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Role of changes in plasma prolactin concentrations on ram and buck sperm cryoresistance

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ABSTRACT

Seasonal endocrine changes may modify sperm cryoresistance in certain small ruminant species. The present work examines the effect of prolactin (PRL) on ram and buck sperm cryoresistance. A dopamine agonist (bromocriptine [BCR] 60 mg i.m. twice per week from May 15 to June 15, that is, approaching the summer solstice) or antagonist (sulpiride [SLP] 100 mg s.c. daily from December 15 to January 15, that is, around the winter solstice) was administered under solstice-appropriate photoperiod conditions to modify PRL secretion. Control animals received the vehicle only. Compared to the corresponding controls, BCR reduced PRL secretion to basal levels in both the rams and bucks. In rams, the cryoresistance ratios for sperm curvilinear velocity ($P < 0.05$) and lateral head displacement ($P < 0.01$) were higher for the BCR-treated animals. In bucks, neither the characteristics of fresh nor frozen-thawed sperm were affected by BCR treatment. After the administration of SLP, PRL levels increased and remained high for more than 5 h in the rams though they immediately began to fall in the bucks. By 24 h, PRL had returned to basal concentrations in both species. In rams treated with SLP, the cryoresistance ratios for sperm progressive motility, straight line velocity, sperm mean path velocity, cross beat frequency, and the progression ratios linearity, straightness and oscillation, were all lower compared to the controls ($P < 0.05$), while the amplitude of lateral head displacement was higher ($P < 0.01$). In bucks, sperm cryoresistance was not affected by SLP administration. Together, these results suggest that high levels of PRL negatively affect the cryoresistance of ram sperm, while buck sperm seems unaffected.

1. Introduction

Protocols for cryopreserving the sperm of small ruminants have been widely implemented [1]. However, they remain suboptimal, and in sheep the fertility rates achieved when using frozen-thawed sperm are usually low [2,3]. Changes in endocrine status appear to modify sperm cryoresistance in some small ruminants. For example, in the ibex (*Capra pyrenaica*) [4], the mouflon (*Ovis musimon*), and rams (*Ovis aries*) [5], the high levels of testosterone seen at the peak of the rutting season exert a negative effect on it. Levels of the hormone prolactin (PRL) also show strong seasonal variation in these species [6–8], and might also affect sperm cryoresistance. For example, in ibexes, cryoresistance has been reported higher at the end of the rutting season [4] when both low plasma testosterone and PRL concentrations are seen. A recent study performed at our laboratory also revealed that *in vitro* supplementation with PRL reduces the post-thaw acrosome integrity of ram and buck sperm (*Capra hircus*) [9]. It may therefore be that seasonal

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changes in PRL secretion influence the cryoresistance of small ruminant sperm.

The main environmental factor modulating the seasonal pattern of PRL is the photoperiod [10,11]. The long days of spring-summer are associated with high PRL concentrations, and the short days of autumn-winter with low PRL concentrations [7,12]. Although the seasonal pattern of testicular activity is not related to annual changes in PRL secretion [13,14], PRL receptor (PRL-R) expression in ram testes has been detected in the interstitial and seminiferous tubular compartments, as well as in Leydig cells, pachytene spermatocytes, and round and elongated spermatids [15,16]. This suggests a role for PRL in spermatogenesis and steroidogenesis [17,18]. In addition, PRL-R have seen detected in the epithelium of the vas deferens, the epididymis, the prostate and seminal vesicles [17–19]. In rams it is known that the chronic suppression of PRL leads to a reduction in the size and fructose content of the seminal vesicles, with no change in testosterone secretion [20]. Hence PRL might affect the sperm milieu, and in turn sperm cryoresistance. It may be that PRL exerts an antiapoptotic effect, at least in human sperm, by suppressing the activation of the intrinsic apoptotic pathway [21].

Given the above information, the aim of the present study was to examine the influence of PRL on ram and buck sperm cryoresistance, replicating seasonal changes in PRL secretion via the administration of a dopamine agonistic or antagonist under appropriate photoperiod conditions. Since sperm head size has been suggested a predictor of sperm freezability [22], the associations between sperm head dimensions and cryoresistance were also examined.

2. Materials and methods

All reagents used in the different media, diluents and procedures were purchased from Panreac Química S.A. (Barcelona, Spain) or Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Bromocriptine (BCR; Parlodel, a dopamine agonist) (Mylan Pharmaceuticals, SL, Barcelona, Spain) was prepared for injections by dissolving 1 g in a vehicle containing 6 g of Dextran 70 and 100 mL of saline serum. Sulpiride (SLP; Dogmatil, a dopamine antagonist) (Sanofi Aventis, SA, Barcelona, Spain) for injections was prepared by dissolving 5 g in a vehicle containing 1.5 g of tartaric acid and 100 mL of saline serum.

2.1. Animals

Fifteen Spanish Merino rams and 15 Murciano-Granadina bucks, all sexually mature and all aged between 3 and 4.5 yrs, were housed in the Department of Animal Reproduction of INIA in Madrid (latitude, 40° 25′ N) in adjacent 250 m^2 enclosures under natural light and temperature conditions. All were fed a balanced diet of Visan K59 (Visan Ind. Zoot., Madrid, Spain) based on barley grain, barley straw and dried alfalfa. Free access was provided to vitamin/mineral blocks and water. All experimental procedures were approved by the INIA Ethics Committee and carried out in accordance with the Spanish Animal Protection Policy (RD53/2013), which complies with the European Union Directive 2010/63/EU regarding the protection of animals used in scientific experiments. To determine possible stress interferences by animal management and treatments with dopamine agonist/antagonist on results, plasma cortisol concentrations were assessed in the different experimental groups.

2.2. Experimental design

Two experiments were performed in the seasonal periods of maximum and minimum PRL secretion (ie, approaching the summer solstice and around the winter solstice respectively) of 2 consecutive years.

2.2.1. Experiment 1

Ten animals of each species were randomly divided into two groups: (1) Control group: 5 animals of each species were intramuscularly (i.m) administered 1 ml of a 0.9% NaCl solution containing 60 mg of Dextran 70 twice per week from May 15 to June 15; (2) BCR group: 5 animals of each species were given intramuscular (i.m) injections of 10 mg of BCR in the same Dextran 70-containing vehicle twice weekly from May 15 to June 15. This administration protocol induces baseline concentrations of PRL in blood plasma [8].

