Title: **Sperm Subpopulations in Iberian Red Deer Epididymal Sperm and their Changes Through the Cryopreservation Process**

Short title: Sperm subpopulations in red deer epidydimal sperm

Key Words: Iberian red deer, cryopreservation, epididymal sperm, CASA, sperm subpopulations, cluster analysis

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ABSTRACT

We have applied a statistical protocol based on principal component analysis, clustering methods and discriminant analysis for the identification of sperm subpopulations in CASA data. Samples were obtained from the cauda epididymis of 11 Iberian red deers, and cryopreserved following a standard protocol. Motility by CASA was analyzed just after sperm recovery, just before freezing and after thawing, and eight motility descriptors for each individual spermatozoon were recorded. Sperm viability and acrosomal status were also assessed. Subpopulation analysis was performed in four sequential steps: principal component analysis using the 8 motility descriptors; non-hierarchical clustering analysis (k-means) using the first two principal components; hierarchical clustering analysis (UPGMA); and selection of the final number of clusters. Three clusters were obtained for each motility analysis: slow and non-linear; rapid and linear; and rapid, high ALH, non-linear. We detected variations in the clusters between treatments (initial, pre-freezing and post-thawed). Indeed, motility increased and linearity decreased in the pre-freezing analysis. A discriminant analysis isolated three descriptors that were used again in the same statistical analysis, giving four clusters that resembled the pattern found in the first classification. We also performed a clustering analysis of the males according to pre-freezing/post-thawed variation of total motility, viability and acrosomal status. The proportion of the linear subpopulations in the pre-freezing treatment, in both clustering analyses, correlated positively with post-thawed viability recovery. Our results show that clustering analysis of CASA data gives useful and practical information that is not obtained by conventional sperm analysis.

INTRODUCTION

The presence of several distinct subpopulations within sperm samples is nowadays widely accepted by the scientific community. Although the mechanism of formation of these subpopulations and their physiological role are not yet clear [1, 2], many authors have found that susceptibility to capacitation and fertilizing ability varies depending on the subpopulation studied [3–5]. Therefore, the development of experimental techniques and statistical analyses aimed at identifying and isolating subpopulations depending on several characteristics is of great interest, not only because of the study of sperm biology, but also because of practical and economical reasons [6, 7]. In fact, many sperm separation methods, such as density gradient centrifugation or the swim-up are based on the isolation of sperm subpopulations with certain abilities, specially related to fertility [2].

Subpopulation identification has been carried out according to very different sperm characteristics, such as biochemical parameters [8–11], functional tests [12–18] or depending on of sperm morphology [2, 19–21]. These studies have frequently reported a relationship between detected subpopulations and sperm quality, fertility or its ability to resist cryopreservation.

Improvements in CASA devices have also enabled individual sperm to be distinguished in motility analysis, and subpopulations characterised by motility descriptors to be identified by means of clustering algorithms. However, few studies have considered the existence of sperm subpopulations [22], implying that useful information may have been overlooked. The use of the mean values of motility descriptors oversimplifies the motility analysis, considering the whole sample as being an homogeneous one. Consequently, its internal variability is not taken into account, thus impairing the analysis of the relationship between motility and sperm quality, and, ultimately, fertility [6, 23].

Many researchers have reached interesting conclusions after carrying out subpopulation studies. A large number of these studies were performed using the PATN software, a powerful collection of statistical procedures aimed to the extraction and display of patterns in multivariate data. For instance, Abaigar et al. [22] showed that the addition of caffeine or bicarbonate or cryopreservation with different extenders altered the subpopulation pattern of boar and gazelle semen. Another study on gazelle semen [24] indicated that the subpopulation pattern was altered

depending on the voltage used during electroejaculation, seminal fraction, body weight and storage time. Thurston et al. [25] included a multivariate analysis of boar semen, obtaining three subpopulations with different motility qualities. In general, the analysis performed with this software consisted of a so-called non-hierarchical clustering step followed by a hierarchical one, which rendered the final clusters.

Other researches have used other statistical methods based on less specific software, but not less efficient. Most of them performed non-hierarchical clustering on the CASA data, using the k-means model, although others [6] also explored some hierarchical procedures. Amongst the studied species are human [26, 27], common marmoset [6], stallion [28] and boar [6, 7]. These studies reported some interesting data on sperm subpopulation variations with regard to resistance to cryopreservation [27], presence of stimulants [6], storage and fertility [28] and between-boar variability [7].

The aim of the present study was mainly to develop a multi-step statistical protocol that would enable the subpopulation composition of a sperm sample to be determined utilizing the motility data obtained from CASA analysis. We also intended this protocol not to be dependent on one particular statistical package, but to be easily portable to any software capable of performing principal component analysis and clustering procedures. We based the protocol on two successive steps of non-hierarchical and hierarchical clustering procedures, in order to combine the advantages of both techniques. However, as stated by other authors [22, 28], most motility descriptors are highly correlated, and present different scales of measure, which are considerable problems when performing certain statistical clustering methods. Therefore, prior to the clustering steps, we performed a principal component analysis, reducing the number of descriptors to a few, uncorrelated and standardized, variables (principal components). We then used a statistical method based on the clustering history of some statistics to determine the more suitable number of final clusters. Furthermore, a discriminant analysis determined which motility descriptors were the most important discriminating between the found clusters, and another clustering analysis was performed in order to determine if reliable subpopulations could be obtained by reducing the initial amount of descriptors.

For this study, we used Iberian red deer epididymal sperm, which was analyzed for motility just after being

obtained, before freezing and after thawing. Our intention was not only to determine if we could separate sperm subpopulations, but also study the variation of the subpopulation structure amongst the three treatments (initial, pre-freezing and post-thawed), and its relationship with the whole sperm population, the subpopulation of "progressive" spermatozoa, and the subpopulation of "rapid" spermatozoa (obtained apart from the clustering analysis). Finally, we investigated the relationship between sperm "freezability" (taken as the difference between post-thawed and pre-freezing values of some parameters) and the motility subpopulations found in the study.

MATERIAL AND METHODS

All chemicals were obtained from Sigma (Madrid, Spain). Media were not bought as such, but prepared in our laboratory as referred. Table 1 shows a list of acronyms frequently used throughout this work.

Genitalia collection and sperm recovery

Genitalia were collected from 11 Iberian red deer (*Cervus elaphus hispanicus*) harvested in several private hunting reserves in the region of Cáceres (Spain). All the animals were adults and lived in a free-ranging regime. Sample collection was carried out during the first fortnight of December.

Harvest plans followed the Spanish Harvest Regulation, Law 19/01 of Extremadura, which conforms to European Union Regulation. Furthermore, species and number of individuals that can be hunted, as well as the exact times of the year when hunting can take place, are reviewed each year by the Annual Hunting Regulation of the respective regions. Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD223/1998, which conforms to European Union Regulation 86/609 and adheres to guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the American Society of Andrology.

Scrotum, including testicles and epididymes, was removed from the carcass and refrigerated down to 5 °C as soon as possible. Date and time of death, collection and refrigeration were recorded and attached to the corresponding sample. Refrigerated genitalia were sent to our laboratory at the Veterinary Clinic Hospital of the University of León

(Spain), arriving about 24 hours postmortem.

Sample manipulation was carried out in a walk-in fridge (5 °C). Testicles with epididymes and vas deferens attached were isolated from scrotum and other tissues. Epididymes were dissected free from the testicles, and cleaned of connective tissue. To avoid blood contamination, superficial blood vessels were previously cut, wiping their contents and drying thoroughly the surface of the cauda. Sperm was collected making several incisions on the cauda epididymis with a surgical blade, and taking the liquid emerging from the cut tubules with the aid of the blade.

Cryopreservation protocol

Sperm (still at 5 °C) was diluted 1:1 with Tes-Tris-Fructose extender, containing 10% egg yolk and 4% glycerol [29]. After resting 2 h at 5 °C, the sample was further diluted with the same extender down to 100×10^6 sperm/mL and packed in 0.25 mL French straws. Freezing was carried out using a a programmable biofreezer (Planner MRII®), at -20 °C/min down to -100 °C, and then transferred to liquid nitrogen containers. Thawing was performed by dropping the straws in water at 65 °C for 6 s.

Computer assisted sperm analysis

Samples were analyzed just after recovery (initial assessment), just before freezing (pre-freezing assessment), and after thawing (post-thawed assessment). We will refer to these three stages as treatments.

Sperm were diluted down to $10–20 \times 10^6$ spermatozoa/ml in a buffered solution (20 mmol/L Hepes, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose; pH 7, 400 mOsm/kg), and warmed on a 37 °C plate for 20 minutes. Then, a prewarmed Makler counting chamber (10 μm depth) was loaded with 5 μL of sample. The CASA system consisted of an optical phase contrast microscope (Nikon Labophot-2) (endowed with negative phase contrast objectives and a warming stage at 37 °C), a Sony XC-75CE camera, and a PC with the Sperm Class Analizer software (SCA2002, Microptic, Barcelona, Spain). The magnification was $\times 10$. All samples were analyzed at least twice, in order to discard errors due to incorrect sampling. At least 5 fields per sample were acquired, recording at least 100 motile sperm. Image sequences were saved and analyzed afterwards. CASA acquisition parameters were:

25 images acquired, at an acquisition rate of 25 images per second.

