Title

Influence of bull age, ejaculate number and season of collection on semen production and sperm motility parameters in Holstein Friesian AI bulls

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ABSTRACT: In the current era of genomic selection, there is an increased demand to collect semen from young genomically-selected elite sires at a young age. The objective of this study was to assess the effect of bull age, ejaculate number and season of collection on semen production (ejaculate volume, sperm concentration and total sperm number; TSN) and sperm motility (pre-freeze and post-thaw total and gross motility) parameters in Holstein Friesian bulls in a commercial artificial insemination (AI) centre. The study involved the interrogation of a large dataset collected over a four year period, (n=8,983 ejaculates; n=176 Holstein Friesian bulls aged between 9 months and 8 years). Bulls aged less than one year had the poorest semen production and sperm motility values for all parameters assessed compared to older bulls ($P < 0.01$). First ejaculates had a higher semen production and greater pre-freeze motility values than second consecutive ejaculates ($P < 0.01$), but despite this, there was no difference in post-thaw motility. When subsequent ejaculates were collected from bulls aged less than one year, semen production and sperm motility did not differ significantly compared to mature bulls. Semen collected in Winter was poorest in terms of sperm concentration and TSN, but best in terms of post-thaw motility ($P < 0.01$). In conclusion, second ejaculates can be collected from bulls aged less than one year without a significant decrease in post-thaw sperm motility, thus may be a useful strategy to increase semen availability from young genomically selected AI bulls in high demand.

INTRODUCTION

Artificial insemination (AI) is one of the most influential biotechnologies in the dairy breeding industry as it allows for the wide dissemination of superior genetics and promotes faster genetic gain (Oliveira et al. 2013). The success of an AI centre depends largely on the genetic merit of the bulls for economically-relevant traits as well as the quality and fertility of semen used (Lemma and Shemsu 2015). Therefore, AI studs need to manage their bulls and semen processing accordingly. While over the last decade the 'tool box' of semen evaluation techniques has expanded to include more sophisticated technologies such as computer-assisted sperm analyses (CASA) and flow cytometry, these measures in combination can still only explain, at best, approximately 40% of the variation in bull fertility (Sellem et al. 2015). Therefore, most AI studs worldwide still rely heavily on the routine evaluation of ejaculate volume, sperm concentration, motility (pre-freezing and post-thawing) and morphology via standard microscopic techniques (Chenoweth and McPherson 2016). Indeed, a number of studies have reported a significant relationship between semen traits, specifically sperm motility, and field fertility (Christensen and Lehn-Jensen 1999, Christensen et al. 2005). In recent years, the advent of genomic selection has allowed the dairy industry to reliably select AI bulls at a younger age and has thereby hastened genetic progress by reducing the generation interval (Goddard and Hayes 2007). However, there are major challenges to collecting sufficient high quality semen to meet demand from these elite young bulls. Therefore, identifying the relationships between factors affecting semen production and quality in both young and mature bulls would be useful for AI studs.

Semen quality can be affected by a wide range of genetic and environmental factors including bull age, collection interval, collection frequency and season (Fuerst-Waltl et al. 2006, Snoj et al. 2013, Brito et al. 2002a, Fiaz et al. 2010). It is widely acknowledged that the age of a bull at collection affects semen characteristics such as ejaculate volume and sperm concentration (Brito et al. 2002a, Mathevon et al. 1998), with older mature bulls having greater semen volume and quality than younger bulls (Fuerst-Waltl et al. 2006, Brito et al. 2002a). This increase is primarily believed to be due to physiological changes such as an increase in body mass (Balić et al. 2012) and the simultaneous development of the testis and accessory glands post-puberty and during sexual maturation which consequently leads to an increase in semen production (Almquist 1978). However, peak ejaculate volumes and total sperm number are achieved at different ages in different breeds (Snoj et al. 2013).

While the first ejaculate of a bull collected on a given day is typically of higher volume and sperm concentration compared to subsequent collections on the same day (Everett and Bean, 1982), collection of multiple ejaculates on the same day did not affect post-thaw sperm motility (Boujenane and Boussaq 2013). The reduction in semen production seems to be largely related to the short collection interval between consecutive ejaculates as longer intervals produce higher ejaculate volumes and total sperm number (TSN; Fuerst-Waltl et al. 2006, Mathevon et al. 1998). However, although in general, second ejaculates are of lower volume and sperm concentration, the collection of sequential ejaculates increases productivity per unit time, as more insemination doses can be obtained on a given day.

The effect of season on bovine semen production has been widely assessed (Mathevon et al. 1998, Stälhammar et al. 1989, Brito et al. 2002a, Malama et al. 2017); however, data are conflicting, perhaps due to the range of climatic conditions under which these studies have been carried out (Wildeus and Hammond 1993, Brito et al. 2002b). Studies reporting seasonal variation in semen characteristics have mainly attributed these changes to compromised scrotal thermoregulation and heat dissipation mechanisms (Menegassi et al. 2015) as well as the endocrine profile and the differential response of bull testes to gonadotropins (Jiménez-Severiano et al. 2003). Seasonal variations associated with photoperiod, in particular luteinising hormone, testosterone concentrations and melatonin levels, have been shown to affect spermatogenesis (Tatman et al. 2004, Lincoln et al. 1996, Godfrey et al. 1990) as sperm output, libido, semen volume and fertility are under direct endocrine control (Convey et al. 1971). Moreover, the adaptability of a bull to local microclimatic conditions may have consequences for semen quality (Nichi et al. 2006) and therefore, could account for differences in their reproductive capacity throughout the year.

For younger bulls in particular, age at puberty and subsequent sexual maturity is paramount for their success in an AI programme. Due to the rapid advancement in genomics, there is an interest in AI bull production systems which hasten the onset of maturity (Byrne et al. 2017, Dance et al. 2015), thus enabling AI centres to collect semen from bulls at a younger age, reducing production costs, shortening the generation interval and increasing genetic gain. However, the reproductive performance of young bulls varies greatly mainly due to the large variation in the age of onset of puberty among and within breeds (Barth et al. 2008). Thus, increasing semen output of bulls, particularly young bulls and those in high demand, by adapting management practices such as determining appropriate sexual preparation and collection frequency would enable AI centres to increase the dissemination of genetic merit of superior bulls.

