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THE VALUE OF TRANS-SCROTAL ULTRASONOGRAPHY AT BULL BREEDING SOUNDNESS EVALUATION (BBSE): THE RELATIONSHIP BETWEEN TESTICULAR PARENCHYMAL PIXEL INTENSITY (PI) AND SEMEN QUALITY.

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Abstract

Bull Breeding Soundness Evaluation (BBSE) is commonly undertaken to identify bulls that are potentially unfit for use as breeding sires. Various studies worldwide have found that approximately 20 per cent of bulls fail their routine pre-breeding BBSE, and are therefore considered subfertile. Multiple papers describe the use of testicular ultrasound as a non-invasive aid in the identification of specific testicular and epididymal lesions. Two previous studies have hypothesized a correlation between ultrasonographic testicular parenchymal pixel-intensity (PI) and semen quality, however to date no published studies have specifically examined this link. The aim of this study therefore was to assess the relationship between testicular parenchymal PI (measured using trans-scrotal ultrasonography) and semen quality (measured at BBSE), and the usefulness of testicular ultrasonography as an aid in predicting future fertility in bulls, in particular those that are deemed subfertile at first examination. A total of 162 bulls from 35 farms in the South East of Scotland were submitted to routine BBSE and testicular ultrasonography between March and May 2014, and March and May 2015. Thirty three animals failed their initial examination (BBSE1) due to poor semen quality, and were re-examined (BBSE2) 6 to 8 weeks later. Computer aided image analysis and gross visual lesion scoring were performed on all ultrasonograms, and results compared to semen quality at BBSE1 and BBSE2. The PI measurements were practical and repeatable in a field setting, and although the results of this study did not highlight any biological correlation between semen quality at BBSE1 or BBSE2 and testicular PI, it did identify that gross visual lesion scoring of testicular images is comparable to computer analysis of PI ($P<0.001$) in identifying animals suffering from gross testicular fibrosis.

Keywords

Bull, Fertility, BBSE, Ultrasound, Pixel intensity

1. Introduction

Beef suckler cow enterprises heavily rely on natural service sires to achieve pregnancy in their females, and bulls are also often used to 'sweep up' following a period of artificial insemination (AI) in both dairy and beef herds [1]. Bull Breeding Soundness Evaluation (BBSE) is commonly undertaken to identify bulls that are potentially unfit for use as breeding sires, and thus to avoid poor herd reproductive performance and economic losses [2]. Few male animals are truly infertile, however it is accepted that approximately 20 to 40 per cent of bulls examined as part of routine screening fail their BBSE, and are therefore considered subfertile [3]. However collection and assessment of semen collected via electro-ejaculation (EEJ) may not always be a true representation of the quantity and quality of semen produced by a bull throughout a breeding season [4]. This can lead to difficulties in decision making on farm, and potential misclassification of bulls as unfit for purpose based on the results of a single BBSE conducted using semen collected via EEJ.

Measurement of testicular weight (and a proxy for this; testicular circumference) should be undertaken as part of all BBSE [5] and is widely accepted as a predictor of sperm output [6]. However this measurement involves a gross measurement of the scrotal exterior circumference and does not account for potential (non-palpable) pathology or lesions of the testicular tissue that may affect fertility [7]. Multiple papers describe the use of testicular ultrasound as a non-invasive aid in the identification of specific testicular and epididymal gross lesions [7-12]. However few studies have examined the correlation between ultrasonographic testicular parenchymal pixel intensity (PI) and semen quality [7]. Those that have show little correlation between the two measurements at the time of testing [13]. Three papers have proposed a link between parenchymal PI and future fertility [13-15]. However the results across these studies were not consistent, nor always conducted on sexually active animals. The aim of this field study was to assess the relationship between testicular parenchymal PI (measured using trans-scrotal ultrasonography) and semen quality (measured at BBSE), and thereby assess the usefulness of testicular ultrasonography as an aid in predicting the future fertility of sexually mature bulls in clinical veterinary practice.

2. Materials and Methods

2.1 Farm and bull selection

This field study was conducted in the South East of Scotland using bulls belonging to clients of a single first opinion farm animal veterinary practice, and approved by the Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee (VERC Ref:29-14). The veterinary practice routinely performs 150 to 200 BBSEs per year across approximately 40 beef suckler enterprises. BBSEs of all bulls enrolled in the study were undertaken as part of the routine examination of

animals approximately 8 weeks in advance of the breeding season (BBSE1). Animals that failed BBSE1 and were classified as subfertile due to poor semen quality were re-examined 6 to 8 weeks later (BBSE2), which allowed for one spermatic cycle to be completed between both evaluations. This enabled assessment of persistent or transient subfertility, and therefore decision making by the veterinarian and farmer on whether a bull was deemed suitable as a breeding sire or not. Although BBSE does not guarantee fertility, it provides producers confidence that they are greatly reducing the risk of using bulls that will fail to achieve normal fertility levels due to physical or semen quality problems [16].

2.2 BBSE

All BBSEs were performed on farm by trained and experienced examiners following British Cattle Veterinarian Association (BCVA) guidelines [16]. A 4-stage BBSE was performed at each examination and involved a general physical examination, examination of the external reproductive tract (including scrotal circumference measurement using a Reliabull® measuring tape), examination of the internal reproductive tract, and collection and examination of a semen sample collected via electro-ejaculation (EEJ). If a sample of poor quality was collected upon first EEJ, a second and final semen sample was collected by EEJ after a 20 minute rest period. Gross motility was assessed using a bright field microscope at x10 magnification, and the percentage of progressively motile spermatozoa was estimated using phase contrast microscopy at x40 magnification. Sperm morphology was assessed using eosin-nigrosin stained semen smears at x100 magnification. Percentage of normal spermatozoa, detached heads, proximal cytoplasmic droplets, head defects, coiled tails, distal midpiece reflex, coiled prinicipal piece, white blood cells, “other” and total abnormal spermatazoa were calculated by counting a total of 200 spermatozoa per slide. Bulls were classified as subfertile due to poor semen quality if the ejaculate contained less than 60 per cent progressively motile spermatozoa and/or less than 70 per cent morphologically normal spermatozoa [16].

