Theriogenology 198 (2023) 36–46

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

In vitro approach points to a chemotactic effect of melatonin on ram spermatozoa

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A R T I C L E I N F O

Article history:
Received 17 December 2021
Received in revised form
14 December 2022
Accepted 14 December 2022
Available online 15 December 2022

Keywords:
Ram spermatozoa
Chemotaxis
Chemokinesis
Melatonin
Capacitation

A B S T R A C T

Sperm orientation mechanisms, such as chemotaxis, are essential for the sperm to reach the oocyte and fertilize it. Melatonin is secreted by the cumulus cells and is also present in the follicular fluid in mammals. The presence of membrane receptors for melatonin in ram spermatozoa, and its proven involvement in the sperm functionality, may suggest a possible role in the guided movement towards the oocyte. Hence, the objective of the present work is to study the *in vitro* potential chemotactic action of melatonin on ram spermatozoa, analysing the influence of the season (breeding and non-breeding) and the sperm capacitation state. The first experimental approach consisted in the inclusion of melatonin in the upper layer of a swim-up selection method. During the non-breeding season, the presence of melatonin at 100 pM and 1 μM concentrations significantly increased the cell recovery rate, and induced changes in the sperm location of the MT_2 melatonin receptor, compared with the standard swim-up. Moreover, the selected sperm population with 100 pM melatonin presented a higher percentage of capacitated spermatozoa. The greater recovery rate obtained with melatonin could be due to the stimulation of sperm movement in random directions, i.e., a chemokinetic effect, or due to a guided movement (chemotaxis) towards the gradient of the melatonin. To elucidate this issue, together with the study of the influence of the sperm capacitation status, we performed a second experimental approach which consisted in the use of chemotaxis chambers and an open-source software (Open-CASA) that analyses the sperm trajectories towards the hormone gradient and calculates a chemotaxis index (SL index). There was a significant difference between the SL index in the presence of 1 μM melatonin and the control without hormone. This effect was only observed in capacitated spermatozoa with cAMP-elevating agents (Cap-CK samples) obtained during the non-breeding season. These results would point to an *in vitro* chemotactic effect of melatonin on ram spermatozoa, although chemokinesis cannot be ruled out. Nonetheless, the inclusion of this hormone in the swim-up procedure could enhance the sperm recovery rate.

1. Background

Sperm orientation towards the oocyte is an essential process for fertilization to take place. Many factors and substances could affect the sperm guidance towards the oocyte. Processes such as rheotaxis, thermotaxis and chemotaxis are postulated, along with the muscular contractions of the female reproductive tract. Rheotaxis and thermotaxis are considered “long-range mechanisms”, and seem to play a significant role in the first part of the female reproductive tract, guiding spermatozoa along the oviduct to the fertilization site. Chemotaxis is considered a “short-range mechanism”, which would affect sperm movement in the surroundings of the oocyte (reviewed in Lottero et al., 2017 [1]; Pérez-Cerezales et al., 2015 [2]). Chemotaxis refers to the cell movement to a gradient of a chemical factor, called chemoattractant. This process was first discovered in the mid-1960s in sea animals with external fertilization, such as sea urchins and corals, whose spermatozoa are released into the seawater and swim towards a chemoattractant secreted by the egg [3,4]. However, chemotaxis in mammals has been under debate over the years because it was thought that the

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https://doi.org/10.1016/j.theriogenology.2022.12.022

number of ejaculated spermatozoa was high enough to reach the oocyte accidently without an attractive stimulus from the egg. However, only a few of the millions of ejaculated sperm reach the oviduct [5], and only capacitated and hyperactivated spermatozoa can penetrate the cumulus cells surrounding the oocyte [6,7]. Past studies strongly suggested that chemotaxis must be involved in the sperm guidance towards the oocyte (reviewed by Ref. [3]), and, although extremely difficult to study, chemotaxis in mammalian sperm has finally been proved [8,9].

Chemotaxis requires the sperm to detect the chemotactic signals, and numerous molecules have been proposed sperm attractors. For example, many authors have pointed to the follicular fluid, as it contains secretions from the oocyte and its surrounding cells; moreover, the follicular fluid's *in vitro* chemotactic effect has been demonstrated in human, mouse, stallion and boar [10–14] spermatozoa. Nevertheless, the composition of the follicular fluid changes between species and follicular phases, and the identity of the chemoattractants in this fluid remains unknown. One of the most studied chemoattractant agents has been progesterone, as this hormone is released from the cumulus cells of the preovulatory follicle near the time of ovulation. Some authors have reported its chemoattractant role in human, mouse, pig and rabbit spermatozoa [15–18]. Another hormone, the estradiol, together with cAMP and cGMP, seems to be essential for the chemotaxis process in sperm, increasing the intracellular calcium levels [19–22] responsible for the changes in the flagellar beat and the swimming behaviour of spermatozoa [20,23]. Other substances, such as atrial natriuretic peptide [24], heparin, adrenalin, calcitonin acetylcholine and nitric oxide have also shown chemotactic effects in spermatozoa (reviewed in Refs. [25,26]). Melatonin is present in the female reproductive fluids [27–30], and spermatozoa express melatonin receptors MT_1 and MT_2 [31]; thus, this hormone could be hypothesized as a chemoattractant for sperm orientation. The role of melatonin in chemotaxis has already been described in leukocytes [32] and retinal epithelial cells [33], but, to date, no studies have been made in spermatozoa.

In ovine, the presence of MT_1 and MT_2 on sperm plasma membrane has also been evidenced [34] and the direct effects of melatonin on ram spermatozoa has been reported, especially in modulating capacitation. This effect depends on the melatonin concentration, promoting capacitation at 100 pM and diminishing it at 1 μM, and seems to be mediated by its binding to MT_2 [35,36]. In the present study, we intended to study the putative chemoattractant capacity of melatonin at these two concentrations in ram spermatozoa. For this purpose, two different approaches were proposed: 1) evaluating the cell recovery rate in a dextran/swim-up selection method in the presence of melatonin, also analysing the quality of the recovered spermatozoa; and 2) the recording of the sperm trajectories towards a melatonin gradient on a commercial device, and their analysis with an open software (Open-CASA) [37].

2. Methods

Unless otherwise stated, all reagents were purchased from Merck KGaA (Darmstadt, Germany).

2.1. Sperm collection

The experiments were carried out with fresh semen obtained from eight mature Rasa Aragonesa rams (2–4 years old), using an artificial vagina. All the rams belonged to the National Association of Rasa Aragonesa Sheep Breeders (Asociación Nacional de Criadores de Ganado Ovino Selecto de Raza Rasa Aragonesa, ANGRA) and were housed under uniform nutritional conditions at the Experimental Farm of the University of Zaragoza (Spain). All experimental procedures were performed in compliance with the requirements of the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes under Project License PI19/17 approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (approval date: May 24, 2017).

