THE UNIVERSITY OF MANITOBA

FACTORS AFFECTING THE FLUIDITY OF BOAR SPERM MEMBRANES

BY

© ANDREW T. CANVIN

A Thesis Submitted to the Faculty
of Graduate Studies in Partial Fulfillment
of the Requirements for the Degree
of

MASTER OF SCIENCE

DEPARTMENT OF ANIMAL SCIENCE

WINNIPEG, MANITOBA

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ABSTRACT

Problems in long-term preservation of boar sperm have plagued the swine artificial insemination industry. This study has hypothesized that reduced fertility of stored boar sperm may be a consequence of damage caused to the plasma membrane on the head of the sperm during the storage process. Three experiments were undertaken to identify the effects of cooling plus reheating or constant temperature in the presence or absence of calcium upon the viscosity of the sperm body (SBM) and head plasma (HPM) membranes from three sources of semen: whole ejaculate, sperm rich fraction, extended.

The SBM from all sources underwent an increase in viscosity with variable temperature treatment. Calcium significantly decreased the viscosity of the whole ejaculate SBM, while it increased that of the sperm rich fraction and extended semen SBM under this temperature regimen.

The HPM under variable temperature, displayed the greatest viscosity increase in the whole ejaculate, while the extended semen was least affected. Calcium did not affect viscosity where whole ejaculate or extended semen was used, however there was a calcium effect detected for the sperm rich fraction. Distinct breakpoints were detected across

most sources for the viscosity increases, indicating lipid phase shifts.

At constant temperature, unique viscosity patterns and breakpoints were seen in the SBM from all sources. The viscosity in the presence of calcium was greater for the whole ejaculate and the sperm rich fraction. However, no effect was detected for the extended semen SBM over the time period studied. Unique breakpoints were detected for the SBM from all sources.

Increases in viscosity of the HPM at constant temperature were potentiated by calcium in all sources. Breakpoints were detected in the whole ejaculate HPM in the presence and absence of calcium, and in the sperm rich fraction HPM in the absence of calcium. No breakpoints were detected in the sperm rich fraction HPM in the presence of calcium or in the extended semen HPM in the presence or absence of calcium. Boar sperm membranes from each source were uniquely affected by time, temperature and presence or absence of calcium.

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LITERATURE REVIEW

1.1 INTRODUCTION:

The first documented report of artificial insemination (AI) was in 1780, when Spallanzani inseminated a bitch which resulted in the birth of 3 pups (Foote, 1982). Artificial insemination remained only of scientific interest until the early 1900s, when it began to be applied to livestock breeding in Russia and Japan (Foote, 1982). In 1914, the first artificial vagina, which enabled quick, easy collection of semen, was invented by Amantea at the University of Rome. The discovery of the artificial vagina may be the single most important development in the history of AI, as it continues to be used for collecting semen of bulls, rams, bucks, and stallions (Bearden and Fuquay, 1984). In 1936, the first known AI organization in the world opened in Denmark (Bearden and Fuquay, 1984).

Semen collected by the artificial vagina originally had to be used the same day in order to obtain good fertility. Phillips and Lardy (1940) formulated a yolk-phosphate buffer which allowed the sperm to remain viable and fertile for three to four days. Salisbury et al. (1941) replaced the

phosphates with sodium citrate which allowed greater visibility of the sperm under the light microscope.

Dissemination of reproductive diseases, such as vibriosis, was a problem. Almquist of Pennsylvania State University discovered that the inclusion of penicillin and streptomycin in extenders eliminated the spread of these diseases. This was reflected through increased conception rates with AI (Bearden and Fuquay, 1984).

Polge and colleagues (1949), considered the 'fathers of cryopreservation of semen', were the first to document successful freezing and thawing of semen without total loss of motility. Those experiments were performed on fowl semen using 40% glycerol (Polge et al., 1949). The transfer of this technology to freezing mammalian sperm was at first unsuccessful (Bearden and Fuquay 1984). However, Parkes and Polge eventually did achieve good results by allowing the sperm to equilibrate in the presence of glycerol for a period of time before subjecting it to deep-freezing on dry ice (Bearden and Fuquay, 1984). American Breeders Service introduced liquid nitrogen as a refrigerant for the processes of freezing and storage of semen in 1957. The latest major development was the invention of the straw, requiring less storage space and also enhancing sperm survival in smaller and smaller volumes (Bearden and Fuquay, 1984).

1.2 THE ADVANTAGES OF USING AI:

The use of AI has grown concommitantly with the recognition of certain advantages. These have brought about a rapid expansion of AI with the largest growth being in the cattle industry (Sherman, 1965; Foote, 1982; Bearden and Fuquay, 1984). These advantages include:

1. availability of the best progeny-tested genetically superior sires to all producers at a reasonable cost;
2. reduction in the expense and potential hazards caused by keeping sires on the farm;
3. control of venereal and other diseases which may be spread during natural mating;
4. maximized use of semen, as one ejaculate can be diluted to inseminate many females;
5. alleviation of difficulties in breeding where:

   a) structural abnormalities and deformities in the female reproductive tract may interfere with normal sperm passage;

   b) age or abnormalities of the sire may not allow natural mating;

   c) discrepancies in size of male and female may lead to problems especially when using large old sires to breed small young females.

Intensive experimentation contributed to the greater understanding of optimum conditions under which sperm could be collected, evaluated, diluted and preserved for use in a short time frame, or cooled, frozen, stored, and thawed for use in the future (Foote, 1982). However, when these storage techniques, developed for cattle, were applied to other species, similar results were not easily realized (Foote, 1982). Use of AI for goats, sheep, swine and horses is not as widely practised as it is for cattle because of a host of management problems, economics, breeder's prejudices and also a lack of established methods (Graham et al., 1978).

1.3 SWINE ARTIFICIAL INSEMINATION:

The use of AI in the swine industry has undergone a steady but slow growth, even though boar semen was one of the first used in successful AI experiments in domestic livestock some 50 years ago (Milovanov, 1934).

1.3.1 Use of Fresh Semen for AI

Practical use of swine AI involves a larger input at the producer level to detect estrus for proper timing of insemination (Bearden and Fuquay, 1984). After estrus has been detected the producer must plan insemination times, order semen from a boar stud, and allow time for transportation to the farm, in order to have the semen ready for insemination on the 2nd day of estrus (Milovanov, 1934; Bearden and

Fuquay, 1984). The present state of our knowledge allows producers, under optimal conditions, to successfully obtain farrowing rates of 60 to 70% using a single insemination of 3 billion spermatozoa. A number of different extenders are used for sperm preservation (Pursel et al., 1973; Paquignon et al. 1984; Johnson et al., 1982; Corteel and Paquignon, 1984). In a recent report, farrowing rates of 91.7% with 10.0 piglets/litter were obtained with semen in Beltsville-1 (BL1) extender (Maynard et al., 1987). Inseminations were done at 12 and 24 h after standing heat was detected. These results were equal to those obtained naturally (Maynard et al., 1987).

To achieve these results, much research was conducted, and many diluents were formulated (Polge, 1956; Graham et al., 1971; Pursel et al., 1973; Larsson et al., 1977; Larsson, 1978; Johnson et al., 1980; Pursel, 1983; Paquignon, 1983; Corteel and Paquignon 1984). In 1956, Polge was one of the first to demonstrate and report the extension of boar semen with egg yolk/glucose for AI using fresh semen. The Illinois Variable Temperature (IVT) extender which was developed for fresh semen AI in cattle in 1957 by Van Demark and Sharma was modified for use in pigs by du Mesnil du Buisson and Jondet in 1961 (Reed, 1982). In 1966, Plisko formulated a simple diluent known as Kiev or Guelph. Many other diluents including Kharkov (Serdiuk, 1968), BL1 (Pursel et al., 1973), Beltsville Thawing Solution (BTS; Pursel

and Johnson, 1975) and Zorlesco (Gottardi et al., 1980) have also been developed, providing lesser or greater degrees of successful preservation (Reed, 1982). The Kiev and BL1 extenders are now the most widely used (Paquignon, 1983). Many researchers have confirmed the ability of these diluents to preserve the fertilizing ability of boar sperm for up to 3 days. Bariteau and coworkers (1976) compared the IVT and BL1 extenders and showed that BL1 maintained fertility of boar sperm for one more day than IVT ($p<.01$). Beltsville-1 allowed a greater economy of semen compared to IVT, as inseminations on the day following collection required only one dose of semen to achieve conception (Bariteau et al., 1976). Even though the extenders contain the same components in different concentrations, Bariteau et al. (1976) were unable to confirm which of the chemicals made BL1 a superior extender. The Kiev extender subsequently has been tested against the BL1 extender and found to have superior preserving ability (Johnson et al., 1982). Using Kiev, Johnson and colleagues (1982) obtained significantly higher ($p<.0001$) farrowing rates, compared to the BL1 extender. Farrowing rates also decreased more using BL1 over 1 to 3 days storage as compared to the Kiev. This led Johnson et al. (1982) to conclude that Kiev is a better extender than BL1 for fresh semen. These results were confirmed by Paquignon et al. (1984), who reported that farrowing rate and prolificacy were not decreased when 3 billion spermatozoa extended in Kiev diluent was inseminated on the second day after collection.

Interest has been stimulated recently in BTS, a diluent originally designed for use as a thawing medium for frozen boar semen. The fertility results have shown BTS to have equal or greater sperm preserving ability compared to the Kiev extender (Aalbers et al. 1983; Pursel, 1983). Aalbers and coworkers (1983) showed an increased farrowing rate ($p<.05$) for BTS over that obtained with the Kiev extender. Pursel (1983) confirmed these results in a study which showed sperm preserved in BTS had a higher motility score ($p<.05$) after storage for 7 days than Kiev or other extenders. This measurement was taken after incubation at 37 C for 30 min. According to this study, boar semen motility can be preserved successfully for a period of 7 days at 23 C in BTS (Pursel, 1983) but no fertility results were reported. Based upon the above experiments, six AI centres in the Netherlands now use BTS as the extender for 600,000 primary inseminations per year (Aalbers et al., 1983). Paquignon (1982b in Paquignon, 1983), hypothesized that a lower sodium ion content in BTS and Kiev compared to BL1 and IVT is one of the reasons BTS and Kiev extenders are able to preserve fertilizing ability of boar sperm at a high level for a longer time period.

Because of large distances from boar studs to farms in Canada, it is of advantage to order semen in advance of estrus to ensure semen availability for insemination at the correct time. Advance ordering can be undertaken success-

fully in well-managed swine herds where 75% of sows will exhibit estrus 5 to 6 days after weaning (Alberta Swine AI Handbook, 1985). Use of the unique diluents mentioned above has allowed preservation of the fertilizing ability of the sperm for 3 days. While practised with good results in some operations, weaning to induce estrus does not always work and inseminations therefore can take place at the wrong time, leading to poor results with fresh semen.

1.3.2 Use of Frozen Semen for AI

It would be of great advantage to freeze semen to allow use of valuable proven sires after their prime reproductive period and even after their death (Sherman, 1965; Bearden and Fuquay, 1984). Despite sperm being one of the first mammalian cells to be effectively cryopreserved, preservation of sperm fertilizing ability in most species has only been realized for little more than a decade (Polge et al., 1949; Pursel et al., 1978a). One of the biggest problems in the development of sperm cryopreservation procedures has been the fact that researchers have not realized that techniques developed for preservation of one species' sperm may not work with sperm of a different species. "The fate of ejaculated sperm following dilution, cooling and freezing of extended semen is the most important and yet the most forgotten concern in the preservation of the male gamete" (Corteel and Paquignon, 1984).

Several diluents have been developed for cryopreservation of boar semen including egg-yolk glucose (Polge et al., 1970), Tes NaK glucose egg yolk buffer (Crabo et al., 1972), Beltsville F5 (BF5, Pursel and Johnson, 1975) and Hulsenburg (Westendorf et al., 1975 in Paquignon, 1983). These extenders contain sources of energy, buffers, adjuvants and cryoprotectants (Paquignon, 1983).

Glycerol acts as an excellent cryoprotectant in preservation of bull sperm, but decreases fertilizing capacity of swine sperm (Polge, 1956; Graham et al., 1971). Concentrations of 4 to 8% glycerol retained motility, but not fertilizing capacity (Polge, 1956; King and MacPherson, 1967). Polge and coworkers (1970) were the first to show a retention of sperm fertilizing capacity using surgical insemination of cryopreserved sperm. This renewed interest in long term preservation of boar sperm (Graham et al., 1971; Crabo and Einarsson, 1971; Pursel and Johnson, 1971a,b; Pursel and Johnson, 1975). With a 0 to 2% glycerol concentration in frozen-thawed boar semen, pregnancy was established (Crabo and Einarsson, 1971; Graham et al., 1971). Boar sperm were found to be very sensitive to glycerol when exposed at 20 C during the freezing and thawing process (Wilmut and Polge, 1974). In order to retain good fertilizing ability, boar sperm require unique extenders with a 2% glycerol concentration being optimal (Salamon et al., 1973; Wilmut and Polge, 1974; Pursel et al., 1978b). Many of the same procedures

and extenders discovered in these experiments are still in use today.

Egg yolk, an original ingredient in semen extenders for almost all species, increases motility and percentage of normal acrosomes in sperm after thawing (Visser and Salamon, 1974). Watson and Martin (1975) speculated that the egg yolk stabilizes the sperm membrane and protects it during the freezing process. Concentrations as low as 6% egg yolk protected bull, ram and boar sperm from injury during sharp temperature declines (Otashko et al., 1984). Kichev (1976), however, reported poor deep-freezing results using egg yolk with boar sperm, and suggested these were due to the inability of the seminal plasma proteins and egg-yolk to undergo physical-chemical binding. Generally, 20% egg yolk is utilized (Larsson et al., 1977; Pursel and Johnson, 1975). Graham and Crabo (1972) found that a synthetic detergent, sodium lauryl sulphate or Orvus Es Paste (O.E.P), increased the percentage of motile sperm with normal acrosomes after freezing and thawing. Pursel and colleagues (1978a) determined the optimum concentration of O.E.P. to be 1 to 2%. The effect of O.E.P., although not completely understood, is thought to be alteration of the composition of the egg-yolk rather than direct influence on the sperm membrane (Pursel et al., 1978a).