2.3 Testicular ultrasound and pixel intensity (PI)

A B-mode ultrasound scanner equipped with a 4.5MHz-8MHz linear array transducer (Easi-Scan; BCF Technology, Strathclyde, Scotland) was used to image the testes of each bull submitted for BBSE before EEJ was carried out. The same equipment was used for every examination and the settings for focus, gain, brightness and contrast standardised at the machine median settings. All images were taken by the same examiner (MT). The testicles were prepared before each examination using disposable paper towels so that they were clean and dry. A conductive ultrasound gel was used as a coupling material between the scrotum and transducer, and pressure applied until minor scrotal skin indentation occurred. The ultrasound transducer was held vertically (parallel to the long axis of the testes) on the caudal surface of the scrotum. The image was aligned until the mediastinum of the testes was clear and apparent. The image was then frozen and stored. This process was repeated

with the ultrasound transducer in the horizontal plane (at the widest part of the testicle) and both views were repeated for the other testicle. Each ultrasound examination therefore comprised of four images from each bull (Figure 1 a, b).

Computer analysis of each image was undertaken using image analysis software (Image J, U. S. National Institutes of Health, Maryland, USA [17]). The examiner was blinded to the bulls and testicular ultrasonographic images by anonymous numbering of the images. Testicular PI of images in the vertical plane was determined by drawing 6 circles 10mm in diameter in the parenchyma of the testicle within 10mm of the mediastinum of the testicle (3 medially and 3 laterally to the mediastinum testes) where the parenchyma appeared homogenous. The same method was used for images in the horizontal plane using 4 circles 10mm in diameter (2 cranially and 2 caudally to the mediastinum testes) (Figure 1 c, d). PI within the drawn areas was measured according to shade on a 1 to 255 grey-scale (1 corresponding to black and 255 corresponding to white). A macro was established to calculate the mean, mode, minimum, maximum and standard deviation (a proxy for testicular homogeneity) of PI within the selected areas. The entire process (with new areas of assessment selected) was repeated 3 times for each image, at intervals separated by a minimum of one week, and an average of the 3 data calculations used to prevent bias in the drawing of the circles on each image. In summary each testicle had 30 areas of measurement (6 in the vertical plane, 4 in the horizontal plane, repeated separately 3 times). A gross visual scoring of fibrotic lesions within the testicular parenchyma was carried out to give a gross testicular fibrosis score [18]. This used a six-point scale of fibrosis per image, with 0 indicating a normal homogenous echotexture throughout the testicular parenchyma and 5 indicating severe fibrosis throughout the testicle (Figure 2). This measurement was done at a separate time to the computer PI scoring. Once all images were assessed, the data from the vertical and horizontal images from each testicle were combined to give an overall mean, mode, minimum, maximum and standard deviation of PI as well as a gross testicular fibrosis score for each bull. This was then placed into one dataset alongside the corresponding BBSE data for each bull for analysis.

2.4 *In vitro* assessment of the repeatability of the testicular ultrasonography and pixel intensity (PI) measurements

The repeatability of the PI assessment of testicular images was assessed *in vitro* via blinded image collection by 4 vets, each collecting 10 vertical images of testicular parenchyma from the same cadaver testicle. The testicle was obtained from the castration of a 12 month old Holstein Friesian bull, the tunic albuginea was removed at the time of castration and the testicle stored at 4^{o}C in a refrigerator. All images were collected within 24 hours of testicular removal. Analysis of variance (ANOVA) of mean PI collected from each image (as described in section 2.3) showed no significant differences between vets (P= 0.625).

2.5 Statistical Analysis

All data were entered into an Excel (Microsoft®) spreadsheet for subsequent analyses. Scatter plots were used to visually assess the correlation between PI mean, mode and standard deviation and the percentage of progressively motile spermatozoa, percentage of morphologically normal spermatozoa and gross visual fibrosis. Simple linear regression models using statistical software (Minitab® and R® [19]) were used to identify any statistical correlation. This was done comparing testicular parenchymal image analysis values (e.g. PI mean) and semen quality values taken at BBSE1. Testicular parenchymal image data taken at BBSE1 were also compared to semen quality at BBSE2 (6 to 8 weeks later) and the change in semen quality between BBSE1 and BBSE2 in animals requiring a second BBSE was assessed. Box and whisker plots and two sample t-tests were undertaken to investigate the relationship of BBSE pass or fail outcomes with ultrasound variables. Multivariable general linear regression models with backwards selection were used to investigate the association between progressive motility and PI mean, testicular lesion score whilst controlling for any effect of age.

3. Results

Of 162 bulls tested in this study, 61 animals (37%) failed BBSE1, with 33 (20%) failing due to poor semen quality (less than 60 percent progressively motile spermatozoa and/or less than 70 per cent morphologically normal spermatozoa). Twenty one of the 33 animals that failed BBSE1 (64%) also failed BBSE2 6 to 8 weeks later. Reasons for failure of BBSE and semen associated abnormalities recorded are described in Table 1.

Number of animals failing BBSE1 and reasons for failure :	n=61	Number of animals undergoing BBSE2 and reasons for failure :	n=33
<60% progressively motile spermatozoa and <70% morphologically normal spermatozoa	25	<60% progressively motile spermatozoa and <70% morphologically normal spermatozoa	14
<60% progressively motile spermatozoa only	11	<60% progressively motile spermatozoa only	4
<70% morphologically normal spermatozoa only	5	<70% morphologically normal spermatozoa only	4
Lameness	11		
Inadequate scrotal circumference	4		
Seminal vesiculitis	1		
Epididymitis	1		
Testicular mass	1		
Brisket abscess	1		
Eye ulcer	1		
Number of animals with <70% morphologically normal spermatozoa	30	Number of animals with <70% morphologically normal spermatozoa	18
Predominant morphological abnormality recorded:-		Predominant morphological abnormality recorded:-	
Detached heads	12	Detached heads	5
Mid piece reflex	10	Mid piece reflex	8
Proximal Droplets	5	Proximal Droplets	4
Coiled Tails	3	Coiled Tails	1

Table 1. Reasons for bull failure at BBSE1 and BBSE2.

