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Comparison between primary sex ratio in spermatozoa of bulls and secondary sex ratio in the deriving offspring

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ABSTRACT

The objectives of the present work were to compare the primary sex ratio in sperm with the secondary sex ratio recorded in the offspring produced by artificial insemination (AI) with the same sperm and assess whether the primary sex ratio is influenced by sperm survival and motility after thawing. Calving data of 98 Holstein Friesian bulls used in AI were collected during 4 years, and commercial semen of the same bulls was analyzed immediately after thawing and after swim-up using a real-time polymerase chain reaction method developed and validated in our laboratory. Calving data relative to single bulls did not reveal any significant deviation between genders from the theoretical 1:1 for none of the bulls, being the mean values of male and female calves born 52.1 ± 2.80% and 47.9 ± 2.71%, respectively. Thereafter, calving events of bulls were classified and analyzed according to four classes of years: 2009 (n = 13,261), 2010 (n = 21,551), 2011 (n = 24,218), and 2012 (n = 41,726), and seasons categorized as winter, spring, summer, and fall. When data aggregated per years were analyzed, the difference between the two sexes was significant ($P < 0.005$) in favor of the male gender, whereas no influence of the season was evidenced. Real-time polymerase chain reaction did not evidence any difference between the mean values of frequency of Y chromosome–bearing sperm detected in three sperm batches of the same bulls analyzed immediately after thawing (51.1 ± 2.1), nor a difference with respect to the theoretical 1:1 ratio was reported after sperm analysis of one batch of sperm of the bulls analyzed after swim-up and immediately after thawing (50.1 ± 2.1 and 49.8 ± 1.8, respectively). The results are consistent with the observation of the farmers who often report a skewed sex ratio of the calves being born with AI in favor of the male gender. However, we have not evidenced differences in the primary sex ratio with respect to the theoretical 1:1 ratio both at thawing and after swim-up, thus demonstrating that the freezing procedure itself does not impact selectively on the survival of the X or Y chromosome–bearing sperm. Therefore, we hypothesize that the difference between genders observed after AI is more likely due to the events occurring after fertilization, which can comprise an impaired function of the X- or Y-bearing sperm with consequences on embryo development or a maternal influence.

1. Introduction

In mammals, the presence or absence of the Y chromosome in the fertilizing spermatozoa determines the sex of the offspring. The primary sex ratio is thus defined as the ratio of male to female zygotes or the percentage of sperm bearing the Y chromosome in the ejaculate, whereas the secondary sex ratio is calculated at birth.

Since 1970, several approaches have been experienced with the attempt to evaluate the primary sex ratio in the ejaculate of mammals. This interest arose from the

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evidence that the secondary sex ratio, defined as the proportion of male to female offspring in a given population at birth, can vary significantly from the expected 1:1 ratio [1]. The bases of this imbalance are still unclear, but some studies suggested the environmental and physiological factors [2], the time of insemination or mating during estrus [3,4], with an early insemination resulting in more females and a late insemination resulting in more males, and a distortion of the proportion of X and Y chromosome–bearing spermatozoa in the ejaculate [5–7] as possible causes of this disequilibrium. Furthermore, in cattle, a skewed sex ratio in favor of the male gender was attributed at the use of artificial insemination (AI) with respect to natural mating [8]. On the contrary, other studies in cattle did not evidence any relationship between timing of service and sex ratio [9,10]. In the zootechnical industry, the possibility to alter the secondary sex ratio is attractive, thanks to the economic benefits of planning mating for a specific sex, and if a naturally skewed sex ratio is confirmed under certain conditions, this would be of great interest for the breeders to implement selective schemes in favor of a desired gender. This financial interest to manipulate the secondary sex ratio has stimulated the development of semen sex-sorting technologies. Up to now, the only commercially viable method able to alter the primary sex ratio and consequently to determine the sex of the offspring with 90% accuracy is the sperm sorting by flow cytometry [11]. Different attempts have been undertaken for separating X- and Y-bearing spermatozoa based on sperm motility, but controversial results have been reported. For example, a modified swim-up method was indicated as capable of altering the sex ratio of *in vitro*–produced bovine embryos [12], whereas no clinically significant change from a 1:1 ratio was found in the distribution of X- or Y-bearing spermatozoa following a similar approach [13]. At the same time, the sex-sorting technologies required the development of sensible and accurate methods to verify the yield in sperm separation. At present, some of the methods proven to be applicable in this respect are the flow cytometric reanalysis of the sexed semen [14], the analysis by capillary electrophoresis [15], the multicolor fluorescence *in situ* hybridization [16], and the TaqMan real-time polymerase chain reaction (PCR) detection. This last method, which establishes the frequencies of X and Y chromosome–bearing spermatozoa in bovine semen samples, was developed and validated in our laboratory [17] and has been demonstrated to be rapid and reliable, with a very high level of sensitivity and accuracy, providing a valid support to the sperm-sorting technologies.

