

The predictive value of routine semen evaluation and IVF technology for determining relative boar fertility

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Received 1 November 2005; accepted 1 December 2005

Abstract

Practical techniques for assessing semen quality in order to predict male fertility are still needed. The principal objective of this experiment was to evaluate routine laboratory evaluation and in vitro fertilization (IVF) techniques as predictors of relative boar fertility using a low-dose AI protocol. Nine boars were evaluated during a 6.5 ± 1 mo period, beginning at 29–32 wk of age. Ejaculates were evaluated for motility, morphology and concentration, diluted to 1.5 billion sperm in 50 mL extender, and used to breed 50 ± 5 gilts over the same period. On nine occasions, a specific aliquot of the ejaculate's first sperm-rich fraction was evaluated using IVF procedures. Boars differed ($P < 0.001$) consistently for pregnancy rate (from 73 to 98%), farrowing rate (71–98%) and total born (8.8–12.0). Routine semen evaluation and IVF parameters that presented significant differences between boars, but no differences in time and no boar by time interaction, were used to correlate in vivo fertility. A multiple regression model based on routine semen evaluation parameters accounted for up to 27 and 22% of the variation of fertility index and total piglets born, respectively, whereas male pronuclear formation rate was the IVF variable that accounted for 17 and 12% of the variation in farrowing rate and fertility index, respectively. Collectively, we inferred that the use of low sperm numbers for AI, determination of pregnancy rate at Day 30, motility of extended semen after 7 and 10 d, and specific IVF parameters may be useful for identifying relatively infertile boars that are not currently excluded from use in existing commercial boar studs.

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Keywords: Semen quality; Boar fertility; In vitro fertilization; Artificial insemination; Swine

1. Introduction

In the swine industry, a single male has a more profound impact on efficiency and productivity than an individual female, and this impact is even higher with the use of artificial insemination (AI). In commercial AI centers, routine semen assessment generally includes

the evaluation of ejaculate characteristics such as sperm concentration, morphology, viability, and motility. Although some of these characteristics can be used to detect male reproductive disorders that result in low fertility, they are not useful for predicting relative fertility in healthy boars with ejaculate quality that meets normal industry standards ($>70\%$ motility and $<30\%$ abnormal sperm), even though the productivity of these boars may be substantially different. Due to the complexity of the fertilization process, several sperm attributes are required for successful fertilization, such as the ability to undergo

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capacitation, hyperactivation, the acrosome reaction, binding to the zona pellucida (ZP), and oocyte penetration. A range of *in vitro* tests have been used to evaluate sperm characteristics directly related to the fertilization process, including the hypoosmotic-swelling test [1–3], sperm–ZP binding test [4,5], and the *in vitro* fertilization (IVF) of homologous zona-intact, *in vitro* matured (IVM) oocytes [6]. However, only ZP penetration rate [7] and oocyte penetration rate [8–10] have been successfully used to identify fertile versus subfertile boars, ejaculates, or both. It is unclear whether these techniques would be effective for predicting the relative fertility of boars with ejaculates that meet normal laboratory criteria for extension and use for AI. Standardized IVM and IVF techniques have previously been used in our laboratory to assess boar semen quality [11,12] and Xu et al. [6] reported that the estimated potential embryo production rate (an integrated measure of sperm quality *in vitro*), and number of sperm attached per oocyte, accounted for up to 53% of the variation in litter size, when 2 billion sperm per AI dose were used to determine relative boar fertility *in vivo*. The importance of using low sperm doses for AI to initially determine relative boar fertility *in vivo* has been confirmed in a number of subsequent studies [13–15] and this approach likely avoids the compensatory effect of using high sperm numbers per dose for AI.

Previous studies in our group [12] reported inter-boar differences in the motility of extended semen at Day 7, and found a high correlation ($r = 0.8985$, $P = 0.0001$) between these characteristic and penetration rate *in vitro* (IVF). Furthermore, Juonala et al. [16] found positive correlations with *in vivo* fertility and diluted storage semen. Therefore, evaluation of motility of extended semen at different days of storage represents an inexpensive indicator of boar fertility *in vivo*. Practical techniques for assessing semen quality in order to predicting male fertility are still needed.

The principal objective of the present study was to further evaluate effective predictors of relative boar fertility using: (1) a population of boars that would be considered acceptable for use in AI programs on the basis of ejaculate/sperm characteristics measured in most commercial AI centers; (2) only 1.5 billion sperm per AI dose to make comparisons of relative boar fertility *in vivo*. Results from the use of IVF with homologous, zona-intact, *in vitro* matured pig oocytes, and from routine semen evaluation for assessing semen quality, are reported in the present paper.

2. Materials and methods

2.1. Boar fertility evaluation *in vivo*

This experiment was conducted at the Swine Research and Technology Center of the University of Alberta, according to the guidelines of Canadian Council for Animal Care and with approval from the Faculty Animal Policy and Welfare Committee.

A total of nine Genex Large White boars were received at 26–28 wk of age (Genex Swine Group, Regina, SK, Canada), evaluated in three groups of three boars each, and identified by color and group (Blue-1, Red-1, Green-1; Red-2, Green-2, Yellow-2; Blue-3, Red-3 and Purple-3). After adapting and training each group of boars for a period of 3–4 wk, semen collections were standardized to twice per week using the gloved hand technique. A complete single ejaculate was collected into sterile pre-warmed 15-mL Falcon tubes (VWR Canlab, Mississauga, Ont., Canada), as described by Xu et al. [11]. Tubes containing the first sperm-rich fraction were visually identified and the concentration of the last tubes of the first sperm-rich fraction measured using a calibrated colorimeter (Model 254; Sherwood Scientific Ltd., Cambridge, UK) to identify the last tube with a concentration of $\geq 100 \times 10^6$ spermatozoa per mL; this and previous tubes were included as part of the first sperm-rich (SR) fraction. The tubes containing less than 100×10^6 spermatozoa per mL were considered to be part of the sperm-free fraction (SF) and discarded with any subsequent sperm-rich fractions collected from the same ejaculate (Fig. 1). All the tubes from the first sperm-rich fraction were then combined in a pre-warmed thermos by filtering through gauze to eliminate any gel component, to create the complete first SR fraction for routine semen evaluation (twice a week during the evaluation period) and breeding (every 3 wk out of 4).