Given this background, the aim of this study was to assess the effect of bull age, ejaculate number and season of collection on semen production and quality characteristics in Holstein Friesian AI bulls.

MATERIALS AND METHODS

Animal management

Semen collection data from Holstein Friesian bulls (n = 176 bulls, n = 8,983 ejaculates), ranging between 9 months to 8 years of age (Figure 1), from Ireland's largest AI centre (National Cattle Breeding Centre, Naas, County Kildare) were used in this study. Bull semen production and quality records over a period of four years (2012 to 2016) were analysed. Data were categorised according to season of collection: Spring (February, March, April), Summer (May, June, July), Autumn (August, September, October) and Winter (November, December, January). Mean temperature over the four years during these periods was 7, 14, 13 and 6 ^{o}C, respectively (Met Éireann, 2017) with a mean number of collections per day of 33, 25, 23 and 17 for Spring, Summer, Autumn and Winter, respectively. Bulls were individually housed in a barn with ambient (i.e., unregulated) temperature, fed and maintained under similar management and feeding conditions. Bulls were fed a standard ration of 85% dry matter haylage as well as approximately 5 kg of a 14% protein cereal-based ration daily with *ad libitum* access to water. The mean age of the bulls analysed in Spring, Summer, Autumn and Winter was 24 ± 0.20, 26 ± 0.25 and 29 ± 0.29 and 28 ± 0.31 months, respectively. Typically, semen was collected from a bull on one to three days per week, depending on demand. In cases where semen from a particular bull was in very high demand, regardless of bull age, a second ejaculate was collected within 1 h of the first collection (as described below).

Semen Collection and Processing

All bulls were sexually stimulated using a teaser bull and allowed to false mount a minimum of three times. The time between each false mount varied between individual bulls but was determined by an experienced barn technician and typically took no longer than 2-3 min. Semen was collected from all bulls using an artificial vagina, once bulls were deemed to be sufficiently stimulated. This method of stimulation and collection was similar for all collections, regardless of the ejaculate number, and remained constant from year to year. Ejaculates were kept separate throughout and were initially partially diluted in 10 mL pre-warmed (37 ^{o}C) BullXcell extender (IMV Technologies, L'Aigle, France) and transported in a temperature-regulated box at 18 ^{o}C to the laboratory (within 3 h). On arrival, the ejaculate was assessed for weight to determine volume, sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland), total motility (% of the total sperm population both motile and non-motile) and gross motility (5-point scale: 1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard. Initial quality control cut-off values were a total and gross motility of $\geq$70% and a score of $\geq$3, respectively. Any ejaculates failing to meet these criteria were rejected for commercial production but the data were still included in this study. Although morphology was not accurately assessed, consideration was given to the percentage of morphological abnormalities within an ejaculate by the experience laboratory technician and the degree of abnormality was reflected upon when assessing motility.

Following *in vitro* assessments, the ejaculate was fully extended in BullXcell to a final concentration of 15 x 10^6 sperm per 0.25 mL semen straw (IMV Technologies). Straws were filled, sealed and printed as per routine procedures using the IS4 instrument (IMV Technologies). Straws from each ejaculate were then cooled to 4 ^{o}C over 3 h and were frozen to -140 ^{o}C as follows: -5 ^{o}C per min from +4 ^{o}C to -10 ^{o}C, -40 ^{o}C per min from -10 ^{o}C to -100 ^{o}C and thereafter -20 ^{o}C per min from -100 ^{o}C to -140 ^{o}C in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at -196 ^{o}C until use (Murphy et al. 2017). Four straws from each ejaculate of each bull were assessed immediately post-thaw via standard microscopic techniques for total and gross motility. Post-thaw quality control cut-off values were a total and gross motility of $\geq$50% and a score of $\geq$3, respectively.

2.2.1 Assessment of sperm motility

Sperm motility (total and gross) was assessed pre-freezing and post-thawing using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of 400 X. A droplet (5 µL) of diluted semen was placed on a pre-warmed glass slide and covered with a pre-warmed coverslip (18 x 18 mm; 37 ^{o}C). Total motility was assessed by counting a minimum of 100 sperm over at least five different fields of view, while gross motility was evaluated by assessing the swimming pattern of the entire sperm sample on a scale of 1 to 5 as described above. Total motility was expressed as a percentage of the total sperm population (motile and non-motile).

Statistical Analysis

Data were checked for normality and homogeneity of variance using histograms, QQ plots, and formal statistical tests in the Univariate procedure (version 9.1.3; SAS Institute, Cary, NC, USA). Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS. Semen production (ejaculate volume, sperm concentration per mL and TSN and sperm quality (pre-freeze and post-thaw total and gross motility) parameters were analysed using the MIXED procedure of SAS with a model that included fixed effect of bull age, season and ejaculate number. All two- and three-way interactions were tested for among the main factors. Bull was included as a random effect. Differences among means were determined by F-tests using Type III sums of squares. The PDIFF option and the Tukey test were applied to evaluate pairwise comparisons between means. Where appropriate, spearman partial correlation analysis was carried out between variables using the PROC CORR accounting for year and GLM procedure of SAS was also used to determine the relationships responses between the main factors and semen production and quality variables.