Comparison of PI of images and semen quality parameters at BBSE1 are shown in Figure 3. No visual correlation was observed when comparing mean PI or standard deviation of PI to percentage of progressively motile spermatozoa or percentage of morphologically normal spermatozoa at BBSE1. Statistically significant correlation was observed between PI standard deviation and progressive motility (P= 0.022) (r2=3.2%) and morphology (P=0.008) (r2= 4.3%) (Figure 3b, d). However examination of the plots suggests this is driven by outliers, and is not biologically significant.

Fibrotic lesion scoring of images had no association with percentage of progressively motile spermatozoa, or percentage of morphologically normal spermatozoa at BBSE1. Gross visual fibrotic lesion scoring was compared to PI parameters. Fibrotic lesion scoring of testicles had an association effect of 40.5% (P<0.001) of variance in PI standard deviation in a linear regression model (Figure 4). Therefore visual assessment of images and fibrotic lesion scoring may be as useful as computer aided assessment of testicular homogeneity. Gross testicular fibrosis can be associated with reduced potential daily sperm output [14].

No correlation was observed between PI measurements with pass or fail outcomes of bulls at BBSE1 (Figure 5). Significant statistical correlation was observed between gross visual fibrotic lesion scoring and pass or fail outcomes (P< 0.001)(T= 3.92) (Figure 5d).

Comparison of the PI of images taken at BBSE1 and semen parameters at BBSE2 are shown in Figure 6. No visual correlation was observed between the mean PI or standard deviation of PI when compared to the percentage of progressively motile spermatozoa or the percentage of morphologically normal spermatozoa. Statistically significant correlation was observed between PI standard deviation and progressive motility (P= 0.044))(r2= 16.1%), (Figure 6b). However examination of the plots suggests this is driven by outliers, and is not biologically significant.

Figure 7 shows the comparison of the PI of images taken at BBSE1 and the change in semen parameters between BBSE1 and BBSE2. No visual or statistical correlation was observed between the mean PI or standard deviation of PI when compared to change of sperm motility and change of sperm morphology.

To assess whether age was confounding results and masking significant associations, a multivariable general linear regression model was carried out. The outcomes of progressive motility and sperm morphology were investigated for their association with PI mean. Age was included in the model, and no significant association was identified from the maximal model or following backwards

selection [20]. The maximal model progressive motility ≈ PI mean + age + testicular lesion score and the parsimonious model sperm morphology ≈ PI mean + age + testicular lesion score was used (Table 2)

	Progressive motility			Sperm morphology		
Variable	Coefficient	Standard error	P	Coefficient	Standard error	P
PI mean (grey scale)	0.03322	0.10630	0.755	0.09213	0.08389	0.2743
Age (years)	0.14872	0.98996	0.881	1.09453	0.75720	0.1509
lesion score	-0.91115	0.75450	0.23	-1.03588	0.59265	0.0831

Table 2. Results of multivariable general linear regression model, investigating the association between outcomes of progressive motility and sperm morphology with PI mean.

4. Discussion

Although previous studies have assessed the correlation between testicular PI and semen quality (as assessed by measurement of sperm motility and morphology), this is the first field study to investigate the correlation between testicular PI, gross testicular fibrosis score and future semen quality in commercial bulls of breeding age. The PI measurements were practical to collect and repeatable in a field setting. Although the results of this study did not highlight any significant correlation with semen quality at BBSE1 or BBSE2 and testicular PI, it did identify that gross visual lesion scoring of testicular images is comparable to computer analysis of PI in identifying animals potentially suffering from gross testicular fibrosis.

Previous studies [13-15] have suggested a link between testicular PI and future fertility [7]. This study however found no significant correlation between testicular PI at BBSE1 and semen quality of bulls at BBSE2. One study using scrotal insulation as a research model concluded that PI was correlated to semen quality of ejaculates two to four weeks after initial examination [14]. Brito et al 2012 [13] observed similar results in a study examining bulls at four week intervals with correlations between testicular PI and sperm morphology identified 4 to 8 weeks after initial examination. Interpretation of these results has been difficult however, as correlation between semen parameters and PI has been low and often conflicting in different studies [13]. This is the first field study to investigate the correlation between testicular PI and future fertility of animals with abnormal sperm motility and/or morphology at initial examination. In this study no significant correlation was identified between testicular PI of images taken at BBSE1 and semen parameters at BBSE2 6 to 8 weeks later. Additionally no correlation was observed between testicular PI assessment and the

change in semen parameters between BBSE1 and BBSE2. Therefore the results of this study suggest that testicular PI is not useful as an aid in predicting current and future semen parameters of bulls in the field setting.

The design of this study used equipment and image analysis software readily available to the general veterinary practitioner. Preliminary *in vitro* work suggested standardisation of equipment and testicular PI assessment between different veterinary practitioners was possible. However environmental factors in the field, including the preparation and collection of testicular images, alongside undertaking a full BBSE may have resulted in a variation of image quality.

Increased testicular echogenicity is associated with Sertoli cell differentiation, increased seminiferous tubule diameter and a higher proportion of the testicular parenchyma occupied by seminiferous tubules [21]. An increase in testicular echogenicity has been observed in bulls during development of sexual maturity [13]. However variation of testicular PI in sexually mature bulls has proven difficult to explain [13]. In agreement with previous studies, testicular PI in beef bulls had no association with semen parameters at the time of testing [3, 21]. This is likely to be due to the fact that testicular parenchyma at any given time does not correlate with the semen within an ejaculate until several weeks later [7]. In this study fibrotic lesion scoring of testicles had an association effect of 40.5% ($P<0.001$) of variance in PI standard deviation. Therefore visual assessment of images and fibrotic lesion scoring may be as useful as computer aided assessment of testicular homogeneity in identifying animals with gross testicular fibrosis which could be expected to reduce daily sperm output [14].