There have been many procedures developed for freezing boar semen, but the proven and most used methods are those

of Pursel and Johnson (1975), Paquignon and Courot (1976), Larsson et al. (1977) and Westendorf et al. (1975 in Paquignon, 1983). Each of these methods have some unique features, but they all require equilibration of the spermatozoa in the seminal plasma before centrifugation. This time allows for development of a resistance to damage caused during freezing and thawing (Paquignon, 1983). Pavelko and Crabo (1976) also found that during the equilibration period, sperm became coated with seminal plasma proteins. This may have a role in the fertilization process (Crabo and Hunter, 1975 in Larsson and Einarsson, 1976). All methods centrifuge sperm to remove the seminal plasma before the sperm are cooled below 15 C. This step increases the percentages of motile sperm and sperm with normal acrosomes after thawing (Pursel and Johnson, 1971b). There has been some concern about centrifugation causing physical damage to the sperm. Ibrahim and Kovacs (1982) were able to visually identify physical damage to the sperm which occurred during centrifugation. The amount of tail damage and disappearance of agglutination and motility upon centrifugation at 540xg (Ibrahim and Kovacs 1982) were said to be alleviated by decreasing the force applied and(or) centrifugation time (Larsson 1978).

After centrifugation the sperm are resuspended in a diluent without glycerol to a specified concentration of between 400 and 800 million sperm/ml (Paquignon and Courot,

1976). Above or below these concentrations, motility and percent normal acrosomes were affected (Graham and Crabo, 1972; Westendorf et al., 1975 in Paquignon, 1983). Most methods today use a concentration between 450 and 600 million sperm/ml (Paquignon, 1983).

Last, low concentrations of glycerol are added to the diluted sperm at temperatures of 5 to 7 C, immediately before freezing (Larsson et al., 1977; Pursel and Johnson, 1975). Semen is then quickly frozen by dropping small volumes of semen on dry ice (Nagase and Niwa, 1964 in Paquignon 1983). Lindemann et al. (1982) reported that freezing rate determined the amount of damage occurring in the plasma membrane of the sperm. The appearance of intracellular ice crystals within the cells during freezing caused damage to the structural elements of the sperm. Three of the four methods (Pursel and Johnson, 1975; Paquignon and Courot, 1976; Larsson et al., 1977) used the pellet technique on dry ice, whereas Westendorf et al. (1975 in Paquignon, 1983) utilized 5ml straws. Regardless of technique, the semen can then be stored indefinitely in liquid nitrogen (Larsson et al., 1977; Pursel and Johnson, 1975).

Thawing of frozen boar semen has been done by several methods including thawing pellets on a heated metal plate (Kozumplik, 1980), in a thawing solution (Pursel and Johnson, 1975; Larsson and Einarsson, 1976) or thawing semen preserved in straws in a water bath at 50 C (Paquignon,

1983). The procedure used to thaw semen has an important role in the ability of the sperm to retain good fertilizing capacity and may be one of the major causes of sperm death (Vincente, 1972). Survival rates measured by motility were 60 to 75% when semen was thawed at 85 C over a 10 second period. Vincente (1972) reported that this is understandable since sperm are damaged more by crystallization if exposed to the critical temperature zone of -35 to -15 C for longer periods of time. Thawing solutions promote faster thermal exchanges and greater semen quality (Pursel and Johnson, 1976). Larsson and coworkers (1977) recommended only a 30 second holding period for pellets at room temperature before dumping the pellets into a thawing solution. Rapid thawing was demonstrated to reduce damage to sperm (Larsson et al., 1977; Polge, 1976). Seminal plasma was used as a thawing solution with some success (Crabo and Einarsson, 1971; Crabo et al., 1972), but was judged impractical (Pursel and Johnson, 1975).

Pursel and Johnson (1975) outlined criteria for selection of an ideal thawing solution. These included maintenance of maximum sperm fertility, economy and simplicity of manufacture and storage (Pursel and Johnson, 1975). Beltsville Thawing Solution was found to be significantly superior to a host of other solutions, by allowing better post thaw acrosome morphology and sperm motility (Pursel and Johnson, 1974). Saline based solutions such as BTS (Pursel and John-

son, 1975), OLEP (Larsson and Einarsson, 1976) and INRA-ITP (Paquignon and Courot, 1976) and their respective procedures are now the most commonly used with sperm survivability in these solutions ranging from 32 to 85%, based upon farrowing results (Pursel and Johnson, 1975; Larsson et al., 1977; Ibrahim and Kovacs, 1982; Maynard et al., 1987).

1.3.3 Results and Problems with Frozen Semen

The freeze-thaw preservation of boar semen has not been perfected to date. Conception rates and litter sizes using frozen semen under ideal conditions are close to those required by producers (Paquignon et al., 1980; Kozumplik, 1980; Ibrahim and Kovacs, 1982), but do not equal those achieved with fresh semen insemination (Corteel and Paquignon, 1984; Maynard et al., 1987). Many factors contribute to the decreased fertility of frozen semen, including poor survivability of semen after it has been frozen, and practical problems at the producer level.

Insemination times are again of crucial importance, and more pressure is placed upon proper estrus detection when using frozen semen (Larsson and Einarsson, 1976; Paquignon, 1983). The inability of producers to estimate estrus and therefore predict insemination times of sows and gilts, dictates that sperm must be maintained for a long time period or that inseminations be done twice during the estrous period (Larsson et al., 1977; Maynard et al., 1987). Using two

inseminations per estrous period however, effectively cuts in half the number of females settled per ejaculate (Paquignon et al., 1980). Paquignon and coworkers (1980) were able to obtain good fertility results using single inseminations of frozen semen when accurate detection of estrus was practised.

Differences in inseminator techniques were also identified when using frozen semen (Johnson et al., 1979; Paquignon et al., 1980; Maynard et al., 1987). The farrowing rates obtained by different inseminators varied significantly ($p<.05$) from 44.3 to 62.4% (Paquignon et al., 1980).

1.4 DETERMINING SPERM FERTILIZING ABILITY INVITRO:

When determining the quality of a semen sample, the two initial criteria which are examined are motility and morphological characteristics (Paquignon, 1983). Motility, even though used routinely as an indicator of sperm quality, is not at all correlated with fertility of boar semen (Larsson, 1978). Measuring motility was found to be of limited use as good motility was observed in a semen sample which had no fertilizing capacity (Larsson, 1978). Pursel and colleagues (1972a) also reported that sperm which had good motility but damaged acrosomes lost their fertilizing ability. When storing semen, the techniques used should always consider preservation of sperm fertilizing ability (Corteel and Paquignon, 1984). The ultimate fertility test for comparing

the effects of the many diluents and extenders is the farrowing rate and prolificacy (Paquignon, 1983; Corteel and Paquignon, 1984).

An accurate method to estimate the in vivo sperm fertilizing ability needs to be developed to assess frozen-thawed sperm (Corteel and Paquignon, 1984). The procedure of determining motility and other characteristics of sperm after incubation at 37 C for several hours has been a standard practice (Paquignon and Courot, 1976; Paquignon, 1983). This involves the incubation of spermatozoa at 37 C for several hours, exposing the sperm to conditions which are similar to those encountered in the female reproductive tract. After this incubation, decreased survival and motility of sperm has been correlated with depressed fertilizing ability (Pursel et al., 1978c). Consequently, it was found that boars with poor conception rates produce sperm that lack an ability to withstand the effects of incubation (Larsson et al., 1980). However, the correlation between the incubation results and fertility, while the best available, is still low (Paquignon, 1983). The counting of sperm flagella and head abnormalities aids in obtaining an accurate assessment of fertility (Larsson et al., 1980).

Schilling and Vengust (1984) found a correlation between characteristics of sperm after fresh storage and and prospective freezability of the same boars' semen. They reported a positive although insignificant correlation

(r=.50) for motility and normal apical ridge (NAR) between frozen-thawed and fresh stored semen samples (Schilling and Vengust, 1984). It was therefore hypothesized that the motility of fresh stored semen could be used as an indicator of the freezability of that same boar's semen(Schilling and Vengust, 1984). Pursel and coworkers (1972b) also found motile boar sperm can develop swollen apical ridges even when stored at room temperature. Semen samples which were greatly affected by this defect were not fertile (Pursel et al., 1972b).

Acrosome morphology, identifying damage to the apical ridge of the acrosome, has often been used as a semen quality test in AI programmes for bulls (Aalseth and Saacke, 1985), rams (Jones and Martin, 1973) and boars (Ibrahim and Kovacs, 1982). Ibrahim and Kovacs (1982) found that the acrosome was the main component of boar sperm damaged during deep-freezing. Damage, visible by light microscopy, was observed at the apical ridge and the outer acrosomal membrane (OAM). Damage to the apical ridge appeared to be one of the reasons for decreased fertility of stored semen (Aalseth and Saacke, 1985; Ibrahim and Kovacs, 1982). It appeared that changes in the structure of the OAM released enzymes that lowered fertilizing capacity (Ibrahim and Kovacs, 1982; Lindemann et al., 1982).

Another major reason for decreased fertilizing capacity could be decreased viability of sperm in the female repro-

ductive tract (Larsson, 1978). Frozen and thawed sperm had a depressed fertilization rate because of a low survivability and could be rendered non-functional before they reach the oviduct (Pursel et al., 1978c; Polge et al., 1970; Wishart, 1985). Polge et al. (1970) hypothesized that the sperm were subjected to phagocytosis within the uterine environment, and could not enter the oviducts.

Lindemann and Gibbons (1975) showed that bull sperm remain motile following removal of the cell membrane with Triton X-100 detergent. In 1982, Lindemann and coworkers found that the processes of freezing and thawing do not decrease the motility of the de-membranated sperm. Therefore, the observed loss of motility was not due to damage to the motility apparatus of the sperm but rather due to membrane damage. This damage to the plasma membrane (PM) increased its permeability, allowing endogenous ATP to escape out of the sperm (Lindemann et al., 1982). Even though motility can be stimulated in bull sperm without the PM, the sperm PM must be maintained for proper cellular integrity as well as to undergo the events leading to fertilization (Holt and North, 1984). The structure of the PM must therefore be preserved in order for the sperm to be motile, undergo capacitation and the acrosome reaction and complete the process of fertilization (Holt and North, 1984).

1.5 CHANGES IN THE SPERM DURING EVENTS LEADING TO FERTILIZATION:

1.5.1 Capacitation

Capacitation takes place after the sperm have spent some time in the female reproductive tract; this time varies among species (Peterson and Russell, 1985). Capacitation can also be stimulated in vitro (Peterson and Russell, 1985). Pursel (1980) reported that processing of boar sperm by removing seminal plasma and extending in BF5 and BTS diluents decreased the time required for capacitation. However, fresh extended semen was found to have a longer capacitation time than frozen semen (Wheeler and Seidel, 1986). This may explain the importance of inseminating as closely as possible to the time of ovulation in order to obtain good fertility with frozen semen.

The processes of capacitation and acrosome reaction are closely linked, but capacitation involves several specific biochemical and physiological changes (Berruti and Franchi, 1986; Nikolopoulou et al., 1986a). It also involves changes of both integral and surface constituents within the PM (Holt and North, 1984). There is a relocation of surface proteins from the head of the sperm to the flagellar region (Saxena et al., 1986a), and a loss of the quilt pattern in the PM on the head of the sperm as seen by electron microscopy (Friend, 1977).

These changes in the makeup of the boar sperm PM that occur with capacitation were enhanced by the presence of calcium (Saxena et al., 1986a). Modifications in the transport of calcium ions across the PM were an integral part of capacitation (Peterson and Russell, 1985). This was mediated by a calcium pump or Ca^{2+} and Mg^{2+} ATPase present in the PM of boar sperm (Ashraf et al., 1982; Ashraf et al., 1984). Calmodulin, a calcium binding protein, has also been identified in boar sperm membranes (Casale et al., 1986). Capacitation was allowed to occur after removal of a calcium transport inhibitory protein (Rufo et al., 1984). During capacitation, calcium is hypothesized to stimulate calmodulin which then causes an increase in adenylyl cyclase and cAMP synthesis (Rogers and Bentwood, 1982). Capacitation also appears to be a reversible process, as migration of the surface proteins back to the head region of the PM occurred when seminal plasma was added to capacitated sperm (Peterson et al., 1986).

Calcium increases similar to those of capacitation were also found to occur in ejaculated ram sperm after dilution and(or) cold shock (Robertson and Watson, 1986). These influxes of calcium were reversed upon rewarming (Robertson and Watson, 1986). This effect was seen if the calcium pump, important in maintenance of intracellular calcium levels, was not damaged by the treatment. Damage to the calcium pump was characterized by a steady and irreversible cal-

cium build up inside the cell (Robertson and Watson, 1986). Peterson and Russell (1985) reported that an increase in intracellular calcium triggered the acrosome reaction.

1.5.2 Acrosome Reaction

The acrosome is an organelle, underlying the PM which covers the head of the sperm (Yanagimachi, 1981). The acrosome reaction involves fusion of the outer acrosomal membrane with the PM, exocytosis to lose these two vesiculated membranes (Yanagimachi, 1981), followed by a further influx of calcium into the cell (Vijayasarathy et al., 1982). There is conflicting information about where within the female reproductive tract the acrosome reaction takes place. Some researchers have reported it to occur after the sperm has bound to the zona pellucida, whereas others have said zona binding was not necessary, and the acrosome reaction occurred throughout the female tract (Shapiro et al., 1981). Yanagimachi (1981) has also found that the acrosome reaction occurred in vitro. The acrosome reaction ends with the release of hydrolases that disperse cells surrounding the ovum and allow sperm to penetrate the zona pellucida and bind to the vitelline membrane of the female ovum (Peterson and Russell, 1985).

1.6 THE SPERM PLASMA MEMBRANE:

One of the biggest errors made by many early reseachers was the failure to account for species-specific differences in biophysical characteristics, composition, and function of the plasma and intracellular sperm membranes (Amann, 1984). The PM of the sperm surrounds the whole sperm except for a small portion of the tail region. The mammalian sperm PM contains hundreds of polypeptides (Peterson and Russell, 1985). Proteins occur in the membrane in an asymmetrical manner, with some only protruding into the bilayer while others extend all the way through the bilayer (Bretscher and Raff, 1975). These latter proteins may act as messengers, relaying information to other organelles inside the sperm (Saxena et al., 1986a). While in the epididymis, the sperm undergo significant changes in structure and activity of certain regions (Peterson and Russell, 1985).