Semen evaluations were performed by two well-trained and experienced individuals. Preliminary studies were performed to ensure that morphology and motility scores obtained by these individuals were comparable. The SR fraction was processed as follows: (1) total volume (mL) was measured by weighing the SR fraction using an electronic balance and assuming a density of 1 g per mL; (2) sperm morphology was evaluated using an eosin–nigrosin vital stain under a microscope (Olympus CH30, Japan) fitted with a 40 \times phase-contrast lens. A smear was prepared by mixing a drop of semen with a drop of stain on a preheated glass slide. The smear was air-dried and at least 100 sperm cells were evaluated and the percentage of spermatozoa

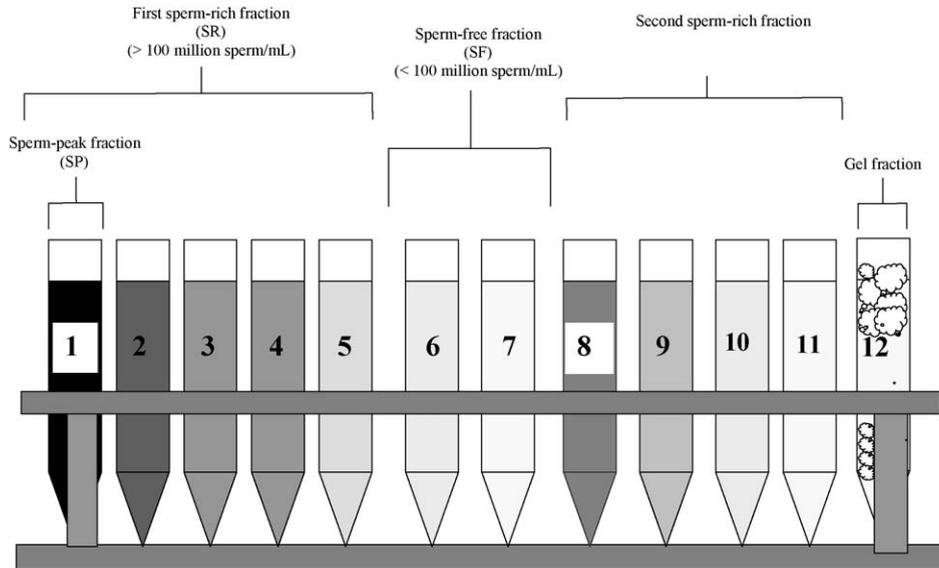


Fig. 1. Different fractions of an ejaculate used for in vivo and in vitro analysis of boar semen. A complete single ejaculate was collected into tubes and different fractions were identified using a calibrated colorimeter. Fractions were identified as follows: the first sperm-rich fraction (SR), the sperm-peak (SP) fraction (tube containing the highest sperm concentration within the SR fraction), the sperm-free fraction (SF) (as the tubes after SR and before the second sperm-rich fractions started that contain less than 100×10^6 spermatozoa per mL) and the gel fraction.

with abnormal heads, cytoplasmic droplets (both distal and proximal), abnormal tails and total of abnormal sperm were calculated. If a high proportion of detached heads were found a second smear was prepared in order to confirm the results; (3) sperm concentration was measured using the calibrated colorimeter mentioned above. To ensure accurate sperm counts, calibration was checked every 2–3 wk using a haemocytometer chamber. Also, when the concentration of the ejaculates exceeded the critical point, samples were re-diluted and measured again to confirm ejaculate concentration. Periodically, sperm concentration from extended semen was verified using the haemocytometer chamber; (4) progressive motility on the day of collection (Day 0) was evaluated at 37°C using the same microscope with a final magnification of $400\times$, screening at least five different fields. The estimate for progressive motility was subjectively scored to the nearest 5%. Diluted semen was stored at 17°C in aliquots of 3 mL (5 mL glass tubes) for further motility evaluations. On Days 3, 7, and 10, a sample of diluted semen (3 mL aliquot) was gently mixed and warmed up to 37°C for 20 min. Caffeine was added to the sample prior to examination and motility assessment was carried out following the same protocol used above; (5) finally, on breeding weeks, the SR fraction was diluted with Beltsville Thawing Solution containing antibiotics (BTS; Minitube of America, Inc., Verona, WI, USA) to 1.5×10^9 morphologically normal, motile sperm per 50-mL dose

(calculated as the average between the percentage of motile sperm and the percentage of normal sperm minus 100, giving the proportion of additional sperm per AI dose). Diluted semen from each boar was identified by color (Minitube of America, Inc.) and used to breed approximately equal numbers of gilts over the same breeding week. The purpose of using relatively low sperm numbers per dose was to improve the ability to detect differences in proven fertility in vivo. Extended semen was stored at 17°C and gently agitated once a day until the time of insemination. Each group of boars was evaluated during a 6.5 ± 1 mo period (from May to February) and semen from each boar was used to breed 50 ± 5 gilts during this time, with breeding occurring 3 wk out of 4, depending on gilt availability.

Genex hybrid gilts (Genex Swine Group) were housed in individual stalls and fed to appetite with a standard gestation (dry sow) diet. All gilts were bred at second or third estrus. Estrus was synchronized during the previous estrus cycle with the oral progestagen, Altrenogest (Regumate; Hoechst-Roussel Vet., Regina, SK, Canada), ensuring that the majority of gilts were bred with semen no older than 3 days. Gilts were checked twice daily (07:00 and 19:00) for standing estrus using the backpressure test during fence line contact with a mature boar, and inseminated with semen from the same test boar 24 h after first detection of standing heat and again 12 h later if the animal still displayed a strong standing reflex. Quality of insemina-

tion and duration of standing heat were recorded for all gilts. Test boars were collected Tuesday morning and Friday afternoon. The majority of breedings (95%) were completed from Tuesday to Friday. Animals with poor standing heat and/or inseminations were removed from the experiment. Pregnancy was confirmed by ultrasonography at Day 30. Pregnancy rate (% of bred animals pregnant at Day 30), farrowing rate (% of bred animals that farrowed) and total litter size (total number of piglets born alive or dead in a litter) were recorded. A boar fertility index (FI) was also calculated as the total piglets born divided by the number of gilts initially bred per boar. Both gilts and boars were kept under controlled conditions (temperature and hours of light) in order to reduce any seasonal effect.

2.2. Semen evaluation *in vitro*

Semen quality from each boar was also evaluated using an oocyte *in vitro* maturation and fertilization procedure described previously [11,12] with substantial modifications described below.

2.2.1. Culture media

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO, USA). Oocyte maturation medium consisted of modified M199 (Gibco, Grand Island, NY, USA), supplemented with 861 µg/mL glucose; 155 µg/mL sodium pyruvate; 155 µg/mL polyvinyl alcohol; 70 µg/mL cysteine; 70 µg/mL L-ascorbic acid; 35 µg/mL insulin (I1882); 2.5 µg/mL pLH-BIO (AFP-12389A, NIDDK, Torrance, CA, USA); 2.5 µg/mL pFSH-BIO (AFP-9400D, NIDDK, Torrance, CA, USA); 10 ng/mL EGF. The maturation media was also supplemented with 10% (v/v) of porcine follicular fluid (pFF) obtained from clear follicles 5–8 mm in diameter, centrifuged at 1680 × *g* for 30 min, filtered with a 0.22 µm syringe filter and stored at –15 °C until use.

