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**FUNCTIONAL AND DEVELOPMENTAL
STUDIES OF THE PRIMATE TESTIS**



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**FUNCTIONAL AND DEVELOPMENTAL STUDIES
OF THE PRIMATE TESTIS**

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY**

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CERTIFICATE

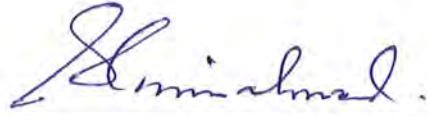
The Thesis titled “Functional and Developmental Studies of the Primate Testis” submitted by **Mr. Shahzad Irfan** accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Doctor of Philosophy in Endocrinology.

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DECLARATION

I hereby declare that the material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Shahzad Irfan

DEDICATION

*I dedicate my thesis to my loving parents and my
mentor*

Professor Dr. Muhammad Shahab

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List of abbreviations

GnRH	Gonadotropin Releasing Hormone
LH	Luteinizing Hormone
FSH	Follicle Stimulating Hormone
HPG axis	Hypothalamus Pituitary Gonadal axis
GABA	γ-Amino Butyric Acid
NPY	Neuropeptide Y
GPR54	G-protein Coupled Receptor- 54
Kiss1	Kisspeptin
Kiss1r	Kisspeptin receptor
TGFβ	Transforming growth factor Beta
IGF	Insulin like growth factor

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General Abstract

Primate testis acts as the main source for androgen and sperm under the functional regulation of pituitary gonadotropins. The understanding of the functional regulation of adult primate testis as well as the developmental aspects of maturation during puberty in primates is still not complete. The present thesis aimed to assess the localization and functional significance of kisspeptin signaling in adult non human primate testis as well as to characterize the pubertal and testicular development in a New World monkey. Rhesus macaque (*Macaca mulatta*) and common marmoset (*Callithrix jacchus*) were employed as primate models. To sort out the functional significance of kisspeptin signaling in the primate testis *in vivo* pharmacological approach was used by employing chemical hypophysectomy by using acyline (a GnRH receptor antagonist) in adult rhesus monkeys (n=4). Animals were given iv boluses of kisspeptin (50µg) and kisspeptin (50µg) with hCG (50IU). Blood samples were taken and testosterone and LH was analyzed in the plasma by specific RIAs. To find out the testicular localization of kisspeptin receptor immunocytochemistry was performed on paraffin embedded testis tissue from adult rhesus (n=2) and marmoset monkey (n=2) whereas for testicular localization of kisspeptin immunocytochemistry was performed on paraffin embedded testis tissue from adult rhesus monkey (n=2). 12 sections from each testis were used for immunocytochemical analysis. To characterize the pubertal testicular development, immature common marmoset (n=48) were observed for a period of 13 months for monthly changes in body weight, testis volume and serum testosterone. At the end of the study animals were sacrificed and testis tissue was collected and processed for paraffin embedding. PAS staining was conducted. Histological and morphometric data were determined. Results of the experiments demonstrated that kisspeptin, given as an intravenous bolus enhanced stimulated plasma testosterone in pituitary clamped adult male rhesus monkeys whereas the same bolus had no effect on the basal levels of plasma testosterone. The immunocytochemical localization revealed that kiss1r positive areas were present at the periphery of the seminiferous tubules in the adult rhesus testis. Same

pattern of localization for kiss1r was found in adult marmoset testis tissue. Whereas the kisspeptin like immunoreactivity was found to be present in the interstitial area of the adult rhesus testis tissue. The detailed examination revealed that the kiss1r localization was present around the spermatogonial stem cells (A spermatogonia) which are present at the periphery of the seminiferous tubules. While the kisspeptin like immunoreactivity was observed in the peritubular myoid cells, Leydig cells and underlying layers of basement membrane. Developmental studies showed that the start of pubertal activation in common marmoset occur around 7 months of age and was characterized by sudden peaks of serum testosterone accompanied by a rapid increase in the testis volume. Morphometric analysis revealed that the pubertal activation created a lumen in the center of seminiferous tubules. The germ cell compliment was observed to divide mitotically after the initial increase in serum testosterone depicted by presence of B spermatogonia in the seminiferous tubules after 7 months of age. The complete spermatogenesis characterized by the presence of sperm in epididymis was first observed around 12 month of age in the common marmoset testis. Although testis volume continued to grow after 12 months of age, the qualitative maturity of testicular functions was achieved at one year of age in the male common marmoset.

The present findings implicate that an active paracrine kisspeptin-kisspeptin receptor signaling is present in the adult primate testis; where the kisspeptin can enhance stimulated testosterone thus hinting towards an indirect action on the Leydig cell. The finding of the tubular immunolocalization of the kisspeptin receptor in primate testis where Sertoli cells were found to be positive further extends our *in-vivo* results and affirms the assumption of an indirect action of kisspeptin on Leydig cell via the Sertoli cells. However to understand the true nature of this signaling cascade further investigations at the cellular level are required. The developmental kinetics of the process of puberty including pubertal activation and testicular development in common marmoset mimics higher primate like pattern. Although the endocrine correlates for these developmental events need to be further substantiated in common marmoset.

Chapter 1

General Introduction

General Introduction

Reproductive functionality in primates is achieved by the endocrine communication between hypothalamus, pituitary and gonads. This interplay of hormones imposes a functional regulation which ensures the maintenance of reproductive capacity. Gonadotropin releasing hormone (GnRH) a decapeptide, is synthesized in hypothalamic neurons called GnRH neurons, and is released in the pituitary portal circulation (Silverman et al., 1977; Silverman et al., 1982). At the pituitary, GnRH acts on the gonadotrophs which expresses GnRH-receptor (GnRH-R). The GnRH-GnRH-R coupling at the gonadotroph results in synthesis and secretion of gonadotropins into the portal circulation (Knobil et al., 1980). Two types of gonadotropins are secreted in response towards a specific tone and frequency GnRH release namely luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Kamberi et al., 1971; Neill et al., 1977). LH and FSH once released from pituitary reach gonads via peripheral circulation and acts on the Leydig cells and Sertoli cells, respectively. LH ensures the synthesis and secretion of sex steroids (steroidogenesis) from Leydig cells (Ascoli et al., 2002). FSH on the other hand is critical for proliferation of the developing Sertoli cell and affects the spermatogenesis quantitatively (Hackert and Griswold, 2002; Plant and Marshall, 2001). Testosterone is the primary steroid synthesized and released by the Leydig cells in response to the LH (Eik-Nes and Hall, 1965). Constant high levels of serum testosterone down regulates hypothalamic pituitary axis in terms of LH and the low serum levels of testosterone acts as a positive inducer of GnRH-LH secretion in male primates. Apart from the endocrine regulation a particular type of local intercellular communication also exists in the primate testis known as paracrine signaling. Paracrine signaling ensures the local interaction of testicular cells in delivering coordinated state of action to achieve the fine balance between the processes of steroidogenesis and spermatogenesis (Schlatt et al., 1997).

The functional competence of an adult primate testis depends upon the developmental events during fetal and postnatal life. The postnatal phases of

development in higher primates include infantile, juvenile, pre-pubertal and pubertal and adult stages (Plant and Witchel, 2006). The major developmental events occur in infantile, juvenile and pre-pubertal phases whereas the main maturational events occur during the pre-pubertal and pubertal phase (Marshall and Plant, 1996; Waites et al., 1985). Developmental events include cellular proliferation and rearrangement (Simorangkir et al., 2012). The maturational events include the initiation of the functional capacity of the testicular cells. The functional capacity is in turn characterized by the expression of nuclear receptors and the secretory response towards particular intratesticular stimuli (Griswold, 1988; McKinnel et al., 2001; Majumdar et al., 2012). The testicular developmental and maturational periods are generally characterized by intensive cellular proliferation, cell movement, cellular rearrangement in the juvenile phase of development whereas the attainment of functional capacity is observed after the onset of puberty (Schlatt et al., 1995). All these above mentioned events lead to a considerable increment in the testicular size during pubertal development and are anatomically depicted by a significant increase in the testicular volume (Plant et al., 2005).

The current thesis focuses on functional and developmental aspects of primate testis and accordingly the next sections will focus on the functional anatomy of the adult primate testis, the primate pubertal development, involvement of kisspeptin-kisspeptin receptor signaling in puberty and the postnatal and pubertal testicular development in primates.

Primate testis

Primate testis serves as a fundamental source for the production of male gametes (sperm) and testosterone through the process of spermatogenesis and steroidogenesis, respectively, under the influence of pituitary gonadotropins. Anatomically primate testis is divided into two functionally distinct compartments namely tubular and interstitial compartment (Schlatt et al., 1997). Tubular compartment contains Sertoli cell and developing spermatogonia whereas the interstitial compartment bears Leydig cells,

macrophages, sympathetic innervations and blood vessels. Sertoli cell serves as nurse cell for the developing spermatogonia whereas Leydig cell acts as the main source of steroidogenesis. Sertoli cells and Leydig cells are functionally regulated by FSH and LH, respectively. LH acts on Leydig cells through LH receptors (LHR) and initiates the process of steroidogenesis (Ascoli et al., 2002). Testosterone is the principal androgen synthesized through the side chain cleavage of cholesterol in the mitochondria of Leydig cell by the steroidogenesis (Preslock and Steinberger, 1977). The principal action for FSH is the control of Sertoli cell proliferation during development and maintenance of spermatogenesis quantitatively during adulthood (Plant and Marshall, 2001). Testosterone is the prime regulator of the testicular microenvironment during pubertal development and is also responsible for the maintenance of spermatogenesis qualitatively (Marshall et al., 1986; Schlatt et al., 1995; Weinbauer et al., 1988).

Apart from endocrine regulation of testicular functions via pituitary gonadotropins, a distinctive intercellular communication exists between the testicular cells. This intra-testicular intercellular communication which is also known as paracrine signaling, plays an important part in the testicular development as well as the normal functioning of the adult testis (Schlatt et al., 2007). The Leydig cells inside the interstitial compartment release testosterone, the Sertoli cells which are present in the tubular compartment express androgen receptor (Suarez-Quian et al., 1999). In order to act on the Sertoli cell, the testosterone must reach the tubular compartment by crossing peritubular myoid cells and basement membrane. Spermatogenesis, a testicular process through which male spermatogonial stem cells, which are present at the periphery of the seminiferous tubules, are transformed through mitotic and meiotic division into motile sperm with the capacity to fertilize the ovum (de Kretser et al., 1998). During spermatogenesis, the developing spermatozoa are in direct communication with the Sertoli cells and the developmental requirements of the germ cells are met through the secretory activity of the Sertoli cells (de Kretser et al., 1998). Sertoli cell expresses androgen receptor as the key events during the process of spermatogenesis are dependent and/or regulated by testicular androgens indirectly through Sertoli cells (Rey et al., 2009). Testosterone and dihydrotestosterone (DHT) are the main androgens types which are present at high concentration inside the seminiferous tubules ((Preslock and Steinberger,

1977). This high intra-tubular concentration of androgens is mainly achieved by androgen binding protein secreted by Sertoli cells (Ritzen et al., 1982; Galdieri et al., 1984).

The functional aspect of compartmentalization in the primate testis is to provide the proper environment and suitable conditions for the successful completion of steroidogenesis and spermatogenesis separately (Dym and Fawcet, 1970; Schlatt et al., 1997). Although these two compartments are present in close proximity, a cytological barrier exists between these two compartments which not only separates them functionally but is also responsible for highly sensitive inter-compartmental communication through the exchange of chemicals via an active transport mechanism (Pelletier and Byers, 1992). The cell types and extracellular structure responsible for this barrier include peritubular myoid cells, basement membrane and Sertoli cells. Peritubular myoid cell acts as the first layer of this barrier and completely encircles the tubular compartment or the seminiferous tubules. Peritubular myoid cells are followed by basement membrane and Sertoli cells. Adjacent Sertoli cell creates junctional complexes and thus form another barrier known as blood testes barrier (Lui et al., 2003; Setchell, 2008). Sertoli cell performs multiple functions in the tubules and is the main cell type responsible for establishing specific intra-tubular environment. Sertoli cells along with peritubular myoid cells and basement membrane work together to achieve the highly sensitive equilibrium created by this barrier for the proper functioning of the testis (Dym and Fawcet, 1970).

Apart from testosterone, numerous proteins and growth factors have been shown to act as paracrine factors in testis. Like the Sertoli cell secreted factors which include transforming growth factor (TGF), insulin like growth factor (IGF), interleukines (IL), fibroblast growth factor (FGF) and stem cell factor (SCF) and peritubular myoid cell secreted factors like TGF β , IGF, leukemia inhibiting growth factor (LIF) and peritubular factor that modulates Sertoli cell function (PModS) (Mullaney and Skinner, 1991; Skinner, 1991; Skinner et al., 1991). The proposed actions of these growth factors in the testis include modulation of cellular growth and proliferation as well as the modulation of steroidogenesis. Thus paracrine communication is highly important for the proper

functioning of the adult testis, if this inter-compartmental communication is disrupted or compromised; the adult testis ceases to function properly.

Primate puberty is a complex process of temporal growth involving integration of hypothalamus, pituitary and testis (Ojeda et al., 1980; Plant and Berker-Gibb, 2004). Pubertal testicular development significantly affects the future functional capacity of the testis during the adulthood. A timely progression into pubertal phase of development is vital for the normal functionality of the adult primate testis both qualitatively and quantitatively.

Puberty in male primates

Puberty is the temporal process of development which is characterized by the activation of hypothalamic GnRH neurons at a specific age during postnatal development (Ojeda et al., 1980; Plant, 2001; Plant and Berker-Gibb, 2004). The juvenile phase is characterized by a hypogonadotropic state. The level of GnRH and the GnRH mRNA in the hypothalamus of juvenile monkey were found to be similar to those in adult monkey brain implying that the release of GnRH, instead of the synthesis, in the juvenile period of development is compromised (Fraser et al., 1989). As the control of pulsatile GnRH release occurs within the hypothalamus (Plant, 1985). The neurobiological mechanism by which this restraint is implied is known as pre-pubertal hypothalamic restraining brake (Plant and Berker-Gibb, 2004). This brake is achieved by the combined action of many putative neurotransmitters and neuropeptides. γ -Amino butyric acid (GABA, an inhibitory neurotransmitter), neuropeptide Y (NPY) and glutamate (an excitatory amino acid) have been shown to play major role in establishing this pre-pubertal hypothalamic restraining brake on GnRH neurons (Terasawa and Fernandez, 2001).

Although it was found that the pubertal activation can be achieved by the chemical stimulation of the hypothalamus (Plant et al., 1989). The precise neurobiological trigger was yet to be discovered until 2003. In 2003, a neuropeptide named kisspeptin along with its G-protein coupled receptor was demonstrated to be

involved in the pubertal activation of GnRH neurons in humans and primates (Terasawa et al., 2013). It has been shown that the kisspeptin action, via a G-protein coupled receptor (GPR54) present on the GnRH neurons, results in the pulsatile release of GnRH (Shahab et al 2005). This mode of release of GnRH is entrained by a neuro-biological setup known as GnRH pulse generator. Activation of GnRH pulse results in the generation of corresponding LH pulses from anterior pituitary gland (Plant, 2008). By binding to LH receptors, present on the Leydig cell membrane, these LH pulses are responsible for the activation of the process of steroidogenesis inside the Leydig cells (Ascoli et al., 2002). The sudden rise in serum testosterone acts on Sertoli cells present in the seminiferous tubules. Sertoli cells are expressing androgen receptor during this specific time of pubertal activation (Majumdar et al., 2012). This intra-testicular action of testosterone is very significant as the Sertoli cells show rapid response by attaining functional activity which is characterized by an increase in the cytoplasmic area and the initiation of secretory activity. Secretory activity is characterized by fluid secretion from Sertoli cells (Sharpe et al., 2003). Expression of androgen receptor by Sertoli cells during the pubertal onset is very important as the levels of testosterone are also high during early postnatal period (neonatal period) but Sertoli cells do not express steroid receptors during that period (Majumdar et al., 2012). The formation of lumen and the rapid increase in the Sertoli cell size eventually increase the seminiferous tubule diameter (Plant et al., 2005). The first sign of puberty at the testicular level is the rapid increase in the testicular volume. In parallel the A-spermatogonia, the spermatogonial stem cells present at the periphery of the seminiferous tubules also start to divide mitotically and the presence of B spermatogonia are observed. These events at the testicular axis are subsequent to the hypothalamic activation of the GnRH neurons (Plant, 2008).

However, from testicular perspective the process of puberty is defined as the attainment of the ability of the testes to produce and release mature sperm. The process of spermatogenesis is characterized by mitotic and meiotic cell divisions of the spermatogonia. The process of meiosis needs to be performed in an immunologically controlled environment as the haploid sperm can initiate an immunogenic response from the macrophages present in the interstitial area of the testes (Meinhardt and Hedger, 2011). To create the immunologic barrier inside the seminiferous tubule, Sertoli cell

creates tight junctional complexes between adjacent Sertoli cell membrane, these tight junctions forms the blood testes barrier (Lui et al., 2003; Setchell, 2008). Testosterone is responsible for the formation of this blood testes barrier by the Sertoli cells (McLachlan et al., 2002). Until the establishment of the blood testes barrier, the process of spermatogenesis could not proceed beyond the first mitotic divisions. For these reasons after the hypothalamic GnRH pulse activation, the presence of sperm either in the tubule lumen or in the epididymis is not observed immediately (Schlatt et al., 1995).

Initiation of puberty in primates is triggered by resurgence of GnRH pulsatility which then leads to pulsatile LH release and gonadal development (Plant, 2001). GnRH pulsatility itself is regulated by a number of neuronal signals including glutamate, GABA and NPY. However recently, there has been much excitement about the central role of kisspeptin signaling which has been shown to be the most potent drive to the GnRH neurons (Terasawa et al., 2013). Therefore the next section represents a brief description on the basic biology of the kisspeptin-kisspeptin receptor signaling.

Basic biology of kisspeptin and kisspeptin receptor signaling

Kisspeptin is a 145- amino acid precursor peptide encoded by *KISS1* in humans. Endogenous forms of kisspeptin comprising 54, 14 and 13 amino acids in length have been isolated from human placenta (Murphy, 2005; Hilden et al., 2007). Chemically kisspeptins are included in RFamide peptide family; a structurally related group of peptides terminating with an arginine-phenylalanine-amide at their carboxy terminal (Dockery, 2004). The kisspeptin receptor in humans (KISS1R) is a 398 amino acid G-protein coupled receptor with a short extracellular domain, seven transmembrane domains linked by extracellular and intracellular loops and an intracellular domain (Lee et al., 1999).

Three concurrent discoveries revealed the natural ligand to this receptor as the peptide product of the metastasis suppressor gene *Kiss1*, termed kisspeptin (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). The minimum sequence necessary to

activate KISS1R is the common C-terminal decapeptide which is commonly shared by all endogenous forms of kisspeptin peptide having different fragment length. *Kiss1* is expressed in the CNS, pituitary, ovary, testis, and pancreas but is highly concentrated in placenta whereas *Kiss1R* is expressed in the CNS, testis, ovary, pancreas, intestine, liver, pituitary and placenta (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Funes et al., 2003; Terao et al., 2004; Mead et al., 2007). *Kiss1* is expressed by neurons in the hypothalamus and KISS1R is found to co-localize with GnRH (Irwig et al., 2004; Han et al., 2005; Kirilov et al., 2013). The stimulatory effect of kisspeptin on HPG axis is mediated via hypothalamic GnRH, as central or peripheral effects of kisspeptin are blocked by GnRH antagonist administration (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Shahab et al., 2005).

The nomenclature for the kisspeptin and kisspeptin receptor has been officially recommended by the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification. Human kisspeptin gene was designated as *KISS1* whereas non human kisspeptin gene was designated as *Kiss1*. The kisspeptin protein names for human and non human were given as KISS1 and Kiss1, respectively. For the kisspeptin receptor, *KISS1R* was designated for human gene whereas *Kiss1r* was designated for non-human gene (Kirby et al., 2010)

Kisspeptin-Kiss1r signaling in primate puberty

Role of kisspeptin-kisspeptin receptor signaling in primate pubertal activation was discovered in 2003, when it was shown that mutations in *KISS1R* (GPR54) gene caused idiopathic hypogonadotropic hypogonadism (IHH) in humans (DeRoux et al., 2003; Funes et al., 2003; Seminara et al., 2003). Humans with inactivating mutations (loss of function mutation) in *KISS1R* have low gonadotropin levels, lack LH pulses and fail to undergo puberty (Seminara et al., 2003). Since 2003, a number of studies have shown that IHH is caused by inactivating mutations of *KISS1R* in humans (Lanfranco et al., 2005; Semple et al., 2005; Pallais et al., 2006). On the other hand it has also been shown that activating mutations (gain of function mutations) of *KISS1R* cause precocious

puberty in humans, where the subjects experience puberty at much early age (Teles et al., 2008).

The hypothalamic *Kiss1* expression is increased during puberty in castrated non human primates and the intravenous (iv) administration of kisspeptin significantly enhances the serum LH levels in juvenile agonadal male rhesus monkeys (Shahab et al., 2005). The involvement of kisspeptin-Kiss1r signalling, as a mediator in the negative feedback action of the testosterone on the GnRH release in monkeys has also been shown and kisspeptinergic neurons upstream to the GnRH neurons are involved in the negative feedback action of testicular testosterone on LH secretion in the adult male rhesus monkey (Shibata et al., 2007). The structural interactions between kisspeptinergic and GnRH neurons in the mediobasal hypothalamus (MBH) of the adult male rhesus monkeys has been demonstrated (Ramaswamy et al., 2008). It has been shown that kisspeptin is secreted in a pulsatile manner in the stalk median eminence and also stimulates GnRH release in vivo and the pubertal increase in the kisspeptin is accompanied by GnRH release in a pulsatile manner (Keen et al., 2008).

Postnatal and pubertal testicular development in primates

Postnatal development of primate testes until the end of juvenile period is characterized by a slow and steady increase in Sertoli cell numbers which is then followed, in pre-pubertal phase, by a burst of proliferation and an increase in cytoplasmic volume, mostly referred to as functional maturation of Sertoli cells (Sharpe et al., 2003). Quantitative increase of Sertoli cells results in the significant lengthening of the seminiferous cords (Marshall and Plant, 1996). Further into puberty a dramatic rise in germ cells occurs resulting in an enlargement of seminiferous tubule diameter and consequently a rapid increment in the size and weight of the testis. Seminiferous tubule length and Sertoli cell number stabilize during this period (Plant et al., 2005). It is concluded that testicular volume presents a valid readout of testicular maturation during puberty. In rhesus macaques the Sertoli cell number increases from approximately 40 million at birth to 2.000 million in each testis in the adult (Sharpe, 1994), whereas the

undifferentiated spermatogonia increase from one million at birth to 600 million in the adult (Simorangkir et al., 2005). In human there is a 6-fold increase in testicular volume during the first year of life (Muller and Skakkebaek, 1983), whereas in new world monkeys a 13 fold increase is observed within the first 5 month (Abbot and Hearn, 1978; Rey et al., 1993). The distinctive 4-fold increase in Sertoli cell number in infancy, when gonadotropin levels are high, is then followed by an abated rate of proliferation in the juvenile phase of development when the serum levels of gonadotropins are very low (Plant et al., 2005). Until the onset of puberty, while the cords are slowly extending in length, no significant increase in the seminiferous cord diameter is detectable and the cords are devoid of a lumen. Thus the incremental rise in Sertoli cells number in the infancy and juvenile period, when Sertoli cells account for 95% of the seminiferous tubule mass, seems to be an innate feature of this period.

The next section reviews the general notion of developmental characteristics and regulation of different cell types present in the testis.

Sertoli cell

Enrico Sertoli, an Italian scientist first described the testicular cells in 1865 which bear his name. He described them as the large branched cells which are linked to the production of spermatozoa. The overall number of adult Sertoli cells establishes the upper limit of sperm production in the adult testis, and spermatogenic efficiency is highly associated with Sertoli cell supportive capacity, which in turn is the best indication of Sertoli cell function in the adult testis (Hess and Franca, 2005). Sertoli cells are columnar cells and extend from the basement membrane of the seminiferous epithelium to the lumen (Fawcet and Burgos, 1956). When fully differentiated they are mitotically arrested and act as nurse cells (Griswold, 1998). Forming a syncytium-like epithelial structure, Sertoli cells are embedding and holding the interconnected cohorts of germ cells (Russell, 1980). The Sertoli cells are interconnected by specialized junctional complexes which are responsible for establishing the blood testis barrier (Fawcet and Burgos, 1956; Dym, 1974; Russell, 1980). Approximately 40% of the Sertoli cell surface is in contact with the

surface of elongated spermatids (Griswold, 1995; Russell et al., 1986). In contrast, immature Sertoli cells form a simple columnar epithelium with limited cytoplasmic connections sharing the basement membrane with few A-spermatogonia. The immature Sertoli cell proliferates significantly in the infantile period owing to the high level of pituitary gonadotropins during this phase and thus creating nearly 95 % of the total seminiferous tubule cell mass along with germ cells which constitute 5 % (Rey et al., 1993; Simorangkir et al., 2012). This numerical density of Sertoli cells in seminiferous tubule cross section diminishes during pre-pubertal stage as the Sertoli cell cytoplasm volume increases considerably along with an increase in germ cell population (Dang and Meusy-Dessolle, 1985). During the infant and juvenile period Sertoli cells increase in number without any significant increase in size and morphology (Simorangkir et al., 2003). Apart from being mitotically active immature Sertoli cells are also characterized by their ability to secrete estrogens and anti-mullerian hormone (AMH) and presence of functional FSH receptors. At initiation of puberty a significant increase in cytoplasmic volume occurs apart from the mitotic proliferation (Marshall and Plant, 1996). This morphological change in Sertoli cells signifies an important first sign of puberty. An important and histological obvious feature is a re-arrangement of Sertoli cells at the beginning of puberty. While Sertoli cell nuclei are randomly distributed in the seminiferous cords prior to puberty, they are organized more epithelial-like with all nuclei being close to the basement membrane early into puberty. This epithelial re-arrangement as well as the increase in number are associated with longitudinal expansion of the cords and are augmented by an increase in the number and volume of peritubular cells (Fawcett, 1975; Marshall and Plant, 1996).

The pubertal onset is characterized by an intense proliferation of Sertoli cells and hence the testis size increases 10 fold (Marshall and Plant 1996; Plant et al., 2005). In the peri-pubertal time period, tubule diameters also increase to accommodate the growth of Sertoli cells during differentiation and the increasing number of germ cells. Now, Sertoli cells become closely interconnected and form the blood testes barrier. In consequence, the fluid accumulation occurs, which creates a lumen thus transforming the cords into seminiferous tubules (Fawcett, 1975) and evoking additional diametrical growth. These processes are coincident with the expansion of the germ cell population. At first,

spermatogonia populate the basement membrane below Sertoli cells. When meiotic differentiation starts, germ cells become engulfed in Sertoli cell extensions. The multiplied germ cells lose the contact to the basal lamina and move towards the centre of the tubules. The precedence of Sertoli cell proliferation prior to germ cell expansion is physiologically important. The maximum number of germ cells in the adult testis depends on the number of Sertoli cells because each Sertoli cell supports a limited number of germ cells (Orth et al., 1988). Therefore, adequate Sertoli cell expansion at the beginning of puberty is essential to provide the structural and functional basis in establishing the capacity of the testes to generate sperm later in life (Plant, 2005). In this respect the early phase of puberty is more relevant for future fertility than the second phase of puberty when germ cell differentiation starts after Sertoli cells have reached peak numbers and established the mitotic arrest.

The ability of Sertoli cells to respond to gonadotropins is achieved much earlier than the actual pubertal onset (Arslan and Qazi, 1976; Arslan et al., 1981; Arslan et al., 1993; Schlatt et al., 1995; Sameshima and Hamana, 1996; Ramaswamy et al., 2000). During the juvenile period male monkeys respond to exogenous FSH or hCG/testosterone treatment (Arslan et al., 1993; Schlatt et al., 1995). This response pertains to an almost immediate and robust proliferation and maturation of immature Sertoli cells along with an increase in A-spermatogonia. Thus a persistent low pre-pubertal level of gonadotropins is the limiting and critical factor maintaining the testes in a passive state. This signifies that immature Sertoli cells are highly dependent on gonadotropins to induce proliferation and differentiation and that the ability and potential of Sertoli cell to proliferate and differentiate is established through mechanisms independent from puberty and already established long before pubertal induction (Schlatt et al., 1995). On the other hand high levels of gonadotropins in infancy cannot induce germ cell differentiation revealing that some important changes must have occurred during the early juvenile period creating the capacity of the testes to not only expand its somatic components but to kick start spermatogenesis in the more mature seminiferous epithelium. These molecular and cellular changes are attributed to the lack of androgen receptor expression by the Sertoli cell during infancy (Majumdar et al., 2012).

Germ cell

Although many changes occur in the early stages of puberty in the seminiferous epithelium, the first emergence of meiotic spermatocytes after a peak of spermatogonial proliferation is considered often as a first sign of pubertal maturation of the seminiferous tubules (Plant, 2005). The increment in the total germ cell number before puberty is much slower as compared to Sertoli cells or longitudinal outgrowth of tubules. Consequently, the number of germ cells expressed as a function of Sertoli cell number or per tubule cross section decreases during juvenile period. This relative decline does not reflect the actual progressive increment in germ cells per testis before puberty (Chemes, 2001; Marshall et al., 2005; Albert et al., 2010). It is important to note that only unbiased morphometric procedures provide actual and valid values on cell counts. In contrast to adult animals in which changes in germ cell counts can be expressed in relation to a fixed number of Sertoli cells, it is mandatory to use more complex evaluation strategies in immature testes as all cellular components expand. The pubertal proliferation of germ cell results in a loading of the seminiferous tubules with high numbers of germ cells until at the end of the puberty the germ cell mass accounts for more than 90% of seminiferous tubule volume (Plant, 2005).

In primates the undifferentiated spermatogonia (Type A) have been classified into two categories, dark (Ad) and pale (Ap) based on their nuclear staining pattern (Ehmcke et al., 2005). Ad spermatogonia are mitotically quiescent in adulthood and hence designated as reserve stem cell. Ap spermatogonia on the other hand are mitotically active with self renewal capacity. Their clonal expansion and splitting is responsible for the generation of differentiated spermatogonia (type B) and maintenance of a progenitor pool (Ehmcke and Schlatt, 2006). In rhesus monkeys Ad and Ap spermatogonia are mitotically active in pre pubertal primate testis as evident by robust S-phase labelling of A-spermatogonia in the infantile and juvenile testis. This expansion of both spermatogonial subtypes occurs irrespective of a hypogonadotropic environment. The post-natal pattern of Ad spermatogonial proliferation is similar to Ap spermatogonial expansion (Simorangkir et al., 2005) which reflects the expansion of Ap spermatogonia

in hypogonadotropic adult monkeys (Marshall et al., 2005). The regulatory mechanisms of the gonadotropin independent proliferation of Ad (in immature monkeys) and Ap spermatogonia (in immature and adult monkeys) is still unclear but indicates an autonomous regulation often found in stem cell systems. Nevertheless, the juvenile testis can attain the potential to successfully undergo spermatogenesis well before the onset of actual puberty (Marshall et al., 1984, 1986; Arslan et al., 1993; Schlatt et al., 1995). Random but rare appearance of type B spermatogonia has also been reported in the pre-pubertal macaque testis (Cavicchia and Dym, 1978; Kluin et al., 1983). However, significant numbers of differentiated spermatogonia are only evident after the onset of puberty.

Leydig cell

Leydig in 1850 first reported masses of cells containing vacuoles and pigmented inclusions. These cells were later called after his name as Leydig cells. Leydig cells reside in the interstitial compartment of the testis and serve as the main source of testosterone production. Postnatal production of testosterone by the Leydig cell is regulated by the pituitary gonadotropin LH (Saez, 1994). The Leydig cell responsiveness towards pituitary LH is achieved early in primates i.e, during neonatal phase, as the gonadotropins levels are high during this phase and testosterone levels correspond to LH peaks (Frawley and Neill, 1979; Fouquet et al., 1984). Leydig cell number increases in a linear trend from birth towards puberty. The morphological differentiation of Leydig cells is a long process during pubertal development in primates, the peculiar correlation of pituitary LH and testosterone remains throughout this developmental period as after a long juvenile hiatus the rise in the levels of pituitary gonadotropin during the prepubertal period is followed by high levels of testosterone (Steiner and Bremner, 1981; Meusy-Dessolle and Dang, 1985; Bernstein et al., 1991; Plant, 2006).

The levels of testosterone are in turn correlated to the number and volume of Leydig cells. As shown by the Fouquet et al., 1984, rhesus monkeys show a strong positive correlation between Leydig cell number and plasma testosterone during postnatal

development. In rhesus macaques the post natal levels of plasma testosterone demonstrate four distinct phases, 1) a neonatal phase with moderate levels of testosterone and large Leydig cells, 2) a juvenile phase with very low levels of testosterone along with a significant reduction in Leydig cell size and volume, 3) a pre-pubertal phase with a rise in testosterone concentration along with a significant increase in Leydig cell size and volume and 4) a pubertal phase with increasing levels of testosterone but relatively unchanged Leydig cell volume (Steiner and Bremner, 1981; Fouquet et al., 1984; Meusy-Dessolle and Dang, 1985; Arslan et al., 1986).

The same pattern of postnatal testosterone secretion has been demonstrated in new world monkeys (Abbott and Hearn, 1978; Kholkute et al., 1983; Lunn et al., 1994; Rey et al., 1995; Kelnar et al., 2002; Chandolia et al., 2006). Immature and mature Leydig cells are present from birth to late puberty in the Cebus, a new world monkey. At birth and during infancy, immature Leydig cells represent the highest proportion, with mature Leydig cells comprising only 12%. The quantitative analysis of the postnatal testicular development in this monkey shows that in neonatal phase the interstitial tissue represents nearly half of the testicular parenchyma, with a progressive decrease during postnatal life to reduce to half of its size in late puberty. However, the absolute volume of interstitial tissue increases during development i.e. there is a 7 fold increase in the Leydig cell number during neonatal period which is followed by a stable period during juvenile until late puberty when there is a two fold increase. There are two periods of interstitial tissue growth, the first during the neonatal and early infantile periods, and the second, in mid to late puberty (Rey et al., 1991; Rune et al., 1994, 1996). As the pubertal activation of spermatogenesis is dependent on high intratesticular levels of testosterone, the physiological insensitivity of the neonatal testis towards high intratesticular testosterone is due to the absence of Sertoli cell androgen receptor expression during this period (McKinnel et al., 2001; Majumdar et al., 2012).

