Morphology, membrane integrity, and mitochondrial function in sperm of crossbred beef bulls selected for residual feed intake

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| Keywords:              | acrosome, beef cattle, bull fertility, feed efficiency, semen quality |

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Running head: Residual feed intake and bull sperm quality

Morphology, membrane integrity, and mitochondrial function in sperm of crossbred beef bulls selected for residual feed intake

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ABSTRACT

The objectives of this study were to compare morphology, plasma and acrosome membrane integrities, and mitochondrial function in sperm of bulls selected for low- vs. high-residual feed intake (RFI). Semen samples obtained from 10 low- and 8 high-RFI yearling crossbred beef bulls were evaluated. Assessment of sperm morphology was performed by microscopy, and sperm membrane integrity and mitochondrial membrane potential were evaluated by flow cytometry. Parameters of sperm morphology evaluated did not differ between low- and high-RFI bulls. Compared to high-RFI bulls, low-RFI bulls had an increased proportion (LSM ± SE) of sperm with actively respiring mitochondria (54.2 ± 2.9 vs. 43.6 ± 3.3%, \( P = 0.03 \)). However, a greater proportion of sperm from low-RFI bulls had low mitochondrial membrane potential (34.4 ± 4.2 vs. 19.0 ± 4.7%, \( P = 0.03 \)). Results indicate that selection for improved feed efficiency do not compromise bull sperm morphology and viability. However, despite greater mitochondrial activity, the increased proportion of mitochondria with low membrane potential in sperm of low-RFI bulls warrants further investigation to rule out any potential negative effects on fertility.

Key words: acrosome, beef cattle, bull fertility, feed efficiency, semen quality
As a business enterprise, the beef cattle industry is highly dependent on efficiency and profitability of cattle operations. Koch et al. (1963) introduced the concept of residual feed intake (RFI) as a measure of feed efficiency, and of late, RFI has become an important trait not only evaluated in beef cattle (Nkrumah et al. 2006; Blair et al. 2013) but also in other species such as pigs (Barea et al. 2010), chicken (Aggrey et al. 2010), and fish (Eya et al. 2011).

Residual feed intake is estimated as the deviation of the actual feed intake from the predicted feed intake required for body weight maintenance and gain (Arthur and Herd 2008). Thus, a high feed-efficient animal will consume less feed relative to the expected feed intake for the given level of production, partitioning less energy to a residual portion (i.e. lower or negative RFI). On the other hand, a low feed-efficient animal will consume more feed relative to the expected intake, partitioning more residual energy (i.e. higher or positive RFI).

In beef cattle, RFI has been reported to be a moderately heritable trait (heritability estimates from 0.16 to 0.39), and highly correlated with traits such as feed intake and feed conversion ratio (correlation coefficients from 0.41 to 0.85; Arthur and Herd 2008). Thus, genetic selection to reduce the RFI will potentially result in improved efficiency in feed intake per unit of body weight gained. As feed efficiency is positively correlated to production traits, understanding physiological factors associated with variations in RFI becomes important. Before using high feed efficiency (i.e. low-RFI) as a trait for genetic selection it is important to determine whether selecting bulls for low-RFI will have a negative impact on fertility. Studies on different species have reported inconsistent outcomes on fertility in animals selected for feed efficiency. For instance, while selection for improved feed efficiency was associated with reduced reproductive performance in female mice (Nielsen et al. 1997), it was associated with improved fertility in cocks (Morisson et al. 1997). However, pregnancy outcomes of Angus
heifers were neither associated with their own RFI nor with their sire’s RFI values (Blair et al. 2013). The number of progeny per sire in low-RFI bulls was higher than in high-RFI bulls on pasture-based multi-sire mating (Wang et al. 2012); however, the proportion of bulls that failed to meet the 60% minimum sperm motility requirement during breeding soundness evaluation tended to be greater in the low- than in the high-RFI group (10 vs. 4%). Moreover, Wang et al. (2012) and Awda et al. (2013) reported decreased sperm motility in bulls selected for higher feed efficiency (i.e., low-RFI bulls).

