

## Objectively Measured Sperm Motility and Sperm Head Morphometry in Boars (*Sus scrofa*): Relation to Fertility and Seminal Plasma Growth Factors

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**ABSTRACT:** This study was conducted to investigate the relationships between results of computer-assisted semen analysis (spermatozoal motility and sperm head morphometry) and fertility of boars. In addition, concentrations of insulin-like growth factor (IGF)-I and IGF-II in seminal plasma were determined. The nonreturn rate (NRR) and the number of live-born piglets were compatible with the requirements of artificial insemination for all boars included in this study. Semen samples of 12 boars (Pietrain; 3 ejaculates each) were evaluated for spermatozoal motility and sperm head dimensions using computer-assisted methods. Native semen samples were centrifuged, and seminal plasma was frozen at  $-20^{\circ}\text{C}$  until assayed for IGF-I and IGF-II by specific radioimmunoassays. Sper-

matozoa of boars with a higher NRR ( $>86\%$ ) had a significantly slower average velocity of motile spermatozoa when compared with that of boars with an NRR below  $86\%$ . High-fertility boars (NRR  $>86\%$ ) had significantly smaller sperm heads than did boars with an NRR below  $86\%$ , and their sperm heads were less elongated. Substantial concentrations of IGF-I ( $8.4\text{--}22.2\text{ ng/mL}$ ) and IGF-II ( $12.1\text{--}19.8\text{ ng/mL}$ ) could be measured in porcine seminal plasma; however, there was no correlation between IGF levels and semen parameters or individual fertility.

Key words: Artificial insemination, semen parameters.

**J Androl 2001;22:104–110**

The use of artificial insemination (AI) in the pig industry has grown considerably during the last years. It is therefore of both scientific and economic interest to identify boars with suboptimal fertility. Advances in computer technology have led to considerable progress in the quantitative analysis of sperm characteristics. Spermatozoal motility in boars has been evaluated by computer-assisted semen analysis (CASA) systems. In earlier studies, no relationship of motility parameters evaluated by computer-assisted systems with fertility of boars could be found (Aumüller and Willeke, 1988; Rath et al, 1988). More recently, results of a fertility trial demonstrated a correlation of objectively measured boar sperm motility parameters with the outcomes of on-farm inseminations (Holt et al, 1997).

Meanwhile, automated sperm morphology analysis (ASMA) has been applied in a number of species, including cattle (Gravance et al, 1996b, 1998b), goat (Gravance et al, 1995), sheep (Gravance et al, 1998a; Sancho

et al, 1998), horse (Ball and Mohammed, 1995; Gravance et al, 1996a; Casey et al, 1997), rabbit (Gravance and Davis, 1995), and humans (Davis et al, 1993; Kruger et al, 1995, 1996). To date, there has been only 1 study describing the use of ASMA in the boar (Schmidt, 1997), and no information is available about the results of ASMA and fertility of individual boars. With the steady increase of AI in pigs, interest in the application of ASMA for the prediction and control of fertility is expected to rise significantly.

Components of seminal plasma affecting semen quality, such as the insulin-like growth factor (IGF) system, deserve increasing interest. A receptor for IGF-I has been identified on bovine spermatozoa, and addition of IGF-I and, to a lesser extent, IGF-II in physiological concentrations stimulated spermatozoal motility in washed semen samples (Henricks et al, 1998). A positive correlation between IGF-I in seminal plasma and semen quality has been demonstrated in human (Glander et al, 1996). Likewise, significantly aberrant levels of growth factors were observed in bulls of unknown fertility with poor semen quality when compared with the case of animals with normal semen quality and proven fertility (Hoeftlich et al, 1999). These results suggest a physiological role of growth factors in posttesticular spermatozoa.

Supported by a DAAD scholarship (M.H.).

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Received for publication March 13, 2000; accepted for publication July 31, 2000.