Rationale

Kisspeptin in primate testis

While the central role of kisspeptin signaling in the control of reproductive axis is thoroughly demonstrated, the functional role of kisspeptin at the primate testis is yet to be defined. The available data from human and non human primate studies indicates an active kisspeptin-Kiss1r signaling in primate testis (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Ramaswamy et al., 2007). The high expression levels of kisspeptin and moderate expression levels of kisspeptin receptor in adult human testis indicate a possible intratesticular role of kisspeptin signaling in primate testis. As the testicular paracrine signaling, featuring tens of molecules is a distinct feature of the adult primate testis (Schlatt et al., 1997) and it has been shown previously that hypothalamic neuropeptide, GnRH and GnRH receptors are present in the testis and affect steroidogenesis through paracrine signaling (Bambino et al., 1980; Bourne et al., 1980; Petersson et al., 1989). The possible involvement of kisspeptin in enhancing testicular steroidogenesis in adult male rhesus monkeys given an iv infusion of kisspeptin has been indicated, where the serum levels of testosterone were high for a given concentration of LH in these monkeys (Ramaswamy et al., 2007). The immunohistochemical localization of the kisspeptin and kisspeptin receptor in rodents and vertebrates testis has been reported recently (Anjum et al., 2012; Chianese et al., 2013; Hua et al., 2013), although the testicular localization in primate testis has not been reported to date.

Pubertal testicular development in common marmoset (*Callithrix jacchus*) a New World monkey

The pubertal and testicular development in New World monkeys is not well characterized to date. Whereas the specific age of puberty, the prepubertal developmental pattern as well as the hormonal correlates of pubertal activation and the subsequent testicular developmental patterns are thoroughly depicted in old world monkeys (Plant,

2005). Despite the fact that common marmoset (*Callithrix jacchus*) is a representative New World monkey; a certain degree of controversy exists regarding its pubertal age and subsequent testicular development. Common marmosets have a unique reproductive endocrinology as compared with higher primates where the LH is replaced by a chorionic gonadotropin (CG) like molecule having a shorter half life as compared with LH (Muller et al., 2004) as well the testicular steroidogenesis utilizes $\Delta 4$ pathway whereas in higher primates $\Delta 5$ pathway is utilized (Preslock and Steinberger, 1977). Apart from these physiological diversity, the high individual variation in the timing of pubertal onset, the possible impact of social status on the pubertal onset and the subsequent testicular development in common marmoset has not been assessed systematically in a large number of cohorts. Apart from the above mentioned physiological variation, an important physiological aspect of adult common marmoset is the resemblance of its testicular tubular organization towards human testis. It has been shown that adult marmoset testis shows highly similar seminiferous tubule organization with human (Millar et al., 2000; Wistuba et al., 2003) as well as the fetal and neonatal germ cell development in common marmoset mimic human like pattern (Mitchell et al., 2008). While the use of common marmoset as a laboratory primate in toxicological and biomedical research has increased enormously in the recent years, owing to its small size, high breeding rate and longer life span in captivity (Carrion and Patterson, 2012; Hart et al., 2012), the need for a better understanding of pubertal and testicular development in common marmoset is mandatory.

Objectives

The present study was designed for three objectives:

1. To elucidate the possible involvement of kisspeptin in testicular steroidogenesis in adult rhesus monkeys (*Macaca mulatta*) through an *in vivo* approach.
2. To determine the immunohistochemical localization of kisspeptin and kisspeptin receptor in adult primate testis.
3. To characterize the pubertal onset and the subsequent testicular development in Common marmoset (*Callithrix jacchus*) through in vivo and histomorphometric approaches.

Chapter 2

Intratesticular action of kisspeptin in adult rhesus monkey (*Macaca mulatta*): An *in vivo* study

Intratesticular action of kisspeptin in adult rhesus monkey (*Macaca mulatta*): An *in vivo* study

Abstract

Kisspeptin-kisspeptin receptor signaling in mammals has been implicated as an integral part of the reproductive cascade. Kisspeptinergic neurons upstream of GnRH neurons are involved in the activation of hypothalamic GnRH pulse generator during the pubertal onset. Thus, the major research focus either in rodents or in primates has been on the central effects of kisspeptin. The demonstration of the presence of *KISS1R* expression in human testes suggests additional, as yet unknown, physiological actions of kisspeptin-kisspeptin receptor signaling at the distal component of the male reproductive axis. In this study we tried to explore the impact of kisspeptin at testis in the adult male rhesus monkey, a representative higher primate. We employed the pituitary gonadotropin hypophysectomised monkey to assess the intratesticular actions of iv bolus of kisspeptin. Plasma testosterone and plasma LH levels were monitored in a group of four adult male monkeys. The peripheral administration of human kisspeptin-10 (50 μ g, iv bolus) caused a single LH pulse followed by robust increase in plasma testosterone levels which sustained for the next 180 minutes. This potent increase in testosterone was abolished when kisspeptin was administered to acyline (a GnRH receptor antagonist) pretreated animals (n=4) (Acyline: 60 μ g/kg and 120 μ g/kg BW, sc, morning and evening, respectively, 1 day before kisspeptin injection). However kisspeptin administration significantly ($p < 0.005$) elevated hCG-stimulated testosterone levels in acyline-pretreated monkeys as compared with saline+hCG treatment. These results revealed a novel facet of kisspeptin-Kiss1r signaling at the testis, where kisspeptin administration significantly enhanced stimulated testosterone secretion in adult intact male monkeys suggesting a possible paracrine action of kisspeptin in amplifying the responsiveness of the primate Leydig cell towards luteinizing hormone.

Introduction

In male primates, GnRH pulses activate pituitary gland to release LH in a pulsatile fashion, activating the LH receptor present on the Leydig cell in such a manner that a response in the form of testosterone is produced. The pulsatile fashion of LH release is physiologically important as consistent high serum levels of LH down regulates the LH receptor on the Leydig cells which results in diminished testicular androgenic response. A single LH pulse will give rise to a corresponding testosterone peak. This LH-testosterone loop shows a diurnal rhythm in adult male rhesus monkeys (Schlatt et al., 2008). Rhesus monkey also shows seasonality in terms of reproductive efficiency. Levels of testosterone and testicular functionality are greatly reduced in non breeding season as compared with the breeding season (Wickings and Nieschlag, 1980).

Since the initial discovery of the role of kisspeptin-kiss1r signalling in primate puberty, most of the studies were focused on the central role of kisspeptin. Although the presence of kisspeptin and kiss1r transcripts in adult primate testis hints towards the possible role of kisspeptin signalling at the testis (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). On the other Ramaswamy found that in adult male rhesus monkeys (*Macaca mulatta*) the iv administration of kisspeptin given as a continuous infusion resulted in an increase in circulating LH levels which peaked after 2-4 hours of initial treatment and then followed by a decline (Ramaswamy et al., 2007). The interesting revelation made in this study was the disproportional relationship between circulating testosterone and LH, where testosterone levels were constantly high for a given concentration of LH during iv infusion of kisspeptin thus raising the possibility of an endocrine aspect of kisspeptin-kiss1r signaling within the primate testis. These results hint towards a possible role of kisspeptin signalling in regulating the testicular steroidogenic response towards LH.

In the light of the findings by Ramaswamy (2007), to further explore the possible role of kisspeptin at the testis, we decided to investigate the possibility of a kisspeptin iv bolus in enhancing basal testosterone levels in adult male monkey. As the kisspeptin has a central effect in enhancing hypothalamic GnRH release, the kisspeptin iv bolus would increase the serum levels of LH. This increased level of LH would enhance the testosterone levels in result of the kisspeptin bolus via the central mechanism.

In order to explore the role of kisspeptin in enhancing the basal levels of testosterone, we needed to establish an endocrine model which can assure the possibility of direct testicular effect of kisspeptin on the basal testosterone levels which is independent of serum LH and also the central effect of kisspeptin in enhancing the serum LH levels should also be compromised. In order to block the pituitary secretion of LH we used Acyline, a GnRH receptor antagonist (Shahab et al., 2005). Acyline competes with GnRH for binding with GnRH receptor present at the pituitary thus blocking the effect of GnRH at the pituitary levels. After achieving this chemical hypophysectomy in terms of gonadotropins, any effect observed in the basal testosterone levels after the administration of kisspeptin iv bolus can be positively attributed to the kisspeptin signaling at the testis level. We selected adult male rhesus monkeys to test our hypothesis in exploring the testicular effect of kisspeptin signalling.

Materials and Methods

Monkeys

Four, 4-6 years old, intact male rhesus monkeys (*Macaca mulatta*), weighing 6.0-8.0 kg were used in this study. Animals were housed in individual cages, under standard colony conditions, fed with monkey diet at 1300-1330 hours daily and supplemented with fresh fruits in the morning in the Primate Facility of the Department of Animals Sciences, Quaid-i-Azam University, Islamabad. Water was available *ad libitum*. The monkeys were trained for chair-restraint prior to the experiments in order to sample these animals without sedation or anaesthesia. Under ketamine sedation (5 µg/kg bw, im) animals were placed in primate chairs. After recovery from sedation the animals were allowed to sit on the chair for gradually increasing periods of time. The animals were habituated to chair restraint over several months. All experiments were approved by the Departmental Committee for Care and Use of Laboratory Animals and were performed in accordance with regulations of the Ethics Committee of the university.

Catheterization

To permit sequential withdrawal of the blood samples and iv administration of drugs, the animals were anesthetized with ketamine hydrochloride (Ketamax, Rotexmedica, Trittau, Germany, 5 µg/kg bw, im), and a teflon cannula (Vasocan Branule, B. Braun Melsungen AG, Belgium; 0.8mm/22G O.D) was inserted in the cephalic vein before initiation of sampling and the animals were restrained to the chair. The free end of the cannula was attached to a syringe via a butterfly tubing (20G diameter and 300mm length). Blood sampling and infusion of treatments were carried out when the animals had fully regained consciousness.

Pharmacological agents

Human kisspeptin-10 (112-121) (Phoenix pharmaceuticals, Belmont, CA, USA) and GnRH receptor antagonist acyline (Bioqual, USA) were kindly provided by Dr. Tony M. Plant. hCG (Pregnyl®, N.V Organon Oss Holland) and heparin (Rotexmedica, Germany) were purchased locally. Working solutions of kisspeptin and hCG were made in normal saline while acyline was dissolved in 5% aqueous mannitol. Ketamine hydrochloride (Ketamax, Rotexmedica, Trittau, Germany) was purchased locally.

Dosage

Based upon previously reported centrally effective iv kisspeptin doses (10µg, 30µg and 50 µg) in adult intact male rhesus monkeys (Ramaswamy et al., 2007; Wahab et al., 2008, 2011) the highest dose (50µg) of kisspeptin-10 was selected for exploring a possible direct testicular action in the present study. In our one of previous studies this dose of kisspeptin-10 was also found to be peripherally effective as it caused significant enhancement of plasma adiponectin levels in adult intact monkeys (Wahab et al., 2010).

In-Vivo experimental design and blood sampling

In order to examine our hypothesis of an intra-testicular action of kisspeptin-10 iv bolus in male monkeys, we used pituitary gonadotropin-clamped monkey model. The primary site for kisspeptin action is at hypothalamic GnRH neurons expressing kiss1r, this receptor coupling increase the GnRH pulse activity and subsequently increases the gonadotropin levels in blood circulation. To prevent this central action of kisspeptin administration being transduced at the pituitary level, chemical hypophysectomy was achieved by treating the animals with a potent GnRH receptor antagonist, acyline (Herbst et al., 2002; Shahab et al., 2005). Acyline competes with GnRH to bind with GnRH receptors at gonadotropes and blocking a secretory response from pituitary in response to GnRH and/or kisspeptin bolus.

The different sets of *in-vivo* experiments along with the treatments and pretreatments in this study are shown in Table 1. Same group of animals were used in each of these experiments. All the animals received the same treatments in each experiment. Animals were treated with acyline 24 and 12 hours before the experiment 1(c), 2(a) and 2(b) in the Table 1. The morning (24hr prior) and evening (12 hour prior) treatment was given subcutaneously with two different doses (60µg/kg and 120 µg/kg BW, sc, 9:00 am and 9:00 pm respectively). Blood sampling for all experiments started between 0900-0930 hours, and samples (2.5-3 ml) were obtained at 30-min intervals. First sample (-30 min) was collected 30 min before the treatment (vehicle/ kisspeptin/ hCG/ hCG + kisspeptin). Samples were taken in heparinised syringes and immediately transferred into culture tubes kept on ice. After completion of the blood sampling, these culture tubes were centrifuged at 3000 rpm for 30 minutes and plasma was extracted and stored at -15°C until assayed. After taking every sample, approx 1 ml of normal saline containing 5IU of heparin was injected to compensate the lost blood volume to prevent hypovolumic shock to the animals and blood clotting.

Table 2.1 Details of the experiments.

Experiment #	Administration (iv)	Time of the year	Pretreatment	Animal number
1. (a) <i>Vehicle Control</i>	Normal saline (1ml)	May	None	4 Male monkeys
(b) <i>Kisspeptin</i>	Kisspeptin-10 (50µg)	May	None	4 Male monkeys
(c) <i>Kisspeptin</i>	Kisspeptin-10 (50µg) hCG (50IU) at 240min sample	July	Acyline (sc) 12 hrs and 24 hrs prior sampling	3 Male monkeys
2. (a) <i>hCG+Saline</i>	hCG (50IU) + Normal saline	August	Acyline (sc) 12 hrs and 24 hrs prior sampling	4 Male monkeys
(b) <i>hCG+Kisspeptin</i>	hCG (50IU) + Kisspeptin-10 (50µg)	August	Acyline (sc) 12 hrs and 24 hrs prior sampling	4 Male monkeys

Effect of kisspeptin-10 on plasma testosterone and plasma LH in normal adult intact male monkeys

Impact of peripheral administration of kisspeptin-10 (50 μ g) on basal testosterone and LH was monitored in normal adult intact male monkeys (n = 4). Kisspeptin-10 or normal saline (vehicle control, 1 ml) was administered intravenously (iv) immediately after taking 0 min sample.

Effect of kisspeptin-10 on plasma testosterone in acyline pretreated adult intact male monkeys

Impact of kisspeptin-10 administration (50 μ g) on plasma testosterone was monitored in pituitary clamped monkey by the help of acyline pre-treatment (12 and 24 hr prior). Kisspeptin-10 (50 μ g) was administered intravenously (iv) immediately after taking 0 min sample and hCG was administered intravenously (iv) at 240 min sample.

Effect of kisspeptin-10 on plasma testosterone and plasma LH in acyline pretreated hCG challenged adult intact male monkeys

Impact of peripheral intravenous (iv) administration of kisspeptin-10 (50 μ g) on hCG stimulated testosterone secretion was determined in acyline pretreated adult monkeys (n = 4). Acyline pretreatment was given 12 and 24 hours before the start of the experiment to ensure the complete suppression of HPG axis. hCG (50 IU) and saline were given immediately after taking 0 min sample; and in the second set of experiment hCG (50 IU) and kisspeptin-10 (50 μ g) were administered after taking 0 min sample.

Plasma LH and testosterone measurement by specific RIA

Plasma LH concentrations were determined by double antibody radioimmunoassay (RIA) with reagents supplied by NHPP (National Hormone & Peptide Program). The standard preparation used was rec-moLH-RP-1, tracer was prepared from AFP-6936A and antiserum was AFP 342994. The tubes were counted in a Gamma counter (LKB Gamma Master 1277) for 1 minute. The sensitivity of the assay was 0.2 ng/ml and the intra- and inter assay coefficients of variation were 8.8 % and 5.8 %, respectively.

Plasma testosterone concentrations were determined by using specific solid phase competitive radioimmunoassay. The testosterone kits were purchased from Immunotech (Prague, Czech Republic). The assays were done according to the instructions given by the manufacturer. Tubes for testosterone were incubated at 37°C. Tubes were then carefully decanted and placed in a Beckman Gamma counter (Gamma5500) for counting in-bound radioactivity. The counting time for each tube was one minute. The sensitivity of the assay was 0.025 ng/ml and intra- and inter assay coefficients of variants were 14.8% and 15%, respectively.

Statistics

Student's t-test was employed using Microsoft Excel to determine differences between means of hCG+saline and hCG+kisspeptin stimulated testosterone values. Statistical significance was set at $P < 0.05$.

Results

Impact of peripheral administration of kisspeptin-10 on plasma testosterone and LH in normal adult intact male monkeys

Peripheral administration of 50 μ g kisspeptin-10 in acyline untreated group induced a potent increase in plasma testosterone levels (Fig. 2.1). Within 30 min after peripheral administration of kisspeptin-10, a single LH pulse was observed in all the animals. Subsequently plasma testosterone level increased 2 fold and remained elevated for the next 2 to 3 hours, while vehicle administration which served as a negative control did not influence the LH or testosterone levels (Fig. 2.2). The animals were not showing any LH pulsatile activity during the sampling hours (Fig. 2.2) mainly because of the quiescent hypothalamic pituitary axis in the morning hours in late pubertal animals.

Impact of peripheral administration of kisspeptin-10 on plasma testosterone in acyline pretreated adult intact male monkeys

In acyline pretreated group the kisspeptin treatment in all the animals did not induce an increment in the plasma testosterone levels (Fig 2.3), The testicular tissue was responsive in terms of testosterone synthesis in the acyline pretreated group as hCG administration caused a sudden increase in plasma testosterone levels in all animals (Fig. 2.3).

Impact of peripheral administration of hCG and hCG+kisspeptin-10 plasma testosterone and LH in acyline pretreated monkeys

Administration of hCG (50IU)+saline in acyline pretreated monkeys caused a moderate but sustained increase in plasma testosterone levels (Fig 2.4). Administration of kisspeptin-10 (50 μ g) + hCG (50 IU) amplified plasma testosterone levels as compared to the hCG induced increase in saline+hCG treatment in all the individual animals without effecting plasma LH values (Fig 2.5). Pituitary gonadotropin clamping was successful by Acyline treatment was obvious as kisspeptin (50 μ g) treatment could not induce a LH peak in the animals (Fig 2.5).

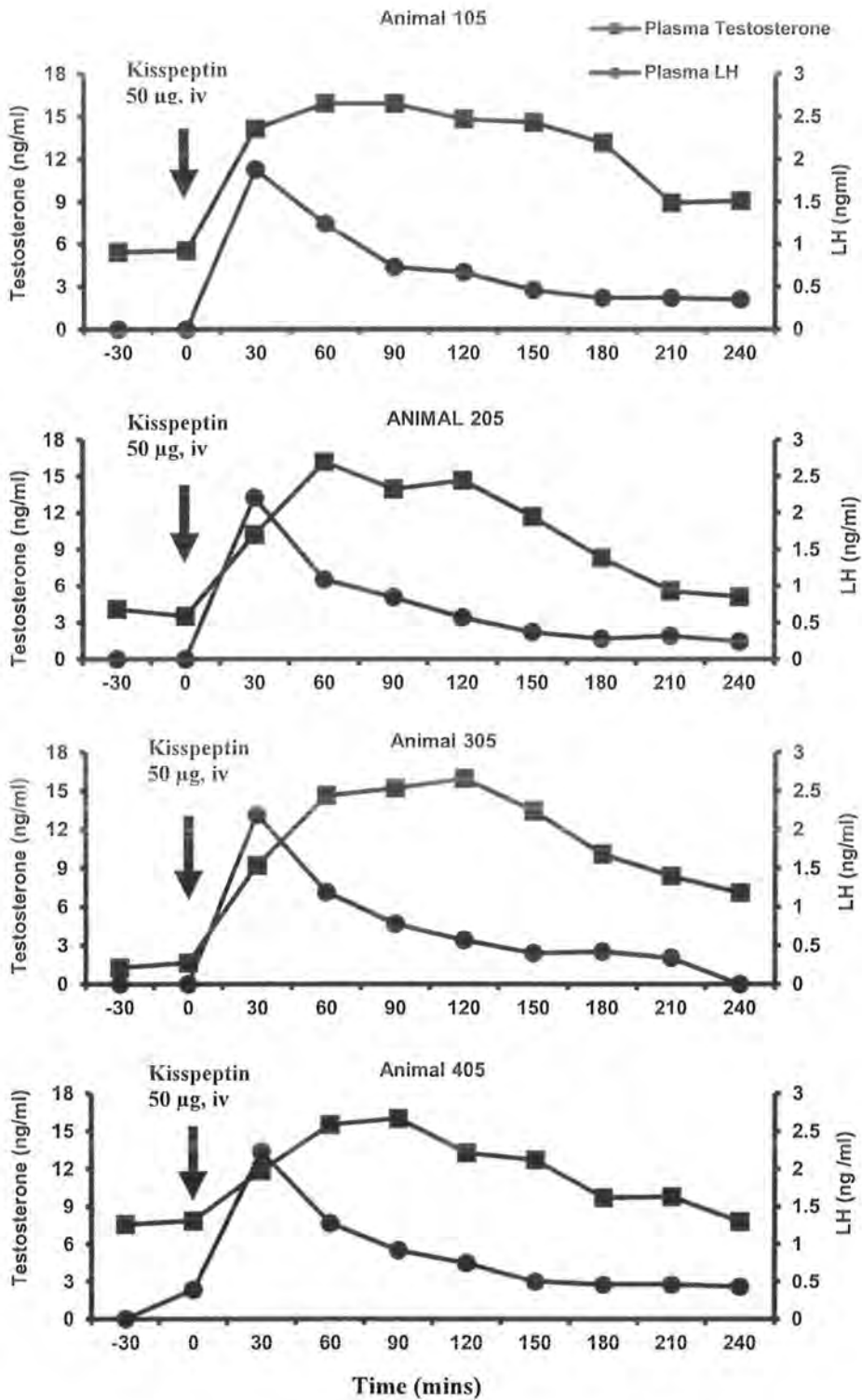


Figure 2.1 Individual plasma LH and testosterone concentration in adult intact rhesus monkeys before and after iv administration of kisspeptin (arrow).

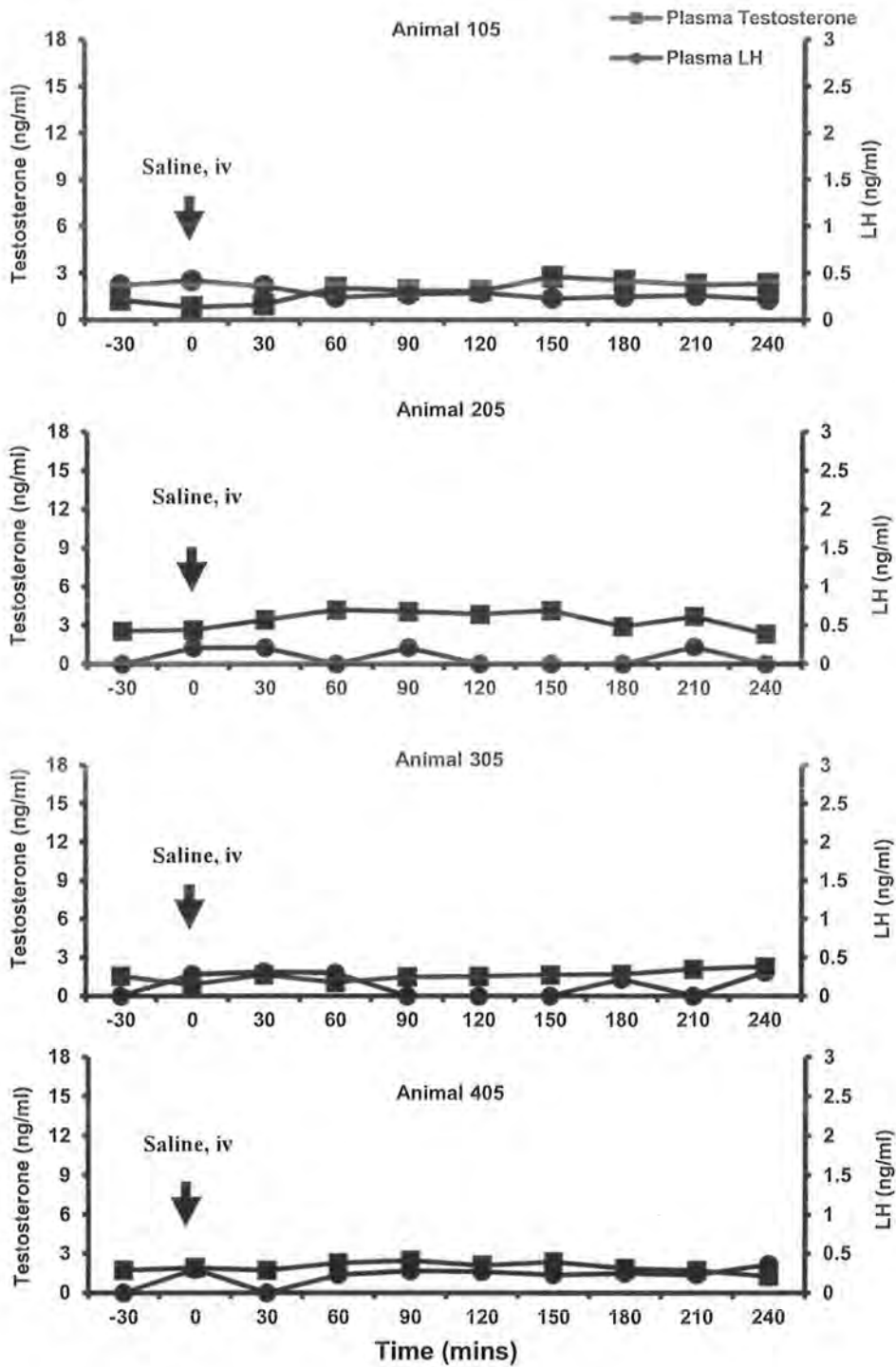


Figure 2.2 Individual plasma LH and testosterone concentration in intact rhesus monkeys before and after iv administration of saline (arrow).

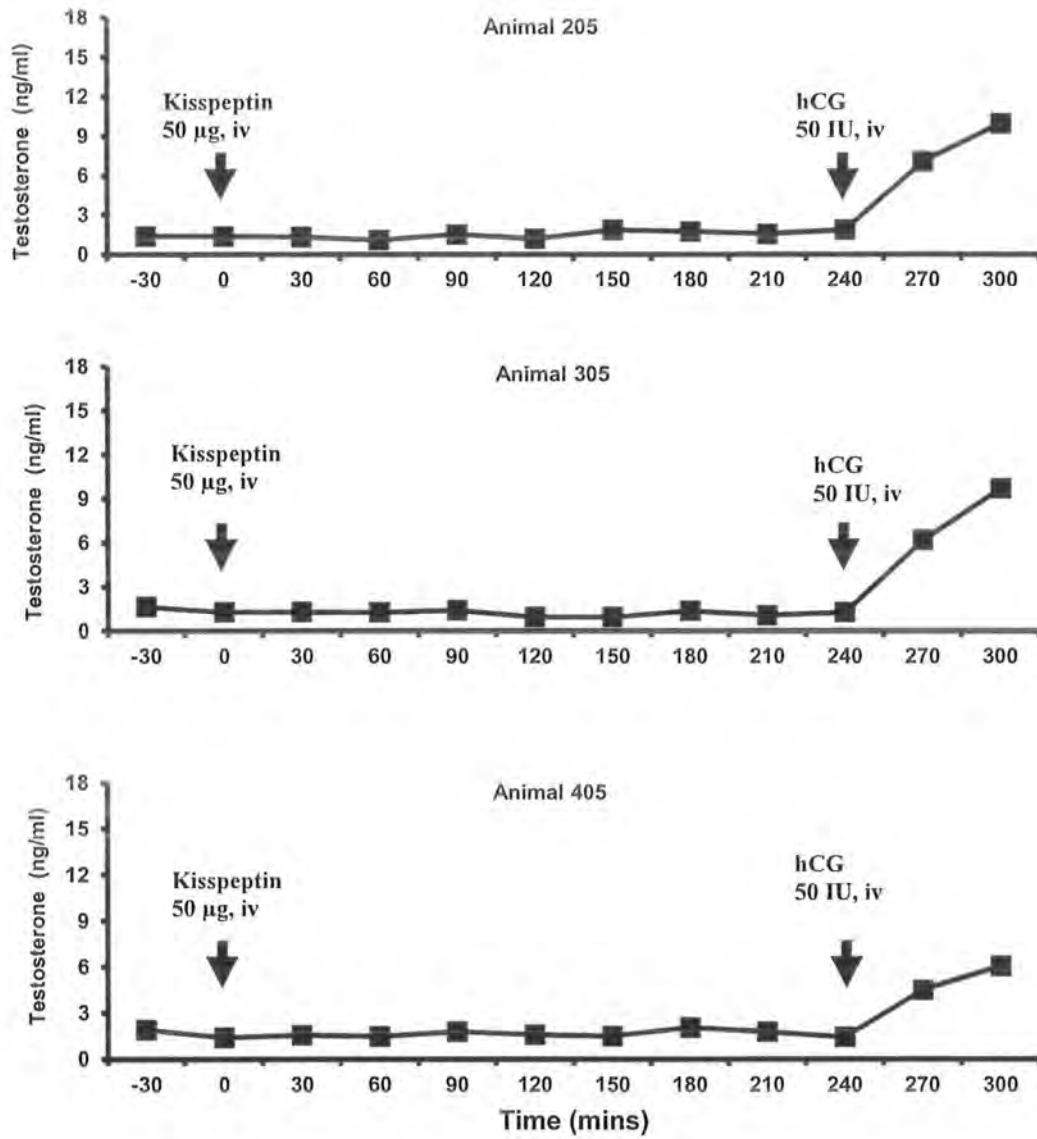


Figure 2.3 Individual plasma testosterone concentration in acyline pretreated adult intact monkeys before and after the iv administration of kisspeptin and hCG (arrows).

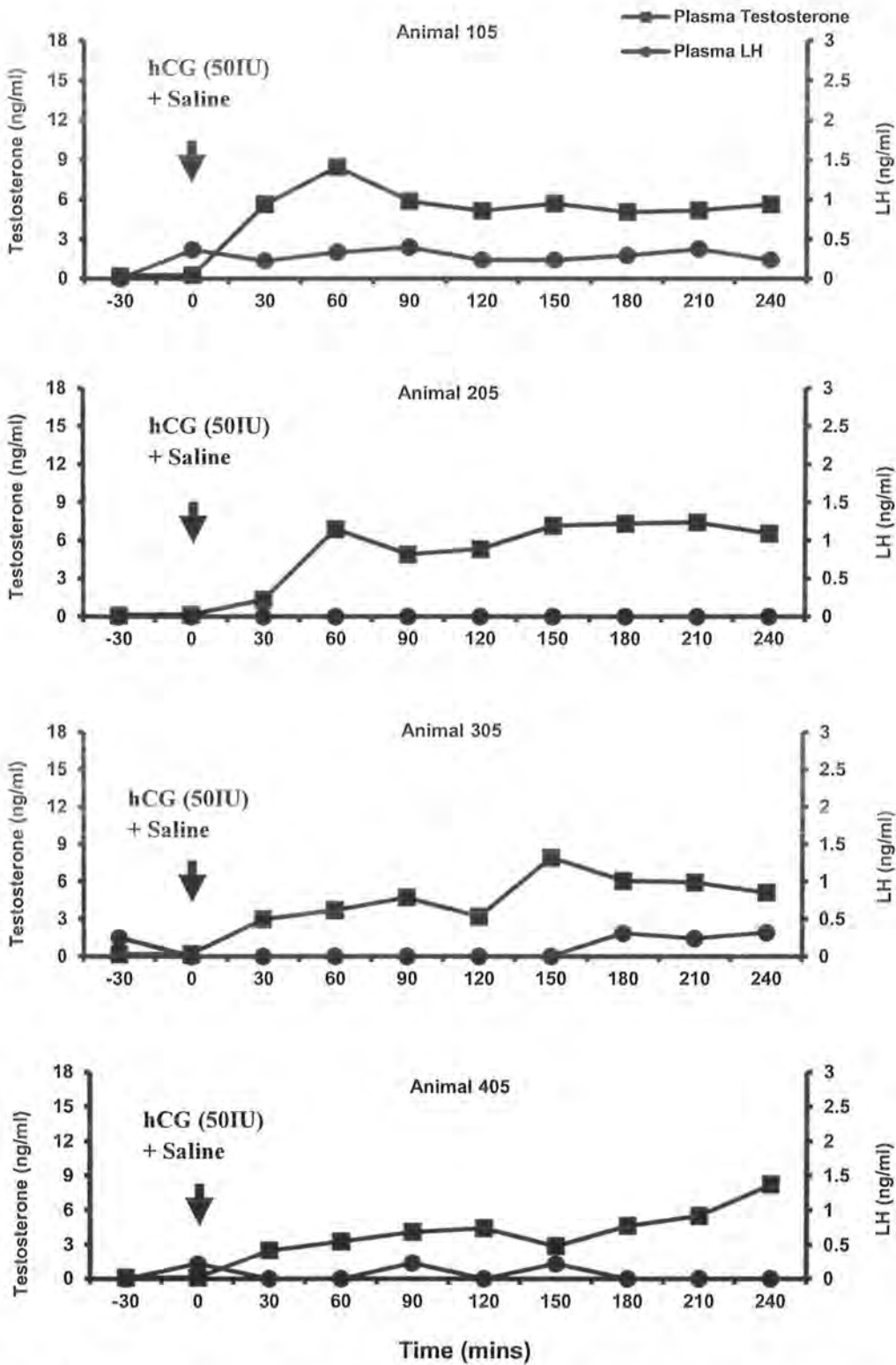


Figure 2.4 Individual plasma LH and testosterone concentration in acyline pretreated adult intact monkeys before and after iv administration of saline+hCG (arrow).

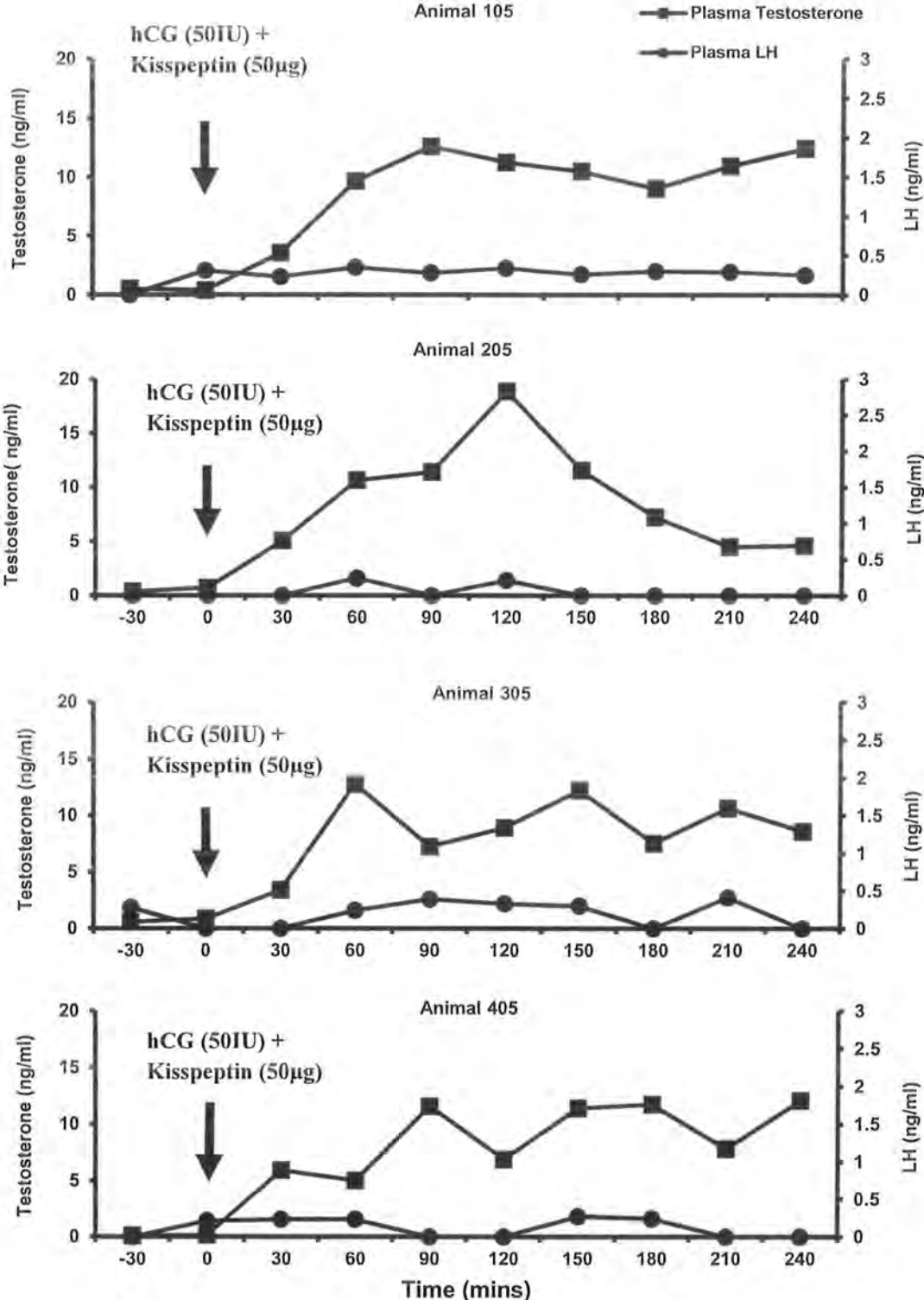


Figure 2.5. Individual plasma LH and testosterone concentration of acyline pretreated intact monkeys before and after iv administration of kisspeptin+hCG (arrow).