Previous studies reported that semen quality parameters such as morphology (Tartaglione and Ritta 2004), plasma and acrosome membrane integrities (Tartaglione and Ritta 2004; Morrell et al. 2017), and mitochondrial function (Oliveira et al. 2014) were associated with fertility. For instance, Tartaglione and Ritta (2004) reported that 82% of the variation in in vitro fertilization rates of bovine oocytes was attributable to sperm head, tail, acrosome and plasma membrane integrities, combined, in Angus bulls. Furthermore, Oliveira et al. (2014) reported that semen of mature Nellore bulls with intact plasma membrane and acrosome, and high mitochondrial membrane potential (MMP), a measure of mitochondrial function, resulted in increased pregnancy rates (65 vs. 36%) compared to semen with reduced membrane integrity and mitochondrial function. As an overall relationship between semen quality and fertility is well established (Fitzpatrick et al. 2002; Chenoweth and McPherson 2016), exploring potential associations between RFI and sperm quality parameters in crossbred bulls that are used for breeding purposes in the Canadian beef industry is important. Although previous studies have reported that young bulls with low-RFI have decreased sperm motility (Wang et al., 2012; Awda et al., 2013), sperm viability and scrotal circumference (Awda et al., 2013), sperm morphology, membrane integrity and mitochondrial function have not been evaluated previously.
Therefore, the objectives of this study were to determine if crossbred bulls selected for higher feed efficiency (low-RFI) have compromised sperm morphology, plasma and acrosome membrane integrities, or mitochondrial function, compared to bulls of lower feed efficiency (high-RFI). Accordingly, we tested the hypotheses that (1) low-RFI bulls do not have increased defects in sperm morphology compared to high-RFI bulls, and (2) sperm plasma membrane and acrosome integrities and mitochondrial function are not decreased in low- compared to high-RFI bulls.

MATERIALS AND METHODS

Animals and semen collection

Eighteen yearling crossbred beef bulls from two bull stations, one situated in Ontario (n = 9; 65 to 80% Angus and 20 to 35% Simmental or Gelbvieh) and one situated in Alberta (n = 9; TX, a terminal cross paternal line, Beefbooster, Inc. Calgary, Alberta), were used in the study. Bulls were born between 2004 and 2008 and tested for RFI performance over a 84 to 112-d period starting at approximately 8 mo of age, as previously described (Basarab et al. 2003). Of the total 18 bulls, 10 and 8 were classified as low- and high-RFI, respectively. Values of RFI ranged from -2.42 to -0.98 kg dry matter per day in low-RFI bulls and from 1.43 to 2.57 kg dry matter per day in high-RFI bulls. Rations at the two locations were not identical but comparable in composition, DM and energy content (55 to 60% concentrate, 35 to 40% forage, ~5% protein + mineral supplement; ~70% DM and 2.35 to 2.65 Mcal/kg ME). Major ingredients were barley grain, oats, wheat middlings and alfalfa silage (Alberta) or high moisture corn, alfalfa silage and corn silage (Ontario).

Semen collection was performed at (mean ± SD) 398 ± 33 d of age by electro-ejaculation (Pulsator IV Auto Adjust, Lane Manufacturing, Denver, USA) by a certified veterinarian. Up to
three ejaculates were pooled (to increase volume and eliminate variability in sperm parameters among ejaculates), packaged and frozen in 0.5 mL⁻¹ French-straws using standard procedures (final concentration range of 5 to 15 x 10⁶ living spermatozoa per straw) and stored in liquid nitrogen until used for evaluation. All animals were cared for according to the Canadian Council of Animal Care (1993) guidelines and animal use was approved by the Animal Care and Use Committee at either the University of Alberta (Protocol # WANG-2007-19) or the University of Guelph (Protocol # 06R075).