Samples were corrected and analyzed using the editing facilities provided by SCA2002. Events other than spermatozoa were removed, and settings were adjusted in each case in order to assure a correct track analysis. Whenever a field was considered to have incorrect analyzed tracks and correction was not suitable, it was removed. After each analysis, data were saved in an Excel file (Microsoft, Redmon, WA). For each sperm analyzed, the SCA2002 rendered the following data: VCL (velocity according to the actual path; μm/s), VSL (velocity according to the straight path; μm/s), VAP (velocity according to the average —smoothed— path; μm/s), LIN (linearity; %), STR (straightness; %), WOB (wobble; %), ALH (amplitude of the lateral displacement of the sperm head; μm), and BCF (frequency of the flagellar beat; Hz). Detailed explanation of these descriptors of sperm movement is provided elsewhere [27, 30–32]. Spermatozoa were considered motile when VCL$>10\mu$m/s.

CASA data preprocessing

Data from SCA were processed with the help of Excel 4 macros programmed ad hoc. Excel files from SCA2002 were modified in order to give each observation (individual spermatozoa) two labels, identification of the animal and treatment. Then, files were concatenated and the resulting file was used in further analysis. Data produced by statistical procedures were also processed with the aid of macros in order to assign cluster ownership. Also, we defined other two sperm subpopulations, independently of cluster analysis: “rapid” sperm subpopulation (each spermatozoa with VCL$>75\mu$m/s) and “progressive” sperm subpopulation (progressive spermatozoa, each spermatozoa with VCL$>25\mu$m/s and STR>80%). These subpopulations will be referred to as PSP and RSP, respectively. We chose these parameters and these values because they were assigned in that way in the configuration of our CASA system for deer. For each subpopulation, we calculated the proportion of spermatozoa it comprised respect to the total number of motile spermatozoa in the sample (SM), or respect to the total number of spermatozoa (either motile or inmotile) in the sample (ST).

Evaluation of sperm viability and acrosomal status

Samples (pre-freezing and post-thawed) were diluted in buffered media (1:100, same composition that the one used for motility analysis), and stained with prodidium ioide (PI; 25 μg/L) and PNA lectin conjugated with FITC (1 μg/mL). After 10 min, the samples were analyzed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). We used a 15 mW argon-laser that provided an excitation wavelength of 488 nm, using the FL3 photodetector channel to read the red emission light of PI (650 long pass filter), and the FL1 photodetector channel to read the green emission light of FITC (530/30 band pass filter). At least 10000 events (spermatozoa detected, after discarding debris) were acquired. Considering that PI stains cells with damaged plasma membrane, and that PNA-FITC stains acrosome-reacted cells, viability and acrosomal status were defined as the percentage of PI-unstained and FITC-unstained cells in the sample, respectively.

Statistical analysis

All statistical analyses were carried out using the SAS/STAT™ package V. 8 (SAS Institute, Cary, NC) [33]. The main objective of the analysis was to extract sperm subpopulations using the motility data obtained from each treatment, by means of a clustering procedure, and then compare subpopulations and treatments using χ^2 and general linear models. $P<0.05$ was used for significance.

As a first step, we used the PRINCOMP procedure in order to perform a principal component analysis (PCA) of the motility data. The purpose of PCA is to derive a small number of linear combinations (principal components) from a set of variables, that retain as much of the information in the original variables as possible. This allows to summarize many variables in few, jointly uncorrelated, principal components. A good result is when we obtain few principal components accounting for a high proportion of the total variance. In order to select the number of principal components that should be used in the next step of our analysis, we followed the criterion of selecting only those with an eigenvalue (variance extracted for that particular principal component) higher than 1 (Kaiser criterion).

As a second step, we performed a non-hierarchical cluster analysis using the FASTCLUS procedure, and the

selected principal components as variables. This procedure performs a disjoint cluster analysis on the basis of distances computed from one or more quantitative variables, using euclidean distances (k-means model) to calculate cluster centers. This clustering method is often used with large data sets before trying a hierachical one, in order to reduce the data to a few initial clusters and then pass them to the hierarchical procedure. After trying many options, we selected a maximum number of clusters of 15, as this number represents a fairly high number of initial clusters for the subsequent hierarchical procedure (too few initial clusters may have impaired the results of the last clustering step), but is not high enough to complicate the follow-up of the hierarchical clustering. We also took advantage of this step to detect outliers, since this clustering procedure is very sensitive to them, which appear as clusters with only one member. Following detection, outliers can be removed and the procedure rerun.

The third step of the clustering analysis was carried out using the CLUSTER procedure, which performed a hierarchical clustering on the clusters obtained by the previous step, using the average linkage method (UPGMA) for joining the clusters. The procedure displays a history of the clustering process, giving useful statistics for estimating the number of clusters in the population from which the data are sampled. In order to determine the final number of clusters, we studied the evolution along the clustering process of three statistics provided by CLUSTER: the pseudo t^2, the pseudo F and the cubic clustering criterion (CCC). We looked for certain kind of consensus among the three variables, to be precise local peaks of the CCC and pseudo F statistics combined with a small value of pseudo t^2 and a larger pseudo t^2 for the next cluster fusion. It must be noted that other methods can be used, but this one has the advantage of being simple and gives a good estimate of the clustering trend (Figure 1). The output data set of CLUSTER were passed to the TREE procedure, that drew a tree diagram (dendrogram) describing the clustering process, and produced the n-clusters solution in a final data set, which was utilized in the subsequent statistical analysis.

After clustering analysis, we compared treatments within subpopulations and subpopulations within treatments (we include clusters, PSP, RSP and the whole motile sperm population within the term “subpopulation”). We used the GLM (general linear models) procedure in order to carry out an ANOVA on the data. Viability and acrosomal status

before and after cryopreservation were also compared with this procedure. Previously, percentages were subjected to arc sine transformation and absolute measures to log transformation. The Student-Newman-Keuls test was used for pairwise comparison when results were significant.In order to study the distributions of observations (individual motile spermatozoa) between subpopulations within treatments and between treatments within subpopulations, we used the χ^2 test, included in the FREQ procedure. When assumptions for χ^2 test were violated, the exact Fisher's test was used instead.

In order to determine whether we could reduce the original number of variables and still obtain informative results, we carried out a stepwise discriminant analysis (STEPDISC procedure) in the eight variables and the three clusters (separately for each treatment). This kind of analysis indicates which of the original variables best reveal differences among the given groups. Thus, we selected those variables with high discriminatory power in the three treatments, and repeated the whole clustering analysis, entering only the selected variables. This time, we selected the number of principal components by the variance explained, rather than by their eigenvalues, since we expected only one principal component with an eigenvalue higher than one due of entering less variables in the PCA.

Finally, we studied some aspects of the "freezability" of the individual sperm samples. We defined "freezability" as the difference between post-thawed and pre-freezing sperm quality. Therefore, we used three variables for determining the "freezability" of each sample: total motility, sperm viability and acrosomal status, substracting their post-thawed value from the pre-freezing one. We called the new variables TMdiff, VIABdiff and ACRdiff, respectively. Then, we carried out a clustering analysis in order to classify the males according to the values of these variables. This cluster analysis was performed by carrying out a PCA and hierarchical clustering on "freezability" parameters in an identical manner as described above, but omitting the non-hierarchical clustering step (in this case there were few initial objects —males—, and this step was not necessary). Also, we carried out a correlation analysis between pre-freezing variables (including those derived from the subpopulation study) and "freezability" variables. This analysis was performed using the Spearman correlation coefficients (CORR procedure).

RESULTS

General results and preliminary analysis

Samples acquired with CASA were analyzed and corrected. When a field could not be analyzed due to sampling errors (incorrect contrast or bright, causing most of the tracks to be incorrectly analyzed by the software), it was removed. After correcting the samples, we pooled all the data in a common database, with 893 observations for the initial analysis, 1526 observations for pre-freezing, and 919 observations for post-thawed analysis. Each observation was identified by three codes, the number of the deer, the treatment (initial, pre-freezing, or post-thawed), and the number of the sperm within each acquisition.

In general, motility parameters were good, even after thawing, considering the whole population, PSP and RSP subpopulations (Table 2). Comparison of the proportion of motile, progressive and rapid spermatozoa (χ^2 test) indicated significant differences amongst all treatments. Total motility had the lowest values initially, improved greatly in the pre-freezing, and decreased post-thawed, but were still higher than in the initial treatment. Conversely, PSP decreased pre-freezing and returned to initial values post-thawed, which also gave good numbers of RSP (in fact, sperm velocity was higher post-thawed). On the other hand, viability decreased significantly from a pre-freezing value of 84.5$\pm$1.5% to a post-thawed value of 67$\pm$1.8%, whereas the percentage of spermatozoa with damaged acrosomes, as indicated by PNA-FITC staining, increased significantly from 3.2$\pm$0.7% to 8.1$\pm$1.2%.

First cluster analysis

For each treatment, PCA rendered two principal components with eigenvalues above 1 (PRIN1 and PRIN2; Table 3), which accounted for more than 80% of the variance. Considering the scores of CASA parameters, the first principal component was related to fast and linear movement, whereas the second principal component was related to fast erratic movement, including wide head lateral displacement.