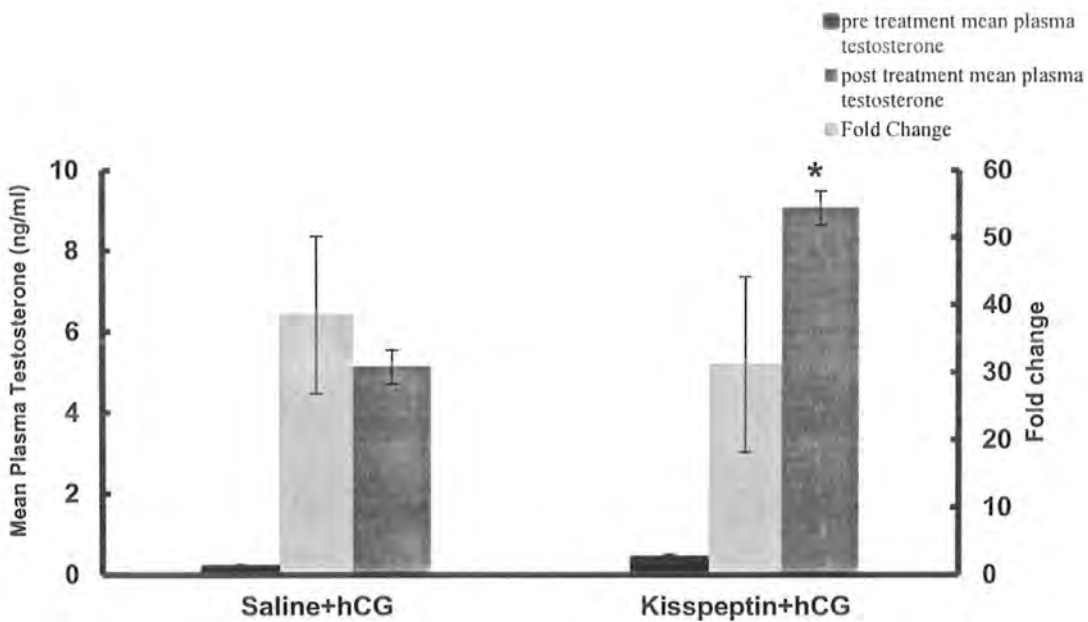


Figure 2.6 Comparison of the mean plasma testosterone values of pre and post kisspeptin+hCG and saline+hCG treatment as well as the fold change observed between pre and post treatment for kisspeptin+hCG and saline+hCG. Mean plasma testosterone concentration were significantly higher ($*P<0.005$) in post kisspeptin+hCG treatment as compared to post saline+hCG treatment. The fold change in plasma testosterone levels observed in saline+hCG and kisspeptin+hCG was not found to be significant.

Discussion

Loci of kisspeptin action on levels other than hypothalamus have not been systematically assessed in primates. Our study aimed at assessing actions of kisspeptin at the testicular level in terms of testosterone production in the adult male rhesus monkey, a representative higher primate. In order to investigate the intratesticular action of kisspeptin without the influence of pituitary gonadotropic drive, we used pituitary gonadotropic clamped monkey model with pretreatment of acyline, a GnRH receptor antagonist. As the hypothalamic/pituitary influence on the testis is blocked in the acyline pretreated animals, seasonality does not play a role in our experiments. Despite the blockade of central influences at the hypothalamic pituitary level it was previously shown that seasonality does not affect the ability of the rhesus monkey testis to respond towards LH and the capacity of Leydig cells to secrete testosterone (Higashi et al., 1984; Wickings et al., 1981).

In the Acyline untreated animals a single bolus of kisspeptin resulted in testosterone discharge which is preceded by a single LH pulse. Although baseline pretreatment levels differed the testicular testosterone response towards kisspeptin bolus was present in all four animals and showed similar amplitude (16ng/ml) with comparable kinetics with the maximum testosterone level reaching one hour after kisspeptin treatment. Regardless of basal testosterone levels our observation of an equivalent and analogous testosterone response in terms of the time and intensity towards a given dose of kisspeptin (50 μ g) is quite remarkable.

In acyline treated animals the lack of testosterone response towards kisspeptin-10 bolus affirmed the absence of pituitary responsive towards GnRH but also negated our hypothesis that kisspeptin might have a direct endocrine action on testes in terms of testosterone release. That the testicular tissue was responsive is confirmed by hCG bolus which resulted in testosterone release.

The significant amplification of hCG-induced testosterone release by co-treatment with kisspeptin in clamped monkeys indicates a novel indirect action of kisspeptin on testosterone release. The amplified testosterone secretion was evident in all the animals used in this experiment and this response sustained for almost 2 hours. This observation reveals a thus far unknown intratesticular effect of kisspeptin and indicates a functional kisspeptin receptor signalling cascade in the primate testis. The potential mechanism through which kisspeptin enhances stimulated testosterone would likely to involve a direct action on Leydig cell or an indirect action via Sertoli cells or germ cells. Such notions are supported by presence of a certain level of paracrine signaling in the primate testis (Schlatt et al., 1997). It has been shown that testosterone production can be enhanced by secretory factors from the tubular compartment (Johnson and Ewing, 1971). FSH treatment of hypophysectomized rats not only increased testicular LH receptor number but also augmented the steroidogenic response of Leydig cells to LH (Kerr and Sharpe, 1985). Sertoli cells have also been shown *in-vitro* to secrete factors which increase Leydig cell steroidogenesis and this effect is augmented in the presence of LH or hCG (Papadopoulos et al., 1987; Papadopolus, 1991; Sharpe, 1985). Studies on the testicular localization of kisspeptin receptor are needed in order to specify the site of action of kisspeptin in the primate testis. It is likely that kisspeptin through a direct or indirect action leads to enhancement of sensitivity of LH receptors towards LH/hCG on the Leydig cells.

It is important to mention that GnRH receptors are present in the testis and it has been demonstrated, though in a rodent model, that GnRH has a negative effect on *in vitro* testicular steroidogenesis (Bambino et al., 1980; Bourne et al., 1980; Petersson et al., 1989). This raises the possibility of a direct action of acyline in affecting testicular steroidogenesis in the current study and possibly confounding the testicular action of kisspeptin. However this notion appears slim because testicular action of acyline was expected to actually stimulate testosterone while in the current study no such effect of acyline was observed on basal testosterone levels in acyline pretreated monkeys. Therefore we tend to hold that there were no functional effects of antagonism of testicular GnRH receptors by acyline at least on testosterone secretion. Hence a

confounding effect of acyline on kisspeptin testicular action was less likely in the present study.

Previous kisspeptin studies on primates have either utilized castrated animals (Shahab et al., 2005; Seminara et al., 2006) or the terminal signal for the HPG axis in males i.e., testosterone has not been assessed with regards to a direct testicular action of kisspeptin iv administration in higher primates (Plant et al., 2006). The observation of disproportional relationship between circulating testosterone and LH during continuous iv infusion of kisspeptin, when testosterone levels were constantly high for a given concentration of LH by Ramaswamy et al (2007) indicated about the possible action of kisspeptin within the testis. But the animals used in that study were not chemically hypophysectomised and were given a continuous kisspeptin infusion. The intratesticular action of kisspeptin can only be assessed by using chemical hypophysectomy as continuous kisspeptin infusion down regulates kiss1r (Seminara et al., 2006). We assume that the intratesticular action of kisspeptin can only be assessed by using gonadotropic hypophysectomised model given bolus injections of kisspeptin as performed in the present study. Our finding has implications for humans as the human testes have been shown to express *KISS-1* and *KISS1R* transcripts (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Clinical studies in humans are needed to explore the intratesticular role of kisspeptin during pubertal growth and senescence.

Our results demonstrate that kisspeptin exerts an intratesticular action in primate testes. This intratesticular action is in terms of an increased steroidogenic response towards plasma LH/hCG. Our results also demonstrate that kisspeptin cannot effect the basal androgen production in male rhesus monkey. While the mechanism through which kisspeptin enhance Leydig cell response to LH/hCG needs to be investigated at the cellular level. Immunocytochemical and cell culture approaches will help to further explain the peculiar pathway through which kisspeptin modulates Leydig cell's steroidogenesis in primate testes.

Chapter 3

Immunocytochemical
localization of kisspeptin and kisspeptin
receptor in primate testis

Immunocytochemical localization of kisspeptin and kisspeptin receptor in primate testis

Abstract

Hypothalamic kisspeptin-kisspeptin receptor signaling in primates ensures the successful progression of juvenile phase into puberty during development and maintenance of reproductive activity during adulthood. Human testis has been shown to express high to moderate levels of *KISS1* and *KISS1R* expression. We have recently demonstrated a novel intratesticular role of kisspeptin in enhancing stimulated testosterone in pituitary clamped adult rhesus monkeys. In the present study we aimed to sort out the immunocytochemical localisation of kisspeptin and kisspeptin receptor in adult primate testis tissue. Immunocytochemistry was performed on paraffin embedded testis tissue from adult rhesus monkey (*Macaca mulatta*), a representative higher primate (n=2) and common marmoset (*Callithrix jacchus*) a representative new world monkey (n=2). The immunolocalisation of kisspeptin receptor was found to be present inside the seminiferous tubules and concentrated around the peripheral areas rich in A spermatogonia. The localisation pattern for kisspeptin receptor was consistent in new world and old world monkey testis. The kisspeptin immunocytochemistry revealed an interstitial pattern of localisation where the Leydig cells along with testicular macrophages and peritubular myoid cells were found to be positive for the kisspeptin. These results demonstrate that primate testis is positive for kisspeptin receptor and kisspeptin localisation and imply that kisspeptin-kisspeptin receptor signalling might have an active role in primate testis involving the interplay of tubular and interstitial cells.

Introduction

We have demonstrated a novel intratesticular role of kisspeptin in primate testis in enhancing stimulated testosterone production in pituitary gonadotropin clamped adult male monkeys (Chapter 1). Our *in vivo* results promise the presence of an active kisspeptin-kisspeptin receptor signaling at the testis. The specific localization of kisspeptin and its receptor in the primate testis is vital to understand the precise cellular type through which kisspeptin exerts its possible role in the testicular microenvironment. The testis contains different somatic cell types where each cell performs different functions. The intercellular communications between somatic cells as well as the communications between the germ cells and somatic cells are key features of the normal testicular functionality. The kisspeptin and kisspeptin receptor localisation has been recently reported for rodent and vertebrate testis (Anjum et al., 2012; Chianese et al., 2013; Hua et al., 2013). In primate testis the cellular localisation for kisspeptin and kisspeptin receptor still need to be sorted out. The precise testicular cellular localisation would help further to delineate the role of kisspeptin-kisspeptin receptor signalling in primate testis. In the present study we aimed to carry out the immunohistochemical localisation of kisspeptin and kisspeptin receptor in adult primate testis. In order to be concise we used testis tissue from Old World monkey (*Macaca mulatta*) and New World monkey (*Callithrix jacchus*) to assess the localisation pattern of kisspeptin and kisspeptin receptor across the primate testis.

Previously the presence of GnRH, a hypothalamic neuropeptide has been reported in the testis. GnRH peptide had been isolated from the interstitial fluid and the functional GnRH receptors have been found to be present on the Sertoli cells and functionally GnRH affect the testicular steroidogenesis in a negative manner (Bambino et al., 1980; Bourne et al., 1980; Petersson et al., 1989).

Materials and Methods

Animals

Two adult male rhesus monkeys (*Macaca mulatta*) were employed for testicular immunohistochemical analysis. These animals were obtained from the University of Pittsburgh Primate Facility's breeding colony and aged 6-6.5 years and weigh around 10 kg at the time of castration. Testicular tissues were obtained by castration via an inguinal incision. Surgeries were performed at the operation room of the facility. Anaesthesia was induced with Ketaject (Ketamine Hcl Inj., Usp; Phoenix Pharmaceuticals, MO, USA) via intramuscular injection and maintained by isoflurane inhalation. Animal husbandry and all experimental procedures involving the animals were performed in compliance with the University of Pittsburgh Guidelines for the Care and Use of Laboratory Animals and after IACUC approval were obtained.

Two adult male common marmoset (*Callithrix Jacchus*) were also employed for testicular immunohistochemical analysis. The animals were obtained from University Hospital Munster (UKM) animal facility and were aged 3-4 years and weigh 400-500 grams at the time of sacrifice. Animal husbandry and all experimental procedure were performed in compliance with the University of Munster and UniKlinikum Munster (UKM) guidelines.

Testicular biopsy and tissue fixation

Tissue samples from each testis were fixed overnight at room temperature in Bouin's fixative; tissues were then washed and stored in 70% ethanol. Subsequently, tissues were embedded in paraffin, and were sectioned to 3µm thick section employing a Leica SM 2000R microtome. A total of 12 sections were taken from adult rhesus and marmoset monkey testis.

Antibodies and chemicals

Primary antibody for kisspeptin (GQ2) was kindly provided by Dr. Stephan R Bloom. The primary antibody for KISS1R/GPR54 (Anti AXOR 12) was purchased from Phoenix Pharmaceuticals, Belmont, CA, USA. Chicken anti rabbit horse reddish peroxidase conjugated (Cat-Nr. sc 2955, Santa Cruz Biotechnologies, Heidelberg, Germany) was used as secondary antibody for kiss1r immunocytochemistry. Donkey anti sheep biotin was used as secondary and Streptavidin-HRP (horse reddish peroxidase) was used as tertiary antibody for kisspeptin immunocytochemistry. DAB (Diaminobenzidine, Cat.-Nr. D-4168) tablets were purchased from Sigma-Aldrich, Munich, Germany.

Dilutions and concentrations

KISS1R/GPR54 primary antibody (Anti AXOR 12) and secondary antibody (chicken-anti-rabbit-HRP-conj. Sc-2955) both were used at a dilution of 1:100. The antibodies were diluted in 25% chicken serum in 0.5% BSA in TBS. For kisspeptin the primary antibody (GQ2) was used at a dilution of 1:120,000 as previously described (Ramaswamy et al., 2008) and the secondary and tertiary antibodies were used at a dilution of 1:100 and 1:500 respectively. The primary, secondary and tertiary antibodies for kisspeptin were diluted in 25% donkey serum in 0.5% BSA in TBS.

Controls

Human placental tissue was used as a positive control and secondary antibody omission was used as negative controls for kisspeptin and kiss1r. Human placental tissue was kindly provided by the Gynaecology Clinic of the University Hospital Muenster, Germany. Placental tissues were fixed in sera fixative, embedded in paraffin and were sectioned to 3µm thick section employing a Leica SM 2000R microtome.

Immunocytochemistry for kisspeptin receptor (kiss1r)

A total of 6 sections were used from each testis from adult rhesus and marmoset monkeys were used for kisspeptin receptor immunocytochemistry. The tissue sections were deparaffinated in paraclear, rehydrated in an ethanol row, washed in running tap water and rinsed with distilled water. Antigen retrieval was performed using citrate buffer (pH 6.0) and heating in a microwave to approx. 80°C for 2 minutes. Tissue sections were washed in tris buffered saline (TBS), incubated in 3 % H₂O₂, washed in distilled water then again washed in TBS. Blocking of unspecific binding sites was performed using 25 % chicken serum and 0.5 % bovine serum albumin (BSA) in TBS for 30 mins. After blocking the sections were incubated with primary antibody Anti AXOR 12 (anti-GPR54; Cat.-Nr. 375-398, Phoenix Pharmaceuticals, Belmont, CA. USA, dilution 1:100) in humid chamber at 4°C over night. Next morning sections were washed in TBS, incubated with secondary antibody (chicken-anti-rabbit Horse raddish-peroxidase conjugated, Cat.-Nr. sc 2955, Santa Cruz Biotechnologies, Heidelberg, Germany; diluted at 1:100) for 1 hour in wet chamber. Sections were then washed with TBS and then stained with DAB was performed as specified by the manufacturer. Sections were then counterstained using Mayer's hemalaun. After counterstaining sections were dehydrated and mounted using permanent mounting medium.

Immunocytochemistry for kisspeptin

A total of 6 sections from each testis from adult rhesus and marmoset were used for kisspeptin immunocytochemistry. Tissue sections were treated the same way as mention in the previous sections. The blocking was performed using 25% donkey serum in 0.5% BSA in TBS for 30 mins. Sections were then incubated with primary antibody (GQ2, dilution 1:120,000) in humid chamber at 4°C overnight. Next morning sections were washed in TBS and then incubated with secondary antibody (Donkey- anti-sheep biotin, dilution 1:100) for 1 hour in wet chamber. Section were again washed with TBS and incubated with tertiary antibody (Streptavidin-HRP conj, dilution 1:500) for 30 mins. Section were then washed with TBS, stained with DAB as per manufacturer's

instructions, counterstained using Mayer's hemalaun, dehydrated and mounted using permanent mounting medium.

Microscopy

Slides were analyzed using an Olympus BX61 microscope (Melville, NY, USA) with an attached Retiga 4000R camera (QImaging, Surrey, BC, Canada). All images were acquired digitally using QCapture imaging software (QImaging, Surrey, BC, Canada).

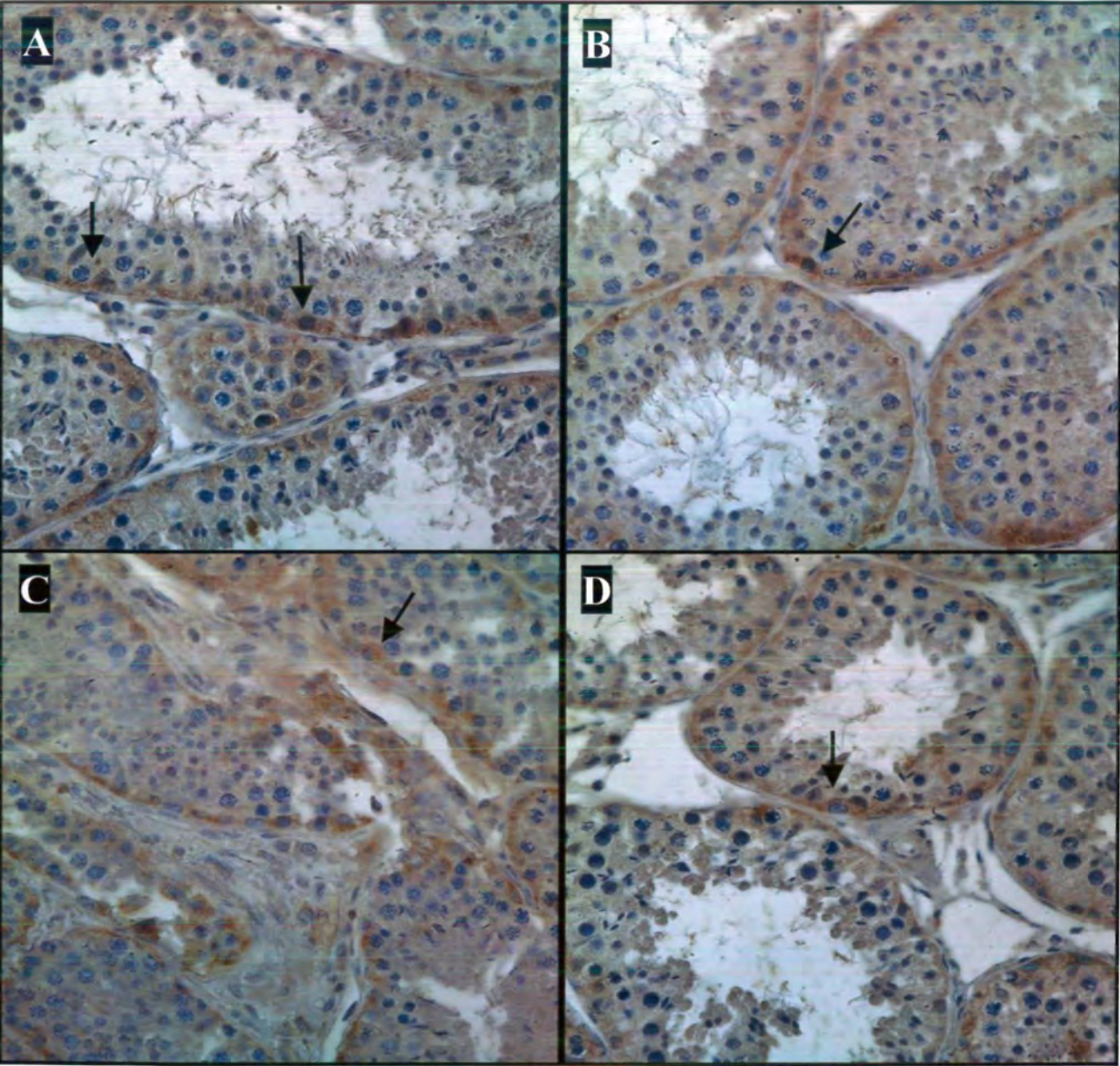


Figure 3.1 Immunocytochemistry for kisspeptin receptor (Kiss1r) in adult rhesus monkey testis sections (40x) (A,B,C,D). Positive kiss1r-like immunoreactivity at the periphery of the seminiferous tubule (arrows).

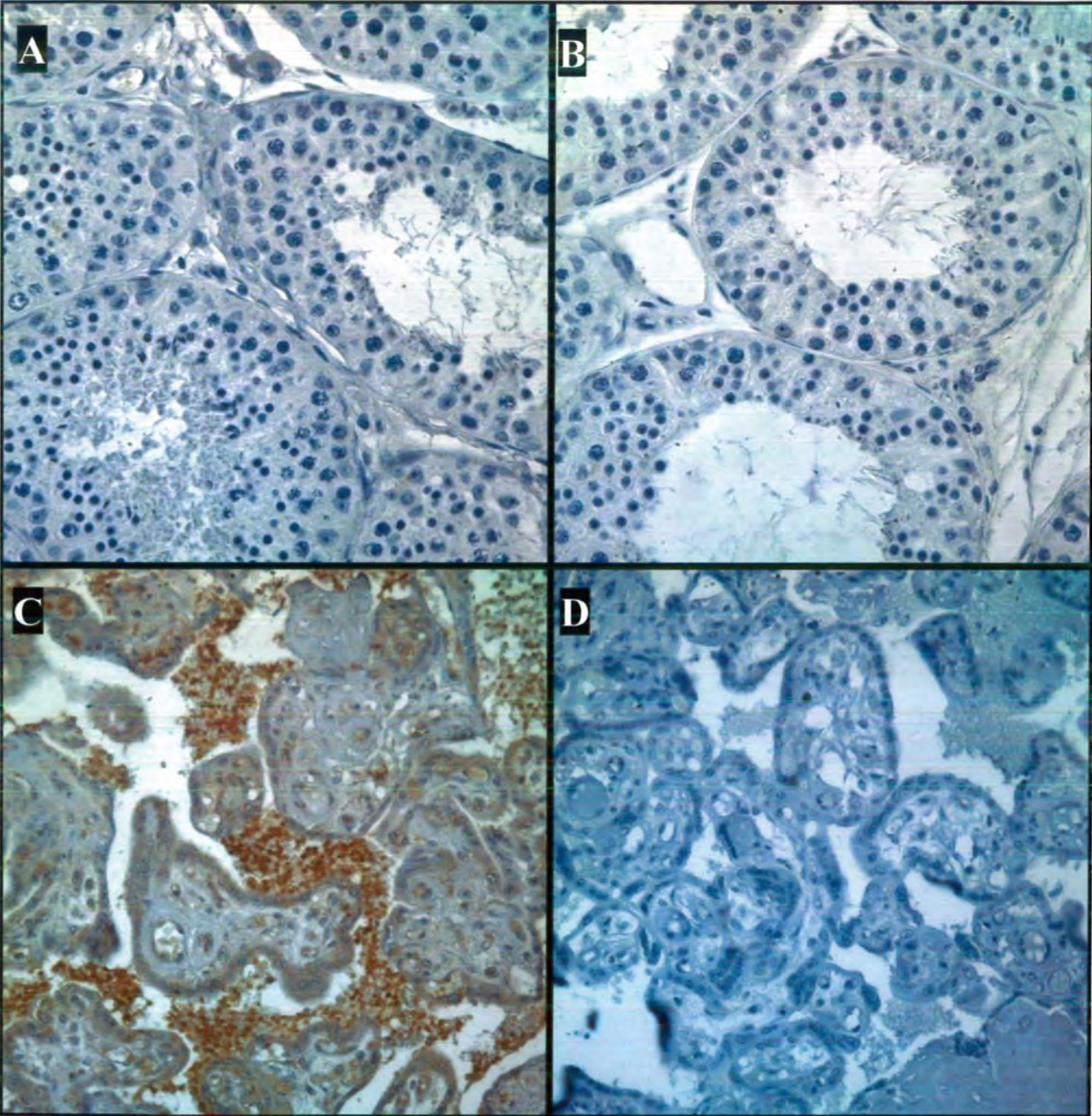


Figure 3.2 Controls for kisspeptin receptor immunocytochemistry. A) Primary antibody omitted negative control, adult rhesus testis (40x). B) Isotype control, adult rhesus testis (40x). C) Positive control, human placenta (40x). D) Primary antibody omitted control, human placenta (40x).

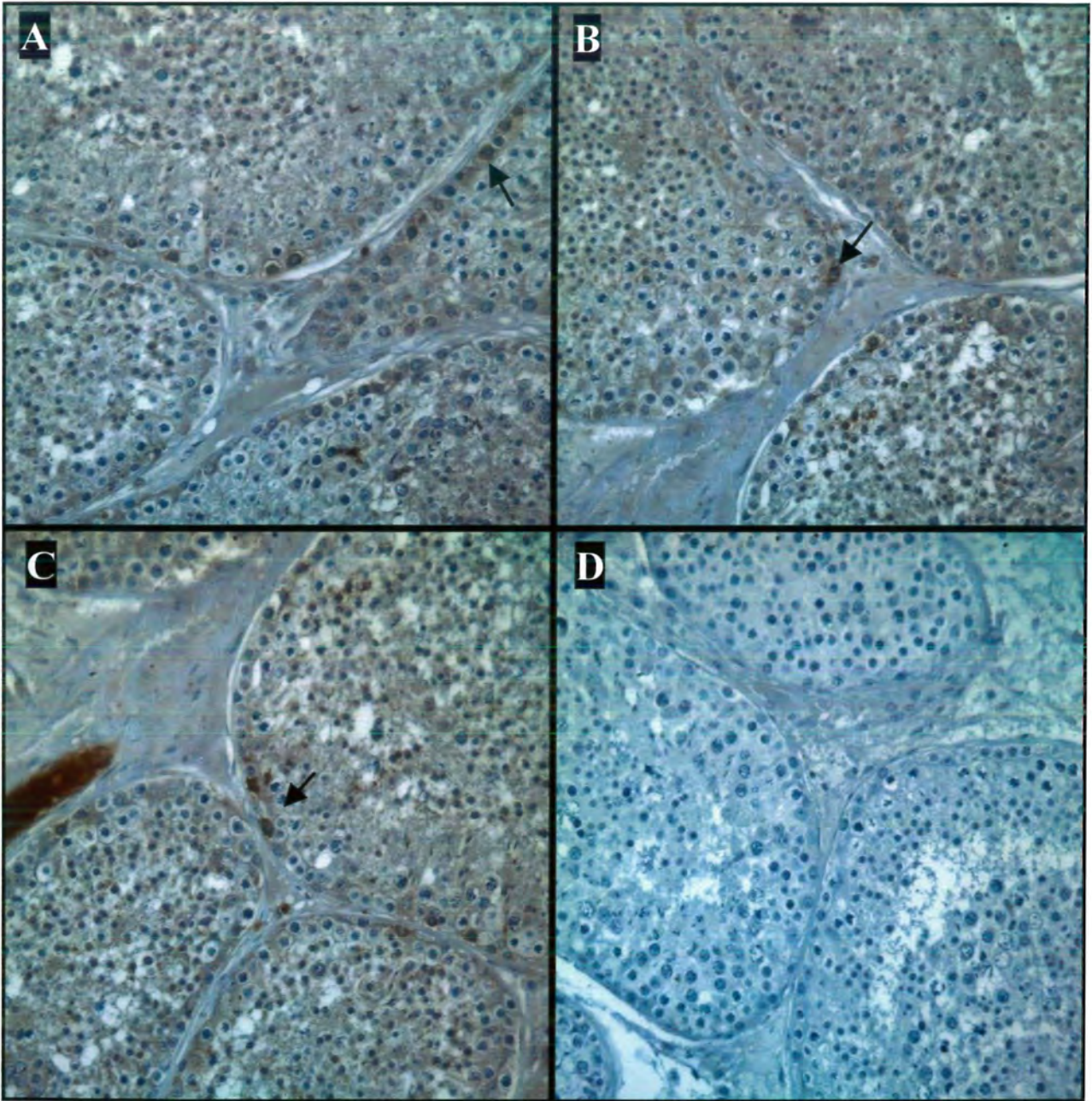


Figure 3.3 Immunocytochemistry for kisspeptin receptor (Kiss1R) in adult common marmoset testis (A, B and C) (40X). Primary antibody omitted negative control (D) (40X). Note the peripheral localisation of the kiss1r-like immunoreactivity at the periphery of the seminiferous tubules (Arrows).

Testicular immunocytochemistry for kisspeptin

The interstitial area was found positive for kisspeptin immunolocalisation in adult male rhesus monkey testis. The different cell types in interstitial area were found positive for the kisspeptin. Based on the location and the shape of these cells, we concluded that the most persistent and strong localisation was shown by the peritubular myoid cell along with the underlying layers of the basal lamina which forms the basement membrane around the seminiferous tubules (Fig. 3.4, Thin arrows). The other interstitial cells types were also seem to show positive kisspeptin-like immunoreactivity (Fig 3.4, thick arrows). However the precise identification of the cell type of these positive cells needs further labelling. Surprisingly the tubular area failed to show any immunoreactivity for kisspeptin in adult male rhesus testis.

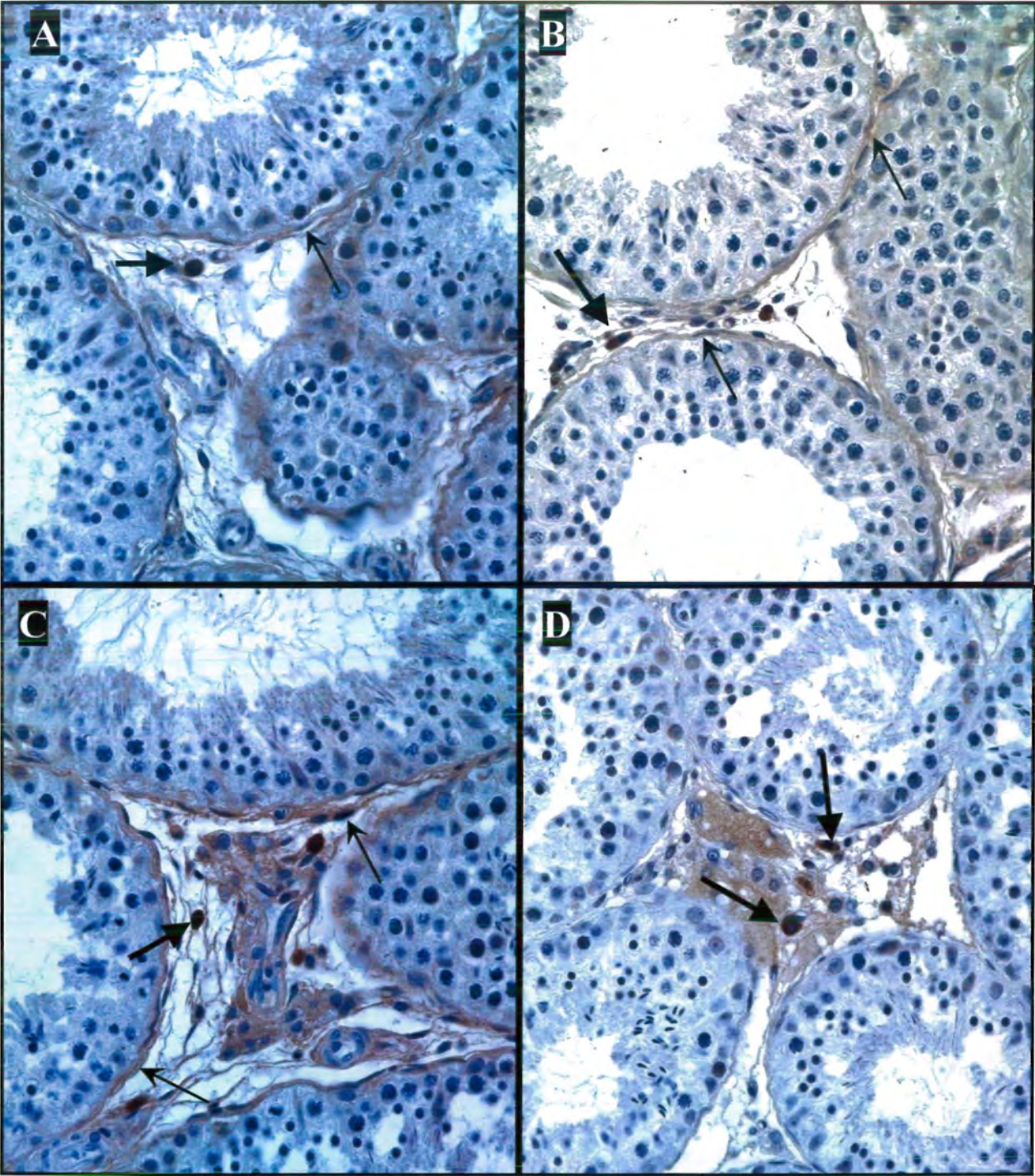


Figure 3.4 Immunocytochemistry for kisspeptin in adult rhesus testis A, B, C, D. (A, B, D 40x) (C, 60x). Peritubular myoid cells and basement membrane (Thin arrow), Interstitial cells (thick arrows).

