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THE RELATIONSHIP BETWEEN IN VIVO HETEROSPERMIC FERTILITY AND IN VITRO TESTS OF SEMINAL QUALITY

Iowa State University PH.D. 1985

The relationship between *in vivo* heterospermic fertility and *in vitro* tests of seminal quality

by

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ABSTRACT

The relative fertilizing potential of frozen-thawed semen from four black and four white boars, whose offspring were distinguishable, was determined following heterospermic insemination. A heterospermic index was computed for each of the 16 possible pairs of black and white boars, as the ratio of offspring sired by the black boar minus the ratio of offspring sired by the white boar multiplied by 100. Correlation coefficients were computed between the heterospermic indices and the differences (black boar minus white boar) in several in vitro tests of seminal quality before and after cryopreservation of the semen. For the in vitro tests before cryopreservation, the heterospermic indices were negatively correlated (-0.57, $P<0.05$) with the differences in spermatozoan motility before cooling the semen but were not correlated ($P>0.05$) with the differences in spermatozoan motility after cooling to 5°C. The heterospermic indices were not correlated ($P>0.05$) with the differences in the following in vitro tests after freezing and thawing: spermatozoa with normal apical ridges, damaged apical ridges, and loose acrosomal caps, extracellular and maximum releasable glutamic oxaloacetic transaminase, and the number of penetrations per zona-free hamster oocyte. The heterospermic indices were correlated ($P<0.05$) with the differences in the following in vitro tests after freezing and thawing: spermatozoan motility (0.50), spermatozoa with either normal or damaged apical ridges (0.31), spermatozoa with missing apical ridges (-0.51), spermatozoa filtered through sephadex columns (0.32), spermatozoa with

acrosin-activity (0.38), percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly (0.54), spermatozoan intracellular glutamic oxaloacetic transaminase (-0.57), spermatozoa bound per zona-free hamster oocyte (0.64), and percentage of zona-free hamster oocytes penetrated (0.75). The relatively low correlations between the heterospermic indices and the differences in most in vitro tests indicated that the fertilizing potential of the semen used in this study could not be predicted with reasonable accuracy with any single in vitro test evaluated. In contrast, the multiple regression correlation coefficient between the heterospermic indices and the differences in four parameters from three in vitro tests (motility, spermatozoa with either normal or damaged apical ridges, number of spermatozoa bound per oocyte, and percentage of oocytes penetrated) was 0.94. This high correlation indicated that the fertilizing potential of the semen could be accurately predicted with four parameters that appeared to measure different properties of the spermatozoa.

INTRODUCTION

Pregnancies have been reported following cervical insemination of frozen-thawed porcine semen since 1971 (Crabo and Einarsson, 1971; Graham et al., 1971a,b; and Pursel and Johnson, 1971a,b). However, compared with fresh semen, frozen-thawed porcine semen has not been used extensively because (1) conception rates are reduced by 20% to 30%, (2) litter size is reduced by one to two pigs per litter, (3) the minimum number of spermatozoa needed to establish pregnancies is increased by three-fold, and (4) the variation in fertility among boars is increased markedly. Little progress has been made towards improving the methods for freezing porcine semen because expensive and time-consuming field trials have to be used to measure the fertilizing potential of the semen after manipulating extenders and freezing methods. In vitro tests that accurately predict the fertilizing potential of frozen-thawed porcine semen would be invaluable for improving freezing methods and for identifying boars of high fertility following freezing and thawing of their semen.

The relationship between in vitro tests of seminal quality and homospermic fertility, that is, fertility after inseminating females with semen from one male, has been investigated in several species. However, this relationship has been studied extensively only in the bovine. In this species, the correlation coefficients between tests of seminal quality and homospermic fertility have varied markedly among studies (review by Graham et al., 1979). Saacke et al. (1980) suggested two reasons for the inconsistency among studies designed to

correlate seminal quality with homospermic fertility. The first reason was that homospermic fertility could not be measured with acceptable precision because of the large variation in fertility among the female population due to differences in season, age, breed, disease, and management. The second reason for the inconsistency was the large number of spermatozoa used in these studies to ensure that optimal conception rates were maintained. Elliot (1979) reported that as certain traits of seminal quality increased, fertility also increased until the optimum or average fertility of the female population was reached. However, once this optimum level of fertility was reached, further increases in seminal quality were not detected because there were no corresponding increases in fertility. On this basis, Saacke et al. (1980) suggested that to accurately detect the relationship between homospermic fertility and seminal quality, the number of spermatozoa should be kept lower than the number required for optimum fertility of the female population.

A potential method of eliminating the above problems is through the use of tests of heterospermic fertility, that is, fertility after inseminating females with semen from two or more males (Saacke et al., 1980). Tests of heterospermic fertility have been shown to be more efficient for determining the relative fertility of males than tests of homospermic fertility (Beatty et al., 1969). Therefore, by using tests of heterospermic fertility, rather than tests of homospermic fertility, it should be possible to establish correlations

between *in vitro* tests of seminal quality and fertility with greater accuracy.

The first objective of this study was to correlate heterospermic fertility in swine following heterospermic insemination of frozen-thawed semen with several *in vitro* tests of seminal quality before and after cryopreservation. The *in vitro* tests of seminal quality that were evaluated before cryopreservation were motility of the spermatozoa before and after cooling to 5°C. The *in vitro* tests of seminal quality that were evaluated following freezing and thawing were motility of the spermatozoa without caffeine stimulation, acrosomal morphology, sephadex filtration of the spermatozoa without caffeine stimulation, glutamic oxaloacetic transaminase released from the spermatozoa, acrosin activity of the spermatozoa, and the spermatozoan penetration assay of zona-free hamster oocytes. The second objective of this study was to examine how much of the total variation in heterospermic fertility could be accounted for using selected *in vitro* tests of seminal quality.

LITERATURE REVIEW

Heterospermic Insemination

Heterospermic insemination with an equal number of spermatozoa from two or more males resulted in one male producing a disproportionate number of offspring in the rabbit (Beatty, 1960), mouse (Edwards, 1955), cow (Beatty et al., 1969), and chicken (Martin et al., 1974). Beatty (1960) reported that heterospermic insemination with an equal number of spermatozoa from two different rabbits resulted in a significantly higher ($P<0.005$) number of offspring from one of the rabbits. However, after natural mating, the conception rates of the two rabbits were similar. Beatty (1960) concluded that rabbits may appear to be of equal fertility following natural mating, but of unequal fertility following heterospermic inseminations. He also concluded that the differences in heterospermic fertility could be largely attributed to differences in the viability of the spermatozoa in the ejaculates.

Beatty et al. (1969) examined the correlation between heterospermic and homospermic fertility. Spermatozoa from five Friesian bulls were placed in competition with that of five Hereford bulls so that 25 mixtures of spermatozoa from one Friesian and one Hereford bull were possible. Heterospermic indices that expressed the relative power of each bull to sire offspring were calculated by computing 10 maximum likelihood constants. The correlation between the logarithm of the heterospermic indices and the 16-week homospermic nonreturn rates was 0.69 ($P<0.05$). The equation for this relationship

was $\hat{Y} = 61.95 + 4.23(\log X)$, where $\hat{Y}$ was the homospermic nonreturn rate and X the heterospermic index. A rise of 1.0 in the logarithm of the heterospermic index, that is, a ten-fold increase in the actual heterospermic index, predicted a rise of 4.23 in the homospermic index. The range in nonreturn rates following homospermic insemination for the ten bulls was 60.1% to 69.3%; the heterospermic indices ranged from 1.25 to 26.72. The test of heterospermic fertility, when compared with the test of homospermic fertility, appeared to increase markedly the variance in fertility among males, making it easier to detect differences in fertility. Linford et al. (1976) demonstrated the importance of maximizing the variation in fertility among bulls when correlating the results of laboratory assays for seminal quality with homospermic fertility. Correlations between measures of seminal quality and homospermic fertility increased as the variation in fertility among the bulls increased.

In the study by Beatty et al. (1969) that was just reviewed, the test of heterospermic fertility was calculated to be at least 170 times more efficient in estimating fertility than the test of homospermic fertility. They concluded that (1) homospermic insemination was an inefficient means of testing for fertility because, over a wide range of seminal quality, there would be enough spermatozoa to fertilize the majority of oocytes and, consequently, differences in fertility between males would be small and (2) heterospermic insemination was more efficient because (a) spermatozoa were placed in direct competition with each other, thereby

exaggerating differences in fertility between males and (b) comparisons were made within rather than between females.

A relationship similar to that observed in bulls between heterospermic and homospermic fertility was also reported for the chicken (Martin and Dziuk, 1977a). When hens were inseminated with a mixture containing an equal number of spermatozoa from one of three Leghorn and one of three Columbian cocks, using nine different Leghorn-Columbian combinations, the hierarchy of heterospermic fertility was similar to the hierarchy of homospermic fertility.

The repeatability of the ratios of offspring sired by different males after heterospermic insemination has been investigated in three species. The ratios were repeatable over several months in the rabbit (Beatty, 1960), bull (Beatty et al., 1969 and Stewart et al., 1974), and chicken (Martin et al., 1974).

Martin et al. (1974) demonstrated the importance of keeping a known ratio of spermatozoa in the inseminate used for tests of heterospermic fertility. The percentage of chicks sired by Columbian cocks increased as the percentage of Columbian spermatozoa in the inseminate increased. However, the total number of spermatozoa in the inseminate had no effect on the percentage of offspring sired when the ratio of spermatozoa in the inseminate was held constant.

Heterospermic insemination has been used to examine differences in seminal quality. Roche et al. (1968) used heterospermic insemination to demonstrate the decreased fertilizing ability of aged spermatozoa that competed with freshly ejaculated spermatozoa from

rabbits. The ability of different extenders to preserve the fertilizing potential of frozen and nonfrozen spermatozoa was investigated heterospermically in the rabbit (O'Reilly et al., 1972), pig (Pursel and Johnson, 1976), and chicken (Martin and Dziuk, 1977b). Heterospermic insemination was also used to study the effect of exposure to toxins on fertility (Cole and Davis, 1914 and Hagen and Dziuk, 1981) and to demonstrate that the relative fertilizing potential of a male is often not the same for fresh and frozen semen (Stewart et al., 1974 and Beatty et al., 1976). In all these studies, differences in seminal quality were detected by inseminating relatively few females.

The ratio of offspring sired by males that compete heterospermically may be due to the preferential removal of spermatozoa from one male by the female reproductive tract (Overstreet and Adams, 1971 and Ferreira and Graves, 1972). In these two studies, the ratios of offspring following heterospermic insemination were similar to the ratio of spermatozoa recovered from the female reproductive tract. As will be reviewed later, the preferential removal of spermatozoa from the female reproductive tract appears to be related to abnormalities in seminal quality.

Saacke et al. (1980) investigated the correlation between heterospermic fertility in the bovine and several <u>in vitro</u> tests of seminal quality. Spermatozoa from five bulls were placed in competition with spermatozoa from five other bulls. One ejaculate of frozen semen from each bull was used for all the tests of

heterospermic fertility and tests of seminal quality. Heterospermic indices were calculated as described earlier in Beatty et al.'s study (1969). The correlations between the tests of seminal quality and the heterospermic indices were much higher than those reported previously for the correlations between seminal quality and homospermic fertility. The correlation coefficients ranged from 0.44 ($P>0.05$) for their assay of head-to-head agglutination at 1.0 hour post-thaw to 0.93 ($P<0.01$) for photographic assessment of motility at 0 hour post-thaw. The average correlation over all assays and post-thaw times was 0.73. The correlation coefficients between assays of seminal quality and homospermic fertility in the bovine have ranged from 0.002 ($P>0.05$) to 0.83 ($P<0.01$) and have averaged around 0.42 (review by Graham et al., 1979).

Assays of Seminal Quality

Concentration of spermatozoa Beatty et al. (1969) reported a positive correlation ($r = 0.84$, $P<0.01$) between the concentration of spermatozoa in the ejaculate and the heterospermic indices following the insemination of nonfrozen bovine semen. In contrast, these parameters were not correlated for frozen bovine semen (Saacke et al., 1980). The correlation between these two parameters has not been reported for porcine semen.

Motility of spermatozoa The estimate of spermatozoan motility is the most widely used measure of seminal quality. Following the successful freezing of bovine semen (Polge and Rowson, 1952), attempts were made to freeze porcine semen using similar techniques. Although

motility after thawing was good, pregnancies were not achieved (review by Watson, 1979). It was shown later that concentrations of 7% to 11% glycerol, used to freeze bovine semen, were toxic to spermatozoa from boars (review by Graham et al., 1978). Because the percentage of motile porcine spermatozoa increased as the concentration of glycerol increased from 0% to 7% (Pursel et al., 1978b) and because fertility was affected adversely as the concentration of glycerol increased, it was assumed that estimates of motility were of little value for determining the fertilizing potential of frozen-thawed semen from boars (review by Graham et al., 1978). However, Yanagimachi (review, 1984) reported that vigorous motility is necessary for fertilization of zona-intact oocytes and, therefore, motility should be evaluated when estimating the fertilizing potential of semen. In addition, Hunter (1980) stated that in the porcine, "motility is almost certainly important for negotiation of the utero-tubal junction by sufficient numbers of spermatozoa to ensure subsequent fertility." One of the problems associated with determining the fertilizing potential of semen by estimating the percentage of motile spermatozoa is that live spermatozoa are often immotile after freezing and thawing (Aitken et al., 1983). There are two effective methods for bringing these immotile but live spermatozoa out of their low metabolic state. The first is incubation of the spermatozoa at 37^{o}C for several hours post-thaw. The relationship between spermatozoan motility and fertility increased when motility determinations were made after incubating frozen-thawed porcine semen for 3 hours at 37^{o}C, compared

with determinations made immediately post-thaw (Larsson and Einarsson, 1976). The second method for stimulating spermatozoa out of their low metabolic state is incubation with phosphodiesterase inhibitors such as caffeine. The loss in motility following freezing and thawing of human spermatozoa was partially overcome by the addition of caffeine in all (Barkay et al., 1977; Schill et al., 1979; and Aitken et al., 1983) but one study (Read and Schnieden, 1978). Phosphodiesterase inhibitors, such as caffeine, theophylline, and aminophylline, stimulated motility and respiration of spermatozoa by increasing the intracellular concentrations of cAMP (Garbers et al., 1973 and Haesungcharern and Chulavatnatol, 1973).

Tash and Mann (1973) reported that agents that depressed motility also decreased the concentration of cAMP in spermatozoa. Using multiple exposure photography and a supravital stain, Makler et al. (1980) demonstrated that immotile but live spermatozoa were activated by caffeine. Damage to the plasma membrane caused by freezing or cold shock resulted in loss of cAMP from the spermatozoa (Tash and Mann, 1973). Because it is known that a certain threshold of cAMP is required for the initiation of motility during spermatozoan maturation in the epididymis (Amann et al., 1982), the reduction in cAMP in frozen-thawed spermatozoa may be responsible for some of the loss in motility following freezing and thawing.

Zaneveld and Polakoski (1977) hypothesized that the female reproductive tract may provide the spermatozoa with a stimulus similar to that provided <u>in vitro</u> by the caffeine and that spermatozoan

motility following caffeine stimulation may be a better indicator of spermatozoan motility in the female reproductive tract. Graham et al. (1978) reported that the addition of caffeine to porcine semen thawed in fluids known to maintain the fertility of spermatozoa resulted in higher estimates of motility when compared with the motility of spermatozoa thawed in a fluid that did not maintain the fertility of spermatozoa. Without the addition of caffeine, motility did not differ. Bamba and Kojima (1978) reported that placing nonfrozen porcine spermatozoa on slides coated with caffeine provided a convenient method for stimulating spermatozoan motility.

Acrosomal morphology Abnormalities in the structure of the acrosome have been associated with reduced fertility in the boar (Buttle and Hancock, 1965 and Pursel et al., 1972b) and in several other species (review by Watson, 1979). However, in field trails, correlations between acrosomal morphology and fertility in the bovine have ranged from reasonably high and significant to low and nonsignificant (review by Graham et al., 1979). Talbot (1979) reported that exposing spermatozoa from hamsters to cold shock caused changes in acrosomal morphology, a decrease in *in vivo* and *in vitro* fertility, and a decrease in the number of spermatozoa binding to and penetrating the zona pellucida. Similarly, spermatozoa from boars with the acrosomal knobbed defect were unable to bind *in vivo* to the zona pellucida (Buttle and Hancock, 1965). Pursel et al. (1972b) reported that porcine spermatozoa stored in a diluent that maintained

motility, but resulted in acrosomal deterioration, were no longer fertile.

When compared with normal, viable spermatozoa, fewer dead or injured spermatozoa from pigs were found in the female reproductive tract several hours after insemination (Baker and Degan, 1972 and Pursel et al., 1978a). In the rabbit, Bedford (1965) demonstrated selective phagocytosis of spermatozoa with damaged acrosomes by uterine leucocytes. In addition, porcine (Pursel et al., 1978a and Hunter, 1980) and murine (Krzanowska, 1974; Nestor and Handel, 1984; and Redi et al., 1984) spermatozoa with abnormal morphology appeared to be disadvantaged in reaching the site of fertilization. After mating or artificial insemination, a lower percentage of abnormal spermatozoa was found in the oviducts than in the uterus. It is not known whether this phenomenon is due to an active selection against the abnormal spermatozoa by the female reproductive tract, or an inherent physiological inability of the abnormal spermatozoa to ascend the female reproductive tract (Hunter, 1980 and Nestor and Handel, 1984). Gaddum-Rosse (1981) reported that only motile spermatozoa were capable of passing through the utero-tubal junction of rats; neither immotile spermatozoa nor inert material passed through the junction. The hypothesis that only motile spermatozoa enter the oviduct was supported by other reports in the rat (Leonard and Perlman, 1949) and sheep (Dauzier, 1955, as cited by Gaddum-Rosse, 1981). However, other investigators demonstrated that inert material and immotile spermatozoa passed through the utero-tubal junction with equal

efficiency when compared with normal, motile spermatozoa (Glover and Patterson, 1963 and First et al., 1968). In addition, Nestor and Handel (1984) and Pursel et al. (1978a) demonstrated that some abnormal spermatozoa gained access to the oviduct. It was also possible for some abnormal spermatozoa to fertilize oocytes in mice (Olds-Clarke and Becker, 1978 and Krzanowska and Lorenc, 1983).

Sephadex filtration of spermatozoa The subjectivity in estimating motility and acrosomal morphology often results in inaccurate and imprecise values for these measurements (Diebel et al., 1976). A variety of assays of objective motility which increased the accuracy and precision of the estimate of motility has been reported but the cost of the equipment and labor for these assays has made them impractical for routine evaluation of semen (review by Graham et al., 1979). Fayemi et al. (1979) reported that filtration of frozen-thawed porcine semen through a sephadex column resulted in a spermatozoan population that was 89% motile and possessed 98% normal apical ridges. In the unfiltered samples, 45% of the spermatozoa were motile and 67% had normal apical ridges. Therefore, it appeared that filtration of semen through a sephadex G-15 column provided an objective means of separating spermatozoa to facilitate analysis of spermatozoan motility and acrosomal morphology. The sephadex columns appeared to remove damaged and dead but not inactive, live bovine spermatozoa (Graham et al., 1979). Correlations between this assay and fertility in the bovine ranged from 0.51 for homospermic fertility (Graham et al., 1979) to 0.82 for heterospermic fertility (Saacke et al., 1980). The

correlation between sephadex filtration and in vivo fertility has not been established in other species.

Acrosin activity of spermatozoa Acrosin, a trypsin-like proteinase, is an important functional constituent of mammalian spermatozoa. The acrosin system consists of the nonzymogen form of the enzyme (acrosin), the zymogen form of the enzyme (proacrosin), and the acrosin inhibitor. By quantifying components of the acrosin system it is possible to evaluate the integrity of the acrosome. Several studies demonstrated an appreciable loss of proacrosin and acrosin from spermatozoa damaged by cold shock and freezing with and without cryoprotectants; the harshest treatments produced the greatest damage to the membranes and caused the greatest alterations in the acrosin system (Harrison and Flechon, 1980; Goodpasture et al., 1981; and Froman et al., 1984).

Acrosin has been implicated in the following: penetration of the zona pellucida (Stambaugh et al., 1969), the acrosomal reaction (Meizel and Lui, 1976), spermatozoan transport through cervical mucus (Schumacher and Zaneveld, 1974), enhancement of spermatozoan migration in the female reproductive tract (Fritz et al., 1972), binding of spermatozoa to the zona pellucida (Saling, 1981), incorporation of spermatozoa into the oocyte (Wolf, 1977), and dispersion of chromosomes from spermatozoa following fertilization (Marushige and Marushige, 1978). Although the exact function(s) of acrosin is unknown, there is little doubt that it is an important functional

constituent of mature spermatozoa because inhibitors of acrosin prevent fertilization (Stambaugh et al., 1969).

Pace et al. (1981) reported a positive correlation (0.33) between homospermic fertility and acrosin activity of frozen-thawed spermatozoa from bulls. The extender in which canine spermatozoa were frozen influenced the acrosin activity of the spermatozoa (Froman et al., 1984). In addition, the acrosin activity of the spermatozoa was correlated (0.97) with the percentage of spermatozoa with intact acrosomes. Froman et al. (1984) concluded that although the percentage of proacrosin and acrosin activity associated with the spermatozoa were indices of cellular damage, acrosin activity alone could not be used to predict fertility. This conclusion was based on the observation that acrosin activity of the spermatozoa was reduced by only 37% following cryopreservation, while the ability of the frozen-thawed spermatozoa to penetrate oocytes was reduced by 65%. However, Froman et al. (1984) stated that "the assay of acrosin activity, in conjunction with motility estimates, provides a more complete evaluation of the efficacy of seminal extenders in attenuating freezing injury than do motility estimates alone."

Significant differences in acrosin activity of spermatozoa were reported between the spermatozoa from fertile and infertile men (Mohsenian et al., 1982). Spermatozoan motility was not related to changes in acrosin activity of spermatozoa after cryopreservation of human semen (Goodpasture et al., 1981). Cechova et al. (1984) also reported that for nonfrozen porcine spermatozoa stored in different

extenders, motility was not related to the concentration of proacrosin and acrosin released from the spermatozoa. Motility following storage of semen for 96 hours in Merck, Sadovnikova, and BL-M diluents was 50%, 70%, and 50%, respectively, and the concentration of proacrosin/acrosin (IU/7.5 x 10^9 spermatozoa) released from the spermatozoa was 7.7/0.3, 0.2/0.2, and 0.7/1.4, respectively. The proacrosin/acrosin activity of the spermatozoa, but not the motility of the spermatozoa, appeared to predict the fertilizing potential of the spermatozoa stored in the different extenders.

The acrosin activity of the spermatozoon can be quickly determined by the gelatin-substrate-film method (Ficsor et al., 1983). Incubation of spermatozoa on the film caused disintegration of the acrosome and digestion of the gelatin around the head of the spermatozoa, provided the acrosomal contents were still present when the spermatozoa were applied to the film. Acrosin appeared to be responsible for this digestion because the average diameter of the halo and the average spermatozoan content of acrosin were correlated (0.83, $P<0.01$; Wendt et al., 1975a) and because digestion was completely inhibited when inhibitors specific for acrosin were present in the film (Wendt et al., 1975b). The gelatin-substrate-film technique has been used for monitoring the acrosin activity of nonfrozen spermatozoa from the boar (Muller-Esterl et al., 1983), man, dog, squirrel monkey, mouse, and rat (Ficsor et al., 1983).

Glutamic oxaloacetic transaminase released from the spermatozoa

The release of enzymes from spermatozoa has been used to estimate damage to the cellular membrane after storage of frozen-thawed and nonfrozen semen from the boar, (Brown et al., 1971; Crabo et al., 1972; Bower et al., 1973; and Larsson and Einarsson, 1976) and other species (review by Watson, 1979). Graham et al. (1970) reported an increase in the release of glutamic oxaloacetic transaminase, lactate dehydrogenase, hyaluronidase, and acid and alkaline phosphatase from the spermatozoa that was proportional to the cellular damage in frozen-thawed semen of the boar, bull, stallion, and turkey. Larsson et al. (1976) reported that the release of glutamic oxaloacetic transaminase from porcine spermatozoa was a more sensitive indicator of cellular integrity than was ultrastructural evaluation of the spermatozoa. Similarly, Osinowo (1981) reported significant release of enzyme activity from spermatozoa in the bull and ram in the absence of "apparent" cellular damage to the spermatozoa. The extracellular levels of glutamic oxaloacetic transaminase and lactate dehydrogenase increased after storage of human semen at 5°C, increased further after cryostorage, and were highest following quench freezing (Zavos et al., 1980 and Mortimer and Bramley, 1981). In the buffalo, a positive correlation was reported between extracellular glutamic oxaloacetic transaminase and acrosomal damage following cryopreservation (Kakar and Anand, 1984).

Of the several enzymes investigated, glutamic oxaloacetic transaminase was the most highly correlated with spermatozoan

concentration in the boar, turkey, and man (Graham and Crabo, 1978). Brown et al. (1971) reported that extracellular glutamic oxaloacetic transaminase was a better indicator of spermatozoan damage than lactate dehydrogenase, cholinesterase, and acid and alkaline phosphatase in the boar, bull, and turkey. Motility and homospermic fertility appeared to be inversely related to the amount of glutamic oxaloacetic transaminase released from frozen-thawed porcine spermatozoa (Crabo et al., 1972 and Larsson and Einarsson, 1976). Of the four boars used in the study by Larsson and Einarsson (1976), Boar A had the highest conception rate and embryonic survival rate, boars B and C were about equally fertile, and boar D had a 0% conception rate. The extracellular glutamic oxaloacetic transaminase for Boars A, B, C, and D was 172, 280, 316, and 564 mIU/10^9 spermatozoa, respectively. Due to the small size of the study, a statistical analysis of the relationship was not possible.

Spermatozoan penetration assay of zona-free hamster oocytes

The incidence of idiopathic infertility in men was relatively high when diagnosis was based on standard measures of seminal quality such as spermatozoan concentration, motility, and morphology (review by Yanagamachi, 1984). Thus, other *in vitro* tests were needed to reduce the incidence of unexplained infertility in men. In 1976, Yanagimachi et al. reported *in vitro* penetration of zona-free hamster oocytes by human spermatozoa capacitated *in vitro*. Successful penetration of the oocytes depended on the ability of the spermatozoa to undergo capacitation and the acrosomal reaction. Before spermatozoa can

fertilize oocytes *in vivo*, the spermatozoa must undergo a series of biochemical and biophysical changes, collectively referred to as capacitation (Chang, 1951; Austin, 1951; and Bedford, 1983). Capacitation makes it possible for spermatozoa to undergo the acrosomal reaction, penetrate the zona pellucida, and bind to the vitelline membrane *in vivo* and *in vitro* (Bedford, 1983). Thus, the assay reported by Yanagimachi et al. (1976) appeared to offer an additional means of estimating the fertilizing potential of a male's semen by measuring the ability of the spermatozoa to complete certain functional requirements of fertilization. The penetration of zona-free hamster oocytes by heterologous spermatozoa is most commonly referred to as the "spermatozoan penetration assay".

The plasma membrane of the hamster oocyte is unusual in its inability to prevent extensive polyspermic penetration (Binor et al., 1982), despite the release of cortical granules from the oocyte at fertilization (Austin, 1956). Zona-free hamster oocytes have been penetrated by spermatozoa from the boar, bull, human, goat, bat, dog, dolphin, monkey, hamster, mouse, rat, rabbit, guinea pig, and stallion (review by Yanagimachi, 1984).

Although the spermatozoan penetration assay has been used extensively in the diagnosis of male infertility, controversy still exists about the ability of the test to predict a given man's chance for completing fertilization *in vivo*. The percentage of zona-free hamster oocytes penetrated by spermatozoa from fertile men ranged from 0 to 100% and the average was 57% (review by Yanagimachi, 1984).

Approximately 30 to 50 zona-free hamster oocytes are usually used per seminal sample to determine penetration rates. Most workers reported a positive relationship between the ability of human spermatozoa to penetrate zona-free hamster oocytes and their ability to complete fertilization in vivo (review by Yanagimachi, 1984). However, in most reports, there were cases in which the zona-free hamster oocytes were either penetrated by spermatozoa from men of longstanding infertility (false positives) or failed to be penetrated by spermatozoa from fertile men (false negatives). In most laboratories that deal with human fertility, men whose spermatozoa fail to penetrate at least 15% of the zona-free hamster oocytes were diagnosed as infertile (review by Yanagimachi, 1984). The average percentage of zona-free hamster oocytes penetrated by spermatozoa from infertile men was 17% (review by Yanagimachi, 1984).

According to Yanagamachi (1984), the requirement for very high numbers of spermatozoa in the spermatozoan penetration assay implies that the test is not very sensitive for estimating the fertilizing capacity of the spermatozoon. However, he suggested that this lack of sensitivity may be beneficial for estimating the in vivo fertilizing capacity of spermatozoa "because spermatozoa that are able to penetrate hamster eggs may represent a highly selected population of spermatozoa with excellent fertilizing capacity" and "therefore the spermatozoa potentially capable of fertilizing oocytes in vivo may be among those that are able to penetrate zona-free hamster eggs."

The first successful penetration of zona-free hamster oocytes by spermatozoa from domestic species was accomplished when porcine spermatozoa were capacitated in the gilt's isolated reproductive tract (Imai et al., 1977, 1979). When the spermatozoa were removed from the tract, a high percentage of the spermatozoa had undergone the acrosomal reaction and only these spermatozoa fused with the zona-free hamster oocyte (Imai et al., 1980). Therefore, it appeared that porcine spermatozoa, like human spermatozoa (Yanagimachi et al., 1976), must complete the acrosomal reaction before penetrating zona-free hamster oocytes. Porcine spermatozoa that were incubated in modified Krebs Ringer Bicarbonate failed to undergo the acrosomal reaction and failed to penetrate zona-free hamster oocytes (Imai et al., 1977, 1979, 1980). Kim et al. (1980) reported similar results for caprine spermatozoa.