RESULTS

Effect of bull age on semen production and quality

There was an effect of bull age on ejaculate volume, TSN ($P < 0.01$) and sperm concentration (Figure 1; $P < 0.05$) as well as pre- and post-thaw total and gross sperm motility (Figure 2; $P < 0.01$). Semen quality, as assessed by pre-freeze and post-thaw total and gross motility was lowest for bulls collected at less than one year of age compared to all other age categories; however, the difference in motility scores between bulls aged less than one year and older bulls was small (~2%) and unlikely to be of biological or commercial importance (Figure 2; $P < 0.01$). Ejaculate volume was strongly positively correlated with bull age ($r = 0.62$; $P < 0.01$) and increased by approximately 0.5 mL per year. As a result TSN also increased with age, with bulls aged less than one year producing the lowest ejaculate volume and TSN, while bulls aged more than four years produced the largest semen volume and TSN (Figure 1; $P < 0.01$). There was a linear increase in TSN with increasing ejaculate volume ($r = 0.71$; $P < 0.01$). Bulls aged between one and two years had a greater sperm concentration per ejaculate than bulls aged less than one or more than two years old (Figure 1; $P < 0.05$).

Effect of ejaculate number on semen production and quality

There was an ejaculate number by bull age interaction on volume ($P < 0.05$), sperm concentration and TSN ($P < 0.01$) as bulls aged greater than one year had a reduced ejaculate volume, sperm concentration and TSN in their second ejaculate (Figure 3; $P < 0.01$); however, there was no effect of ejaculate number on bulls aged less than one year (P

> 0.05). There was an effect of ejaculate number on ejaculate volume, sperm concentration, TSN (Figure 3: P < 0.01) as well as pre-freeze total and gross motility (Figure 4; P < 0.01). First ejaculates exhibited higher pre-freeze total and gross motility scores (P < 0.01) and had a higher ejaculate volume and sperm concentration than second ejaculates, by approximately 15 and 40%, respectively (P < 0.01). However, ejaculate number did not affect post-thaw total and gross motility (Figure 4; P > 0.05). Overall, first ejaculates resulted in more than twice the number of total sperm than a second consecutive collection, with a TSN of 5.5 and 2.6 x 10^9, respectively, primarily as a result of the large difference in sperm concentration between subsequent ejaculates.

Effect of season of collection on semen production and quality

There was a season by bull age interaction for ejaculate volume, sperm concentration, TSN, post-thaw gross motility (P < 0.01) and pre-freeze gross and post-thaw total motility (P < 0.05). Although bulls aged less than one year had a reduced ejaculate volume, sperm concentration and TSN in Winter than any other season, there was no clear biological pattern for any other age category. There was an effect of season on sperm concentration, TSN (Figure 5; P < 0.01) as well as post-thaw total and gross motility (Figure 6; P < 0.01). There was a tendency for season to affect ejaculate volume (P = 0.065) with semen collections in Spring having the lowest volume. Semen collections in Winter had the greatest post-thaw total and gross motility score compared to semen collections in Spring (Figure 6; P < 0.01). Ejaculates collected in Summer and Autumn had higher sperm concentration and TSN in comparison to Spring and Winter (Figure 5; P < 0.01). Thus, regardless of the parameter assessed, semen collections in Winter resulted in the poorest semen production output, while collections in Summer and Autumn had the best semen production characteristics in terms of sperm concentration and TSN.

DISCUSSION

This study involved the interrogation of a large dataset collected over a period of four years, involving a total of 8,983 ejaculates from 176 Holstein Friesian bulls aged between 9 months and 8 years of age, thereby facilitating an in-depth assessment of sperm motility of AI bulls in a comprehensive attempt to identify factors affecting semen production and quality in a commercial AI setting. The main novel findings of this study were that second ejaculates can be collected from young bulls without a concomitant significant decrease in post-thaw sperm motility and thus may be a useful strategy to increase semen availability from AI bulls in high demand. This study also clearly illustrates the challenges surrounding the collection of ejaculates of sufficient volume and quality from bulls of less than one year of age.

The observed increase in volume and TSN associated with increasing bull age is consistent with a number of other reports (Everett and Bean 1982, Mathevon et al. 1998, Taylor et al. 1985, Brito et al. 2002a). Since sperm concentration remained constant after 1 year of age the increase in TSN with age was being driven by increases in ejaculate volume up to four years of age, consistent with the findings of Everett and Bean (1982). Not surprisingly, bulls aged less than one year had the lowest values (Al-Kanaan et al. 2015). It is widely acknowledged (Perumal 2014, Karabinus et al. 1990, Mathevon et al. 1998) that peri-pubertal bulls have lower ejaculate volumes than mature bulls, which is in agreement with the findings in this study, and that the pre-pubertal period is generally characterised by rapid increases in both body and testicular weight (Aponte et al. 2005). Therefore, the increase in ejaculate volume with age may be related to an increase in activity of the hypothalamic-pituitary-testicular axis and the concurrent development of the testis and accessory glands with sexual maturity, which are believed to continue to develop for up to 5 years post-puberty (Almquist 1978). Following the onset of puberty (as defined by Wolf et al., 1965), at approximately 9-11 months of age in Holstein Friesian bulls (Byrne et al. 2017; Dance et al. 2015), the reproductive capacity of a bull increases for several years until sexually maturity is reached (Amann 1983)

In the current study, there was an ejaculate number by bull age interaction for ejaculate volume, sperm concentration and TSN for bulls older than one year of age, with the collection of a subsequent ejaculate resulting in lower semen production values. First ejaculates had a greater volume, sperm concentration and TSN for bulls older than one year in comparison to second ejaculates collected on the same day; however there was no effect of ejaculate number on bulls aged less than one year. This finding is similar to that of Fuerst-Waltl et al. (2006) and Bhakat et al. (2011) as first ejacuales recorded greater semen production values for all age categories; however, these studies did not investigate the effects on bulls aged less that one year with the lowest bull age category for each study of 16-18 months and less than three years, respectively. Conversely, although volume increased with bull age, ejaculate number did not significantly affect ejaculate volume for all ages as bulls aged between 2-3 years and greater than 4 years recorded similar volumes for both collections. Surprisingly, the effect of ejaculate number was not significant in bulls aged less than one year for any semen production parameter. This may be primarily due to the significantly lower semen production values associated with young bulls and the large variation within their analysis compared to more mature bulls. Furthermore, although semen production decreases with the collection of multiple ejaculates on the same day, the overall production of semen increases, resulting in an increase in the number of semen doses produced per bull per day. To place it in perspective, for an average bull, the first and second consecutive ejaculates typically produce approximately 400 and 200 straws, respectively, with a concentration of 15 x 10^6 sperm per 0.25 mL straw. Therefore, a bull collected twice a day, twice a week (1,200 straws) compared to once a day, twice a week

(800 straws) would result in increasing overall production by 400 straws. Thus, a second collection may be justified for bulls which are in high demand, particularly those less than one year of age, as semen production from these young bulls was not negatively impacted by the collection of a second ejaculate.