Discussion

Previously a novel *in vivo* intratesticular action of kisspeptin in enhancing Leydig cell steroidogenesis in the presence of LH/hCG in the pituitary gonadotropin clamped adult monkey model has been demonstrated (Chapter 2). In the current study we aimed to sort out the cellular localisation of kisspeptin and kisspeptin receptor in the adult primate testis. Immunocytochemistry as a tool was employed to identify the peculiar testicular cell type positive for kisspeptin and its receptor. The Sertoli cell localisation for kisspeptin receptor (*kiss1r*) was the novel finding of this study. The same pattern of localisation for *kiss1r* was observed in adult rhesus and marmoset testis. According to our *in vivo* data (Chapter 2), the finding of Sertoli cell localisation for kisspeptin receptor makes sense at least from an androgenic perspective. The Sertoli cell is known to secrete factors which enhance Leydig cell steroidogenesis in the presence of LH (Saez et al., 1989). It seems that kisspeptin indirectly enhances Leydig steroidogenesis via Sertoli cells instead of a direct endocrine action at the Leydig cell membrane.

On the other hand the kisspeptin positive areas were found to be present in the interstitial region of the adult rhesus monkey testis. Surprisingly all the interstitial cell types were found positive. The cells which were found positive for kisspeptin like immunoreactivity included Leydig cells, testicular macrophages and peritubular myoid cells along with layers of basement membrane. The finding of non tubular localisation for kisspeptin in the current study is not surprising as it has been shown recently that kisspeptin like immunoreactivity is present in the interstitial compartment in mouse and frog testis where Leydig cells are found positive for kisspeptin like immunoreactivity (Anjum et al., 2012; Hua et al., 2013; Chianese et al., 2013). Although human sperm were shown positive for the kisspeptin like immunoreactivity (Pinto et al., 2012), but in our experiment we failed to observe any tubular areas positive for kisspeptin like immunoreactivity.

It seems that the interstitial compartment of the primate testis is responsible for the kisspeptin secretion which in turn acts on the Sertoli cells and induces the release of factors. These factors act back on the Leydig cells and enhance the responsiveness of the LH receptor present on the Leydig cell membrane towards the LH. This idea is supported by previous studies where Sertoli cell produced factors that influenced testosterone secretion from the Leydig cell or affect Leydig cell response towards gonadotropins (Lejuene et al., 1992).

Apart from the androgenic perspective, another possible functional role of the kisspeptin-kisspeptin receptor signaling in the primate testis could be its involvement in defining the spermatogonial stem cell niche environment. As the peripheral area of the seminiferous tubules which was found positive for kisspeptin receptor like immunoreactivity in adult primate testis, harbours A spermatogonia which are also known as the undifferentiated spermatogonia or spermatogonial stem cells (Ehmcke and Schlatt, 2006; Ehmcke et al., 2006). This same site is involved in aiding the mitotic multiplication of the Ap spermatogonia to become B spermatogonia as well as the self renewal activity of A spermatogonia. This anatomical area had been suggested as the spermatogonial stem cell niche region because of its fundamental involvement in the initiation of spermatogenesis and spermatogonial stem cell renewal activity (de Rooij, 2009). The niche region is defined as the area which provides all the required factors needed by the spermatogonial stem cells for their mitotic multiplication and self renewal activity. The specialised niche region in testis is anatomically composed of Sertoli cell membrane encircling the A spermatogonia. It seems that kisspeptin through its receptor might be involved in playing a role in this niche region. Parenthetically, kisspeptin was initially found to be involved in the metastatic cancer as an anti metastatic peptide (also known initially as metastatin) where the primary action of kisspeptin was suggested to either block or stop metastasis (Lee et al., 1996; Lee and Welch, 1997; Murphy, 2004). It has been shown that the antimetastatic effects are achieved by kisspeptin in metastatic cancer by inhibiting chemotaxis and cell migration (Hori et al., 2001; Yan et al., 2001; Harms et al., 2003; Navenot et al., 2009).

During the postnatal testicular development, the germ cells migrate from the centre of the seminiferous tubules to the peripheral area. During their migration these cells are called gonocytes and once they establish a physical contact with the basement membrane, these cells are known as A spermatogonia or spermatogonial stem cells (Culty, 2009). A spermatogonia do not show any migratory property in the primate testis. The peculiar localisation of kisspeptin and kisspeptin receptor in adult primate testis leads us to propose that kisspeptin via kisspeptin receptor signaling might be involved in hindering the migratory property of A spermatogonia in the adult testis. The migratory male germ cells known as gonocytes in postnatal primate testis must reach their niche regions in order to exhibit self renewal and mitotic capacity (Culty, 2009). The kisspeptin signaling by restraining the germ cells inside the niche regions might assist in establishing the self renewal ability and aid in establishing the spermatogenic capacity of the primate testis. Further studies employing *in vitro* tissue cultures on juvenile, pubertal and adult testis tissue is required to sort out the physiological significance of the presence of kisspeptin signalling in the primate spermatogenic dynamics.

Nonetheless the present study highlights a novel testicular site of localization of both kisspeptin and its receptor in higher primates. Present findings also suggest a role for kisspeptin-kisspeptin receptor signalling in the primate testis relevant to testicular endocrine action or possibly spermatogenesis, which might be supplementary to already well established central kisspeptin signalling relevant to control of the reproductive axis.

Chapter 4

Pubertal and testicular
development in common marmoset
(*Callithrix jacchus*): a New World
monkey model

Pubertal and testicular development in common marmoset (*Callithrix jacchus*): a New World monkey model

Abstract

The common marmoset (*Callithrix jacchus*) became an attractive non-human primate model due to its small size and long life span in captivity. Adult marmoset share high testicular organizational similarity with the human testis but the details of pubertal testicular development in this monkey has been poorly explored. Aims of the present study were to describe onset and kinetics of pubertal testis growth and function in common marmosets. Immature common marmosets (n=48) were observed for 13 months either starting from birth or at an age of less than 12 months. Monthly changes in body weight and testicular volume were recorded. Testosterone was assayed by radioimmunoassay in serum samples. At the end of the study the testis tissue was collected, fixed and embedded in paraffin. Histological and morphometric data were determined. In the first six months a rapid rise in body weight but not in testis volume was observed. At this point some animals entered puberty (rise in testis volume (TV), increase in testosterone) reaching adult testis size ($> 200\text{mm}^3$) already at 10 month of age. Others showed delayed entry into puberty until 12 or 13 months. While testosterone levels corresponded with early or late onset of puberty, body weight did not. At 15-18 months of age, body weights were similar in all monkeys but testis size and testosterone levels were highly variable. Histological analysis reveals that the onset of puberty is accompanied by an increase in the tubule diameter and the formation of a tubular lumen. Increase in the diameter and length of seminiferous tubules leads to a continuous increase in tubule weight. Motile sperm were first observed in the epididymis at 12 months of age. Marmoset puberty begins earliest at 6 month of age. In contrast to a uniform increase of

body weight, pubertal onset is highly variable between individual animals. Onset of testicular growth and steroidogenesis are synchronized.

Our data indicate that the hypothalamus in marmosets controls growth and gonadal function independently. Whether body weight gain is a prerequisite for pubertal activation is unclear. The high variability of pubertal development appears to depend on the timing of the GnRH-pulse generator as morphological and functional parameters develop normally once initiated. Preliminary observations are indicative for a control of the hpg-axis during puberty via social factors. This observation needs to be substantiated in future studies.

Introduction

Common marmoset (*Callithrix jacchus*) a New World primate, has become an attractive laboratory animal in biomedical research and toxicology owing to its small size, high breeding rate and long life span in captivity (Abbott et al., 2003; Mansfield, 2003; Zühlke and Weinbauer, 2003; 't Hart et al., 2012). Common marmoset has been proposed as animal model in research areas which include aging, arthritis, multiple sclerosis, neuroscience and Parkinson's disease (Eslamboli, 2005; Austad and Fischer, 2011; Petersen and Yu, 2011; Carrion and Patterson, 2012). The benefit of a non human primate model over the rodent model is very important for medical research as the basic physiology of postnatal reproductive development is different between rodents and primates (Plant, 2012).

Apart from being a model animal for medical research an important physiological aspect of adult common marmoset is the resemblance of its testicular tubular organization towards human testis. It has been shown that adult marmoset testis show highly similar seminiferous tubule organization with human (Millar et al., 2000; Wistuba et al., 2003) as well as the fetal and neonatal germ cell development in common marmoset mimic human like pattern (Mitchell et al., 2008). These findings highlight common marmoset as an attractive non human primate for the study of testicular morphogenesis and fertility research (Gassei and Schlatt, 2007).

The process of primate puberty and testicular development on the other hand has been characterized on the basis of data generated in higher primates (Plant et al., 2005; Plant, 2006, 2008; Plant and Witchel, 2006). In comparison with Old World monkeys (2.5-3 years), the New World monkeys need a lot less post natal time to reach sexual maturation (12 months) (Plant and Witchel, 2006). Apart from a significant difference in the time period required for the sexual maturation the question that how similar is the process of puberty in terms of hormonal and gonadal parameters between Old World monkeys and New World monkeys has never been addressed.

On the contrary, the limited data available on the pubertal and testicular development in common marmoset have been controversially argued thus fabricating a perplexed picture in terms of the hallmarks of the pubertal testis development (Li et al., 2005). However recent findings in common marmoset points towards a unique reproductive endocrinology which involve the presence of a chorionic gonadotropin (CG) like molecule instead of LH in HPG axis interplay. This CG like molecule has a much shorter serum half life as compared with LH (Muller et al., 2004). The reason for the dissension regarding pubertal testicular ontogeny mainly arise due to the fact that nearly all of the reports regarding pubertal and testicular development in common marmoset were conducted well before the findings of the presence of CG like molecule instead of LH, except for Chandolia (2006). The unique reproductive endocrinology in the common marmoset is also present in terms of steroid hormone resistance where the unbound plasma testosterone in new world primates were found to be around 20-40 ng/dl as compared to 3-9 ng/dl in old world monkey and 10 ng/dl in humans (Puggeat et al., 1984). Adult common marmosets have also been shown to exhibit a strong social regulation of fertility both in male and females and to date it is unclear whether this phenomenon also impacts the timing of pubertal onset in common marmoset (Abbot et al., 1999; Baker et al., 1999).

The general notion to date is that in common marmoset the pubertal activation of HPG axis occurs between 6-12 months of age (Li et al., 2005). This high variation of time is critical for selecting animals for developmental and testicular research and might affect the outcome of these studies. While keeping the above mentioned background, we aimed to investigate the pubertal and testicular development in common marmoset by using a simplistic approach of observing immature common marmoset monthly for body weight, testis volume and blood samples for a period of 13 months. At the end of the study animals were sacrificed and testis tissue was collected, fixed and embedded in paraffin for histological and morphometric analysis.

Materials and Methods

Animals and sampling

Immature common marmosets (n=48) were observed for 13 months either starting from birth or at an age of less than 12 months. Animals were housed in the central animal facility of the University Hospital Muenster (UKM). Animals were either housed in their respective family cages or were housed in the cages where all animals were males and were of the same age approximately (± 1 month). Animals were fed fresh fruits in the morning and feed ration in the evening. Water was *ad libitum*.

Body weight and testicular volume samples were recorded monthly. Also the blood samples were collected from the femoral vein of each animal during the collection of body weight and testicular volume. All the samples were collected between 0800 and 1030. The serum was separated from the collected blood samples and stored at -20 C till assayed. For the testis volume the testis length and width was measured using vernier caliper and the testicular volume was calculated using an established ellipsoid formula.

Hormone assay

Testosterone (T) was assayed in serum samples using an in-house (CeRA, Muenster, Germany) developed RIA method. Serum testosterone was measured by a solid-phase, double-antibody RIA technique, using a commercially available iodinated tracer (testosterone-3-(0-carboxymethyl) oximino-2-[12T] iodohistamine, (Amersham International, Braunschweig, Germany) and an antiserum raised in rabbit against testosterone-3(carboxymethyloxime)-BSA. The bound/free separation was performed by addition of a solution of solid-phase antirabbit immunoglobulins (Immunobead Second Antibody, Biorad, Munich, Germany). The recovery after ether extraction was monitored by addition of trace amounts of [$1\beta,2\beta$ - 3H]testosterone (NET-187, NEN, Boston, MA) and the final results were corrected accordingly. The sensitivity was 2 pg/tube (0.07

nmol/l). In 10 consecutive assays the intra-assay coefficients of variation (mean \pm SEM) were 8.43 \pm 1.42, 4.2 \pm 0.59 and 4.37 \pm 0.63% for control sera with low, middle and high testosterone concentrations, respectively. The corresponding inter-assay coefficients of variations were 16.62, 6.26 and 3.85%, respectively.

Tissue collection, fixation and histology

At the end of the study each animal was anesthetized with ketamine and body weight and testicular volume are measured, subsequently the animals were sacrificed by exsanguinations. The testis tissues were removed and weights of testes were recorded immediately. Testis tissues were then placed in Bouin's solution overnight. The next day the testis tissues were dehydrated in ascending grades of alcohol (70%, 80%, 90%, 100% for 1 hour each) and then two xylol washes each for one hour in histomaster. The tissues were then embedded in liquid paraffin and allowed to cool. 2.5-3 μ m thick sections were cut. Total of six sections were used from the left testis of each animal. Periodic Acid Schiff staining was conducted.

Microscopy and histo-morphometry

Slides were analyzed using an Olympus BX61 microscope (Melville, NY, USA) with an attached Retiga 4000R camera (QImaging, Surrey, BC, Canada). All images were acquired digitally using QCapture imaging software (QImaging, Surrey, BC, Canada). Pictures were taken from five independent positions from each section at 20X (5 pictures/section) using the camera fitted on the microscope. A total of 30 pictures from each testis were used for point counting and morphometric measurements.

Volume densities, expressed as the percent of testicular parenchyma were determined by point counting. Randomness and sufficient sampling were ensured by the use of 6 sections from each testis. A total of 120 points were scored for each testis. Volume density of various components multiplied by testis weight yielded the total weight of that component per testis. For tubule diameter 60 measurements were taken from each testis (10 measurements from each section). Round tubules were selected for

measurements. For point counting square shaped grid was used and four corners of grid were observed in terms of their location on interstitium, tubule epithelium and tubule lumen.

Statistics

All data were expressed as mean \pm SEM unless stated otherwise.

Results

Mean body weight, mean bi-testis volume and mean plasma testosterone

The mean body weight in immature common marmoset showed accelerated growth rise during the first seven months of age which was followed by a passive rise (Fig. 4.1). The mean testis volume shows an abated increase till the first six months of age, which is then followed by relatively sharp increase up till 16 months of age (Fig 4.2). The testis volume of the individual animal shows a period of quiescent developmental up till 24 weeks of age (Fig 4.3). The increase in testis volume was first observed around 28 weeks (7 months) which then followed a highly diverse individual pattern where some animals reach adult size around 44 weeks (9-10 months) while others show a slow rise in the testis volume. Mean serum testosterone levels of male common marmoset from birth till 18 months showed a sudden rise in serum testosterone at 7 month of age (Fig 4.4). The sudden rise in serum testosterone at 7 month of age is the first sign of the pubertal activation of hypothalamic pituitary gonadal axis and it also correlates with the rise in the testicular volume.

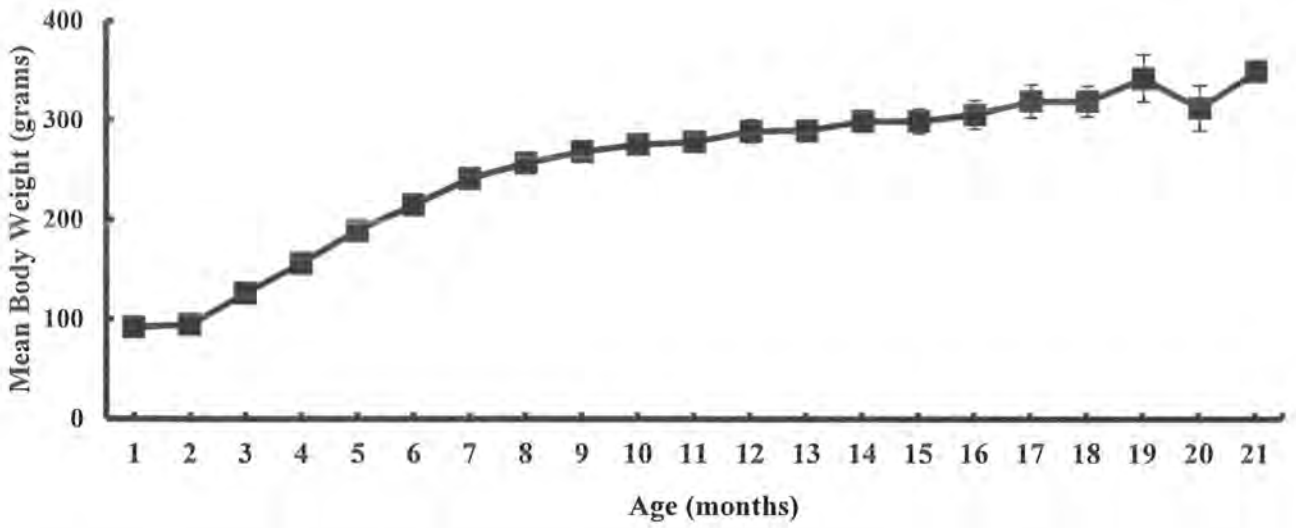


Figure 4.1 Mean body weight in common marmoset from birth till 21 months of age (n=48).

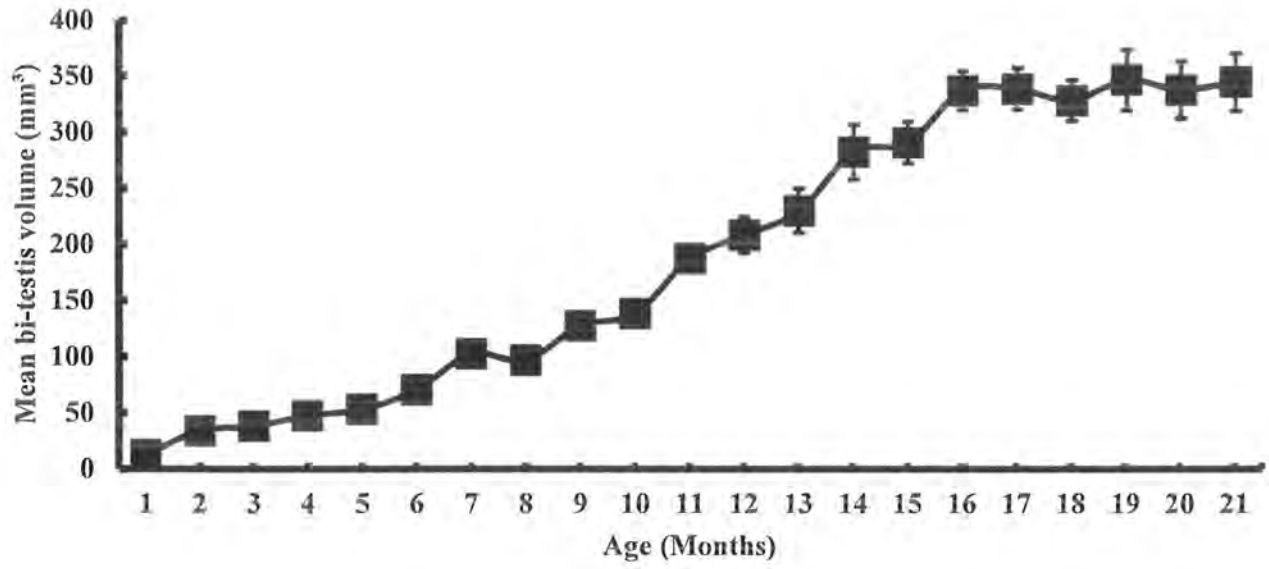


Figure 4.2 Mean bi-testis volume in common marmoset from birth till 21 months of age (n=48).

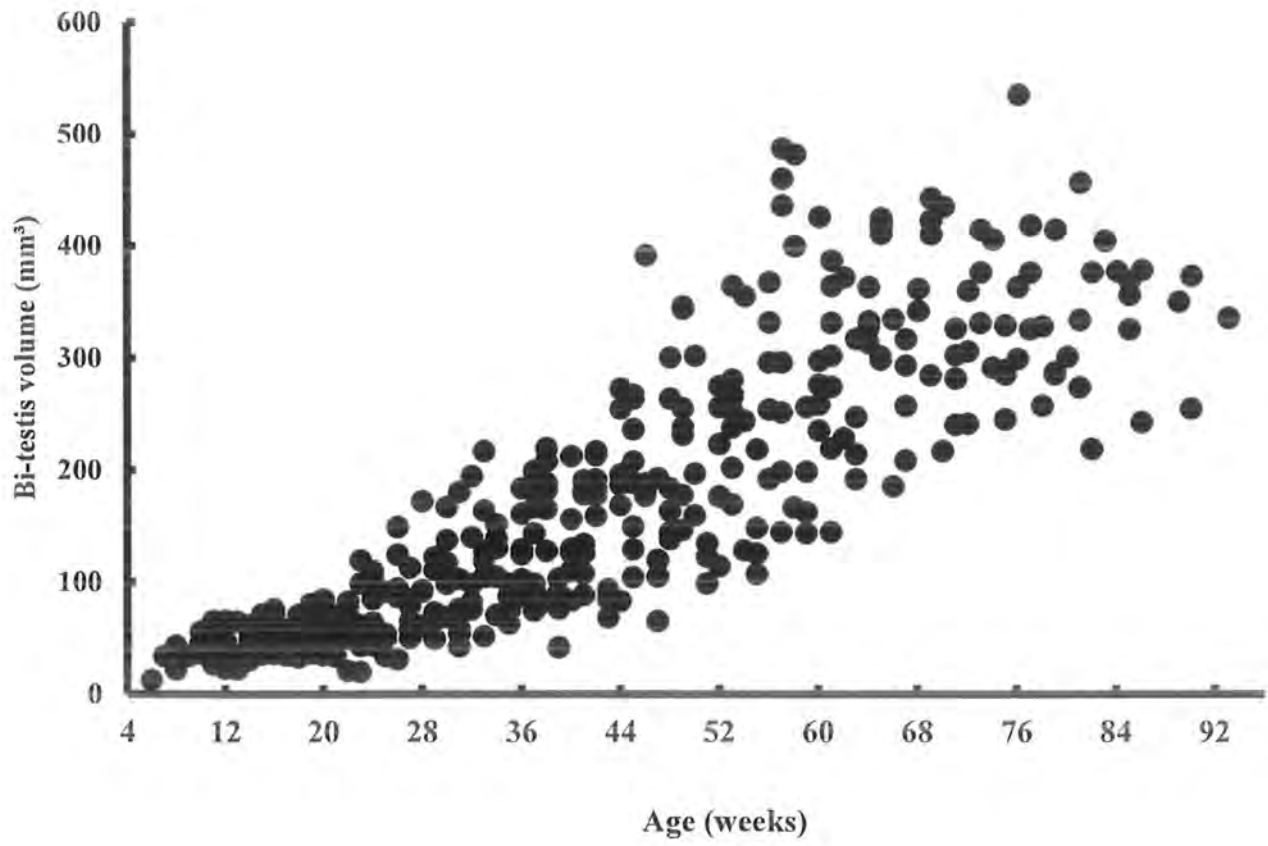


Figure 4.3 Bi-testis volume of the individual common marmoset (n=48).

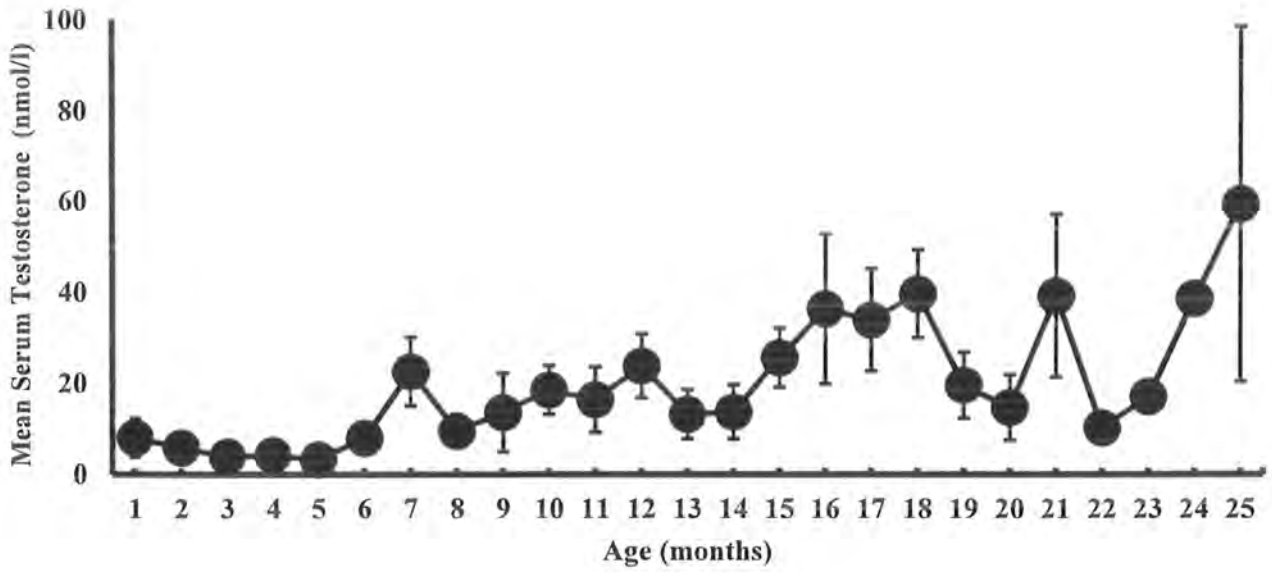


Figure 4.4 Mean serum testosterone levels from birth till 25 months of age in common marmoset (n=48).

Testicular histo-morphometric analysis

The seminiferous tubule diameter showed an increasing trend after 24 weeks (6 months) onwards up till 68 weeks of age (16 months) (Fig 4.5). The timing of the initial increase in the seminiferous tubule diameter correlates with serum levels of testosterone (Fig 4.4). The presence of a tubule lumen was also observed from 24 weeks (6 months) onwards (Fig 4.6 and 4.7) which in turn is the testicular tubular response towards the higher levels of serum testosterone. With advancing age the percentage of the seminiferous tubules exhibit a decreasing trend and the percentage of the lumen demonstrated an increasing trend (Fig 4.6), While the weight of the seminiferous tubules increased from 28 weeks onwards (Fig 4.7). Our testicular morphometric analysis completely correlates with increasing levels of serum testosterone observed from 28 weeks onwards in immature male common marmoset. Histological analysis for the Sertoli cell arrangement demonstrated that randomly placed Sertoli cell are observed up till 5 months of age, at 6 month Sertoli cells arranged themselves towards the periphery in epithelial like fashion around (Table. 4.1). Gonocytes were observed around 5 months of age while the A spermatogonia were first observed around 6 month of age and the occasional B spermatogonia were first observed around 7 month of age, whereas sperm were found in the epididymis at 12 months of age (Table. 4.1).

Our manual measurements of the testis volume in common marmoset were quite accurate as the testis weight and the volume were highly comparable as seen in the (Fig. 4.8). The relative testis weight also show an increasing trend after 28 weeks (Fig. 4.9). Note the sudden increase in the bi-testis weight and bi-testis volume in the age groups of 44 weeks and 52 weeks (Fig 4.9). This sudden increase in testis weight and testis volume is also observed in the seminiferous tubule diameter of the same age group (Fig.4.5). The reason for this two fold increase in the testis weight in these two groups might be due the spermatogonial expansion which was observed at 52 weeks (12 months) of age in terms of the presence of elongated spermatids as well as the epididymal sperm (Table 1).

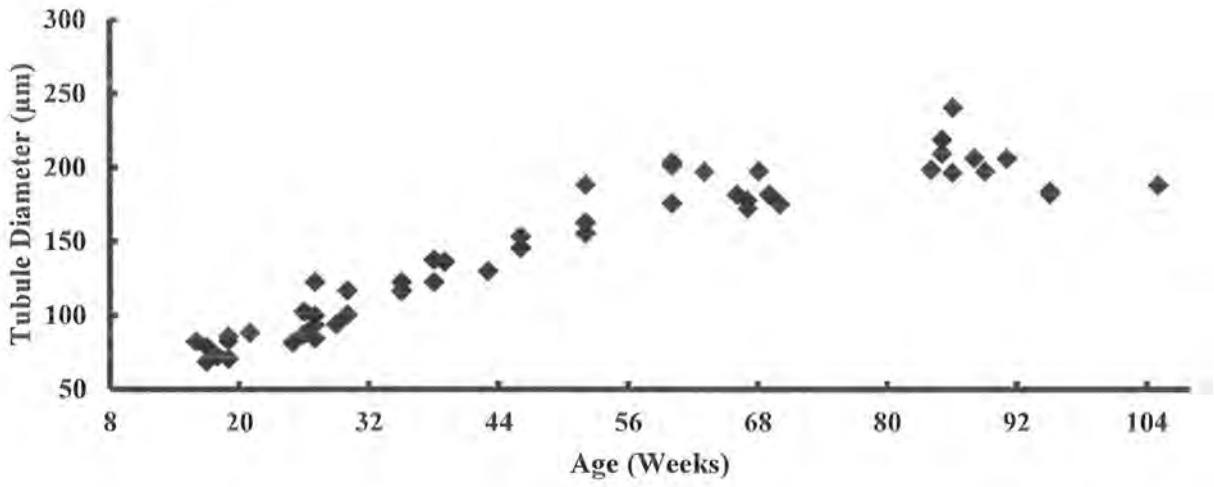


Figure 4.5 Seminiferous tubule diameter from 16 weeks onwards till 96 weeks in common marmoset (n=48).

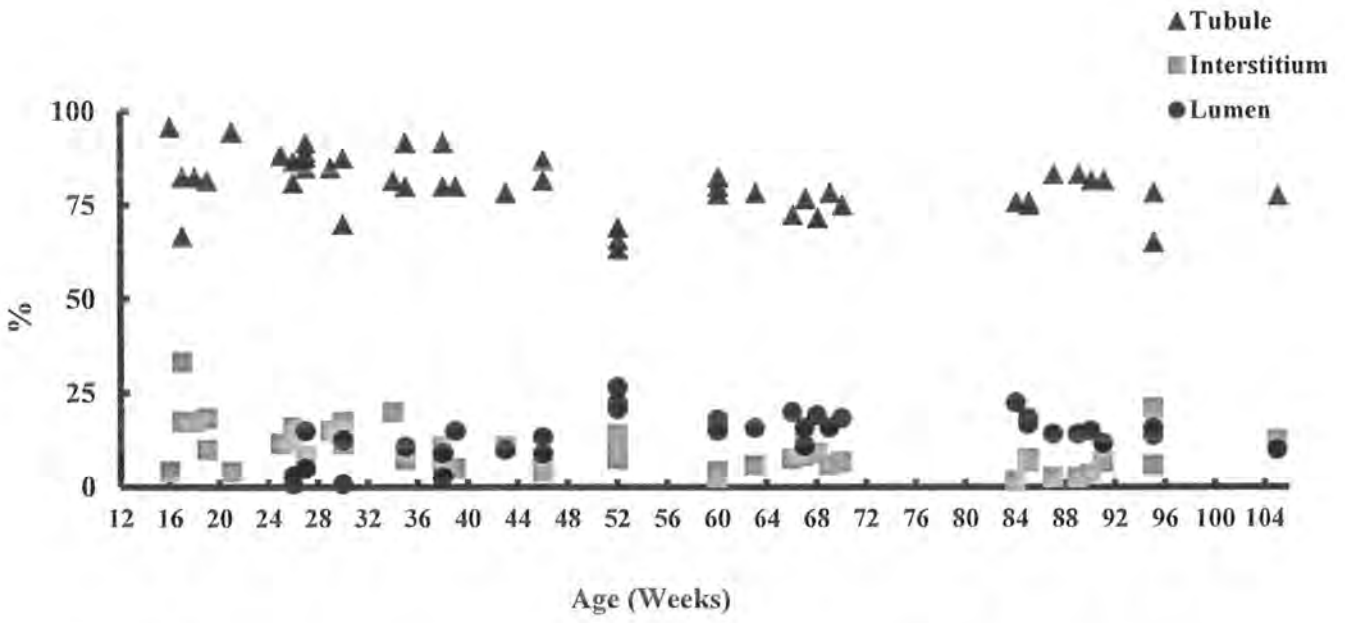


Figure 4.6 Percentage of seminiferous tubules, interstitial area and tubule lumen during development in common marmoset (n=48).

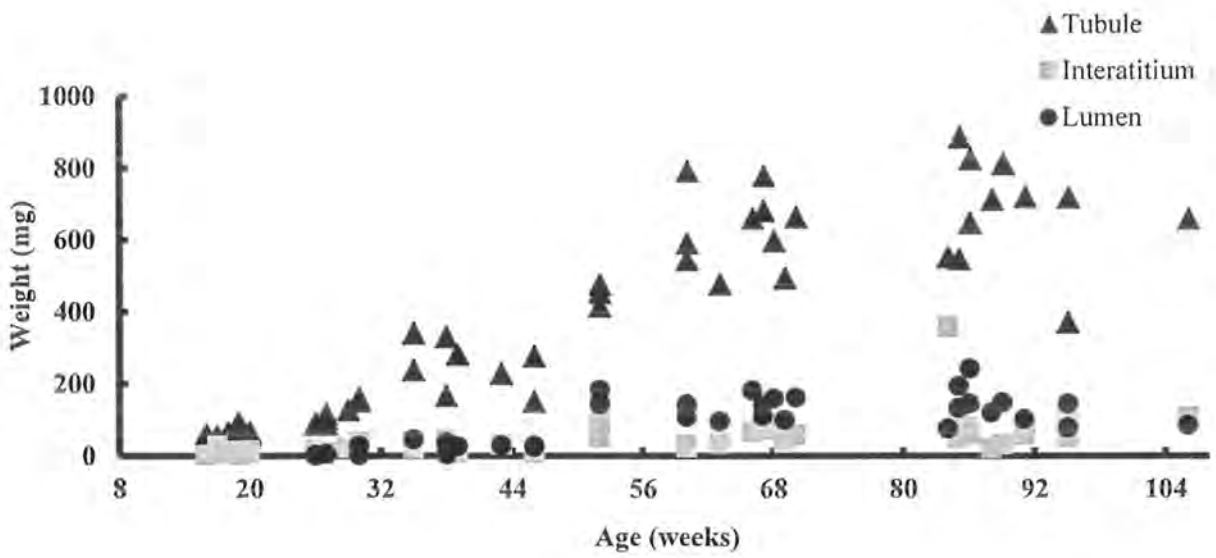


Figure 4.7 Calculated weight of seminiferous epithelium, interstitial area and the tubule lumen during development in common marmoset (n=48).