Successful penetration of zona-free hamster oocytes has also been achieved following incubation of spermatozoa in the heterologous isolated reproductive tract. Spermatozoa from the boar and bull were successfully capacitated for the spermatozoan penetration assay after incubation in the isolated uterus of rabbits (Hanada and Nagase, 1981). Once again, the hamster oocytes were not penetrated if the spermatozoa were incubated in a defined medium (Hanada and Nagase, 1981).

The acrosomal reaction was induced in noncapacitated porcine spermatozoa by adding the divalent cation ionophore A23187 to the incubation medium (Smith et al., 1983). These spermatozoa were then

capable of immediately penetrating zona-free hamster oocytes. When spermatozoa from the same population were incubated for up to 12 hours in the same medium that did not contain ionophore, the hamster oocytes were not penetrated.

The first successful penetration of zona-free hamster oocytes by nonfrozen porcine spermatozoa that were capacitated in a defined medium was reported by Pavlok (1981). Zona-free, but not intact, porcine oocytes were also penetrated using the same procedures (Pavlok, 1981). Recently, in vitro fertilization of intact porcine oocytes in Pavlok's medium was achieved when incubation temperatures were increased from 37°C to 39°C (Cheng and Polge, 1983). Fresh (Brackett et al., 1982a,b) and frozen (Bousquet and Brackett, 1982) spermatozoa from bulls have been successfully capacitated in vitro for the spermatozoan penetration assay. Zona-free (Fulka et al., 1982) and zona-intact (Brackett et al., 1982a) bovine oocytes have also been penetrated by bovine spermatozoa capacitated in vitro.

The relationship between the spermatozoan penetration assay and in vivo fertility in domestic animals is largely unknown. Bousquet and Brackett (1982) compared the ability of spermatozoa from bulls A and B, with established 60-day nonreturn rates of 68.2% and 64.3%, respectively, to interact with zona-free hamster oocytes. Bull A excelled in the average number of spermatozoa attached per oocyte, number of penetrations per oocyte, and percentage of oocytes penetrated. However, the percentage of penetrated oocytes with male pronuclei was significantly greater for bull B than for bull A.

In swine, the relationship between in vivo fertility of nonfrozen porcine spermatozoa and the spermatozoan penetration assay has been evaluated indirectly (Cechova et al., 1984). Semen diluted with Merk, Sadovnikova, or BL-M extenders was stored at 16° to 18°C for four days. After storage, the percentage of zona-free hamster oocytes penetrated was 37% for the Merk extender and 0% for the other two extenders. Semen stored for four days in the Merk, but not in the Sadovnikova or BL-M extenders, maintained its in vivo fertilizing potential according to the Minitub Company (Germany) and veterinary practitioners in Czechoslovakia (Cechova et al., 1984). Correlation coefficients between the spermatozoan penetration assay and in vivo fertilty have not been reported.

It appears that some events differ during fertilization of zona-free and zona-intact oocytes. Pavlok (1981) and Brackett et al. (1982a) demonstrated that conditions suitable for penetration of zona-free hamster oocytes were not always suitable for in vitro fertilization of homologous zona-intact oocytes. Penetration of zona-free hamster oocytes by spermatozoa does not appear to require the vigorous spermatozoan motility and zona lysins that are essential for successful penetration of intact oocytes (review by Yanagimachi, 1984).

Only limited information exists on the correlation between assays of seminal quality and the spermatozoan penetration assay in domestic animals. High correlations were reported between the percentage of zona-free hamster oocytes penetrated by bovine spermatozoa and the

percentage of spermatozoa with intact acrosomes and progressively motile spermatozoa (Calcote, 1979; Bousquet and Brackett, 1982). In contrast, the spermatozoan penetration assay and percent motility were not related following storage of porcine semen in different diluents for four days (Cechova et al. 1984).

There is also a paucity of information on how cryopreservation of semen affects the spermatozoan penetration assay. Cryopreservation of human spermatozoa usually results in a reduction in the percentage of zona-free hamster oocytes penetrated when compared with nonfrozen spermatozoa (review by Yanagimachi, 1984). The effect of freezing on the time required for _in vitro_ capacitation has not been studied. In swine, Pursel (1983) demonstrated that frozen-thawed spermatozoa were capacitated sooner _in vivo_ than nonfrozen spermatozoa. This effect was due to the processing of the semen before freezing on dry ice (Pursel, 1983).

The pH of the medium affects the spermatozoan penetration assay. When washed human spermatozoa were incubated with zona-free hamster oocytes either without preincubation (Yanagimachi et al., 1976) or after 18 hours of preincubation (Tyler et al., 1981), penetration occurred sooner at pH 8.1 to 8.3 (normal atmospheric air) than at pH 7.2 to 7.6 (5% CO_2 in normal atmospheric air). It appeared that capacitation and the acrosomal reaction occurred sooner at higher pH than at lower pH. During _in vitro_ capacitation of spermatozoa from the guinea pig, low pH (6.1, 6.4, 6.7, and 7.0), when compared with

high pH (7.5 and 8.2), was also found to inhibit motility and the acrosomal reaction (Murphy and Yanagimachi, 1984).

Procedures and diluents have a profound effect on the spermatozoan penetration assay. For example, Johnson et al. (1984) reported a 2.5-fold increase in the percentage of zona-free hamster oocytes penetrated by spermatozoa that were preincubated for 42 hours rather than 18 hours in buffered egg yolk at 4°C. The higher penetration rates for spermatozoa preincubated for 42 hours may be related to the fact that the acrosomal reaction within any given population of spermatozoa is asynchronous (Bedford, 1983). Because spermatozoa have a short lifespan after they undergo the acrosomal reaction and because buffered egg yolk prolongs the viability of spermatozoa, Johnson et al. (1984) reasoned that the buffered egg yolk permitted capacitation to proceed only to the point of the acrosomal reaction. This synchronization of the spermatozoan acrosomal reaction increased the percentage of competent spermatozoa that could penetrate the zona-free hamster oocytes. The proteins in egg yolk may also help remove decapacitation factors from the surface of the spermatozoa (Johnson et al., 1984). Other factors which appear to affect the spermatozoan penetration assay are (1) the interval between seminal collection and removal of the seminal plasma from the spermatozoa, (2) the concentration of the spermatozoa in the preincubation and insemination medium, and (3) the composition of the medium and the atmosphere for incubation of spermatozoa and oocytes (review by Yanagimachi, 1984).

The correlation between the spermatozoan penetration assay and *in vivo* fertilty may be increased if the number of spermatozoa used in the assay are adjusted so that approximately 50% of the oocytes are fertilized. Robl and Dziuk (1984) reported that differences in fertility among three strains of mice were detected only when approximately 50% of the oocytes were fertilized *in vivo*. The percentage of oocytes fertilized was varied by adjusting the number of spermatozoa in the inseminate. When the percentage of oocytes fertilized was either high or low, the differences in fertility among the three strains were not detected. They concluded that the optimal number of spermatozoa in the inseminate for estimating differences in fertility was the number that resulted in a fertilization rate of approximately 50%.

MATERIALS AND METHODS

Processing of Semen

Two ejaculates, A and B, were collected from each of eight boars and were evaluated for seminal quality before and after cryopreservation. Each ejaculate was collected into an insulated centrifuge glass using the gloved-hand technique. A double layer of cheesecloth was fastened over the opening of the glass to remove the gel portion of the ejaculate during collection. For cryopreservation purposes, the ejaculate was first diluted two parts semen to three parts Prediluter (Appendix A). The extended semen was then cooled gradually from 32°C to 18°C over a four-hour period in a programmable incubator. The cooled semen was centrifuged for 10 minutes at 800 x g and the supernatant removed by aspiration. The pellet was resuspended with Cool Diluter (Appendix A) to a concentration of 2×10^9 spermatozoa/ml. The temperature of this suspension was decreased gradually from 18°C to 5°C during a 2.25-hour period. Immediately before freezing, the spermatozoa were further diluted with Deep Freeze Diluter (Appendix A) to a concentration of 1.2×10^9 spermatozoa/ml. German Makrotub straws were filled with 5 ml of extended spermatozoa and sealed with one plastic and one metal ball. The straws were frozen in an automated freezing device (Appendix B) and stored in liquid nitrogen until required for an insemination or an assay. The straws were thawed in a 52°C water bath for 0.87 minute and the contents were added to 80 ml of Olep (Appendix A) prewarmed to

25°C for the test of heterospermic fertility and for all the assays of seminal quality that were performed post-thaw except for the spermatozoan penetration assay, in which undiluted frozen-thawed semen was used.

Experimental Designs for the Assays of Seminal Quality Before Cryopreservation

The assays of seminal quality before cryopreservation were performed on the individual A and B ejaculates from each of the eight boars. A two-factor crossed design in which there were eight boars and two ejaculates was used for the following assays: concentration of spermatozoa, volume of the ejaculate, and total number of spermatozoa in the ejaculate. A two-factor crossed design in which there were eight boars, two ejaculates, and four subsamples was used for the assays of motility of the spermatozoa. The subsamples are referred to as replicates in the analysis of variance tables.

Methods for the Assays of Seminal Quality Before Cryopreservation

Concentration of spermatozoa and volume of the ejaculate The volume of the ejaculate was determined with a 500-ml graduated cylinder. The concentration of the spermatozoa in the ejaculate was determined with a hemacytometer according to the methods of the World Health Organization (1980).

Motility of spermatozoa The motility of the spermatozoa was evaluated once before cooling and once after cooling the semen. The first evaluation was made immediately following dilution with

Prediluter. The second evaluation was made after the semen was cooled to 5°C and extended with Deep Freeze Diluter. The cooled semen was extended 1:10 with Modena (Appendix C) and warmed to 37°C for 10 minutes before estimating motility.

Experimental Designs for the *In Vitro* Tests of Seminal Quality Following Freezing and Thawing

Before performing the *in vitro* tests of seminal quality following freezing and thawing of the semen from a particular boar, one straw from ejaculate A and one straw from ejaculate B were pooled and mixed thoroughly. A two-factor crossed design in which there were eight boars, five post-thaw incubation times (0, 1, 3, 5, and 7 hours), and four subsamples was used for the following assays: motility of the spermatozoa with and without caffeine stimulation, acrosomal morphology, sephadex filtration of the spermatozoa with caffeine stimulation, acrosin activity of the spermatozoa, and extracellular, intracellular, and percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly. A two-factor crossed design in which there were eight boars, four post-thaw incubation times (1, 3, 5, and 7 hours), and two subsamples was used for the assay of sephadex filtration of spermatozoa without caffeine stimulation. The subsamples are referred to as replicates in the analysis of variance tables. The spermatozoa were incubated post-thaw in the Olep diluent at 37°C. The seminal samples were coded, so that the identity of the boar was not known during the evaluation.

The in vitro test of maximum releasable glutamic oxaloacetic transaminase from the spermatozoa and the spermatozoan penetration assay were performed at 1 and 0 hour post-thaw, respectively. A completely randomized design in which there were eight boars and four replicates was used for these two in vitro tests.

Methods for the In Vitro Tests of Seminal Quality Following Freezing and Thawing

Motility of spermatozoa Percent motility was estimated to the nearest 5% at 400X with a phase contrast microscope that had a stage heated to 37°C. Wet mounts were prepared by placing an 8 ul drop of semen on a clean slide and covering the drop with a No. 1-1/2, 22 x 22 mm, cover slip.

Slides coated with caffeine were used for the assay of motility following caffeine stimulation. Slides with caffeine were prepared by spreading 0.05 ml of 1% caffeine in ethanol (w/v) between two slides (Bamba and Kojima, 1978). The slides were air-dried and stored in slide boxes until needed. The average time between placing 0.01 ml of semen on the slide and the first observation was 15 seconds. Samples were observed continuously until the motility began to decline to ensure that maximal motilities were always recorded. From previous experience, it was known that maximal motility occurred approximately 45 seconds following the addition of the semen to the slide.

Acrosomal morphology A 0.5-ml sample of semen was diluted 1:1 with 10% buffered formalin and 0.01 ml of the diluted sample was covered with a No. 1-1/2, 22 x 22 mm, cover slip and evaluated with a

phase contrast microscope at 1,000X. One hundred acrosomes per slide were categorized into one of four classes of acrosomal morphology (Pursel et al., 1972a; Appendix D).

Sephadex filtration of spermatozoa The columns for the assays of sephadex filtration were prepared according to the procedures of Fayemi et al. (1979) and Graham et al. (1979; Appendix E). A 1.0-ml aliquot of semen was added to each column and eluted three times with 3.0 ml of flushing fluid. The flushing fluid consisted of 0.12 M sodium citrate with 5 mM caffeine or 0.12 M sodium citrate without caffeine. The replicates were performed simultaneously within each time period. Filtrates were collected in 30-ml beakers, the volume measured with a 10-ml graduated cylinder, and transferred to test tubes containing 0.5 ml of buffered formalin. The spermatozoa in the filtrates were counted with a hemacytometer within 48 hours. The number of spermatozoa added to each column was also determined using a hemacytometer. The percentage of spermatozoa filtered through the column was calculated as follows:

$$\% \text{ filtered} = \frac{\text{total number spermatozoa in filtrate}}{\text{total number spermatozoa added to column}} \times 100$$

During the preliminary studies, frozen-thawed semen was quench frozen to disrupt the spermatozoa for the assays of validation. A 1.5-ml aliquot of semen was placed in a plastic ampoule and plunged directly into liquid nitrogen. After removing the aliquot from the liquid nitrogen, it took 0.5 hour at room temperature for the aliquot to thaw, at which time it was returned to the liquid nitrogen. The aliquot was frozen and thawed three times.

Acrosin activity of spermatozoa The gelatin-substrate-film method for the detection of acrosin activity was performed according to the procedures of Ficsor et al. (1983; Appendix F). To remove extracellular acrosin, a 0.5-ml aliquot of frozen-thawed semen was centrifuged twice at 600 x g for 5 minutes, and the pelleted spermatozoa were resuspended with 1.5 ml of phosphate buffered saline. A 1.5-ul sample of washed spermatozoa was spread evenly over the gelatin with an L-shaped glass spreader. The liquid was evaporated from the surface of the gels by placing the gels 7 inches away from a 1,000-watt heater (Model 30H33-1, Arvin Industries Inc., Columbus, IN) for 10 minutes. After drying, the slides were placed horizontally for 60 minutes at 37°C in humidified chambers. Following the 60-minute incubation, the gels were cooled for 5 minutes at room temperature, dipped once in distilled water, and stained for 2 minutes in 0.3% toluidine blue O in 0.1 M borate buffer. The gels were rinsed twice in distilled water, dried vertically at room temperature for a minimum of 24 hours, and examined with a phase contrast microscope at 200X. Four hundred spermatozoa per slide were examined for the presence or absence of halos of digestion. The diameters of the halos surrounding ten acrosin-positive spermatozoa, from ten different microscopic fields, were measured with an ocular micrometer.

Quench frozen spermatozoa were used to validate the assay of acrosin activity. The technique for quench freezing was identical to the technique described earlier.

The relationship between acrosin activity and acrosomal morphology following in vitro storage of nonfrozen porcine semen

To gain further information on which class(es) of acrosomes possessed acrosin activity, the acrosomal morphology and the acrosin activity of nonfrozen porcine spermatozoa were examined. Semen was collected from one Hampshire crossbred boar and one Landrace crossbred boar that were different from the other eight boars in this study. Immediately following seminal collection the semen was diluted 1:3 with Prediluter. The diluted semen was stored in the dark at room temperature and the spermatozoa were examined for acrosomal morphology and acrosin activity on days 0, 2, 4, 7, 9, 13, 15, 18, 20, 22, 26, 33, and 47 post-collection.

Glutamic oxaloacetic transaminase released from the spermatozoa

The spermatozoa were separated from the extenders according to the procedures of Brown et al. (1971; Appendix G). During the preliminary studies, quench frozen and sonicated frozen-thawed semen were compared to determine the better method of achieving maximum release of glutamic oxaloacetic transaminase from the spermatozoa. Samples were quench frozen by the method described earlier. Sonicated samples (5 ml) were processed for 40 seconds at 30% maximum power (Biosonik IV, VWR Scientific). The quench freezing and sonication procedures were always initiated one hour following thawing of the semen. Quench frozen and sonicated samples were layered on the density gradient, centrifuged, and stored as described in Appendix G.

The extracellular glutamic oxaloacetic transaminase of the normal frozen-thawed spermatozoa was determined at each of the five post-thaw times. Maximum releasable glutamic oxaloacetic transaminase was determined 1.0 hour post-thaw. Intracellular glutamic oxaloacetic transaminase was calculated for each of the five post-thaw times as the difference between maximum releasable glutamic oxaloacetic transaminase and extracellular glutamic oxaloacetic transaminase. The percentage of the maximum releasable glutamic oxaloacetic transaminase that was present extracellularly at each of the five post-thaw times was calculated as the extracellular glutamic oxaloacetic transaminase x 100/maximum releasable glutamic oxaloacetic transaminase.

All samples were analyzed within three weeks of storage by a kinetic procedure (Appendix G) at the Veterinary Clinical Pathology Laboratory, Iowa State University. Results were converted from IU/l to mIU/10^9 spermatozoa.

Spermatozoan penetration assay of zona-free hamster oocytes

Supplemented medium 199 was prepared according to the procedures of Pavlok (1981; Appendix H). This medium was used for all the handling procedures for the gametes and for the 0.2% trypsin and 0.1% hyaluronidase.

Prepuberal Syrian Golden hamsters, 5 to 7 weeks of age, were superovulated according to the regimen of Berger et al. (1983; Appendix H). Immediately before the oocytes were collected, the animals were anesthetized with ether and killed by cervical

dislocation. The oviduct and a small piece of the adjacent uterine horn were removed and placed in 2 ml of 0.1% hyaluronidase under paraffin oil. A tear was made at the ampullary-isthmus junction of the oviduct with a 25-gauge needle, and a 30-gauge blunt-end needle was inserted into the infundibulum. Small forceps were used to hold the needle in the oviduct. The cumulus masses containing the oocytes were flushed out of the ampullary region with 0.3 ml of 0.1% hyaluronidase within 10 minutes of cervical dislocation. The cumulus masses were incubated for 2 minutes in 0.1% hyaluronidase to free the oocytes from the cumulus cells. Next, the oocytes were washed three times in 0.2-ml aliquots of medium under equilibrated paraffin oil. The oocytes were then incubated in 0.2% trypsin for 2 minutes to remove the zona pellucida and washed three more times in 0.2 ml of medium. Four replicates of 12 oocytes each from a pool of oocytes from two to four hamsters were transferred in 0.05 ml of medium to multiwell culture plates that contained equilibrated paraffin oil.

Four replicates of frozen-thawed spermatozoa were prepared for the spermatozoan penetration assay by adding 0.02 ml of undiluted frozen-thawed semen to 0.98 ml of supplemented medium 199 at 25°C. Each of the four groups of oocytes was inseminated with 0.05 ml from one of the four replicates of frozen-thawed spermatozoa. The final concentration of spermatozoa in the microdroplet was 1×10^7 spermatozoa/ml. Sterile procedures were used for all the steps described above.

The oocytes were incubated with the spermatozoa for 3 hours at 39°C in a humidified desiccator under normal atmospheric air. At the end of the incubation period, 0.1 ml of buffered formalin was added to each 0.1 ml microdroplet. Formalin appeared to strengthen or harden the vitelline membrane and prevented rupture of the oocyte during the washing and mounting procedures. The 0.2 ml microdroplet was then transferred to 1.5 ml of 0.1% gelatin in saline. The gelatin prevented the oocytes from sticking to the bottom of the petri dish and pipette. Loosely bound spermatozoa were removed from the oocytes by pipetting the oocytes five times through a 9-inch Pasteur pipette that was pulled by hand to a bore of 80 to 100 um.

All the oocytes from one replicate were mounted on one slide. The oocytes were transferred in 6 ul of medium to a microscope slide. Two strips of a 1:1 vaseline-paraffin mixture were placed along the long edges of the slide. The oocytes were compressed with a No. 1 1/2, 22 x 22 mm, cover slip until the oocytes were flat and translucent. The oocytes were fixed overnight in 25% acetic acid in undenatured absolute ethanol (v/v) and stained the following morning for 10 minutes in 0.5% lacmoid in 45% acetic acid (w/v), filtered through No. 2 Whatman filter paper. The edges of the coverslip were sealed with clear fingernail polish. Immediately after sealing, the oocytes were observed under phase contrast microscopy at 400X for the number of bound spermatozoa and the number of male pronuclei or swollen spermatozoan heads. The average number of spermatozoa bound to all oocytes, the number of penetrations per oocyte for those

oocytes that were penetrated, and the percentage of oocytes penetrated were calculated for each replicate.

Estrous Synchronization and Artificial Insemination

Purebred Duroc sows were synchronized for the test of heterospermic fertility by using one of the following schedules.

Schedule for aborted sows

Treatment	Hour	
10 mg $PGF_2\alpha$ (i.m.) at 15-45 days of gestation	0	
10 mg $PFG_2\alpha$ (i.m.)	12	
750 IU PMSG (s.c.)	24	78-hour interval
750 IU hCG (i.m.)	102	34-hour interval
Insemination	136	

Schedule for weaned sows

Treatment	Hour	
Weaned at 3-6 weeks of lactation	0	
750 IU PMSG (s.c.)	8	72-hour interval
750 IU hCG (i.m.)	80	34-hour interval
Insemination	114	

Sows were synchronized and artificially inseminated in groups of four. The inseminates were prepared by pooling one straw from ejaculate A and one straw from ejaculate B from each of the two boars and dividing the pool into four equal volumes.

Design for the Test of Heterospermic Fertility

A 4 x 4 design was used for the test of heterospermic fertility. Spermatozoa from one Spotted (B1), Hampshire (B2), or Berkshire (B3 and B4) boar were placed in competition with spermatozoa from one Yorkshire (W1 and W3) or Landrace (W2 and W4) boar so that 16 pairs of boars were possible. This design is illustrated below.

	B1	B2	B3	B4
W1				
W2				
W3				
W4				

The design permitted identification of the paternity of the offspring by the color of their coat, because, in swine, black is dominant to red (Duroc sows) and white is dominant to black and red.

Statistical Analyses

A two-way analysis of variance was used to test for main effects for the assays in which a two-factor crossed design was used. A one-way analysis of variance was used to test for main effects for the assays in which a completely randomized block design was used. The expected mean squares for the source lines in the analysis of variance tables are given in Appendix I. The denominator for the F tests in the one-way analysis of variance tables was the replicate(boar) mean square. The denominator for the F tests in the two-way analysis of

variance tables was the boars x ejaculates or boars x times mean square. The replication mean square in the two-way analysis of variance tables could not be used as an error term to test for interaction between the main effects because, as stated earlier, the replicates were actually subsamples.

Valid F tests could not be made for the time effects for the assays of seminal quality in which a two-factor boar x time crossed design was used because the residuals (errors) from the model were not normally and independently distributed with the same variance. In experiments in which a unit is measured repeatedly over time, the residuals are not normally and independently distributed, but rather the residuals for a given treatment are correlated with each other (Gill and Hafs, 1971 and Snedecor and Cochran, 1980). Therefore, conservative degrees of freedom were used for the tests of significance for time (Gill and Hafs, 1971). Conservative degrees of freedom were formed by dividing the times and boars x times normal degrees of freedom by the normal degrees of freedom for times.

The times and boars x times sums of squares were partitioned into their linear and lack of fit components ($time_{linear}$, $time_{lack\ of\ fit}$, boars x $times_{linear}$, boars x $times_{lack\ of\ fit}$). The $time_{linear}$/boars x $times_{linear}$ F ratio was used to test for a significant linear relationship between the assay values (averaged over all boars) and time. The F test indicated whether the <u>in vitro</u> laboratory assays significantly increased or decreased in a linear manner during the post-thaw incubation period. The F ratio for

$time_{lack\ of\ fit}$/boars x $times_{lack\ of\ fit}$ was used to determine whether the assay x time deviations from linearity were significant. Significant deviations from linearity indicated that the relationship between the assay and time might be described better with a higher degree polynomial.

The standard errors for the boar, time, and overall means for the assays with a two-factor crossed design, in which eight boars and four or five times were used, could not be computed with the usual equation ($\sqrt{s^2/n}$) because the residual errors were correlated. The equations for calculating the standard errors for the boar, time, and ejaculate means are given in Appendix J. The Tukey's Studentized range test (Snedecor and Cochran, 1980) was used to test for differences among the boar means for the assays of seminal quality.

The repeatability of the assays was estimated by calculating 95% confidence intervals for boar or boar x time assay means. The equations for these calculations are given in Appendix J.

Pertinent correlation coefficients were calculated between the in vitro tests of seminal quality.

The model used for calculating the 16 heterospermic indices for the 4 x 4 design is given in Appendix K. The heterospermic indices were calculated as the ratio of offspring sired by the black boar minus the ratio of offspring sired by the white boar multiplied by 100. A Chi-square analysis (Snedecor and Cochran, 1980) was used to test whether the number of offspring sired by the black and white boars differed significantly from a 1:1 ratio for each of the 16

combinations of boars. An overall or average heterospermic index was also calculated for each of the eight boars, according to the statistical model of Saacke et al. (1980; Appendix L).

The differences (black boar minus white boar) for each test of seminal quality were calculated for each of the 16 combinations of two boars. The correlation coefficients between the 16 heterospermic indices and the 16 differences calculated for each of the in vitro tests were then computed to establish the relationship between heterospermic fertility and the in vitro tests of seminal quality.

A multiple regression analysis was used to establish how much of the total variation in heterospermic fertility could be accounted for using selected in vitro tests. Four parameters were selected from three different in vitro tests. A "backward elimination procedure" was used to determine the parameters that contributed significantly to the multiple regression model.

RESULTS

Seminal Quality Before Cryopreservation

The results of the tests of seminal quality before cryopreservation for each of the eight boars are shown in Table 1. There was a significant effect of boar ($P<0.025$) on the total number of spermatozoa in the ejaculate (Table 2). However, there was no significant effect of boar ($P>0.05$) on spermatozoan concentration, volume of the ejaculate, motility following dilution, and motility following cooling to 5°C. There were no differences ($P>0.05$) between ejaculates for any of the *in vitro* tests (Table 2).

In Vitro Tests of Seminal Quality Following Freezing and Thawing

Motility of spermatozoa

Motility without caffeine stimulation The results of all the *in vitro* tests in which a boar x time design was used are presented in tabular form as boar x time means ± the standard deviation of the replicates. The estimates of the percentage of motile spermatozoa without caffeine stimulation following cryopreservation and thawing are given in Table 3. The two-way analysis of variance for this assay is shown in Table 4. There was a significant effect of boar ($P<0.005$) and time ($P<0.025$) on motility. In addition, there was a linear decrease ($P<0.005$) in motility, averaged over all boars, with increasing incubation time post-thaw. The regression equation that predicted the change in spermatozoan motility ($\hat{y}$, %) with time

(x, hours) was $\hat{y}$ = 35.9 - 2.6 hours (r = -0.48, P<0.0001). The deviations from linearity were not significant (P>0.05).

Motility with caffeine stimulation The results of the assay of motility following caffeine stimulation are shown in Table 5. The analysis of variance for this assay is presented in Table 6. There was a significant effect of boar (P<0.001) and time (P<0.05) on motility following caffeine stimulation. The motility following caffeine stimulation decreased (P<0.025) linearly with increasing incubation time post-thaw. The regression equation that predicted the change in spermatozoan motility ($\hat{y}$, %) with time (x, hours) was $\hat{y}$ = 45.8 - 1.6 hours (r = -0.30, P<0.002). The deviations from linearity were not significant (P>0.05).

Acrosomal morphology

The percentages of spermatozoa with normal apical ridges, damaged apical ridges, missing apical ridges, and loose acrosomal caps are given in Tables 7, 9, 11, and 13, respectively, and the analysis of variance in Tables 8, 10, 12, and 14, respectively. For spermatozoa with normal and missing apical ridges there was a significant effect of boar (P<0.001 and P<0.005), time (P<0.01 and P<0.025), and $time_{linear}$ (P<0.005 and P<0.005), respectively. The deviations from linearity were not significant (P>0.05). The regression equations that predicted the change in spermatozoa with normal and missing apical ridges ($\hat{y}$, %) with time (x, hours) were $\hat{y}$ = 44.4 - 3.8 hours (r = -0.40, P<0.0001) and $\hat{y}$ = 20.6 + 1.6 hours (r = 0.46, P<0.0001),

respectively. For spermatozoa with damaged apical ridges and loose acrosomal caps there was a significant effect of boar ($P<0.005$ and $P<0.001$, respectively). The percentages of spermatozoa with damaged apical ridges and loose acrosomal caps were not affected by time ($P>0.05$).

Sephadex filtration of spermatozoa

Preliminary studies Table 15 shows the results of the validation of the assay of sephadex filtration. Known percentages of immotile, damaged spermatozoa were added to normal, frozen-thawed spermatozoa. Quench frozen spermatozoa, with 0% motility and 0% normal and damaged apical ridges, were used for the immotile, damaged population. The seminal samples that were added to the sephadex columns were prepared with the following ratios of normal, frozen-thawed to quench frozen spermatozoa: 100:0, 75:25, 50:50, 25:75, and 0:100. The expected percentage of filtered spermatozoa was calculated by multiplying the percentage of normal, frozen-thawed spermatozoa in the sample by the observed percentage of spermatozoa in the filtrate for the sample with 100 normal frozen-thawed:0 quench frozen spermatozoa. The differences between the observed and expected percentages ranged from 0% to 1.84%. The linear correlation between the observed and expected percentages was 0.99 ($P<0.001$).