**Morphology assessment**

Two straws per bull were thawed in a pre-warmed (38º C) water bath for 30 s, pooled, and three slides per bull were prepared, and stained with Eosin-Nigrosin for morphology assessment (Hopkins and Spitzer 1997). At least 300 sperm were counted in each slide and morphological defects evaluated according to classifications described by Barth (2000) using an inverted microscope (Eclipse TE2000-U, Nikon Instruments Inc., Melville, USA) at 600 x magnification. The defects identified in mid-piece (distal mid-piece reflex or bowed mid-piece), head (pyriform, detached, micro or macro head and other defects), tail (coiled, bent, reversed), and cytoplasmic droplets (proximal or distal) were recorded.

**Membrane integrity and mitochondrial function assessment**

Assessment of sperm viability (plasma membrane integrity), acrosome integrity and mitochondrial function measured as MMP were performed by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, USA) as per previous reports (Garner et al. 1994; Hossain et al. 2011). For the evaluation of plasma and acrosome membrane integrities and
mitochondrial function, frozen-thawed semen samples were first diluted (1:12 ratio) in a pre-warmed commercial medium (Sperm-TL; Specialty Media, Phillipsburg, NJ, USA). Then, 0.25 mL⁻¹ of this diluted sperm suspension was added to an equal volume of the same medium (Sperm-TL) in pre-warmed 2.5 mL⁻¹ amber tubes containing the corresponding dye combinations, for a final dilution ratio of 1:24 to obtain a flow rate of 1 to 2 x 10³ events per second during flow cytometry.

Plasma membrane integrity was assessed as a proxy for sperm viability using a combination of SYBR-14 and propidium iodide (PI) nucleic acid stains (Live/Dead Sperm Viability Kit L-7011, Molecular Probes Inc., Eugene, OR, USA), as previously described (Garner et al. 1994). In brief, SYBR-14 is a membrane-permeable nuclear stain which will enter both live and dead sperm and stain the nucleus, whereas PI is membrane-impermeable nuclear stain, hence excluded from viable sperm (intact plasma membrane). It enters only non viable sperm (damaged plasma membrane) and stains the nucleus and is a preferred counterstain in multicolour fluorescent imaging. When excited at 488 nm, nuclei fluorescing bright green (SYBR-14) will represent the live sperm population and nuclei fluorescing red (PI) will represent the dead sperm population. Moribund sperm subpopulations are stained with green-red. The proportion of SYBR-14 and PI-stained sperm was quantified by flow cytometry.

To assess acrosome membrane integrity, peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) was used in combination with PI (L-7381, Sigma-Aldrich, St. Louis, USA), as previously described (Januskauskas et al. 2000). The outer acrosome membrane of acrosome-damaged and acrosome-reacting sperm will fluoresce green upon FITC-PNA labeling whereas acrosome intact sperm will remain unlabeled by FITC-PNA, hence non-fluorescent. Using PI in combination with FITC-PNA allowed for distinction between live and
dead sperm. The proportion of live sperm with intact acrosome was used as indicator of acrosome integrity and accessed by flow cytometry.

Sperm mitochondrial function was assessed using 5,5’6,6’-tetrachloro-1,1’3,3’-tetracythylbenzimidazolcarbocyanine iodide (JC-1, MMP Detection Kit, Biotium, Fremont, USA), as previously described (Garner et al. 1997; Amaral and Ramalho-Santos 2010). The JC-1 stain produces populations of fluorescent cells with green monomers indicating mitochondria with low membrane potential (low MMP), and red-orange aggregates indicating mitochondria with high membrane potential (high MMP) (Garner et al. 1997) in addition to the remaining population of dead or moribund cells. In the present study, variables used for evaluation of mitochondrial function were the proportion of low MMP, high MMP, and the sum of low MMP and high MMP, indicating total actively respiring mitochondria. All flow cytometer results were obtained using the software BD CellQuest Pro (BD Biosciences, San Jose, USA).