This study was conducted to investigate the relationships between spermatozoal parameters evaluated by computer-assisted systems, fertility traits, and concentrations of IGF-I and IGF-II in seminal plasma of boars with known fertility (nonreturn rate [NRR], litter size). The proportion of motile spermatozoa and the average velocity of motile spermatozoa were determined in freshly diluted semen samples. Sperm smears were evaluated for area, length, width, and elongation (width to length ratio) of sperm heads.

## Materials and Methods

### Animals and Semen Collection

The boars (12 animals; Pietrain, aged 22–44 months) used in this study were housed in an AI center according to German and European regulations for AI centers. The boars had a 60–90-day NRR (defined as percentage of sows not appointed for a second insemination within a period of minimum 60 to maximum 90 days after the first insemination) of 79.8% to 88.7% (median, 86.0%) based on a mean of 553 first inseminations (range, 216–930). The average number of live-born piglets (referred to as *litter size*) ranged from 9.1 to 11.7 (median, 10.0). Whole ejaculates were collected once a week during routine AI operations in August and September 1998 (3 ejaculates per boar). All ejaculates fulfilled the minimum requirements for use in AI (70% motile spermatozoa, <20% cytoplasmic droplets, total sperm number  $>10 \times 10^9$ ). Immediately after semen collection, native semen was centrifuged at  $1000 \times g$  for 10 minutes, and the supernatant was removed and stored at  $-20^\circ\text{C}$  until analyzed for IGF-I and IGF-II. Aliquots of the semen were diluted with a Beltsville Thawing Solution (Weitze, 1991) to a total volume of 100 mL (concentration,  $50 \times 10^6$  cells per milliliter), packed in a Styrofoam container, and shipped at ambient temperature by courier to the laboratory within 3 hours.

### Sperm Motility Analysis

A Mika Motion Analyzer (Medical Technologies Montreux SA, Clarens/Montreux, Switzerland) was used for the evaluation of sperm motility. The recording unit consisted of a microscope (Nikon Optiphot) equipped with a negative phase-contrast  $20\times$  objective and a heated stage (Minitüb, Tiefenbach, Germany), a video camera (black and white; RCA model TC 1910X), and a video recorder (Panasonic model 7500). A 5- $\mu\text{L}$  drop of the diluted sample was filled in a prewarmed ( $37^\circ\text{C}$ ) Makler chamber (Sefi Medical Instruments, Haifa, Israel), and 3 fields were videotaped for 10 seconds per field. Four chamber fillings per ejaculate were recorded. The tapes were analyzed later with the video recorder fitted to a personal computer with the Motion Analyzer analysis software (Windows NT-based version 2.0).

The instrument settings were as follows: number of frames acquired, 32; minimum number of frames, 15; minimum area of objects, 20 pixels; maximum area of objects, 300 pixels; minimum velocity for mobile objects, 10  $\mu\text{m/s}$ . Immobile particles within the required pixel range were distinguished from debris by an automatic tail detection feature implemented in the soft-

ware. The track of each sperm was analyzed for the following parameters: the velocity of average path (VAP; micrometers per second) and the velocity calculated for the straight line between start and endpoint (VSL; micrometers per second).

The percentage of motile spermatozoa included all spermatozoa with a VAP of  $\geq 10 \mu\text{m/s}$ . For a further analysis of the motility pattern, the software calculated the straightness index (straightness =  $\text{VSL}/\text{VAP} \times 100$ ) of each motile spermatozoon. Motile spermatozoa with a straightness index  $\geq 90$  were classified as linear motile spermatozoa.

In this study, the following parameters describing different aspects of spermatozoal motility were used: MOT, the percentage of all motile spermatozoa (VAP  $\geq 10 \mu\text{m/s}$ ); LIN, the percentage of linear motile spermatozoa; V-MOT, the velocity (VAP) of all motile spermatozoa; and V-LIN, the velocity (VAP) of linear motile spermatozoa.