Sephadex filtration with caffeine stimulation The percentages of spermatozoa filtered through the sephadex columns following flushing with 0.12 M sodium citrate with 5 mM caffeine are shown in Table 16. The analysis of variance for this assay is presented in

Table 17. There was a significant effect of boar ($P<0.001$), time ($P<0.005$), and $time_{linear}$ ($P<0.001$) on the percentage of spermatozoa filtered. The regression equation that predicted the change in spermatozoa filtered ($\hat{y}$, %) with time (x, hours) was $\hat{y} = 25.5 - 1.5$ hours ($r = -0.40$, $P<0.0001$). The deviations from linearity were not significant ($P>0.05$).

Sephadex filtration without caffeine stimulation The percentages of spermatozoa filtered through sephadex columns following flushing with 0.12 M sodium citrate without caffeine are presented in Table 18. The analysis of variance for this assay is shown in Table 19. There was a significant effect of boar ($P<0.001$), but not time ($P>0.05$), on the percentage of spermatozoa filtered.

Acrosin activity of spermatozoa

Preliminary studies A minor modification of the Ficsor et al. (1983) technique was required to achieve acrosin-positive zones of digestion on the gelatin-substrate-films with frozen-thawed porcine semen. Ficsor et al. (1983) applied 5 to 10 ul of a suspension of washed spermatozoa to gelatinized slides. The slides were then allowed to dry for 5 to 7 minutes at room temperature. When this procedure was used with frozen-thawed porcine spermatozoa, acrosin-positive zones of digestion were seldom observed. However, the addition of smaller volumes (1.5 ul) of the suspension of washed spermatozoa to the gel, along with longer drying times (10 minutes) in front of a heater, consistently resulted in numerous spermatozoa with acrosin-positive halos.

The results of the validation of the assay of acrosin are shown in Table 20. The validation procedures were the same as those used previously for validation of the assay of sephadex filtration. The expected percentage of acrosin-positive spermatozoa was calculated by multiplying the percentage of normal, frozen-thawed spermatozoa in the sample by the observed percentage of acrosin-positive spermatozoa in the sample with 100 normal, frozen-thawed:0 quench frozen spermatozoa. The difference between the observed and expected percentages ranged from 0% to 10%. The correlation between the observed and expected percentages was 0.97 ($P<0.001$).

Percentage of acrosin-positive spermatozoa The percentages of spermatozoa with acrosin-positive zones of digestion following incubation on gelatin-substrate-films are shown in Table 21. There was a significant effect of boar ($P<0.005$), time ($P<0.001$), and $time_{linear}$ ($P<0.001$) on the percentage of spermatozoa with acrosin activity (Table 22). The deviations from linearity were significant ($P<0.025$; not shown in the Table), indicating that a polynomial of a higher degree might better describe the relationship between the percentage of acrosin-positive spermatozoa and hours post-thaw. The quadratic fit between the percentage of acrosin-positive spermatozoa and hours post-thaw was significant ($P<0.005$). The deviations from the quadratic fit were not significant ($P>0.05$). The regression equation that predicted the change in acrosin activity ($\hat{y}$, %) with time (x, hours) was $\hat{y} = 88.2 - 7.2 \text{ hours} + 0.4 \text{ hours}^2$ ($r = -0.73$, $P<0.006$).

Diameter of the halos of acrosin-positive spermatozoa The diameters of the halos surrounding the acrosin-positive spermatozoa are given in Table 23. There was a significant effect of time ($P<0.05$), but not boar ($P>0.05$), on diameter of the halos (Table 24). A linear relationship was observed between the diameters and hours post-thaw ($P<0.001$). The regression equation that predicted the change in diameter of the halos ($\hat{y}$, um) with time (x, hours) was $\hat{y} = 25.2 - 2.2$ hours ($r = -0.71$, $P<0.0001$). The deviations from linearity were not significant ($P>0.05$).

The relationship between acrosin activity and acrosomal morphology following in vitro storage of nonfrozen porcine semen The results of the study with nonfrozen porcine semen are shown in Table 25. From the data in Table 25, means were calculated for the two boars. Immediately following collection, the percentage of spermatozoa that were acrosin-positive and had normal apical ridges were 91% and 94%, respectively. After forty-seven days at room temperature, only 1% of the spermatozoa had normal apical ridges, but 39.3% of the spermatozoa were acrosin-positive. Therefore, a class(es) of acrosomal morphology other than normal apical ridge class appeared to possess some acrosin activity. The percentage of acrosin-positive spermatozoa declined ($P<0.005$) abruptly from 68.1% to 39.5% between days 15 and 20 post-collection but the percentage of spermatozoa with normal apical ridges did not decrease ($P>0.05$) during this period (12.5% and 9.0%, respectively). However, during this five-day period, the percentage of spermatozoa with missing apical

ridges increased ($P<0.005$) from 7.3% to 27.5% and the percentage of spermatozoa with damaged apical ridges decreased ($P<0.005$) from 77.3% to 58.3%.

There was a negative correlation between the percentage of acrosin-positive spermatozoa and the percentage of spermatozoa with missing apical ridges ($r = -0.87$, $P<0.001$). A positive correlation was found between the percentage of acrosin-positive spermatozoa and the percentage of spermatozoa with either normal or damaged apical ridges ($r = 0.82$, $P<0.001$). The percentage of spermatozoa with acrosin activity was not correlated with the percentage of spermatozoa with loose acrosomal caps ($P>0.05$).

Glutamic oxaloacetic transaminase released from the spermatozoa

Preliminary studies Two different techniques for cellular disruption were compared to determine the better method to achieve maximum release of glutamic oxaloacetic transaminase from the spermatozoa. Tables 26 and 27 demonstrate the effect of sonication and quench freezing on the glutamic oxaloacetic transaminase released from the spermatozoa. Different ratios of sonicated (Table 26) or quench frozen (Table 27) to normal, frozen-thawed spermatozoa were prepared as discussed earlier. The maximum releasable glutamic oxaloacetic transaminase from the spermatozoa following sonication and quench freezing are shown in Tables 26 and 27 in the three rows for the 0 normal, frozen-thawed:100 sonicated and 0 normal, frozen-thawed:100 quench frozen ratios, respectively. The maximum releasable glutamic oxaloacetic transaminase was not significantly different

($P>0.05$; not shown in the Table) for the two methods of cellular disruption. However, there appeared to be a consistent tendency for greater glutamic oxaloacetic transaminase following quench freezing when compared with sonication. The quench freezing technique was selected for achieving maximum release of glutamic oxaloacetic transaminase from the spermatozoa.

The expected extracellular glutamic oxaloacetic transaminase in a sample was calculated with the following equation: (percentage of normal, frozen-thawed spermatozoa in the sample x observed glutamic oxaloacetic transaminase in the sample with 100 normal, frozen-thawed:0 quench frozen spermatozoa) + (percentage of quench frozen spermatozoa in the sample x observed glutamic oxaloacetic transaminase in the sample with 0 normal, frozen-thawed:100 quench frozen spermatozoa). The difference between the observed and expected glutamic oxaloacetic transaminase ranged from 5.3 to 74.7 and 2.5 to 52.5 $mIU/10^9$ spermatozoa for the sonication and quench freezing techniques of cellular disruption, respectively. The linear correlations between the observed and expected activities for the sonication and quench freezing techniques were 0.95 ($P<0.001$) and 0.98 ($P<0.001$), respectively.

The background glutamic oxaloacetic transaminase present in the egg yolk and Olep extenders were within the range of the background reagent used by the Veterinary Clinical Pathology Laboratory, Iowa State University. Freezing and storage of the extracellular fluid for 2 weeks at $-20^{\circ}C$ following centrifugation appeared to have no effect

on glutamic oxaloacetic transaminase, when compared with nonfrozen samples evaluated immediately following centrifugation.

Extracellular activity The extracellular glutamic oxaloacetic transaminase for the eight boars in this study is given in Table 28. The analysis of variance is shown in Table 29. There was a significant effect of boar ($P<0.001$), but not time ($P>0.05$), on extracellular glutamic oxaloacetic transaminase.

Maximum releasable activity The glutamic oxaloacetic transaminase for the eight boars following quench freezing is shown in Table 30. There was a significant effect of boar ($P<0.001$) on maximum releasable glutamic oxaloacetic transaminase (Table 31).

Percentage of maximum releasable activity present extracellularly The percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly is shown in Table 32. There was a significant effect of boar ($P<0.005$), but not time ($P>0.05$), on the percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly (Table 33).

Intracellular activity The glutamic oxaloacetic transaminase remaining inside the spermatozoa post-thaw is shown in Table 34. The analysis of variance for intracellular glutamic oxaloacetic transaminase is given in Table 35. There was a significant effect of boar ($P<0.001$), but not time ($P>0.05$), on intracellular glutamic oxaloacetic transaminase.

Spermatozoan penetration assay of zona-free hamster oocytes

Preliminary studies During the preliminary studies, it was found that frozen-thawed porcine spermatozoa could not be capacitated by the same procedures used by Pavlok (1981) for nonfrozen porcine spermatozoa. However, when frozen-thawed porcine spermatozoa were added to zona-free hamster oocytes immediately post-thaw, a high percentage of the oocytes were penetrated. Frozen-thawed porcine spermatozoa are apparently capacitated very quickly following their addition to supplemented medium 199.

Binding of spermatozoa to and penetration of zona-free hamster oocytes The number of spermatozoa bound per zona-free hamster oocyte, the number of penetrations per oocyte, and the percentage of oocytes penetrated are shown in Table 36. There was a significant effect of boar on the number of penetrations per oocyte ($P<0.01$; Table 37) but the boar effect for the number of spermatozoa bound per oocyte and for the percentage of oocytes penetrated was not significant ($P>0.05$; Table 37).

Pertinent Correlations Between the In Vitro Tests of Seminal Quality

The pertinent correlations between the in vitro tests of seminal quality are presented in Table 38. The assay of sephadex filtration with caffeine stimulation was correlated with (1) spermatozoan motility with caffeine stimulation ($r = 0.67$, $P<0.001$) and (2) spermatozoa with (a) normal apical ridges ($r = 0.80$, $P<0.001$), (b) damaged apical ridges ($r = -0.56$, $P<0.01$), (c) missing apical

ridges ($r = -0.54$, $P<0.01$), and (d) loose acrosomal caps ($r = -0.37$, $P<0.01$). The assay of sephadex filtration without caffeine stimulation was correlated with (1) spermatozoan motility without caffeine stimulation ($r = 0.46$, $P<0.01$) and (2) spermatozoa with (a) normal apical ridges ($r = 0.70$, $P<0.001$), (b) damages apical ridges ($r = -0.42$, $P<0.01$), and (c) missing apical ridges ($r = -0.48$, $P<0.01$). The assay of sephadex filtration without caffeine was not correlated with spermatozoa with loose acrosomal caps ($P>0.05$). The assay of acrosin-positive spermatozoa was correlated with spermatozoa with (1) either normal or damaged apical ridges ($r = 0.76$, $P<0.001$), (2) missing apical ridges ($r = -0.65$, $P<0.001$), and (3) loose acrosomal caps ($r = -0.34$, $P<0.01$). The number of spermatozoa bound per zona-free hamster oocyte and the percentage of zona-free hamster oocytes penetrated were not correlated with spermatozoan motility without caffeine stimulation ($P>0.05$) or spermatozoa with either normal or damaged apical ridges ($P>0.05$).

Repeatability of the Assays

The repeatability of the assays was estimated by calculating 95% confidence intervals for boar or boar x time assay means. The 95% confidence intervals for the boar or boar x time assay means are presented in Table 39. The 95% confidence intervals are also expressed as a percentage of the overall assay mean. For example, the 95% confidence interval for the assay of motility without caffeine stimulation was 7.1% and the 95% confidence interval as a percentage of the overall assay mean was 25.8% (7.1 x 100/27.5). This

calculation was performed so that the relative repeatabilities, among the different assays, could be compared.

Fertility Following Heterospermic Insemination

Ratio of offspring and heterospermic indices The ratios of offspring sired by the black and white boars for each litter are shown in Table 40. A minimum of 6 litters was farrowed for all but three of the 16 combinations (B3 x W2, B4 x W3, and B4 x W4). More inseminations were not possible because of insufficient semen from boars W2 and B4.

The heterospermic indices for the 16 combinations of boars are shown in Table 41. Because the heterospermic indices were calculated as the ratio of offspring sired by the black boar minus the ratio of offspring sired by the white boar multiplied by 100, a negative heterospermic index indicated that the white boar sired more than 50% of the offspring, whereas a positive heterospermic index indicated that the black boar sired more than 50% of the offspring. The heterospermic indices for each of the 16 combinations of boars ranged from -66.1 to 97.3. The number of offspring sired by the black and white boars differed significantly from a 1:1 ratio for 11 of the 16 combinations of boars (Table 41). The average heterospermic index, the percentage of offspring sired, the rank by the average heterospermic index, and the rank by the percentage of offspring sired are shown in Table 42. The average heterospermic indices for the eight boars ranged from -21.7 to 80.4. The rank by the average

heterospermic index and the percentage of offspring sired per boar were identical.

Sixty-day conception and farrowing rates Of the 229 sows inseminated, nine were excluded because: (1) five sows died before 60 days of gestation without exhibiting estrus, (2) three sows were not in estrus on the day of insemination, and (3) one sow failed to abort following administration of $PGF_2\alpha$ and farrowed a litter of pigs from nonfrozen semen. Of the remaining sows, 21 aborted between 60 to 90 days of gestation. The average conception rate at 60 days was 60.9% and ranged from 39.1% to 86.7% (Table 43) for sows inseminated to the 16 pairs of boars. The etiology for eight of the abortions was confirmed to be pseudorabies by the Veterinary Diagnostic Laboratory, Iowa State University. Because the aborted fetuses of the remaining 13 sows were usually necrotic when found, the cause of most of these abortions could not be determined. However, in a few instances, fresh fetuses were obtained and the cause of abortion was attributed to hemolytic E. coli or streptococcus. The average farrowing rate was 51.4% and ranged from 26.1% to 81.8% (Table 44). The average litter size for the 113 sows that farrowed was 8.8 (998/113) and ranged from 1 to 18 (Table 40).

Pertinent Correlations Between the Tests of Seminal Quality and the Heterospermic Indices

The correlations between the differences (black boar minus white boar) in the in vitro tests and the heterospermic indices are shown in Table 45. Correlations between the heterospermic indices and the

differences in the in vitro tests were calculated for each of the five post-thaw times, where applicable. The correlations between the differences in the in vitro tests, using the data for all the post-thaw times and the heterospermic indices, were also established.

The differences in the following assays were not significantly correlated ($P>0.05$) with the heterospermic indices: motility following cooling to 5°C, percentage of spermatozoa with normal apical ridges, damaged apical ridges, or loose acrosomal caps, extracellular glutamic oxaloacetic transaminse, maximum releasable glutamic oxaloacetic transaminase, and the number of penetrations per zona-free hamster oocyte. A negative correlation ($r = -0.57$, $P<0.02$) was found between the heterospermic indices and the differences in prefreeze motility following dilution. The relationship between these two parameters is illustrated in Figure 1. Significant correlations were found between the heterospermic indices and the differences in percent motility without caffeine stimulation at 0 ($r = 0.50$, $P<0.05$), 1 ($r = 0.62$, $P<0.01$), and 7 ($r = 0.67$, $P<0.005$) hours post-thaw and when the data from all five post-thaw times were used ($r = 0.50$, $P<0.0001$). The relationship between the heterospermic indices and the differences in percent motility without caffeine stimulation is shown in Figure 2. Negative correlations were found between the heterospermic indices and the differences in the percentage of spermatozoa with missing apical ridges at 1 ($r = -0.51$, $P<0.05$), 3 ($r = -0.52$, $P<0.04$), 5 ($r = -0.74$, $P<0.001$), and 7 ($r = -0.65$, $P<0.007$) hours post-thaw and when the data from all five times were

used ($r = -0.51$, $P<0.0001$). The relationship between the heterospermic indices and the differences in the percentage of spermatozoa with missing apical ridges is presented in Figure 3. The differences in the percentage of spermatozoa with either normal or damaged apical ridges were correlated with the heterospermic indices at 3 hours ($r = 0.51$, $P<0.05$) post-thaw and when the data from all five post-thaw times were used ($r = 0.31$, $P<0.006$). This relationship is illustrated in Figure 4. The differences in the percentage of spermatozoa filtered through sephadex columns without caffeine stimulation were correlated ($r = 0.32$, $P<0.009$) with the heterospermic indices only when the data from all four post-thaw times were used. This relationship is shown in Figure 5. The differences in the percentage of acrosin-positive spermatozoa were correlated with the heterospermic indices at 3 hours ($r = 0.49$, $P<0.05$) post-thaw and when the data from the five times were used ($r = 0.38$, $P<0.0006$). This relationship is shown in Figure 6. The differences in the diameter of the halos were correlated with the heterospermic indices at 0 hour ($r = 0.74$, $P<0.001$) post-thaw and when the data from the five times were used ($r = 0.30$, $P<0.007$). The relationship between these two parameters is shown in Figure 7. The heterospermic indices were correlated with the differences in the percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly at 0 ($r = 0.57$, $P<0.02$), 1 ($r = 0.55$, $P<0.03$), 3 ($r = 0.63$, $P<0.01$), 5 ($r = 0.49$, $P<0.05$), and 7 ($r = 0.50$, $P<0.05$) hours post-thaw and when the data from all five post-thaw times were used ($r = 0.54$,

$P<0.0001$). The heterospermic indices were negatively correlated with the differences in intracellular glutamic oxaloacetic transaminase at 0 ($r = -0.60$, $P<0.01$), 1 ($r = -0.61$, $P<0.01$), 3 ($r = -0.62$, $P<0.01$), 5 ($r = -0.52$, $P<0.04$) and 7 ($r = -0.52$, $P<0.04$) hours post-thaw and when the data from all five post-thaw times were used ($r = -0.57$, $P<0.0001$). The relationship between the heterospermic indices and the differences in the percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly and the differences in intracellular glutamic oxaloacetic transaminase are shown in Figures 8 and 9, respectively. The differences in the number of spermatozoa bound per zona-free hamster oocyte ($r = 0.64$, $P<0.007$) and percentage of zona-free hamster oocytes penetrated ($r = 0.75$, $P<0.0008$) were correlated with the heterospermic indices. The relationship between the differences in the number of spermatozoa bound per oocyte and the differences in the percentage of oocytes penetrated and the heterospermic indices are shown in Figures 10 and 11, respectively.

The parameters included in the multiple regression analysis with the heterospermic indices were the differences in (1) percent motility without caffeine at 7 hours post-thaw, (2) percentage of spermatozoa with either normal or damaged apical ridges at 3 hours post-thaw, (3) number of spermatozoa bound per zona-free hamster oocyte, and (4) percentage of zona-free hamster oocytes penetrated. The results of this analysis are not shown in the Tables. All four parameters contributed significantly to the multiple regression model. The

multiple regression correlation between the differences in these four parameters and the heterospermic indices was 0.94 ($P<0.0001$). The regression equation that predicted the change in the heterospermic index ($\hat{y}$) with the differences in these four parameters (x) was

$\hat{y} = -26.96 + 3.67$(motility) + 1.36(normal or damaged apical ridges) + 7.03(oocytes penetrated) - 31.86(spermatozoa per oocyte).

TABLES

Table 1. Seminal quality before cryopreservation

Boar	Sperm/ml ($x10^8$)[a,b]	Volume of ejaculate (ml)[a,b]	Total number of sperm/ejaculate ($x10^9$)[a,b]	% Motility following dilution[a,c]	% Motility following cooling[a,d]
B1	4.0±1.1	361.4± 96.7	139.0± 2.2[efg]	95.0±0.0	88.1±0.9
B2	5.5±0.5	385.1± 36.6	212.8± 1.3[e]	92.5±0.0	88.1±0.9
B3	5.0±0.4	322.8± 45.6	163.8±34.5[efg]	93.1±0.9	90.6±2.7
B4	5.7±0.6	166.3± 34.5	94.6± 9.2[f]	92.5±0.0	90.0±3.5
W1	4.8±2.0	396.1± 49.3	185.3±54.8[efg]	93.1±0.9	92.5±1.8
W2	3.2±0.7	353.5± 19.5	112.3±18.7[fg]	93.8±1.8	86.3±5.3
W3	5.2±1.8	394.8±105.0	198.0±14.7[eg]	95.0±0.0	90.0±0.0
W4	4.7±0.1	409.5± 36.1	192.7±22.8[eg]	93.1±0.9	85.6±0.9

[a] $\bar{X}$±S.D. of two ejaculates.

[b] Undiluted ejaculate.

[c] Determinations made immediately following dilution with Prediluter and before cooling.

[d] Determinations made after cooling to 5°C and immediately following dilution with Deep Freeze Diluter.

[e,f,g] Means in the same column without a common superscript are different ($P<0.05$).

Table 2. ANOVA for seminal quality before cryopreservation

Source	df	SS	MS	F	Probability
Concentration of spermatozoa					
Boars	7	10	1	1.25	P<0.500
Ejaculates	1	2	2	1.49	P<0.500
Boars x Ejaculates	7	8	1		
Total	15	19			
Volume of the ejaculate					
Boars	7	87058	12437	3.02	P<0.100
Ejaculates	1	312	312	0.08	P<0.750
Boars x Ejaculates	7	28789	4113		
Total	15	116159			
Total number of spermatozoa					
Boars	7	25803	3686	6.59	P<0.025
Ejaculates	1	1452	1452	2.59	P<0.250
Boars x Ejaculates	7	3916	559		
Total	15	31171			
Motility following dilution					
Boars	7	56	8	3.04	P<0.100
Ejaculates	1	4	4	1.34	P<0.500
Boars x Ejaculates	7	18	3		
Replication (Boars x Ejaculates)	48	256	5		
Total	63	334			
Motility following cooling					
Boars	7	298	43	1.45	P<0.500
Ejaculates	1	6	6	0.21	P<0.750
Boars x Ejaculates	7	206	29		
Replication (Boars x Ejaculates)	48	313	7		
Total	63	823			

Table 3. Percent motility without caffeine stimulation

Boar	Hours post-thaw[a]				
	0	1.0	3.0	5.0	7.0
B1	26.1± 7.5[b]	18.8±4.8[b]	18.8± 6.3	16.1± 4.8	12.5± 6.5
B2	73.8± 7.5[c]	63.8±4.8[c]	48.8± 2.5	36.1± 4.8	37.5± 5.0
B3	23.8± 4.8[b]	25.0±4.1[b]	35.0±12.9	22.5±12.6	15.0± 4.1
B4	37.5± 9.6[b]	26.1±4.8[b]	46.1±12.5	36.1± 4.8	16.1± 2.5
W1	28.8± 8.5[b]	31.1±7.5[b]	25.0± 5.8	18.8± 8.5	12.5± 5.0
W2	23.8± 4.8[b]	17.5±5.0[b]	21.1± 6.3	13.8± 7.5	12.5± 6.5
W3	41.1±16.5[b]	32.5±5.0[bc]	27.5± 5.0	18.8± 2.5	12.5± 5.0
W4	37.5± 5.0[b]	30.0±7.1[b]	22.5± 6.5	22.5± 5.0	15.0±10.0

[a] $\bar{X}$±S.D. of four replicates.

[b,c] Means in the same column without a common superscript are different (P<0.05).

Table 4. ANOVA for percent motility without caffeine stimulation

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	16699	2386	14.53		P<0.005
Times	4	7585	1896	11.55	1	P<0.025
Linear	(1)	7104	7104	25.93		P<0.005
Lack of fit	(3)	481	160	1.26	1	P<0.500
Boars x Times	28	4597	164		7	
Linear	(7)	1920	274			
Lack of fit	(21)	2677	127		7	
Replication (Boars x Times)	120	6119	51			
TOTAL	159	35000				

Table 5. Percent motility following caffeine stimulation

Boar	Hours post-thaw[a] 0	1.0	3.0	5.0	7.0
B1	53.8± 7.5^{bcd}	26.1±4.8^{b}	22.5± 2.9^{b}	27.5±2.9^{b}	31.1±2.5
B2	76.1± 4.8^{d}	65.0±7.1^{c}	62.5± 6.5^{d}	62.5±6.5^{c}	53.8±4.8
B3	58.8±10.3^{bcd}	56.1±7.5^{bc}	55.0±13.5^{cd}	55.0±5.8^{bc}	52.5±8.7
B4	40.0±10.8^{bc}	31.1±2.5^{b}	37.5± 2.9^{bcd}	41.1±6.3^{bc}	41.1±4.8
W1	66.1± 4.8^{cd}	27.5±9.6^{b}	35.0± 7.1^{bcd}	32.5±2.9^{bc}	28.8±8.5
W2	33.8± 4.8^{b}	31.1±4.8^{b}	30.0± 7.1^{bc}	23.8±2.5^{b}	26.1±4.8
W3	46.1±11.1^{bcd}	35.0±7.1^{bc}	27.5± 5.0^{bc}	35.0±5.8^{bc}	35.0±4.1
W4	41.1± 8.5^{bc}	48.8±3.1^{bc}	30.0± 4.1^{bc}	25.0±7.1^{b}	30.0±0.0

[a] $\bar{X}$±S.D. of four replicates.

[b,c,d] Means in the same column without a common superscript are different ($P<0.05$).

Table 6. ANOVA for percent motility following caffeine stimulation

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	20799	2971	15.31		P<0.001
Times	4	5062	1265	6.52	1	P<0.050
Linear	(1)	2796	2796	12.05		P<0.025
Lack of fit	(3)	2266	755	4.17	1	P<0.100
Boars x Times	28	5433	194		7	
Linear	(7)	1622	232			
Lack of fit	(21)	3811	181		7	
Replication (Boars x Times)	120	5581	47			
TOTAL	159	36875				

Table 7. Percentage of spermatozoa with normal apical ridges

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	29.5 ± 6.3^{bc}	23.1 ± 2.1^{bcd}	15.5 ± 2.1^{bc}	16.5 ± 4.5	10.8 ± 0.5
B2	50.1 ± 2.5^{bcd}	59.5 ± 2.1^{e}	52.5 ± 3.1^{d}	39.5 ± 5.3	37.1 ± 3.3
B3	70.1 ± 5.4^{d}	63.1 ± 5.3^{e}	46.0 ± 5.0^{cd}	38.0 ± 5.9	27.8 ± 2.6
B4	65.0 ± 5.0^{d}	42.8 ± 3.6^{bcde}	20.8 ± 4.5^{bcd}	21.5 ± 4.5	13.5 ± 4.6
W1	65.5 ± 3.4^{d}	48.5 ± 1.3^{cde}	36.0 ± 2.9^{bcd}	31.1 ± 1.5	20.5 ± 1.9
W2	16.5 ± 3.0^{b}	18.0 ± 3.8^{bc}	11.8 ± 3.3^{b}	12.1 ± 3.8	7.8 ± 2.4
W3	56.8 ± 4.6^{cd}	56.0 ± 5.0^{de}	40.5 ± 4.0^{bcd}	29.5 ± 1.9	22.0 ± 3.6
W4	17.0 ± 2.4^{b}	9.0 ± 1.8^{b}	7.1 ± 1.5^{b}	10.1 ± 3.5	10.1 ± 4.9

[a] $\bar{X}\pm$S.D. of four replicates.

[b,c,d,e] Means in the same column without a common superscript are different ($P<0.05$).

Table 8. ANOVA for percentage of spermatozoa with normal apical ridges

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	33193	4742	18.47		P<0.001
Times	4	16252	4063	15.83	1	P<0.010
Linear	(1)	15556	15556	21.19		P<0.005
Lack of fit	(3)	696	232	2.37	1	P<0.250
Boars x Times	28	7188	257		7	
Linear	(7)	5137	734			
Lack of fit	(21)	2051	98		7	
Replication (Boars x Times)	120	1673	14			
TOTAL	159	58306				

Table 9. Percentage of spermatozoa with damaged apical ridges

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	43.0±1.8^{bc}	50.5±3.1^{c}	40.1±1.9	45.0±6.5	35.8±5.0
B2	16.1±3.6^{b}	13.1±3.4^{b}	18.8±6.8	29.5±5.2	30.5±2.4
B3	13.1±3.2^{b}	12.8±1.9^{b}	25.5±4.0	30.1±5.5	38.0±2.9
B4	19.1±2.4^{bc}	21.5±4.9^{bc}	27.0±2.4	19.1±2.9	25.1±1.7
W1	17.8±2.5^{bc}	27.1±1.7^{bc}	38.0±3.6	39.0±4.2	41.0±5.3
W2	47.1±5.6^{c}	48.1±6.3^{c}	42.8±2.9	46.8±5.4	48.5±4.8
W3	13.8±5.1^{b}	13.0±2.8^{b}	16.1±4.8	28.8±3.3	38.8±5.4
W4	33.5±4.2^{bc}	42.5±8.2^{bc}	40.1±3.6	34.4±4.1	29.5±8.3

[a] $\bar{X}$±S.D. of four replicates.

[b,c] Means in the same column without a common superscript are different (P<0.05).

Table 10. ANOVA for percentage of spermatozoa with damaged apical ridges

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	14877	2125	12.30		P<0.005
Times	4	1869	467	2.70	1	P<0.250
Linear	(1)	1738	1738	3.48		P<0.250
Lack of fit	(3)	131	44	0.69	1	P<0.500
Boars x Times	28	4839	173		7	
Linear	(7)	3503	500			
Lack of fit	(21)	1336	64		7	
Replication (Boars x Times)	120	2351	20			
TOTAL	159	23937				

Table 11. Percentage of spermatozoa with missing apical ridges

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	23.5±4.2^{bc}	23.8±3.2	39.0±3.6^{cd}	31.8±3.4^{bc}	49.8±6.2^{b}
B2	20.1±2.1^{bc}	15.5±4.4	20.8±6.0^{bc}	18.8±3.4^{bc}	22.0±1.8^{b}
B3	12.5±4.4^{b}	19.8±3.8	22.8±3.0^{bcd}	27.5±2.5^{bc}	30.8±1.0^{bcd}
B4	13.5±2.9^{bc}	22.8±1.3	24.8±1.3^{bcd}	28.5±3.4^{bc}	24.5±4.0^{bc}
W1	12.8±2.5^{bc}	16.0±3.3	17.5±4.4^{b}	12.5±3.7^{b}	24.1±6.9^{bc}
W2	33.5±6.0^{c}	31.5±4.8	42.8±2.6^{d}	37.8±4.3^{c}	38.5±4.4^{bcd}
W3	21.0±2.9^{bc}	22.5±2.5	32.5±3.1^{bcd}	36.0±4.7^{c}	43.1±3.5^{cd}
W4	23.1±2.1^{bc}	21.5±4.1	26.5±8.3^{bcd}	24.1±8.3^{bc}	25.0±5.2^{bc}

[a] $\bar{X}$±S.D. of four replicates.