The final dye concentrations used were 100 nM SYBR 14, 12 µM PI, 10µg of FITC- PNA, or 1 x JC-1 reagent solution at 10uL mL⁻¹. Non-sperm events were excluded (gated out) by adjusting side and forward light scatter parameters in the flow cytometer so that only cells emitting light scatter were used for fluorescence assessment, and 10,000 sperm were analyzed per sub-sample.

**Statistical analyses**

Data were analyzed with SAS 9.4 (Studio 3.5 platform, SAS Institute Inc., Cary, NC, USA). Descriptive statistics were obtained using the MEANS and UNIVARIATE procedures, and variables presented as means ± standard error of the mean (SE). Analysis of variance models were used to evaluate the effects of RFI (low- vs. high-RFI) on each variable with the
GLIMMIX procedure. Dependent variables relative to sperm morphology (proportion of head, mid-piece and tail defects, and cytoplasmic droplets), plasma membrane integrity (proportion of cells with intact plasma membrane), acrosome integrity (proportions of cells with intact acrosome), proportions of low MMP, high MMP, and proportion of actively respiring mitochondria (the sum of low MMP and high MMP) were modelled against the fixed effect of RFI group, location (Alberta, Ontario), and interaction between RFI group and location, including age as a constant covariate. Bull was the experimental unit and included as the subject of the random statement. The covariance structure with the lowest Bayesian information criterion (BIC) was chosen, which was either compound symmetry or auto-regressive. Normality and homoscedasticity of the standardized residuals were assessed graphically. Location and its interaction with RFI were removed from the final models as their effects were non-significant. Exclusively for the head defects model, location had an initial tendency effect (\( P = 0.10 \)) so it was considered as a random variable for this model. Descriptive statistics are reported as means ± SD, while results are reported as least squares means ± SE. Significant differences were defined as \( P \leq 0.05 \) whereas \( P > 0.05 \) and \( \leq 0.10 \) were considered as tendency.

A multiple correlation analysis and the respective Pearson’s correlation coefficients among continuous variables were conducted using the CORR procedure, where the average value of the three slides of each bull for sperm parameters were used. For the multiple correlation coefficients, Bonferroni’s correction was applied for the probability value and significance was considered when \( P \leq 0.004 \).
RESULTS AND DISCUSSION

Residual feed intake is considered a very important trait, that if incorporated in genetic improvement schemes, can provide economic benefits for beef cattle operations through improved feed efficiency (Arthur and Herd 2008). As RFI might be associated with inherent variation in basic metabolic processes, understanding potential associations between RFI and biological parameters, such as semen quality and function, that affect bull fertility (Fitzpatrick et al. 2002), is of great importance. Sperm morphology is considered to be an important criterion for bull breeding soundness evaluations (Barth and Oko 1989; Hopkins and Spitzer 1997); therefore, the present study evaluated variables of sperm morphology in yearling crossbred beef bulls classified as either low- or high-RFI. The overall descriptive statistics for morphological defects are presented in Table 1. The total proportion of sperm with at least one morphological defect was 34.3%, which was greater than previous reports. For instance, Menon et al. (2011) reported 22.8% of morphological defects in semen of yearling bulls of different beef breeds, while Brito et al (2002) reported 17.8% of defects in semen of crossbred beef bulls of 18 to 36 mo of age. Both studies (Brito et al. 2002; Menon et al. 2011) reported no effects of breed or age on total sperm abnormalities. Regardless of breed and age, bulls classified as having greater proportion of normal sperm will result in improved reproductive and economical outcomes in beef operations (Chenoweth and McPherson 2016).