A demonstrable increase and decrease in concentrations of various PM proteins was shown as the sperm migrate through the epididymis (Peterson and Russell, 1985). Immunofluorescence was used to show marked changes in sperm PM constituents (Saxena et al., 1986b). Differences depended upon whether the sperm were obtained from the various regions of the epididymis or were ejaculated sperm (Saxena et al., 1986b; Schlegel et al., 1986; Russell et al., 1984).

Lipid composition of the PM is not fully known (Peterson and Russell, 1985). Most lipid existed as phospholipids, with only a small proportion being sterols (Clegg, 1983). Phospholipid molecules consist of a polar head group and two hydrophobic hydrocarbon tails of variable lengths and degrees of saturation (Bretscher and Raff, 1975). The major unsaturated fatty acid in the phospholipids of ram, bull and boar sperm is docosahexaenoic acid (22:6) and the predominant saturated fatty acid is palmitic acid (16:0) (Poulos et al., 1973; Darin-Bennett et al., 1974). The ratio of unsaturated to saturated fatty acids in ram, bull and boar sperm is high (3.0), while other species such as humans and fowl have a ratio of about 1.0 (White and Darin-Bennett, 1976). This may have a significant influence on the different freezability among species since the former have poor fertilizing capacity and the other has high fertilizing capacity after freezing (White and Darin-Bennet, 1976).

1.7 FLUIDITY OF MEMBRANES:

The fluidity of a membrane bilayer at various temperatures is influenced by its composition (Bretscher and Raff, 1975). The characteristics of the phospholipid head group and the degree of saturation of the hydrocarbon side chains determine the temperature at which the membrane will undergo transitions from the liquid to gel phase (Bretscher and Raff, 1975). As the membrane lipids undergo phase tran-

sition from liquid to gel, there is a parallel drop in enzyme activity which does not appear to be linked to the aggregation of proteins within the membrane (Silvius and McElhaney, 1982). Barber and Thompson (1980) demonstrated a permeability increase during liquid-gel phase shifts in bean cotyledon membranes. This resulted in breaks in the membrane and loss of intracellular compartmentation (Barber and Thompson, 1980). Carlson and colleagues (1982) reported that a loss of cell function was associated with a lipid phase transition.

Presence of proteins and cholesterol, as well as phospholipid heterogenity influences the fluidity of the membranes (Bretscher and Raff, 1975; White and Darin-Bennett, 1976). Cooper and coworkers (1977) found that an increased sphingomyelin/ lecithin ratio in patients with a blood disorder caused a decrease in fluidity of red blood cell membranes. It was found that differences in lipoprotein and therefore lipid composition ultimately lead to modifications in fluidity and function of these membranes (Cooper et al., 1977). Calcium was found to have a role in binding and distribution of fatty acids on the PM of liver cells (Schroeder and Soler-Argilaga, 1983). Adding calcium to bovine brain PM caused a fluid-to-gel phase shift in fatty acyl chains at physiological temperatures (Sklar et al., 1979b). Sea urchin eggs at fertilization undergo changes in membrane fluidity influenced by increases in cytoplasmic pH and cal-

cium (Scandella et al., 1982). Decreases in function related to changes in membrane fluidity were also observed in hormone secreting cells (Carlson et al., 1984). Holt and North (1984) used freeze fracture analysis to detect extensive reorganization of proteins in ram sperm PM as the sperm were prepared to be frozen. These changes in the constituents of the PM were not all reversible (Holt and North, 1984).

1.8 FACTORS AFFECTING FLUIDITY OF BOAR SPERM MEMBRANES:

This project hypothesized that reduced fertility of boar sperm may be caused by structural changes in the HPM during the storage process. These changes in the PM could interfere with the ability of the sperm to bind to and penetrate the ovum. Membrane fluidity and therefore structural changes can be measured by fluorescence polarization (FP, Shinitzky and Barenholz, 1978; Vijayasarathy and Balaram, 1982) and therefore sperm membrane fluidity should reflect any changes in membrane structure induced by temperature variation.

Chapter II

MATERIALS AND METHODS

2.1 SEMEN COLLECTION:

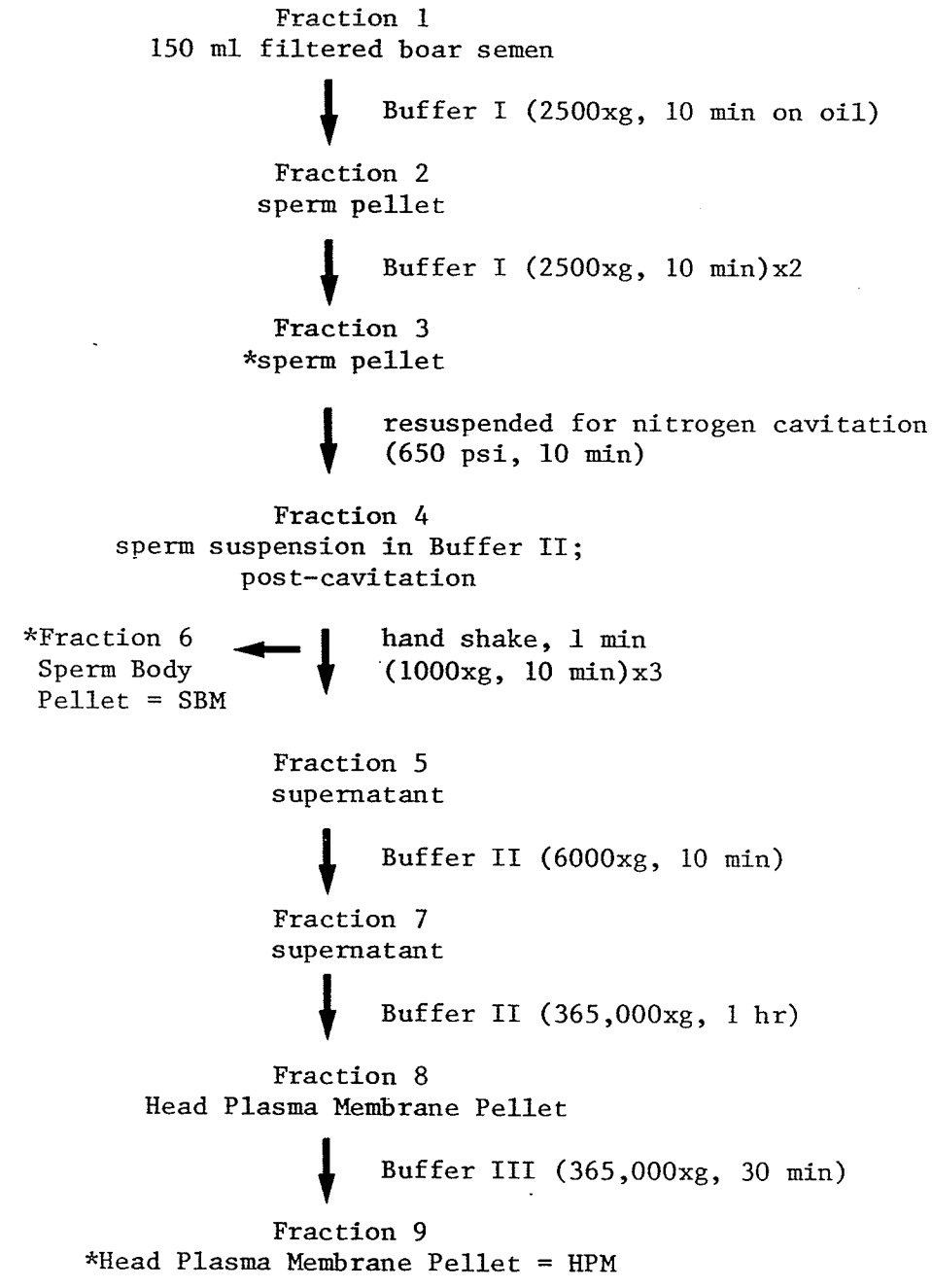
For Exp. 1 and 2 semen was collected one to two times per week from Yorkshire or Managra boars of proven fertility, maintained in university barns. A boar's ejaculate can be divided into 3 fractions: preputial, sperm rich, and seminal fluid. On the day of the experiment, the whole ejaculate was collected for Exp. 1 and the sperm rich fraction was collected for Exp. 2. Semen was strained during the collection through two layers of J-cloth (Johnson and Johnson Inc. Toronto, Ontario). In Exp. 3, semen was obtained from the Alberta Swine Breeding Centre at Leduc, Alberta. The sperm rich fraction was collected from Yorkshire, Duroc, Hampshire or Landrace boars of proven fertility one to two times per week, and extended in BL1 extender (Pursel et al., 1973) as per established methods in that laboratory. The sperm were maintained in this extender for approximately 24 h until membrane isolation began. Ejaculates from at least 3 boars were pooled together in all experiments. This was done to reduce the individual variability which may be due to semen quality among different boars.

2.2 MEMBRANE ISOLATION:

For Exp. 1 and 2, semen was filtered twice through Miracloth (Calbiochem, LaJolla, CA), once into a flask at room temperature, and then once into a flask in a water bath maintained at 25 C. All preparations after this point in all experiments were carried out at 25 C. In Exp. 3, the extended semen was filtered and then was centrifuged in six 250 ml bottles (2500xg, 10 min) to obtain a pellet of concentrated sperm. All but approximately 25 ml of the supernatant in each bottle was discarded. This remaining supernatant was used to resuspend the pellets up to a total volume of 150 ml. Membrane preparations proceeded identically for all three experiments from this point.

Semen (150 ml) was diluted 1:1 (semen:buffer) at 25 C in buffer I (5mM tris-chloride, 0.25M sucrose, pH 7.4) and layered 1:1 (v/v) on oil (Fig. 1, Fraction 1). The oil was the supernatant resulting from the centrifugation (1500xg, 10 min) of a 1:1 (v/v) mixture of two highly refined light oils, 550 and 1107 (Dow Corning, Mississauga, Ont.). This oil:semen combination, which had a total volume of approximately 50 ml, was centrifuged (2500xg, 10 min) to produce a sperm pellet free of seminal gel (P.F. Watson, personal communication). The sperm pellet (Fraction 2) was retained and the supernatant (gel:liquid combined) was discarded. The pellets were resuspended in the original volume of buffer I and centrifuged (2500xg, 10 min). twice to remove residual oil.

Figure 1. BOAR SPERM MEMBRANE ISOLATION



* indicates aliquot taken for enzyme analysis

The pellets were combined over these washes into two tubes. After the final wash, the pellets (Fraction 3) were resuspended in buffer I to a total volume of 25 ml in a graduated cylinder and placed in a Parr bomb to allow for nitrogen cavitation. The graduated cylinder was rinsed with three 2 ml aliquots of buffer I, and the rinses were added to the bomb. A 1 ml sample of this initial sperm mixture was taken and frozen immediately for enzyme analysis, leaving a total of 30 ml in the bomb. The sperm were subjected to a nitrogen pressure of 650 psi for 10 min (Gillis et al., 1978). Sperm suspension was then slowly extruded (over a 60-90 sec interval), into an equal volume of buffer II (5mM tris-chloride, 0.25M sucrose, pH 5.0) in order to inhibit proteolysis (variation of Gillis et al., 1978). The extrusion of the sample removes the plasma membrane from the head of the sperm (HPM) leaving the sperm body membranes (SBM) attached to the sperm. The SBM has been shown to consist of the acrosome and remaining sperm body plasma membranes (Peterson et al., 1980). The cavitate (Fraction 4) was then shaken vigorously by hand for 1 min in order to separate the membrane vesicles (Kaplan et al., 1984). The cavitate was split into two tubes and washed in buffer II three times (1000xg, 10 min). The supernatants (Fraction 5) were combined into a graduated cylinder to a total volume of approximately 250 ml. A sample of the pellet containing SBM (Fraction 6) was frozen for enzyme analysis, and an additional aliquot of the same pellet was held at 25 C for

fluorescence polarization. The combined supernatant (Fraction 5) was centrifuged (6000xg, 10 min) to further remove any intact cells. The supernatant (Fraction 7), which contained HPM, was then pipetted into quickseal ultracentrifuge tubes (Beckman Instruments (Canada) Inc. Burnaby, British Columbia) and centrifuged on a Beckman L8-M ultracentrifuge (365,000xg, 1 h). The pellets (Fraction 8) were resuspended, combined and washed in buffer III (10mM tris-chloride, 0.9% sodium chloride, pH 7.4), and then centrifuged (365,000xg, 30 min). The HPM pellet (Fraction 9) was resuspended in 2 ml of buffer III and transferred to a glass-teflon tissue homogenizer. The tube was rinsed with 1 ml of buffer III and the rinse added to the homogenizer. The pellet was broken up into a homogeneous solution with approximately 10 to 15 strokes of the pestle. All of the sample (Fraction 9) was saved for fluorescence and enzyme analysis. Protein content of this final HPM sample (Fraction 9) and that of the SBM pellet (Fraction 6) was determined by the method of Bradford (1976), using bovine gamma globulin (Sigma Chemical Co.) as a standard. For Fluorescence analysis 300 ug of protein from the SBM or HPM were placed in individual quartz cuvettes. In order to assess the effect of calcium upon these membranes two cuvettes of each membrane sample were prepared with one designated for calcium inclusion.

2.3 FLUORESCENCE POLARIZATION:

The SBM and the isolated HPM were subjected to fluorescence analysis in an LS5 Perkin-Elmer fluorescence spectrophotometer (Perkin-Elmer, Oak Brook, Illinois) fitted with a polarizing accessory. Excitation slit and emission slit widths were 5 and 10 nm respectively.

The fluorescence probe trans-Parinaric Acid (tPNA, Molecular Probes Inc. Junction City, OR) is a molecular probe which has been used to measure viscosity of different membranes (Sklar et al., 1979c). A tPNA stock solution of 2mM was made fresh every 3 months using pharmaceutical grade ethanol as a solvent and stored in the dark under nitrogen at -20 C. The stock solution was diluted 1000-fold into membranes in the cuvette and mixed thoroughly. This brought the final protein concentration in the cuvette to 100ug/ml. For those measurements taken in the presence of calcium, 10 ul of a 3M solution of calcium chloride was added to obtain a final concentration of 10mM in the cuvette. The calcium was mixed in by pipette and allowed to incubate for 15 min. The wavelengths providing the maximal excitation and emission intensities were determined daily. These highly consistent wavelengths averaged 324±1 nm (mean±S.E.) and 420±1 nm for excitation and emission, respectively.