The reproducibility of motility parameters evaluated with this CASA system has been investigated and compared with 4 other systems for computerized evaluation of sperm motility (Holt et al, 1994). Operator expertise and sample handling was the biggest source of variability. In this study, all samples were evaluated by the same operator, and sample handling followed the same protocol throughout the study. Variance component estimates for the effect of the random factors of boar and ejaculate on V-MOT and V-LIN showed that these factors contributed between 1.1% and 3.9% to the total variability observed in this study.

### Sperm Head Morphometry

A Mika Morphology Analyzer (Medical Technologies Montreux SA) was used for the automated sperm morphometry analysis (ASMA) as described previously (Boersma et al, 1999). Briefly, the system comprised a microscope (Zeiss Photo microscope II) and a video camera (Panasonic TV camera) attached to a personal computer (DELL Optiplex 466/MX). The slides for ASMA were prepared by staining air-dried smears from the diluted semen samples by using a modified Farelly staining procedure (Boersma et al, 1999). The staining times were prolonged (aniline blue, 5 minutes; crystal violet, 1 minute) to obtain dark sperm heads against a clear background. The sperm heads were observed using a bright-field  $100\times$  oil immersion objective with a green filter. Video images of the sperm heads were transmitted to the frame grabber of the computer. The computer software (version 2.0) performed the analysis of the digitized sperm images and calculated the following morphometric parameters of each sperm head: area (in square micrometers), length (in micrometers), width (in micrometers), and width as a proportion of length (as percentage;  $\text{width}/\text{length} \times 100$ ). For the morphometric analysis of a sample, 4 slides with 50 sperm heads each were analyzed, resulting in 200 sperm heads per ejaculate. In previous experiments with bull spermatozoa, the coefficient of variation for repeated measurements of a single sperm was found to be 0.54% to 0.89% (Boersma, unpublished data). A maximum number of 60 spermatozoa per slide was sufficient to minimize variation between slides (Boersma et al, 1999).

Variance component estimates for the effect of the random factors of the ejaculate and slide on sperm head dimensions in

Table 1. Results of computer-assisted sperm motility analysis (mean ± SEM of 18 ejaculates per group)

Parameter	Nonreturn Rate		Litter Size	
	<86%*	>86%*	<10*	>10*
MOT (%)	86.5 ± 3.3	87.5 ± 2.9	80.9 ± 3.8	93.2 ± 0.9†
LIN (%)	33.7 ± 2.4	39.9 ± 3.1†	36.9 ± 3.8	36.2 ± 1.7
V-MOT (μm/s)	40.1 ± 1.6	36.4 ± 1.3†	37.0 ± 1.6	39.5 ± 1.4
V-LIN (μm/s)	45.5 ± 1.4	42.9 ± 1.2	44.1 ± 1.4	44.4 ± 1.2

\* Groups defined using the median value of 12 boars (6 boars with 3 ejaculates each per group).

† Denotes significant differences ( $P < .05$ ) within fertility parameter.

boars revealed that these factors contributed between 1.7% to 3.7% and 0.1% to 0.9%, respectively to the total variability.

#### Quantification of IGF-I and IGF-II in Seminal Plasma

Concentrations of IGF-I and IGF-II in seminal plasma samples were determined by specific radioimmunoassay as described elsewhere (Hoefflich et al, 1999), according to the recommendations of the Third International Symposium on IGFs (Sydney, Australia, 1994; Blum and Breier, 1994). For both assays, dilution curves of seminal plasma samples were linear and paralleled those of human IGF standards.

#### Statistical Analysis and Methods

All data recorded (fertility, sperm motility, morphometry, and IGF values) were imported into a database and analyzed by the statistical software package SPSS for Windows (release 8.0, SPSS Inc, Munich, Germany). To examine relationships between boar fertility and the parameters in semen and seminal plasma, the data were split into 2 sets. The median value for NRR (86%) and litter size ( $n = 10$ ) was used to allocate boars into groups above (set 1) or below (set 2) the respective median value (Holt et al, 1997). Besides descriptive statistics, a general linear model (GLM type III) was employed to conduct a multivariate analysis of variance with eight dependent variables (area, length, width, and width/length, as well as MOT, LIN, V-MOT, and V-LIN) and the between-subject (independent) variables: litter size, with 2 levels (<10 or >10); NRR, with 2 levels (<86% or >86%); and boar, with 12 levels. The litter size × NRR interaction was nested within the boar factor.

