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Biochemical composition of the spermatozoal plasma membrane in normal and heat-stressed boars

Gary Carl Althouse
Iowa State University

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Althouse, Gary Carl, Ph.D.

Iowa State University, 1992

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Biochemical composition of the spermatozoal plasma membrane in normal and heat-stressed boars

by

Gary Carl Althouse

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LIST OF ABBREVIATIONS

ABP	Androgen Binding Protein
ABP-T	Androgen Binding Protein-Testosterone Complex
AMP	Adenosine-5′-Monophosphate
APz	Zona Pellucida Adhesion Protein
Ca^{2+}	Calcium
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicle Stimulating Hormone
GAG	Glycosaminoglycans
GLC	Gas-liquid chromatography
HEPES	4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid
HPLC	High Performance Liquid Chromatography
IMP	Integral Membrane Protein
KCl	Potassium Chloride
KDa	Kilodalton
KH_2PO_4	Potassium Phosphate Monobasic
LH	Luteinizing Hormone
LPC	Lyso-phosphatidylcholine
mol%	Percentage Based on moles of Individual Substances
MPa	Mega Pascal
N_2	Nitrogen Gas
NaCl	Sodium Chloride
$Na_2HPO_4 \cdot 7H_2O$	Disodium Monophosphate Heptahydrate
PAGE	Polyacrylamide-gel-electrophoresis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerides
PI	Phosphatidylinositol
PMP	Peripheral Membrane Protein
PS	Phosphatidylserine
RH	Relative Humidity
SM	Spermatozoal Morphology
SPH	Sphingomyelin
TRIS	Tris-hydroxymethylaminoethane Chloride
TRIS-HCl	TRIS Hydrochloride
TSC	Total Spermatozoal Concentration
TSM	Total Spermatozoal Motility

I. INTRODUCTION

A. Statement of Problem

Fertility of a spermatozoal population in an ejaculate is dependent upon the capability of the spermatozoa in that population to undergo the necessary biological events of capacitation, the acrosome reaction, fusion and penetration of an oocyte for fertilization to occur (Moore and Bedford, 1983). These processes have been shown to involve cellular modifications in the plasma membrane lipid bilayer by polar and neutral lipid efflux, production of protein-free zones through protein-lipid migration, and lipid-lipid interactions leading to hexagonal phase formation (Yanagimachi et al., 1981; Ahuji et al., 1985; Austin, 1985; Hammerstedt and Parks, 1987; Phelps et al., 1990; Ravnik et al., 1990).

In addition, the ability of a spermatozoal population to be successfully involved in fertilization is dependent upon a minimum number of biologically competent spermatozoa being available at the potential fertilization site for a sufficient period of time (Walton, 1938). Consequently, if spermatozoa in an ejaculate are deficient in undergoing any of the aforementioned described processes and if the number of these abnormal sperm cells is increased in an ejaculate, the required number of physiologically normal spermatozoa for fertilization proportionally decreases, resulting in conception failure.

Review of the literature on studies addressing porcine spermatozoal plasma membranes have provided basic information with respect to their

lipid and protein contents (Peterson et al., 1983; Peterson et al., 1984; Russell et al., 1983; Russell et al., 1984; Nickolopoulou et al., 1985; Nickolopoulou et al., 1986; Parks and Lynch, 1992); however, each researcher utilized different techniques for plasma membrane isolation and subsequent analyses. Additionally, and more importantly, the only study which examined lipid and protein components concurrently, analyzed only epididymal spermatozoa and only quantitated protein concentration rather than performing an individual protein profile analysis (Nickolopoulou et al., 1985).

Boars exposed to increased ambient temperatures, or heat stress, have been found to exhibit a decrease in reproductive efficiency (McNitt and First, 1970; Wettemann et al., 1976; Wetteman et al., 1979; Cameron and Blackshaw, 1980; Stone, 1981/1982; Malmgren and Larsson, 1984; Malmgren, 1989). When examining the effects of heat-stress more closely, it was found that the decrease in reproductive efficiency could be associated with decreases in both animal libido and semen quality. The decrease in boar libido was hypothesized to be due to the observed alterations in the hormonal parameters (i.e., testosterone) induced by the heat-stress (Wettemann and Desjardins, 1979). With examination of semen samples obtained from heat-stressed boars in several other studies, it was found that a significant decrease in spermatozoal motility and an increase in abnormal sperm cell morphology (i.e., high numbers of head and midpiece defects) occurred (Wettemann et al., 1979; Malmgren and Larsson, 1984; Malmgren, 1989).

Spermatogenesis, the formation of spermatozoa from undifferentiated male germ cells known as spermatogonia, is primarily controlled in mammals by the Leydig cells, Sertoli cells, and accessory reproductive structures which are regulated through the gonadotrophin-steroidal axis (deKretser et al., 1971; Steinberger, 1971; Hagenas and Ritzen, 1976; Lee et al., 1976; Orth and Christensen, 1977, Tendall et al., 1977; and others). This gonadotrophin-steroidal axis which supports spermatogenesis can be described in the following simplified model. Functioning Sertoli cells, which are directly involved in the development of spermatozoa, are regulated partially by follicle stimulating hormone (FSH) which is released from the adenohypophysis; subsequently, FSH stimulates production of androgen binding protein (ABP) within the Sertoli cells of the testicle. Androgen binding protein primarily functions in the binding of testosterone (T), a product of the Leydig cells under the influence of luteinizing hormone (LH), a substance also secreted by the adenohypophysis. The ABP-T complex is then transported within the testis in order to maintain high concentrations of T, the primary stimulus needed for initiation and maintenance of spermatogenesis. Lower peripheral blood levels of unbound T, found in the remaining body and especially in the brain, are responsible for the overt libido exhibited by the male.

Studies have shown that an increase in testicular temperature produces a change in blood hormonal concentrations including an increase in peripheral blood levels of FSH and LH, a decline in ABP and T, and a decrease of T within the testis (Wettemann and Desjardins, 1979; deKretser et al., 1982). Additionally, histological examinations of mature boars

with retained testicles from the abdominal cavity (i.e., cryptorchidism) and, thus, continual exposure of the testicular parenchyma to an increase in temperature, showed a significant decrease in the number of spermatozoal precursors (Waites and Ortavant, 1968; Wettemann and Desjardins, 1979), with no active spermatogenesis taking place. Further observations by other researchers on the characteristics of cryptorchid testes showed an increase in total testicular lipids (Parvinen, 1973), lipid metabolism (Fleeger et al., 1968; Johnson et al., 1969), and alterations in testicular parenchyma membrane structure (Blackshaw, 1973; Lee, 1974).

These aforementioned findings all support the concept that alterations in the gonadotrophin-steroidal axis, via an increase in temperature, affects spermatogenesis. These fluctuations in hormonal concentrations have traditionally been thought to be the primary cause of both decreased libido and seminal quality in heat-stressed boars; these hormonal fluctuations, however, may play only an indirect role in the effects of temperature on seminal quality, with temperature itself having a direct effect on the Sertoli cells which modify and nurture the developing spermatozoa.

An important phenomena observed in cell physiology is the ability of cells to maintain a constant fluidity of the plasma membrane which helps to maintain optimal physical properties in the presence of an ever changing environment (Thompson, 1980). This "survival mechanism", known as homeoviscous adaptability, has been shown to exist in cells when fluctuations in temperature have been induced in their surrounding environment. It has been observed that when cells of fibroblastic,

intestinal, hepatic, or neuronal origin were exposed to increased environmental temperatures, alterations of the lipid bilayer in the cells plasma membrane occurred so as to maintain the cell membrane in a state of its most functionally desirable condition (Thompson, 1980). This adaptability was brought about by changes in the lipid bilayer accompanying cell endocytosis/exocytosis which is partially regulated by the golgi apparatus. Cells exposed beyond their limits of homeoviscous adaptability showed a disruption in membrane fluidity, resulting in perturbation of the cell and death (Cossins et.al., 1978). Subsequently, cells which adapted to the increase in temperature were capable of continual growth, with new cells having the same membrane constituency as its mother cell. Most cells have some type of homeoviscous adaptability within a finite limit (for review, see Houslay and Stanley, 1982); therefore spermatogonia, like other cells, are capable of modifying the plasma membrane to adapt to a change in environmental temperature(s) within a prescribed limit. These spermatogonia which are undergoing spermatogenesis may then produce terminal cells (spermatozoa) which may contain membrane characteristics that are representative of the environmental conditions imposed at the time of development for cell survival.

During normal spermatogenesis, the developing spermatozoa are modified to the extent that cellular organelles are discarded or modified so that all available space is utilized to its best efficiency (Bloom and Fawcett, 1975; Garner and Hafez, 1980). During this modification, the golgi apparatus is incorporated into the formation of the acrosome, a membrane-bound structure containing enzymes used by the sperm for

penetration of the cumulus investments surrounding the oocyte. This modification leads to a loss of the sperm cells capability of actively altering its membrane and, thus, its ability of homeoviscous adaptability early in its' development. This irreversible change induced by increased temperatures on the developing membranes of spermatozoa during spermatogenesis may be the primary factor as to how temperature may have its effect on the ability of spermatozoa to undergo capacitation, the acrosome reaction and its eventual ability to fuse and fertilize an oocyte.

The duration of spermatogenesis and spermatozoal maturation is approximately 51 days in the boar (Swierstra, 1968). When further divided, 35 days are needed for spermatogenesis to occur in the testis, 6 days are needed for the spermatozoa to traverse through the seminiferous tubule lumen, rete testis and into the head of the epididymis, and approximately 10 days are needed for maturation of the spermatozoa in the epididymis prior to becoming the fertile gametes emitted during ejaculation. Maturation of spermatozoa in the epididymis has been found to consist of insertion of peripheral and integral membrane proteins, insertion of cholesterol, and modifications in lipid composition in the plasma membrane (Houslay and Stanley, 1982; Nikolopoulou et al., 1985; Saxena et al., 1986; Petersen et al., 1987). It could, thus, be feasible to suggest that alterations in the structure of the spermatozoal plasma membrane during spermatogenesis may have an untoward effect on the sperm cells' ability to undergo final maturation in the epididymis. In support of this proposed hypothesis is the observation that boars exposed to continual heat-stress conditions showed no increase in spermatozoal abnormalities and reduced

fertility until the second week after initiation of exposure to increased ambient temperatures (Wettemann et al., 1976; Wettemann et al., 1979; Stone, 1981/1982)). In these studies, it was observed that once the boars were reintroduced to normal environmental temperatures, it was not until approximately 5-6 weeks after the end of exposure to heat-stressed conditions that the boars again produced normal "appearing" ejaculates (i.e., sperm motility, normal sperm morphology, sperm concentration). These observations tend to strongly suggest that the spermatozoa in the epididymis are not grossly or physiologically affected by an increase in ambient temperatures, but that heat-stress exerts its effect on the developing spermatozoa within the testis.

Previous research has established that the lipid bilayer of the spermatozoal plasma membrane is made up chiefly of polar/neutral lipids and proteins. It is also well documented and commonly accepted that this membrane is directly involved in the necessary events leading up to and including fertilization of an oocyte. Membrane changes induced by heat-stress on the boar would then be expected to affect some or all of the above listed membrane components, leading to a decrease in functionality and potential fertility. Additionally, previous research has established that increased ambient temperatures exert an effect on the Sertoli cells, Leydig cells and the developing sperm cells within the testis, but no visual or physiological effect has been observed on the maturing epididymal spermatozoa.

The problem of how a controlled, short-interval, of increased ambient temperatures alters the biochemical, structural and physical integrity of

the plasma membrane of ejaculated porcine spermatozoa over an 8-week post-treatment period is addressed in this work. Unequivocal evidence supporting the hypothesis that major biochemical alterations in the spermatozoal plasma membrane occurs in response to a short-term increase in ambient temperature will be presented. The potential effects of these membrane alterations on the fertilizing capabilities of spermatozoa will be discussed.

B. Effects of Elevated Ambient Temperature on Male Reproductive Function

An increase in ambient temperatures or heat-stress, whether acute or chronic, has been found to affect the overall reproductive performance in both male and female mammals. These affects have been attributed to both alterations in physiological and endocrinological parameters. In the male, heat-stress has been shown to adversely affect semen quality in the boar (Okauchi and Hirokata, 1962; Thibault et al., 1966; Signoret and du Mesnil du Bursson, 1968; Lawrence et al., 1970; McNitt and First, 1970; Christenson et al., 1972; Wettemann et al., 1976; Stone, 1977; Stork, 1979; Wettemann et al., 1979; Wettemann and Desjardins, 1979; Cameron and Blackshaw, 1980; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; and Malmgren, 1989), bull (Anderson, 1945; Phillips et al., 1945; Casady et al., 1953; Skinner and Louw, 1966; Venter et al., 1973; Zaremba, 1975; Stähr et al., 1987), ram (McKenzie and Phillips, 1934; McKenzie and Berliner, 1937; Dutt and

Hamm, 1957; Moule and Waites, 1963; Rathore and Yeates, 1967; Cameron, 1971), and human (Kandeel and Swerdloff, 1988; Levine et al., 1990). Similarly, localized heat-stress on the scrotum and testes has been shown to have an adverse affect on semen quality in the boar (Holst, 1949; Mazzauri et al., 1968; Malmgren and Larsson, 1984; Malmgren, 1989), bull (Singleton, 1974), and ram (Braden and Mattner, 1970; Samisoni and Blackshaw, 1971). Along with a decrease in semen quality, heat-stress has also been shown to decrease libido in the boar (Winfield et al., 1981; Stone, 1981/1982). A significant reduction in fertility rates from the use of heat-stressed semen has been shown to occur in the boar (Christenson et al., 1972; Wetteman et al., 1976; Stone, 1981/1982; Malmgren and Larsson, 1984), ram (Howarth, 1969), and bull (Venter et al., 1973), and has induced endocrinological changes via decreased testosterone in the bull (Rhymes and Ewing, 1973), along with decreased cortisol and testicular androgen biosynthesis in the boar (Wettemann and Desjardins, 1979; Einarrson and Larsson, 1980; Larsson et al., 1983).

All of the aforementioned studies are in agreement that ejaculate volume is unaltered in acute or chronically heat-stressed boars (McNitt and First, 1970; Wettemann et al., 1979; Cameron and Blackshaw, 1980; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989). Additionally, non-significant alterations were observed in the non-cellular seminal plasma components (e.g., sodium, potassium, chloride, magnesium, total protein and fructose) (Larsson and Einarrson, 1984).

When examining spermatozoal motility, a significant decrease in

sample motility occurred at 2-3 weeks post-treatment, with the heat-stressed boars regaining their pre-treatment spermatozoal motility values by 5-6 weeks post-treatment (McNitt and First, 1970; Wettemann et al., 1979; Cameron and Blackshaw, 1980; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989). When evaluating spermatozoal morphology, different morphological abnormalities appeared and disappeared at different times post-treatment. Abnormal heads (including pyriform, micro/macrocephalic, double, abnormal acrosomes, and nuclear pouches), and proximal cytoplasmic droplets generally appeared at 14-15 days post-treatment, peaking in numbers at 21-28 days post-treatment with a gradual disappearance in their presence by six weeks post-treatment (McNitt and First, 1970; Wettemann et al., 1979; Cameron and Blackshaw, 1980; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989). Other spermatozoal abnormalities such as midpiece abnormalities, kinked/coiled tails, detached heads, double midpieces, abaxial tail attachments, swollen midpieces, bent midpieces, double tails, distal cytoplasmic droplets and bent tails appeared anywhere from 14-21 days and disappeared at approximately 35-42 days post-treatment (McNitt and First, 1970; Wettemann et al., 1979; Cameron and Blackshaw, 1980; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989).

Conflicting results are published as to whether total spermatozoal concentration is affected by heat-stress. Work in Sweden, where they exposed boars to 35°C and 40% relative humidity (RH) for 4 days, did not observe any fluctuations in total sperm concentration (Larsson and

Einarrson, 1984; Malmgren and Larsson 1984); in contrast, other work where boars were exposed to 34.5° C, 25-40% RH for 8 hours/day for 11 weeks (Wettemann et al., 1979), or 35° C for 4 days (Malmgren, 1989), or 33-37° C, 40-80% RH for 6 hours/day for 4, 5 or 7 days (Cameron and Blackshaw, 1980), or 33° C, 50% RH for 72 continuous hours (McNitt and First, 1970), found that 1 in 7 boars (Malmgren, 1989), 5 in 12 boars (Cameron and Blackshaw, 1980), 12 of 12 boars (Wettemann et al., 1979), and 6 of 6 boars (McNitt and First, 1970) had a significant decrease in total sperm concentration, starting by day 21-28 post-treatment and disappearing by days 40-56.

In these previous studies, all mature boars (> 11 months of age) exposed to heat-stress regained their pre-treatment spermiogram values by day 49 post-treatment (McNitt and First, 1970; Wettemann et al., 1979; Cameron and Blackshaw, 1980; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989). Additionally, those studies that performed macroscopic and microscopic examinations of the testes and epididymides at the conclusion of their experiment, found no significant lesions produced as a result of the heat-stress treatment (Wettemann and Desjardins, 1979; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989). Peripubertal boars (3.3 months of age when exposed to 100 continuous hours of scrotal heat-stress), however, had a more pronounced effect - taking up to five months post-treatment before the animals produced a normal spermiogram when compared to their contemporary controls (Malmgren, 1989). This treatment, in effect, delayed the onset of puberty - and even with a normal spermiogram, at

necropsy, all of the heat-stressed peripubertal boars exhibited damaged seminiferous tubules in all testes, indicating lasting pathological changes.

When examining the fertility potential of the ejaculate produced by heat-stressed boars, the fertility rate was decreased 2-7 weeks after heat-stress, with the greatest decrease observed during weeks 2-4 post-treatment (Christenson et al., 1972, Wettemann et al., 1976; Wettemann et al., 1979 and Stone, 1981/1982) when using gilts bred using either artificial insemination or natural coverage. Of particular interest was a study by Malmgren and Larsson (1984) where they collected ova from the uterine horns 2 days after artificial insemination. In this study, it was observed that when this low quality heat-stressed semen was utilized in an insemination program, few ova had any spermatozoa attached to their zona pellucida. This finding indirectly indicated that there is an alteration that occurs to spermatozoa which may decrease their ability to get through the uterotubal junction into the oviduct or to attach to the zona pellucida for fertilization to occur.

From a review of the literature, several conclusions on the effects of heat-stress in boars can be made. First, heat-stress alters boar spermiograms causing an increase in spermatozoal morphological abnormalities, a decrease in spermatozoal motility, and a possible decrease in total spermatozoal output. Second, spermatozoal abnormalities are not seen until approximately 2 weeks post-treatment; indirectly, this supports the hypothesis that spermatozoa undergoing maturation in the epididymides are more tolerant to heat-stress than

spermatozoa in the testis, since it is known that epididymal transit time of spermatozoa in the boar lasts approximately 10 days (Swierstra, 1968). Third, heat-stress disrupts spermatogenesis (i.e., primary/secondary spermatocytes and early spermatids) at the time of the insult, but this insult appears to be transient since normal spermiograms are produced from the following new spermatogenic cycle. Fourth, lower fertility rates occur as a result of using heat-stressed semen; additionally, few ova recovered from gilts in which heat-stressed semen was used had any spermatozoa attached to their zona pellucida, indicating that the fertilizing ability was impaired, even with the "normal appearing" spermatozoa present in the heat-stressed ejaculate under microscopic examination. Fifth, a majority of the previous studies discussed the presence of individual boar variation with regards to heat-stress sensitivity. This heat-stress resistance and/or adaptability can additionally be supported with work comparing boars raised and transferred between temperate and tropical climates (Egbunike and Steinbach, 1976; Egbunike, 1979) or when comparing within and/or between breeds (McNitt and First, 1970); from these studies, a general consensus can be drawn that a genetic influence exists upon these aforementioned characteristics. Lastly, both whole body heat-stress and local heat-stress (i.e., scrotal insulation) produced identical alterations in the spermiogram; from this observation, it can be assumed that heat-stress exhibits its primary effect upon the testicular parenchyma itself and that its effects are not solely mediated via secondary mechanisms (e.g., gonadrotrophins).

C. The Spermatozoan

The spermatozoan is a haploid gamete produced by the male. Although its primary function is to serve as the transmitter of paternal genetic information, it varies in both size and form between and among species. The spermatozoan is formed in the testicle from its precursor cell - the spermatogonium. Once formed, immature spermatozoa traverse through the testicles, seminiferous tubules, rete testes, and the efferent ductules to arrive at the epididymis. In the epididymis, spermatozoa undergo a maturation phase where insertion and/or extraction of various proteins, neutral and polar lipids occur in the spermatozoa's plasma membrane.

When viewed under a light microscope, the typical porcine spermatozoan is a cell consisting of a large elongated, ovoid, broad and flat head with a long, circular flagellum attached at its base (Appendix A). In the boar, the head is 8-10 microns long by 4-4.5 microns in width; the tail is 45-60 microns in length by 0.4-1.0 microns in diameter. The tail is 5 1/2 to 6 times as long as the head, and makes up approximately 86% of the total length of the cell. Within the head of the sperm cell are two organelles, the acrosome and the haploid nucleus. The acrosome is located at the apex of the sperm head and is an enveloped membrane consisting of both an outer and inner acrosomal membrane. The acrosome contains enzymes (e.g., hyaluronidase, esterases) which aid the sperm cell in its penetration through the mucoproteinaeous investments surrounding the oocyte during the

fertilization process. The nucleus, lying central to the acrosome, is contained by the nuclear membrane and is composed primarily of deoxyribonucleoprotein - the nucleus makes up the majority of the area with which the head encompasses.

The long flagellum, or tail, of the spermatozoan is used for forward propulsion and is attached to the head via the neck. It can be divided into three distinct regions, the midpiece, principal piece and endpiece. The midpiece is attached to the base of the head and makes up approximately 25% of the tail's length. Examined under a light microscope, the midpiece looks like a thickened portion of the tail which bluntly ends and at which point the remainder of the tail continues on as the principal piece. This thickened midpiece portion is due to the fact that all of the sperm's mitochondria are located in this region and are arranged in a helical arrangement around a central axial filament; these mitochondria produce the energy which is required for the cell's motility. The central axial filament, which extends from the base of the head to the tail endpiece, consists of 9 pair of peripheral and 2 central fibrils, therefore, it is the main contractile element of the flagellum. The central axial filament extends centrally through the principal piece, however, here it is surrounded by a fibrous coil sheath which is absent in the region where the mitochondria are found surrounding the midpiece region. Both the mitochondrial and fibrous coil sheath help to protect the central fibrils from mechanical damage which may occur from its whip-like action when generating propulsion for motility. The entire spermatozoan is enveloped in a semi-permeable,

continuous plasma membrane which, in configuration and function, appears analogous to the plasma membrane of other cells (for a general review, see Fawcett and Bedford, 1979).

Although the spermatozoan is simple in structure, containing few organelles when compared to its diploid somatic cell counterparts, it must undergo two unique physiological events, capacitation and the acrosome reaction, before it can fertilize an oocyte (reviewed by Bavister, 1980; Pineda, 1989). Capacitation, put simply, consists primarily of a physicochemical, biochemical and/or physiological change(s) in the plasma membrane which will potentially allow the plasma membrane to fuse with the underlying, outer acrosomal membrane at the apex of the head at a later time. Once capacitation has been completed, the acrosome reaction can proceed, involving progressive fusion, vesiculation and/or exocytosis of the acrosomal membrane to the overlying plasma membrane; this vesiculation/exocytosis allows for the concomitant release of the acrosomal enzymes which are subsequently involved in the sperm's penetration of the mucoproteinaeous investments surrounding the oocyte. Both capacitation and the acrosome reaction have been linked to cellular modifications in membrane structure of the lipid bilayer to produce protein-free zones via protein alteration and/or migration which may also include polar/neutral lipid efflux (Ahuji et al., 1975; Yanagimachi, 1981; Austin, 1985; Hammerstedt and Parks, 1987; Phelps et al., 1990; Ravinik et al., 1990).

D. The Spermatozoal Plasma Membrane

1. Isolation techniques

Over the past three decades, numerous techniques have been performed in an attempt to isolate spermatozoal plasma membranes and/or its components from several mammalian species by disrupting the intact spermatozoan. Such techniques included the use of acidic or basic solutions (Hathaway and Hartree, 1963; Bernstein and Teichman, 1973; Schill, 1973), extraction with detergents (Srivastava et al., 1970; Multamaki et al., 1975), physical removal using glass beads (Hathaway and Hartree, 1963; Morton and Lardy, 1967; Multamaki and Niemi, 1972), freeze-thawing (Pedersen, 1972; Brown and Hartree, 1974), sonication (Lunstra et al., 1974; Esbenshade and Clegg, 1976; Clegg et al., 1975; Hinkovska et al., 1986), homogenization (Moore and Hibbit, 1976; Soucek and Vary, 1984; Holt and North, 1985; Casali et al., 1985; Holt and North, 1986), hypotonic shock (Ivanov and Profirov, 1981; Rana and Majumder, 1989) and nitrogen cavitation (Gillis et al., 1978; Peterson et al., 1980; Noland et al., 1983; Nikolopoulou et al., 1985; Parks and Hammerstedt, 1985; Parks et al., 1987; Agrawal et al., 1988; Canvin and Buhr, 1989; Buhr et al., 1989; Robertson et al., 1990; Parks and Lynch, 1992).

