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THE SEASONAL AND STAGE-RELATED CYCLES OF LIPID DROPLETS IN  
SERTOLI CELLS IN THE SEASONAL BREEDING MINK *Mustela vison*:

by

Lyndon E. Keeping, BSc.

A thesis submitted to the School of  
Graduate Studies and Research  
in partial fulfilment  
of the requirements for the degree of  
Master of Science in Anatomy

Department of Anatomy  
University of Ottawa  
Ottawa, Ontario

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## ABSTRACT

The accumulation of lipid inclusions in Sertoli cells have typically been associated with interruptions of spermatogenic activity and have therefore been considered as storage organelles for either hormone precursors or waste material. It is our contention that the amount of Sertoli cell lipid inclusions varies with the degree of spermatogenic activity and that a seasonal arrest of spermatogenesis coincides with a reduction in the Sertoli cell lipid droplet content. The relationship between Sertoli cell lipid inclusions and spermatogenic activity was investigated using light microscopy and morphometric analysis of the lipid droplet content of Sertoli cells and spermatids from the seasonal breeding mink. Tissue was obtained and analyzed each month during a twelve month period to establish the presence of seasonal and stage-related cycles of lipid droplet content in these cells. The tissue was fixed by perfusion and treated with the potassium-ferrocyanide-tannic acid uranyl-acetate *en bloc* staining technique (PFTA) to optimize contrast of cytoplasmic details, thereby facilitating the distinction of germ cells and Sertoli cells. During the height of spermatogenic activity, when the complete contingent of germ cells was present in the seminiferous epithelium, lipid droplets were studied in spermatids and Sertoli cells. Lipid droplets were observed in spermatids at all phases of spermiogenesis. From the relatively low lipid droplet volume density observed during the Golgi and cap phases of spermiogenesis, during the acrosome phase, the lipid droplet content increased by approximately three fold but then decreased again during the maturation phase. At spermiation this reduced amount of lipid was localized in the cytoplasmic droplet and in the residual body. When separated by stage of the cycle, spermatid lipid droplet content varied in relation to the

stage. Stages in which both round and elongated spermatids were found i.e., I-VII, had more spermatid lipid droplets than stages in which only one population of spermatids i.e., VIII-XII was present. Lipid droplets in Sertoli cells also varied in a stage-dependent manner during the breeding season. Sertoli cells that were associated with populations of both round and elongated spermatids had significantly higher amounts of lipid inclusions than Sertoli cells associated with only one population of spermatids. The lipid droplet content of spermatids and Sertoli cells combined also varied in relation to the stage of the cycle. Prior to the release of mature spermatids into the lumen, the combined lipid droplet content remained approximately constant due to the increasing contribution of Sertoli cell lipids and the decreasing contribution of germ cell lipids. Following spermiation however, the combined value decreases abruptly owing equally to a decrease of lipids in spermatid and a decrease in lipids in Sertoli cell.

During testicular regression when only an incomplete number of classes of germ cells was present, lipid droplets were studied in Sertoli cells only. At the onset of testicular regression, the lipid droplet content of Sertoli cells increased by 25% over the average of breeding season levels. Later in testicular regression, when spermatogenic activity was lowest, Sertoli cell lipid droplet content was less than 30% of breeding levels.

Preliminary investigations were also conducted to investigate the involvement of cholesterol in the spermatogenic process. Cytochemical studies of the mink testis using cholesterol oxidase and cholesterol esterase in conjunction with 3,3-diaminobenzidine revealed that cholesterol esters are a component of lipid droplets in Sertoli cells, spermatids, and Leydig cells. In addition, the Sertoli cells, germ cells and Leydig cells were separated at strategic times of the mink's seasonal breeding cycle and analyzed biochemically for free and esterified cholesterol.

During the height of spermatogenic activity, the cholesteryl esters in the enriched Sertoli cell population was 30  $\mu\text{g}/\text{mg}$  of protein. This value increased briefly during the onset of testicular regression but then decreased to only 6  $\mu\text{g}/\text{mg}$  protein when spermatogenic activity was at its lowest.

It is concluded that the amount of lipid inclusions in Sertoli cells varies with the degree of spermatogenic activity being lowest after the release of mature spermatids in the lumen during the active spermatogenic phase and at the time of maximal testicular regression. Furthermore, cholesterol esters are a component of these lipid inclusions and the seasonal changes in lipid inclusions of Sertoli cells are reflected by seasonal changes in the amount of Sertoli cell cholesterol esters.

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## 1. INTRODUCTION

The testis is made up of about eighteen hundred meters of convoluted tubules (De Graaf, 1668) bound together by interstitial tissue. The tubules and the interstitium constitute two functionally distinct compartments: namely the site of male germ cells production i.e., spermatogenesis, and the site of male sex hormones production i.e., androgenesis. Although lipids, particularly cholesterol, are known to play an important role in androgenesis, the present work is directed towards the evaluation of lipids as structural participants in germ cell differentiation and this aspect will therefore receive the most attention. However, because spermatogenesis is dependent on testicular hormones from steroid lipids, a brief exposé dealing with some of the endocrine aspects of spermatogenesis will be included.

### 1.1 Endocrine aspects of testicular activity

#### **1.1.1 Endocrine Factors Effecting Spermatogenesis**

The most important regulatory hormones for spermatogenesis are follicle stimulating hormone (FSH) and testosterone (Steinberger, 1971). Follicle stimulating hormone is a dimeric glycoprotein secreted from the adenohypophysis in pulses of high biological activity (Ellis and Desjardins, 1982; Ellis et al., 1983) in response to the pulsatile signal of gonadotropin releasing hormones (GnRH) from the hypothalamus. Changes in reproductive state, such as those imposed by seasonal regression or orchidectomy, are usually associated with changes in the amplitude and frequency of these secretory pulses (reviewed by (Ellis and Desjardins, 1984; Lincoln, 1981)). FSH binds to specific receptors on the Sertoli cell plasma membrane (Orth and

Christensen, 1977; Reichert and Dattatreya Murty, 1989). Signal transduction occurs through guanine nucleotide-mediated adenylate cyclase activation (Rodbell et al., 1971) and the consequent activation of cAMP-dependent protein kinases (Abou-Issa and Reichert, 1976; Dufau et al., 1980; Means et al., 1976; Mendelson et al., 1975; Podesta et al., 1976).

Testosterone is a steroid hormone synthesized by the interstitial Leydig cells from stored cholesterol (Catt et al., 1974; Federman, 1981). Synthesis and secretion of testosterone are promoted by luteinizing hormone (LH) (Christensen, 1975; Ewing et al., 1983; Neaves, 1975; Neaves, 1978; Pokel et al., 1972; Sandler and Hall, 1966; Sandler and Hall, 1966) which, like FSH, is secreted from the adenohypophysis in response to GnRH. Testosterone binds to specific receptors found within the Sertoli cell nucleus (Sanborn et al., 1981), where it may be involved in the modification of transcription (Mills, 1990).

### 1.1.2 Local Control of Testosterone Synthesis

Luteinizing hormone is not the only factor to effect steroidogenesis in Leydig cells (Aoki and Fawcett, 1978; Bergh, 1982; Bergh, 1983; Parvinen et al., 1984; Sharpe et al., 1983). Paracrine or local factors from interstitial macrophages and/or Sertoli cells as well as catecholamines may modify the cell's ability to respond to LH secretion (for review see Sharpe, 1984; Sharpe, 1986; Sharpe et al., 1990; Tähkä, 1986). Leydig cells possess stereospecific receptors for luteinizing hormone releasing hormone (LHRH) -like peptides, and LHRH and its agonists have been shown to have direct stimulatory and inhibitory effects on the testicular microcirculation of blood (Sharpe et al., 1983; Sharpe et al., 1982), LH receptors (Sharpe and Harmor, 1983) and steroidogenesis (Bourne et al., 1982; Hsueh and Jones, 1981; Hunter et al., 1982; Sharpe and Cooper, 1982; Sharpe and

Cooper, 1982). There is evidence that LHRH-like peptide may be formed by the Sertoli cell (Cooke et al., 1989; de Jong et al., 1979; Nagendranath et al., 1983; Sharpe et al., 1981; Sharpe et al., 1982; Sharpe and Harmar, 1983). In addition, two other Sertoli cell peptides which influence Leydig cells have been described (Benahmed et al., 1985). LHRH from Sertoli cells influences the Leydig cell's LH receptor number and steroidogenic activity as well as the vascular resistance and capillary permeability in the immediate area (Damber et al., 1985; Veijola and Rajaniemi, 1985). This provides the Sertoli cell with an effective tool for the regulation of local testosterone concentrations (Sharpe, 1984).

The field of paracrine regulation however, is still in its infancy with most theories on mechanisms and even principles, requiring additional studies for verification. The consequences of local control of Leydig cells *in vivo* has not been well documented (Aoki and Fawcett, 1978; Cooke et al., 1981). A few reports (Bergh, 1982; Paniagua et al., 1988) describe a variation in the size of Leydig cells coincidental with the stage of the cycle in the juxtaposed tubule. No communication mechanisms or signal molecules were proposed. Kasuga (Kasuga et al., 1989) reported a difference in Leydig cells ability to respond to hCG dependent on the presence of germ cells. It is evident from these investigations that the day-to-day function of Leydig cells are modulated by local influences from testicular macrophages, germ cells and Sertoli cells and not just pituitary hormones.

### **1.1.3 Steroidogenesis and the Sertoli cell**

Steroid hormones are synthesized from stored cholesterol (Federman, 1981). The presence of lipid droplets and an abundance of smooth endoplasmic reticulum are cytological features commonly found in cells that synthesize steroid hormones. These features were reported in Sertoli cells of

the rat (Bell et al., 1971; Brökelmann, 1963; Christensen and Fawcett, 1961; Lacy, 1962; Lacy and Pettitt, 1969; Lacy and Pettitt, 1970; Nagano, 1966). This led to the proposal that lipid droplets in Sertoli cells were stores of steroid hormone precursors (Lacy, 1962). During normal spermatogenic activity, the precursor levels would decline, because steroid anabolism and export are active. Restocking of the stores would however, occur cyclically as lipids from the residual bodies are phagocytosed and brought down to the base of the Sertoli cells. The residual bodies would then serve as a signal triggering further steroid synthesis by the Sertoli cell. Because most residual bodies were reported to be released into the lumen with the spermatozoa, it was proposed that lipids may also serve as a negative feedback to the pituitary (Johnsen, 1964).

The mechanical separation of tubule from interstitium (Christensen and Mason, 1965) allowed for evaluation of the respective enzymatic activities of the two testicular compartments. Initial reports had proclaimed androgen production by seminiferous tubules from progesterone precursors (Christensen and Mason, 1965), but the enzymatic activity was too low to exclude the possibility of contamination by the interstitium. Androgenesis by seminiferous tubules was shown to proceed through different biosynthetic pathways than did the interstitial tissue (Bell et al., 1968; Bell et al., 1971; Lacy et al., 1969). This, coupled with the presence of lipid droplets and an abundance of smooth endoplasmic reticulum, established the Sertoli cell as a *bona fide* steroid-producing cell (Fawcett, 1975; Lacy and Pettitt, 1970; Maddocks and Setchell, 1988; Wiebe et al., 1980). Infusion of radio-labelled progesterone into the testicular artery resulted in the appearance of labelled compounds in the rete testis fluid. These compounds displayed a behavior on thin layer chromatography that was similar to androgens (Setchell and Waites, 1975). This was presented as evidence for androgenic activity by Sertoli

cells although there is no indication that the conversion from progesterone to androgen occurred within the Sertoli cell rather than the Leydig cell. Other evidence has suggested that estrogens are the most likely end product to the Sertoli cell's steroidogenic activity (Cooke et al., 1972; de Jong et al., 1974; Huggins and Moulder, 1945; Lewis and Stockard, 1950; Teilum, 1949). Interestingly, Leydig cells have been shown to have estrogen receptors (Steinberger and Steinberger, 1977). Sertoli cell steroidogenic activity also produces intermediates which are not formed by Leydig cells (Bell et al., 1971; Lacy, 1973). These intermediates may also be considered as potential regulators, acting locally on germ cells (Chemes et al., 1976) or on Leydig cells (Maddocks and Setchell, 1988; Tähka, 1986). Alternatively, the Sertoli cell-produced intermediate may act as a feedback signal to regulate the production and/or secretion of pituitary hormones (Fan et al., 1974; Kraulis et al., 1978; Steinberger and Fecher, 1973; Steinberger et al., 1973). One of the metabolites, 3HP ( $3\alpha$ -hydroxy-4-pregnen-20-one) has been shown to stimulate spermatocyte production in prepubertal and estradiol-benzoate treated rats (Campbell and Wiebe, 1989).

Sertoli cells have the capacity to synthesize cholesterol (Wiebe J.P., 1979), but not biologically active steroid hormones such as androgens progesterones or estrogens from acetate or from endogenous precursors (Parvinen and Niemi, 1971; Setchell and Waites, 1975). Radio-labelled progesterone precursors were converted into androgens by tubule fractions (Bell et al., 1968; Bell et al., 1971; Christensen and Mason, 1965; Cooke et al., 1972; Hall et al., 1969; Lacy et al., 1969; Wisner et al., 1975). The rate limiting enzyme in the process of steroidogenesis, and the one required for the conversion of cholesterol to pregnenolone is P450<sub>scc</sub> (cholesterol side-chain cleavage cytochrome) (Eik-Nes, 1970; Simpson, 1979). This enzyme is found in the tubular cristae of typical "steroidogenic

mitochondria" (Hall, 1984) of steroid producing cells (Waterman and Simpson, 1985) such as Leydig cells (Anderson and Mendelson, 1984; Bass et al., 1973; Mason et al., 1984; Payne, 1990; Scott et al., 1990), cells of the corpus luteum (Arthur and Boyd, 1974; Rodgers et al., 1986; Rodgers et al., 1988), the ovarian follicle (Farkash et al., 1986; Rodgers et al., 1986), the theca intima (Farkash et al., 1986), the adrenal cortex (Farkash et al., 1986; Jefcoate, 1975; Shimizu et al., 1961; Simpson, 1979), and the placenta (Nestler and Williams, 1987; Shimizu et al., 1961; Waterman and Simpson, 1985). Sertoli cells' mitochondria have the orthodox folliate cristae of non-steroid producing cells, although occasional tubular cristae have been reported in the rat (reviewed by (de Kretser and Kerr, 1988): In any case, rat seminiferous tubule fractions were shown to have only about 2% of the cholesterol side-chain cleavage ability of the accompanying interstitium (Bass et al., 1973). The enzymes responsible for the conversion of pregnenolone to progesterone, P450  $3\beta$ -hydroxysteroid dehydrogenase and  $17\alpha$ -hydroxylase, are also absent from seminiferous tubules (Sasano et al., 1989; van der Molen and Rommerts, 1981; van der Vusse et al., 1974; van der Vusse et al., 1975).

The peak of steroidogenic activity by the Sertoli cell occurs at puberty (Welsh and Wiebe, 1978; Wiebe et al., 1980) during the initiation of spermatogenesis, when signals to the pituitary and the interstitium for the promotion of gonadotropins and androgens would be of highest importance. In adult animals, the steroidogenic capacity of Sertoli cells is minimal (Armstrong et al., 1975; Dorrington and Armstrong, 1975; Dorrington et al., 1976; Raeside and Lobb, 1984), which raises the possibility that in adults, the steroidogenic machinery i.e., smooth endoplasmic reticulum and cholesterol containing lipid droplets, is involved in activities other than the conversion or synthesis of new steroid hormones. These may include sequestration Leydig cells-produced androgens by the Sertoli cells, or *de novo* synthesis of

cholesterol. Although cholesterol as a precursor to androgens plays an important role in spermatogenesis, the present work is directed towards the evaluation of lipids as structural participants in germ cell differentiation and this aspect will therefore receive the most attention in the following sections.

## 1.2 Spermatogenesis

The seminiferous epithelium has been viewed as a population of proliferating, differentiating, and migrating germ cells occupying the intercellular spaces of a non-proliferating, simple columnar epithelium of sessile Sertoli cells (Fawcett, 1979). Spermatogenesis incorporates three distinct cytological processes and each one involves a distinct class of germ cells (Clermont, 1972). Along the basement membrane of the epithelium, spermatogonia are involved in mitotic divisions to perpetuate their own numbers and to give rise to spermatocytes (de Rooij et al., 1989; Leblond and Clermont, 1952; Oakberg, 1956). A blood-testis barrier made up of Sertoli cell occluding junctions (Dym and Fawcett, 1970) separates the spermatogonia below from the spermatocytes above. After about eight divisions (Clermont, 1969; Clermont and Bustos-Oregon, 1968; Clermont and Leblond, 1953; Huckins and , 1971; Huckins, 1978) the cells move off the basement membrane and enter the long prophase of meiosis. Early in meiosis, (Connell, 1980; Dym and Cavicchia, 1977; Pelletier, 1986; Russell, 1978) spermatocytes appear above the barrier (Setchell, 1967) (Setchell et al., 1969) and become insulated from the plasma. The final process of spermatogenesis, spermiogenesis, involves a series of cytological transformations or "steps" which end with the formation of a highly differentiated cells: the spermatozoon, whose shape is species specific.

At a given step of their development, spermatids become consistently associated with the same classes of spermatogonia and spermatocytes and thus define a number of typical cellular associations that were first described by Brown in 1885 (Brown, 1885). The associations succeed one another in time (Regaud, 1901; von Ebner, 1888). The complete series of associations or "stages" was referred to as the "spermatogenic cycle" (Regaud, 1901; von Ebner, 1888) or as the "cycle of the seminiferous epithelium" (Leblond and Clermont, 1952). Roosen-Rünge and Giesel (1950) introduced a method of identification of the cellular associations that was based on the nuclear shape of spermatids and on the changes in meiotic figures. In the mink, this method was used to identify eight stages of the cycle (Deguchi, 1978; Onstad, 1967; Sakai, 1981; Tiba, 1973; Tiba et al., 1968). Leblond and Clermont (Leblond and Clermont, 1952) introduced a method based on the changes in acrosome conformation. The method allows for the identification of a greater number of stages of the cycle and is therefore more precise than the one proposed by Roosen-Rünge and Giesel (1950). Using a modified version of the stage identification method proposed by Leblond and Clermont (1952), Pelletier (1986) described 19 steps of spermiogenesis and twelve stages of the cycle in the mink, (see fig.1). This classification is the one used throughout the present text.

### **1.3 The Sertoli cell and Spermatogenesis**

The supporting cells were first recognized by Sertoli (1865). Von Ebner (1871) introduced the concept of a symbiotic relationship between Sertoli cells and germ cells, and described the "spermatoblast: a cluster of synchronously developing germ cells embedded in the apical portion of the supporting cell, as the functional unit of the germinal

epithelium". This section will review some aspects of this relation.

Sertoli cells do not require the presence of germ cells for survival (Bergh, 1981; Lacy and Lofts, 1962). However, they are affected by their presence. This suggests the existence of some form of communication between the two cell types. The Sertoli cell displays features which are dependent on the development of the adjacent germ cells. For instance, its nuclear morphology (Leblond and Clermont, 1952) and relative cell volume (Bugge and Ploen, 1986; Caviccia and Dym, 1977) have been reported to change in relation to the stages of the cycle in the rat. Its smooth endoplasmic reticulum was noted to be more abundant during stages which contain late spermatids (Brökelmann, 1961; Brökelmann, 1963; Flickinger and Fawcett, 1967) (Dym and Fawcett, 1970) (Ross, 1976). Its lipid droplet population apparently fluctuates in a stage-dependent manner in the rat (Kerr and de Kretser, 1975; Lacy, 1960; Niemi and Korman, 1965; Posalaki et al., 1968), mouse (Dietert, 1966), mink (Onstad, 1967) and human (Paniagua et al., 1987), but not in the monkey (*Macaca anulata*) (Dym, 1973), domestic boar (*Sus scrofa*) or African wart hog (*Phocochoerus aethiopicus*) (Fawcett, 1975). Stage-related variations in the volume of lipid droplets (Kerr et al., 1984) smooth endoplasmic reticulum, mitochondria, lysosomes, Golgi complex, granular endoplasmic reticulum and plasma membrane surface area of the Sertoli cells have also been demonstrated by morphometric analyses (Kerr, 1988; Ueno and Mori, 1990).

The introduction of a new method of identification of the stages of the cycle in fresh rat testicular tissue by transillumination (Parvinen and Vanha-Perttula, 1972), opened the door to the investigation of a crowd of stage-dependent biochemical events. Specifically, acid phosphatase IV (p-nitrophenyl phosphate active), was shown to have maximum activity at stages VI-VII, and minimum activity at stages XI-XII in the rat (Parvinen and Vanha-Perttula, 1972). The

list of biochemical events that display a cyclic, stage-dependent pattern has grown to include aminopeptidase III (Parvinen and Vanha-Perttula, 1972), FSH responsiveness (Kangasniemi et al., 1990; Parvinen et al., 1980), plasminogen activator (Lacroix et al., 1981), aromatase inhibitor (Boitani et al., 1981), FSH receptor numbers (Parvinen, 1982), androgen binding protein (ABP) (Ritzén et al., 1982), a mitogen-like factor (Johnsonbaugh et al., 1982), meiosis inducing substances (MIS) (Parvinen, 1982), transferrin (Mather et al., 1983), total protein production (Wright et al., 1983), proteins S70, S45 and S35 (Shabanowitz et al., 1986), cyclic protein 2 (Wright, 1988), pertussis toxin sensitivity (Huhtaniemi et al., 1989), and adenosin receptors concentration (Conti et al., 1989). Most non-secreted proteins do not show a stage-dependent variation in synthesis (Wright et al., 1983).

Each stage of the spermatogenic cycle presumably have different functional requirements (Parvinen et al., 1986; Sharpe, 1983; Sharpe et al., 1990). When testosterone levels were reduced by gonadotrophin deprivation, germ cells that are characteristically present at Stage VII of the cycle, namely mid-pachytene primary spermatocytes and step 7 and step 19 spermatids, degenerated (Dym and Madhwa Raj, 1977; Russell and Clermont, 1977; Russell et al., 1981). Plasminogen activator (PA) is secreted by Sertoli cells principally at Stage VII and VIII in the rat (Lacroix et al., 1981). It has been proposed that this factor is linked to the migration of spermatocytes and the release of mature spermatids (Lacroix et al., 1977). These reports indicate that germ cells have special function-related requirements for precise developmental steps to occur and that Sertoli cells are able to respond to those changing needs.

### 1.3.1 Germ cell-Sertoli cell Communication

Sertoli cells support the proliferating germ cell population mechanically with lateral cytoplasmic processes, and alimentary by supplying germ cells with their special nutritive requirements (Bishop and Griswold, 1987; Griswold, 1989; Jutte et al., 1981; Jutte et al., 1982). There is growing evidence that germ cells dictate their needs to Sertoli cells and that these merely respond to them (Grootegoed et al., 1989). The synthetic activities of Sertoli cells (de Philip and Kierszenbaum, 1982; Jutte et al., 1982) but not germ cells (Clermont and Harvey, 1965; Grootegoed et al., 1979) is modulated by the incubation milieu and the extracellular environment. Pachytene spermatocytes maintain a stage specific pattern of protein secretion even in the absence of Sertoli cells (Jutte et al., 1985). The plasminogen activator secreted by Sertoli cells is proportional to the number of preleptotene spermatocytes (Vihko et al., 1984). The phosphorylation of particular Sertoli cell proteins *in vitro* is stimulated by germ cells' presence (Ireland and Welch, 1987). The synthesis and secretion of total protein, transferrin and specific peptides by Sertoli cells is stimulated by proteins secreted by pachytene spermatocytes (Djakiew and Dym, 1988). Androgen binding protein secretion by Sertoli cells was correlated with the presence of late spermatids and spermatozoa in neutron and  $\gamma$ -irradiated rats (Pineau et al., 1989). The secretion of specific proteins (testins) by Sertoli cells may be suppressed by germ cells (Cheng et al., 1989). Inhibin production by Sertoli cells is modulated by spermatocytes (Pineau et al., 1989). These reports suggest that the Sertoli cells support rather than direct germ cell differentiation. An active role for the Sertoli cell may be required during initiation or reinitiation of spermatogenesis (Kancheva et al., 1990).

The form taken to communicate the germ cells' requirements to the Sertoli cells remains unclear. The gap junctions observed between Sertoli cells and germ cells (McGinley, 1979; Szollosi and Marcaillou, 1980) presumably allow for transmission of information by intracellular molecules making a responsive and intimate interaction between the two cell types. In addition to the gap junctions, tight junctions (McGinley et al., 1977; Pelletier and Friend, 1983), and adhering junctions (Kaya and Harrison, 1976; Russell, 1977) have also been observed between germ cells and Sertoli cells. Presumably, cell recognition precedes the formation of any shared junctional membrane specialization. This recognition may involve cell surface signals. In this regard, Pelletier and Byers (Pelletier and Byers, 1990) have proposed the involvement of germ cell specific adhesion molecules that would act in concert with Sertoli cell adhesion molecules to synchronize blood-testis barrier function with germ cell development. In addition, molecules on the surface of pachytene spermatocytes have been shown to promote adhesion to Sertoli cells (D'Agostino and Stefanini, 1987). It is possible that these molecules are similar to those described in the nervous system where specific cell adhesion molecules have been shown to affect development of particular neurons (Rutishauser et al., 1988). Alternatively, the message may be carried by a secreted signal that would enter into the space which separates the plasma membranes, reminiscent of neurotransmitters in the synaptic cleft (Wright, 1988).

### **1.3.2 Germ cell Influence on Sertoli cell Lipids**

Lipids can be divided into two groups: neutral lipids and acidic lipids. Neutral lipids, namely triacylglycerides and cholesteryl esters, are storage forms of high energy metabolites and steroid hormone precursors, whereas acidic lipids, namely fatty acids and free cholesterol, are the more

active and typically transitional forms of those metabolites. Lipid soluble dyes such as Sudan black and Oil red O are used to nonspecifically localize lipids in frozen sections. Nile blue is used to distinguish neutral lipids from acidic lipids. High concentrations of cholesteryl esters can be identified using the combination of techniques, namely sudanophilia, birifringence, and acetone solubility. The presence of free cholesterol in frozen tissue sections is indicated by phenylhydrazine reactivity and the formation of digitonides with digitonin. Cholesterol can also be localized using the Schultz test for unsaturated sterols (Schultz, 1924) (Kiernan, 1981).

#### 1.3.2.1 Sertoli cell lipids in relation to the spermatogenic cycle.

Von Ebner (1888), found that lipids were more abundant in the basal region of seminiferous tubules where elongated spermatids were absent in the rat. Observations performed on frozen sections stained with Oil Red O to estimate the variation in the lipid content of the tubules at different stages of the spermatogenic cycle also substantiate the same findings (Lynch and Scott, 1951). In short, they showed that when mature spermatids were present in a given tubule, lipids occupied the apex of the epithelium. When they were absent, the lipids were located next to the base. Since both instances never occurred at the same point in time, it was proposed that lipids may form a nutritive support for germ cells probably contained within the Sertoli cell and eliminated towards the lumen upon completion of spermatid maturation (Lynch and Scott, 1951). One obvious limitation of these early studies was precisely the difficulty to decide whether the lipid under observations belonged to the Sertoli cells or to the germ cells.

Histochemical and electron microscopy studies revealed the presence of lipidic inclusions within the caudal cytoplasm of rat elongated spermatids (maturation phase i.e.,

step 15 to step 19) (Kingsley Smith and Lacy, 1959). Furthermore, the same studies also revealed the appearance of lipid droplets at the base of Sertoli cells following the release of mature spermatids. These observations led to the suggestion that this accumulation of lipid droplets in Sertoli cells was derived from the phagocytosis of lipids contained in residual bodies of the mature spermatids (Kingsley Smith and Lacy, 1959). When the variation in lipid content of the tubules determined by Sudan black B was correlated with the stages of the cycle in the rat, the basal lipid inclusions present in Sertoli cells were most abundant just after spermiation (Stage IX) and fewest just prior to the completion of meiosis (Stage IV) (Lacy, 1960). From these observations it was proposed that most lipids seen within the Sertoli cells after spermiation (i.e., from Stage IX to Stage XIV) were derived from lipids that had once belonged to spermatids' residual bodies. A smaller fraction of lipids would be converted into a Sertoli cell hormone that would locally stimulate the completion of meiosis (Lacy, 1960; Lacy, 1962). However this proposal has not been confirmed.

By comparing frozen sections stained with Oil Red O, to adjacent sections stained with periodic acid Schiff (PAS), Niemi and Kormanio (1965) established the lipid content for some of the stages of the cycle that were not previously well identified. Their results showed that the decrease in the number of Sertoli cell lipid droplets was gradual from just after spermiation (Stage IX) to just before spermiation (Stage VII). They showed that during spermiation, most residual bodies and their lipids were sloughed into the lumen of the seminiferous tubules and passed down to the epididymis. Only a small proportion of them were phagocytosed by Sertoli cells and accumulated at their base.

Histochemical techniques were used to compare the localization of lipids to the activities of lysosomal enzymes such as acid phosphatase, non-specific esterase and aryl

sulfatase in rat Sertoli cells (Posalaki et al., 1968). This study showed that lipid content and the activity of hydrolytic enzymes were cyclic but in opposite directions. Specifically, Sertoli cell lipid content was low when the Sertoli cell hydrolytic activity was high and when mature spermatids were present. Conversely, at times when mature spermatids were absent, Sertoli cell hydrolytic activity was low, and Sertoli cell lipid content was high. The results suggested that a relationship existed between phagocytosis of residual bodies by Sertoli cells and accumulation of lipids in Sertoli cells, but that the decrease in Sertoli cell lipid droplets was related to an increase in the lysosomal activity of the supporting cells.

In more recent studies, identification of stages of the cycle was conducted on 0.5  $\mu\text{m}$  semithin sections stained with toluidine blue. The use of thinner sections allowed a better resolution and thus favour a more accurate localization of lipid droplets. Hence, lipid droplets were recorded in oldest primary spermatocytes and in step 15 to step 19 rat spermatids only (Kerr and de Kretser, 1975). Lipids in Sertoli cells were reported to decrease in number before spermiation and to increase after this process. This accumulation of lipids in Sertoli cells was found to occur one complete stage after spermiation in the rat (Stage X) and gradually reaching maximum levels at Stage XIII suggesting that these lipids are not derived from the phagocytosis of residual bodies alone. Furthermore, the decrease in the lipid droplets occurred long after meiosis was completed (i.e., Stage II) indicating that a hormone derived from this lipid would not be directly involved in determining the completion of meiosis as had been proposed (Lacy, 1960; Lacy, 1962). Quantitation of lipid droplets by morphometric techniques indicated that lipids began to increase immediately at spermiation and gradually reached a maximum level during meiosis (Kerr et al., 1984). This accumulation of lipids was attributed to the phagocytosis of residual

bodies by the Sertoli cells after lipid resynthesis from the hydrolyzed components. Kerr and his colleagues (Kerr et al., 1984) reasoned that esterases and lipases in Sertoli cell phagosomes would digest lipids and ester complexes present in residual bodies but that the basic subunit components thus liberated would provide the "building blocks" for the synthesis of new lipids by Sertoli cells. The observed cyclic decrease in the amount of lipid droplets in the Sertoli cells may indicate utilization of lipidic material by the Sertoli cell. Moreover a correlation between the timing in the decrease of the amount of lipid droplets in Sertoli cells and germ cell translocation, meiotic completion, or spermiation is not apparent.

At present, most published reports suggest that lipid droplets in rat Sertoli cells, increase in number after spermiation. This conclusion is consistent with the idea that these lipids originate from residual bodies of the mature spermatids. Sertoli cells would seemingly dispose of the lipids in a relatively rapid way since from when they are maximal (Stage XIV) to when they are minimal (Stage IV) only four days have been calculated to elapse in the rat (Clermont and Harvey, 1965).

#### 1.3.2.2 Sertoli cell lipids and seasonal spermatogenic activity.

Testicular seasonal regression is associated with a decline in spermatogenic activity and a decrease in sperm production. The peak of spermatogenic activity is referred to as the *zenith*, and the trough or minimum of spermatogenic activity is referred to as the *nadir*. (Lincoln, 1981) broke down seasonal breeding males into three categories according to the degree to which spermatogenesis declines at the nadir. In category 1, a reduced number of germ cells complete spermatogenesis; in category 2, some germ cells enter meiosis but they do not complete spermatogenesis; in category 3, no germ cells enter meiosis, and the germinal epithelium

consists of only spermatogonia and Sertoli cells. In this text, these categories will be referred to as *mild*, *moderate* and *emphatic* seasonal breeders respectively.

In most seasonal breeders, the annual reproductive cycle is partially echoed by variations in the content of lipids in seminiferous tubules. During testicular regression of birds (Marshall, 1955), the teleost fish (Lofts and Marshall, 1957), amphibians (Lofts, 1964; Lofts and Boswell, 1960), and reptiles (Lofts and Boswell, 1961; Sanyal and Prasad, 1965), an accumulation of sudanophilic material formed a lipid "plug" that occluded the depleted tubules (see review by (Lofts, 1972). In the frog *Rana esculenta*, the plug remained until reinitiation of spermatogenesis (Lofts, 1964). In mammalian seasonal breeders there is apparently no plug but variations in the amount of lipids within the Sertoli cell has been documented. In moderate seasonal breeders such as the Japanese deer (*Cervus nippon*) and the Virginia deer (*Odocoileus virginianus borealis*), coarse droplets of sudanophilic material were found at the periphery of the tubules while fine droplets were found scattered throughout the tubules during the breeding season (Wislocki, 1949). The lipid droplets were reported to be fewer in number and smaller in size during testicular regression (Wislocki, 1949). Conversely, they were more numerous and larger during testicular regression in *Rattus fuscipes* (Hodgson et al., 1979), rhesus monkey (Sehgal et al., 1986) and golden hamster (Sinha Hikim et al., 1989) also moderate seasonal breeders. Therefore, most studies on mild and moderate seasonal breeders agreed that the number of lipid droplets increases in Sertoli cells when spermatogenic activity is reduced.

In emphatic seasonal breeder such as the civet cat (*Paguma l. larvata*), large lipid droplets accumulate at the periphery of the tubules at the onset of testicular regression but during the regression itself and during and recrudescence of spermatogenesis, no lipid droplets were reported in the tubules (Tsui et al., 1974). Onstad (1967)

demonstrated a variation in the presence of sudanophilic material in the emphatic seasonal breeding mink. At the peak of spermatogenesis, the amount of lipid droplets in the tubules varied according to the stage of the cycle being in greatest amount next to the base of the Sertoli cells following spermiation. In the germ cells, lipid droplets seemed to increase with spermiogenesis as evidenced by the appearance of a dark sudanophilic zone near the centre of the tubules. The intratubular content of lipid droplets was reduced during testicular regression.

### 1.3.2.3 Sertoli cell lipids in Cryptorchid Testis

Neutral lipid content was reported higher in congenital cryptorchid whole testis homogenates (Hanes and Rosenbloom, 1911). Although the absolute amount of neutral lipid per testis was not different in cryptorchid than in normal testes, because the size of each testis was reduced by fifty percent, the amount of lipid per gram of tissue was twice as high in animals with cryptorchidism. Frozen sections stained with Sudan III and hematoxylin, revealed that the increased concentration of lipids was localized in the seminiferous tubules of these cryptorchid animals. Moore (1924) proposed that the purpose for the localization of the testis in the scrotum is to maintain a lower the temperature of 34°C. He explained that the absence of spermatogenic activity in the cryptorchid testis was due to the elevated ambient temperature of the abdominal cavity i.e., 37°C.

Experimentally induced cryptorchidism and externally applied heat to the testis will cause arrest of spermatogenesis at the spermatocyte stage in the adult rat. Heat will cause an increase in testicular neutral lipids as detected by the Nile blue test (Collins and Lacy, 1969). With mild heat treatment (less than twenty minutes at 43°C) spermatogenesis and lipid content in Sertoli cells returned to normal within six weeks. When treatment eliminated all

meiotic and post-meiotic germ cells (thirty minutes at 43°C), spermatogenesis did not recover after six weeks and the high contents of lipid in Sertoli cells remained unchanged. Collins and Lacy (1969) suggested that the lipid accumulation in Sertoli cells was due to the increased availability of degenerating germ cells and the decreased secretion of a lipidic Sertoli cell hormone. Adult rats that had been irradiated in utero to destroy all germ cells and made unilaterally cryptorchid at birth, had more lipid droplets in the Sertoli cells of the abdominal testis than in the Sertoli cells of the scrotal testis (Bergh, 1981). When the testis is made cryptorchid before puberty, Sertoli cells may not achieve complete maturation (Hadziselemovic, 1977). Therefore, they may respond to heat stimulation differently than adult Sertoli cells would.