The cuvette holder was hooked up to a Lauda RC3 circulating water bath and fluorescence measurements were taken

while the samples were cooled from 25 C to 5 C and subsequently reheated from 5 C to 40 C at a rate of approximately .4 C/min. In separate trials within each experiment, fluorescence readings were also taken on similar samples at a constant temperature of 25 C over an equivalent period of time. The sample temperature was continuously monitored with a thermocouple placed in a cuvette. Polarization values were calculated from the fluorescence measurements by using the Perrin equation (Sklar et al., 1979a):

$$\text{Polarization Value}=\frac{IVpara-IVperp*G}{IVpara+IVperp*G} \qquad \text{where } G=\frac{IHpara}{IVperp}.$$

The I represents the intensity of the exciting light. The V indicates the exciting light was vertically polarized, H indicates the exciting light was horizontally polarized, while para and perp refer to components of the emission light parallel and perpendicular, respectively, to the vertical component.

2.4 ENZYME ANALYSIS:

All samples that were taken during the experiments were frozen (-20 C) overnight. The activity of alkaline phosphatase, which is a HPM marker enzyme (Soucek and Vary, 1984a), was measured by the method of Linhardt and Walter (1963). From these measurements specific activities were calculated and used in determining the purity of the membrane sample. Nitrophenylphosphate substrate was obtained from Sigma Chemical Co., (St. Louis, Mo.).

2.5 CALCIUM ANALYSIS:

Calcium content of each of the 3 ejaculate fractions plus an entire ejaculate was analyzed by the method of Thompson and Blanchflower (1971).

2.6 STATISTICAL ANALYSIS:

Initial polarization values from all experiments were analyzed for differences using least square means and their predicted differences. Treatments were grouped together for this analysis as differences in time and temperature measurements were found to be non-significant.

Polarization values within each replicate ($n>4$) in each treatment were adjusted by subtracting the initial polarization value from all values. This eliminated variation due to differences in start values. These adjusted polarization values were analyzed by stepwise regression on a polynomial equation with time and the powers of time as the dependent variables. If data proved to be nonlinear ($p<.05$) then a procedure to fit a series of straight-line segments to the data was imposed. The NLIN procedure (SAS Inc. 1985) was used to fit the series of line segments, with the criteria of minimum residual sums of squares used to estimate the various slopes and intercepts. Since several straight lines were fitted, the points on the x-axis at which the lines changed direction (breakpoints) were also estimated by the

procedure. For the temperature data, times at which break-points occurred were converted into temperature values. Anderson and Nelson (1975) in explaining their results have used a similar method of imposing nonlinear regression on lines to obtain linear segments within one line. Lack of overlap of 95% confidence limits were used to determine significant differences when comparing different membrane types and treatments within membrane types. If slopes were linear within an experiment, a t-test for parallelism (Kleinbaum and Kupper, 1978) was used to determine differences. ANOVA was used to determine differences between calcium levels in the boars' ejaculate.

Chapter III

RESULTS

3.1 FLUORESCENCE POLARIZATION:

Measureable differences in initial polarization values, which indicate viscosity, were detected between the membrane types and among the sources of semen. Significant differences in HPM among sources were identified by analysis of the unadjusted initial polarization values. However, source had no effect on SBM (Table 1). The SBM and HPM differed significantly in the whole ejaculate and the extended semen whereas, the differences approached significance for the sperm rich fraction (Table 1).

Further analysis of the membranes by fluorescence polarization detected viscosity changes which were influenced by calcium, time and temperature, in a manner unique to each membrane.

3.1.1 Exp. 1. Whole Ejaculate

The changes which occurred in the viscosity of the sperm membranes (SBM, HPM) with temperature are represented in Fig. 2 and 3. In the presence of calcium, the SBM underwent a linear increase in viscosity, whereas in the absence of

Table 1. Initial polarization values of SBM and HPM

	Source		
	1	2	3
SBM	.3744±.0075*[a]	.3616±.0075[a]	.3697±.0068[a]
HPM	.4295±.0075[b]	.3920±.0075[ca]	.3354±.0068[d]

*values given are LSM±S.E.
different superscripts indicate significant differences

Figure 2. TEMPERATURE-RELATED CHANGES IN VISCOSITY OF SPERM BODY MEMBRANES

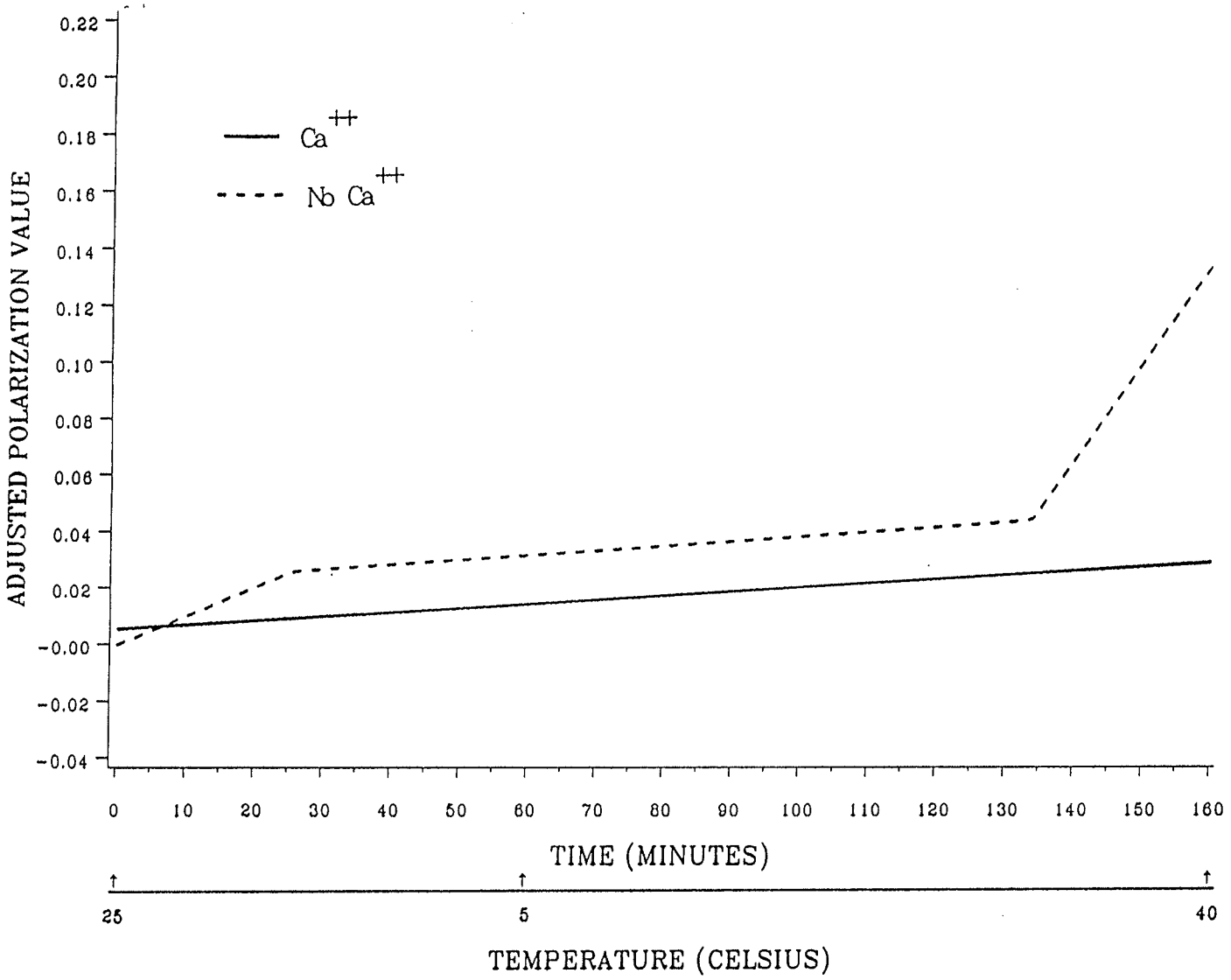
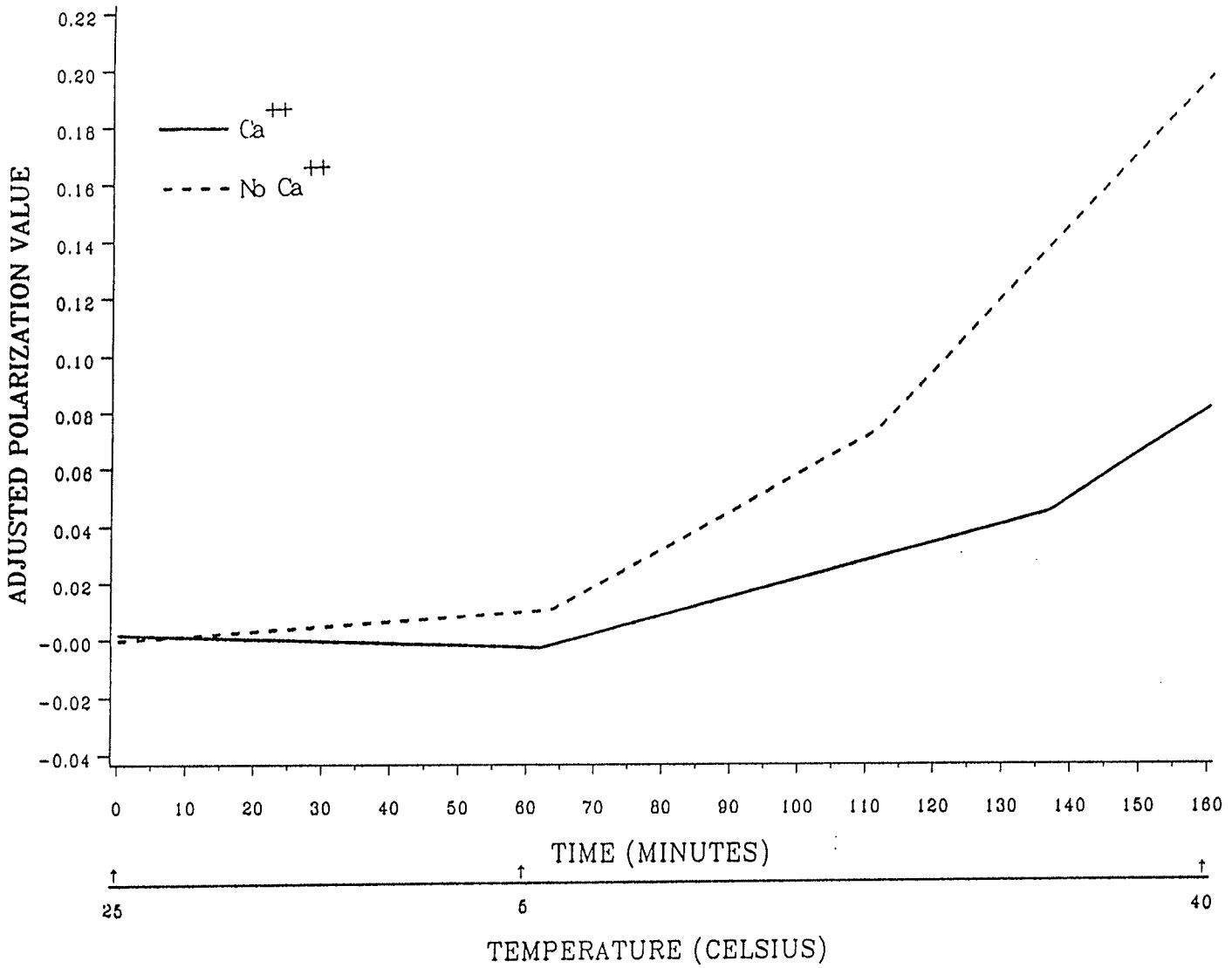


Figure 3. TEMPERATURE-RELATED CHANGES IN VISCOSITY OF HEAD PLASMA MEMBRANES
WHOLE EJACULATE



calcium the SBM demonstrated a nonlinear increase in viscosity. There were three different slopes identifiable in the absence of calcium (Table 2). The viscosity of the HPM increased in presence or absence of calcium in nonlinear patterns which differed from those of the SBM. The HPM viscosity described three slopes which did not differ in the presence or absence of calcium (Table 2).

Viscosity changes which occurred in the SBM and HPM with time are represented in Fig. 4 and 5. In the presence of calcium the viscosity of the SBM underwent a linear increase. In the absence of calcium, the viscosity decreased with two different slopes over the same time period (Table 3). The HPM in the presence or absence of calcium also had an overall viscosity increase, described by two different slopes within the time period studied.

The viscosity of the SBM in the presence of calcium increased linearly when under either variable or constant temperature treatment, although the slopes were significantly different ($p<.05$). Breakpoints defining where the slopes of data sets changed are given in Table 4. For the SBM in the absence of calcium, two breakpoints were detected under the variable temperature regime. Viscosity of the membranes increased rapidly after the second breakpoint. However, when the temperature was held constant, there was only one breakpoint detected, and the viscosity decreased after this point. Two breakpoints were detected for the HPM in the

Table 2: Line characteristics for Exp. 1 (whole ejaculate) with variable temperature.