When examining initial purification techniques, a good percentage of this past work has been performed in the boar (Lunstra et al., 1974; Gillis et al., 1978; Peterson et al., 1980; Soucek and Vary, 1984; Nikolopoulou et al., 1985; Canvin and Buhr, 1989; Parks and Lynch, 1992)

with less done in the ram and goat (Hathaway and Hartree, 1963; Srivastava et al., 1970; Srivatava et al., 1970; Brown and Hartree, 1974; Ivanov and Propfirov, 1981; Holt and North, 1985; Parks and Hammestedt, 1985; Hinkovska et al., 1989; Rana and Majumder, 1989), bull (Hathaway and Hartree, 1963; Multamaki and Niemi, 1972; Berstein and Teichman, 1973; Multamaki et al., 1975; Noland et al., 1983; Casali et al., 1985; Parks et al., 1987; Parks and Lynch, 1992), stallion (Parks and Lynch, 1992) and rat (Jones, 1986; Agrawal et al., 1988).

As one can readily detect, numerous techniques have been employed in the attempt to isolate spermatozoal plasma membranes from mammalian species. Since the introduction of these aforementioned techniques, and through the technological advancements of science, mounting evidence has shown that the majority of these aforementioned techniques in some way(s) alter the plasma membrane and its constituents. Although juxtapositional analyses of these techniques have never been performed, through our increased understanding of protein-protein, protein-lipid and lipid-lipid interactions, we have become increasingly aware of how these interactions (e.g., electrostatic, hydrophobic, van der Waal's forces) can become disrupted merely through variations in media pH, ionic strength, or the addition of chelating agents for calcium and magnesium (e.g., EDTA). These variations tend to dissociate proteins and lipids on/or within the membrane (for review, see Houslay and Stanley, 1982); because of this, techniques such as the use of acidic or basic solutions, detergents, and hypotonic shock have become obsolete. Additionally, techniques which are non-specific in their membrane

disruption (i.e., sonication, homogenization, physical removal by glass beads), generally have a decreased yield and are of a lesser membrane purity than those that can be membrane-specific like nitrogen cavitation (i.e., exposure to pure, pressurized N_2 for 10 minutes followed by rapid decompression to atmospheric conditions which allows N_2 to form gas vesicles which disrupt the membrane when N_2 escapes into the surrounding atmosphere).

At this time, it is important to note that if nitrogen cavitation is used, it cannot be assumed that an unadulterated, pure plasma membrane preparation can be collected from which further analyses are performed. The importance in the cell preparation before and after cavitation is of equal concern. This concern is warranted since previous research has demonstrated that something as simple as the number of centrifugation/washing steps (i.e., greater than five) can cause large numbers and a variety of polypeptides of the boar plasma membranes to be easily removed and lost (Russell et al., 1985). Collectively, the damaging effects from the extensive washing, as well as any other additional treatments which could inhibit enzyme activity, denature membrane proteins, oxidize lipids and/or disrupt the functional integrity of the plasma membrane, provide an immediate and justifiable concern when assessing a plasma membrane isolation technique and the way in which the technique was validated before its implementation into a research protocol.

When critically examining past plasma membrane isolation techniques on boar spermatozoa, it becomes readily evident that each has

inherent problems which could lead to altered plasma membrane samples. Lunstra et al. (1974) described an isolation technique on ejaculated boar spermatozoa involving the use of packed glass wool filtration, media containing EDTA, disruption of the cells by sonication, and validated membrane purity using only microscopy. Although the technique is antiquated by today's standards, the authors also failed to offer a complete validation of their membrane purity either through the use of enzyme markers, antibodies, or binding proteins specific for polypeptides of the plasma membrane. These two factors, along with the aforementioned problems inherent to sonication and EDTA-containing media, preclude the use of such a technique in current research protocols.

Four years later, Gillis et al. (1978) published an article on the isolation and characterization of boar spermatozoal plasma membrane vesicles. In this article, the authors described the use of nitrogen cavitation to isolate plasma membranes from ejaculated porcine spermatozoa; they followed up the isolation procedure with appropriate validation using both electron microscopy and several enzyme markers. However, with further critical analysis of their procedure, it was estimated that a minimum of 10 washing/centrifugation steps were utilized, and the media used in the study contained a buffer which was outside of its effective range (e.g., TRIS), along with the chelator EDTA. Because of the aforementioned and the previously discussed problems inherent with each, the procedure as originally described would most likely produce altered plasma membranes with respect to the

membrane proteins. It is worthy to note, however, that this procedure is probably the one most presently used, with modifications, for isolation of plasma membranes from mammalian species.

Two years later, Peterson et al. (1980) published a modification of the Gillis procedure for isolating plasma membranes from ejaculated boar spermatozoa. Major modifications in the procedure included the use of a phosphate-buffered saline solution rather than the originally used TRIS-buffered sucrose solution, the inclusion of several antiproteases in the medium, and a reduction in the number of washings/centrifugations to at least 6 steps. Validation of their plasma membrane preparation was by electron microscopy only. Potential problems with this described procedure were that a chelating agent (EDTA) was used in the medium, and the numerous washing steps utilized in the isolation of plasma membranes tend to suggest that an altered final plasma membrane sample stripped of membrane proteins, was harvested; additionally, no enzyme markers, antibodies, or binding proteins specific to the plasma membrane were used in their validation protocol.

In 1985, Nikolopoulou et al. published a study examining the changes in the lipid content of porcine spermatozoal plasma membranes during epididymal transit. Using a modification of the Soucek and Vary (1984) technique, the authors used a Percoll® gradient and nitrogen cavitation on minced epididymal segments in a EDTA-HEPES-buffered sucrose solution. As described in the article, their technique included the use of 12 washing/centrifugation steps to complete their plasma membrane isolation. Biochemical enzyme marker analyses were used to

assess membrane purity. Since their protocol called for multiple washing steps and all medium contained chelating agents, it is highly probable that the recovered plasma membranes were altered in some way or form.

Several years later, others examining the effects of temperature on the fluidity of boar spermatozoal plasma membranes described another modification of the Gillis procedure (Canvin and Buhr, 1989). After collecting semen from boars using the gloved-hand technique, the semen was filtered, diluted with a TRIS-buffered sucrose solution and then washed through two different types of an industrial grade silicon oil. Sperm were harvested from this wash and then re-washed in a TRIS-buffered sucrose solution (pH 5.0) three additional times prior to undergoing nitrogen cavitation for membrane disruption. Following cavitation, 5 additional washing/centrifugation steps were performed before obtaining the final product. Consequently, this final product was assessed for membrane purity using only one enzyme marker specific for the plasma membrane - alkaline phosphatase. Although the authors did show an overall increase in membrane purity, they failed to test for any other intracellular organelle contaminants (e.g., acrosomal, mitochondrial, nuclear membranes); additionally, a total of 9 washing/centrifugation steps were performed with one step allowing exposure to a silicon-based oil. This author can only suspect the alterations that the plasma membranes underwent when exposed to this oil treatment (i.e., lipid oxidation, protein and/or lipid leaching, etc.), and the use of 9 washing steps only substantiates the possibility that

the plasma membranes used in their experiments were not the same membranes that were present when on intact, boar spermatozoa.

Recently, Parks and Lynch (1992) examined lipid composition and thermotropic phase behavior in spermatozoa of several mammalian species, including the boar. Spermatozoal preparation and plasma membrane isolation were performed using modifications, once again, of the Gillis procedure. In their procedure, a Tyrode's solution, nitrogen cavitation, and a six-step centrifugation/washing process using different centrifugation speeds and times were used on ejaculated boar spermatozoa. No determination as to the yield and purity of their plasma membrane preparation was made. And although a significant reduction in the number of washing/centrifugations steps and prevention, on the part of possible media components which could alter plasma membranes was made, it can only be speculated as to the true purity of their membrane preparation on which further analyses were made. Additionally, in vitro tests on human spermatozoa have shown that Tyrode's solution functionally alters the sperm cell so that it is less successful at penetrating human cervical mucus (Overstreet et al., 1980). No further work was performed to characterize where the direct effects of Tyrode's solution may be on or within the spermatozoan, however, this finding does warrant concern when analyzing plasma membranes which have been exposed to a solution known to have a negative effect on sperm physiology.

Collectively, numerous procedures have been used to isolate plasma membranes from either epididymal or ejaculated porcine spermatozoa.

Although each of the aforementioned techniques deserve merit in the evolution of obtaining a high yield, highly purified plasma membrane preparation, each equally warrants speculation as to their true representation of boar plasma membranes due to the potentially serious flaws innate to each of the described techniques.

2. General structure

As described earlier, the plasma membrane (which surrounds the cell) is a selectively permeable barrier which separates the internal milieu of a cell from its outside environment. Besides defining the outer boundaries of the cell, the plasma membrane also functions as part of the cell's cytoskeleton and acts as a mediator and/or initiator of physiological stimuli between the internal and surrounding cell environment.

Our overall conceptualization of the structure of the plasma membrane comes from the hypothesis proposed in the published works of Singer and Nicholson in 1972. In this landmark article, a fluid-mosaic model for cell membranes was proposed. In this model, the authors combined the concepts of membrane fluidization as described by Frye and Edidin (1970) who observed the free movement of fluorescent dyes in the cell membrane, and they added the inclusion of proteins within the lipid bilayer, a hypothesis originally proposed by Danielli and Davson (1935). The two major factors of this proposal which allowed for its succession over past proposals on cell membrane structure was that is postulated from the fluidity study that; 1) membrane components could diffuse

laterally in the plane of the membrane, and 2) included the presence of proteins which penetrated into or through the membrane's lipid bilayer leading to its mosaicism; these two factors still remain the predominant characteristics in the evolution of the cell membrane model.

It has become commonly accepted that the primary biochemical components which make up the molecular hetergeneity of the plasma membrane are proteins (integral and peripheral) and lipids (polar and neutral). Covalently attached to these components on the outer bilayer surface are carbohydrates which provide a "shield" from the outside environment. This glycocalyx "shield" normally gives the cell surface a net negative charge and, thus, contributes to the selective permeability of the membrane. For convenience, each of these membrane components will be addressed separately.

a. Lipids Lipids can be subdivided into polar and neutral lipids. One feature shared by either of these lipids are the fact that they exhibit certain degrees of amphipathicity (i.e., two different charges on the same molecule) in nature. The major polar lipids of biological membranes are the phospholipids. These phospholipids contain phosphorus as phosphoric acid which is esterified to the third hydroxyl group of a glycerol molecule giving us a compound known as phosphatidic acid. Additionally, two long-chain fatty acid molecules (14-24 carbons long which may be either saturated or unsaturated) are esterified to this same glycerol molecule but at its first and second hydroxyl groups - these fatty acyl chains lend hydrophobicity to the molecule.

Attached to the phosphatidic acid at another site is a hydrophilic base, which gives the molecule its dipolar ionic headgroup. The most abundant headgroups attached to the phosphatidic acid in sperm membranes are choline, ethanolamine, serine and inositol. Since these polar headgroups generally carry a net negative charge, they are commonly called the anionic phospholipids.

Not all phospholipids contain diacyl side-chains from the glycerophospholipid molecule. These molecules instead are monoacyl and are identified by the prefix lyso- attached to its name. A monoacyl compound commonly found in biomembranes, especially in spermatozoa, is lyso-phosphatidylcholine (LPC).

Structurally related to phospholipids and commonly found in biological membranes are the sphingolipids. In spermatozoal plasma membranes, sphingomyelin (SPH) has been found to be a major lipid component. The sphingomyelin molecule contains a phosphate ester which is condensed from sphingosine with an amino group linked to an acyl group via an amide linkage; this molecule of course exhibits amphipathicity.

The neutral lipids most often present in the cell's plasma membrane are the sterols. In spermatozoa, cholesterol is the major class of sterol, with desmosterol making up a minor component of the overall sterol composition in the plasma membrane. Sterols usually contain a large planar steroid hydrophobic region with the exception of one hydroxyl group on the A-ring which is hydrophilic; these two characteristics give sterols an all but minor degree of amphipathicity.

At this point, a differentiation must be made between the plasma membranes found in mammalian spermatozoa and the plasma membranes of mammalian somatic cells with regards to their stoichiometric and biophysical properties. Unlike somatic cells, spermatozoa lose the ability to alter the plasma membrane via intracellular organelles early in spermatogenesis. The spermatozoal plasma membrane, therefore, is generally left with only a passive means of modifying its membrane through external environmental influences. Indeed, post-testicular modifications of spermatozoa have been shown to exist and are significant both during their maturation in the epididymis, during ejaculation when exposed to seminal fluid, and when in contact with the female reproductive tract.

Delineation of the lipids and their composition in porcine spermatozoa was first documented in 1954, where Mann reported that 6 mg of lipid phosphorous was present per 100 ml of boar semen. It wasn't until approximately a decade later (1965), that Komarek et al. published work which analyzed lipids of porcine spermatozoa, seminal plasma and the gel. These researchers found that boar spermatozoa contained approximately 12% (w/w) lipid which was made up of phospholipids (74.7%), cholesterol (12.6%), diglycerides (5.7%), triglycerides (4.5%) and wax esters (2.4%). Only a small proportion of the dry matter of both seminal plasma (0.32%) and gel (0.32%) contained lipid.

One year later, Grogan et al., (1966) analyzed phospholipid content of whole boar spermatozoa using freeze-dried techniques. From their results, they reported the presence of 5 different phospholipids;

these were PC, PE, SPH, choline and ethanolamine plasmalogens.

Johnson et al. (1969) performed a quantitative analysis of boar spermatozoa and its seminal plasma phospholipids by thin layer chromatography with phosphorus analysis in relation to frequency of ejaculation. Major phospholipids found in this study were in the following decreasing order of PC (41.4%), PE (26%), SPH (15.2%), PS (5.9%), phosphatidic acid and/or polyglycerol phosphatide (5.5%); non-migrating phospholipids (i.e., those remaining at the origin of application) made up the remaining 6%. No difference in phospholipid content and composition was observed with respect to ejaculate collection frequency except for PE, which was observed to be significantly different throughout the study among the boars. With regards to seminal plasma phospholipids, in addition to the aforementioned phospholipids found in the spermatozoa, LPC was additionally found to be present in the seminal plasma.

The first study assessing components of the boar spermatozoal plasma membrane, rather than whole spermatozoa, via a comprehensive analysis of the caput, corpus, and cauda epididymal spermatozoal plasma membrane was done by Nikolopoulou and co-workers (1985). In this study, epididymides were harvested from freshly slaughtered boars and then processed by dividing the epididymides into their distinct segments, combining the same segments, mincing them, and then decanting and straining the suspended tissue to obtain spermatozoa from the respective segments. Spermatozoal plasma membranes were then harvested via washing, nitrogen cavitation and isolation using a discontinuous sucrose

gradient. Using thin layer and gas chromatography techniques, the authors found that polar phospholipids accounted for approximately 70% of the total membrane lipid, with sterols accounting for the majority of the remaining lipid material. As spermatozoa traversed through the epididymis, sterols remained constant in concentration while plasma membranes decreased in their amounts of PE (7.5 mol%), PS (3.4 mol%), and PI (2.7 mol%), with a concomitant increase in PC (7.4 mol%) and SPH (5.3 mol%). PC (39.9 mol%) was found to make up the majority of total polar phospholipid in caudal epididymal sperm, followed by PE (27.7 mol%) and SPH (23 mol%) in the plasma membrane. Phosphatidylserine (3.3 mol%), PI (2.9 mol%) and LPC (2.1 mol %) made up the remainder of total polar phospholipid.

Two sterols were found to be present in porcine spermatozoal plasma membranes, cholesterol and desmosterol, with cholesterol being the major sterol of the two. Additionally, it was observed that cholesterol decreased (approximately 10.8 nmol/10^9 sperm) as spermatozoa traversed the epididymis, with desmosterol showing an increase (5 nmol/10^9 sperm) in its membrane concentration. When membrane components were analyzed through the use of ratios, cholesterol/phospholipid, total sterol/phospholipid and phospholipid/protein remained constant throughout the different sections of the epididymis. Combined, these results provided the first evidence that porcine spermatozoa undergo post-testicular plasma membrane modifications. These results were also in agreement with previous work in which they observed a decrease in the amount of whole sperm lipid during transit through the epididymis in the

boar (Grogen et al., 1966; Evans and Setchell, 1979).

Later, using epididymal harvested spermatozoal plasma membranes, Nikolopoulou et al., (1986) analyzed changes in vitro of the plasma membrane phospholipids during an induced acrosome reaction over a 2-hour period. It was observed that as spermatozoa underwent the acrosome reaction, diacylglycerols (63 nmol/mg protein) and free fatty acids (11.8 nmol/mg protein) significantly increased, whereas a decrease was found with regards to PI (7 nmol/mg protein) and SPH (41.9 nmol/mg protein) membrane content. The largest alteration in spermatozoal plasma membrane content during the acrosome reaction occurred with cholesterol, where a 34% increase was observed to occur over a one-hour period. Fluctuations in other lipids were seen (e.g., PC, PE, PS, LPC and desmosterol), however, they were found not to be significant. When examining possible causes for these individual lipid changes, it was observed that a correlation existed with an uptake of extracellular calcium by the spermatozoa (using $^{45}Ca^{2+}$). These observations lended support to that which was originally described by Yanagimachi (1981) who observed a correlation with Ca^{2+} uptake and the acrosome reaction in hamster spermatozoa. Therefore, from their results, it was postulated that anionic phospholipids underwent a calcium-induced phase transition in the plasma membrane which eventually produced disorder in the organization and character of the membrane lipids. This disorganization in the membrane would then promote vesiculation/exocytosis leading to the acrosome reaction. In conclusion, no significant change in total lipid content during the acrosome reaction was observed over the 2-hour

period. These results were, once again, in agreement with lipid analyses of whole sperm where no change was observed in lipid composition before or after capacitation and the acrosome reaction (Elliott and Higgins, 1983; Takei et al., 1984).

From the aforementioned research and that of others (for general review see Yeagle, 1985), cholesterol has become an intensely studied topic with regards to its role in membrane stability and permeability and, thus, its relation to spermatozoal capacitation and the acrosome reaction. Cholesterol is known to occur at higher concentrations in the cell membrane of mammalian cells than in any other membrane found within cells. Cholesterol is believed to orient itself parallel to the hydrocarbon chains of phospholipids and, thus, becomes perpendicular to the membrane surface. Since cholesterol is primarily located in the hydrophobic region of the lipid bilayer, it exerts its affect on the fatty acyl chains rather than directly on the polar head regions of the membrane phospholipids. Because of this association between the cholesterol molecule and the phospholipid acyl chains, it is currently hypothesized that an overall decrease in the fluid motion of the phospholipid chains occurs; this decreased motion, along with an increase in hydrophobicity in the interior of the bilayer, results in an overall decrease in the membrane's permeability. Therefore, it seems that cholesterol gives a membrane stability through this "special ordering" effect on the bilayer. This is not to say that cholesterol exerts its effect only on phospholipids, cholesterol/protein interactions most probably exist, however, little research has been

performed addressing such a question.

Hypotheses and documentation lending support to the idea that cholesterol is somehow involved with spermatozoal membrane modifications and stability can be found in the early works of White and Darin-Bennett (1976) in which they examined lipids in relation to sperm cold shock (i.e., a sudden drop in environmental temperature in vitro on spermatozoa). From their observations, they felt that cholesterol imparted some type of cohesiveness to the membrane, since spermatozoa with high cholesterol content tended to be less susceptible to cold shock than spermatozoa exhibiting a low membrane cholesterol content.

David (1981) demonstrated that mammalian spermatozoa underwent a cholesterol efflux during capacitation in vitro by the way of lipid-binding uterine fluid proteins. From these observations and a critical review of the literature, he formed a logical extension of current theories that this loss in cholesterol perturbated the plasma membrane causing a concomitant increase in membrane fluidity along with the facilitation of calcium influx which would induce membrane fusion between the plasma membrane and the outer acrosome membrane and, thus, initiate the acrosome reaction. He further posulated that when spermatozoa are ejaculated as semen, to prevent premature capacitation and the acrosome reaction, a cholesterol donor membrane vesicle (i.e., a decapacitation factor) was present in the semen which was eventually lost in the female reproductive tract. These hypotheses, as well as findings presented by various other researchers (Evans et al., 1980; Friend, 1980; Yanagimachi, 1981; Llanos et al., 1982; Llanos and Meizel,

1983; Clegg, 1983; Farooqui, 1983; Go and Wolf, 1985) have all lead to a molecular model for capacitation in which the lipid bilayer of the mammalian spermatozoal plasma membrane is destabilized in a manner consistent with current theories on membrane fusion (for general review, see Cullis et al., 1985; Langlais and Roberts, 1985). To date, further work examining cholesterol efflux in mammalian spermatozoal plasma membranes (Ehrenwald et al., 1988a & b; Seki et al., 1992) have only reinforced this elegant hypothesis.

Along with the effects of cholesterol efflux on the spermatozoal plasma membrane, other studies have gathered supportive data to suggest a change in phospholipid composition during capacitation and the acrosome reaction. A review by Cullis and deKruijff (1979) examining the role of lipids in biological membranes, reiterated a consensus from previous researchers that along with cholesterol, PC and SPH also play a major, functional role in the stabilization of the lipid bilayer configuration in biological membranes; minor experimental support was also shown which suggested that PS and PE can also function as membrane stabilizers. Additional support in these discussions can be found in the work done by Snider and Clegg (1975) in which they observed an alteration in phospholipid composition (i.e., decrease in PC, increase in PI) when whole porcine spermatozoa were incubated in the uterus and oviduct over a 2 hour period. Later studies have all lended to suggest or support that phospholipid composition changes (i.e., PC, PI, SPH) indeed do occur after ejaculation and before fertilization of the oocyte (Evans et al., 1980; Nikolopoulou et al., 1986; Evans et. al., 1987).

In recent years, observations of the different phospholipids and their individual preferences for specific orientations at the molecular level in the plasma membrane have been examined (For review, see Houslay and Stanley, 1982; Cullis et al., 1985; Hammerstedt et al., 1990). It has been observed that the phospholipids PC, SPH and LPC tend to prefer to be outwardly oriented (i.e., extracellular-facing leaflet of the bilayer), while PG, PE, PI and PS prefer to be located in the inner surface (cytoplasmic-facing leaflet) of the bilayer. In addition, cholesterol has been found to prefer to interact with PC and SPH in contrast to PE, PS and PI (Yeagle, 1985); therefore, it would seem that cholesterol content is of an asymmetric proportion in relation to the outer vs. the inner surface of the bilayer. This asymmetry may be one of the many key factors which play a role in the physiological events which mammalian spermatozoa must undergo prior to oocyte fertilization.

From the evidence and hypotheses presented here, it is proposed that because of these aforementioned unique orientations, interactions between the lipid bilayer and the external environment are highly feasible and probable, especially given the evidence presented earlier which showed significant fluctuations in both polar and neutral lipids during the normal physiological events of capacitation and the acrosomal reaction in mammalian spermatozoa. With an efflux and/or loss of lipids in the outer surface of the bilayer, instability of the outer bilayer results. An ensuing alteration in the bilayer causes a change, via kinetic relationships between the factors involved in the inner surface of the bilayer (either through lipid compartmentalization,

lateral/translational/rotational lipid motion, forced alternate orientations or phase transitions), which promote eventual fusion of the membranes. Other events (i.e., lipid-protein interactions, protein modifications and/or migrations, or other presently unknown components) may be integral to the overall process of plasma membrane alterations; at this point in time, it would be premature to state that the events involved in spermatozoal plasma membrane alterations are of a discrete and ordered nature.

b. Proteins Proteins are polypeptides made up of long chains of amino acids and are macromolecules which constitute a significant proportion of the plasma membrane. Membrane proteins can be classified as either peripheral or integral proteins based upon whether they can be dissociated from the membrane using various ionic strength buffers (peripheral) or if a detergent or organic solvent is needed to extract them from the membrane (integral). Peripheral membrane proteins (PMP) interact with the bilayer primarily by electrostatic or hydrophilic linkages with either the glycocalyx, phospholipids, or integral membrane proteins, and does not usually contain a significant apolar region which would be buried deep inside the hydrophobic region of the bilayer. Integral membrane proteins (IMP), on the other hand, do contain a considerable surface area which is strongly apolar in nature. Due to this apolar region, IMPs have a major portion of their moiety within the hydrophobic region of the bilayer; this apolar moiety can completely traverse the bilayer with polar components exposed on both

sides of the bilayer (transmembrane) or, as is normally the case, only unilateral exposure of a polar component occurs (ecto-/endo-proteins). Integral membrane proteins can be further subdivided as to whether only a single strand of polypeptide chain spans the membrane (fibrous protein) or whether many loops of the polypeptide traverse through the membrane (globular protein).

Basically, the traditional functions ascribed to proteins (i.e., structural, channels, pumps, receptors, and enzymes), can also apply to the membrane proteins found in the spermatozoan. Specifics include forward motility protein (bovine - Acott and Hoskins, 1978), adhesion protein for binding sperm to oocytes prior to the acrosome reaction (porcine - Peterson and Hunt, 1989), fucose-binding protein (porcine - Töpfer - Petersen et al., 1985), zona-binding protein (porcine - Jones and Brown, 1987), voltage-dependent pore-forming protein (human - Young et al., 1988), actin (porcine - Peterson et al., 1990), protein and tyrosine kinases (goat - Dey and Majumder, 1990; porcine - Berrute and Mastegani, 1989; Chaudry and Casillas, 1989).

Early studies using radioisotopes examining spermatozoal membrane proteins established that upon ejaculation, the seminal plasma contained proteins which immediately adhered and became part of the PMP component (Moore and Hibbit, 1976). During this same time period, a different research group published results characterizing proteins of the porcine spermatozoa plasma membrane using polyacrylamide gel electrophoresis [PAGE] (Esbenshade and Clegg, 1976); from this study, the authors found 11 prominent bands (14.5 to 260 kDa) which were most likely PMPs, due to

the author's use of EDTA and low pH during their isolation procedure. Further characterization of these proteins showed that at least 6 of these bands were glycoprotein in nature, 4 of which were of a high molecular weight (140-260 kDa) and 2 of a low molecular weight (14.5-22.5 kDa). It was also noted that some protein material remained at the top of their gels and, therefore, this indirectly showed that very large proteins (> 260 kDa) are also present in boar spermatozoal plasma membranes.