#### 1.3.2.4 Nature of lipids in Sertoli cells.

Frozen sections were investigated for the histochemical demonstration of cholesterol (Pollock, 1942). The presence of cholesterol-like material determined by phenylhydrazine reactivity, acetone soluble birefringence, and formation of digitonides, was not observed in the seminiferous tubules although it was observed in the interstitial tissue. Acidic lipids (Nile Blue) were observed in rat Sertoli cells (Lacy, 1962), but again cholesterol, as determined by the Schultz test for unsaturated sterols, was not. In the mink (Onstad, 1967) lipid droplets within the Sertoli cells displayed some cholesterol-like properties (acetone-soluble birefringence) during the peak of spermatogenesis and at the onset of testicular regression but they were negative for the Schultz test for unsaturated sterols (Onstad, 1967).

### 1.3.2.5 Changes in the nature of testicular lipids related to changes in spermatogenic activity

The concentration of testicular neutral lipids i.e., cholesterols and triacylglycerides, increased when spermatogenesis was impaired. This was demonstrated biochemically on homogenized testicular tissue after experimental cryptorchidism (Fleeger et al., 1968; Johnson et al., 1968) (rabbit) (Johnson et al., 1971) (rat), vitamin A deficiency (Butler et al., 1968) (rat), efferent duct obstruction (Sheriff and Govindarajulu, 1977) (human), progesterone treatment (Sheriff, 1980; Sheriff and Govindarajulu, 1975) (rat), gossypol treatment (Sheriff, 1988) (rat), in quaking mice (Coniglio et al., 1975), after Klinefelter's syndrome (Sheriff and Govindarajulu, 1977) (human), testicular feminization syndrome (Chung and Hamilton, 1975; Sheriff and Govindarajulu, 1978) (mouse), and bilateral varicocele (Sheriff, 1982) (human). In addition, the activity of the cholesterol side chain cleavage enzyme was reduced in rats deprived of vitamin A (Jayaram et al., 1973), and the incorporation of  $^{14}\text{C}$  acetate into free and esterified cholesterol increased following hypophysectomy (Hafiez and Bartke, 1972) (rat and mouse). Sudanophilia and acetone soluble birefringence used together localized sterols in the interstitial Leydig cells but not in the tubules of the Japanese deer and the Virginia deer during the nadir and zenith of spermatogenic activity (Wislocki, 1949). The use of frozen sections stained with oil red O demonstrated that experimental manipulations that interrupt the hypophysis-testis axis i.e., hypophysectomy, estrogen treatment, testosterone propionate treatment, or starvation, resulted in an increase in the amount of lipid droplets in the Sertoli cells together with an arrest in spermatogenesis (Lynch and Scott, 1951). FSH administration to estrogen treated rats restored normal amounts of lipid droplet in the

Sertoli cells simultaneously with normal spermatogenesis (Lacy and Lofts, 1962). Mice with testicular feminization syndrome have more cholesterol in both their Sertoli cells and Leydig cells as determined on frozen sections by the Folch method (Chung and Hamilton, 1975). These reports suggest the existence of a correlation between an arrest of spermatogenesis and an increase in neutral lipids within the Sertoli cells themselves and/or within the whole testis. However, heat treatment caused an increase in the amount of unsaturated sterols (Schultz test), and in the amount of neutral lipids (Nile blue test) (Collins and Lacy, 1969) prior to a reduction in the germ cell population. An accumulation of neutral lipids in Sertoli cells therefore may not necessarily be a direct consequence of spermatogenic disruption but a reflection of a pathological state.

#### **1.4 Aims and rationale for the thesis project**

Using electron microscopy, lipid droplets in the basal region of the seminiferous tubules have been localized to the Sertoli cell (Lacy, 1960). At this location, the amount of lipid droplets has been documented to change during the cycle of the seminiferous epithelium in relation with the release of spermatids at spermiation in the rat (Lacy, 1960; Niemi and Ikonen, 1962; Pollock, 1942) and mink (Onstad, 1967). The variations in the amount of lipid droplets present in Sertoli cells had been studied by morphometric methods in the rat (Kerr et al., 1984). This led to the proposal that Sertoli cell lipids arise as a consequence of spermatid contribution. Specifically, residual bodies would be phagocytosed by Sertoli cells and their lipid constituents would be used as building blocks for the synthesis of new lipids by the Sertoli cells (Kerr et al., 1984)

To investigate whether there was indeed a contribution of lipid droplets from spermatids to Sertoli cells, I

performed the morphometric quantitation of the lipid inclusions in both cell types for each of the twelve stages of the mink cycle of the seminiferous epithelium during the active phase. This represented the first attempt at quantifying lipid droplets in germinal cells at each step of their development. The data were compared to the stage-dependent variations in the lipid droplets present in the Sertoli cells. Special attention was focused on evaluating the effect of spermiation on the Sertoli cell lipid content.

The consequences of an arrest of spermatogenesis on the lipid droplets population in Sertoli cells seem to differ according to whether or not some germ cells are still allowed to enter meiosis following this arrest. Specifically, when spermatogenesis is arrested at the spermatid or spermatocyte stage as in mild and moderate seasonal breeders (Hodgson et al., 1979; Irby et al., 1984; Lofts, 1960; Lofts, 1964; Lofts and Boswell, 1961; Lofts and Marshall, 1957; Marshall, 1955; Sanyal and Prasad, 1965; Sehgal et al., 1986; Sinha Hikim et al., 1989), or following experimental manipulations, (Collins and Lacy, 1969; Hanes and Rosenbloom, 1911; Lynch and Scott, 1951) the number of lipid droplets in Sertoli cells increased. When spermatogenesis is arrested at the spermatogonial step allowing no germ cell to enter meiosis, for instance in emphatic seasonal breeders, lipid droplets in Sertoli cells decreased (Hilton, 1961; Lofts, 1960; Marshall and Wilkinson, 1956; Onstad, 1967; Tsui et al., 1974; Wislocki, 1949). I wanted to evaluate a potential contribution of lipid droplets from germ cells to Sertoli cells in emphatic seasonal breeding mink. Therefore, I undertook to do morphometric quantitation of the variation in the lipid droplets of the Sertoli cells throughout the mink's annual seasonal reproductive cycle. My study had the advantage over the previous ones of recording the quantified lipid variations at each month of the year rather than only during specific periods of the annual reproductive cycle

namely the peak in spermatogenic activity and the testicular regression.

Fig. 1. The characteristic cellular associations found in the cycle of the seminiferous epithelium of the mink. The first 12 steps (Arabic numerals) of spermiogenesis define the 12 stages (Roman numerals) of the cycle. Type A, B, and intermediate spermatogonia are respectively indicated "A", "B" and "in". "PL", "L", "Z", "P" and "Di" refer to the preleptotene, leptotene, zygotene, pachytene, and diplotene primary spermatocytes respectively. the secondary spermatocyte "II", is indicated. (Redrawn from Pelletier, 1986).

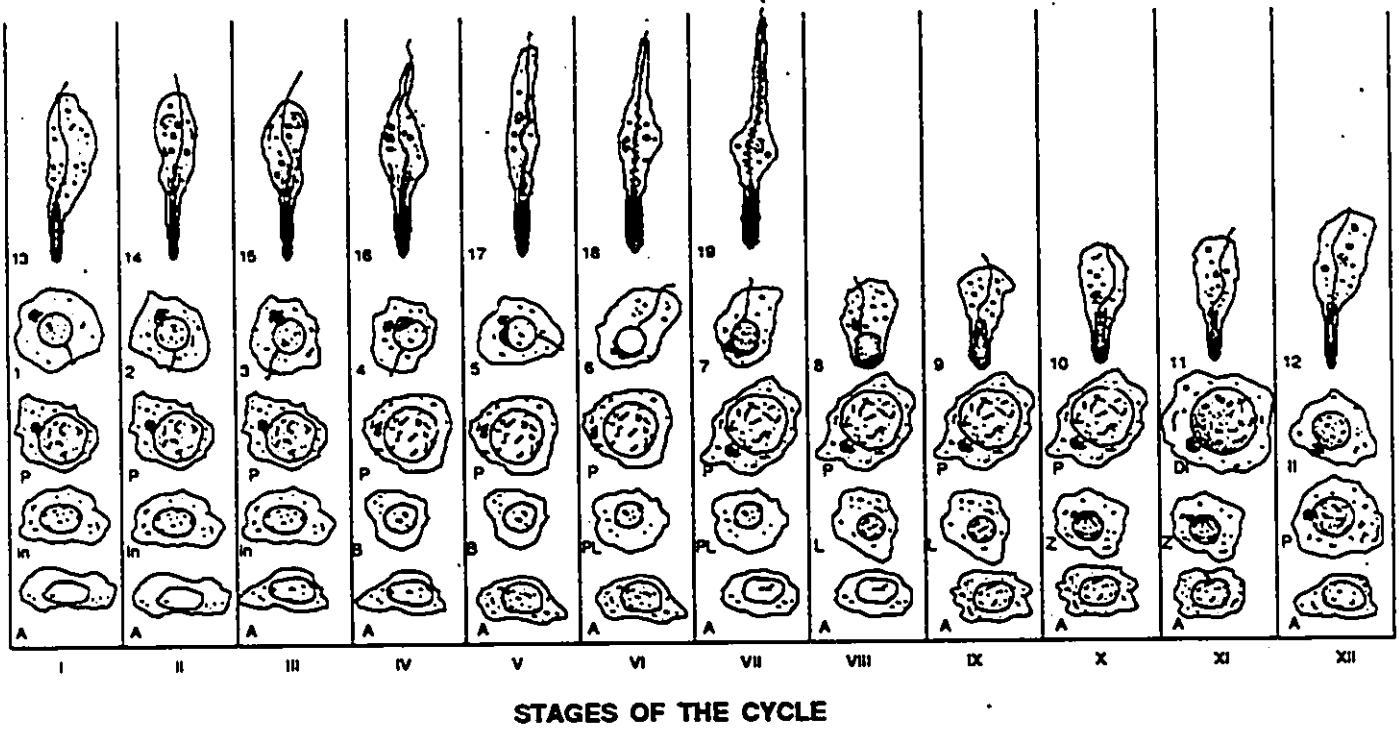
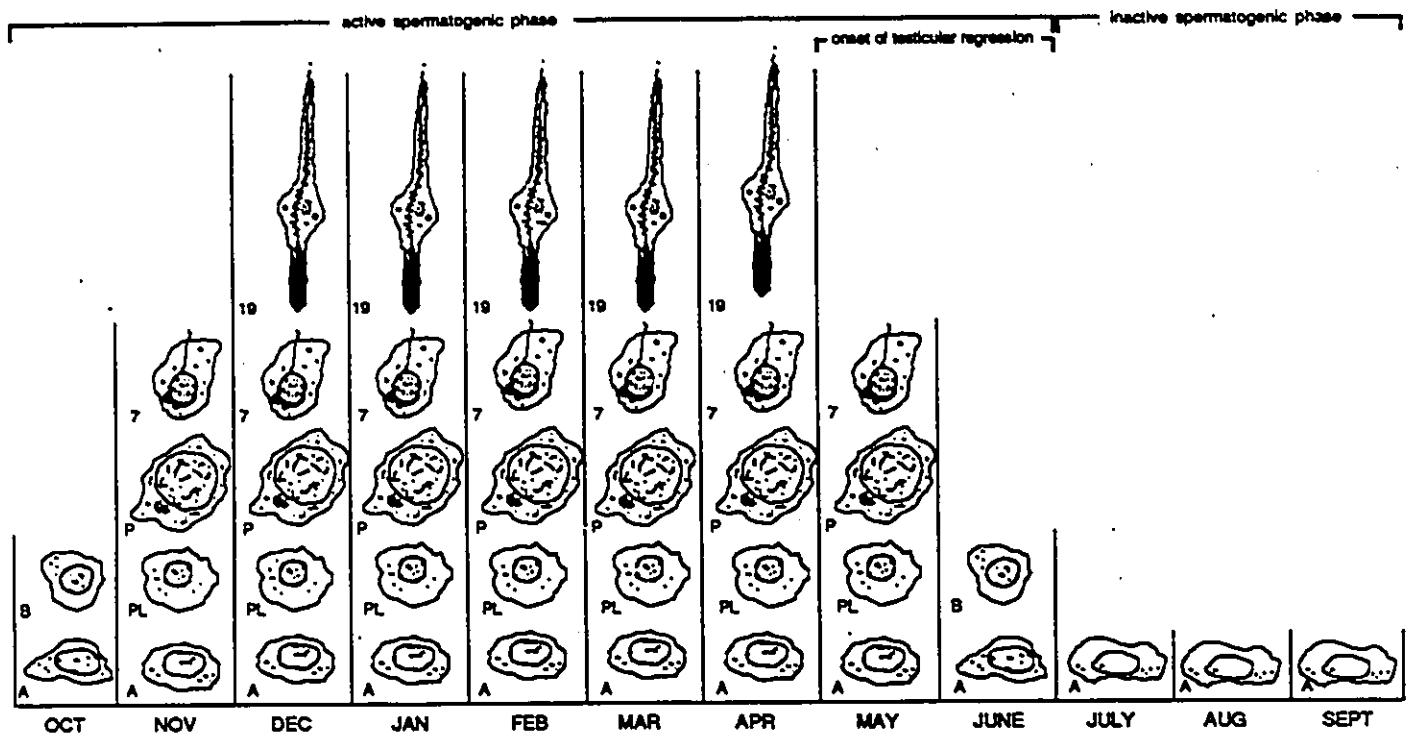


Figure 1

Fig. 2. The changes in the germ cell composition during the annual reproductive cycle for the mink. For each of the twelve months of the year, the most advanced generation of germ cells found in the seminiferous epithelium is illustrated. Although spermatogenic activity is present from October to June, the full compliment of germ cells from type A spermatogonia to elongated spermatids, are only observed between December and April. (Redrawn from Pelletier, 1986).



CALENDAR OF THE GERM CELL POPULATION

Figure 2

## 2. MATERIALS AND METHODS

### 2.1 Morphological studies of lipid droplets.

#### 2.1.1 Animals

Fertile adult male mink between the ages of two and four years were used. Fertility was determined at every mating season, i.e., in March, from vaginal smears examined under the light microscope for the morphology, mobility and number of spermatozoa. In addition, the breeding history of each animal was recorded. Only males that had successfully sired five litters of no fewer than four kits were used. These precautions were necessary because of the documented high frequency of spontaneous autoimmune orchitis in some breeds of mink associated with the colour of the fur and the resulting high incidence of secondary infertility (Tung et al., 1983; Tung et al., 1981; Tung and Fritz, 1984). Therefore, four mink were sacrificed during the last week of each month during a 12 month period. Additional animals were sacrificed at the onset of the active and inactive spermatogenic phases, respectively November and April. Spermatogenesis is complete from December to March, and maximal testicular regression occurred from July to September. A calendar of the germ cell associations has already been documented (Pelletier, 1986) and is presented in Figure 2 and to which reference will be made throughout the text. Each animal was individually caged in an unheated shed and provided with a high protein diet. An extra two hours of daylight was allowed throughout the month of February. The mink were purchased from RBR Fur Farms, St. Mary's, Ontario, and Ray's Fur Farm, Limoge, Ontario, and Lajeunesse Fur Farms, St.-Jacques, Quebec.

All animals used in this project were cared for in accordance with the principles outlined in the "Guide to the Care and Use of Experimental Animals" as published by the Canadian Council on Animal Care and provincial legislation entitled "The Animal for Research Act of the Province of Ontario".

### 2.1.2 Processing of tissue.

The mice were anesthetized by intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceutical, Mississauga, Ontario). Once removed, the testes were perfused through the testicular artery (Christensen, 1965) with 60 ml of diluted Karnovsky's fixative (Karnovsky, 1965) in 0.1 M sodium cacodylate buffer, pH 7.35, using a syringe pump (Sage Instrument Orion Research Inc., Cambridge, MA.) set at 3.0 ml/min. The tissue was then cut into 1 mm<sup>3</sup> blocks and further immersed in the same fixative for an additional 12 hours at room temperature. The potassium-ferrocyanide-tannic acid-uranyl-acetate *en bloc* staining or PFTA technique (Pelletier, 1988) was used. This technique combines two carbohydrate staining methods namely, glycogen staining by potassium ferrocyanide-reduced osmium tetroxide, and glycolyx staining by tannic acid, with phosphate and nucleic acid enhancement by uranyl acetate *en bloc* staining, to optimize the resolution of cytoplasmic details. In accordance with this technique, after being washed in buffer, the tissue pieces were post-fixed for 2-12 hours in a 1% solution of osmium tetroxide (Fisher Co. or Stevens Metalurgical Corp., New York NY.) and a 1.5% solution of potassium ferrocyanide (Karnovsky, 1971), followed by immersion in a 1% solution of tannic acid (Mallinckrodt Inc., Paris, KY.), and staining *en bloc* in 2-5% uranyl acetate. The tissue was then dehydrated in graded concentrations of ethanol, cleared in propylene oxide, and embedded in Polybed 812 (Luft, 1961) (Polysciences). During complete testicular

regression, the tannic acid treatment was reduced in concentration and time (Pelletier, 1988) to avoid excessive contrast of the cell membranes.

### **2.1.3 Light and electron microscopy.**

One half micron, toluidine blue stained, semi-thin sections were examined under oil immersion. Lipid inclusions were identified on toluidine blue-stained plastic sections according to their deep blue to black staining characteristic or their green iridescence. A circular profile with a fine even texture and radially uniform staining were used as criteria for recognizing lipid droplets under the electron microscope. Photomicrographs were taken through a Carl Zeiss D-7082 Oberkochen light microscope. For electron microscopy, silver to gray thin sections were cut with a diamond knife on a Porter-Blum Sorvall MT-2 microtome, mounted on Formvar-coated and carbon-stabilized grids. Aqueous uranyl acetate (Ryter, 1958) and alkaline lead (Reynolds, 1963) were used as contrast stains prior to examination under a Phillips EM 300 at 80 kV. Stages of the cycle were classified according to conformational changes in the spermatids' acrosome as proposed for the mink by Pelletier (1986).

### **2.1.4 Morphometric Studies**

The amount of lipid droplets in germ cells and Sertoli cells was investigated using morphometric techniques. When spermatogenesis was complete (December to March), a total of fifty tubules from each of the twelve stages of the spermatogenic cycle was counted. For each stage, no greater than ten tubules was counted from any one animal, thus fifty tubules required the contribution from a minimum of five animals. During the periods of the year when spermatogenesis was not complete (April to November), all tubules were pooled

into a single group because they did not possess the distinct germ cell associations that allowed staging of the cycle of the seminiferous epithelium. Twenty five tubules were selected at random from each animal, making a total of 100 tubules counted per month.

The morphometry reported here, exploited the principles of stereology. Stereology, a type of quantitative morphology, includes a group of mathematical equations that calculate three dimensional data from a two dimensional data source, such as tissue sections. Specifically, the relationship between areas of profiles of different tissue components is equal to the relationship between the volumes of those same components (Delesse, 1848). From this, information about 3-dimensional structures can be elucidated from the quantitative analysis of flat images of that structure (see review by (Elias and Hyde, 1980)). The volume density ( $V_v$ ) of lipid droplets and Sertoli cell nuclei, relative to the volume of the seminiferous tubule, was determined by the point counting method (Glagolev, 1934) on 0.5  $\mu$ m semithin sections. This method utilizes the equation:

$$P_p = V_v \quad (\text{Glagolev, 1934})$$

where  $P_p$  is the number of points that hit profiles of the structure being investigated, divided by the total number of test points, in this case the total number of points that hit the seminiferous tubule of interest. The basement membrane was taken as the boundary of the tubule. Points which laid on the lumen, artifactual spaces, or the basement membrane were not counted. Thus, measurements were made of cellular elements only.

The numerical density ( $N_v$ ) of Sertoli cell nuclei, or the number of nuclei per unit volume of tubule, was derived using the equation:

$$N_v = V_v / (D + t - 2h) \quad (\text{Floderus, 1944});$$

where  $D$  is the caliper diameter,  $t$  the thickness of the tissue section and  $h$  the height of lost polar caps i.e., the height of the smallest visible grazing section of the

structure being investigated. The Sertoli cell nucleus is a prolate rotatory ellipsoid which makes it impossible to measure D directly as one would for a spherical structure. The caliper diameter was therefore calculated using the equation:

$$D = (a+b(\sinh^{-1}(a^2/b^2-1)^{1/2})/(a^2b^2-1)^{1/2})/2 \quad (\text{Mack, 1956}).$$

Fifty nuclei were used to determine the mean measurements of the major (a) and minor (b) axes of the Sertoli cell nuclei. The caliper diameter thus obtained was calculated for each stage of the cycle during the active spermatogenic phase and for each month of the year during the inactive spermatogenic phase. The height of the smallest visible cap (h) was estimated to be  $1/10$  of the diameter of the nucleus (Mori and Christensen, 1980).

The volume  $V_L$  and number  $N_L$  of a particular structure per unit length of tubule were derived as the product of the volume density  $V_V$  or numerical density  $N_V$  of that structure and the average cross sectional area (A) of the seminiferous tubule (Wing and Christensen, 1982):

$$V_L = V_V \times A, \text{ and } N_L = N_V \times A.$$

The average cross-sectional area of the epithelium was calculated from the mean of the diameters as measured across the minor axis of ten epithelial profiles for each stage and for each month of the year (Wing and Christensen, 1982).

For each stage of the cycle and for each month of the year, these formulae were used to determine the volume of lipid droplets in Sertoli cells and in spermatids per cross section of the tubule and in relation to the number of Sertoli cell nuclei. In this way, the volume density i.e., volume of lipid droplets within a given cell class in  $\mu\text{m}^3$  per volume of epithelium in  $\mu\text{m}^3$ , is multiplied by the mean of the cross-sectional area of the epithelium in  $\mu\text{m}^3$  to calculate the volume of lipid droplets in that given cell class per unit length-of epithelium.

## **2.2 Biochemical assay of cholesterol content.**

### **2.2.1 Reagents.**

Medium 199 with Earle's Balanced salt and L-glutamine (M199), N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid (Hepes), N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), N,N-bis[hydroxymethyl]-2-aminoethanesulfonic acid (BES), bovine serum albumin (BSA, fraction V), collagenase (type IV), hyaluronidase (type I-S, from bovine testes), deoxyribose nuclease (DNase, type I from bovine pancreas), trypsin, trypsin inhibitor (lyophilized from soybean), Percoll (colloidal PVP coated silica), N-tris[hydroxymethyl]-aminoethane hydrochloride (Tris-HCl), cholesterol oxidase (from *Pseudomonas fluorescens*), cholesterol esterase (from *Pseudomonas fluorescens*), cholic acid (Sodium salt from ox or sheep bile), p-hydroxyphenylacetic acid (HPA), cholesterol (chromatography grade), and cholesteryl myristate, were purchased from Sigma Chemical Company, St. Louis MO.

### **2.2.2 Isolation of cell sub-types.**

Three mink were sacrificed at each of the following periods of the annual reproductive cycle: the zenith (January) the nadir (July) and the transition periods (May and October) (see fig. 2). Table 1 outlines a general description of the procedure used for isolating Sertoli cells, germ cells and Leydig cells. The tunica albuginea and attached blood vessels were peeled away from the testis and placed in ice cold M199E (M199 with 3.575 g/l HEPES, 2.292 g/l TES, 2.133 g/l BES, 1.1 g/l sodium bicarbonate, and 1.0 g/l BSA, pH 7.4, (Shaw et al., 1979)). The tubules were gently teased to allow free access of media to the interior of the testis. In general, a modification of the procedure of Bucci (Bucci et al., 1986) was followed to isolate the

Table 1.

Procedure for the isolation of Testicular Cells

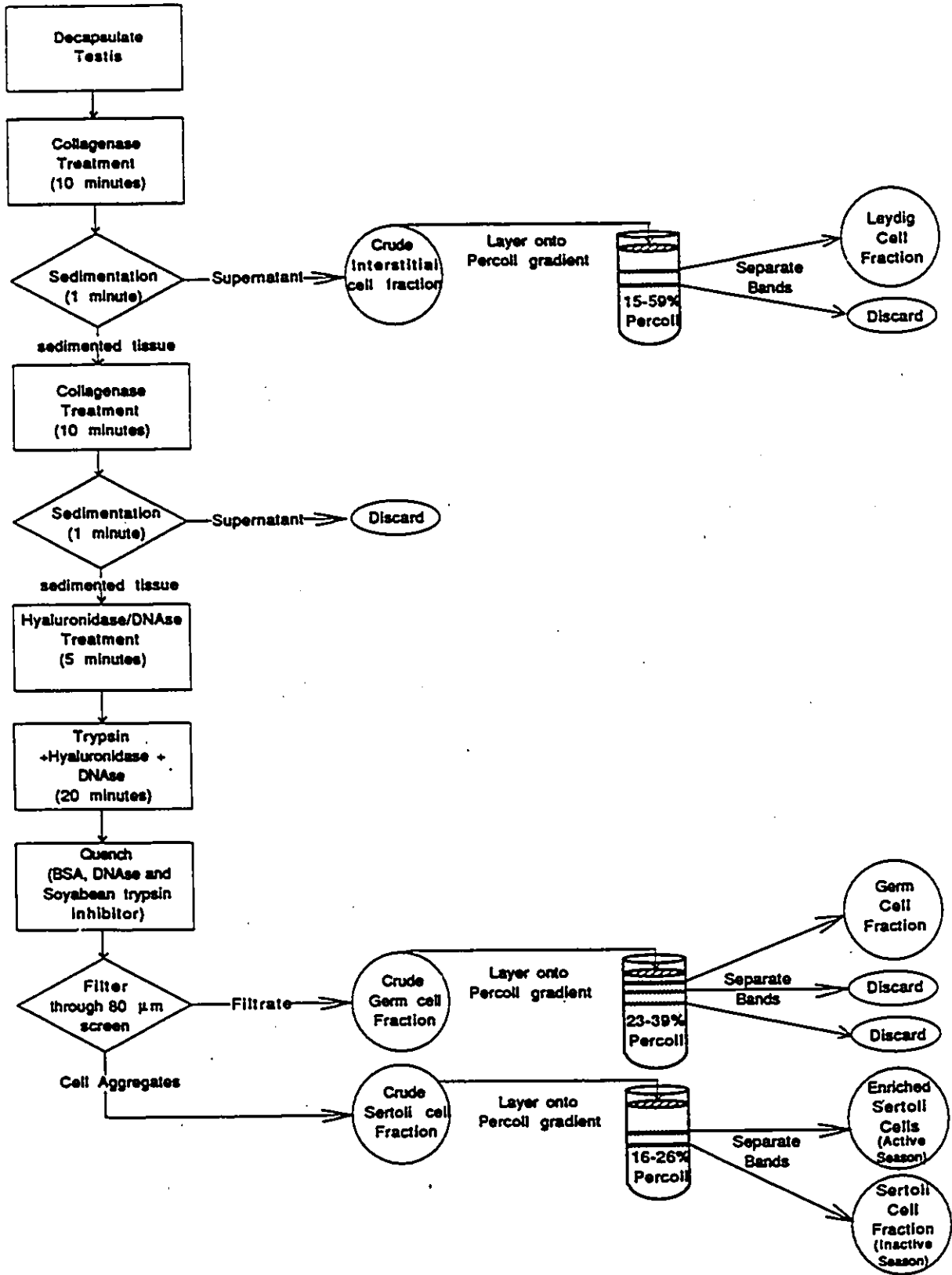


Table 1

various cell subtypes. The tissue was incubated in 10 ml of M199E + collagenase (120 units/ml) at 37°C. in a shaking water bath set at 80 cycles/min., for ten minutes to break down the affiliation between interstitial tissue and seminiferous tubules (Romrell et al., 1976; Shaw et al., 1979). After a brief vortexing, the mixture was allowed to settle for one minute. The crude interstitial cell fraction remained in suspension and was removed (Romrell et al., 1976). The tubules fell to the bottom of the test tube and were subject to a second 10 minute collagenase treatment, to ensure removal of all interstitial tissue (Hon et al., 1983). The cells of the tubules were dissociated by a five minute incubation under the same conditions but with hyaluronidase (300 units/ml.), and DNase (120 units/ml) instead of collagenase. A second incubation for the dissociation of the cells lasting 20 minutes followed. In this incubation however, trypsin (200 units/ml) was added to the hyaluronidase/DNase medium. When a uniform suspension of cells was obtained, the reaction was quenched by the addition of BSA (8% final concentration), soybean trypsin inhibitor (0.01%) and DNase (120 units/ml). The cell suspension was then filtered through an 80  $\mu$ m nylon screen. During the breeding season, the filtrate was composed of the crude germ cell fraction. The enriched population of Sertoli cells remained in aggregates and did not pass through the screen (Beckman and Coniglio, 1979). Immediately after this step, the germ cell and Sertoli cell fractions were washed twice with cold M199E, to remove digestive enzymes, and then resuspended in 5% BSA, 120 units/ml of DNase, and 0.025% soybean trypsin inhibitor in ice cold M199E. A solution of 80% Percoll in M199E was added to the cell suspension. The volume of 80% Percoll was calculated to bring the concentration of Percoll in each cell suspension to 5% below that of the low density end of the final gradient (see below).

During initial studies using guinea pig testes, non-linear Percoll gradients were performed by centrifugation of 50 and 90% Percoll in M199E (Hon et al., 1983) at 30,000 g for one hour. The use of these gradients did not result in adequate separation of the cell subtypes, and for this reason, the linear gradients reported by Bucciet al. (1986) and Slaughter et al. (1989) were used in all subsequent experiments performed on mink testes. For experiments performed in October and in January, each cell fraction was layered onto a 23-50% linear gradients of Percoll in M199E (Bucci et al., 1986). In May and July, to further enrich the cell fractions, linear gradients were tailored for each cell class to be purified (Slaughter et al., 1989). For Sertoli cells, a 16-26% linear gradient was prepared; for germ cells the gradient was 23-39% , and for Leydig cells the gradient was 15-59% Percoll in M199E. Gradients were then centrifuged at 10,000 g for ten minutes at 4°C. in a Beckman L8-70 Ultracentrifuge fitted with a Ti70 fixed angle rotor. The resulting layers were separated, washed twice in fresh M199E, and sampled for identification under the light microscope using 0.5% orcein 50% acetic acid as a quick fix and stain (Welsh and Wiebe, 1975). Fractions of highest purity as determined by the quick fix and stain procedure, were also tested for viability using 0.1% trypan blue. Samples were taken from each group for characterization under the electron microscope. At this point, the isolated cell suspensions were frozen at -80°C. for storage prior to further processing.

### **2.2.3 Subcellular fractionation.**

The plasma membrane isolation techniques of Atkinson and Summers (1971) and Brake et al. (1978) as modified by Marzowski et al. (1985), were utilized for the subcellular fractionation of Sertoli cells and that of Millette et al. (1980) was used for the subcellular fractionation of germ cells and Leydig cells. All steps were performed on ice or

in a cold room (4°C). Cells were washed twice in Tris-buffered saline solution (TBSS, 160 mM. sodium chloride, 3.0 mM. magnesium chloride, 5.0 mM. potassium chloride and 10mM Tris HCl, pH 7.4 (Brake et al., 1978). Swelling of the cells was achieved by resuspending them in 1.5 ml. of a hypotonic buffer (10% TBSS in 10 mM Tris HCl pH 7.4) for 5 minutes prior to homogenization. Cell suspensions were homogenized in a tight fitting Dounce homogenizer with 3 strokes for germ cells and Leydig cells (Millette et al., 1980), and 12 strokes for Sertoli cells (Marzowski et al., 1985). After a light centrifugation (30 seconds at 1000 g) to remove unbroken cells, the supernatant was mixed with one part 80% sucrose in TBSS to produce 3.0 ml at 40% sucrose. The 40% sucrose was then layered on top of 45% sucrose (w/v in TBSS) in a polyallomer centrifuge tube (Beckman). Five ml of 30% sucrose was layered on top of the 40% sucrose, followed by straight TBSS to fill the tube. The tubes were then centrifuged at 100,000 g for two hours in a Beckman L8-70 ultracentrifuge equipped with an SW40 swinging bucket rotor. The interfacial layers were removed and washed twice in TBSS to clear excess sucrose. Samples were taken and processed for electron microscope characterization (see section 2.1.3). The remaining material was used for cholesterol determination (Gamble et al., 1978) and protein determination.

#### **2.2.4 Cholesterol determination.**

Lipid was extracted from the isolated cell subtypes (section 2.2.2) and their respective subcellular fractions (section 2.2.3) according to the method of Folch (Folch et al., 1957). Washed material in 2 ml of TBSS, was extracted twice with 2 ml of 2:1 chloroform:methanol. The aqueous phase was removed and retained for protein determination. The volume of the organic (heavy) phase was reduced by evaporation under a stream of N<sub>2</sub>, and completely evaporated in a vacuum at room temperature for 12 hours. Each sample was

then dissolved in chloroform to allow for accurate division of the material into two equal portions, which were then transferred to assay tubes. Standard solutions were prepared from cholesterol and cholesteryl myristate in chloroform. To remove all traces of chloroform, which may otherwise have affected the assay, the standards and duplicate samples were evaporated under a stream of N<sub>2</sub> and then heated to 100°C for one hour. Dissolution in 2 ml of 95% ethanol required vigorous vortexing and heating at 50°C for 30 minutes. Without this step, cholesteryl myristate in the standard is virtually undetectable.

For the determination of total cholesterol, 2 ml of the assay solution (Table 2) was added to each sample. A similar solution lacking cholesterol ester hydrolase was used for the determination of free cholesterol. After incubation at 37°C in a shaking water bath for 30 minutes in the dark, the fluorescence was measured at an excitation wavelength of 325 nm and an emission wavelength of 415 nm in an Aminco-Bowman J4-8962 spectrophotometer (American Instrument Co., Inc., Silversprings MD. 20910, USA.). Fluorescence was measured a second time after an additional 12 hours of incubation in the dark at room temperature.

Table 2. Composition of assay solution for total cholesterol

Cholesterol oxidase	0.11 U/ml
Horseradish peroxidase	1.05 U/ml
Cholesterol esterase	0.11 U/ml
Triton X-100	0.025 %
Sodium cholate	0.45 mg/ml
p-Hydroxyphenylacetic acid	0.63 mg/ml

Buffer was 0.1M potassium phosphate buffer, pH 7.4.

## 2.2.5 Protein determination

The protein content of each sample was determined from the aqueous phase retained after lipid extraction. A 0.1 ml aliquot of vortexed solution was placed in each assay tube. Protein standards were prepared from a stock solution of 1mg/ml of BSA in distilled water determined by absorbance at 280 nm; 1 mg/ml = 0.6A<sub>280</sub>. To each tube, 2.0 ml of working reagent was added according to instructions with the BCA Protein Assay Reagent Kit (Pierce Chemical Company, Box 117, Rockford, Il., 61105, USA). The samples were vortexed and incubated for 30 minutes at 37°C in a shaking water bath. Absorbance of light at a wavelength of 562 nm was determined on an SP8-100 Ultraviolet Spectrophotometer (Pye Unicam Ltd. Cambridge, England).

## 2.2.6 Statistical Analysis

One-way analysis of variance (ANOVA) was performed on each cell subtype for the free and esterified cholesterol levels separately. This determined if there was a significant difference in the levels of either cholesterol moiety with relation to the time of the year. Significant variation was observed in both the interstitial and Sertoli cell fractions. These two cell subtypes were further investigated using a two-way ANOVA with the Duncan pairwise comparison as a post hoc test. Because the sample sizes were unequal, the Duncan test was considered to be the most appropriate test to determine where the significant differences lay (Nair, R. personal communication).

## 2.2.7 Tissue processing for characterization.

Isolated cell sub-types were pelleted at 16,000 g for 5 minutes in 400 µl tubes using an Ependorf 5415

Microcentrifuge (Brinkmann Instruments Inc. Sybron Corp., Catingue Rd., Westbury, NY., 11590). Subcellular fractions were centrifuged at 100,000 g for two hours in the L8-70 equipped with a type 50 rotor adapted for 2ml centrifuge tubes (Ultra-clear Beckman Instruments Inc., Palo Alto, CA.) Pellets were fixed by immersion in Karnovsky's fixative (Karnovsky, 1965) and processed as previously described in section 2.1.2.

## **2.3 Cytochemistry**

### **2.3.1 Reagents.**

Cholesterol oxidase from *Streptomyces sp.*, *Pseudomonas fluorescens*, and horseradish peroxidase (HRP) type II were purchased from Sigma Co. The cholesterol oxidase from *Streptomyces sp.* gave the most consistent results. EM grade 3,3'-diaminobenzidine tetrahydrochloride (DAB), and filipin were bought from Polysciences. The source of the other reagents were previously described (section 2.2.1).

