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The contribution of different sperm parameters to better explain ram semen cryoresistance

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ABSTRACT

In order to determine an effective procedure for explaining ram sperm cryoresistance and develop a new model for breeders classification, a retrospective study was conducted using sperm analysis data obtained over two consecutive years from a total of 82 sessions of ram semen cryopreservation. In each session, fresh ejaculates from eight males were collected via artificial vagina, pooled and frozen in liquid nitrogen vapors. After thawing, a total of 19,084 sperm tracks and 11,319 morphometric measurements were analysed. Clustering analyses were applied to establish motile and morphometric sperm subpopulations. Additionally, plasma and acrosome membrane integrity, as well mitochondrial activity using flow cytometry immediately after sperm thawing and following hypoosmotic shock test (HOST) was assessed. To develop a Ram Sperm Cryoresistance Index, Principal Component Analyses (PCA) using 22 variables were conducted. In the first PCA, the parameters that best explain cryoresistance include total motility (TM), motile subpopulation 2 (motSP2, which groups slow, very linear spermatozoa with low lateral head displacement), morphometric subpopulation 1 (morphSP1, grouping spermatozoa with the smallest head size and lowest shape values), sperm plasma membrane integrity immediately after thawing and following hypoosmotic shock test. These parameters collectively account for 77.34 % of the accumulated variance. To emphasize their importance, a second PCA was performed, revealing significant higher weighting coefficients for the quantity (TM) and quality (motSP2) of sperm movement after thawing, compared to the head size and shape of the thawed sperm (morphSP1). Furthermore, HOST Viability played a more decisive role than what was observed under isotonic conditions.

Introduction

Ram spermatozoa have low capacity to resist the cryopreservation process, constituting a fertility problem that is reflected in low pregnancy rates often below 40 % after conventional artificial insemination using frozen sperm (Salamon and Maxwell, 1995). Interestingly, individual differences in ram sperm cryoresistance have been observed (Thurston and Watson, 2002).

In order to create useful tools for the classification of breeders as "good" or "bad freezers", researchers have studied different species to identify key sperm parameters related to sperm cryoresistance (Casas et al., 2009; Ramón et al., 2013; Rego et al., 2016). For instance, Yeste et al. (2013), in boars, proposed a hierarchical conglomerate analysis to group breeders based on sperm viability and progressive motility post-thawing, meanwhile Jiang et al. (2017) used multiple logistic regression analysis in humans to predict freezing rates based on rectilinear, mean and progressive motilities of the spermatozoa, establishing *cut-off* values for breeder classification.

However, in rams, the classification of breeders as "good" or "bad freezers" has not been thoroughly studied, considering all available information. Furthermore, a proper statistical approach for this classification has not been established. Therefore, the aim of the present work is to determine the most effective data treatment procedure from a retrospective study conducted in our laboratory selecting the sperm parameters that better explain ram sperm cryoresistance with the final purpose of designing a tool to classify the breeders.

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Materials and methods

Data origin

This retrospective study was conducted using data from the sperm analyses performed during the cryopreservation process of 82 ejaculate mixture collected in autumn through artificial vagina from 8 rams (between 1 and 2.5 years old approximately) during 2 consecutive years in Caldes de Montbui (Spain). Sperm cryopreservation process and analyses were performed following the protocols described in elsewhere by Garcia et al. (2017). Briefly, the sperm quality parameters included as data in this study are described as follow:

Freezing Resistance Index (FRI) represents the percentage of sperm that survive the freezing and thawing stages estimated by eosine/nigrosine vital staining (Hancock, 1951).

FRI= (sperm viability after thawing / sperm viability before freezing) x 100

Sperm kinematic parameters were obtained using the computer-assisted sperm analysis (CASA) of system ISAS® (PROISER SL, Valencia, Spain). Total motility (TM), progressive motility (PM), curvilinear velocity (VCL), rectilinear velocity (VSL), mean velocity (VAP), linearity coefficient (LIN=[VSL/VCL]×100), straightness coefficient (STR=[VSL/VAP]×100), wobble coefficient (WOB=[VAP/VCL]×100), lateral head displacement (ALH), beat crossover frequency (BCF) and dance (DNC=[VCL×ALH]) were evaluated as described Garcia et al. (2017).