Controversial studies reported differences in secondary sex ratio among bulls [18,19], and the results were often attributed to relative small number of sires used in most studies. The objectives of the present work were to compare the secondary sex ratio in the offspring produced by AI using the sperm of 98 bulls with the primary sex ratio assessed by real-time PCR in sperm of the same bulls and assess whether the primary sex ratio is influenced by sperm survival and motility after thawing, to establish if a naturally skewed sex ratio can be detected in commercial doses and evaluate possible association with the secondary sex ratio.

2. Materials and methods

2.1. Calving records

Field data relative to calving events of 98 Holstein Friesian bulls undergoing progeny testing (aged 18–36 months), which were used in AI, were collected from January 2009 to December 2012 by Inseme-CIZ, La Serra-San Miniato, Pisa, Italy. Inseminations were conducted in Holstein Friesian dairy herds located in northern Italy. Cows were farmed intensively and fed using the totally mixed rations technique mixing all the feed together and making it available 20 to 22 hours per day. Information for individual calving events including sire identification, sire age, calving date, and calf gender was included in the data set. Calving events of bulls were further classified and analyzed according to four classes of years: 2009 (n = 13,261); 2010 (n = 21,551); 2011 (n = 24,218); and 2012 (n = 41,726), and seasons categorized as winter (December, January, and February); spring (March, April, and May); summer (June, July, and August); and fall (September, October, and November). Frozen semen samples of the same bulls were subsequently used for the determination of the primary sex ratio.

2.2. Determination of primary sex ratio in sperm

2.2.1. Ejaculate

To determine the primary sex ratio in the ejaculate, three batches of each of the 98 bulls were randomly chosen to span over the entire period of AI. The selected sperm batches were, therefore, comprised in the period between January 2008 and February 2012. Sperm was analyzed by real-time PCR immediately after thawing.

2.2.2. Swim-up

To verify if the primary sex ratio is influenced by the survival and motility after thawing, one batch of sperm of each of the 98 bulls was selected and subjected to self-migration (swim-up) method as described [20], as follows: sperm was thawed for 10 minutes in water bath at 37 °C. An aliquot was used for the determination of the total motility (TM), progressive motility, and mean velocity *via* computer-assisted sperm analysis system (HTM-IVOS version 12.3; Hamilton Thorne) and for the determination of the primary sex ratio by real-time PCR. Thereafter, 1 mL of sperm was placed on the bottom of a conic plastic tube containing 4 mL of HEPES-buffered, Ca^{2+}-free, TALP medium (H-TALP; pH 7.4) supplemented with 0.6% BSA and kept at 38 °C in a water bath for 40 minutes. Three milliliter of the medium was then aspirated from the upper layer and centrifuged for 10 minutes at ×800 *g*. The pellet was recovered and transferred into 500 μL of the same H-TALP medium, and the sperm sample was divided into two aliquots: the first aliquot was stored at −20 °C for the real-time PCR analysis, and the second aliquot was placed in water bath at 37 °C for the determination of the TM, progressive motility, and mean velocity after swim-up. Only samples that after swim-up showed a TM greater than 80% were selected and used for the determination of the primary sex ratio. The results were then compared with the