[b,c,d] Means in the same column without a common superscript are different ($P<0.05$).

Table 12. ANOVA for percentage of spermatozoa with missing apical ridges

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	7107	1015	12.34		P<0.005
Times	4	3203	801	9.73	1	P<0.025
Linear	(1)	2842	2842	14.88		P<0.005
Lack of fit	(3)	361	120	2.61	1	P<0.250
Boars x Times	28	2304	82		7	
Linear	(7)	1334	191			
Lack of fit	(21)	970	46		7	
Replication (Boars x Times)	120	2147	18			
TOTAL	159	14761				

Table 13. Percentage of spermatozoa with loose acrosomal caps

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	4.0 ± 1.6^{bc}	2.8 ± 2.7^{b}	5.1 ± 2.5^{bc}	6.8 ± 4.6^{b}	3.8 ± 2.6^{b}
B2	13.1 ± 2.1^{bc}	11.8 ± 5.4^{bc}	8.1 ± 4.2^{bc}	12.5 ± 5.7^{bc}	10.1 ± 3.6^{b}
B3	4.1 ± 2.6^{bc}	4.1 ± 2.4^{b}	5.8 ± 2.9^{bc}	4.5 ± 1.9^{b}	3.8 ± 1.3^{b}
B4	2.5 ± 1.9^{b}	13.0 ± 2.8^{bc}	27.5 ± 4.6^{c}	30.8 ± 4.6^{c}	37.0 ± 3.9^{c}
W1	4.1 ± 0.5^{bc}	8.1 ± 2.7^{bc}	8.5 ± 3.1^{bc}	17.5 ± 4.4^{bc}	15.0 ± 3.2^{bc}
W2	2.8 ± 2.1^{b}	2.7 ± 1.0^{b}	3.1 ± 1.3^{b}	3.5 ± 2.4^{b}	5.1 ± 2.1^{b}
W3	10.1 ± 3.8^{bc}	8.5 ± 1.7^{bc}	11.0 ± 2.0^{bc}	5.8 ± 1.7^{b}	6.0 ± 0.8^{b}
W4	26.5 ± 3.1^{c}	27.8 ± 6.9^{c}	26.5 ± 6.8^{bc}	31.5 ± 4.2^{c}	35.5 ± 5.9^{c}

[a] $\bar{X}\pm$S.D. of four replicates.

[b,c] Means in the same column without a common superscript are different ($P<0.05$).

Table 14. ANOVA for percentage of spermatozoa with loose acrosomal caps

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	12229	1747	15.21		P<0.001
Times	4	888	222	1.93	1	P<0.250
Linear	(1)	848	848	1.85		P<0.250
Lack of fit	(3)	40	13	0.52	1	P<0.750
Boars x Times	28	3216	115		7	
Linear	(7)	2688	459			
Lack of fit	(21)	528	25		7	
Replication (Boars x Times)	120	1445	12			
TOTAL	159	17778				

Table 15. Validation of sephadex filtered spermatozoa

F:QF[a]	Boar[b]	Observed percentage[c]	Expected percentage[d]	Difference between observed and expected
100:0	1	21.17±0.78	---	---
75:25	1	17.71±2.43	15.87	1.84
50:50	1	11.61±0.18	10.59	1.02
25:75	1	4.99±0.34	5.29	-0.30
0:100	1	0.16±0.23	0	0.16
100:0	2	15.35±0.96	---	---
75:25	2	13.24±2.16	11.51	1.73
50:50	2	9.02±0.20	7.67	1.35
25:75	2	4.46±0.48	3.84	0.62
0:100	2	0	0	0
100:0	3	31.82±1.84	---	---
75:25	3	25.73±2.02	23.90	1.83
50:50	3	16.89±0.34	15.91	0.98
25:75	3	7.73±0.07	7.95	-0.22
0:100	3	0	0	0

[a]Ratio of frozen-thawed (F) to quench frozen-thawed (QF) spermatozoa.

[b]One straw of frozen-thawed semen selected at random from three boars.

[c]$\bar{X}$±S.D. of two replicates.

[d]Expected percentage = (percentage F in mixture)(observed percentage of 100F:0QF).

Table 16. Percentage of sephadex filtered spermatozoa after flushing with sodium citrate and caffeine

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	20.1±1.5^{bc}	16.3± 0.6^{bc}	14.4±0.8^{bc}	11.4±0.5^{b}	7.7±1.4^{b}
B2	32.7±4.5^{de}	27.9± 2.4^{c}	29.4±1.5^{de}	24.0±1.6^{cd}	23.8±1.9^{c}
B3	43.1±4.9^{e}	40.3± 3.8^{d}	32.7±4.8^{e}	34.2±1.8^{d}	35.9±3.6^{d}
B4	32.0±4.2^{de}	23.7±12.8^{c}	25.5±1.4^{cde}	16.1±0.8^{bc}	13.0±2.8^{bc}
W1	22.9±1.2^{bcd}	22.8± 1.8^{bc}	17.2±9.8^{bc}	18.1±2.9^{bc}	17.1±1.8^{bc}
W2	21.6±1.5^{bcd}	20.7± 1.7^{bc}	14.1±1.2^{bc}	10.8±2.0^{b}	7.2±0.8^{b}
W3	25.4±3.5^{cd}	19.1± 1.2^{bc}	18.7±1.2^{bcd}	11.7±1.6^{b}	13.9±2.1^{bc}
W4	12.3±1.1^{b}	11.6± 0.7^{b}	9.4±0.5^{b}	9.3±1.8^{b}	8.6±2.4^{b}

[a] $\bar{X}$±S.D. of four replicates.

b,c,d,e Means in the same column without a common superscript are different (P<0.05).

Table 17. ANOVA for percentage of sephadex filtered spermatozoa after flushing with sodium citrate and caffeine

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	10508	1501	53.93		P<0.001
Times	4	2405	601	21.59	1	P<0.005
Linear	(1)	2313	2313	32.58		P<0.001
Lack of fit	(3)	92	31	2.21	1	P<0.250
Boars x Times	28	779	28		7	
Linear	(7)	495	71			
Lack of fit	(21)	284	14		7	
Replication (Boars x Times)	120	651	5			
TOTAL	159	14343				

Table 18. Percentage of sephadex filtered spermatozoa after flushing with sodium citrate

Boar	Hours post-thaw[a]			
	1.0	3.0	5.0	7.0
B1	10.86±1.06^{bc}	7.13±2.55^{b}	6.63±0.07^{b}	8.07±1.06^{b}
B2	19.77±0.57^{cd}	24.80±3.82^{cd}	25.31±1.48^{d}	20.25±1.48^{cd}
B3	27.52±8.56^{d}	29.94±4.74^{d}	23.37±0.78^{cd}	24.23±2.69^{d}
B4	7.26±2.05^{b}	10.30±1.27^{b}	12.51±3.54^{bc}	7.90±0.14^{b}
W1	12.46±0.92^{bc}	18.32±2.19^{bc}	13.68±1.70^{bc}	13.43±0.50^{bcd}
W2	9.45±0.92^{bc}	9.20±0.42^{b}	8.01±0.57^{b}	6.94±1.20^{b}
W3	12.37±0.35^{bc}	11.21±0.50^{b}	11.30±0.71^{b}	7.47±1.06^{b}
W4	8.50±0.14^{bc}	9.07±0.78^{b}	8.93±0.57^{b}	10.22±1.27^{bc}

[a] $\bar{X}$±S.D. of two replicates.

[b,c,d] Means in the same column without a common superscript are different ($P<0.05$).

Table 19. ANOVA for percentage of sephadex filtered spermatozoa after flushing with sodium citrate

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	2748	393	44.45		P<0.001
Times	3	58	19	2.20	1	P<0.250
Linear	(1)	20	20	2.86		P<0.250
Lack of fit	(2)	38	19	0.28	1	P<0.750
Boars x Times	21	185	9		7	
Linear	(7)	50	7			
Lack of fit	(14)	135	10		7	
Replication (Boars x Times)	32	166	5			
TOTAL	63	3158				

Table 20. Validation of the assay for acrosin activity

F:QF[a]	Boar[b]	Observed percentage[c]	Expected percentage[d]	Difference between observed and expected
100:0	1	69.5± 4.2	---	---
75:25	1	56.5± 5.7	52.1	4.4
50:50	1	44.7± 7.4	34.7	10.0
25:75	1	17.5± 0.0	17.4	0.1
0:100	1	0	0	0
100:0	2	92.0± 2.1	---	---
75:25	2	69.0± 3.5	68.4	0.6
50:50	2	50.5± 0.7	45.6	4.9
25:75	2	29.5± 3.5	22.8	6.7
0:100	2	0.7± 1.1	0	0.7
100:0	3	65.7± 4.6	---	---
75:25	3	46.5±14.1	48.0	1.5
50:50	3	37.0± 0.0	32.0	5.0
25:75	3	14.5± 7.8	16.0	1.5
0:100	3	1.7± 0.4	0	1.7

[a]Ratio of frozen-thawed (F) to quench frozen thawed (QF) spermatozoa.

[b]One straw of frozen-thawed semen selected at random from three boars.

[c]$\bar{X}$±S.D. of two replicates.

[d]Expected percentage = (percentage F in mixture)(observed percentage 100F:0QF).

Table 21. Percentage of spermatozoa with acrosin activity

Boar	Hours post-thaw[a]				
	0	1.0	3.0	5.0	7.0
B1	80.1±3.7	78.0±4.7	59.0±4.1	53.7±1.7	55.2±1.4
B2	93.0±0.7	88.6±3.6	79.1±3.6	70.6±1.6	67.6±1.2
B3	92.5±1.9	92.4±2.5	78.7±4.8	75.9±2.3	68.4±3.7
B4	86.2±3.7	86.9±1.8	63.2±5.6	59.2±3.7	53.2±6.5
W1	91.1±1.8	87.1±2.8	82.0±4.1	70.5±9.4	64.2±3.1
W2	83.7±2.7	87.6±7.5	63.7±4.7	50.2±3.2	46.3±4.7
W3	88.0±4.0	81.4±3.6	71.2±2.8	59.4±1.6	61.0±1.5
W4	74.5±6.7	77.4±7.2	61.1±2.2	53.6±4.9	58.3±4.8

[a] $\bar{X}$±S.D. of four replicates.

Table 22. ANOVA for percentage of spermatozoa with acrosin activity

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	6672	953	14.79		P<0.005
Times	4	20519	5130	79.59	1	P<0.001
Linear	(1)	19200	19200	143.28		P<0.001
Quadratic	(1)	717	717	16.67		P<0.005
Lack of fit	(2)	602	301	0.40	1	P<0.750
Boars x Times	28	1805	64		7	
Linear	(7)	938	134			
Quadratic	(7)	299				
Lack of fit	(21)	568	27		7	
Replication (Boars x Times)	120	2032	17			
TOTAL	159	31027				

Table 23. Diameter (um) of the halos of spermatozoa with acrosin activity

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	16.2± 4.3[b]	24.7± 9.1	11.5± 0.8	11.3± 0.6	9.3±1.8
B2	54.2± 2.1[c]	16.8± 3.6	12.3± 1.9	12.2± 4.6	9.2±0.9
B3	20.0± 9.2[b]	30.0± 7.0	17.4±12.5	23.5± 5.3	2.5±3.0
B4	24.7±13.5[bc]	16.2± 7.7	23.3± 6.9	14.8± 6.4	11.0±1.9
W1	32.7±25.3[bc]	22.1± 4.5	26.5± 8.4	21.2±12.9	9.5±1.3
W2	23.0± 3.1[bc]	29.4± 3.3	26.9± 5.8	15.2± 4.4	8.4±1.1
W3	26.2± 8.3[bc]	18.6± 6.0	9.6± 0.3	11.2± 1.9	8.1± .9
W4	14.4± 4.9[b]	20.5±13.5	11.3± 3.2	10.8± 2.5	8.1± .4

[a] $\bar{X}$±S.D. of four replicates.

[b,c] Means in the same column without a common superscript are different (P<0.05).

Table 24. ANOVA for the diameter of the halos of spermatozoa with acrosin activity

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	1775	254	1.25		P<0.500
Times	4	5450	1363	6.72	1	P<0.050
Linear	(1)	5317	5317	31.10		P<0.001
Lack of fit	(3)	133	44	0.21	1	P<0.750
Boars x Times	28	5677	203		7	
Linear	(7)	1194	171			
Lack of fit	(21)	4483	213		7	
Replication (Boars x Times)	120	6353	53			
TOTAL	159	19255				

Table 25. Acrosomal morphology and acrosin activity following *in vitro* storage of nonfrozen porcine semen

Boar[c]	Days post-collection	Acrosomal morphology (%)[a,b] NAR	DAR	MAR	LAC	Halo-positive spermatozoa[a] (%)
H	0	94.0± 0.0	6.0±0.0	0.0±0.0	0.0±0.0	92.1± 2.8
L	0	94.0± 1.4	6.0±1.4	0.0±0.0	0.0±0.0	89.9± 5.1
H	2	92.5± 4.9	6.5±4.9	0.0±0.0	1.0±0.0	88.7± 3.0
L	2	90.5± 0.7	7.5±2.1	0.0±0.0	2.0±1.4	78.8± 1.8
H	4	87.0± 2.8	10.0±0.0	1.5±2.1	1.5±0.7	77.4± 4.2
L	4	86.0± 1.4	11.0±1.4	1.0±1.4	2.0±1.4	71.1± 2.8
H	7	70.0± 4.2	27.5±3.5	0.0±0.0	2.5±0.7	82.1± 4.6
L	7	80.0± 5.7	18.5±6.4	1.0±0.0	0.5±0.7	83.3± 8.9
H	9	61.0± 1.4	34.5±0.7	2.5±0.7	2.0±1.4	84.5± 1.2
L	9	68.0± 2.8	27.5±0.7	0.5±0.7	4.0±2.8	75.3± 0.3
H	13	15.0± 1.4	70.5±2.1	7.5±2.1	7.0±2.1	77.4± 3.5
L	13	26.5± 7.8	64.0±5.7	5.0±1.4	4.5±2.1	71.8± 2.8
H	15	8.5± 0.7	80.0±8.5	9.5±7.8	2.0±0.0	65.6± 3.4
L	15	16.5± 4.9	74.5±3.5	5.0±0.0	4.0±1.4	70.7± 1.6
H	18	2.5± 0.7	74.0±4.2	18.5±3.5	5.0±0.0	56.7±12.8
L	18	8.0± 2.8	75.5±7.8	12.0±5.7	4.5±0.7	68.5± 0.0
H	20	3.0± 1.4	53.5±2.1	37.0±1.4	6.5±2.1	34.3± 0.6
L	20	15.0± 0.7	63.0±1.4	18.0±1.4	4.0±2.8	44.8±10.9
H	22	3.0± 1.4	62.5±6.4	30.5±4.9	4.0±2.1	35.3± 4.6
L	22	12.0± 0.0	51.5±6.4	33.0±7.1	3.5±0.7	29.9± 1.3
H	26	2.0± 1.4	50.0±2.8	44.0±5.7	3.0±0.0	31.8± 4.0
L	26	11.5± 2.1	46.0±0.0	34.5±2.1	8.0±0.7	43.5± 1.6
H	33	4.5± 2.1	33.5±7.8	55.0±7.1	8.0±1.4	20.1± 7.5
L	33	10.0± 2.8	36.0±4.2	48.5±0.7	5.5±1.4	37.6± 8.2
H	47	1.0± 1.4	26.0±8.5	67.5±6.4	5.5±0.7	23.1± 4.5
L	47	0.5± 0.7	18.5±3.5	74.0±2.8	7.0±0.7	55.6± 1.3

[a] $\bar{X}$±S.D. of two replicates.

[b] NAR (normal apical ridges), DAR (damaged apical ridges), MAR (missing apical ridges), and LAC (loose acrosomal caps).

[c] One ejaculate from a Hampshire (H) crossbred boar and one ejaculate from a Landrace (L) crossbred boar.

Table 26. Validation of glutamic oxaloacetic transaminase assay using sonicated spermatozoa

F:S[a]	Boar[b]	Observed activity (mIU/10^9 sperm)[c]	Expected activity (mIU/10^9 sperm)[d]	Difference between observed and expected
100:0	1	466.4±18.4	---	---
75:25	1	454.0± 3.0	505.4	-51.4
50:50	1	479.1± 3.8	544.3	-65.2
25:75	1	508.6±55.1	583.3	-74.7
0:100	1	622.3± 5.3	---	---
100:0	2	564.3±22.4	---	---
75:25	2	623.0± 1.1	595.7	27.3
50:50	2	621.8±18.3	627.1	-5.3
25:75	2	626.9±29.8	658.4	-31.5
0:100	2	689.8± 8.7	---	---
100:0	3	398.3±26.3	---	---
75:25	3	384.3± 3.3	405.1	-20.7
50:50	3	405.4±17.4	411.8	-6.4
25:75	3	406.6± 6.3	418.5	-11.9
0:100	3	425.3±11.8	---	---

[a]Ratio of frozen-thawed (F) to sonicated (S) spermatozoa.

[b]One straw of frozen-thawed semen selected at random from three boars.

[c]$\bar{X}$±S.D. of two replicates.

[d]Expected percentage = (percentage F in mixture)(observed activity for 100F:0S) + (percentage S in mixture)(observed activity for 0F:100S).

Table 27. Validation of glutamic oxaloacetic transaminase assay using quench frozen spermatozoa

F:QF[a]	Boar[b]	Observed activity (mIU/10^9 sperm)[c]	Expected activity (mIU/10^9 sperm)[d]	Difference between observed and expected
100:0	1	466.4±18.4	---	---
75:25	1	535.9± 7.1	538.7	-2.9
50:50	1	613.6± 4.1	611.1	2.5
25:75	1	690.8± 5.3	683.5	7.3
0:100	1	755.8±23.6	---	---
100:0	2	564.3±22.4	---	---
75:25	2	573.9±11.5	622.9	-48.9
50:50	2	628.9± 3.3	681.4	-52.5
25:75	2	701.9±13.4	739.9	-38.1
0:100	2	798.5±12.2	---	---
100:0	3	398.3±26.3	---	---
75:25	3	357.0± 2.9	408.7	-51.7
50:50	3	388.5± 4.3	419.1	-30.5
25:75	3	408.7± 6.7	429.4	-20.7
0:100	3	439.8± 6.7	---	---

[a]Ratio of frozen-thawed (F) to quench frozen thawed (QF) spermatozoa.

[b]One straw of frozen-thawed semen selected at random from three boars.

[c]$\bar{X}$±S.D. of two replicates.

[d]Expected percentage = (percentage F in mixture)(observed activity for 100F:0QF) + (percentage QF in mixture)(observed activity for 0F:100QF).

Table 28. Extracellular glutamic oxaloacetic transaminase ($mIU/10^9$ spermatozoa)

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	1079±48[c]	1116±64[c]	979±147[d]	1060±99[c]	1036±107[c]
B2	780± 3[b]	765±63[b]	824± 56[cd]	774±64[b]	777± 72[b]
B3	675±13[b]	688±19[b]	659± 43[bc]	729±29[b]	727± 48[b]
B4	675±12[b]	683±19[b]	624± 29[b]	671±29[b]	683± 69[b]
W1	734±19[b]	754±19[b]	689± 53[bc]	680±67[b]	679± 26[b]
W2	637± 8[b]	666± 3[b]	626± 48[b]	676±46[b]	730± 23[b]
W3	689±49[b]	687±51[b]	682± 32[bc]	686±43[b]	660± 22[b]
W4	654±48[b]	636±81[b]	710± 42[bc]	725±34[b]	768± 24[b]

[a] $\bar{X}$±S.D. of four replicates.

[b,c,d] Means in the same column without a common superscript are different ($P<0.05$).

Table 29. ANOVA for extracellular glutamic oxaloacetic transaminase

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	2387638	341091	64.23		P<0.001
Times	4	20981	5245	0.99	1	P<0.750
Linear	(1)	4249	4249	0.34		P<0.750
Lack of fit	(3)	16732	5577	1.90	1	P<0.250
Boars x Times	28	148703	5311		7	
Linear	(7)	86908	12415			
Lack of fit	(21)	61795	2943		7	
Replication (Boars x Times)	120	338311	2819			
TOTAL	159	2895633				

Table 30. Maximum releasable glutamic oxaloacetic transaminase following quench freezing

Boar	GOT activity ($mIU/10^9$ sperm)[a]
B1	1442 ± 20^d
B2	940 ± 36^b
B3	1092 ± 11^c
B4	852 ± 8^b
W1	856 ± 28^b
W2	867 ± 35^b
W3	862 ± 7^b
W4	866 ± 96^b

[a] $\bar{X} \pm$ S.D. of four replicates.

[b,c,d] Means in the same column without a common superscript are different ($P<0.05$).

Table 31. ANOVA for maximum releasable glutamic oxaloacetic transaminase following quench freezing

Source	df	SS	MS	F	Probability
Boars	7	1195149	170735	103.29	P<0.001
Replication(Boar)	24	39662	1653		
Total	31	1234811			

Table 32. Percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly

Boar	Hours post-thaw[a] 0	1.0	3.0	5.0	7.0
B1	74.8±3.3^{bc}	77.4±4.4^{bc}	67.9±10.2^{bc}	73.5±6.9	71.8±7.4^{bc}
B2	82.9±0.4^{c}	81.4±6.7^{bc}	87.7± 5.9^{d}	82.3±6.8	82.7±7.7^{bc}
B3	61.8±1.2^{b}	63.0±1.8^{b}	60.3± 3.9^{b}	66.7±2.7	66.6±4.4^{b}
B4	79.2±1.5^{bc}	80.2±2.3^{bc}	73.2± 3.4^{bcd}	78.7±3.5	80.2±8.1^{bc}
W1	85.8±2.2^{c}	88.1±2.2^{c}	80.5± 6.2^{cd}	79.4±7.8	79.4±3.0^{bc}
W2	73.5±1.0^{bc}	76.8±0.3^{bc}	72.2± 5.6^{bcd}	78.0±5.3	84.2±2.6^{bc}
W3	80.0±5.7^{bc}	79.7±5.9^{bc}	79.1± 3.7^{bcd}	79.7±5.0	76.6±2.6^{bc}
W4	75.5±5.5^{bc}	73.4±9.4^{bc}	81.9± 4.9^{cd}	83.7±3.9	88.7±2.8^{c}

[a] $\bar{X}$±S.D. of four replicates.

[b,c,d] Means in the same column without a common superscript are different (P<0.05).

Table 33. ANOVA for percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	5689	813	14.08		P<0.005
Times	4	206	51	0.89	1	P<0.750
Linear	(1)	66	66	0.45		P<0.750
Lack of fit	(3)	140	47	1.68	1	P<0.750
Boars x Times	28	1617	58		7	
Linear	(7)	1039	148			
Lack of fit	(21)	578	28		7	
Replication (Boars x Times)	120	3089	26			
TOTAL	159	10601				

Table 34. Intracellular glutamic oxaloacetic transaminase (mIU/10^9 spermatozoa)

	Hours post-thaw[a]				
Boar	0	1.0	3.0	5.0	7.0
B1	363±48^{cd}	326±64^{cd}	463±147^{d}	382±99^{d}	406±107^{d}
B2	160± 3^{b}	175±63^{bc}	116±56^{b}	166±64^{b}	163±72^{b}
B3	417±13^{d}	404±19^{d}	433±43^{cd}	363±29^{cd}	365±48^{cd}
B4	177±12^{bc}	169±19^{bc}	229±29^{b}	181±29^{bc}	169±69^{b}
W1	121±19^{b}	102±19^{b}	167±53^{b}	176±67^{bc}	176±26^{bc}
W2	230± 8^{bcd}	201± 3^{bc}	241±48^{bc}	191±46^{bcd}	137±23^{b}
W3	173±49^{bc}	175±51^{bc}	180±32^{b}	175±43^{bc}	202±22^{bc}
W4	212±48^{bc}	230±81^{bcd}	156±42^{b}	141±34^{b}	98±25^{b}

[a] $\bar{X}$±S.D. of four replicates.

[b,c,d] Means in the same column without a common superscript are different ($P<0.05$).

Table 35. ANOVA for intracellular glutamic oxaloacetic transaminase

Source	df	SS	MS	F	Conservative df	Probability
Boars	7	1481561	211652	39.85		P<0.001
Times	4	20981	5245	0.99	1	P<0.750
Linear	(1)	4249	4249	0.34		P<0.750
Lack of fit	(3)	16732	5577	1.90	1	P<0.250
Boars x Times	28	148703	5311		7	
Linear	(7)	86908	12415			
Lack of fit	(21)	61794	2943		7	
Replication (Boars x Times)	120	338311	2819			
TOTAL	159	1989556				

Table 36. Number of spermatozoa bound per oocyte, number of penetrations per oocyte, and percentage of oocytes penetrated following in vitro insemination of zona-free hamster oocytes

Boar	Spermatozoa bound per oocyte[a]	Number of penetrations per oocyte[a]	Percentage of oocytes penetrated[a]
B1	9.3±1.3	1.6 ± 0.2^{cd}	86.3 ± 4.3^{bc}
B2	11.5±1.5	1.8 ± 0.1^{d}	91.3 ± 6.8^{c}
B3	10.2±1.2	1.6 ± 0.1^{cd}	84.4 ± 7.5^{bc}
B4	9.2±2.2	1.5 ± 0.2^{bc}	85.7 ± 11.9^{bc}
W1	9.8±1.2	1.4 ± 0.1^{bc}	86.9 ± 8.0^{bc}
W2	9.6±1.2	1.3 ± 0.3^{b}	84.1 ± 8.7^{bc}
W3	8.8±0.7	1.5 ± 0.1^{bc}	73.0 ± 5.5^{b}
W4	10.6±0.8	1.6 ± 0.1^{bc}	86.9 ± 5.1^{bc}

[a] $\bar{X}\pm$S.D. of four replicates.

[b,c,d] $\bar{X}$ Means in the same column without a common superscript are different ($P<0.05$).

Table 37. ANOVA for the spermatozoan penetration assay

Source	df	SS	MS	F	Probability
Spermatozoa bound per oocyte					
Boar	7	21	3	1.69	P<0.250
Replications(Boar)	24	44	2		
Total	31	65			
Number of penetrations per oocyte					
Boar	7	0.61	0.09	3.55	P<0.010
Replication(Boar)	24	0.59	0.02		
Total	31	1.19			
Percentage of oocytes penetrated					
Boar	7	772	110	1.93	P<0.250
Replication(Boar)	24	1372	57		
Total	31	2144			

Table 38. Pertinent correlations between the *in vitro* tests of seminal quality

In vitro tests that were correlated		Correlation
First test	*Second test*	
Sephadex filtration with caffeine	Motility with caffeine	0.67
	Normal apical ridges	0.80
	Damaged apical ridges	-0.56
	Missing apical ridges	-0.54
	Loose acrosomal caps	-0.37
Sephadex filtration without caffeine	Motility without caffeine	0.46
	Normal apical ridges	0.70
	Damaged apical ridges	-0.42
	Missing apical ridges	-0.48
	Loose acrosomal caps	NS[a]
Acrosin-positive spermatozoa	Normal & damaged apical ridges	0.76
	Missing apical ridges	-0.65
	Loose acrosomal caps	-0.34
Spermatozoa bound per oocyte	Motility without caffeine	NS
	Normal & damaged apical ridges	NS
Percentage of oocytes penetrated	Motility without caffeine	NS
	Normal & damaged apical ridges	NS

[a]Not significantly correlated ($P>0.05$).

Table 39. Repeatability of the assays estimated by 95 percent confidence intervals for boar or boar x time assay means

Assay	95% CI for the assay mean[a]	95% CI as a percentage of the overall assay mean[b]	Rank[c]
Motility without caffeine	7.1	25.8	13
Motility with caffeine	6.7	16.5	11
Normal apical ridge	3.7	11.7	7
Damaged apical ridge	4.4	14.2	9
Missing apical ridge	4.2	16.2	10
Loose acrosomal cap	3.4	29.1	14
Sephadex filtration with caffeine	2.3	11.2	6
Extracellular glutamic oxaloacetic transaminase	52.6	7.1	3
Percentage of maximum releasable glutamic oxaloacetic transaminase	5.0	6.5	2
Intracellular glutamic oxaloacetic transaminase	52.6	23.1	12
Acrosin-positive spermatozoa	4.1	5.6	1
Diameter of halos of acrosin-positive spermatozoa	7.2	39.8	15
Spermatozoa bound per oocyte	1.4	14.1	8
Number of penetrations per oocyte	0.2	10.7	5
Percentage of oocytes penetrated	7.8	9.2	4

[a]95% confidence intervals (CI) calculated according to the equations given in Appendix J.

[b]95% CI x 100/overall assay mean.

[c]Rank from the highest to the lowest 95% CI, expressed as a percentage of the overall mean.