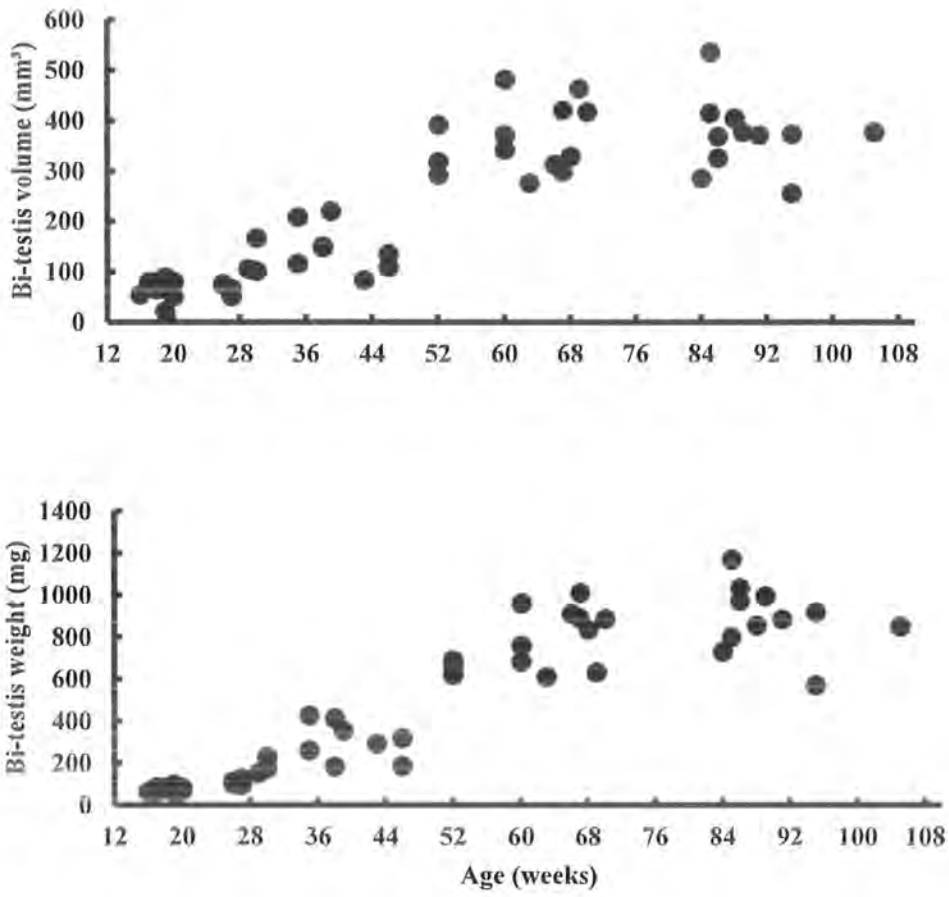


Figure 4.8 Bi-testis weight and bi-testis volume of the sacrificed common marmoset (n=48).

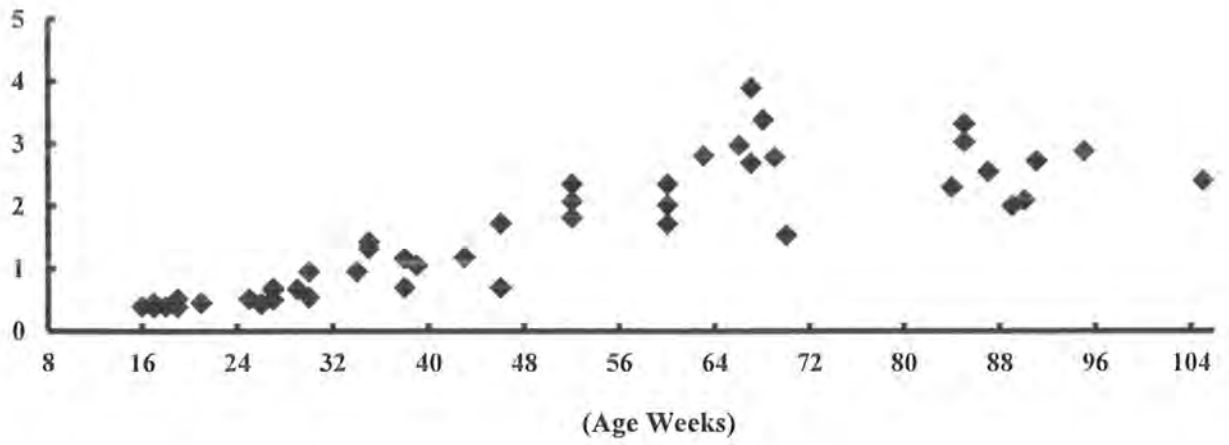


Figure 4.9 Relative testis weight of the sacrificed common marmoset (n=48).

Table 4.1 Description of advanced germ cell type, Sertoli cell arrangement and the presence or absence of sperm in the epididymis of common marmoset over the course of development (n=48).

Age	Most advanced Germ cell type	Sertoli cell nuclei arrangement	Epididymal sperm
4 Months <i>n= 6</i>	Gonocytes	Centrally placed Randomly arranged	No
5 Months <i>n= 3</i>	A spermatogonia	Centrally placed Randomly arranged	No
6 Months <i>n= 6</i>	A spermatogonia	Peripherally placed epithelial arrangement	No
7 Months <i>n= 3</i>	B spermatogonia	Epithelial-like arrangement	No
8 Months <i>n= 5</i>	Secondary spermatocytes	Epithelial-like arrangement	No
10 Months <i>n= 3</i>	Round spermatids	Epithelial-like arrangement	No
12 Months till 20 months <i>n= 22</i>	Elongating spermatids	Epithelial-like arrangement	Yes

Body weight, bi-testis volume, serum testosterone in individual animals from birth till the sacrifice and the bi-testis weight, seminiferous tubule diameter and most advanced germ cell type on the day of sacrifice

The individual animal's body weight, bi-testis volume and serum testosterone concentration are presented from birth till the day of sacrifice. The bi-testis weight which was measured after the removal of the testis, the serum testosterone concentration on the day of sacrifice along with the histomorphometric data including the seminiferous tubules diameter and the most advanced germ cell type present are also shown. Note the sudden rise in serum testosterone at 7 month of age (Fig 4.10, 4.11, 4.13, 4.14, 4.15, 4.16, 4.18, 4.18, 4.26, 4.28, 4.36, 4.38) while some animals fail to show a substantial rise in serum testosterone at 7 months of age. In these animals the substantial rise in serum testosterone is seen either before 7 months of age (6 months) (Fig 4.17, 4.20, 4.25, 4.32) or after 7 months of age (Fig 4.21, 4.22, 4.23, 4.24, 4.27, 4.30, 4.31, 4.33, 4.35, 4.37, 4.39, 4.40). Interestingly the seminiferous tubule diameter and most advanced germ cell type were found to be similar in the animals from same age group but having different testosterone levels. Animal # 596 (Fig 4.12) did not show a testosterone rise at 7 month of age but the seminiferous diameter is comparable along with the most advanced germ cell type with those animals which show substantial rise at 7 month of age and were in the same age group as animals# 596 at the sacrifice day (Fig 4.10, 4.11, 4.13). Few animals did not show any testosterone rise during the complete sampling period (Fig 4.19, 4.29), but the testis weight and seminiferous tubule diameter of these animals were comparable with the other animals from the same age group. The significant rise in bi-testis weight was observed in 12 month old group (Fig 4.21, 4.22, 4.23) as the testis weight doubled as compared with the 11 month old group (Fig 4.19, 4.20). This sudden increase in testis weight was mainly due to the spermatogonial expansion as the sperms were observed in the epididymis in the 12 month old group (Table. 1). Although the seminiferous tubules diameter did not show a remarkable difference in these two age groups.

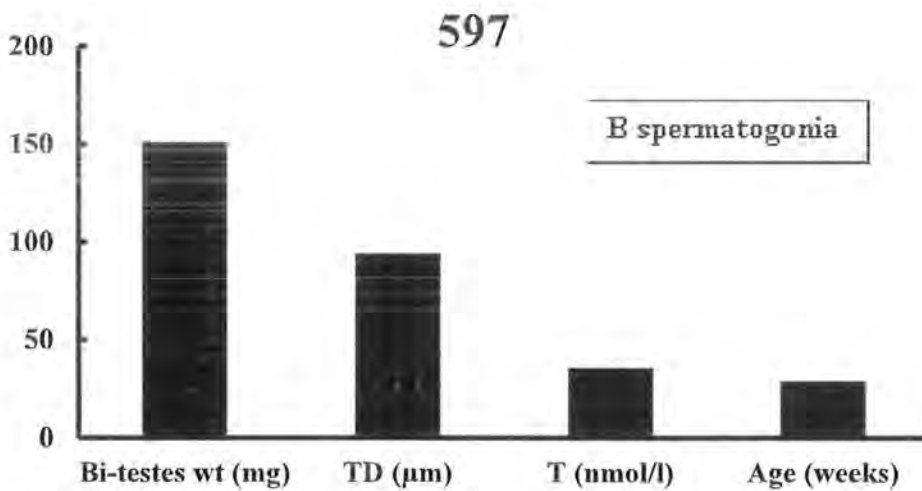
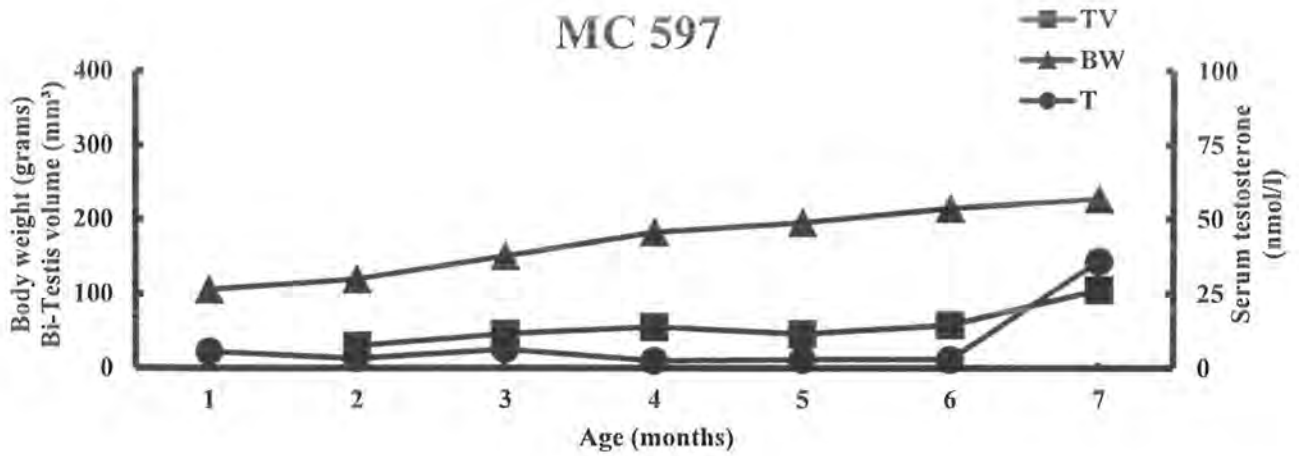


Figure 4.10 Body weight, serum testosterone and bi-testis volume from birth till 7 month of age in common marmoset (#597) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

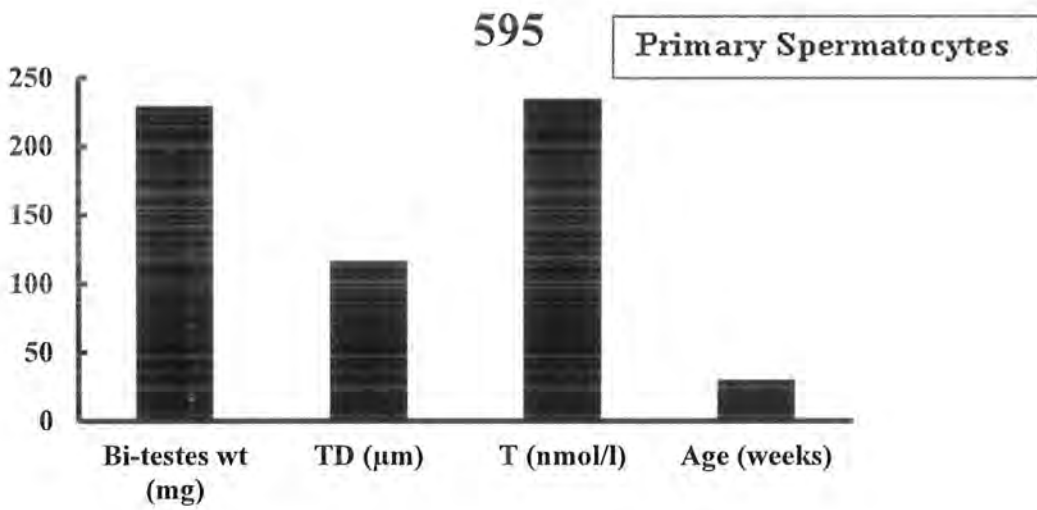
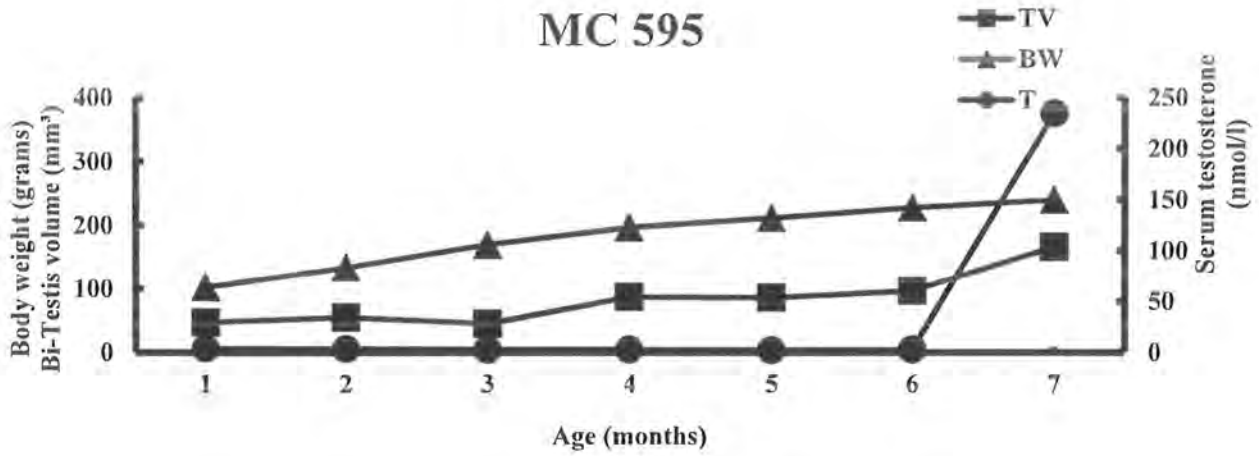


Figure 4.11 Body weight, serum testosterone and bi-testis volume from birth till 7 month of age in common marmoset (#595) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

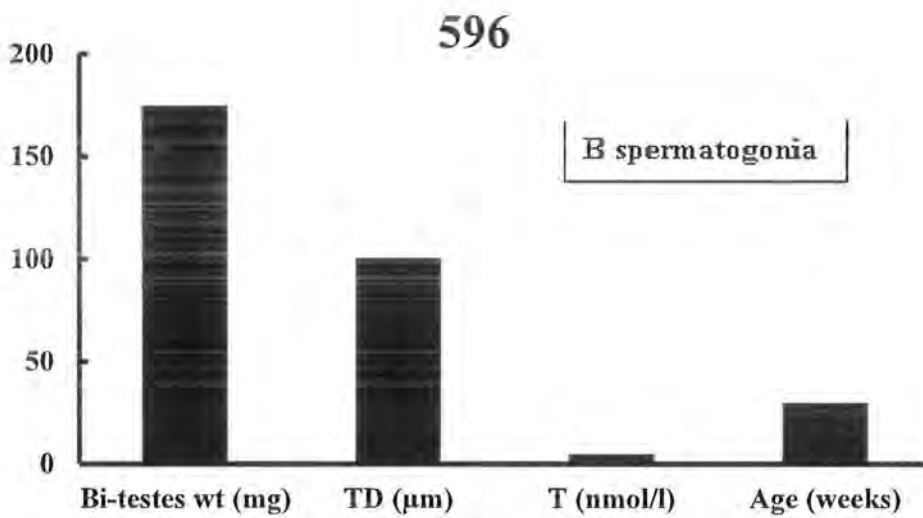
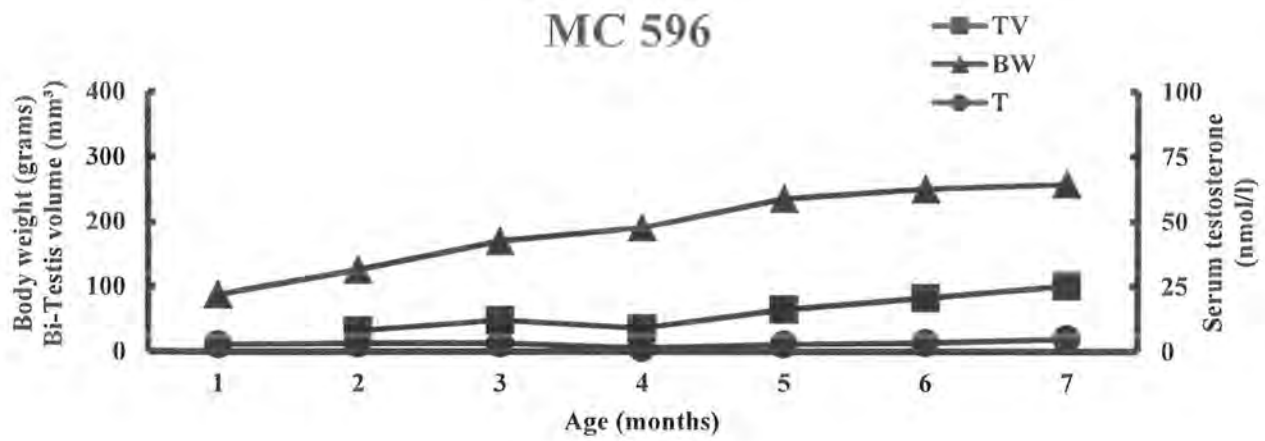


Figure 4.12 Body weight, serum testosterone and bi-testis volume from birth till 7 month of age in common marmoset (#596) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

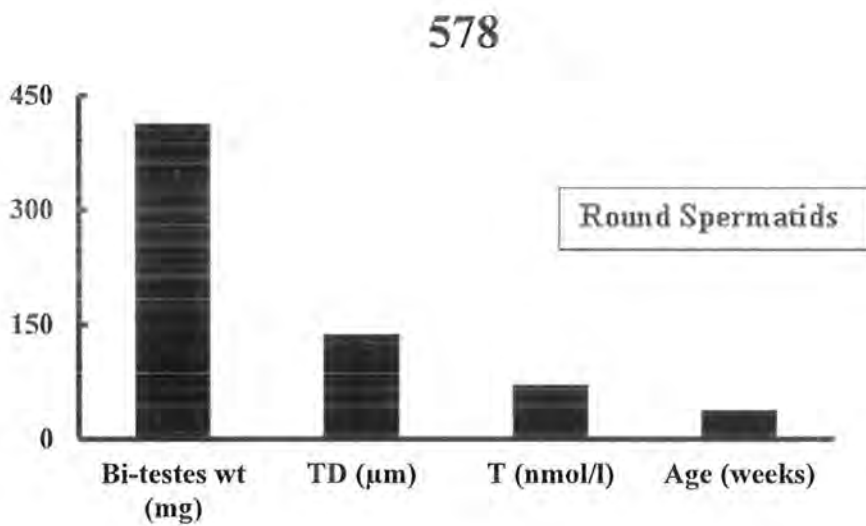
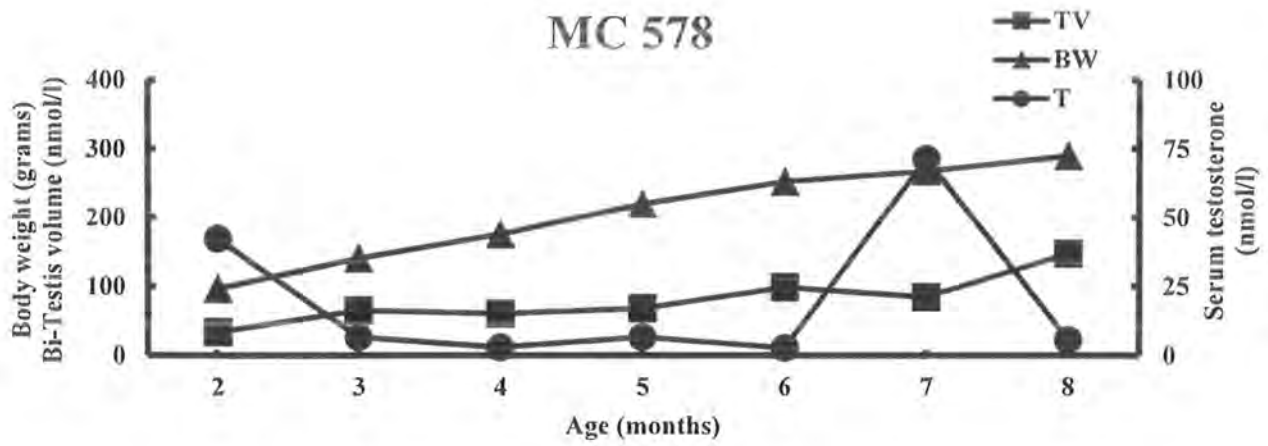


Figure 4.13 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#578) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

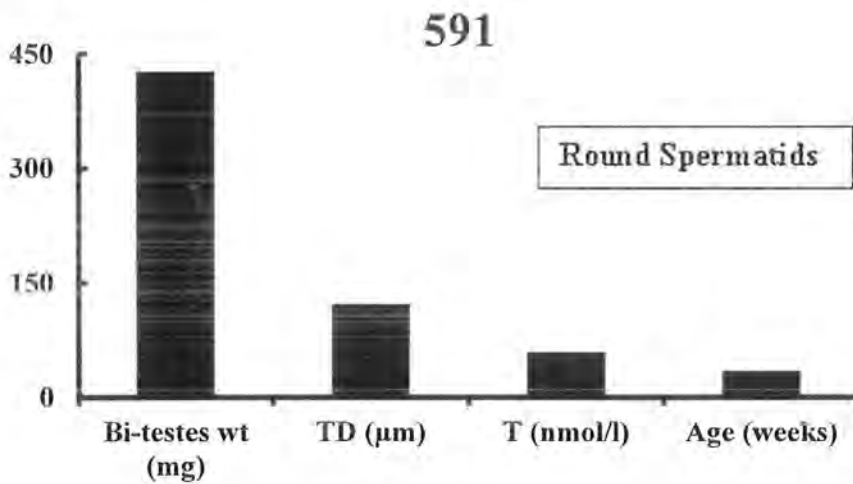
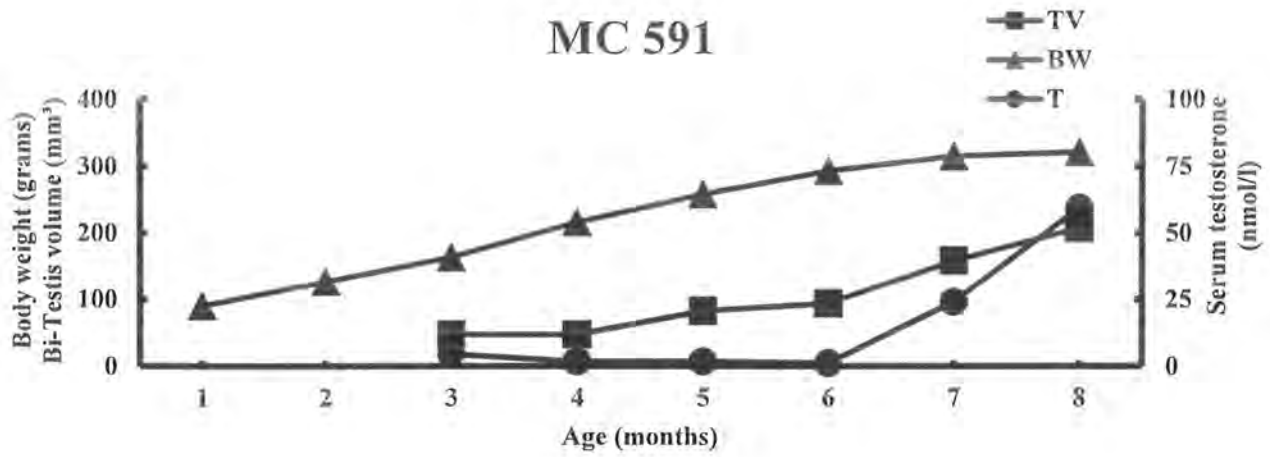


Figure 4.14 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#591) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

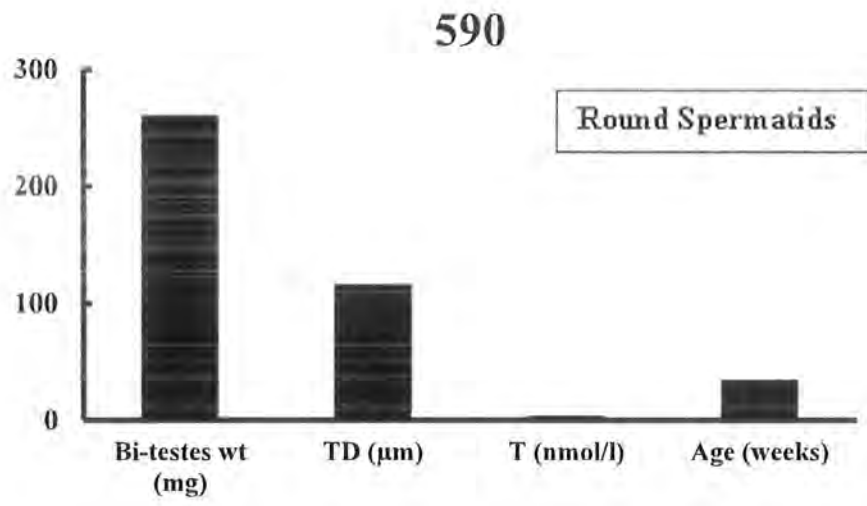
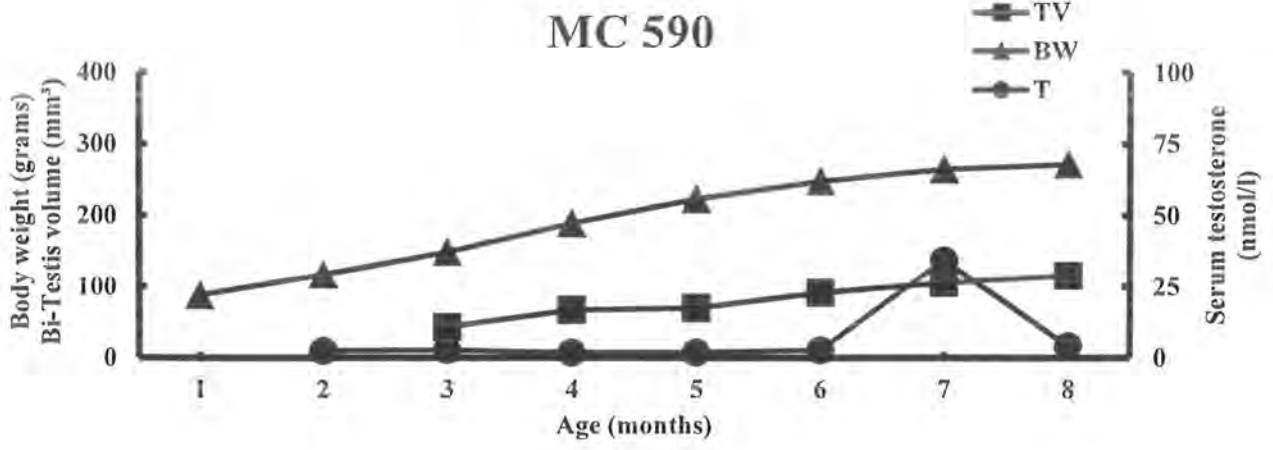


Figure 4.15 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#590) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

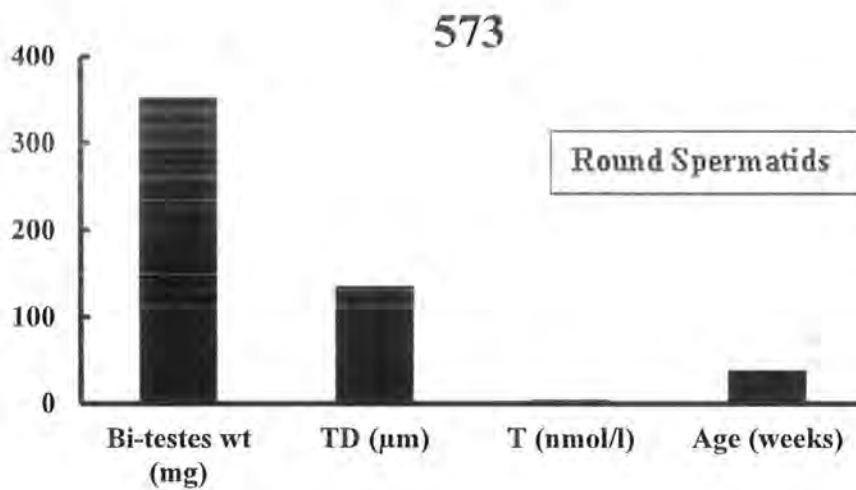
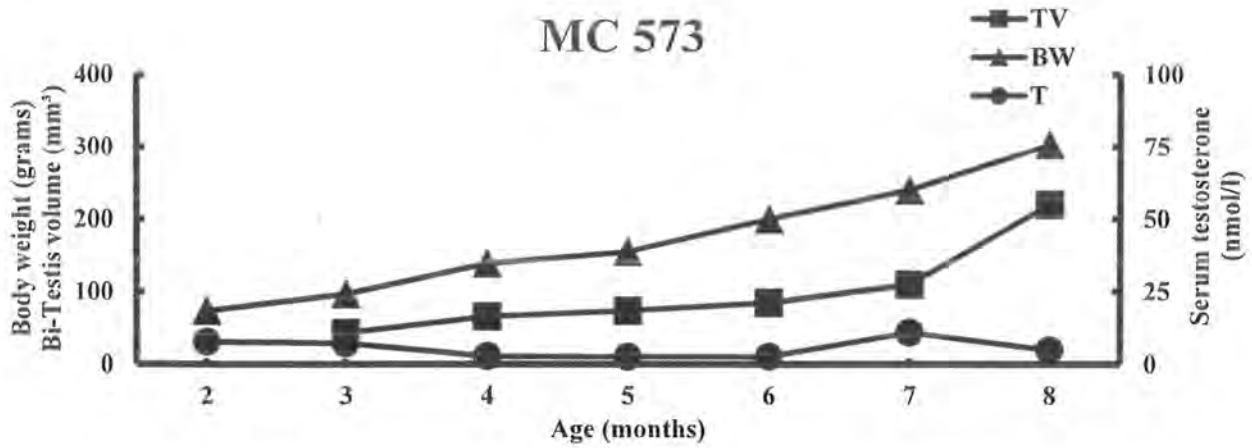


Figure 4.16 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#573) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

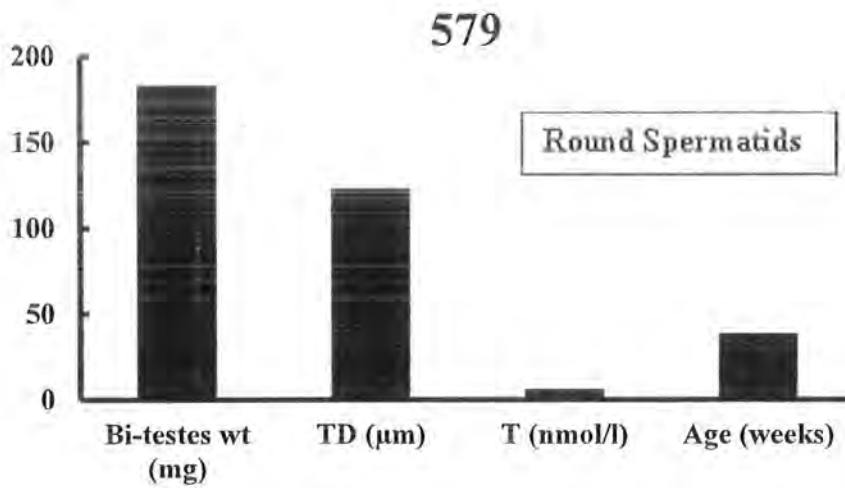
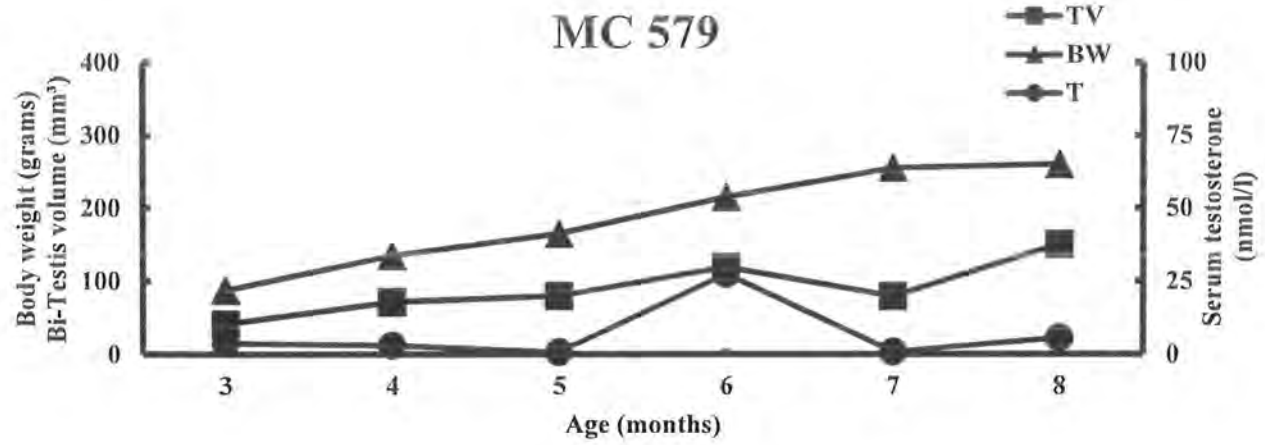


Figure 4.17 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#579) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