Two successive ejaculates were collected every 2 days, and the second ejaculates from four ramps per day were pooled and used for each assay to avoid individual differences [38]. The mean time between semen collection and the start of the experiments in the laboratory was no longer than 15 min. In order to determine the effect of seasonality on the results, seminal samples were obtained during the breeding season (October to February) and non-breeding season (April to June). Samples were kept at 37 °C until their use.

2.2. Experiment 1

2.2.1. Sperm selection and experimental design

In order to determine the effect of melatonin on sperm selection, the dextran/swim-up method [39] was used. The medium used in this procedure (swim-up medium, SM) was composed of 50 mM NaCl, 10 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM K_2HPO_4, 2.8 mM glucose, 21 mM HEPES, 0.3 mM sodium pyruvate, 18.6 mM sodium lactate, 200 mM sucrose, 1.5 UI/mL penicillin, and 15 μg/mL streptomycin, at pH 6.5.

For the swim-up method, 0.5 mL of semen were placed in a round-bottomed 15 mm diameter tube, then carefully overlaid with 0.5 mL of Dx-SM (30 mg dextran/mL SM). Finally, 1.5 mL of BSA-SM (5 mg bovine serum albumin BSA/mL SM) were added on top. The tubes were kept in a vertical position at 37 °C. After 15 min of incubation, 750 μL were collected from the upper layer and replaced with the same volume of BSA-SM added to the upper layer. The process was repeated three more times. Four supernatants were obtained, but the first was discarded to avoid seminal plasma contamination. The three remaining ones were pooled and named swim-up (sw) samples.

The melatonin was included in the BSA-SM at two different concentrations, 100 pM and 1 μM [40], and the swim-up recovered samples were named “sw-Mel 100 pM” and “sw-Mel 1 μM”, respectively. Melatonin was diluted in DMSO and PBS (final DMSO concentration 0.01% v/v); thus, 0.01% DMSO was added in the BSA-SM of the control sample and named “sw-control”. Sperm samples were evaluated before (fresh semen) and immediately after the swim-up procedure (sw-control, sw-Mel 100 pM or sw-Mel 1 μM) by determining the cell recovery rate, motility, viability, phosphatidylserine translocation, capacitation state and distribution of the melatonin receptor MT_2.

2.2.2. Sperm concentration and cell recovery rate determination

The sperm concentration was calculated in duplicate using a Neubauer's chamber (Marienfeld, Lauda-Königshofen, Germany) and a microscope (Nikon Eclipse 50i, Nikon Instruments Inc, Tokyo, Japan) equipped with a 10x negative phase contrast lens. An aliquot of 10 μL of the ejaculate was diluted at 1:2000 (v/v) with water, whereas 1:100 (v/v) dilution in water was applied to an aliquot of 10 μL the swim-up samples. The use of water as dilution medium stop the sperm movement and allows to easily count them in the Neubauer's chamber. The cell recovery rate was defined as:

$$cell\,recovery\,rate = \frac{swim-up\,sample\,concentration\,x\,3\,x\,0.75\,mL}{ejaculated\,sample\,concentration\,x\,0.5\,mL} x\,100$$

2.2.3. Sperm motility evaluation

Total and progressive motility and sperm kinematic parameters (curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), percentage of linearity (LIN), percentage of straightness (STR), wobble coefficient (WOB), mean amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF)) were evaluated using the *Motility Module* of OpenCASA, a free and open-source software for sperm analysis that we have recently developed [37]. Two drops of 2 μL of each sample, diluted to a final concentration of 3 x 10^7 cells/mL in a medium composed of 0.25 M sucrose, 0.1 mM EGTA, 10% (v/v) HEPES buffer and 4 mM phosphate buffer, with a pH of 7.5, were placed in a pre-warmed Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and maintained at 37 °C during all the analyses by a heated slide holder. Spermatozoa were recorded with a video camera (Basler acA1920; Basler Vision Components, Ahrensburg, Germany) mounted on a microscope (Nikon Eclipse 50i, Nikon Instruments Inc, Tokyo, Japan) equipped with a 10x negative-phase contrast lens.

Recorded videos were evaluated with the following settings: 60 frames per second, 120 frames, 800 x 600 pixels image resolution, 10 μm^2 minimum cell size, 100 μm^2 maximum cell size, STR (straightness coefficient) > 80% and VAP (mean velocity) > 90%, 10 μm/s minimum VCL (curvilinear velocity), 100 μm/s VCL lower threshold, 200 m/s VCL upper threshold, 30 frames minimum track length and 20 μm maximum displacement between frames.

2.2.4. Evaluation of sperm membrane integrity

All the analyses were performed on a Beckman Coulter FC 500 (Beckman Coulter Inc., Brea, CA, USA) with CXP software, equipped with two excitation lasers (air-cooled argon ion laser 488 nm and solid-state laser 633 nm) and 5 absorbance filters (FL1-525, FL2-575, FL3-610, FL4-675 and FL5-755, ±5 nm each bandpass filter). A minimum of 20,000 events was recorded in all the experiments. The sperm population was identified for further analysis by the specific forward (FS) and side scatter (SS) properties; thus, other non-sperm events were excluded. A flow rate stabilized at 200–300 cells/sec was used.

Cell viability (membrane integrity) was analysed by using double staining with propidium iodide (PI) and carboxyfluorescein diacetate (CFDA) [41]. Sperm samples were loaded with 3 μL of 1 mM carboxyfluorescein diacetate (CFDA), 3 μL of 1.5 mM propidium iodide (PI) and 5 μL of formaldehyde (0.5% (v/v) in water) to a final concentration of 5 x 10^6 cells/mL in a 300 μL volume, and then incubated at 37 °C in darkness for 15 min. Samples were assessed by flow cytometry using the argon laser and filters FL1-525 ± 5 nm (CFDA) and FL4-675 ± 5 nm (PI) to avoid overlapping. The monitored parameters were FS log, SS log, FL1 log (CFDA) and FL4 log (PI), and for the gated sperm cells, percentages of viable spermatozoa (CFDA+/PI-) were evaluated.

2.2.5. Assessment of capacitation status by chlortetracycline (CTC) staining

Capacitation status was evaluated by a chlortetracycline (CTC) fluorescent assay [42], which was previously validated for ram spermatozoa by our group [43]. A CTC solution (750 μM; Sigma-Aldrich Corp., St. Louis, MO, USA) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 μM cysteine, with a pH of 7.8, and filtered through a 0.22 μm filter (Merck Millipore, Darmstadt, Germany). For each sample, 18 μL (1.6 x 10^8 cells/mL) of sperm sample were stained with 20 μL of CTC solution, fixed with 5 μL of 1.25% (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.8) and incubated at 4 °C in the dark for 30 min. Six microliters of the stained sample were placed onto a glass slide and mixed with 2 μL of 0.22 M 1,4-diazabicyclo[2.2.2]octane (DABCO) in glycerol:PBS (9:1 v/v). The samples were covered with 24 × 60 mm coverslips, sealed with transparent enamel and stored in the dark at −20 °C until evaluation with a Nikon Eclipse E−400 microscope (Nikon Corporation, Kanagawa, Japan) under epifluorescence illumination with a V-2A filter. Spermatozoa were classified in subtypes following three staining patterns [44]: NC (non-capacitated; fluorescence on all the head), C (capacitated; fluorescence in the anterior region of the head) and AR (acrosome-reacted; with fluorescence only at the equatorial segment or without fluorescence on the head). All samples were processed in duplicate, and at least 200 spermatozoa were classified per slide.