## Results

#### Spermatozoal Motility

The mean percentage of motile spermatozoa as evaluated by computerized systems was 87.0%. The percentage of

motile spermatozoa (MOT) was not significantly different between boars with a NRR above or below the median value of 86% (Table 1), but boars with higher NRR had significantly more spermatozoa with a linear movement type. Boars with a litter size >10 had a higher percentage of motile spermatozoa compared with boars with a smaller litter size (93.2% vs 80.9%;  $P < .05$ ).

The average path velocity of motile spermatozoa (V-MOT) across all boars was 38.2 μm/s. V-MOT was significantly ( $P < .05$ ) lower in boars with higher NRR than in boars with lower NRR (Table 1). However, this parameter was not influenced by litter size.

#### Sperm Head Morphometry

Sperm head morphometry revealed significant differences in all 4 parameters evaluated (area, length, width, and width/length ratio) when boars were grouped above and below the median value of 86% NRR (Table 2). Spermatozoa in boars with a lower NRR had a more elongated shape of the sperm head. Splitting morphometry data by litter size again showed differences between subgroups in all parameters investigated (Table 2). Although the absolute differences were small, boars with a litter size >10 had significantly ( $P < .05$ ) more elongated spermatozoa than did boars with less than 10 piglets per litter. A significant correlation ( $P < .01$ ) was found between the NRR of individual boars and morphometric parameters of their sperm heads (length:  $r = -.85$ ; width to length ratio:  $r = .87$ ).

#### Seminal Plasma IGF-I and IGF-II Concentrations

Substantial concentrations of IGF-I ( $14.8 \pm 4.1$  ng/mL; mean ± SD) and IGF-II ( $15.3 \pm 2.4$  ng/mL; mean ± SD)

Table 2. Results of computer-assisted sperm head morphometry (mean ± SEM for 18 ejaculates per group)

Sperm Head	Nonreturn Rate		Litter Size	
	<86%*	>86%*	<10*	>10*
Area (μm <sup>2</sup> )	35.7 ± 0.22	35.1 ± 0.22†	35.1 ± 0.24	35.7 ± 0.21†
Length (μm)	9.27 ± 0.05	8.97 ± 0.02†	9.06 ± 0.05	9.18 ± 0.05†
Width (μm)	4.66 ± 0.02	4.73 ± 0.02†	4.70 ± 0.01	4.68 ± 0.02†
Width/length (%)	50.4 ± 0.42	52.8 ± 0.18†	52.1 ± 0.34	51.1 ± 0.45†

\* Groups defined using the median value of 12 boars (6 boars with 3 ejaculates each per group).

† Denotes significant differences ( $P < .05$ ) within fertility parameter.