The most extensive study done to date examining polypeptides of ejaculated boar spermatozoal plasma membranes was performed by Russell and co-workers (1983). Using single-dimensional PAGE, two-dimensional PAGE, isoelectric focusing and nonequilibrium pH gradient electrophoresis, the authors detected well over 250 polypeptides using silver-staining techniques. Ninety of these 250 polypeptides routinely were detected using Coommassie-stained gels; twenty of these consistently stained heavy and, therefore, were considered major proteins of the spermatozoal plasma membrane, with the remaining being classified as minor proteins. The majority of the major proteins were either strongly acidic (pH 3.5-5.5) or moderately basic (pH > 7.5). Spermatozoal plasma membrane proteins observed in this study ranged in molecular weight from 14 to >300 kDa. The major proteins were of an approximate molecular weight of 14, 16, 16.5, 17.5, 18, 18+, 18.5, 20, 43, 44, 47, 50, 64, 67, 72, 82, 110, 115, 120 and 300 kDa; of these, eight were considered to be glycopeptides (20, 43, 44, 64, 72, 110, 120 and 300 kDa). Using fluorescent monoclonal antibodies to these major

glycoproteins, it was found that these major glycoproteins were localized primarily in the head region of the sperm cell - this is entirely understandable given the fact that the authors' previous work showed that their plasma membrane isolation technique routinely isolated only those plasma membranes surrounding the sperm head above the equatorial segment (Peterson et al., 1980). Later, Hunt et al. (1985) quantitated that the 44 and 47 kDa major proteins made up approximately 20% of the total plasma membrane protein component that Coomassie-stained.

In 1984, Russell's group published results on the plasma membrane proteins of epididymal spermatozoa, and described which proteins were added to the membrane during epididymal transit and during ejaculation from the accessory sex gland fluids. Coomassie-stained gels indicated that only one or two major polypeptides (a previously unreferenced protein of approximately 19 kDa and one at 40 kDa) were added to the plasma membrane during epididymal transit. Numerous proteins were added to the plasma membrane at ejaculation, with the majority coming from seminal vesicle fluid (15 neutral or basic polypeptides ranging from 14 to 20 kDa, an acidic polypeptide of approximately 150 kDa, and a large glycoprotein at 300 kDa). Only one other major polypeptide (115 kDa) was added by the remaining accessory sex glands (prostate or bulbourethral gland). Using silver-staining techniques, evidence was obtained to show that several other proteins were added to the spermatozoal plasma membrane during epididymal transit and from the seminal plasma during ejaculation; these, however, were considered minor

proteins since staining intensity was not consistent among and between samples. Overall, it was found that approximately 35% of the total plasma membrane protein content was added to the spermatozoan at the time of ejaculation.

In order to gain a better understanding of the function of the aforementioned major proteins, Russell et al. (1985) performed a study to determine which proteins were peripheral and which were integral in the plasma membrane. Using both epididymal and ejaculated spermatozoa, plasma membranes were isolated using nitrogen cavitation. Numerous and variable washing steps were performed using hyper/hypotonic salt medias to detect peripheral proteins. Integral proteins were extracted using a detergent (Triton X-100). Examination for the presence/absence of plasma membrane proteins during the various steps was performed on extracts from the plasma membranes using 2-D PAGE. Approximately 20 proteins (via silver-staining) were found to be released, although not completely, from the plasma membrane when exposed to a hypertonic salt solution; of these, 3 were considered to be the major proteins of 47, 67 and 110 kDa. Using hypotonic salt solutions, 40 proteins were found to be released (silver-staining), with 4 of these being major proteins (47, 67, 110 and +200 kDa). As with the hypertonic salt solutions, complete extraction of these proteins from the plasma membrane did not occur. Using detergents, a total of 12 proteins were extracted from the plasma membrane. Major polypeptides found to be extracted from this procedure were at 47, 110 and 300 kDa; major glycoproteins extracted were of 43, 44, 64, 82 and 120 kDa. From this study, it was concluded that the

major proteins of 47, 67 and 110 kDa were peripheral membrane proteins and major proteins of 43, 44, 64, 82 and 120 kDa were integral membrane proteins.

Saxena et al. (1986b) further investigated the addition of plasma membrane proteins to boar spermatozoa during maturation in the epididymis. Using monoclonal antibodies, the author found that the 44 kDa integral glycoprotein and the 47 kDa peripheral polypeptide were both synthesized and secreted by the epididymis with subsequent absorption, in increasing concentrations, by the spermatozoa as they progressed through the epididymis. During this same year (1986a), Saxena et al. published work in which they examined boar spermatozoal plasma membranes using immunofluorescence with monoclonal antibodies to specific plasma membrane proteins. It was revealed that an asymmetric distribution of the proteins existed in the plasma membrane, with limited redistribution of these surface antigens occurring during maturation and at the time of ejaculation. Five domains, areas of restricted protein mobility in the plasma membrane, were proposed; these were the peri-acrosomal segment, equatorial segment, postacrosomal segment, midpiece, and principal piece (Appendix A). This study lended substantial support to previous studies which described a restricted topographical distribution of the spermatozoal plasma membrane protein composition in other mammalian species (Sazuki and Nagano, 1980; Myles et al., 1981; Friend, 1982; Bellve and Moss, 1983), and the observations discussed by Peters (1985) who described in detail, restricted lateral mobility of proteins within cellular plasma membranes.

Further classification of these domains into subdomains was proposed by Peterson et al. (1987). Using indirect immunofluorescence microscopy and freeze fracture, it was found that within the principal or acrosomal segment of the plasma membrane, there was an area containing very few integral membrane proteins when compared to the remaining regions of the sperm head segment. From this observation, it was hypothesized that the absence of IMPs and the proposed presence of normal membrane anionic phospholipids found in this region, may be the facilitating factor from which fusion of this part of the plasma membrane to the underlying outer acrosomal membrane may help to initiate that which we know as the acrosome reaction.

Individual analyses including the characterization and functions of these membrane polypeptides have been extremely limited with respect to mammalian spermatozoa. Both acid and alkaline phosphatase activities have been found to exist in the isolated plasma membrane of boar spermatozoa (Soucek and Vary, 1984). Detergents were needed for isolation of these enzymes; therefore, it was believed that these polypeptides, which hydrolyze phosphate esters under acid or basic conditions, reside as integral membrane proteins. Other enzymes isolated from spermatozoal plasma membranes, along with the boar, include α-D-Mannosidase (rat-Tulsiani et al., 1989; mouse-Cornwall et al., 1989; human-Tulsiani et al., 1990), galactosyltransferase (mouse-Lopez et al., 1985; human-Sullivan et al., 1989), fucosyltransferase (mouse-Ram et al., 1989), cyclic AMP phosphodiesterase I (bull-Stephens et al., 1979), adenylate cyclase and phosphodiesterase I (human-Tulsiani

et al., 1990), and arylsulfatases (boar, dog, stallion, bull, rabbit-Gadella et al., 1991).

A 300 kDa glycoprotein complex from boar sperm plasma membranes was found to be a major calcium-binding protein of seminal plasma (Peterson et al., 1989). This complex is believed to be a peripheral membrane protein added to the sperm head during ejaculation, and is somehow involved in the acrosome reaction as an initiator of this event. Peterson and Hunt (1989) characterized a 55 kDa protein associated with the boar spermatozoal plasma membrane skeleton which functioned in the binding of the spermatozoan to the zona pellucida prior to the acrosome reaction. This adhesion protein (AP_z) was found to be inserted in the epididymis, and makes up $\leq$ 1% of the total membrane protein composition. Enzymatic activities of this AP_z were not detected in this study. As an added note, Jones et al. (1990) found that rat spermatozoa in vitro contained a 55 kDa protein which migrated from the plasma membrane of the tail to the head during capacitation. In this article, Jones proposed that this protein was involved as a sperm-egg recognition molecule; it was also demonstrated that under certain conditions, redistribution of membrane proteins is possible under an external stimulus (i.e., Ca^{2+}). Lastly, it has been recently demonstrated that functional ion channels are present in the boar spermatozoal plasma membranes (Cox and Peterson, 1989; Cox et al., 1991); continuing efforts are currently underway to further characterize this newly identified polypeptide.

Intrinsic to a number of membrane proteins and possibly

glycolipids are glycosaminoglycans (GAG), which constitute part of the glycocalyx of the plasma membrane. These glycoproteins can associate with a membrane either by intercalating (core protein inserted directly into the membrane) or be peripherally bound (core protein or the polysaccharide bound on the outside of the cell via noncovalent linkages). It has been postulated that due to the unique location of these GAGs on the surface of the cell, they may function in cell-to-cell and cell-to-substrate interactions.

Although a relatively young field of study when compared to lipid and protein research in spermatozoa, fundamental insight has been elucidated as to the function(s) of GAGs during spermatozoal development, capacitation, acrosome reaction and fertilization. In the ram, changes in lectin-binding features associated with epididymal maturation and ejaculation on the spermatozoal surface has been investigated (Magargee et al., 1988). Using epilumenescent-fluorescence microscopy and flow cytometry, it was found that only minor nonsignificant changes occurred in the number of mannosyl and/or glucosyl residues during epididymal transit and at ejaculation. A decrease in the amount of galactosyl residues occurred during transit through the epididymis and, therefore, this residue was believed to be involved in the maturation process of spermatozoa. With regards to N-acetylglucosamine dimers or sialyl residues, an increase in their numbers were found to occur during epididymal transit and subsequent to ejaculation; it was believed that these residues may provide a greater net negative surface charge so that enhanced binding of proteins present

in the seminal plasma and the reproductive tract fluids occurs.

Examination for the presence of heparin-binding proteins from seminal plasma which bind to bovine spermatozoa was performed by Miller et al. (1990). The theory to support the presence of heparin-binding proteins on spermatozoa revolves around the observation that incubation of bovine spermatozoa in heparin-containing media (heparin acting like a GAG-like substitute) induces a zonae pellucidae initiated exocytosis of the acrosome prior to fertilization and, thus, may act as a regulatory step for the acrosome reaction via an extracellular matrix glycoprotein(s) (Parrish et al., 1988). The authors indeed found that a series of acidic 15-17 kDa, a basic 24 kDa, and a 31 kDa protein became attached to the spermatozoa after incubation in heparin-containing media and then seminal plasma. These proteins were peripherally attached to the plasma membrane and subsequently, significantly potentiated the sensitivity of the incubated spermatozoa to zonae pellucidae with regards to onset of the acrosome reaction. Therefore, from these observations, further support was provided to establish that GAGs somehow play an integral role in the sperm's quest for fertilization with an oocyte. As an added note, similar binding sites have been recently reported to be present as a plasma membrane component of the boar spermatozoal plasma membrane (Delgado et al., 1990).

In summary, a multitude of proteins have been found to be present in the plasma membrane of boar spermatozoa. These proteins are acquired by the spermatozoan during spermatogenesis, epididymal maturation, and from reproductive tract fluids during ejaculation. Both peripheral and

integral membrane proteins can be inserted from the outside directly into or on the lipid bilayer of the spermatozoan. These modifications in number, type, and redistribution of membrane proteins by external stimuli are entirely understandable given the simple fact that spermatozoa cannot synthesize new polypeptides due to the lack of the normal cellular organelles. The available evidence also establishes the spermatozoal plasma membrane as a dynamic structure which undergoes reorganization as a precursor to the events of capacitation and the acrosome reaction. These reorganizational events may, in same way, alter presumptive barriers through the molecular restructuring of the plasma membrane via protein and lipid mobilization, protein function (through their attached polysaccharides and/or calcium binding), insertion/deletion of proteins/lipids, and through lipid-lipid/lipid-protein interactions. From a review of the literature, it is readily seen that we are just beginning to elucidate upon the mechanisms which are involved in the complex physiological events mammalian spermatozoa must go through prior to its final task of fertilization with an oocyte.

II. MATERIALS AND METHODS

A. Boar Selection

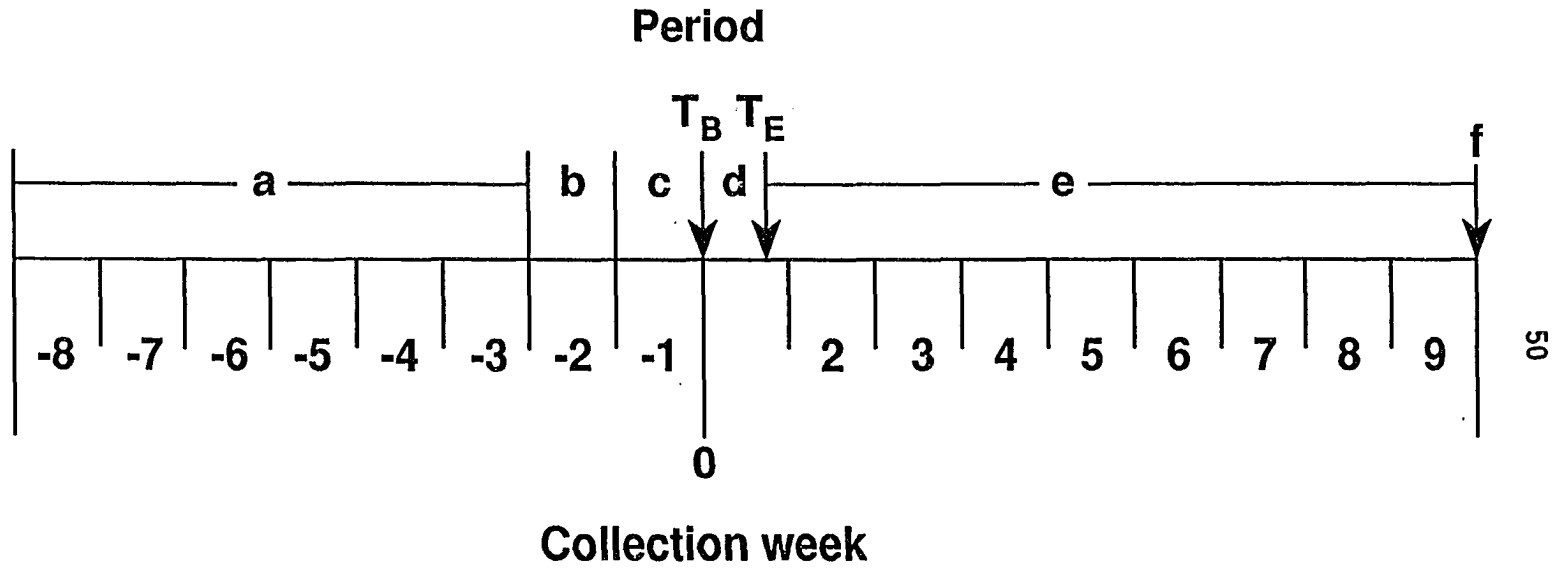
Eight healthy, 14-16 month, sibling-paired, Yorkshire-Hampshire-Duroc crossed boars from 3 different litters in the enclosed Department of Veterinary Clinical Sciences herd were used in this study. After weaning, boars were reared in 10′ 6" x 11′ 10" confined stalls until approximately 6 months of age; at that time, boars were physically grouped, with each group being placed in a separate 10′ 6" x 11′ 10" confined stall for the remainder of the study (except during exposure of the treated boars to increased ambient temperatures). Between 4-14 months of age, boars were trained for gloved-hand semen collection using a mounting dummy. After the initial training period, boars were placed on a weekly routine semen collection schedule. Immediately prior to inclusion in the study, all boars were given a complete physical and a breeding soundness examination as described in the guidelines for the Society of Theriogenology (Hurtgen, 1984). All animals throughout the study were kept on a 11 hours/13 hours (light:dark) schedule of incandescent lighting, had water available ad libitum, and were fed two times per day (AM and PM) with a 14% protein corn/soybean oil meal feed.

B. Experimental Design to Examine the Effects of Environmental Heat Stress on the Spermiogram and Plasma Membrane Components of Porcine Spermatozoa

Provided is a schematic (Figure 1) outlining the design of this study. This study was sub-categorized and identified by period (a-f) and collection week (-8 thru +9); references to period and collection week will be made in the ensuing text.

A complete spermatogenic cycle and maturation phase (51 days) were characterized in all paired boars via individual spermiograms (Periods a-c) prior to the onset of the experiment. During period (a), ejaculates were collected from all paired boars on a single day and analyzed for total spermatozoal motility (TSM), normal spermatozoal morphology (SM) and total sperm concentration (TSC). During period (b), along with ejaculate collection and analysis, boars within each pair were randomly assigned to either the control (n=4) or treatment group (n=4), with the pair also being assigned to a set collection day. In period (c), paired boars were collected on their set collection day; so 4 consecutive days of semen collection were performed, with no paired-boars collected on the 5th, 6th or 7th day. Ejaculates were analyzed for total spermatozoal motility, spermatozoal morphology, total sperm concentration and plasma membranes were isolated for determination of their pre-treatment lipid and protein concentration. At the beginning of period (d) (T_b), the designated treatment boars from each pair were separated from their sibling control boars and placed in a 12'9" x 8'10"

Figure 1. A schematic diagramming the experimental design of this study by period and collection week: (a) weekly collection of boar ejaculates, (b) sibling boars paired and assigned to either treated or control group with assignment to a set collection day, (c) paired boars collected on their set collection day, (d) paired boars split and exposed to heat-stress treatment or maintained as a control for 5 days, (e) semen from paired boars collected on their set collection day for 7 weeks post-treatment, (f) completion of study, (T_B) paired boars separated and treatment begins, (T_E) end of treatment period, boars re-paired.


Period
T_B T_E
a
b
c
d
e
f
-8 -7 -6 -5 -4 -3 -2 -1 0 2 3 4 5 6 7 8 9
Collection week

controlled environmental chamber for a 5-day (120-hour) heat-stress treatment period. Treatment boars were exposed to 35-37°C, 20-30% RH for 8 consecutive hours each 24-hour period, with the remaining 16 hours being exposed to 30-32°C, 20-30% RH. During treatment, rectal temperatures were taken at approximately 12 hour intervals with a mercury thermometer. Control boars were kept at the area of origin, separate from the treatment group, and were maintained at ambient temperature (20-22°C) and RH (20-30%); this area of origin was where both control and treated animals were located when the treated group was not in the environmental chamber. After 120 hours (T_E), treated boars were taken back to their area of origin and placed with their paired sibling; no semen collections were performed during period (d).

Starting 48 hours post-treatment, (168 hours after initiation of the heat-stress treatment), semen was collected from a pair of boars on their set collection day of the first week and the schedule repeated for 7 additional weeks (period e).

To avoid the expected individual variability of boars with regards to undefined seasonal effects and possible genetic heat resistance among sibling groups, sibling boars were paired and data were pooled within the control and treatment groups over the 4 day periods as "collection weeks" for analysis; therefore, all analyses for treatment versus control boars were based on pair differences rather than on individual boar differences. Collection weeks in this study were defined as follows:

Collection week -1 = week of collection immediately prior to boar separation and heat-stress treatment

Collection week 2 = 7-13 days post-T_B

Collection week 3 = 14-20 days post-T_B

Collection week 4 = 21-27 days post-T_B

Collection week 5 = 28-34 days post-T_B

Collection week 6 = 35-41 days post-T_B

Collection week 7 = 42-48 days post-T_B

Collection week 8 = 49-55 days post-T_B

Collection week 9 = 56-62 days post-T_B

C. Methods Used in Determining the Effects of Increased Ambient Temperatures on the Spermiogram and Plasma Membrane Component of Porcine Spermatozoa

1. Semen collection and analysis

a. Semen collection A complete ejaculate (i.e., containing pre-sperm, sperm-rich, and post- sperm-rich fractions only) from each boar were collected into sterile, pre-warmed containers held at 37°C. A separate container with an attached filter (Miracloth®, Calbiochem, LaJolla, CA) was used to collect the sperm-rich fraction of the ejaculate, with the pre- and post- sperm-rich fractions collected into a second container. As soon as the ejaculates were collected, they were taken immediately to the laboratory and the sperm-rich fraction filtered a second time through Miracloth®. The semen was analyzed for TSM, SM,

TSC, sperm velocity, and ejaculate volume.

b. Motility of spermatozoa For estimation of TSM, a 5 μl drop of semen was placed on a prepared slide (37°C) and a coverslip applied. A minimum of four visual fields were examined at 100X and 400X, using a phase-contrast microscope with a warmed stage (37°C). For this study, motile spermatozoa were those considered to be independently moving within the microscopic field during visual examination, and was assessed as a percentage in 5% increments. In an attempt to avoid interpretive bias during motility examinations, random samples were selected and analyzed by a second person. In no one instance was there greater than a 10% variation between individuals, therefore, the readings of the first party were used in the experimental analysis.

c. Velocity of spermatozoa The sperm velocity is a measurement that describes the speed of progression of spermatozoa in a sample. This laboratory used a subjective ordinal scale from 0 through 4 to record a semen samples overall velocity: 0 = all dead, no motion in entire sample; 1 = slow side-to-side motion with no forward progression; 2 = slow forward progression; 3 = rapid side-to-side forward progression, and; 4 = fast forward progression.

d. Morphology of spermatozoa Spermatozoal morphology was assessed by placing a 10 μl drop of semen and a similar sized drop of Hancock's stain on one end of a glass slide. The two drops were gently

mixed together with a pusher slide and then drawn out as a thin film similar to that of a blood smear. The sample was allowed to air dry and then, using differential interference contrast microscopy at 1,250X (oil immersion), a total of 200 spermatozoa were morphologically assessed. These 200 spermatozoa were divided into the following categories: normal sperm, abnormal sperm heads, abnormal sperm midpieces, abnormal/bent sperm tails, proximal cytoplasmic droplets, distal cytoplasmic droplets, coiled sperm tails, detached sperm heads, and other (i.e., spheroids, double-headed sperm, detached acrosomes, etc.). If multiple abnormalities were present on any one individual spermatozoa, major abnormalities took precedence over minor abnormalities; additionally, major abnormalities were further categorized as to their importance with head > midpiece > principal piece abnormalities.

e. Concentration of spermatozoa and volume of the ejaculate

Determination of TSC was performed by diluting semen to a 1:200 ratio using a red-cell Unopette® (Becton-Dickinson Co., Rutherford, NJ). This mixture was then slowly agitated to expose all spermatozoa to the solution to disrupt motility. A sample of this mixture was then transferred to both sides of a hemacytometer, with the hemacytometer subsequently being placed inside a humidified chamber for 5 minutes to allow spermatozoa to settle into one visual field. Under 200X, spermatozoa within 5 squares on the diagonal of the central 1-mm square on both sides of the hemacytometer were individually counted; final

readings between the 2 squares were required to be within 10% of one another, otherwise, the whole procedure was repeated. If within 10% of each other, the two counts were averaged and the final number multiplied by 10 to get the numbers of spermatozoa found in each ml of semen. The TSC of the ejaculate was then calculated by multiplying the count per ml by the total volume of the sperm-rich portion of the ejaculate. Total ejaculate volume was measured by using a calibrated container in which both the sperm-rich and the pre- and post- sperm-rich portions were individually handled. Occasionally, spermatozoa were found to be present in the pre- and post- sperm-rich combined portions, however, these were generally found to be of little significance with regards to sperm concentration when compared to the sperm-rich fraction; therefore, a separate TSC was not performed on this fraction.

2. Plasma membrane isolation

A preliminary study was undertaken to develop a validated procedure that would benefit plasma membrane yield with minimal contamination or alterations to membrane integrity. Over a 7-month period during 1990, various techniques were performed using modifications of a procedure initially described by Gillis and co-workers (1978) as a starting point. Variations in nitrogen cavitation pressures (600 or 650 psi), times (10 or 15 minutes), media (5 mM HEPES or 5 mM TRIS-phosphate buffered saline solutions) and alterations in the number and length of centrifugation steps were performed. Plasma membrane purity was quantitated spectrophotometrically using the enzyme

markers alkaline phosphatase (E.C. 3.1.3.1) (Nikolopoulou et al, 1986; Appendix B) and 5'-nucleotidase (E.C. 3.1.3.5) (Gillis et al., 1978; Appendix C). Acrosomal membrane contamination was assessed using the enzyme marker acrosin (E. C. 3.4.21.10) as described by Zaneveld et al. (1973; Appendix D), and succinate dehydrogenase (E. C. 1.3.99.1) was assayed as previously described (Pennington, 1961; Appendix E) for detecting mitochondrial membrane contamination. In addition, light and electron microscopy was used to visually confirm membrane purity. For electron microscopy, the spermatozoal homogenate and the final membrane pellet were prepared by transferring and fixing a portion of the sample into 3.0% glutaraldehyde in Sorenson's phosphate buffer (pH 7.4). After 30 minutes incubation at room temperature, the glutaraldehyde-fixed material was transferred to vials containing 2% uranyl acetate/Reynold's lead citrate in methanol for staining. The tissue was then dehydrated using increasing concentrations of ethanol, infiltrated with propylene oxide and embedded (EmBED812®, Electron Microscopy Sciences, Fort Washington, PA) for sectioning, viewing, and photographing.

From these preliminary studies, the following technique was developed, validated, and used throughout the remainder of the study for isolating spermatozoal plasma membranes (Figure 2). Immediately after semen collection and filtration, 20 ml of semen was gently layered on top of a 1.0M sucrose gradient and centrifuged at 1,200 x g for 25 minutes at 20-22°C in a swinging bucket rotor. After centrifugation, the supernatant was discarded and the clean sperm pellet resuspended in a HEPES-phosphate buffered saline solution which contained 5 mM HEPES,

Figure 2. A flowchart outlining the procedure used in this study to isolate plasma membranes from ejaculated boar spermatozoa.