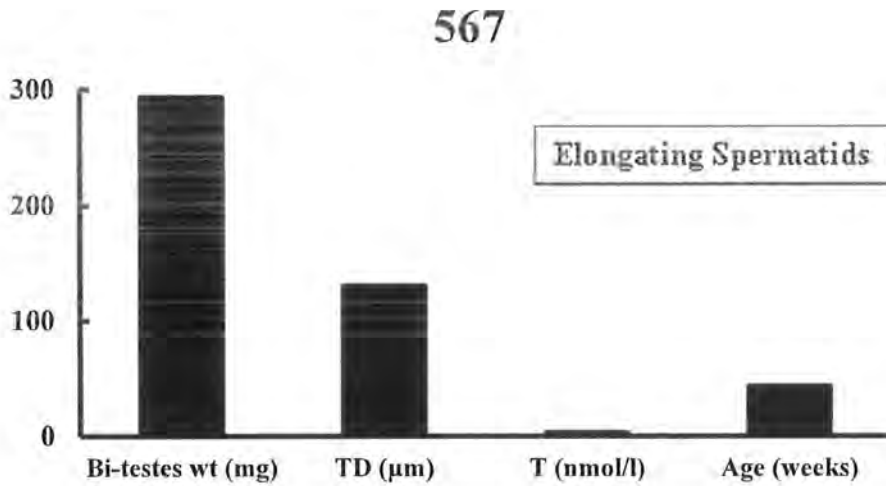
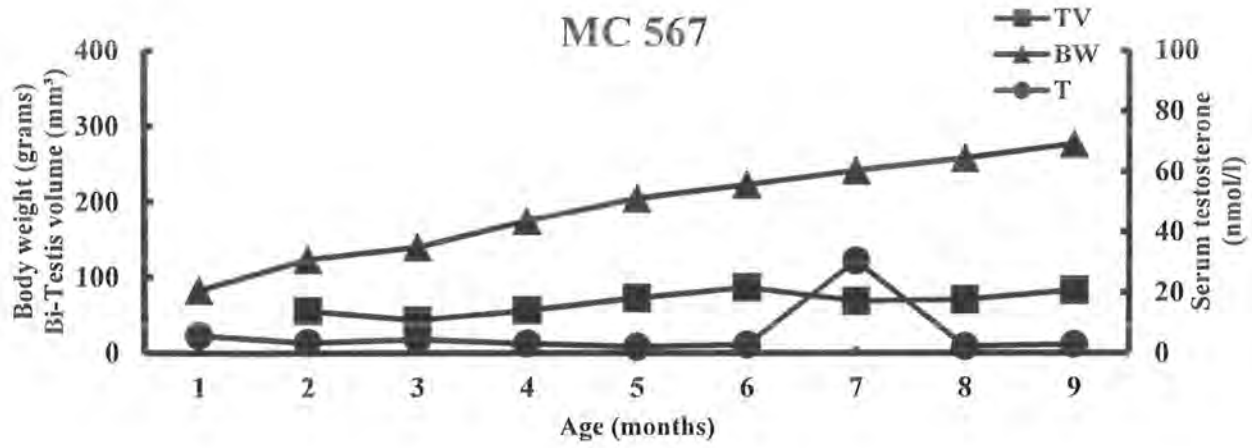


Figure 4.18 Body weight, serum testosterone and bi-testis volume from birth till 9 month of age in common marmoset (#567) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

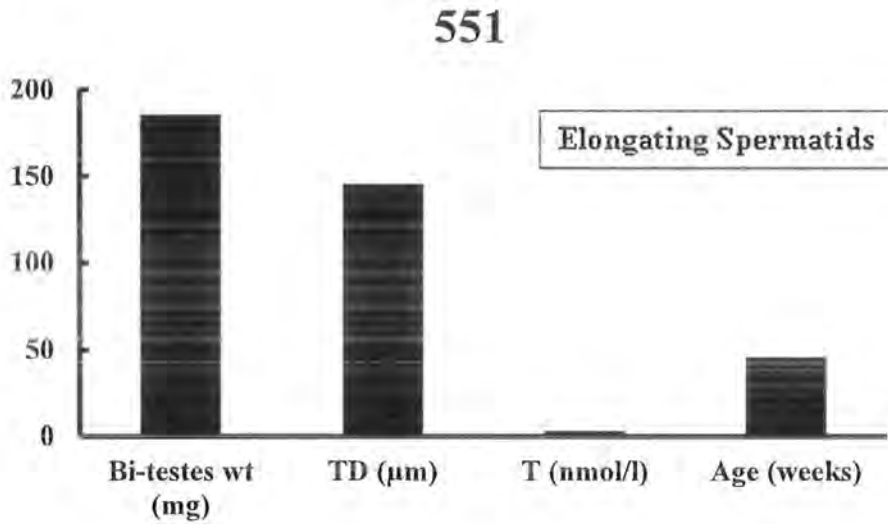
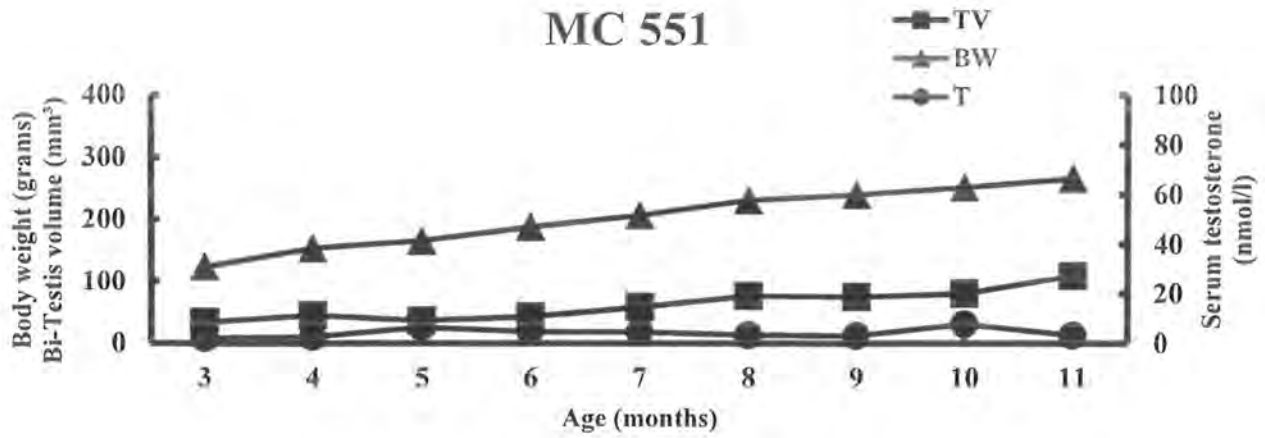


Figure 4.19 Body weight, serum testosterone and bi-testis volume from birth till 11 month of age in common marmoset (#551) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

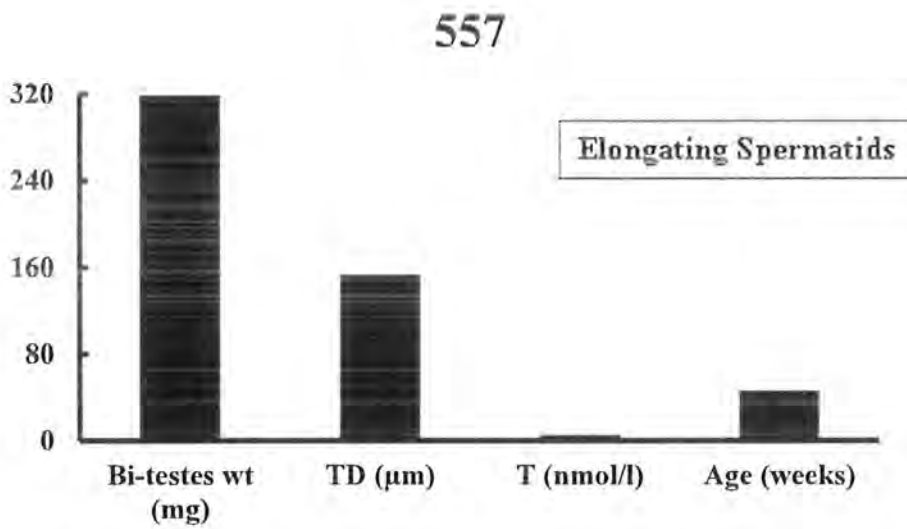
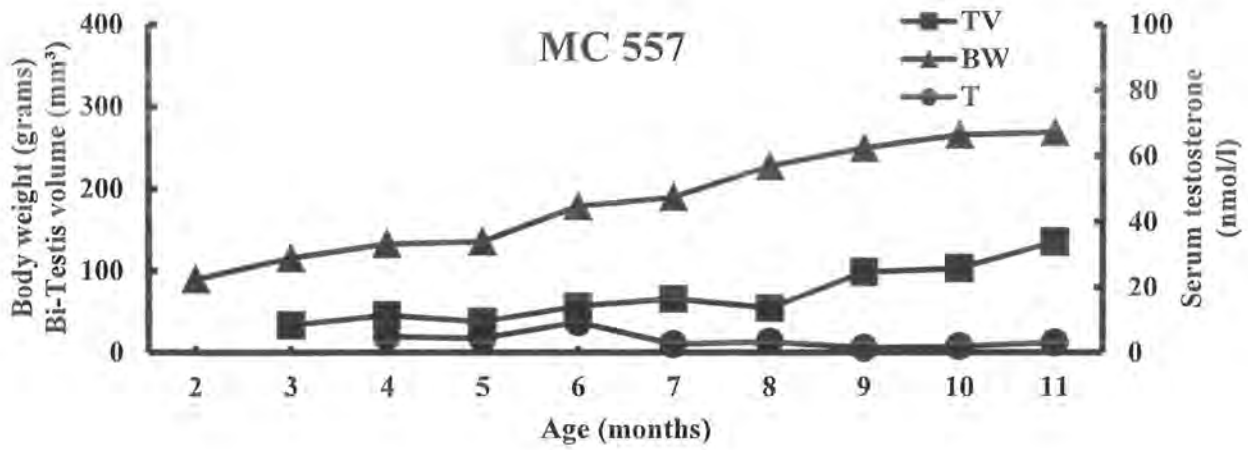


Figure 4.20 Body weight, serum testosterone and bi-testis volume from birth till 11 month of age in common marmoset (#557) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

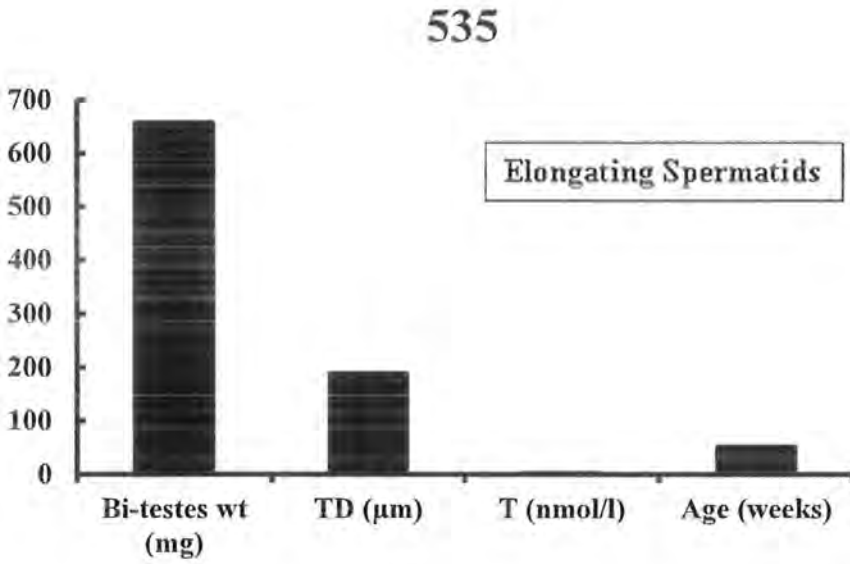
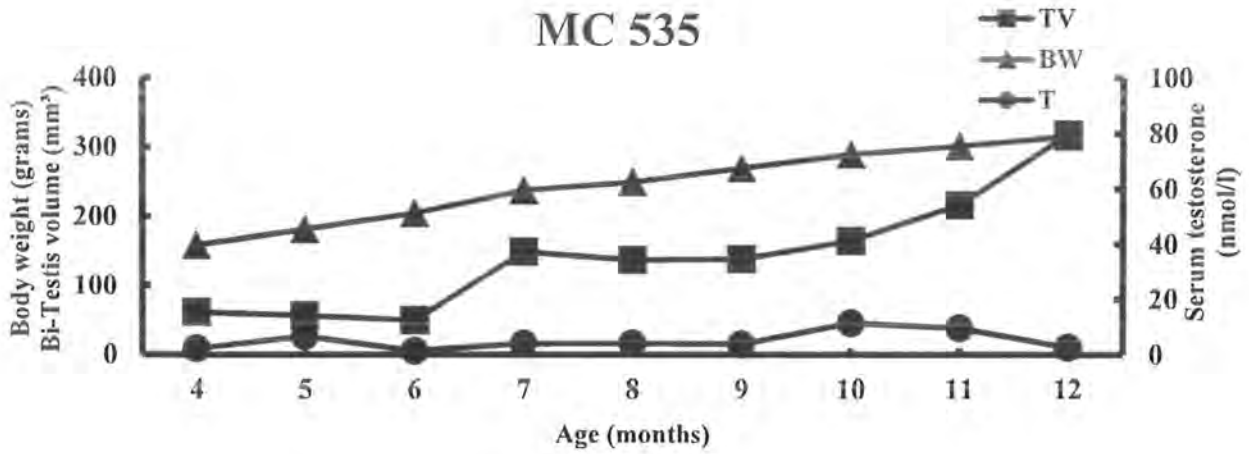


Figure 4.21 Body weight, serum testosterone and bi-testis volume from birth till 12 month of age in common marmoset (#535) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

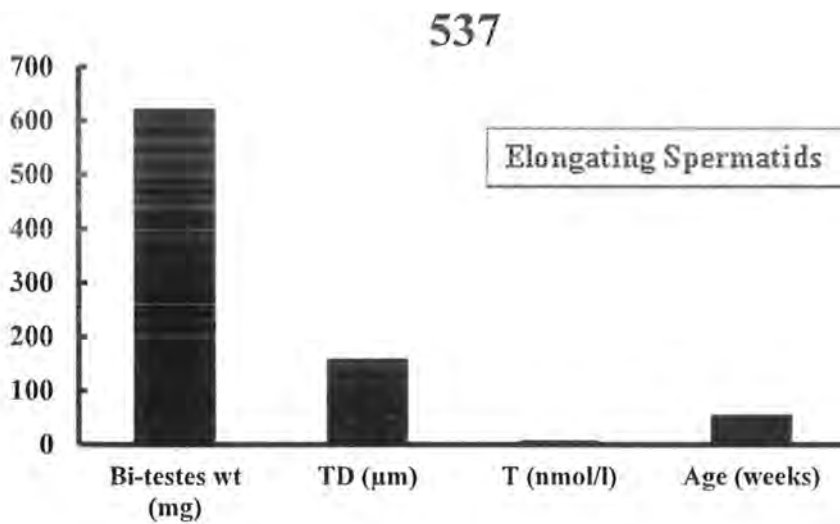
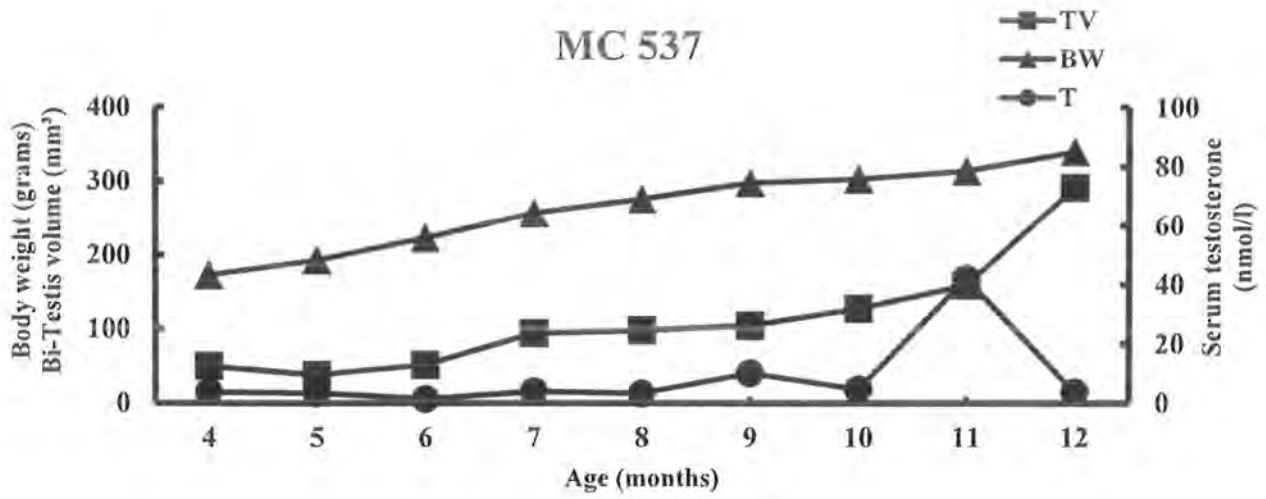


Figure 4.22 Body weight, serum testosterone and bi-testis volume from birth till 12 month of age in common marmoset (#537) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

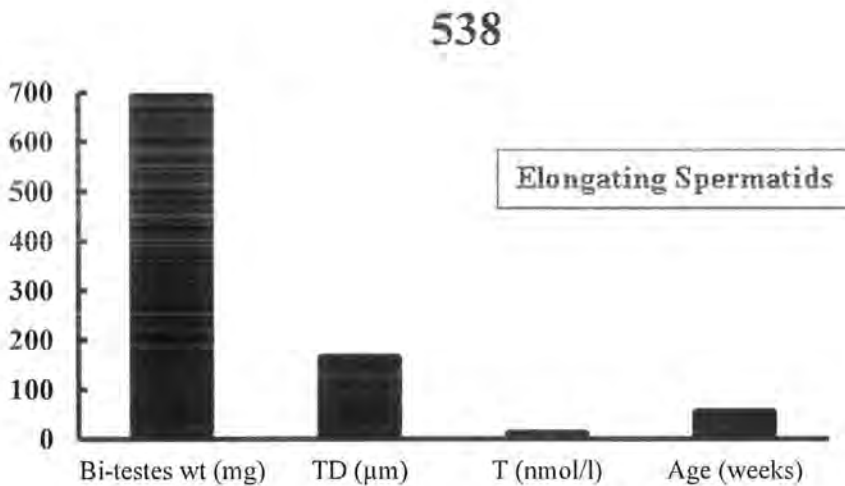
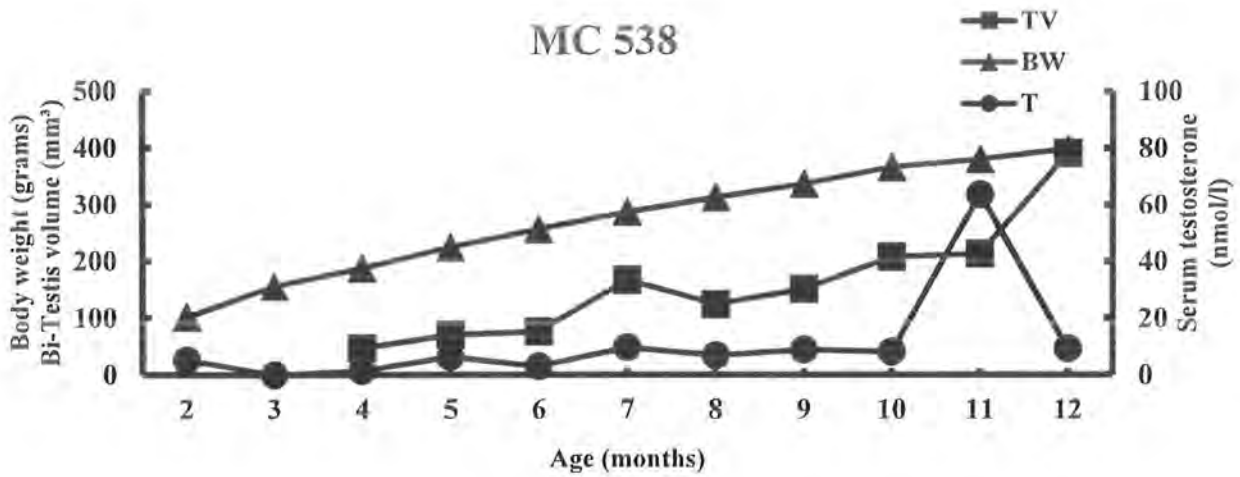


Figure 4.23 Body weight, serum testosterone and bi-testis volume from birth till 12 month of age in common marmoset (#538) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

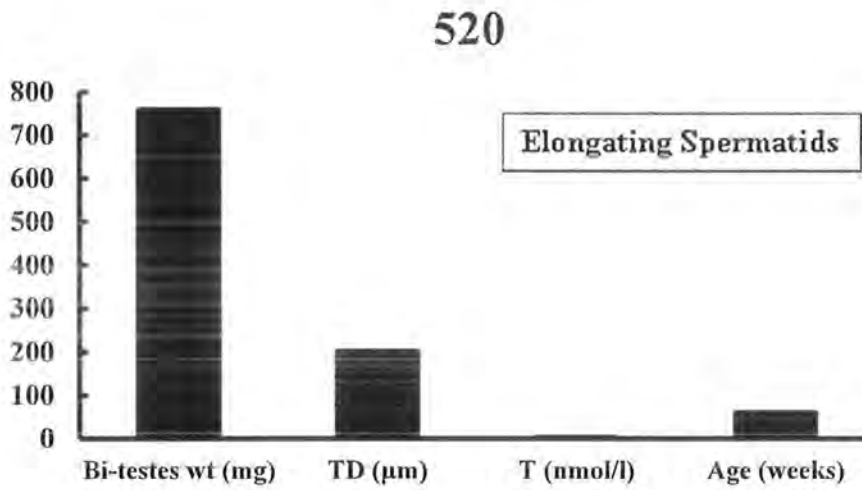
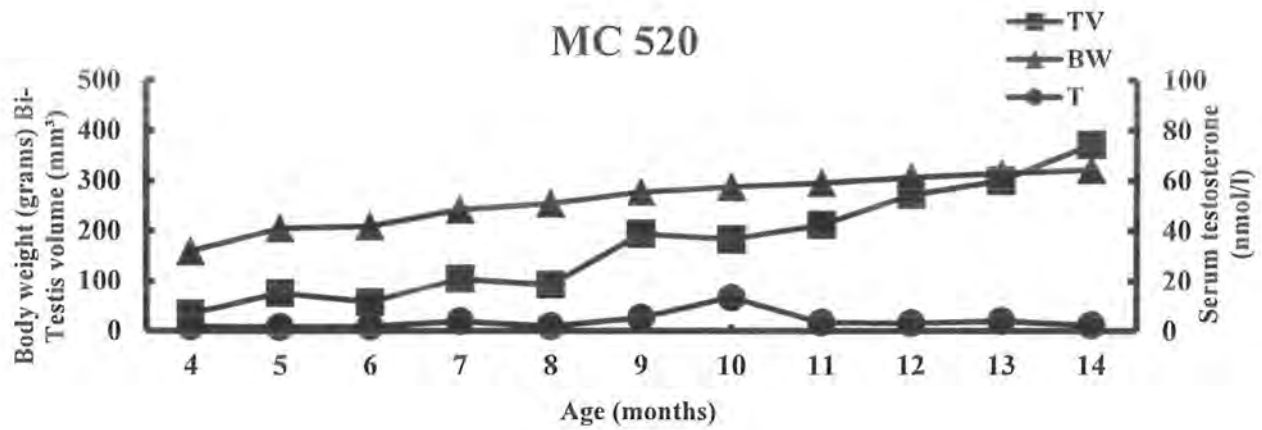


Figure 4.24 Body weight, serum testosterone and bi-testis volume from birth till 14 month of age in common marmoset (#520) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

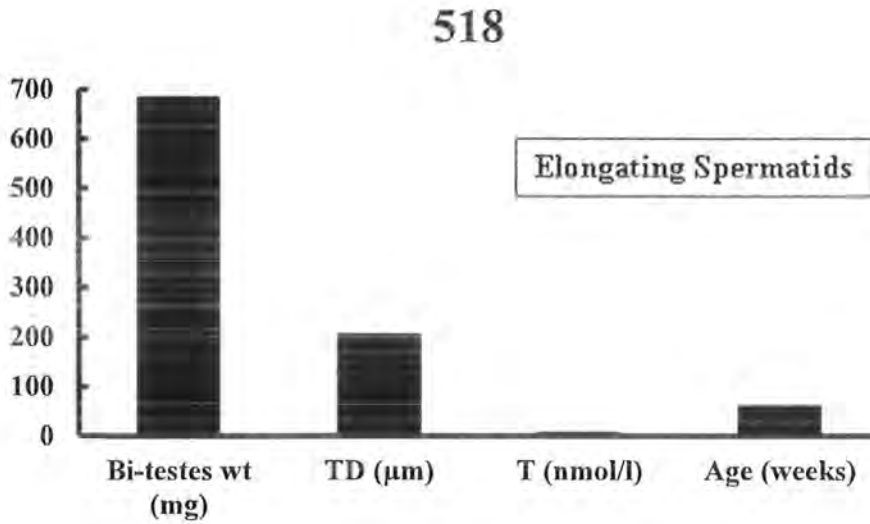
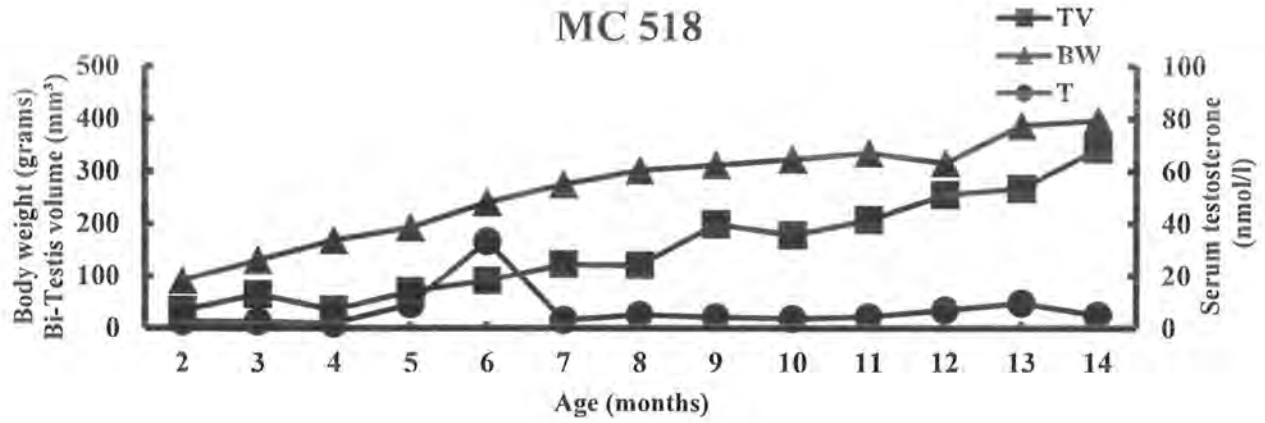


Figure 4.25 Body weight, serum testosterone and bi-testis volume from birth till 14 month of age in common marmoset (#518) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

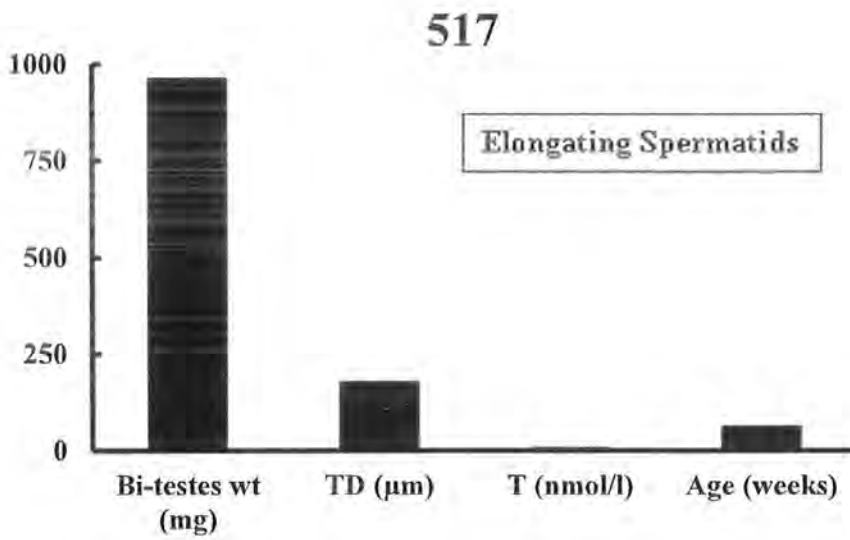


Figure 4.26 Body weight, serum testosterone and bi-testis volume from birth till 14 month of age in common marmoset (#517) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

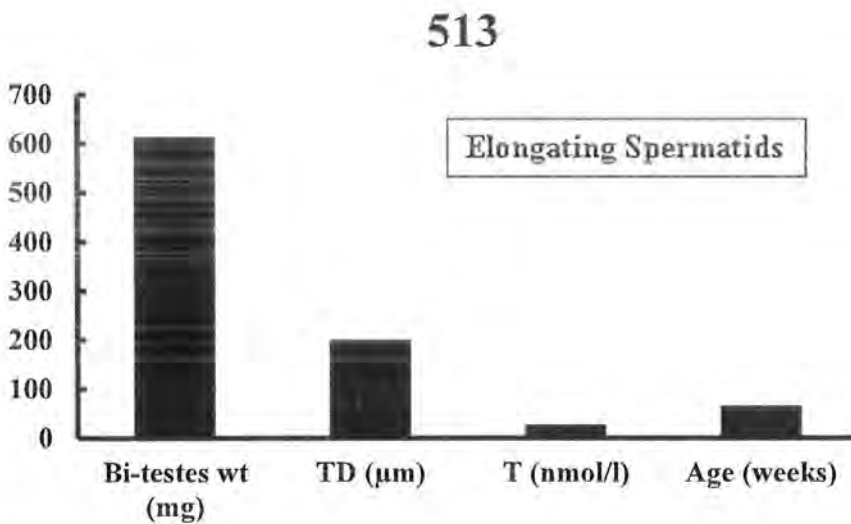
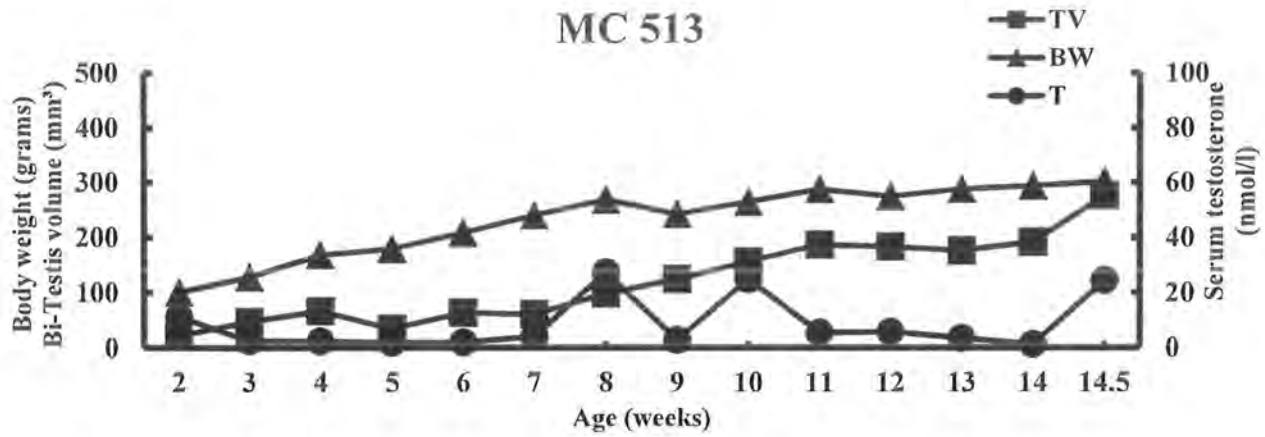


Figure 4.27 Body weight, serum testosterone and bi-testis volume from birth till 14.5 month of age in common marmoset (#513) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

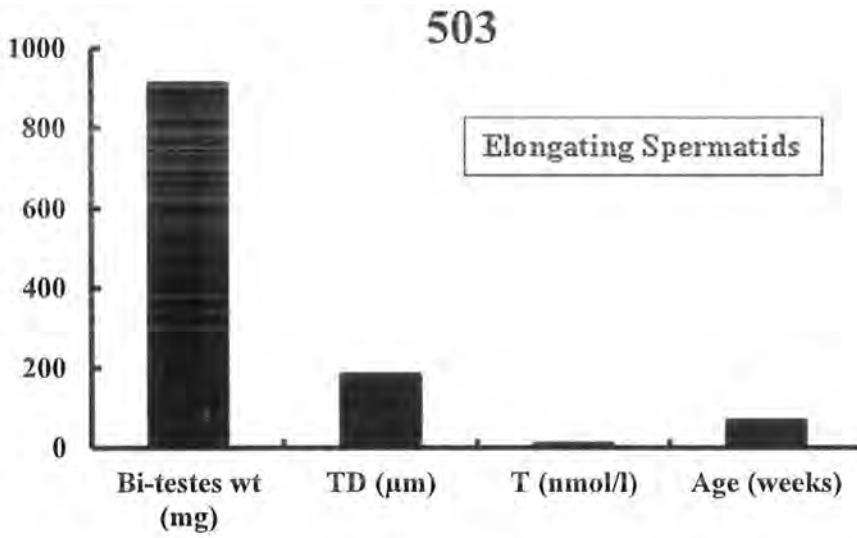
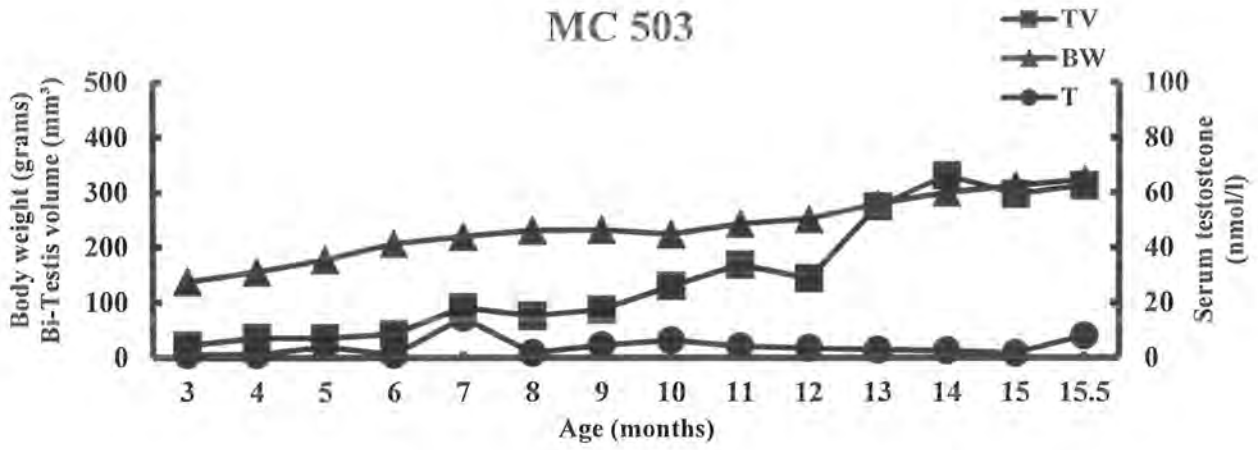