2.2.6. Indirect immunofluorescence

Melatonin receptor (MT_2) localization was revealed by indirect immunofluorescence analyses (IIF), as previously described for ram spermatozoa by Casao et al. (2012) [34]. Sperm samples were diluted (4 x 10^6 cells/mL) in PBS and fixed in 0.5% (v/v) formaldehyde at room temperature for 20 min. Then, cells were centrifuged at 900×*g*, and the pellet was resuspended in 500 μL PBS. Forty microliters of cell suspension were placed onto Superfrost slides (Superfrost Plus; Thermo Fisher Scientific, Waltham, MA, USA) and washed three times with PBS. Non-specific binding sites were blocked with 5% (w/v) BSA in PBS for 5 h in a wet chamber. The slides were rewashed in PBS and incubated at 4 °C overnight in a wet chamber with the primary antibody anti-MT_2 (RRID: AB_1619198, from Acris Antibodies GmbH, Herford, Germany) diluted 1/50 v/v) in PBS with 1% (w/v) BSA. The next morning, the samples were washed three times with PBS and incubated with the secondary antibody (Alexa Fluor 488 chicken anti-rabbit; Thermo Fisher Scientific; Cat#A-21441, RRID: AB_2535859), diluted 1/600 (v/v) in PBS with 1% (w/v) BSA for 90 min at room temperature in a wet chamber. The slides were then washed three times with PBS before the addition of 6 μL of 0.22 M 1,4-diazabicyclo[2.2.2]octane (DABCO) in glycerol:PBS (9:1 v/v) to enhance and preserve cell fluorescence. The slides were covered with a coverslip and sealed with transparent enamel. Cells were visualized with a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) under epifluorescence illumination using a B-2A filter (X 1000). All samples were processed in duplicate, and at least 150 spermatozoa were scored per slide.

2.3. Experiment 2

2.3.1. In vitro capacitation

In order to investigate the effect of the sperm capacitation status on the chemotactic potential response to melatonin, swim-up selected (by standard dextran/swim-up) spermatozoa (1.6 x 10^8 cells/mL) were incubated for 3 h under capacitating conditions: 39 °C in a humidified incubator with 5% CO_2 in air. Incubations were performed in a complete TALP medium [45] containing 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM Na lactate, 3 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1 mM Na pyruvate, 5 mM glucose, and 5 mg/mL bovine serum albumin (BSA), with a pH of 7.2. Ram spermatozoa are difficult to capacitate *in vitro*, and high levels of intracellular cAMP are required. Thus, a specific cocktail of substances, already proven for triggering ram sperm capacitation [43,46], was added to the TALP medium. This cocktail was composed of 1 mM dibutyryl (db)-cAMP, 1 mM caffeine, 1 mM

theophylline, 0.2 mM okadaic acid and 2.5 mM methyl-b-cyclodextrin. The samples incubated in TALP medium without or with cAMP-elevating agents were named Cap-TALP and Cap-CK samples, respectively.

Swim-up samples were evaluated just after the procedure, and *in vitro* capacitated samples were assessed after 3 h incubation.

2.3.2. Influence of melatonin on sperm orientation

As it has been reported that only capacitated spermatozoa can respond to chemotactic stimuli [15,47–50], all experiments were carried out separately with swim-up selected spermatozoa just after recovery (swim-up samples) or after incubation in capacitating conditions with or without cAMP elevating agents (Cap-CK and Cap-TALP samples, respectively).

Chemotaxis assays were performed using the disposable μ-Slide Chemotaxis device (ibidi GmbH, Martinsried, Germany). The μ-Slide Chemotaxis includes three different chambers for three parallel assays (Fig. 1). Each chamber consists of two large reservoirs connected by a narrow observation area. These compartments, the large reservoirs and the narrow one, have two wells each where a pipette tip can be placed for filling them. The chambers were filled as follows: first, 6 μL of sperm sample was injected into the narrow observation area, and immediately afterwards, the large two-sided reservoirs were each filled with 65 μL of the same sample (2.5 x 10^6 cells/mL) [8]. Once the chamber was completely filled, 30 μL of sperm suspension (2.5 x 10^6 cells/mL) without (control) or with melatonin (100 pM and 1 μM) was then applied on one of the reservoirs to avoid the diminishing of the cell concentration by the application of the chemoattractant (Fig. 1). By the opposite well of the reservoir, 30 μL were removed. Following loading, the slides were incubated at 37 °C for 5 min to establish a concentration gradient of the hormone. The swimming of the spermatozoa in the observation area was video-recorded at 200 frames per second for a total of 3 s using a video camera (acA1920-155uc, Basler, Exton, PA) connected to a microscope (Nikon Eclipse 50i, Nikon, Tokyo, Japan) equipped with a 10x negative-phase contrast lens.

Sperm chemotaxis was analysed through the Chemotaxis Module included in the free, open-source software OpenCASA [37]. This software detects the trajectory coordinates of each spermatozoon and normalizes them to the same reference point. A region (chemotactic zone of influence) is selected to determine the hormone gradient region, in this case, 180°. Based on these data, the software calculates the SL (Straight Line) index, as the percentage of sperm whose trajectory enters the chemotactic area based on their initial and final positions.

2.4. Statistical analysis

Differences between the groups in the cell recovery rate, motility, viability, CTC staining, MT_2 immunotypes and SL index were analysed by means of the chi-square test. Differences in the kinematic parameters were analysed by one-way ANOVA after the evaluation of normality and homoscedasticity by the Kolmogorov-Smirnov test and Levene test, respectively. All statistical analyses were performed using GraphPad Prism 8 (v. 8.0.1; GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Experiment 1

3.1.1. Cell recovery rate

The inclusion of melatonin (100 pM and 1 μM) in the upper layer of the dextran/swim-up did not result in differences during the breeding season. However, a significant ($p < 0.05$) increase was observed in the sperm recovery rate during the non-breeding season (52.05% ± 10.47% in sw-control vs 59.33% ± 6.57% in sw-Mel 100 pM and 64.83% ± 7.35% in sw-Mel 1 μM samples; Fig. 2).