### **2.3.2 Animals**

The protocol for the localization of cholesterol at the ultrastructural level is outlined in Table 3 and 5. In February and April, adult mink testes were fixed by perfusion with 60 ml of 0.5% gluteraldehyde and 4% para-formaldehyde in 0.1 M. sodium phosphate buffer, at 4°C pH 7.35. The reduced concentration of gluteraldehyde was used to maximize the reaction between tissue and enzymes (Lange and Ramos, 1983). After washing in phosphate buffer (isoosmolarity was maintained using sucrose), the tissue blocks were cut into 70  $\mu$ m thick sections on a vibratome (series 1000, Pelco), and collected into 0.17 M phosphate buffer to maintain isoosmolarity and remove the sucrose.

Table 3

# Enzymatic Determination of Cholesterol

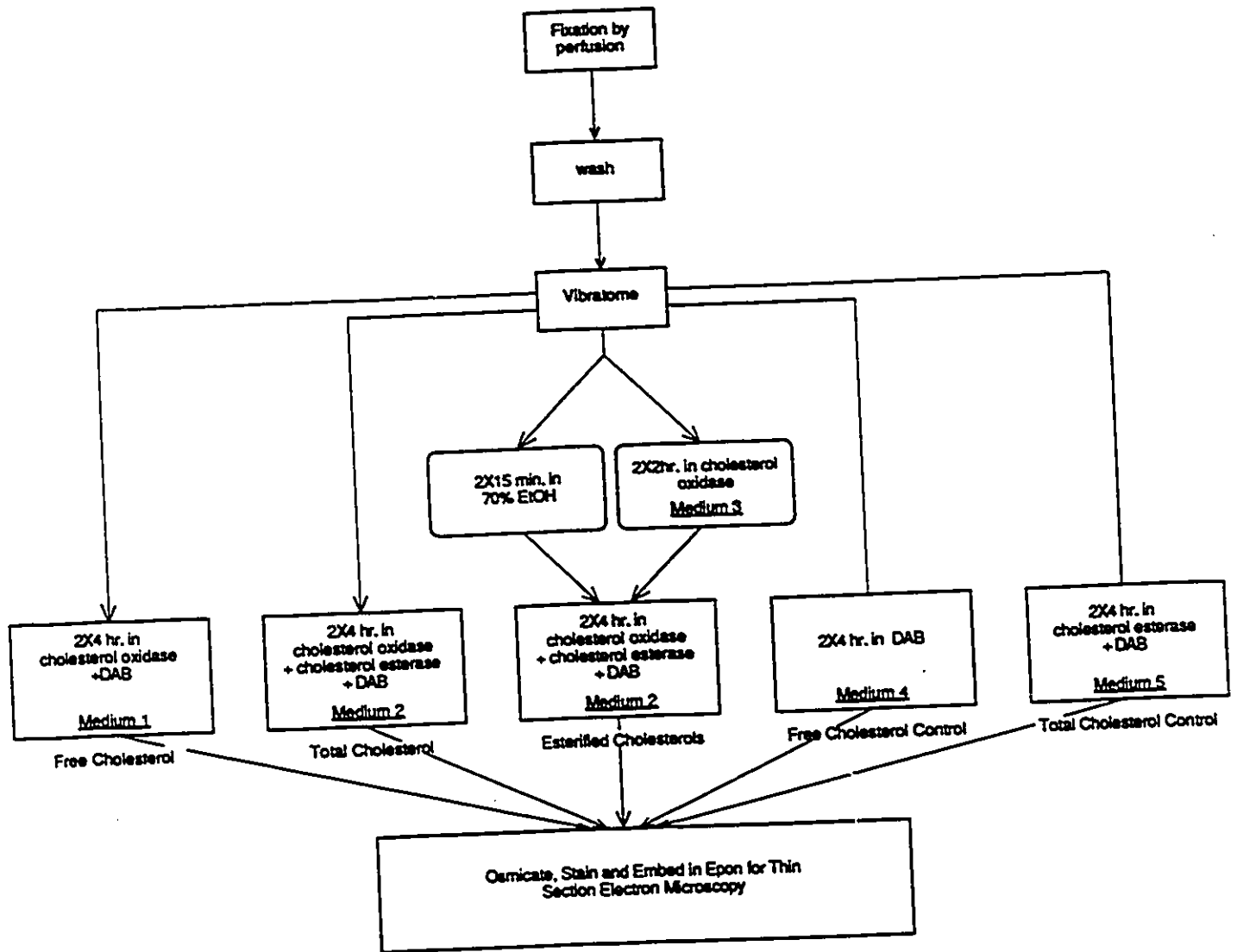


Table 3

### 2.3.3 Incubation.

Two different cytochemical procedures were applied to these vibratome sections 1) **enzymatic determination of cholesterol** using DAB as the marker; and 2) **specific ligation of cholesterol** to the antibiotic filipin. Each procedure was used for the determination of A) free, B) total and C) esterified cholesterol. Esterified cholesterol was distinguished from free cholesterol using: i) selective enzymatic oxidation of free cholesterol, and ii) selective solubilization of free cholesterol (Kruth, 1984).

For the **enzymatic determination of cholesterol**, the procedure of Allain et al. (1974) for serum cholesterol levels as modified for histochemistry by Emeis et al. (1977) and Jones and Miyai, (1981) was used. Table 3 outlines the general procedures followed, and Table 4 summarizes the composition of each incubation medium used for the demonstration of cholesterol with DAB.

Table 4

	Medium				
	M1	M2	M3	M4	M5
0.4 units/ml cholesterol oxidase	*	*	*		
1.4 units/ml cholesterol esterase		*			*
0.5 mg/ml DAB	*	*		*	*
50 units/ml HRP	*	*		*	*
0.1% (v/v) TX100	*	*	*	*	*
0.05% TRIS-HCl	*	*	*	*	*

The tissue sections were immersed in 2 ml of media as described in Table 4, and incubated at 37°C under a 95% oxygen and 5% carbon dioxide atmosphere in a shaking water bath at 80 agitations per minute. A) Free cholesterol determined using DAB. The tissue was incubated for 2 X 4 hours in fresh medium M1. B) For the demonstration of total cholesterol

using DAB, tissue sections were incubated as for 1a but in medium M2. Ci) Esterified cholesterol determination using DAB: the tissue was preincubated in fresh medium M3 for 2 X 2 hours to enzymatically oxidize the free cholesterol. The preincubation was followed by two five minute rinses in buffer and two four hour incubations in medium M2 as described for 1b. Cii) Esterified cholesterol determination using ethanol for the selective solubilization of free cholesterol. The tissue was rinsed for 2 X 15 minutes in 70% ethanol to extract unesterified cholesterol (Kruth, 1984). This was followed by an incubation as for total cholesterol: two four hour incubations in medium M2. In control experiments, cholesterol oxidase was omitted from the incubation medium as described for media M4 and M5. Strips of filter paper were infiltrated with 0.1 mg/ml of either free cholesterol or cholesteryl myristate in chloroform and allowed to dry in a vacuum. The test strips were incubated in the same media as the tissue sections in order to evaluate the specificity of the localization technique.

**Specific ligation of cholesterol to filipin.** Table 5 outlines the procedure followed for the localization of cholesterol using filipin. Filipin is a polyene antibiotic which induces distinctive membrane perturbations by complexing specifically to 3 $\beta$ -hydroxysterols (Kinsky, 1970). These perturbations can be visualized in thin sections as corrugations (Bittman et al., 1974; Kinsky et al., 1967; Robinson and Karnovsky, 1980), which correspond in freeze-fracture replicas to 25 nm protuberances on the membrane (Elias et al., 1979; Friend and Elias, 1978; Tillack, 1973; Verkleij et al., 1973). Because both filipin and cholesterol oxidase have the same substrate namely, free cholesterol, filipin can be used as a control. Using this reasoning, the effectiveness of cholesterol oxidase at converting free cholesterol into cholestenone, an inappropriate substrate for filipin, was checked. The vials containing the vibratome sections were wrapped in tin foil to exclude possible

Table 5

Specific Ligation of Cholesterol to the Antibiotic Filipin

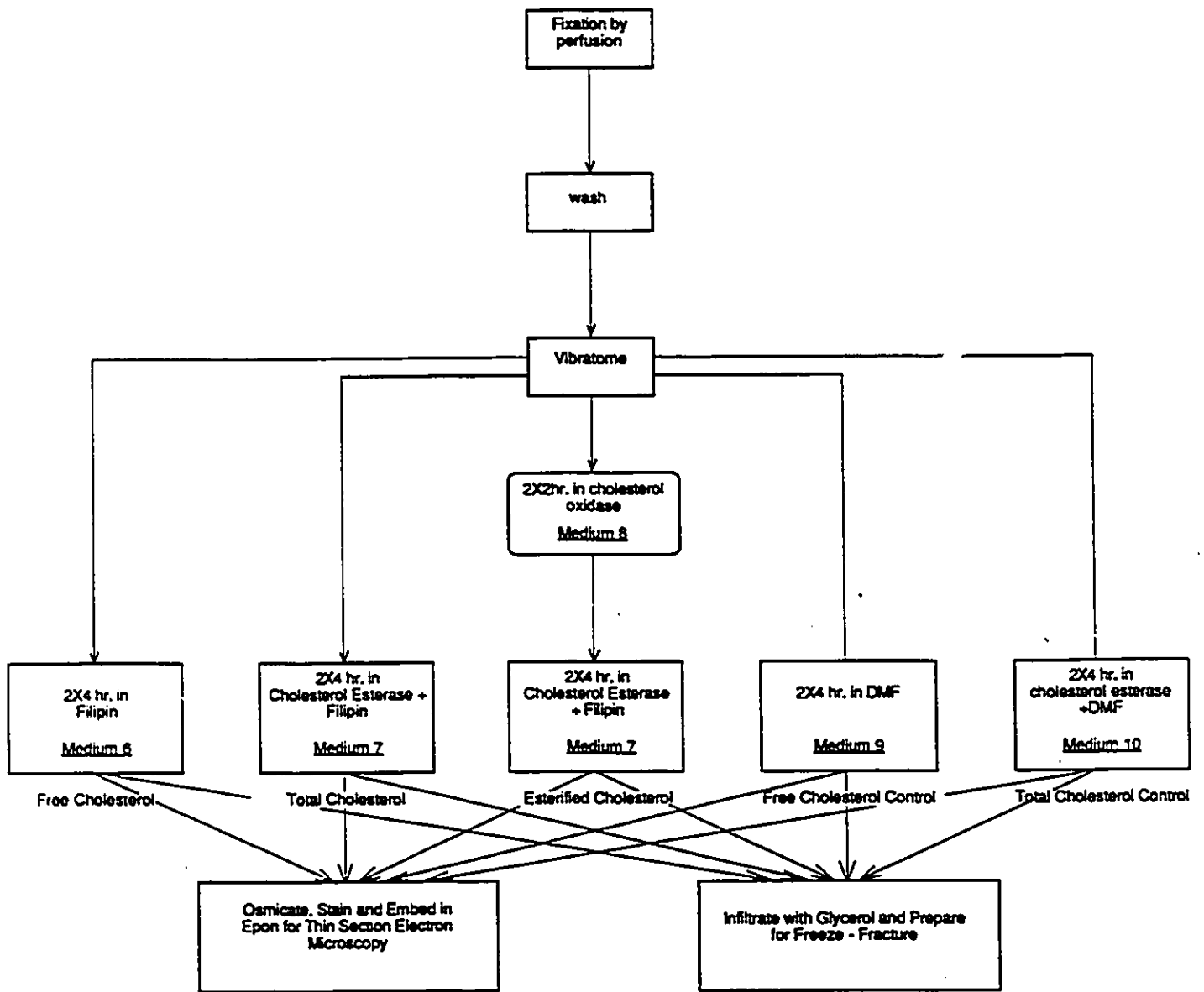


Table 5

photodegradation of the antibiotic. Filipin was solubilized in two drops (<1% v/v) of dimethyl formamide (DMF) (Sigma). Table 6 summarizes the composition of each medium used for cholesterol demonstration with filipin.

Table 6

	Medium				
	M6	M7	M8	M9	M10
0.4 units/ml cholesterol oxidase			*		
1.4 units/ml cholesterol esterase	*				*
0.1% (v/v) TX100	*	*	*	*	*
400 mg/ml filipin (+ 1% DMF)	*	*		*	*
0.1M phosphate buffer	*	*	*	*	*

A) Filipin labeling of free cholesterol. The tissue was incubated for 2 X 4 hours in fresh medium M6. B) Total cholesterol demonstration using filipin: tissue sections were incubated for 2 X 4 hours in fresh medium M7. C) For the demonstration of esterified cholesterol using filipin, tissue sections were preincubated for 2 X 2 hours in medium M8 to promote the enzymatic oxidation of free cholesterol. The preincubation was followed by two four hour incubations in medium M7. In control experiments, cholesterol oxidase was omitted from the incubation medium as described for media M9 and M10.

### 2.3.4 Tissue processing

The tissue exposed to DAB was thoroughly rinsed for 3 X 5 minutes in phosphate buffer containing sucrose to remove unreacted DAB (Seligman et al., 1973), prior to osmication and processing as previously described in section 2.1.2. Thin sections were viewed both with and without contrast staining in uranyl acetate and lead acetate. Reaction

product was easier to identify in the absence of contrast staining.

The tissue exposed to filipin was processed for electron microscopy of both thin sections (as described in section 2.1.2 and 2.1.3), and freeze-fracture. The vibratome sections destined for freeze-fracture were cryoprotected with 30% glycerol in 0.2 M cacodylic buffer, pH 7.3 for three hours at room temperature and at 4°C for the next 12 - 24 hours (Pelletier and Friend, 1983). The tissue was rapidly frozen in Freon 22 and fractured at -118°C in a vacuum of at least  $1.0 \times 10^{-6}$  Torr. in a Balzer's BAF 300 freeze-fracture apparatus. The replicas were obtained by shadowing the freshly exposed fracture surface with platinum at an angle of 45°, followed by carbon at 90°. Replicas were cleaned in filtered (0.22µm Millipore filter, Millipore Corp., Bedford, MA. 01730, USA.) sodium hypochlorite (Javex) and rinsed through several changes of distilled water prior to mounting on uncoated copper grids.

### **3 RESULTS**

#### **3.1 Morphology and Morphometry**

To determine with precision, to which cell a lipid droplet belonged at the light microscope level, the cell membrane and cytoplasmic details had to be more clearly visible than they are after toluidine blue staining of routine plastic sections. The PFTA technique followed by toluidine blue staining, fulfilled this goal and made it possible not only to visualize the limits of each cell but also to distinguish the cytoplasm of germ cells from that of Sertoli cells by the difference in their staining characteristics. Germ cell cytoplasm had a light homogeneous texture. The Sertoli cell cytoplasm had a darker, somewhat granular appearance (Figs. 3-8).

Lipid droplets were clearly visible in all classes of germ cells, from pre-meiotic and meiotic cells (Figs. 3, 7, 8, 15-20 and 25) to round and elongated spermatids (Figs. 3, 8, 15, 16, 21, 26 and 27). Lipid droplets were also observed in Sertoli cells in the infranuclear or basal region, as well as in the cell trunk and processes or apical region (Figs. 3-8, 15-21, 29, 30 and 31). Variations in the amount of lipid droplets were studied and evaluated for spermatids and Sertoli cells as observed during the period of complete spermatogenic activity. The studies were also carried out during the inactive spermatogenic phase but only on Sertoli cell lipids since spermatids were absent during this phase.

### 3.1.1 During Complete Spermatogenic Activity

#### 3.1.1.1 Variations in lipid droplets in Germ cells

##### 3.1.1.1.1 Morphology

Small 1-2  $\mu\text{m}$  lipid droplets were present in step 1 spermatids (Fig.3). Throughout spermiogenesis, lipid droplet content within spermatids gradually increased. During the Golgi (steps 1-3) (Fig.3) and cap phases (steps 4-7) (Figs. 4 to 6), droplets were more frequent, and by step 8, four to five medium size (2-4  $\mu\text{m}$ ) droplets were observed in each cell (Figs. 7 and 9). The acrosome phase (steps 8-12) (Fig. 7 and Fig. 8) and the maturation phase (steps 13-19) (Figs. 3 and 4) of germ cell differentiation are associated with a redistribution of spermatid cytoplasm and it is difficult, on morphological basis alone, to determine if the potential increase in lipid droplets during these phases is real. It is clear that there are larger lipid inclusions in the later step spermatids than there are in step 8 (compare Fig. 7 to Fig. 4) but in order to appreciate the total amount of lipid, morphometric techniques were applied.

##### 3.1.1.1.2 Morphometric analysis of lipid droplets in developing spermatid

Variations in the amount of lipid droplets within the germ cells and Sertoli cells were evaluated by morphometry. This required first the establishment of stage and season-independent features that could be used as parameters in the comparison of the quantity of lipid droplets from different cell populations and at various periods in their development. One such parameter is a defined length of tubule. When a specific ratio of lipid droplet volume per total volume of epithelium is multiplied by the cross-sectional area of that epithelium, the product is an expression of the lipid droplet

volume per given length of tubule. Thus,

$$\frac{V_{\text{lipid}} (\mu\text{m}^3) / V_{\text{epithelium}} (\mu\text{m}^3) \times A_{\text{epithelium}} (\mu\text{m}^2)}{V_{\text{lipid}} (\mu\text{m}^3) / L_{\text{epithelium}} (\mu\text{m})} =$$

where V is the volume, A the area and L the length. Because Sertoli cells are considered not to divide in adult mammals, the number of Sertoli cell nuclei can also be used as an independent parameter. Our data shows that the area of the epithelium (Figs. 9 and 10') and the number of Sertoli cell nuclei per unit length of tubule (Figs. 10 and 10') did not vary in a stage-dependent manner during the active spermatogenic period.

Graphs using the length of the epithelium as the independent parameter will be presented to the left of those using the number of Sertoli cell nuclei as the independent parameter in Figures 11 to 14 and in Figure 24. However, because of the similarity between these two sets of graphs, the description of graphs will be restricted to those using the number of Sertoli cell nuclei as their independent parameter. Since the number of Sertoli cells throughout the cycle of the seminiferous epithelium (Wing and Christensen, 1982) (also Figs. 10 and 10'), and the number of spermatids from step to step during spermiogenesis remains approximately constant (Bustos-Obregon, 1970; Wing and Christensen, 1982), the total volume of lipid droplets in spermatids per number of Sertoli cells (Figs. 11 and 14') is proportional to the total volume of lipid droplets per spermatid. Figure 11 illustrates that the volume of lipid droplets per spermatid increased steadily throughout the first three quarters of spermiogenesis: that is from step 1 at  $0.3 \mu\text{m}^3$  per Sertoli cell, to step 14 at  $5.0 \mu\text{m}^3$  per Sertoli cell. During the last quarter of spermiogenesis, i.e., from step 15, the volume of lipid droplets in spermatid decreased to  $2.1 \mu\text{m}^3$  per Sertoli cell by step 18. At Stage VII, lipid droplets of the step 19 spermatid were found in the caudal cytoplasm and in the residual body totaling  $2.5 \mu\text{m}^3$  per Sertoli cell (see also Figs. 5, 6 and 28).

Figs. 3-8. Light micrographs of toluidine blue-stained 0.5  $\mu\text{m}$  thick sections. Taken from adult mink sacrificed during the breeding season.

Fig. 3. Lipid droplets are observed at the base (large arrow) and within the trunk and cytoplasmic processes (small arrows) of Sertoli cells. A prominent location for lipid droplets is within the caudal cytoplasm of elongated spermatids (arrowhead). Sertoli cells (S), type A spermatogonia (A), pachytene spermatocytes (P), step 1 spermatid (1), and elongated spermatid (E) are identified. The tubule is at Stage I in the spermatogenic cycle. X 600.

Fig. 4. Large lipid droplets (arrow) are found at the base of Sertoli cells (S). Lipid droplets (open arrow) are observed within the cytoplasm of round spermatids (R), and in aggregates (arrowhead) in the cytoplasmic droplet of the elongated spermatids. The tubule is at Stage IV of the spermatogenic cycle. X 600.

Fig. 5. Lower magnification comparing two tubules with a cellular association at Stage VII, and one at VIII. Large lipid droplets (arrowheads) are common in Sertoli cells at Stage VII, but less so in Stage VIII (open arrow). Step 8 spermatids have abundant lipid droplets (arrow). X 450.

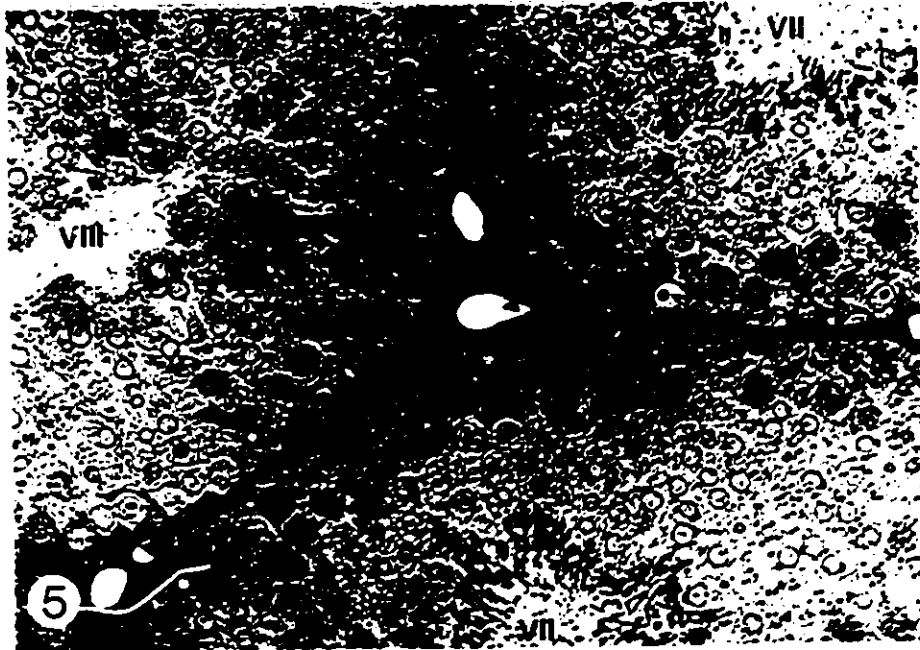
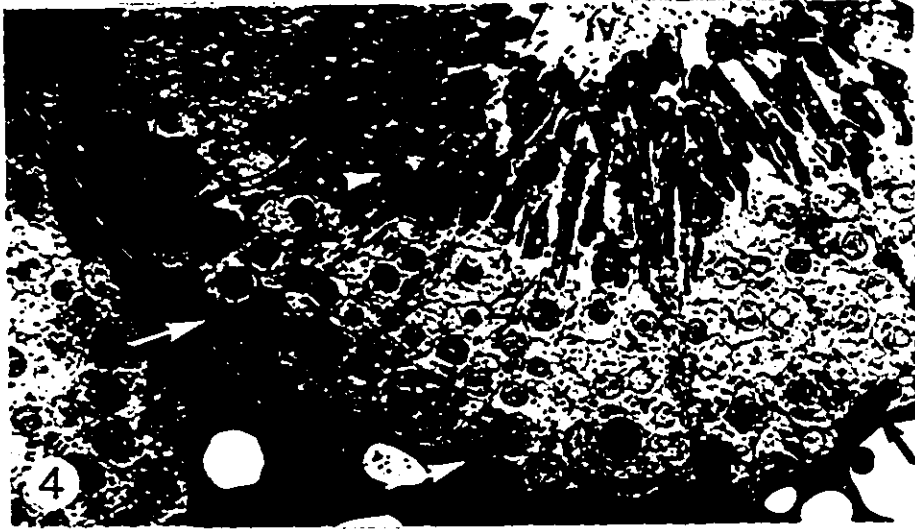
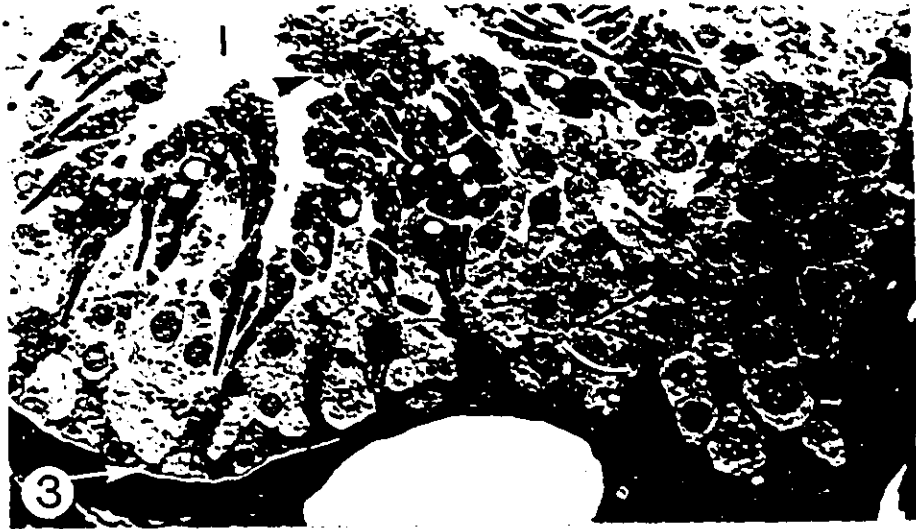


Fig. 6. Lipid droplets (open arrowhead) are seen in residual bodies (arrow 1) next to the lumen. Residual bodies void of lipid (arrows 2 and 3) are observed closer to the base of the epithelium. A Sertoli cell nucleus (S) is indicated. The tubule is at Stage VII of the spermatogenic cycle. X 600.

Fig. 7. Stage VIII seminiferous epithelium illustrating the abundance of lipid droplets (open arrowhead) within step 8 spermatids. Large lipid droplets (closed arrowhead) are labeled within the body of a Sertoli cell (S). A small lipid droplet is indicated (arrow) within a spermatogonium (G). X680.

Fig. 8. The seminiferous epithelium at Stage X. Lipids are indicated (small arrowhead) within the caudal cytoplasm of step 10 spermatids. Zygotene (Z) and pachytene (P) spermatocytes also have lipids (open and closed arrowheads respectively). The nucleus of a Sertoli cell (S) with many lipid droplets (arrows) in its trunk and basal cytoplasm is indicated. X 770.



Figs. 9-10. Stage-dependent variations of the seminiferous epithelium. Error bars indicate standard errors of the mean.

Fig. 9. Stage-related changes in the area of the seminiferous epithelium during the breeding season. Values indicate the difference between the total area of the tubule (excluding the limiting membrane) and the area of the lumen.

Fig. 10. Changes in the number of Sertoli cell nuclei observed in a unit length of seminiferous tubule as determined by numerical density stereology.

**Areas of the Seminiferous Epithelium at the different Stages of the Cycle**

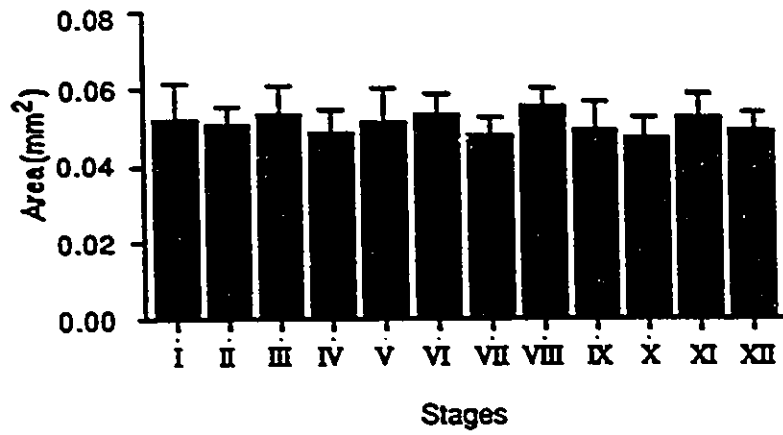


Figure 9

**Number of Sertoli Cell Nuclei per Length of Epithelium at different Stages of the Cycle**

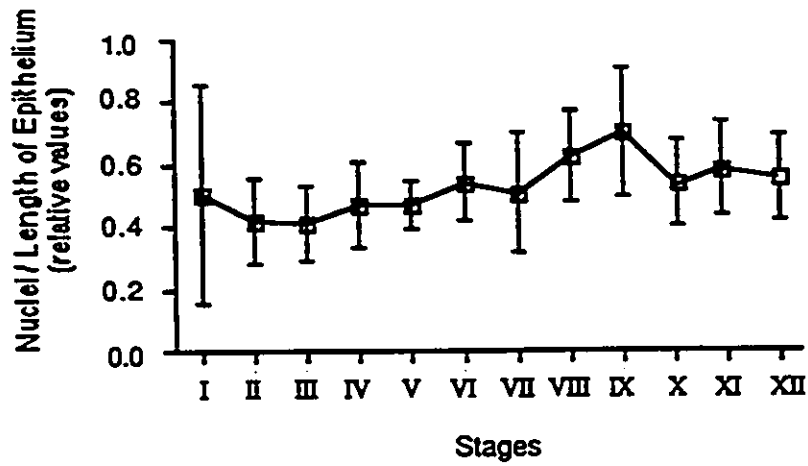


Figure 10

Fig. 10'

**Figure 10' Stage-Related Changes within the Seminiferous Epithelium**

Stages of the Cycle	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Epithelial Area (mm <sup>2</sup> X10 <sup>-2</sup> )	5.2	5.1	5.3	4.9	5.1	5.4	4.8	5.6	4.9	4.8	5.2	5.3
Standard Error of the Area	0.9	0.5	0.8	0.6	0.9	0.6	0.5	0.5	0.7	0.5	0.6	0.5
# of Sertoli cells (Relative)	0.54	0.46	0.42	0.50	0.50	0.58	0.54	0.67	0.75	0.54	0.63	0.58
Standard Error	0.37	0.14	0.13	0.15	0.08	0.13	0.20	0.15	0.22	0.14	0.15	0.14
Number of Animals (n)	7	6	8	7	7	14	13	10	8	6	9	7

Figs. 11-12. Volume density measurements of germ cell lipid droplets observed during the breeding season. In a, values are expressed as volume density in  $\mu\text{m}^3/\mu\text{m}^3$  (that is, cubic micrometers of lipid per cubic micrometer of epithelium) multiplied by the cross-sectional area of the seminiferous epithelium in  $\mu\text{m}^2$ . The product of this multiplication is the volume of lipid per unit length of epithelium in  $\mu\text{m}^3/\mu\text{m}$ . In b, the values are expressed as volume density in  $\mu\text{m}^3/\mu\text{m}^3$  divided by the numerical density of Sertoli cell nuclei (nuclei/ $\mu\text{m}^3$ ). The resulting quotient is the volume of lipid per Sertoli cell nucleus in  $\mu\text{m}^3/\text{nuclei}$ .

Fig. 11. Differences in the volume density of lipid droplets found in spermatids as they progress through the 19 steps of spermiogenesis.

Fig. 12. Stage-dependent variations in germ cell lipid droplet volume density.

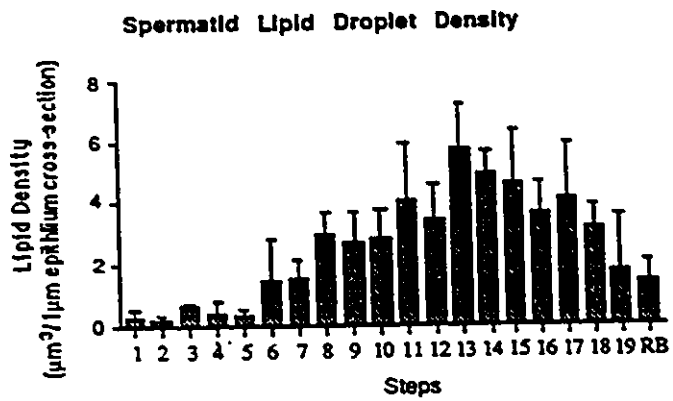


Figure 11a

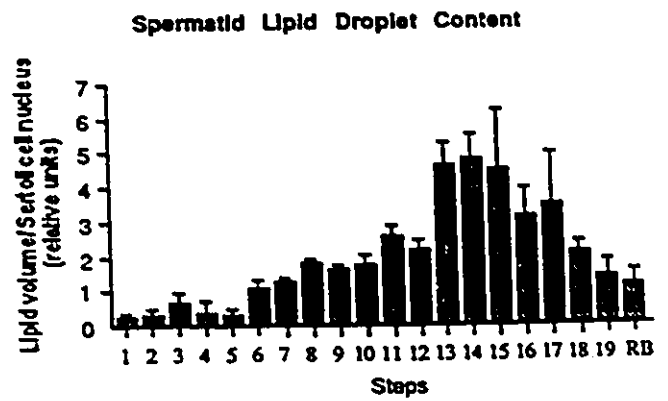


Figure 11 b

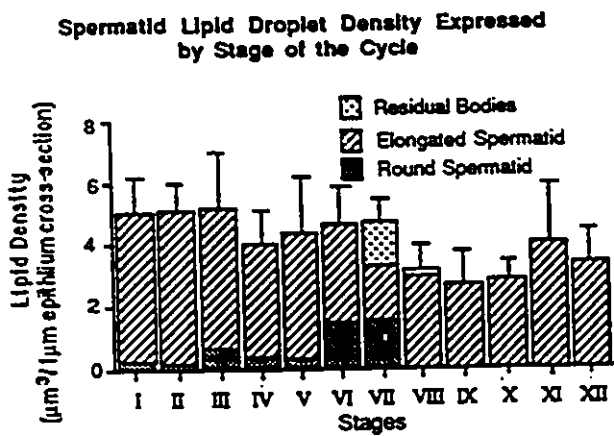


Figure 12a

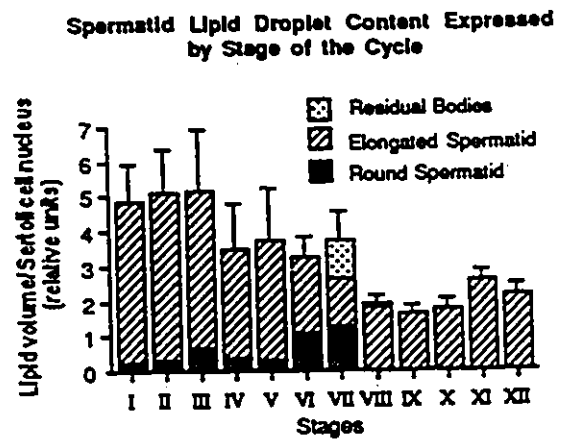


Figure 12b

The volume of lipid droplets in round and elongated spermatids of each cellular association, was added together to express the variation in lipid droplets by stage of the cycle rather than by step of spermiogenesis (Figs. 12 and 14'). A gradual, but insignificant, decrease of lipid droplets in germ cells was observed during the first seven Stages (I-VII) (Figs. 12 and 14') followed by a fifty percent decrease at Stages VIII.

### 3.1.1.2 Variations in lipid droplets in Sertoli cells

#### 3.1.1.2.1 Morphology

At Stage I, scarce and small (1-2  $\mu\text{m}$ ) lipids are found at the base of Sertoli cells, and larger ones (2-3  $\mu\text{m}$ ) in the supranuclear region (Fig. 3). Through the next five stages, the number and size of lipid droplets increased (Fig. 4) until Stage VI when several large (4-6 $\mu\text{m}$ ) droplets are found within each cell. Spermiation, coincides with the onset of a prolonged decline in Sertoli cell lipid droplets (Fig. 6). Residual bodies with lipid droplets, were observed at the apex of the Sertoli cell, but towards the base of the cell, these lipids disappeared and the volume of the residual bodies decreased (Figs. 7 and 8).

#### 3.1.1.2.2 Morphometric analysis of Sertoli cell lipid droplets

The volume of lipid droplets in Sertoli cells was expressed for each stage of the cycle. The value gradually increased from 2.0  $\mu\text{m}^3$  per Sertoli cell during Stage I, to 5.0  $\mu\text{m}^3$  per Sertoli cell during Stage VI (Figs. 13 and 14'). The lipid volume then decreased from 4.2  $\mu\text{m}^3$  per Sertoli cell during Stage VII, to 1.5  $\mu\text{m}^3$  per Sertoli cell during Stage XII. The most significant decrease in the volume of lipid droplets in Sertoli cells occurred between Stage VII and

Stage VIII, that is, just before and just after spermiation. This marked decrease is exemplified in figure 5 which shows two tubules at Stage VII that are adjacent to a tubule at Stage VIII. Levels of lipid droplets in Sertoli cells were maximal prior to spermiation. The infranuclear or basal lipids were small (1-2 $\mu$ m) and scarce at Stage I, increased in size and number until Stage VI, and then decreased during the second half of the cycle. The supranuclear lipids were larger and more frequent at Stage VII, but other than that seemed to follow the same general pattern as the basal lipids.