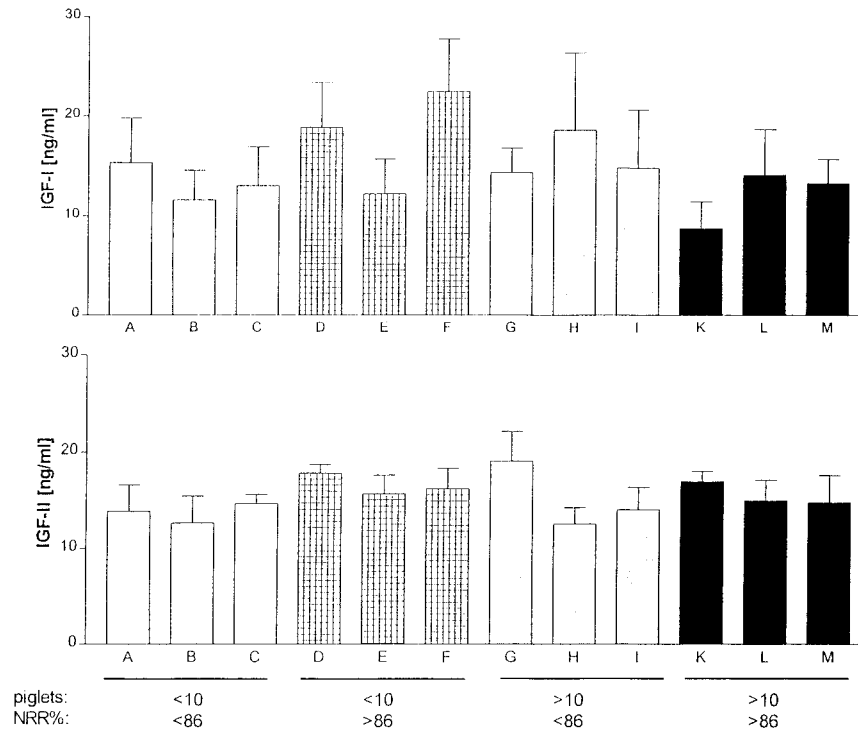


Figure 1. Concentrations of IGF-I (upper panel) and IGF-II (lower panel) in seminal plasma from 12 boars (A–M). The figure shows means and standard errors of the means of 3 samples per boar.

were detected in porcine seminal plasma (Figure 1). Range of concentrations in individual boars (mean of 3 ejaculates) was 8.4 to 22.2 ng/mL for IGF-I and 12.1 to 19.8 ng/mL for IGF-II. Levels of IGF-I and IGF-II in seminal plasma were not related to semen parameters or individual fertility.

## Discussion

In routine AI operations, subjective estimation of spermatozoal motility is the main parameter used to select ejaculates. All ejaculates examined in this study fulfilled the requirements concerning spermatozoal motility ( $\geq 70\%$  motile spermatozoa). With the computer-assisted method applied here, a high percentage of motile spermatozoa (87.0%) could be observed. Splitting data by litter size, but not by NRR, revealed a significant difference in MOT. Conversely, the average velocity of all motile spermatozoa (V-MOT) was significantly different only when boars were grouped according to high or low NRRs (Table 1). In bulls, no significant correlation between the percentage of motile spermatozoa evaluated by CASA and the 59-day NRR could be found, whereas highly significant correlations were detected when CASA parameters describing the velocity of motile spermatozoa or the trajectory line of motile spermatozoa were included (Farrell et al, 1998). In earlier studies on boars, no relation-

ship of motility parameters evaluated by computer-assisted systems with fertility was found (Aumüller and Willeke, 1988; Rath et al, 1988). More recent results of a fertility trial demonstrated a correlation between the outcomes of on-farm inseminations and objectively measured boar sperm motility parameters determined in semen samples processed for AI and stored for 24 hours at ambient temperatures (Holt et al, 1997); only CASA parameters describing the velocity of motile spermatozoa (eg, VAP, VSL) or the trajectory line of motile spermatozoa (eg, amplitude of lateral head displacement) were considered in this study. The 2 field trials produced different associations between fertility and spermatozoal motility; significant effects were found in the litter-size data in the first trial and in the NRR data during the second trial. In the present study, a significant difference in the percentage of motile spermatozoa between boars with high or low litter size (93.2% vs 80.9%) could be demonstrated, whereas this parameter was not significantly different when data was split by NRR. A high percentage of motile spermatozoa in processed semen samples may indicate that these spermatozoa have not been damaged by the process of dilution and storage. It is well known that premature capacitation occurs during processing of bull semen samples, ultimately leading to a reduced fertility in comparison to fresh semen samples (Cormier et al, 1997). In the multiparous species pig, such events could lead to a lower litter size while the NRR remains

unaffected because of the high number of spermatozoa used for AI. The different aspects of fertility in a multiparous species (conception and number of live-born piglets) and their relation to specific sperm functions deserve further attention.