Figure 4.28 Body weight, serum testosterone and bi-testis volume from birth till 15.5 month of age in common marmoset (#503) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

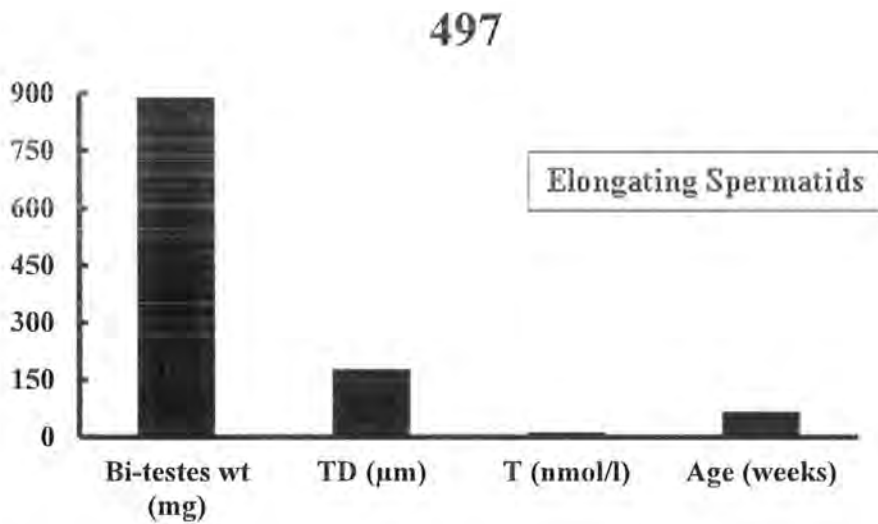
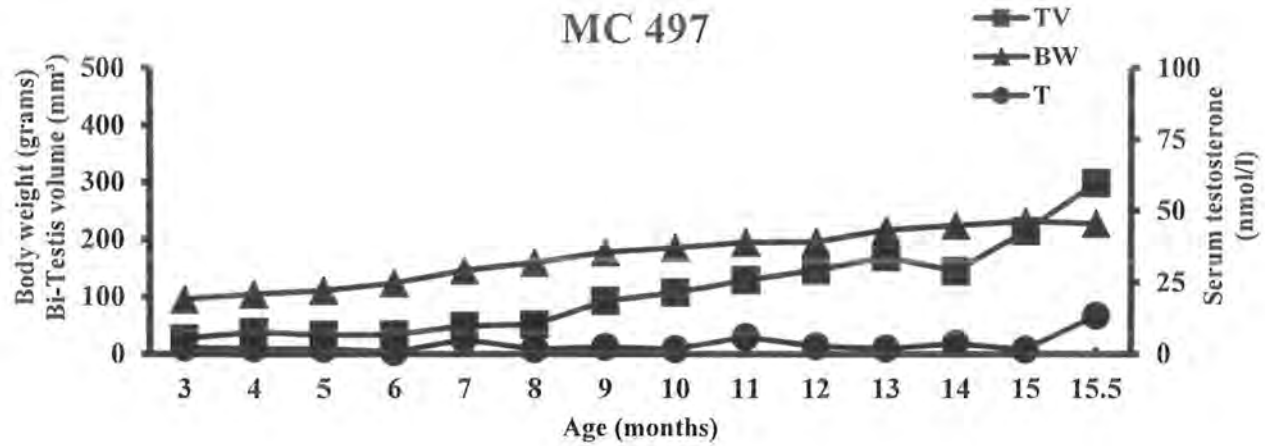


Figure 4.29 Body weight, serum testosterone and bi-testis volume from birth till 15.5 month of age in common marmoset (#497) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

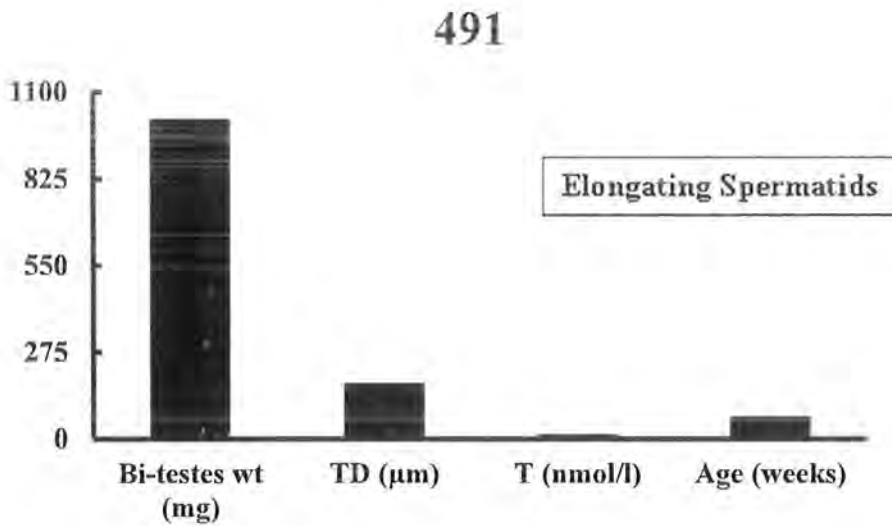
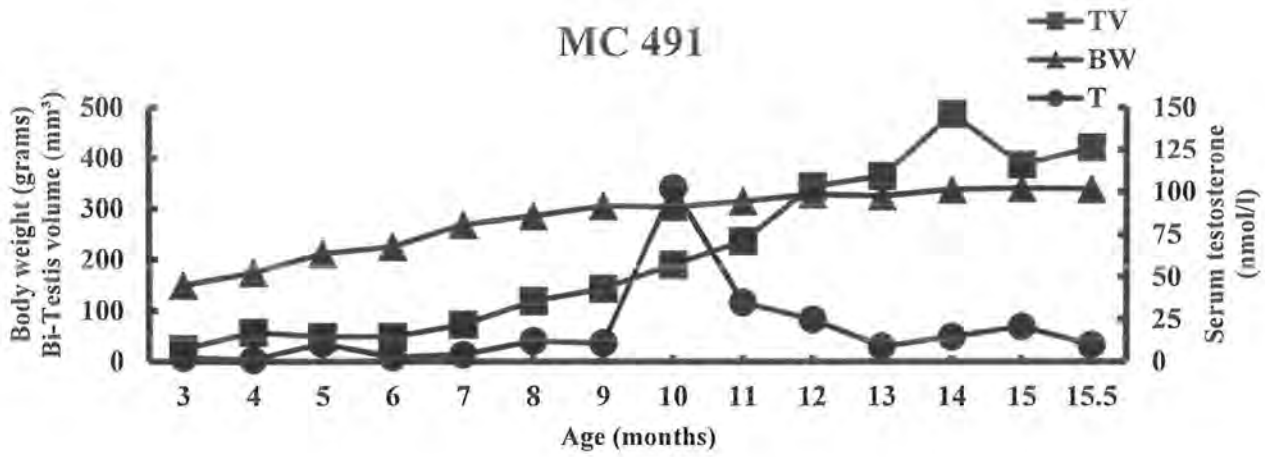


Figure 4.30 Body weight, serum testosterone and bi-testis volume from birth till 15.5 month of age in common marmoset (#491) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

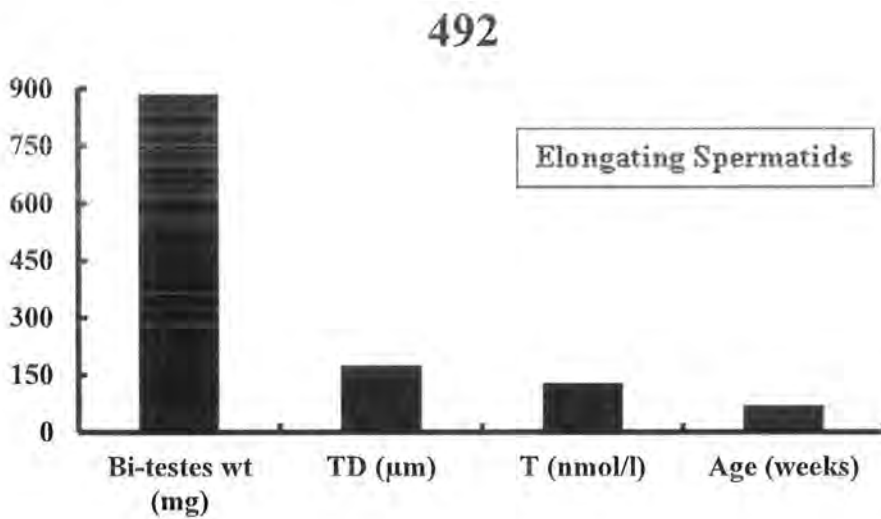
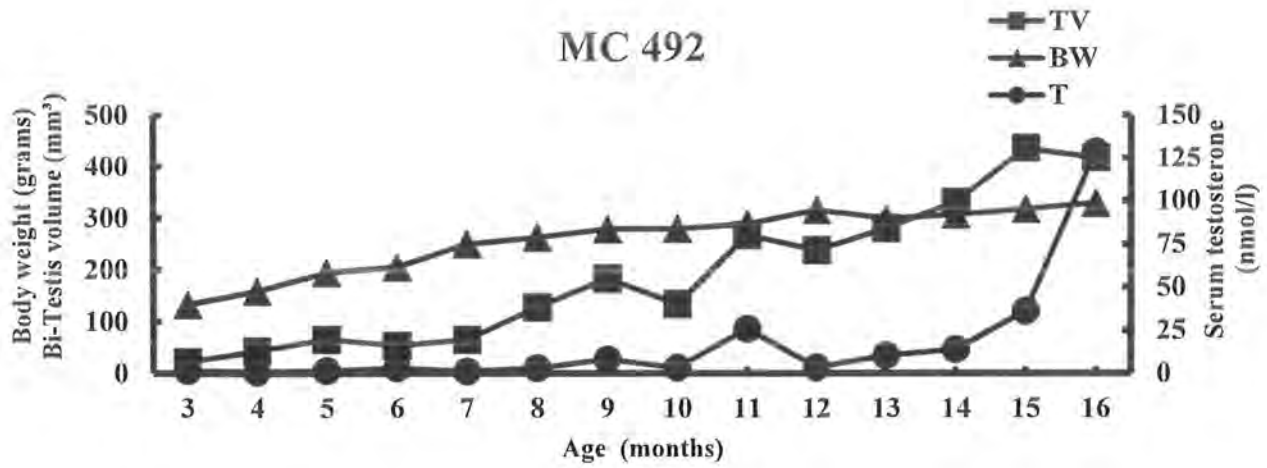


Figure 4.31 Body weight, serum testosterone and bi-testis volume from birth till 16 month of age in common marmoset (#492) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

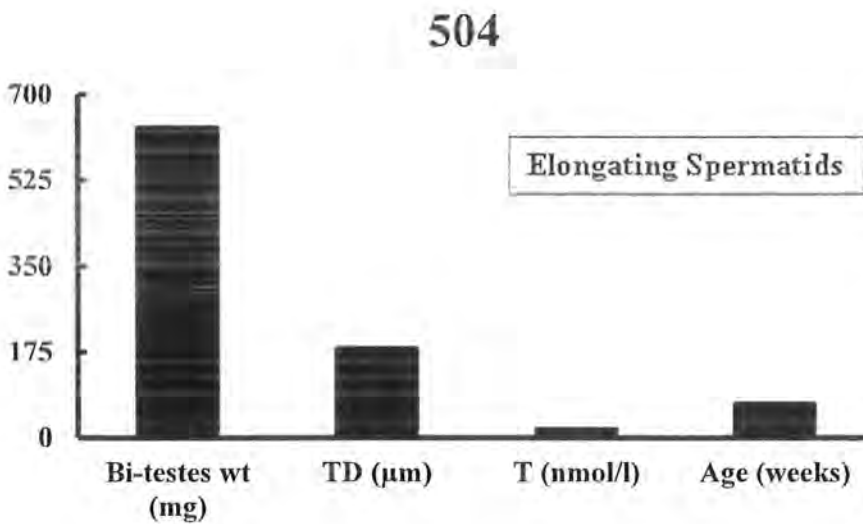
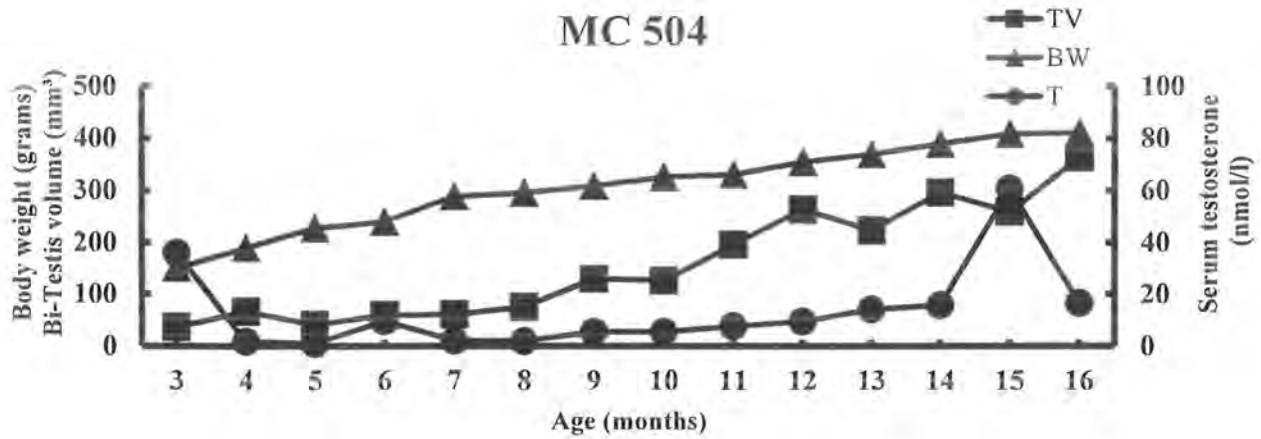


Figure 4.32 Body weight, serum testosterone and bi-testis volume from birth till 16 month of age in common marmoset (#504) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

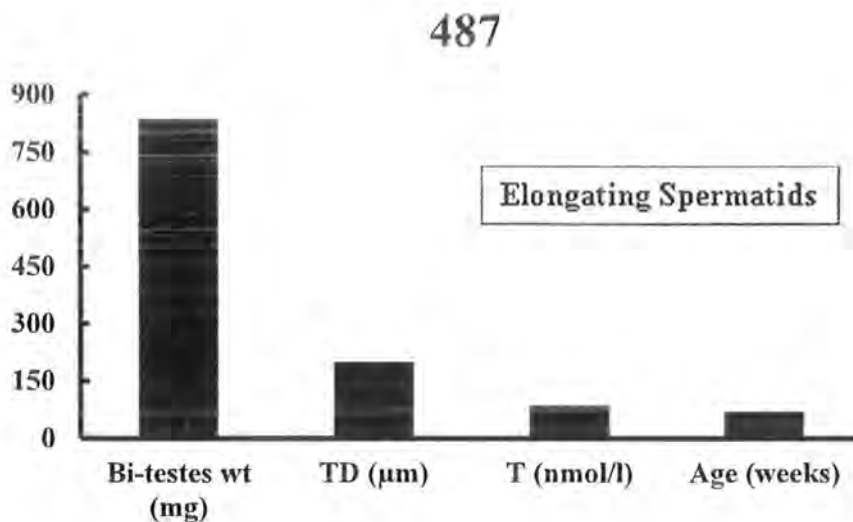
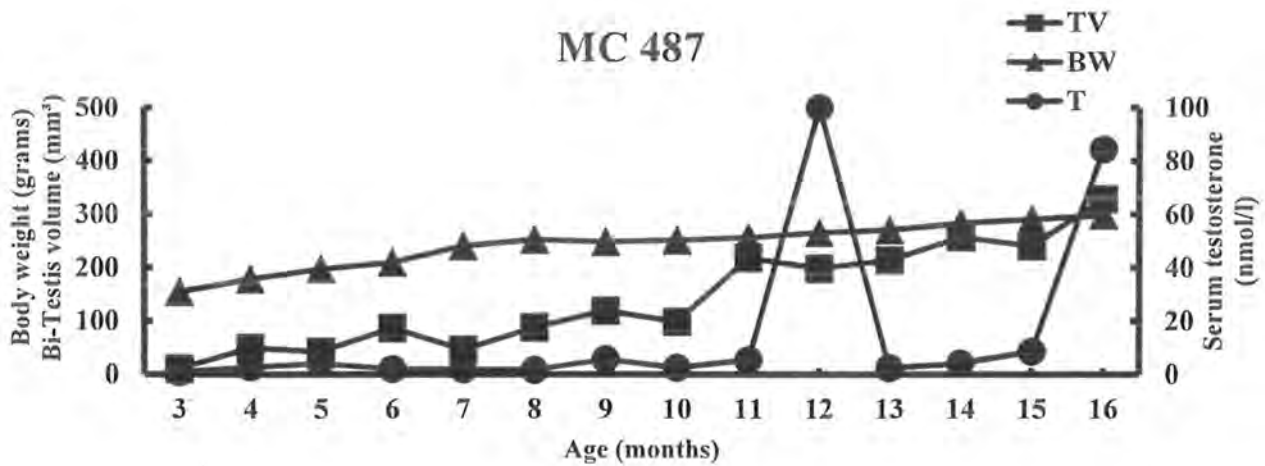


Figure 4.33 Body weight, serum testosterone and bi-testis volume from birth till 16 month of age in common marmoset (#487) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

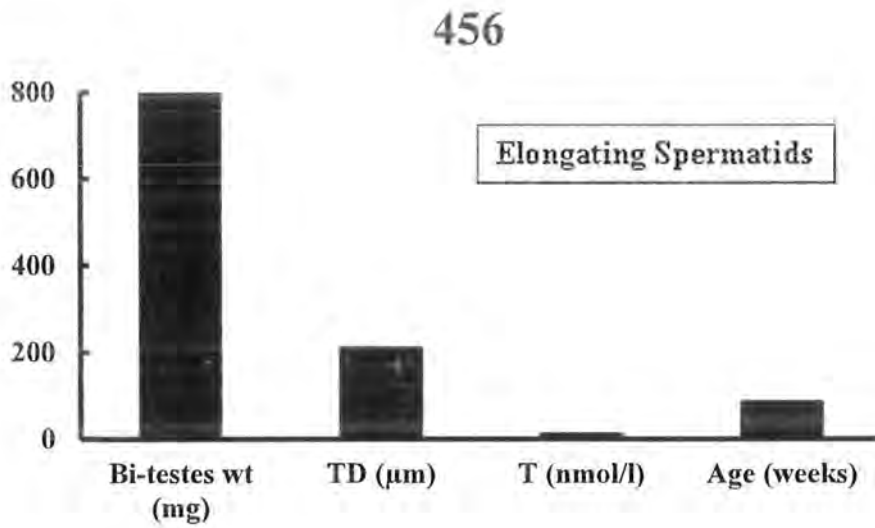
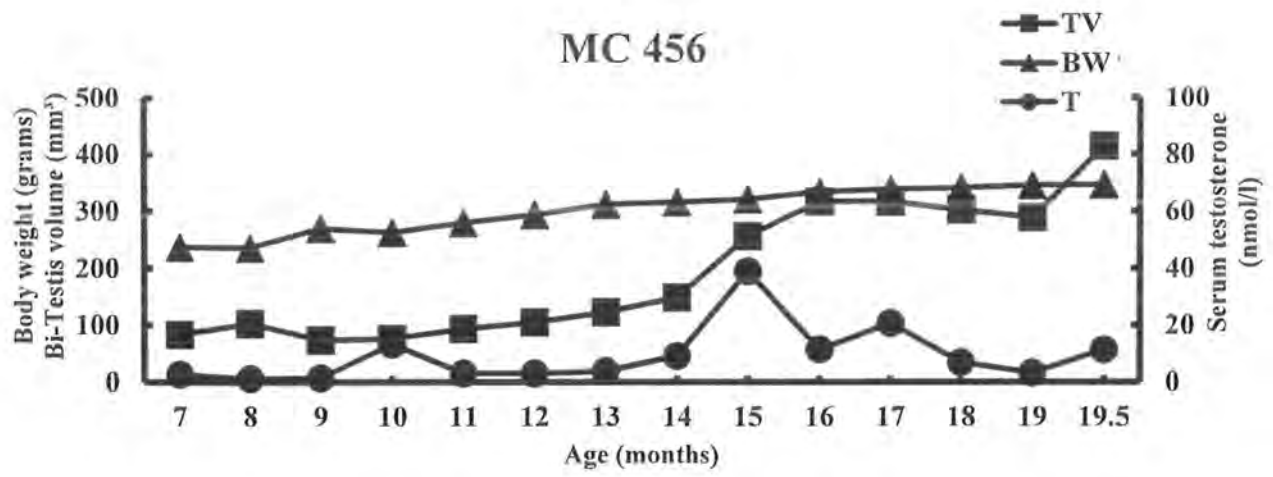


Figure 4.34 Body weight, serum testosterone and bi-testis volume from birth till 19.5 month of age in common marmoset (#456) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

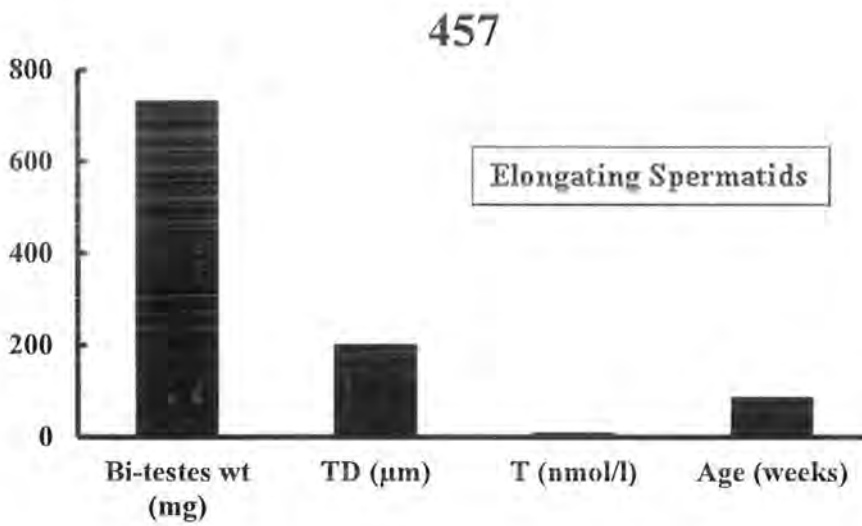
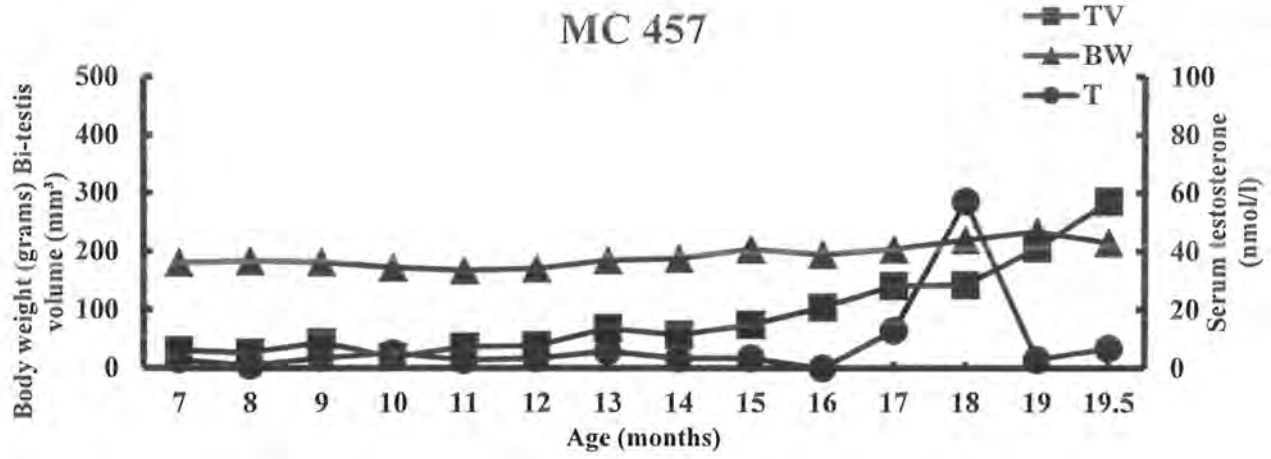


Figure 4.35 Body weight, serum testosterone and bi-testis volume from birth till 19.5 month of age in common marmoset (#457) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

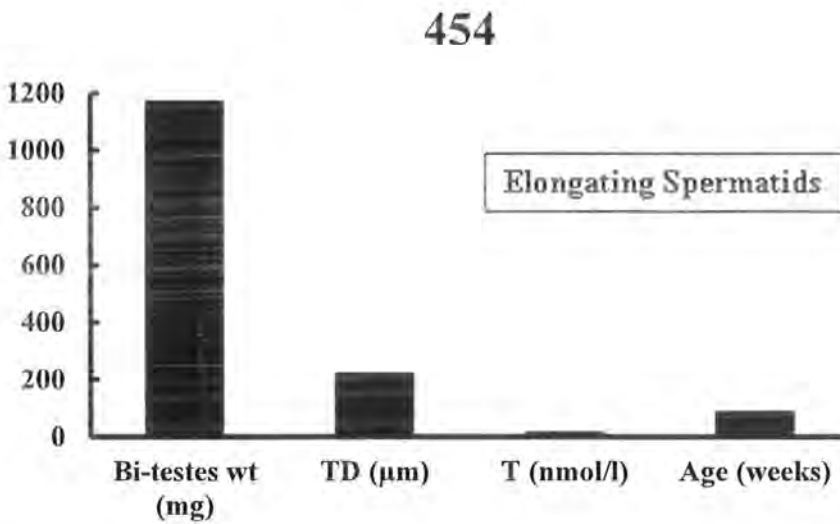
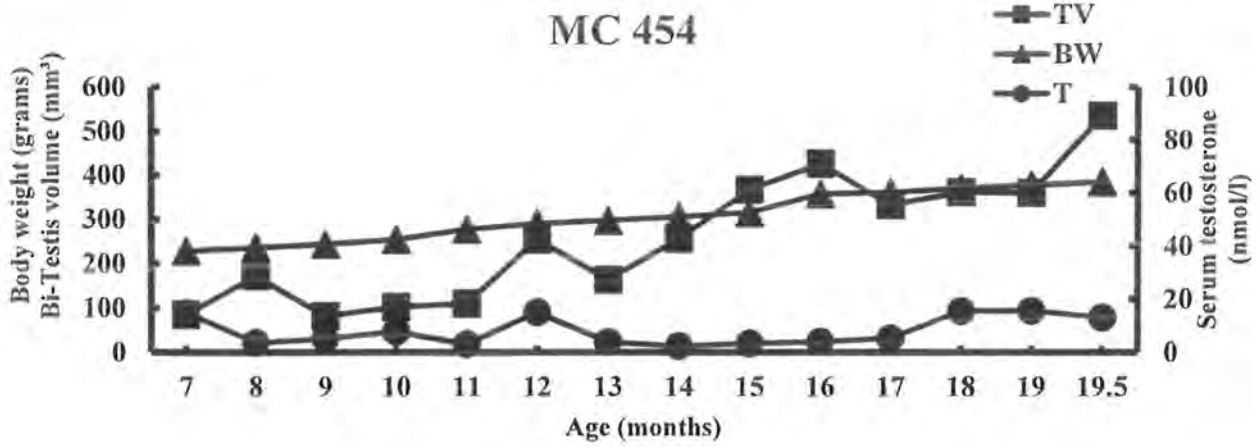


Figure 4.36 Body weight, serum testosterone and bi-testis volume from birth till 19.5 month of age in common marmoset (#454) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

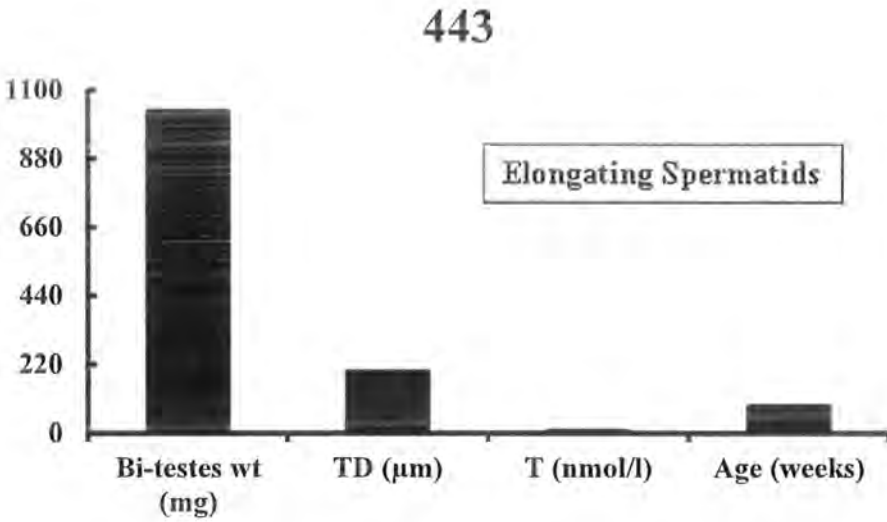
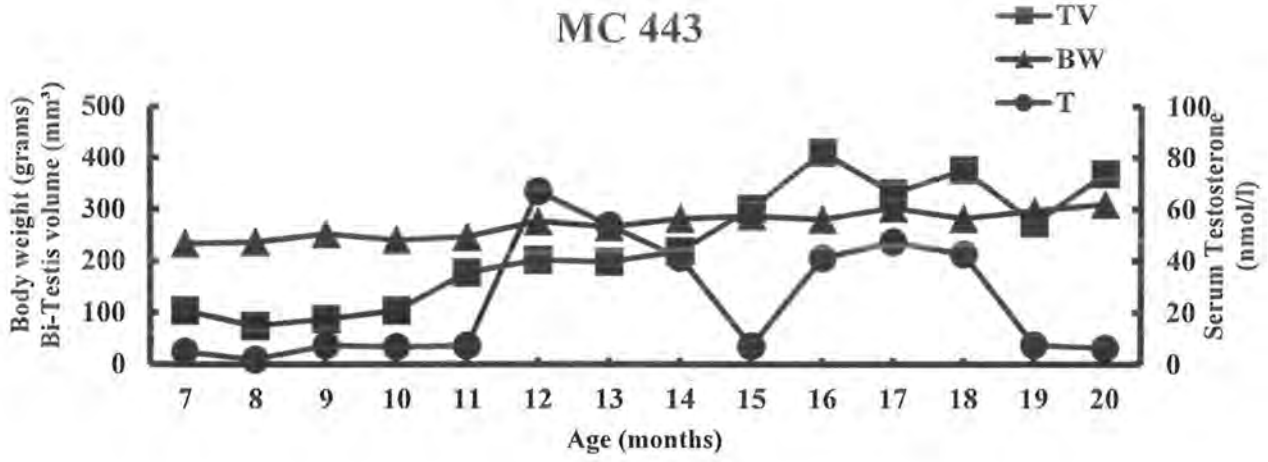


Figure 4.37 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#443) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

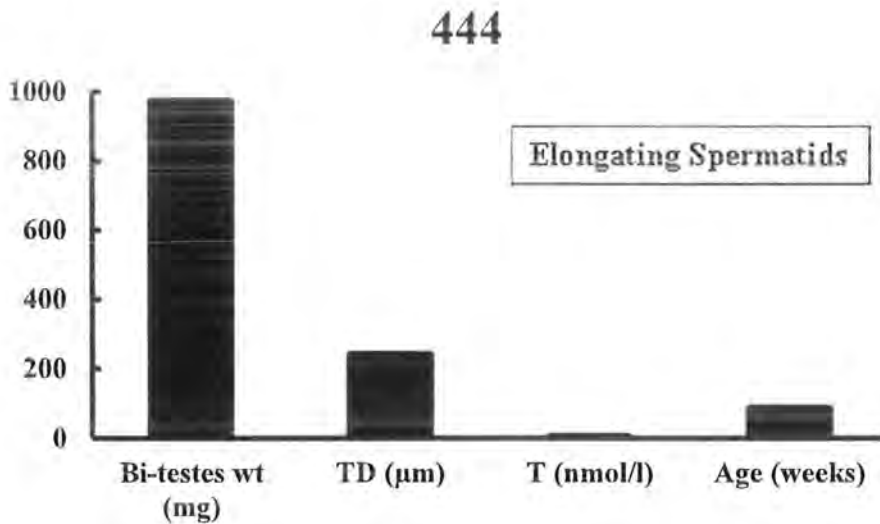
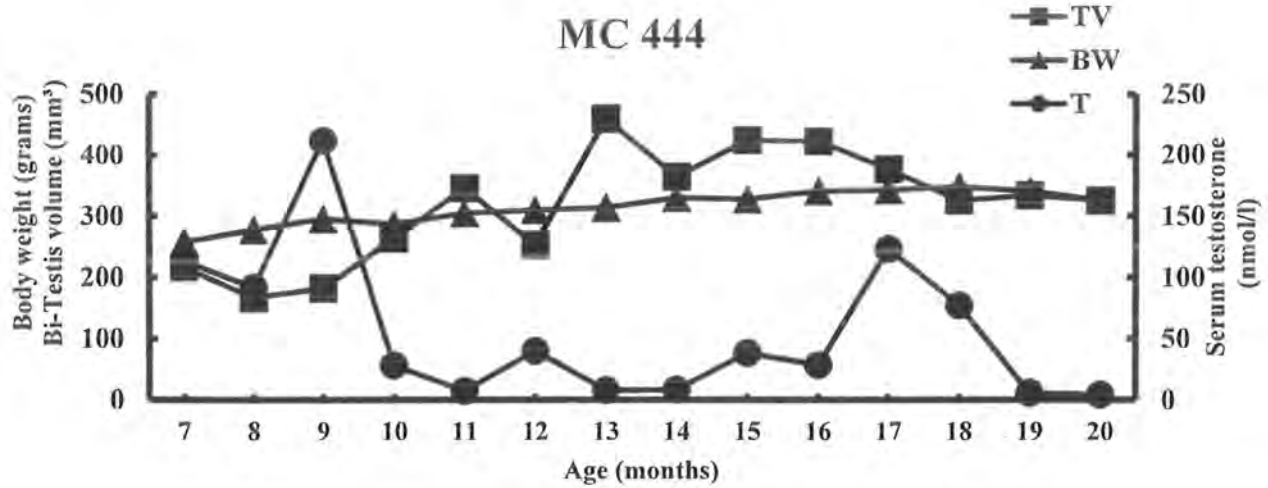


Figure 4.38 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#444) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