Table 40. Number of offspring in each litter sired by the black and the white boar following heterospermic insemination

	B1			B2			B3			B4		
W1	0/8[a]	2/4	1/8	9/0	8/2	7/1	3/2	0/7	0/10	3/9	4/13	1/6
	4/8	2/9	1/12	5/3	10/1	11/2	0/8	2/10	4/5	5/10	1/5	0/1
							1/8	0/7	3/6			
							0/5	4/3	5/5			
W2	4/3	11/3	3/1	11/2	4/0	5/1	3/0	1/6	3/6	5/7	2/3	6/4
	2/4	0/2	4/6	9/0	3/1	7/1	5/1	3/6		7/5	6/1	2/3
	3/5	3/4										
W3	4/1	8/1	2/1	7/0	10/0	12/0	9/3	7/3	6/1	14/1	8/1	7/3
	7/1	15/3	4/1	9/1	6/0	10/0	7/3	4/5	2/2	5/0		
	7/0	12/1	5/6	10/0	10/0							
W4	2/0	6/1	2/8	8/0	11/1	4/0	1/3	4/7	3/1	8/3	7/5	8/2
	5/9	1/1	4/10	9/3	13/3	7/0	3/4	2/3	5/0	9/7		
	4/5			11/1	15/0	14/1	11/1	7/4	5/0			
							5/8	4/3				

[a]For each litter, the ratio represents the number of piglets sired by the black (B) boar/number of piglets sired by the white (W) boar.

Table 41. Heterospermic index (HI) for each of the sixteen combinations of boars in the 4 x 4 design

Boar combination	HI±S.E.[a]
B1W1	-66.1± 9.8
B1W2[b]	3.4±13.1
B1W3	62.0±8.8
B1W4[b]	-17.2±12.9
B2W1	69.5± 9.4
B2W2	77.3± 9.6
B2W3	97.3± 2.6
B2W4	82.2± 5.7
B3W1	-55.1± 8.4
B3W2[b]	-11.8±17.0
B3W3	34.6±13.0
B3W4[b]	19.1±10.7
B4W1	-51.7± 5.6
B4W2[b]	9.6±13.9
B4W3	74.4±10.7
B4W4	30.6±13.6

[a]The HI were calculated as the ratio of offspring sired by the black boar minus the ratio of offspring sired by the white boar multiplied by 100.

[b]The ratio of piglets differed significantly ($P<0.05$) from 1:1 for all boar combinations without a superscript.

Table 42. Ranking by fertility based on the percentage of offspring sired and the average heterospermic index

Boar	Offspring sired by designated boar/total offspring	Percentage offspring sired by designated boar	Rank by % offspring sired	Average heterospermic index	Rank by average heterospermic index
B1	128/254[a]	50.4	4	16.7	4
B2	255/279	91.4	1	80.4	1
B3	122/268	45.5	5	9.2	5
B4	110/197	55.8	3	21.3	3
W1	178/274	65.0	2	62.2	2
W2	75/187	40.1	6	6.4	6
W3	38/245	15.5	8	-21.7	8
W4	94/292	32.2	7	0.0	7

[a]Number of offspring sired by B1/the total number of offspring produced with all mixtures of semen that contained spermatozoa from B1.

Table 43. Sixty-day conception rates following heterospermic insemination

Boar	B1	B2	B3	B4	$\bar{X}$ for W boars
W1	7/10[a] (70.0%)	7/12 (58.3%)	14/19 (73.7%)	7/10 (70.0%)	35/51[b] (68.6%)
W2	11/13 (84.6%)	6/8 (75.0%)	6/12 (50.0%)	6/12 (50.0%)	29/45 (64.4%)
W3	13/15 (86.7%)	11/19 (57.9%)	8/17 (47.1%)	5/12 (41.7%)	37/63 (58.7%)
W4	9/23 (39.1%)	9/11 (81.8%)	11/18 (61.1%)	4/9 (44.4%)	33/61 (54.1%)
$\bar{X}$ for B boars	40/61[b] (65.6%)	33/50 (66.0%)	39/66 (59.1%)	22/43 (51.2%)	134/220 (60.9%)

[a]Number of pregnant sows at 60-days of gestation/number of sows inseminated for the B1W1 combination (percent pregnant).

[b]Total number of pregnant sows at 60-days of gestation/total number of sows inseminated following the use of four different mixtures of semen containing spermatozoa from the designated boar (percent pregnant).

Table 44. Farrowing rates following heterospermic insemination

Boar	B1	B2	B3	B4	$\bar{X}$ for W boars
W1	6/10[a] (60.0%)	6/12 (50.0%)	12/19 (63.2%)	6/10 (60.0%)	30/51[b] (58.8%)
W2	8/13 (61.5%)	6/8 (75.0%)	6/13 (38.5%)	6/12 (50.0%)	25/46 (54.3%)
W3	9/15 (60.0%)	8/19 (42.1%)	7/16 (43.7%)	4/12 (33.3%)	28/62 (45.2%)
W4	6/23 (26.1%)	9/11 (81.8%)	11/18 (61.1%)	4/9 (44.4%)	30/61 (49.2%)
$\bar{X}$ for B boars	29/61[b] (47.5%)	29/50 (58.0%)	35/66 (53.0%)	20/43 (46.5%)	113/220 (51.4%)

[a]Number of sows that farrowed/number of sows inseminated for the B1W1 combination (percent farrowing).

[b]Total number of sows that farrowed/total number of sows inseminated following the use of four different mixtures of semen containing spermatozoa from the designated boar (percent farrowing).

Table 45. Pertinent correlations between the in vitro tests of seminal quality and the heterospermic indices

	Prefreeze	Hours post-thaw 0	1	3	5	7	For all times[a]
Motility following dilution	-.57[b]						
Motility following cooling to 5°C	NS[b]						
Motility without caffeine		.50	.62	NS	NS	.67	.50
Normal apical ridge		NS	NS	NS	NS	NS	NS
Damaged apical ridge		NS	NS	NS	NS	NS	NS
Missing apical ridge		NS	-.51	-.52	-.74	-.65	-.51
Loose acrosomal cap		NS	NS	NS	NS	NS	NS
Normal and damage apical ridge		NS	NS	.51	NS	NS	.31
Sephadex filtration without caffeine			NS	NS	NS	NS	.32
Acrosin-positive spermatozoa		NS	NS	.49	NS	NS	.38
Diameter of halos of acrosin-positive sperm		.74	NS	NS	NS	NS	.30
Extracellular GOT[c]		NS	NS	NS	NS	NS	NS
Maximum releasable GOT[c]			NS				
Percentage of maximum releasable GOT[c]		.57	.55	.63	.49	.50	.54
Intracellular GOT[c]		-.60	-.61	-.62	-.52	-.52	-.57
Spermatozoa bound per oocyte		.64					
Number of penetrations per oocyte		NS					
Percentage of oocytes penetrated		.75					

[a]Correlations between the heterospermic indices and the differences (black boar minus white boar) in the in vitro tests using all the data from the five post-thaw times.

[b]Not significantly correlated ($P>0.05$).

[c]GOT (glutamic oxaloacetic transaminase).

FIGURES

Figure 1. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percent motility of their spermatozoa before freezing. The differences (black boar minus white boar) in the percent motility after dilution with Prediluter were correlated with the heterospermic indices ($r = -0.57$)

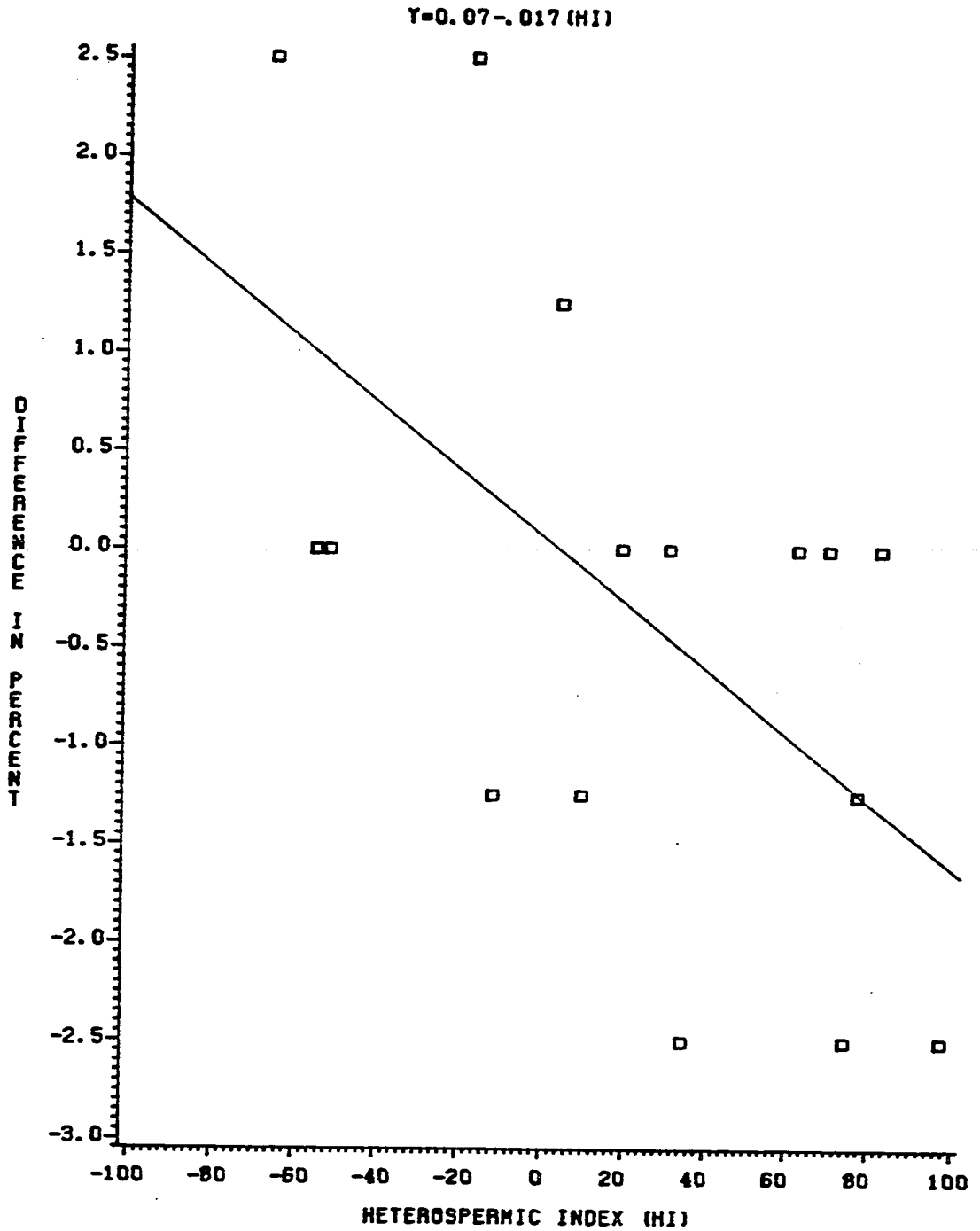

Y=0.07-.017(HI)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
DIFFERENCE IN PERCENT
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 2. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percent motility of their spermatozoa without caffeine stimulation. The percentage of spermatozoa with motility was determined at 0 (□), 1 (Δ), 3 (●), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in the percent motility were correlated with the heterospermic indices at 0, 1, and 7 hours post-thaw, and when the data from all five post-thaw times were used (r = 0.50, 0.62, 0.67, and 0.50, respectively)

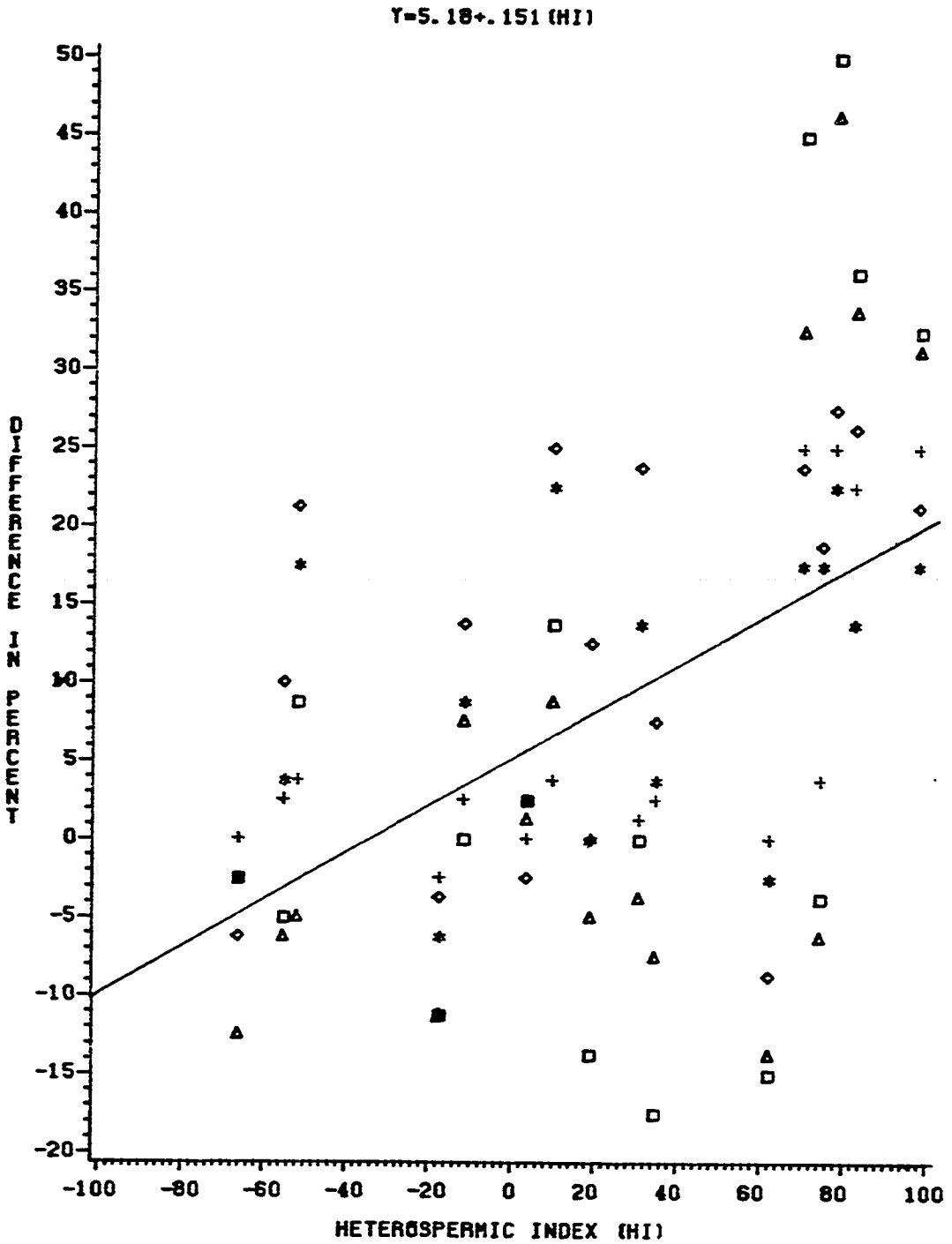

Y=5.18+.151(HI)
DIFFERENCE IN PERCENT
HETEROSPERMIC INDEX (HI)
50
45
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-100
-80
-60
-40
-20
0
20
40
60
80
100

Figure 3. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percentage of their spermatozoa with missing apical ridges. The percentage of spermatozoa with missing apical ridges was determined at 0 (□), 1 (Δ), 3 (◇), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in the percentage of spermatozoa with missing apical ridges were correlated with the heterospermic indices at 1, 3, 5, and 7 hours post-thaw and when the data from all five post-thaw times were used ($r = -0.51, -0.52, -0.74, -0.65$, and -0.51, respectively)

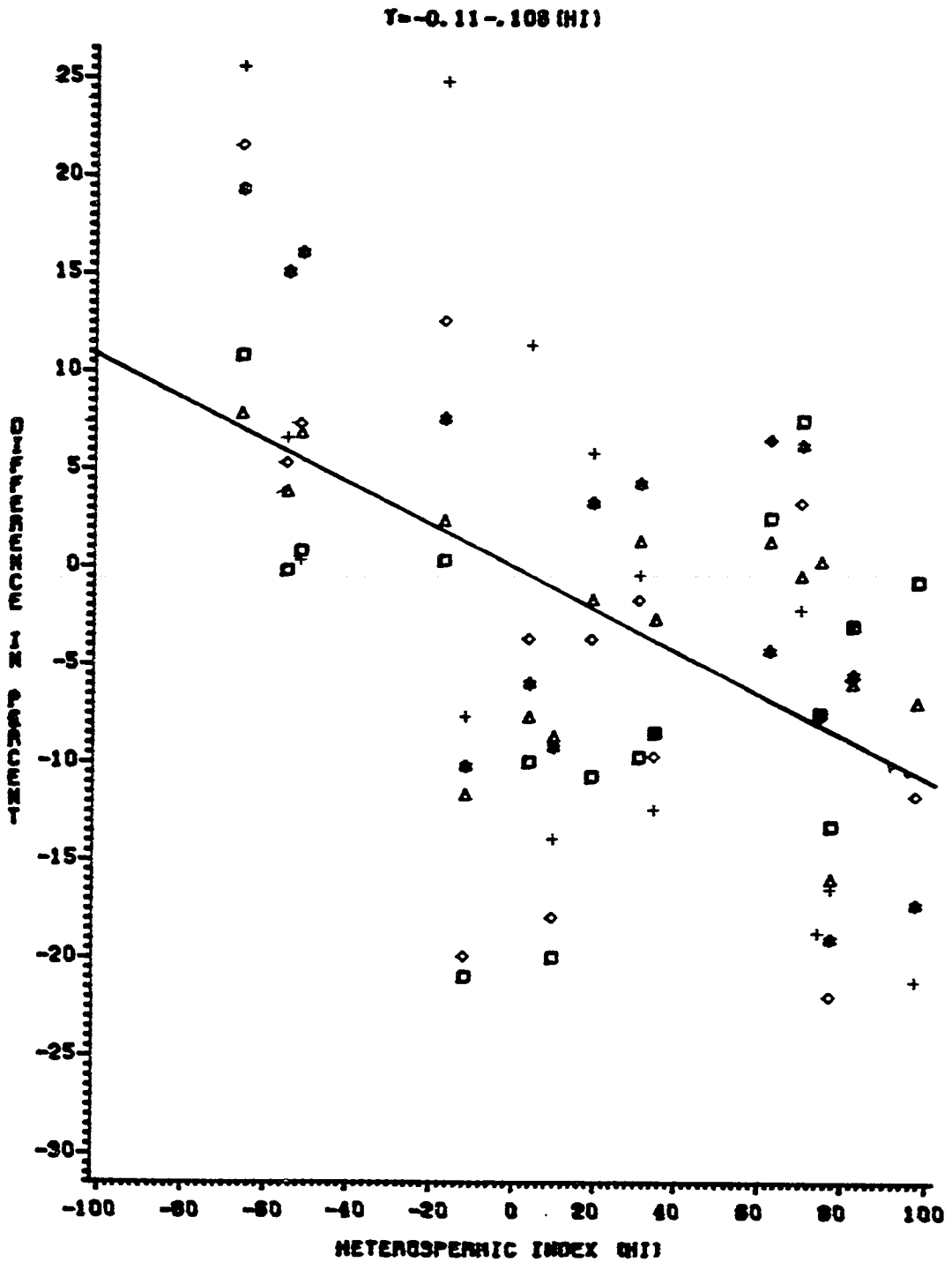

Y=-0.11-.108(HI)
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 4. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percentage of their spermatozoa with either normal or damaged apical ridges. The percentage of spermatozoa with normal and damaged apical ridges was determined at 0 (□), 1 (Δ), 3 (◇), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in the percentage of spermatozoa with either normal or damaged apical ridges were correlated with the heterospermic indices at 3 hours post-thaw and when the data from all five post-thaw times were used (r = 0.51 and 0.31, respectively)

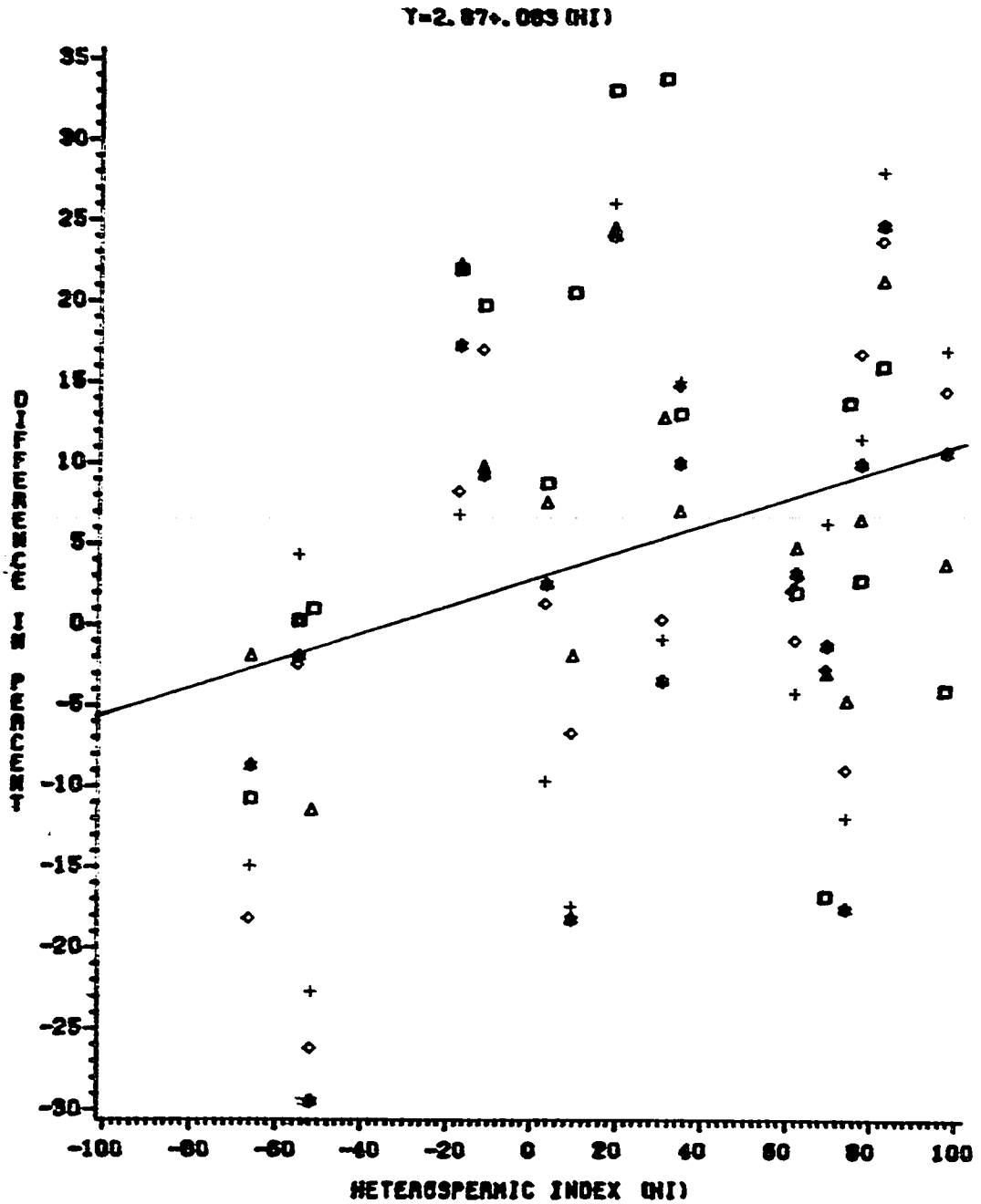

Y=2.87+.083 (HI)
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 5. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percentage of their spermatozoa filtered through sephadex columns following flushing with 0.12 M sodium citrate without caffeine. The percentage of filtered spermatozoa was determined at 1 (Δ), 3 (◇), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in the percentage of filtered spermatozoa without caffeine stimulation were correlated with the heterospermic indices when the data from all five post-thaw times were used ($r = 0.32$)

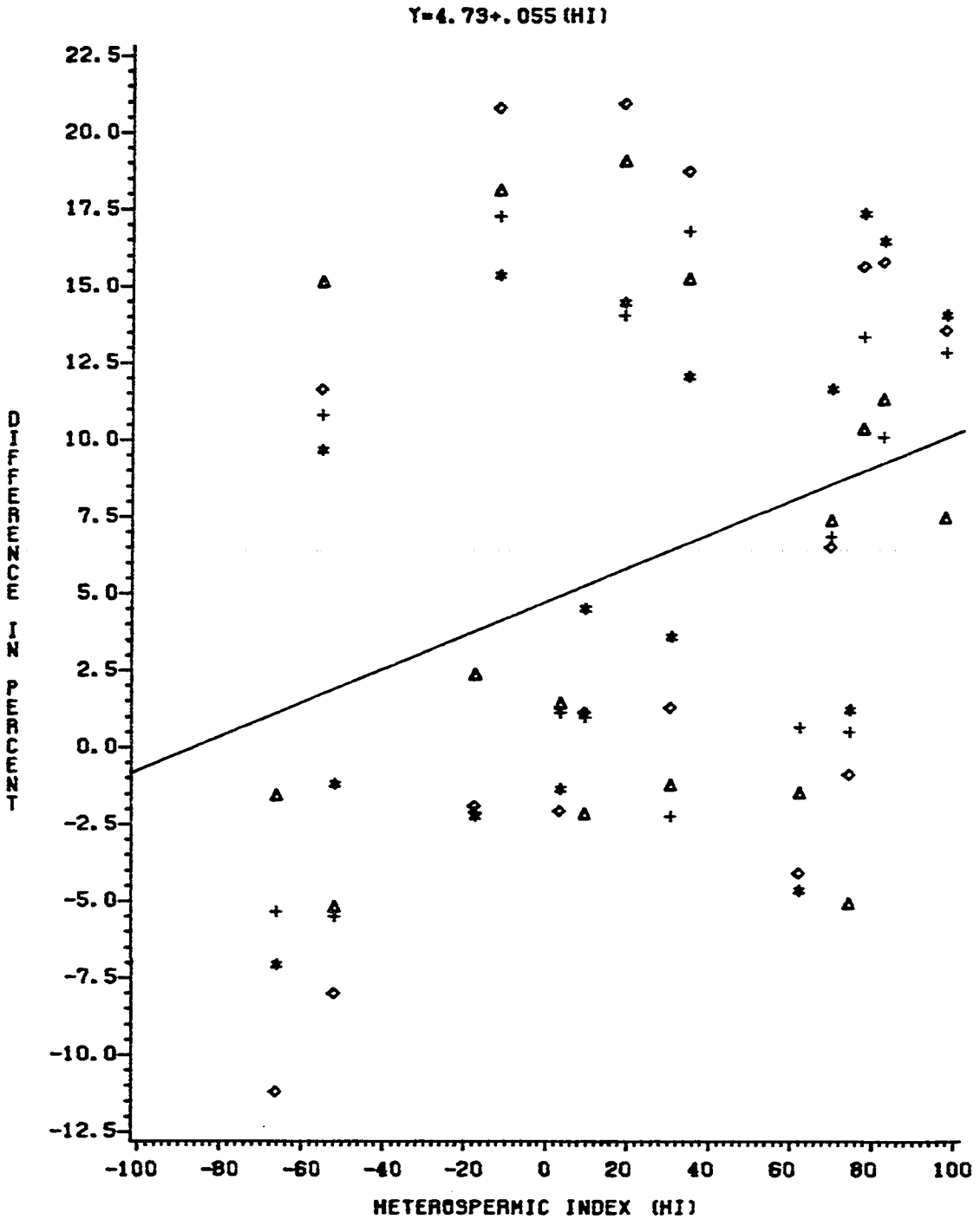

Y=4.73+.055(HI)
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
-2.5
-5.0
-7.5
-10.0
-12.5
DIFFERENCE IN PERCENT
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 6. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percentage of their spermatozoa with acrosin activity. The percentage of spermatozoa with acrosin activity was determined at 0 (□), 1 (Δ), 3 (◇), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in the percentage of spermatozoa with acrosin activity were correlated with the heterospermic indices at 3 hours post-thaw and when the data from all five post-thaw times were used (r = 0.49 and 0.38, respectively)

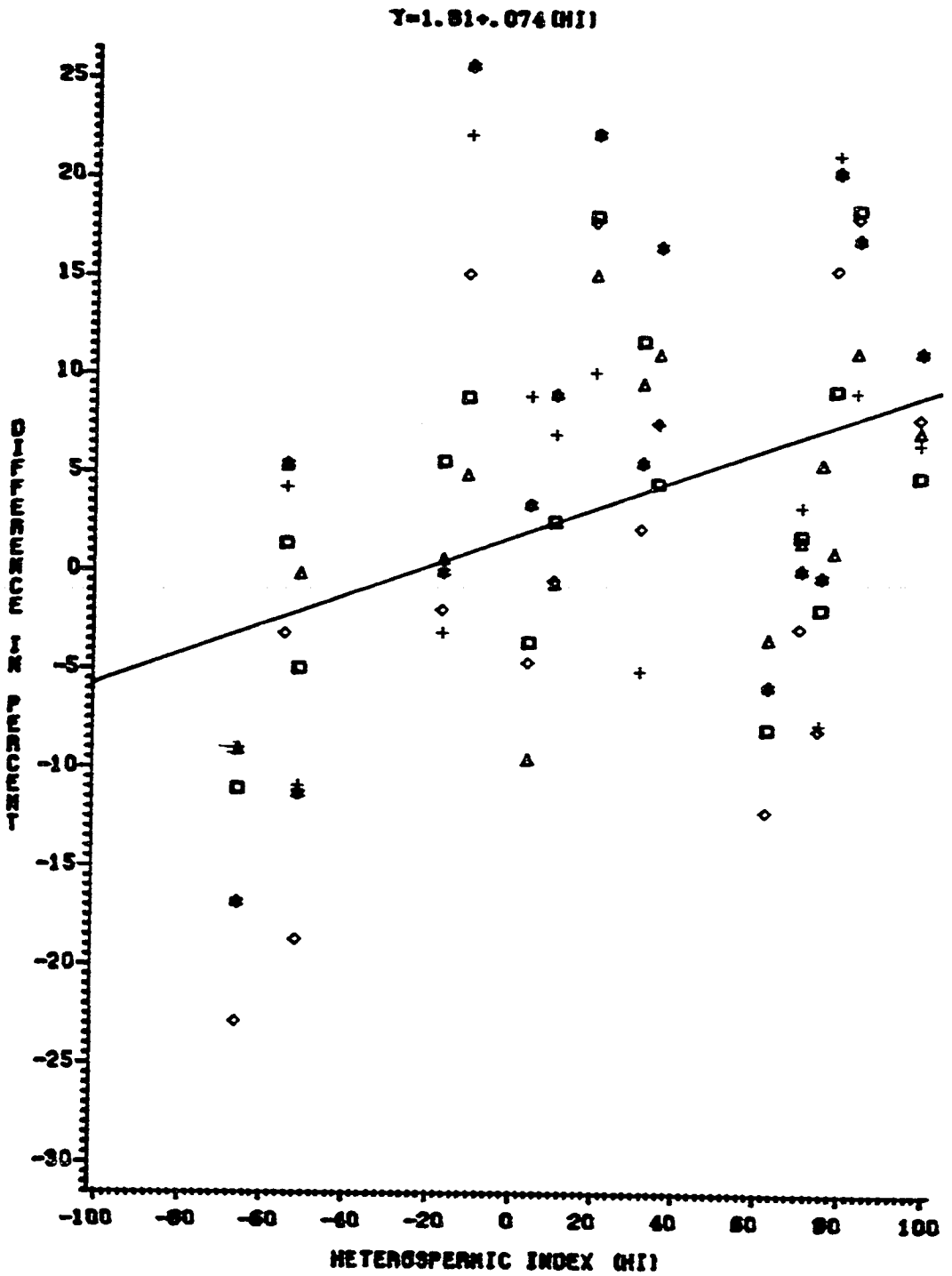

Y=1.81+.074(HI)
DIFFERENCE IN PERCENT
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 7. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the diameter of the halos of their spermatozoa. The diameter of the halos was determined at 0 (□), 1 (Δ), 3 (◇), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in the diameter of the halos were correlated with the heterospermic indices at 0 hour post-thaw and when the data from all five post-thaw times were used (r = 0.74 and 0.30, respectively)