2.2.2. Experiment 2

Again, 10 animals of each species were randomly divided into two groups: 1) Control group: 5 animals of each species were subcutaneously (s.c) administered 2 ml of 0.9% NaCl solution containing 30 mg of tartaric acid (vehicle) daily from December 15th to January 15th; 2) SLP group: 5 animals of each species were administered 100 mg of s.c SLP in the same vehicle daily from December 15 to January 15. Preliminary work performed with three rams and bucks each confirmed that SLP administration increases plasma PRL (blood samples were taken at 0, 1, 2, 3, 5, and 24 h post administration).

2.3. Collection of samples and measurements

Ejaculates were collected using the artificial vagina technique (Cassou IMV Technologies, L'Aigle, France). Samples were collected in 15 mL polypropylene centrifuge tubes (Sterilin, Stone, UK). Ram ejaculates were immediately diluted 1:1 (v:v) in TTG medium (210.6 mM Tes, 95.8 mM Tris, 10.1 mM glucose, 320 mOsm/Kg, pH 6.8-7.2); buck ejaculates were immediately diluted 1:1 (v:v) in TCG medium (313.7 mM Tris, 104.7 mM citric acid, 30.3 mM glucose, 345 mOsm/kg, pH 6.8). Four samples were collected from each animal at 15-day intervals.

Blood samples were collected by jugular venipuncture in 5 mL plastic serum tubes (BD Vacutainer, Becton Dickinson Co., Plymouth, United Kingdom) once every week over the experimental period.

2.4. Hormone analysis

Plasma PRL concentrations were determined by radioimmunoassay in duplicate 100 µl plasma aliquots as previously described [23], using rabbit anti-ovine PRL

serum (NIDDK-anti-oPRL-2 [AFP-C358106]) and highly purified ovine prolactin antigen (NIDDK-oPRL-I-3 [AFP-10789B]) (both supplied by Dr. A F. Parlow, Pituitary Hormones & Antisera Ctr., Harbor-UCLA Medical Center, CA, USA). All samples were analysed in a single assay (sensitivity 0.2 ng/mL, intra-assay coefficient of variation 5%; n = 10).

Plasma cortisol concentrations were measured by using commercial enzyme-immunoassay kits (DEH-3388, Demeditec Diagnostics GmbH, Kiel, Germany). The samples were measured in duplicate according to the protocol provided by the kit. The intra and inter-assay coefficient of variation were 6.9% and 5.5% respectively. Sensitivity of the assay was 3.79 ng/ml.

2.5. Sperm quality

The concentration of sperm was determined using an SMD1® photometer (Minitüb, Tiefenbach, Germany) at 546 nm. Sperm motility was evaluated using the computer-assisted analysis system (CASA) running Sperm-Class Analyzer software v.5.3.0.1 (Microptic SL, Barcelona, Spain) coupled to a Nikon Eclipse model 50i negative phase contrast microscope (Nikon Corporation, Tokyo, Japan). The samples were diluted 1:200 (v:v) in a medium identical to that used during collection and loaded into an 8-compartment Leja chamber (20 µm) (Leja Products B.V., Nieuw-Vennep, The Netherlands) at 37°C. The percentage of motile sperm and the percentage showing progressive motility were recorded. In addition, sperm curvilinear velocity (VCL; µm/s), straight line velocity (VSL; µm/s), mean path velocity (VAP; µm/s), amplitude of lateral head displacement (ALH; µm/s), and the cross beat frequency (BCF; Hz) were recorded. Three progression ratios were then calculated: linearity (LIN = VSL / VCL x 100), straightness (STR = VSL / VAP x 100) and oscillation (WOB = VAP / VCL x 100). Three fields and 500 sperm tracks were examined at 100x in each sample chamber (image acquisition rate 25 frames/s) [24].

The percentage of viable sperms and acrosome status were determined by fluorescence microscopy (analysing 200 cells) employing a fluorochrome combination of propidium iodide (PI; Sigma P-4170) and peanut (*Arachis hypogaea*) agglutinin conjugated with fluorescein isothiocyanate (PNA-FITC; Sigma L7381), as described by Soler et al. [25]. Samples were examined using a Nikon Eclipse E200 epifluorescence microscope (excitation 450 to 490 nm, emission 520 nm) (Nikon Instruments Inc., NY, USA).

Morphological abnormalities were assessed in 200 sperm cells by phase contrast microscopy (X 400), using samples fixed in 2% glutaraldehyde solution. Normal sperms and eight types of sperm with abnormalities in the head, intermediate piece and tail (decapitated, altered intermediate piece, broken neck, whip tail, altered head, double tract, broken or broken tail and cytoplasmic gout) were counted [26].

Sperm head morphometric analyses were undertaken for fresh sperm (only) employing a Nikon Eclipse model 50i microscope with a clear field objective at X 60 (Nikon Corporation, Tokyo, Japan) and using the morphometry module of Sperm-Class Analyzer v.5.3.0.1 software (Microptic SL Barcelona, Spain). A total of 100 sperms were analysed on slides previously fixed and stained with Hemacolor. Width (µm), length (µm), area (µm^2) and head perimeter (µm) were determined as previously described by Esteso et al. [27].

2.6. Sperm cryopreservation

Ram sperm was diluted with TTG-ey-gly medium (TTG plus 6% clarified egg yolk [v/v] and 5% glycerol [v/v]). Buck sperm was first washed by diluting in TCG (1:9 v:v), centrifuging at 800 X *g* for 30 min, and the supernatant removed; the pellet was then diluted in TCG-ey-gly medium (TCG plus clarified egg yolk 6% [v/v] and glycerol 5% [v/v]). This was done to reduce the effects of the interaction of phospholipases with the egg yolk lecithin present in the freezing medium [4]. The final concentration was 100 × 10^6 sperm/mL for both the ram and buck samples. These samples were then equilibrated for 3 h at 5°C before loading into 0.25 mL French straws (Minitüb, Landshut, Germany). The latter were then suspended in the vapour 5 cm above a liquid nitrogen bath for 10 min, before being plunged into the liquid nitrogen itself. After a storage time of 4 to12 months, the straws were thawed at 37°C for 30 s in a water bath.

