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# An exploratory study on the sperm ratio in dogs: repeatability over time and some reproductive effects

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**Abstract:** Mechanisms that regulate sexual chromosome distribution in the canine ejaculate are not quite understood with no data about both its variation over time and the effect on reproductive parameters. With the aim to deepen these aspects, ten purebred male dogs, aged between 1.5 and 8.5 years, underwent digito-digital semen collection every three months throughout one year. A quantitative real-time PCR method was used to measure the sperm-sex ratio. The sperm-sex ratio was slightly female-biased without a significant difference throughout the year, albeit with some individual dynamic fluctuation over time. The total number of spermatozoa ($P < 0.05$) but not serum testosterone concentration nor the age of the dog was related to the sperm-sex ratio. In particular, dogs with a lower spermatozoa rate showed a greater number of spermatozoa in the ejaculate than dogs with a higher rate. A decreasing trend of X spermatozoa rate was also observed in dogs younger than 5 years of age. Quantitative real-time PCR proved to be an accurate, practical, and reliable method for determining the sperm-sex ratio in dogs. Further studies on a large scale could help to deepen the factors involved in the sperm-sex ratio and consequently in the offspring-sex ratio, opening new frontiers in canine andrology.
**Keywords:** dog, sex ratio, sperm, PCR, SRY.

## 1. Introduction

Although the standard meiotic model forecasts an almost equal and constant number of spermatozoa carrying the X and the Y chromosome, equivocal and no consensual findings have been reached so far (Lobel et al., 1993; Grant et al., 2007; Garcia-Herreros et al., 2010). Being the heterogametic sex in mammals, the male should theoretically influence the offspring sex ratio during spermatozoa production. However, the paternal implication remains largely unknown. Many aspects seem to affect the sperm-sex ratio, among which parental hormonal setting and age (Ein-Mor et al., 2007; Douhard and Geffroy, 2020; Firman et al., 2020; Rahman et al., 2020).

Few studies on dogs have been published so far, mainly about sperm sorting biotechnologies rather than the physiological distribution of sexual chromosomes at conception. A new quantitative real-time PCR method previously validated in the bovine species (Parati et al., 2006; Nix et al., 2023) has been recently transposed in dogs with successful results (Barros Mothé et al., 2018). Based on these premises, the first aim of the present study was to verify the stability over time of the sperm-sex ratio measured by quantitative real-time PCR in dogs. Then we focused on investigating some aspects that may be related to the sperm sex ratio, such as serum testosterone concentration, the total number of spermatozoa in the ejaculate, and the dog's age. Due to serum testosterone levels increasing at ejaculation while remaining equal pre- and post-sampling (Alonge et al., 2018; Kobori et al., 2020), blood was collected after semen sampling.

## 2. Materials and Methods

### 2.1. Animals and sampling

Ten purebred male-owned dogs intended for breeding were studied over 12 months. One French bulldog, two German shepherd dogs, two Golden retriever, two Labrador retriever, and three American Bully dogs constituted our caseload. Age at the start of the survey was 1.5 to 8.5 years (4.5 ± 2.4). Body weight ranged from 15.2 to 40.0 kg (31.7 ± 7.4). Ejaculate collection was performed every three months throughout one year. Namely, a total of 4 evaluations for each dog was planned. The ejaculate was collected by digital manipulation in the presence of a bitch in estrus or using swabs soaked in estrus vaginal secretions according to the usual procedures in canine andrology (Kutzler, 2005). The total number of spermatozoa in the ejaculate was calculated according to the volume of the sperm-rich fraction and the sperm concentration was measured with a photometer SDM 1 calibrated for the canine species (Spermacue MiniTube, Tiefenbach, Germany). At each examination, serum testosterone was also measured with an immunoassay system validated in dogs (Quartuccio et al., 2021) and based on the enzyme-linked fluorescent assay principles (ELFA technique (MiniVidas Biomerieux, Marcy l'Etoile, France). Blood samples were taken about 15 minutes after the semen collection.