Treatment	Start (min)	End (min)	Slope±S.E. (PV/min) $x10^{-4}$	Overall Model R^2
SBM				.53175
Slope 1	0	25	10.2±6.6	
2	25	134	1.6±1.1	
3	134	160	34.8±8.6	
SBM+Calcium				..13960
Total Slope (linear)	0	160	1.5±.3	
HPM				.83704
Slope 1	0	64	1.7±1.7	
2	64	111	13.2±3.3	
3	111	160	25.5±3.3	
HPM+Calcium				.84723
Slope 1	0	62	-.7±.8	
2	62	137	6.4±.6	
3	137	160	15.7±3.2	

Figure 4. TIME-RELATED CHANGES IN VISCOSITY OF SPERM BODY MEMBRANES
WHOLE EJACULATE

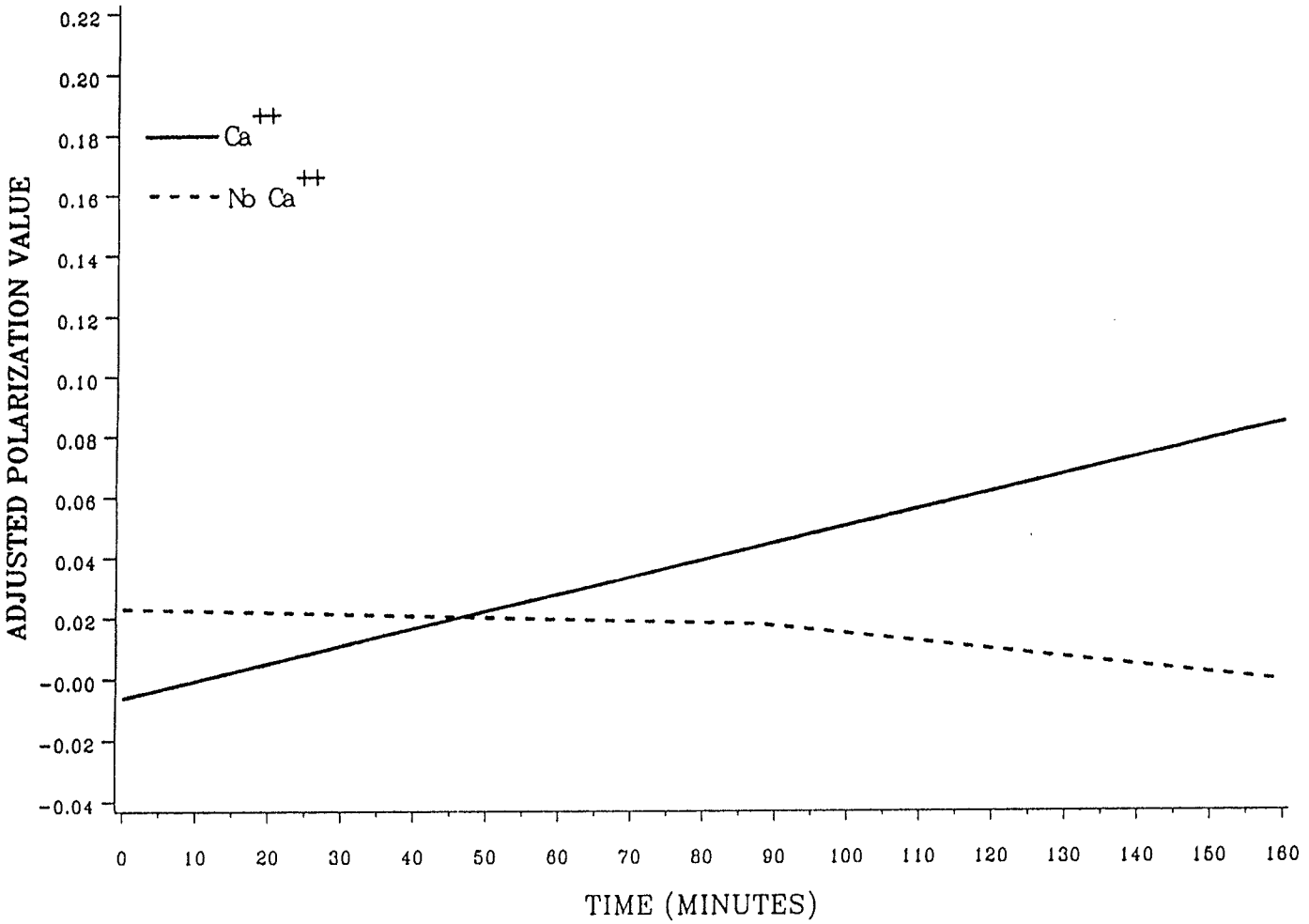


Figure 5. TIME-RELATED CHANGES IN VISCOSITY OF HEAD PLASMA MEMBRANES
WHOLE EJACULATE

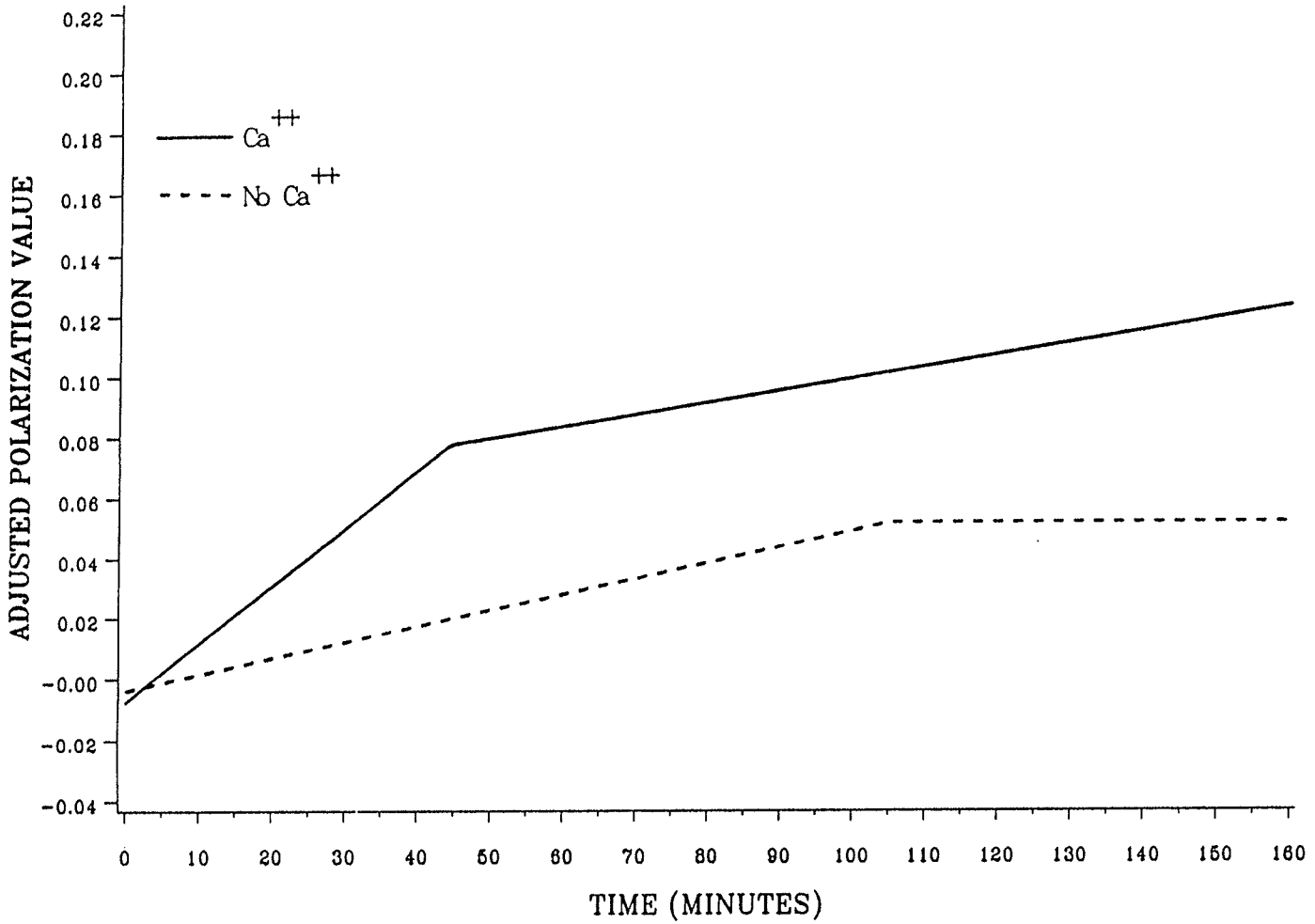


Table 3: Line characteristics for Exp. 1 (whole ejaculate) with constant temperature.

Treatment	Start (min)	End (min)	Slope±S.E. (PV/min) $x10^{-4}$	Overall Model R^2
SBM				.11971
Slope 1	0	88	-.5±.7	
2	88	160	-2.6±1.1	
SBM+Calcium				.37063
Total Slope (linear)	0	160	5.6±.5	
HPM				.55360
Slope 1	0	105	5.3±0	
2	105	160	0±1.3	
HPM+Calcium				.40626
Slope 1	0	44	19.2±5.5	
2	44	160	3.9±1.0	

Table 4. Breakpoints in regression lines for Exp. 1 (whole ejaculate).

Treatment	Breakpoints±S.E. at variable temperature		at constant temperature
	min	equivalent C	min
SBM	25±12	16(d)±4	88±30
	134±5	31(i)±2	
SBM+Calcium	ND*	-	ND
HPM	64±10	7(i)±3	105±8
	111±11	23(i)±4	
HPM+Calcium	62±4	6(i)±1	44±10
	137±6	32(i)±2	

*ND - non-detectable
(d)=decreasing temperature
(i)=increasing temperature

presence of calcium under variable temperature treatment but only one breakpoint occurred when the temperature was held constant. The HPM without calcium reacted similarly, having two breakpoints when the temperature was continuously changed and one breakpoint when the temperature was constant.

3.1.2 Exp. 2. Sperm Rich Fraction

Viscosity of the SBM changed with temperature (Fig. 6) in a manner that was significantly affected by calcium (Table 5). The temperature-induced changes in the HPM viscosity were alike in overall direction in the presence or absence or calcium (Fig. 7). Calcium effect was insignificant as assessed by 95% confidence limit overlap (Table 5).

Viscosity of the SBM and HPM also changed with time at 25 C (Fig. 8 and 9). In the presence of calcium the viscosity of the SBM underwent a distinct change in slope, while the viscosity of the SBM in the absence of calcium described a minimal linear increase (Table 6). The viscosity of the HPM in the absence of calcium increased in a manner that was different from the linear increase observed in the presence of calcium (Table 6).

Breakpoints which occurred in the sperm rich viscosity measurements are given in Table 7. The breakpoints seen in SBM viscosity in the presence of calcium differed signifi-

Figure 6. TEMPERATURE-RELATED CHANGES IN VISCOSITY OF SPERM BODY MEMBRANES

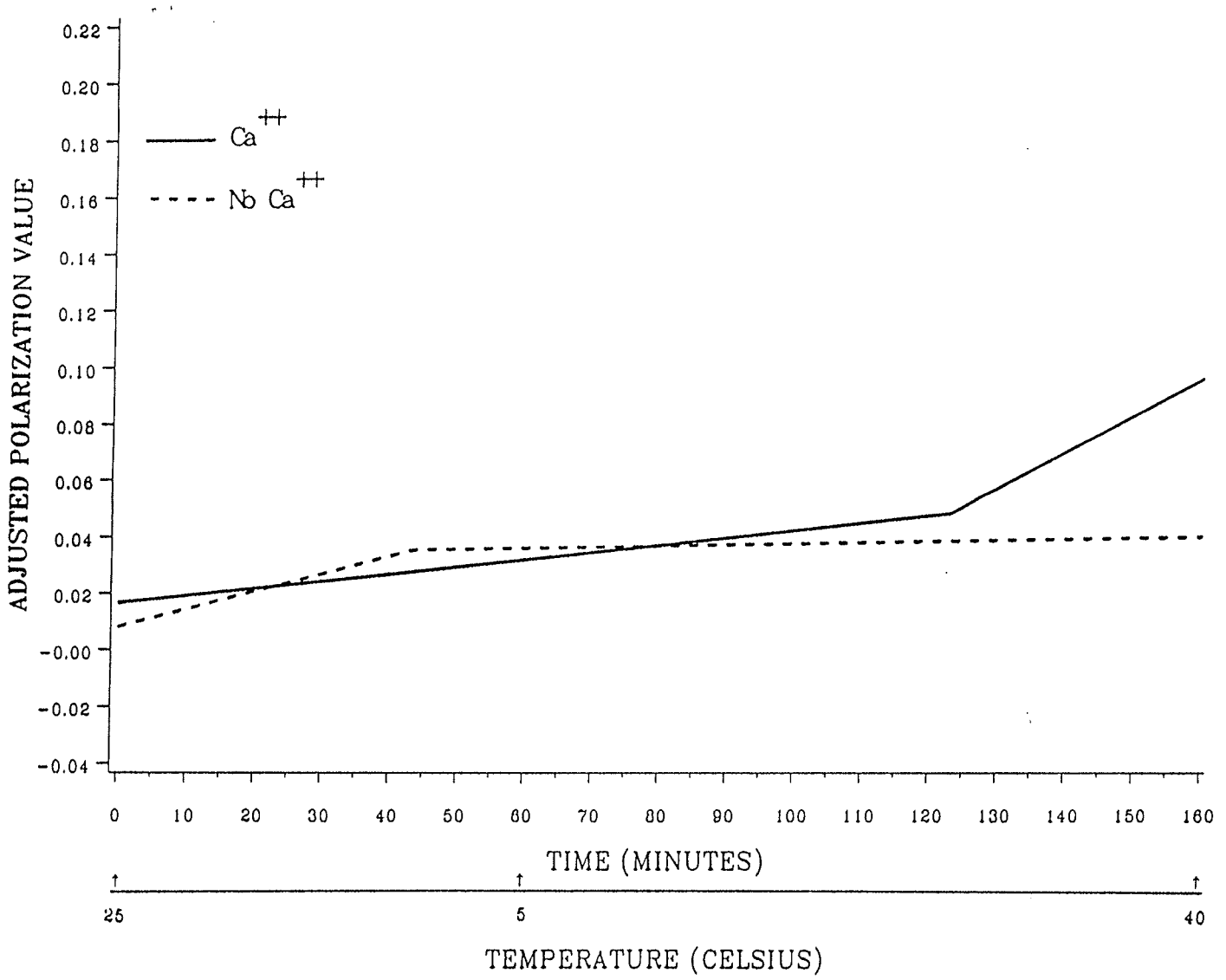


Table 5: Line characteristics for Exp. 2 (sperm rich fraction) with variable temperature.