Fertilization medium was prepared using a modified TRIS solution (2.30 mg/mL TRIS, 6.28 mg/mL sodium chloride, 0.21 mg/mL potassium chloride, 0.52 mg/mL glucose, 0.52 mg/mL sodium pyruvate, and 1.05 mg/mL calcium chloride), supplemented with 1.04 mg/mL caffeine/sodium benzoate and 3.80 mg/mL BSA (A7888).

Sperm-free medium consisted of North Carolina State University (NCSU) 23 media [17] containing 75 µg/mL penicillin, 50 µg/mL streptomycin and supplemented with 4 mg/mL BSA (A8022) immediately before use.

2.2.2. Semen collection

At least nine times during the boar evaluation period, ejaculates were collected into sterile pre-warmed 15-mL Falcon tubes and 5 mL aliquots of the first sperm-peak (SP) fraction were used for *in vitro* assessment. The SP fraction was defined as the tube containing the highest sperm concentration within the first SR fraction. The SP fraction was initially determined visually as the tube with the greatest density and confirmed colorimetrically by comparing the sperm concentration of this tube to the next tube collected. The remainder of the SP fraction and the other tubes in the SR fraction were combined and used for routine semen evaluation and AI as described in Section 2.1 (Fig. 1).

2.2.3. Oocyte collection and *in vitro* maturation

Ovaries with well-developed Graafian follicles were routinely collected from prepubertal gilts immediately after slaughter through the cooperation of staff of a local abattoir (Olymel; Red Deer, Alberta, Canada). To minimize temperature changes and bacterial contamination, the ovaries were transported to the laboratory within 90 min of collection in thermos flasks containing sterile physiological saline at 30 °C with added antibiotics (75 µg/mL of penicillin G potassium; 50 µg/mL of streptomycin sulphate). Ovaries were then washed three or four times with pre-warmed saline solution before aspiration of follicles. Modifications to established oocyte collection and *in vitro* maturation procedures were based on information provided by Dr. B. Day (Department of Animal Science, University of Missouri-Columbia, Columbia, MO, USA) as follows: cumulus–oocyte-complexes (COC's) were aspirated from clear follicles with a diameter of 3–6 mm using a 10 mL syringe fitted with an 18 gauge needle. The COC's were washed twice with PBS and then once with PBS containing 1% (v/v) of pFF (PBS–pFF). Only COC's with a uniformly dark cytoplasm and with several compact layers of cumulus cells were selected and washed twice with PBS–pFF, and then washed twice more with oocyte maturation medium. Approximately 40 COC's were then transferred to a Falcon culture dish (Becton Dickinson Labware, USA) containing 2 mL of oocyte maturation media, and incubated for 40–44 h at 39 °C in an atmosphere of 5% (v/v) CO₂ in air.

2.2.4. Sperm capacitation

Freshly ejaculated samples representing the SP fraction of the ejaculate were kept at room temperature

(20 °C) for approximately 16 h after collection. A 2 mL aliquot of the sperm sample was then transferred into a 15 mL Falcon tube, and diluted with 5 mL sperm washing medium (PBS supplemented with 0.1% BSA, w/v) and washed by centrifugation for 5 min at $1000 \times g$. The supernatant was discarded and the sperm pellet resuspended in sperm washing medium. This washing procedure was repeated twice. The supernatant was then discarded and the sperm pellet resuspended at 4×10^8 sperm/mL in fertilization medium and incubated at 39 °C under an atmosphere of 5% (v/v) CO₂ in air for 150 min. Our own serendipitous observations, subsequently confirmed by observations in other laboratories (personal communication from Dr. B. Day, Department of Animal Science, University of Missouri–Columbia, Missouri), led us to increase the time of sperm incubation from 90 to 150 min to substantially increase the oocyte penetration rate in vitro.

2.2.5. *In vitro* fertilization

After in vitro maturation, COC's with fully expanded cumulus cells were selected and washed twice in fertilization medium. Ten oocytes were transferred to each well of a 4-well culture dish (Nucleon 176740, Mississauga, Ont., Canada) containing 0.95 mL of fertilization medium per well and incubated pending in vitro fertilization.

After capacitation, sperm motility was evaluated and the sperm concentration adjusted to produce a final ratio of 5×10^4 motile sperm per oocyte. Sperm and oocytes were incubated at 39 °C under 5% (v/v) CO₂ in air for 6 h. After fertilization the oocytes were washed three times with sperm free medium. They were then transferred into a 4-well culture dish containing sperm free medium and cultured for a further $6 \text{ h} \pm 30 \text{ min}$ at 39 °C under 5% (v/v) CO₂ in air. Finally, 12 h after fertilization, the oocytes were transferred from the incubator to a refrigerator at approximately 5 °C for $10 \pm 2 \text{ h}$ until examination.

2.2.6. *Sperm penetration assessment*

Zona pellucida penetration was examined by initially mounting the unstained oocytes on slides, evaluating them under a phase-contrast microscope (Dialux 20 EB Leitz Wetzlar, Germany) at a magnification of 200 and 400 \times , and recording both the zona pellucida penetration rate (percentage of oocytes with sperm within the zona pellucida) and the number of sperm penetrating the zona (all sperm with both head and tail within the zona pellucida viewed in the sagittal focal plane). The oocytes were then fixed for at least 48 h in slide fixing solution (1:3

acetic acid:ethanol), stained with lacmoid, and examined 1 d later under a phase-contrast microscope at magnifications of 200 and 400 \times for penetration rate (percent of mature oocytes penetrated), number of sperm per oocyte (average number of penetrated sperm, from slightly swollen sperm head(s) to male pronuclei, per oocyte), monospermy rate, polyspermy rate, male pronuclear formation rate (MPN-f); (estimated as the percentage of penetrated oocytes with at least one male pronucleus), potential embryo production rate (% of penetrated oocytes with both a female and a single male pronuclei), and the percentage of penetrated oocytes with zero (zeroMPN), one (oneMPN), and more than one male pronucleus (>1MPN).

2.3. *Statistical analysis*

A total of 15 or 16 groups of gilts were bred during the evaluation period. To increase the number of gilts bred and farrowed per boar during specific breeding periods that were subsequently used to correlate in vivo estimates of boar fertility to laboratory and in vitro assessment of semen quality, data were analyzed as seven time intervals (boar age), representing successive periods from the start of breeding (time 1; 29–32 wk old) to the last evaluation period (time 7; 64–68 wk old). The results of in vitro fertilization and routine semen evaluation were grouped on the same basis for the statistical analysis.

