

Andrological characterisation of Chilean purebred stallions

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ABSTRACT. Chilean purebred horses are widely used in Chile for rodeo and farm work. Despite the importance of the breed, systematic studies characterising their andrological variables are lacking, making stallion evaluation and selection difficult. This study aimed to determine whether age and seasonality affect seminal, spermatogenic, gonadal and endocrine variables of 15 Chilean purebred stallions. The animals were studied over a year and were separated into a young group (n=6) (5 to 12 years) and an aged group (n=9) (13 to 25 years). The variables evaluated were: total seminal volume (mL), free gel seminal volume (mL), sperm concentration (106 x spermatozoa/mL using Spermacue refractometer TM), sperm progressive motility (conventional microscopy (%)), sperm vitality (Eosine-Nigrosine stain (%)) and sperm morphology (Hematoxylin Eosin stain (%)), testicle volume (cm³) (ultrasound Weld 3000V, 5MHz), plasmatic testosterone (ng/mL) and estrogen (pg/mL) concentration determined by radioimmunoassay. To determine the statistical difference and correlation between variables, ANOVA and Pearson's correlation were used, respectively ($P < 0.05$). Most of the andrological variables were affected by age and season and although not significant, the values obtained were higher in young stallions during summer. Progressive motility (70 ± 12.1 %), sperm normal morphology (90 ± 1.7 %) and plasmatic estrogen (6.49 ± 1.8 pg/mL) reached statistical significance to plasmatic testosterone (0.3 ± 0.08 ng/mL) ($P=0.01$), sperm concentration ($253 \pm 59.5 \times 10^6$ sperm/mL) ($P=0.03$) and testicular volume (463.4 ± 90 cm³) ($P=0.002$) variables. Despite the results, we suggest that all these variables should be considered during stallion selection since andrological variables were neither correlated with testosterone nor the oestrogen plasmatic concentration. The results provide parameters that should be considered during the evaluation and selection of Chilean purebred stallions to avoid the extrapolation of data from other breeds.

Key words: Chilean purebred, stallion, semen, age, seasonality, andrology.

INTRODUCTION

The andrological variables and seminal quality in aged stallions (*Equus caballus*) may suffer damage due to metabolic, endocrinological and degenerative processes that normally occur during ageing. Arabian and Thoroughbred stallions are two breeds considered as the biological reference for equine species because they have been spread out worldwide and extensively studied, also, both breeds have reported significant decreases of andrological variables starting at eleven years old, for example (mean \pm SD before 12 years old; after 12 years old): seminal volume (48.00 ± 5.59 ; 45.83 ± 5.90 mL), spermatogenic concentration (359.9 ± 17.41 ; $315.25 \pm 18.96 \times 10^6$ /mL), abnormal sperm (11.80 ± 1.30 ; 17.58 ± 2.02 %) motile sperm (76.50 ± 2.69 ; 67.92 ± 3.23 %) and the oxygen consumption spermatogenic mitochondrial activity (0.007; 0.005 % Mitox) (Dowsett and Knott 1996, El-Maaty *et al* 2014, Darr *et al* 2017). In Arabian and Thoroughbred stallions, the seminal quality also decreases during winter mainly because its reproductive functionality depends on the photoperiod and the daily light hours, for example: testicle volume (summer 380 ± 20 ; winter 220 ± 20 cm³), seminal volume (summer 92 ± 11 ; winter 61 ± 8 mL), spermatogenic motility (summer 73 ± 4 ; winter 63 ± 3 %) (Janett *et al* 2003, Pereira *et al*

2012, Aurich 2016). Moreover, testosterone and oestrogen values are frequently used as fertility indicators during andrological examination of the stallions, however, they also vary over a year showing lower testosterone plasmatic concentration during winter in Arabian stallions (summer 798.62 ± 47.24 ; winter 572.42 ± 95.13 pg/mL), therefore, its clinical interpretation should be done carefully (Waheed *et al* 2015). Despite these reference values, it is necessary to make an objective evaluation of each patient to know the differences of andrological variables between particular breeds of stallions. To do this, it is necessary to create referential values for each breed, also considering that the andrological variables depend on domestication grade of each one of them, as well as the latitude and photoperiod of the geographic place where they reside (Pereira *et al* 2012). Thus, contradictory situations might be seen such as in Spanish stallions that surprisingly show higher spermatogenic concentration during winter (Adamou *et al* 2013).