The total volume of lipid droplets in the seminiferous epithelium (Figs. 14 and 14') was defined as the contribution of lipid droplets from Sertoli cells and spermatids. The contributions from meiotic and pre-meiotic germ cells was not included because the lipid droplet content of these cells was found to account for less than five percent of the total volume of lipid droplets in germ cells and because the standard error for very small values is proportionally larger using the applied morphometric techniques. The volume of lipid droplets in spermatids and Sertoli cells combined (Figs. 14 and 14') remained nearly constant (6.6 to 8.4  $\mu$ m<sup>3</sup> per Sertoli cell) until just before spermiation. After spermiation however, i.e., Stages VIII to XI, the lipid droplets were significantly reduced in volume (3.5 to 4.5  $\mu$ m<sup>3</sup> per Sertoli cell), indicating a net loss of lipid droplets from the epithelium at spermiation.

Figs. 13-14. Volume density measurements of lipid droplets in the seminiferous epithelium observed during the breeding season. In a, values are expressed as volume density in  $\mu\text{m}^3/\mu\text{m}^3$  (that is, cubic micrometers of lipid per cubic micrometer of epithelium) multiplied by the cross-sectional area of the seminiferous epithelium in  $\mu\text{m}^2$ . The product of this multiplication is the volume of lipid per unit length of epithelium in  $\mu\text{m}^3/\mu\text{m}$ . In b, the values are expressed as the volume density in  $\mu\text{m}^3/\text{mm}^3$ , divided by the numerical density of Sertoli cell nuclei (nuclei/ $\mu\text{m}^3$ ). The resulting quotient is the volume of lipid per Sertoli cell nucleus in  $\mu\text{m}^3/\text{nuclei}$ .

Fig. 13. Stage-dependent variations in Sertoli cell lipid droplet volume densities.

Fig. 14. Spermatids' and Sertoli cells' contributions are distinguished but compiled to illustrate stage-dependent changes in the total lipid droplet volume densities within the seminiferous epithelium.

**Stage-Related Variation in Sertoli Cell Lipid Droplet Density**

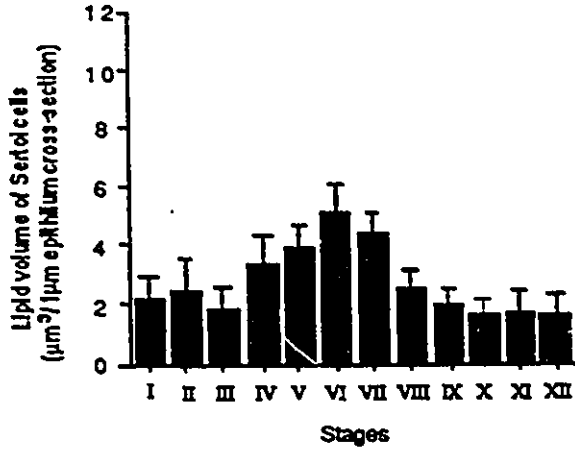


Figure 13a

**Stage-Related Variation in Sertoli Cell Lipid Droplet Content**

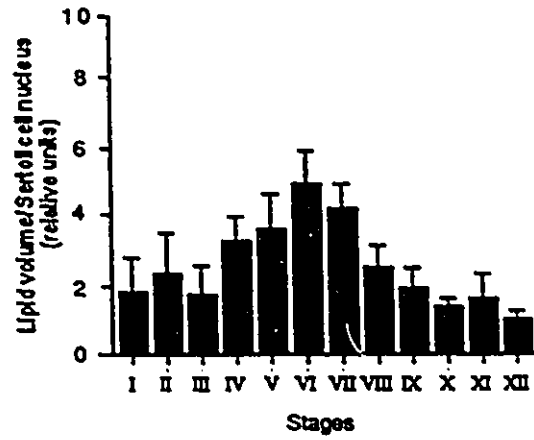


Figure 13b

**Stage-Related Variations in the Spermatid and Sertoli cell lipid Droplet Density**

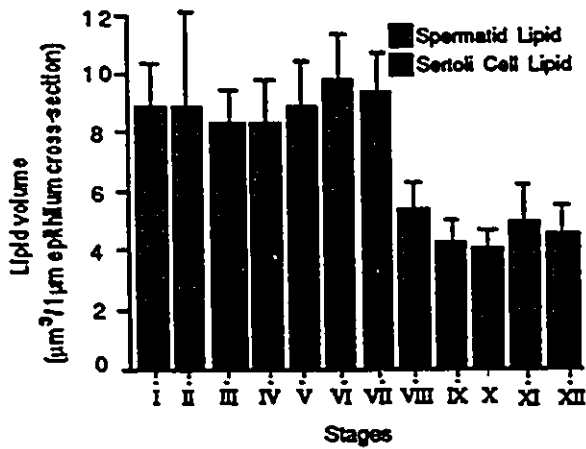


Figure 14a

**Stage-Related Variations in the Sertoli cell and Spermatid Lipid Droplet Content per Sertoli Cell**

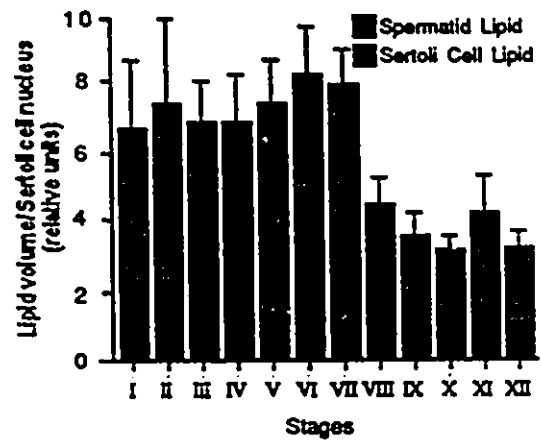


Figure 14b

Fig.14'

Figure 14' Stage-Related Variations in the Volume of Lipid Droplets in Spermatids and Sertoli cells per Sertoli cell Nucleus ( $\mu\text{m}^2/\text{nuc.}$ )

Stages of the Cycle	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Round Spermatids	0.32	0.29	0.65	0.35	0.27	1.09	1.23					
Round Spermatids S.E.	0.15	0.19	0.08	0.36	0.21	0.22	0.16					
Elongated Spermatids	4.97	4.77	4.47	3.10	3.45	2.12	1.39	1.73	1.61	1.75	2.56	2.47
Elongated Spermatids S.E.	0.67	0.75	1.75	0.86	1.54	0.26	0.54	0.15	0.13	0.32	0.34	0.55
Residual bodies							1.11	0.14				
Total Lipids in Spermatids	5.29	5.06	5.12	3.45	3.72	3.21	3.74	1.92	1.61	1.75	2.56	2.47
Spermatids Lipid S.E.	0.76	0.93	1.63	1.06	1.50	0.47	0.74	0.19	0.13	0.32	0.34	0.55
Sertoli Cells	2.08	2.33	1.73	3.38	3.67	5.00	4.20	2.50	1.94	1.57	1.64	1.28
Sertoli Cell Lipid S.E.	0.81	1.19	0.82	0.60	0.99	0.92	0.74	0.71	0.60	0.41	0.69	0.44
Spermatids & Sertoli cells	7.37	7.39	6.63	6.98	7.44	8.40	7.97	4.54	3.48	3.33	4.39	3.85
Combined Volumes S.E.	1.30	2.73	1.20	1.36	1.23	1.38	1.05	0.86	0.71	0.73	1.08	0.97
Number of Animals (n)	7	6	8	7	7	14	13	10	8	6	9	7

### 3.1.2 Throughout the Seasonal Reproductive Cycle

#### 3.1.2.1 Morphology

The breeding season ends in the last week of March for the mink. Germ cell loss progressed from the most mature to the least mature germ cells (Figs. 15-19) until the period of maximal testicular regression in July and August at which time the seminiferous epithelium contained only spermatogonia and Sertoli cells, in accordance with previous observations (Pelletier, 1986). During the last week of April, the lumen of the seminiferous tubule was usually closed. The closure of the lumen marks the onset of spermatogenic regression along that particular length of tubule. Tubules at various stages of the cycle were found with a typical complement of lipid inclusions even when the lumen was closed (Fig. 15). At the onset of testicular regression (Fig. 16), the lumen closed and elongated spermatids were no longer present within the epithelium. Normal round spermatids however, still occupied the central region of the tubule. Large 5-10  $\mu\text{m}$  lipid droplets formed a distinct ring between the younger germ cells and the apically located round spermatids. This location of the lipids was maintained even when testicular regression was more advanced (Fig. 17). The lipid ring was still observed just above the Sertoli cell nuclei in tubules that had no post-meiotic germ cells (Fig. 18). Clusters of small (1-3 $\mu\text{m}$ ) dense bodies were also observed above Sertoli cell nuclei which had migrated away from the limiting membrane (Fig. 18). By the end of June, the tubules no longer contained meiotic or post-meiotic germ cells. The once prominent supranuclear lipids were now scarce and small (rarely larger than 4  $\mu\text{m}$  in diameter).

A few small 2-3  $\mu\text{m}$  lipid droplets were seen during the period of complete testicular regression in July (Fig. 19) and August (Fig. 20). These were usually seen next to a Sertoli cell nucleus or associated with clusters of small (1-

3 $\mu$ m) dense bodies (Fig. 20). The appearance of Sertoli cell lipids remained unchanged until the onset of the new spermatogenic season in November.

The frequency and size of lipid droplets in Sertoli cells in November depended on the presence meiotic activity. Lipids were unchanged in tubules that did not contain meiotic germ cells. However, tubules in which meiosis had been initiated (Fig. 21) as indicated by the presence of leptotene and pachytene spermatocytes, contained large (4-8  $\mu$ m diameter) lipid droplets. These droplets formed a distinct ring between the centrally located pachytene spermatocytes and the basal layer of germ cells and Sertoli cell nuclei similar to that observed in May (Fig. 18). Smaller (2-4  $\mu$ m diameter) basal lipids were found within the Sertoli cell cytoplasm. At this time, spermatogenesis in some tubules had progressed as far as step 6 spermatid, but these tubules typically had a slightly reduced population of lipid droplets.

In late December, spermatogenesis was complete and the presence of lipid droplets returned to the stage-dependent distribution already described.

#### 3.1.2.2 Morphometric Analysis of Sertoli cell lipids throughout the seasonal reproductive cycle

The closure of the lumen and loss of elongated spermatids coincided with a sharp decrease in the cross-sectional area of the seminiferous epithelium (Figs. 22 and 23'). The decrease in area continued until the period of maximal testicular regression in August. In contrast, Sertoli cell numbers per length of tubule remained approximately constant throughout the year (Figs. 23 and 23').

During the breeding season, the average volume of lipid droplets in Sertoli cells was 2.8  $\mu$ m<sup>3</sup> per cell (Figs. 24 and 24'). This value increased briefly to 4.3  $\mu$ m<sup>3</sup> at the onset of

testicular regression, but then gradually declined to below  $1.0 \mu\text{m}^3$  per cell during the period of maximal testicular regression (Figs. 24 and 24'). Prior to the return to full spermatogenic activity, the Sertoli cell lipid droplet volume recovered to  $1.8 \mu\text{m}^3$  per cell. The relative differences in the volume of lipid droplets in Sertoli cells was reflected in infranuclear and supranuclear regions. Infranuclear lipid droplets became almost non-existent during the inactive spermatogenic phase in contrast to their relative frequency during the active spermatogenic phase.

In summary, after an initial increase in lipid droplets at the beginning of testicular regression, the volume of lipid droplets in Sertoli cells was dramatically reduced later during testicular regression. This reduction coincided with a decrease in spermatogenic activity and the eventual disappearance of the post-meiotic and meiotic germ cell population.

Figs. 15-21. Light micrographs of toluidine blue-stained 0.5mm thick sections. Taken from adult mink sacrificed during testicular regression.

Fig. 15. Lipid droplets are indicated within Sertoli cells (arrows) and in the caudal cytoplasm of step 9 spermatids (open arrowhead). Tubule from a mink sacrificed in the last week of April. The lumen is closed. X 680.

Fig. 16. Round spermatids (R) with lipid droplets in their caudal cytoplasm (arrowhead) are still present, but no elongated spermatids are found. Large lipid droplets (arrows) enscribe a circle just apical to the Sertoli cell nuclei (S). A pre-meiotic germ cell (G) is indicated. The lumen is also closed in this tubule which was taken from a mink sacrificed in the last week of May. X 680.

Fig. 17. No post-meiotic germ cells are present in this tubule. Large lipid droplets (arrows) found within Sertoli cell (S) cytoplasm, form a ring between the spermatogonia and spermatocyte (P). This seminiferous tubule was taken from a mink sacrificed in the last week of May. X 680.

Fig. 18. Sertoli cell nuclei (S), along with the large lipid droplets (arrows), have migrated away from the limiting membrane of the tubule. Clusters of small (1-3 $\mu$ m) dense bodies are also noted (arrowhead). Spermatogonia (G) are located near the base of the epithelium. This tubule was also taken from a mink sacrificed in the last week of May. X 680.

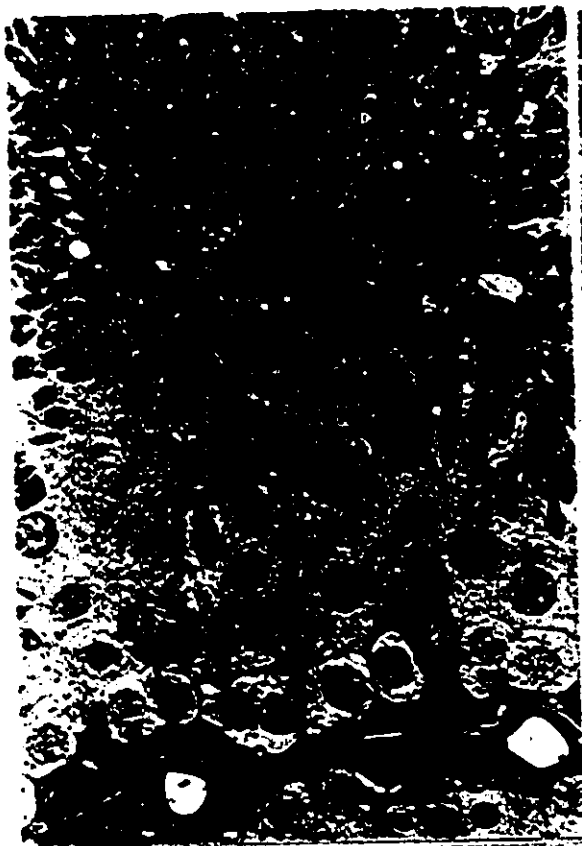


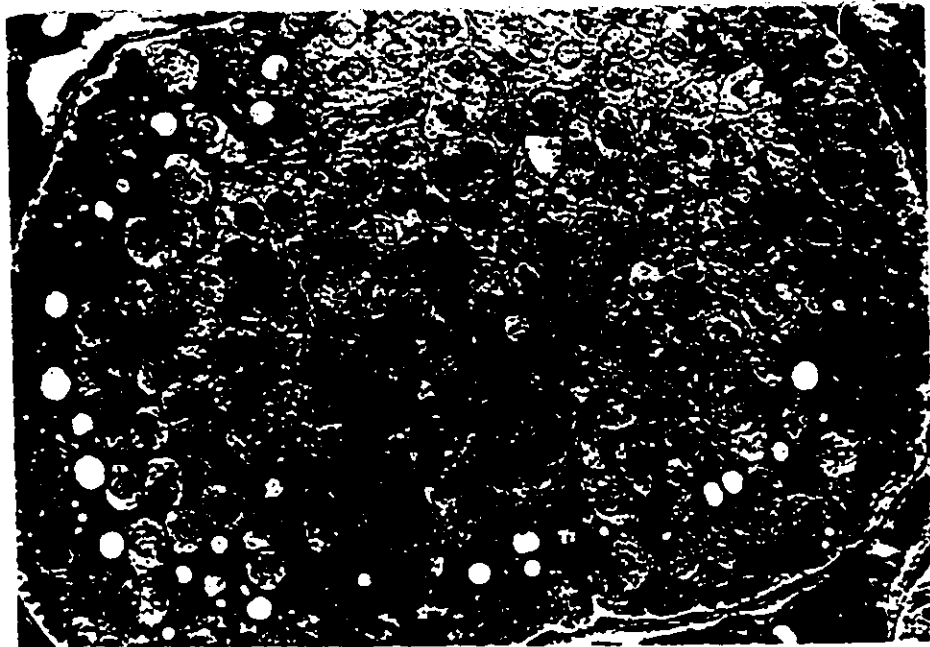
Fig. 19. Two distinct types of lipid droplet are recognized: dark (open arrow) and pale (closed arrow). The overall number and size of lipid droplets however, is dramatically reduced as is the number of meiotic and post-meiotic germ cells. The seminiferous epithelium is primarily composed of Sertoli cells (S) interspersed by a few spermatogonia (G). The tubule was taken from a mink sacrificed in the last week of July. X 680

Fig. 20. The population of type A spermatogonia (G) seems to have increased and in the process, displaced many of the Sertoli cell nuclei (S) away from the limiting membrane. Only small, dark lipid droplets are present. The seminiferous tubule of a mink sacrificed in the last week of August. X 680.

Fig. 21. In November, spermatogenesis is reinitiated, as illustrated in this light micrograph. Large lipid droplets once again form a prominent ring just apical to the Sertoli cell nuclei. Round spermatids (R) and pachytene spermatocytes (P) are present. X 450.



19



Figs. 22-24. Graphs representing of the seasonal changes within the seminiferous epithelium. Error bars indicate standard error of the mean.

Fig. 22. A histogram depicting the changes in the cross-sectional areas of the seminiferous epithelium. The values refer to the average area of the tubule, minus the luminal areas.

Fig. 23. Variation in the number of Sertoli cell nuclei observed in a 1mm length of seminiferous tubule.

**Areas of the Seminiferous Epithellum at the different Months of the Year**

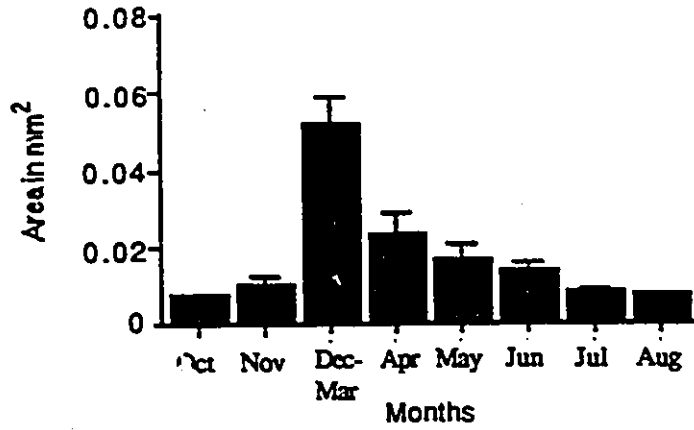


Figure 22

**Number of Sertoli cell Nuclei per Length of Epithellum at different Months of the Year**

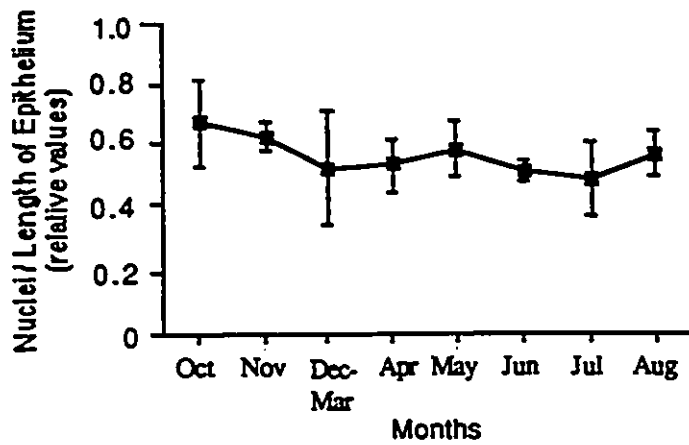


Figure 23

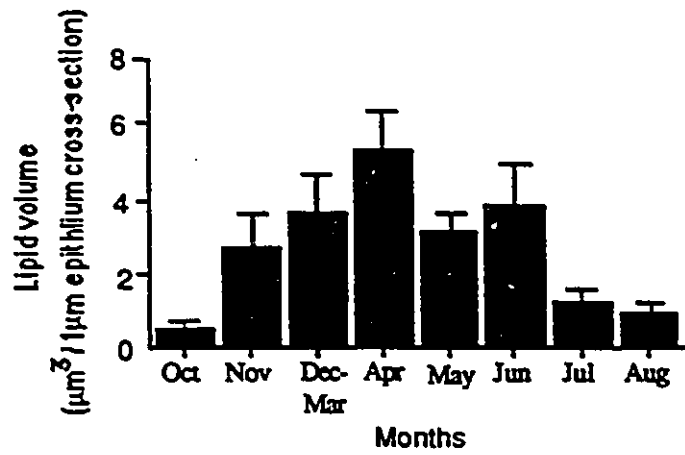
Fig. 23'

**Figure 23' Seasonal Changes within the Seminiferous Epithelium**

<b>Months of the Year</b>	<b>Oct</b>	<b>Nov</b>	<b>Dec-Mar</b>	<b>Apr</b>	<b>May</b>	<b>Jun</b>	<b>Jul</b>	<b>Aug</b>
<b>Epithelial Area (mm<sup>2</sup>X10<sup>-2</sup>)</b>	<b>0.74</b>	<b>1.02</b>	<b>5.12</b>	<b>2.23</b>	<b>1.66</b>	<b>1.43</b>	<b>0.78</b>	<b>0.66</b>
<b>Standard Error</b>	<b>0.01</b>	<b>0.23</b>	<b>0.68</b>	<b>0.6</b>	<b>0.39</b>	<b>0.18</b>	<b>0.11</b>	<b>0.03</b>
<b># of Sertoli cells (Relative)</b>	<b>0.71</b>	<b>0.63</b>	<b>0.54</b>	<b>0.54</b>	<b>0.63</b>	<b>0.54</b>	<b>0.50</b>	<b>0.58</b>
<b>Standard Error</b>	<b>0.15</b>	<b>0.06</b>	<b>0.19</b>	<b>0.08</b>	<b>0.10</b>	<b>0.04</b>	<b>0.10</b>	<b>0.08</b>
<b>Number of Animals (n)</b>	<b>4</b>	<b>4</b>	<b>14</b>	<b>4</b>	<b>4</b>	<b>4</b>	<b>4</b>	<b>4</b>

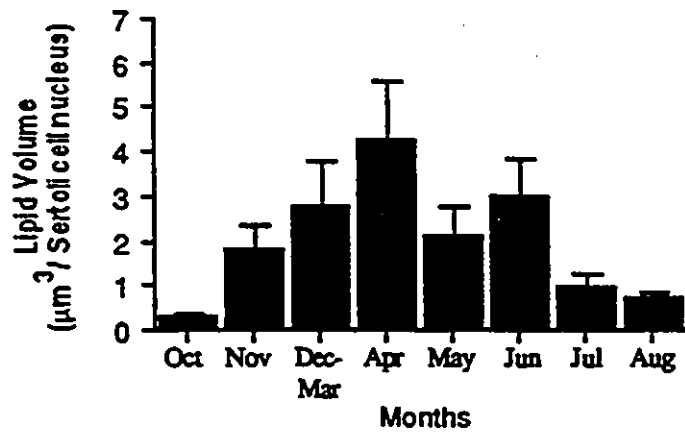
Fig. 24. Seasonal changes in the volume density of Sertoli cell lipid droplets. In a, values are expressed as the volume density in  $\mu\text{m}^3/\mu\text{m}^3$  (that is, cubic micrometers of lipid per cubic micrometer of epithelium) multiplied by the cross-sectional area of the seminiferous epithelium in  $\mu\text{m}^2$ . The product of this multiplication is the volume of lipid per unit length of epithelium in  $\mu\text{m}^3/\mu\text{m}$ . In b, the values are expressed as volume density in  $\mu\text{m}^3/\mu\text{m}^3$ , divided by the numerical density of Sertoli cell nuclei (nuclei/ $\mu\text{m}^3$ ). The resulting quotient is the volume of lipid per Sertoli cell nucleus in  $\mu\text{m}^3/\text{nuclei}$ .

**Seasonal Variation in Sertoli Cell  
Lipid Droplet Density**



**Figure 24a**

**Seasonal Variation in Sertoli cell Lipid  
Droplet Content**



**Figure 24b**

Fig. 24'

Figure 24: Seasonal Variations in the Volume of Lipid Droplets in Sertoli cells per Sertoli cell Nucleus ( $\mu\text{m}^2/\text{nuc.}$ )

Months of the Year	Oct	Nov	Dec-Mar	Apr	May	Jun	Jul	Aug
Lipid Volume/Nucleus	0.29	1.78	2.75	4.27	2.09	3.02	0.97	0.70
Standard Error	0.11	0.59	1.05	1.34	0.74	0.88	0.35	0.22
Number of Animals (n)	4	4	14	4	4	4	4	4

### 3.1.3. Electron microscopy

During the active spermatogenic phase, lipid droplets were found in all germ cell classes (Figs. 25, 26, and 27). The lipids were more abundant in older germ cells (Fig. 29). At Stage VII, residual bodies (Fig. 28) containing eccentrically located lipid droplets were observed near the apex of the seminiferous epithelium. During the middle stages of the cycle (Stage IV to X), large lipid droplets were found within the Sertoli cell, either at the base or just above the nucleus (Fig. 29). Basal lipids were occasionally accompanied by concentric cisternae of smooth endoplasmic reticulum, along with small membrane bound vesicles. Although not common, these assemblages were observed in all stages. A frequent location for Sertoli cell lipid droplets was the thin cytoplasmic processes between germ cells. At this location, the droplet could occupy the entire width of the process (Fig. 44). Mitochondria were often observed in close proximity to lipid droplets. Most mitochondria were the orthodox variety with shelf-like cristae. They were scattered throughout the Sertoli cell cytoplasm but were concentrated just apical to the nucleus (Fig. 29). Lipid droplets found inside occasional degenerating cells appear to contribute to the Sertoli cell's lipid content.

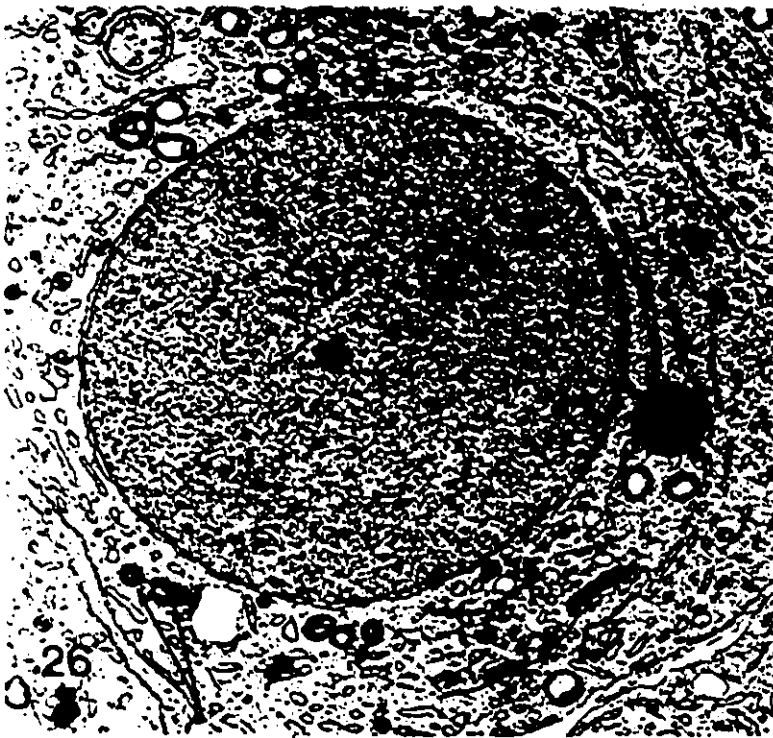
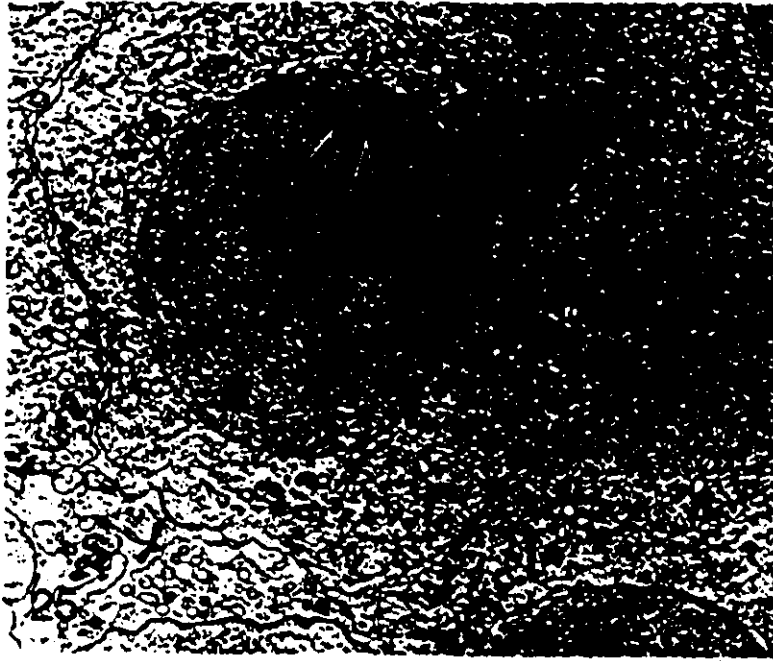
During testicular regression, small 2-3 $\mu$ m, pale, supranuclear lipids were found in Sertoli cells (Fig. 30). Clusters of small (1-2 $\mu$ m) dense bodies were found in close proximity to these lipids prior to the onset of the new spermatogenic period (Fig. 31). Concentrated clusters of elongated mitochondria were also observed in the supranuclear region. The apical cytoplasm was attenuated, with extensive lamellae of smooth endoplasmic reticulum.

Figs. 25-29. Electron micrographs from adult mink testes sacrificed during the active spermatogenic period.

Fig. 25. Lipid droplet (L) observed within the cytoplasm of a Stage V pachytene spermatocyte (P). Synaptonemal complex is indicated (arrowheads). Sertoli cell cytoplasm (S) encompasses the spermatocyte. X 9,600.

Fig. 26. Lipid droplet (closed arrowhead) observed within the cytoplasm of a step 1 spermatid (Std 1). The Golgi complex (open arrowhead), centriole, and flagellum (arrows) are indicated. X 10,800.

Fig. 27. Lipid droplets (arrows) observed within the caudal cytoplasm of a step 10 spermatid (Std 10). Apical extensions of the Sertoli cell cytoplasm are indicated (S). X 9,050.



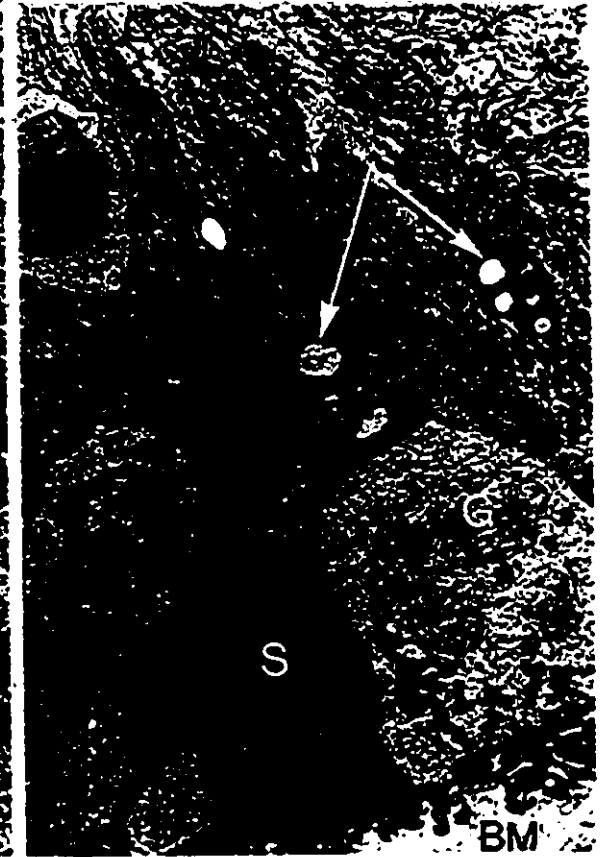
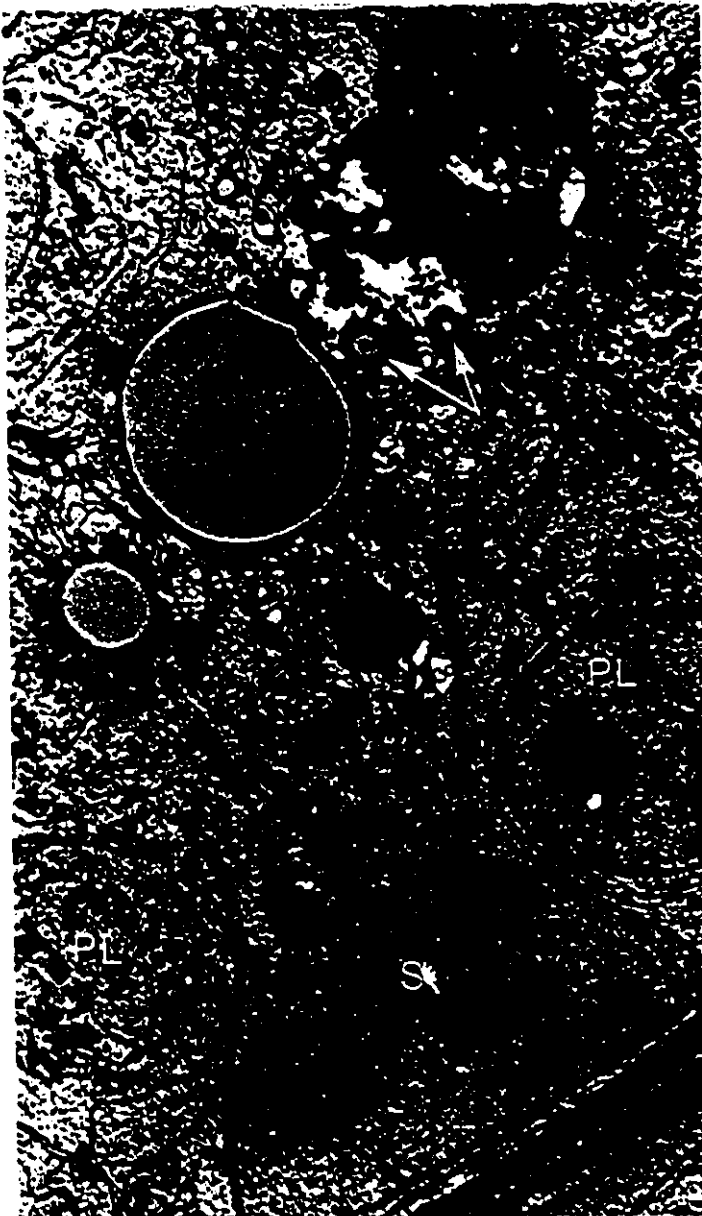
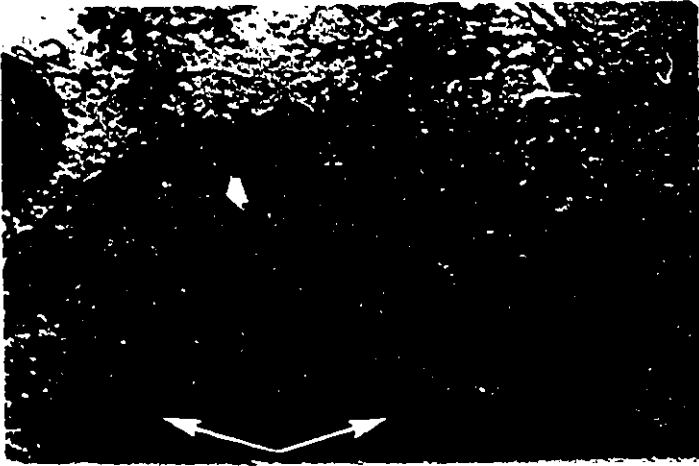
0.26

Fig. 28. Residual bodies (RB) observed within the apical cytoplasm of a Sertoli cell at Stage VII of the cycle. Lipid droplets (arrowhead) are seen restricted to one side of the residual body. X 7,400.

Fig. 29. Photomicrograph showing the basal region of a Stage VII seminiferous epithelium. Small lipid droplets (arrows) opposite the condensed nucleus (N) of a phagocytosed germ cell, are immediately adjacent to a large lipid droplet (L) in the trunk of a Sertoli cell (S). Preleptotene spermatocytes (PL) are close to the limiting membrane (LM). X5,400.