The two principal components entered in the non-hierarchical clustering, and the resulting 15 clusters were grouped into three clusters, after applying the hierarchical procedure and studying the plots of CCC, pseudo-F and

pseudo-t^2 vs. number of clusters (Figure 1). The dot plots of the two principal components for each treatment showed that the positions of the three clusters were similar in the multidimensional space defined by these principal components (Figure 2), thus we related each cluster in each treatment to its equivalent in the other two treatments and denominated them CL1, CL2 and CL3. CL1 and CL2 were well defined by their PRIN1 values (in general, CL1 with negative values and CL2 with positive ones). Therefore, accordingly to the eigenvalues of the motility descriptors for PRIN1, CL1 would include slow and non-linear spermatozoa, whereas CL2 would include fast and linear spermatozoa. In the same sense, CL3 has positive values for PRIN2, so this cluster would include fast, non-linear sperm, with high ALH. These characteristics are reflected in the mean values of the motility descriptors (Table 2). Observing the variation in the clouds of dots between treatments, we observed a higher dispersion of CL3 in the initial treatment, whereas CL2 was compact in the initial and post-thawed treatments, but more scattered in the pre-freezing treatment. These changes correspond with alterations in their motility descriptors, as can be observed in table 2.

In general, the distribution of spermatozoa amongst the three clusters followed the trend found for the whole population, and PSP and RSP subpopulations. Interestingly, PSP included 93% and 99% of CL2 (rapid and linear) in the initial and post-thawed treatments, respectively, but in the pre-freezing one, only 74% of CL2 was included into PSP, indicating a drop in the proportion of high-linear spermatozoa contained in CL2 (Figure 3). On the other hand, RSP (Figure 4) comprised the totality of CL3 (rapid and non linear) in the three treatments, plus a constant part of CL2 (around 75%). Although spermatozoa in CL1 were considered slow and non-linear, 20–25% were included in PSP, and around 11% (initial) and 18% (pre-freezing and post-thawed) were included in RSP.

Clustering analysis with a reduced set of variables

We selected VCL, VSL and LIN after carrying out the discriminant analysis. In the three treatments, these variables were among the four with the highest F and R^2 values (indicating good discriminant power). BCF in the initial, and VAP in the post-thawed treatment had also good discriminant power, but were rejected because they did not get good values in all the three treatments. The PCA rendered three principal components, and the two with the highest eigenvalues passed to the clustering analysis (Table 4). Although PRIN2 was lower than 1, we included it in the

analysis because it contributed with an appreciable proportion of the total variance. PRIN1 represented one variable related to both good velocity and linearity, whereas PRIN2 represented low velocity (VCL), but even better linearity than PRIN1.

We obtained four clusters in each treatment, which were called CL1b, CL2b, CL3b and CL4b, plus an extra cluster in the post-thawed treatment, CL5b. Considering their characteristics (Table 5), CL1b and CL2b were similar to CL1 and CL3, respectively. CL3b and CL4b were similar to CL2, sharing good linearity, but CL3b was faster and had higher ALH. Moreover, the evolution of the proportions of CL1 and CL2 compared with CL1b and CL3b+CL4b, respectively, were very similar. However, CL2b behaved differently than CL3, peaking in the pre-freezing treatment instead. The positions of each cluster in the space defined by the two principal components (Figure 5) coincided in the three treatments, and it was possible to carry out an interpretation similary to the one in the first analysis. CL1b and CL3b were mainly defined by PRIN1 (slow and non-linear vs. rapid and linear), and CL2b and CL4b by PRIN2 (rapid and non-linear vs. —comparatively— slow and linear). CL5b could be considered as a residual subpopulation, considering its low proportion and that its position coincides with part of CL3b in the other treatments. The proportion of each subpopulation included in PSP was similar to the numbers reported for the first clustering analysis, CL3b and CL4b being almost completely included. Although CL3b drop from above 90% to 87% in the pre-freezing treatment, this decrease was less important that the one undergone by CL2. Regarding RSP, it comprised totally both CL2b and CL3b totally. There were interesting variations between treatments considering CL4b, being its proportions in RSP being 71, 39 and 25%, for the initial, pre-freezing and post-thawed treatments, respectively. This matched the decrease of the mean values of VAP throughout the treatments, such as showed in Table 5. CL1b also underwent also a consequent but less dramatic variation (12, 17 and 14%, for each treatment).

Study of sperm "freezability"

Table 6 shows the values for "freezability" parameters and the proportions of the subpopulations obtained in the first part of this study (pre-freezing values) for each group of males. TMdiff, Viabdiff and ACRdiff were introduced in the PCA, which rendered two principal components (eigenvalues 1.38 and 1.06), taking account of 0.46 and 0.35 of total

variance for each. The eigenvectors of the three “freezability” variables, for PRIN1 and PRIN2 respectively, were: TMdiff, 0.06 and 0.93; VIABdiff, 0.70 and -0.30; and ACRdiff, 0.72 and 0.20. In this case, PRIN1 was clearly related to viability and acrosomal status, whereas PRIN2 was mostly related to motility, and, to a lesser degree, to viability (inversely) and acrosomal status. The classification of the males depending on “freezability” parameters is shown in Figure 6. According to the interpretation of the principal components, cluster 1 would contain the samples that better resisted cryopreservation (its location, with positive PRIN1 values suggested better VIABdiff and ACRdiff), whereas cluster 2 represented samples with worse (lower) VIABdiff and ACRdiff. TMdiff seemed little affected in both cases, since these clusters have low absolute values for PRIN2. On the other hand, cluster 3 was characterized by bad VIABdiff and ACRdiff, but good motility recovery, because of PRIN2 positive values. Cluster 4 represented an interesting case, because its position indicated bad motility and acrosomal status recovery, but, because of VIABdiff negative eigenvector for PRIN2, viability recovery would be better than those of clusters 2 or 3. In fact, as shown in the dendrogram, male 11 may be an outlier, which would be removed from the analysis as more males were included in the study.

Interestingly, clusters 1 and 4 seemed to have a higher CL2 (rapid and linear) pre-freezing proportions, whereas clusters 2 and 3 had higher CL1 (slow and no linear) pre-freezing proportions (due to the low number of males, no significance test were performed). Cluster 4, with the poorest TMdiff and ACRdiff, had the highest CL3 (rapid and no linear) proportion. PSP and RSP proportions did not show any apparent relation with better or worse recovery of ‘freezability’ variables. The correlation analysis rendered a significant correlation between CL2 and VIABdiff ($r=0.71$, $P=0.015$). No significant correlations were found between the other parameters. However, the sum of CL3b (very rapid and linear) and CL4b (rapid and linear) (considered roughly equivalent to CL2) also correlated with VIABdiff ($r=0.81$, $P=0.003$).

DISCUSSION

Considerations on the statistical procedure

Multivariate clustering methods for the study of CASA data are not of general usage in sperm work. However, a review on articles that apply this kind of analysis show a number of interesting findings from this kind of analysis. We can also appreciate that different authors have chosen a great variety of combinations of different statistical test. For instance, Davis et al. [27] carried out a multi-step iterative procedure, combining the k-means model with multivariate discriminate analysis. On the other hand, Holt [6] compared a non-hierachical clustering method (k-means model) with two hierarchical ones (UPGMA and WPGMC). However, these authors compared these methods, but did not use them sequentially, such as in the present work. Abaigar et al. [22] utilized a different approach, using the PATN software. This software package provides many statistical tools which allow to extract and displaying patterns in multivariate data. These authors performed a non-hierarchical clustering carried out using non-parametric algorithms, its output was redirected to a hierarchical clustering analysis (UPGMA), and the final number of clusters was selected with the help of the FUSE module of the statistical package. To facilitate interpretation of data, they completed the analysis performing principal coordinates analysis and principal axis correlation, which allowed them to present multivariate data in a simpler format. This statistical package and a similar statistical procedure has been followed in other articles [24, 25, 34].

Other authors have followed simpler methods, which have provided useful information, though. Quintero-Moreno et al. [7 28] selected the motility descriptors that should pass to the clustering step by means of applying the VARCLUS procedure of the SAS package, which divides a set of numeric variables into either disjoint or hierarchical clusters. This classi?cation, enables one to select the most representative parameters for the data to be studied. After selecting the most representative parameters, they performed a non-hierarchical cluster analysis using the k-means model (FASTCLUS procedure), obtaining the corresponding clusters.

The main objective of this work was to combine several statistical procedures in order to extract several clusters (sperm subpopulations) from CASA data. We based it on the cited studies, specially the one by Abaigar et al.