The Chilean purebred stallion has the oldest genealogical records in South America and is recognised by its adaptability to a wide range of different environments and working conditions, being considered as the genetic enhancer to other breeds, characteristics that spark new interest in other countries (Perez and Huber 2004). In Chile, purebred stallions are mainly used for rodeo, an equestrian sport closely related to Chilean culture and folklore and, for this reason, the breed was declared a natural monument by the Chilean government¹. Nevertheless, despite the social and

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¹ Ministerio de Agricultura. 2011. Declara monumento natural al caballo de pura raza chilena. Biblioteca del Congreso Nacional de Chile. <https://www.leychile.cl/N?i=1024797&f=2011-04-26&p=>; accessed August 8, 2019.

cultural importance of the Chilean purebred horse, there are no systematic studies characterising its andrological variables, making difficult the evaluation and selection of stallions. In addition, the extensive use of reproductive reference values from other breeds might lead to underestimate or overestimate its reproductive variables. The Chilean purebred stallions are subjected to reproductive management along the year and the population of aged stallions is increasing due to the extension of their useful lifetime, therefore the aim of this study was to determine whether the parameters of age and seasonality affect the andrological variables of this particular breed.

MATERIAL AND METHODS

All the procedures performed were revised and approved by the Institutional Animal Care and Use Committee of the Universidad Santo Tomás, authorisation N° 02-2017.

Fifteen 15 fertile Chilean purebred stallions were used. The animals were registered by the Ministry of Agriculture of Chile and the ages ranged between 5 to 25 years old, with a mean pregnancy rate $90.2 \pm 4.1\%$ (using fertility criteria proposed by Pickett *et al* 1989 for fertile stallions: reaching pregnancy rate over 75% last season, covering at least 10 mares). During the study, the selected stallions were kept in DSO condition (Daily Sperm Output) with the purpose of standardising the sperm emissions of each stallion. The stallions were subjected to the andrological study for a year at the Centre of Studies of Chilean Purebred Horse ("Centro de Estudios del Caballo Chileno"), located in the Maule Region ($35^{\circ}25' S$, $71^{\circ}39' W$). Two groups were formed, a young group ($n=6$) (5 to 12 years old) and an aged group ($n=9$) (13 to 25 years of age), according to Darr *et al* 2017. Six semen samples were obtained from each stallion using an artificial vagina (Missouri model, Minitub) (n total = 90 ejaculates). The ejaculates were obtained every 30 days during summer and winter. The andrological variables registered in each seminal sample were: total seminal volume (mL), free gel seminal volume (mL), sperm concentration ($10^6 \times$ spermatozoa/mL obtained by SpermaCue Photometer 12300/0500 (Minitub Ibérica S.L., La Selva del Camp, Spain)), sperm progressive motility (by conventional microscopy (%)), sperm vitality (by Eosine-Nigrosine stain (%)) and sperm morphology (by Hematoxylin Eosin stain (%)) according to Brito *et al* 2011. All the recordings were carried out by the same person. Before obtaining each seminal sample, the testicle volume (cm^3) was calculated by using ultrasound 5MHz (Wed 3000v, Welld, Shenzhen, China) according to Love 2014, and a 10 mL blood sample was obtained from the jugular vein to determine plasmatic testosterone (ng/mL) and oestrogen (pg/mL) concentration using radioimmunoassay, according to the manufacturer's recommendation (Double Antibody RIA kits SL-4000 or DSL-43100, respectively; Diagnostic System Laboratories, Inc, USA).

STATISTICAL ANALYSIS

The data were grouped according to age and seasonality and, subsequently, mathematical mean and standard deviation were calculated. Data were analysed for normal distribution by Shapiro-Wilk test and Two-way ANOVA, while Bonferroni test and Pearson's correlation were used to determine statistical differences between groups and correlation between variables. The statistical analysis was performed with the SPSS statistics program (version 20.0, IBM-SPSS, Armonk, NY, USA). A P -value of less than 0.05 was considered significant.