No relationship between PI, semen quality and testicular lesion scoring and age were identified by multivariable models. Aravindakshan et al. described differences in echogenicity between early and late maturing bull breeds prior to puberty [22]. These differences may not have been observed as the bulls in this field study were considered to be post-pubertal by their owners before presentation for BBSE.

The proportion of bulls failing at BBSE1 due to poor semen quality parameters in this study was 20 per cent and an overall failure rate at BBSE1 of 37% was identified. This is similar to the figures of 20 to 40 per cent reported previously [3]. Semen parameters that showed the greatest improvement between BBSE1 and BBSE2 and resulted in 14 animals that failed BBSE1 yet passed BBSE2 were percentage of progressively motile spermatozoa only (64%, 7 of 11 bulls) and percentage of morphologically normal spermatozoa with a predominant abnormality of detached heads only (59%, 7 of 12 bulls). The improvement in progressive motility only and proportion of spermatozoa with detached heads only seen between BBSE1 and BBSE2 suggest that these abnormalities may improve over time, and a repeat BBSE may be warranted to avoid unnecessary culling of potentially fertile bulls with these abnormalities. Improvement in the percentage of progressively motile spermatozoa as the only abnormality observed could be explained by the influence of semen handling on sperm

viability and the fact that this is a subjective assessment must not be overlooked [23]. The reliability of semen progressive motility assessment in relation to number of calves born per cow appears limited and requires further investigation [2, 24]. More accurate assessment of semen motility and morphology can be performed by the use of computer aided semen assessment (CASA) [23]. However this equipment is not readily available in general veterinary practice in the UK and may have economic constraints. Semen with a high percentage of detached heads (stress spermiogram or 'rusty load') can relate to abnormal storage and maturation time in the epididymis, and is commonly seen in bulls that have had an extended period of time without expressing sexual behaviour (as may be the case prior to the breeding season) or have suffered an inflammatory insult [25].

Testicular weight as part of a BBSE is still the only proven assessment to reliably predict the future fertility of bulls [7]. Other modalities such as ultrasonography, scrotal thermography and testicular biopsy can be used in the diagnosis and assessment of gross testicular pathology [7]. These modalities may be helpful to predict future fertility of bulls, but their application in the field appears limited. Brito et al. reported that a lower scrotal temperature and a bigger top-to-bottom temperature gradient was correlated with a greater sperm production and better semen quality [19]. However Gabor [26] reported a negative effect of top-to-bottom temperature gradient. Considering the variations in environmental temperature in the UK, trying to standardise such measurements may limit their practical application by the general veterinary practitioner. One study by Heath et al. [4] observed no long term effects of testicular biopsy in 6 bulls and concluded that testicular biopsies may provide a valuable tool for evaluating future breeding ability. However this method of assessment should be reserved for animals with questionable breeding potential and not used as a regular screening tool.

5. Conclusion

This study found no correlation between testicular ultrasonographic PI at BBSE1 and semen quality of bulls at BBSE2. Ultrasonographic assessment of the testicle still remains useful for the assessment of gross testicular pathology or research purposes [13], but no evidence was found to support its use as an additional screening tool as part of BBSE in general veterinary practice. Reliable predictors of future fertility assessed using ultrasonography of the testes remain elusive and problematic [2]. Further work is needed to develop tools useful for guiding decision making on bulls of questionable fertility at BBSE, as well as the interaction of individual bull assessment parameters and herd level fertility.