Additionally, in the current study first ejaculates recorded a higher pre-freeze total and gross motility score than second ejaculates which is in agreement with Fuerst-Waltl et al. (2006) who reported a higher percentage of motile sperm in first ejaculates. However, similar to the findings of Boujenane and Boussaq (2013), there was no difference observed in post-thaw motility. One possible explanation behind a reduction in semen production and pre-freeze sperm quality associated with second ejaculates may be due to the shortened collection interval of the second ejaculate (although not formally assessed in this study the norm was within 1 h). Longer collection intervals have been reported to result in greater semen production and quality, however, these collection interval vary from 3-4 to 10 days (Mathevon et al. 1998, Everett and Bean 1982, Fuerst-Waltl et al. 2006) but are unrealistic in a commercial environment setting. Due to the high demand, it is impractical for AI centres to allow up to 10 days between collections. In the current study, critically there was no effect of ejaculate number on post-thaw sperm motility in which a collection interval of approximately 1 h was implemented. This is important as the ability of sperm to maintain their functional status post-thaw in both first and second ejaculates is essential considering that AI in cattle is primarily implemented with the use of cryopreserved semen (Thibier and Wagner 2002). Therefore, the results of the current study highlight that semen production and quality can be maintained with a shorter collection interval of approximately 1 h, hence increasing productivity.

The effect of season on semen production has been widely assessed in the bull (Malama et al. 2017, Snoj et al. 2013, Bhakat et al. 2014, Al-Kanaan et al. 2015).

Spermatogenesis has been shown to be susceptible to temperature variation (Rahman et al. 2011) and as it takes approximately 61 days in the bull (Johnson et al. 2000), the quality of sperm in an ejaculate may reflect conditions to which the bull was exposed 8-9 weeks prior to collection. The impact of many environmental factors, however, is reduced when bulls are maintained in temperature-controlled barns (Haugan et al. 2005) as other studies have shown that neither temperature nor humidity affected sperm production or semen quality (Brito et al. 2002b, Taylor et al. 1985). Under the temperate climatic conditions of the current study, there was a season by bull age interaction on semen production; ejaculate volume, sperm concentration, TSN and semen quality; post-thaw total motility and pre- and post-thaw gross motility. Bulls aged less than one year recorded poorest semen production values in terms of volume, sperm concentration and TSN in Winter than any other season; however, there was no clear biological pattern for any other age category. Semen collections in Summer recorded the highest values for sperm concentration and TSN; however, this did not differ from collections in Autumn. This result is broadly in agreement with Stälhammar et al. (1989) and Snoj et al. (2013) as they observed greater sperm concentration and TSN during the Summer months than in any other season in AI centres located in Sweden and Slovenia, respectively. Furthermore, Winter collections recorded significantly higher post-thaw sperm motility values; but, while there was a statistical difference in sperm motility, the difference between seasons was relatively small and is unlikely to be of biological importance or have a significant impact on quality control in a commercial environment as all values recorded were sufficient to pass quality control analysis. The results of the current study are consistent with Sullivan and Elliott (1968) who reported that semen collections in the US in Winter resulted in higher non-return rates than those in Spring, which may be related to better semen quality in line with the current study. Similarly, Boujenane and Boussaq (2013) reported that semen collected in a Moroccan AI centre in Winter was of higher quality than Summer collections.

CONCLUSION

In conclusion, this study characterised the challenges surrounding the collection of young Holstein Friesian bulls in a commercial AI setting. The low semen ejaculate volume typically associated with young bulls not only reduces sperm numbers but also pre-freeze and post-thaw sperm motility. As these young bulls are typically of a higher genomic value compared to older bulls, AI centres require large quantities of their semen in order to meet demand and therefore, need to minimise the amount of inferior quality semen being handled. The collection of a second consecutive ejaculate, although having a significantly lower volume and TSN, with the exception of bulls less than one year, does not affect post-thaw sperm quality and therefore, should be considered, particularly for bulls in high demand.

DECLATRATION OF INTEREST

The authors declare that they have no financial or personal relationship with other people or organisations that could inappropriately influence or bias the paper entitled "Influence of bull age, ejaculate number and season of collection on semen production and sperm quality characteristics in Holstein Friesian AI bulls".