Differences among boars ($n = 9$) for total litter size, routine semen evaluation and IVF characteristics were analyzed as a repeated measures analysis using a mixed procedure of Statistical Analysis System (SAS version 8.2, SAS Institute Inc., Cary, NC, USA). The fixed effects were time (seven levels), boars ($n = 9$), and their interaction, and the boar group as a random effect. In all statistical models, the Kenward–Roger option was used to calculate the denominator degrees of freedom. The variance–covariance matrix was chosen for each statistical model by an interactive process wherein the best fitting model was based on Schwarz's Bayesian criteria. Least square means and standard errors were generated and separated using a pdiff adjusted by Tukey option for significant, fixed effects. All IVF percentile data were subjected to arcsine transformation before analysis. All data are presented as $\text{LSM} \pm$ standard error of LSM.

As the IVF results from the second breeding time interval for one group of three boars were inadvertently lost due to technical problems, data from this time interval were removed for all the boars, in order to be able to run the statistical program, leaving data

from six time periods per boar for analysis of in vitro differences. Differences among boars for conception rate and farrowing rate were evaluated by Chi-square analysis using boar and time as independent variables.

A stepwise linear regression model was used to determine the association between in vivo characteristics (conception rate, farrowing rate, total litter size, and fertility index) and in vitro fertilization variables, and between in vivo characteristics and routine semen evaluation characteristics (SAS version 8.2, SAS Institute Inc.). Parameters, significant at the 0.15 level, were included in the regression model. Independent association between selected variables (IVF and routine semen evaluation variables) and in vivo fertility were evaluated using INSIGHT procedure (SAS, 2003).

Data from 38 gilts that were identified as having problems during artificial insemination were removed in order to reduce the effect of mis-breeding on estimates of boar fertility in vivo. Data from the last group of gilts (11 gilts) bred with the first group of boars that were identified with problems related to an outbreak of circovirus disease were also removed from the analysis.

3. Results

3.1. Fertility evaluation in vivo

The in vivo performance of boars is shown in Tables 1 and 2. There were differences among boars in pregnancy rate and farrowing rate but no effect of time for these characteristics. For pregnancy rate and

Table 1
In vivo results from the nine boars during the evaluation period

| Boar | Number of gilts | | | Fertility | | | |
|------|-----------------|----------|----------|--------------------|--------------------|----------------------------|---------------------------------|
| | Bred | Pregnant | Farrowed | Pregnancy rate (%) | Farrowing rate (%) | Total born (mean ± S.E.M.) | Fertility index (mean ± S.E.M.) |
| R-2 | 51 | 50 | 50 | 98 x | 98 x | 11.7 ± 0.4 ab | 11.4 ± 0.6 a |
| Y-2 | 53 | 48 | 47 | 91 xy | 89 xy | 12.0 ± 0.5 a | 10.9 ± 0.6 ab |
| Pu-3 | 57 | 54 | 52 | 95 xy | 91 xy | 11.2 ± 0.5 abc | 10.2 ± 0.6 ab |
| B-1 | 55 | 54 | 52 | 98 x | 94 xy | 10.7 ± 0.5 abcd | 10.2 ± 0.6 ab |
| R-3 | 55 | 52 | 52 | 94 xy | 95 xy | 10.9 ± 0.4 abcd | 10.1 ± 0.6 ab |
| G-2 | 45 | 42 | 41 | 93 xy | 91 xy | 10.1 ± 0.5 abcd | 9.5 ± 0.6 ab |
| B-3 | 55 | 51 | 51 | 93 xy | 93 xy | 9.6 ± 0.5 cd | 8.8 ± 0.6 abc |
| R-1 | 56 | 48 | 47 | 86 yz | 84 yz | 10.0 ± 0.4 bcd | 8.4 ± 0.6 bc |
| G-1 | 51 | 37 | 36 | 72 z | 71 z | 8.4 ± 0.6 d | 6.0 ± 0.6 c |
| | | | | $P = 0.0003$ | $P = 0.0003$ | $P < 0.001$ | $P < 0.0001$ |

Means with different letters (x–z) within each column were different ($P < 0.05$). LSM with different letters (a–d) within each column were different ($P < 0.05$). Values in the table are least means (LSM) ± standard errors (S.E.M.) of LSM. P : probability of main effect of boar.

Table 2
Combined in vivo results from all nine boars for each breeding period (time) during the evaluation

| Time | Number of gilts | | | Fertility in vivo | | | |
|------|-----------------|----------|----------|--------------------|--------------------|--------------------------------|-----------------|
| | Bred | Pregnant | Farrowed | Pregnancy rate (%) | Farrowing rate (%) | Total born ^a (mean) | Fertility index |
| 1 | 78 | 65 | 63 | 83 | 81 | 9.5 ± 0.4 | 7.8 ± 0.5 a |
| 2 | 59 | 53 | 52 | 90 | 88 | 10.9 ± 0.5 | 10.0 ± 0.5 ab |
| 3 | 65 | 58 | 58 | 89 | 89 | 10.6 ± 0.4 | 9.5 ± 0.5 ab |
| 4 | 70 | 67 | 65 | 96 | 92 | 11.1 ± 0.4 | 10.4 ± 0.5 b |
| 5 | 72 | 71 | 70 | 99 | 97 | 11.0 ± 0.4 | 10.7 ± 0.5 b |
| 6 | 58 | 54 | 53 | 93 | 91 | 10.5 ± 0.4 | 9.7 ± 0.5 ab |
| 7 | 76 | 68 | 67 | 89 | 88 | 9.7 ± 0.4 | 8.7 ± 0.5 ab |
| | | | | $P = 0.13$ | $P = 0.14$ | $P = 0.04^a$ | $P = 0.01$ |

LSM with different letters (a and b) within each column were different ($P < 0.05$) using a pdiff adjusted by Tukey. Values in the table are least means (LSM) ± standard errors (S.E.M.) of LSM. P : probability of main effect of time.

^a Total born presented significant main effect but no significant differences were found when LSM were compared using pdiff adjusted by Tukey.

Table 3

Semen characteristics of ejaculates collected from the nine boars during the evaluation period

| Boar | N | Total sperm per ejaculate ($\times 10^9$) | Sperm with cytoplasmic droplets (%) | Motility Day 3 (%) | Motility Day 7 (%) | Motility Day 10 (%) |
|------|----|---|-------------------------------------|--------------------|--------------------|---------------------|
| R-2 | 45 | 19 \pm 1.5 cd | 0.24 \pm 0.12 a | 79 \pm 1.3 a | 71 \pm 1.9 a | 60 \pm 2.0 a |
| Y-2 | 45 | 27 \pm 1.5 ab | 0.17 \pm 0.12 a | 77 \pm 1.3 ab | 70 \pm 1.9 a | 61 \pm 2.0 a |
| Pu-3 | 48 | 21 \pm 1.6 bcd | 0.17 \pm 0.12 a | 72 \pm 1.3 bc | 64 \pm 1.9 abc | 55 \pm 2.1 ab |
| B-1 | 48 | 21 \pm 1.6 db | 0.27 \pm 0.12 a | 71 \pm 1.3 bc | 61 \pm 1.9 bc | 52 \pm 2.3 abc |
| R-3 | 48 | 23 \pm 1.6 abc | 0.19 \pm 0.12 a | 76 \pm 1.3 ab | 67 \pm 1.8 ab | 60 \pm 2.1 a |
| G-2 | 45 | 18 \pm 1.5 cd | 0.38 \pm 0.12 a | 76 \pm 1.3 ab | 67 \pm 1.9 ab | 55 \pm 2.0 ab |
| B-3 | 48 | 29 \pm 1.5 a | 0.15 \pm 0.12 a | 73 \pm 1.3 abc | 66 \pm 1.9 ab | 59 \pm 2.1 a |
| R-1 | 48 | 14 \pm 1.5 d | 0.35 \pm 0.13 a | 62 \pm 1.5 d | 55 \pm 2.0 c | 47 \pm 2.5 bc |
| G-1 | 48 | 20 \pm 1.5 cd | 1.25 \pm 0.12 b | 69 \pm 1.3 c | 59 \pm 1.9 bc | 42 \pm 2.3 c |
| | | $P < 0.0001$ | $P < 0.0001$ | $P < 0.0001$ | $P < 0.0001$ | $P < 0.0001$ |