3.1.2. Motility and membrane integrity

Compared with the fresh sample, the dextran/swim-up procedure improved the total motility ($p < 0.05$), but only during the non-breeding season (Fig. 3). The inclusion of melatonin in the upper layer during this season resulted in a higher percentage of motile sperm compared with the control swim-up, especially with melatonin 100 pM ($p < 0.05$) (Fig. 3B). Progressive motility

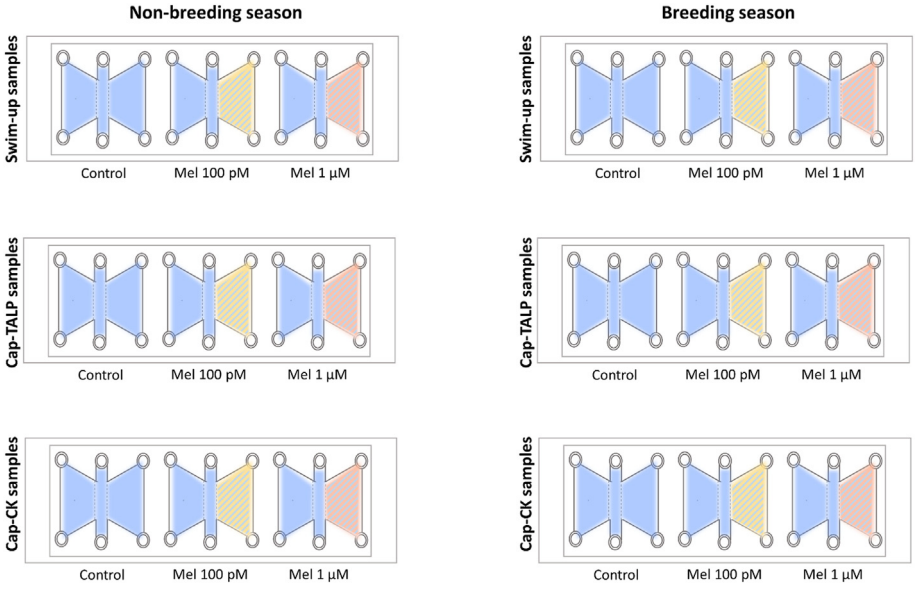

Fig. 1. Graphic scheme of the experimental design. In blue, the sperm suspension of swim-up, Cap-TALP or Cap-CK samples, filling the central narrow observation area and the two lateral reservoirs. In yellow and light red, blue striped, the sperm suspension with 100 pM and 1 μM melatonin, respectively, added to one of the reservoirs. The experiment was performed 4 times in each season (non-breeding and breeding). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

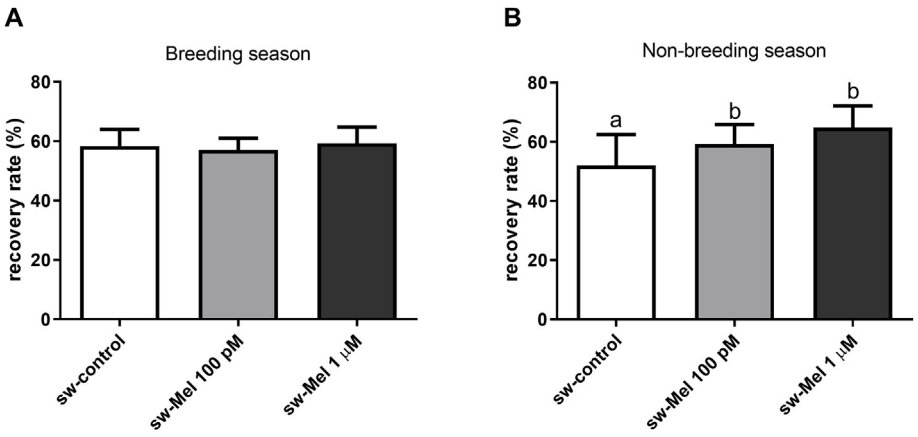

Fig. 2. Cell recovery rate after dextran/swim-up procedure without (sw-control) and with 100 pM and 1 μM melatonin (sw-Mel) in the upper layer during breeding (A) and non-breeding (B) seasons. Data are shown as mean ± SEM (n = 6). Different letters indicate significant differences ($p < 0.05$).

decreased after the dextran/swim-up procedure in all experimental samples and both seasons, although this decrease was lower when melatonin 100 pM was added during the non-reproductive season (Fig. 3B). No significant differences between treatments were found in the sperm kinematic parameters (Supplementary material).

Sperm viability was improved by the dextran/swim-up procedure when performed during the non-breeding season ($p < 0.01$), in comparison to the fresh sample (Fig. 4). However this method did not improve the viability of samples collected in the breeding season. Nonetheless, no differences were observed among the three experimental swim-up procedures, demonstrating that melatonin did not affect sperm viability throughout the selection process (Fig. 4B).

3.1.3. Capacitation status

The spermatozoa selection by the standard dextran/swim-up procedure (sw-control) did not modify the capacitation status compared to the fresh ejaculate semen. Also, the addition of melatonin in the upper layer of the dextran/swim-up procedure during the breeding season did not alter the capacitation status (Fig. 5A). Interestingly, during the non-breeding season (Fig. 5B), the selection of sperm in the presence of melatonin at 100 pM concentration promoted a significant change in the capacitation state of the recovered cells ($p < 0.05$). Specifically, an increase in the percentage of capacitated spermatozoa was detected, along with a concomitant decrease of non-capacitated sperm. However, no changes in the capacitation state were observed with melatonin 1 μM. Finally, the sperm selection with melatonin at any concentration did not affect the percentage of acrosome reacted spermatozoa.

3.1.4. Localization of MT_2 receptor in ram spermatozoa

Since significant differences were observed in sperm capacitation between the different experimental groups during the non-breeding season, and previous works have evidenced changes in the localization of the melatonin receptor MT_2 during the capacitation process [51], we decided to study the distribution of this receptor in both the initial sample as those selected by swim-up in this season. As shown in Fig. 6, three immunotypes were observed and classified as [51]: Type A, greater staining intensity at the acrosome than the post-acrosome; Type P, greater staining intensity at the post-acrosome region than the acrosome; and Type