Sperm-rich fraction collected, filtered and sperm motility, morphology and concentration assessed.

↓

Filtered semen layered on top of a 1.0 M sucrose gradient and centrifuged (1,200xg, 25 min).

↓

Sperm pellet resuspended in a HEPE-PBS solution (pH 7.4, 280-300 mOsm) at $1x10^8$ sperm cells/ml.

↓

Spermatozoa subjected to nitrogen cavitation (650 psi, 10 min). Extrude sample into HEPES-PBS (pH 5.0) and agitate.

↓

Wash sample twice (6,000xg, 10 min) to remove disrupted cells and small particulate matter from plasma membrane enriched supernatant

↓

Centrifuge supernatant (35,000xg, 15 min) to remove remaining subcellular debris.

↓

Ultracentrifuge supernatant (300,000xg, 70 min) to pellet sperm plasma membranes.

2.7 mM KCl, 1.5 mM KH_2PO_4, 0.137 M NaCl, and 8 mM $Na_2HPO_4 \cdot 7 H_2O$ (pH 7.4, 280-300 mOsm), to achieve a final concentration of 1 x 10^8 sperm cells/ml; a total of 60 ml was then subjected to nitrogen cavitation at 650 psi for 10 minutes at 20-22°C. The cavitate was slowly extruded into a clean container which contained HEPES-phosphate buffered saline solution at a pH 5.0 to inhibit protease activity, followed by gentle agitation for approximately 5 minutes. The cavitate was then centrifuged (6,000 x g, 10 minutes, 5°C) twice to remove disrupted cells and small particulate matter. The subsequent supernatant was then centrifuged at 35,000 x g for 15 minutes at 5°C to remove remaining subcellular debris, yielding a post-subcellular fraction. The post-subcellular fraction was lastly ultracentrifuged at 300,000 x g for 70 minutes at 5°C to pellet the remaining membranes within the sample. The harvested membrane pellet was resuspended in 1.0 ml of HEPES-phosphate buffered saline (pH 5.0) and placed into storage at -80°C until further analysis.

3. Plasma membrane lipid extraction and fractionation

a. Lipid extraction Lipid extraction from the spermatozoal plasma membrane was performed using a modification of the technique previously described by Folch et al., (1957). The semen phospholipids phosphatidylglycerides (PG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidycholine (LPC) and sphingomyelin (SPH) were separated and quantified using a modification of the high

performance liquid chromatography (HPLC) technique first described by Engen and Clark (1990). Briefly, a proportion of the harvested membrane material was mixed with 10 ml of 2:1 chloroform/methanol (v/v). A total of 2.5 ml of a 0.74% potassium chloride solution was then added to precipitate membrane protein and separate liquid phases. The preparation was then vigorously mixed using a vortex mixer and then immediately centrifuged at 400 x g for 10 minutes at 20-22°C. The upper phase was discarded and the lower phase harvested and immediately filtered through a 0.45 μm mesh filter (Acrodisc® 13 CR-PTFE, Gelman Sciences, Ann Arbor, MI). The filtered sample was then dried and stored under nitrogen at -80°C until reconstitution in a chloroform:methanol:hexane (5:4:1) mixture for subsequent HPLC analysis.

Neutral lipids were eluted from the extracted lipid material by using a prepared strong cation exchange silicic acid column (Supelco Inc., Bellefonte, PA). Neutral lipid elution was performed by flushing the prepared and sample loaded column with 5 ml of a 2:1 chloroform/methanol (v/v) wash, and collected in an amber glass vial. The collected neutral lipids were then dried under nitrogen, resuspended in 400 μl of chloroform, and stored under nitrogen at -80°C until analysis using gas liquid chromatography at a later date.

b. <u>High performance liquid chromatography</u> Membrane phospholipid composition (PG, PI, PS, PC, PE, LPC and SPH) was determined using an HPLC system consisting of a programmable pump (Model 2350 pump, Instrumentation Specialty Co., Lincoln, NE), a variable

wavelength recorder (V^4 Absorbance Detector, Instrumentation Specialty Co., Lincoln, NE), and a chart recorder (Model 1242, Soltec Corp., Sun Valley, CA), all of which were interfaced to a computer using ChemResearch® (Instrumentation Specialty Co., Lincoln, NE) as a systems controller, data collector, and analyzer. Membrane phospholipids were separated on a ISCO 5μSI column (5 μm, silica). The mobile phase through the column consisted of a 100:5:0.05 acetonitrile/methanol/sulfuric acid solution (v/v/v) which had been previously filtered and degassed. Instrument conditions included a mobile phase flow rate of 1.5 ml/min, a column pressure of < 6.0 MPa, and a column effluent detector setting of 202 nM. Peak elution times and quantitation of unknowns by integration of peak areas were determined using purified external standards of PG, PI, PS, PE, PC, LPC, and SPH.

c. Gas liquid chromatography Neutral lipid composition (i.e., cholesterol and desmosterol) was determined using a Varian Model 3700-GC gas chromatograph. Neutral lipids, suspended in degassed chloroform, were separated on a 60 cm X 2 mm i.d. glass column packed with SP-2250 on a 100/120 mesh with support (Supelco, Bellefonte, PA). Column temperature was maintained at 250°C, with the injection port and detector maintained at 280°C. The chromatograph was integrated to a computer using LabTech Chrom® (IMI, State College, PA) as a systems controller, data collector and analyzer. Sterols were quantitated relative to the addition of cholestane as an internal standard. Peaks

were identified based on retention times corresponding to those for known purified standards of cholesterol and desmosterol.

4. Protein determination

a. Quantification of protein Protein concentration in plasma membrane preparations were quantified using a micro-Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA), with bovine serum albumin (Fraction V) used to establish a standard curve. This assay was designed for sensitivity detection ranges of 1-20 μg of protein.

b. Identification of membrane proteins Identification of membrane proteins were performed using one-dimensional PAGE. A total of 20 μg of protein from each plasma membrane preparation was resuspended in 4.0% sodium dodecyl sulfate, 0.125 M TRIS-HCl, 20% glycerol, 10% 2-mercaptoethanol (pH 8.0), and heated at 100°C for 3 minutes. The sample was then loaded onto a 1-D PAGE gel using a 10-20% gradient and electrophoresed for 3 hours at 20 mAmp. Gels were stained using Coomassie blue, and the gel patterns analyzed by a computer (PDI, Quantity I, Huntington Station, NY) by comparing to known standards (14.2, 20.1, 24, 29, 36, 45 and 66 KDa) as well as with previously published reference maps (Russell et al., 1983; Russell et al., 1984). Similarities in overall migratory properties and in shapes of specific polypeptides on the gels were used to identify proteins according to their published molecular weights.

D. Statistical Analysis

Data from this study were expressed/analyzed using fundamental descriptive statistics. A general linear models procedure of SAS (SAS Institute, Inc., Cary, NC) was used for statistical computation.

To ascertain if there was a natural effect of time on the spermatozoal plasma membrane components (i.e., polar/neutral lipids, total protein), an analysis of variance was initially performed on all quantified variables collected on the ejaculates of control boars using boar (df=3) and collection week (df=8) as main effects.

Previous studies examining the effects of heat-stress on boar spermatozoal variables (e.g., TSM, SM, and TSC) have repeatedly demonstrated that heat-stress treatment significantly affects the spermiogram from 7 through 56 days after initiation of treatment (see literature review), therefore, to determine if heat-stress concurrently affected plasma membrane lipid composition and protein concentration, data collected on all boar ejaculate variables from day 7 through day 56 after initiation of treatment (corresponding to collections weeks 2 through 8) were computed collectively as control value vs. treated value. Data collected prior to the onset of treatment (collection week -1) and beyond 56 days after initiation of treatment (collection week 9) were computed together as control value vs. treated value to assess whether plasma membrane components returned to within their normal pre-treatment values. The ejaculate variables TSM, SM and TSC were analyzed in this study primarily to assess the success of our

treatment in relation to previously published reports (see literature review).

Collection weeks 2 through 8 were examined using Analysis of Variance with boar pair (df=3), treatment (df=1), collection week (df=6), and the interactions pair X treatment (df=3) and collection week X treatment (df=6) as sources of variation to determine if heat-stress affected plasma membrane polar/neutral lipid composition and protein concentration as well as TSM, SM and TSC. The interaction collection week X treatment was used as an error term for effect by week and the interaction pair X treatment used as the error term for the effect of treatment. The same ejaculate and plasma membrane variables were collectively examined for collection weeks -1 and 9 by Analysis of Variance using boar pair (df=3) and treatment (df=1) as main effects, to ascertain if boar ejaculates recovered completely to their pre-treatment variables at the conclusion of the experimental period. In this study, differences were considered to be statistically significant if the probability of error was less than 0.05.

III. RESULTS

A. Examination of Boars

All the boars used in this study satisfactorily passed the breeding soundness examination which included a physical examination, an assessment of libido and an analysis of their semen quality. All boars had satisfactory external genitalia and readily serviced a mounting dummy. For assessment of semen quality, each of the 8 boars used in this study exceeded the minimal criteria for consideration as a reproductively normal boar ($\geq$ 70% motility, > 20 X 10^9 sperm/ejaculate, and $\geq$ 75% normal spermatozoal morphology), and maintained their semen quality throughout the pre-treatment period (periods a - c).

B. The Effects of Increased Ambient Temperature on the Spermiogram and the Plasma Membrane Component of Porcine Spermatozoa

The effect of increased ambient temperature on rectal temperature differences between treated and control boars was almost immediate after initiation of treatment (Table 1). While the control boars maintained a rectal temperature of 38.9 $\pm$ 0.2°C, treated boars had a sharp increase in rectal temperature to 41.1 $\pm$ 0.4°C after their first 8-hours of exposure to increased ambient temperatures. Thereafter, treated boars averaged 39.7 $\pm$ 0.6°C during the 8-hours of heat exposure at 35-38°C,

Table 1. Rectal temperatures of boars (n = 4) prior to, during and after exposure to increased ambient temperatures over a 5-day trial period (mean ± SEM)

	TIME OF DAY RECTAL TEMPERATURE (°C) TAKEN	
Day of Exposure	AM[a]	PM[b]
Day 1	--------	41.1 ± 0.35
Day 2	39.4 ± 0.4	39.8 ± 0.6
Day 3	39.9 ± 0.4	39.6 ± 0.35
Day 4	39.2 ± 0.2	39.9 ± 0.9
Day 5	39.5 ± 0.6	39.5 ± 0.4
Day 6	--------	38.7 ± 0.25[c]

[a] Mean rectal temperature following exposure to 16 hours at 30-32°C, 20-30% RH.

[b] Mean rectal temperature following exposure to 8 hours at 35-37°C, 20-30% RH.

[c] Mean rectal temperature 16 hours following reintroduction to 20-22°C, 20-30% RH with control boars.

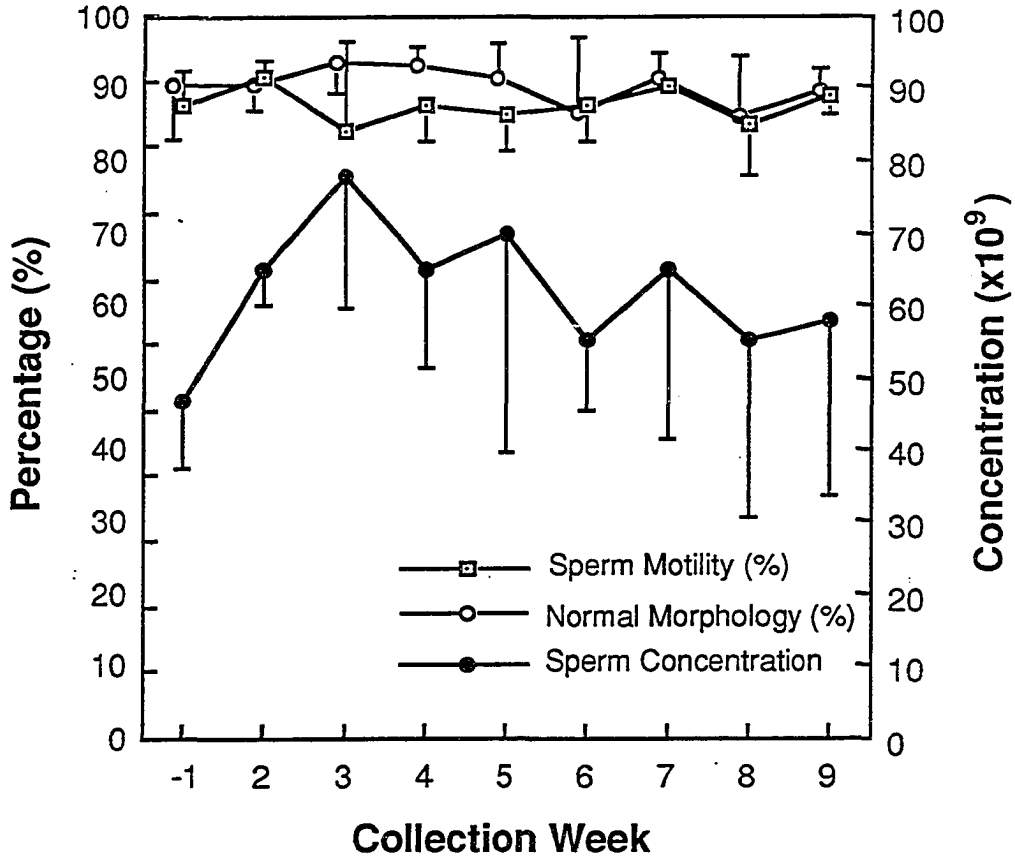
20-30% RH, with an average subsequent drop to 39.5 ± 0.5°C during the remaining 16-hours at 30-32°C, 20-30% RH out of the 24 hour rotational period. Upon completion of the heat-stress treatment, treated boars dramatically decreased their body temperatures over a 12-hour period to 38.7 ± 0.3°C after return to their area of origin with the control boars - a temperature not significantly different from that exhibited by the paired-control animals.

1. Semen collection and analysis

A total of 72 semen collection attempts were made throughout the 9 collection weeks; 69 complete ejaculates were successfully collected from the 8 boars. The remaining 3 failed attempts at semen collection occurred within the heat-stress treated group, with 2 failures accounted for by one boar during collection weeks 6 and 7, and the remaining failure from a different boar during collection week 8.

Within the control group, little variation ($P > 0.05$) was observed with regards to TSM (Figure 3), sperm velocity and ejaculate volume. A significant difference was found between, but not within, control boars for normal SM ($P < 0.01$) and TSC ($P = 0.01$). Spermatozoal morphological differences between the control boars existed with regards to the presence of proximal cytoplasmic droplets ($P < 0.01$) and detached heads ($P < 0.01$); no significant differences between the control boars occurred with respect to numbers of abnormal heads, abnormal midpieces, bent/coiled tails or distal cytoplasmic droplets ($P > 0.05$).

Figure 3. Total sperm motility, normal sperm morphology and total sperm concentration in ejaculates of control boars exposed to an ambient temperature of 20-22°C, 20-30% RH, throughout the 9 collection weeks. A bar graph depicting the spermatogenic cell type at the onset of the study is provided.

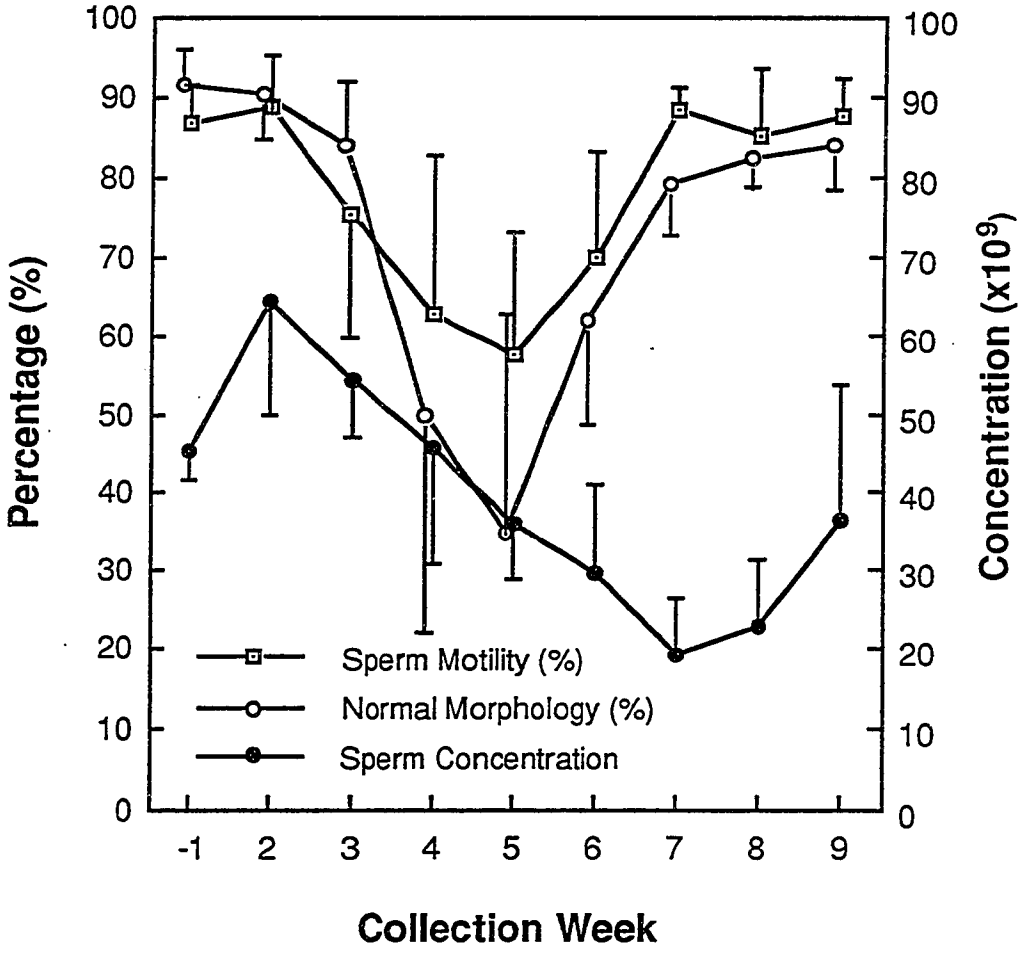


Spermatozoa	Sperm-atid	Spermatocyte	Spermatogonia

When comparing spermiograms (i.e., TSM, SM, TSC, sperm velocity and ejaculate volume) for collection weeks 2 through 8, a significant difference was observed between treated and control pairs for thevariables TSM ($P < 0.01$), numbers of morphologically normal sperm ($P < 0.01$), TSC ($P < 0.01$) and sperm velocity ($P < 0.01$). Differences in TSM and numbers of morphologically normal sperm were most prominent during collection weeks 4 through 6 (Figure 4). During collection weeks 7 through 9, TSM and numbers of morphologically normal sperm for the treated group were found to be comparable to the values obtained from their paired controls. A gradual decrease in TSC was found to occur in treated boars throughout collection weeks 2 through 7 (Figure 4); at week 7, TSC constituted only 42% of the treated boars pre-treatment values, and was only 30% of the TSC produced by the paired controls. An increase in TSC in the treated group did occur during collection weeks 8 and 9; by collection week 9, no observable differences in TSC were present between the treated and control paired groups. Differences in sperm velocity were most pronounced at collection week 4 (data not shown); these differences were minimal during the other weeks of collection.

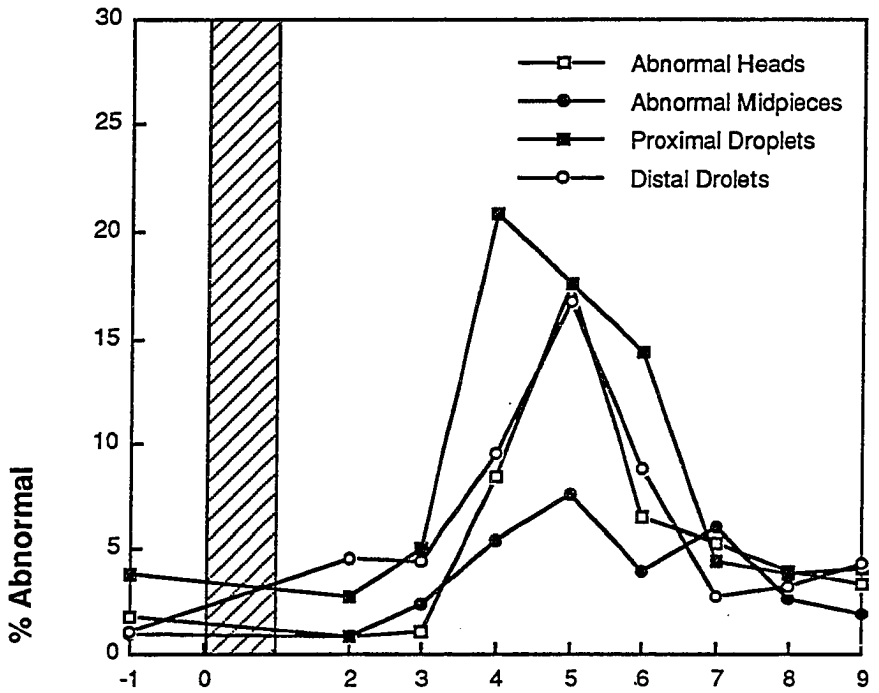
When assessing treated and control boar ejaculates for abnormal spermatozoa, treated boars were found to exhibit a significantly higher number of abnormal heads ($P< 0.01$), abnormal midpieces ($P < 0.01$), proximal cytoplasmic droplets ($P < 0.01$), and distal cytoplasmic droplets ($P < 0.01$) in their ejaculates during collection weeks 3 through 6 (Figure 5). The presence of abnormal spermatozoa decreased to

Figure 4. Total sperm motility, normal sperm morphology and total sperm concentration observed in boar ejaculates 1-week prior to, and 8-weeks after exposure to increased ambient temperatures. A bar graph depicting the spermatogenic of cell type at the time of treatment is provided to ascertain a possible origin to the semen variable alterations.



Spermatozoa	Sperm-atid	Spermatocyte	Spermatogonia

Figure 5. Spermatozoal morphological abnormalities observed in boar ejaculates 1-week prior to, and 8-weeks after exposure to increased ambient temperatures. A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain the possible origin of the sperm abnormalities.



	Treat-ment	Spermatozoa	Sperm-atid	Spermatocyte	Spermatogonia

non-significant values by collection week 8 in most paired boars. No significant differences were observed between treated and control pairs with respect to the presence of bent tails, coiled tails, and detached heads in the ejaculates.

Ejaculate volume was variable throughout the experimental period. Although a significant difference ($P < 0.01$) was observed between treated and control groups, neither treatment nor week could be implicated as the primary reason for this observation.

2. Analysis of purity of the plasma membrane isolate

A total of 3 different ejaculates were processed and subsequently analyzed for membrane purity using established enzyme markers, light and electron microscopy. Table 2 shows results of the enzyme marker analyses for comparison of the post-cavitate homogenate with the final plasma membrane preparation. Acrosin, a polypeptide associated with the acrosomal membrane, showed a 54% decrease in activity during the isolation procedure; therefore, it was concluded that the presence of acrosomal membranes diminished significantly and, thus, indicated minimal contamination in the final membrane preparation. Additionally, succinate dehydrogenase, an enzyme found only in mitochondrial membranes, dramatically decreased in its specific activity to non-detectable levels over the course of our plasma membrane isolation protocol, indicating that no mitochondrial contamination existed in our final plasma membrane preparation. Specific activities for the plasma membrane enzyme markers alkaline phosphatase and 5′-nucleotidase

Table 2. Specific activities[a] selected of enzyme marker assays for determination of plasma membrane purity (n = 3)

Enzyme Marker	Homogenate vs. Membrane	Enrichment
Acrosin	348 vs. 186 nmol/min mg protein	0.5
Aklaline phosphatase	28 vs. 75 nmol/min mg protein	2.7
5′-nucleotidase	6 vs. 31 nmol/min mg protein	5.2
Succinate dehydrogenase	7 vs. 0 nmol/min mg protein[b]	0

[a] Nanomoles benzoic acid/min/mg protein for acrosin, nmol p-nitrophenol/min/mg protein for alkaline phosphatase, nmol P_i/min/mg protein for 5′-nucleotidase, and nmol formazan/min/mg protein for succinate dehydrogenase.

[b] Beyond sensitivity of spectrophotometric assay

significantly increased in the membrane isolation protocol, indicating an enrichment in plasma membrane harvest. On an average, a 3-fold (range 2-4 fold) increase in specific activity of alkaline phosphatase in the plasma membrane preparation was observed in relation to the post-cavitate homogenate; even greater increases in 5′-nucleotidase (3 to 9-fold) activity, another enzyme marker reportedly used for detecting plasma membrane purity, were present.

Transmission electron microscopy showed that nitrogen cavitation generally removed plasma membranes from the anterior, peri-acrosomal portion of the head (Figure 6); the outer acrosomal membrane was not removed by the described cavitation and isolation procedure, leaving the acrosome proper intact. Comparison of the post-cavitate homogenate (Figure 7 and 8) to the final plasma membrane preparation (Figure 9 and 10) showed that the procedure isolated unilamellar vesicles which were relatively devoid of electron-dense material (an indication of acrosomal membrane contamination). No other subcellular fragments were visually observed in the final plasma membrane preparation.

All of the above analyses consistently suggest that the recovered vesicles from the plasma membrane isolation procedure represent the plasma membrane of the boar's spermatozoa, with the majority of these plasma membranes coming from the anterior, peri-acrosomal portion of the sperm head. Further evidence supporting uniformity of our plasma membrane preparation is provided by the consistency of the ratio for the plasma membrane components (e.g., phospholipids, sterols and proteins) in our control animals over the entire 9-week study.