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Figure 1

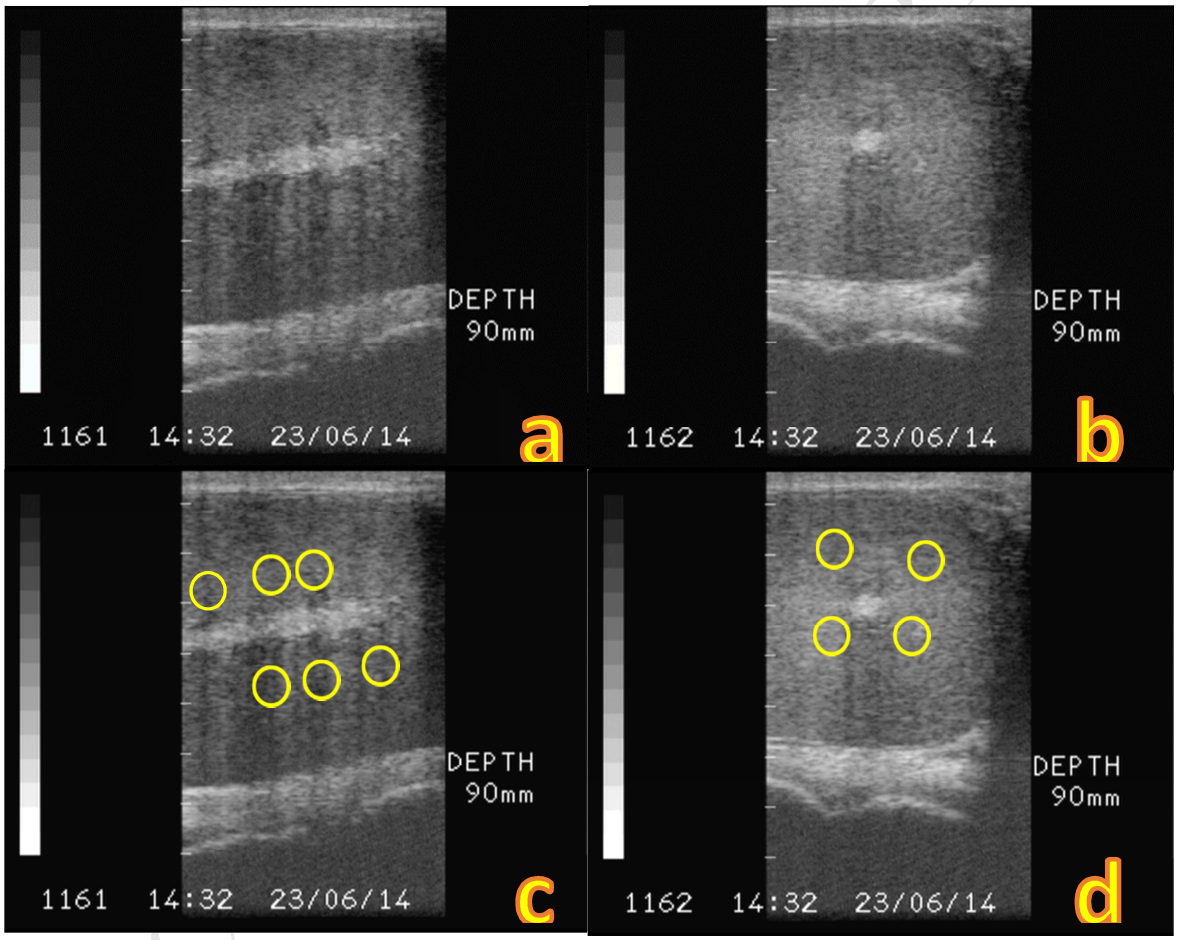

Figure 1. Ultrasonographic appearance of testicular images in a) + c) the vertical plane and b) + d) the horizontal plane. The areas selected for PI analysis corresponding to pictures a) and b) can be seen in c) and d).

Figure 2.

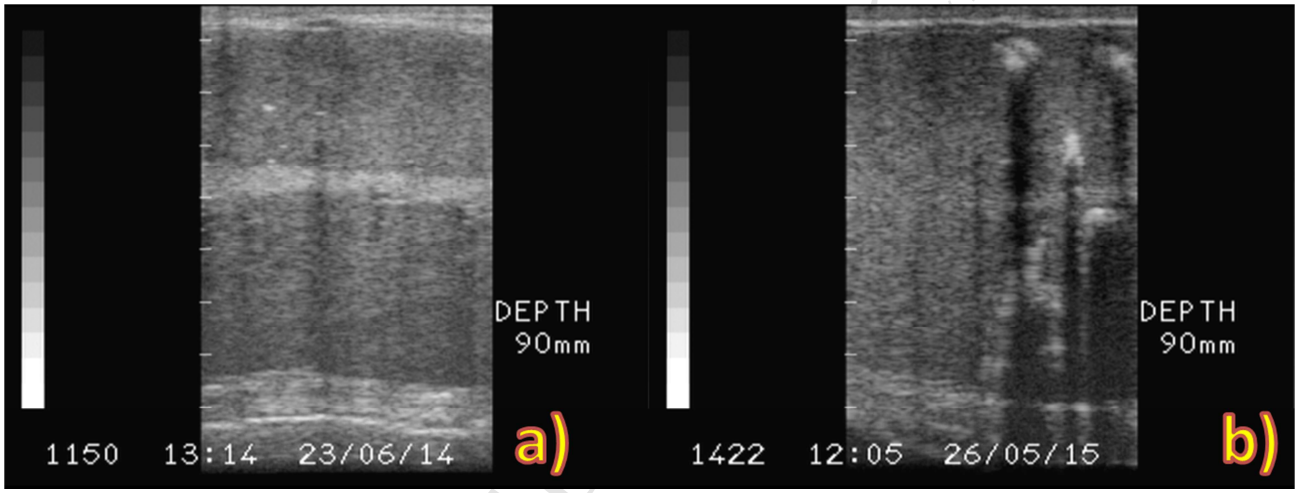

Figure 2: Ultrasonagraphic appearance of a testicular image in the vertical plane with a gross visual fibrosis score of a) 1 and b) 4 [18].

Figure 3.