Sperm morphometric parameters were assessed using the automated sperm morphometric analysis (ASMA) of system ISAS® (PROISER SL, Valencia, Spain) using a Diff-Quick® procedure (Hidalgo et al., 2006). The morphometric dimensions for head: area (A), perimeter (P), length (L), width (W) and the parameters derived of head shape: ellipticity (L/W), rugosity ($P^2/4\pi A$), elongation ([L-W]/[L+W]), and regularity ($\pi LW/4$ A) were measured from 150 sperm heads.

Sperm viability, acrosome integrity and mitochondrial activity of thawed sperm were evaluated by flow cytometry using quadruple-staining described by Tabarez et al. (2017). The fluorescent probes used were LIVE/DEAD® sperm viability kit (SYBR-14 and Propidium Iodide (PI); L-7011, Invitrogen, SA, CA, USA) for plasma membrane integrity, PE-PNA (GTX01509, AntibodyBcn SL, Barcelona, Spain) for acrosome integrity and Mitotracker deep red (M22426, Invitrogen, SA, CA, USA) for the detection of mitochondrial activity. The equipment used was the BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) and samples were analyzed using BD FACSDiva (BD Biosciences, San Jose, CA, USA). The sperm analysis was performed after sperm thawing in an isotonic solution and following the hypoosmotic shock test (HOST) as described by Forouzanfar et al. (2010). After evaluation, apart from the percentage of total spermatozoa with intact plasma membrane immediately after thawing (Viability) and the percentage of total thawed spermatozoa with intact plasma membrane after the hypoosmotic shock test (HOST Viability), four sperm populations with intact plasma membrane were considered in each sample: intact acrosome and active mitochondrial (IAAM), damaged acrosome and active mitochondrial (DAAM), intact acrosome and inactive mitochondrial (IAIM), and damaged acrosome and inactive mitochondrial (DAIM) sperm.

Statistical analysis

The kinematic and morphometric parameters of the spermatozoa obtained from CASA and ASMA after thawing were analyzed through the FASTCLUS procedure (Quintero-Moreno et al., 2003), using the SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA). The subpopulations were obtained by means of a non-hierarchical multivariate cluster analysis using the k-means model based on Euclidean distances calculated from kinematic and morphometric parameters.

The number of subpopulations was established by the elbow method described by Bravo et al. (2014), using the R software (Version 4.0.3, 2020, R Core Team, Vienna, Austria). The frequency distribution of the motile and morphometric sperm subpopulations was performed for each replicate through the FREQ procedure. Then an adjustment was made on the proportion of each subpopulation based on the total motility (TM) observed in each sperm sample (motSP=[SP/100]×TM). In this manner, a novel subpopulation was obtained, precisely grouping static spermatozoa (motSP4=100 %-TM). An ANOVA was used to evaluate parameters that characterize each motile and morphometric sperm subpopulation through GLM procedure. Differences between means were analyzed using Tukey test, considering them significant at the $P<0.05$ level.

Afterward, motile and morphometric sperm subpopulations frequencies were combined with parameters obtained through flow cytometric analysis. These parameters included Viability immediately after thawing, HOST Viability, and the frequency of the four intact membrane sperm subpopulations previously described based on the state of their acrosome and its mitochondrial activity under isosmotic and hypoosmotic conditions. The different sperm categories with damaged plasma membrane were not included in the analysis. In addition, the percentages of TM and PM from the different thawed sperm samples were considered, along with the following three parameters: Progression Index which is calculated as PI=[PM/TM]×100), HOST Survival Index as HSI=[Viability HOST/Viability]×100 and Freezing Resistance Index above described.

In summary, 22 sperm variables were considered in the statistical analysis approach, including the frequencies of four motile subpopulations (motSP1, motSP2, motSP3 and motSP4) and three morphometric subpopulations (morphSP1, morphSP2 and morphSP3) after thawing, the frequencies of four intact plasma membrane subpopulations (IAAM, DAAM, IAIM and DAIM) after thawing and following hypoosmotic stress as well total intact plasma membrane sperm percentages (Viability and HOST viability). Additionally, percentage of total and progressive motility (TM and PM) after thawing, the Progression Index (PI), HOST Survival Index (HSI) and Freezing Resistance Index (FRI) were considered and determined in every single sample included in the retrospective study. SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA) was used for the following analyzes:

A normality analysis of the whole set of parameters was performed out, applying the Kolmogorov-Smirnov test through the UNIVARIATE procedure. When necessary, the values were transformed with the square root of the arcsine to approximate a normal distribution.