[22]. However, we intended to develop a protocol that would not depend on one specific type of software, but could be easily implemented elsewhere (SPSS, Statistica, S/STAT, R, etc). Thus we have given a detailed description of the SAS procedures that we utilized. Since information on this statistical package is widely available [33], anybody with some knowledge of statistics should be able to apply the described analysis to their own statistical software. Firstly, we had to decide which clustering analysis to use. Taking into account the huge amount of data that a CASA analysis can produce, we decided to perform the clustering in two steps: a previous non-hierarchical clustering, which would reduce the data to a relative small number of clusters, followed by a hierarchical clustering step. Holt [6] recommended hierarchical clustering methods to the non-hierarchical k-means model, because the former provide more information and allows the clustering process history to be thoroughly studied (in order to select the final number of clusters, for instance). Moreover, the use of the k-means model implies a previous knowledge of the clustering structure, since the final number of clusters must be provided before performing the analysis (although some statistics can be used to test the efficacy of the clustering procedure). A good reason for the use of a non-hierarchical method as an intermediate step is the detection of outliers, as explained in Material and Methods.

On the other hand, an important issue is the selection of the motility descriptors that may enter in the clustering analysis. Quintero-Moreno et al. [28] pointed out that most motility descriptors are often highly correlated, and that the relative importance of these parameters may vary between species. In fact, these authors used the VARCLUS procedure to select the variables that would enter in the clustering analysis. Another problem is the need of transforming motility parameters prior to the clustering analysis, at least by performing a standardization step. Otherwise some descriptors would outweigh other because of different scales. It is important to note that Abaigar et al. [24] overcame this problem without having to of transforming their data because they used non-parametric algorithms in their non-hierarchical clustering method. Our proposal for resolving these issues was to perform a principal component analysis before carrying out any clustering analysis. We thus converted a number of unstandardized, highly correlated parameters into few variables, representing linear combinations of the former parameters, standardized and uncorrelated. This analysis not only serves to simplify the interpretation of our data,

because of the parameter reduction, but also provides abundant information that can be useful for the interpretation and representation of the results of the clustering analysis. We also carried out a discriminant analysis, from which we selected a subset of variables that were used successfully in the second clustering analysis. In fact, results obtained with that subset even provided more detailed information on some aspects, indicating the suitability of this kind of analysis when performing in-depth studies with CASA data.

Another fundamental issue in the clustering analysis is the selection of the final number of clusters obtained from the analysis. The study of the cluster distances and the dendrograms produced by hierarchical cluster analysis can greatly help in this step, especially when we have a previous knowledge of the internal structure of our data. The method used in this work, based on the evolution of pseudo-F, CCC and pseudo-t^2 statistics in the clustering process, has many limitations (it works better with compact or slightly elongated clusters [33]), but provides an objective and flexible way to determine the final number of clusters. Although many studies analyze significant differences amongst the mean values of clusters, these results should be carefully considered, especially if the analyzed variables were those that entered in the clustering analysis. The reason is that clustering algorithms intend to maximize the variability between clusters, thus significant differences are not surprising, but rather expected. Signi?cant differences amongst clusters may be used as an aid to explain clusters characteristics or, such as in this study, to showing the differences between the clusters and other groups of motile sperm obtained by different means.

Subpopulation structure and cryopreservation effects

The clustering analysis allowed us to obtain three distinct subpopulations. It may be interesting to test whether CL3 or CL2b (rapid and no linear spermatozoa) contained spermatozoa with some kind of hiperactivation, but unfortunately the acquisition conditions (chamber only 10 μm deep) were not adequate to perform such an analysis. It is important to point out that we obtained the same number of subpopulations in the three treatments, in both analyses, and, although mean values were different for some parameters, their general characteristics remained similar. This indicates that the internal structure of the CASA data was mostly preserved, undergoing few changes through the processing and freezing-thawing. Interestingly, a similar pattern was obtained by Holt [6] in boar sperm after applying

a hierarchical clustering method. However, other reports indicate different characteristics, especially considering sperm progressivity. This may have happened because of different clustering techniques, different species, and different sources of sperm. However, there is a general coincidence on the number of subpopulations (three or four, in general). Quintero-Moreno et al. [28] highlighted this coincidence between studies, and suggested that this pattern could be a widespread fact.

On the other hand, the source of spermatozoa could be a very important factor regarding differences between works, since epididymal and ejaculated spermatozoa are different in many senses. Although sperm from the cauda epididymis is almost analogous to ejaculated semen, it has not had contact with seminal plasma and its diverse factors, which are known to alter many characteristics of sperm [35, 36]. For instance, Holt [6] and Abaigar et al. [22] represented the location of the different clusters in plots in a multidimensional space defined by canonical or factor variables, where the clusters appeared mostly as clearly separated groups, whereas in our study it was difficult to visually separate the cloud of points defined by PRIN1 and PRIN2 in well-defined groups. The lack of separation between clusters may be a characteristic of epididymal sperm, although we need to compare with ejaculated deer sperm in order to confirm this.

One interesting fact in our data is the higher proportion of motile sperm in thawed samples when compared with the initial sampling. This may seem surprising if we do not analyze the pre-freezing sampling. In order to explain the variations in the motility characteristics in the three treatments, we have to consider many factors, the most important being the source of sperm. The samples (still in the cauda epididymis) arrived at our laboratory after being stored many hours at 5 °C, and the first evaluation of motility took place in a relative simple medium. Our experience with epididymal sperm has showed us that spermatozoa recently salvaged from the cauda epididymis generally are slower, compared with those from ejaculates, and frequently many of them just present a weak the tail beat, with very slow displacement or none at all, and are therefore not detected as motile by CASA. Only after warming the sample for some time (generally from 20 to 40 min) did we obtain a stable and representative motility pattern that could be reliably measured with the CASA equipment. The situation was very different for the pre-freezing samples, possibly

412 because the sperm had been diluted with the extender. This is a more complex medium including egg yolk, which is
413 known to alter sperm motility [37–39]. Epididymal sperm may undergo some changes when in contact with the
414 extender, activating the motility of some sperm [40], and therefore altering the characteristics and proportions of the
415 subpopulations, as we have observed. In fact, the increasing in the proportion of CL1 (slow, non-linear sperm) may be
416 due mostly to the activation of spermatozoa that were not detected as motile in the initial sampling, rather than the
417 conversion of CL2 (rapid and linear) or CL3 (rapid and no linear) spermatozoa to CL1 spermatozoa. Furthermore,
418 after being in contact with the extender, long warming times were not necessary anymore, and motility could be
419 acquired at 5 or 10 min of warming time with no difference. Nevertheless, we kept 20 min as warming time, in order
420 to respect the initial protocol, and considering that there was no alteration with regard to shorter times.
421 The discriminant analysis and the subsequent clustering analysis showed that it is possible to reduce the
422 number of initial variables and still obtain a good subpopulation study. Still, the similarity of the subpopulation
423 structure of both analysis (with the exception of the different number of subpopulations) suggest that most of the
424 motility descriptors were redundant and may be removed from the analysis without problems. However, the use of
425 more complex descriptors, such as those based on hyperactivation, angular and oscillation parameters, may improve
426 this kind of study. However, the correct analysis of these parameters implies certain conditions that we were unable to
427 achieve, such as sufficient chamber depth to allow the free movement of spermatozoa [41]. In the second part of the
428 study we obtained even more extensive information. For instance, assuming that CL3b+CL4b (very rapid and linear,
429 and rapid and linear) were equivalent to CL2 (rapid and linear), that means that there were some variations in the
430 internal structure of CL2 between different treatments. That is, CL3b was stable considering its motility parameters,
431 but the mean velocity of CL4b dropped in the pre-freezing and post-thawed treatments when compared to the initial
432 one, which was not evident in the mean values of CL2. The reason was that, in the pre-freezing treatment, a
433 considerable part of CL2b (rapid and non linear) was included in CL2, and, in the post-thawed treatment, CL3b
434 prevailed over CL4b. Although we considered CL2b equivalent to CL3 (rapid and non linear), it had a more complex
435 nature, since in the initial treatment many CL3 members (the most linear ones) were included in CL3b, and in the

pre-freezing treatment, as noted before, CL2b included not only CL3, but also part of CL2.

It is noteworthy that, without considering sperm subpopulations, all this interpretation would have not been possible. In fact, the inclusion of PSP (progressive) and RSP (rapid), although not as informative as the subpopulations derived from cluster analysis, provided some useful information that is not obtained when only the mean values of motility descriptors are considered. These subgroups have the advantage that they can be readily extracted from the CASA data. However, clusters provided more extensive results, and there was not a clear identity between them and PSP or RSP. On the other hand, the definition of “progressive” or “rapid” spermatozoa allowed to deepen the internal structure of the clusters.

Considering the “freezability” analysis, both sperm viability and acrosomal status were clearly affected by the cryopreservation process, since these parameters are highly dependent on the status of sperm membranes. The clustering of the males according to our “freezability” parameters grouped effectively those samples with similar ability for maintaining these parameters after thawing. On the other hand, the correlation between the fre-freezing proportion of CL2 and VIABdiff suggested some kind of relationship between the predominance of a sperm subpopulation and the outcome of the cryopreservation process. This seemed to be further supported by the correlation found between VIABdiff and the sum of the pre-freezing proportions of CL3b and CL4b. The lack of correlation with CL3b or CL4b separately, but with CL3b+CL4b, may indicate that the presence of a subpopulation with linear spermatozoa, irrespective of its velocity, may be related to good post-thawed recovery of sperm viability. Unfortunately, our limited data did not allow us to present a thorough analysis on this issue, including the relationship of subpopulations with fertility. Anyway, this was not one of the objectives of this study, which were aimed at applying the studied statistical methods and describing the subpopulations obtained. However, further studies should be carried out to test the real meaning of these subpopulations in relation to fertility, as suggested by other authors [27]. In this sense, Quintero-Moreno et al. [28], working with stallion sperm, found that ejaculates with high fertility shared a special subpopulation pattern. Nevertheless, the same authors [7] did not find such a relationship when studying boar semen. The exact nature of sperm subpopulations and their influence on the fertility of a sample may be

a complex issue, and the use of different CASA protocols and statistical analysis may complicate the comparison between different studies.