Fig. 30. Electron micrograph from the testis of a mink sacrificed in July. Lipid droplets (arrows) are apical to the Sertoli cell nuclei (S). A spermatogonia (G) is identified on the basement membrane (BM). X 4,200.

Fig. 31. Electron micrograph from the testis of a mink sacrificed in October. Lipid droplets (arrows) are associated with clusters of small 1-2 $\mu$ m dense bodies (open arrowheads) found just apical to germ cells (G) and Sertoli cell nuclei (S). The centre of the tubule (C) and the basement membrane (BM) are defined. X 3,000.



## **3.2 Biochemistry**

### **3.2.1 Isolation of cell sub-types**

Percoll density gradient centrifugation of the crude Leydig cell fraction taken during the active spermatogenic period, resulted in the formation of two bands (see Table 1). The upper band was composed primarily of Leydig cells as determined by the quick fix and stain method and further characterized by light and electron microscopy. The lower band contained both germ cells and Leydig cells and was discarded. Three layers were formed from the germ cell separation, of which the top layer was determined by light microscopy to be principally made up of germ cell sub-types.

The crude Sertoli cell fraction was separated into two layers of cells by Percoll density gradient centrifugation. During the active phase, the top layer contained small clumps of Sertoli cells and isolated germ cells in nearly equal portions. The lower layer contained short lengths of broken tubules that were stripped of germ cells. During testicular regression, the upper layer contained Sertoli cells only, while the lower layer still contained short lengths of tubules. Both layers were retained for further investigation. Trypan blue exclusion showed viability of cells at greater than 70% for all fractions that were accepted for further analysis. Germ cell fractions obtained during the active spermatogenic phase typically had highest viability (about 90%).

Light microscope observations revealed that the Leydig cell fractions were composed of large cells (15-20  $\mu\text{m}$  in diameter) with a spherical nucleus (Fig. 32b). Electron microscope observations revealed the presence of an extensive smooth endoplasmic reticulum, large mitochondria, lysosome-

like bodies, and numerous lipid droplets in the cytoplasm of these cells (Fig. 34a).

Optical and ultrastructural characterization of the germ cell layer revealed the presence of all classes of germ cells found in the intact testes except for spermatogonia (Figs. 33a and b). Sertoli cell cytoplasmic processes still attached to older spermatids, were the only non-germ cell contaminant but these were found to compose only about 5% of the total volume.

From the Sertoli cell fractionation, during the breeding season, the top layer (see Table 1) was highly contaminated with germ cells and was discarded, but during the inactive spermatogenic phase, this fraction was used as the Sertoli cell fraction being almost free of all other cell subtypes. The bottom layer (Table 1) was made up of short segments of broken tubules composed primarily of Sertoli cells with spermatogonia, preleptotene spermatocytes and myoid cells making up about 20% of the total volume. In the active season, this was used as the Sertoli cell enriched fraction. Ultrastructurally, the Sertoli cells had long attenuated cytoplasmic processes (Fig. 34a). The nucleus was typical of the Sertoli cell, being often highly invaginated, with a prominent nucleolus and accompanying satellite. When the cells were found in clumps, Sertoli cell junctional complexes were usually still intact (Fig. 34b). Subcellular fractionation of Sertoli cells revealed the presence of tight and gap junctions in the plasma membrane fraction (Fig. 35).

## **3.2.2 Cholesterol determination**

### **3.2.2.1 Subcellular Fraction**

Subcellular fractions occasionally had cholesterol or protein levels that were below the limit of sensitivity of the assay applied. This limitation made discernment of the

cholesterol content statistically invalid and so those results will not be discussed further.

### 3.2.2.2 Whole Cell

The analysis of variance performed on the determined free and esterified cholesterol content, revealed that the seasonal change of cholesterol content in Sertoli cells was highly significant. Within germ cells however, neither the free nor the esterified cholesterol content was significantly different from one phase of spermatogenic activity to the next. The Leydig cell population was shown to vary with a high level of significance in its content of esterified cholesterol only.

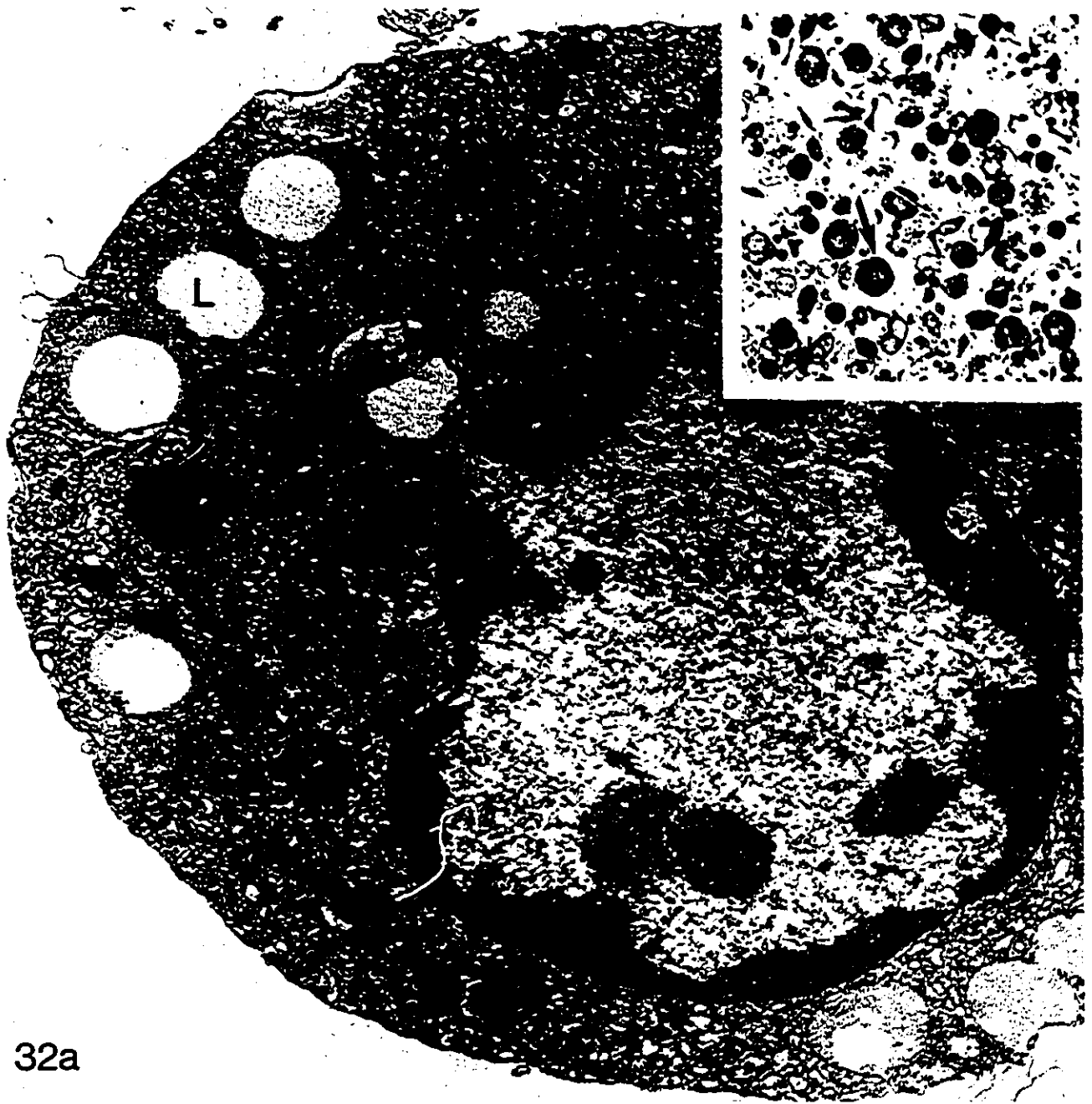
For whole Sertoli cells, there was a significant increase in the content of esterified cholesterol accompanying the onset of spermatogenesis in November. This increase was maintained throughout the active spermatogenic phase and peaked at the onset of testicular regression (late April to early May). The peak was of short duration since by July, esterified cholesterol levels in Sertoli cells had declined, reaching levels similar to those recorded at the onset of spermatogenesis. In short, the cholesteryl ester values in Sertoli cells followed the annual cyclic variations in the germ cell population. The free cholesterol levels varied in a similar fashion but with greater moderation.

The germ cell population showed a modest, but not significant, increase in esterified cholesterol content as spermatogenesis proceeded (Fig. 36 and Fig. 36').

In the Leydig cell fraction, the esterified cholesterol values were at their lowest levels at the onset of spermatogenesis and during the active spermatogenic phase. Maximum levels were attained at the onset of testicular regression. There was no significant change in the content of free cholesterol throughout the year.

Fig. 32a. Electron micrograph showing an isolated Leydig cell. Mitochondria (Mit), endoplasmic reticulum (ER), lysosome-like bodies (Ly) and lipid droplets from which the lipid was partially extracted during processing (L) are identified. X 18,000.

Fig. 32b. Light micrograph taken of a representative field from the isolation of interstitial tissue. A Leydig cell is identified (arrow). X 500.



32a

Fig. 33a. Electron micrograph taken of isolated germ cells. A step 5 (Std 5) and step 8 (Std 8) spermatid are shown. The Golgi complex (GB) is indicated. X 7,300.

Fig. 33b. Light micrograph taken of an isolated germ cell fraction. A step 5 (5) and step 8 (8) spermatid and a pachytene spermatocyte (P) are identified. X 500.

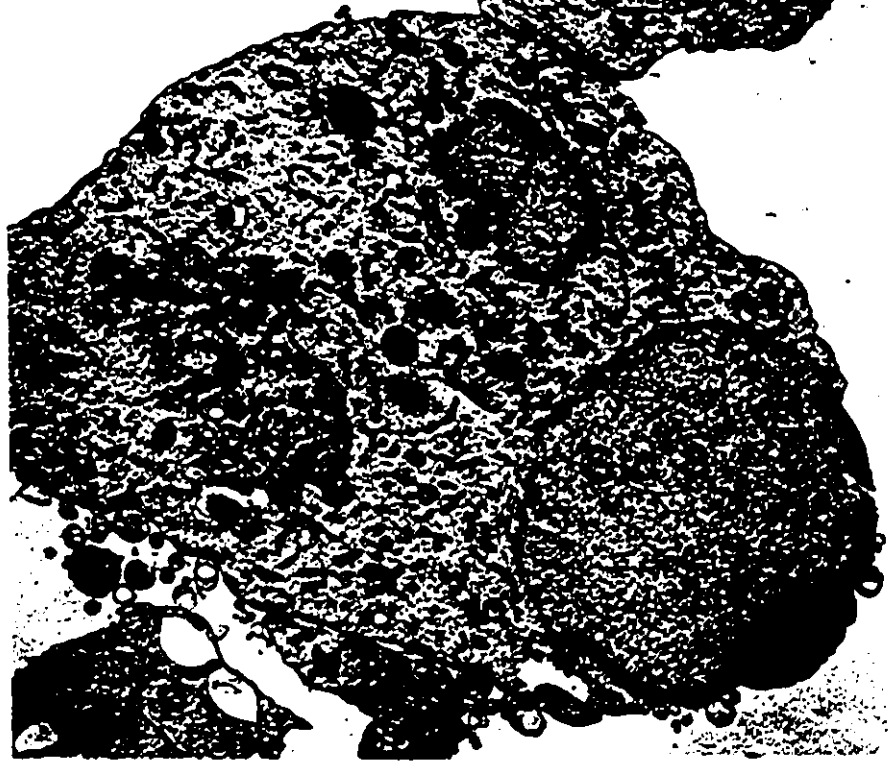
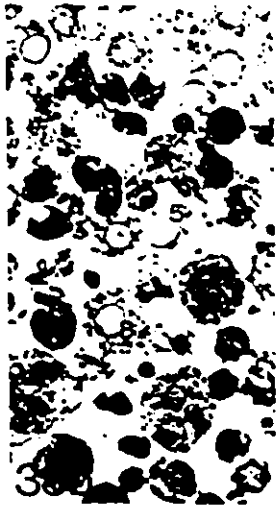


Fig. 34a. Electron micrograph showing an isolated Sertoli cell. Note the presence of lipid droplets (L and arrow) and the extensive apical cytoplasmic processes (cp). A prominent nucleolus (Nu) within the nucleus is indicated. X 9,180.

Fig. 34b. Electron micrograph showing Sertoli cell junctions from isolated Sertoli cell fraction. The tight junctions (arrows), subsurface filaments and cisternae are intact. X 56,000.

Fig. 34c. Light micrograph taken of an isolated Sertoli cell fraction. Sertoli cells (arrow) are seen individually and in clumps. X 500.

Fig. 35. Electron micrograph taken from an isolated Sertoli cell membrane fraction. Gap junctions (open arrow) and tight junctions (closed arrows) are identified. X 67,500.



Fig. 36. A diagram, line-graph and histogram combined to facilitate the comparison of the seasonal spermatogenic activity of the testis with the variation in cholesterol content within the three cellular compartments (Sertoli cells, germ cells, and interstitial cells) and changes in the volume density of Sertoli cell lipid droplets. On the diagram, A and B are respectively type A and B spermatogonia, PL and P are respectively the preleptotene and pachytene spermatocytes, and R and E refer to the round and elongated spermatid. The letters on the line-graphs indicate tests that were different at a 0.05 level of significance as determined by the Duncan pairwise comparison. Uppercase letters refer to esterified cholesterol and lower case refer to free cholesterol. C is different than A at a high level of significance ( $p < 0.01$ ).

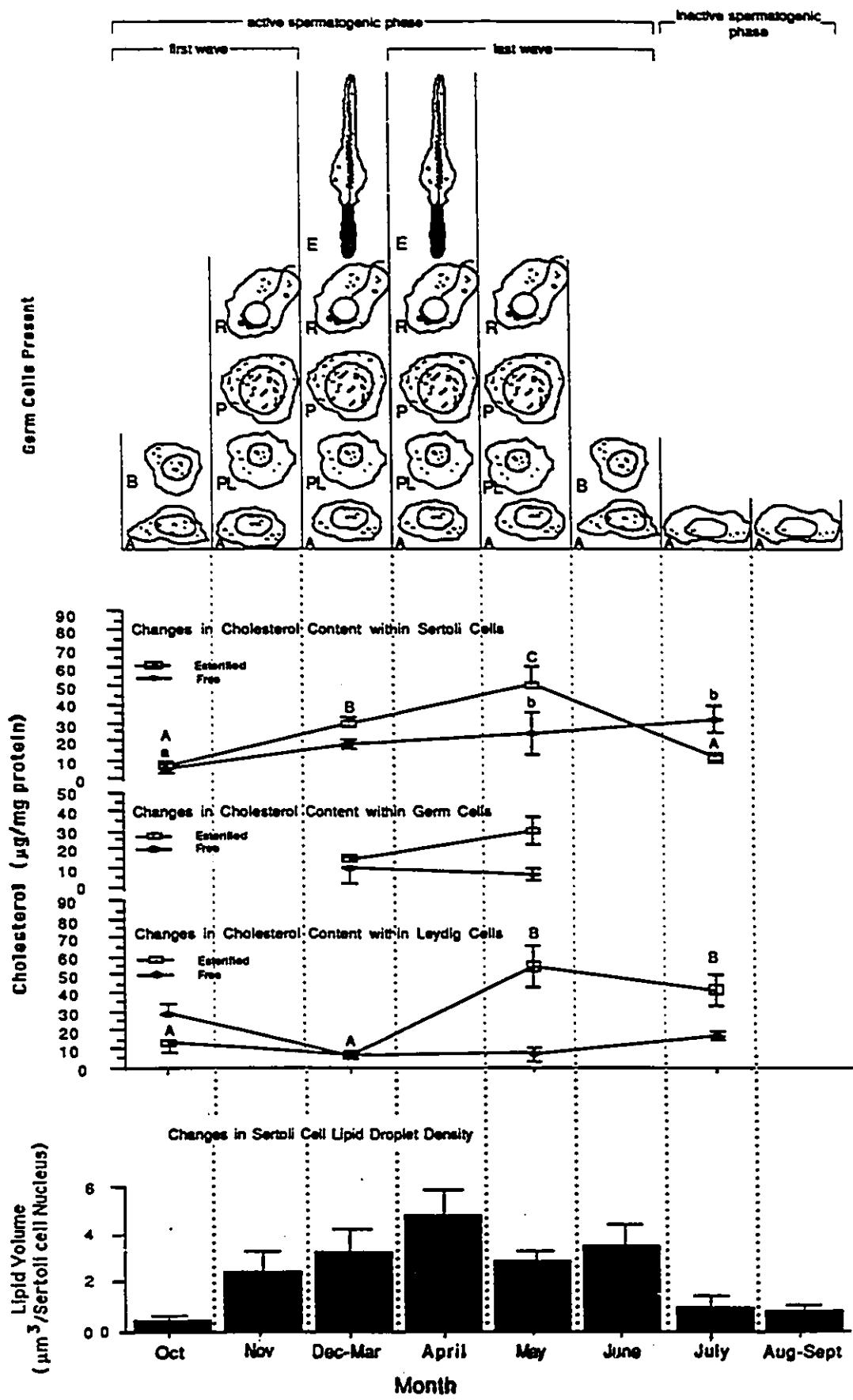


Figure 36

Fig. 36'

Figure 36' Seasonal Variations in the Cholesterol content of Sertoli cells, Germ cells and Leydig cells ( $\mu\text{g}/\text{mg}$  protein  $\pm$  standard error)

Month of the Year	October	January	May	July
Esterified Cholesterol in Sertoli cells	5.8 $\pm$ 1.4	30.0 $\pm$ 2.8	50.5 $\pm$ 29	10.9 $\pm$ 2.4
Free Cholesterol in Sertoli cells	4.8 $\pm$ 1	22.2 $\pm$ 3	23.9 $\pm$ 12	31.8 $\pm$ 8
Esterified Cholesterol in Germ cells		14.6 $\pm$ 3.1	30.3 $\pm$ 8.7	
Free Cholesterol in Germ cells		11.2 $\pm$ 8	6.3 $\pm$ 2	
Esterified Cholesterol in Leydig cells	13 $\pm$ 5	6.5 $\pm$ 2	54 $\pm$ 13	41 $\pm$ 9
Free Cholesterol in Leydig cells	28.9 $\pm$ 5	5.4 $\pm$ 1	6.4 $\pm$ 2	16.8 $\pm$ 2
Number of Animals (n)	3	3	3	3
Number of Determinants	6	6	6	6

### **3.3 Cytochemistry**

#### **3.3.1 Diaminobenzidine**

##### **3.3.1.1 Characteristics of the DAB Reaction**

After performing the procedure of Jones and Miyai (1981) for the ultrastructural localization of free and esterified cholesterol, electron dense reaction product was observed within lipid droplets (Fig. 37a) and was usually associated with a small region of lipid extraction (Fig. 37b). Typically, large single droplets would have several small, electron lucent vacuoles inside, with an electron opaque deposit lining the border of the vacuoles or forming clumps at their edges (Figs. 38 and 39). In tissue that showed the best preservation of lipid morphology, large single droplets had several small, electron lucent vacuoles inside, with an electron opaque deposit lining the border of the vacuoles or forming clumps at their edges (Fig.39). In some instances however, the lipid droplets assumed an irregular shape and a blotchy texture, with electron opaque reaction product associated with the least electron dense regions of the droplet (Figs.41 and 43). These "labeled profiles" were found when both cholesterol oxidase and cholesterol esterase were present in the incubation medium. Lysosome-like bodies labelled positively (Figs. 40 and 43b), but without the characteristic extraction zones described for lipid droplets.

##### **3.3.1.2 Location of Reaction Product**

Reaction product was observed in the lipids of spermatids and also in lipids that were free in the lumen (Fig. 41).

The most conspicuous location for reaction product was in the basal cytoplasm of Sertoli cells where large labeled lipids were often seen (Figs. 37b, 38, 39, and 42). This

was particularly true at Stages V to VIII when lipid droplets were largest. Lysosome-like bodies (Fig. 40) and lipidic components associated with clusters of small 1-2 $\mu$ m dense bodies (Fig. 37a) also labeled positive. Small droplets within residual bodies were labeled. Small, membrane-bound vesicles and cisternae (10-20 nm in diameter) often appeared to take on an increased electron density (Fig. 42) reminiscent of a positive reaction product. These vesicles however, often labelled positive even in the absence of cholesterol oxidase.

The Leydig cells of the interstitial tissue were labeled in two different cytoplasmic organelles. The numerous lipid droplets of the mink Leydig cell labelled in a manner that is similar to those of the Sertoli cells and spermatids (Fig. 43a). In addition, membrane bound, lysosome-like bodies were labeled (Figs. 43a and 43b) however, as in Sertoli cell lysosome-like bodies, the cleared regions which were characteristic of enzyme-digested lipid droplets, were not always seen.

In control experiments, cholesterol oxidase or cholesterol esterase was omitted from the incubation media in order to evaluate the necessity of free cholesterol or cholesteryl esters as substrates in the reaction sequence. In these experiments, no deposition of reaction product was observed within lipid droplets (Figs. 44 and 45). Removal of free cholesterol by preincubation of the tissue in cholesterol oxidase (Figs. 38, 41 and 43), or 70% ethanol (Fig. 37) does not adversely affect the appearance of the reaction product.

The demonstration of a positive reaction for membranous cholesterol could only be found on the luminal surface of endothelial cells. In tissue that had been processed for the demonstration of free or total cholesterol, an electron dense thickening of the luminal plasma membrane surface of endothelial cells was observed (Fig. 46). Fuzzy-coated vesicles which were in continuity with, or in close proximity

to this membrane also labeled. No specific reaction product was found on other plasma membranes. The plasma membrane of mature spermatids appeared to be somewhat reaction positive but this was also observed in tissue which had been incubated in the absence of cholesterol oxidase.

Fig. 37a. Electron micrograph showing the basal region of a Sertoli cell (S) and a Leydig cell. Reaction product (arrows) is indicated in lipid bodies (L) next to a cluster of small 1-2 $\mu$ m dense bodies reminiscent of lipofuscin bodies (Lf), and in lipid droplets within the Leydig cell. The limiting membrane (LM) of the seminiferous tubule is identified. The mink was killed during the breeding season and the tissue was washed in 70% ethanol prior to treatment for localization of total cholesterol. X 12,400.

Fig. 37b. Electron micrograph showing a lipid droplet in the basal third of a Sertoli cell. The reaction product (arrows) is found in electron dense clumps at the rim of vacuoles inside the lipid droplet. X 6,700.

Fig. 38. Reaction product is seen in dense clumps (arrows) and fine granules (open arrowhead) inside these Sertoli cell lipid droplets. The tissue was treated for the DAB method of labelling esterified cholesterol. X 54,000.

Fig. 39. Electron micrograph showing a Sertoli cell lipid droplet next to the basement membrane (BM). Reaction product is found as dense clumps (arrows) or granular material (open arrowhead) at the rim of intra-droplet vacuoles. Tissue was treated with the DAB method of labelling total cholesterol. X 43,000.

Fig. 40. Electron micrograph showing lysosome-like vesicles within the basal cytoplasm of a Sertoli cell. Electron dense reaction product is indicated (arrows). Tissue was treated with the DAB method of labelling esterified cholesterol. X 73,000.

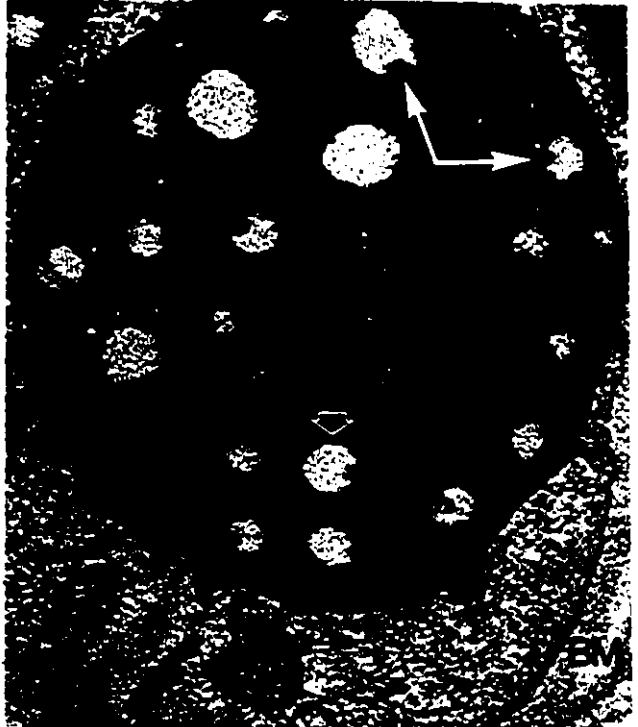
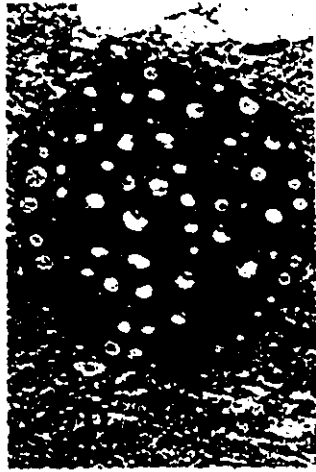


Fig. 41. Electron micrograph showing the apical region of a Stage I of the cycle seminiferous epithelium. Electron dense reaction product is indicated (arrows) within lipidic bodies found free in the lumen and within the caudal cytoplasm of step 13 spermatids (Std 13). An apical Sertoli cell cytoplasmic process is identified (S). X 27,000.

Fig. 42. Electron micrograph showing the basal third of a Sertoli cell (S). Large lipid droplets are shown with electron dense reaction product (arrows) in pale regions of the droplet. Less pronounced electron dense profiles of endoplasmic reticulum seem also to be positively labeled (open arrowhead) near the basement membrane (BM). X 17,300.

Lumen

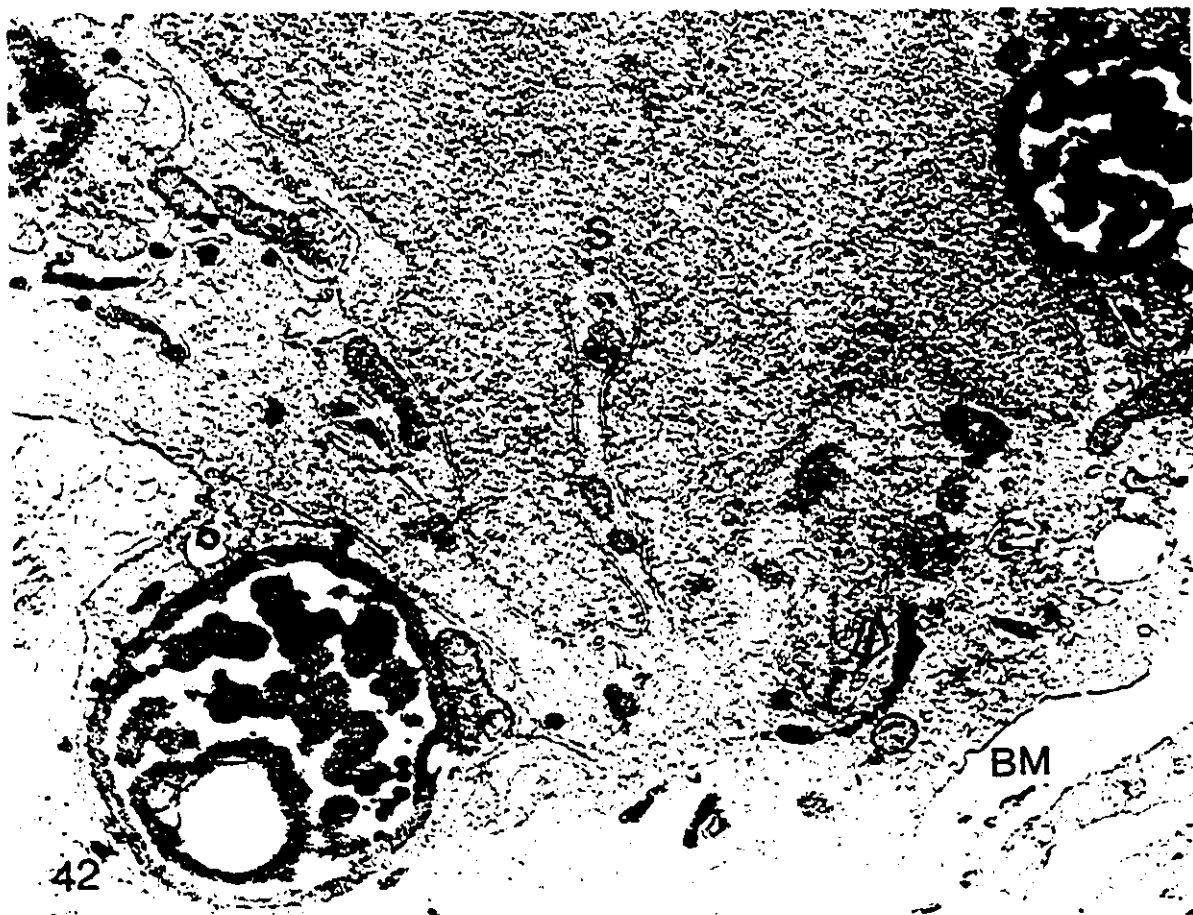


Fig. 43a. Electron micrograph of a Leydig cell taken from a mink sacrificed during the breeding season. Electron dense reaction product is seen as large clumps (arrows) within irregular shaped lipid droplets, and as fine granules (open arrowhead) within lysosome-like membrane bound vesicles. X 54,000.

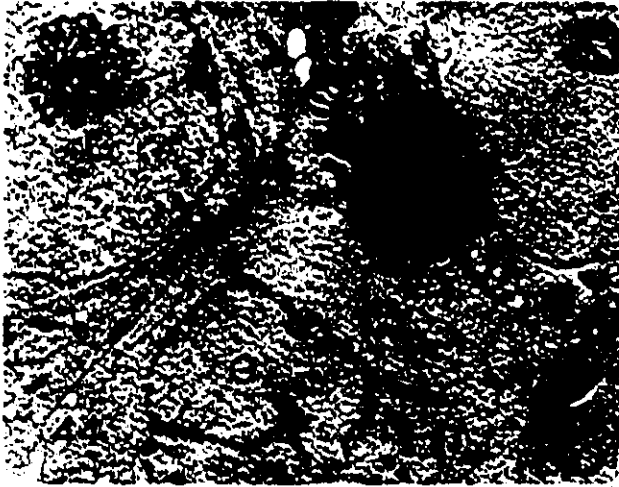
Fig. 43b. Electron micrograph showing a lysosome-like body (Ly) in the cytoplasm of a Leydig cell. Reaction product is indicated (arrow). Tissue was treated for the DAB labelling of esterified cholesterol. X 60,000.



Fig. 44. Electron micrograph showing an unlabelled lipid droplet (open arrowhead) within Sertoli cell cytoplasm (S) next to the cellular bridge (arrow). Two type B spermatogonia (G) are near by. The tissue was treated as a control in the absence of cholesterol oxidase. X 7,200.

Fig. 45. Electron micrograph showing an unlabelled lipid droplet (open arrowhead) within the cytoplasm of a step 7 spermatid (Std 7). The tissue was treated for the DAB localization of free cholesterol. X 12,500.

Fig. 46. Reaction product is indicated along the plasma membrane (closed arrowheads), vesicles still attached to the plasma membrane (open arrowheads) and vesicles free within the cytoplasm (arrow). Electron micrograph showing an endothelial cell from testicular tissue treated for the DAB localization of free cholesterol. X 54,000.



### 3.3.2 Filipin

Treatment of tissue with filipin can result in the formation of 25 nm membrane protuberances (filipin-sterol complexes) in membranes that contain a sufficient amount of free cholesterol (Kinsky, 1970). The effects of treating tissue with filipin in the presence of cholesterol esterase was investigated in electron microscopy of freeze-fracture replicas and thin sections. The plasma membrane of germ cells were highly corrugated in thin sections (Fig. 47). In freeze-fracture replicas, the corrugations corresponded to 25 nm. protuberances (Fig. 48) characteristic of filipin-sterol complexes. Filipin-sterol complexes were also recognized on the plasma membrane of Sertoli cells (Figs. 49 and 50).

In order to test the effectiveness of cholesterol oxidase at reacting with membranous cholesterol, some tissue was treated with cholesterol oxidase prior to incubation with filipin and cholesterol esterase. Membranes viewed in thin sections (Fig. 51) were free from corrugations. However, fracture surfaces that exposed the cytoplasm revealed the presence of filipin-sterol complexes on intracellular structures (Figs. 52a and 52b) but not on the plasma membrane. This suggests that filipin had gained access to the cell and its membranes, but that the preincubation with cholesterol oxidase had successfully prevented it from reacting with the free cholesterol in the plasma membrane.

Fig. 47. Thin section of testicular tissue treated with filipin. Filipin-induced membrane perturbations (arrows) are seen on the plasma membrane of a spermatid (Std) and the adjacent Sertoli cell (S). Sertoli cell plasma membrane (open arrowhead) near the junctional complex, is not visibly affected. X 52,000.

Fig. 48. Filipin-sterol complexes (arrow) are extremely abundant on the plasma membrane of a step 6 spermatid. They are less abundant on the anterior (Aa) and posterior (Ap) segments of the acrosomal membrane. The cytoplasm (C) is identified. X 20,000.

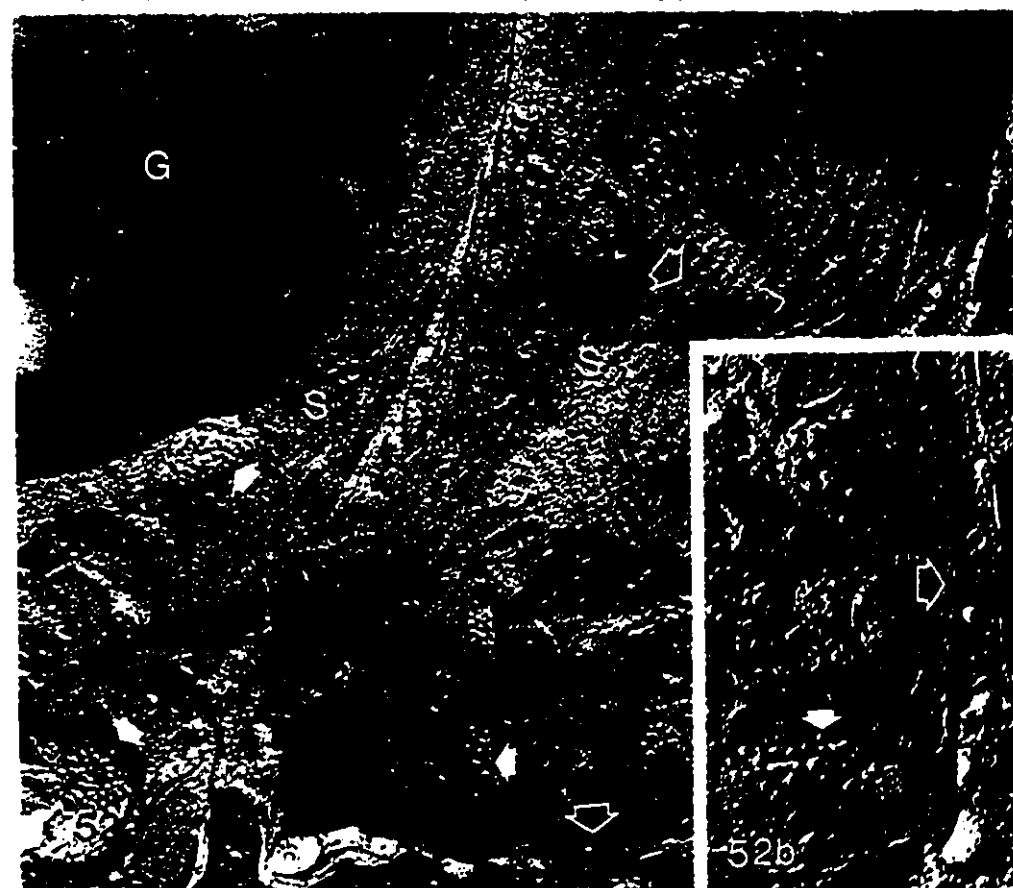
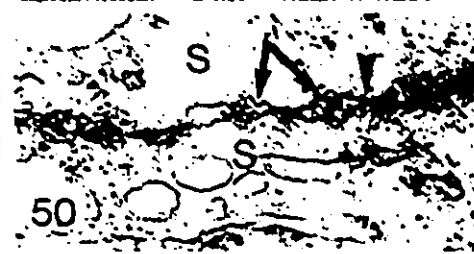
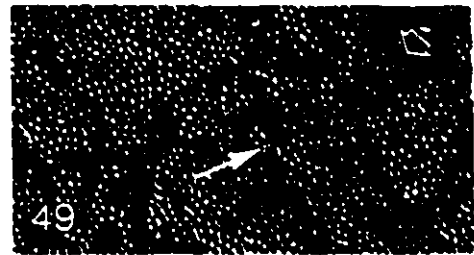
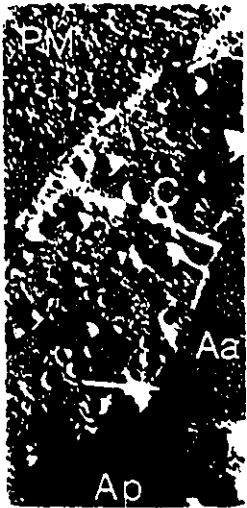
Fig. 49. Freeze-fracture replica of a Sertoli cell plasma membrane treated with filipin. Perturbations in the membrane caused by filipin-sterol complexes (arrow) are seen between the tight junctional strands (open arrowhead). X 27,000.