First, a Principal Component Analysis (PCA) was conducted on the 22 parameters using the PRINCOMP procedure (Martinez-Pastor et al., 2005). The optimal number of principal components was determined based on the Kaiser criterion, selecting only those with an eigenvalue (representing variance extracted for this principal component) greater than 1 (Yániz et al., 2015). Subsequently, the parameters that best explain cryoresistance were identified through VARCLUS procedure (Quintero-Moreno et al., 2003), using the principal component number obtained in the previous step (based on the Kaiser criterion) as our criteria. To refine the parameter selection, the methodology described by Luna et al. (2017) was followed but with a modification, establishing the determination coefficient (R^2) greater than 0.8 within its own PCA and lesser than 0.2 in the ratio of its R^2 to the next closest R^2 within the same principal component. This approach ensures meaningful clustering.

After selecting the parameters that best explained cryoresistance, a Pearson correlation was conducted between these parameters using the CORR procedure of the SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA). Subsequently, a second PCA was performed on these selected parameters through VARCLUS procedure, to obtain the eigenvectors (used as weighting coefficients) from the

correlation matrix for each parameter within the principal component, expressing the greatest amount of the accumulated variance. Finally, Ram Sperm Cryoresistance Index (CRI) was constructed by summing the product of each value of the cryoresistance parameters obtained through laboratory analysis of the spermatozoa and their respective weighting coefficients.

Results

A total of 19,084 sperm were analyzed to establish motile subpopulations. Among these, 43.12 % of the sperm (8229 tracks) exhibited some form of movement. Through cluster analysis, three motile sperm subpopulations based on kinematic parameters obtained from the CASA analysis of thawed sperm samples were identified. Interestingly, static sperm constituted the fourth motile subpopulation (motSP4). The motion patterns characterizing each motile sperm subpopulation are presented in Table 1. Motile subpopulation 1 (motSP1) represented the fastest spermatozoa but exhibited the least linear movement, showing the lowest STR and the highest ALH, and statistically different ($P<0.05$) from the other two subpopulations in terms of kinematic parameters, except for BCF, which was similar to subpopulation 2 (motSP2). Motile subpopulation 2 (motSP2) was characterized by containing the slowest spermatozoa, but with highly linear movement, showing the greatest STR and the lowest ALH. Meanwhile, motile subpopulation 3 (motSP3) displayed intermediate values between motSP1 and motSP2, having fast and very linear movement, high STR, moderate ALH and high BCF, and similar to motSP2 ($P>0.05$) in terms of LIN and WOB.

Similarly, three morphometric sperm subpopulations were defined after conducting cluster analysis on the morphometric parameters obtained from ASMA analysis of a total of 11,319 thawed ram sperm heads. The summary of statistics for the head size and shape patterns characterizing the spermatozoa in each morphometric subpopulation is shown in Table 2. Morphometric subpopulation 1 (morphSP1) comprised the smallest spermatozoa, exhibiting the lowest values for head dimensions (Length, Width, Area, Perimeter; $P<0.05$) compared to the other two subpopulations. Morphometric subpopulation 3 (morphSP3) grouped spermatozoa with the largest head size dimensions, meanwhile morphometric subpopulation 2 (morphSP2) represented spermatozoa with intermediate head size dimensions between morphSP1 and morphSP3 ($P<0.05$).

Table 3 presents the results obtained from the initial Principal Component Analysis (PCA) performed on all 22 sperm parameters included in the study. This analysis defined seven principal components with eigenvalues greater than 1, representing 77.34 % of the accumulated variance. Based on the R^2 within its own component, the first principal component was strongly associated with sperm plasma membrane integrity immediately after thawing (Viability) and after the hypoosmotic shock test (HOST Viability). The second principal component was highly related to TM and to motSP4, since both parameters are complementary. TM was subsequently selected as a cryoresistance parameter because it reflects the overall quantity of sperm movement in a sperm sample. The second component was also associated with motSP2, which describes the quality of the sperm movement characterized by grouping slow, very linear spermatozoa with high STR and low ALH. Meanwhile, the third principal component exhibited a strong relationship with the morphSP1, providing information on the size and shape of sperm heads after thawing. Specifically, morphSP1 grouped the smallest spermatozoa with the lowest values for head size and shape. The remaining principal components did not show significant relationships with the other analysed parameters; therefore, no further details are provided in Table 3.