N: number of ejaculates evaluated. P: probability of main effect of boar. LSM with different letters (a–d) within each column differ ($P < 0.05$). Values in the table are least means (LSM) \pm standard errors (S.E.M.) of LSM.

farrowing rate, eight boars showed superior fertility, among which boar R-2 and B-1 demonstrated the highest fertility and were significantly superior to boars R-1 and G-1 in terms of pregnancy rate. Boar G-1 had reduced pregnancy and farrowing rates compared to all other boars studied, except R-1. There were significant effects of both boar and time on total litter size (Tables 1 and 2), but no boar by time interaction ($P = 0.88$). Boars R-2 and Y-2 had a higher litter size born than boars G-1 and B-3. Boar G-1 produced the lowest litter size born but was not different to five other boars. There were significant differences between boars in fertility index, with R-2 having the highest, and R-1 and G1 the lowest fertility index. The fertility index was also affected by time. Across boars, pregnancy rate and farrowing rate were highly correlated ($r = 0.95$; $P < 0.0001$). There were much weaker, albeit highly significant, correlations between both pregnancy rate and farrowing rate, and total litter size ($r = 0.42$; $P < 0.0005$ and $r = 0.44$; $P < 0.0004$, respectively).

3.2. Routine optical evaluation of semen quality

There were differences ($P < 0.0001$) among the nine boars in total sperm number per ejaculate, the percentage of progressively motile spermatozoa in extended semen on Days 3, 7, and 10, and in the percentage of sperm with cytoplasmic droplets (Table 3), but no time effect was found for these characteristics. As shown in Table 3, sperm motility in diluted semen on Days 3–10 on boars R-1 and G-1 was consistently lower than other boars. The percentage of detached heads showed a time effect ($P = 0.010$) but no boar effect (data not shown). Significant interactions between boar and time were observed for the total volume of ejaculate, sperm numbers (concentration) per mL, percentage of progressively motile spermatozoa in raw and extended semen on Day 0, percentage of morphological normal sperm (Table 4), and the percentage of bent tails in fresh semen (data not shown). Boars G-1 and R-1 presented the lowest values

Table 4

Boar by time effect ($P = 0.02$) on percentage of morphologically normal sperm on the day of collection (Day 0)

| Time | Boars | | | | | | | | |
|------|----------------|-----------------|-----------------|-----------------|----------------|-----------------|----------------|-----------------|-------------------|
| | R-2 | Y-2 | Pu-3 | B-1 | R-3 | G-2 | B-3 | R-1 | G-1 |
| 1 | 97 \pm 1.1 a | 99 \pm 1.1 a | 98 \pm 1.5 a | 95 \pm 1.3 a | 98 \pm 1.5 a | 96 \pm 1.1 a | 99 \pm 1.3 a | 87 \pm 1.3 b | 86 \pm 1.3 by |
| 2 | 99 \pm 1.2 a | 98 \pm 1.2 a | 99 \pm 1.3 a | 95 \pm 1.3 ab | 98 \pm 1.3 a | 99 \pm 1.2 a | 98 \pm 1.3 a | 89 \pm 1.3 b | 90 \pm 1.3 bxy |
| 3 | 99 \pm 1.3 a | 99 \pm 1.3 a | 98 \pm 1.1 a | 96 \pm 1.2 ab | 99 \pm 1.1 a | 98 \pm 1.3 a | 98 \pm 1.1 a | 89 \pm 1.2 b | 95 \pm 1.2 abx |
| 4 | 98 \pm 1.2 a | 96 \pm 1.2 ab | 99 \pm 1.2 a | 97 \pm 1.2 a | 99 \pm 1.1 a | 98 \pm 1.2 a | 98 \pm 1.1 a | 88 \pm 1.2 b | 93 \pm 1.2 abxy |
| 5 | 99 \pm 1.2 a | 97 \pm 1.2 a | 98 \pm 1.1 a | 98 \pm 1.2 a | 98 \pm 1.1 a | 99 \pm 1.2 a | 99 \pm 1.1 a | 92 \pm 1.2 ab | 87 \pm 1.2 by |
| 6 | 99 \pm 1.2 a | 97 \pm 1.2 a | 97 \pm 1.2 ab | 99 \pm 1.2 a | 99 \pm 1.2 a | 98 \pm 1.2 ab | 99 \pm 1.2 a | 94 \pm 1.3 ab | 92 \pm 1.2 bxy |
| 7 | 98 \pm 1.3 a | 99 \pm 1.3 a | 99 \pm 1.3 a | 98 \pm 1.0 a | 99 \pm 1.3 a | 97 \pm 1.3 a | 99 \pm 1.3 a | 90 \pm 1.1 b | 94 \pm 1.0 abx |

LSM \pm S.E.M. with different letters (a and b) within each row were different ($P < 0.05$). LSM \pm S.E.M. with different letters (x–z) within each column were different ($P < 0.05$). Values in the table are least square means (LSM) \pm standard errors (S.E.M.) of LSM.