Figure 6. Photomicrograph (16,000X) showing the typical absence of the plasma membrane around the periacrosomal region of the sperm head after nitrogen cavitation (arrows). Note that integrity of the acrosome is maintained.

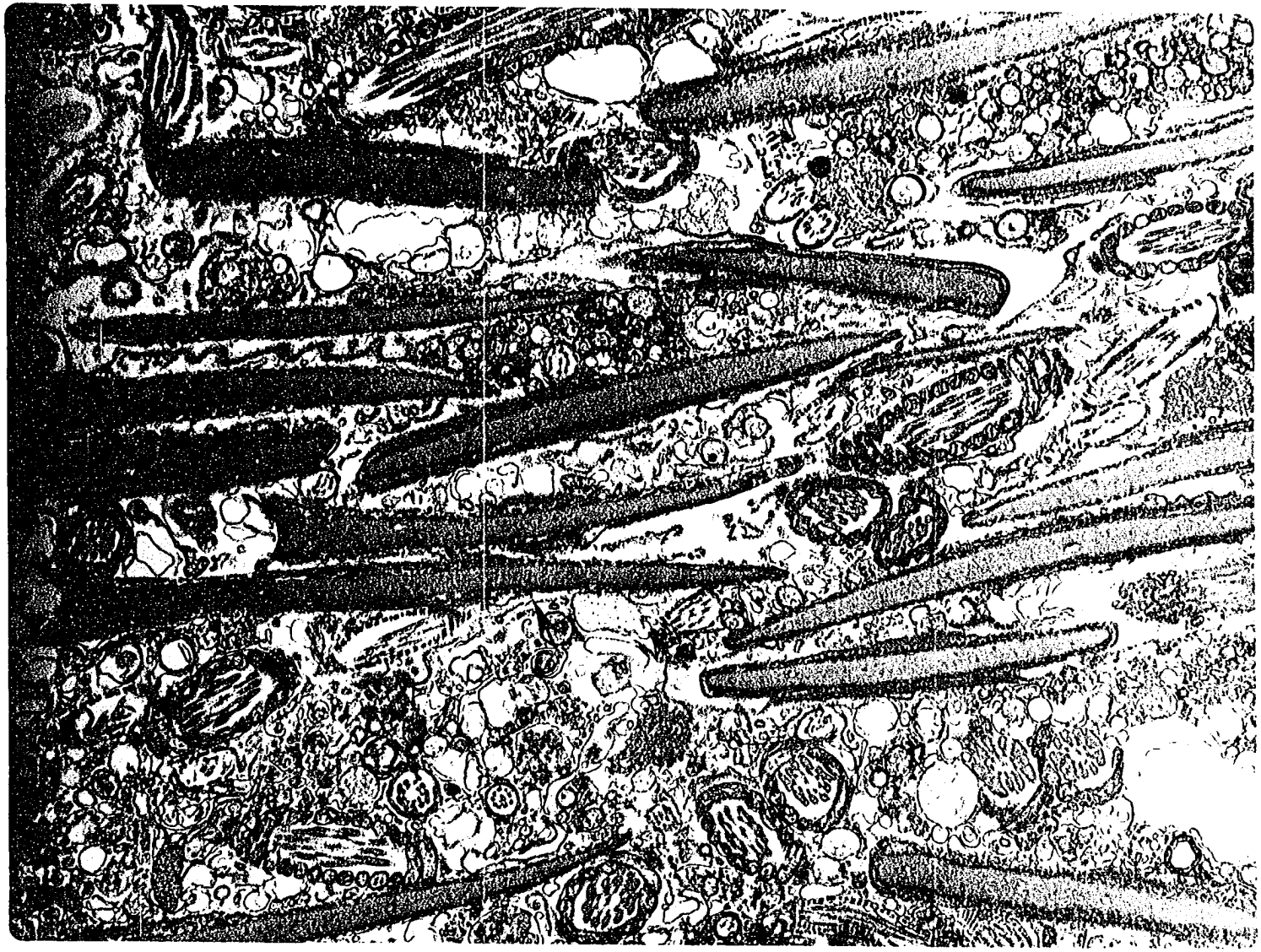


Figure 7. Photomicrograph depicting the variety of sperm cell constituents in the immediate post-cavitate homogenate (10,680X).

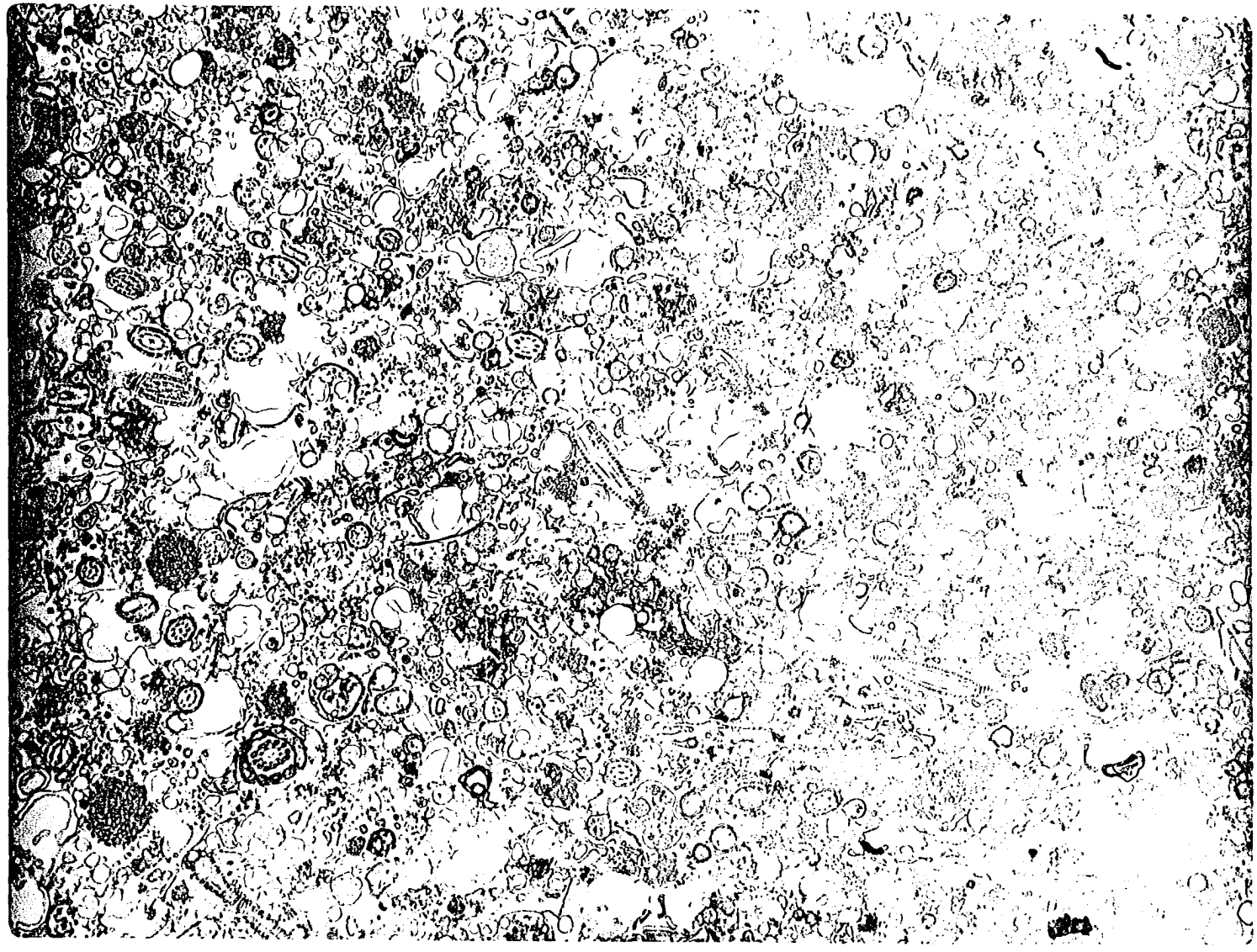


Figure 8. Photomicrograph depicting a higher magnification of the post-cavitate homogenate (46,700X).

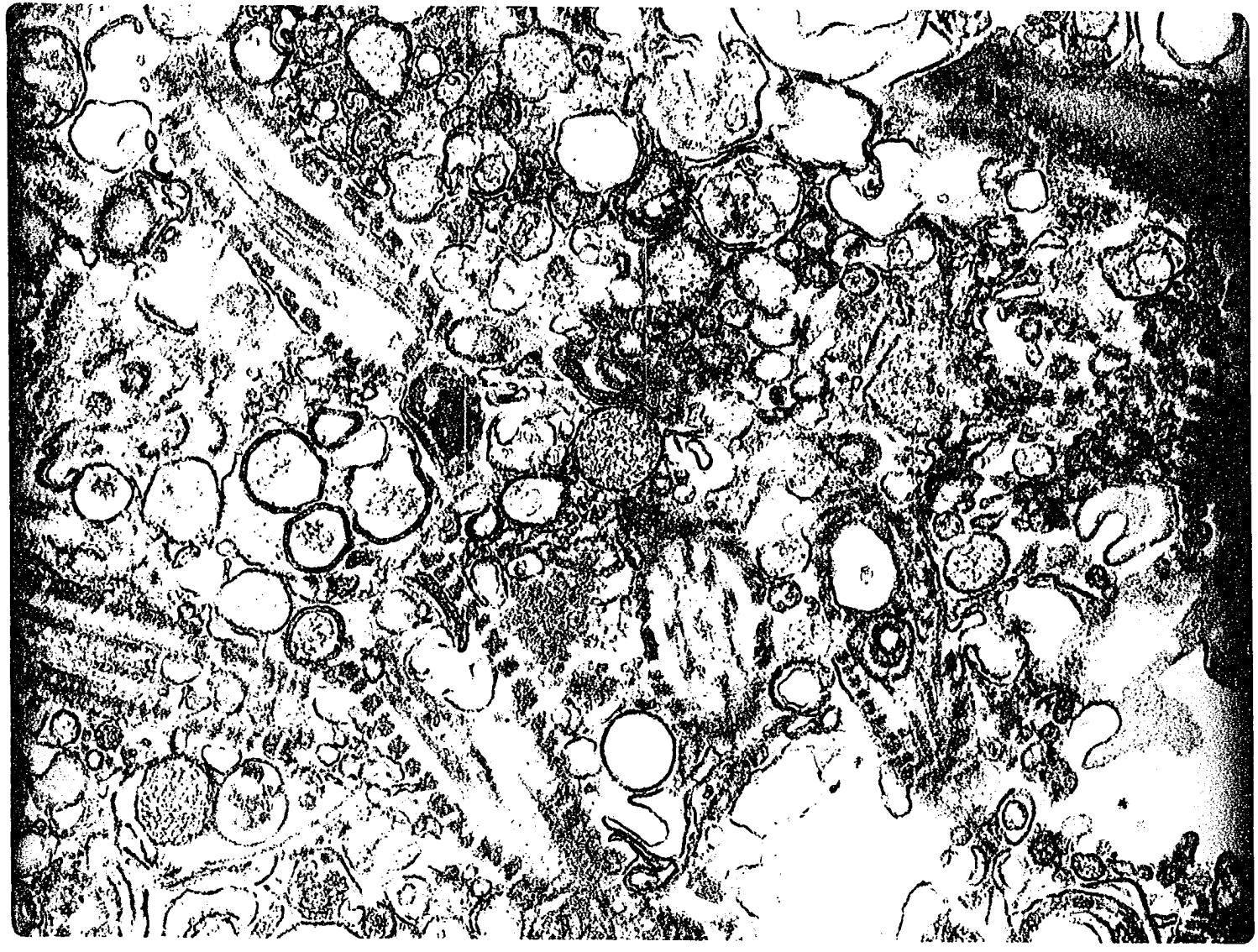


Figure 9. Photomicrograph showing unilamellar plasma membrane vesicles at the conclusion of our isolation procedure (23,200X).

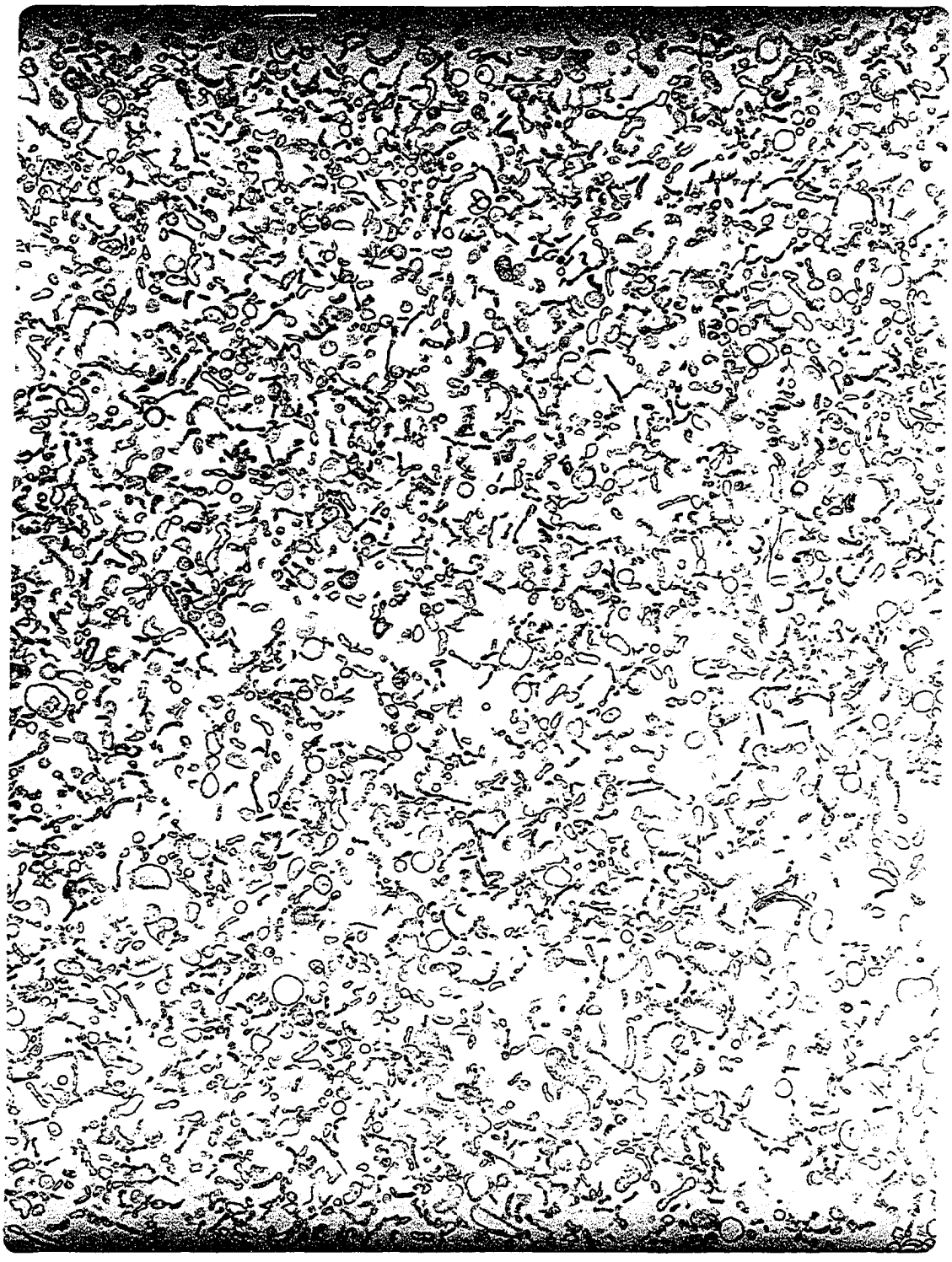
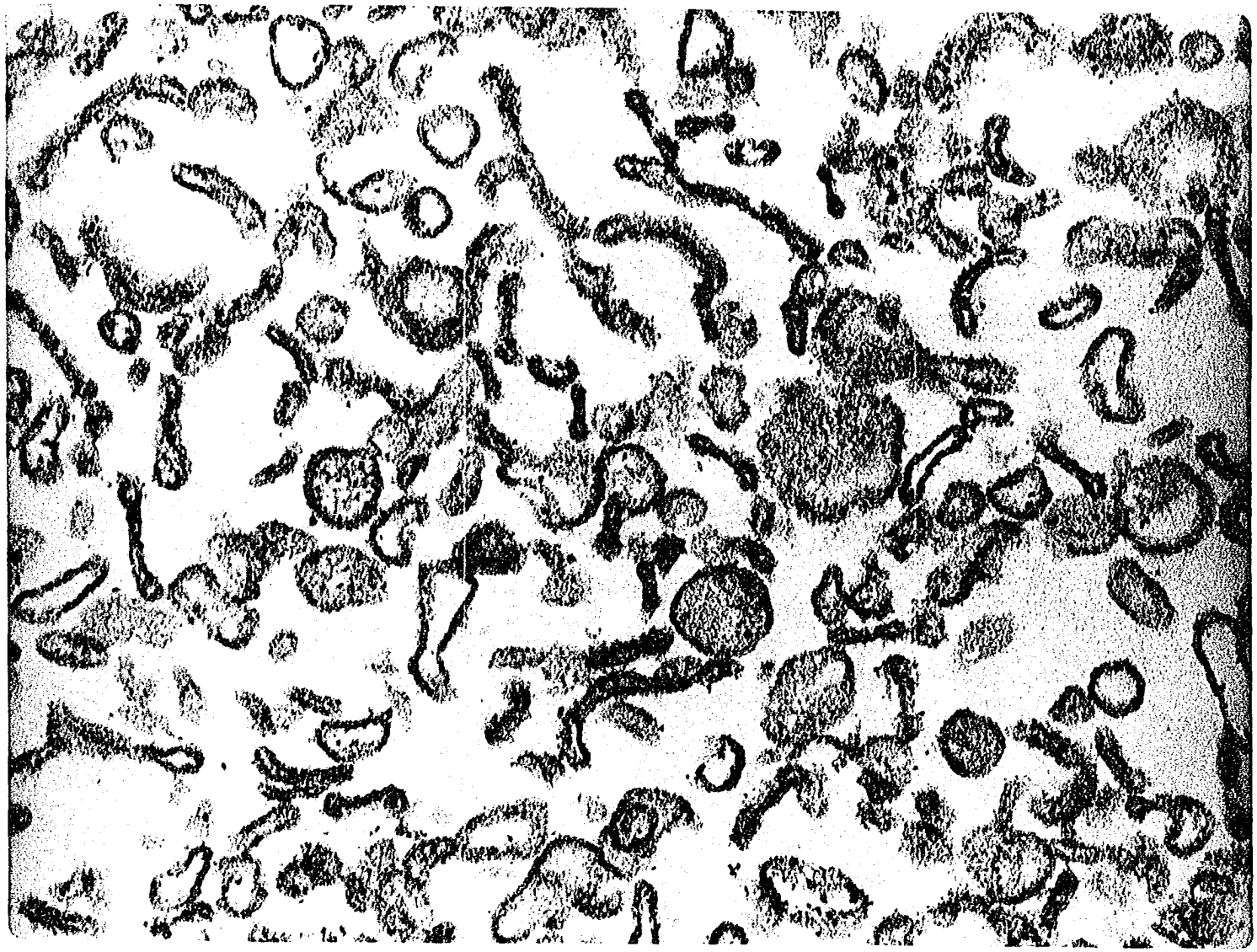


Figure 10. Photomicrograph showing a higher magnification of the isolated unilamellar plasma membrane vesicles (106,800X).



C. Biochemical Composition of the Boar Spermatozoal Plasma Membrane

Lipid composition and protein concentration in control boars were initially analyzed to assess whether natural changes occurred in the biochemical composition of the spermatozoal plasma membrane over time. Phospholipids accounted for approximately 83.5% of the total lipid content of the plasma membrane; of this percentage, PC was the most abundant phospholipid (56.5 $\pm$ 1.9 %), followed by SPH (16.6 $\pm$ 1.8 %), PG (11.0 $\pm$ 3.9 %), LPC (9.0 $\pm$ 1.3 %), PE (5.8 $\pm$ 1.1 %), and PI (1.04 $\pm$ 0.4 %). Only negligible amounts of PS were found in any of the spermatozoal plasma membrane samples. The second major class of membrane lipids were the sterols which accounted for approximately 16.5% of the membrane lipid composition. In this study, cholesterol was the major sterol found in the sperm's plasma membrane (72.1%), with desmosterol making up the remaining 27.9%. When plasma membrane amounts of phospholipids and sterols were analyzed per 10^9 sperm cells in control boars, cholesterol ($P = 0.15$), desmosterol ($P = 0.13$) and total sterols ($P = 0.23$) remained constant throughout the study. In the controls, total phospholipids did show an ever so slight fluctuation in membrane composition ($P = 0.047$); this fluctuation was shown to be primarily the effect of boar ($P = 0.01$) rather than by week ($P = 0.26$). With further analysis of the individual phospholipids, PC ($P = 0.024$), LPC ($P = 0.03$) and SPH ($P = 0.03$) fluctuated more between boars than did PG ($P = 0.41$), PI ($P = 0.55$) and PE ($P = 0.35$) over the 9 collection weeks.

Protein concentration was observed to fluctuate between ($P < 0.05$) and within the control boars ($P < 0.05$); however, upon further examination, it was determined that these fluctuations were random in order rather than of a noticeable trend. Total protein concentration per 10^9 sperm cells averaged 0.33 ± 0.03 mg (mean $\pm$ SEM).

A maximum of 19 different molecular weight proteins were found to be present in plasma membranes from control boar spermatozoa. Numbers of different proteins varied considerably between and within boars. Due to this extreme variability, no statistical analysis was performed on the data. A typical gel pattern over a 9-week period for plasma membrane proteins from control boar spermatozoa is presented in Figure 11. Average molecular weights for these 19 membrane polypeptides were 14, 15, 17, 20, 23, 26, 30, 35, 39, 44, 49, 55, 66, 73, 82, 90, 98, 103 and 111 kDa.

The effects of increased ambient temperatures on the biochemical composition of boar spermatozoal plasma membranes were dramatic and long lasting. During collection weeks 2 through 8 (7-55 days after initiation of or 2-50 days after completion of heat-stress treatment), a significant effect of treatment was observed with respect to plasma membrane sterol composition ($P < 0.01$), but not in the total phospholipid composition ($P = 0.068$) in the treated boar spermatozoal plasma membranes versus their paired controls (Figure 12). An initial, but non-significant, decrease in total phospholipids was observed at collection week 3; this decrease remained evident throughout the entire

Figure 11. Electrophoretic separation of proteins from the plasma membrane of control boar spermatozoa. After SDS-denaturing, 20 μg protein was loaded into each of the lanes which represented weeks -1 through 9. Molecular weight standards are indicated by arrows.