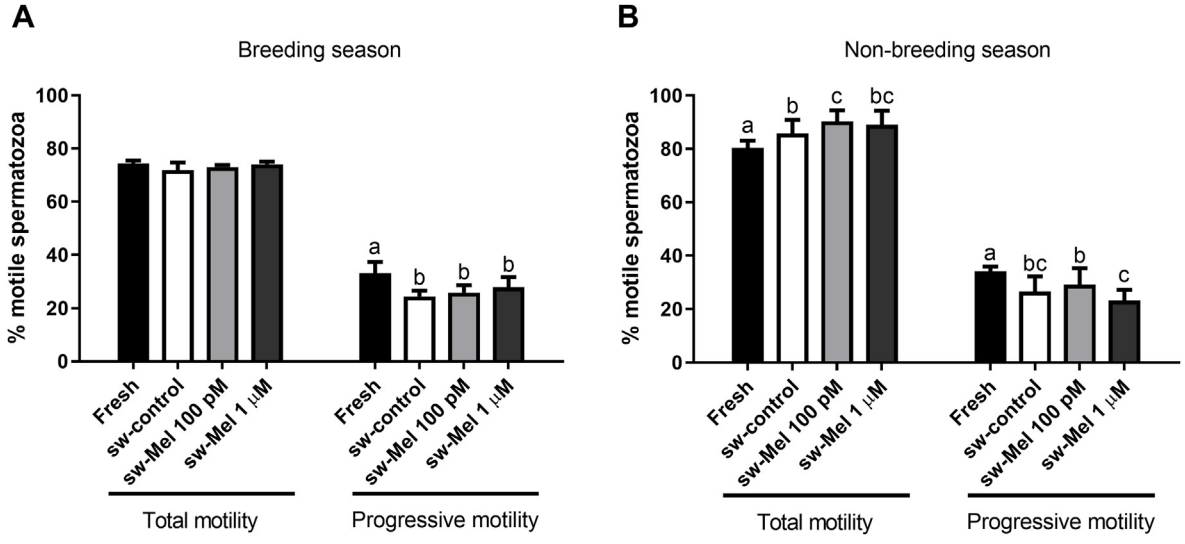

Fig. 3. Percentage of total motile and progressive motile spermatozoa before (fresh) and after the dextran/swim-up procedure, without (sw-control) and with melatonin (sw-Mel 100 pM and sw-Mel 1 μM) during the breeding (A) and non-breeding (B) seasons. Data are shown as mean ± SEM (n = 6). Different letters indicate significant differences ($p < 0.05$).

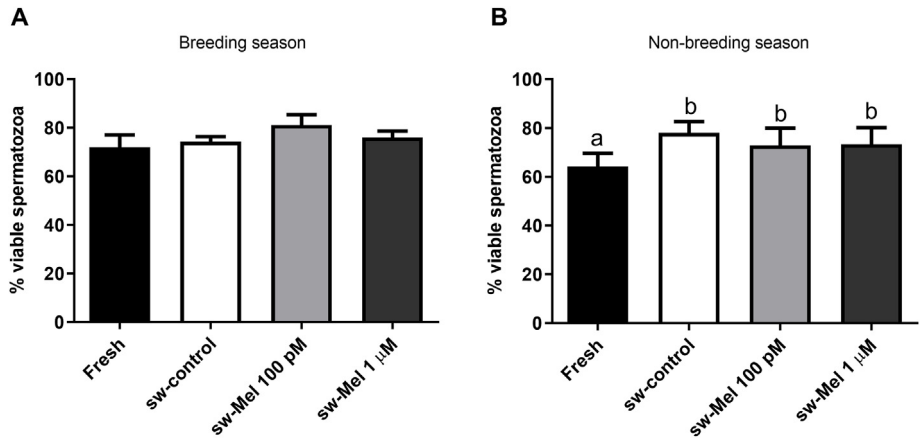

Fig. 4. Percentage of live spermatozoa (CFDA+/PI-) before (fresh) and after the dextran/swim-up procedure, without (sw-control) and with melatonin (sw-Mel 100 pM and sw-Mel 1 μM) during the breeding (A) and non-breeding (B) seasons. Data are shown as mean ± SEM (n = 6). Different letters indicate significant differences ($p < 0.01$).

AP, same immunostaining intensity at both the acrosome and post-acrosome. The selected samples obtained in the presence of low melatonin concentration (sw-Mel 100 pM) showed a significantly ($p < 0.01$) higher percentage of Type A spermatozoa. In contrast, the selection with the higher melatonin concentration (sw-Mel 1 μM) decreased this percentage compared to fresh and standard swim-up samples (sw-control). Opposite results were found in the presence of both melatonin concentrations when analysing the Type P sperm population, where a significantly lower percentage ($p < 0.05$) was observed with melatonin 100 pM compared to the fresh sample. However, no differences were found in the Type AP subpopulation between treatments.

3.2. Experiment 2

3.2.1. Evaluation of sperm in vitro capacitation

The induction of *in vitro* capacitation in ram spermatozoa affected motility, capacitation status, and cell viability related to membrane integrity. Viability decreased in the capacitated samples ($p < 0.05$; Fig. 7) in both seasons, probably due to the increase of acrosome-reacted sperm (Fig. 9), but remained at suitably high values. Also, total and progressive motility significantly declined ($p < 0.05$) after *in vitro* capacitation in both seasons (Fig. 8). The most notable effect ($p < 0.001$) was observed when incubation was carried out in TALP with cAMP-elevating agents (Cap-CK), with a decrease of around 20% in total and progressive motility, irrespective of the season.

In order to test that the *in vitro* capacitation was correctly induced, the capacitation status was evaluated by chlortetracycline staining (CTC) (Fig. 9). During the breeding season, the percentage of non-capacitated spermatozoa was significantly ($p < 0.05$) lower in the Cap-TALP and Cap-CK than in the swim-up samples (69.75% ± 3.03% in swim-up vs 59.25% ± 4.13% in Cap-TALP and 29.25% ± 2.53% in Cap-CK; Fig. 9A). In concordance with these results, the percentage of capacitated spermatozoa increased after the incubation in capacitating conditions, especially when cAMP-elevating agents were added to the medium (Cap-CK sample), even doubling the rate ($p < 0.001$) in comparison with the swim-up sample (22.75% ± 2.17% in swim-up vs 49.50% ± 3.80% in Cap-CK). Similar results were observed during the non-breeding season (Fig. 9B), except for the lack of statistical significance in the percentage of capacitated spermatozoa in the Cap-TALP compared to the swim-up samples. Conversely, the percentage of non-capacitated spermatozoa decreased after *in vitro* capacitation in the TALP medium without cAMP-elevating agents ($p < 0.05$). Regarding the acrosome reaction, *in vitro* capacitation with high cAMP triggered a significantly higher percentage of spermatozoa undergoing this process ($p < 0.001$) than in the swim-up and Cap-TALP samples, in both non-breeding and breeding seasons.

3.2.2. Influence of melatonin on sperm orientation

In regarding the chemotaxis analysis, no changes were detected

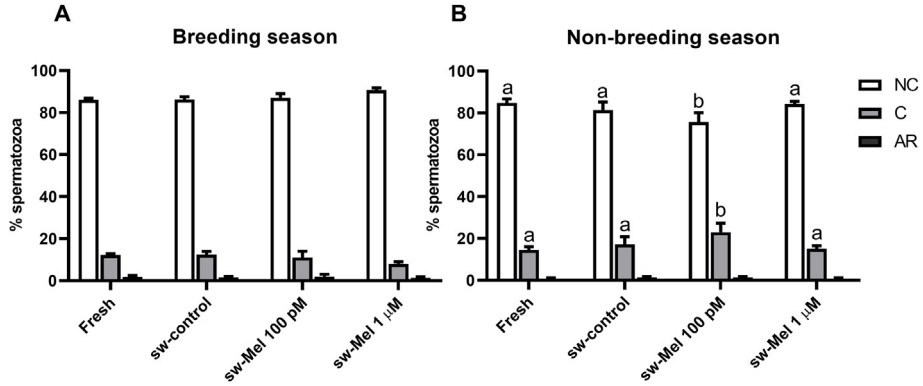

Fig. 5. Assessment of capacitation status, evaluated by CTC, in ram spermatozoa before (fresh) and after dextran/swim-up selection without (sw-control) and with melatonin (sw-Mel 100 pM and sw-Mel 1 μM) during the breeding (A) and non-breeding (B) seasons. Data of non-capacitated (NC), capacitated (C) and acrosome-reacted (AR) spermatozoa in each sample are shown as mean ± SEM (n = 6). Different letters within the same sperm subtype indicate significant differences between experimental groups ($p < 0.05$).