Treatment	Start (min)	End (min)	Slope±S.E. (PV/min) $x10^{-4}$	Overall Model R^2
SBM				.15905
Slope 1	0	43	6.3±1.2	
2	43	160	.4±.6	
SBM+Calcium				.42061
Slope 1	0	123	2.6±.2	
2	123	160	13.1±3.6	
HPM				.67804
Slope 1	0	117	3.5±.6	
2	117	160	22.6±2.5	
HPM+Calcium				.79863
Slope 1	0	86	2.2±1.1	
2	86	133	12.2±2.8	
3	133	160	30.9±4.6	

Figure 7. TEMPERATURE-RELATED CHANGES IN VISCOSITY OF HEAD PLASMA MEMBRANES

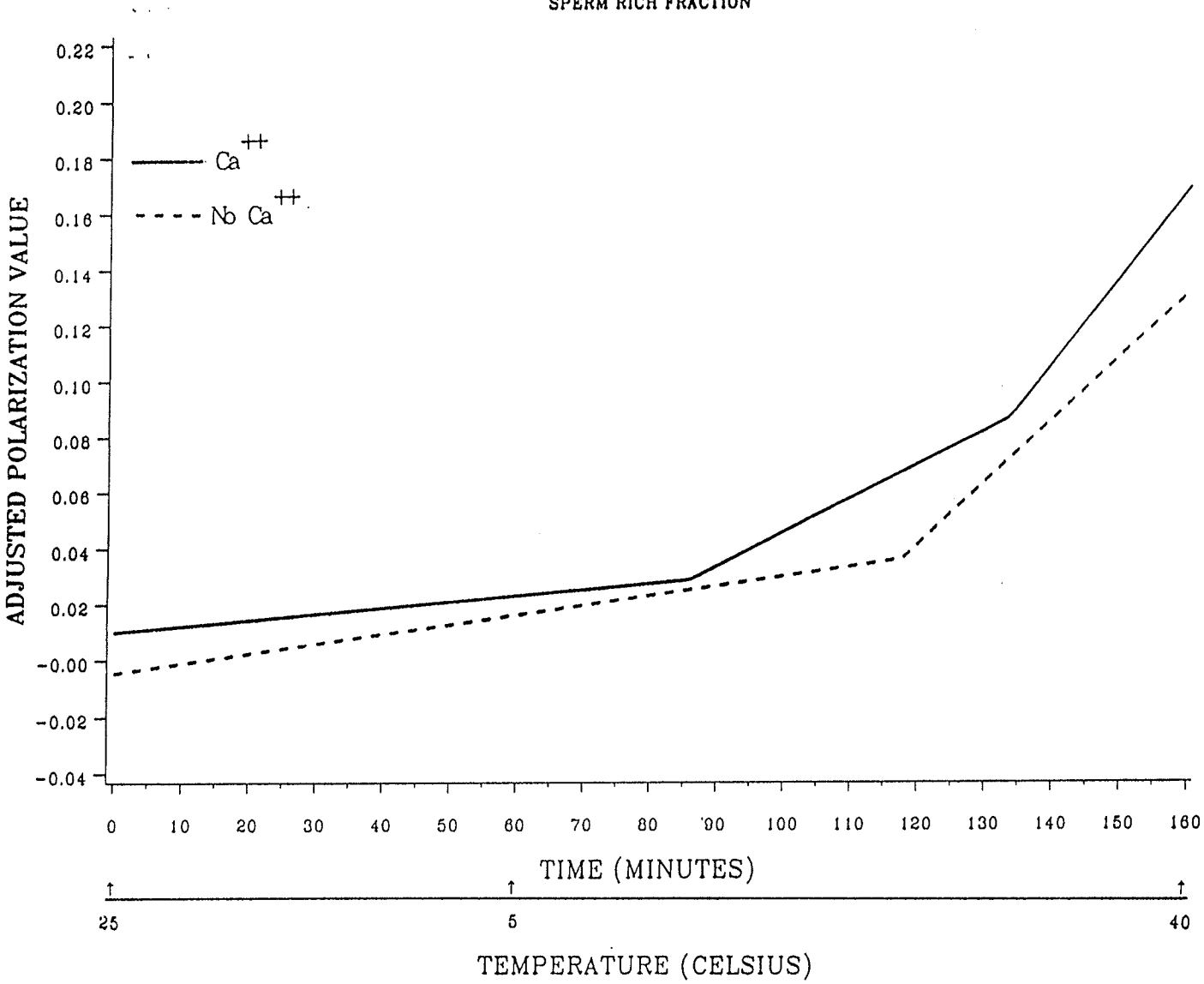


Figure 8. TIME-RELATED CHANGES IN VISCOSITY OF SPERM BODY MEMBRANES

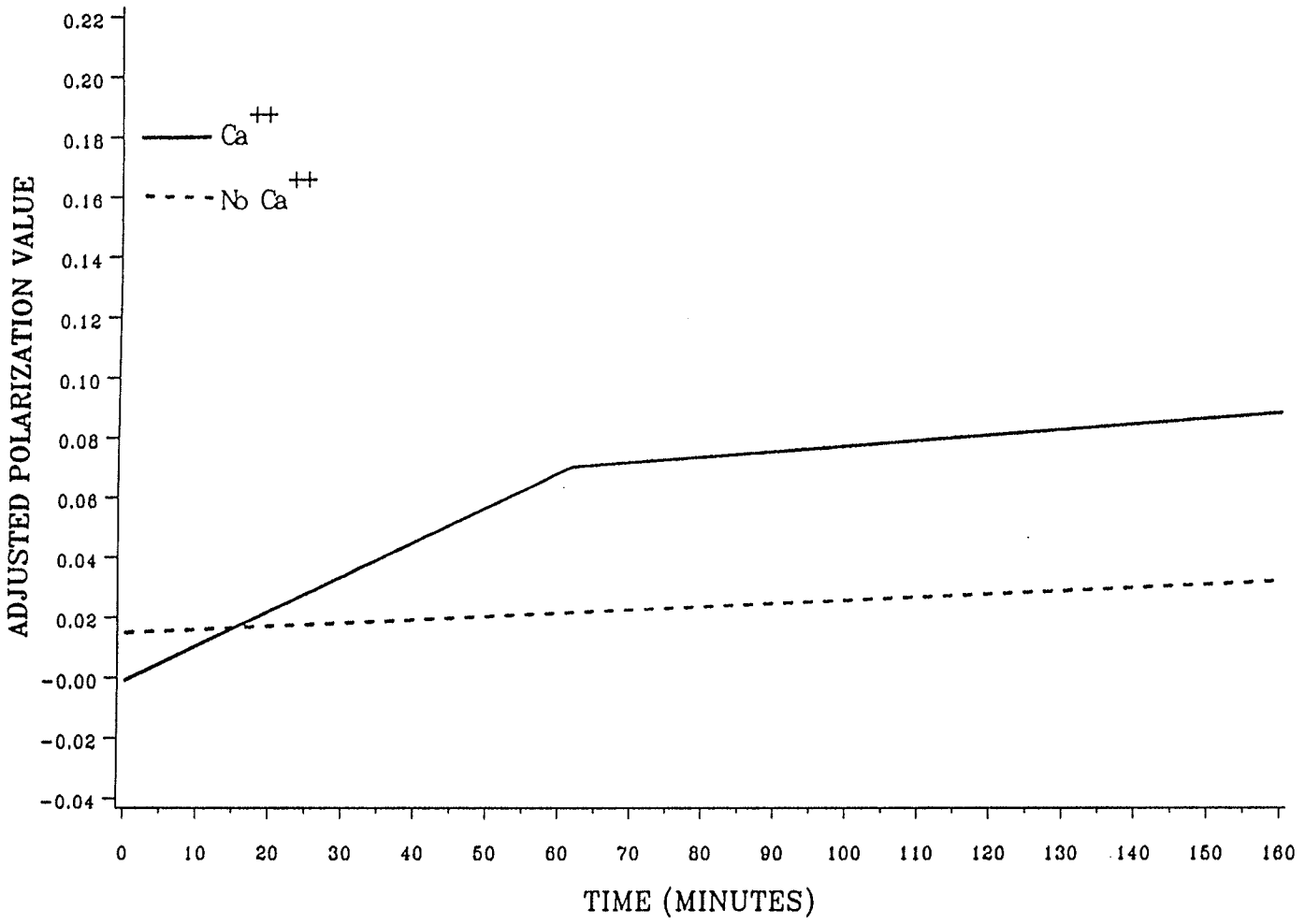


Figure 9. TIME-RELATED CHANGES IN VISCOSITY OF HEAD PLASMA MEMBRANES

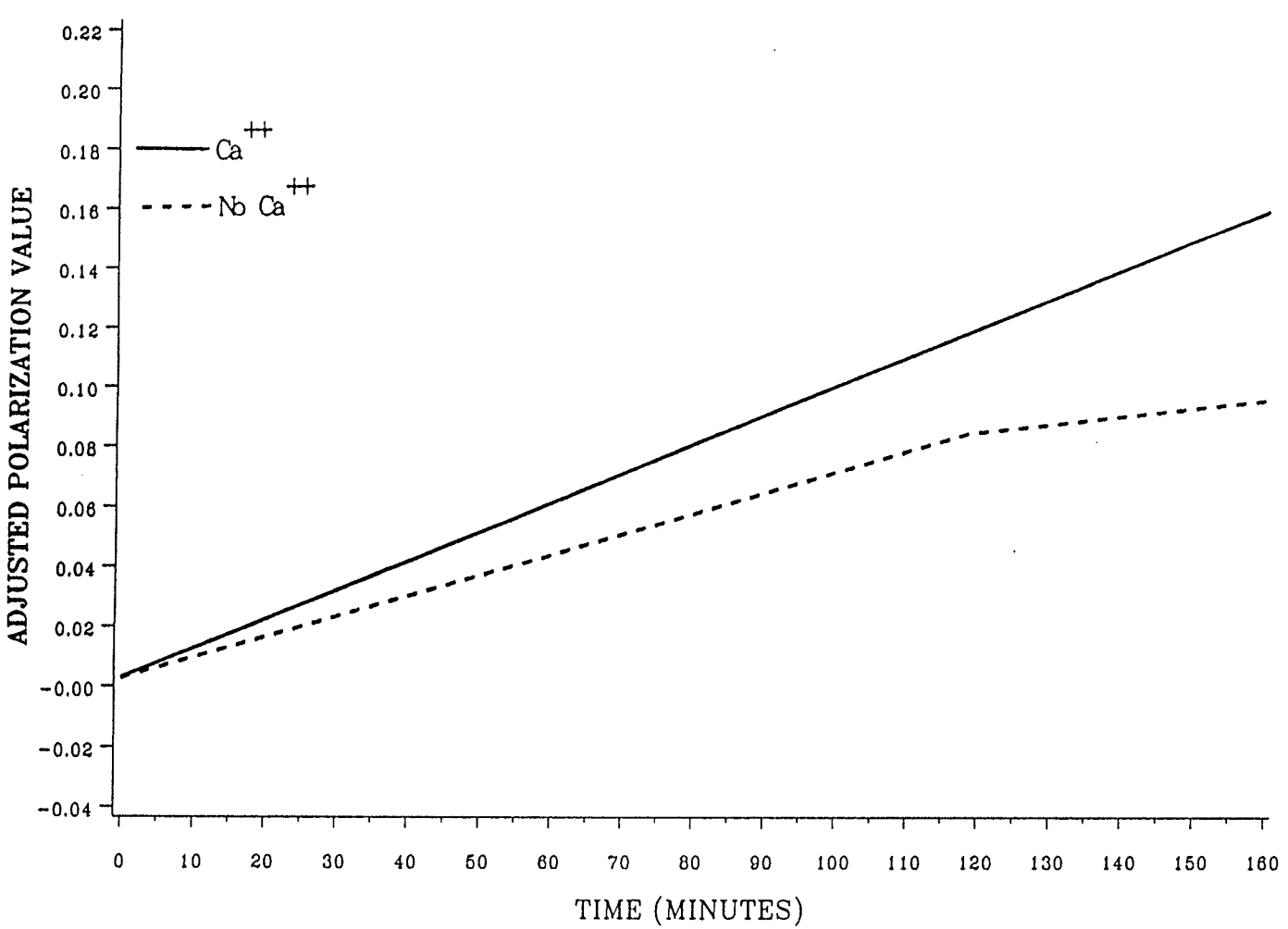


Table 6: Line characteristics for Exp. 2 (sperm rich fraction) with constant temperature.

Treatment	Start (min)	End (min)	Slope±S.E. (PV/min) $x10^{-4}$	Overall Model R^2
SBM				.06201
Total Slope (linear)	0	160	1.1±.3	
SBM+Calcium				.19072
Slope 1	0	62	11.5±4.5	
2	62	160	1.9±2.2	
HPM				.82420
Slope 1	0	119	6.9±.4	
2	119	160	2.6±2.1	
HPM+Calcium				.53404
Total Slope (linear)	0	160	9.8±.8	

cantly between the variable and constant temperature treatments. In the absence of calcium the SBM had one breakpoint when temperature was varied, but no breakpoints with constant temperature treatment. The HPM in the presence of calcium had two breakpoints in the viscosity measurements when temperature was varied, but no breakpoints were detected when the measurements were taken while temperature was constant. Similar breakpoints were found for the HPM in the absence of calcium under variable and constant temperature treatment (Table 7). However viscosity increased with variable temperature treatment and tended to plateau with constant temperature treatment.

3.1.3 Exp. 3. Extended Semen

The SBM in the presence of calcium at variable temperature underwent a significant increase in viscosity, as compared to the SBM in the absence of calcium (Fig. 10). The slope of the viscosity measurements for the SBM in the presence of calcium changed once over the temperature range studied, whereas the slope of the viscosity measurements for the SBM in the absence of calcium underwent a linear increase (Table 8). The HPM viscosity changes at variable temperature did not differ with the presence or absence of calcium (Fig. 11). The slope changed twice over the viscosity measurements, and had a similar pattern in both cases (Table 8).

Table 7. Breakpoints in regression lines for Exp. 2 (sperm rich fraction).