The response to cryopreservation in each experiment was examined by calculating a cryoresistance ratio (CR) for each test variable as follow: CR = (value after thawing/value before thawing) x 100 [28].

2.7. Statistical analysis

All data were expressed as means ± SEM, with the exception of the morphometric variables which were expressed as means ± SDM. Prior to analysis, the percentage values for sperm motility, progressive motility, LIN, STR, WOB, the percentage of viable sperms, acrosome integrity and morphological abnormalities were arcsine transformed since they were not normally distributed according to the Shapiro-Wilk test. For the same reason, the numerical values for plasma PRL, cortisol, VCL, VSL, VAP, ALH, BCF, and morphometric variables were log-transformed. The influence of the treatments on the concentration of PRL and sperm variables was examined by repeated measures ANOVA. The effect of the interaction *species x treatment* on the CR was examined by 2-way ANOVA. The *post hoc* Tukey test was used to compare PRL values between groups at different times over the experimental period, and to compare CR between species. The differences in the cortisol levels were examined by repeated measured ANOVA. Fresh and frozen-thawed sperm variable values were compared using paired t-tests. All calculations were performed using STATISTICA software for Windows v.12 SP3 (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Experiment 1

Figure 1 shows the PRL levels during and after BCR treatment in both species. The treatment affected the

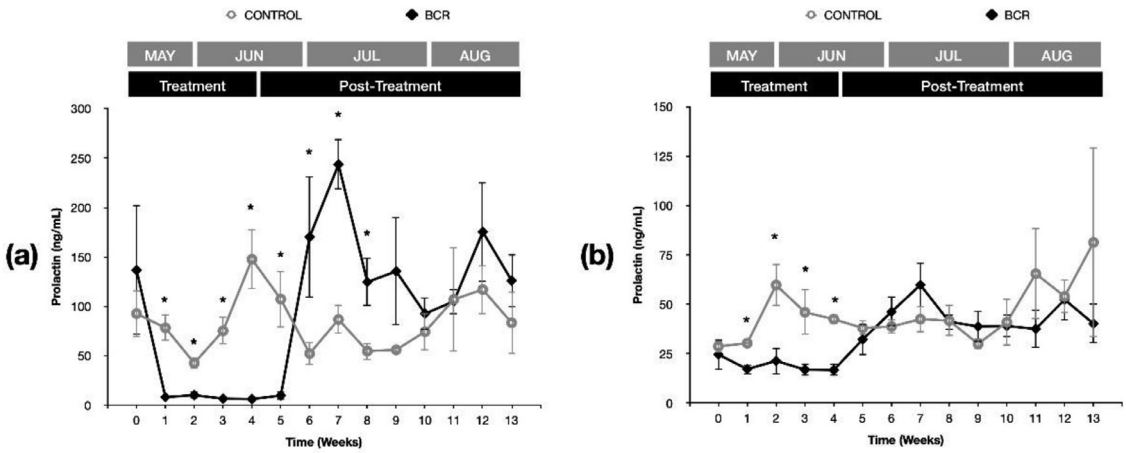

Fig. 1. Weekly change in plasma prolactin concentration (ng/mL; means ± SEM) in rams (a) and bucks (b) during the experimental period; control group (○), bromocriptine (BCR) group (●). Asterisks indicate differences ($P < 0.05$, ANOVA) between groups.

Table 1
Fresh and frozen-thawed ram sperm characteristics in the control and bromocriptine (BCR) groups (mean ± S.E.).

SPERM CHARACTERISTICS	FRESH		FROZEN-THAWED	
	CONTROL	BCR	CONTROL	BCR
Motile sperm (%)	$90.72 \pm 1.08_{A}$	$81.94 \pm 3.91_{A}$	$58.13 \pm 6.57_{B}$	$61.65 \pm 5.19_{B}$
Progressive motility (%)	$31.28 \pm 2.87_{A}$	$29.49 \pm 2.82_{A}$	$23.73 \pm 2.97_{B}$	$23.62 \pm 1.79_{B}$
VCL (μm/s)	$156.81 \pm 4.32_{A}$	$155.85 \pm 6.42_{A}$	$99.16 \pm 3.71_{B}$	$113.14 \pm 5.03_{B}$
VSL (μm/s)	$74.33 \pm 5.60_{A}$	79.79 ± 7.07	$56.83 \pm 3.12_{B}$	64.33 ± 3.55
VAP (μm/s)	$110.18 \pm 6.40_{A}$	$116.36 \pm 7.81_{A}$	$77.40 \pm 4.04_{B}$	$88.22 \pm 4.21_{B}$
LIN (%)	$46.73 \pm 2.73_{B}$	50.53 ± 3.32	$57.07 \pm 1.97_{A}$	56.89 ± 1.77
STR (%)	$66.49 \pm 1.98_{B}$	67.56 ± 2.39	$73.43 \pm 1.15_{A}$	72.82 ± 1.34
WOB (%)	$69.48 \pm 2.43_{B}$	73.78 ± 2.78	$77.56 \pm 1.98_{A}$	77.88 ± 1.13
ALH (μm)	$5.34 \pm 0.16_{aB}$	$4.58 \pm 0.23_{bA}$	$3.02 \pm 0.14_{A}$	$3.23 \pm 0.12_{B}$
BCF (Hz)	$7.94 \pm 0.21_{b}$	$8.66 \pm 0.22_{aA}$	7.59 ± 0.13	$7.61 \pm 0.15_{B}$
Viability (%)	$88.69 \pm 2.01_{bA}$	$94.75 \pm 0.91_{aA}$	$49.44 \pm 5.06_{B}$	$55.13 \pm 4.15_{B}$
Intact acrosome (%)	$95.88 \pm 0.88_{b}$	$99.00 \pm 0.32_{a}$	92.00 ± 2.07	94.94 ± 1.78
Morphological abnormalities (%)	3.13 ± 0.82	5.13 ± 1.16	3.19 ± 0.81	5.13 ± 1.29

Different lower case letters indicate significant differences (P<0.05, ANOVA) between the control and BCR group for fresh and frozen thawed sperm (a-b). Different capital letters indicate significant differences (P<0.05, paired t tests) between fresh and frozen-thawed sperm within each group (A-B).