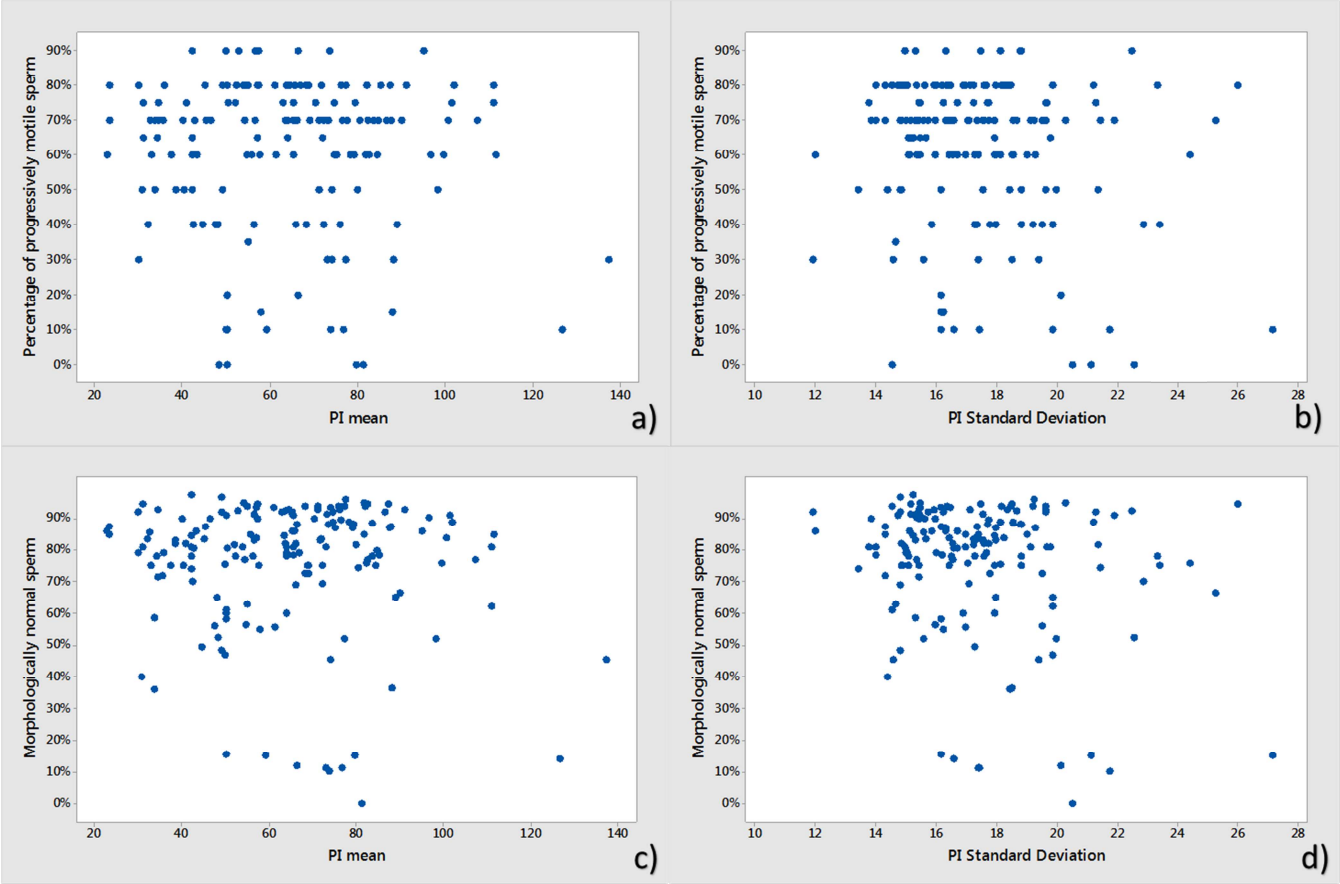

Figure 3. Comparison of PI at BBSE1 and semen parameters at BBSE1 for 162 bulls. a) PI mean and percentage of progressively motile spermatozoa (P= 0.448). b) PI standard deviation and percentage of progressively motile spermatozoa (P= 0.022)(r2=3.2%). c) PI mean and percentage of morphologically normal spermatozoa (P= 0.355). d) PI standard deviation and percentage of morphologically normal sperm (P=0.008)(r2= 4.3%).

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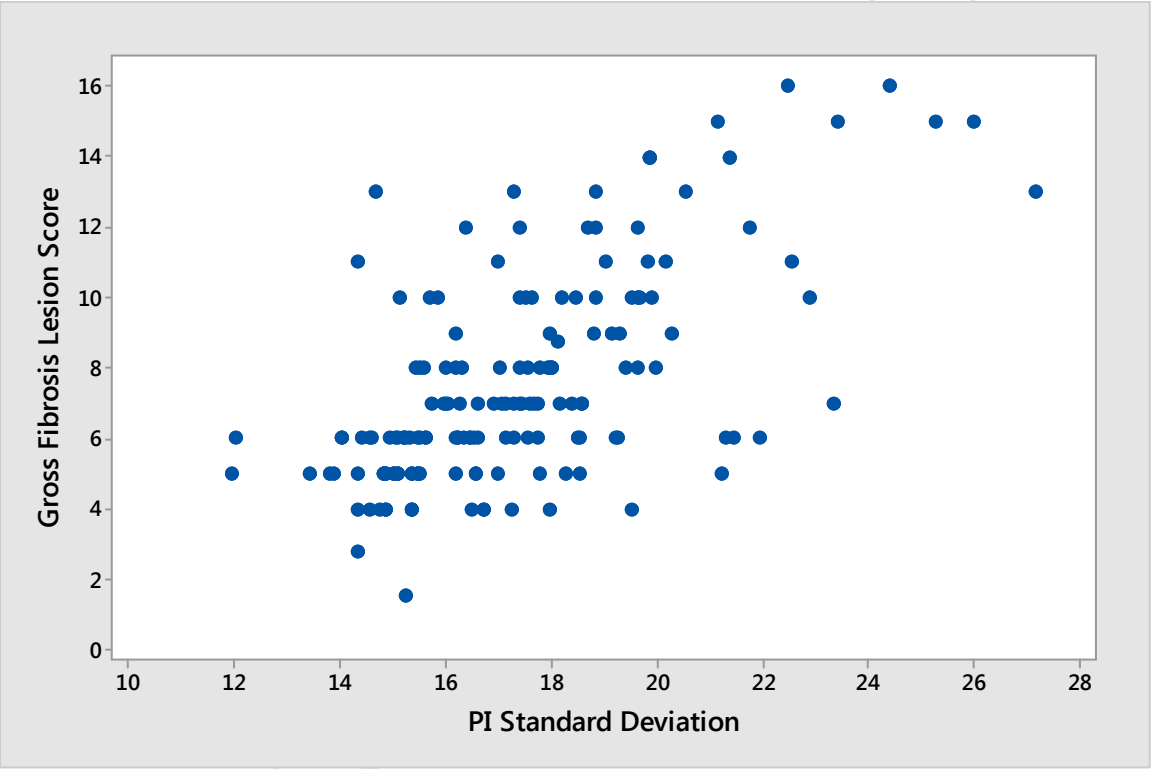
391 Figure 4.



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393 Figure 4. Correlation of gross fibrotic lesion score and PI standard deviation (P<0.001)(r2= 40.5%).

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Figure 5.

