainly depends on micro-environmental cues like water and food availability, temperature, rainfall, etc. European moles (Gorman and Stone, 1990), lemmings (MacLean Jr *et al.*, 1974), hamsters (Hoffmann, 1979), deers (Blottner *et al.*, 1996), brown bears (Dahle and Swenson, 2003), and many other species living in central and northern Europe and similar latitudes in North America and Asia, probably belong to the first group. The greater white-toothed shrew and the Mediterranean pine vole and probably many other species whose area of distribution extends toward lower latitude regions, belong to the second group.

6. Conclusions

1. Males of *Crocidura russula* do not undergo testis regression at any time of the year and retain full testis function even when most females are not sexually receptive in the summer. We suggest that in southern populations the non-breeding period is short enough to make testis regression inefficient in terms of energy savings because testes of *C. russula* are very small, due to monogamy, and the spermatogenic cycle is slow and long.

2. In southern Iberian populations of *Mus spretus*, testes maintain the spermatogenic function during the entire year, but undergo an apoptosis-mediated, slight size reduction. During this period, the blood-testis barrier is permeated, an unexpected finding in apparently functional testes.

3. The lack of testis regression exhibited by males in both *C. russula* and *M. spretus* represents a new adaptive response to the very short periods of sexual inactivity occurring in southern populations.

4. Complete testis regression occurs in males of *Apodemus sylvaticus* during the summer in populations located in south-eastern Iberian Peninsula. It is mediated by reduced levels of serum testosterone and probably induced by germ-cell desquamation derived from impaired expression of genes encoding cell-adhesion molecules.

5. Males from populations of *Microtus duodecimcostatus* located in wastelands and cereal crop lands of south-eastern Iberian Peninsula undergo complete testis regression during the summer, whereas those from natural populations located in poplar groves and those maintained in captivity in an animal house retain full testicular function at the same time, showing that testis regression and seasonal breeding in this species does not depend on photoperiod.

6. The conserved spermatogenic stage-specific expression pattern of both SOX9 and DMRT1 in the testes of males from all seasonal breeding mammalian species studied to date, clearly suggest that these two genes play important roles in the control of spermatogenesis.

7. Our results in *A. sylvaticus* and *M. duodecimcostatus*, together with those we previously reported on *Talpa occidentalis*, indicate that the role of apoptosis in the regressed testes of seasonally breeding males is to eliminate the germ cells that continue entering meiosis during the non-breeding period.

8. Repeated observation that seasonal testis involution is associated with reduced levels of circulating testosterone in all species studied to date suggests that reduced concentration of androgens is probably the hormonal signal inducing seasonal germ-cell depletion and BTB permeation in mammals.

9. Our findings in southern populations of four mammalian species evidence seasonal breeding is not a species-specific feature at this latitude. Rather, it depends on the life conditions that every particular individual is facing at each moment. Unlike populations located at higher latitudes, those from the south in many species probably experience photoperiod-independent seasonal breeding.

10. Our study of four micro-mammal species in south-eastern Iberian Peninsula evidences the existence of multiple species-specific models of circannual testis variation, showing that he mechanisms controlling seasonal reproduction are in fact very plastic and fast evolving. Hence, mammalian populations probably have available multiple ways to get adapted to the unstable environmental conditions that the climate change will probably cause in the future.

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