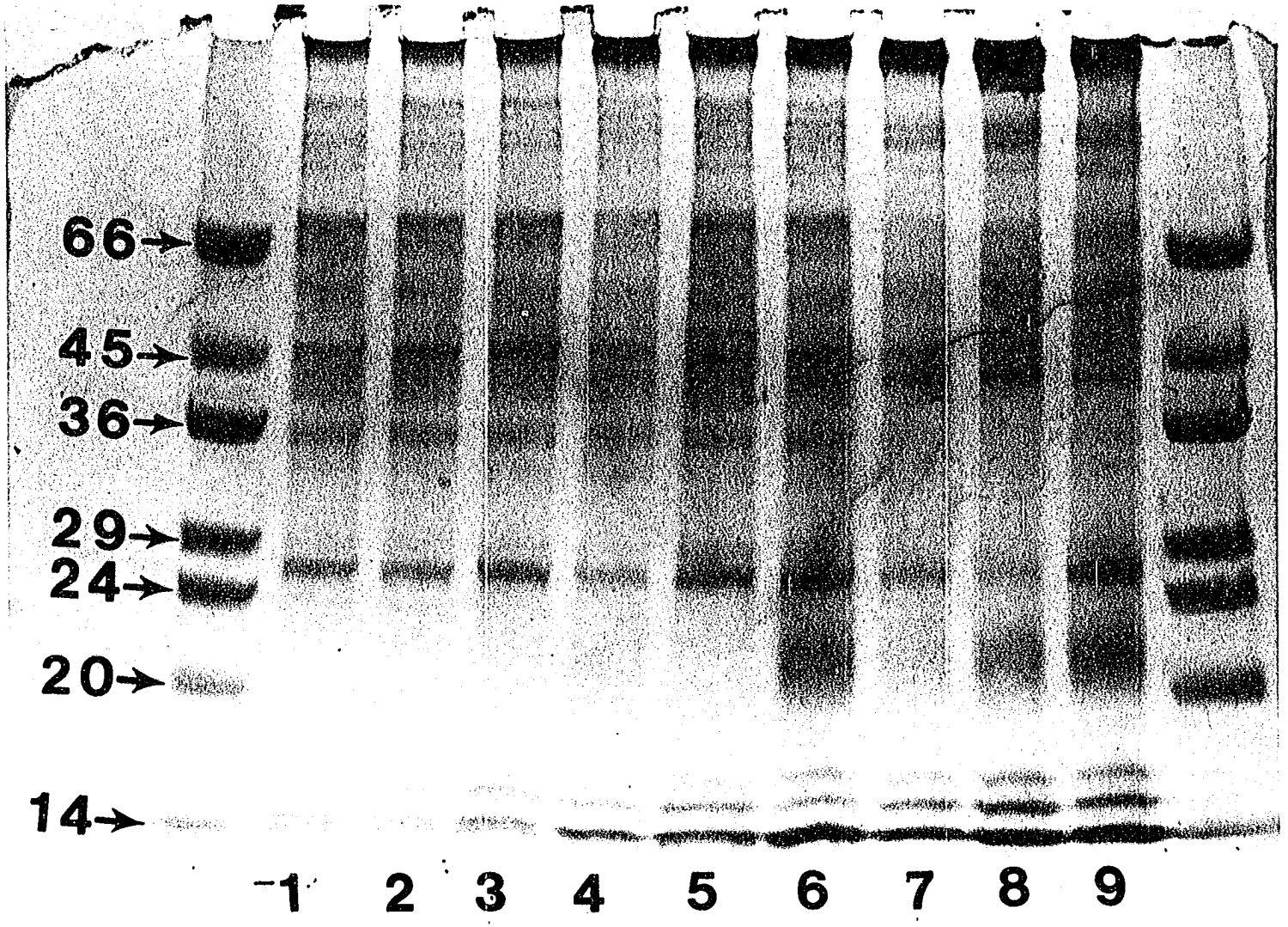
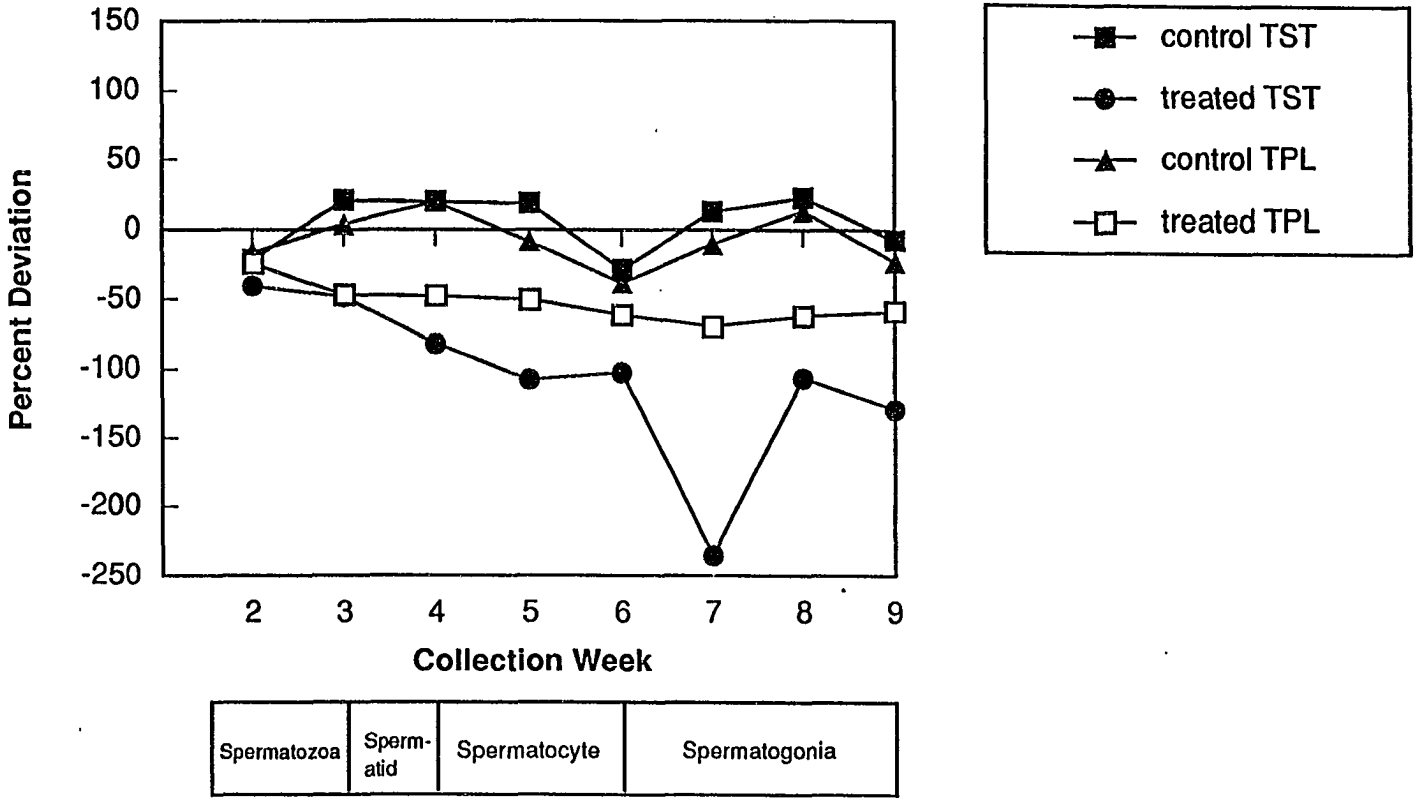

66
45
36
29
24
20
14
1
2
3
4
5
6
7
8
9

Figure 12. Collection week mean group differences as a percentage of pre-treatment mean group values for plasma membrane total sterols and total phospholipids in the paired-control/treatment groups. A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin to the biochemical alterations.


150
100
50
0
-50
-100
-150
-200
-250
Percent Deviation
2
3
4
5
6
7
8
9
Collection Week
control TST
treated TST
control TPL
treated TPL
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

experiment. Total plasma membrane sterols showed a gradual decrease which reached its lowest level during collection week 7 (Figure 12). Asubsequent rebound in the amount of total sterols in the plasma membranes of treated boar spermatozoa was observed during collection weeks 8 and 9, however, membrane sterols were still considerably depressed when compared to either the paired-control or pre-treatment values (Figure 12).

When examining total sterol:phospholipid (w/w) and cholesterol:phospholipid (w/w) ratios for treated versus control pairs (Table 3), the greatest alterations in either of these ratios occurred during collection weeks 5, 7 and 9 for total sterol-to-phospholipid, and collection weeks 5 and 7 for cholesterol-to-phospholipid.

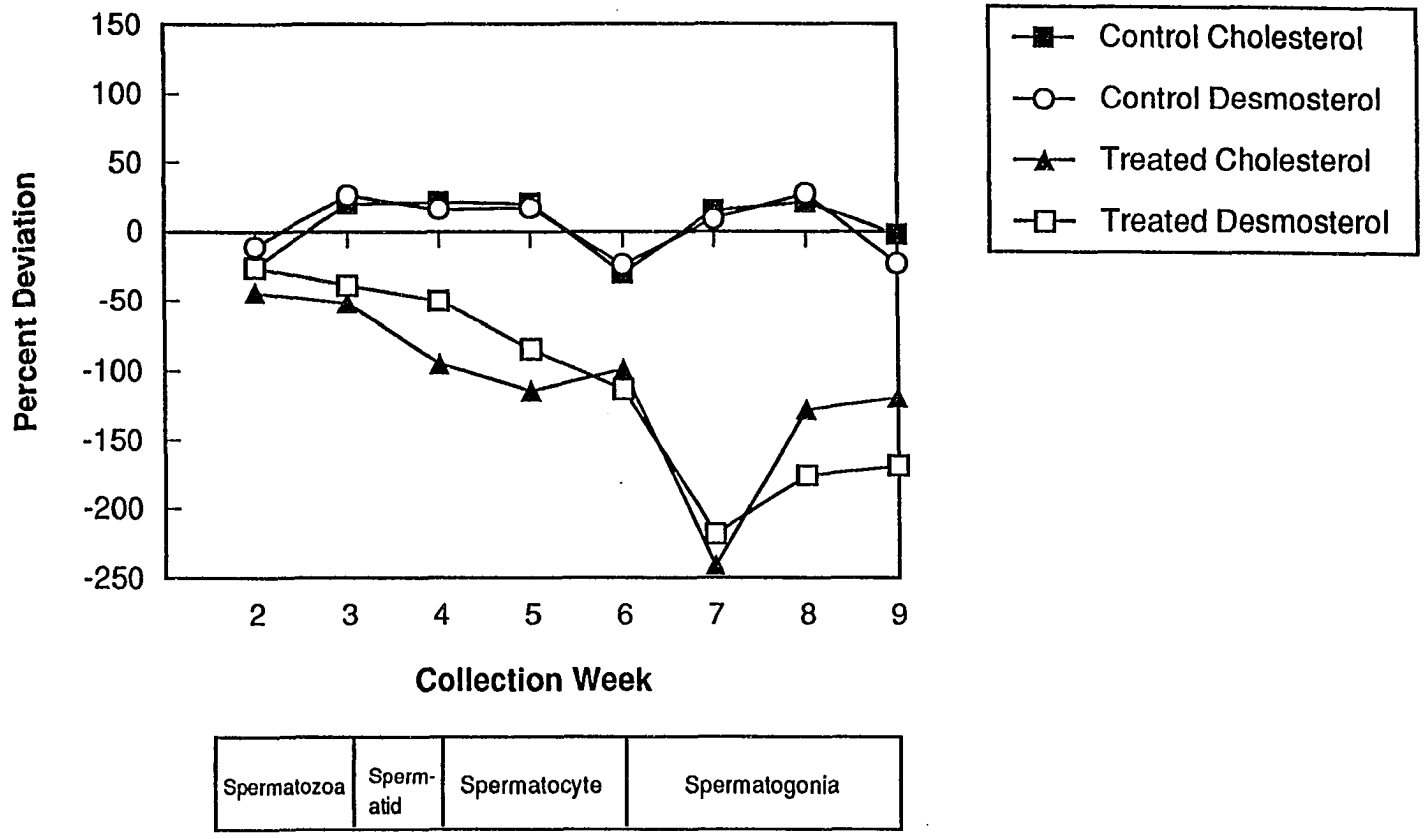
The individual sterols, cholesterol and desmosterol, were analyzed to ascertain if one or both were responsible for this significant decrease in plasma membrane total sterol composition. The content of both cholesterol ($P < 0.04$) and desmosterol ($0 < 0.01$) were found to be significantly affected by heat-stress treatment (Figure 13). Both sterols showed a gradual decrease in their mole concentrations within the plasma membrane of treated boars, reaching their lowest concentrations during collection week 7. During collection weeks 8 and 9; the desmosterol:cholesterol ratio reached its lowest value (0.24) when compared to that of the paired controls (0.30).

When assessing the relative amounts of the individual phospholipids in the treated and paired control groups, PC remained the

Table 3. Ratio (w/w) of cholesterol to phospholipid and total sterol to phospholipid in plasma membranes harvested from control (n = 4) and treated (n = 4) boar ejaculates over a 9-week collection period (-1 = 1-week prior to, and weeks 2-9 after exposure of treatment group to increased ambient temperatures)

Collection Week	Cholesterol:Phospholipid		Total Sterol:Phospholipid	
	Control	Treated	Control	Treated
-1	0.24	0.23	0.34	0.30
2	0.23	0.21	0.33	0.28
3	0.29	0.29	0.42	0.38
4	0.26	0.23	0.36	0.32
5	0.33	0.22	0.46	0.29
6	0.30	0.30	0.43	0.39
7	0.32	0.22	0.44	0.30
8	0.28	0.27	0.39	0.39
9	0.31	0.26	0.41	0.32

Figure 13. Collection week mean group differences as a percentage of pre-treatment mean group values for plasma membrane cholesterol and desmosterol in the paired-control/treatment groups. A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin of the biochemical alterations.


150
100
50
0
-50
-100
-150
-200
-250
Percent Deviation
2
3
4
5
6
7
8
9
Collection Week
Control Cholesterol
Control Desmosterol
Treated Cholesterol
Treated Desmosterol
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

most abundant phospholipid in the plasma membrane. A significant effect of treatment was observed with respect to PC ($P < 0.05$) and LPC ($P < 0.01$) content. The effects of heat-stress on PC content were most pronounced during collection weeks 7 and 8 (Figure 14); whereas, LPC was primarily decreased only during collection week 7 (Figure 15). This resulted in a decrease in the LPC:PC ratio from 0.20 (pre-treatment value) to a low of 0.09 during collection week 7, and quickly rebounding to its original value of 0.20 for final collection week 9. The remaining phospholipids PG, PI, PE and SPH were found to not change dramatically ($P > 0.05$) in the plasma membrane from that of their paired controls as a result of heat-stress (Figures 16 thru 19, respectively).

Fluctuations in the order of decreasing phospholipid content in the plasma membrane were found to occur in the study within the treated group. During collection week 2, LPC rather than PG made up a greater proportion (10.9 vs. 10.1 %) of the phospholipid content; additionally, during collection weeks 5 and 7, PG contributed more to the phospholipid component of the plasma membrane than did SPH (16.8 and 26.3 % vs. 14.9 and 14.7 %, respectively). During the remaining collection weeks, phospholipid compositions were proportional to that observed in the plasma membranes from the paired controls.

No significant difference in protein concentration was observed between the paired treated and control boars. Complete analysis of spermatozoal plasma membrane proteins from the heat-stress treatment group was performed on only 2 out of the 4 boars used in this study; technical failure resulted in the unrecoverable loss of the protein

Figure 14. Phosphatidylcholine (PC) composition (%) of sperm plasma membranes harvested from control and treated boar ejaculates (n=69) over a 9-week collection period. (-1 = 1-week prior to and weeks 2-9 after exposure of treatment group to increased ambient temperatures). A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin of the biochemical alterations.

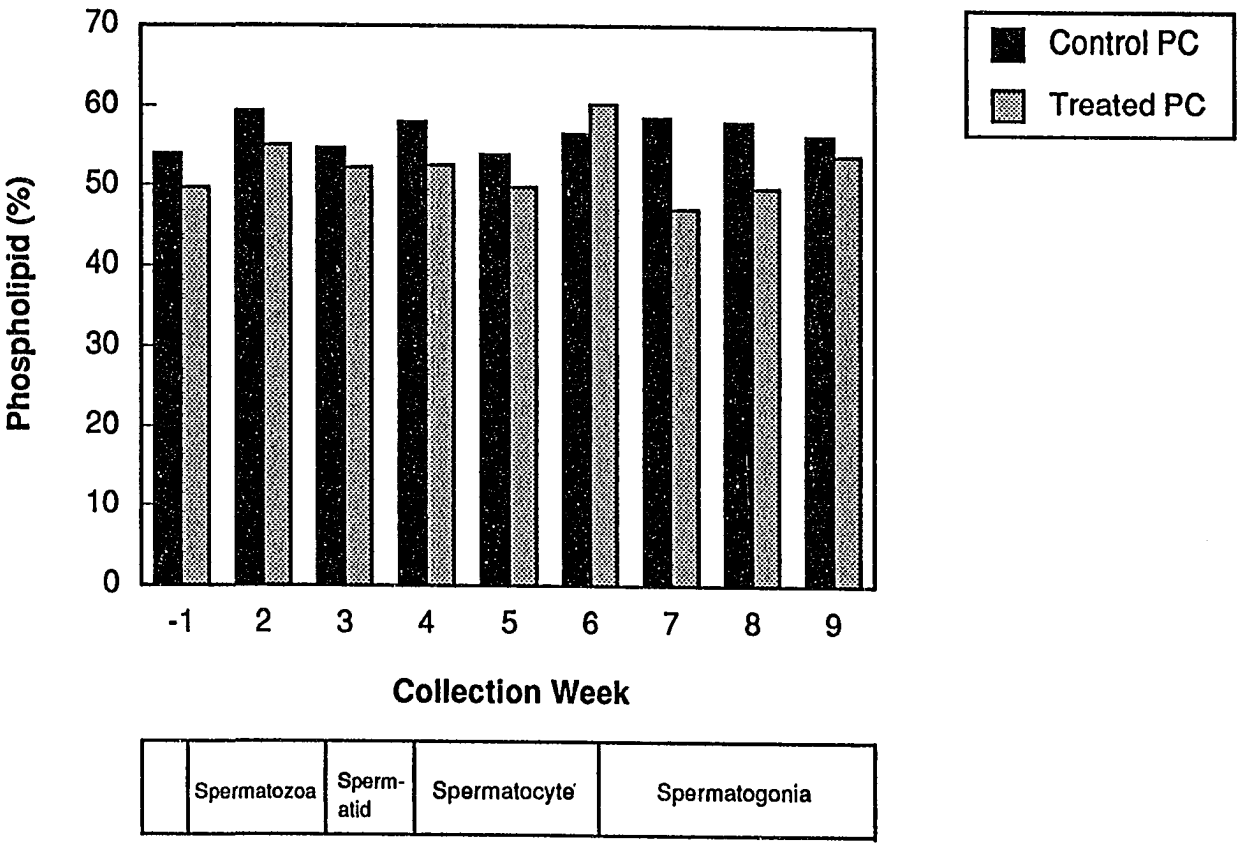

70
60
50
40
30
20
10
0
Phospholipid (%)
-1
2
3
4
5
6
7
8
9
Collection Week
Control PC
Treated PC
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

Figure 15. Lysophosphatidylcholine (LPC) composition (%) of sperm plasma membranes harvested from control and treated boar ejaculates (n=69) over a 9-week collection period. (-1 = 1-week prior to and weeks 2-9 after exposure of treatment group to increased ambient temperatures). A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin of the biochemical alterations.

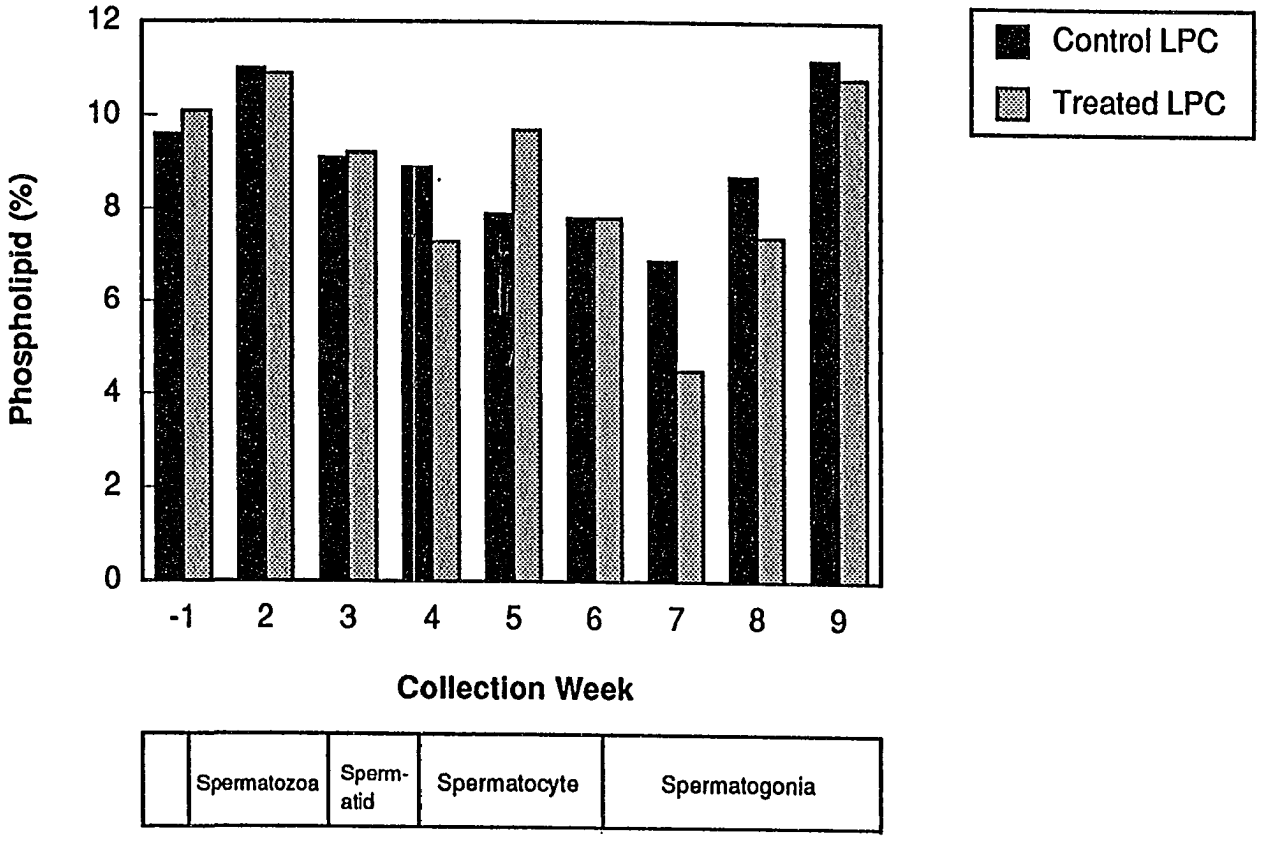

12
10
8
6
4
2
0
Phospholipid (%)
-1
2
3
4
5
6
7
8
9
Collection Week
Control LPC
Treated LPC
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

Figure 16. Phosphatidylglycerol (PG) composition (%) of sperm plasma membranes harvested from control and treated boar ejaculates (n=69) over a 9-week collection period. (-1 = 1-week prior to and weeks 2-9 after exposure of treatment group to increased ambient temperatures). A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin of the biochemical alterations.

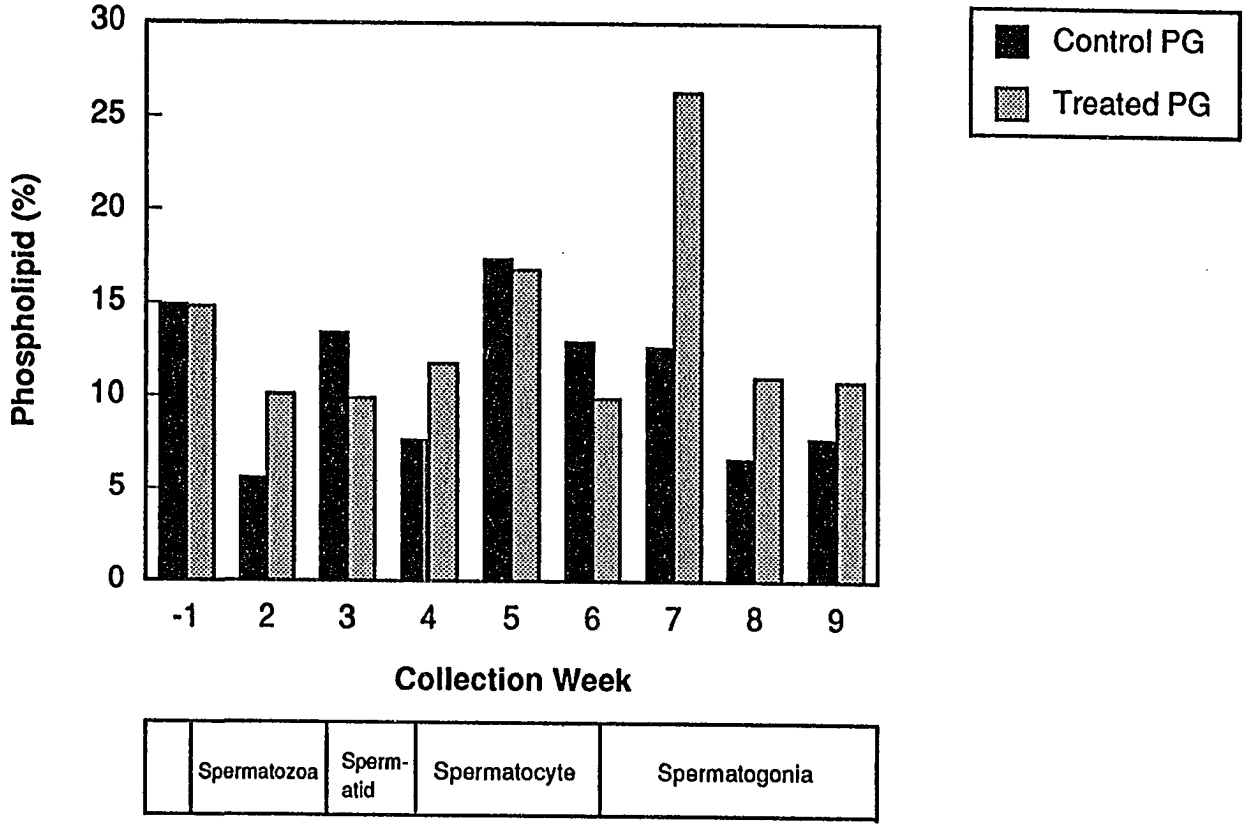

Control PG
Treated PG
Phospholipid (%)
30
25
20
15
10
5
0
-1
2
3
4
5
6
7
8
9
Collection Week
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

Figure 17. Phosphatidylinositol (PI) composition (%) of sperm plasma membranes harvested from control and treated boar ejaculates (n=69) over a 9-week collection period. (-1 = 1-week prior to and weeks 2-9 after exposure of treatment group to increased ambient temperatures). A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin of the biochemical alterations.