### 2.2. Analysis of sperm-sex ratio by quantitative real-time PCR

Quantitative real-time PCR was performed to estimate the ratio between sperm with proteolipid protein (PLP) and the sex-related Y (SRY) genes located on the non-homologous regions of X and Y chromosomes, respectively. The analyses to determine the ratio of spermatozoon X and Y were performed following the instructions previously described by Parati et al. (2006). DNA was extracted from the spermatozoon using the NucleoSpin tissue kit following the supplier's instructions (Macherey-Nagel, Dürer, Germany).

The extracted DNA was then diluted to a concentration of 10 ng/µl and then 2 µl was used for each PCR reaction. To identify the content of Y chromosomes, a portion of the SRY gene was amplified, using the following primers: SRY-frw 5'- agcctcctcctccatgctat - 3' and SRY-rew 5'- gtcctccattcgtgtgtgtg - 3'. This amplification produces a 106 bp fragment. The content of X chromosomes was obtained by amplifying a portion of the PLP1 gene with the following primers: PLP1-frw 5' - GCTTGTTAGAGTGCTGTGCAA - 3' and PLP1-rew 5' – CTTCATGTCCACAGCCACAG - 3'. This amplification produces a 111 bp fragment.

To create a calibration, these loci were amplified into DNA obtained from the blood of a male dog. In this way, we are sure that at any dilution the content of Y fragments is identical to that of X fragments. We used 4 successive dilutions: 1000, 100, 10, and 1. The lowest dilution corresponds to a concentration of 1 ng/µl of DNA. For each PCR reaction, 3 µl of DNA was used. All amplifications were performed in triplicate using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Segrate, Italy). The percentages of spermatozoa Y and X were obtained following the formula previously reported (Parati et al., 2006).

### 2.3. Statistical analysis

All data were analyzed using IBM SPSS 27.0 for Windows software (Armonk, USA). Descriptive statistics were reported as mean ± Standard deviation. The sex ratio of spermatozoa and the age of the dogs were analyzed as categorical variables as described below: age (< 5 years; ≥ 5 years), sex ratio (low: < 54.11 % of X spermatozoa; high: ≥ 54.11 % of X spermatozoa. These thresholds are the median value. Data distribution was assessed through the Shapiro-Wilk test. The sperm-sex ratio was compared to testosterone concentration and the age of the dogs using a parametric analysis of variance (ANOVA). A non-parametric test was applied in the comparison of the sperm count (U-Mann Whitney). Statistical significance was accepted when $P < 0.05$.

## 3. Results

The amplifications obtained on the blood at the different dilutions allowed the determination of the following regression lines and correlation values ($R^2$) (Figure 1).

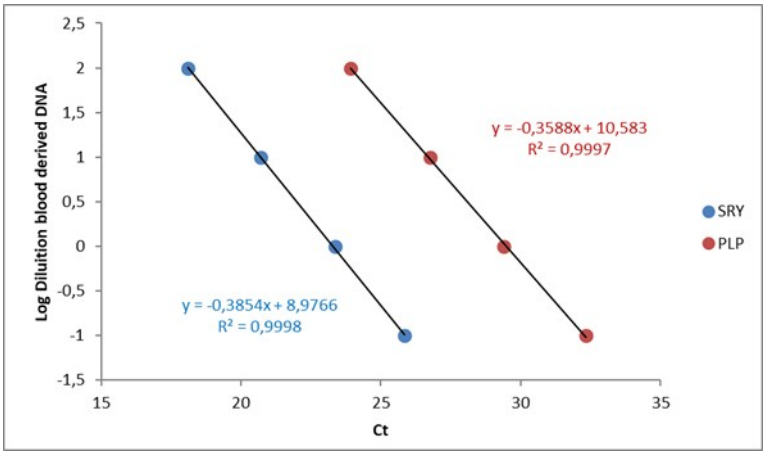


**Figure 1**: Quantitative real-time PCR regression curves of extracted DNA from dog blood for the SRY and PLP genes.

The mean percentage of the X spermatozoa was 54.8±3.0 (median 54.11; min 49.9; max 59.8%) and of the Y spermatozoa was 45.2 ± 3.0 (median 45.9; min 40.2 max 50.1). There were no significant differences in the percentage of X chromosomes over time. The mean serum testosterone concentration was 4.2±3.9 ng/mL. Serum testosterone concentrations were not correlated to sperm sex ratio. However, in ejaculates with a low rate of X spermatozoa (< 54.11%) testosterone tended to be less than in samples with a higher rate of X spermatozoa (≥ 54.11%; Figure 2).