Fig. 50. Thin section illustrating filipin-induced perturbations (arrows) of Sertoli cell (S) plasma membranes, near a tight junction (arrowhead). X 48,000.

Fig. 51. Thin section of tissue treated with cholesterol oxidase prior to incubation with filipin and cholesterol esterase. No filipin-sterol complexes are observed on the plasma membrane (arrow), Golgi saccules (G at arrowhead) or acrosomal membrane (A at arrow) in this step 6 spermatid (Std6). The nucleus (N) is identified. X 27,000.

Fig. 52a. Freeze-fracture replica of a tissue treated with cholesterol oxidase prior to incubation with filipin and cholesterol esterase. No filipin-sterol complexes are observed on the plasma membrane of the germ cell (G) or the Sertoli cell (S). Filipin-sterol complexes are observed on the surface of intracellular vesicles (closed arrowheads) revealed by fractures through the Sertoli cell cytoplasm. Junctional strands are indicated (open arrowheads). X 27,000.

Fig. 52b. Electron micrograph showing the enclosed region in 52a at a higher magnification. A filipin-sterol complex (arrow) and a tight junctional strand (open arrowhead) are indicated. X 54,000.



## **4. DISCUSSION**

### **4.1 Methodology**

#### **4.1.1 Morphology**

We chose first to establish a correlation between germ cell population and lipid droplets. For this, we used 0.5  $\mu\text{m}$  semithin plastic sections stained with toluidine blue. The tissue was fixed by perfusion and processed for electron microscopy using the potassium-ferrocyanide-tannic acid-uranyl-acetate *en bloc* staining or PFTA technique. This technique provides optimal preservation cell membranes, thus enabling distinction of all cell sub-types within the testis. Pelletier (1986) used this technique to develop a method of identification of the stages of the cycle of the seminiferous epithelium in the mink (fig. 1), and to identify the germ cells that were present at various times of the annual reproductive cycle (fig. 2). In addition, because Sertoli cell cytoplasm is distinguishable from germ cell cytoplasm due to the difference in their staining intensity, lipid droplets can be clearly defined as indwelling one or the other cell class. Kerr and de Kretser (1975) compared toluidine blue-stained plastic sections to frozen sections that had been treated with Sudan Black to establish the validity of using toluidine blue-stained plastic sections for identifying lipid droplets.

#### **4.1.2 Biochemistry**

Biochemical studies for cholesterol have been performed on testicular tissue by several investigators (Beckman and Coniglio, 1979; Davis et al., 1966; Fleeger et al., 1968; Fleeger et al., 1968; Kinson et al., 1964). Only one of these studies separated interstitial cells from tubular cells

(Beckman and Coniglio, 1979). Quantitative data on testicular cell cholesterol levels in seasonal breeders or even in animals with experimentally induced aspermatogenesis, is nonexistent. It was pertinent therefore, to isolate the three major cell classes within the testis of mink i.e., Sertoli cells, germ cells, and Leydig cells, at times of the year that coincide with the zenith and nadir of spermatogenic activity, and to determine the amounts of cholesterol that each cell fraction contained.

Protein was used as the independent parameter in these studies because it is known that the Sertoli cell itself is reduced in size during testicular regression (Sinha Hikim et al., 1989; Sinha Hikim et al., 1988). With the reduction in most components associated with this overall volume reduction, one would expect a decrease in all Sertoli cell parameters if Sertoli cell number or DNA content had been used as the denominator. Thus, changes in the amount of cholesterol per mg. of protein reflects changes in the proportion of the Sertoli cell that is cholesterol rather than changes in the absolute amount of cholesterol.

#### **4.1.3 Cytochemistry**

It was the intention of this investigation to show the coincidental association of testicular cholesterol content with spermatogenic activity in the seminiferous epithelium. A good approach is the application of a histochemical technique to localize cholesterol and cholesteryl esters. Two classical techniques have been used for localizing cholesterol in histological sections: acetone soluble birefringence under polarized light, and the Schultze test (Schultz, 1924) for unsaturated sterols. Both procedures however, require that the tissue is in the relatively native state achieved only with cryostat sections. Cryosections do not retain adequate structural preservation to allow accurate identification of the stages of the cycle, or distinction of

the cell class in which the reactive material resides. Furthermore, although neither test is specific to cholesterol, the more sensitive one (acetone-soluble birefringence) is also the least specific, allowing for possible misinterpretation due to the presence of glycerides in their crystalline state in addition to non-cholesterol steroid molecules (Pearse, 1968).

In order to circumvent the short falls associated with cryo-sections and to confirm that cholesterol is a component of lipid droplets, we chose to localize cholesterol histochemically and ultrastructurally. To map cholesterol, most cytochemical studies have used either filipin or saponins which deform the cell membrane to elicit their binding to cholesterol. For determining cholesterol in lipid inclusions or in membranes that are resistant to deformation, filipin is not appropriate (see Karnovsky, 1982; Severs and Simons, 1983). Recent development of an immunological probe for cholesterol has been developed (Swartz et al., 1988), however the application of this technique has thus far been restricted to non-biological material since highly concentrated cholesterol is required for recognition (Blanchette-Mackie et al., 1989). For these reasons, the innovative method of Emeis et al. (1977) as modified for electron microscopy by Jones and Miyai (1981) was used. This technique specifically localizes cholesterol esters under conditions that permit precise identification of cell types and of the subcellular structure in which cholesterol is housed. Hydrogen peroxide released during the oxidation of free cholesterol to cholestenone (see appendix I) is used in the oxidation of DAB to an osmiophilic, water insoluble polymer. It is our belief our recent failures in employing this technique stemmed from the use of distilled water. The low dissolved oxygen content in distilled water blocks completion of the reaction sequence, thus preventing the formation of an insoluble, osmiophilic compound at sites of free or esterified cholesterol (see appendix 1, also

(Cheillan et al., 1989; Hesselink et al., 1990; Khmel'nitsky et al., 1988; Nakamura et al., 1988; Smith and Brooks, 1976). This experiment was attempted again using water that has been fully oxygenated. Preliminary results from this experiment suggest that we were successful (see appendix II).

## **4.2 Germ cells**

### **4.2.1 Overview**

Morphometric results indicate a progressive build-up of lipids in spermatids during the first 13 steps of spermiogenesis and then a progressive decline until spermiation (fig. 5-11). The results were corroborated using two independent parameters: the cross-sectional area of the seminiferous epithelium, and the numerical density of Sertoli cell nuclei. The epithelial area in the rat (Wing and Christensen, 1982) has been shown to vary from  $0.033 \text{ mm}^2$  at Stage XI (corresponds to Stage X in the mink) to  $0.044 \text{ mm}^2$  at Stage V (IV in mink). This stage dependent variation was not substantiated by a more recent study on the rat (Sharpe, 1989) nor in the present study on the mink. The number of Sertoli cells and germ cells per length of tubule, have been shown to remain roughly constant throughout the cycle in the rat (Sinha Hikim et al., 1988; Wing and Christensen, 1982). We also found that the number of Sertoli cells remained roughly constant throughout the cycle in the mink (fig. 4). Germ cell death preferentially at specific stages has however, been reported for several animals (Roosen-Rünge, 1973) including rat (Huckins, 1978; Roosen-Rünge and Giesel, 1950; Wing and Christensen, 1982) and mink (Sundqvist, 1985).

## 4.2.2 Germ cell Lipids

To our knowledge, this is the first comprehensive study on germ cell lipids in relationship to cellular differentiation. Numerous morphological observations have been made regarding germ cell lipids (Breucker et al., 1985; Kerr and de Kretser, 1975; Kerr et al., 1984; Kingsley Smith and Lacy, 1959; Lacy, 1960; Niemi and Kormano, 1965; Sapsford et al., 1969; Sapsford et al., 1969). These reports, however, have usually been with regards to the influence of germ cell lipids on Sertoli cell lipids and have rarely discussed germ cell lipids themselves except for during the last few steps prior to spermiation. Germ cell lipids in the rat, have been reported in pachytene spermatocytes at Stage XII (Kerr and de Kretser, 1975) and after step 16 in elongated spermatids (Kerr and de Kretser, 1975; Niemi and Kormano, 1965). An early report on the mink (Onstad, 1967) demonstrated a progression of sudanophilic material from a basal location (presumably within the Sertoli cell) in Stage IX-X, to a central site near the lumen (presumably within germ cells) in Stages VI and VII. That germ cells contain and apparently accumulate lipid during spermiogenesis has therefore been established (Kerr and de Kretser, 1975; Niemi and Kormano, 1965) (Onstad, 1967). The composition, purpose, and fate of that lipid however, merits further discussion.

### 4.2.2.1 Germ cell Cholesterol

Germ cells in mature rats have been shown to contain relatively high levels of cholesterol (4.0  $\mu\text{mol/g}$ . wet weight) (Beckman and Coniglio, 1979), a finding that was corroborated by our biochemical results (fig. 36). In addition, using cytochemical techniques, we have localized cholesteryl esters to lipid droplets within the caudal

cytoplasm of elongated spermatids (fig. 41). That free cholesterol was not also identified at these sites (fig. 45) is in agreement with reports that free cholesterol is primarily associated with membranes (Lange and Ramos, 1983; Lange et al., 1989), whereas the bulk of esterified cholesterol is found in lipid droplets (Massa and Aoki, 1976). Steric hinderance or a concentration of substrate that was too low may explain the lack of marker associated with the plasma membrane except on the endothelial cell exposed to a larger amount of serum cholesterol. Possibly, the surface of some endothelial cells have supplemented levels of cholesterol due to either increased LDL receptors or their direct contact with blood-born lipoproteins (Collet et al., 1988) and albumin (Deliconstantinos et al., 1986) (see also reviews by (Goldstein and Brown, 1977) (Mahley, 1988; Mahley and Innerarity, 1983)).

#### 4.2.2.2 Germ cell Lipid Dynamics

If lipid droplet volume density is a reflection of the content of cholesteryl esters within a given class of germ cell, then a moderate depletion in lipid droplets within elongated spermatids prior to spermiation may be due to a conversion of esterified cholesterol into free cholesterol which is amphipathic and typically found in membranes (Lange et al., 1979; Lange and Ramos, 1983). Cholesterol ester hydrolase has been shown to have three times the specific activity in the tubular compartment that it has in the interstitium (Renston et al., 1977). It is conceivable that a portion of this activity resides in elongated spermatids.

Cholesterol is a major constituent of cell membranes and is known to regulate fluidity and permeability of lipid bilayers (reviews by (Demel and DeKruyff, 1976; Yeagle, 1985). Sperm capacitation - the acquisition of the ability to fertilize the ovum - involves removal of cholesterol from the sperm plasma membrane (Davis, 1978; Go and Wolf, 1983;

Hoshi et al., 1990; Langlais and Roberts, 1985; Suzuki and Yanagimachi, 1989). Although a rapid diminution in the cholesterol/phospholipid ratio is an important early step in capacitation (Langlais and Roberts, 1985), an elevated ratio may be required to ensure cell viability prior to arrival in the female reproductive tract. Cholesterol may act to protect the male germ cell by decreasing the fluidity and permeability properties of the plasma membrane. It has been suggested that spermatozoa with higher cholesterol/phospholipid ratios may be better suited in fertilizing eggs when longevity is an asset, such as when coitus takes place long before ovulation (Hoshi et al., 1990). In addition, sperm migration through, and maturation within the epididymis results in a general decrease in sterol (2,300 pmole cholesterol /  $10^6$  spermatozoa in the caput compared to 200 at cauda) (Legault et al., 1979; Quinn and White, 1967) such that ejaculated spermatozoa have lower cholesterol levels than testicular spermatozoa (Scott et al., 1967). In anticipation of this sterol depletion, germ cells may acquire an inordinate amount of cholesterol while still in the testis and still in contact with Sertoli cells.

As an alternative to metabolizing lipids, spermatids might simply exocytose their lipids during the maturation phase. Because the entire spermatid population is above the blood-testis barrier, exocytosis of any germ cell product must be to either a neighboring cell or the tubular lumen. The transfer of lipid from one class of germ cells to another in the same cellular association, such as from step 13 spermatids to step 1 spermatids or pachytene spermatocytes at Stage I, is also a possibility. This in light of the fact that little change is observed in the overall spermatid lipid volume density between Stage II and VII (fig. 12) during the period when there is a depletion in mature spermatid lipid droplet amount (steps 14-19 in fig.11). A luminal destination for lipid droplets is supported by the observation of cholesteryl ester-rich lipid droplets in the

lumen above step 13 spermatids (fig. 41). From which cell type and at which stage of the cycle the luminal lipids were secreted is unknown since the lumen is continuous between stages of the cycle, and since both Sertoli cells and germ cells are found at its border.

### **4.3 Sertoli cells**

#### **4.3.1 Overview**

During the height of spermatogenic activity, the volume density of Sertoli cell lipid inclusions shows a stage dependent cycle (figs. 5-10, and 13). The stage-dependent variations in lipid droplets of the seminiferous epithelium have been reported for over a hundred years (von Ebner, 1888). Although not all species studied so far have shown cyclic changes in Sertoli cell lipid abundance (Dym, 1973; Osman and Plöen, 1978; Schulze, 1984), those that have, generally reported maximum levels in stages which closely follow spermiation (Kerr and de Kretser, 1975; Kerr et al., 1984; Lacy, 1960; Niemi and Korman, 1965; Sapsford et al., 1969). That the mink Sertoli cell should reach peak abundance before spermiation requires additional explanation.

#### **4.3.2 Germ cell - Sertoli cell interactions**

##### **4.3.2.1 Early Accumulation of Lipid**

Lipid cyclicity (the stage-dependent waxing and waning of lipids) is the result of two events: lipid build-up involving either lipid biosynthesis or endocytosis, and lipid depletion involving either lipid catabolism, bioconversion, or exocytosis. Classical theories suggested that germ cell-derived lipids renew Sertoli cell lipid stores periodically at spermiation following a recycling of the residual bodies lipid content, and that Sertoli cells in turn deplete those

lipids for steroid hormones biosynthesis (Lacy, 1962; Niemi and Kormano, 1965). The endocytosis by the Sertoli cells of residual cytoplasm from germ cells does not appear to be that simple. In an excellent review on Sertoli cell function, Fawcett expressed wonderment at the Sertoli cell's ability to deal so quickly and efficiently with a "burden of protoplasmic disposal far greater than that of tissue macrophages" (Fawcett, 1975). This particularly in light of the fact that there is an unremarkable accumulation of lipochrome pigment and an apparent absence of exocytotic activity.

Quantitative studies on mammalian (Sprando and Russell, 1987) and submammalian (Sprando et al., 1988; Sprando and Russell, 1988) species revealed a huge reduction in the cytoplasmic volume of mature spermatids prior to spermiation. In an elegant experiment by Sakai et al. (1988) Sertoli cell processes were shown to invade spermatids at step 13 of spermiogenesis. These processes formed a complex canal system that intertwined with spermatid vesicles (Sakai and Yamashina, 1989) compiling what had previously been defined as a "mixed body" (Morales and Clermont, 1982). This may represent an organelle that is responsible for the early transfer of spermatid cytoplasm into the Sertoli cell (Sakai and Yamashina, 1989; Wrobel and Schimmel, 1989). The task of residual body disposal at spermiation is thereby alleviated. It is interesting to note that mixed bodies become prevalent in mouse spermatid at step 15 (Stage III) (Sakai and Yamashina, 1989), the same time in the mink at which the lipid droplet content of spermatid decreases (Fig. 11) and that of the Sertoli cell increases (Fig. 13).

Lipid droplets depletion may also be stage-dependent, with periods of decreasing lipid droplet content of the Sertoli cells corresponding to periods of heightened neutral lipid exocytosis or catabolism (Kerr et al., 1984). Excretion of lipid would presumably be to satisfy a stage-specific need of the germ cell population (see Parvinen et

al., 1986; Sharpe et al., 1990 for review). The non-polar lipids may be bioconverted into a steroid hormone (Lacy, 1960) or they may be transferred directly to the pachytene spermatocytes (Kerr and de Kretser, 1975) to control or in some other way participate in meiosis.

#### 4.3.2.2 Contribution to Sertoli cell lipids by the Residual Body

The residual body does however, still contain lipids (figs. 8 and 28) when it is phagocytosed by the Sertoli cell at spermiation (Breucker et al., 1985; Dietert, 1966; Posalaki et al., 1968). These lipids however, do not likely contribute directly to the Sertoli cell lipid droplet pool as had originally been assumed (Lacy, 1960). It was suggested by Dietert (1966) and Kerr et al. (1984) that the lipids in the residual body are degraded to their fatty acids and glycerol components after the residual body fuses with Sertoli cell lysosomes (Morales et al., 1985). The subsequent appearance of lipid droplets in the Sertoli cell cytoplasm, suggests resynthesis of neutral lipids from these components (Kerr et al., 1984). Alternatively, the build-up of Sertoli cell lipids may be independent of material contributed by germ cells.

#### 4.3.2.3 Endogenous Lipid Cycles

In rats that had been irradiated in utero to eliminate all germ cells and then either made experimentally cryptorchid or sham treated, Sertoli cells of the cryptorchid testes had more lipids than sham treated testes (Bergh, 1981). Thus, even in the absence of germ cells, the Sertoli cell is able to synthesize lipids. Furthermore, the Sertoli cell may display an endogenous lipid cycle. Pelletier (1988) suggested that Sertoli cell membrane specializations may be recycled in concert with the migration of germ cells towards the apex of the spermatogenic

epithelium. In this scenario, lipid components of the membrane, may contribute to the Sertoli cell lipid pool after being internalized and degraded in autophagic vacuoles. The Sertoli cell membrane specialization most likely participate in this cycle is the tight junction or zonula occludens. The discovery of cholesterol as a component of Sertoli cell lipid droplets during the active spermatogenic phase (figs. 37, 38, 39, and 42) may indicate an involvement of cholesterol in tight junctions.

Artificial phospholipid membranes exhibit short-range repulsive interactions that prevent intimate contact (McIntosh et al., 1987; Simon et al., 1988). Reducing these repulsive forces is a prerequisite for cell membranes to fuse as they do at the site of tight junctions. Cholesterol has been shown to do just that (McIntosh, 1989). The addition of cholesterol to phosphatidylcholine membranes reduced the amount of osmotic pressure required to force those membranes together. Thus, rather than being cholesterol-poor as had been suggested by cytochemical studies using filipin (Chailley, 1981; Robenek et al., 1982), tight junctional membranes might be cholesterol-rich. The lack of filipin-induced membrane perturbations in the vicinity of tight junctions was more likely due to increased membrane rigidity caused by the abundance of subsurface filaments (Brown et al., 1982; Feltkamp and van der Waerden, 1982; Hirsch et al., 1989; Severs and Simons, 1983), including the tight junction specific cytoskeleton-plasma membrane bridge molecules ZO-1 (Stevenson, 1986; Stevenson et al., 1989) and cingulin (Citi et al., 1988; Citi et al., 1989), than to the absence of cholesterol. The lipids and cholesterol removed from apical junctional membrane sites as they are dismantled would then be used by the Sertoli cell to refurbish tight junction formation in the basal third of the lateral surface (Pelletier, 1988).

The cyclic accumulation and diminution of Sertoli cell lipids (probably including cholesterol), is not therefore,

exclusively a reflection of the timing of residual body phagocytosis and degradation, but rather is the visual manifestation of several processes. These include lipid anabolism by the Sertoli cell and subsequent dispersement or utilization of these lipids as dictated by the stage-specific needs of the germ cells and/or the Sertoli cell itself, in addition to lipid contribution from the residual body.

That both germ cells and Sertoli cells contain cholesterol in their respective lipid droplets (figs. 37-42) allows for the possibility of exchange of material between these two cell types. However, only a portion may be exchanged. Most may be retained by each cell type for their own use: germ cells during their maturation and transit through the reproductive tract, and Sertoli cells in membrane recycling.

The stage-related changes in lipid droplet volume are probably not directly produced by circulatory hormones since these would cause a similar effect in all Sertoli cells. Furthermore, it would seem that the lipid cycle is not related to the synthesis of lipid soluble factors responsible for the coordination of meiotic division since meiosis is completed at the time when Sertoli cell lipid content is still at maximum levels. If the stage-dependent waxing and waning in lipid droplet amount reflects, periods of decreased and increased neutral lipid utilization respectively, it is conceivable that the fact that lipid droplet amount reaches maximum levels in the mink earlier in the cycle than it does in the rat (Kerr et al., 1984), indicates that lipid droplets are involved in a spermatogenic process that is flexible in its scheduling.

#### **4.3.3 Seasonal Variation in Sertoli cell Lipid**

We have investigated the influence of germ cells on Sertoli cell lipid dynamics and function by studying the Sertoli cell during various phases of spermatogenic

activities. Our results indicate that cholesterol esters and lipid droplet abundance decrease in the absence of spermatogenic activity (fig. 36). This is in general agreement with earlier studies on mammalian emphatic seasonal breeders (Onstad, 1967; Tsui et al., 1974; Wislocki, 1949). However, it is in contradiction with other reports (Hodgson et al., 1979; Irby et al., 1984; Pudney and Lacy, 1977; Sinha Hikim et al., 1988) that showed an accumulation of lipid in the regressed testis. The studies on the australian bush rat (Hodgson et al., 1979; Irby et al., 1984) and golden hamster (Sinha Hikim et al., 1988) were performed at only two times of the year: during spermatogenic activity and during testicular regression. For this reason generalizations on the fate or significance of the lipid accumulation could not be evaluated. Still, depletion of germ cells is generally believed to be accompanied by an accumulation of lipid inclusions and cholesterol (reviews by de Kretser and Kerr, 1988; Lofts, 1972). The significant feature seems to be the stage at which spermatogenesis is arrested. If pachytene spermatocytes are still present, then the amount of Sertoli cell lipid droplets increases, if spermatogenesis is arrested to the point where only spermatogonia and Sertoli cells are present, then the amount of lipid droplets decreases.

#### 4.3.3.1 Effects of Germ cell Depletion on Sertoli cell Lipid Content

Lipid accumulation in Sertoli cells is associated with pathological conditions, not just the depletion of germ cells. Lipid and cholesterol esters accumulation have been observed in humans in relation to old age (Lynch and Scott, 1950; Paniagua et al., 1985; Paniagua et al., 1987) varicocele (Sheriff, 1982; Sultan and D., 1984), prostatic neoplasia (Lynch and Scott, 1950), Klinefelter's syndrome (Sheriff and Govindarajulu, 1977), and testicular feminization (Sheriff and Govindarajulu, 1978). Elevating the ambient temperature of the testis either by

cryptorchidism (Hanes and Rosenbloom, 1911; Perlman, 1950) or immersion in hot water (Collins and Lacy, 1969) has been used to induce spermatogenic arrest. The treatment was assumed to effect only the germ cells, with any effect on somatic cells being a result of germ cell depletion. A direct effect of cryptorchidism on Sertoli cells however, has been reported. Lipid accumulation in Sertoli cells was observed prior to the degeneration of germ cells in cryptorchidism (Jansen, 1970). Also, in rats irradiated in utero with ionizing radiation to kill all germ cells, cryptorchidism was found to induce an accumulation of lipid in Sertoli cells (Bergh, 1981). This indicates that elevated temperature has a direct effect on the Sertoli cell, independent of germ cell depletion.

#### 4.3.3.2 Sertoli cell Free Cholesterol

Free cholesterol unexpectedly increased during the first half of testicular regression (fig. 36). An increase in free cholesterol per milligram of protein would be observed if the absolute amount of free cholesterol stayed the same while the absolute amount of protein declined. With the seasonal loss of germ cells, the Sertoli cell is left with an expanse of redundant apical membrane (fig. 31). Conceivably, membrane cholesterol per cell does not decline at this time. Total Sertoli cell protein however, does very likely decline since the volume of the Sertoli cell in the golden hamster decreases by 75% during testicular regression (Sinha Hikim et al., 1989), reflected as well by a thirty percent decrease in nuclear volume (Hochereau-de Reviers et al., 1985; Sinha Hikim et al., 1988). The ratio of membranes i.e., free cholesterol, to cytoplasm i.e., total protein, would therefore be expected to increase (fig. 36). Presumably, this increase would not have been recognized if the value had been represented as a quotient of free cholesterol by Sertoli cell numbers or milligrams of DNA. That the ratio of

cholesteryl esters to protein was shown to decrease during testicular regression (fig. 36) reinforces the validity of our claims regarding a decrease in cholesterol esters not only in concentration but also most certainly in amount per cell.

#### **4.4 Leydig cells / Interstitial Tissue**

The increase in Leydig cell cholesteryl esters at the onset of testicular regression (fig. 36) is consistent with most reports on seasonal breeding mammalian (Bostrom et al., 1968; Gustafson, 1987; Onstad, 1967; Pudney and Lacy, 1977; Tsui et al., 1974) and submammalian species (Lofts, 1968; Lofts and Bern, 1972; Lofts et al., 1972; Tam et al., 1969). These reports describe an accumulation of lipidic inclusions during testicular regression. Studies which included more than two data points, indicated a profound accumulation of sudanophilic material in the interstitial tissue at the onset of testicular regression, followed by a gradual waning until the recrudence of spermatogenic activity (Onstad, 1967; Tsui et al., 1974), when most of the lipid is depleted. In the bat however, acetone soluble sudanophilic material reaches maximum levels in Leydig cells during the early phases of the seasonal recrudescence of spermatogenesis. Periods of increasing lipid and cholesterol content were very well correlated with periods of decreasing androgen synthesis (Gustafson, 1987; Lofts, 1968; Lofts and Bern, 1972; Pudney and Lacy, 1977; Tsui et al., 1974). Thus supporting the classical axiom that in the absence of steroidogenesis, the lipid precursor of steroid hormones (cholesterol) accumulates (Armstrong, 1968; Bartke, 1971; Dailey et al., 1963). Cholesterol side-chain cleavage (the rate limiting step in steroidogenesis) does not procede until ester link of cholesteryl esters has been hydrolyzed (Bartke et al., 1973;

Moyle et al., 1970) indicating that cholesterol esters are the storage form of steroid hormone precursors.

In the mink, circulating testosterone levels begin to increase at the onset of spermatogenic activity (October) reaching maximum levels at the peak of spermatogenesis (end of January) (Boissin-Agasse et al., 1981; Sundqvist et al., 1984). Spermatogenic activity is coincidental with steroidogenic activity (Webley et al., 1985) but not with lipid droplets/cholesterol since these are catabolized for the synthesis of steroid hormones. The administration of LH (the gonadotrophin responsible for testosterone synthesis (Chaudhary and Stocco, 1988; Dufau et al., 1980; Dufau et al., 1981; Freeman and Ascoli, 1982; Freeman, 1989; Purvis et al., 1981).) decreases cholesteryl esters in Leydig cells (Pokel et al., 1972). The activity of enzymes involved in converting pregnenolone to progesterone were modulated by specific phospholipids (Cooke, 1989). Since total phospholipid content of rat Leydig cells were altered by LH and cAMP (Lowitt et al., 1982), modifying phospholipid content appears to be a mechanism for controlling androgenesis.

#### **4.5 Conclusions**

Our cytochemical observations have localized cholesteryl esters in Sertoli cell, germ cell, and Leydig cell lipid droplets during the active spermatogenic phase. This has not previously been demonstrated. A positive correlation was observed between the amount of Sertoli cell cholesteryl esters and the degree of spermatogenic activity. In addition, we showed that the volume density of Sertoli cell lipid droplets varied coincidentally with the presence of older germ cells (pachytene spermatocytes and spermatids). During periods of full germ cell compliment, stage-dependent fluctuations in Sertoli cell and germ cell lipids were observed. Lipid droplets were present in all classes of germ cells. In spermatids, the lipids accumulated during spermiogenesis but decreased in amount prior to spermiation. The decline of lipid in spermatids (Stages I-VII, Fig. 12b) corresponds to the stages during which lipid droplets increased in Sertoli cells (Stages I-VII, Fig. 13b). Sertoli cell processes which invade spermatids during the maturation phase of spermiogenesis may actually remove excess cytoplasm, and presumably, lipids. Sertoli cell lipid droplet content peaks before, not after spermiation in the mink. The presence of cholesteryl esters in Sertoli cells lipid droplets (Figs. 36-43) is consistent with a role in steroid hormone production. However, the lack of evidence in adult Sertoli cells for enzymes required in steroid hormone synthesis and the lack of correlation between lipid droplet volumes in Sertoli cells (Fig.24) and published levels of plasma testosterone (Boissin-Agasse and Boissin, 1979; Sundqvist et al., 1984) are inconsistent with steroidogenic activity. Cholesterol's participation in Sertoli cell membrane recycling and the process of capacitation, indicate that perhaps its role in spermatogenesis is associated with

its properties as a membrane constituent rather than as a precursor for steroid hormone production. Finally, seasonal variations in cholesterol in Leydig cells is consistent with the role of the Leydig cell as a steroid hormone producing cell.

**BIBLIOGRAPHY**

**Abou-Issa, H. and L.E.J. Reichert 1976** Properties of the follitropin receptor interaction: characterization of the interaction of follicotropin with receptors in purified membranes isolated from mature rat testes tubules. *J. Biol. Chem.*, 251:3326-3327.

**Allain, C.C., L.S. Poon, C.S.G. Chan, W. Richmond and P.C. Fu 1974** Enzymatic determination of total serum cholesterol. *Clin. Chem.*, 20:470-475.

**Anderson, C.M. and C.R. Mendelson 1984** Regulation of the synthesis of cholesterol side-chain cleavage cytochrome P-450 and adrenodoxin in rat Leydig cell in culture. *Ann. N.Y. Acad. Sci.*, 438:259-268.

**Aoki, A. and D.W. Fawcett 1978** Is there a local feedback from the seminiferous tubules affecting activity of the Leydig cells? *Biol. Reprod.*, 19:144-158.

**Armstrong, D.T. 1968** Gonadotropins, ovarian metabolism and steroid biosynthesis. *Rec. Prog. Horm. Res.*, 24:255-308.

**Armstrong, D.T., Y.S. Moon, I.B. Fritz and J.H. Dorrington 1975** Synthesis of estradiol-17 $\beta$  by Sertoli cells in culture: stimulation by FSH and dibutyl cyclic AMP. *Curr. Top. Molec. Endocr.*, 2:85-96.

**Arthur, J.R. and G.S. Boyd 1974** The effect of inhibitors of protein synthesis on cholesterol side-chain cleavage in mitochondria of luteinized rat ovaries. *Eur. J. Biochem.*, 49:117-127.

Atkinson, P.H. and D.F. Summers 1971 Purification and properties of HeLa cell plasma membranes. J.Biol. Chem., 246:5162-5175.

Bartke, A. 1971 Concentration of free and esterified cholesterol in the testes of immature and adult mice. J.Reprod. Fert., 25:153-156.

Bartke, A., N. Musto, B.V. Caldwell and H.R. Behrman 1973 Effects of a cholesterol esterase inhibitor and of prostaglandin  $F_2\alpha$  on testis cholesterol and on plasma testosterone in mice. Prostaglandins, 3:97-104.

Bass, J.J., J.B. Bell and D. Lacy 1973 Side-chain cleavage of (26- $^{14}$ C) cholesterol by rat testicular tissues and their subcellular fractions. J. Endocrinol., 56:321-2.

Beckman, J.K. and J.G. Coniglio 1979 A comparative study of the lipid composition of isolated rat Sertoli and germinal cells. Lipids, 14:262-267.

Bell, J.B.G., G.P. Vinson, D.J. Hopkin and D. Lacy 1968 Pathways for androgen biosynthesis from [ $7\alpha$ - $^3$ H] pregnenolone and [ $4$ - $^{14}$ C] progesterone by rat testis interstitium *in vitro*. Biochim. Biophys. Acta, 164:412-420.

Bell, J.B.G., G.P. Vinson and D. Lacy 1971 Studies on the ultrastructure and function of the mammalian testis III: *In vitro* steroidogenesis by the seminiferous tubules of the rat testis. Proc. Roy. Soc. Biol., 176:433-443.

Benahmed, M., C. Grenot, E. Tabone, P. Sanchez and J.M. Morera 1985 FSH regulates cultured Leydig cell function via Sertoli cell proteins: an *in vitro* study. Biochem. Biophys. Res. Comm., 132:729-734.

**Bergh, A.** 1981 Morphological signs of a direct effect of experimental cryptorchidism on the Sertoli cells in rats irradiated as fetuses. *Biol.Reprod.*, 24:145-152.

**Bergh, A.** 1982 Local differences in Leydig cell morphology in the adult rat testis: evidence for a local control of Leydig cells by adjacent seminiferous tubules. *Int.J.Androl.*, 5:325-330.

**Bergh, A.** 1983 Paracrine regulation of Leydig cells by the seminiferous tubules. *Int. J. Androl.*, 6:57-65.

**Bishop, P.D. and M.D. Griswold** 1987 Uptake and metabolism of retinol in cultured Sertoli cells: evidence for a kinetic model. *Biochemistry*, 26:7511-7518.

**Bittman, R., W.C. Chen and O.R. Anderson** 1974 Interaction of filipin III and amphotericin B with lecithin-sterol vesicles and cellular membranes. Spectral and electron microscope study. *Biochemistry*, 13:1364.

**Blanchette-Mackie, E.J., N.K. Dwyer and L.A. Amende** 1989 Cytochemical studies of lipid metabolism: Immunogold probes for lipoprotein lipase and cholesterol. *Am.J.Anat.*, 185:255-263.

**Boissin-Agasse, L. and J. Boissin** 1979 Variations saisonnieres du volume testiculaire et de la testosteronemie chez deux mustelides: le furet (*Mustela furo*) et le vison (*Mustela vison*). *J. Physiol., Paris*, 75:227-232.

**Boissin-Agasse, L., C. Maurel and J. Boissin** 1981 Seasonal variations in thyroxine and testosterone levels in relation to the moult in the adult male mink (*Mustela vison* Peale and Beauvois). *Can. J. Zool.*, 59:1062-1066.

- Boitani, C., E.M. Ritzen and M. Parvonen 1981** Inhibition of rat Sertoli cell aromatase by factor(s) secreted specifically at spermatogenic Stages VII and VIII. *Mol.Cell.Endocrinol.*, 23:11-22.
- Bostrom, R.E., R.J. Aulerich, R.K. Ringer and P.J. Schaible 1968** Seasonal changes in the testes and epididymides of the ranch mink (*Mustela vison*). *Q. Bull.Mich. Agric. Exp. Stn.*, 50:538-558.
- Bourne, G.A., M.R. Dockrill, S. Regiani, J.C. Marshall and A.H. Payne 1982** Induction of testicular gonadotropin-releasing hormone (GnRH) receptors by GnRH: effects of pituitary hormones and relationship to inhibition of testosterone production. *Endocrinology*, 110:727-733.
- Brake, E.T., P.X. Will and J.S. Cook 1978** Characterization of HeLa5'-nucleatidase: A stable plasma membrane marker. *Membrane Biochem.*, 2:17-46.
- Braucker, H., E. Schäfer and A.-F. Holstein 1985** Morphogenesis and fate of the residual body in human spermiogenesis. *Cell Tiss. Res.*, 240:303-309.
- Brökelmann, J. 1961** Surface modification of Sertoli cells at various stages of spermatogenesis in the rat. An electron microscope study. *Anat. Rec.*, 131:211 Abst.
- Brökelmann, J. 1963** Fine structure of germ cells and Sertoli cell during the cycle of the seminiferous epithelium in the rat. *Z. Zellforsch. Mikroskop. Anat.*, 59:820-850.
- Brown, D., R. Montesano and L. Orci 1982** Patterns of filipin-sterol complex distribution in intact erythrocytes and intramembrane particle aggregated ghost membranes. *J. Histochem. Cytochem.*, 30:702.