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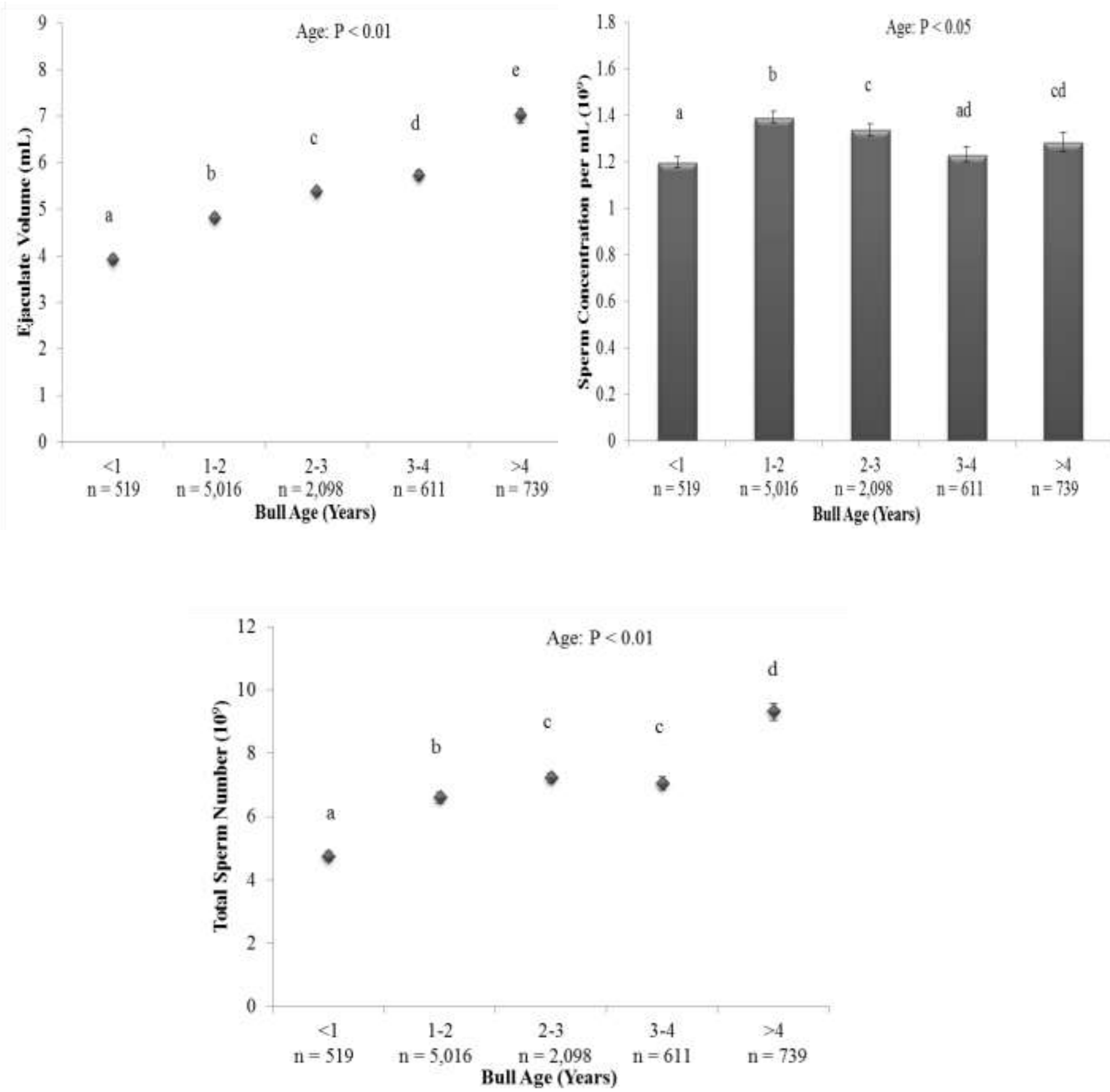

Figure 1: The effect of bull age on ejaculate volume (upper left panel), sperm concentration (upper right panel) and total sperm number (lower panel). Vertical bars represent s.e.m. [abcde]Differing superscripts differ between bull ages within each parameter ($P < 0.01$). n = number of ejaculates.