The most prevalent morphological abnormality in the current study was mid-piece defect (16.7%), followed by tail defects (10.5%), head defects (4.4%), and cytoplasmic droplets (2.7%). The proportion of total defects observed (34.3%) was greater than the maximum of 30.0% for a bull semen sample to be considered satisfactory quality (Barth and Oko 1989; Hopkins and Spitzer 1997), and greater than the 14.1% of abnormal sperm in post-thaw semen from mature
crossbred bulls of 36 to 54 mo of age reported by Vyas et al. (1992). However, the proportion of head defects (4.4%), tail defects (10.4%), and cytoplasmic droplets (2.5%) were within the acceptable range as defined by Barth and Oko (1989). It has been reported that yearling bulls have high total sperm abnormalities (> 30%) and the percentage of abnormal sperm decreases progressively from 11 to 14 mo of age (Arteaga et al. 2001). Given this, the higher-than-acceptable proportion of total sperm abnormalities found in the present study is most likely related to the young age of the bulls at semen collection, and likely inconsequential. Although in the present study the bulls evaluated from Alberta were younger (mean ± SD; 368 ± 6 d) than those from Ontario (429 ± 14 d; \( P < 0.001 \)), there was no significant effect of location on sperm quality parameters. Furthermore, no significant correlation between age and sperm defects was observed (Table 2).

Our first hypothesis was that low-RFI bulls do not have increased sperm morphological defects than high-RFI bulls. In support of our hypothesis the proportions of sperm morphological defects did not differ between low- and high-RFI bulls (Figure 1). Similarly, Fox et al. (2004) evaluated net feed intake (a measure similar to RFI) in yearling Bonsmara bulls and reported no associations with sperm abnormalities, semen concentration, or overall breeding soundness. Also, Wang et al. (2012) reported no difference in the proportion of sperm with normal morphology between low- and high-RFI crossbred bulls. The present findings support our hypothesis that low-RFI bulls do not have compromised sperm morphology (i.e. increased proportion of sperm defects) compared to high-RFI bulls.

The assessment of plasma membrane and acrosome integrities and mitochondrial function was performed using flow cytometry, which is an established approach to evaluate sperm characteristics with high repeatability and accuracy compared to other techniques (Amaral and...
Ramalho-Santos 2010; Hossain et al. 2011). Descriptive statistics for sperm plasma membrane and acrosome integrities, low MMP, high MMP, and actively respiring mitochondria, are summarized in Table 3. Literature reporting these parameters in sperm of crossbred beef bulls, assessed by flow cytometry, is lacking. Regardless, we observed a similar proportion of plasma membrane integrity (38.3%), but smaller proportion of acrosome integrity (39.7%) and of high MMP (21.9%) than that reported in a previous study (Oliveira et al. 2014), which used a similar method and reported 35.6% plasma membrane integrity, 63.3% acrosome integrity and 38.2% high MMP. That study (Oliveira et al. 2014) evaluated commercial semen samples from mature Nellore (Bos indicus) bulls, which might have different proportion of sperm abnormalities than the ones evaluated in the present study that represented crossbred lines of yearling Bos taurus cattle. For instance, Simmental bull sperm reportedly have increased oxidative stress and greater proportion of defects than that of Nellore bulls (Nichi et al. 2006), possibly explaining the reduced proportion of sperm acrosome integrity and high MMP observed in the present study compared to that of Oliveira et al. (2004).

Our second hypothesis was that plasma membrane and acrosome integrities and mitochondrial function are not decreased in low- compared to high-RFI bulls. Previous studies have demonstrated that plasma membrane and acrosome integrities are associated with both in vitro (Tartaglione and Ritta 2004) and in vivo (Oliveira et al., 2014) sperm fertilizing potential. For instance, in vitro fertilization rates of bovine oocytes (varying from 52.3 to 75.6%) were highly influenced ($r^2 = 0.82$) by sperm head, tail, plasma membrane and acrosome integrities combined in Angus bulls (Tartaglione and Ritta 2004). In addition, Oliveira et al. (2014) reported that sperm samples from semen straw batches of mature Nellore bulls that had a greater proportion (44.5%) of intact plasma membrane, intact acrosome, and high MMP combined,
resulted in increased pregnancy rates (64.7 vs. 36.2%) compared to straw batches with lower proportion (8.5%) of intact plasma membrane, intact acrosome, and high MMP combined. However, the study by Oliveira et al. (2014) did not report evaluations of low MMP or of total actively respiring mitochondria.