Conclusions

In conclusion, we have applied a statistical method that enabled us to find sperm subpopulations defined by motility parameters. By examining the obtained subpopulations, we were also able to determine some characteristics of red deer epididymal sperm, and allowed us to study the variations it underwent through a cryopreservation protocol. One interesting fact was the conservation of the subpopulation pattern between the different treatments. We could relate one of these subpopulations, characterized by rapid and linear spermatozoa, to good post-thawed viability recovery. The study of two motility subgroups defined by us independently of the clustering analysis, one of rapid and other of progressive sperm, helped to obtain useful information on the internal composition of the clusters. This study was necessarily limited, including the lack of fertility data, but the clustering analysis gave interesting information not available using conventional motility analysis. Due to the possibilities of this kind of analysis, we consider that the study of sperm subpopulations defined by motility descriptors should be widely considered, especially when including fertility results.

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FIG. 1. Example of the dendrogram derived from the hierarchical clustering analysis, and of the plot used for the determination of an adequate final number of clusters (data from the first clustering analysis, post-thawed treatment). The plot on the left shows the dendrogram resulting from the hierarchical clustering of the 15 clusters derived from the PCA and subsequent non-hierarchical clustering of post-thawed motility data. The plot on the right shows the line plot of CCC, pseudo-F and pseudo-t^2 statistics produced by the hierarchical clustering procedure, which helped to find possible cut places in the dendrogram (the three variables were standardized in order to show all three in the same plot). Each step in the hierarchical clustering joins two clusters in a new one, and generates new values for these three statistics. Suitable number of clusters are indicated by local high values of the CCC and pseudo-F statistics and low values of pseudo-t^2 statistic, followed by decreasing CCC and pseudo-F and increasing pseudo-t^2 in the next cluster fusion. In this case, the plot clearly indicates 3 final clusters, although numbers of 5 or 8 may have been eligible for study.

FIG. 2. Dot plots corresponding to the clusters obtained from the first analysis, defined by the two first principal components (PRIN1 and PRIN2). In order to follow the evolution of each subpopulation between treatments, each original dot plot was decomposed into three, showing only one cluster each. Each event represents one individual spermatozoa. The PCA enabled the resulting eight-dimensional space to be shown in a two-dimensional plot, not only preparing the data for the clustering analysis, but also helping in its interpretation. Events are represented by different symbols in order to identify which cluster they belong to (CL1: ○; CL2: □; CL3: △).

587 FIG. 3. Representation of the “progressive” (PSP) subpopulation in the space defined by the ?rst two principal
588 components (PRIN1 and PRIN2, from the ?rst clustering analysis). In order to follow the evolution of each
589 subpopulation between treatments, each original dot plot was decomposed into three, showing only one cluster each.
590 Each event represents one individual spermatozoon. Events are represented by different symbols in order to identify
591 which cluster they belong to (CL1: ○; CL2: □; CL3: △).

FIG. 4. Representation of the “rapid” (RSP) subpopulation in the space defined by the two first principal components (PRIN1 and PRIN2, from the first clustering analysis). In order to follow the evolution of each subpopulation between treatments, each original dot plot was decomposed into three, showing only one cluster each. Data from the initial, pre-freezing and post-thawed analysis are presented from left to right. Each event represents one individual spermatozoon. Events are represented by different symbols in order to identify which cluster they belong to (CL1: ○; CL2: □; CL3: △).

598 FIG. 5. Clusters obtained after the discriminant analysis. Dot plots of the motility data defined by the two first
599 principal components (PRIN1 and PRIN2). In order to follow the evolution of each subpopulation between
600 treatments, each original dot plot was decomposed in three, showing only one cluster each. Data from the initial,
601 pre-freezing and post-thawed analysis are presented from left to right. Each event represents one individual
602 spermatozoon. Events are represented by different symbols in order to identify which cluster they belong to
603 (CL1b: ○; CL2b: □; CL3b: △; CL4b: +). CL5b (•) is shown together with CL3b and CL4b in the corresponding plot.

FIG. 6. Clustering of the males depending on the “freezability” of their sperm samples. 6A show the dendrogram with the males progressively joining in clusters, and the position where it was cut to form the final clusters. 6B show the males in the multidimensional space defined by the two first principal components extracted from PCA (PRIN1 and PRIN2). Ellipses indicate belonging to a concrete cluster. Compare the joining process in the dendrogram with the distances between males in the plot.

TABLE 1. List of some acronyms frequently used in this work.

Acronym	Meaning
ST	Proportion of spermatozoa in a subpopulation respect to the total number of spermatozoa (either motile or inmotile) in the whole sample.
SM	Proportion of spermatozoa in a subpopulation respect to the number of motile spermatozoa in the whole sample.
PSP	Subpopulation comprising progressive sperm (VCL$>10\mu$m/s and STR$>80\%$).
RSP	Subpopulation comprising rapid sperm (VCL$>75\mu$m/s).
PCA	Principal components analysis.
CCC	Cubic clustering criterion.
PRIN1	First principal component extracted from a PCA.
PRIN2	Second principal component extracted from a PCA.
CL1, CL2 and CL3	Clusters obtained from the first clustering analysis.
CL1b, CL2b, CL3b, CL4b and CL5b	Clusters obtained from the second clustering analysis, after performing the discriminant analysis.

TABLE 2. Summary of selected motility parameters obtained in this study (SM, ST, VAP, LIN, ALH and BCF), considering subpopulations and treatments. Subpopulations include: the whole population of motile spermatozoa, PSP, RSP, and the three subpopulations defined by the first clustering analysis (CL1, CL2 and CL3). The treatments are I: initial, PF: pre-freezing and PT: post-thawed. Data are expressed as Mean±SD.

Subpopulation	Treatment	SM (%)[1]	ST (%)[1]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz/s)
	I	100	68.6^{a}	68.4 ± 40.9^{aA}	64.3 ± 27.1^{aA}	2.8 ± 1.9^{aA}	7.8 ± 4.1^{A}
Motile[2]	PF	100	82.0^{b}	58.7 ± 37.8^{bA}	47.9 ± 24.1^{bA}	3.4 ± 2.1^{bA}	8.0 ± 3.5^{A}
	PT	100	77.6^{c}	74.6 ± 45.3^{aA}	47.9 ± 27.6^{bA}	3.4 ± 1.5^{bA}	8.0 ± 3.6^{A}
	I	69.5^{aA}	47.7^{aA}	81.4 ± 34.3^{aB}	78.4 ± 17.5^{aA}	2.6 ± 1.8^{aA}	8.1 ± 3.8^{A}
PSP	PF	42.8^{bA}	35.1^{bA}	69.0 ± 35.6^{bB}	69.8 ± 17.0^{bB}	2.9 ± 1.7^{aB}	9.3 ± 3.5^{B}
	PT	64.9^{cA}	50.4^{aA}	88.2 ± 39.8^{aB}	80.2 ± 16.2^{aB}	2.5 ± 1.2^{bA}	8.9 ± 3.5^{A}
	I	61.0^{aB}	41.9^{aB}	94.0 ± 29.0^{aC}	73.2 ± 22.3^{aC}	3.5 ± 2.0^{aB}	8.7 ± 4.0^{B}
RSP	PF	51.8^{bB}	42.5^{abB}	88.3 ± 27.0^{bC}	50.7 ± 23.7^{bA}	4.8 ± 2.0^{bC}	8.2 ± 3.5^{A}
	PT	58.6^{aB}	45.5^{bB}	106.2 ± 29.0^{cB}	73.1 ± 23.9^{aC}	3.3 ± 1.5^{aB}	8.6 ± 3.7^{A}
	I	27.9^{aC}	19.1^{aC}	20.3 ± 13.9^{aD}	31.7 ± 13.8^{aD}	1.9 ± 1.1^{aC}	4.9 ± 3.1^{aC}
CL1	PF	45.6^{bC}	37.4^{bAC}	26.8 ± 15.9^{bD}	32.9 ± 14.1^{aC}	2.4 ± 1.2^{bD}	7.2 ± 3.3^{bC}
	PT	33.0^{cC}	25.6^{cC}	28.2 ± 21.1^{bD}	35.8 ± 15.7^{bD}	2.2 ± 1.1^{bC}	7.0 ± 3.2^{bB}
	I	50.9^{aD}	34.9^{aD}	80.8 ± 28.8^{aB}	86.1 ± 11.2^{aE}	2.0 ± 0.8^{aD}	9.6 ± 3.8^{aB}
CL2	PF	45.9^{bC}	37.6^{aC}	82.6 ± 29.6^{aC}	67.3 ± 18.7^{bB}	3.6 ± 1.8^{bE}	9.0 ± 3.5^{bB}
	PT	60.2^{cB}	46.7^{bB}	96.1 ± 35.3^{bBC}	83.1 ± 13.0^{cB}	2.5 ± 1.3^{cA}	8.9 ± 3.6^{bA}
	I	21.2^{aE}	14.6^{aE}	101.8 ± 33.5^{C}	54.8 ± 17.4^{aF}	5.6 ± 1.8^{aE}	7.3 ± 3.6^{A}
CL3	PF	8.6^{bD}	7.0^{bD}	100.7 ± 20.9^{E}	23.9 ± 8.4^{bD}	7.7 ± 1.6^{bF}	7.1 ± 3.1^{C}
	PT	6.9^{bD}	5.3^{cD}	108.2 ± 37.3^{C}	30.3 ± 16.1^{cE}	5.9 ± 1.1^{cD}	6.9 ± 3.0^{B}

[1]: χ^2 on raw data (rest of comparisons by ANOVA+SNK test).