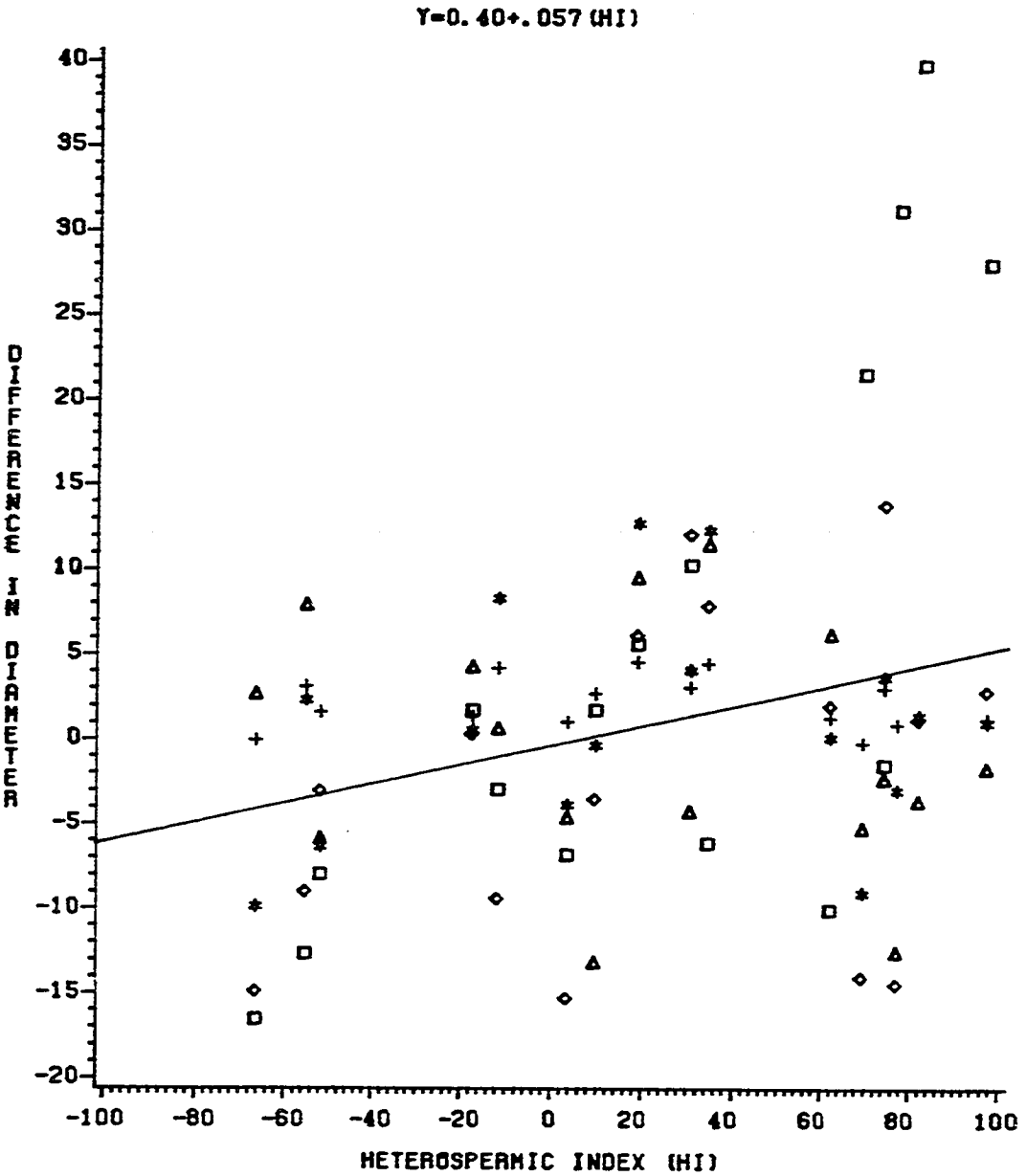

Y=0.40+.057(HI)
DIFFERENCE IN DIAMETER
HETEROSPERMIC INDEX (HI)
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-100
-80
-60
-40
-20
0
20
40
60
80
100

Figure 8. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percentage of maximum glutamic oxaloacetic transaminase activity present extracellularly. The percent activity was determined at 0 (□), 1 (Δ), 3 (◇), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in the percent activity were correlated with the heterospermic indices at 0, 1, 3, 5, and 7 hours post-thaw, and when the data from all five post-thaw times were used (r = 0.57, 0.55, 0.63, 0.49, 0.50 and 0.54, respectively)

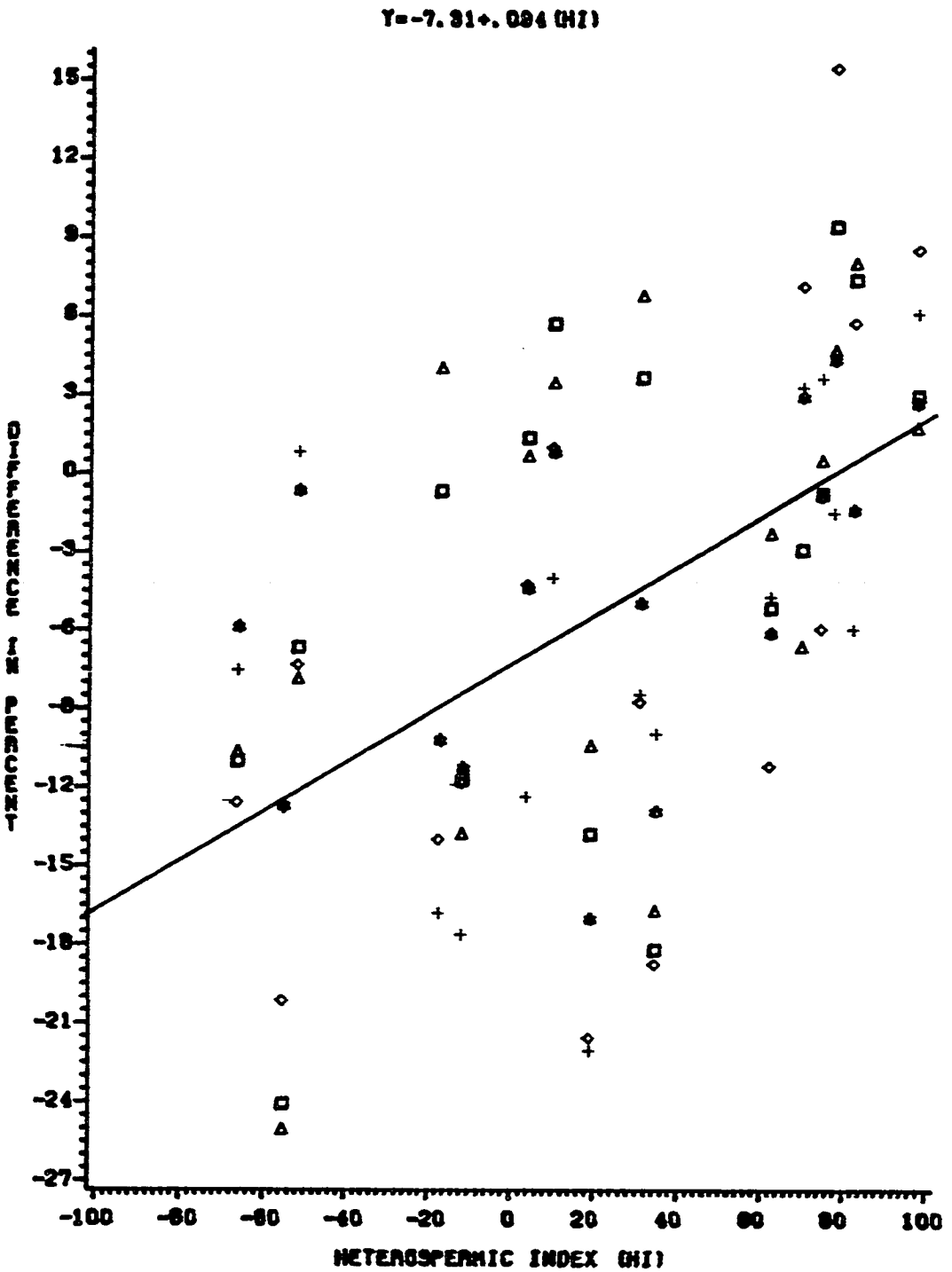

Y=-7.31+.094 (HI)
15
12
9
6
3
0
-3
-6
-9
-12
-15
-18
-21
-24
-27
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 9. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the intracellular glutamic oxaloacetic transaminase activity of their spermatozoa. The activity was determined at 0 (□), 1 (Δ), 3 (◇), 5 (*), and 7 (+) hours post-thaw. The differences (black boar minus white boar) in activity were correlated with the heterospermic indices at 0, 1, 3, 5, and 7 hours post-thaw, and when the data from all five post-thaw times were used ($r = -0.60, -0.61, -0.62, -0.52, -0.52$ and -0.57, respectively)

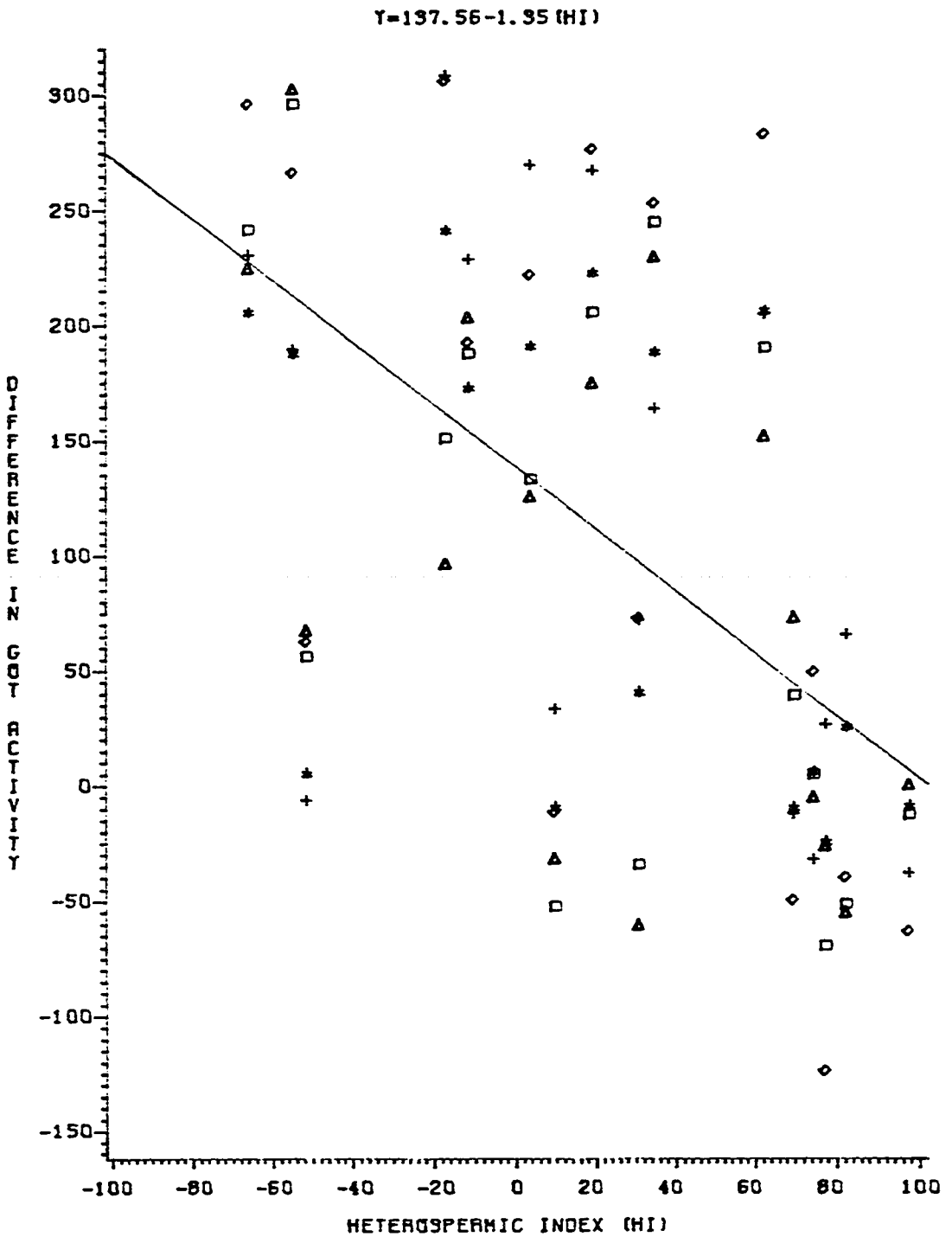

Y=137.56-1.35(HI)
DIFFERENCE IN GOT ACTIVITY
300
250
200
150
100
50
0
-50
-100
-150
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 10. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the number of their spermatozoa bound per zona-free hamster oocyte. The differences (black boar minus white boar) in the number of spermatozoa bound were correlated with the heterospermic indices ($r = 0.64$)

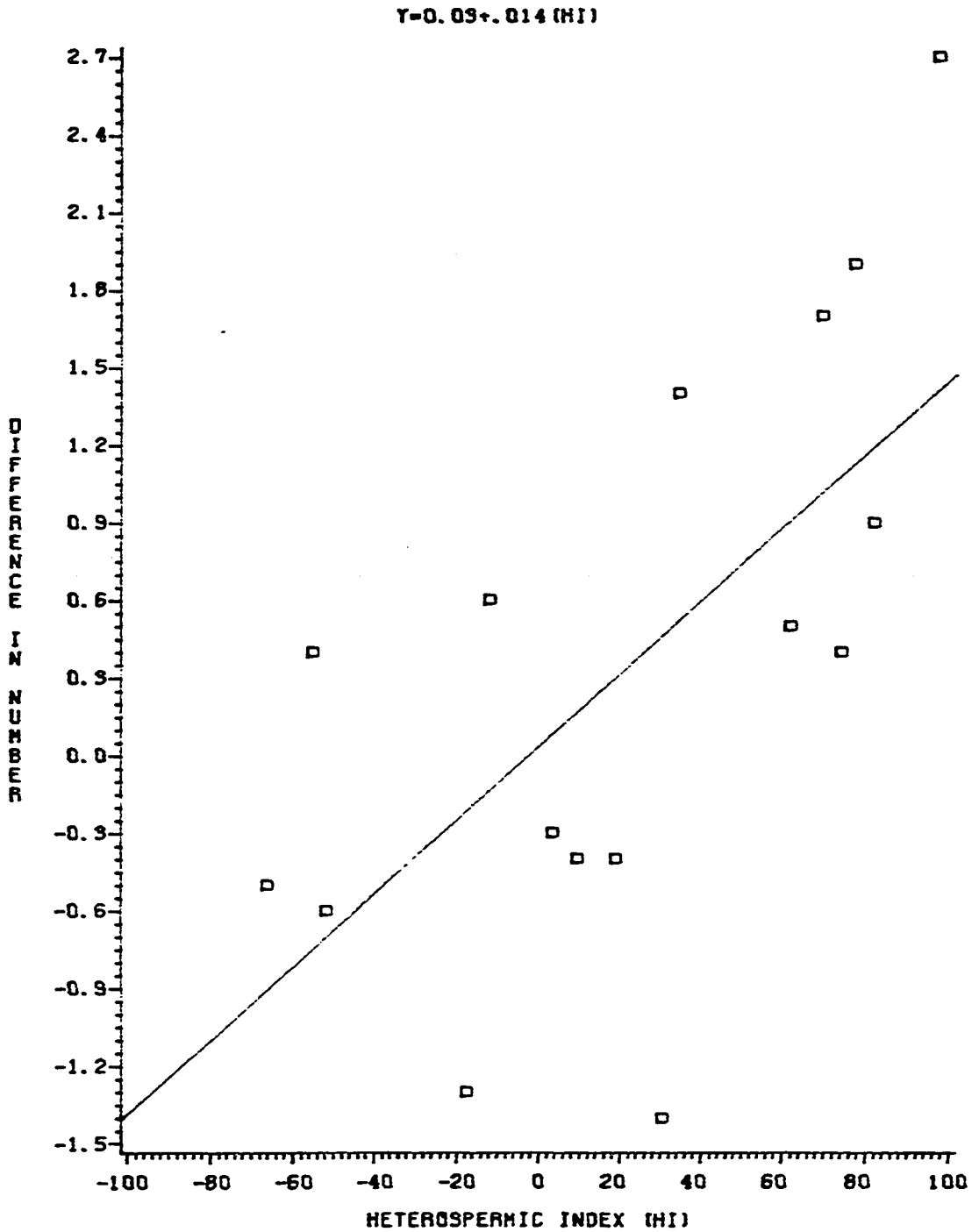
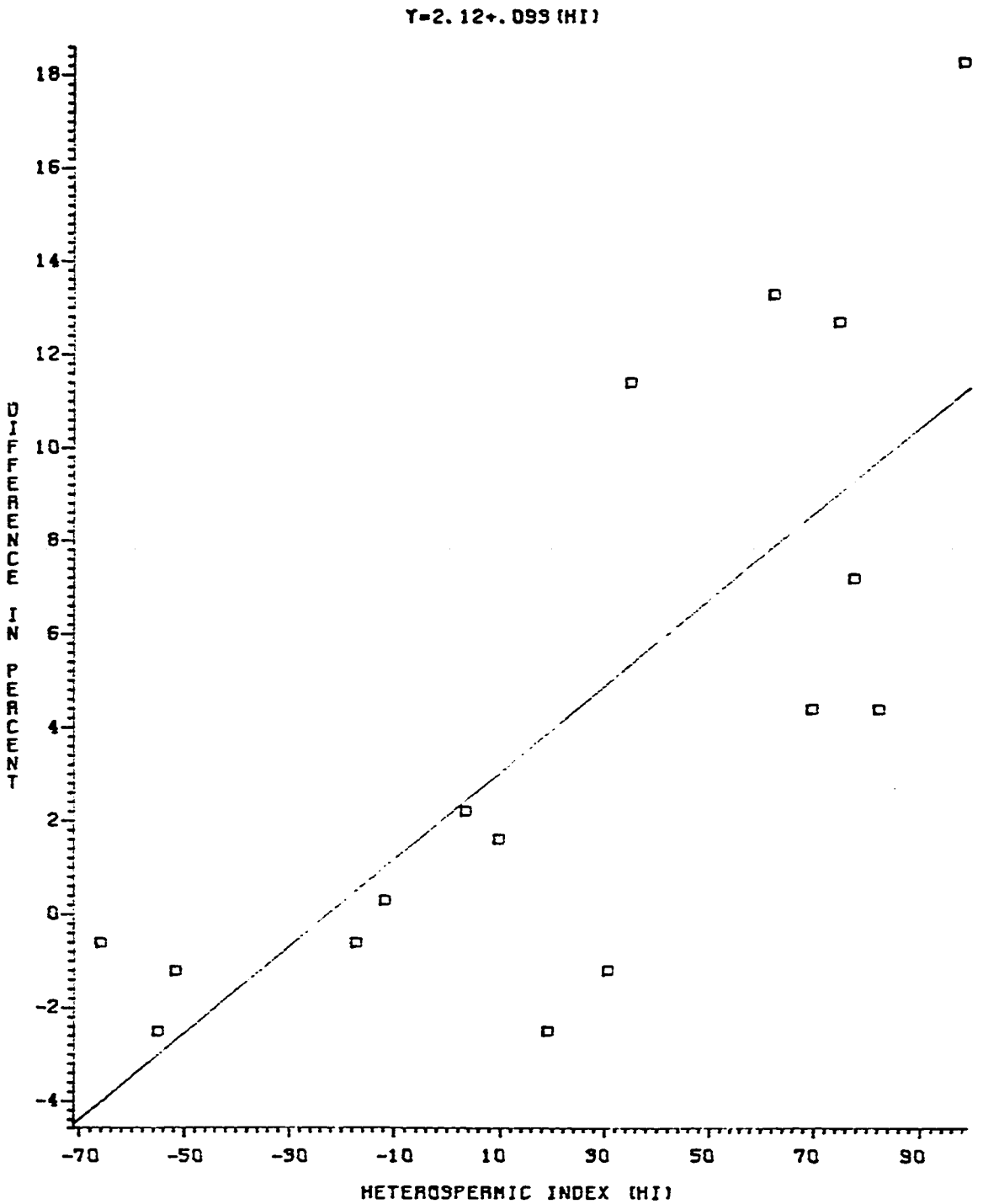

Y=0.03+.014(HI)
DIFFERENCE IN NUMBER
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
-0.3
-0.6
-0.9
-1.2
-1.5
-100
-80
-60
-40
-20
0
20
40
60
80
100
HETEROSPERMIC INDEX (HI)

Figure 11. Correlation between the heterospermic index of each of the 16 pairs of boars and the difference in the percentage of zona-free hamster oocytes penetrated by their spermatozoa. The differences (black boar minus white boar) in the percentage of zona-free hamster oocytes penetrated were correlated with the heterospermic indices ($r = 0.75$)


Y=2.12+.093(HI)
DIFFERENCE IN PERCENT
18
16
14
12
10
8
6
4
2
0
-2
-4
-70
-50
-30
-10
10
30
50
70
90
HETEROSPERMIC INDEX (HI)

DISCUSSION

Ratio of Offspring and Heterospermic Indices

Heterospermic insemination with an equal number of frozen-thawed spermatozoa from two boars, resulted in one of the boars siring a significantly greater ($P<0.05$) number of offspring for 11 of the 16 combinations of boars (Table 41). These results were similar to those of other studies in which equal numbers of spermatozoa from two or more males were inseminated in the rabbit (Beatty, 1960), mouse (Edwards, 1955), cow (Beatty et al., 1969), and chicken (Martin et al., 1974), and with one study in which sows were mated sequentially to two different boars (Martin and Dziuk, 1977a).

In this study, the heterospermic index was a measure of the relative fertility between two males. The heterospermic index for each pair of boars was calculated as the ratio of offspring sired by the black boar minus the ratio of offspring sired by the white boar multiplied by 100. The absolute value of the heterospermic index was large - close to 100 - when one boar sired nearly all the offspring. For example, B2 sired 99% of the offspring when his semen was mixed with that of W3 (Table 40) and the heterospermic index for this combination was 97.3 (Table 41). The absolute value of the heterospermic index was small - close to 0 - when each of the boars sired approximately equal numbers of offspring. For example, B4 sired 55% of the offspring when his semen was mixed with that of W2 and the heterospermic index for this combination was 9.6.

Beatty et al. (1969) and Saacke et al. (1980) computed an average heterospermic index for each male in their trials of heterospermic fertility, rather than a heterospermic index for each pair of males. When an average heterospermic index is computed for each male in which spermatozoa from n number of males from group A are placed in competition with spermatozoa from n number of males from group B, the relative differences in fertility among the competing males may not always be totally accounted for. For example, if a male in group A is far less fertile than the other males in the study, an average heterospermic index will tend to underestimate the fertility of the males in group A and overestimate the fertility of the males in group B because males in group B, but not those in group A, competed with the male of lowest fertility. Conversely, if a male in group A is far superior in fertility to all the other males in the study, an average heterospermic index for each male will tend to overestimate the fertility of the males in group A and underestimate the fertility of the males in group B because the males in group B, but not those in group A, had to compete with the male of highest fertility. The latter example may have occurred in the present study because the B2 boar appeared to be much more fertile than the other seven boars. When B2 competed with W1, who ranked second overall in fertility (Table 42), B2 sired 84.7% of the offspring (Table 40). Therefore, in this study, the heterospermic index for each of the 16 combinations of boars (Table 41) appeared to be a better indicator of a male's relative fertility than the average heterospermic index for each of

the eight boars (Table 42). For this reason, the heterospermic index for each pair of boars, rather than the average heterospermic index, was used to establish the correlations between heterospermic fertility and the in vitro tests of seminal quality.

Seminal Quality Before Cryopreservation

There were no differences in seminal quality before cryopreservation between the two ejaculates collected from each boar (Table 2). There were significant differences among the eight boars for the total number of spermatozoa in the ejaculate, but not for the concentration of spermatozoa, volume of the ejaculate, motility following dilution with Prediluter, and motility following cooling to 5°C (Table 1). These results indicated that for the parameters of seminal quality that were examined before cryopreservation, the two ejaculates collected from each boar were quite similar as were the 16 ejaculates from the eight boars used in this study.

As will be discussed below, following freezing and thawing, there were significant differences among the boars for most of the in vitro tests of seminal quality. Others have also reported that the quality of semen from boars (Larsson, 1978) and bulls (Stewart et al., 1974) was similar before but disparate after freezing and thawing. The results of the present study agreed with the results of several other studies (review by Watson, 1979) and indicated that boars differed widely in the ability of their spermatozoa to withstand damages during freezing and thawing. Furthermore, Larsson (1978) reported that the extent of the damages during freezing and thawing influenced the

lifespan of the spermatozoa in the female reproductive tract and the fertility of a boar's frozen-thawed spermatozoa. It is not known why spermatozoa from certain boars are damaged more extensively than spermatozoa from other boars during freezing and thawing.

All the boars in this study had excellent motility immediately following dilution with Prediluter and following cooling to 5°C (Table 1). During the prefreezing procedures, the average motility declined by 4.5% ($P<0.001$; not shown in the Tables). This decline was probably due to aging of and damage to the spermatozoa during cooling and centrifugation.

The differences (black boar minus white boar) in percent motility following dilution with Prediluter were correlated negatively with the heterospermic indices (Table 45). A negative correlation was not expected between these two parameters because boars of high fertility would be expected to have a higher percentage of motile spermatozoa than boars of lower fertility. I was not able to find a physiological explanation for this negative correlation but others have also observed correlations of the opposite sign to that expected between parameters of seminal quality and *in vivo* fertility (Linford et al., 1976). The differences (black boar minus white boar) in percent motility following cooling to 5°C were not correlated with the heterospermic indices. The lack of correlation between these parameters indicated that the ability of the spermatozoa from different boars to withstand damages during freezing and thawing was not related to the motility of their spermatozoa immediately before

freezing. In future studies, factors which could affect the ability of the spermatozoa to withstand damages during freezing and thawing should be investigated. The identification of factors that affect spermatozoan survival during freezing and thawing could lead to the development of methods for improving the resistance of spermatozoa to damages during freezing and thawing.

In Vitro Tests of Seminal Quality Following Freezing and Thawing

Motility of spermatozoa After freezing and thawing, there was a significant effect of boar on spermatozoan motility without and with caffeine stimulation (Tables 4 and 6, respectively). As expected, spermatozoan motility without and with caffeine stimulation decreased significantly during the 7-hour incubation period (Tables 4 and 6, respectively). The differences (black boar minus white boar) in percent motility without caffeine stimulation for the 16 pairs of black and white boars were significantly correlated with the heterospermic indices when the assays were performed at 0, 1 and 7 hours post-thaw and when the data from all five post-thaw times were used. However, the correlations were not high (Table 45). These results agreed with those of Larsson and Einarsson (1976) and suggested that estimates of motility alone did not accurately predict the fertilizing potential of frozen-thawed porcine semen. Spermatozoan motility has been considered to be a poor indicator of the fertilizing potential of frozen-thawed porcine semen because, in numerous studies, sows inseminated with frozen-thawed semen did not

produce pigs though the motility of the frozen-thawed spermatozoa in the inseminate was good (review by Watson, 1979). However, because motility is necessary for (1) transport of sufficient numbers of spermatozoa to the site of fertilization to ensure subsequent fertility (Hunter, 1980) and (2) penetration of the zona-pellucida (review by Yanagimachi, 1984), it would seem that motility is one parameter that should be evaluated when estimating the fertilizing potential of spermatozoa. Saacke et al. (1980) found a higher correlation between heterospermic fertility and motility of frozen-thawed bovine spermatozoa when motility was evaluated objectively (photographic technique; $r = 0.93$) rather than subjectively ($r = 0.78$). In future studies, objective and subjective methods for estimating the motility of porcine spermatozoa should be compared to determine which method is most highly correlated with fertility.