PRL concentrations in both rams ($P < 0.001$) and bucks ($P < 0.05$), falling to basal levels during the treatment period in both species.

In rams, BCR treatment increased fresh sperm BCF, the percentage of viable sperms and acrosome integrity ($P < 0.05$), and reduced sperm ALH, compared to the control group. Within the control animals, sperm motility, VCL, VAP, ALH, the percentage of viable sperms (all $P < 0.01$), progressive motility, and VSL ($P < 0.05$) values were all reduced by freeze-thaw. Within the BCR-treated animals, the following variables decreased their values due to freezing-thawing: sperm motility, VCL, VAP, ALH, BCF, the percentage of viable sperms (all $P < 0.01$) and progressive motility ($P < 0.05$) (Table 1). The cryoresistance ratios for VCL ($P < 0.05$) and ALH ($P < 0.01$) were higher in the BCR animals than in the controls (Table 2).

No differences were seen between the fresh sperm of BCR-treated and control bucks, nor between the frozen-thawed sperm of treated and control animals. Within the control group, sperm motility, VCL, VSL, VAP, ALH, percentage of viable sperms, acrosome integrity (all $P < 0.01$) and progressive motility ($P < 0.05$) values were all reduced by freeze-thaw. Within the BCR group, freezing-thawing decreases the values of sperm motility, progressive motility, VCL, ALH, percentage of viable sperms, acrosome integrity (all $P < 0.01$), VSL, VAP, and BCF ($P < 0.05$) (Table 3). No differences were seen in the cryoresistance ratios for any variable between the control and BCR groups (Table 2).

The mean length, area and perimeter of the sperm heads were reduced ($P < 0.05$) in animals treated with BCR. In bucks, however, no differences were seen between the treatment and control groups with respect to these variables (Table 4).

The interaction BCR treatment x species did not have significant effect on the CR for any sperm variable. Ram and buck sperm showed the same CR in both control and BCR treated groups (Table 2).

No significant differences were found between groups throughout the experimental period in the plasma cortisol levels, neither in rams nor in buck. The monthly mean (means ± SEM) in rams was 14.94 ± 0.75 ng/mL in the control group and 21.19 ±2.31 in the treated

Table 2
Ram and buck sperm cryoresistance ratios - control and bromocriptine (BCR) groups (mean ± S.E.).

CR BCR	RAMS		BUCKS	
	CONTROL	BCR	CONTROL	BCR
Motile sperm (%)	64.08 ± 7.18	75.59 ± 6.44	53.49 ± 8.14	51.08 ± 5.64
Progressive motility (%)	78.09 ± 7.84	88.70 ± 9.70	64.49 ± 14.88	61.25 ± 11.39
VCL (μm/s)	63.60 ± 2.41	72.95 ± 2.42*	68.29 ± 3.46	68.93 ± 5.00
VSL (μm/s)	81.81 ± 6.73	90.26 ± 9.10	76.04 ± 4.76	77.20 ± 8.69
VAP (μm/s)	71.97 ± 3.85	79.70 ± 5.51	71.15 ± 4.33	73.31 ± 7.48
LIN (%)	128.44 ± 8.82	121.90 ± 10.07	112.63 ± 7.29	109.02 ± 7.23
STR (%)	111.98 ± 3.93	110.12 ± 4.79	108.04 ± 6.32	104.02 ± 3.43
WOB (%)	113.01 ± 3.72	108.28 ± 5.12	104.16 ± 3.12	103.94 ± 4.30
ALH (μm)	57.24 ± 3.14	73.76 ± 4.76**	71.57 ± 6.20	74.93 ± 5.70
BCF (Hz)	96.97 ± 3.67	88.48 ± 2.17	90.86 ± 5.81	87.77 ± 3.87
Viability (%)	56.05 ± 5.78	58.18 ± 4.47	47.25 ± 7.88	46.00 ± 6.24
Intact acrosome (%)	96.09 ± 2.36	95.93 ± 1.90	85.17 ± 3.83	85.71 ± 3.84
Morphological abnormalities (%)	105.77 ± 25.54	92.90 ± 10.94	85.00 ± 23.63	84.72 ± 41.36

Cryoresistance ratio (CR) = (Post-thaw value / Fresh value) x 100. Asterisks indicate significant differences (*P<0.05, **P<0.01, ANOVA) between the control and BCR groups within each species.

Table 3
Fresh and frozen-thawed buck sperm characteristics - control and bromocriptine (BCR) groups (mean ± S.E.).

SPERM CHARACTERISTICS	FRESH		FROZEN-THAWED	
	CONTROL	BCR	CONTROL	BCR
Motile sperm (%)	$83.55 \pm 1.74_A$	$84.90 \pm 2.93_A$	$44.34 \pm 6.82_B$	$43.52 \pm 5.33_B$
Progressive motility (%)	$50.95 \pm 4.77_A$	$50.23 \pm 3.75_A$	$29.28 \pm 4.89_B$	$28.78 \pm 4.30_B$
VCL (μm/s)	$166.86 \pm 5.94_A$	$153.63 \pm 8.20_A$	$113.40 \pm 5.74_B$	$102.77 \pm 5.26_B$
VSL (μm/s)	$112.29 \pm 7.66_A$	$105.25 \pm 9.24_A$	$84.46 \pm 6.19_B$	$76.06 \pm 6.28_B$
VAP (μm/s)	$140.02 \pm 8.02_A$	$127.88 \pm 10.42_A$	$98.68 \pm 6.49_B$	$88.30 \pm 6.36_B$
LIN (%)	67.45 ± 4.22	67.67 ± 3.32	73.62 ± 2.31	72.30 ± 3.53
STR (%)	80.47 ± 3.40	82.06 ± 1.89	85.09 ±1.13	84.84 ± 1.85
WOB (%)	83.58 ± 3.01	82.13 ± 2.96	86.36 ± 1.82	84.60 ± 2.84
ALH (μm)	$3.88 \pm 0.27_A$	$3.56 \pm 0.28_A$	$2.66 \pm 0.13_B$	$2.53 \pm 0.12_B$
BCF (Hz)	9.76 ± 0.56	$10.31 \pm 0.33_A$	8.60 ± 0.23	$8.93 \pm 0.25_B$
Viability (%)	$90.70 \pm 2.76_A$	$95.67 \pm 0.43_A$	$41.70 \pm 6.01_B$	$44.00 \pm 5.99_B$
Intact acrosome (%)	$97.80 \pm 1.48_A$	$99.58 \pm 0.23_A$	$83.10 \pm 3.50_B$	$85.33 \pm 3.79_B$
Morphological abnormalities (%)	1.10 ± 0.18	1.42 ± 0.29	1.10 ± 0.23	1.17 ± 0.41