[2]: for the whole population of motile sperm, % (motile) is 100% (not included in between-subpopulations comparison).

^{a,b,c}: rows (treatments within subpopulations) with different superscripts differ $P<0.05$.

^{A,B,C,D,E}: rows (subpopulations within treatments) with different superscripts differ $P<0.05$.

TABLE 3. Summary of the results of the PCA performed on the CASA data. The first two principal components obtained for each treatment (eigenvalues higher than 1) are showed. Variance explained is the proportion of the total variance explained by each principal component. The eigenvectors are a measure of association of the original parameters with the resulting principal components.

		Initial		Pre-freezing		post-thawed	
		PRIN1	PRIN2	PRIN1	PRIN2	PRIN1	PRIN2
Eigenvalues		4.57	2.05	3.82	2.63	4.47	2.02
Variance explained		0.57	0.26	0.48	0.33	0.56	0.25
Eigenvectors	VCL	0.35	0.46	0.35	0.44	0.37	0.44
	VSL	0.45	0.12	0.49	0.03	0.46	0.07
	VAP	0.43	0.24	0.45	0.27	0.43	0.26
	LIN	0.40	-0.34	0.38	-0.40	0.40	-0.35
	STR	0.35	-0.26	0.29	-0.41	0.33	-0.38
	WOB	0.37	-0.31	0.38	-0.27	0.40	-0.22
	ALH	0.12	0.64	0.20	0.54	0.11	0.64
	BCF	0.25	-0.18	0.16	-0.18	0.18	-0.09

TABLE 4. Summary of the second PCA, performed only with the variables selected after the discriminant analysis. Variance explained is the proportion of the total variance explained by each principal component. The eigenvectors are a measure of association of the original parameters with the resulting principal components.

		Initial		Pre-freezing		post-thawed	
		PRIN1	PRIN2	PRIN1	PRIN2	PRIN1	PRIN2
Eigenvalues		2.26	0.70	1.98	0.96	2.30	0.65
Variance explained		0.75	0.23	0.66	0.32	0.77	0.22
Eigenvectors	VSL	0.66	-0.04	0.70	0	0.65	-0.02
	VCL	0.55	-0.66	0.52	-0.69	0.55	-0.68
	LIN	0.52	0.75	0.49	0.72	0.52	0.73

TABLE 5. Clustering obtained after the discriminant analysis. Summary of selected motility parameters obtained in this study (SM, ST, VAP, LIN, ALH and BCF), considering subpopulations and treatments. The treatments are I: initial, PF: pre-freezing and PT: post-thawed. Data are expressed as Mean±SD.

Subpopulation	Treatment	SM (%)[1]	ST (%)[1]	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz/s)
	I	32.2^{a}	22.1^{a}	22.8 ± 14.9^{a}	35.9 ± 17.2	2.0 ± 1.1^{a}	5.5 ± 3.4^{a}
CL1b	PF	48.8^{b}	40.0^{b}	27.6 ± 15.7^{b}	36.4 ± 16.3	2.3 ± 1.1^{b}	7.5 ± 3.5^{b}
	PT	33.7^{a}	26.1^{c}	26.0 ± 17.6^{c}	36.9 ± 16.5	2.1 ± 1.0^{a}	7.2 ± 3.3^{b}
	I	10.9^{a}	7.5^{a}	81.2 ± 22.1^{a}	43.5 ± 14.2^{a}	5.3 ± 1.7^{a}	7.2 ± 3.6
CL2b	PF	21.8^{b}	17.8^{b}	88.5 ± 21.8^{b}	33.8 ± 12.5^{b}	6.1 ± 1.7^{b}	7.6 ± 3.2
	PT	7.0^{c}	5.4^{c}	99.3 ± 28.6^{c}	27.7 ± 10.5^{c}	5.5 ± 1.2^{a}	6.9 ± 3.3
	I	20.8^{a}	14.2^{a}	123.9 ± 18.8^{a}	80.1 ± 14.2^{a}	4.0 ± 2.2^{a}	8.8 ± 3.8
CL3b	PF	16.6^{b}	13.6^{a}	110.8 ± 17.3^{b}	71.6 ± 12.9^{b}	4.3 ± 1.6^{b}	9.1 ± 3.3
	PT	40.7^{c}	31.6^{b}	114.4 ± 20.2^{b}	83.4 ± 13.3^{c}	2.9 ± 1.2^{c}	8.9 ± 3.9
	I	36.1^{a}	24.8^{a}	72.7 ± 18.2^{a}	86.8 ± 10.2	1.9 ± 0.8^{a}	9.5 ± 3.9^{a}
CL4b	PF	12.8^{b}	10.5^{b}	59.0 ± 17.4^{b}	85.3 ± 8.8	1.8 ± 0.8^{b}	9.1 ± 3.2^{a}
	PT	16.7^{c}	12.9^{c}	55.5 ± 17.3^{b}	85.9 ± 9.2	1.5 ± 0.5^{c}	8.5 ± 3.0^{b}
CL5b	PT	2.0	1.5	156.2 ± 19.2	64.5 ± 10.3	6.0 ± 1.1	9.1 ± 2.4

[1]: χ^2 on raw data (rest of comparisons by ANOVA+SNK test).

[a,b,c]: rows (treatments within subpopulations) with different superscripts differ $P<0.05$.

TABLE 6. Description of the clusters obtained in the "freezability" clustering analysis (Figure 6). We have indicated the number of males included in each cluster, and the Mean±SD of the "freezability" parameters (TMdiff, VIABdiff and ACRdiff), total motility (TM) and the percentages of the previously studied subpopulations (CL1, CL2, CL3, PSP and RSP).

Cluster	Males	TMdiff	VIABdiff	ACRdiff	TM	CL1	CL2	CL3	PSP	RSP
1	5	-3.3±6.6	-16.3±3.5	-2.7±0.9	81.6±5.9	38.7±12.6	51.0±8.4	10.3±10.5	30.9±7.8	28.3±17.2
2	3	-6.1±5.5	-22.1±4.4	-5.5±1.0	80.4±3.8	46.3±7.7	45.8±3.7	7.8±4.8	21.4±2.9	48.1±16.4
3	2	6.9±1.2	-25.6±1.3	-6.9±2.9	76.5±0.0	65.1±19.6	25.9±6.9	9.0±12.7	25.3±7.1	34.2±4.2
4	1	-22.3	-17.4	-9.1	87.2	24.6	53.8	21.5	20.4	46.4
Total	11	-4.0±9.1	-19.7±4.9	-4.8±2.5	80.8±5.0	44.3±16	45.3±11.6	10.4±8.9	26.3±7.3	36.4±16.1

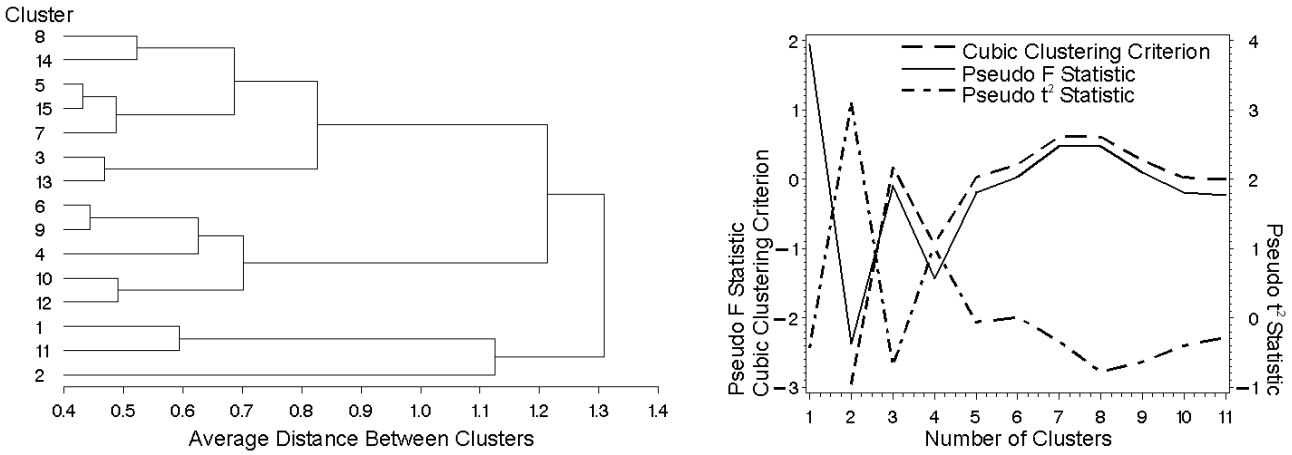

Cluster
8
14
5
15
7
3
13
6
9
4
10
12
1
11
2
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
Average Distance Between Clusters
Cubic Clustering Criterion
Pseudo F Statistic
Pseudo t² Statistic
Pseudo F Statistic
Cubic Clustering Criterion
Pseudo t² Statistic
Number of Clusters

FIGURE 1.