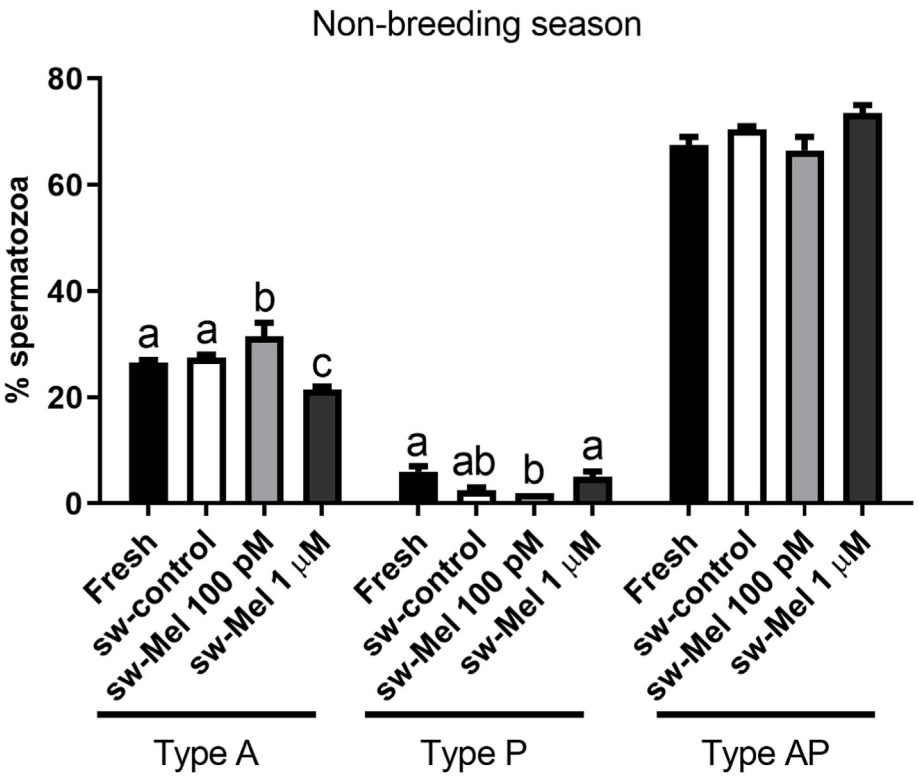

Fig. 6. Percentages of MT_2 melatonin receptor immunotypes in ram spermatozoa before (fresh) and after dextran/swim-up sperm selection without (sw-control) and with melatonin (sw-Mel 100 pM and sw-Mel 1 μM) during the non-breeding season. Data of Type A sperm (more intensity of acrosome staining than post-acrosome), Type P sperm (more intense staining on post-acrosome than acrosome), and Type AP sperm (equal staining on both acrosome and post-acrosome) are shown as mean ± SEM (n = 4). Different letters indicate statistical differences between experimental groups within the same immunotype ($p < 0.05$).

in the SL index in the presence of melatonin during the breeding season (Fig. 10A–C). However, in the non-breeding season, an increment in the SL index was observed when the Cap-CK samples were exposed to the influence of 1 μM melatonin (0.48 ± 0.01 in the control and 0.52 ± 0.01 with melatonin 1 μM, $p < 0.01$; Fig. 10F). No chemotactic behaviour was observed when 100 pM melatonin was added or the cap-TALP samples were tested (Fig. 10E).

4. Discussion

Sperm cells must finish their maturation during their transit across the female reproductive tract to reach their fertilizing ability [52]. The so-called capacitation process involves membrane remodelling and motility hyperactivation, among other changes [53–55]. What is more, only those spermatozoa that are capacitated and hyperactivated are able to penetrate the cumulus cells surrounding the oocyte [6]. Thus, there are few possibilities for the sperm to successfully fertilize the egg, and guidance mechanisms must be involved in achieving the sperm-egg fusion. Previous studies have demonstrated the chemoattractant capacity of the follicular fluid in mammalian spermatozoa [11,13,56,57], but the question of which substances act in this chemotaxis response remains unresolved. In the present study, we propose melatonin as a possible candidate to attract ram spermatozoa, since it is present in

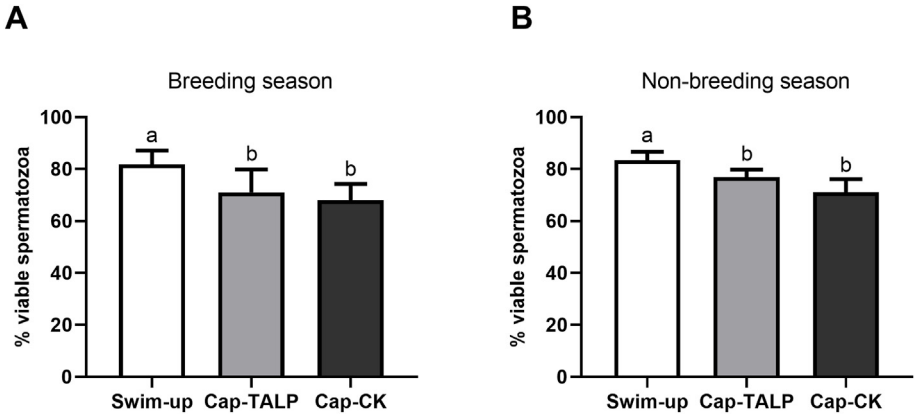

Fig. 7. Percentage of viable spermatozoa (CFDA+/PI-) before (swim-up) and after *in vitro* capacitation without (Cap-TALP) and with cAMP-elevating agents (Cap-CK) in breeding (A) and non-breeding (B) seasons. Data are shown as mean ± SEM (n = 4). Different letters indicate significant differences ($p < 0.05$).