The motility of the spermatozoa increased following incubation on slides coated with caffeine (Table 5). The increase in motility in response to caffeine stimulation appeared to be boar dependent (Tables 3 and 5). The motility over all times and boars increased from 27.5% for noncaffeine stimulated to 41.0% for caffeine stimulated spermatozoa ($P<0.001$; not shown in the Tables). These results are in agreement with those of Larsson et al. (1976). Following incubation of frozen-thawed porcine semen in 2 mM caffeine for 1 to 2 minutes at 0, 0.5, and 3 hours post-thaw they demonstrated an increase in motility when compared with nonstimulated samples and varying degrees of stimulation depending upon the boar.

Caffeine is a phosphodiesterase inhibitor and stimulates spermatozoan motility and respiration by increasing intracellular cAMP concentrations (Garbers et al., 1973 and Haesungcharern and Chulavatnatol, 1973). The loss in cAMP following cryostorage may be responsible for some of the loss in motility because some frozen-thawed human spermatozoa regain motility following incubation with caffeine (Barkay et al., 1977; Schill et al., 1979; and Aitken et al., 1983). Some investigators have hypothesized that the female reproductive tract provides the spermatozoa with a stimulus similar to that provided by the caffeine in vitro (Zaneveld and Polakoski, 1977). However, if the female reproductive tract does not provide the frozen-thawed porcine spermatozoa with a stimulus similar to that provided by the caffeine in vitro, reversibly immotile spermatozoa may not be transported to the site of fertilization (Hunter, 1980). Thus, the addition of caffeine or other agents that stimulate spermatozoan motility to the thawing extenders should be investigated as a means of improving the motility and transport of the frozen-thawed porcine spermatozoa following insemination.

Acrosomal morphology There was a significant effect of boar on all the classes of acrosomal morphology (Tables 8, 10, 12 and 14). The percentage of spermatozoa with damaged apical rides and loose acrosomal caps appeared to increase during the post-thaw incubation period (Tables 9 and 13, respectively), but the increase was not significant (Tables 10 and 14, respectively). The percentage of spermatozoa with normal apical ridges decreased significantly and the

percentage of spermatozoa with missing apical ridges increased significantly during the post-thaw incubation period (Tables 8 and 12, respectively). This indicated that acrosomes deteriorated as the spermatozoa aged post-thaw. The incubation of semen for several hours post-thaw appeared to be beneficial for revealing latent freeze-thaw injury to the porcine acrosome. Saacke and White (1972) also reported that post-thaw incubation of bovine semen revealed latent acrosomal damage.

The differences (black boar minus white boar) in the percentage of spermatozoa with normal apical ridges, damaged apical ridges, or loose acrosomal caps were not correlated with the heteropsermic indices (Table 45). The differences (black boar minus white boar) in the percentage of spermatozoa with missing apical ridges were correlated negatively with the heterospermic indices at 1, 3, 5, and 7 hours post-thaw and when the data from all five post-thaw times were used (Table 45). The correlation between the differences (black boar minus white boar) in the percentage of spermatozoa with either normal or damaged apical ridges and the heterospermic indices was computed because, as will be discussed later, both of these classes of acrosomal morphology appeared to contain acrosin activity and because Pursel (1979) noted that spermatozoa with normal and damaged apical ridges are capable of exhibiting motility. When the normal and damaged apical ridge classes of acrosomal morphology were combined, a positive correlation was computed with the heterospermic indices at

3 hours post-thaw and when the data from all five post-thaw times were used (Table 45).

The low correlations observed in this study between heterospermic fertility and acrosomal morphology were in agreement with a study by Larrson and Einarsson (1976) in which acrosomal morphology of frozen-thawed porcine spermatozoa was not related to homospermic fertility. In addition, in another study, Larsson et al. (1976) reported that the percentage of spermatozoa with normal apical ridges appeared to be higher after thawing in isotonic glucose than after thawing in Olep; however, fertility appeared to be higher for spermatozoa thawed in Olep. The low correlations between fertility and acrosomal morphology for frozen-thawed porcine spermatozoa may be due to the loss of motility and deterioration of the acrosome which appeared to occur independent of each other during freezing and thawing (review by Watson, 1979). As will be discussed later, motility and acrosomal integrity both appear to be important for fertilization in vivo. Thus, not all spermatozoa with normal apical ridges would be capable of completing fertilization because not all of these spermatozoa would be motile.

In the present study, the percentage of spermatozoa with missing apical ridges, but not with loose acrosomal caps, appeared to increase as the fertility of the boar decreased (Table 45). Pursel (1979) noted that spermatozoa with missing apical ridges, but not with loose acrosomal caps, were capable of exhibiting motility. Because motility was necessary for penetration of the zona pellucida (review by

Yanagimachi, 1984), it did not seem that spermatozoa with loose acrosomal caps could complete fertilization *in vivo*. The absence of a negative correlation between spermatozoa with loose acrosomal caps and heterospermic fertility was an unexpected finding. During this study, I observed a tendency for extracellular debris to adhere preferentially to spermatozoa with loose acrosomal caps *in vitro*. When too much debris was agglutinated to the spermatozoa, the acrosomes were not always visible under phase contrast microscopy and, therefore, they could not be categorized into one of the four classes of acrosomal morphology. The preferential binding of extracellular debris to spermatozoa with loose acrosomal caps may be responsible for the low repeatability observed among the replicates for spermatozoa with loose acrosomal caps (Table 39). In future studies, attempts should be made to develop procedures that eliminate or minimize the binding of debris to spermatozoa *in vitro*, to increase the accuracy and precision of the assay of acrosomal morphology.

Sephadex filtration of spermatozoa The assays of sephadex filtration of spermatozoa were correlated with the assays of motility and acrosomal morphology (Table 38). The repeatability of the assay of sephadex filtration seemed to be higher than the repeatability of the assay of motility or the assay of acrosomal morphology (Table 39). Fayemi et al. (1979) reported that, for porcine spermatozoa filtered through sephadex columns, 89% $\pm$ 4% were motile and 97.6% $\pm$ 1.6% had normal apical ridges, while for unfiltered spermatozoa 45% $\pm$ 8% were motile and 66.6% $\pm$ 7.1%, had normal apical ridges. They concluded

that the assay of sephadex filtration was a rapid and objective *in vitro* test that reflected the percentage of motile spermatozoa with normal apical ridges.

There was a significant effect of boar on the percentage of spermatozoa filtered through the sephadex columns with (Table 17) and without caffeine stimulation (Table 19). A significant effect of boar on sephadex filtration was expected because there was a significant effect of boar on motility and spermatozoa with normal apical ridges, and as stated above, sephadex filtration of frozen-thawed porcine spermatozoa appeared to be a good method for separating motile spermatozoa with normal apical ridges. When caffeine was included in the flushing fluid, the percentage of spermatozoa filtered through the sephadex columns declined significantly during the post-thaw incubation period (Table 17). In contrast, when caffeine was not included in the flushing fluid, the percentage of spermatozoa filtered did not decline significantly during the post-thaw incubation period (Table 19). A significant effect of time was expected for both assays of sephadex filtration because the time effects for motility without and with caffeine stimulation and for spermatozoa with normal apical ridges were significant (Tables 4 ,6 and 8, respectively). I was not able to find a reason for the lack of a significant effect of time on the assay of sephadex filtration without caffeine stimulation.

The percentage of spermatozoa filtered through sephadex columns when caffeine was included (Table 16) in the flushing fluid was significantly greater ($P<0.001$; not shown in the Tables) than when

caffeine was not included (Table 18) in the flushing fluid (20.7% ± 0.5% and 13.6% ± 0.5%; $\bar{X}$ ± S.E., respectively). Fayemi et al. (1979) also reported that the addition of caffeine to the flushing fluid increased ($P<0.01$) the percentage of frozen-thawed porcine spermatozoa filtered through the sephadex columns (22.8% ± 2.3% and 15.2% ± 1.2%; $\bar{X}$ ± S.D., respectively). It seems likely that the percentage of spermatozoa filtered through the sephadex columns increased when caffeine was included in the flushing medium because of the stimulating effect of caffeine on spermatozoan motility.

There was a low correlation between the heterospermic indices and the differences (black boar minus white boar) in the percentage of spermatozoa filtered through the sephadex columns without caffeine stimulation (Table 45). In contrast to the low correlation observed in this study, Saacke et al. (1980) reported a high correlation ($r = 0.82$) between heterospermic fertility and sephadex filtration using frozen-thawed bovine semen. However, in contrast to the present study, their correlations between heterospermic fertility and the percentage of spermatozoa with motility ($r = 0.78$) and intact acrosomes ($r = 0.90$) were high.

Acrosin activity of spermatozoa The percentage of frozen-thawed porcine spermatozoa with acrosin activity at 0 hour post-thaw (Table 21) was similar ($P>0.05$; not shown in the Tables) to the percentage of nonfrozen porcine spermatozoa with acrosin activity immediately following ejaculation (Table 25). In contrast, the

acrosin activity of frozen-thawed spermatozoa from the dog (Froman et al., 1984) and buffalo (Kakar and Anand, 1984) was reduced by 37% and 23%, respectively, compared with nonfrozen spermatozoa.

Porcine spermatozoa appeared to lose most of their acrosin activity as the acrosome deteriorated from the damaged to the missing apical ridge class of acrosomal morphology. There were three lines of evidence that supported this observation. First, the percentage of spermatozoa that had either normal or damaged apical ridges approximated the percentage of spermatozoa with acrosin activity following freezing and thawing (Tables 7, 9, and 21, respectively). Second, during a five-day period (days 15 to 20 post-collection; Table 25) in which the percentage of acrosin-positive nonfrozen porcine spermatozoa decreased markedly ($P<0.005$; not shown in the Table), the percentage of spermatozoa with damaged apical ridges also decreased markedly ($P<0.005$; not shown in the Table), but the percentage of spermatozoa with missing apical ridges increased markedly ($P<0.005$; not shown in the Table). Third, the percentage of frozen-thawed (Table 38) and nonfrozen (not shown in the Tables) porcine spermatozoa with acrosin activity was correlated positively with the percentage of spermatozoa with either normal or damaged apical ridges and were correlated negatively with the percentage of spermatozoa with missing apical ridges.

The observation that frozen-thawed porcine spermatozoa in the present study appeared to lose most of their acrosin activity as the acrosome deteriorated from the damaged to the missing apical ridge

class of acrosomal morphology appeared to explain (1) the positive correlations (Table 45) between the percentage of spermatozoa with either normal or damaged apical ridges and heterospermic fertility, (2) the negative correlations (Table 45) between the percentage of spermatozoa with missing apical ridges and heterospermic fertility, and (3) the low correlation (Table 45) between the percentage of spermatozoa filtered through sephadex columns and heterospermic fertility. Spermatozoa must be motile (Hunter, 1980 and Yanagimachi, 1984) and possess acrosin activity (Stambaugh et al., 1969) to fertilize oocytes in vivo. Pursel (1979) noted that spermatozoa with normal, damaged, and missing apical ridges were capable of motility. However, from the present study it appeared that only those spermatozoa with normal and damaged apical ridges contained appreciable acrosin activity. Thus, it seemed likely that only spermatozoa with either normal or damaged apical ridges had the potential for fertilizing oocytes in vivo. These observations may explain the positive correlations between heterospermic fertility and the percentage of spermatozoa with either normal or damaged apical ridges and the negative correlations between heterospermic fertility and the percentage of spermatozoa with missing apical ridges. The former correlation coefficients were probably lower than the latter correlation coefficients (Table 45) because only a portion of the spermatozoa with either normal or damaged apical ridges would be capable of fertilization, while most of the spermatozoa with missing apical ridges would be capable of fertilization. Lastly, the low

correlation between the percentage of spermatozoa filtered through sephadex columns and heterospermic fertility may have been due to the retention of motile spermatozoa with damaged apical ridges in the sephadex columns. However, it should be noted that the results of a study by Pursel et al. (1972b) did not appear to support the hypothesis that spermatozoa with both normal and damaged apical ridges had the potential of fertilizing oocytes in vivo. Pursel et al. (1972b) reported that nonfrozen spermatozoa stored in an extender that allowed the acrosomes to deteriorate from normal apical ridges to damaged apical ridges (96.3% damaged apical ridges) could not fertilize oocytes in vivo. Furthermore, only 7 of 92 unfertilized oocytes contained accessory spermatozoa. Further research is needed to clarify the fertilizing potential of porcine spermatozoa with damaged apical ridges.

There was a significant effect of boar on the percentage of acrosin-positive spermatozoa (Table 22). The percentage of acrosin-positive spermatozoa declined significantly during the post-thaw incubation period (Table 22). A significant effect of boar and time on the percentage of acrosin-positive spermatozoa was expected because there was a significant effect of boar on spermatozoa with normal and damaged apical ridges and a significant effect of time on spermatozoa with normal apical ridges, and as stated above, spermatozoa with either normal or damaged apical ridges appeared to be the only classes of spermatozoa that contained acrosin activity. The differences (black boar minus white boar) in the percentage of acrosin-positive

spermatozoa were correlated with the heterospermic indices at 3 hours post-thaw and when the data from all five post-thaw times were used. As might be expected, these correlations were similar to the correlations observed between the differences (black boar minus white boar) in the percentage of spermatozoa with either normal or damaged apical ridges and the heterospermic indices (Table 45). A low correlation ($r = 0.33$) between spermatozoan acrosin activity and homospermic fertility was also reported for frozen-thawed bovine semen (Pace et al., 1981). Froman et al. (1984) reported that, although the acrosin activity of canine spermatozoa was an index of cellular damage following semen cryopreservation, acrosin activity alone could not be used to accurately predict the fertilizing potential of the spermatozoa. The low correlations observed in the present study between the difference (black boar minus white boar) in the percentage of spermatozoa with acrosin activity and the heterospermic indices also indicated that the acrosin activity of the spermatozoa alone could not be used to accurately predict the fertilizing potential of frozen-thawed porcine semen.

From the present study, it appeared that damage to the acrosomal region could be evaluated with the assay of acrosomal morphology or by determining the percentage of spermatozoa with acrosin activity. However, the assay of acrosomal morphology may be the preferred method of estimating damage to the acrosomal region of porcine spermatozoa because this assay is less time consuming and less expensive than the assay of acrosin activity. It should be noted that the porcine

acrosome can be clearly observed with a phase contrast microscope. In species in which the acrosome cannot be clearly seen with a light microscope, the gelatin technique for the detection of acrosin activity may be extremely useful for estimating acrosomal damage.

There was no significant effect of boar on diameter of the halos of the acrosin-positive spermatozoa (Table 24). The diameter of the halos declined during the post-thaw incubation period (Table 24). The differences (black boar minus white boar) in the diameter of the halos of acrosin-positive spermatozoa were correlated with the heterospermic indices (Table 45). However, because of the extreme variability between replicates for the diameter of the halos (Table 39) and the lack of a significant effect of boar on the diameter of the halos (Table 24), this assay did not appear to be a useful assay for estimating the fertilizing potential of porcine semen.

Glutamic oxaloacetic transaminase released from the spermatozoa

There was a significant effect of boar on extracellular glutamic oxaloacetic transaminase, maximum releasable glutamic oxaloacetic transaminase, percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly, and intracellular glutamic oxaloacetic transaminase (Tables 29, 31, 33, and 35, respectively). The effect of time was not significant for any of the assays of glutamic oxaloacetic transaminase (Tables 29, 33, and 35, respectively). The lack of a significant effect of time on glutamic oxaloacetic transaminase suggested that the leakage of glutamic oxaloacetic transaminase from the spermatozoa occurred immediately

post-thaw and that incubation of the semen at 37°C did not reveal latent damage to the spermatozoan membrane.

Several investigators have reported that leakage of glutamic oxaloacetic transaminase from spermatozoa was caused by cellular damage during freezing and thawing (Brown et al., 1971; Crabo et al., 1972; Bower et al., 1973; and Larsson and Einarsson, 1976). The differences (black boar minus white boar) in the percentage of maximum releasable glutamic oxaloacetic transaminase present extracellularly were positively correlated with the heterospermic indices (Table 45). A negative correlation was expected between these two parameters because spermatozoa from boars of high fertility should have less damage to their cell membranes and, therefore, less leakage of glutamic oxaloacetic transaminase from their spermatozoa than for spermatozoa from boars of low fertility. The differences (black boar minus white boar) in intracellular glutamic oxaloacetic transaminase were negatively correlated with the heterospermic indices (Table 45). A positive correlation was expected between these two parameters, because spermatozoa from boars of high fertility should have less damage to their cell membranes and, therefore, more glutamic oxaloacetic transaminase remaining inside their spermatozoa than for spermatozoa from boars of low fertility. I was not able to find a physiological explanation for these unexpected observations. The differences (black boar minus white boar) in the extracellular and maximum releasable glutamic oxaloacetic transaminase were not correlated with the heterospermic indices (Table 45). In contrast,

Larsson and Einarsson (1976) reported an inverse relationship between extracellular glutamic oxaloacetic transaminase and fertility following homospermic insemination of frozen-thawed porcine semen. However, in the bovine, the correlations between extracellular glutamic oxaloacetic transaminase and fertility following homospermic insemination were low or nonsignificant (review by Graham et al., 1979). Therefore, the results of the present study were in agreement with the studies using frozen-thawed bovine semen, but were not in agreement with the results of a relatively small study with frozen-thawed porcine semen. In future studies, the extracellular glutamic oxaloacetic transaminase present immediately following ejaculation should be determined along with the glutamic oxaloacetic transaminase following freezing and thawing. If boars differ greatly in extracellular glutamic oxaloacetic transaminase before freezing, it may be possible to increase the correlation between extracellular glutamic oxaloacetic transaminase and fertility of frozen-thawed semen by correcting for this prefreeze extracellular glutamic oxaloacetic transaminase.

Spermatozoan penetration assay of zona-free hamster oocytes

The present study appeared to be the first in which the spermatozoan penetration assay was used to predict the fertilizing potential of frozen-thawed porcine spermatozoa. The parameters of the spermatozoan penetration assay that were measured were the number of penetrations per oocyte, the number of spermatozoa bound per oocyte, and the percentage of oocytes penetrated (Table 36). There was a significant

effect of boar on the number of penetrations per oocyte, but not on the number of spermatozoa bound to the oocytes or on the percentage of oocytes penetrated (Table 37). However, the heterospermic indices were significantly correlated with the differences (black boar minus white boar) in the percentage of oocytes penetrated and the differences in the number of spermatozoa bound per oocyte but not with the differences in the number of penetrations per oocyte (Table 45). The percentage of oocytes penetrated by the spermatozoa from the boars ranked first and eighth overall in this study (Table 42) were 91.3% $\pm$ 6.8% and 73.0% $\pm$ 5.5%, respectively (Table 36). Spermatozoa from the remaining six boars penetrated approximately the same percentage of oocytes (84.1% to 86.9%). Therefore, under the experimental conditions used in this study, it appeared that it was possible to predict which boar was the most and the least fertile with the spermatozoan penetration assay. The lack of a significant effect of boar on the percentage of oocytes penetrated was probably due to the relatively small variation among the boars ranked second through seventh inclusive.

There may be four ways to improve the predictive value of the spermatozoan penetration assay for estimating the fertilizing potential of frozen-thawed porcine spermatozoa. First, it may be necessary to decrease the number of spermatozoa used in this assay. Robl and Dziuk (1984) demonstrated that differences in _in vivo_ fertility among three strains of mice were detected only when approximately 50% of the oocytes were fertilized. The percentage of

fertilized oocytes was varied by adjusting the number of spermatozoa in the inseminate. When the percentage of fertilized oocytes was either high or low, the differences in fertility among the three strains were not detectable. They concluded that the optimal number of spermatozoa for detecting differences in fertility was the number that resulted in a fertilization rate of approximately 50%. The accuracy of the spermatozoan penetration assay for predicting the fertilizing potential of frozen-thawed porcine spermatozoa may also be increased by adjusting the number of spermatozoa in the inseminate so that approximately 50% of the zona-free hamster oocytes are penetrated. In the present study, a high percentage of zona-free hamster oocytes were penetrated by spermatozoa from all eight boars (Table 36).

Second, it may be important to improve the techniques for the in vitro capacitation of frozen-thawed porcine spermatozoa. Little is known about the conditions required for the in vitro capacitation of frozen-thawed porcine spermatozoa. Improved methods for capacitating human spermatozoa in vitro have increased the diagnostic value of the spermatozoan penetration assay by decreasing the number of false negatives (review by Yanagimachi, 1984). The effect of pH, osmolarity, and concentration of serum in the capacitation medium on the percentage of oocytes penetrated by porcine spermatozoa were not determined in this study and should be investigated.

Third, it may be important to minimize the binding of noncapacitated porcine spermatozoa to the surface of the zona-free

hamster oocyte when estimating male fertility, to maximize the surface area of the oocyte available for the attachment of acrosome-reacted spermatozoa, because only acrosome-reacted porcine spermatozoa can fuse with the vitelline membrane (Imai et al., 1979, 1980 and Smith et al., 1983). Koehler et al. (1984) reported that uncapacitated spermatozoa with intact acrosomes from the hamster, stallion, bull, rabbit, and man attached firmly to the microvilli of the zona-free hamster oocyte but were not capable of fusing with the vitelline membrane. However, the avidity of human and hamster spermatozoa for the microvilli was low compared to that of spermatozoa from the bull, horse, and rabbit. Koehler et al. (1984) also noted that the avidity of the spermatozoa for the oocyte's surface was inversely related to the tendency of acrosome-reacted spermatozoa to fuse with zona-free hamster oocytes. Other investigators have reported extensive binding of porcine spermatozoa with intact acrosomes to zona-free hamster oocytes in media that did not permit penetration of the oocytes (Imai et al. 1979, 1980 and Smith et al. 1983). In the present study, frozen-thawed porcine spermatozoa were added to zona-free hamster oocytes immediately following thawing. At the end of the incubation period, the oocytes were always covered with hundreds of spermatozoa. The surface of the oocytes may have been covered by numerous noncapacitated and dead spermatozoa shortly after insemination and this may have limited the surface area available for the attachment of spermatozoa that had undergone the acrosomal reaction. In future studies, the interval from thawing to maximal capacitation of the

spermatozoa should be determined and the oocytes should then be inseminated at that time.

Fourth, it may be important to determine if spermatozoan motility modulates the number of penetrations per zona-free hamster oocyte and the percentage of oocytes penetrated. Aitken and Elton (1984) demonstrated that the Poisson distribution accurately described the relationship between the motility of human spermatozoa and penetration of zona-free hamster oocytes. The authors stated that the "fixed nature" between these parameters "enables the results of such in vitro fertilization experiments to be corrected for the number of motile spermatozoa in the incubation media"..."thus permitting analysis of the penetrating ability of the spermatozoa in isolation from defects in motility." From the results of the present study, there was no way of determining whether the motility of frozen-thawed porcine spermatozoa had a similar modulating effect on penetration of the oocyte, because the motility of the spermatozoa was not determined in the capacitating medium immediately before insemination of the oocytes, as was the case in the Aitken and Elton (1984) study. In view of the report by Koehler et al. (1984) that human spermatozoa bound to the zona-free hamster oocyte with less avidity than spermatozoa from several other species it would seem prudent to determine, for each species, the modulating effect of motility on penetration of the oocytes. It seems likely that the modulating effect of porcine spermatozoan motility on penetration of zona-free hamster oocytes may not be as great as for human spermatozoa because,

immediately following insemination, porcine spermatozoa bind to the oocytes in large numbers while few human spermatozoa bind to the oocytes.

The Variation in Heterospermic Fertility Accounted for by Selected *In Vitro* Tests

A multiple regression analysis was used to examine the variation in heterospermic fertility that was accounted for with selected *in vitro* tests. Four parameters from three different *in vitro* tests were used in the multiple regression model. The four parameters were the percentage of motile spermatozoa without caffeine stimulation, the percentage of spermatozoa with either normal or damaged apical ridges, the number of spermatozoa bound per zona-free hamster oocyte, and the percentage of zona-free hamster oocytes penetrated.

Motility was selected as one of the parameters because in this study, motility was correlated with fertility and because others (review by Yanagimachi, 1984) have shown that strong motility was needed for spermatozoa to penetrate the zona pellucida. Motility may also be important for transport of porcine spermatozoa to the site of fertilization (Hunter, 1980). The percentage of spermatozoa with either normal or damaged apical ridges was selected because, in this study, these classes of spermatozoa were correlated with fertility, possessed acrosin activity, and because Pursel (1979) noted that spermatozoa with normal and damaged apical ridges often were motile. The number of spermatozoa bound per zona-free hamster oocyte and the percentage of oocytes penetrated were selected because the correlation

coefficients between these two parameters and heterospermic fertility were relatively high. In addition, there was the potential that these two parameters would offer additional information on the fertility of the eight boars over that provided by the estimates of motility and acrosomal morphology because these two parameters were not correlated with either motility or acrosomal morphology (Table 38). The assay of sephadex filtration was not selected because it did not provide additional information about the fertilizing potential of the eight boars over that provided by the assays of motility and acrosomal morphology. The assay of acrosin activity was not selected because it did not provide additional information about the fertilizing potential of the eight boars over that provided by estimating the percentage of spermatozoa with either normal or damaged apical ridges. The assays of glutamic oxaloacetic transaminase were not selected because they did not accurately reflect the fertilizing potential of the eight boars.

The multiple regression correlation between the differences (black boar minus white boar) in the four parameters that were selected and the heterospermic indices was 0.94. This high correlation indicated that the heterospermic fertility of the semen used this study could be predicted with reasonable accuracy with four parameters from three different in vitro tests. The correlation between each of the selected parameters and the heterospermic indices was much lower than the multiple regression correlation between the heterospermic indices and all four of the parameters. The results of

this study agree with the suggestion of Froman et al. (1984) and Yanagimachi (1984) that no single *in vitro* test of seminal quality can accurately predict the fertilizing potential of a semen sample, because no single *in vitro* test can measure all the qualities and functions of the spermatozoa that are necessary for *in vivo* fertilization.

Suggested Future Studies

In the present study, the extracellular glutamic oxaloacetic transaminase was not determined before the spermatozoa were frozen. In future studies, the correlations between heterospermic fertility and extracellular glutamic oxaloacetic transaminase following freezing and thawing should be computed after correcting for extracellular glutamic oxaloacetic transaminase before freezing.

In future studies, efforts should also be made to increase the accuracy and precision of the assay of motility, the assay of acrosomal morphology, and the spermatozoan penetration assay to further increase the correlations between these assays and *in vivo* fertility. There are several objective methods presently available for the assessment of spermatozoan motility that could be used for frozen-thawed porcine spermatozoa (review by Watson, 1979). Attempts should be made to decrease the quantity of debris that binds to porcine spermatozoa post-thaw and interferes with the evaluation of the acrosome. Hypertonic media appeared to decrease the quantity of debris that bind to frozen-thawed porcine spermatozoa *in vitro* (personal observation). However, the effect of hypertonic media on

acrosomal morphology should be determined. Lastly, attempts should be made to determine the (1) optimal number of porcine spermatozoa for the insemination of zona-free hamster oocytes, (2) optimal conditions for the in vitro capacitation of frozen-thawed porcine spermatozoa, and (3) modulating effect of porcine spermatozoan motility on the penetration of zona-free hamster oocytes.

Importance of This Study to the Swine Industry

When compared with natural mating, artificial insemination with nonfrozen or frozen semen allows for the more widespread use of boars of superior genetic value and decreases the chance of transmitting diseases. However, the shipment of nonfrozen porcine semen is difficult because (1) the volume of the inseminate is large, (2) during the winter the spermatozoa are likely to be subjected to cold shock, and (3) nonfrozen spermatozoa have a relatively short lifespan. Therefore, it is necessary to ship spermatozoa frozen, rather than fresh, to maximize the widespread use of semen from boars of superior genetic value.

The results of this study indicated that a large percentage of the total variation in heterospermic fertility was accounted for with four parameters from three in vitro tests. These in vitro tests could be used in two ways to increase the use of frozen-thawed porcine semen. First, these in vitro tests could be used to improve freezing methods and thereby increase conception rates and litter sizes following insemination of frozen-thawed semen. As the freezing methods are improved, it may be possible to reduce the number of

spermatozoa in an inseminate and thereby increase the number of inseminates from each boar of superior genetic value and decrease the cost of an inseminate.

Second, these in vitro tests could be used to increase the use of frozen-thawed porcine semen by commercial producers through the development of an index of fertility for each boar in an artificial insemination stud. Presently, frozen-thawed semen is used extensively by the purebred producer only because, economically, the commercial producer does not benefit enough from the genetic gain to offset the losses due to the decrease in conception rate and litter size. Providing an index of fertility for each boar in an artificial insemination stud along with the other indices that are based on feed efficiency, average daily gain, backfat, and loin eye area will allow the producer to select boars based on the economics that are indigenous to his operation.

This is the first report on the fertilizing potential of porcine semen packaged in straws and frozen at a controlled rate in liquid nitrogen vapors. This method of packaging and freezing offers an alternative to the pellet method which is widely used by boar studs. Porcine semen has been frozen in straws above the surface of liquid nitrogen on an experimental basis (Larsson, 1978). With this method, I noted that the rates of freezing among the straws were inconsistent when more than 20 straws were frozen simultaneously. Therefore, a program was developed for freezing up to 200 straws of semen simultaneously using an automated freezing chamber (Appendix B).

The 60-day conception rate, farrowing rate, and litter size following the insemination of semen frozen by this method were 60.9%, 51.4%, and 8.8, respectively (Tables 43, 44, and 40, respectively). Johnson (1980) summarized the fertility obtained following the insemination of porcine semen frozen by different methods. Pregnancy rates ranged from 53% to 72% and litter sizes ranged from 8.3 to 9.7. Therefore, the pregnancy rate and the litter size obtained with semen frozen by the method I developed was comparable to the pregnancy rates and litter sizes obtained with semen frozen by other methods. The development of a method for freezing porcine semen in straws on a commercial basis is important because this method offers the following advantages when compared with the pellet method (1) more inseminates can be stored in a given space, (2) there is less chance of contaminating the semen, and (3) there is less labor involved in the preparation of the inseminate.