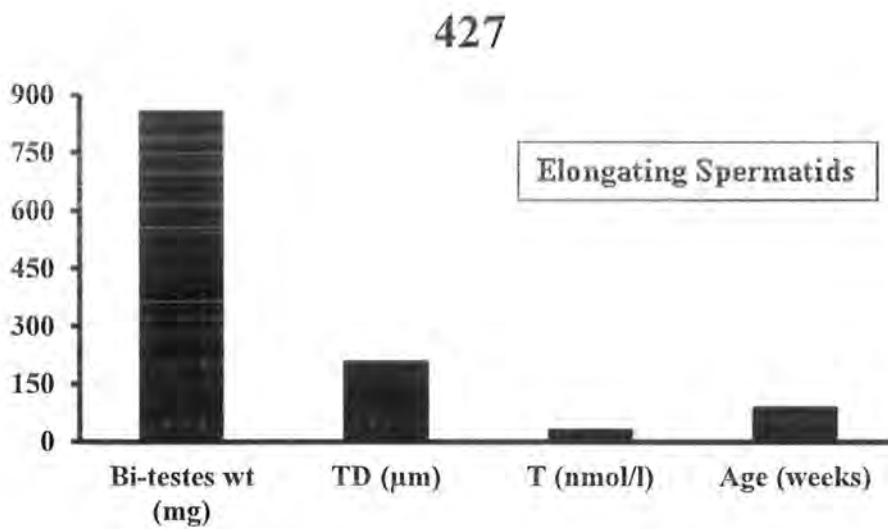
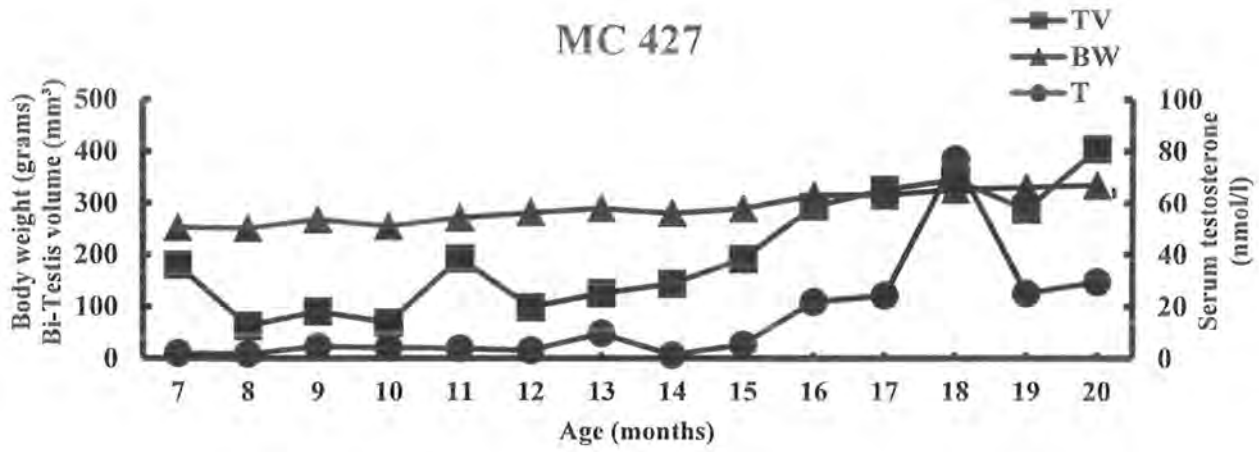


Figure 4.39 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#427) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

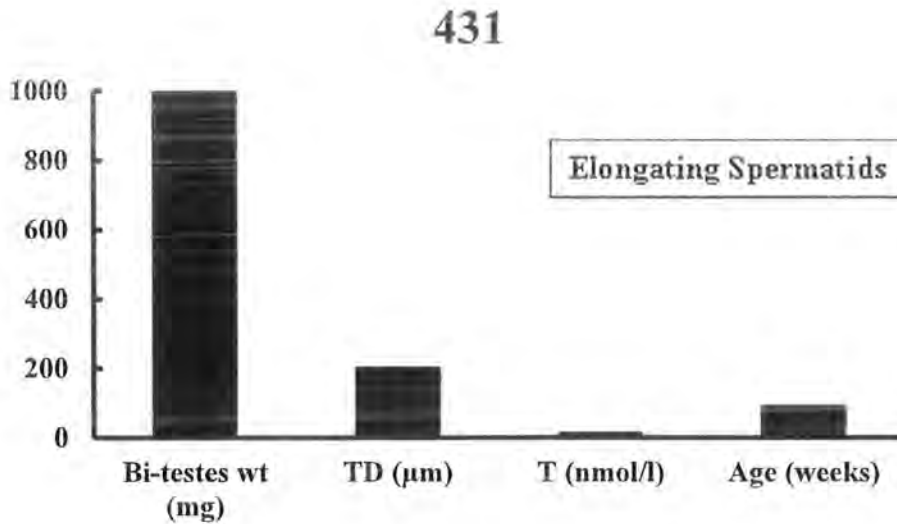
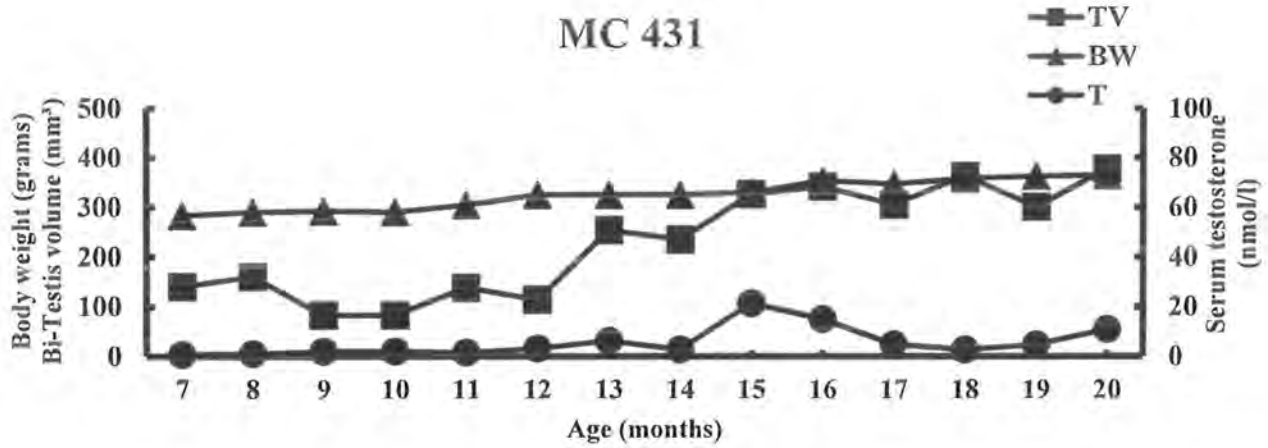


Figure 4.40 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#431) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

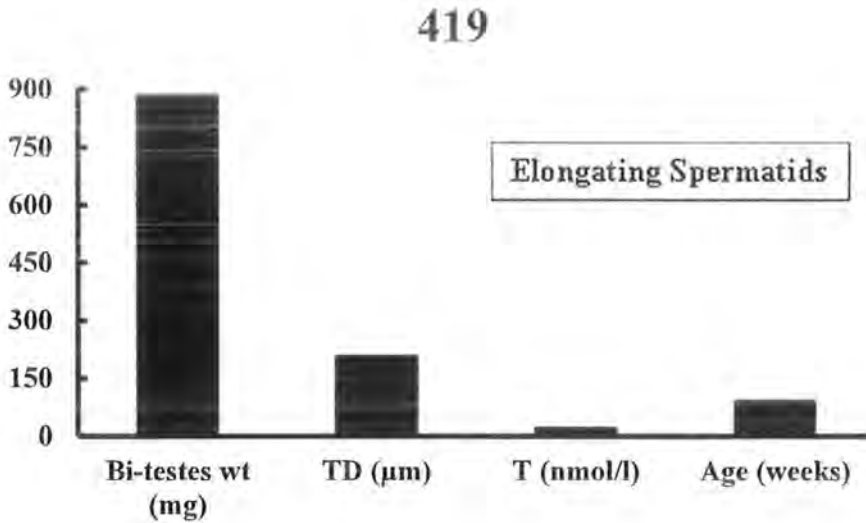
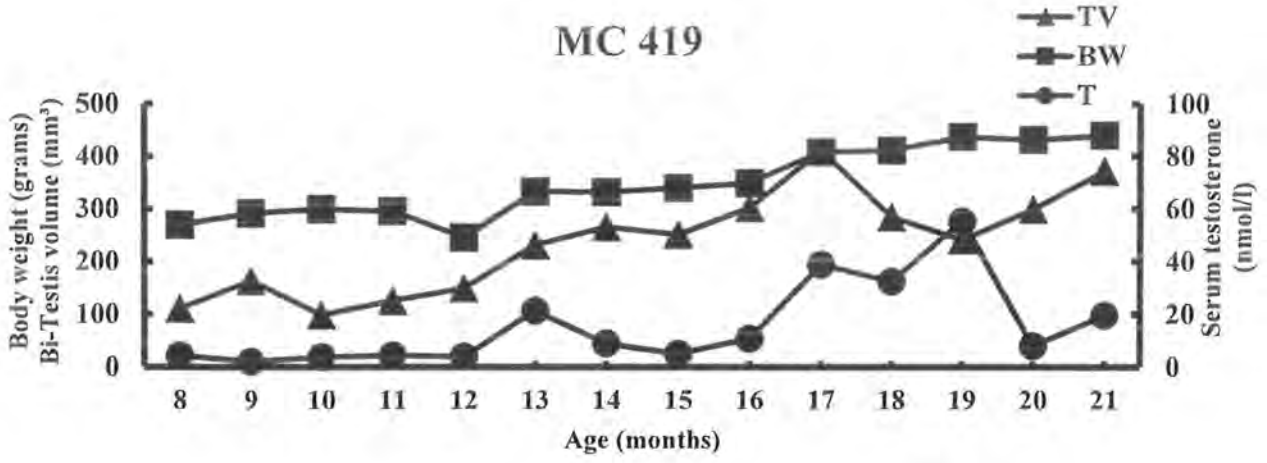


Figure 4.41 Body weight, serum testosterone and bi-testis volume from birth till 21 month of age in common marmoset (#419) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

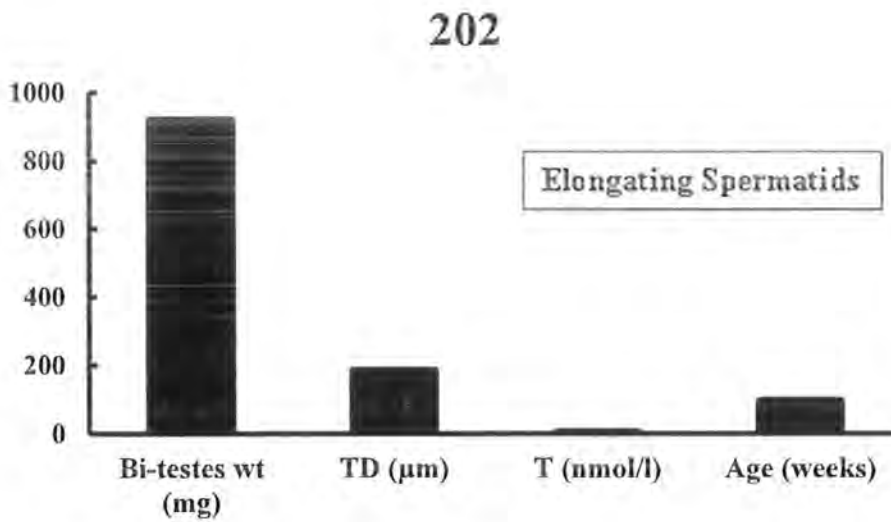
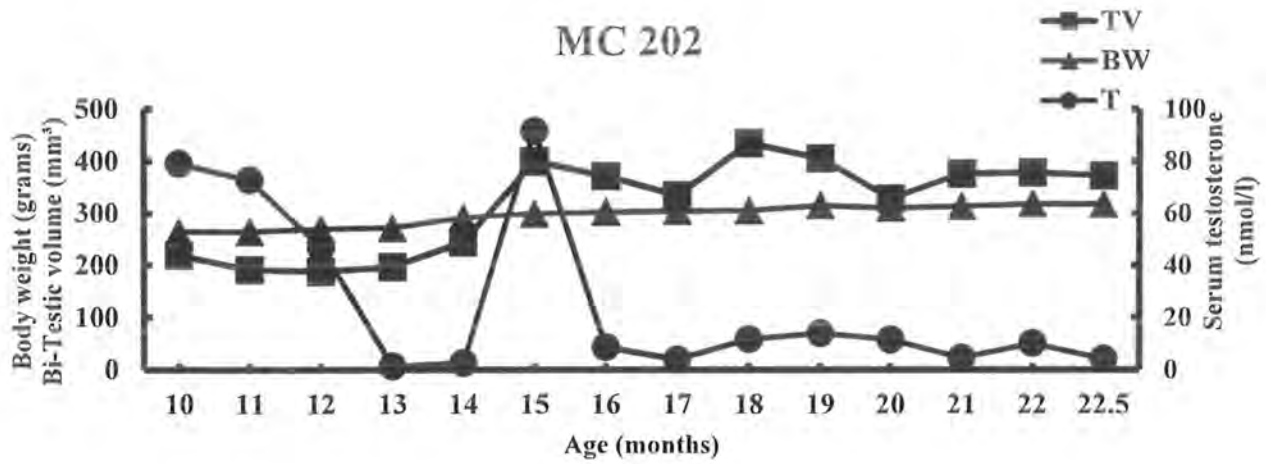


Figure 4.42 Body weight, serum testosterone and bi-testis volume from birth till 22.5 month of age in common marmoset (#202) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

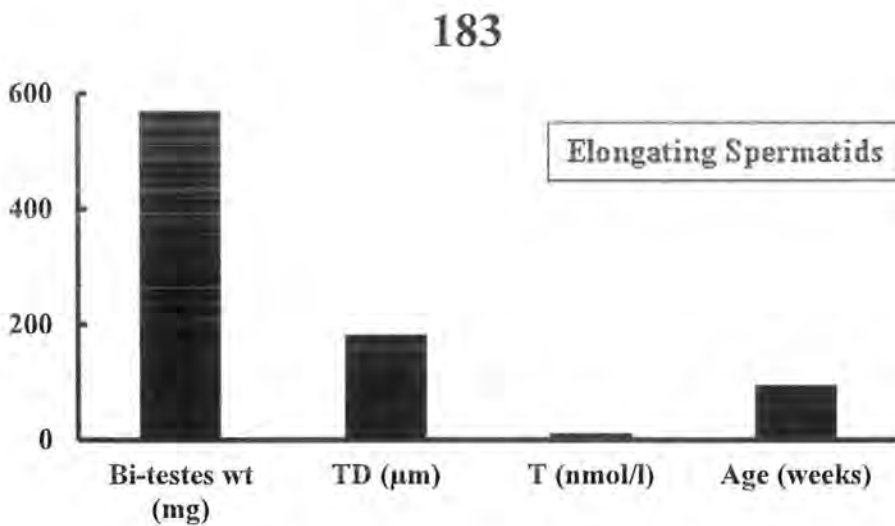
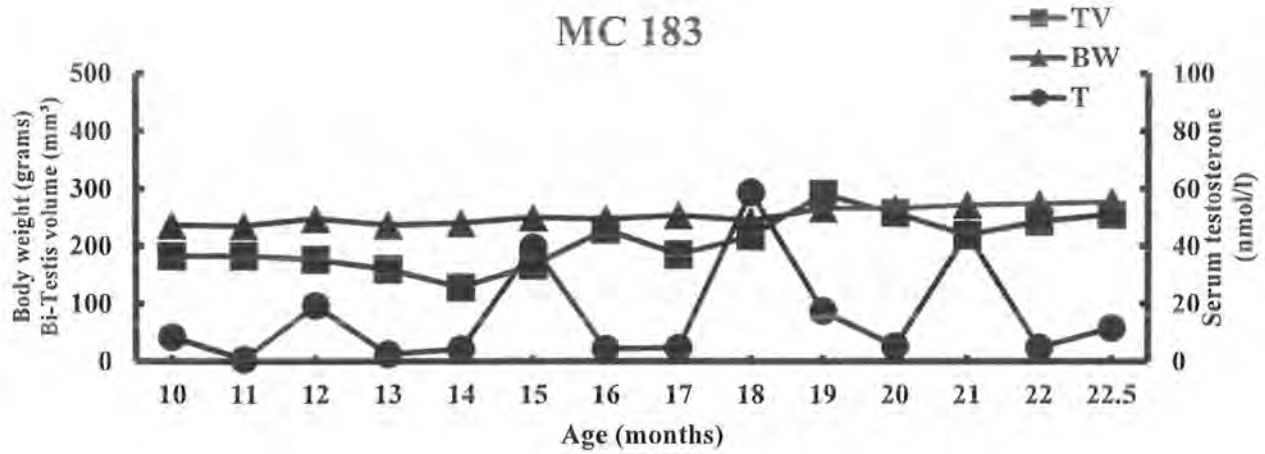
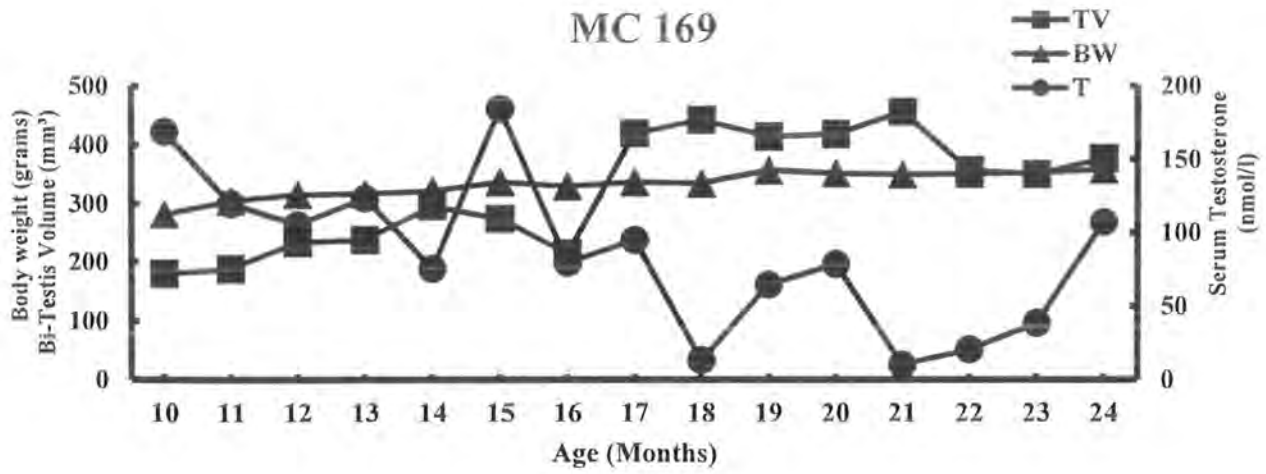


Figure 4.43 Body weight, serum testosterone and bi-testis volume from birth till 22.5 month of age in common marmoset (#183) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



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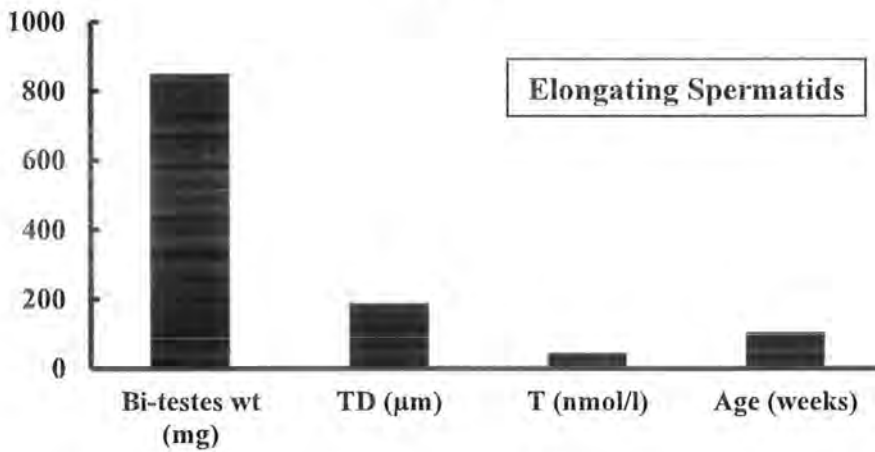


Figure 4.44 Body weight, serum testosterone and bi-testis volume from birth till 24 month of age in common marmoset (#169) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

Discussion

The pubertal and testicular development in common marmoset was highlighted almost 30 years ago (Abbot and Hearn, 1978; Jackson and Edmonds, 1984). Since these classical reports the testicular characterization during development in common marmoset has been attempted regarding Sertoli cells and Leydig cell morphology (Rune et al., 1991; Rune et al., 1992). The observational attempts to unravel the ontogeny of pubertal onset in common marmoset has been scarce (Dixon, 1986; Chandolia et al 2006). Apart from these above mentioned studies, it is interesting to note here that nearly all of the other studies conducted on common marmoset to unravel the postnatal aspect of sexual development at the testis level were manipulative instead of observatory (Lunn et al., 1990, 1992, 1994, 1997; Sharpe et al., 2000, 2003).

The scarcity of the available observational data creates poor understanding of the pubertal and testicular development and poised a question on the validity of the data generated by studies involving hormonal manipulation. For example, Abbot and Hearn reported that testis volume and plasma testosterone began to rise on 250th day or 7th month of age (Abbott and Hearn, 1978). This postnatal time is considered as start of puberty and the end of the juvenile phase and is characterized by night time increase in LH pulses followed by a sudden increase in testis volume in higher primates (Plant, 2005). Jackson and Edmonds pointed 60th week (14 months) of age to be considered as testicular maturity in terms of accomplishment of spermatogenesis where all stages of spermatogonia were present (Jackson and Edmonds, 1984). On the other hand Kholkute et al (1983) considered 12-16 month old marmosets as prepubertal animals.

As a clear understanding of the process of primate puberty and the precise time period required for different post natal developmental stages in common marmoset is still under debate and vividly argued. We attempted to unravel the pubertal ontogeny in terms of testicular growth in common marmoset. We selected an observatory approach where

the animals were not manipulated in any way. Thus a normal postnatal growth pattern was observed by collecting monthly samples for body weight, testis size and serum testosterone. We found out that the activation of hypothalamic pituitary gonadal axis in terms of the presence of high serum levels of testosterone in common marmoset occurs around (28 weeks \pm 2). Few animals did not show any testosterone rise till 10 or 12 months, although their testis size was comparable with animals which have shown testosterone rise at 7 month of age. As in common marmoset the predominant trigger for testosterone is a CG-like molecule having a short serum half life, unlike the LH in higher primates. We assumed that the animals, which failed to show any testosterone rise up till 10th or 12th of age, have actually already gone through the pubertal activation of testosterone but the testosterone peaks were missed in once a month blood sampling regime, as the CG-like molecule is active for a very short time in serum as compared with LH. Our assumption is also supported by the histomorphometric data where the animals with low serum testosterone depicted ongoing spermatogenesis at 10 and 12 month of age. We propose that for the correct analysis of the testosterone status of the 6-12 months old marmoset monkeys, weekly blood sampling regime should be employed because normally it is not possible to histologically analyze the testis tissue if the respective animals is to be employed for further experimentation. Our testosterone data also mimicked the classical findings documented by Abbot and Hearn (Abbott and Hearn, 1978). Although it was shown previously that in common marmoset the quiescent juvenile period lasts till 35 weeks (8.5 months) of age (Lunn et al., 1997; Kelnar et al., 2002). Our data concerning the body weight gain pattern depicted an important aspect of postnatal development in common marmoset. The maximum body weight gain had been observed well within the first 6 months of age, or in other words before the pubertal activation. After 6 months the animals showed a slow rise in the body weight. Either this initial weight is a prerequisite for the pubertal activation is unknown. It seems that growth and reproductive axis are controlled independently in common marmoset where the time courses for somatic and reproductive development do not overlap.

At the testicular level the major germ cell migration towards the periphery and the arrangement of Sertoli cell nuclei in an epithelial like fashion seems to be completed well within the first 6 months of development. After 6 month of age the seminiferous tubules

in common marmoset showed a rapid response towards the high serum levels of testosterone by the formation of a lumen around 28 weeks (7 month) of age. The formation of the lumen is indicative of the fluid secretion from Sertoli cells which attain functional maturity characterized by an increase in Sertoli cell cytoplasm (Sharp et al., 2003). As depicted by the point counting, due to the increase in size the of the Sertoli cells, the percentage of the epithelial area also increased sharply after 7 month of age. The formation of lumen and the increase in the Sertoli cell size collectively increase the seminiferous tubule diameter at the start of the puberty. The increase in tubule diameter as well as in the testis volume observed after 44 week (10 months) was mainly due to the proliferation of germ cell as the B spermatogonia, primary spermatocytes and secondary spermatocytes were observed during this time. So the initial increase in testis size observed at 7 month of age is caused by somatic activation while the increase in testis size observed after 8-10 months of age is due to the increase in the germ cell compliment. Sperm were first observed in the epididymis at 12 months of age and the testis weight and testis volume increased almost two fold as compared with the 10 month old age group. This sudden increase in testis volume at 12 month of age is not the start of puberty in common marmoset. Thus a one year old marmoset should be considered a pubertal animal, although the testis volume continues to increase after this age but the spermatogenesis is qualitatively achieved at this time. So the time from the normal onset of puberty till the presence of epididymal sperm in common marmoset seems to be around 5 months. This time period is very short as compared with higher primates and also shows that testicular cells in common marmosets are normally capable of a swift response towards pituitary gonadotropins. Our endocrine, orchidectomy and histological data in common marmoset suggest a similar pattern of pubertal testicular development towards higher primates but requiring a significantly less time to achieve these developmental features as their higher primate counterparts.

In conclusion the puberty in common marmoset is not initiated before 6 months of age. Pubertal onset is preceded by a period of accelerated somatic growth. The hypothalamic control of the somatic and reproductive axis seems to be in a chronological order where the pubertal growth comes later. Either this early weight gain is a prerequisite for the normal pubertal initiation remains to be answered. The Sertoli

arrangement and germ cell migration seems to be completed before the onset of puberty in common marmoset. Further studies on the involvement of kisspeptin in pubertal activation, dynamics of GnRH and CG interplay at the pituitary level, the timing of the androgen expression by developing Sertoli cells and the hormonal regulation of germ cell development are needed to fully characterize hypothalamic and gonadal ontogeny of pubertal development in common marmoset.

Chapter 5

General Discussion

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The current thesis focuses on functional and developmental aspects of the primate testis. The specific aims and objectives of the present study were firstly to sort out a possible functional role of kisspeptin-kisspeptin receptor signaling in adult primate testis, secondly to sort out the cytological localization of the kisspeptin receptor in the adult primate testis and thirdly to characterize the pubertal testicular development in common marmoset: a new world monkey. To achieve these objectives, three separate experiments were conducted using an in vivo approach. For the first two objectives adult animals were employed whereas for the third objective immature animals were used. The animal models selected in the current study included Old World monkeys and New World monkeys. Rhesus monkey (*Macaca mulatta*): a representative higher primate was employed for the first and second objective whereas common marmoset (*Callithrix jacchus*) was employed in the second and third objective.

The primate testis performs two main functions namely spermatogenesis and steroidogenesis where successful spermatogenesis is dependent on the availability of testosterone through the process of steroidogenesis. The first objective which is the functional aspect of kisspeptin-kisspeptin receptor signaling in the adult primate testis was sorted out in terms of the ability of the kisspeptin to affect the testosterone secretion (steroidogenesis). It is interesting to note that the kisspeptin has the ability to enhance the serum levels of testosterone but this increase in the testosterone levels is due an increase in the LH levels. Increased LH secretion is in turn occasioned by action of kisspeptin at the hypothalamus resulting in an enhanced GnRH release. In general, to observe an effect of a given iv bolus on the testosterone secretion which is solely at the testis level, it is a prerequisite that the testis should not be exposed to pituitary gonadotropins.

In the first experiment, to observe an effect of kisspeptin which is solely at the testis level and to prevent the kisspeptin to influence pituitary LH secretion, pituitary gonadotropin clamped monkey model was employed. The chemical hypophysectomy was employed and achieved by pretreating the animals with acyline, a GnRH receptor antagonist. In the pituitary clamped monkey model, the kisspeptin (50µg) given as an iv bolus significantly enhances stimulated testosterone levels while having no effect on the basal testosterone levels. This result demonstrates that kisspeptin does not affect Leydig cells directly, as the direct action of kisspeptin at the Leydig cells would result in an effect on the basal testosterone levels. Although the observation of an effect on the stimulated testosterone levels indicates the presence of a functional kisspeptin-kisspeptin receptor signaling cascade which can affect the Leydig cell responsiveness towards LH/hCG. However, it seems that kisspeptin-kisspeptin receptor signaling can only influence the Leydig cell steroidogenesis in the presence of LH/hCG. These results also hint towards an indirect action of kisspeptin on Leydig cells via another testicular cell type. As the paracrine interaction exist between different testicular cell types and this local communication is necessary for the proper functioning of the testis (Schlatt et al., 1997). So it is plausible that kisspeptin-kisspeptin receptor signaling affect Leydig cell steroidogenesis indirectly through another testicular cell type. Sertoli cell, germ cell and peritubular myoid cell are the other cell types present in the testis where Sertoli cells has been shown to secrete factors which have the ability to modify Leydig cell steroidogenesis (Saez et al., 1989). It has been demonstrated in vitro that Sertoli cell factors increased Leydig cell steroidogenesis and this effect was augmented in the presence of LH or hCG (Papadopoulos et al., 1987; Papadopolus, 1991; Sharpe, 1985).

The second objective was to find out the testicular cell type positive for kisspeptin receptor. To localize the specific cell type immunocytochemistry was employed on the testis tissue obtained from two adult rhesus monkeys as well as two adult common marmoset monkeys. The testicular immunocytochemistry revealed that the kisspeptin receptor is localized inside the seminiferous tubules. The area found positive for the kisspeptin receptor was the outer periphery of the seminiferous tubules. This area contains the undifferentiated A spermatogonia along with the basal surface of the Sertoli cells. The detailed analysis revealed

that Sertoli cell membrane around the A spermatogonia exhibited strong staining for kisspeptin receptor. The same staining pattern was also found in the new world monkey testis. The kisspeptin like immunoreactivity was found to be present in the interstitial area. It has been shown previously that interstitial area was positive for kisspeptin like immunoreactivity in mouse and vertebrate testis (Anjum et al., 2012; Chianese et al., 2013; Hua et al., 2013). In particular Leydig cell were found positive for kisspeptin in these above mentioned studies. These immunocytochemical findings not only support our previous finding of an indirect action of kisspeptin on Leydig cell steroidogenesis but might also reveal a much unexpected and important aspect of kisspeptin-kisspeptin receptor signaling at the testis level. As kisspeptin was initially discovered as an anti-metastatic peptide, initially called metastin found in the metastatic cancerous tissue (Lee et al., 1996; Lee and Welch, 1997). Kisspeptin has been shown to influence metastasis through its antimetastatic effects by inhibiting chemotaxis and cell migration (Hori et al., 2001; Yan et al., 2001; Harms et al., 2003; Nash and Welch, 2006; Navenot et al., 2009). During the testicular development the germ cell migrate from the center of the tubules towards the periphery. During migration, these cells are called gonocytes (Culty, 2009) and once they reach the periphery and establish contact with the basement membrane they are known as undifferentiated A spermatogonia (Plant, 2010). It is not clear that whether these cells inherently lost the migratory ability once they establish the contact with basement membrane or this migratory ability is somehow compromised through the action of another cell i.e. Sertoli cell. As the peripheral area of seminiferous tubules which exhibited strong signal for kisspeptin receptor has also been implicated as spermatogonial stem cell niche (de Rooij, 2009). It seems that kisspeptin receptor is the new candidate for the spermatogonial stem cell niche region. Further studies are needed to delineate the precise role of kisspeptin receptor as a niche player in primate testis. Keeping in view the finding of an efficacious intratesticular action of kisspeptin on stimulated testosterone and the immunocytochemical finding of a tubular localization for kisspeptin receptor where Sertoli cells were found to be the main cell type positive for kisspeptin

receptor signal, it is probable that kisspeptin might act on the Sertoli cells via kisspeptin receptor and the receptor activation initiates a secretory response from the Sertoli cells which in turn enhances the responsiveness of the Leydig cells towards LH/hCG. This notion is supported by previous studies where Sertoli cell produced factors have been shown to influence testosterone secretion from the Leydig cell or affect Leydig cell response towards gonadotropins (reviewed in Lejuene et al., 1992).

The third objective of the study was to characterize the pubertal and subsequent testicular development in common marmoset (*Callithrix jacchus*): a representative New World monkey model. Common marmoset exhibits unique reproductive endocrinology in terms of the presence of a chorionic gonadotropin (CG) like molecule instead of LH in HPG axis interplay. This CG-like molecule has a much shorter serum half life as compared with LH (Muller et al., 2004). Combined with the classical report about the different steroidogenic pathway ($\Delta 4$) used by marmoset Leydig cells compared with higher primates including man (Preslock and Steinberger, 1977). And the lack of the availability of functional data on the dynamics of hypothalamic-pituitary-gonadal axis during puberty in the presence of CG instead of LH not only obscure the recent efforts to associate pituitary and testicular interplay during pubertal development in common marmoset (Chandolia et al., 2006) but also demands different approach towards understanding the pubertal ontogeny in common marmoset. Our results demonstrated that the earliest pubertal onset characterized by high serum testosterone levels in common marmoset was found to occur around 7 months of age. The major body weight gain was observed in the first 6 months employing that the somatic growth and pubertal growth do not coincide in this monkey. The puberty in higher primates is preceded by a juvenile phase of development characterized by stable testis volume, low serum testosterone and a rapid body weight gain (Plant, 2005, 2006). Common marmoset shows a somewhat higher primate like pattern of postnatal development although higher primates require much longer time to reach puberty whereas the common marmoset require significantly less time, depicting a highly responsive testicular tissue towards pituitary gonadotropins. At the testis level the time period from the first

testosterone peak to the presence of epididymal sperm was around 5 months. These 5 months are characterized by rigorous somatic and germ cell activity at the testis. While the major cellular activity shown by a prepubertal and juvenile testis is in terms of gonocyte migration from the center of the seminiferous tubules towards the periphery. This migratory activity was completed before the onset of puberty indicating that the germ cell migration is an innate ability of gonocytes to reach the peripheral areas of the seminiferous tubules and is also independent of HPG axis interplay. This feature had been documented before and found to be highly similar with gonocytes migration in human testis (Mitchell et al., 2008). In conclusion common marmoset has a swift progression from an immature state to a mature stage and one year old marmoset testis show qualitatively established spermatogenesis although the quantitative spermatogenic out continue to increase after one year of age.

The overall conclusion of the current thesis is that the non human primates offer an excellent model to study the primate testis biology as the testicular morphology and functionally is preserved across non-human primate models. The findings of the current thesis emphasize that further studies are needed to delineate the involvement of kisspeptin-kisspeptin receptor signaling in the Sertoli cell functionality by using pharmacological approaches in *in vitro* and organ culture model. Also the possible involvement of kisspeptin receptor in spermatogonial stem cell niche in the context of germ cell migratory ability in the immature testis as well as its role as an anti migratory signal for the A spermatogonia in the adult primate testis needs to be examined. In common marmoset, studies are needed to characterize the endocrine requirements for the pubertal onset in terms of kisspeptin signaling at hypothalamic level as well as the kinetics of the gonadotropic secretory activity from the pituitary gland during pubertal activation and the downstream requirement by LH/CG receptor present on Leydig cells for activation and producing an adequate androgenic response during pubertal activation.

Chapter 6

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