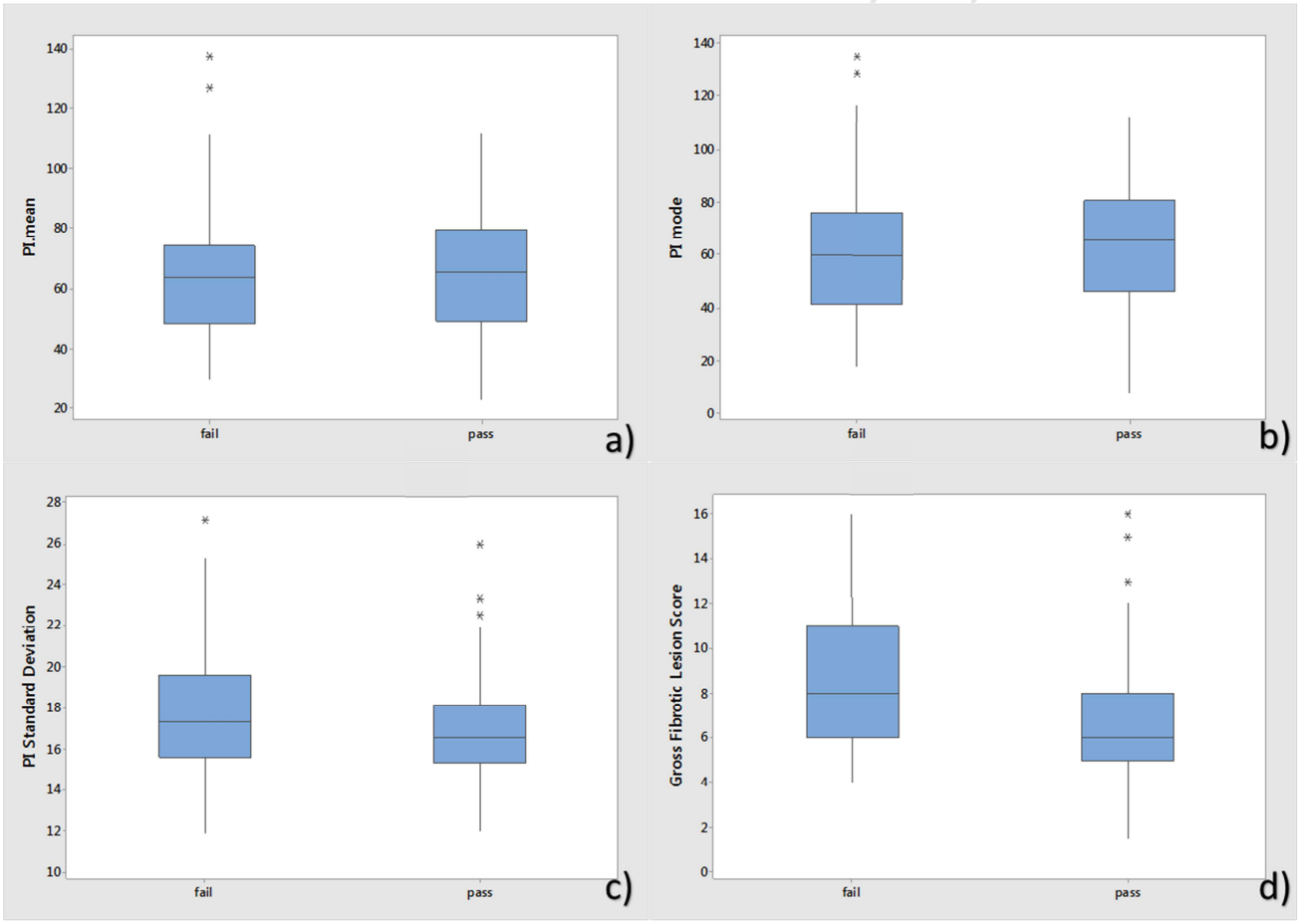

Figure 5. Pass/fail interactions between BBSE1 outcome and a) PI mean (P= 0.916), b) PI mode (P=0.785), c) PI standard deviation (P=0.052) and d) fibrotic lesion scores (P< 0.001) (T= 3.92) for 162 bulls.

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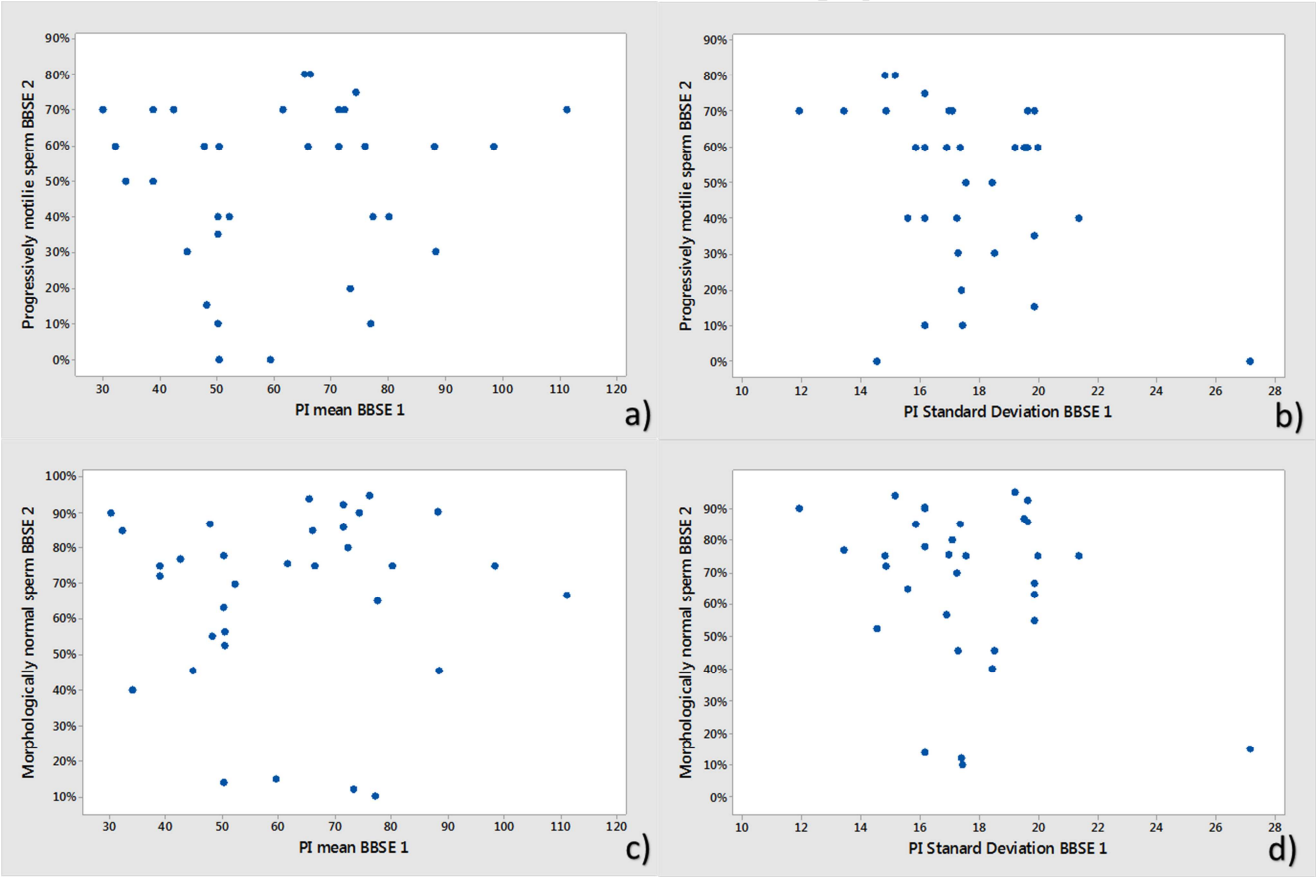
410 Figure 6.