**Brown, H.H. 1885** On spermatogenesis in the rat. *Quart. J. Microscop. Sci.*, 25:343-370.

**Bucci, L.R., W.A. Brock, T.S. Johnson and M.L. Meistrich 1986** Isolation and biochemical studies of enriched populations of spermatogonia and early primary spermatocytes from rat testes. *Biol. Reprod.*, 34:195-206.

**Bugge, H.P. and L. Ploen 1986** Changes in the volume of Sertoli cells during the cycle of the seminiferous epithelium in the rat. *J. Reprod. Fertil.*, 76:39-42.

**Bustos-Obregon, E. 1970** On Sertoli cell number and distribution in rat testis. *Arch. Biol. (Liege)*, 81:99-108.

**Butler, W.R., A.D. Johnson and W.R. Gomes 1968** Effect of short-term vitamin A deficiency on testicular lipids in the rat. *J. Reprod. Fert.*, 15:157-9.

**Campbell, S.M.C. and J.P. Wiebe 1989** Stimulation of spermatocyte development in prepubertal rats by the Sertoli cell steroid, 3 $\alpha$ -hydroxy-4-pregnen-20-one. *Biol. Reprod.*, 40:897-905.

**Catt, K.J., T. Tsuruhara, C. Mendelson, J.-M. Ketelslegers and M.L. Dufau 1974** Gonadotropin binding and activation of the interstitial cells of the testis. In: *Hormone Binding and Target Cell Activation in the Testis*. Vol. M. L. Dufau and A. R. Means, eds. Plenum Press, New York, pp. 1-30.

**Caviccia, J.C. and M. Dym 1977** Relative volume changes of Sertoli cells in monkey seminiferous epithelium: A stereological analysis. *Am. J. Anat.*, 150:501-507.

Chailley, B. 1981 Absence of filipin-sterol complexes from tight junctions of oviduct cells in differentiation. *Biol. Cell*, 42:189-192.

Chaudhary, L.R. and D.M. Stocco 1988 Stimulation of cholesterol side-chain cleavage enzyme activity by cAMP and hCG in MA-10 Leydig tumor cells,. *Biochimie*. 70:1799-1806.

Cheillan, F., H. Lafont, E. Termine, F. Fernandez, P. Sauve and G. Lesgards 1989 Molecular characteristics of the cholesterol oxidase and factors influencing its activity. *Biochim. Biophys. Acta.*, 999:233-238.

Chemes, H.E., E. Podesta and M.A. Revarola 1976 Action of testosterone, dihydrotestosterone, and S- $\alpha$  androstane-3 $\alpha$ ,17 $\beta$ -diol on spermatogenesis of immature rats. *Biol. Repr.*, 14:332-338.

Cheng, C.Y., J. Grima, M.S. Stahler, R.A. Lockshin and C.W. Bardin 1989 Testins are structurally related Sertoli cell proteins whose secretion is tightly coupled to the presence of germ cells,. *J. Biol. Chem.*, 264:21386-21393.

Christensen, A.K. 1965 The fine structure of testicular interstitial cells in guinea pigs. *J. Cell Biol.*, 26:911-935.

Christensen, A.K. 1975 Leydig cells. In: *Handbook of Physiology*. Vol. 5, Male Reproductive System. R. O. Greep, eds. Williams and Wilkins, Baltimore, pp. 335-378.

Christensen, A.K. and D.W. Fawcett 1961 The normal fine structure of opossum testicular interstitial cells. *J. Biophys. Biochem. Cytol.*, 9:653-670.

Christensen, A.K. and N.R. Mason 1965 Comparative ability of seminiferous tubules and interstitial tissue of

rat testes to synthesize androgens from progesterone-4-<sup>14</sup>C in vitro. *Endocrinology*, 76:646-656.

**Chung, K.W. and J.B. Hamilton** 1975 Testicular lipids in mice with testicular feminization. *Cell Tiss. Res.*, 150:69-80.

**Citi, S., H. Sabanay, R. Jakes, B. Geiger and J. Kendrick-Jones** 1988 Cingulin, a new peripheral component of tight junctions. *Nature*, 333:272-276.

**Citi, S., H. Sabanay, J. Kendrick-Jones and B. Geiger** 1989 Cingulin: Characterization and localization. *J. Cell Sci.*, 93:107-122.

**Clermont, Y.** 1969 Two classes of spermatogonial stem cells in the monkey (*Cercopithecus aethiops*). *Am.J.Anat.*, 126:57-72.

**Clermont, Y.** 1972 Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.*, 52:198-236.

**Clermont, Y. and E. Bustos-Oregon** 1968 Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto". *Am.J.Anat.*, 122:237-248.

**Clermont, Y. and S.C. Harvey** 1965 Duration of the cycle of the seminiferous epithelium of normal, hypophysectomized, and hypophysectomized-hormone treated albino rats. *Endocrinology*, 76:80-89.

**Clermont, Y. and C.P. Leblond** 1953 Renewal of spermatogonia in the rat. *Am.J.Anat.*, 93:475-502.

- Collet, X., B. Perret, F. Chollet, F. Hullin, H. Chap and L. Douste-Blazy 1988 Uptake of HDL phospholipolysis and cell cholesterol content. *Biochim. Biophys. Acta*, 958:81-92.
- Collins, P. and D. Lacy 1969 Studies on the structure and function of the mammalian testis. II. Cytological and histochemical observations on the testis of the rat after a single exposure to heat applied for different lengths of time. *Proc. Roy. Soc. B.*, 172:17-38.
- Coniglio, J.G., W.M.J. Grogan, D.G. Harris and M.L. Fitzhugh 1975 Lipid and fatty acid composition of testes of quaking mice. *Lipids*, 10:109-12.
- Connell, C.J. 1980 Blood-testis barrier formation and the initiation of meiosis in the dog. In: *Testicular Development, Structure and Function*. Vol. A. Steinberger and E. Steinberger, eds. Raven Press., New York, pp. 71-78.
- Conti, M., C. Boitani, D.E. deManno, S. Migliaccio, L. Monaco and C. Szymczek 1989 Characterization and function of adenosine receptors in the testis. *NY. Acad. Sci.*, 564:39-47.
- Cooke, B.A., F.H. de Jong, H.J. Van der Molen and F.F.G. Rommerts 1972 Endogenous testosterone concentrations in rat testis interstitial tissue and seminiferous tubules during *in vitro* incubation. *Nature*, 237:255-256.
- Cooke, B.A., C.J. Dix, A.D. Habberfield and M.H.F. Sullivan 1989 Control of steroidogenesis in Leydig cells: Roles of  $Ca^{2+}$  and lipoxxygenase products in LH and LHRH agonist action. *Proc.Natl.Acad.Sci.*, 269-282.

Cooke, B.A., F. Magee-Brown, M. Golding and C.J. Dix 1981 The heterogeneity of Leydig cells from mouse and rat testis-evidence for a Leydig cell cycle? *Int. J. Androl.*, 4:355-366.

Cooke, G.M. 1989 Identification and mechanism of action of phospholipids capable of modulating rat testicular microsomal  $3\beta$ -hydroxysteroid dehydrogenase-isomerase activity *in vitro*. *Biol. Reprod.*, 41:438-445.

D'Agostino, A. and M. Stefanini 1987 A rat spermatocyte surface protein is involved in adhesion of pachytene spermatocytes to Sertoli cells *in vitro*. *Mol. Cell. Biol.*, 7:1250-1255.

Dailey, R.E., L. Swell and C.R. Treadwell 1963 Hydrolysis and utilization of cholesterol esters for steroid synthesis by canine adrenal homogenates. *Arch. Biochem. Biophys.*, 100:360-363.

Damber, J.-E., A. Widmark and A. Bergh 1985 Changes of testicular vascular resistance after treatment of rats with an LHRH-agonist. *Acta Endocr., Copenh., Suppl.*, 270:205 Abst.

Davis, B.K. 1978 Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles. In: *Symposium on the Pharmacological Effects of Lipids*. Vol. 5. eds. Am. Oil Chem. Soc., Champaign, IL., pp. 145-157.

Davis, J.T., R.B. Bridges and J.G. Coniglio 1966 Changes in lipid composition of the maturing rat testis. *Biochem. J.*, 98:342.

de Graaf, R. 1668 *Tractatus de virorum organis generationi inservientibus*. Hackiana, Ludg. Batav. et. Roterod.

de Jong, F.H., A.H. Hey and H.J. van der Molen 1974 Oestradiol-17 $\beta$  and testosterone in rat testis tissue: effect of gonadotrophins, localization and production in vitro. J. Endocr., 60:409-419.

de Jong, F.H., R. Welschen, W.P. Hermans, S.D. Smith and H.J. van der Molen 1979 Effects of factors from ovarian follicular fluid and Sertoli cell culture medium on in vivo and in vitro release of pituitary gonadotrophins in the rat: an evaluation of systems for the assay of inhibin. J. Repr. Fert., Suppl., 26:47-59.

de Kretser, D.M. and J.B. Kerr 1988 The cytology of the testis. In: The Physiology of Reproduction. E. Knobil and J. Neill et al, eds. Raven Press. Ltd., New York, pp. 837-929.

de Philip, R.M. and A.L. Kierszenbaum 1982 Hormonal regulation of protein synthesis, secretion, and phosphorylation in cultured rat Sertoli cells. Proc. Natl. Acad. Sci. U.S.A., 79:6551-6555.

de Rooij, D.G., F.M.F. van Dissel-Emiliani and A.M.M. van Pelt 1989 Regulation of spermatogonial proliferation. Ann. NY Acad. Sci., 564:140-153.

Deguchi, E. 1978 Histological studies on the kinetics of the spermatogenesis in the mink (*Mustela vison*). VIII. Cellular association in the seminiferous epithelium in the pre-breeding season (16 months old). Jpn. J. Vet. Res., 26:1-10.

Delesse, A. 1848 Procédé mécanique pour déterminer la composition des reches. Annales eds Mines, 13(IV<sup>e</sup>):379.

Deliconstantinos, G., C. Tsopanakis, F. Karayiannakos and G. Skalkeas 1986 Evidence for the existence of non-

esterified cholesterol carried by albumin in rat serum. *Atherosclerosis*, 51:67-75.

**Demel, R.A. and B. DeKruyff 1976** The function of sterols in membranes. *Biochim. Biophys. Acta*, 558:257-266.

**Dietert, S.E. 1966** Fine structure of the formation and fate of the residual bodies of mouse spermatozoa with evidence for the participation of lysosomes. *J. Morphol.*, 120:317-346.

**Djakiew, D. and M. Dym 1988** Pachytene spermatocyte proteins influence Sertoli cell function. *Biol.Reprod.*, 39:1193-120.

**Dorrington, J.H. and D.T. Armstrong 1975** Follicle stimulating hormone stimulates estradiol-17 $\beta$  synthesis in cultured Sertoli cells. *Proc. Natl. Acad. Sci. USA*, 72:2677-2681.

**Dorrington, J.H., I.B. Fritz and D.T. Armstrong 1976** Site at which FSH regulates estradiol-17 $\beta$  biosynthesis in Sertoli cell preparations in culture. *Molec. Cell. Endocr.*, 6:117-122.

**Dufau, M.L., A.J. Baukal and K.J. Catt 1980** Hormone-induced guanyl nucleotide binding and activation of adenylate cyclase in the Leydig cell. *Proc.Natl.Acad.Sci.USA.*, 77:5837-5841.

**Dufau, M.L., S.H. Sorrell and K.J. Catt 1981** Gonadotropin-induced phosphorylation of endogeneous proteins in the Leydig cell. *FEBS Lett.*, 131:229-234.

**Dym, M. 1973** The fine structure of the monkey (*Macaca*) Sertoli cell and its role in maintaining the blood-testis barrier. *Anat. Rec.*, 175:634-656.

- Dym, M. and J.C. Cavicchia 1977 Further observations on the blood-testis barrier in monkeys. Biol. Reprod., 17:390-403.
- Dym, M. and D.W. Fawcett 1970 The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol. Reprod., 3:308-326.
- Dym, M. and H.G. Madhwa Raj 1977 Response of adult rat Sertoli cells and Leydig cells to depletion of luteinizing hormone and testosterone. Biol. reprod., 17:676-696.
- Eik-Nes, K.B. 1970 Synthesis and secretion of androstenedione and testosterone. In: The Androgens of the Testis. K. B. Eik-Nes, ed. Marcel Dekker, Inc., New York, pp. 1-47.
- Elias, H. and D.M. Hyde 1980 An elementary introduction to stereology (quantitative microscopy). Am.J.Anat., 159:411-446.
- Elias, P.M., D.S. Friend and J. Goerke 1979 Membrane sterol heterogeneity: Freeze-fracture detection with saponin and filipin. J. Histochem. Cytochem., 27:1247-1260.
- Ellis, G.B. and C. Desjardins 1982 Male rats secrete luteinizing hormone and testosterone episodically. Endocrinology, 110:1618-1627.
- Ellis, G.B. and C. Desjardins 1984 Orchidectomy unleashes pulsatile luteinzing hormone secretion in the rat. Biol. Reprod., 30:619-627.

**Ellis, G.B., C. Desjardins and H.M. Fraser 1983** Control of pulsatile Luteinizing hormone release in male rats. *Neuroendocrinology*, 37:177-183.

**Emeis, J.J., C.M. Van Gent and C.M. Van Sabben 1977** An enzymatic method for the histochemical localization of free and esterified cholesterol separately. *Histochem. J.*, 9:197-204.

**Ewing, L.L., T.-Y. Wing, R.C. Cochran, N. Kromann and B.R. Zirkin 1983** Effect of luteinizing hormone on Leydig cell structure and testosterone secretion. *Endocrinology*, 112:1763-1769.

**Fan, D.F., H. Oshima, B.R. Tröen and P. Tröen 1974** Studies of human testis. 4. Testicular 20 $\alpha$ -hydroxysteroid dehydrogenase and steroid 17 $\alpha$ -hydroxylase. *Biochim. Biophys. Acta.*, 360:88.

**Farkash, Y., R. Timberg and J. Orly 1986** Preparation of antiserum to rat cytochrome P-450 cholesterol side-chain cleavage, and its use for ultrastructural localization of immunoreactive enzymes by protein-A gold technique. *Endocrinology*, 118:1353-1365.

**Fawcett, D.W. 1975** Ultrastructure and function of the Sertoli cell. In: *Handbook of Physiology. Section 7: Male Reproductive System. Vol. 5.* C. W. Hamilton and R. O. Greep, eds. American Physiological Society, Washington, pp. 21-55.

**Fawcett, D.W. 1979** The organization of the seminiferous epithelium, Its relevance to fertility control. In: *Contraception: Science, Technology and Application. Proceedings of a Symposium of the National Academy of Sciences.*, Washington, D.C. eds. Vol. pp. 158-179.

**Federman, D.D.** 1981 General principles of endocrinology. In: Textbook of Endocrinology. Vol. R. H. Williams, eds. W. B. Saunders Company, Toronto, pp. 1-14.

**Feltkamp, C.A. and A.W.M. van der Waerden** 1982 Membrane-associated proteins affect the formation of filipin-cholesterol complexes in viral membranes. *Exp. Cell Res.*, 140:289-297.

**Fleeger, J.L., J.P. Bishop, W.R. Gomes and N.L. van Demark** 1968 Testicular lipids. I. Effects of unilateral cryptorchidism on lipid classes. *J.Reprod.Fert.*, 15:1-7.

**Fleeger, J.L., J.P. Bishop, W.R. Gomes and N.L. van Demark** 1968 Testicular lipids: II. Effect of unilateral cryptorchidism on fatty acids of testicular phospholipids and triglycerides. *J.Reprod.Fert.*, 15:9-14.

**Flickinger, C.J. and D.W. Fawcett** 1967 Junctional specializations of the Sertoli cells in the seminiferous epithelium. *Anat. Rec.*, 158:207-222.

**Floderus, S.** 1944 Untersuchungen uber den Bau der menschlichen Hypophyse mit besonderer Berucksichtigung der quantitativen mikromorphologischen Verhaltnisse. *Acta path. microgiol.Scand.*, suppl. 53:1-276.

**Folch, J., M. Lees and G.A. Sloan-Stanley** 1957 A simple method for the isolation and purification of total lipides from animal tissues. *J.Biol.Chem.*, 226:497.

**Freeman, C.A. and M. Ascoli** 1982 Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cell. *J. Biol. Chem.*, 257:4231-4238.

**Freeman, D.A. 1989** Plasma membrane cholesterol: Removal and insertion into the membrane and utilization as substrate for steroidogenesis. *Endocrinology*, 124:2527-2534.

**Friend, D.S. and P.M. Elias 1978** Heterogeneity of filipin-sterol complexes in the guinea pig sperm plasma membrane. *J. Cell Biol.*, 79:216a (abstr.).

**Gamble, W., M. Vaughan, H.S. Kruth and J. Avigan 1978** Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.*, 19:1068-1070.

**Glagolev, A.A. 1934** Quantitative analysis with the microscope by the point method. *Eng.Min.J.*, 135:399-402.

**Go, K.J. and D.P. Wolf 1983** The role of sterols in sperm capacitation. *Adv. Lipid Res.*, 20:317-330.

**Goldstein, J.L. and M.S. Brown 1977** Low density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.*, 46:897-930.

**Griswold, M.D., P.D. Bishop, K.-H. Kim, R. Ping, J.E. Siiteri, C. Morales 1989** Function of vitamin A in normal and synchronized seminiferous tubules. *Ann. NY Acad. Sci.*, 564:154-172.

**Grootegoed, J.A., P.J. Den Boer and P. Mackenbach 1989** Sertoli cell-germ cell communication. *Ann.NY Acad.Sci.*, 564:232-242.

**Grootegoed, J.A., L.M. van Meerkerk, F.F.G. Rommerts and H.J. van der Molen 1979** Effect of hypophysectomy on ribonucleic acid synthesis in primary spermatocytes isolated from rat testis. *Int. J. Androl.*, 2:330-342.

**Gustafson, A.W. 1987** Changes in Leydig cell activity during the annual testicular cycle of the bat *Myotis lucifugus lucifugus*: Histology and lipid histochemistry. *Am. J. Anat.*, 178:312-325.

**Hafiez, A.A. and A. Bartke 1972** Effect of hypophysectomy on cholesterol metabolism in the testes of rats and mice. *J. Endocrinol.*, 52:321-326.

**Hall, P.F. 1984** Cellular organization for steroidogenesis. *Int. Rev. Cytol.*, 86:53-95.

**Hall, P.F., D.C. Irby and D.M. de Kretser 1969** Conversion of cholesterol to androgens by rat testes: comparison of interstitial cells and seminiferous tubules. *Endocrinology*, 84:488-96.

**Hanes, F.M. and J. Rosenbloom 1911** A histological and chemical study of the fatty matter of normal and cryptorchid testes. *J. Exp. Med.*, 13:355.

**Hesselink, P.G., A. Kerkenaar and B. Witholt 1990** Inhibition of microbial cholesterol oxidases by dimethylmorpholines. *J Steroid Biochem*, 35:107-113.

**Hilton, F.K. 1961** Relationships of testicular cholesterol to hormonal activity and behavior in the Starling. *Proc. Soc. Exp. Biol. Med.*, 107:653-655.

**Hirsch, M., J. Tassin, W. Noske and Y. Courtois 1989** Filipin-induced deformations in plasma membranes of cultured bovine corneal endothelial cells with incomplete belts of tight junctions. *Exp Eye Res*, 49:717-727.

Hochereau-de Reviere, M.T., C. Perreau and G.A. Lincoln 1985 Photoperiodic variations of somatic and germ cell populations in the Soay ram testis. J. Reprod. Fert., 74:329-334.

Hodgson, Y.M., D.C. Irby, J.B. Kerr and D.M. deKretser 1979 Studies on the structure and function of the Sertoli cell in a seasonally breeding rodent. Biol.Reprod., 21:1091-1098.

Hon, C.A., P.J. Gardner and M.P. Leuschen 1983 Immunocytochemical staining of isolated rat Sertoli cells for anti-FSH $\beta$ . Am.J.Anat., 167:441-450.

Hoshi, K., T. Aita, K. Yanagida, N. Yoshimatsu and A. Sato 1990 Variation in the cholesterol/phospholipid ratio in human spermatozoa and its relationship with capacitation. Hum Reprod, 5:71-74.

Hsueh, A.J.X. and P.B.C. Jones 1981 Extrapituitary actions of gonadotropin-releasing hormone and its antagonist on androgen biosynthesis by cultured testicular cells. Endocr. Rev., 2:437-461.

Huckins, C. 1971 The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat. Rec., 169:533-558.

Huckins, C. 1978 The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of germinal epithelium. Anat. Rec., 190:905-926.

Huggins, C. and P.V. Moulder 1945 Estrogen production by Sertoli cell tumours of the testis. Cancer Res., 5:510-514.

Huhtaniemi, I., H. Nikula and M. Parvinen 1989 Pertussis toxin enhances follicle-stimulating hormone-stimulated cAMP production in rat seminiferous tubules in a stage-dependent manner. *Mol.Cell.Endocrinol.*, 62:89-94.

Hunter, M.G., M.H.F. Sullivan, C.J. Dix, I.F. Aldred and B.A. Cooke 1982 stimulation and inhibition by LHRH analogues of cultured rat Leydig cell function and lack of effect on mouse Leydig cells. *Mol. Cell. Endocrinol.*, 27:31-44.

Irby, D.C., J.B. Kerr, G.P. Risbridger and D.M. de Kretser 1984 Seasonally and experimentally induced changes in testicular function of the Australian bush rat (*Rattus fuscipes*). *J. Reprod. Fert.*, 70:657-666.

Ireland, M.E. and M.J. Welch 1987 Germ cell stimulation of Sertoli cell protein phosphorylation. *Endocrinology*, 120:1317-1326.

Jansen, R.P. 1970 Electronmicroscopic observations on the Sertoli cell in the experimentally cryptorchid rat testis. *J. Anat.*, 106:192 Abstr.

Jayaram, M., S.K. Murthy and J. Ganguly 1973 Effect of vitamin A deprivation on the cholesterol side-chain cleavage enzyme activity of testes and ovaries of rats. *Biochem. J.*, 136:221-3.

Jefcoate, C.R. 1975 Cytochrome P-450 of adrenal mitochondria. *J. Biol. Chem.*, 250:4663-4670.

Johnsen, S.G. 1964 Studies on the testicular-hypophyseal feed-back mechanism in man. *Acta Endocr.*, Suppl. 90:99-124.

Johnson, A.D., W.R. Gomes, M.J. Free and N.L. VanDemark 1968 Testicular lipids. 3. Effect of surgery and unilateral or bilateral cryptorchidism. J. Reprod. Fert., 16:409-14.

Johnson, A.D., W.R. Gomes and N.L. VanDemark 1971 Testicular lipids. IV. Effect of unilateral and bilateral cryptorchidism on the fatty acids of esterified cholesterol in the rat and rabbit. J. Reprod. Fert., 25:425-30.

Johnsonbaugh, R.E., E.M. Ritzén, K. Hall, M. Parvinen and W.W. Wright 1982 Secretion of a somatotropin-like compound from Sertoli cells in vitro. Proc, Eur. Testis, Workshop, 2nd:Abstr.D12.

Jones, H.M. and K. Miyai 1981 Ultrastructural localization of cholesterol by enzyme histochemistry. Histochem. J., 13:1017-1028.

Jutte, N.H.P.M., J.A. Grootegoed, F.F.G. Rommers and H.J. van der Molen 1981 Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. J. Reprod. Fert., 62:399-405.

Jutte, N.H.P.M., R. Jansen, J.A. Grootegoed, F.F.G. Rommerts, O.P.F. Clausen and H.J. van der Molen 1982 Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. J. Reprod. Fert., 65:431-438.

Jutte, N.H.P.M., R. Jansen, J.A. Grootegoed, F.F.R. Rommerts and H.J. van der Molen 1985 Protein synthesis by isolated pachytene spermatocytes in the absence of Sertoli cells. J. Exp. Zool., 233:285-290.

Kancheva, L.S., Y.S. Martinova and V.D. Georgiev 1990 Prepubertal rat Sertoli cells secrete a mitogenic factor(s) that stimulates germ and somatic cell proliferation. Mol Cell Endocrinol, 69:121-127.

Kangasniemi, M., A. Kaipia, P. Mali, J. Toppari, I. Huhtaniemi and M. Parvinen 1990 Modulation of basal and FSH-dependent cyclic AMP production in rat seminiferous tubules staged by an improved transillumination technique. Anat Rec, 227:62-76.

Karnovsky, M.J. 1965 A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol., 27:137A-138.

Karnovsky, M.J. 1971 Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. Proc. 11th Am. Soc. Cell Biol., 184:146a.

Karnovsky, M.J. 1982 Intramembranous cytochemistry: a perspective. Lab. Invest., 46:637-639.

Kasuga, F., K. Shiota and M. Takahashi 1989 Significance of germ cells for the expression of testicular endocrine function in rats. J. Reprod. Fertil., 87:179-186.

Kaya, M. and R.G. Harrison 1976 The ultrastructural relationship between Sertoli cells and spermatogenic cells in the rat. J. Anat., 121:279-290.

Kerr, J.B. 1988 An ultrastructural and morphometric analysis of the Sertoli cell during the spermatogenic cycle of the rat. Anat. Embryol., 179:191-203.

**Kerr, J.B. and D.M. de Kretser 1975** Cyclic variations in Sertoli cell lipid content throughout the spermatogenic cycle in the rat. *J. Reprod. Fert.*, 43:1-8.

**Kerr, J.B., R.A. Mayberry and D.C. Irby 1984** Morphometric studies on lipid inclusions in Sertoli cells during the spermatogenic cycle in the rat. *Cell Tiss. Res.*, 236:699-709.

**Khmelnitsky, Y., L., R. Hilhorst and C. Veeger 1988** Detergentless microemulsions as media for enzymatic reactions; Cholesterol oxidation catalyzed by cholesterol oxidase. *Eur. J. Biochem.*, 176:265-271.

**Kiernan, J.A. 1981** *Histochemistry and Histochemical Methods*. Pergamon Press, New York.

**Kingsley Smith, B.V. and D. Lacy 1959** Residual bodies of seminiferous tubules of the rat. *Nature, Lond.*, 184:249-251.

**Kinsky, S.C. 1970** Antibiotic interaction with model membranes. *Annu.Rev.Parmacol.*, 10:119.

**Kinsky, S.C., S.A. Luse, C. Zopf, L.L.M. Van deenen and J. Haxby 1967** Interaction of filipin and derivatives with erythrocyte membranes and lipid dispersions: electron microscope observations. *Biochim Biophys. Acta*, 135:844.

**Kinson, G.A., D. Lacy and B. Lofts 1964** Examination of various fractions obtained from the testes of normal and oestrogen-treated rats. In: *Proceedings of the V International Congress on Animal Reproduction and Artificial Insemination, Trento, Italy*. eds. La Lucciola. Vol. 2. pp. 440-445.

**Kraulis, I., H. Traikov, M. Sharpe, K.B. Ruf and F. Naftolin 1978** Steroid induction of gonadotropin surges in

immature rat. 1. Priming effects of androgens. *Endocrinology*, 103:1822-1828.

**Kruth, H.S. 1984** Histochemical detection of esterified cholesterol within human atherosclerotic lesions using the fluorescent probe filipin. *Atherosclerosis*, 51:281-292.

**Lacroix, M., M. Parvinen and I.B. Fritz 1981** Localization of testicular plasminogen activator in discrete portions (stage VII and VIII) of the seminiferous tubule. *Biol. Reprod.*, 25:143-146.

**Lacroix, M., R.E. Smith and I.B. Ritz 1977** Secretion of plasminogen activator by Sertoli cell enriched cultures. *Mol. Cell. Endocr.*, 9:227-236.

**Lacy, D. 1960** Light and electron microscopy and its use in the study of factors influencing spermatogenesis in the rat. *J.R.Microsc.Soc.*, 79:209-225.

**Lacy, D. 1962** Certain aspects of testis structure and function. *Brit. Med. Bull.*, 18:205-208.

**Lacy, D. 1973** Androgen dependency of spermatogenesis and the physiological significance of steroid metabolism *in vitro* by the seminiferous tubules. In: *Endocrine Function of the Human Testis*. Vol. 1. V. H. T. James, M. Serio and L. Martini, eds. Academic Press, New York, pp. 493-532.

**Lacy, D. and B. Lofts 1962** The use of ionizing radiation and estrogen treatment in the detection of hormone synthesis by the Sertoli cell. *J. Physiol., Lond.*, 161:23-24 Abstract.

**Lacy, D. and A.J. Pettitt 1969** Transmission electron microscopy and the production of steroids by the Leydig and Sertoli cells of the human testis. *Micron*, 1:15-33.

Lacy, D. and A.J. Pettitt 1970 Sites of hormone production in the mammalian testis, and their significance in the control of male fertility. [Review]. Brit. Med. Bull., 26:87-91.

Lacy, D., G.P. Vinson, P. Collins, J. Bell, P. Fyson, J. Pudney and A.J. Pettitt 1969 The Sertoli cell and spermatogenesis in mammals. In: Progress in Endocrinology. Vol. 1. C. Gual, eds. Excerpta Medica Foundation, Amsterdam, pp. 1019.

Lange, Y., J.S. O'Alessandro and D.M. Small 1979 The affinity of cholesterol for phosphatidylcholine and sphingomyelin. Biochem. Biophys. Acta, 556:388-398.

Lange, Y. and B.V. Ramos 1983 Analysis of the distribution of cholesterol in the intact cell. J. Biol. Chem., 258:15130-15134.

Lange, Y., M.E. Swaisgood, B.V. Ramos and T.L. Steck 1989 Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. J. Biol. Chem., 264:3786-3793.

Langlais, J. and K.D. Roberts 1985 A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamete Res., 12:183-224.

Leblond, C.P. and Y. Clermont 1952 A definition of the stages of the cycles of the seminiferous epithelium in the rat. Ann.N.Y.Acad.Sci., 90:167-214.

Legault, Y., M. Bouthillier, G. Bleau, A. Chapdelaine and K.D. Roberts 1979 The sterol and sterol sulfate

content of the male hamster reproductive tract. Biol. Reprod., 20:1213-1219.

**Lewis, L.G. and C.G. Stockard 1950** Feminizing testis tumours. J. Urol., 64:518-523.

**Lincoln, G.A. 1981** Seasonal aspects of testicular function. In: The Testis. H. Burger and D. de Kretser, eds. Raven Press, New York, pp. 255-302.

**Lofts, B. 1960** Cyclical changes in the distribution of testis lipids of a seasonal mammal (*Talpa europea*). Q. J. Microsc. Sci., 101:199.

**Lofts, B. 1964** Seasonal changes in the functional activity of the interstitial and spermatogenic tissue of the green frog, *Rana esculenta*. Gen. Comp. Endocrinol., 4:550-562.

**Lofts, B. 1968** Patterns of testicular activity. In: Hormones in the lives of lower vertebrates. E. J. W. Barrington and C. B. Jørgensen, eds. Academic Press, New York and London, pp. 239.

**Lofts, B. 1972** The Sertoli cell. Gen. Comp. Endocr., suppl. 3:636-648.

**Lofts, B. and H.A. Barn 1972** The functional morphology of steroidogenic tissues. In: Steroids in non-mammalian vertebrates. Vol. D. R. Idler, eds. Academic Press, New York and London, pp. 37.

**Lofts, B. and C. Boswell 1960** Cyclical changes in the distribution of the testis lipids in the common frog, *Rana temporaria*. Nature, 187:708-709.

**Lofts, B. and C. Boswell 1961** Seasonal changes in the distribution of the testis lipids of the caspian terrapin *Clemmys caspia*. Proc. Zool. Soc. London, 136:581-592.

**Lofts, B. and A.J. Marshall 1957** Cyclical changes in the distribution of the testis lipids of a teleost fish *Exocoelacanthus*. Quart.J. Microsc. Sci., 98:79-88.

**Lofts, B., J.J. Wellen and T.J. Benraad 1972** Seasonal changes in endocrine organs of the male common frog, *Rana temporaria*. 3. The gonads and cholesterol cycles. Gen. Comp. Endocrinol., 18:344-63.

**Lowitt, S., R.V. Farese, M.A. Sabir and A.W. Root 1982** Rat Leydig cells phospholipid content is increased by luteinizing hormone and 8-bromo-cyclic-AMP. Endocrinology, 111:1415-1417.

**Luft, J.H. 1961** Improvements in epoxy embedding methods. J. Biophys. Biochem. Cytol., 9:409-414.

**Lynch, K.M. and W.W. Scott 1950** The lipid content of the Leydig cell and Sertoli cell in the human testis as related to age, benign prostatic hyperplasia, and prostatic cancer. J. Urol., 64:767-776.

**Lynch, K.M.J. and W.W. Scott 1951** Lipid distribution in the Sertoli cell and Leydig cell of the rat testis as related to experimental alterations of the pituitary-gonad system. Endocrinology, 49:8-14.

**Mack, C. 1956** On clumps formed when convex laminae or bodies are placed at random in two or three dimensions. Proc.Camb.Phil.Soc., 52:246-250.

**Maddocks, S. and B.P. Setchell 1988** The physiology of the endocrine testis. *Ox. Rev. Reprod. Biol.*, 10:53-123.

**Mahley, R.W. 1988** Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science*, 240:622-630.

**Mahley, R.W. and T.L. Innerarity 1983** Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta*, 737:197-222.

**Marshall, A.J. 1955** Reproduction in birds: the male. *Mem. Soc. Endocrinol.*, 4:75-89.

**Marshall, A.J. and O. Wilkinson 1956** Reproduction in the Orkney vole (*Microtus orcandensis*) under a six hour daylength and other conditions. *Proc. Zool. Soc. London*, 126:391-395.

**Marzowski, J., S.R. Sylvester, R.R. Gilmont and M.D. Griswold 1985** Isolation and characterization of Sertoli cell plasma membranes and associated plasminogen activator activity. *Biol. Reprod.*, 32:1237-1245.

**Mason, J.I., A.A. MacDonald and A. Lupton 1984** The activity and biosynthesis of cholesterol side-chain cleavage enzyme in cultured immature pig testis cells. *Biophys. Biochim. Acta.*, 795:504-512.

**Massa, E.M. and A. Aoki 1976** Differentiation of cholesterol compartments in the immature chick testis. *J. Reprod. Fert.*, 47:313-8.

**Mather, J.P., G.L. Gunsalus, N.A. Musto, C.Y. Cheng, M. Parvinen, W. Wright, V. Pérez-Infante, A. Margioris, A. Liotta, R. Becker, D.T. Kriger and C.W.**

**Barkin 1983** The hormonal and cellular control of Sertoli cell secretions. *J. Steroid Biochem.*, 19:41-51.

**McGinley, D., Z. Posalaky and M. Porvaznik 1977** Intercellular junctional complexes of the rat seminiferous tubules: A freeze-fracture study. *Anat. Rec.*, 189:211-232.

**McGinley, D.M., Z. Posalaky, M. Porvaznik and L. Russell 1979** Gap junctions between Sertoli and germ cells of rat seminiferous tubules. *Tissue Cell*, 11:741-754.

**McIntosh, T.J., Magid, A.D., and Simon, S.A. 1989** Cholesterol modifies the short-range repulsive interactions between phosphatidylcholine membranes. *Biochemistry*, 28:17-25.

**McIntosh, T.J., A.D. Magid and S.A. Simon 1987** Steric repulsion between phosphatidylcholine bilayers. *Biochemistry*, 26:7325-7332.

**Means, A.R., J.L. Fakunding, C. Huckins, D.J. Tindall and R. Vitale 1976** Follicle stimulating hormone, the Sertoli cell and spermatogenesis. *Rec. Prog. Horm. Res.*, 32:477-527.

**Mendelson, C., M.L. Dufau and K.J. Catt 1975** Gonadotropin binding and stimulation of cyclic adenosine 3',5'-monophosphate and testosterone production in isolated Leydig cells. *J. Biol. Chem.*, 250:8818-8823.

**Millette, C.F., D.A. O'Brian and M. C.T. 1980** Isolation of plasma membranes from purified mouse spermatogenic cells. *J. Cell Sci*, 43:279-299.

**Mills, N.C. 1990** Androgen effects on Sertoli cells. *Int. J. Androl.*, 13:123-134.

**Moore, C.R. 1924** Properties of the gonads as controllers of somatic and physical characteristics. VIII. Heat applications and testicular degeneration; the function of the scrotum. *Am. J. Anat.*, 34:337-358.

**Morales, C. and Y. Clermont 1982** Evolution of Sertoli cell processes invading the cytoplasm of rat spermatids. *Anat. Rec.*, 203:233-244.

**Morales, C., Y. Clermont and L. Hermo 1985** Nature and function of endocytosis in Sertoli cells of the rat. *Am.J.Anat.*, 173:203-217.