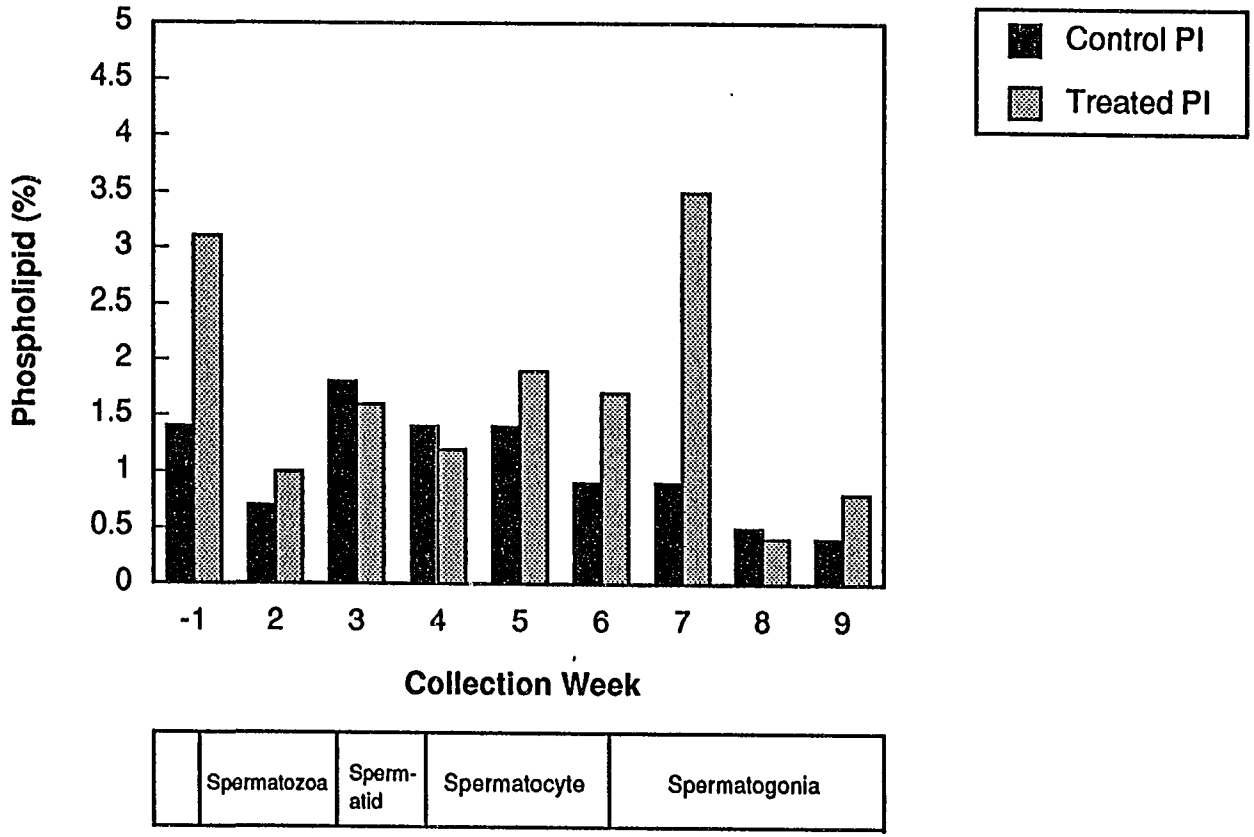

Control PI
Treated PI
Phospholipid (%)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-1
2
3
4
5
6
7
8
9
Collection Week
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

Figure 18. Phosphatidylethanolamine (PE) composition (%) of sperm plasma membranes harvested from control and treated boar ejaculates (n=69) over a 9-week collection period. (-1 = 1-week prior to and weeks 2-9 after exposure of treatment group to increased ambient temperatures). A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin of the biochemical alterations.

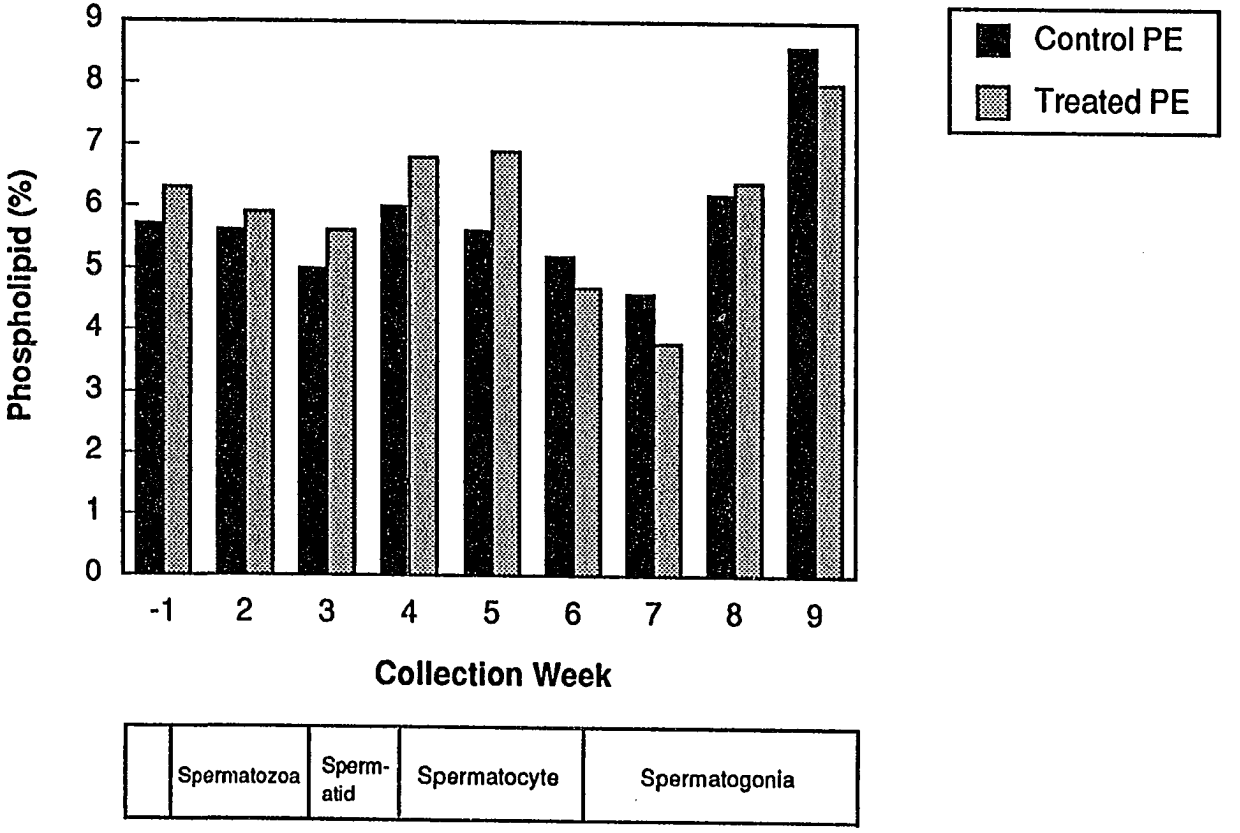
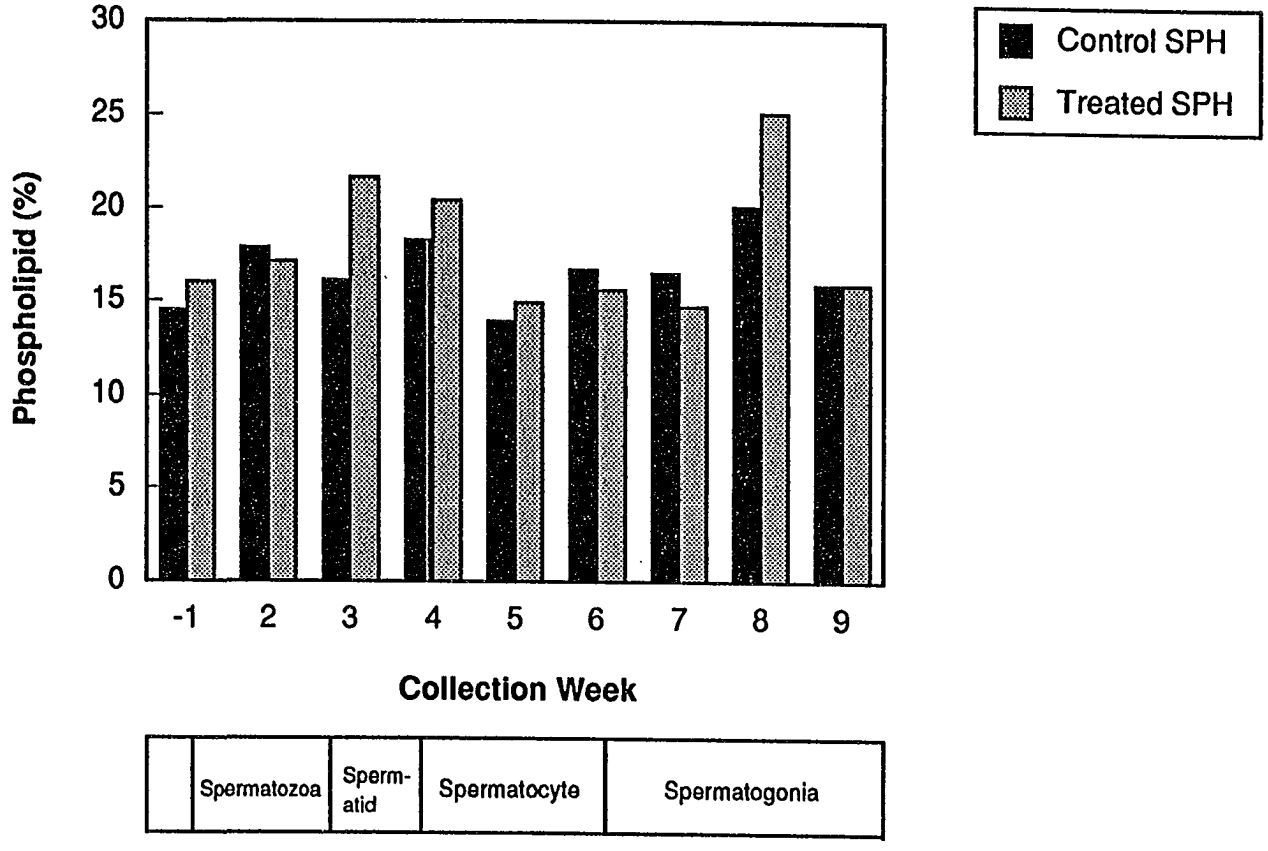
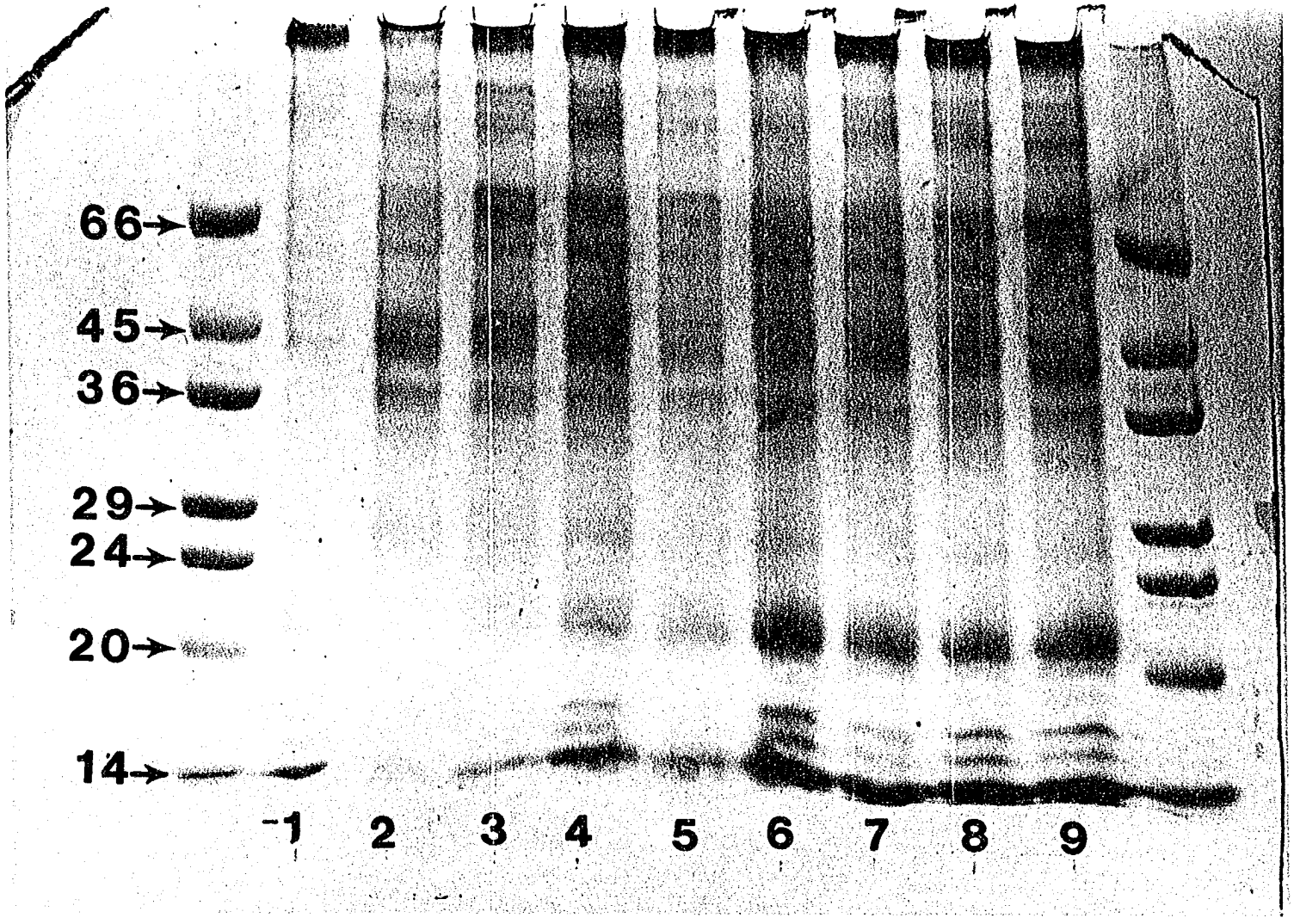

Phospholipid (%)
0
1
2
3
4
5
6
7
8
9
-1
2
3
4
5
6
7
8
9
Collection Week
Control PE
Treated PE
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

Figure 19. Sphingomyelin (SPH) composition (%) of sperm plasma membranes harvested from control and treated boar ejaculates (n=69) over a 9-week collection period. (-1 = 1-week prior to and weeks 2-9 after exposure of treatment group to increased ambient temperatures). A bar graph depicting the spermatogenic cell type at the time of treatment is provided to ascertain a possible origin of the biochemical alterations.


30
25
20
15
10
5
0
Phospholipid (%)
-1
2
3
4
5
6
7
8
9
Collection Week
Control SPH
Treated SPH
Spermatozoa
Sperm-atid
Spermatocyte
Spermatogonia

samples from the remaining 2 treatment boars. Within the treated group, variability in numbers of proteins observed between and within boars was evident. Because of this variability, no statistical analysis wasperformed on the data within the treatment group or for statistical comparison to the paired-control group. A maximum of 19 different membrane proteins were found to be present in plasma membranes from heat-stressed boar spermatozoa. These proteins were identified as 15, 16, 18, 23, 27, 31, 36, 39, 43, 46, 50, 56, 61.5, 69, 77, 91, 95, 101 and 107 kDa. No gross differences in protein number and estimated molecular weight, within expected variation, was found between the treated and control boar spermatozoal plasma membranes. A typical gel pattern over a 9-week period for plasma membrane proteins from heat-stress treated boar spermatozoa is presented in Figure 20.

Figure 20. Electrophoretic separation of proteins from the plasma membrane of heat-stress treated boar spermatozoa. After SDS denaturing, 20 μg protein was loaded into each of the lanes which represented weeks -1 through 9. Molecular weight standards are indicated by arrows.


66
45
36
29
24
20
14
1
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9

IV. DISCUSSION

The ambient temperatures used in this study were selected to simulate a typical diurnal pattern in the State of Iowa during the summer months (June through August). Boars exposed to increased ambient temperatures showed the most dramatic signalment within the first 8 hours of exposure (Table 1) when compared to the control boars. Thereafter, boar rectal temperatures generally followed a diurnal pattern in concert with the ambient temperatures which they were exposed too (i.e., elevated rectal temperatures after 8 hours of exposure to 35-37°C, 20-30% RH; less elevated rectal temperatures after 16 hours of exposure to 30-32°C, 20-30% RH). By the end of the second full day of heat-stress treatment, boars generally adapted to the increase in ambient temperature as evidenced by their near normal average rectal temperatures over the remaining treatment period (Table 1). Variations in individual rectal temperatures were observed throughout the 5-day treatment period among boars, however, these variations could not be attributed to any one boar. Along with an elevated rectal temperature, treated boars exhibited overt signs of heat-stress through an increase in respiratory rates, amount of water consumed, and a decrease in overall activity among the treated group. It is generally known that pigs are ineffective in the ability of decreasing an elevated body temperature through surface sweating as is commonly observed in the majority of other homeoisotherms. Because of this physiological impediment, pigs rely primarily on convection of body heat by direct

contact to an inert surface of decreased temperature or through surrounding environmental ambient temperature, either by the movement of air across their body surface and/or by dissipation of body heat through increased respiratory rates. At an ambient temperature of greater than 32°C, scrota of boars fail to dissipate body heat either by convection or conduction because the ambient temperature exceeds scrotal surface temperature (Setchell, 1970). Since blood perfusing the testis flows on the surface of the testis prior to traversing towards the mediastinum and into the testis, temperature of the testis tends to approximate the scrotal skin surface-air interface temperature which is more than likely proportional to the surrounding ambient temperature. So, even though the boar has the ability to reduce the temperature of the blood from the body core before it enters the testis, this temperature reduction mechanism will most likely fail to decrease the temperature of the blood below that of its surrounding ambient temperature; therefore, it is this direct perfusion of blood of an elevated temperature which exerts the primary effects of heat-stress upon the testis of the boar.

The effects of a short-term increase in ambient temperature on TSM, SM, and TSC in this study are in general agreement with past studies examining the effects of increased ambient temperature or an increased localized scrotal temperature (i.e., scrotal insulation) in the boar (McNitt and First, 1970; Christenson et al., 1972; Wettemann et al., 1976; Cameron and Blackshaw, 1980; Stone, 1981/82; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989). As depicted in Figure 4, the effects of heat-stress on TSM and SM were

initially evident 14-20 days (collection week 3) after initial heat-stress exposure; the effects of heat-stress were most pronounced on these variables 28-34 days after initiation of treatment (collection week 5), and were comparable to their pre-treatment values and those of the control group by day 48 (collection week 7). Similar results have been previously reported in the boar (McNitt and First, 1970; Wettemann et al., 1976; Cameron and Blackshaw, 1980; Larsson and Einarsson, 1984; Malmgren, 1989).

The effects of heat-stress on TSC were not as acute as observed with the other seminal variables. Although heat-stress did cause a significant ($P < 0.01$) reduction in TSC in the treated group, this decrease was not noticeably different from that of its pre-treatment values until approximately 42-48 days (collection week 7) after initial heat-stress exposure. As can be readily observed in Figure 4, a gradual decline in TSC is evident throughout collection weeks 3-7, however, the significance of this decline is most likely overpowered by the individual variability in TSC exhibited among the treated boars. This individualism among boars for TSC, with regards to heat-stress sensitivity, is not unusual in this type of study. In fact, heat-stress resistance has confounded results in past studies examining the effects of elevated ambient temperatures on the boar's spermiogram (Cameron and Blackshaw, 1980; Malmgren, 1989). This problem was attempted to be circumvented by using sibling paired boars in this study, however, it can and should be assumed that this observation is an individual expression of heat-stress resistance/adaptability and, as such, may need

to become a more scrutinized consideration in the design of future studies. As an added note, breed differences with respect to the effects of heat-stress on boar spermiograms has been previously elucidated too by McNitt and First (1970) when comparing purebred Yorkshire, Poland-China and reciprocal F1-cross semen quality characteristics after exposure to an increased ambient temperature.

Several different types of spermatozoal morphological abnormalities were observed in response to heat-stress. The most abundant abnormality observed in ejaculates from all the treated boars was the presence of proximal cytoplasmic droplets (Figure 5). A slight increase in the presence of proximal droplets was observed 14-20 days (collection week 3) after initiation of heat-stress exposure. A dramatic increase in proximal droplet numbers was observed during the following week (collection week 4), with a gradual drop to non-significant levels by collection week 7. Spermatozoal midpiece abnormalities (including distal midpiece reflexes, pseudodroplet defects, bowed midpieces) were noticeably present by day 14-20 (collection week 3); a gradual increase occurred over the next 14 days (collection weeks 4 and 5), with a gradual fluctuating decrease in their presence occurring throughout the remainder of the study. The other two predominant spermatozoal abnormalities seen, distal cytoplasmic droplets and abnormal heads (i.e., knobbed acrosomes, nuclear pouches, pyriform, microcephalic, macrocephalic, and round-short heads) were observed to increase in number during collection week 4 (22-27 days after initiation of heat-stress exposure); both of these aforementioned defects peaked in

their numbers within the ejaculate during collection week 5 (28-34 days), and then decreased to non-significant levels when compared to the control group by collection week 7 (42-48 days).

The presence and types of spermatozoal abnormalities seen in this study are comparable to that reported in previous studies examining the effects of heat-stress on the boar spermiogram (McNitt and First, 1970; Christenson et al., 1972; Wettemann et al., 1976; Cameron and Blackshaw, 1980; Stone, 1981/82; Larsson and Einarsson, 1984; Malmgren and Larsson, 1984; Malmgren, 1989). All boars in this study were found to exhibit the same types of spermatozoal abnormalities within the same temporal relationship, however, the degree of severity (i.e., number) was found to be dependent upon boar.

Except for distal cytoplasmic droplets, all the aforementioned spermatozoal abnormalities seen in this study have been shown to correlate with a decreased fertility. When comparing the interval between onset of heat-stress treatment and the appearance of morphological abnormalities with the spermatogenic cycle described by Swierstra (1968) in the boar, it becomes evident that the developing primary spermatocytes, secondary spermatocytes, round spermatids and elongating spermatids are most affected morphologically by elevated ambient temperatures. Along with these developing spermatozoa intimately attached to the Sertoli cell, it is of interest to note that a slight increase in numbers of midpiece and proximal cytoplasmic droplet defects seem to also occur with the detached developing spermatozoa that are located within the seminiferous tubule lumen and

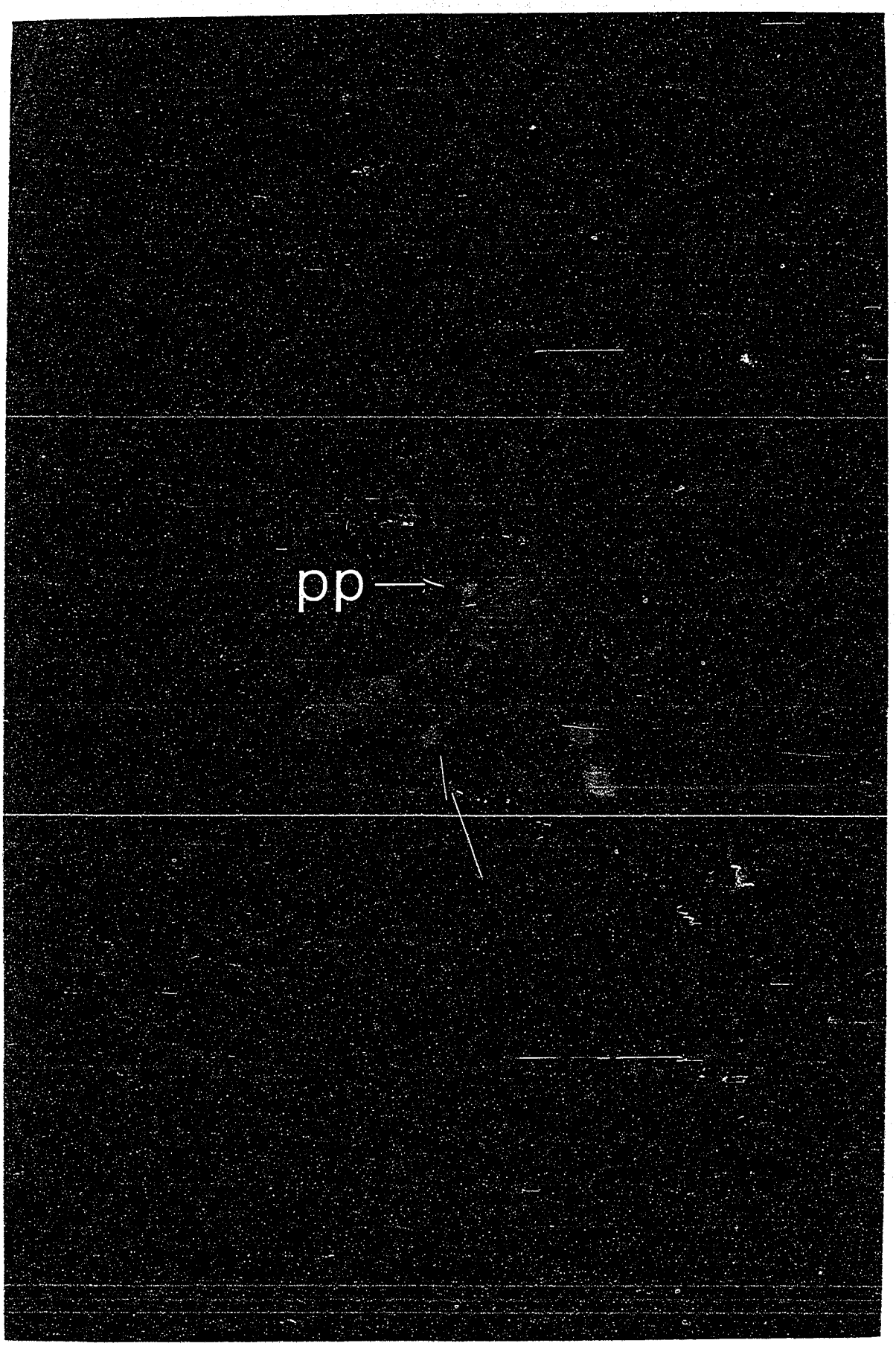
rete testis of the testis. It also appears that possibly Type B_2 spermatogonia may, somehow, be sensitive to heat-stress since all the aforementioned defects are still present, albeit in minor amounts, in the ejaculate during collection week 7 (42-48 days). It is difficult to say, however, whether the Type B_2 spermatogonia are directly and permanently affected by heat-stress or whether the Sertoli cells alter an otherwise normal daughter cell of the Type B_2 spermatogonia (i.e., primary spermatocytes).