RESULTS

Data was normally distributed. The average age and standard deviation in both young and aged groups were 6.67 ± 3.01 years old and 20.44 ± 4.28 years old, respectively, observing significant differences between groups ($P=0.03$). Data had a normal distribution ($P=0.07$). For all the variables studied there were no statistical differences between the monthly values belonging to the same season. Spermatic progressive motility also did not show differences when affected by age or seasonality. However, the variables of seminal volume, sperm morphology, sperm vitality, and testicular volume, only showed differences when affected by seasonality, decreasing these values significantly during winter in young animals ($P=0.01$) ($P=0.04$) ($P=0.02$) ($P=0.01$) and the aged animals ($P=0.02$) ($P=0.04$) ($P=0.02$) ($P=0.04$), respectively, suggesting that age has no influence over these variables (table 1). Moreover, semen quality was not affected by seasonality but in the young group it was significantly higher than in the aged group during winter and summer ($P=0.02$ and $P=0.001$ respectively), suggesting that age has an influence on sperm concentration throughout the year. Likewise, whereas testosterone concentration showed no difference between the young and the aged group in winter, it showed a significant difference during summer, being significantly higher in the young group ($P=0.01$). Furthermore, the oestrogen concentration showed significant differences only in the young group during the summer and in the aged group during the winter ($P=0.01$). The correlation between testosterone and oestrogen concentration was weak ($r = 0.31$) and neither showed a strong correlation with the other variables considered in this study (table 2).

DISCUSSION

In general, the studied group showed andrological variations similar to those described for other breeds according to age and seasonality, observing better seminal quality, endocrine and gonadal values in the young group during the summer season (Dowsett and Knott 1996, Janett *et al* 2003, Pereira *et al* 2012, El-Maaty *et al* 2014, Aurich 2016, Darr *et al* 2017). However, it must be noted that the different variables studied in this breed responded unequally

Table 1. Summary table of mathematical mean and standard deviations of seminal, spermatic, endocrine and testicular variables, in a group of purebred Chilean stallions, according to age and seasonality.

	Young summer group	Aged summer group	Young Winter group	Aged Winter group
Seminal volume (mL)	62 ± 15.3 ^a	66 ± 16.2 ^a	43 ± 12.8 ^b	50 ± 10.2 ^b
Free gel seminal volume (mL)	44 ± 9.6 ^{ab}	50 ± 11.5 ^b	36 ± 10.8 ^a	43 ± 11.3 ^{ab}
Sperm concentration (10 ⁶ x spermatozoa/mL)	253 ± 59.5 ^a	186 ± 69.5 ^b	233 ± 39.8 ^a	179 ± 38.9 ^b
Sperm Progressive motility (%)	70 ± 12.1 ^a	64 ± 9.3 ^a	65 ± 9.2 ^a	63 ± 9.1 ^a
Sperm vitality (%)	93 ± 4.0 ^a	93 ± 4.8 ^a	86 ± 9.3 ^b	88 ± 6.5 ^b
Sperm normal morphology (%)	90 ± 1.7 ^a	89 ± 1.6 ^a	88 ± 1.2 ^b	87 ± 1.7 ^b
Testosterone (ng/ml)	0.3 ± 0.08 ^a	0.21 ± 0.06 ^{bc}	0.27 ± 0.10 ^{ac}	0.17 ± 0.06 ^{bc}
Oestrogen (pg/ml)	6.49 ± 1.8 ^a	5.02 ± 1.5 ^{ac}	5.29 ± 1.6 ^{ac}	3.9 ± 1.2 ^{bc}
Testicular volume (cm ³)	463.4 ± 90.6 ^a	432.7 ± 101.4 ^a	319.7 ± 74.2 ^b	363.9 ± 74.2 ^b

^{a,b,c}Values with different superscripts differ within a file ($P < 0.05$).

Table 2. Correlation (r) between endocrine, spermatic and seminal variables of a group of Chilean purebred stallion.