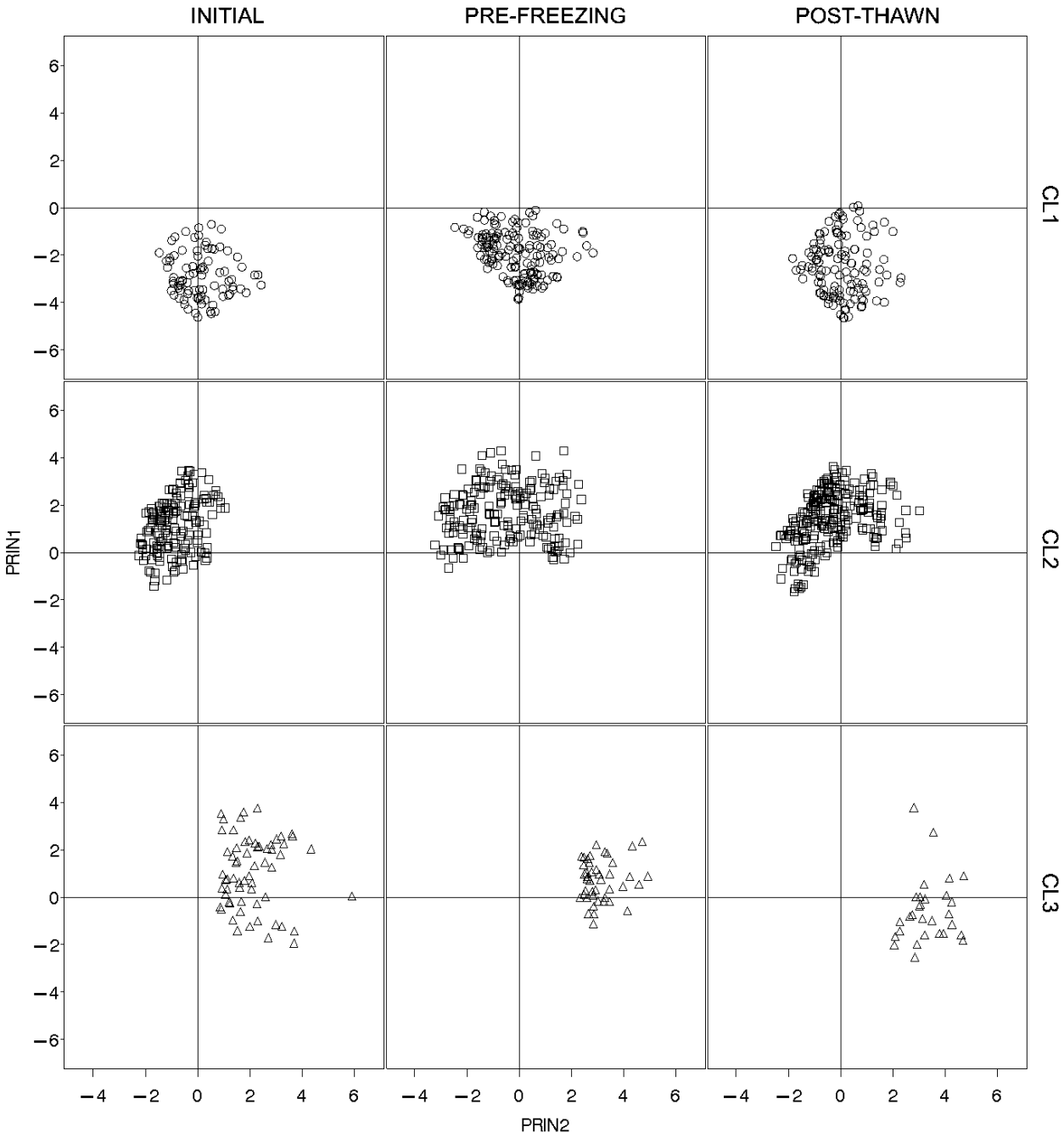


FIGURE 2.

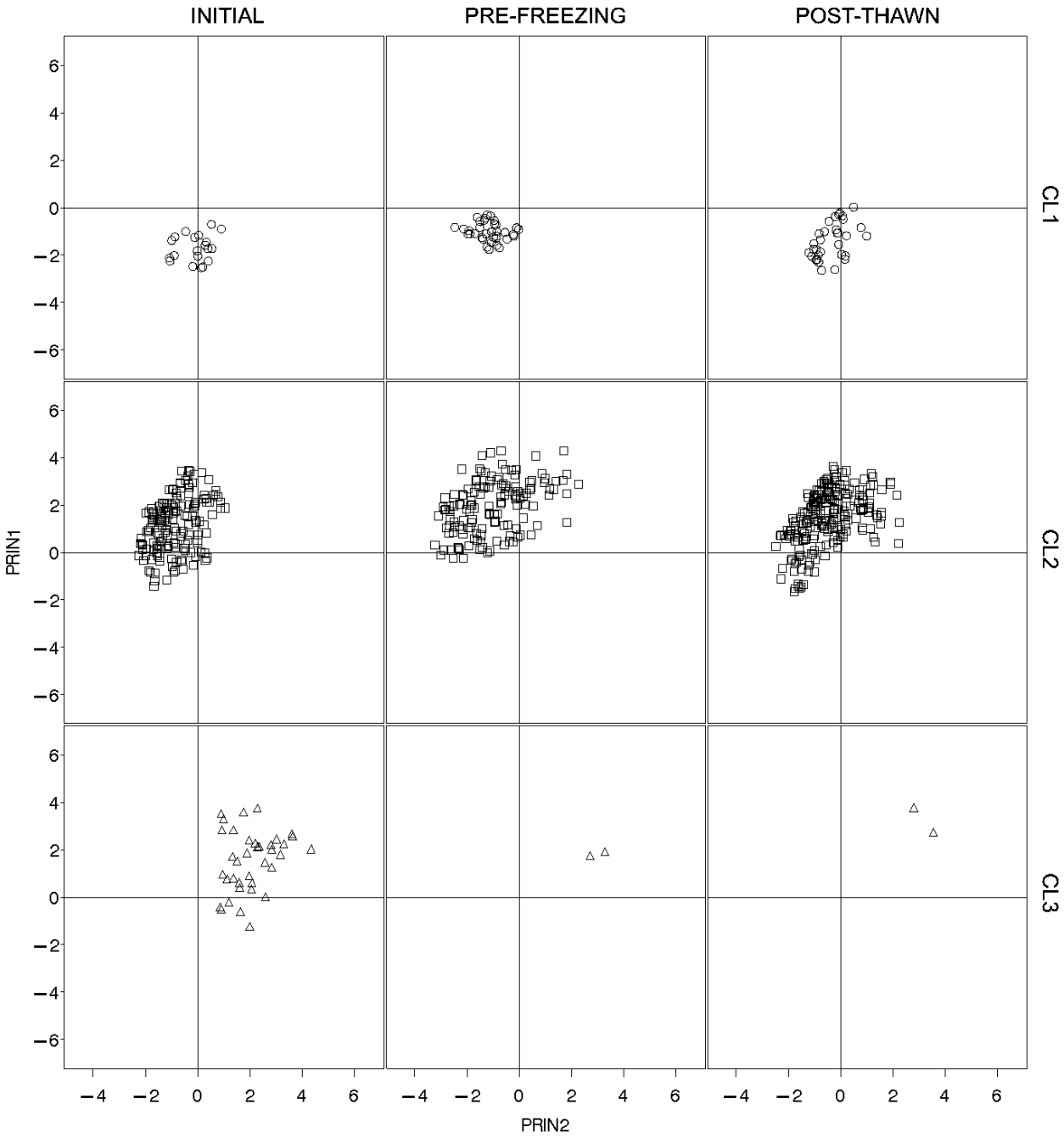


FIGURE 3.