Table 4 presents the Pearson correlations between the selected parameters that better explain ram sperm cryoresistance. Notably, all correlation coefficients were positive among the five cryoresistance parameters. Specifically, TM significantly correlated with motSP2 and

Table 1
Mean values (±SD) of the kinematic parameters that define the four motile sperm subpopulations (motSP) identified in ram sperm samples after thawing.

Kinematic parameter[1]	Motile sperm subpopulations			Static subpopulation motSP4
	motSP1	motSP2	motSP3	
VCL (μm/s)	166.00 ± 23.39[a]	57.80 ± 26.35[c]	117.59 ± 20.23[b]	N/D
VSL (μm/s)	48.77 ± 31.75[b]	28.73 ± 22.79[c]	56.69 ± 32.20[a]	N/D
VAP (μm/s)	85.41 ± 26.93[a]	37.84 ± 23.64[c]	77.13 ± 28.45[b]	N/D
LIN (%)	29.23 ± 18.22[b]	47.56 ± 24.04[a]	46.46 ± 22.54[a]	N/D
STR (%)	54.42 ± 26.50[c]	70.79 ± 24.55[a]	69.30 ± 24.20[b]	N/D
ALH (μm)	7.49 ± 1.41[a]	2.34 ± 0.92[c]	4.50 ± 0.93[b]	N/D
BCF (Hz)	7.68 ± 4.83[b]	7.92 ± 4.71[b]	9.73 ± 4.72[a]	N/D
n	290	5466	2473	10,855
%	1.52	28.64	12.96	56.88

[a-c] Different letters indicate significant differences between motile sperm subpopulations ($P<0.05$)
[1] VCL: Curvilinear velocity; VSL: Rectilinear velocity; VAP: Mean velocity; LIN: Linearity coefficient; STR: Straightness coefficient; ALH: Lateral head displacement; BCF: Beat crossover frequency; n: Sperm number; %: Sperm percentage in each subpopulation; N/D: No data.

Table 2
Mean values (±SD) of the morphometric parameters that define the three morphometric sperm subpopulations (morphSP) identified in ram sperm samples after thawing.

Morphometric parameter	Morphometric sperm subpopulations		
	morphSP1	morphSP2	morphSP3
Length (μm)	8.02 ± 0.37[c]	8.47 ± 0.36[b]	8.89 ± 0.41[a]
Width (μm)	4.62 ± 0.19[c]	4.88 ± 0.17[b]	5.12 ± 0.19[a]
Area (μm^2)	31.24 ± 1.44[c]	34.62 ± 0.94[b]	37.71 ± 1.30[a]
Perimeter (μm)	22.29 ± 0.64[c]	23.53 ± 0.52[b]	24.70 ± 0.81[a]
Ellipticity (%)	1.74 ± 0.11	1.74 ± 0.11	1.74 ± 0.11
Rugosity	0.79 ± 0.03[a]	0.78 ± 0.03[b]	0.77 ± 0.04[c]
Elongation	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.03
Regularity	0.93 ± 0.04[c]	0.94 ± 0.04[b]	0.95 ± 0.04[a]
n	3512	5450	2357
%	31.03	48.15	20.82

[a-c] Different letters indicate significant differences between morphometric sperm subpopulations ($P<0.05$)
n: Sperm number; %: Sperm percentage in each subpopulation

Table 3
Selected parameters after the first PCA according to the established criteria of having the determination coefficient (R^2) greater than 0.8 with its own PCA and lesser than 0.2 in the ratio of its own R^2.