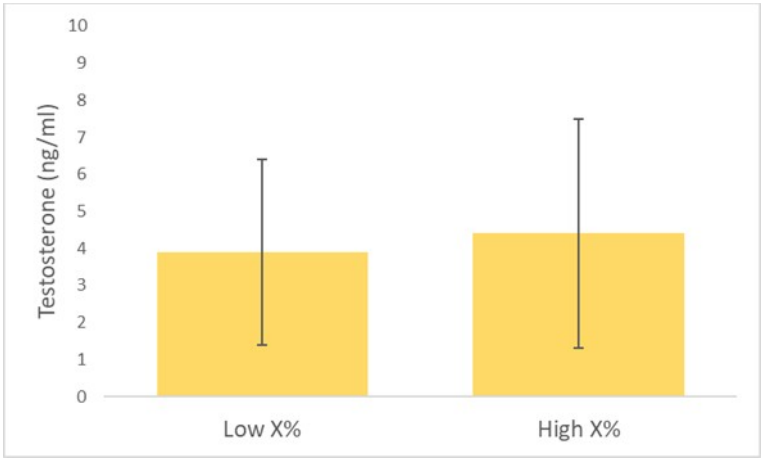


**Figure 2**: Trend of blood testosterone levels based on the X spermatozoa rate.

The mean total number of spermatozoa (TNS) per ejaculate was 576 x$10^6$ (± 319 x$10^6$). The sperm sex ratio was correlated to the total number of spermatozoa (P=0.02). In particular, dogs with a low percentage of X chromosomes showed a greater number of spermatozoa in the ejaculate than dogs with a higher X rate (Figure 3).

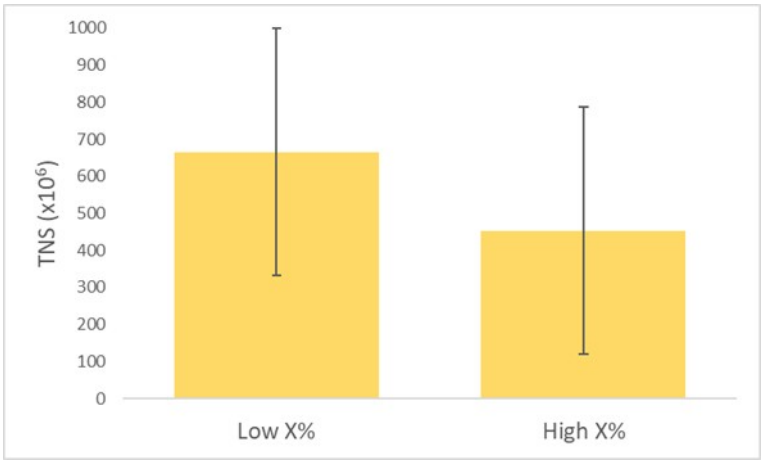


**Figure 3**: Relation between the number of spermatozoa and the X spermatozoa rate.

Even without statistical significance, the X spermatozoa rate tended to be higher in dogs older than 5 years (Figure 4).