At this point, it is important to address the significance of the presence of distal cytoplasmic droplets in the treated boar ejaculates. Although it is generally accepted that proximal cytoplasmic droplets are associated with infertility, distal cytoplasmic droplets have been reported to be common (up to 10-12%) in boar ejaculates and are, thus, not proported to affect fertility (Hurtgen, 1982). During spermiogenesis, as the round spermatid develops into an elongated spermatid while still attached to the Sertoli cell, the excess cytoplasm and its superfluous constituents of the developing spermatid are packaged into a droplet (i.e., proximal droplet) which is initially located at the neck of the spermatozoa when released from the Sertoli cell (Bloom and Nicander, 1961; Dott and Dingle, 1968). After release from the Sertoli cell, as the droplet-ladened spermatozoa undergoes maturation in the epididymis, this proximal droplet somehow undergoes movement from a proximal to a distal position (i.e., distal cytoplasmic droplet). Then, either in the tail of the epididymis or during ejaculation, this distal droplet is sloughed off the spermatozoa through

some type of exocytotic mechanism so that the plasma membrane of the spermatozoa remains intact. When observed under a microscope, the distal cytoplasmic droplet is usually displaced laterally just proximal to Jensen's ring. In this study, a significant percentage of spermatozoal displaying a distal cytoplasmic droplet appeared to have the droplet located more proximal to Jensen's ring and centrally straddling the midpiece rather than being laterally displaced (Figure 21). It is this author's opinion that this type of distal cytoplasmic droplet is unique and different from that which is normally reported as a distal cytoplasmic droplet. This unique type of distal droplet can be correlated to a biochemically altered plasma membrane, as will be discussed in more detail later in this discussion,. It is felt that this alteration in the plasma membrane precludes the normal release of this droplet and, therefore, makes this type of spermatozoa abnormality of major significance with respect to its normalcy and possible fertility potential.

The technique described and used in this study to isolate plasma membranes from ejaculated porcine spermatozoa yielded a highly purified plasma membrane preparation similar to that previously described in more extensive procedures performed by others (Gillis et al., 1978; Peterson et al., 1980; Nikolopoulou et al., 1985). The benefits of the technique described herein, when compared to that of the other published methods, include; 1) a much shorter time frame from semen collection to the final harvest of the sperm's plasma membrane, 2) avoidance of any direct contact of compounds which may or have been previously shown to alter

Figure 21. Phase contrast photomicrograph (1,250X) showing the abnormal distal cytoplasmic droplet (arrow) seen frequently in ejaculates from heat-stressed boars. Note normal appearing distal cytoplasmic droplet in other spermatozoa (dd).


pp

spermatozoa and/or their plasma membrane constituents, 3) a significant reduction in the number of centrifugation/washing steps used in the isolation procedure, in the hope of avoiding any membrane constituent leaching or alteration as described previously (Russell et al., 1985), and 4) a relatively economical, cost effective method requiring limited laboratory materials and technical training.

Light and election microscopy showed that the spermatozoal plasma membranes harvested in this study primarily came from the anterior, periacrosomal region on the sperm head (Figure 6). A relatively homogenous population of unilamellar vesicles ranging in size from 100 μm to 500 μm were observed. Acrosomes appeared to remain intact on the cavitated spermatozoa, and electron microscopic analysis failed to reveal the presence of any thickened, unilamellar electron-dense matrix vesicles which would be suggestive of acrosomal membrane contamination (Gillis et al., 1978; Parks et al., 1987). Other potential sources of membrane contamination are mitochondrial and cytoplasmic droplet membranes. Electron microscopic analysis of cavitated spermatozoa showed that the mitochondrial helix and its surrounding plasma membrane remained intact and retained their normal distribution around the sperm's midpiece, both strongly suggestive of little if any mitochondrial membrane contamination. Centrifugation of our freshly collected sperm-rich sample through the 1.0 M sucrose gradient effectively separated loose cytoplasmic droplets from the spermatozoa. Further microscopic analyses of our final plasma membrane preparation failed to reveal double-walled vesicles, these vesicles being

distinctively indicative of cytoplasmic droplet contamination (Noland et al., 1983; Parks et al., 1987).

Table 2 showed specific enzyme activities for plasma or other membranes of spermatozoa in the final plasma membrane preparation. Enzymes specific for boar spermatozoal plasma membranes, alkaline phosphatase and 5'-nucleotidase, both saw enrichment in the final membrane preparation relative to the post-cavitate homogenate. Alkaline phosphatase showed an average 3-fold increase in the sperm plasma membrane preparation; this enrichment equals or exceeds that which has been previously reported (Soucek and Vary, 1984; Nikolopoulou et al., 1985; Canvin and Buhr, 1989). Similarly, the enzyme 5'-nucleotidase increased in its specific activity an average 6-fold in the final plasma membrane preparation; this enrichment is highly comparable to the enrichment of this enzyme reported by others isolating boar sperm plasma membranes (Gillis et al., 1978; Nikolopoulou et al., 1985). No succinate dehydrogenase activity was observed in our final membrane preparation, an enzyme marker used to detect mitochondrial membranes. This lack in specific activity, along with our microscopic analyses, only reaffirms little if any contamination of our membrane preparation by mitochondria; similar results, again, have been reported in previous studies (Gillis et al., 1978; Nikolopoulou et al., 1985). Lastly, specific activities for the acrosomal membrane marker, acrosin, decreased significantly doing our membrane purification protocol, indicating minimal acrosomal contamination. Overall, the results from our simple, rapid, cost-effective and relatively atraumatic sperm plasma

membrane harvest procedure more than substantiates an effective method for removal and isolation of a relatively purified plasma membrane fraction from the area of the spermatozoa which is intimately involved in the physiological events of capacitation, the acrosome reaction and fertilization.

This study is the first to document the biochemical composition of ejaculated spermatozoal plasma membranes of individual boars (i.e., control boars) over a 9-week collection period. In this study, phospholipids accounted for approximately 83.5% of the total membrane lipid, with sterols making up the remaining 16.5% of the membrane lipid material. In comparison, membrane lipids from cauda epididymal sperm plasma membranes were reported to contain 76.2% phospholipid, 12.7% sterols, and 10.5% glycolipid/diacylglycerols (Nikolopoulou et al., 1985). And although actual percentages were not reported, Parks and Lynch (1992) found phospholipids made up the majority of the membrane lipid, with sterols being the second major lipid source. Although all studies are in agreement as far as with major and minor lipid components, the slight discrepancy in percentages reported by us when compared to Nikolopoulou et al. (1985; Table 4) can probably be attributed to the fact that sperm membrane lipids may undergo modification when exposed to seminal fluid at ejaculation. This alteration, of course, would not be evident if spermatozoa were harvested directly from the epididymides, as was apparently done by Nikolopoulou and co-workers. Additionally, our quantitative techniques were performed using HPLC, a technique more sensitive and specific than

Table 4. Comparison of plasma membrane vesicles obtained from cauda epididymal[a] and ejaculated boar spermatozoa (n.d. = not determined)

COMPONENT	CAUDA EPIDIDYMAL	EJACULATED
Total phospholipids (%)	76.2	83.5
Total sterols (%)	12.7	16.5
Cholesterol (%)	72.9	72.1
Desmosterol (%)	24.1	27.9
Other (%)	3.0	----
Types of Phospholipids		
PC	39.9 mol%	56.5%
SPH	23.0	16.6
PG	n.d.	11.0
LPC	2.1	9.0
PE	27.7	5.8
PI	2.9	1.0
PS	3.3	n.d.
Ratio of:		
Total sterol/phospholipid	0.17	0.40
Cholesterol/phospholipid	0.12	0.28
Desmosterol/cholesterol	0.32	0.39

[a]Data for cauda epididymal boar spermatozoa obtained from Nikolopoulou et al. (1985); data presented in mol% unless otherwise indicated, no formula(s) were provided to ascertain how mol% was calculated

that (i.e., TLC and phosphate spectrophotometric determination) used by Nikolopoulou and co-workers. Lastly, since our study decided not to analyze the plasma membranes for glycolipids or diacylglycerols due to their apparently minor proportion of the overall membrane lipid total and the disproportion in sample size (n = 36 in the present study vs. Nikolopoulou et al. in which only 4 samples were used), a direct and proportional increase in both phospholipid and sterol percentages would be expected to occur in our analyses.

When assessing individual phospholipid composition in ejaculated boar plasma membranes, PC (56.5 ± 1.9 %) was the most abundant phospholipid followed by, in decreasing order, SPH (16.6 ± 1.8 %), PG (11.0 ± 3.9 %), LPC (9.0 ± 1.3 %), PE (5.8 ± 1.1 %) and PI (1.04 ± 0.4 %); only negligible amounts of PS were observed. In the remaining studies, data were presented as mol%; no formula(s) were provided in these articles as to the derivation of mol%, especially given the fact that the attached PL molecular weight fatty acyl side-chains vary dramatically. Therefore, the value of comparing data of the present study to the remaining studies remains questionable at this time. In Nikolopoulou's study using boar cauda epididymal sperm plasma membranes, they also observed PC (39.9 ± 0.2 mol %) as their most abundant phospholipid, with PE (27.7 ± 0.1 mol %), SPH (23.0 ± 0.4 mol %), PS (3.3 ± 0.1 mol %), PI (2.9 ± 0.1 mol %), and LPC (2.1 ± 0.3) making up the remaining phospholipid constituents. Parks and Lynch (1992) reported the approximate phospholipid values of ejaculated boar sperm plasma membranes as choline phosphoglycerides (48 mol%), ethanolamine

phosphoglycerides (30 mol%), SPH (15 mol%), diphosphatidylglycerol (7 mol%), and PS/PI making up the relatively minor remaining proportion. Although our results are in agreement with the others that PC is the major phospholipid constituent in boar sperm plasma membranes, distinct differences in the remaining phospholipid proportions are evident. Possible causes for the variations in the different phospholipid constituents between the present study and the other two published studies are that ejaculated rather than epididymal (Nikolopoulou et al., 1985) spermatozoa were used in this study, and HPLC was utilized for phospholipid identification and quantitation rather than TLC and phosphate spectrophotometric determination (Nikolopoulou et al., 1985; Parks and Lynch, 1992). And along with the implementation of the more sensitive and specific technique (HPLC) in the present study, additional support for our findings can be provided in the aspect that the results from this study were calculated from 36 individual samples; in either of the other studies in question, only 4 samples were collected for data quantitation.

This study supports a recent finding by Parks and Lynch (1992) that PG is present in significant concentrations in the boar spermatozoal plasma membrane. In control boars, PG composed 11.0 $\pm$ 3.9% of the total membrane phospholipid. Only in one other species, the ram, has PG been found to be consistently present as a component of the spermatozoal plasma membrane (Parks and Hammerstedt, 1985; Hinkooska et al., 1989). In our study, it was originally believed that PG was phosphatidylglycerol since it had a similar retention time as that of

our purified standards; however, after publication of the recent article by Parks and Lynch (1992), it was decided to also assess the retention time of diphosphatidylglycerol, the component which they suspected to be present in boar spermatozoal plasma membranes. What we observed was indeed intriguing, retention times for purified phosphatidylglycerol and diphosphatidylglycerol appeared to not be significantly different from each other. Therefore, at this time, it can only be stated that phosphatidylglycerides are a consistent component of the boar spermatozoal plasma membrane; this includes both phosphatidylglycerol and diphosphatidylglycerol, until such time when a more precise distinction can be made.

When analyzing individual phospholipid content among boars and weeks, a slight fluctuation in total membrane phospholipid content ($P = 0.047$) was found to occur. This fluctuation could be attributed to differences among boars ($P = 0.01$) rather than by week ($P = 0.26$). Phosphatidylcholine, LPC and SPH appeared to fluctuate more between boars than did PG, PI and PE. In the only other study comparing the lipid content of porcine spermatozoa in relation to frequency of ejaculation over time using 4 boars, significant ($P < 0.05$) differences were also found to occur among boars but not over time (Johnson et al., 1969); therefore, the results presented in this study examining sperm plasma membranes substantiates that which has been originally reported when analyzing lipid content in whole boar spermatozoa. Additionally, this observation leads one to inquire if these normally observed alterations in the biochemical properties of normal boar sperm PM's have

any affect on their fertility, a common observation when addressing fertility rates between boars with normal spermiograms - future research should address such a question.

Cholesterol (72.1 %) was found to be the major sterol of sperm plasma membranes, with desmosterol (27.9 %) being the only other quantitatively identifiable sterol in our plasma membrane preparation. These results are in agreement with the previous works performed on cauda epididymal boar sperm plasma membranes (Nikolopoulou et al., 1985) and ejaculated boar sperm plasma membranes (Parks and Lynch, 1992). Therefore, it appears that exposure of boar spermatozoa to its seminal plasma upon ejaculation does not significantly affect membrane sterol composition. This is not surprising given the fact that seminal plasma (0.23% lipid dry matter) contains only a small proportion of sterols (17.7%) when compared to its phospholipid (64.7%) content (Komarek et at., 1965). In this study, sterol content remained constant among the 36 ejaculates from the control boars throughout the entire 9-week collection period.

The average total protein concentration of our harvested plasma membrane sample, 0.33 $\pm$ 0.03 mg (mean $\pm$ SE) per 10^9 sperm cells, was lower than that reported by others (Nikolopoulou et al., 1985; Parks and Lynch, 1992). A possible reason for this discrepancy in relation to the Nikolopoulou study, revolves around the fact that random boar epididymides were harvested from a slaughter house for use in their experiment. It is this author's personal experience that at slaughter plants immediately after exsanguination, animals are put through a

scalding tank before introduction into the slaughtering line. It is normally in this slaughtering line that investigators obtain their tissue samples as it is the most appropriate place to avoid interruption in the normal flow of carcasses through the packing plant. If indeed hogs were scalded, normal scalding tank temperatures range from 60-63°C; the porcine epididymides which lie just beneath the scrotal skin would be exposed to extreme thermal variation. Therefore, any dramatic change in external temperature will have a proportionally dramatic temperature change in the epididymides. It is logical to assume that if these boars were scaled prior to epididymide removal, the instantaneous thermotropic increase would most likely alter the tissues with which the heat has conducted through (i.e., protease activation, protein coagulation, lipid-phase transitions, epididymal and sperm cell death, luminal protein fluctuations, etc.), thereby, possibly altering sperm cell plasma membrane biocomposition. Nowhere in their article do they address this scenario.

Identification of individual proteins yielded variable results between and within control boars. Difficulties were encountered during this study when analyzing sperm plasma membrane proteins due, in part, to the high amount of glycoprotein present in the samples. Additionally, sperm plasma membranes contain a substantial amount of protein which is greater than 150 kDa (Russell et al., 1984); this was evident in the present study by the observation that a noticeable amount of protein material remained at the top of each gel lane. When comparing individual proteins in the present study, 19 out of the 20

major proteins reported by Russell et al. (1989) were identified. It is believed that the other major protein (i.e., a 300 kDa glycoprotein), remained at the top of the gels in the present study. Membrane proteins identified in the present study are compared to those reported by Russell and co-workers (1984) in Table 5. Differences in techniques and subsequent analyses of the gels can most likely explain for the slight variation observed in the protein molecular weights of the present study to those reported by Russell and co-workers (1984). No apparent difference was observed between control and heat-stress treated boars sperm plasma membrane proteins with the limited data that were collected in the present study.

Results from this study demonstrate that a transient increase in ambient temperature alters the biochemical composition of the boar spermatozoal plasma membrane. Of greater interest was the observation that even when the spermatozoa from treated boars appeared to recuperate from the short-term heat-stress by regaining normal spermatozoal motility and morphology, biochemically the plasma membranes from these same spermatozoa are still deranged. Sterols seemed to be the most sensitive membrane component to heat-stress; the effects of increased ambient temperatures for 5 days produced a significant effect on membrane sterol composition by the fourth collection period (collection week 4; Figure 13). When compared with the spermatogenic cycle, this would correlate to the spermatid stage in spermiogenesis. A gradual decrease in sterol molar concentrations then occurred throughout

Table 5. Comparison of Coomassie-stained major plasma membrane proteins (kDa) identified in boar spermatozoa[a]

POLYPEPTIDE (R_f)[b]	RUSSELL ET AL. (kDa)	PRESENT STUDY (kDa)
0.1-0.2	300	n.d.
1.0	120	111
1.5	115	103
2.0	110	98
3.0	82	90
3.2	72	82
3.5	67	73
4.0	64	66
4.8	50	55
4.85	47	49
5.0	44	44
5.1	43	39
16.0	20	35
17.2	18.5	30
17.9	18+	26
18.0	18	23
18.2	17.5	20
18.5	16.5	17
18.6	16	15
19.1	14	14

[a]Data for comparison were taken from Russell et al. (1984); present study data obtained from control boar.

[b]Numerical designation of plasma membrane polypeptides according to Russell et al. (1983).

the following three collection weeks (collection weeks 5-7), reaching their lowest membrane concentrations 42-48 days after exposure to heat-stress. And although there was an increase in membrane sterol molar concentrations during the final two collection weeks, this slight increase failed to approach the concentration of either the pre-treatment or paired-control boar values. Both cholesterol and desmosterol were equally found to decrease in proportion to the decrease observed in total membrane sterols. Of additional interest was the observation that during collection weeks 8 and 9, desmosterol to cholesterol ratios reached their lowest value of 0.24 when compared to that of their paired-controls (0.30). Desmosterol appeared to fluctuate more than did cholesterol; at this time, no reason can be expanded upon for this observation. Overall, from these results it appears that along with the effect of heat-stress on spermatids, the developing spermatocytes and spermatogonia also appear to be sensitive to elevated ambient temperatures.

Two possible reasons are offered by the author which may explain for the observed decrease in membrane sterol composition. First, previous work has shown that de novo synthesis and overall conversion of squalene to cholesterol are temperature sensitive events in the rat liver (Bloch, 1985). Therefore, it may be possible that cholesterol and its derivatives become limited in their availability and, therefore, are unable to become utilized in the rapidly dividing spermatogonia cell plasma membranes. Subsequently, Sertoli cells may also have limited sterol availability during spermatogenesis, leading to a maintained

decrease in membrane sterols in the developing sperm cells. Secondly, it is possible that the developing spermatogonia may compensate for an increase in external ambient temperatures by decreasing membrane sterol content to maintain the cell's optimal homeoviscous adaptability. Once this compensation has occurred, the developing spermatocytes, spermatids, and spermatozoa may not have a mechanism by which to insert sterol if a subsequent change in temperature, as performed in our study, occurs; this would be entirely feasible given the fact that each of these aforementioned stages lack the subcellular organelles necessary for biochemical modification of their own plasma membranes; therefore, these cells must rely entirely on external mechanisms for membrane biochemical modification. Either directly or indirectly, these external mechanisms (if they indeed exist) fail to adequately adjust membrane sterol composition and, thus, leave a fully developed sperm cell with a plasma membrane that is functionally of a lesser stability than its normal counterpart. This alteration would then explain for the observed differences in the cholesterol to phospholipid and sterol to phospholipid ratios that are documented in (Table 3).

Increased ambient temperatures caused an observable, but nonsignificant affect on total phospholipid composition ($P = 0.068$) in the plasma membranes. This is not unexpected since a dramatic effect in the amount of membrane phospholipid content would most certainly compromise functional and structural integrity of the sperm's plasma membrane. Assessment of the effects of an increase in ambient temperature on individual membrane phospholipids showed that heat-stress

did significantly effect PC and LPC content, but did not greatly alter that of the remaining membrane phospholipids. The effects of heat-stress on PC content were most prevalent during collection weeks 7 and 8 (Figure 14); whereas LPC was decreased primarily during collection week 7 (Figure 15). Both of these phospholipids are known to prefer localization in the outer leaflet of the membrane bilayer (Hammerstedt et al., 1990). Therefore, this reduction would probably affect the plasma membrane via alterations in the outer bilayer leaflet - What affects this alteration would have on the plasma membrane are unclear at this time, however, it is known that these phospholipids prefer a planar configuration (i.e., polar head groups oriented towards bulk water with their hydrocarbon tails directed inward) which provides a continuous and stable permeability barrier, to that of the cylindrical forming hexagonal configuration (i.e., polar head groups oriented inward and hydrocarbon tails pointed outward), which are known to be extremely unstable and weak permeability barriers since they prefer arrangements similar to that observed in membrane fusion. The phospholipids known to prefer the hexagonal configuration (i.e., PG, PE, PI, and PS) are located primarily in the inner leaflet of the plasma membrane. There was no significant change in the molar concentration of these lipids in the treated group. Therefore, it may be possible that their increase, in proportion to the decrease observed for PC and LPC, may also produce a membrane of lesser stability than its normal counterpart. Collectively, due to the overall changes in membrane neutral and polar lipids, a weak, unstable permeability barrier ensues, leading to a

relatively delicate cell membrane. This instability may allow for perturbation of the sperm cell, and/or allow for an increase in the uptake or exchange of molecules in/out of the membrane which would lead to a premature destabilization of the membrane. Premature destabilization will affect the normal membrane changes which occur over a defined time-course relationship; these include the spermatozoa's physiological events of capacitation, acrosome reaction and fertilization. Therefore, although the freshly ejaculated spermatozoa may appear to be "normal" based upon light microscopic evaluation, these biochemical alterations of the membrane bilayer make the sperm abnormal and, thus, non-functional. The results presented in this study only tend to substantiate a need to develop and implement new techniques for semen analysis which go beyond the visual or microscopic level.

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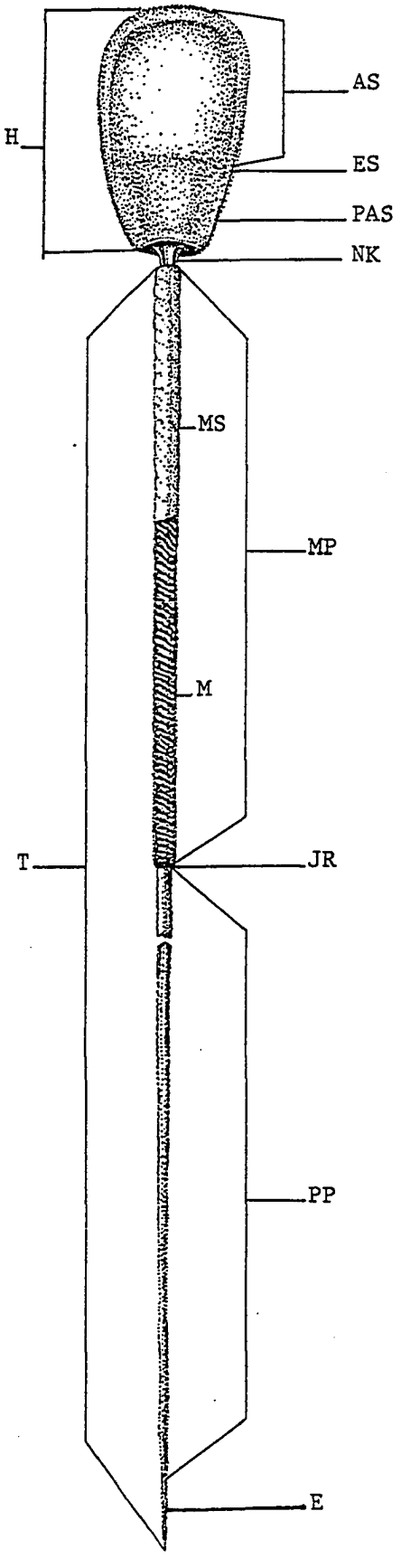
APPENDIX A

MID-SAGITTAL SECTION OF A MAMMALIAN SPERMATOZOAN

A = Acrosome proper
AS = Acrosomal segment of plasma membrane
CAF = Central axial filaments
E = Endpiece
ES = Equatorial segment of plasma membrane
FCS = Fibrous coil sheath
G = Glycocalyx
H = Head of sperm
IAM = Inner acrosomal membrane
JR = Jensen's ring
M = Mitochondria
MP = Midpiece
MS = Middle piece segment of plasma membrane
N = Nucleus (haploid)
NK = Neck
NM = Nuclear membrane
OAM = Outer acrosomal membrane
PAS = Post-acrosomal segment of plasma membrane
PF = Peripheral fibrils
PM = Plasma membrane
PP = Principal piece
T = Tail of sperm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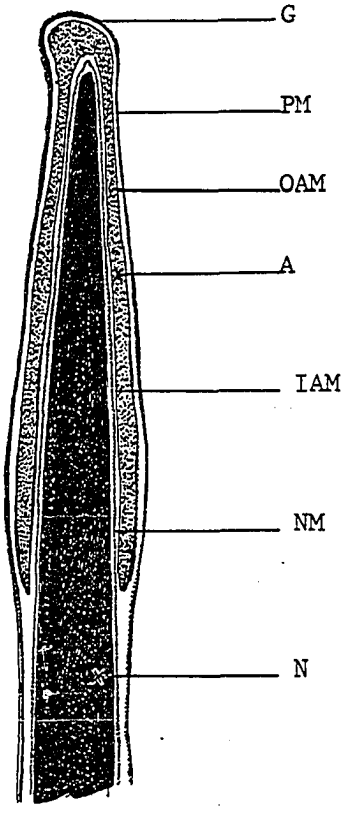
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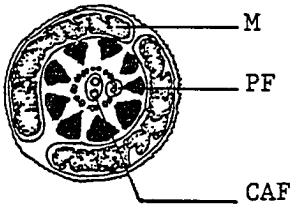
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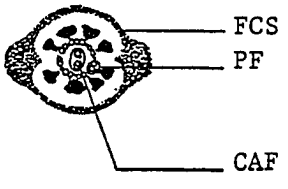
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