	Seminal volume	Free gel seminal volume	Sperm Concentration	Sperm progressive motility	Sperm vitality	Sperm normal morphology
Testosterone	-0.03	-0.16	0.26	0.04	0.29	0.29
Oestrogen	0.02	0.01	0.16	0.01	0.26	0.18

and independently according to age and seasonality. Age and seasonality significantly modified the concentration of testosterone but none of them significantly changed the variables of sperm motility, whereas all the other variables studied here were significantly modified only by one of the two factors. Therefore, we suggest that the average values stated for each andrological variable studied should be considered as preliminary references for the accurate evaluation and reproductive selection of this breed since, based in our data, the clinical interpretation of andrological results in this breed must consider factors of age and seasonality.

One of the aspects to be emphasised in the Chilean purebred stallions is the progressive motility since this variable is considered one of the main indicators of seminal quality, due to its direct relationship with fertility (Malrngren 1997). It is striking that the Chilean purebred does not show significant differences associated with age or time of the year, which can be considered a potential of the breed because it facilitates work with biotechnological purposes such as dose calculation and repetitiveness of results over the year. This characteristic is also present in the Spanish horse (Adamou *et al* 2013) from which the Chilean purebred horse descended directly (Perez and Huber 2004), although the percentage of sperm motility achieved by the Spanish horse is slightly higher. Moreover, in the Arab breed it is possible to recognise a decrease of up to 10% in the sperm motility of aged individuals (El-Maaty *et al* 2014). Although statistical differences were

described for sperm motility in the thoroughbred stallion, according to age and seasonality (<12 years old group = 76.50 ± 2.69 %; > 12 years old group = 67.92 ± 3.23 %) (Summer 73 ± 4; winter 63 ± 3%), respectively (Dowsett and Knott 1996, Janett *et al* 2003), sperm motility in the purebred Chilean stallions group showed no differences related to age or seasonality (young summer group 70 ± 12.1; aged summer group 64 ± 9.3; young winter group 65 ± 9.2; aged winter group 63 ± 9.1%). There were no statistical differences between the groups, the values were similar to those from other breeds.

In the present study, the plasmatic concentration of testosterone in the purebred Chilean stallion decreased significantly during winter and in the aged stallion. In the Brazilian pony (Rua *et al* 2017) and the Arabian horse (Waheed *et al* 2015), the testosterone concentration also showed to decline in winter. Contrary, in the Arabian horse the testosterone concentrations increase in old stallions (El-Maaty *et al* 2014) while also establishing a strong correlation with the fat coverage, variable not yet studied in the purebred Chilean stallion, most probably because Chilean Purebred Horses do not tend to accumulate fat with age. Regarding the concentration of oestrogen, the purebred Chilean stallion show no variation of this variable according to seasonality, however in the Arab breed has been observed a strong decrease of oestrogen in winter reaching half levels of this hormone (Waheed *et al* 2015), demonstrating another relevant difference attributable to the breed.

Based on our data, we suggest that the evaluation of oestrogen and testosterone alone cannot be used as sole variables for estimating fertility in purebred Chilean stallions, being necessary to perform a more complete andrological examination that considers other parameters such as age and seasonality, as demonstrated in this study. It is interesting to recognise that the andrological variables are modified according to age and season in a particular way for each breed. Therefore, considering that sperm cryopreservation is increasingly used throughout the year in Chilean purebred stallions, it would be interesting to assess whether age and season influence the structural and kinetic post thawing variables of sperm undergoing cryopreservation. This would allow generating recommendations that contribute to improving the efficiency of these techniques. Due to the interest of extending the life span of the stallions, the group of old animals is increasingly being represented within the farms, especially in the Chilean purebred because it starts its sporting life late. In this way, further studies are needed to promote studies that allow understanding the decrease in fertility associated with age, allowing adequate management to improve the reproductive management of old stallions.

In conclusion, the Chilean purebred stallion group evaluated in this study showed that the parameters of age and seasonality affected most of the andrological variables analysed. We propose that these parameters should be considered during the evaluation and selection of stallions of this breed. Given that there was no strong correlation between the endocrine variables and the other variables, it is essential to perform a more complete andrological examination in order to improve decision making during the reproductive selection of Chilean purebred stallions.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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