Treatment	Breakpoints±S.E. at variable temperature		at constant temperature
	min	equivalent C	min
SBM	43±13	10(d)±4	ND*
SBM+Calcium	123±7	27(i)±2	62±22
HPM	117±8	25(i)±3	119±14
HPM+Calcium	86±9	14(i)±3	ND
	133±6	31(i)±2	

*ND - non-detectable
(d)=decreasing temperature
(i)=increasing temperature

Figure 10. TEMPERATURE-RELATED CHANGES IN VISCOSITY OF SPERM BODY MEMBRANES

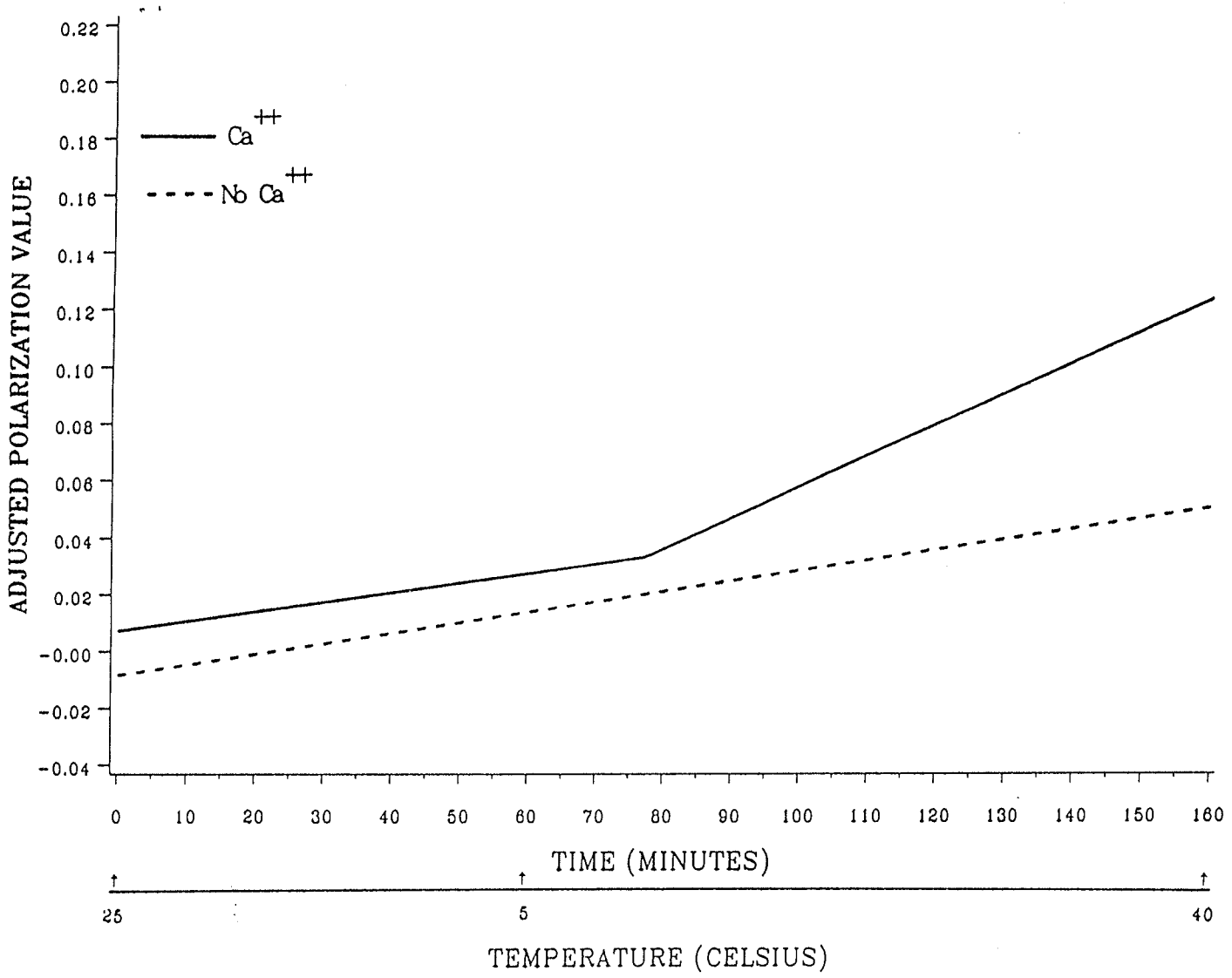
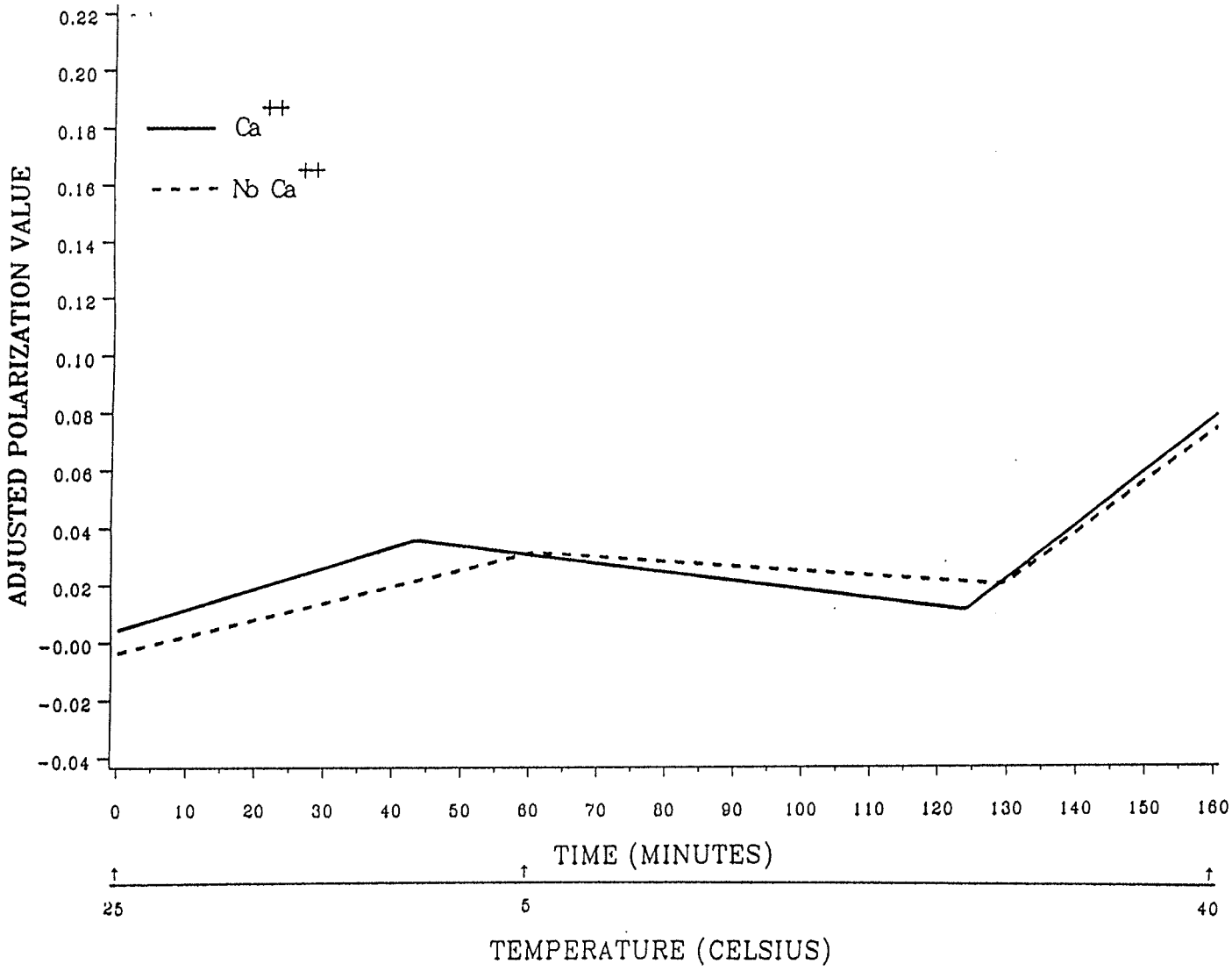


Table 8: Line characteristics for Exp. 3 (extended semen) with variable temperature.

Treatment	Start (min)	End (min)	Slope±S.E. (PV/min) $x10^{-4}$	Overall Model R^2
SBM				.24494
Total Slope (linear)	0	160	3.7±.5	
SBM+Calcium				.48398
Slope 1	0	77	3.4±1.7	
2	77	160	10.9±1.6	
HPM				.59105
Slope 1	0	61	5.8±.9	
2	61	129	-1.7±.9	
3	129	160	17.7±2.5	
HPM+Calcium				.46534
Slope 1	0	43	7.3±2.2	
2	43	124	-3.1±.9	
3	124	160	18.7±2.6	

Figure 11. TEMPERATURE-RELATED CHANGES IN VISCOSITY OF HEAD PLASMA MEMBRANES
EXTENDED SEMEN



Viscosity changes occurred in the SBM and HPM over time at 25 C (Fig. 12 and 13). Calcium did not affect the viscosity of the SBM from this source, with similar decreases in viscosity being found regardless of calcium (Table 9). Viscosity changes of the HPM over time at 25 C in the presence or absence of calcium described linear patterns. However, a significant ($p<.05$) increase in viscosity was detected in the presence of calcium compared to that observed in the absence of calcium.

Table 10 summarizes breakpoints identified in the SBM and HPM from extended semen viscosity measurements. The breakpoint detected for the SBM in the presence of calcium was similar for the variable temperature and constant temperature treatments. However, viscosity increased with variable temperature treatment and decreased with constant temperature treatment. No breakpoints were observed for the SBM in the absence of calcium when temperature was manipulated. However, one breakpoint was observed when the temperature was held constant. For the HPM under variable temperature treatment, two breakpoints were detected in the presence of calcium which were not significantly different from the breakpoints detected in the absence of calcium. No breakpoints were observed in the HPM in the presence or absence of calcium when temperature was held constant (Table 10).