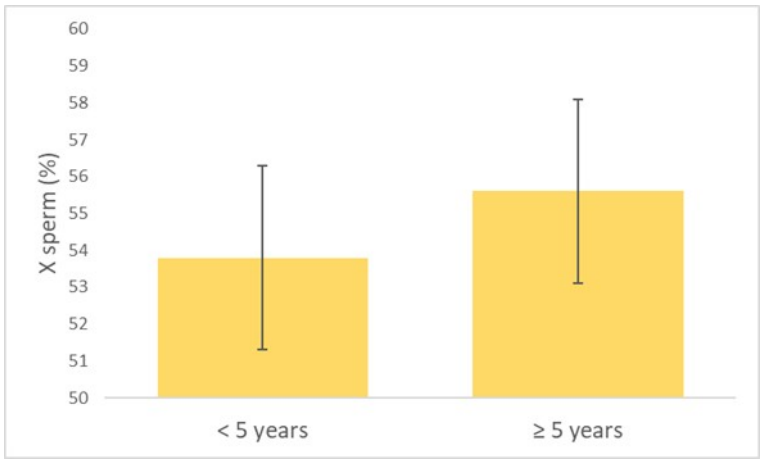

**Figure 4**: Trend of the X spermatozoa rate depending on the dog's age

## 4. Discussion

Quantitative real-time PCR is a very precise and highly sensitive technique that allows simultaneous amplification and measurement of specific DNA segments (Parati et al., 2006; Leong et al., 2007; Tan et al., 2015; Nix et al., 2023). So far, only one study (Barros Mothé et al., 2018) applied to canine species a quantitative real-time PCR method for sperm-sex ratio determination, as previously described in cattle by Parati and colleagues (2006). Our data confirmed that X and Y spermatozoa can be distinguished in dogs by quantitative real-time PCR.

Despite the growing interest in sorted sperm and progress in X and Y spermatozoa separation techniques, to the best of the authors' knowledge, there are no studies on the sperm-sex ratio repeatability over time. We observed a constant sperm-sex ratio in the ejaculates collected four times at 3-month intervals from the same dog (i.e., one per season). Nevertheless, some fluctuation throughout the year was recorded. We postulate that the sperm-sex ratio may be a dynamic status rather than a strictly definite genetic condition. Limited-sized samples prevent us from further speculations on spontaneous variations of the sperm ratio and on the influence of aspects such as the season that may temporarily affect the offspring sex ratio.

The mean percentage of the X spermatozoa in our case was 54.8 (± 2.9) which is similar to the 54.4 (±7.9) value described in humans (Jeyendran et al., 2021). On the contrary, this rate was different from results reported in the only study using quantitative real-time PCR to evaluate sexual chromosome distribution in the canine ejaculate that is, a 49.8 (±2.3) X spermatozoa rate (Barros Mothé et al., 2018). The paucity of publications on the canine species limits further speculations and comparisons.

To date, a possible link between testosterone production and sperm-sex ratio is still debated with conflicting opinions. According to some authors, no evidence supports this data in mammals (Firman et al., 2020), while in humans high testosterone concentrations, both in the mother and father, have been suggested to be associated with an increased proportion of males at birth (Gellatly et al., 2019). In cattle, high testosterone levels are probably related to a high proportion of Y-bearing sperm (Kholghi et al., 2020). Although the precise mechanism by which testosterone influences sperm-sex ratio is currently unknown (Firman et al.,2020). A possible explanation is the role of SRY gene in the viability of the Y chromosome, stimulation of apoptosis of the X-chromosome-bearing sperm during the early stages of spermatogenesis by the PLP gene, and increasing testosterone level (Kholghi et al., 2020). In the present study, the serum testosterone concentration showed an opposite tendency even though without statistical significance. No data have been published on dogs before. It is reasonable to hypothesize a different trend in polytocous compared to a single-born species.

It was found a significant rising in the total number of sperms in samples with a low rate of X spermatozoa (P = 0.02). Again, the literature in human medicine is conflicting and no data on dogs have been published before. Infertile men with less than five million motile sperm have been reported with a significantly lower proportion of Y chromosome-bearing sperm (50.8%), compared to men with higher sperm counts (Eisenberg et al., 2012). Whereas, other authors showed no association between sperm concentration and the sex ratio (Arikawa et al., 2016). A different hypothesis has been advanced to explain the correlation between sperm count and sperm sex ratio such as biological factors acting upon the male reproductive system with a specific effect on X or Y sperm or a natural selection favoring parental ability to adjust the sex ratio according to their reproductive fitness (Eisenberg et al., 2012).

There are contradictory findings also concerning a possible maternal and paternal age effect on the variation in sex ratio (Tremblay et al., 2003; Amadesi et al., 2015). Contrarily to cattle, in which a positive correlation was detected between bull's age and Y sperm ratio Khologhi et al., 2020), we recorded an increasing trend of X spermatozoa rate in dogs older than 5 years. In humans, factors associated with paternal age such as sexual activity frequency (Zarutskie et al., 1898), rather than a direct effect on the frequency of X and Y sperm have been suggested (Martin et al., 1995).

## 5. Conclusion

Quantitative real-time PCR confirmed to be an accurate, practical, and reliable method for determining the sperm-sex ratio in dogs. Even though the limited sample size of the present study requires caution in interpreting the clinical correlations, it should be considered as preliminary data.

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