The present study evaluated associations of RFI groups with plasma membrane and acrosome integrities, low MMP, high MMP, and total actively respiring mitochondria (Figure 2). No differences were observed between low- and high-RFI bulls on the proportions of plasma and acrosome membrane integrities (36.5 ± 2.7 and 36.8 ± 2.7 vs. 40.6 ± 3.1 and 42.1 ± 3.0%, respectively). However, compared to high-RFI bulls, low-RFI bulls had increased proportion of sperm with actively respiring mitochondria (54.2 ± 2.9 vs. 43.6 ± 3.3; \( P = 0.03 \)). However, among the population of sperm with actively respiring mitochondria, there was a greater proportion of sperm with low MMP in low- than high-RFI bulls (34.4 ± 4.2 vs. 19.0 ± 4.7%; \( P = 0.03 \)).

To our knowledge, this is the first study to evaluate associations among plasma and acrosome membrane integrities and sperm mitochondrial function in bulls of low- and high-RFI. An earlier study (Wang et al. 2012) evaluating crossbred bulls reported decreased sperm motility (80.0 vs. 85.0%) in low-RFI (n = 60) compared to high-RFI (n = 55) bulls, with a weak correlation (\( r = 0.11; P = 0.05 \)) between RFI and sperm motility. Intriguingly, low-RFI bulls (n = 18) sired more progeny than high-RFI bulls (n = 18), with an average of 18.3 and 11.8 progeny per sire, respectively. Furthermore, Awda et al. (2013) evaluated 110 young crossbred beef bulls and reported increased sperm viability (38.0 vs. 19.0%), motility (40.0 vs. 18.0%), and progressive motility (22.0 vs. 8.0%) in the 10 bulls with the greatest RFI values compared to the 10 bulls with the lowest RFI values. Although 7 bulls were common between the study by Awda...
et al. (2013) and the present one, the parameters evaluated in the two studies were quite different. Whereas Awda et al. (2013) determined bull scrotal circumference, sperm motility, progressive motility, and viability, the present study evaluated sperm morphology, membrane integrity, and mitochondrial function.

Though not fully understood, it is possible that selecting bulls for RFI leads to differences in sperm mitochondrial function, which is positively associated with epididymal sperm maturation and viability (Peña et al. 2009). In regards to mitochondrial function differences, crossbred steers with low-RFI were reported to produce less heat and less methane energy losses than high-RFI steers (Nkrumah et al. 2006). Thus, a reduced energy requirement in low-RFI than in high-RFI bulls could lead to more stored energy reserves for other biological mechanisms such as sperm mitochondrial function. This would be in accordance with our findings that sperm of low-RFI bulls had greater proportion of actively respiring mitochondria than of high-RFI bulls.

A recent study (Morrell et al. 2017) revealed no differences in the proportion of sperm with both high MMP and low MMP between dairy bulls of low vs. high fertility index. This demonstrates that the biological significance of the proportions of high MMP and low MMP [often referred to as aggregates:monomers ratio (Garner et al. 1997; Garner and Thomas 1999)] on sperm quality and its fertility potential are yet to be fully elucidated, including potential interactions with breed or age. In the present study, no significant correlations among RFI and sperm quality parameters were observed after Bonferroni’s correction for multiple correlations (Table 2).