Different capital letters indicate significant differences ($P < 0.05$, paired t tests) between fresh and frozen-thawed sperm within each group (A-B).

Table 4
Mean length, width, area and perimeter of fresh ram and buck sperm heads in control and bromocriptine (BCR) groups (mean ± SD).

MORPHOMETRY	RAMS		BUCKS	
	CONTROL	BCR	CONTROL	BCR
Length (μm)	$9.00 \pm 0.35_a$	$8.62 \pm 0.33_b$	8.53 ± 0.25	8.52 ± 0.19
Width (μm)	4.79 ± 0.18	4.70 ± 0.14	3.94 ± 0.15	3.96 ± 0.15
Area (μm^2)	$35.59 \pm 2.48_a$	$33.49 \pm 1.96_b$	27.95 ± 1.64	28.01 ± 1.57
Perimeter (μm)	$23.73 \pm 0.86_a$	$22.88 \pm 0.74_b$	21.58 ± 0.67	21.60 ± 0.57

Different lower case letters indicate significant differences ($P < 0.05$, ANOVA) between the control and BCR groups (a-b).

group ($P = 0.09$). In bucks plasma cortisol levels were 16.09 ± 1.41 ng/mL and 52.72 ± 4.31 ng/mL in control and treated group, respectively ($P = 0.61$).

3.2. Experiment 2

After SLP administration, PRL levels increased dramatically; they remained high for >5 h in the rams but fell immediately after reaching their peak in the bucks. Basal PRL concentrations were reached again by 24 h post-administration in both species (Fig. 2).

In rams (Table 5), the values for fresh sperm progressive motility, VCL, VSL, VAP, LIN, WOB (all $P < 0.01$), STR, and the percentage of viable sperms ($P < 0.05$), were all higher in the SLP-treated animals than in the controls. The percentage of morphological abnormalities was also greater ($P < 0.01$) in the SLP group. In contrast, the ALH values were significantly higher in the control group compared to the SLP group. For ram frozen-thawed sperm, values for

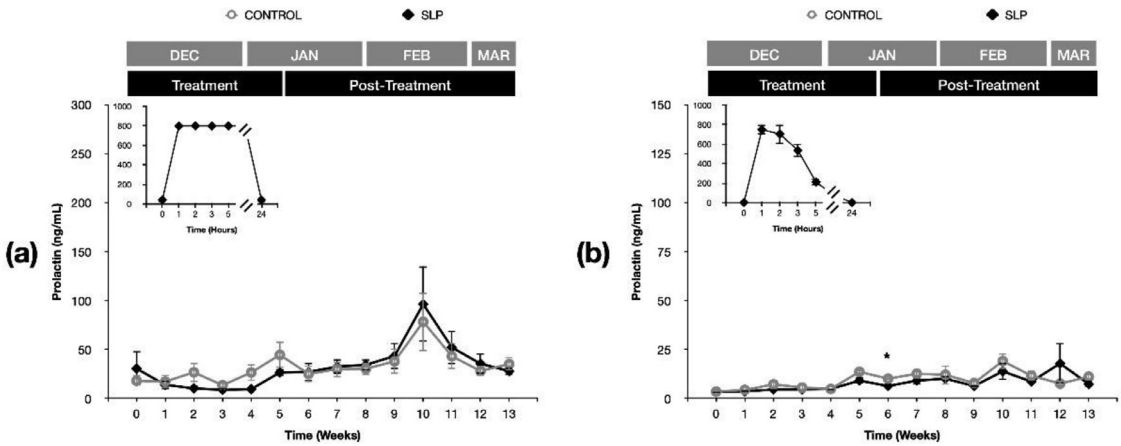

Fig. 2. Weekly change in plasma prolactin concentration (ng/mL; means ± SEM) in rams (a) and bucks (b) during the experimental period; control group (○), sulpiride (SLP) group (●). Asterisks indicate differences ($P < 0.05$, ANOVA) between groups. *Inserted graph*: Change in plasma PRL over the first 5 h and at 24 h following SLP administration.

Table 5
Fresh and frozen-thawed ram sperm characteristics in the control and sulpiride (SLP) groups (mean ± S.E.).