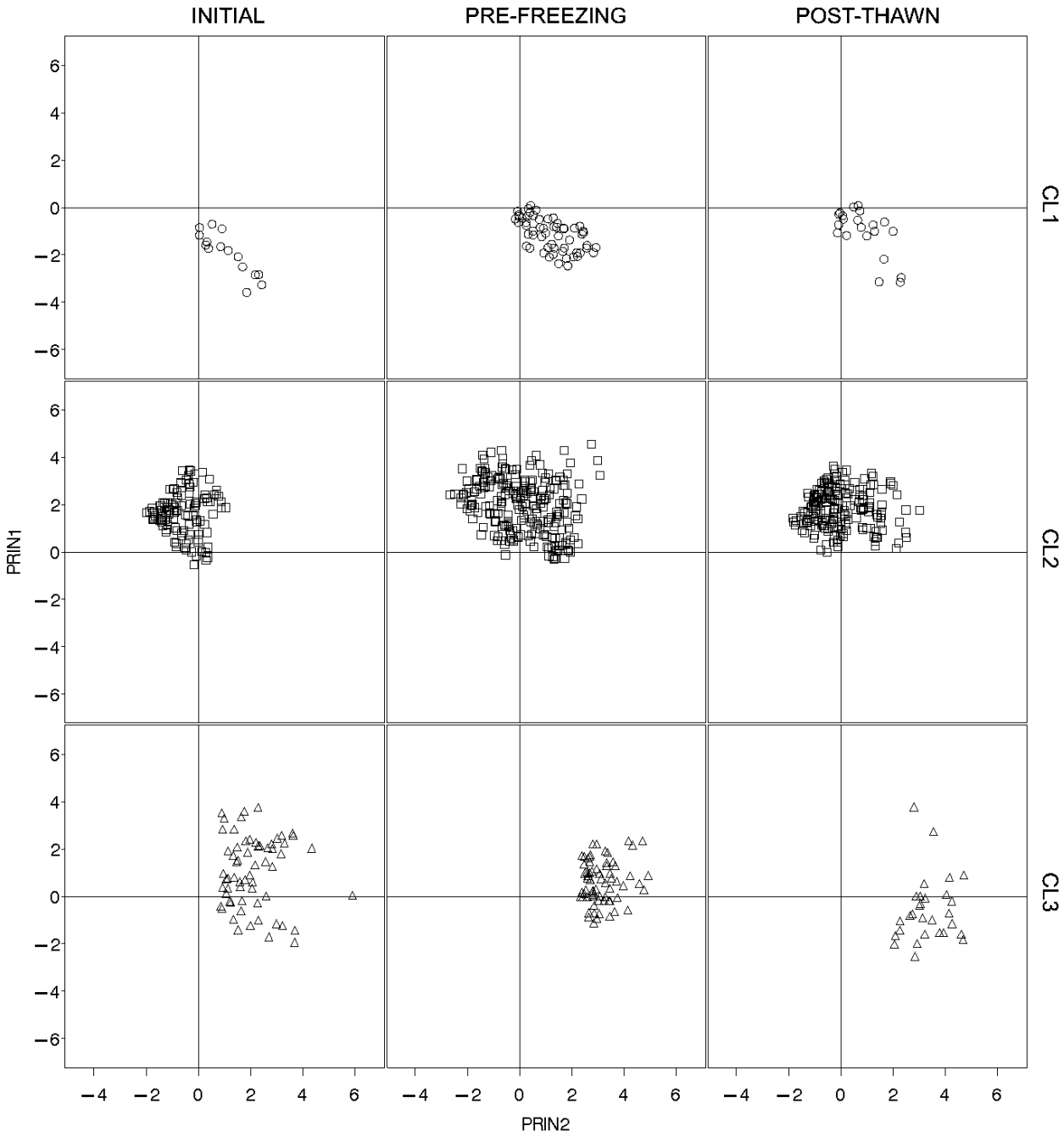


FIGURE 4.

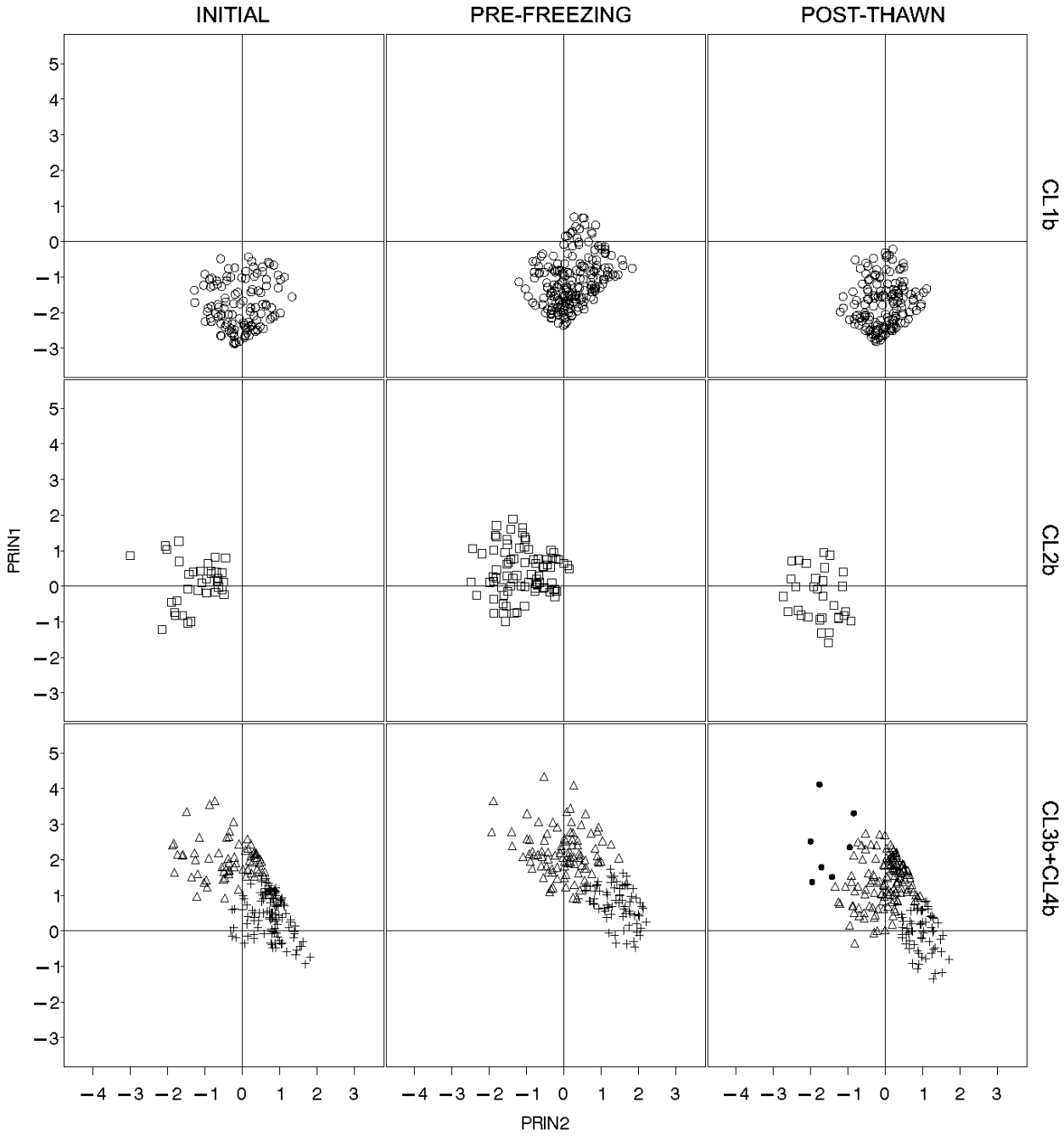
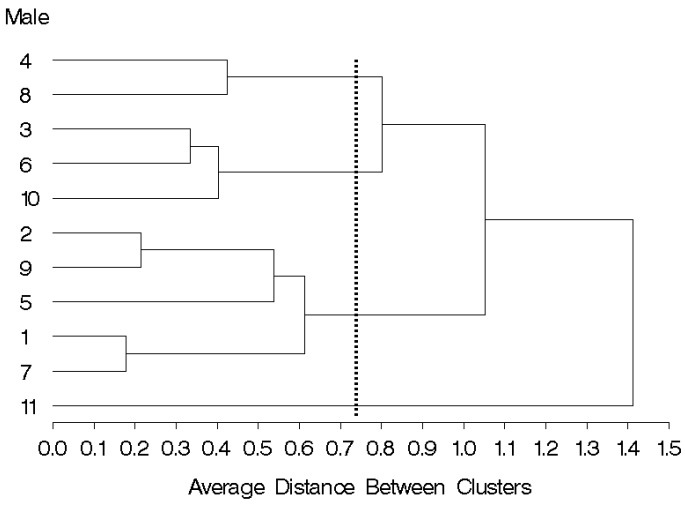
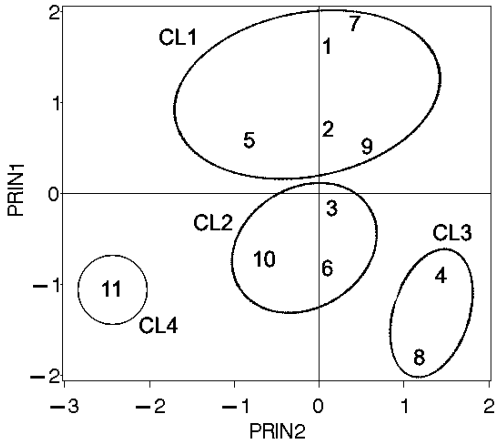

INITIAL
PRE-FREEZING
POST-THAWN
CL1b
CL2b
CL3b+CL4b
PRIN1
PRIN2

FIGURE 5.



6A



6B

FIGURE 6.