Principal component	Parameter[1]	R^2 with its own component (OC)	R^2 with the next closest (NC)	Proportion ($1- R^2_{OC}/1- R^2_{NC}$)
1	Viability	0.86	0.20	0.18
	HOST Viability	0.87	0.19	0.16
2	TM	0.95	0.40	0.10
	motSP2	0.84	0.02	0.17
	motSP4[†]	0.95	0.40	0.10
3	morphSP1	1.00	0.03	0.00

[1] Viability: Total percentage of spermatozoa with intact plasma membrane immediately after thawing; HOST Viability: Total percentage of thawed spermatozoa with intact plasma membrane after the hypoosmotic shock test; TM: Total motility; motSP2: Motile subpopulation 2; motSP4: Subpopulation of static spermatozoa; morphSP1: Morphometric subpopulation 1
[†] Parameter not selected because it corresponds to the subpopulation of static spermatozoa and represents the complementary of TM

Table 4
Pearson correlation of the ram sperm cryoresistance parameters.

Parameter[a]	TM	motSP2	morphSP1	Viability	HOST Viability
TM	1.00	0.82***	0.01	0.21†	0.31**
motSP2		1.00	0.07	-0.04	0.13
morphSP1			1.00	0.13	0.22*
Viability				1.00	0.82***
HOST Viability					1.00

† P<0.1; *P<0.05; **P<0.01; ***P<0.001

[a] TM: Total motility; motSP2: Motile subpopulation 2; morphSP1: Morphometric subpopulation 1; Viability: Total percentage of spermatozoa with intact plasma membrane just after thawing; HOST Viability: Total percentage of thawed spermatozoa with intact plasma membrane after the hypoosmotic shock test

HOST Viability. However, motSP2 did not exhibit any correlation with morphSP1 or with sperm viability percentages after thawing or after HOST. The morphSP1 only shows a statistically significant relationship with sperm viability assessed after HOST, meanwhile this last parameter strongly correlated with sperm viability assessed before HOST.

The second PCA based on the five parameters that best explain the ram sperm cryoresistance (obtained from the first PCA), revealed two principal components. The first principal component represented only 43.60 % of the accumulated variance, while the second principal component accounted for the maximum total variation (74.07 %). The eigenvectors of each parameter served as weighting coefficients for establishing the Ram Sperm Cryoresistance Index (CRI). All the parameters in the second principal component had positive weights. Notably, TM and motSP2 carried the highest weights, followed by HOST Viability. In contrast, morphSP1 and overall sperm Viability had negligible weight.

Based on our data, the resulting equation to determine the sperm Cryoresistance Index in rams is as follows:

$$CRI = 0{,}95 \times TM + 0{,}95 \times motSP2 + 0{,}04 \times morphSP1 + 0{,}09 \times Viability - + 0{,}23 \times HOST\ Viability$$

Discussion

In the present study, a considerable amount of biological information has been combined and objectively characterized according to its importance, in order to better understand the behavior of ram sperm after thawing with the aim of subsequent application. By conducting Principal Component Analysis (PCA) on kinematic and morphometric subpopulations, along with assessing plasma and acrosome membrane integrity, mitochondrial activity, sperm motility and survival after thawing and osmotic stress, five key parameters that best explain ram sperm cryoresistance have been identified, addressing the challenges associated with handling a large volume of available information (Martínez-Pastor et al., 2011).

Based on the aforementioned criteria, spermatozoa movement determines two parameters that best explain cryoresistance: Total motility (TM) and motile subpopulation 2 (motSP2). MotSP2 is characterized by slow and very linear sperm movement, with high STR and low ALH. Notably, motSP2 comprises the highest proportion of motile sperm (28.64 % of total spermatozoa analysed). It is well-established that the reduction in motility resulting from the cryopreservation process significantly impacts sperm structural and functional competence (Watson, 2000; Rhemrev et al., 2001). Freezing induces a series of mechanical, chemical and osmotic changes that can alter sperm movement patterns. According to Santolaria et al. (2015), the largest subpopulation before freezing consists of fast and non-linear sperm, with elevated ALH. Although the sperm subpopulations before freezing were not evaluated in our study, the results after thawing indicate that spermatozoa appear to lose velocity but gain linearity, exhibiting a lower ALH, compared to the findings reported in the aforementioned study.