Table 5
Effect of Boar on IVF variables measured on at least nine occasions during period of fertility assessment in vivo

| Boar | Zona penetration | Number of sperm penetrated the zona pellucida | Oocyte penetration rate | Number of sperm penetrated per oocyte | Number of MPN per oocyte | Polyspermy rate | MPN-f rate | Potential embryo production (%) | IMPN (%) | >IMPN (%) |
|------|------------------|---|-------------------------|---------------------------------------|--------------------------|-----------------|-------------|---------------------------------|--------------|--------------|
| R-2 | 98 ± 2.3 abc | 8.8 ± 4.7 bc | 83 ± 5 b | 3.5 ± 0.7 b | 1.3 ± 0.2 b | 80 ± 5 b | 72 ± 5.7 ab | 14 ± 4 cd | 35.5 ± 4 bc | 37 ± 5.9 a |
| Y-2 | 99 ± 2.3 a | 53.8 ± 4.7 a | 97 ± 5 a | 11.6 ± 0.7 a | 2.3 ± 0.2 a | 99 ± 6 a | 77 ± 5.7 a | 1 ± 4 d | 22.7 ± 4 c | 54 ± 5.9 a |
| Pu-3 | 85 ± 2.6 d | 3.1 ± 5.3 c | 69 ± 5 bc | 1.6 ± 0.8 b | 1.1 ± 0.2 b | 40 ± 6 d | 79 ± 6.4 ab | 43 ± 5 ab | 54.8 ± 4 a | 24 ± 6.7 bc |
| B-1 | 98 ± 2.4 abc | 26.5 ± 5.0 bc | 85 ± 6 ab | 3.1 ± 0.8 b | 0.9 ± 0.2 b | 74 ± 7 bc | 59 ± 6.8 bc | 15 ± 5 cd | 36.4 ± 4 abc | 22 ± 7.1 bc |
| R-3 | 89 ± 2.6 cd | 11.0 ± 5.3 bc | 76 ± 5 bc | 2.1 ± 0.8 b | 1.3 ± 0.2 b | 46 ± 6 cd | 83 ± 6.4 a | 40 ± 4 ab | 54.8 ± 4 a | 26 ± 6.7 a |
| G-2 | 96 ± 2.3 abc | 6.0 ± 4.7 c | 79 ± 5 bc | 2.6 ± 0.7 b | 1.3 ± 0.2 b | 61 ± 5 bcd | 76 ± 5.7 ab | 19 ± 4 cd | 32.7 ± 4 bc | 42 ± 5.9 ab |
| B-3 | 87 ± 2.6 bcd | 6.4 ± 5.3 bc | 81 ± 5 b | 2.7 ± 0.8 b | 1.3 ± 0.2 b | 66 ± 6 bcd | 79 ± 6.4 ab | 24 ± 4 abc | 47.3 ± 4 abc | 32 ± 6.7 abc |
| R-1 | 99 ± 2.6 ab | 29.2 ± 5.3 b | 75 ± 5 bc | 2.9 ± 0.8 b | 1.0 ± 0.2 b | 45 ± 6 cd | 56 ± 6.8 bc | 25 ± 5 abc | 36.8 ± 4 abc | 19 ± 7.1 bc |
| G-1 | 97 ± 2.4 abc | 15.3 ± 5.0 bc | 49 ± 6 c | 1.8 ± 0.8 b | 0.6 ± 0.2 b | 36 ± 6 d | 48 ± 6.8 c | 24 ± 5 abc | 34.5 ± 4 abc | 11 ± 7.1 c |

MPN: male pronuclei per penetrated oocyte; MPN-f: percentage of penetrated oocytes with at least one male pronucleus; IMPN: percentage of penetrated oocytes with one male pronucleus; >IMPN: percentage of penetrated oocytes with more than one male pronucleus. Means with different letters (a–d) within each column were different ($P < 0.001$). Values in the table are least means (LSM) ± standard errors (S.E.M.) of LSM.

for percent normal sperm and were inconsistent over time (Table 4).

3.3. In vitro fertilization data

Boar affected all measures of semen quality evaluated in vitro (Table 5), whereas only oocyte penetration rate ($P = 0.002$), polyspermy rate ($P = 0.001$), potential embryo formation rate ($P < 0.001$), and oneMPN ($P < 0.001$) were different over time (data not shown). Significant interactions between boar and time were observed for monospermy rate and zeroMPN.

3.4. Correlations relating boar fertility to routine semen evaluation and IVF variables

Because the primary objective of this study was to identify semen characteristics that would be predictive of relative boar fertility, and because in vivo measures of boar fertility did not change over time, only those variables measured with IVF and routine laboratory techniques that showed differences among boars, but no difference over time or boar by time interaction, were included in the stepwise linear regression analyses of relationships to in vivo fertility.

On this basis, the routine laboratory variables used to determine correlations to pregnancy rate, farrowing rate, total litter size and fertility index were the percentage of sperm with cytoplasmic droplets (cd) and the percentage of progressively motile spermatozoa in extended semen on Days 3, 7 and 10. Correlation coefficients of the multiple linear regression analyses from selected variables are presented in Table 6. The regression equations obtained were as follows:

$$\text{pregnancy rate} = 61.75 + 0.52(\text{Motility Day 10});$$

$$r^2 = 0.12(P = 0.006),$$

farrowing rate

$$= 44.21 - 5.47(\text{cd}) + 0.72(\text{Motility Day 7});$$

$$r^2 = 0.17(P = 0.0006),$$

total liter size

$$= 4.95 - 0.71(\text{cd}) + 0.10(\text{Motility Day 7});$$

$$r^2 = 0.22(P = 0.004),$$

fertility index

$$= 0.34 - 1.21(\text{cd}) + 0.15(\text{Motility Day 7});$$

$$r^2 = 0.27(P < 0.0001)$$

Similarly, the IVF variables tested for correlations to pregnancy rate, farrowing rate, total litter size and fertility index were: average number of sperm penetrated

Table 6

Multiple linear regression analyses of selected IVF variables (variables that presented differences among boars but not differences in time and no boar by time interactions) and fertility in vivo using stepwise model

| Parameters | Correlation coefficient (<i>r</i>) | | | |
|-------------|--------------------------------------|----------------|------------|-----------------|
| | Pregnancy rate | Farrowing rate | Total born | Fertility index |
| Average SZP | – | – | – | – |
| Average SPO | – | – | – | – |
| Average MPN | – | – | 0.306 | – |
| MPN-f (%) | 0.405 | 0.410 | – | 0.350 |
| >1MPN (%) | – | – | – | – |
| Model r^2 | 0.16** | 0.17** | 0.09** | 0.12** |

Average SZP: average number of sperm penetrated the zona pellucida; average SPO: average number of sperm penetrated per oocyte; average MPN: average number of male pronuclei; MNP-f: percentage of male pronuclear formation; >1MPN: percentage of oocyte with more than one male pronucleus. Variables left in the model were significant at the 0.15 level. (–) Variables that did not meet the 0.15 significance level for entry into the model.

** Model *P* value < 0.005.

Table 7

Multiple linear regression analyses of selected sperm routine evaluation variables (variables that presented differences among boars but not differences over time and no boar by time interactions) and fertility in vivo using stepwise model

| Parameters | Correlation coefficient (<i>r</i>) | | | |
|-------------------------------------|--------------------------------------|----------------|------------|-----------------|
| | Pregnancy rate | Farrowing rate | Total born | Fertility index |
| Cytoplasmic droplets (%) | – | –0.185 | –0.219 | –0.251 |
| Motility at Day 3 ^a (%) | – | – | – | – |
| Motility at Day 7 ^b (%) | – | 0.362 | 0.416 | 0.458 |
| Motility at Day 10 ^c (%) | 0.346 | – | – | – |
| Model r^2 | 0.12** | 0.17** | 0.22** | 0.27** |

Variables left in the model were significant at the 0.15 level. (–) Variables that did not meet the 0.15 significance level for entry into the model.