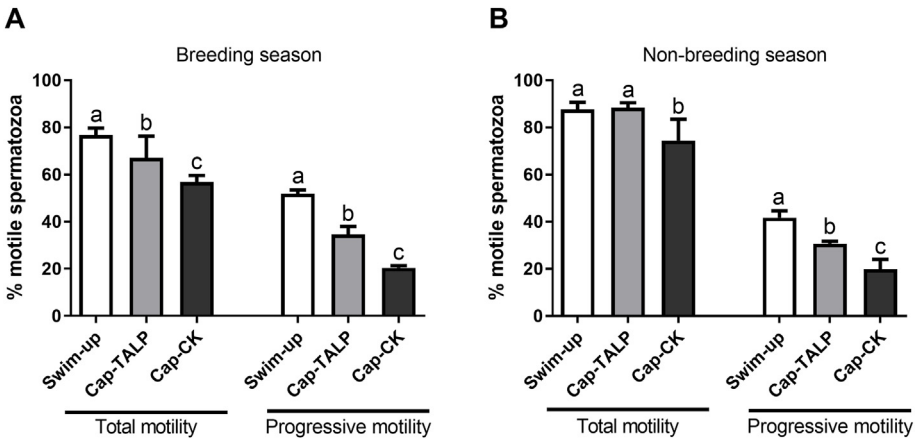

Fig. 8. Percentage of total and progressive spermatozoa before (swim-up) and after *in vitro* capacitation without (Cap-TALP) and with cAMP-elevating agents (Cap-CK) in breeding (A) and non-breeding (B) seasons. Data are shown as mean ± SEM (n = 4). Different letters indicate significant differences ($p < 0.05$).

the oocyte environment [30] and can exert direct effects on spermatozoa [35,36]. Our first approach, consisting of the inclusion of melatonin in the upper layer of the dextran/swim-up procedure (experiment 1), showed a higher sperm recovery rate compared with the standard method without hormone, but only during the non-breeding season. Although the presence of melatonin in the recovery medium did not change the percentage of viability compared to the standard swim-up, taking into account the higher recovery rate achieved with the addition of hormone, a greater number of viable cells in absolute values were selected. On the other hand, the sperm sample selected in the presence of 100 pM melatonin contained a higher percentage of capacitated spermatozoa than other samples. The doubt that arises from this finding is whether capacitated spermatozoa in the fresh sample are more stimulated by 100 pM melatonin than non-capacitated ones or if spermatozoa that swim to the top of the tube become capacitated once they are in the collecting media with melatonin 100 pM. Previous results of our group demonstrated that melatonin at 100 pM, unlike 1 μM, enhances ram sperm capacitation under capacitating conditions [35,36]. Although the incubation conditions were different in the present study, 100 pM melatonin could have induced sperm capacitation during the swim-up process. Moreover, the selected sample obtained in the presence of 100 pM melatonin showed a higher percentage of the sperm subpopulation exhibiting MT_2 receptor in the acrosome region than the other selected samples (standard swim-up or with 1 μM melatonin). This was in agreement with Gonzalez-Arto et al. (2016) [58], who described a positive correlation between the acrosome labelling of MT_2 and the capacitated sperm rate.

The higher sperm recovery rate in the presence of melatonin at both concentrations means that more spermatozoa swam from the bottom to the top of the tube. This fact could be due to the stimulation of movement in random directions, i.e., a chemokinetic effect, or a guided movement provoked by the chemotactic gradient of melatonin formed between the bottom and the upper layer. Considering that it is generally accepted that chemokinesis implies changes in speed or alterations of the amplitude or frequency of the motile character [59], we compared sperm velocities in the three swim-up samples, and no significant differences between them were found. So, the higher sperm recovery in the presence of melatonin could point to a chemoattractant effect of this hormone. To elucidate this issue, as well as the influence of the sperm capacitation status, we performed the second set of experiments. Significant differences, although small, were found between the percentage of spermatozoa moving towards 1 μM melatonin and those swimming to the control without hormone, but only in capacitated spermatozoa with high cAMP levels (Cap-CK samples) obtained during the non-breeding season. This finding is in concordance with other studies that demonstrated that chemoattraction occurs only in capacitated spermatozoa, at least in human, mouse, bull and rabbit samples [15,47–50]. In our experiments, no sperm chemoattraction was evidenced with the gradient formed by 100 pM melatonin in the chamber. Although the experimental approach is very different between experiments 1 and 2, the lack of effect of melatonin 100 pM in the latter would suggest that the increment in the recovery rate in the first experiment was not due to a chemoattractant effect but to the ability of melatonin 100 pM to increase total motility, and probably

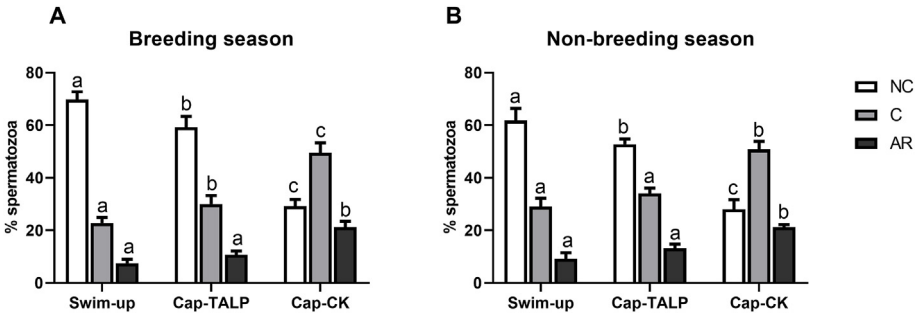

Fig. 9. Assessment of capacitation status, evaluated by CTC, in ram spermatozoa before (swim-up) and after *in vitro* capacitation without (Cap-TALP) and with cAMP-elevating agents (Cap-CK) during breeding (A) and non-breeding (B) seasons. Data of non-capacitated (NC), capacitated (C) and acrosome-reacted (AR) spermatozoa in each sample are shown as mean ± SEM (n = 4). Different letters within the same sperm subtype indicate significant differences between experimental groups ($p < 0.05$).