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APPENDIX A:

EXTENDERS USED FOR THE CRYOPRESERVATION AND THAWING OF SEMEN AND THE BOARS USED FOR THIS STUDY

Ingredients for Prediluter:		
glucose monohydrate	60.044	g
sodium citrate dihydrate	3.703	g
EDTA	3.702	g
sodium bicarbonate	1.201	g
distilled water	1000.000	ml
Ingredients for Cool Diluter:		
11% lactose solution	100.0	ml
egg yolk	25.0	ml
Ingredients for Deep Freeze Diluter:		
11% lactose solution	100.0	ml
egg yolk	25.0	ml
glycerin	9.0	ml
Orvus-Es-Paste	2.0	ml
Ingredients for Olep:		
fructose	5.000	g
sodium pyruvate anhydrous	5.000	g
calcium chloride dihydrate	0.060	g
magnesium chloride hexahydrate	1.847	g
potassium chloride	1.200	g
sodium chloride	3.500	g
sodium bicarbonate	0.840	g
distilled water	1000.000	ml

The boars ranged from 14 to 58 months of age and were sexually rested for 1 to 2 weeks when semen was collected for this study. The *in vitro* tests of seminal quality and the *in vivo* test of fertility were done within two years of seminal collection.

APPENDIX B:

PROGRAM FOR FREEZING PORCINE SEMEN IN VAPORS OF LIQUID NITROGEN USING A CRYO-MED AUTOMATED FREEZING CHAMBER (CONTROLLER MODEL 1010) (Program developed by the author while at Swine Genetics Intl.)

Procedure:

Start temperature: $5^{\circ}C$

Liquid phase freeze rate: $1^{\circ}C$/minute

Start phase change: $-1^{\circ}C$

Phase change temperature drop: $-50^{\circ}C$

End phase change: $-26^{\circ}C$

Solid phase I freeze rate: $5^{\circ}C$/minute

End solid phase I: $-40^{\circ}C$

Solid phase II freeze rate: $10^{\circ}C$/minute

End solid phase II: $-100^{\circ}C$

Straws immersed immediately in and stored in liquid nitrogen ($-196^{\circ}C$).

APPENDIX C:

MODENA EXTENDER USED FOR ESTIMATING MOTILITY IMMEDIATELY BEFORE CRYOPRESERVATION (Swiss Federation for Artificial Insemination, Butchwill, Switzerland)

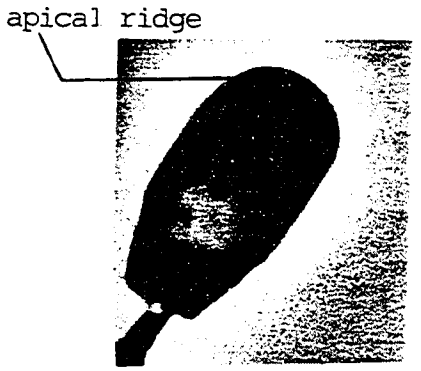
Ingredients for Modena Extender:	g/l
citric acid, trisodium salt	6.90
sodium bicarbonate	1.00
citric acid monohydrate	2.00
EDTA	2.25
glucose	25.00
Tris	5.65
BSA (Cohn Fr. V)	3.00
cysteine	0.05
penicillin (1670 IU/mg)	0.60
streptomycin	1.00

APPENDIX D:

CLASSIFICATION OF ACROSOMAL MORPHOLOGY (Pursel et al., 1972a)

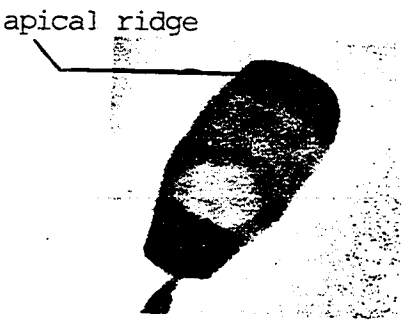
Normal Apical Ridge:

The acrosomal cap is smoothly adhered to the nucleus and possesses a distinct apical ridge that forms a smooth crescent at the apical border of the head.



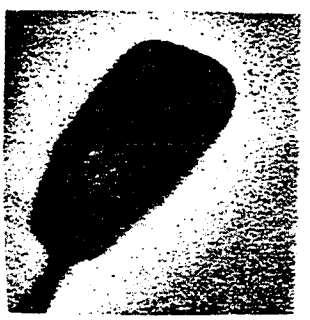
Damaged Apical Ridge:

A spreading of the posterior border of the apical ridge is observed, causing the ridge to take on an irregular shape.



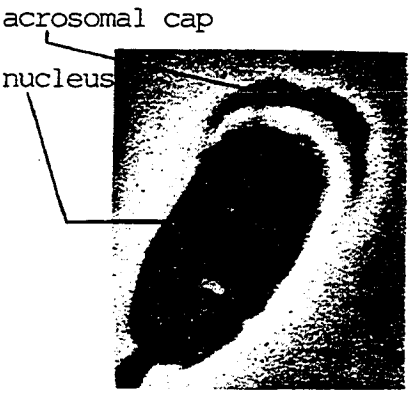
Missing Apical Ridge:

The spermatozoon lacks an apical ridge. The acrosomal cap is still tightly adhered to the nucleus.



Loose Acrosomal Cap:

The anterior acrosomal cap is completely vesiculated or missing.



APPENDIX E:

PREPARATION OF COLUMNS FOR SEPHADEX FILTRATION (Fayemi et al., 1979 and Graham et al., 1979)

Procedure:

a) The luer tip is cut off from disposable plastic syringes (3cc, 1 cm diameter, 6.5 cm height) and a 4.5-mm hole is drilled through the end of the syringe.

b) A disc made of 400-mesh stainless steel screen is placed over the hole and heat-sealed into the plastic with a soldering iron. This creates a permanent filter through which the sephadex cannot pass.

c) A small thin pad of Fiberglas (John-Manville microfiber, code 112, No. 475) is placed over the barrel before placing the plunger into the syringe. This cuts the pad to the exact fit of the barrel and deposits the pad on the screen. The pad keeps the gel from blocking the holes in the screen.

d) A 20% (w/v) slurry of sephadex G-15-120 in 0.12 M sodium citrate is prepared and allowed to swell overnight at $5^{o}C$.

e) Immediately before performing an assay, the columns are filled with 0.6 ml of the well-mixed slurry. Columns are filled and flushed in an incubator at $37^{o}C$.

f) Six ml of prewarmed ($37^{o}C$) 0.12 M sodium citrate is drained through each column, before adding the spermatozoa.

APPENDIX F:

PROCEDURES FOR THE GELATIN-SUBSTRATE-FILM METHOD FOR THE DETECTION OF ACROSIN ACTIVITY OF SPERMATOZOA (Ficsor et al., 1983)

Preparation of Gelatinized Slides:

Number three gels (0.54 g/m^2 gelatin B) were provided gratis by Gyula Ficsor, Ph.D., Department of Biomedical Science, Western Michigan University, Kalamazoo, Michigan, and Eastman Kodak Company, Biosciences Division, Research Laboratories, Rochester, New York. Two weeks before semen was thawed for this assay, the sheets of gelatin were cut into 22 x 22 mm squares and mounted on microscope slides with Protexx (Fisher Scientific). The mounting medium was allowed to harden overnight at room temperature. The slides were placed in a slide box with the lid propped open approximately 2 cm. The slide box was placed in another plastic container with approximately 2 cm of distilled water. This container was tightly sealed and placed in a refrigerator at 5°C. The slides were stored in this manner for a minimum of 2 weeks to ensure proper conditioning of the gelatin. One day before performing an assay, humidified chambers were placed in an incubator at 37°C. The humidified chambers were plastic slide boxes with sponges soaked with distilled water. A different humidified chamber was used for each of the five post-thaw times.

Ingredients for Phosphate Buffered Saline:

	g/l
potassium chloride	0.20
potassium phosphate monobasic	0.20
sodium chloride	8.00
sodium phosphate dibasic	1.69
pH adjusted to 7.4	

Preparation of L-shaped spreaders:

The small end of Pasteur pipettes were bent 3 cm from the tip to an angle of 90°. A new spreader was used for each gelatinized slide.

Preparation of the toluidine blue O stain:

0.3 g of toluidine blue O and 100 ml borate buffer were mixed and filtered through No. 2 Whatman filter paper.

0.1M Borate Buffer:

boric acid	6.18 g
potassium chloride	7.46 g
sodium hydroxide	3.50 g
distilled water	1878.00 ml

APPENDIX G:

KINETIC ASSAY FOR GLUTAMIC OXALOACETIC TRANSAMINASE (Worthington Diagnostic Systems Inc., Freehold NJ)

Preparation of the samples for analysis of glutamic oxaloacetic transaminase (GOT):

To separate the spermatozoa from the extracellular fluid, 1.8 ml of frozen-thawed semen was carefully layered on 2 ml of density gradient fluid in round-bottomed centrifuge tubes. The density gradient consisted of 7.75% raffinose, 15% dextran, 0.02% food coloring, and 0.153% glycerol. The osmotic pressure of the density gradient and the diluted semen were identical (290 mosm/l). The temperature of the density gradient and frozen-thawed semen were equilibrated before layering the semen on the gradient. The layered samples were centrifuged at 12,350 x g for 10 minutes, to completely separate the spermatozoa from the extracellular fluid. A 0.5-ml aliquot of extracellular fluid was removed and stored at -20°C until analyzed.

Contents of reagent for the assay of GOT:

Ingredient	Concentration of Reconstituted Reagent	Final Concentration of Reaction Mixture
L-aspartate	133.0 umol/ml	124 mM/l
2-oxoglutarate	6.5 umol/ml	6.1 mM/l
NADH	0.23 umol/ml	0.22 mM/l
oxamic acid	25.3 umol/ml	23.7 mM/l
malate dehydrogenanase	$\geq$ 1.0 U/ml	$\geq$ 1.0 U/ml
buffer		

Methodology: The assay is based on the following reactions:

$$\text{2-oxoglutarate + L-aspartate} \xrightleftharpoons{\text{GOT}} \text{glutamate + oxaloacetate}$$

$$\text{oxaloacetate + NADH + } H^{+} \xrightleftharpoons{\text{malate dehydrogenase}} \text{malate + NAD}$$

The first reaction is rate limiting because malate dehydrogenase is present in excess amounts. Therefore, the rate of the second reaction is determined by the amount of oxaloacetate formed. The amount of oxaloacetate formed is a function of the GOT in the unknown. In the second reaction, NADH is oxidized to NAD. NAD is monitored by ultraviolet spectrophotometry to give an indirect measure of GOT.

Procedure:

Prewarm 3.0 ml of reagent to 30°C. Add 0.2 ml of the unknown to the reagent and mix by inverting gently several times. While maintaining the temperature of the reaction mixture at 30°C, record the absorbance at 340 nm at 30-second intervals for 2-3 minutes. Determine the change in absorbance per minute ($\Delta A/min$).

Expressing Results:

a) One IU is the amount of enzyme that will oxidize one micromole of NADH per minute at 30°C. The number of

IU/l of an unknown sample is calculated using the following formula:

$$IU/l = \frac{\Delta A/min \times 1000 \times 3.2 \times 1000}{6.22 \times 10^3 \times 0.2}$$

where 1000 converts ml to liter; 3.2 is the total reaction volume; 1000 converts millimoles to micromoles; 6.22×10^3 is the molar extinction coefficient; and 0.2 is the volume of the unknown.

b) The GOT was converted from IU/l to mIU/10^9 spermatozoa before doing the statistical analyses. Frozen-thawed spermatozoa, diluted in Olep, were counted with a hemacytometer to determine the concentration of spermatozoa.

APPENDIX H:

MEDIA AND PROCEDURES FOR THE SPERMATOZOAN PENETRATION ASSAY OF ZONA-FREE HAMSTER OOCYTES

(Pavlok, 1981)

Formulas:

Source of medium 199: Flow Laboratories, McLean VA

Source of chemicals and enzymes: Sigma, St. Louis MO

Supplementation of medium 199:

Ingredient	g/l
sodium pyruvate	0.1001
calcium lactate	0.9002
glucose (anhydrous)	0.5496
potassium penicillin	0.0313
streptomycin sulphate	0.0649

The medium was further supplemented with 20% (v/v) fetal bovine serum (HyClone, Logan UT). The supplemented medium (M199+) was sterilized with a 0.45 um filter (No. 7102, Falcon Plastics, Oxnard CA) and stored in sterile vials at 5^{o}C until needed.

pH and osmolarity of the media:

Media	pH	osmolarity (osm/l)
M199+	7.75	315
0.2% trypsin in M199+	7.31	320
0.1% hyaluronidase in M199+	7.60	315

Preparation of paraffin oil:

Source and type: Fisher Scientific, saybolt viscosity 125/135

Procedure: The paraffin oil was heat sterilized and stored frozen until needed. One ml of M199+ and 7 ml of paraffin oil were mixed thoroughly and allowed to separate overnight at 39°C.

Superovulation regimen for hamsters (Berger et al., 1983):

Day	Time	Procedure	Intervals
0	9 AM	i.p. injection of 35 IU PMSG	55 hr
2	4 PM	i.p. injection of 50 IU hCG	17 hr
3	9 AM	collect eggs	

Cultureware:

Oviductal flushing and dispersal of cumulus: No. 3035 Costar culture plates, Cambridge MA

Washing of the eggs, removal of the zona pellucida, and insemination: Linbro No. 76-058-05 petri dishes, Flow Labs, McLean VA

Dilution of spermatozoa: 12 x 75 mm borosilicate tubes, Fisher Scientific

APPENDIX I:

EXPECTED MEAN SQUARES ASSOCIATED WITH THE SOURCE LINES IN THE ANALYSIS OF VARIANCE TABLES

The relevant source lines in the one-way ANOVA tables for estimating repeatabilities and standard errors are:

Source	Expected Mean Square
Boars	$\sigma_s^2 + 4/7 \sum_{i=1}^{8} (B_i - \bar{B})^2$
Replication (Boars)	σ_s^2

The relevant source lines in the two-way ANOVA tables for the assays of spermatozoan concentration, volume of the ejaculate, and total number of spermatozoa are:

Source	Expected Mean Square
Boars x Ejaculates	σ_s^2

The relevant source lines in the two-way ANOVA tables for the assays of motility before cryopreservation are:

Source	Expected Mean Square
Boars x Ejaculates	$\sigma_s^2 + 4\sigma_\mu^2$
Replication (Boars x Ejaculates)	σ_s^2

The relevant source lines in the two-way ANOVA tables for the assay of sephadex filtration without caffeine stimulation are:

Source	Expected Mean Square
Boars x Times	$(\sigma^2_\mu + 1/2\ \sigma^2_s)[2 - 1/4(6p + 4p^2 + 2p^3)]$
Replication (Boars x Times)	σ^2_s

The relevant source lines in the two-way ANOVA tables for all the other assays where a boar x time design was used are:

Source	Expected Mean Square
Boars x Times	$(\sigma^2_\mu + 1/4\ \sigma^2_s)[4 - 1/5(8p + 6p^2 + 4p^3 + 2p^4)]$
Replication (Boars x Times)	σ^2_s

APPENDIX J:

STATISTICAL PROCEDURES USED FOR CALCULATING THE REPEATABILITY OF ASSAYS AND THE STANDARD ERRORS FOR ASSAY MEANS

Let:

y_{ijk} = observations made on the k^{th} replicate at the j^{th} time for the i^{th} boar

The assumed model was:

$$y_{ijk} = u + B_i + T_j + (BT)_{ij} + e_{ijk} \quad ,$$

where y_{ijk} is decomposed into an overall mean (u), a contribution due to the i^{th} boar (B_i) (i = 1,...,8), a contribution due to the j^{th} time (T_j) (j = 1,...,5), a joint contribution by the i^{th} boar and j^{th} time ($(BT)_{ij}$), and a contribution associated with the k^{th} replicate (k = 1,...,4) in the (i,j)- th boar x time subclass.

The boar and time contributions, B_i and T_j (i = 1,...,8) (j = 1,...,5), were considered to be fixed rather than random. The contributions B_i and T_j were also assumed to be additive for each assay. Their joint contribution BT_{ij} was therefore taken to be a random component of y_{ijk}, namely, one that represents experimental error. Since the four replicates in a boar x time cell were generated from the same experimental unit (a pool of semen from one straw from ejaculate A and one straw from ejaculate B), the contributions

(e_{ijk}) were also assumed to be random but are interpreted as sampling error.

We assumed that $e_{ijk} \sim N(0,\sigma_s^2)\ \forall\ i,j,k$ and that Cov (e_{ijk}, $e_{i'j'k'}$) = 0 if $i \neq i'$ or $j \neq j'$ or $k \neq k'$. Further, we assumed that $(BT)_{ij}$ and e_{ijk} are independently distributed $\forall_{i,j,k}$. Denote the variance of each $(BT)_{ij}$ ($i = 1,\ldots,8$, $j = 1,\ldots,5$) by σ_μ^2, i.e., Var $[(BT)_{ij}] = \sigma_\mu^2\ \forall_{i,j}$ and assume that each $(BT)_{ij}$ has a zero mean. This implies that Var $(Y_{ijk}) = \sigma_\mu^2 + \sigma_s^2$. It is also reasonable to assume that Cov $[(BT)_{ij}, (BT)_{i'j'}] = 0$ whenever $i \neq i'$. However, if $i = i'$, it is likely that $(BT)_{ij}$ and $(BT)_{i'j'}$ are correlated since semen from the same pool is used at each of the five time periods.

Possible correlations in the $(BT)_{ij}$'s, for a fixed i, were considered using the following model. With y_{ij} denoting the average response (over the four replicates) in the (i,j) - th boar x time cell, the above model implies:

$$Y_{ij} = u + B_i + T_j + e_{ij} \quad , \quad i = 1,\ldots,8 \quad , \quad j = 1,\ldots,5$$

where,

$$e_{ij} = (BT)_{ij} + 1/4 \sum_{k=1}^{4} e_{ijk}$$

with this,

$$E(e_{ij}) = 0\ \forall_{i,j}$$
$$\text{Var}(e_{ij}) = \sigma_\mu^2 + 1/4\ \sigma_s^2\ \forall_{i,j} \quad .$$

To account for possible correlations in the e_{ij}'s, assume:

$$e_{ij} = p\, e_{i,j-1} + \delta_{ij}$$

$|p| < 1$ is an unknown parameter

$$\delta_{ij} \sim N(o, \sigma^2) \quad , \quad \text{Cov}(\delta_{ij} , \delta_{i'j'}) = 0$$

if $i \neq i'$ or $j \neq j'$ $\forall$ i,j.

With this model:

$$\text{Corr}(e_{ij}, e_{i,j+k}) = p^k$$

$$\text{Var}(e_{ij}) = \frac{\sigma^2}{1 - p^2}$$

so that,

$$\sigma_\mu^2 + 1/4\, \sigma^2 = \frac{\sigma^2}{1 - p^2} \ .$$

Equation used for calculating the S.E. of the means for the assays of spermatozoan concentration, volume of the ejaculate, total number of spermatozoa, maximum releasable glutamic oxaloacetic transaminase and the spermatozoan penetration assay:

$$\text{S.E. boar and overall mean} = \sqrt{\sigma_S^2/n}$$

Equation used for calculating the S.E. of the means for the assays of motility before cryopreservation:

$$\text{S.E. boar and overall mean} = \sqrt{\sigma_S^2 + 4\, \sigma_\mu^2}$$

Equations used for calculating the S.E. of the means for the assay of sephadex filtration without caffeine stimulation:

$$\text{S.E. boar mean} = \sqrt{(1/16)\ (\sigma^2/1 - p^2)\ (4 + 6p + 4p^2 + 2p^3)}$$

$$\text{S.E. time mean} = \sqrt{(1/8)\ (\sigma^2/1 - p^2)}$$

$$\text{S.E. overall mean} = \sqrt{(1/16)\ (\sigma^2/1 - p^2)\ (4 + 6p + 4p^2 + 2p^3)/8}$$

Equations used for calculating the S.E. of the means for all the other assays with a boar x time design:

$$\text{S.E. boar mean} = \sqrt{(1/25)\ (\sigma^2/1 - p^2)\ (5 + 8p + 6p^2 + 4p^3 + 2p^4)}$$

$$\text{S.E. time mean} = \sqrt{(1/8)\ (\sigma^2/1 - p^2)}$$

$$\text{S.E. overall mean} = \sqrt{(1/25)\ (\sigma^2/1 - p^2)\ (5 + 8p + 6p^2 + 4p^3 + 2p^4)/8}$$

Equations used for calculating the 95% confidence intervals (CI) for boar or boar x time assay means:

$$\text{95\% CI for spermatozoan penetration assay} = \text{boar assay mean} \pm t_{.05} \sqrt{\sigma_s^2/4}$$

$$\text{95\% CI for all other assays} = \text{boar x time assay mean} \pm t_{.05} \sqrt{\sigma_s^2/4}$$

the degrees of freedom associated with t is 24 for the spermatozoan penetration assay and 120 for all of the other assays.

Values for $\hat{p}$, $\hat{\sigma}^2$, $\hat{\sigma}^2/1 - \hat{p}^2$, and $\hat{\sigma}_\mu^2$ for each of the *in vitro* assays

Assay	$\hat{p}$	$\hat{\sigma}^2$	$\hat{\sigma}^2/1 - \hat{p}^2$	$\hat{\sigma}_\mu^2$
Motility without caffeine stimulation	.190	39.22	40.69	27.94
Motility following caffeine stimulation	-.140	47.15	48.09	36.34
Normal apical ridges	.346	54.55	61.97	58.47
Damaged apical ridges	.398	37.35	44.38	39.38
Missing apical ridges	-.058	20.51	20.58	16.08
Loose acrosomal caps	.428	22.54	27.59	24.59
Sephadex with caffeine stimulation	.246	6.57	7.00	5.75
Sephadex without caffeine stimulation	.249	4.06	4.32	1.82
Extracellular and intracellular glutamic oxaloacetic transaminase	.089	1318.66	1329.19	624.44
Percentage of maximum releasable glutamic oxaloacetic transaminase	.193	13.99	14.53	8.03
Acrosin-positive spermatozoa	.100	15.97	16.13	11.88
Diameter of halos of acrosin-positive spermatozoa	-.213	46.90	49.12	35.87

APPENDIX K:

STATISTICAL MODEL USED FOR CALCULATING THE HETEROSPERMIC INDICES FOR EACH OF THE SIXTEEN CELLS IN THE 4 x 4 DESIGN

Notations:

X_{ijk} = the number of <u>black</u> pigs in the litter of the K^{th} sow inseminated with spermatozoa from the i^{th} black boar and the j^{th} white boar

X'_{ijk} = the number of <u>white</u> pigs in the litter of the K^{th} sow inseminated with spermatozoa from the i^{th} black boar and the j^{th} white boar

$N_{ijk} = X_{ijk} + X'_{ijk}$ = total litter size of the K^{th} sow inseminated with spermatozoa from the i^{th} black boar and the j^{th} white boar

$$d_{ijk} = \frac{X_{ijk}}{N_{ijk}} - \frac{X'_{ijk}}{N_{ijk}}$$

$$d_{ij.} = \frac{X_{ij.}}{N_{ij.}} - \frac{X'_{ij.}}{N_{ij.}}$$

where $X_{ij.} = \sum_K X_{ijk}$

$X'_{ij.} = \sum_K X'_{ijk}$

$N_{ij.} = \sum_K N_{ijk}$

Example:

$X_{321} = 3$, $X'_{321} = 0$, $N_{321} = 3$

$X_{322} = 1$, $X'_{322} = 6$, $N_{322} = 7$

$X_{32.} = X_{321} + X_{322} = 4$

$X'_{32.} = X'_{321} + X'_{322} = 6$

$N_{32.} = N_{321} + N_{322} = 10$

$d_{32.} = \frac{4}{10} - \frac{6}{10} = -.20$

Assumptions:

$$X_{ijk} \overset{d}{\sim} \text{Binomial } (N_{ijk}, P_{ij})$$

$$X'_{ijk} \overset{d}{\sim} \text{Binomial } (N_{ijk}, P'_{ij})$$

where

$$P_{ij} + P'_{ij} = 1$$

Assume X_{ijk}'s independent $\forall$ i,j,k.

Under these assumptions,

1) Deriving Var (d_{ijk})

a) $$\text{Var}(d_{ijk}) = \text{Var}\left[\frac{X_{ijk}}{N_{ijk}} - \frac{X'_{ijk}}{N_{ijk}}\right]$$

$$= \text{Var}\left[\frac{X_{ijk}}{N_{ijk}}\right] + \text{Var}\left[\frac{X'_{ijk}}{N_{ijk}}\right] - 2\,\text{Cov}\left[\frac{X_{ijk}}{N_{ijk}}, \frac{X'_{ijk}}{N_{ijk}}\right]$$

$$= \frac{P_{ij}(1-P_{ij})}{N_{ijk}} + \frac{P'_{ij}(1-P'_{ij})}{N_{ijk}} - 2\,\text{Cov}\left[\frac{X_{ijk}}{N_{ijk}}, \frac{N_{ijk} - X_{ijk}}{N_{ijk}}\right]$$

b) since $X'_{ijk} \overset{d}{\sim} \text{Binomial } (N_{ijk}, P'_{ij})$

$\overset{d}{\sim} \text{Binomial } (N_{ijk}, 1-P_{ij})$

c) and $X_{ijk} \overset{d}{\sim} \text{Binomial } (N_{ijk}, P_{ij})$

$N_{ijk} - X_{ijk} \overset{d}{\sim} \text{Binomial } (N_{ijk}, 1-P_{ij})$

d) i.e., $X'_{ijk} \overset{d}{\sim} N_{ijk} - X_{ijk}$

e) $$\text{Var}\ (d_{ijk}) = \frac{P_{ij}\ P'_{ij}}{N_{ijk}} + \frac{P'_{ij}\ P_{ij}}{N_{ijk}} + \frac{2\ N_{ijk}\ P_{ij}\ P'_{ij}}{N_{ijk}^2}$$

$$= \frac{2P_{ij}\ P'_{ij}}{N_{ijk}} + \frac{2\ P_{ij}\ P'_{ij}}{N_{ijk}}$$

$$= \frac{4\ P_{ij}\ P'_{ij}}{N_{ijk}} = \frac{4\ P_{ij}\ (1-P_{ij})}{N_{ijk}} \quad , \forall_{i,j,k}$$

2) Deriving $E\ (d_{ijk})$

$$E\ (d_{ijk}) = E\left[\frac{X_{ijk}}{N_{ijk}} - \frac{X'_{ijk}}{N_{ijk}}\right]$$

$$= \frac{1}{N_{ijk}}\ E\ \left[X_{ijk} - X'_{ijk}\right]$$

$$= \frac{1}{N_{ijk}}\ \left[N_{ijk}\ P_{ij} - N_{ijk}\ P'_{ij}\right]$$

$$= P_{ij} - P'_{ij} \quad , \forall\ K$$

Model:

$$d_{ijk} = \delta_{ij} + e_{ijk}$$

where

$$\delta_{ij} = P_{ij} - P'_{ij} \quad , \quad -1 \leq \delta_{ij} \leq +1$$

$$E\ (e_{ijk}) = 0$$

$$\text{Var}\ (e_{ijk}) = \frac{4\ P_{ij}\ (1-P_{ij})}{N_{ijk}}$$

$i = 1 \ldots, 4$, $j = 1 \ldots, 4$, $K = 1 \ldots,$ (# litters in (i,j)th cell), e_{jik}'s independent.

Strategy:

1) Construct an unbiased estimator of Var (e_{ijk}) and use estimated generalized least squares to estimate δ_{ij} $(i,j = 1, \ldots, 4)$. The estimated δ_{ij}'s will be unique with respect to this particular data set. An estimate δ_{ij} can be interpreted as a measure of the difference in fertility between the i^{th} black boar and the j^{th} white boar.

2) The probability P_{ij} was estimated by

   a) $$\hat{P}_{ij} = \frac{X_{ij.}}{N_{ij.}} = \frac{\sum_K X_{ijk}}{\sum_K N_{ijk}}$$

   b) An unbiased estimator of Var (e_{ijk}), using this estimator of P_{ij} is

$$\hat{\text{Var}}\ (e_{ijk}) = 4\left(\frac{N_{ij.}}{N_{ij.} - 1}\right)\frac{\hat{P}_{ij}\ (1-\hat{P}_{ij})}{N_{ijk}}$$

Results:

1) Can show that the estimates of δ_{ij} $(i,j = 1 \ldots, 4)$ using the above estimate for Var (e_{ijk}) are given by:

$$\hat{\delta}_{ij} = d_{ij} \quad \forall\ i,j = 1 \ldots, 4.$$

2) Note that

$$\delta_{ij} = d_{ij} = \hat{P}_{ij} - \hat{P}'_{ij},$$

which has intuitive appeal since $\delta_{ij} = P_{ij} - P'_{ij}$.

3) The heterospermic indices are the δ_{ij}'s multiplied by 100.

APPENDIX L:

STATISTICAL MODEL USED FOR CALCULATING AN AVERAGE HETEROSPERMIC INDEX FOR EACH OF THE EIGHT BOARS (Saacke et al., 1980)

Heterospermic indices were derived assuming that in n_{ij} competitive encounters between boars i and j, the difference in the proportion of pigs sired by each can be represented as:

$$d_{ij} = p_i - p_j + e_{ij}$$

where p_i and p_j represent the true competitive fertilization abilities for boars i and j and e_{ij} is the sampling error for the specific comparison. Sampling errors were assumed randomly distributed binomial variables with zero expected values and variances, $4p_ip_j/n_{ij}$. Heterospermic indices were estimated generalized least squares solutions for $\hat{p}_i$ and $\hat{p}_j$ multiplied by 100.

VITA

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