SPERM CHARACTERISTICS	FRESH		FROZEN-THAWED	
	CONTROL	SLP	CONTROL	SLP
Motile sperm (%)	$68.46 \pm 6.37_{A}$	$71.87 \pm 4.36_{A}$	$43.54 \pm 4.99_{B}$	$56.49 \pm 5.27_{B}$
Progressive motility (%)	$22.52 \pm 3.12_{b}$	$34.08 \pm 1.83_{aA}$	18.39 ± 2.33	$18.59 \pm 1.83_{B}$
VCL (μm/s)	$140.00 \pm 4.55_{bA}$	$162.24 \pm 3.83_{aA}$	$99.25 \pm 3.58_{bB}$	$116.67 \pm 4.46_{aB}$
VSL (μm/s)	$64.20 \pm 4.59_{b}$	$98.13 \pm 4.71_{aA}$	56.43 ± 3.02	$61.00 \pm 3.79_{B}$
VAP (μm/s)	$98.88 \pm 5.76_{bA}$	$135.47 \pm 4.21_{aA}$	$76.00 \pm 3.86_{bB}$	$88.98 \pm 4.98_{aB}$
LIN (%)	$45.29 \pm 2.38_{bB}$	$60.35 \pm 2.25_{aA}$	$56.51 \pm 1.78_{A}$	$52.24 \pm 2.36_{B}$
STR (%)	$64.27 \pm 1.82_{bB}$	$72.27 \pm 2.19_{a}$	$74.12 \pm 1.05_{aA}$	$68.38 \pm 1.40_{b}$
WOB (%)	$69.98 \pm 2.52_{bB}$	$83.41 \pm 1.37_{aA}$	$76.05 \pm 1.74_{A}$	$75.99 \pm 2.37_{B}$
ALH (μm)	$4.38 \pm 0.20_{aA}$	$3.65 \pm 0.16_{b}$	$3.15 \pm 0.10_{B}$	3.46 ± 0.16
BCF (Hz)	$7.93 \pm 0.23_{A}$	$8.45 \pm 0.21_{A}$	$7.33 \pm 0.13_{B}$	$7.04 \pm 0.13_{B}$
Viability (%)	$73.53 \pm 6.11_{bA}$	$87.56 \pm 1.87_{aA}$	$36.41 \pm 5.20_{bB}$	$57.44 \pm 5.11_{aB}$
Intact acrosome (%)	$97.24 \pm 0.91_{A}$	97.88 ± 0.58	$90.12 \pm 3.22_{B}$	92.63 ± 2.31
Morphological abnormalities (%)	$2.12 \pm 0.48_{bB}$	$7.00 \pm 1.80_{a}$	$2.88 \pm 0.60_{bA}$	$8.06 \pm 1.99_{a}$

Different lower case letters indicate significant differences (P<0.05, ANOVA) between the control and SLP group for fresh and frozen thawed sperm (a-b). Different capital letters indicate significant differences (P<0.05, paired t tests) between fresh and frozen-thawed sperm within each group (A-B).

VCL, the percentage of viable sperm and morphological abnormalities were all higher ($P < 0.05$) in the SLP group than in the control group. In contrast, STR was greater ($P < 0.01$) in the controls than in the SLP group.

Cryopreservation affected the control and SLP-treated groups differently. Within the control group, the following variables decreased their values due to freezing-thawing: percentage of motile sperm, VCL, VAP, AHL, BCF, percentage of viable sperm ($P < 0.01$), and acrosome integrity ($P < 0.05$). Progressive motility and VSL were not affected. In the SLP group, sperm motility, progressive motility, VCL, VSL, VAP, WOB, BCF, the percentage of viable sperms (all $P < 0.01$) and LIN ($P < 0.05$) values were reduced by freeze-thaw process (Table 5). The cryoresistance ratios (Table 6) for progressive motility, VSL, VAP, LIN, STR, WOB and BCF ($P < 0.05$) were lower in the SLP group compared to the controls. The cryoresistance ratio values for ALH were higher ($P < 0.01$) in the SLP group than in the control group.

In fresh buck sperm (Table 7), sperm motility and BCF differed (P<0.05) between the control and SLP-treated animals (motility was higher in the controls and BCF higher in the SLP group). For frozen-thawed buck sperm, no differences were recorded between the groups for any variable. Within the control group, cryopreservation reduced sperm motility, VCL, VSL, VAP, the percentage of viable sperms, acrosome integrity (all P<0.01), and progressive motility ($P < 0.05$). Within the SLP group, cryopreservation reduced sperm motility, progressive motility, VCL, VSL, VAP, ALH, BCF (all $P < 0.01$) and acrosome integrity ($P < 0.05$) (Table 7). No differences were seen in the cryoresistance ratios for any variable between the control and SLP groups (Table 6).

No differences were seen in fresh ram sperm head dimensions between the control and SLP groups. However, in bucks, differences were seen for fresh sperm head width and area (Tables 8), which were larger in the SLP group compared to the controls.

Table 6
Ram and buck sperm cryoresistance ratios - control and sulpiride (SLP) groups (mean ± S.E.).

CR SLP	RAMS		BUCKS	
	CONTROL	SLP	CONTROL	SLP
Motile sperm (%)	67.19 ± 8.22	79.26 ± 7.16	54.67 ± 8.93	57.42 ± 9.46
Progressive motility (%)	96.86 ± 15.27	57.18 ± 6.27*	67.05 ± 12.62	60.54 ± 9.08
VCL (μm/s)	72.07 ± 3.69	72.47 ± 3.08	64.65 ± 5.66	69.68 ± 4.06
VSL (μm/s)	93.46 ± 6.60	65.10 ± 5.93**	71.04 ± 5.66	71.38 ± 4.03
VAP (μm/s)	79.60 ± 4.79	66.59 ± 4.06*	66.33 ± 5.66	69.33 ± 4.21
LIN (%)	129.16 ± 6.65	88.31 ± 5.37**	111.97 ± 8.00	102.72 ± 3.43
STR (%)	116.70 ± 3.49	96.07 ± 3.74**	107.76 ± 4.17	103.20 ± 2.09
WOB (%)	109.98 ± 2.92	91.13 ± 2.53**	103.18 ± 3.99	99.38 ± 1.72
ALH (μm)	73.08 ± 2.49	95.48 ± 3.73**	82.86 ± 11.56	85.12 ± 3.76
BCF (Hz)	93.11 ± 2.02	84.12 ± 2.69*	93.57 ± 3.88	89.54 ± 2.80
Viability (%)	54.86 ± 7.79	65.17 ± 5.33	37.99 ± 8.46	37.30 ± 8.12
Intact acrosome (%)	92.58 ± 3.11	$94.67 \pm 2.38_{A}$	80.03 ± 5.35	$75.60 \pm 9.59_{B}$
Morphological abnormalities (%)	122.06 ± 20.55	116.57 ± 12.69	152.38 ± 43.49	116.67 ± 17.57

Cryoresistance ratio (CR) = (Post-thaw value / Fresh value) x 100.
Asterisks indicate significant differences (*P<0.05, **P<0.01, one way ANOVA) between the control and SLP groups within each species. Different capital letter indicate significant differences (P<0.05) between species.