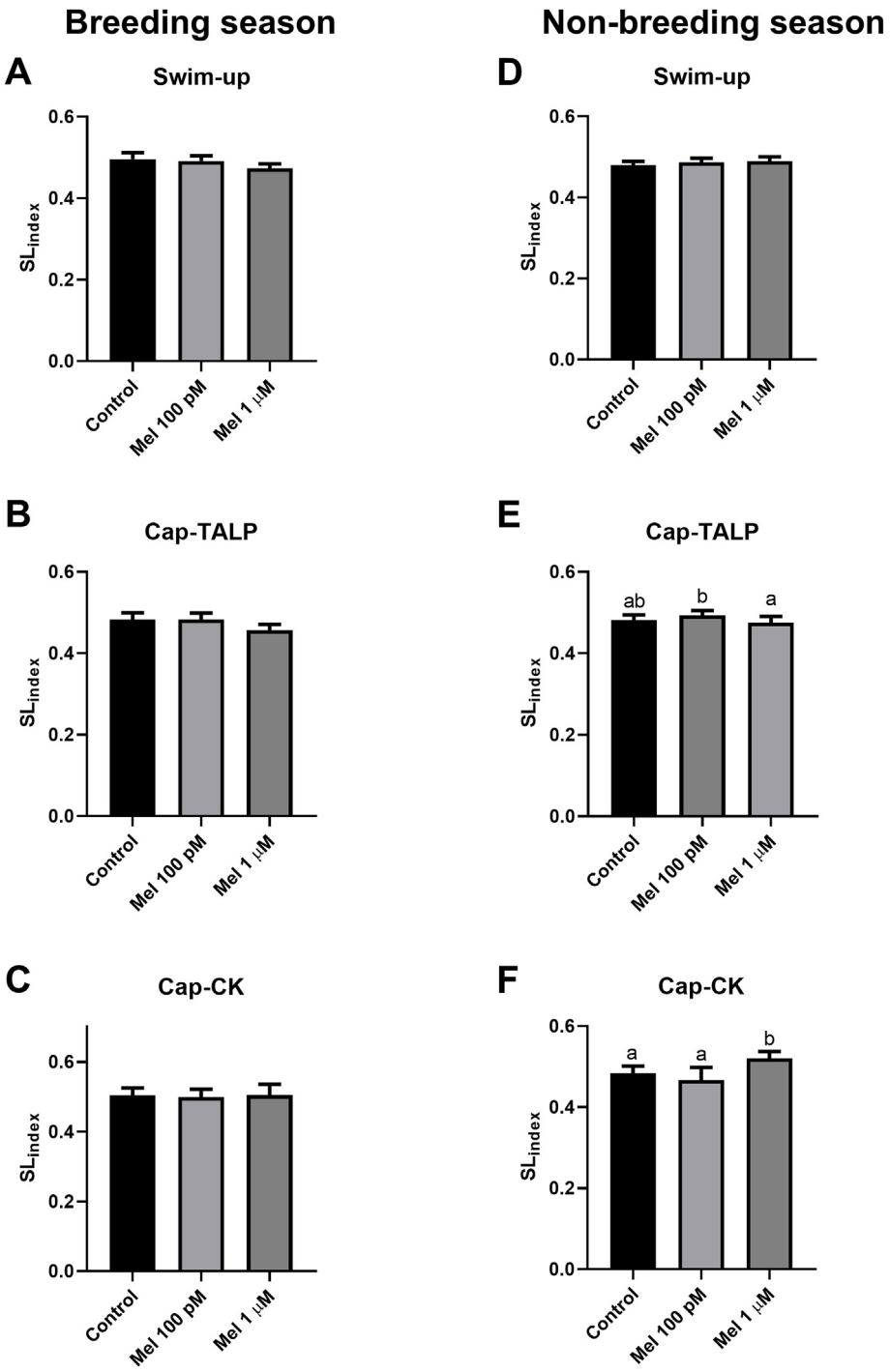

Fig. 10. Chemotactic response (SL index) in the absence (control) or presence of melatonin (Mel 100 pM and 1 μM) in the chamber of ram spermatozoa before (swim-up) and after *in vitro* capacitation without (Cap-TALP) and with cAMP-elevating agents (Cap-CK) during breeding (A–C) and non-breeding (D–F) seasons. Data are shown as mean ± SEM (n = 4). Different letters indicate significant differences ($p < 0.05$).

hyperactivation, linked to the capacitation state so that spermatozoa would swim better towards the upper layer than in the standard swim-up.

In contrast, no differences were observed when experiments were carried out in the breeding season. This may be due to the high melatonin levels found in the seminal plasma during the reproductive season which we reported in a previous work [40]. Melatonin in ram seminal plasma showed monthly variations, with a decrease after the winter solstice and a rise after the summer solstice, reaching maximum levels in October–November, and a marked seasonal variation ($P < 0.01$) with higher levels in the breeding season. Since the seminal plasma is present together with the spermatozoa at the bottom of the tube during the swim-up procedure, this could prevent establishment of the hormone gradient in the medium and, therefore, the possible chemotaxis events. Another possibility could be that spermatozoa had already been exposed to a high melatonin concentration in the ejaculates obtained during the breeding season, so the melatonin receptors

may not perceive the hormone stimuli in the same manner as in the non-breeding season [60]. Also, in experiment 2, sperm samples obtained during the breeding season were not able to respond to melatonin, although *in vitro* capacitation was successful in both seasons. *In vitro* capacitation seems to be a necessary requirement to respond to the melatonin gradient, but not the only one since capacitated sperm samples from the reproductive season do not respond. This could be due to the state of the sperm melatonin receptors. High melatonin concentrations desensitize melatonin receptors by internalization [61,62] or uncoupling [63] in somatic cells. Thus, in the breeding season, sperm melatonin receptors could be totally or partially desensitized due to the previous exposition to the high levels of melatonin present in the seminal plasma after ejaculation, masking the effects of melatonin added in the chemotactic chamber. Nevertheless, the implication of melatonin receptors on the chemotactic process in the spermatozoa remains unknown. Experiments using agonists and antagonists for both receptors, to discern between MT1 and MT2 involvement in this process should be addressed in the future. Finally, it is worth noting that the chambers used for the study of chemotaxis are not specific for sperm, but for slow-moving cells [64]. The use of specific chambers or devices which allow sperm recovery after the chemotaxis assay in the assessment of sperm parameters could shed more light on the study of sperm chemotaxis. In addition, according to some authors [59,65], in order to differentiate between chemotaxis and chemokinesis in devices, a comparative study would have to be carried out that included a uniform concentration of melatonin. As it has not been carried out in this work, we cannot rule out a chemokinetic effect of melatonin. It would be interesting to determine in future studies whether one or both mechanisms are implied.

5. Conclusions

In conclusion, melatonin at 1 μM seems to exert a light chemotactic/chemokinetic attraction on ram spermatozoa *in vitro*. The inclusion of melatonin at this concentration in the recovery layer during the dextran/swim-up procedure leads to a high number of recovered spermatozoa without affecting their capacitation status or kinematic parameters. The assays using chemotaxis chambers would point to this chemotactic effect of 1 μM melatonin on ram spermatozoa obtained in the non-breeding season and after *in vitro* capacitation with high cAMP levels, although chemokinesis cannot be ruled out. Melatonin at 100 pM concentration also results in an increase of the recovered spermatozoa during the dextran/swim-up procedure, but it seems to be due to a capacitation-mediated effect. The inclusion of melatonin in the swim-up procedure could be considered in order to optimize the swim-up-selection protocol depending on whether more sperm, capacitated or not, are needed for their use in assisted reproductive techniques.

Conflicts of interest

The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was funded by Ministerio de Economía, Industria y Competitividad (CICYT AGL-2017-83799-R) and Gobierno de Aragón (DGA A07 20R). S.M.-J. has a predoctoral contract from the Diputación General de Aragón (DGA).

CRediT authorship contribution statement

Sara Miguel-Jiménez: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, All authors have read and agreed to the published version of the manuscript. **Sonia Borao:** Methodology, Formal analysis, Investigation, Data curation, All authors have read and agreed to the published version of the manuscript. **Virginia Portolés-Bayod:** Methodology, Formal analysis, Investigation, Data curation, All authors have read and agreed to the published version of the manuscript. **Adriana Casao:** Conceptualization, Formal analysis, Investigation, Data curation, Writing – review & editing, Supervision, All authors have read and agreed to the published version of the manuscript. **Rosaura Pérez-Pe:** Conceptualization, Investigation, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to acknowledge the use of the Servicio General de Apoyo a la Investigación-SAI (Universidad de Zaragoza), ANGRA for supplying the sires, and the funding by Ministerio de Economía Industria y Competitividad (CICYT AGL-2017-83799-R) and Gobierno de Aragón (DGA A07 20R).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.12.022.

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