In the study by Holt and coworkers (1997), the average velocity of motile spermatozoa (VAP) was 47.2  $\mu\text{m/s}$ , which is slightly higher than that observed in our study (38.2  $\mu\text{m/s}$ ). Results of computer-assisted motility analysis are dependent on the type of equipment and the setting of the CASA instrument (Holt et al, 1994, 1996). Likewise, margins or threshold levels used to define sperm subpopulations vary between laboratories. Although significant associations between spermatozoal motility parameters and fertility could be demonstrated in this study as well as in the study by Holt and coworkers (Holt et al, 1997), the narrow range of fertility in boars that is commonly seen in AI boars may not allow a screening according to spermatozoal motility in the routine laboratory.

In contrast to the sperm motility analysis, only a limited number of well-defined parameters (area, length, and width of sperm head) has been employed so far in ASMA. However, the results of ASMA are influenced by specimen preparation and the staining procedure (Davis and Gravance, 1993; Lacquet et al, 1996; Boersma et al, 1999). Washing and resuspension of spermatozoa to a standard concentration has been demonstrated to increase spermatozoan density and homogeneity on the slide, reducing digitization errors (Davis and Gravance, 1993). In our study, smears for ASMA were prepared from freshly diluted semen samples processed in an AI center. The clear, watery semen extender (Beltsville Thawing Solution) and the fairly constant sperm concentration required for use in AI provided a homogeneous source of semen samples, which allowed the preparation of clean smears with evenly distributed spermatozoa. As a result of this protocol, the effect of the random factor of slide on sperm head dimensions in these boars was small (0.1% to 0.9%) in comparison to the total variability observed.

The staining procedure employed in this study had been previously used for bull spermatozoa (Boersma et al, 1999). The low coefficient of variation after repeated measurements of a single sperm in these experiments demonstrated the quality of the staining procedure. No further modification was necessary to obtain specimens from boar semen samples suitable for ASMA. It has been demonstrated that the size of the sperm head as evaluated in stained smears is largely influenced by the fixation procedure as well as by the staining procedure (Boersma et al, 1999). The results obtained in boar spermatozoa with fixation in 96% methanol, followed by 5 seconds in 0.5% methyl violet (Schmidt, 1997), are clearly different from the measurements obtained in our study. In morphologi-

cally normal spermatozoa from 4 boars, head area ranged from 54.8 to 56.9  $\mu\text{m}^2$  (Schmidt, 1997), whereas an average of 35.4  $\mu\text{m}^2$  was observed in our study. In human spermatozoa, a distinct effect of preparation and staining procedure on length and width but not on the width to length ratio was observed (Davis and Gravance, 1993). Further studies on the effect of staining procedures on head shape and dimensions are warranted to stimulate efforts towards standardization in different laboratories.

The significant influence of head shape on fertility, especially the NRR, of boars is remarkable in the light of the relatively small variation of fertility among boars. In a study investigating morphometric parameters in fertile and subfertile stallions, significantly larger values (length, area, perimeter) were found for subfertile stallions than for fertile stallions (Casey et al, 1997). In our study, significant differences among boars grouped according to their individual fertility were found for all parameters investigated. When contrasting boars with high or low NRR and litter size, the measured parameters shift in opposite directions. No explanation can be given for this phenomenon, and to our best knowledge, no data from the literature is available for discussion. A comparison to the results obtained in horses (Casey et al, 1997) is difficult. In the horse, fertility is a yes or no event, whereas in the pig, the 2 measures of fertility are interrelated and of quantitative nature (litter size) or are dependent on the number of fertilized oocytes (NRR).