Table 7
Fresh and frozen-thawed buck sperm characteristics - control and sulpiride (SLP) groups (mean ± S.E.).

SPERM CHARACTERISTICS	FRESH		FROZEN-THAWED	
	CONTROL	SLP	CONTROL	SLP
Motile sperm (%)	$89.30 \pm 2.60_{aA}$	$74.09 \pm 4.95_{bA}$	$48.48 \pm 7.97_{B}$	$42.43 \pm 7.08_{B}$
Progressive motility (%)	$49.40 \pm 4.77_{A}$	$48.88 \pm 2.87_{A}$	$33.49 \pm 5.84_{B}$	$29.80 \pm 4.69_{B}$
VCL (μm/s)	$144.41 \pm 7.16_{A}$	$147.06 \pm 6.94_{A}$	$92.84 \pm 6.56_{B}$	$101.63 \pm 5.99_{B}$
VSL (μm/s)	$91.71 \pm 6.55_{A}$	$107.99 \pm 7.67_{A}$	$67.95 \pm 5.61_{B}$	$75.96 \pm 5.14_{B}$
VAP (μm/s)	$117.71 \pm 6.34_{A}$	$127.15 \pm 8.15_{A}$	$78.96 \pm 6.53_{B}$	$87.16 \pm 6.16_{B}$
LIN (%)	63.98 ± 4.35	73.16 ± 3.06	72.75 ± 1.89	74.77 ± 2.93
STR (%)	77.77 ± 3.53	84.78 ± 1.63	86.01 ± 0.76	87.32 ± 1.51
WOB (%)	81.69 ± 2.41	86.07 ± 2.33	84.54 ± 1.74	85.45 ± 2.34
ALH (μm)	3.16 ± 0.24	$2.94 \pm 0.18_{A}$	2.42 ± 0.15	$2.50 \pm 0.17_{B}$
BCF (Hz)	$9.31 \pm 0.14_{b}$	$10.02 \pm 0.17_{aA}$	8.68 ± 0.32	$8.96 \pm 0.28_{B}$
Viability (%)	$94.11 \pm 0.72_{A}$	$91.56 \pm 1.24_{A}$	$36.14 \pm 8.79_{B}$	$34.44 \pm 7.68_{B}$
Intact acrosome (%)	$99.22 \pm 0.36_{A}$	$98.67 \pm 0.60_{A}$	$79.29 \pm 5.56_{B}$	$74.67 \pm 9.49_{B}$
Morphological abnormalities (%)	3.00 ± 1.17	2.44 ± 0.53	3.13 ± 0.85	2.67 ± 0.71

Different lowercase letters indicate significant differences (P<0.05, ANOVA) between the control and SLP for fresh and frozen-thawed sperm (a-b). Different capital letters indicate significant differences (P<0.05, paired t tests) between fresh and frozen-thawed sperm within each group (A-B).

Table 8
Mean length, width, area and perimeter of fresh ram and buck sperm heads in control and sulpiride (SLP) groups (mean ± SD).

MORPHOMETRY	RAMS		BUCKS	
	CONTROL	SLP	CONTROL	SLP
Length (μm)	9.03 ± 0.28	8.88 ± 0.33	8.99 ± 0.21	9.08 ± 0.18
Width (μm)	4.80 ± 0.13	4.89 ± 0.21	$4.20 \pm 0.14_{b}$	$4.44 \pm 0.10_{a}$
Area (μm^2)	35.79 ± 1.71	35.88 ± 2.45	$31.25 \pm 1.64_{b}$	$33.28 \pm 1.33_{a}$
Perimeter (μm)	23.77 ± 0.62	23.62 ± 0.81	22.85 ± 0.58	23.36 ± 0.49

Different lower case letters indicate significant differences ($P < 0.05$, ANOVA) between the control and SLP groups (a-b).

The interaction SLP treatment x species did have a significant effect ($P < 0.05$) on the CR for VSL, LIN, STR and WOB. No significant differences were seen in CR values among control rams and bucks. The CR for intact acrosome ($P < 0.05$) was higher in rams treated with SLP than in bucks treated with SLP (Table 6).

In relation to cortisol levels, again no differences were found between groups in both species. The monthly mean (means ± SEM) in rams was 17.74 ± 0.70 ng/mL in the control group and 16.09 ± 1.44 in the treated group ($P = 0.23$). In bucks plasma cortisol levels were 22.29 ± 2.77 ng/mL and 40.53 ± 3.84 ng/mL in control and treated group, respectively ($P = 0.69$).

4. Discussion

Sheep and goats have a robust annual cycle of PRL secretion. This cycle is closely related to day length, with

PRL concentrations highest near the summer solstice and lowest near the winter solstice [7,29]. Earlier work on these species showed that treatment with the dopamine agonist BCR [30,31] and the dopamine antagonist SLP [32,33] respectively reduces and increases PRL secretion. The present results support this, but also reveal that ram and buck sperm (fresh and frozen-thawed) responds differently to BCR treatment around the summer solstice (the time of physiological maximum PRL secretion) and to SLP treatment around the winter solstice (the time of physiological basal PRL secretion). Whereas in the rams BCR treatment affected many fresh and frozen-thawed sperm variables, and their cryoresistance ratios, in bucks it had no effect. Inter-species differences in PRL secretion at the time of maximum secretion (summer solstice) might help explain these findings. In rams, the control group plasma PRL was 150 to 200 ng/mL, but BCR reduced the concentration to a basal 15 ng/mL. In contrast, plasma PRL in the control bucks near the summer solstice was about 60 ng/mL, that is, much lower than in the rams, and BCR treatment smoothly reduced this concentration to <20 ng/mL. This PRL pattern in the present rams agrees with that previously reported for the same breed (Merino) [14] and indeed other breeds [34] living at temperate latitudes. In Murciano-Granadina bucks, the PRL concentrations were similar to those described by Delgadillo et al. [35] in Alpine and Saanen bucks (maximum concentration 62 ng/mL in May), supporting the idea that differences in PRL secretion exist between rams and bucks.