^a Sperm motility (%) of extended semen on Day 3.

^b Sperm motility (%) of extended semen on Day 7.

^c Sperm motility (%) of extended semen on Day 10.

** Model *P* value < 0.005.

the zona pellucida, average number of male pronuclei (aveMPN), average number of sperm penetrated per oocyte, MPN-f and >1MPN rates. Correlation coefficients of the multiple linear regression analyses from selected variables are presented in Table 7. The regression equations obtained were as follows:

pregnancy rate = 73.5 + 23.8(MPNformation);

$r^2 = 0.16(P = 0.001)$,

farrowing rate = 70.4 + 23.8(MPNformation);

$r^2 = 0.17(P = 0.001)$,

total litter size = 9.7 + 0.7(aveMPN);

$r^2 = 0.09(P = 0.01)$,

fertility index = 6.9 + 3.6(MPNformation);

$r^2 = 0.12(P = 0.006)$

Multiple linear regressions including all IVF and routine semen evaluations variables are presented in

Table 8. As well independent relationships between selected variables and in vivo fertility parameters are summarized in Table 9.

4. Discussion

Based on the earlier study of Xu et al. [6], it appears that relationships can be established between in vitro measures of semen quality and differences in relative boar fertility, even when ejaculate/sperm quality meets standard industry criteria for AI use. Therefore, it is important to emphasize that, compared with other published studies [9,10,18]; of ejaculate quality all the boars used in the present study exceeded normal industry standards (>80% progressive motility and >85% morphologically normal sperm).

Several attempts have been made to develop effective techniques for assessing semen quality and predicting male fertility. As discussed earlier, few measures of sperm attributes have been correlated with

Table 8

Multiple linear regression analyses of all selected sperm parameters (routine semen evaluation and IVF variables that presented differences among boars but not differences over time and no boar by time interactions) and fertility in vivo using stepwise model

| Parameters | Correlation coefficient (<i>r</i>) | | | |
|-------------------------------------|--------------------------------------|----------------|------------|-----------------|
| | Pregnancy rate | Farrowing rate | Total born | Fertility index |
| Average SZP | – | – | – | – |
| Average SPO | – | – | – | – |
| Average MPN | – | – | – | – |
| MPN-f (%) | 0.405 | 0.410 | – | 0.187 |
| >1MPN (%) | – | – | – | – |
| Cytoplasmic droplets (%) | – | –0.185 | –0.253 | –0.320 |
| Motility at Day 3 ^a (%) | – | – | – | – |
| Motility at Day 7 ^b (%) | – | 0.260 | – | 0.452 |
| Motility at Day 10 ^c (%) | 0.254 | – | 0.421 | – |
| Model <i>r</i> ² | 0.23** | 0.24** | 0.24** | 0.34** |

Average SZP: average number of sperm penetrated the zona pellucida; average SPO: average number of sperm penetrated per oocyte; average MPN: average number of male pronuclei; MNP-f: male pronuclear formation rate; >1MPN: percentage of oocyte with more than one male pronucleus. Variables left in the model were significant at the 0.15 level. (–) Variables that did not meet the 0.15 significance level for entry into the model.

^a Sperm motility (%) of extended semen on Day 3.

^b Sperm motility (%) of extended semen on Day 7.

^c Sperm motility (%) of extended semen on Day 10.

** Model *P* value < 0.005.

Table 9

Independent linear regressions of selected semen variables (variables that presented differences among boars but not differences over time and no boar by time interactions) with fertility in vivo parameters

| Parameters | Correlation coefficient (<i>r</i>) | | | |
|----------------------------------|--------------------------------------|----------------|------------|-----------------|
| | Pregnancy rate | Farrowing rate | Total born | Fertility index |
| Cytoplasmic droplets (%) | –0.266* | –0.253* | –0.298* | –0.338* |
| Motility Day 3 ^a (%) | 0.314* | 0.352* | 0.277* | 0.379* |
| Motility Day 7 ^b (%) | 0.326* | 0.362* | 0.416* | 0.457* |
| Motility Day 10 ^c (%) | 0.343* | 0.297* | 0.378* | 0.397* |
| Average SZP | 0.032 | 0.043 | 0.228 | 0.159 |
| Average SPO | 0.052 | 0.069 | 0.282* | 0.103 |
| Average MPN | 0.243 | 0.262* | 0.306* | 0.308* |
| MPN-f (%) | 0.405* | 0.410* | 0.231 | 0.350* |
| >1MPN (%) | 0.264* | 0.280* | 0.254* | 0.295* |

Average MPN: average number of male pronuclei; MNP-f: male pronuclear formation rate; >1MPN: percentage of oocyte with more than one male pronucleus; Average SZP: average number of sperm penetrated the zona pellucida; Average SPO: average number of sperm penetrated per oocyte.

^a Sperm motility (%) of extended semen on Day 3.

^b Sperm motility (%) of extended semen on Day 7.

^c Sperm motility (%) of extended semen on Day 10.

* *P* < 0.05.

in vivo and/or in vitro fertility [6,8–10,18]. In earlier studies an obstacle to establishing relationships between laboratory characteristics of an ejaculate and proven boar fertility may be due to the use of high sperm doses for AI, which may partially compensate for differences in fertility among boars. By using only 1.5 billion morphologically normal, and motile, sperm per dose AI in the present study, we were able to demonstrate substantial differences in boar fertility in vivo.

A difference of more than 15% in farrowing rate and more than two total pigs born per litter between the most and least fertile boars, suggests that an ability to predict and exclude boars performing like G-1 and R-1 in the present study, would have a considerable economic impact on production efficiency. Perhaps increased sperm numbers per AI dose could have partially offset the lower fertility of boars G-1 and R-1. However, available evidence (Dr. W. Flowers, personal communication) suggests that only partial

compensation can be achieved by increasing sperm numbers. Furthermore, observed differences in pregnancy rate and farrowing rate in the present study were consistently different among boars over the period of analysis. Perhaps the early results obtained from evaluation of semen collected immediately after the boars have been trained for AI use will be predictive of subsequent relative fertility. Also, as pregnancy rate was highly correlated with farrowing rate, an early indication of relatively low fertility in a small proportion of boars could be identified at an early stage of gestation.