411

412 Figure 6. Comparison of PI measurements at BBSE1 and semen parameters at BBSE2 for 33 bulls that
413 failed BBSE1. a) PI mean and percentage of progressively motile sperm (P=0.614), b) PI standard
414 deviation and percentage of progressively motile sperm (P= 0.044)(r2= 16.1%), c) PI mean and
415 morphologically normal sperm (P = 0.847) and d) PI standard deviation and morphologically normal
416 sperm (P = 0.119).

417

418

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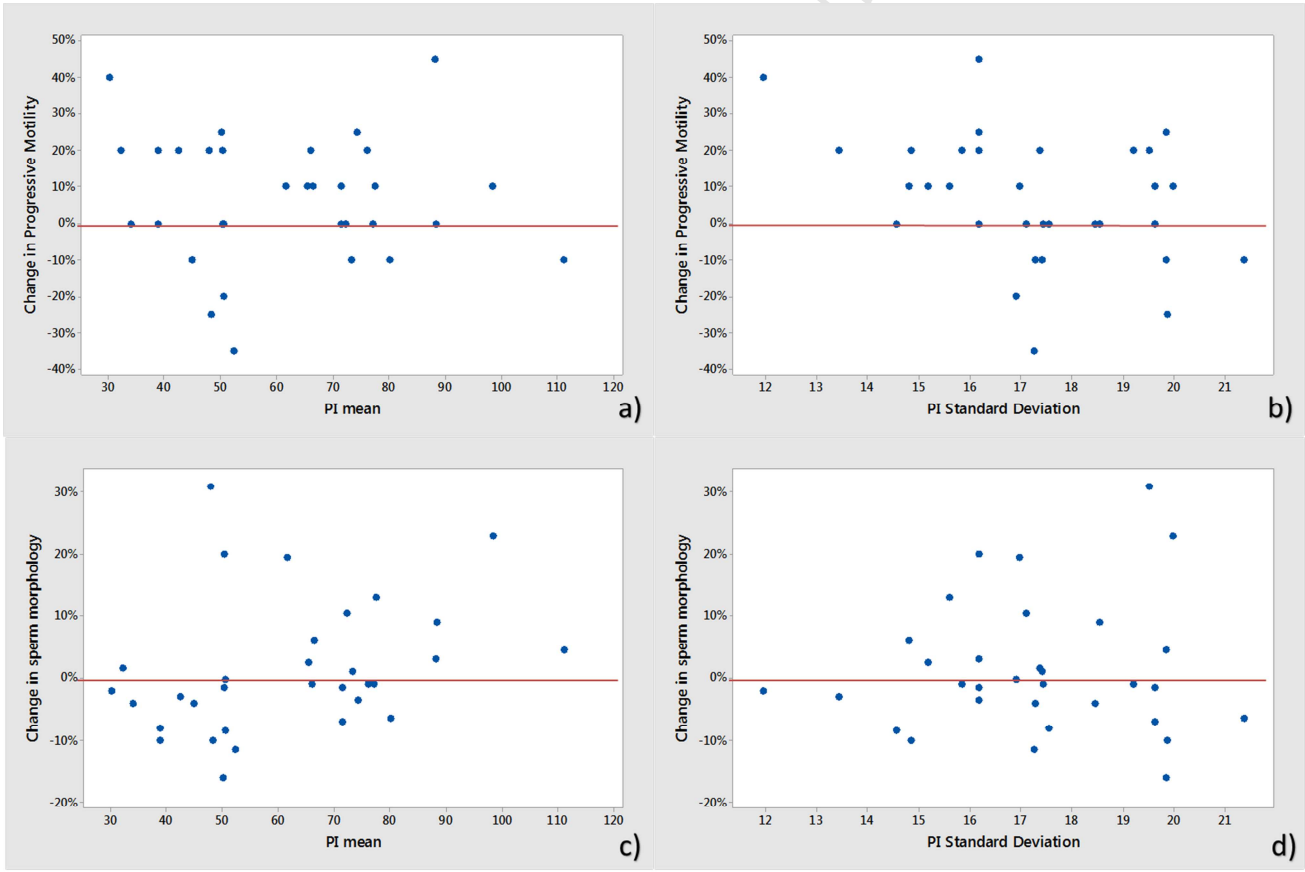
420

421 Figure 7.



422

423 Figure 7. Comparison of PI measurements at BBSE1 and change in semen parameters between
424 BBSE1 and BBSE2 for 33 bulls that failed BBSE1. a) PI mean and change of percentage of
425 progressively motile sperm (P=0.748), b) PI standard deviation and change of percentage of
426 progressively motile sperm (P=0.371), c) PI mean and change in morphologically normal sperm (P=
427 0.235) and d) PI standard deviation and change in morphologically normal sperm (P= 0.325).

- First field study using testicular ultrasound to aid in predicting future fertility
- Measurements were practical and repeatable in a field setting
- No biological correlation between semen quality and testicular pixel intensity
- Manual lesion scores are comparable to computer analysis in identifying fibrosis