BCR treatment approaching the summer solstice improved the ram fresh sperm variables. A similar influence on sperm quality has been reported for humans, in which BCR has been successfully used as a treatment for idiopathic oligo and/or asthenospermia [36]. Although in sheep the role of PRL in LH secretion has been a matter of debate [37], Regisford and Katz [38] reported that the reduction seen in PRL concentrations following BCR administration is associated with an increase in LH secretion. Hyperprolactinaemia also causes reduces LH secretion in male rats [39] and humans [40]. The increase seen in LH after BCR treatment might improve testicular function, and thus sperm quality. It is possible that, in rams, the maximum PRL secretion seen near the summer solstice may have a harmful effect on sperm similar to that exerted by hyperprolactinaemia in humans [41] - which can be reversed by treatment with BCR [42]. In addition to having a direct influence on fresh sperm variables, the present data suggest that high plasma PRL around the summer solstice has a harmful effect on ram sperm freezability.

BCR treatment improved the cryoresistance ratios of VCL and ALH. The positive influence of BCR on ram sperm freezability might be due to an effect on sperm head dimensions. PRL receptors have been detected in spermatogonia, spermatocytes, spermatids and even in Sertoli cells [43], the last of which are involved in determining the final dimensions of sperm heads. It has been suggested that cryodamage in sperm cells is directly related to their head dimensions, and that smaller heads confer greater resistance [22,44]- it should be noted here that, in the present work, the BCR treatment reduced the area of the ram sperm heads, possibly through an indirect influence on PRL receptors expression in Sertoli cells during spermatogenesis. Differences in sperm head dimension may influence sperm water volume, membrane permeability to water and cryoprotectant concentration, and thus sperm freezability [1]. Variations in sperm head size may also be responsible for variations in the velocity of water exchange across the plasma membrane during freezing–thawing [45]. Certainly, less cryodamage during freezing-thawing should mean a smaller production of reactive oxygen species and, subsequently, better kinetic characteristics [46]. However, the influence of the head dimension on sperm cryoresistance is a matter of some debate. A recent study [5] reported better ram sperm freezability to coincide with the larger sperm head dimensions seen at end of the rutting season (December) than in the middle of the rutting season (July). A different endocrine status (eg, related to testosterone secretion) might influence the effect of sperm head dimensions on the response to freezing-thawing.

SLP improved the fresh ram sperm percentage of viable cells and motility variables. Despite the aforementioned improvements, sperm cryoresistance was worse in the SLP-treated animals than in controls. The results of Experiments 1 and 2 reveal a dual effect of PRL on fresh semen quality in rams. Whereas the stimulation of PRL secretion with SLP in Experiment 2 increases the values of fresh sperm motility variables and the percentage of viable sperms - which agrees with the reported protective effect of PRL on sperm function [21,47,48] - the maximum PRL concentrations near the summer solstice in Experiment 1 reduces them, indeed in a manner reminiscent of the effect of hyperprolactinaemia on human sperm [49]. This would appear to indicate that the protective effect of PRL on fresh sperm depends on the concentration of the hormone and the season, that is, PRL favours fresh sperm function in winter, but levels that are too high near the summer solstice have a harmful effect. It should be noted that while high plasma PRL has a negative effect on ram sperm freezability, how this occurs remains unclear. However, PRL stimulates and modulates both steroidogenic and spermatogenic activity in the testes [15,50], and is involved in sperm maturation in the epididymis [51]. It may be that certain changes in membrane composition mediated by PRL during the above determine the changes that occur in sperm freezability. PRL increases the availability of cholesterol for steroidogenesis [52], and thus the amount of cholesterol available for the sperm membrane may be decreased. Low cholesterol content of sperm membranes increases the cell cryodamage [53]. Moreover, the presence of PRL receptors in different areas of mature sperms (post-acrosomal area of the sperm head, neck, and midpiece of human sperms [21]) might determine indirect changes in membrane permeability to water and permeant cryoprotectants. For instance, influencing the expression and localization of water channels (eg aquaporin 3 that functions as a glycerol provider) [54] on membrane sperm, like has been showed in mammary gland cells [55].

The lack of response in buck sperm might be related to the species' lower PRL secretion, or differences in the expression of PRL receptors, or even differences in dopaminergic receptor expression in the central nervous system. In addition, interspecific differences may be ex-

plained by chemical and physical differences in unsaturated fatty acids, phospholipids and cholesterol, which affect sperm membrane fluidity [56].

The findings in control groups revealed that there are not differences in the sperm resistant to freezing-thawing process between rams and bucks. Comparisons in treated animals showed only inter-species differences in the CR for acrosome integrity. The acrosome of bucks treated with SLP appears to be less resistant to the effect of freezing-thawing than rams.

Changes in both ACTH and cortisol secretion are the most common plasma measures that indicate the hypothalamic-pituitary-adrenal axis response to stress. Since PRL secretion is affected by stress, it seems obvious that dopamine would also be implicated in the stress mediated effects, by stimulation or inhibition of PRL secretion depending on the nature of stress [57]. In our study, no significant changes in cortisol levels were observed throughout the experimental period, nor were there significant differences between treated and controls groups in rams and bucks. These results also provide evidence that cortisol concentrations were not affected by BCR administration, as described by Curlewis et al. [58], nor by sulpiride [59]. The experimental design does not allow to know the role of other biochemical or endocrine factors (ie, testosterone) that could affect the present results. The assessment of other biochemical or hormonal parameters that may be affected by dopamine agonists / antagonists should be approached in future studies.

In conclusion, high levels of PRL in rams exert a negative effect on sperm cryoresistance. Reducing PRL levels by BCR near the summer solstice when the seasonal PRL secretion is maximum, leads to an improvement in ram sperm quality and freezability. Buck sperm characteristics and cryoresistance, however, seem to be unaffected.

Declarations of interest

None.

CRediT authorship contribution statement

V.N. Flores-Gil: Data curation, Formal analysis, Investigation, Writing – original draft. **A. Toledano-Díaz:** Investigation, Methodology, Writing – review & editing. **R. Velázquez:** Data curation, Investigation, Resources. **M. Oteo:** Formal analysis, Data curation. **A. López-Sebastián:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **J. Santiago-Moreno:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Writing – review & editing.

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