**Mori, H. and A.K. Christensen 1980** Morphometric analysis of Leydig cells in the normal rat testis. *J.Cell Biol.*, 84:340-354

**Moyle, W.R., R.L. Jungas and R.O. Greep 1970** Apparent need for cholesteryl palmitate (CP) hydrolysis before cholesterol (C) side chain cleavage in intact testicular tumor mitochondria (Mito). *Fed. Proc.*, 29:247.

**Nagano, T. 1966** Some observations on the fine structure of Sertoli cells in the human testes. *Z. Zellforsch. Mikroskop. Anat.*, 73:89-106.

**Nagendranath, N., T.M. Jose and H.S. Juneja 1983** Biosassayable gonadotropin releasing hormone-like activity in the spent nutrient medium of rat Sertoli cells in primary culture. *Horm. Metab. Res.*, 15:99-102.

**Nakamura, K., Y. Inoue, N. Watanabe and T. Tomita 1988** Studies on cholesterol esterase in rat adipose tissue: Comparison of substrates and regulation of the activity. *Biochim.Biophys.Acta*, 963:320-328.

**Neaves, W.B.** 1975 Leydig cells. [Review]. *Contraception*, 11:571-606.

**Neaves, W.B.** 1978 The pattern of gonadotropin-induced change in plasma testosterone, testicular esterified cholesterol and Leydig cell lipid droplets in immature mice. *Biol. Reprod.*, 19:864-71.

**Nestler, J.E. and T. Williams** 1987 Modulation of aromatase and P450 cholesterol side-chain cleavage enzyme activities of human placental cytotrophoblasts by insulin and insulin-like growth factor I. *Endocrinology*, 121:1845-1852.

**Niemi, M. and M. Ikonen** 1962 Cytochemistry of oxidative enzyme systems in the Leydig cells of the rat testis and their function significance. *Endocrinology*, 70:167-174.

**Niemi, M. and M. Kormanen** 1965 Cyclical changes in and significance of lipids and acid phosphatase activity in the seminiferous tubules of the rat testis. *Anat. Rec.*, 151:159-170.

**Oakberg, E.** 1956 A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.*, 99:391-413.

**Onstad, O.** 1967 Studies on postnatal testicular changes, semen quality, and anomalies of reproductive organs in the mink. *Acta Endocr. Copenh.*, Suppl. 117:1-117.

**Orth, J. and A.K. Christensen** 1977 Localization of <sup>125</sup>I-labeled FSH in the testes of hypophysectomized rats by autoradiography at the light and electron microscope levels. *Endocrinology*, 101:262-278.

Osman, D.J. and L. Plöen 1978 The ultrastructure of Sertoli cells in the boar. *Int. J. androl.*, 1:162-179.

Paniagua, R., P. Amat, M. Nistal and A. Martin 1985 Ultrastructural changes in Sertoli cells in ageing humans. *Int. J. Androl.*, 8:295-312.

Paniagua, R., M.C. Rodriguez, M. Nistal, B. Fraile and P. Amat 1987 Changes in the lipid inclusion/Sertoli cell cytoplasm area ratio during the cycle of the human seminiferous epithelium. *J. Reprod. Fert.*, 80:335-341.

Paniagua, R., M.C. Rodríguez, M. Nistal, B. Fraile, J. Regadera and P. Amat 1988 Changes in surface area and number of Leydig cells in relation to the 6 Stages of the cycle of the human seminiferous epithelium. *Anat. Embryol.*, 178:423-427.

Parvinen, M. 1982 Regulation of the seminiferous epithelium. *Endocrin.Rev.*, 3:404-417.

Parvinen, M., R. Marana, D.M. Robertson, V. Hansson and E.M. Ritzen 1980 Functional cycle of rat Sertoli cells: differential binding and action of FSH at various stages of the spermatogenic cycle. In: *Testicular Development: Structure and Fuction*. A. Steinberger and E. Steinberger, eds. Raven Press, New York, pp. 425-432.

Parvinen, M. and M. Niemi 1971 Distribution and conversion of exogenous cholesterol and sex steroids in the seminiferous tubules and interstitial tissue of the rat testis. *Steroidologia*, 2:129-37.

Parvinen, M., H. Nikula and I. Huhtaniemi 1984 Cyclic regulation of rat Leydig cell testosterone production by seminiferous tubules. Ann. N.Y. Acad. Sci., 438:681-683.

Parvinen, M. and Vanha-Perttula 1972 Identification and enzyme quantitation of the stages of the seminiferous epithelial wave in the rat. Anat. Rec., 174:435-450.

Parvinen, M., K.K. Vihko and J. Toppar 1986 Cell-interactions during the seminiferous epithelial cycle. Int. Rev. Cytol., 104:115-151.

Payne, A.H. 1990 Hormonal regulation of cytochrome P450 enzymes, cholesterol side-chain cleavage and 17 $\alpha$ -hydroxylase/C<sub>17-20</sub> lyase in Leydig cells. Biol. Reprod., 42:399-404.

Pearse, A.G.E. 1968 Histochemistry, Theoretical and Applied. J. and A. Churchill Ltd., London, Vol. 1. Ed. 3. pp. 398-446

Pelletier, R.-M. 1986 Cyclic formation and decay of the blood-testis barrier in the mink (*Mustela vison*): a seasonal breeder. Am. J. Anat., 175:91-117.

Pelletier, R.-M. 1988 Cyclic modulation of Sertoli cell junctional complexes in a seasonal breeder: the mink (*Mustela vison*). Am. J. Anat., 183: 68-102.

Pelletier, R.-M. and S. Byers 1990 The blood-testis barrier and the Sertoli cell junctions. J. Elec. Microsc. Tech. (in press).

Pelletier, R.-M. and D.S. Friend 1983 The Sertoli cell junctional complex: structure and permeability to filipin in the neonatal and adult guinea pig. Am. J. Anat., 168:213-228.

**Perlman, P.L. 1950** The functional significance of testis cholesterol in the rat: Histochemical observations on the testis following hypophysectomy and experimental cryptorchidism. *Endocrinology*, 46:341-346.

**Pineau, C., J.F. Velez de la Calle, G. Pinon-Lataillade and B. Jégou 1989** Assessment of testicular function after acute and chronic irradiation: Further evidence for an influence of late spermatids on Sertoli cell function in the adult rat. *Endocrinology*, 124:2720-2728.

**Podesta, E.J., M.L. Dufau and K.J. Catt 1976** Adenosine 3',5'-monophosphate dependent protein kinase of Leydig cells: *In vitro* activation and relationship to gonadotropin action upon cyclic AMP and steroidogenesis. *FEBS Lett.*, 70:212-216.

**Pokal, J.D., W.R. Moyle and R.O. Greep 1972** Depletion of esterified cholesterol in mouse testis and Leydig cell tumors by luteinizing hormone. *Endocrinology*, 91:323-5.

**Pollock, W.F. 1942** Histochemical studies of the interstitial cells of the testis. *Anat. Rec.*, 84:23-29.

**Posalaki, Z., D. Szabó, E. Bácsai and I. Okróss 1968** Hydrolytic enzymes during spermatogenesis in rat an electron microscopic and histochemical study. *J. Histochem. Cytochem.*, 16:249-62.

**Pudney, J. and D. Lacy 1977** Correlation between ultrastructure and biochemical changes in the testis of the American grey squirrel, *Sciurus carolinensis*, during the reproductive cycle. *J.Reprod.Fert.*, 49:5-16.

**Purvis, K., L. Cusan and V. Hansson 1981** Regulation of steroidogenesis and steroid action in Leydig cells. *J. Steroid Biochem.*, 15:77-86.

- Quinn, P.J. and I.G. White 1967 Phospholipid and cholesterol content of epididymal and ejaculated ram spermatozoa and seminal plasma in relation to cold shock. Aust. J. Biol. Sci., 20:1205-1215.
- Raeside, J.I. and D.K. Lobb 1984 Metabolism of androstenedione by Sertoli cell enriched preparations and purified Leydig cells from boar testes in relation to estrogen formation. J.Steroid Biochem., 20:1267-1272.
- Regaud, C. 1901 Etudes sur la structure des tubes séminifères et sur la spermatogénèse chez les mammifères. Arch. Anat. Microsc., 4:101-156;232-380.
- Reichert, L.E. and B. Dattatreyamurty 1989 The follicle-stimulating hormone (FSH) receptor in testis: Interaction with FSH, mechanism of signal transduction, and properties of the purified receptor. Biol.Reprod., 40:13-26.
- Renston, J.P., T.J. Ihrig, R.H. Renston, B. Gondos and R.J. Morin 1977 Cholesterol ester hydrolysis by homogenates of whole testis, seminiferous tubules and interstitial cells of mature rats. Journal of Endocrinology, 75:235-43.
- Reynolds, K.C. 1963 The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Cell Biol., 17:208.
- Ritzén, E.M., C. Boitani, M. Parvinen, F.S. French and M. Feldman 1982 Stage-dependent secretion of ABP by rat seminiferous tubules. Mol.Cell. Endocrin., 25:25-33.
- Robenek, H., W. Jung and R. Gebhardt 1982 The topography of filipin-cholesterol complexes in the plasma membrane of

cultured hepatocytes and their relation of cell junction formation. *J. Ultrastruct. Res.*, 78:95-106.

**Robinson, J.M. and M.J. Karnovsky 1980** Evaluation of the polyene antibiotic filipin as a cytochemical probe for membrane cholesterol. *J. Histochem. Cytochem.*, 28:161-168.

**Rodbell, M., L. Birnbaumer, S.L. Pohl and H.M.J. Krans 1971** The glucagon sensitive adenylyl cyclase system in plasma membranes of rat liver: V. An obligatory role of guanyl nucleotides in glucagon action. *J. Biol. Chem.*, 246:1877-1882.

**Rodgers, R.J., H.F. Rodgers, P.F. Hall, M.R. Waterman and E.R. Simpson 1986** Immunolocalization of cholesterol side-chain cleavage cytochrome P-450 and 17 $\alpha$ -hydroxylase cytochrome P-450 in bovine ovarian follicles. *J. Reprod. Fert.*, 78:627-638.

**Rodgers, R.J., H.F. Rodgers, M.R. Waterman and E.R. Simpson 1986** Immunolocalization of cholesterol side-chain cleavage cytochrome P-450 and ultrastructural studies of bovine corpora lutea. *J. Reprod. Fert.*, 78:639-652.

**Rodgers, R.J., M.R. Waterman, E.R. Simpson and R.R. Magness 1988** Immunoblot analysis of cholesterol side-chain cleavage cytochrome P-450 and adrenodoxin in corpora lutea of cyclic and late-pregnant sheep. *J. Reprod. Fert.*, 83:843-850.

**Romrell, L.J., A.R. Bellvé and D.W. Fawcett 1976** Separation of mouse spermatogenic cells by sedimentation velocity: a morphological characterization. *Dev. Biol.*, 49:119-131.

**Roosen-Rünge, E. 1973** Germinal-cell loss in normal metazoan spermatogenesis. *J. Reprod. Fert.*, 35:339-348.

- Roosen-Rünge, E.C. and L.O. Giesel 1950** Quantitative studies on spermatogenesis in the albino rat. *Am. J. Anat.*, 87:1-30.
- Ross, M.H. 1976** The Sertoli cell junctional specialization during spermiogenesis and at spermiation. *Anat. Rec.*, 186:79-104.
- Russell, L.D. 1977** Desmosome-like junctions between Sertoli and germ cells in the rat testis. *Am. J. Anat.*, 148:301-312.
- Russell, L.D. 1978** The blood-testis barrier and its formation relative to spermatocyte maturation in the adult rat: a lanthanum tracer study. *Anat. Rec.*, 190:99-112.
- Russell, L.D. and Y. Clermont 1977** Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. *Anat. Rec.*, 187:347-365.
- Russell, L.D., J.P. Malone and S.L. Karpas 1981** Morphological pattern elicited by agents affecting spermatogenesis by disruption of its hormonal stimulation. *Tiss. Cell*, 13:369-380.
- Rutishauser, A., B. Acheson, A.K. Hall, D.M. Mann and J. Sunshine 1988** The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science*, 240:53-57.
- Ryter, A., Kellenberger, E. 1958** Etude au microscope électronique de plasma contenant de l'acide desoxyribonucleique. I. Les nucléotides des bactéries en croissance active. *Z. Naturforsch.*, 13b:597.

Sakai, Y. 1981 A quantitative study on the seminiferous epithelium of the adult mink in the post-breeding season. Jpn. J. Vet. Res., 29:30, Abstr.

Sakai, Y., T. Nakamoto and S. Yamashina 1988 Dynamic changes in Sertoli cell processes invading spermatid cytoplasm during mouse spermiogenesis. Anat. Rec., 220:51-57.

Sakai, Y. and S. Yamashina 1989 Mechanism for the removal of residual cytoplasm from spermatids during mouse spermiogenesis. Anat. Rec., 223:43-48.

Sanborn, B.M., A. Steinberger and A. Lee 1981 Subcellular distribution of androgen binding sites in cultured Sertoli cells before and after exposure to androgens. J. Steroid Biochem., 14:133.

Sandler, R. and P.F. Hall 1966 The response of rat testis to interstitial cell-stimulating hormone in vitro. Comp. Biochem. Physiol., 19:833-843.

Sandler, R. and P.F. Hall 1966 Stimulation *in vitro* by adenosine-3',5'-cyclic monophosphate of steroidogenesis in rat testis. Endocrinology, 79:647-649.

Sanyal, M.K. and M.R. Prasad 1965 Seasonal variation in testis and total body cholesterol in the Indian house lizard, *Hemidactylus flaviviridis* Rüppell. Steroids, 6:313-322.

Sapsford, C.S., C.A. Rae and K.W. Cleland 1969 The fate of residual bodies and degenerating germ cells and the lipid cycle in Sertoli cells in the bandicoot *Perameles nasuta* Geoffroy (Marsupialia). Aust. J. Zool., 17:729-753.

Sapsford, C.S., C.A. Rae and K.W. Cleland 1969 Ultrastructural studies on maturing spermatids and on Sertoli

cells in the bandicoot *Perameles nasuta geoffroy* (marsupialia). Aust. J. Zool., 17:195-292.

Sasano, H., J.I. Mason and M. Sasano 1989 Immunohistochemical analysis of cytochrome P-450 17 $\alpha$ -hydroxylase in pig adrenal cortex, testis and ovary. Mol.Cell.Endocrinol., 62:197-202.

Schultz, A. 1924 Eine Methode des mikrochemischen Cholestrin-Nachweises am Gewebsschnitt. Abl. allg. Path. path. Anat., 35:314-317.

Schulze, C. 1984 Sertoli cells and Leydig cells in man. In: Advances in Anatomy, Embryology and Cell Biology. Vol. 88. F. Beck, W. Hild, R. Ortman, J. E. Pauly and T. H. Schiebler, eds. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 1-104.

Scott, I.S., H.M. Charlton, B.S. Cox, C.A. Grocock, J.W. Sheffield and P.J. O'Shaughnessy 1990 Effect of LH injections on testicular steroidogenesis, cholesterol side-chain cleavage P450 mRNA content and Leydig cell morphology in hypogonadal mice. J Endocrinol, 125:131-138.

Scott, T.W., J.K. Voglmayr and B.P. Setchell 1967 Lipid composition and metabolism in testicular and ejaculated ram spermatozoa. Biochem. J., 102:456-61.

Sehgal, A., G.F.X. David, A.K. Dubey, J.S. Pruthi and T.C. Anand Kumar 1986 Circadian and circannual changes in the testicular function of adult rhesus monkeys. J. Biosci., 10:443-452.

Seligman, A., W. Shannon, Y. Hoshino and R. Flapinger 1973 Some important principles in 3,3'-diaminobenzidine

ultrastructural cytochemistry. *J.Histochem. Cytochem.*, 21:756-758.

**Sertoli, E. 1865** De l'esistenza di particolari cellule ramificate nei canalicoli seminiferi dell'testicolo umano. *Morgagni*, 7:31-40.

**Setchell, B.P. 1967** The blood-testicular fluid barrier in the sheep. *J. Physiol. (Lond.)*, 189:63-65.

**Setchell, B.P., J.K. Voglmayr and G.H.M. Waites 1969** A blood-testis barrier restricting passage from blood into rete testis fluid but not into lymph. *J. Physiol. (Lond.)*, 200:73-85.

**Setchell, B.P. and G.M.H. Waites 1975** The blood-testis barrier. In: *Handbook of Physiology*, Vol. 5. K. W. Hamilton and R. O. Greep, eds. Williams and Wilkins, Baltimore., pp. 143-172.

**Seyers, N.J. and H.L. Simons 1983** Failure of filipin to detect cholesterol-rich domains in smooth muscle plasma membrane. *Nature*, 303:637-638.

**Shabanowitz, R.B., R.M. DePhilip, J.A. Crowell, L.L. Tres and A.L. Kierszenbaum 1986** Temporal appearance and cyclic behavior of Sertoli cell-specific secretory proteins during the development of the rat seminiferous tubule. *Biol.Reprod.*, 35:745-760.

**Sharpe, R.M. 1983** Local control of testicular function. *Quart. J. Exp. Physiol.*, 68:265-287.

**Sharpe, R.M. 1984** Intratesticular factors controlling testicular function. *Biol.Reprod.*, 30:29-49.

**Sharpe, R.M. 1986** Paracrine control of the testis. [Review]. Clin. Endocrin. Metabol., 15:185-207.

**Sharpe, R.M. 1989** Possible role of elongated spermatids in control of stage-dependent changes in the diameter of the lumen of the rat seminiferous tubule. J. Androl., 10:304-10.

**Sharpe, R.M. and I. Cooper 1982** The mode of action of LHRH agonists on the rat Leydig cell. Mol. Cell. Endocrinol., 27:199-211.

**Sharpe, R.M. and I. Cooper 1982** Stimulatory effect of LHRH and its agonists on Leydig cell steroidogenesis in vitro. Mol. Cell. Endocrinol., 26:141-150.

**Sharpe, R.M., D.G. Doogan and I. Cooper 1983** Direct effects of luteinizing hormone-releasing hormone agonist on intratesticular levels of testosterone and interstitial fluid formation in intact male rats. Endocrinology, 113:1306-1313.

**Sharpe, R.M., H.M. Fraser, I. Cooper and F.F.G. Rommerts 1981** Sertoli-Leydig cell communication via a LHRH-like factor. Nature, London, 290:785-787.

**Sharpe, R.M., H.M. Fraser, I. Cooper and F.F.G. Rommerts 1982** The secretion, measurement and function of a testicular LHRH-like factor. Ann. N.Y. Acad. Sci., 383:272-294.

**Sharpe, R.M. and A.J. Harnmar 1983** The nature and biological actions of 'testicular LHRH.'. In: Hormones and Cell Regulation. Vol. 7. J. E. Dumont, J. Nunez and R. M. Denton, eds. Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 217-230.

Sharpe, R.M., S. Maddocks and J.B. Kerr 1990 Cell-cell interactions in the control of spermatogenesis as studied using Leydig cell destruction and testosterone replacement. *Am J Anat*, 188:3-20.

Shaw, M.J., L.E. Georgopoulos and A.H. Payne 1979 Synergistic effect of follicle stimulating hormone and luteinizing hormone on testicular  $\Delta^5$ -3 $\beta$ -hydroxysteroid dehydrogenase-isomerase: application of a new method for the separation of testicular compartments. *Endocrinology*, 104:912-918.

Sheriff, D.S. 1980 Influence of hormones on testicular lipids of adult rats. *Horm. Res.*, 13:165-73.

Sheriff, D.S. 1982 The lipid composition of human testis in patients with bilateral varicocele as cause of infertility. *Andrologia*, 14:150-3.

Sheriff, D.S. and P. Govindarajulu 1977 Lipids in human testis. *Horm. Res.*, 8:58-63.

Sheriff, D.S. and P. Govindarajulu 1975 Hormonal influence on testicular lipids. *Horm. Res.*, 6:387-92.

Sheriff, D.S. and P. Govindarajulu 1978 The lipid composition of human testis in testicular feminization syndrome. *Horm. Res.*, 9:107-11.

Sheriff, D.S. 1988 Gossypol and testicular lipids of adult rats. *Horm. Metab. Res.*, 20:691-692.

Shimizu, K., M. Hayano, M. Gut and R.I. Dorfman 1961 The transformation of 20 $\alpha$ -hydroxycholesterol to isocaproic acid and C<sub>21</sub> steroids. *J. Biol. Chem.*, 236:695-699.

Simon, S.A., T.J. McIntosh and A.D. Magid 1988 Magnitude and range of the hydration pressure between lecithin bilayers as a function of headgroup density. J. Colloid Interface Sci., 126:74-83.

Simpson, E.R. 1979 Cholesterol side-chain cleavage, cytochrome P450 and the control of steroidogenesis. Mol. Cell. Endocrinol., 13:213-227.

Sinha Hikim, A.P., A.G. Amador, H.G. Klencke, A. Bartke and L.D. Russell 1989 Correlative morphology and endocrinology of Sertoli cells in hamster testes in active and inactive states of spermatogenesis. Endocrinology, 125:1829-1843.

Sinha Hikim, A.P., A. Bartke and L.D. Russell 1988 Morphometric studies on hamster testes in gonadally active and inactive states: Light microscope findings. Biol. Reprod., 39:1225-1237.

Sinha Hikim, A.P., A. Bartke and L.D. Russell 1988 The seasonal breeding hamster as a model to study structure-function relationships in the testis. Tiss.Cell, 20:63-78.

Slaughter, G.R., M.L. Meistrich and A.R. Means 1989 Expression of RNAs for calmodulin, actins, and tubulins in rat testis cells. Biol.Reprod., 40:395-405.

Smith, A.G. and C.J.W. Brooks 1976 Cholesterol oxidases: properties and applications. J. Sert. Biochem., 7:705-713.

Sprando, R.L., R.L. Heidinger and L.D. Russell 1988 Spermiogenesis in the bluegill (*Lepomis macrochirus*): a study of cytoplasmic events including cell volume changes and cytoplasmic elimination. J. Morphol., 198:165-177.

**Sprando, R.L. and L.D. Russell 1987** Comparative study of cytoplasmic elimination in spermatids of selected mammalian species. *Am.J.Anat.*, 178:72-80.

**Sprando, R.L. and L.D. Russell 1988** Spermiogenesis in the bullfrog (*Rana catesbeiana*): a study of cytoplasmic events including cell volume changes and cytoplasmic elimination. *J. Morphol.*, 198:303-319.

**Steinberger, A. and E. Steinberger 1977** The Sertoli cells. In: *The Testis*. A. D. Johnson and W. R. Gomes, eds. Academic Press, New York., pp. 371-399.

**Steinberger, E. 1971** Hormonal control of mammalian spermatogenesis. *Physiol. Rev.*, 51:1-22.

**Steinberger, E. and M. Fecher 1973** Effect of hypophysectomy and gonadotropin treatment on metabolism of <sup>3</sup>H-progesterone by rat testicular tissue. *Steroids*, 22:425-443.

**Steinberger, E., A. Root, M. Ficher and K.D. Smith 1973** The role of androgens in the initiation of spermatogenesis in man. *J. Clin. Endocrin. Metab.*, 37:746-51.

**Stevenson, B.R., J.D. Silicano, M.S. Mooseker and D.A. Goodenough 1986** Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (Zonula occludens) in a variety of epithelia. *J. Cell Biol.*, 103:755-766.

**Stevenson, B.R., M.B. Heintzelman, J.M. Anderson, S. Citi and M.S. Mooseker 1989** ZO-1 and cingulin: Tight junction proteins with distinct identities and localizations. *Am. J. Physiol.*, 257:C621-C628.

Sultan, S. and S. D. 1984 Further studies on testicular lipids and glycogen in human patients with unilateral varicocele. *Andrologia*, 16:442-5.

Sundqvist, C. 1985 Morphometric studies on mink testicular tissue. *Theriogenology*, 24:713-723.

Sundqvist, C., A. Lukola and M. Valtonen 1984 Relationship between serum testosterone concentrations and fertility in male mink (*Mustela vison*). *J. Reprod. Fert.*, 70:409-412.

Suzuki, F. and R. Yanagimachi 1989 Changes in the distribution of intramembranous particles and filipin-reactive membrane sterols during in vitro capacitation of golden hamster spermatozoa. *Gamete Res.*, 23:335-47.

Swartz, G.M., M.K. Gentry, L.M. Amende, E.J. Blanchette-Mackie and C.R. Alving 1988 Antibodies to cholesterol. *Proc. Natl. Acad. Sci. USA.*, 85:1902-1906.

Szollosi, A. and C. Marcaillou 1980 Gap junctions between germ and somatic cells in the testis of the moth *Anagasta kuehniella* Insecta: Lepidoptera. *Cell Tissue Res.*, 213:137-148.

Tähka, K.M. 1986 Current aspects of Leydig cell function and its regulation. *J.Reprod.Fertil.*, 72:367-380.

Tam, W.H., J.G. Phillips and B. Lofts 1969 Seasonal changes in the in vitro production of testicular androgens by the cobra (*Naja naja*). *Gen. Compar. Endocr.*, 13:117.

Teilum, G. 1949 Estrogen-producing Sertoli cell tumors (Androblastoma Tubulare Lipoides) of human testis and ovary;

homologous ovarian and testicular tumors. J. Clin. Endocrinol. Metab., 9:301-318.

**Tiba, T.** 1973 Histologische Untersuchung der Kinetik der Spermatogenese beim Mink (*Mustela vison*). IV. Zellgemeinschaften in Samenepithel unmittelbar vor den Paarungszeiten (7. und 19. Lebensmonaten). Jpn. J. Vet. Res., 21:112-123.

**Tiba, T., T. Ishikawa and A. Muakami** 1968 Histologische Untersuchung der Kinetik der Spermatogenese beim Mink. I. Samenepithelzyklus in der Paarungszeit. Jpn. J. Vet. Res., 16:159-181.

**Tillack, W.T., Kinsky, S.C.** 1973 A freeze-etch study of the effects of filipin on liposomes and human erythrocyte membranes. Biochim. Biophys. Acta, 323:43-54.

**Tsui, H.W., W.H. Tam, B. Lofts and J.G. Phillips** 1974 The annual testicular cycle and androgen production *in vitro* in the masked civet cat, *Paguma l. larvata*. J. Reprod. Fert., 36:283-93.

**Tung, K.S.K., L.C. Ellis, G. Childs, R.-M. Pelletier and M. Dufau** 1983 The dark mink: a model of male infertility. Clin. Res., 31:473Abst.

**Tung, K.S.K., L.C. Ellis, C. Tensscher, A. Meng, J.L. Bloustein, S. Kohno and R. Howell** 1981 The black mink (*Mustela visa*). A natural model of immunologic male infertility. J. Exp. Med., 154:1016-1032.

**Tung, P.S. and I.B. Fritz** 1984 Extracellular matrix promotes rat Sertoli cell histotypic expression *in vitro*. Biol. Reprod., 30:213-229.

Ueno, H. and H. Mori 1990 Morphometrical analysis of Sertoli cell ultrastructure during the seminiferous epithelial cycle in rats. Biol Reprod, 43:769-776.

van der Molen, H.J. and F.F.G. Rommerts 1981 Testicular steroidogenesis. In: The Testis. Vol. H. Burger and D. de Kretser, eds. Raven Press, New York, pp. 213-238.

van der Vusse, G.J., M.L. Kalkman and H.J. van der Molen 1974  $3\beta$ -Hydroxysteroid dehydrogenase in rat testis tissue. Inter- and subcellular localization and inhibition by cyanoketone and nagarse. Biochim. Biophys. Acta., 380:473-485.

van der Vusse, G.J., M.L. Kalkman and H.J. van der Molen 1975 Endogenous steroid production in cellular and subcellular fractions of rat testis after prolonged treatment with gonadotropins. Biophys. Biochim. Acta., 380:473-485.

Veijola, M. and H. Rajaniemi 1985 Luteinizing hormones activate in testicular interstitial fluid a factor(s) that increases the capillary permeability-a novel effect of hCG and LH. Acta endocr., Copenh., Suppl., 270:205 Abstr.

Verkleij, A.J., B. De Kruijff, W.F. Gerritsen, R.A. Demel, L.L.M. Van Deenen and P.H.J. Ververgaert 1973 Freeze-etch electron microscopy of erythrocytes, *Acholeplasma laidlawii* cells and liposomal membranes after the action of filipin and amphotericin B. Biochim. Biophys. Acta., 291:577.

Vihko, K.K., J.J.O. Suominen and M. Parvinen 1984 Cellular regulation of plasminogen activator secretion during spermatogenesis. Biol.Reprod., 31:383-389.

von Ebner, V. 1871 Untersuchungen über den bau der Samenkanälchen und die Entw'chelung der Spermatozoiden bei den

Säugethürin und Menschen. Leipzig: Untersuchungen aus dem Institute für Physiologie und Histologie im Graz, 2:200-236.

von Ebner, V. 1888 Zur Spermatogenese bei den Säugethieren. Arch. Mikroskop. Anat. Entwicklungsmech., 31:236-292.

Waterman, M.R. and E.R. Simpson 1985 Regulation of the biosynthesis of cytochromes P-450 involved in steroid hormone synthesis. Mol. Cell. Endocr., 39:81-89.

Webley, G.E., G.S. Pope and E. Johnson 1985 Seasonal changes in the testes and accessory reproductive organs and seasonal and circadian changes in plasma testosterone concentrations in the male grey squirrel (*Sciurus carolinensis*). Gen. Comp. Endocrin., 59:15-23.

Welsh, M.J. and J.P. Wiebe 1975 Rat Sertoli cells: a rapid method for obtaining viable cells. Endocrinology, 96:618-624.

Welsh, M.J. and J.P. Wiebe 1978 Sertoli cell capacity to metabolize C<sub>19</sub> steroids: variation with age and the effect of follicle-stimulating hormone. Endocrinology, 103:838-844.

Wiebe J.P., K.S. Tilbe, 1979 De novo synthesis of steroids (from acetate) by isolated rat Sertoli cells. Biochem. Biophys. Res. Comm., 89:1107-1113.

Wiebe, J.P., K.S. Tilbe and K.D. Buckingham 1980 An analysis of the metabolites of progesterone produced by isolated Sertoli cells at the onset of gametogenesis. Steroids, 35:561-577.

Wing, J.-Y. and A. Christensen 1982 Morphometric studies on rat seminiferous tubules. Am.J.Anat., 165:13-25.

Wislocki, G.B. 1949 Seasonal changes in the testes, epididymides and seminal vesicles of deer investigated by histochemical methods. *Endocrinology*, 44:167-189.

Wisner, J.R. Jr. and W.R. Gomes 1975 Comparative ability of rat seminiferous tubules, interstitium, and whole testes to utilize cholesterol-1,5-3H as a substrate for testosterone synthesis and the intratesticular distribution of cholesterol side-chain cleavage enzyme. *Steroids*, 26:769-83.

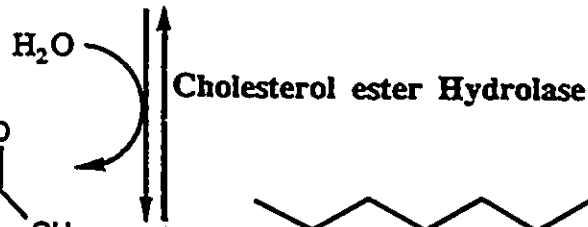
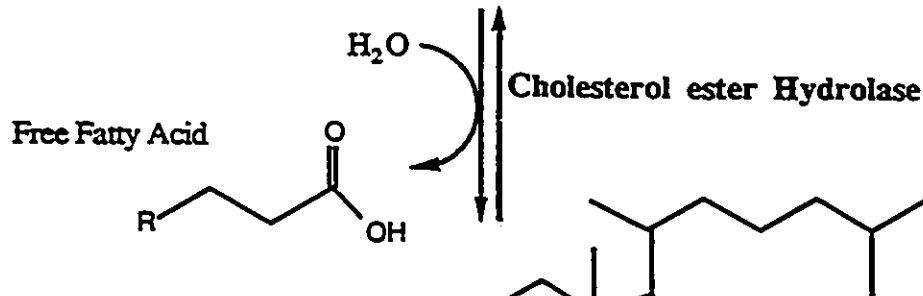
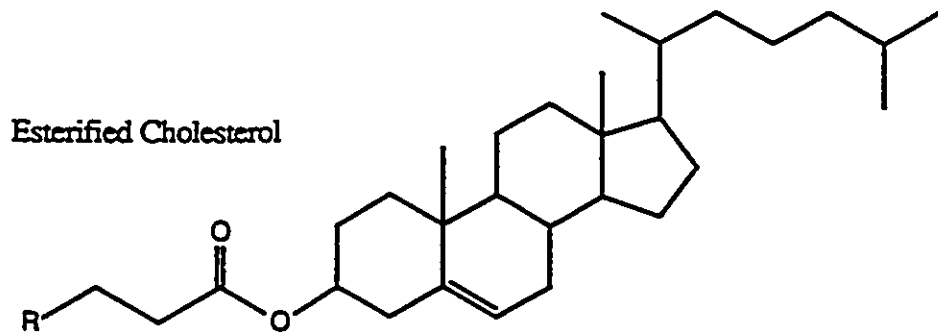
Wright, W.W. 1988 Germ cell-Sertoli cell interactions: analysis of the biosynthesis and secretion of cyclic protein-2. *Dev. Biol.*, 130:45-56.

Wright, W.W., M. Parvinen, N.A. Musto, G.L. Gunsalus, D.M. Phillips, J.P. Mather and C.W. Bardin 1983 Identification of stage-specific proteins synthesized by rat seminiferous tubules. *Biol. Reprod.*, 29:257-270.

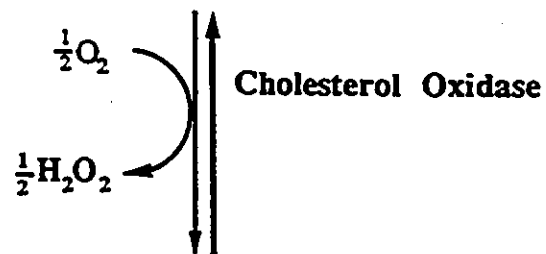
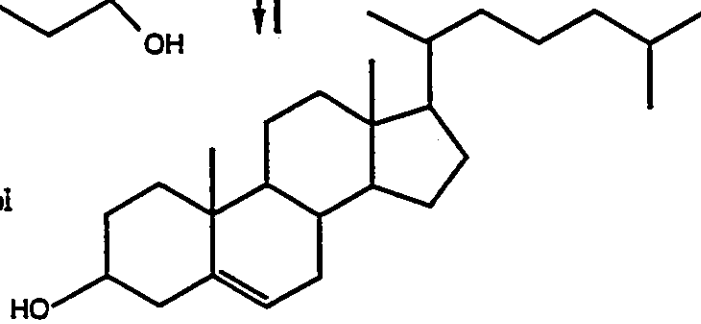
Wrobel, K.-H. and M. Schimmel 1989 Morphology of the bovine Sertoli cell during the spermatogenetic cycle. *Cell Tissue Res.*, 257: 93-103.

Yeagle, P.L. 1985 Cholesterol and the cell membrane. *Biochim. Biophys. Acta.*, 882:267-287.

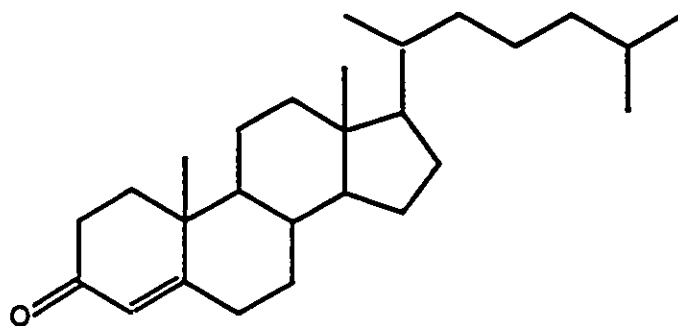
# Appendix I



Free Cholesterol



Cholestenone



Appendix II. Tabulated presentation of filter paper tests for the cytochemical localization of cholesterol using DAB. Triangular pieces were soaked in 0.1 mg/ml free cholesterol in chloroform and the square pieces were soaked in 0.1 mg/ml cholesteryl myristate in chloroform. The test strips were then incubated in media for the indicated cholesterol moiety (control, free, esterified or total cholesterol) or in media for total cholesterol with the addition of dimethyl sulfoxide (DMSO), or sodium cholate, or in the absence of additional oxygen (no oxygen), or with cholesterol oxidase from *Streptomyces sp.* substituting for cholesterol oxidase from *Pseudomonas sp.* (*Streptomyces sp.*)

▲ ■  
Control

▲ ■  
Free

▲ ■  
Esterified

▲ ■  
Total

▲ ■  
DMSO

▲ ■  
Sodium Cholate

▲ ■  
No Oxygen

▲ ■  
Streptomyces Sp.  
c