Total litter size was different among boars and over time. Total litter size, rather than live born, was used as the measure of fertility, on the assumption that the number of stillborn pigs is not likely determined by boar-dependent factors. This was particularly critical when gilts were used for the fertility evaluations, as problems at farrowing of these maiden females might contribute to substantial differences in numbers of stillborn pigs. The lower numbers born in the first breeding time than in subsequent times probably reflected the immaturity of the boars, whereas the lower numbers born in the last replicate may relate to the cumulative effect of the high frequency of collection (twice-weekly) for relatively young animals. However, this aggressive program of collection provided adequate volumes of semen to rapidly identify the relatively less fertile boars.

The correlation between both pregnancy and farrowing rates, and total litter size born was not strong, as previously reported by Juonala et al. [16], suggesting that for the more fertile boars, these fertility characteristics may be differentially affected by semen quality. However, for boars G-1 and R-1 with the lowest relative fertility, all three measures of fertility were affected, suggesting that in less fertile boars a significantly lower pregnancy rate at Day 30 will likely be associated with a reduction in numbers of born.

Based on the fertility data alone, we inferred that these less fertile boars could be identified with as few as 20 single-boar matings, using relatively low sperm numbers for AI. Furthermore, using a standardized breed-abort protocol already established in our research group, cyclic gilts from the gilt development program can be used to provide data on pregnancy rate and potential litter size when aborted at Day 30 of gestation. Thus, meaningful information on relative boar fertility can probably be obtained without waiting for the bred gilts to farrow, and little impact on breeding herd productivity.

The results of routine laboratory evaluation in previous studies confirm that when sperm motility at collection is higher than 60%, it is not a predictive of

boar fertility when 2 billion or higher sperm per AI dose are used [6,18,19–21]. However, Tardif et al. [13] reported that when 0.3×10^9 sperm per AI dose were used, the percent sperm with normal motility was positively correlated with farrowing rate ($r = 0.783$, $P = 0.01$). Similarly, although Berger and Parker [8] and Popwell and Flowers [21] found no correlation between morphology and fertility, Xu et al. [6] demonstrated that the percentage of normal sperm was positively correlated with farrowing rate. In the present study, both the motility of raw semen and percentage of morphologically normal sperm were affected by a significant boar by time interaction. Therefore, given the very consistent differences in relative boar fertility in vivo over time, these results already imply that these laboratory assessments of semen quality will not be useful in predicting boar fertility.

In contrast to motility estimates with raw or extended semen on the day of collection, sperm motility in extended semen at Day 7 was correlated with in vitro fertility estimates [12], but did [16,22] or did not [6] correlate with in vivo fertility. Our results support the suggestion that sperm motility at Days 7 and 10 offers a practical approach for identifying relative boar fertility. However, more studies are needed to confirm this relationship and should involve precise methods of measuring sperm motility and motility characteristics, such as computer-assisted semen analysis (CASA).

Considering the IVF data, the in vitro characteristics that were not affected by time, but were different among boars, were potentially useful as predictors of fertility in vivo. Male pronuclear formation rate was the only IVF variable that explained from 12 to 17% of the variation of fertility in vivo in both independent and multiple linear regressions evaluated. Overall, other IVF characteristics lacked strong correlations with in vivo fertility, suggesting that thresholds for sperm quality were being met when relatively fertile boars are compared. However, the lower fertility boars (G-1 and R-1) showed the lower values for oocyte penetration, MPN-f and >1MPN, and critical thresholds (e.g. >50% oocyte penetration rate) can still possibly be used to identify subfertile boars.

The processes involved in IVF compared to in vivo fertilization may also contribute to the low correlations between in vivo and IVF data. Existing IVM and IVF systems have often been optimized for assessing oocyte quality and embryo production potential [23–31]; as in the present study, there may be a need to improve IVM/IVF techniques for sperm evaluation. The use of standardized total sperm numbers per oocyte for IVF, without any adjustments for motility

after sperm capacitation, *in vitro*, would probably help to identify the variation in sperm quality between the boars that affect the efficiency of fertilization process. Another approach could be to use much lower numbers of sperm per oocyte for IVF, thus placing the sperm in similar challenging situations *in vitro* and *in vivo*. Likewise, the use of the same ejaculate fractions for both *in vivo* and *in vitro* fertility evaluations could confirm the relationship between them. Recent studies by Rodríguez-Martínez et al. [32] demonstrate that the sperm from the SP fraction present better laboratory characteristics (sperm membrane integrity, % of live cells, etc.) than sperm from the bulk ejaculate (including subsequent sperm-rich fractions and sperm-free fraction of the ejaculate). These results could provide a better understanding of why this fraction presents the least variability when used for *in vitro* fertilization [11], and represents the best sperm subpopulation to test in order to obtain a predictor of fertility. The difference between these fractions is likely produced by seminal plasma components [32,33]. Therefore, further investigation need to be done in this area to get a better understanding of the effects of sperm and seminal plasma interactions *in vivo*.

Certain limitations were encountered in evaluating zona pellucida penetration rates. Firstly, a limited area (approximately 30%) of each oocyte was evaluated; secondly, some of the sperm scored as penetrated could have only partially penetrated the zona and be misinterpreted as fully penetrated and finally, oocytes with a high number of sperm were difficult to score. All these technical limitations could be responsible for the lack of correlation between zona penetration rate and fertility *in vivo*. Likewise, this could explain the low correlation presented between zona penetration rate and fertility *in vitro* (oocyte penetration rate $r = 54$, $P < 0.0001$). The use of fluorescence stains to differentiate between the sperm binding to the zona and penetrated sperm could increase the accuracy of this test [7], but unfortunately this technique could be expensive and time consuming. Another option could be to evaluate the number of sperm binding to the zona pellucida. This evaluation has been found as a useful indicator of *in vitro* fertility and embryo quality [34,35] and could be an alternative technique to be tested as a predictor of boar fertility.

In summary, we concluded that: (1) the present study provided compelling evidence that appropriate changes to standard AI procedures, and specifically the use of low sperm numbers per dose for AI, will allow relatively subfertile boars to be effectively

identified; (2) objective methods of assessing progressive sperm motility in stored extended semen may be an effective indicator of relatively less fertile boars; (3) there is still a need to optimize existing IVF techniques for use as good predictors of boar fertility and semen quality; (4) there are opportunities to develop timely and cost-effective procedures for excluding less fertile boars from commercial boar studs and further evaluation of these procedures are warranted.

Acknowledgements

The authors gratefully acknowledge: the Swine Research and Technology Center staff members for care of the animals; Dr. AF Parlow of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK's) National Hormone and Pituitary Program, Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance, CA, USA, for the donation of FSH and LH; Olymel, 7550 40th Av, Red Deer, Alta., Canada for supplying the ovaries; Drs. L. Goonewardene and P. Blenis for statistical advice. Financial support from the Alberta Agricultural Research Institute, NSERC, Alberta Pork and Genex Swine Group. A.L. Ruiz-Sanchez is the beneficiary of a CONACYT scholarship. GR Foxcroft is supported through appointment to a Canada Research Chair in Swine Reproductive Physiology (<http://www.chair.gc.ca>).

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