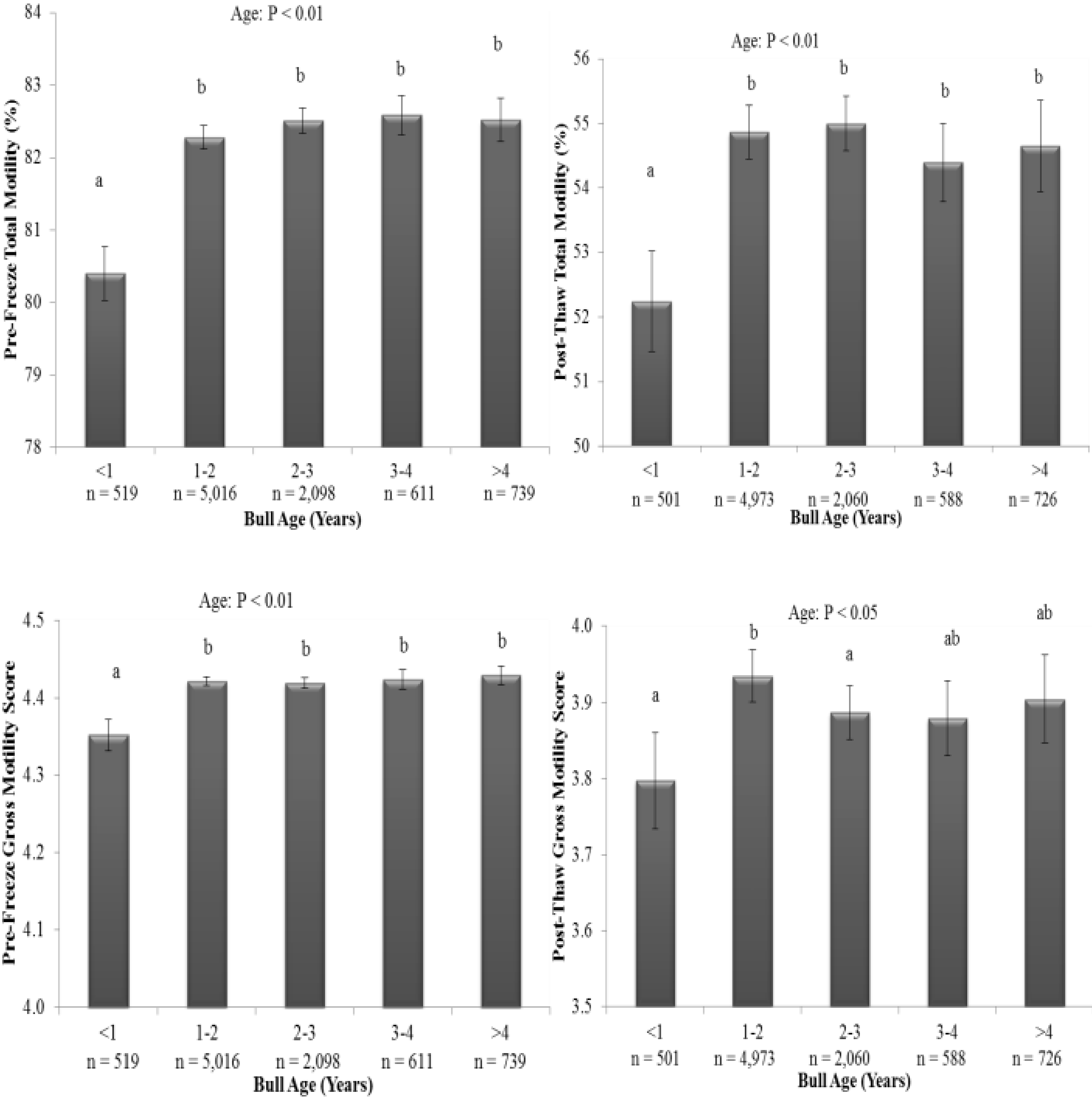

Figure 2: The effect of bull age on pre-freeze (upper left panel) and post-thaw total motility (upper right panel) and pre-freeze (lower left panel) and post-thaw gross motility (lower right panel). Vertical bars represent s.e.m. [ab]Differing superscripts differ between bull ages within each parameter ($P < 0.01$). n = number of ejaculates.

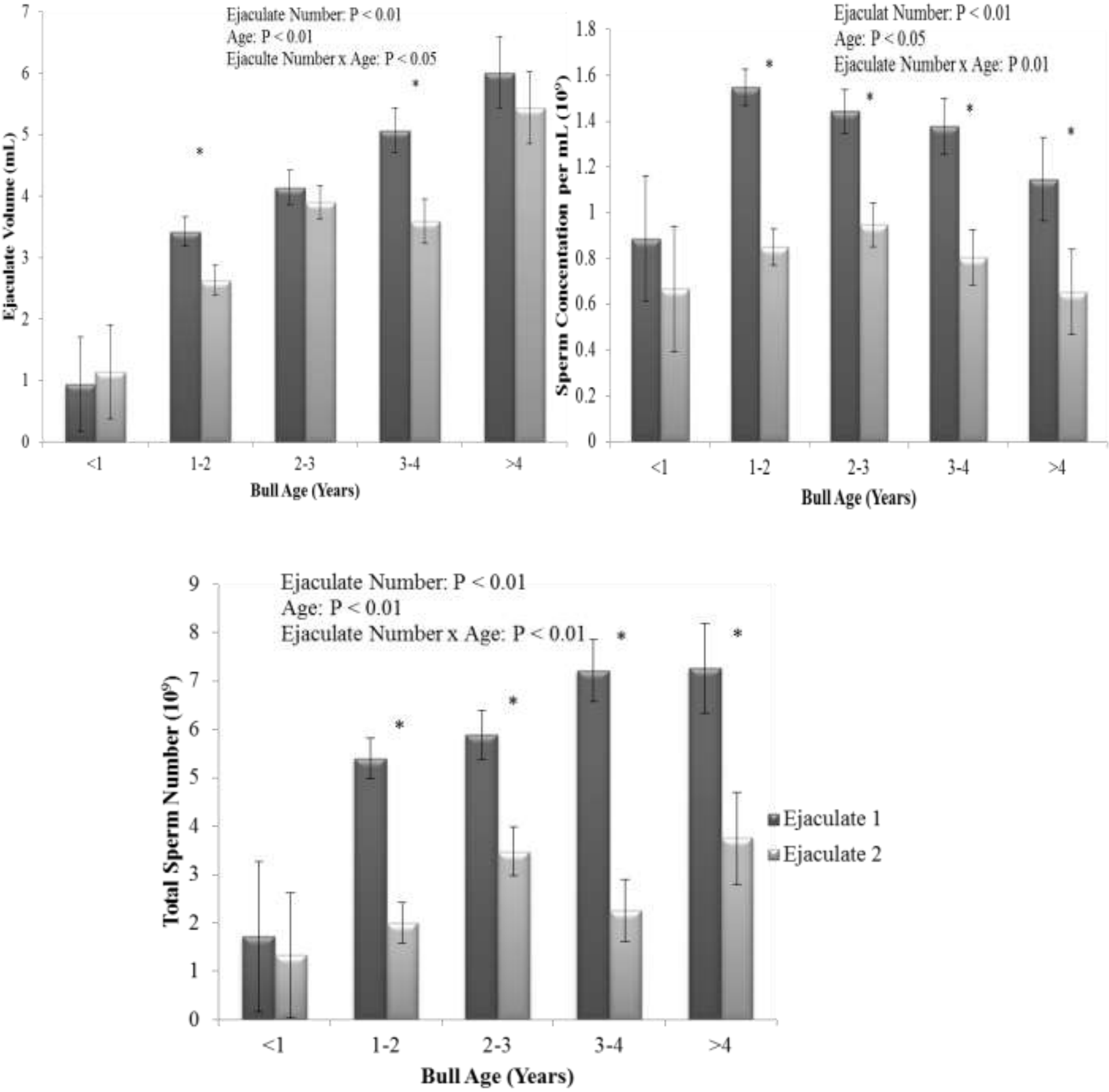

Figure 3: The interaction of ejaculate number and bull age on ejaculate volume (upper left panel), sperm concentration (upper right panel) and total sperm number (lower panel). Vertical bars represent s.e.m. *Asterisk represents differences between ejaculate number within each parameter ($P < 0.01$).