As expected, a negative relationship between low and high MMP ($r = -0.68; P = 0.002$), and a strong positive relationship between low MMP and actively respiring mitochondria ($r = \ldots$)
0.79; \( P < 0.001 \) were observed (Table 2). In this regard, Garner et al. (1997) reported no association between aggregates:monomers ratio and sperm motility, where both aggregates (i.e. high MMP) and monomers (i.e. low MMP) were associated with post-thaw motility (\( r = 0.67 \) and 0.87, respectively). Garner and Thomas (1999) observed a negative correlation (\( r = -0.81 \)) between proportion of monomers (low MMP) and progressive sperm motility in fresh semen, while the proportion of aggregates (high MMP) was positively associated (\( r = 0.60 \)) with motility. Similarly, we observed a positive correlation between high MMP and plasma membrane integrity (\( r = 0.64; \ P = 0.004; \) Table 2). The increased proportion of low MMP observed in sperm of low- than in high-RFI bulls suggests that selecting bulls for improved feed efficiency affect the aggregates:monomers (i.e., high MMP:low MMP) ratio in sperm mitochondria, supporting previous findings (Wang et al. 2012; Awda et al. 2013) of reduced sperm motility in bulls selected for low-RFI. However, mechanisms associating sperm MMP with fertility need further investigations.

In summary, bulls categorized as low-RFI did not have compromised sperm morphology, plasma membrane and acrosome integrities compared to high-RFI bulls. Sperm of low-RFI bulls had, however, increased proportion of low MMP and of actively respiring mitochondria than sperm of high-RFI bulls. Findings indicate that crossbred beef bulls selected for improved feed efficiency do not have compromised sperm morphology and viability compared to less efficient bulls. Nonetheless, the increased proportion of sperm mitochondria with low MMP in feed efficient (low-RFI) bulls suggests a potential negative effect of selecting for RFI on sperm mitochondrial metabolism that warrants further investigation as it could have implications on bull fertility.
ACKNOWLEDGEMENTS

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Table 1. Descriptive statistics [mean, standard deviation (SD), minimum (min) and maximum (max) values] for morphological defects from 18 crossbred beef bulls

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<th>SD</th>
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<td>Head defects</td>
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<td>2.6</td>
<td>1.0</td>
<td>10.3</td>
</tr>
<tr>
<td>Pyriform</td>
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<td>0.2</td>
<td>0.0</td>
<td>0.7</td>
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<tr>
<td>Detached</td>
<td>3.0</td>
<td>2.7</td>
<td>0.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Micro head</td>
<td>0.2</td>
<td>0.3</td>
<td>0.0</td>
<td>0.7</td>
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<tr>
<td>Macro head</td>
<td>0.1</td>
<td>0.2</td>
<td>0.0</td>
<td>0.8</td>
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<tr>
<td>Other (i.e. vacuole and craters)</td>
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<td>2.1</td>
<td>0.0</td>
<td>7.7</td>
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<td>Mid-piece defects</td>
<td>16.7</td>
<td>6.9</td>
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<td>Distal reflex</td>
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<td>3.1</td>
<td>0.7</td>
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<td>Bowed mid-piece</td>
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<tr>
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<td>Proximal</td>
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<td>9.7</td>
<td>18.3</td>
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</table>

* Morphological defects variables were evaluated with Eosin-Nigrosin staining using an inverted microscope at 600x magnification.