Studies investigating chromatin condensation and morphology of spermatozoa suggest that abnormal chromatin condensation may be reflected by or lead to morphological abnormalities. Variation of sperm head measurements was related to fertility potential and abnormal chromatin structure in bulls (Sailer et al, 1996). Moreover, changes of sperm chromatin structure in sperm after scrotal insulation of bulls were paralleled by an increase in the percentage of morphologically abnormal spermatozoa (Karabinus et al, 1997). These findings are supported by studies in cat and human males. Chromatin condensation in epididymal cat spermatozoa, evaluated by aniline blue and acridine orange staining, was significantly higher in cells with head abnormalities than in sperm with only tail defects (Hingst et al, 1995). As much as 95% of human spermatozoa with certain abnormalities showed abnormal chromatin condensation (Dadoune et al, 1988); the percentage of spermatozoa with normal chromatin condensation was lowest in spermatozoa with abnormalities of the sperm head (macrocephalic, elongated). From these and other studies, it can be concluded that variation of sperm head morphology is likely a sensitive biomarker related to abnormal chromatin structure and thus to fertility potential. The detection of subtle yet significant differences in sperm head morphology is only possible with the aid of ASMA instruments.

This is the first study to demonstrate the presence of IGF-I and IGF-II in seminal plasma of boars. These growth factors have been identified so far in seminal plasma of bulls (Henricks et al, 1998; Hoefflich et al, 1999) and in humans (Ramasharma et al, 1986; Glander et al, 1996). The mean concentrations of IGF-I and IGF-II in porcine seminal plasma (14.8 and 15.3 ng/mL, respectively) are considerably lower than that observed in seminal plasma from bulls by Hoefflich and coworkers (1999; IGF-I and IGF-II, 144 ng/mL each), as well as by Henricks and coworkers (1998; IGF-I 116 ng/mL). However, because of the lower sperm concentration in boars, the total amount of IGFs per sperm cell is comparable in bulls and boars.

IGF-I and, to a lesser extent, IGF-II have been shown to stimulate the motility of bovine sperm in the absence of seminal plasma (Henricks et al, 1998), and correlations between seminal plasma IGF levels and semen quality have been observed in humans (Glander et al, 1996). The present study did not reveal a significant association between IGF levels in seminal plasma of boars with normal fertility. These results are consistent with observations in bulls, in which levels of IGF-I and IGF-II were not related to semen quality of animals with normal fertility (Hoefflich et al, 1999). However, bulls with poor semen quality not compatible with AI use had significantly aberrant levels of IGFs. Treatment of oligozoospermic or asthenozoospermic men with growth hormone (GH) produced significant improvement in motility with no changes in sperm count (Ovesen et al, 1996). Likewise sperm motility was improved in GH-deficient dwarf rats after GH treatment (Breier et al, 1996). It is tempting to speculate that these effects of GH in individuals with subnormal semen parameters may have been mediated by IGF because IGF-I levels in seminal plasma of subfertile males increased after GH treatment (Ovesen et al, 1996).

The results obtained so far concerning IGFs and semen quality or fertility have to be related also to the action of IGF-binding proteins (IGFBPs) in seminal plasma. IGFBPs are important regulators of half-life and bioavailability of IGFs and probably modulate IGF actions on spermatozoal function (Zhou and Bondy, 1993). No information is available on the role of IGFBPs in individuals with an impaired semen quality or fertility. The pattern of IGFBPs is markedly different between human and bovine seminal plasma (Hoefflich et al, 1999) and remains to be characterized in porcine seminal plasma.

In conclusion, we have demonstrated significant associations between spermatozoal motility as well as sperm head dimensions evaluated by computer-assisted analysis and fertility. The significant correlation between NRR and sperm head dimensions warrants further studies. The concentrations of IGF-I and IGF-II in seminal plasma of boars were not related to individual fertility and seminal

parameters. Future studies will focus on the characterization of IGF-binding proteins in porcine seminal plasma because this family of proteins has been shown to include important modulators of IGF actions in various body fluids and tissues.

## Acknowledgment

We would like to thank Petra Demleitner for technical assistance.

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