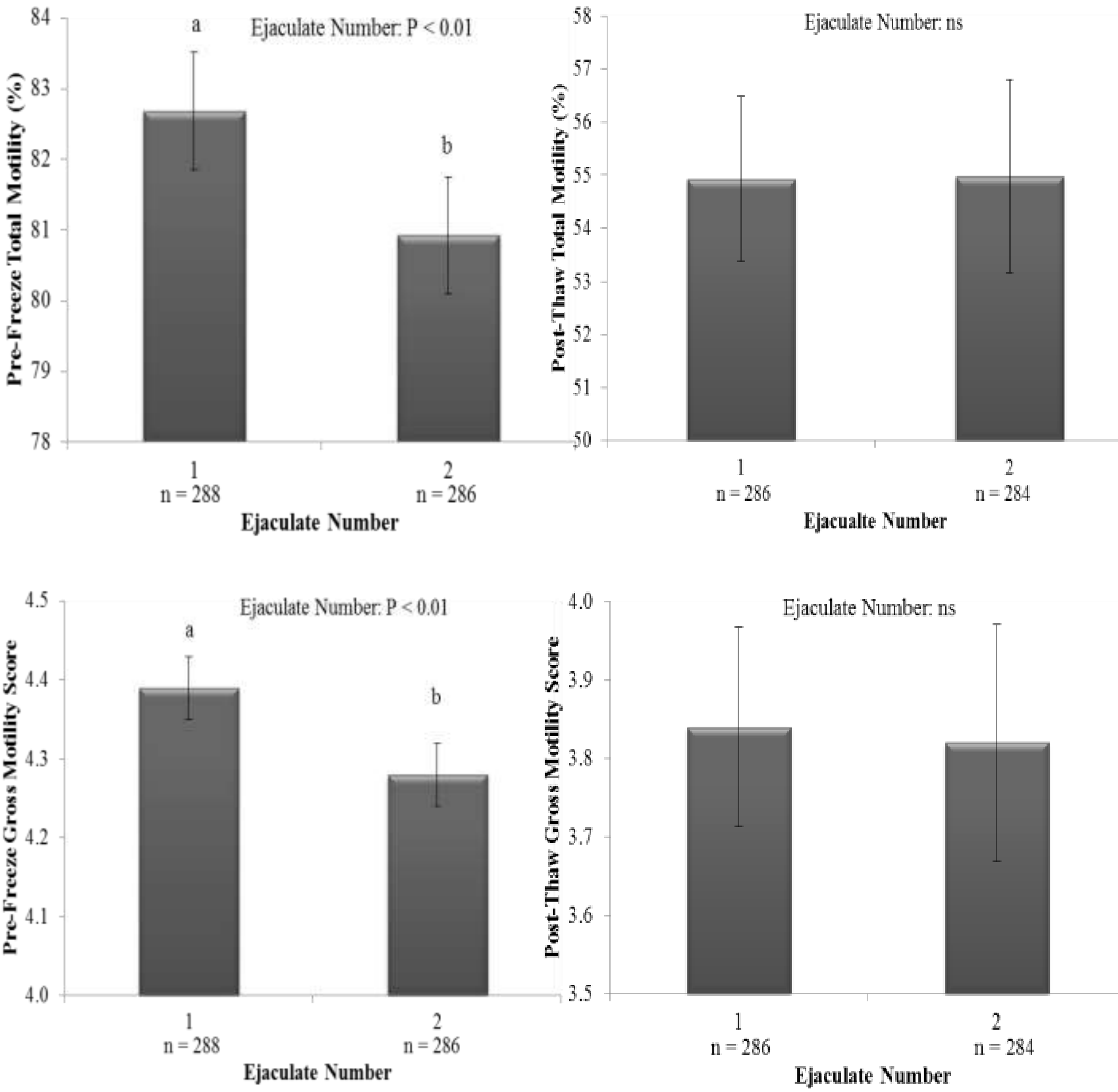

Figure 4: The effect of ejaculate number on pre-freeze (upper left panel) and post-thaw total motility (upper right panel) and pre-freeze (lower left panel) and post-thaw gross motility (lower right panel). Vertical bars represent s.e.m. [ab]Differing superscripts differ between ejaculate numbers within each parameter (P < 0.01). ns = not significant, n = number of ejaculates.