The importance of both quantity (TM) and quality of sperm movement (motSP2) is evident, as they exhibit a strong positive correlation and have similar high weighting coefficient in the Cryoresistance Index. Our data support the notion that sperm motility after thawing is crucial because it may indicate that the sperm have not been damaged by the cryopreservation process (Špaleková et al., 2011). Although fertility was not evaluated in this study, sperm motility is a parameter directly related to it and is considered a fertility prognostic factor, especially when the proportion of motile sperm is less than 40 % (Eliasson, 2010). In this sense, Ledesma et al. (2017) examined the relationship between sperm subpopulations obtained during thawing and fertility data, finding significant differences between the rams with higher and lower fertility.

When considering sperm morphometry after thawing, the most significant subpopulation for explaining cryoresistance is morphometric subpopulation 1 (morphSP1), which comprises the smallest spermatozoa and represents 31.03 % of the total sperm count, aligning with the well-documented observation that cryopreservation reduces sperm head dimensions across various species (Eppleston and Maxwell, 1995; Peña et al., 2005). While our study primarily focuses on sperm cryoresistence rather than fertility, previous research has attempted to establish a connection between individual sperm morphometric parameters (Santolaria et al., 2015) and subpopulations (Yániz et al., 2015) with fertility outcomes after artificial insemination. Unfortunately, these attempts have not yielded conclusive results. Notably, other sperm parameters, such as motility and viability, exert a more influence on the fertilization capacity of the sperm after thawing. These findings align with our results, emphasizing the importance of morphSP1. Interestingly, this subpopulation does not correlate with either quantity (TM) or quality parameter of movement (motSP2), nor does it show a significant association with sperm viability after thawing. Moreover, morphSP1 receives the lowest coefficient weighting in the context of Cryoresistance Index (CRI), suggesting that sperm morphometry has a minimal impact on sperm cryoresistance. In simpler words, alterations in the structure of the sperm morphometric subpopulation would not significant affect the final score, given its very low weight.

Furthermore, sperm viability measured immediately after thawing and following hypoosmotic stress serves as an explanatory parameter for assessing the quality of thawed sperm. The combination of sperm membrane analyses and the HOST provides valuable insights into the tolerance of thawed sperm under new stress conditions. In our study, we observed that 92.16 % of thawed sperm with intact plasma membrane could survive the hypoosmotic shock (HOST). We posit that HOST viability after thawing directly correlates with sperm cryoresistance, as indicated by its significant weight in the index. This correlation is further supported by its strong association with viability immediately after thawing.

According to Godshalk and Timothy (1988) and Wang and Chen (1998), Principal Component Analysis (PCA) allows the establishment of indices by considering parameter importance. It enables the selection and utilization of principal components that capture most of the total variance. Additionally, the eigenvectors associated with the parameters enhance the significance of the results obtained from the analysis. Under the current index, which considers the parameters most relevant for explaining ram sperm cryoresistance, breeders can be classified and compared with one another, excluding classifications that only capture partial information.

In fact, as mentioned earlier, various analyses have been conducted to classify breeders based on semen cryoresistance across different species (Yeste et al. 2013; Jiang et al. 2017) using diverse statistical approaches. However, our results differ from those of the referenced studies, since we propose a single equation, the Cryoresistance Index (CRI), which combines kinematics and morphometric parameters, plasma and acrosome membrane integrity, mitochondrial activity, sperm motility and survival after osmotic stress. This approach assigns a

single weighted value to each male during evaluation, optimizing resources and encompassing all the parameters obtained from semen analyses.

Conclusions

Through principal components analysis, we have identified the parameters that best explain ram sperm cryoresistance from a dataset of 22 sperm parameters analysed in a retrospective study, developing an effective model known as Cryoresistance Index (CRI). This CRI assigns significantly higher weighting coefficients to the quantity (TM) and quality (motSP2) of sperm movement after thawing, compared to the head size and shape of the thawed sperm (morphSP1). Furthermore, HOST Viability plays a more decisive role than what is observed under isotonic conditions.

CRediT authorship contribution statement

MJ Palomo: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **I. Yánez-Ortiz:** Writing – original draft, Methodology, Formal analysis, Data curation. **A. Tabarez:** Methodology, Investigation, Data curation. **W. García:** Methodology, Investigation, Data curation.

Declaration of Competing Interest

Authors have declared no conflict of interest.

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