* Total defects include the sum of head defects, mid-piece defects, tail defects, and cytoplasmic droplets.
<table>
<thead>
<tr>
<th>Variable</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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</thead>
<tbody>
<tr>
<td>1. Residual feed intake value</td>
<td></td>
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<tr>
<td>2. Head defects</td>
<td>0.09</td>
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<tr>
<td>3. Mid-piece defects</td>
<td>0.17</td>
<td>0.05</td>
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<tr>
<td>4. Tail defects</td>
<td>-0.33</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td>5. Cytoplasmic droplets</td>
<td>-0.31</td>
<td>0.02</td>
<td>0.04</td>
<td>0.23</td>
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<tr>
<td>6. Total defects</td>
<td>-0.06</td>
<td>0.33</td>
<td>0.81**</td>
<td>0.57*</td>
<td>0.44</td>
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<tr>
<td>7. Acrosome integrity</td>
<td>0.11</td>
<td>-0.16</td>
<td>0.26</td>
<td>0.25</td>
<td>0.18</td>
<td>0.29</td>
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<tr>
<td>8. Plasma membrane integrity</td>
<td>0.16</td>
<td>-0.15</td>
<td>0.15</td>
<td>0.11</td>
<td>0.35</td>
<td>0.22</td>
<td>0.82**</td>
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<tr>
<td>9. High MMP</td>
<td>0.33</td>
<td>-0.24</td>
<td>-0.05</td>
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<td>-0.01</td>
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<td>0.40</td>
<td>0.64**</td>
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<tr>
<td>10. Low MMP</td>
<td>-0.49*</td>
<td>0.17</td>
<td>-0.17</td>
<td>0.28</td>
<td>0.26</td>
<td>0.11</td>
<td>0.09</td>
<td>-0.15</td>
<td>-0.68**</td>
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<tr>
<td>11. Actively respiring mitochondria</td>
<td>-0.39</td>
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<td>-0.27</td>
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<td>0.35</td>
<td>-0.04</td>
<td>0.46</td>
<td>0.33</td>
<td>-0.08</td>
<td>0.79**</td>
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<tr>
<td>12. Age</td>
<td>0.02</td>
<td>0.05</td>
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<td>-0.09</td>
<td>-0.61*</td>
<td>-0.33</td>
<td>0.00</td>
<td>-0.35</td>
<td>-0.52*</td>
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</tbody>
</table>

*Morphological defects (variables 2 to 6) were evaluated with Eosin-Nigrosin staining using an inverted microscope at 600x magnification. Plasma membrane and acrosome integrities and mitochondrial function (variables 7 to 11) were evaluated using flow cytometry. Variables 2 to 11 represent proportions of sperm.

MMP = Mitochondrial membrane potential

**Indicate significance ($P \leq 0.004$) after Bonferroni’s correction for multiple correlations

* $P \leq 0.05$, but not significant ($P > 0.004$) after Bonferroni’s correction for multiple correlations
Table 3. Descriptive statistics [mean, standard deviation (SD), minimum (min) and maximum (max) values] for plasma membrane and acrosome integrities, high mitochondrial membrane potential (MMP), low MMP, and actively respiring mitochondria in semen from 18 crossbred beef bulls.

<table>
<thead>
<tr>
<th>Variable (%)</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane integrity</td>
<td>38.3</td>
<td>8.9</td>
<td>23.8</td>
<td>56.9</td>
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<tr>
<td>Acrosome integrity</td>
<td>39.7</td>
<td>10.4</td>
<td>22.7</td>
<td>54.0</td>
</tr>
<tr>
<td>High MMP</td>
<td>21.9</td>
<td>9.7</td>
<td>8.9</td>
<td>42.7</td>
</tr>
<tr>
<td>Low MMP</td>
<td>27.6</td>
<td>15.7</td>
<td>4.6</td>
<td>51.3</td>
</tr>
<tr>
<td>Actively respiring mitochondria</td>
<td>49.5</td>
<td>11.6</td>
<td>31.6</td>
<td>66.3</td>
</tr>
</tbody>
</table>

*Plasma membrane and acrosome integrities and mitochondrial function variables were evaluated using flow cytometry. Plasma membrane integrity was assessed using SYBR-14 and propidium iodide (PI); acrosome integrity was assessed using peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and PI; mitochondrial function was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1).*
Figure 1. Comparison of sperm morphological defects (proportion in LSM ± SE) between low- (n = 8) and high- (n = 10) residual feed intake (RFI) bulls. Morphological defects were evaluated with Eosin-Nigrosin staining using an inverted microscope at 600x magnification. No significant differences were observed in the same variable between low- and high-RFI bulls.

Figure 2. Comparison of sperm plasma membrane and acrosome integrities, proportion of low and high mitochondrial membrane potential sperm, and sperm with total actively respiring mitochondria (proportion in LSM ± SE) between low- (n = 8) and high- (n = 10) residual feed intake (RFI) bulls. Variables were evaluated using flow cytometry. *Indicate difference (P = 0.03) in the same variable between low- and high-RFI bulls.