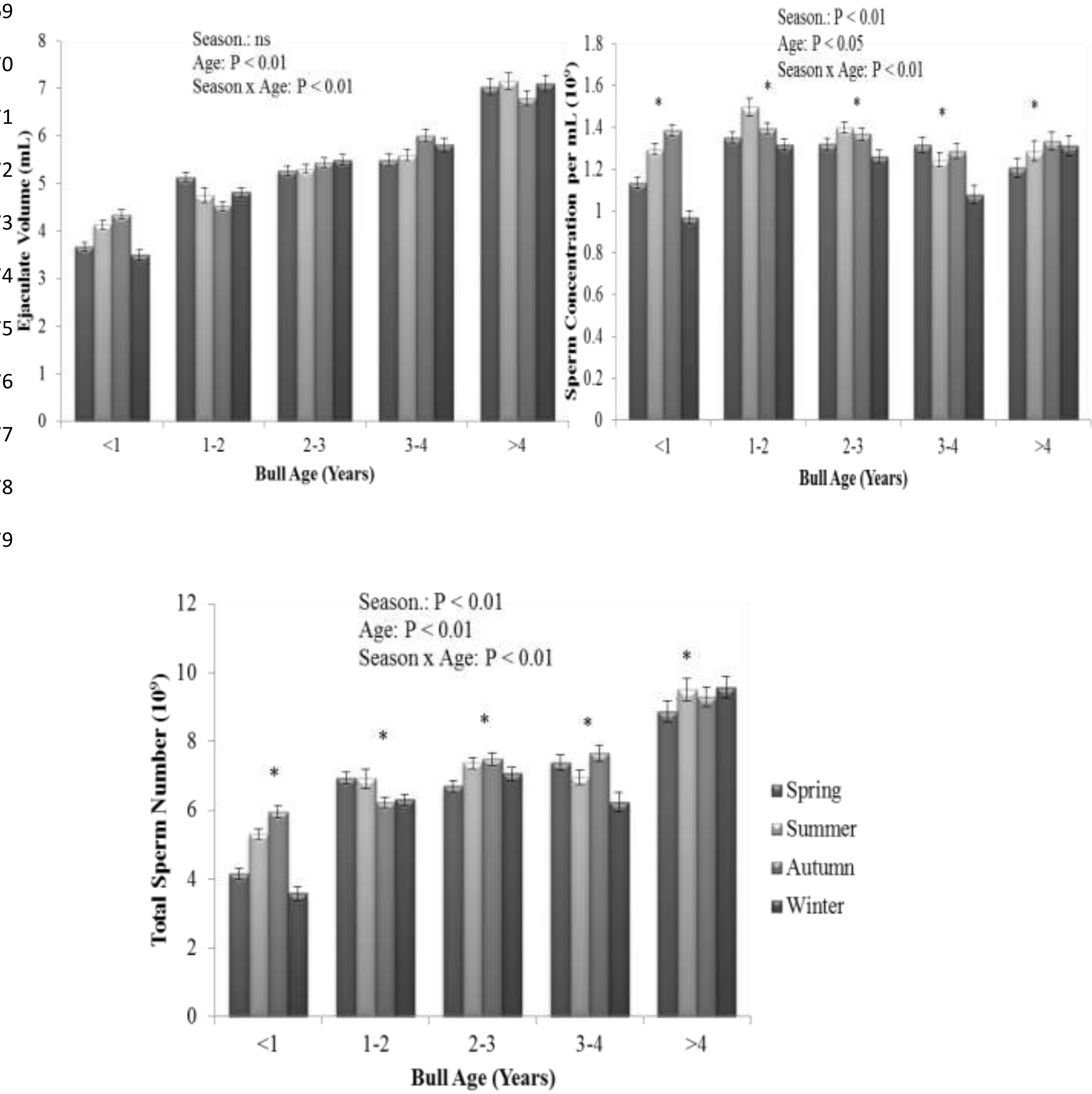

Figure 5: The interaction of season of collection and bull age on ejaculate volume (upper left panel), sperm concentration (upper right panel) and total sperm number (lower panel). Vertical bars represent s.e.m. *Asterisk represents differences between season within each parameter ($P < 0.01$). ns = not significant.

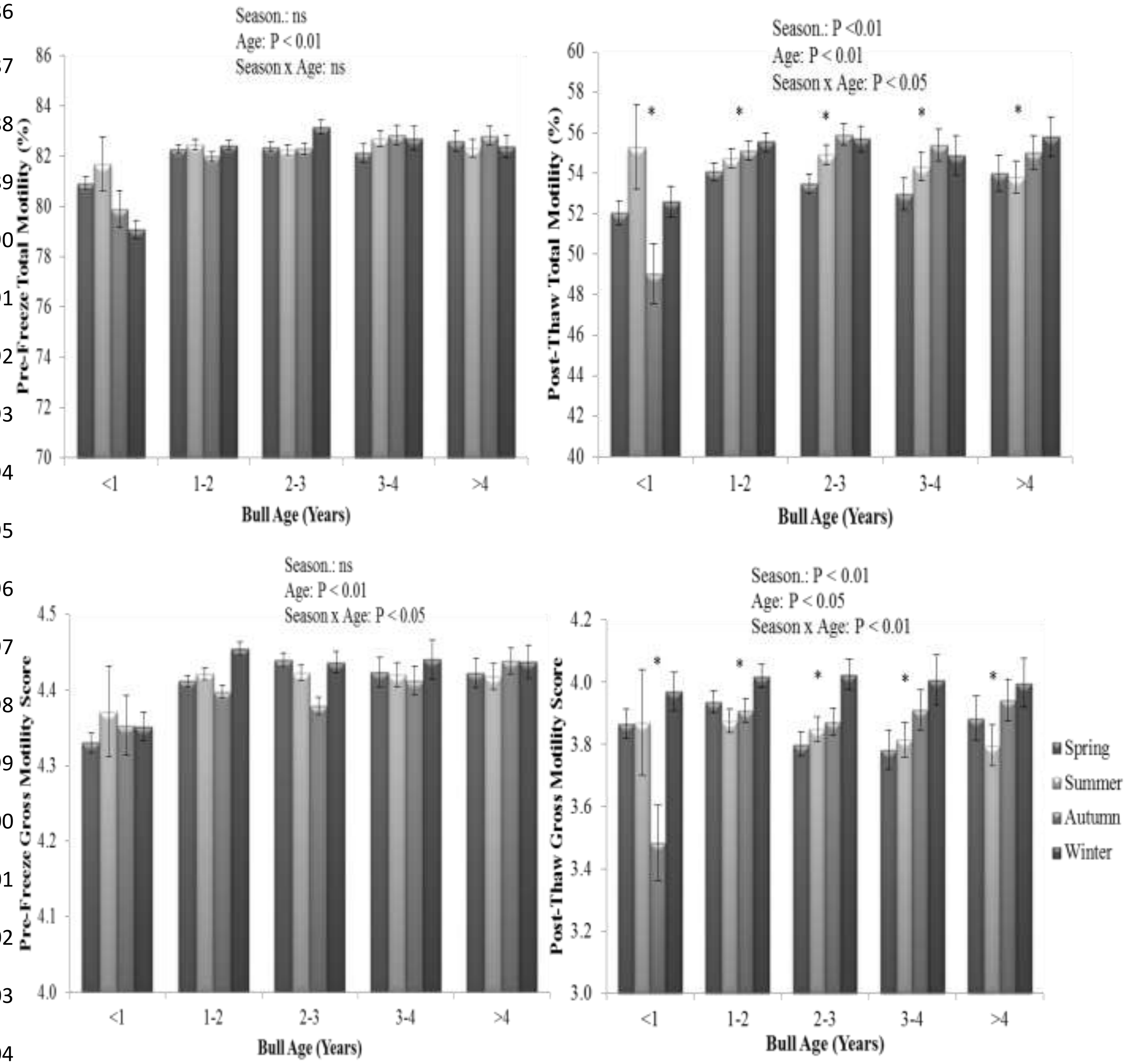

Figure 6: The interaction of season of collection and bull age on pre-freeze (upper left panel) and post-thaw total motility (upper right panel) and pre-freeze (lower left panel) and post-thaw gross motility (lower right panel). Vertical bars represent s.e.m. *Asterisk represents differences between season within each parameter (P < 0.01). ns = not significant.

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