Figure 12. TIME-RELATED CHANGES IN VISCOSITY OF SPERM BODY MEMBRANES
EXTENDED SEMEN

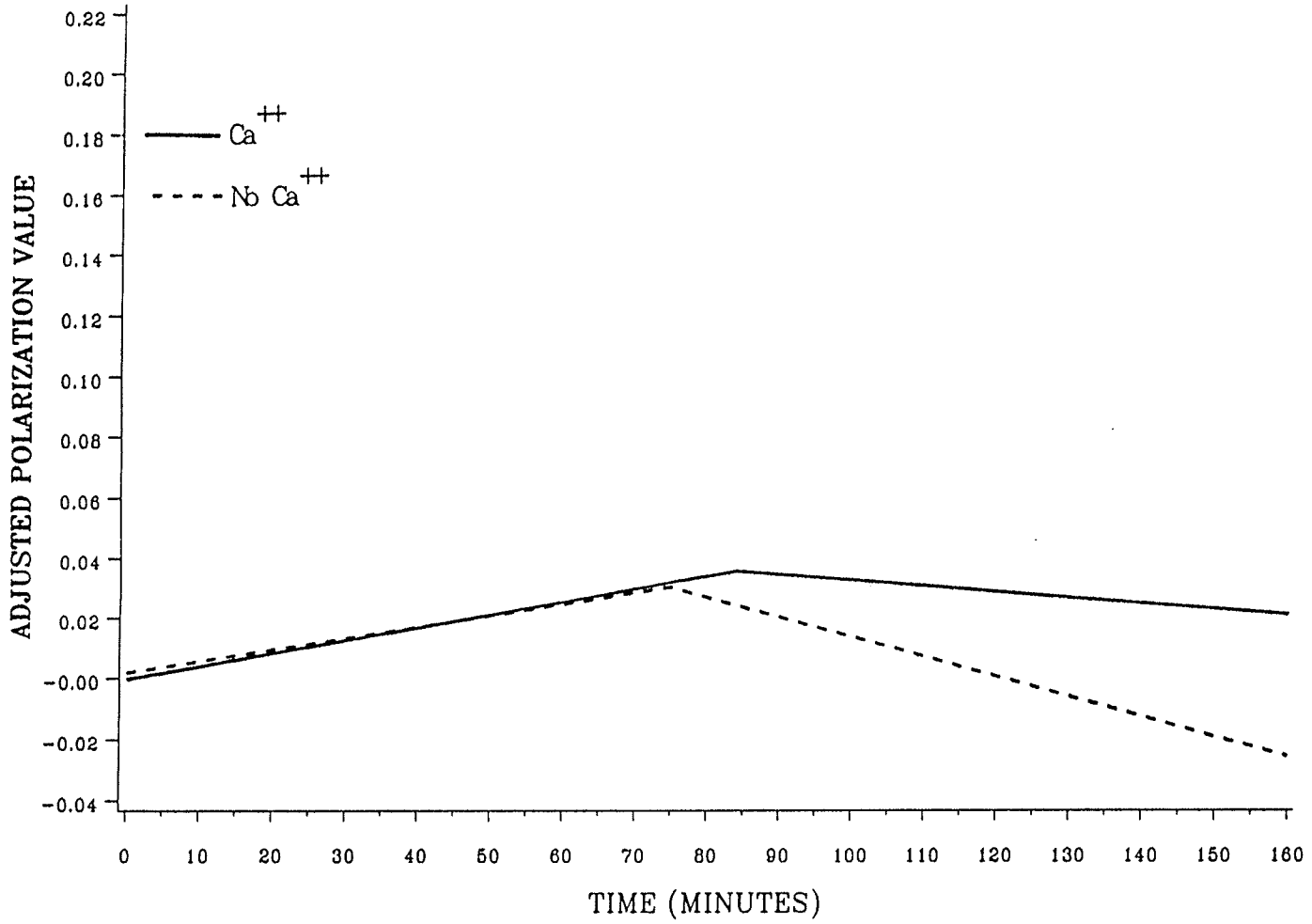


Figure 13. TIME-RELATED CHANGES IN VISCOSITY OF HEAD PLASMA MEMBRANES
EXTENDED SEMEN

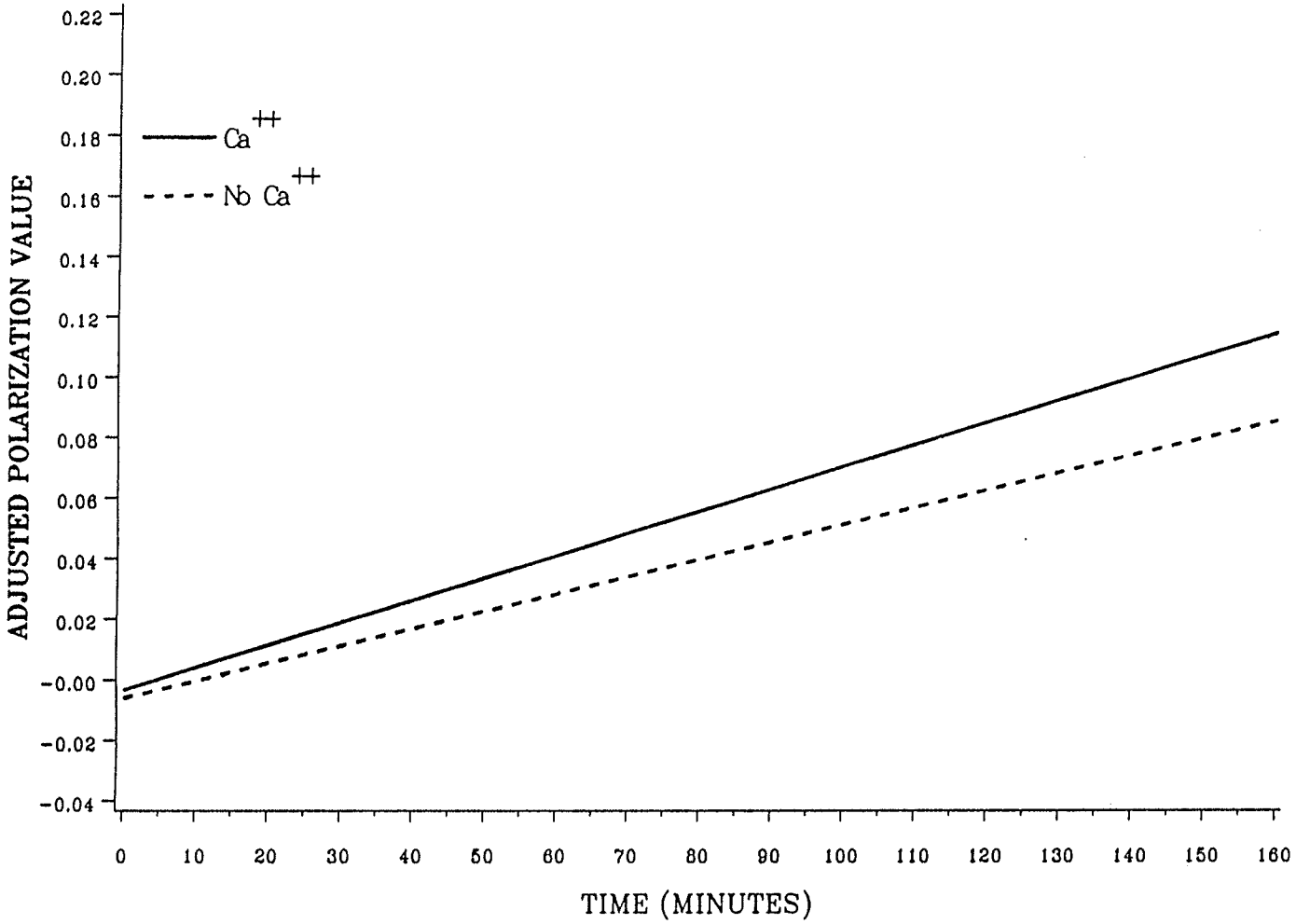


Table 9: Line characteristics for Exp. 3 (extended semen) with constant temperature.

Treatment	Start (min)	End (min)	Slope±S.E. (PV/min) $x10^{-4}$	Overall Model R^2
SBM				.14742
Slope 1	0	75	3.8±1.8	
2	75	160	-6.6±1.6	
SBM+Calcium				.07072
Slope 1	0	84	4.3±1.6	
2	84	160	-1.9±1.9	
HPM				.62008
Total Slope (linear)	0	160	5.7±.4	
HPM+Calcium				.76249
Total Slope (linear)	0	160	7.4±.3	

Table 10. Breakpoints in regression lines for Exp. 3 (extended semen).

Treatment	Breakpoints±S.E. at variable temperature		at constant temperature
	min	equivalent C	min
SBM	ND*	-	75±11
SBM+Calcium	77±15	12(i)±5	84±19
HPM	61±6	6(i)±2	ND
	129±3	30(i)±1	
HPM+Calcium	43±7	10(d)±2	ND
	124±4	28(i)±1	

*ND - non-detectable
(d)=decreasing temperature
(i)=increasing temperature

3.2 ENZYME ANALYSIS:

Measurement of the HPM marker enzyme alkaline phosphatase showed that the HPM sample was enriched 421±55% (mean±S.E.), in comparison to the whole sperm. This enrichment level is comparable to those found in other studies (Soucek and Vary, 1984a; Nikolopoulou et al., 1985).

3.3 CALCIUM ANALYSIS:

Measurement of calcium content in each of the ejaculate fractions as well as an entire ejaculate showed no significant differences ($p>.05$) among these fractions (Table 11). The level of calcium in the ejaculate was approximately 0.72mM.

Table 11. Calcium content of boar ejaculate.

Fraction	Calcium concentration±S.E. (ug/ml)
Preputial	29.30±2.73
Sperm Rich	32.79±1.87
Seminal Fluid	25.84±1.93
Whole Ejaculate	28.72±1.93

Chapter IV

DISCUSSION

4.1 FLUORESCENCE POLARIZATION MEASUREMENTS:

The present study is the first to measure the viscosity changes the sperm undergo when exposed to a temperature regime that mimics some of the swine industry's present semen preservation techniques. This is of importance because semen subjected to these preservation techniques have a reduced fertilizing capacity, which could be caused by temperature-induced damage to membranes crucial to the fertilization process.

Fluorescence polarization measurements taken as temperature changed revealed differences in slopes and the appearance of breakpoints in most samples. This indicates changes in membrane lipid viscosity, since fluorescent polarization has been shown to measure microviscosity in many different membranes (Vijarasarathy et al., 1982; Carlson et al., 1984; Holt and North, 1986). An increase in polarization is equated with an increase in viscosity (Vijarasarathy et al., 1982), which is inversely related to fluidity (Carlson et al., 1984). Breakpoints which were determined within lines are believed to result from one or more species of lipid undergoing a phase-change (Holt and North, 1986).

Fluorescence polarization measurements taken at a constant temperature showed that the boar sperm membrane viscosity changed over time. These membrane changes may represent natural alterations the membranes must undergo in order to be able to bind to and penetrate the female ovum (Friend, 1977; Holt and North, 1984; Saxena et al., 1986a).

4.1.1 Viscosity Changes Within Membrane Domains

Five classical domains, the principal segment, equatorial segment, post-acrosomal segment, midpiece and principal piece are now recognized in all sperm (Friend and Fawcett, 1974; Saxena et al., 1984). Within these domains subdomains have also been identified (Saxena et al., 1986b) through the use of monoclonal antibodies. The HPM in the current study is a heterogeneous, although plasma membrane enriched sample. The fluorescence polarization values of this sample indicate the viscosity of the sperm head plasma membranes. The SBM polarization values indicate the viscosity of the plasma and acrosomal membranes of the sperm body. The HPM of all semen sources underwent significantly different fluorescence changes compared to those recorded for the SBM. Soucek and Vary (1984b) also found fluidity differences between the outer acrosomal and plasma membranes of epididymal boar sperm.

The viscosity of the HPM increased more with variable temperature treatment than did the viscosity of the SBM in

the whole ejaculate and sperm rich fraction. This indicates that the HPM was in a more viscous state in the whole ejaculate and the sperm rich fraction with variable temperature treatment. After temperature manipulation of the extended semen viscosity of the HPM appeared equal to the SBM. Whereas, with constant temperature treatment the HPM was in a more viscous (less fluid) state than the SBM. However, Vijarasarathy et al. (1982) reported that bull sperm acrosomal membrane had a more ordered lipid phase than the plasma membrane. This was indicated by a higher polarization value obtained for the acrosomal membrane using Diphenyl Hexatriene (DPH) as a membrane probe. In the present study of boar sperm, initial polarization values for the HPM were higher than the SBM in the whole ejaculate, tended to be higher in the sperm rich fraction, and were lower in the extended semen. These differences may be attributed to the preserving effect the extender has upon the head membrane. Overall, however the rate of increase in viscosity as assessed by change of slope tended to show a higher polarization value for the HPM.

Differences in biophysical characteristics, composition and function of the plasma and intracellular membranes (Amann, 1984) are of concern when drawing inter-species comparisons. Studies which have utilized different probes are also difficult to compare, since DPH and tPNA insert into the membranes at different regions. The fluorescent probe

tPNA, used in this study, partitions preferentially into gel phase domains within membranes (Carlson et al., 1984).

Significant compositional changes occur in the sperm PM during epididymal migration, ejaculation, capacitation and the acrosome reaction. The amount of lipid (Nikolopoulou et al., 1985; Nikolopoulou et al., 1986a; Nikolopoulou et al., 1986b; Parks and Hammerstedt, 1985; Schlegel et al., 1986) and protein (Saxena et al., 1986a; Saxena et al., 1986b; Hunt et al., 1985; Russell et al., 1983; Russell et al., 1984; Russell et al., 1985) in the sperm PM change during the time period over which these fertilization-readying steps occur. These membrane changes are all accelerated by the presence of calcium (Saxena et al., 1986a). The SBM viscosity changes were affected by calcium in all instances, whereas the HPM viscosity was only affected under constant temperature treatment. This may indicate that the HPM is more sensitive to temperature induced changes than the SBM.

The calcium concentration in the whole ejaculate of the boar was equivalent to 0.72mM, which is much lower than the 10 mM used in these experiments (Table 10). However, Vijay-asarathy et al. (1982) reported that 10mM calcium had no effect upon fluorescence readings of bull sperm membranes using DPH.

The effects produced by time, temperature and calcium depend on the membrane examined and the semen source. This

was as hypothesized, given the different functions of the SBM and HPM, and the different fertilizing capacity of sperm from the three sources.

4.1.2 Sperm Body Membrane Changes

The SBM is a complex variety of different types of membranes. The relative concentration of acrosomal, body plasma and other membranes, could vary from one preparation to the next. Results for the SBM, then, represent a composite of all membranes (other than the head plasma membrane) which are attached to the outer surface of the sperm body. The adjusted polarization values for the SBM were significantly different from those of the HPM, as determined by lack of overlap of the 95% confidence limits for slopes and breakpoints.

The source of semen significantly affected the response of the SBM to cooling and subsequent reheating. The sperm rich SBM had one breakpoint in the absence of calcium, which differed from the two breakpoints of the whole ejaculate SBM and the single straight line of the extended semen. The SBM of the whole ejaculate underwent a rapid, radical increase in viscosity at the highest temperatures. Addition of calcium significantly reduced the viscosity increase of whole ejaculate SBM, while ultimately increasing the viscosity of the SBM from the other two sources. Calcium is known to be a membrane-reactive ion, and is required for normal motility

(Lindemann et al., 1982; Ashraf et al., 1982) capacitation and the acrosome reaction (Fraser, 1987). Thus, it is reasonable that the SBM should be sensitive to calcium. Motility of unextended sperm deteriorates with time. Therefore, it is understandable that SBM from these three sources showed a different sensitivity to calcium when faced with the stress of variable temperatures as compared to constant temperature treatment. That these results are in response to temperature manipulation was confirmed by the fact that the slopes and breakpoints generated in a constant temperature regime were not the same as those seen under variable temperatures. The SBM from the whole ejaculate and sperm rich sources was influenced by calcium, but the SBM from the extended semen did not show a calcium effect. This further indicates that the extender can prevent calcium-induced changes in SBM ultrastructure when temperatures do not reach beyond the protective range of the extender.

Interestingly, breakpoints in SBM viscosity were much more abrupt (smaller S.E.) under variable temperature treatment than under constant temperature. This suggests there are a few species of membrane lipids which undergo rapid phase shifts in the face of changing temperatures, supporting the hypothesis of domains and micro-domains (Friend and Fawcett, 1974; Saxena et al., 1984; Saxena et al., 1986b).

4.1.3 Head Plasma Membrane Changes

Viscosity changes indicate definite membrane differences between HPM and the SBM. Temperature and semen source had major effects while calcium had a minimal effect upon viscosity of the HPM. Cooling the HPM led to a maintenance of initial viscosity. Heating abruptly increased viscosity in the HPM of the whole ejaculate, while that of the sperm rich HPM increased later. The extender essentially prevented major changes in viscosity, except at the highest temperatures.

At variable temperatures calcium had no effect upon the membranes, except for the sperm rich HPM. Even the HPM from this source showed a similar pattern of viscosity change in the presence or absence of calcium. The lack of response to calcium suggests that either the HPM is insensitive to calcium or that temperature-induced structural changes obscure a calcium effect.

At constant temperature, the viscosity of the HPM from all sources increased. The linearity of increase paralleled established fertility estimates. That is, raw semen collected for AI had to be used immediately as the fertility decreased rapidly with time. Use of extenders has greatly lengthened the period over which the semen could be stored and then used without a large decrease in viability. Thus the more fertile semen had a more linear increase in viscosity with time.

The present study demonstrates a positive effect of extender on HPM. It allows viscosity to increase, but in a gradual linear pattern. Particle-free areas on the head PM appear during capacitation but before fusion and vesiculation occur (Flechon, 1985). Fluidity increases are expected in the particle-free areas (Flechon, 1985) and viscosity increases in the remainder. Therefore, the probe tPNA appears to be detecting the viscosity increases seen with achievement of fertilizing capacity. Furthermore, the ultrastructural changes detected at constant temperature are affected by calcium. This supports the suggestion that the lack of a calcium effect in cooling and reheating is a reflection of the magnitude of the stress imposed on the HPM by varying temperature.

The similarity of the viscosity characteristics of the HPM from the extended and sperm rich semen compared to that of the whole ejaculate suggests the ultrastructure of sperm must be protected from temperature shocks but permitted to undergo the natural reorganization necessary in order to complete the process of fertilization.

Chapter V

CONCLUSIONS

1. Fluorescence polarization has proven to be a valid technique for measuring changes in sperm membrane microviscosity.
2. Viscosity of boar SBM and HPM are uniquely affected by variable temperature, constant temperature, calcium and source of semen.
3. Variable temperature treatment increased the viscosity of membranes in a different pattern than did constant temperature treatment.
4. Constant temperature induced distinct membrane viscosity changes that may equate to natural viscosity changes sperm undergo before fertilization.
5. Calcium influences SBM viscosity whereas, its role on HPM viscosity was less consistent.
6. Viscosity differences were detected utilizing different sources of semen. The whole ejaculate was affected differently in comparison to the sperm rich fraction and the sperm rich fraction was affected differently than the extended semen. These comparisons suggest possible reasons for the differences in the fertilizing ability of the three different sources, with the extended semen being representative of

the source most capable of fertilization, regardless of treatment effects.

7. Further research is required in this area to better understand how temperature and time interact to influence the sperm membranes.

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