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Use of a neuroleptic in assisted reproduction of the critically endangered Mohor gazelle (*Gazella dama mhorr*)

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Abstract

Stress is a limiting factor in assisted reproduction in wild animals maintained in captivity and measures to reduce it should improve reproductive success. The effect of the long-acting neuroleptic (LAN) perphenazine enanthate was assessed on ovarian stimulation for the recovery of immature oocytes from Mohor gazelle (*Gazella dama mhorr*) and their subsequent *in vitro* maturation, fertilization and embryo culture. The viability of embryos after transfer was also examined. Perphenazine enanthate decreased activity levels and facilitated handling of treated animals when compared to controls. LAN-treated animals showed a more regular pattern of respiratory and heart rates and body temperature than controls; no major differences were found in hematological and biochemical parameters between groups. Perphenazine-treated females had lower plasma cortisol levels during the days of intense handling. No significant differences were found in the number of punctured follicles and recovered oocytes between groups. The percentage of mature oocytes per female was significantly higher in the LAN-group. Fertilization and cleavage rates were not significantly different between groups. Embryos developed in culture but none reached the blastocyst stage, and those transferred to the oviduct of synchronized recipients did not develop to term. In conclusion, treatment of females with perphenazine enanthate during ovarian stimulation did not have negative effects on maturation, fertilization and embryo development *in vitro*. Moreover, an increase in oocyte maturation rate per female was observed. Thus, the use of LANs could be useful to alleviate the effects of handling-stress during assisted reproductive procedures in wild ungulates.

Keywords: Oocyte; Fertilization; Stress; Cortisol; Long-acting neuroleptic

1. Introduction

The Mohor gazelle (*Gazella dama mhorr*) is extinct in the wild since 1968 due to excessive hunting. It is included in the Red List of Threatened Species as ''critically endangered'' [1]. A captive breeding programme for the Mohor gazelle was established in

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1971 at the Parque de Rescate de la Fauna Sahariana of the Estación Experimental de Zonas Áridas (EEZA, CSIC), in Almería, Spain, with two founding males and nine founding females [2]. A genome resource bank for this and other related endangered gazelles (dorcas and Cuvier's gazelles) is currently being organized at the EEZA to preserve a maximum of genetic variability through the storage of valuable biomaterials and thus complement current captive breeding and future *in situ* conservation. Efforts to develop assisted reproductive techniques (ARTs) for the Mohor gazelle are under way, and they include improvements of semen cryopreservation methods and artificial insemination via laparoscopy with frozen-thawed spermatozoa [3,4]. These efforts have resulted in the first live Mohor gazelle calf born using these techniques [3,4].

In vitro oocyte maturation, fertilization and culture are valuable techniques for the production of offspring from animals of high genetic value. However, efforts to use these assisted reproductive techniques in wild species have had limited success [5,6]. *In vitro* oocyte maturation, fertilization and culture after ovum pick-up have been recently attempted in the Mohor gazelle [4,7,8]. Results revealed only limited development of embryos after *in vitro* fertilization suggesting the possible existence of an *in vitro* developmental block. Failure to develop *in vitro* may be partly due to poor knowledge of factors required by embryos during culture. An alternative to overcome limitations seen during *in vitro* embryo culture could be an early transfer to recipient females. It has been shown that the physiological environment in the oviduct favors the development of cleaved *in vitro* produced cattle embryos [9] and *in vitro* matured and fertilized oocytes were capable of establishing pregnancies when transferred to recipient females [10,11].

Stress generated during repeated handling may be another cause of limited success in *in vitro* production of gazelle embryos. Handling stress and capture myopathy are important consequences of intensive management of wild species. There is evidence suggesting disruptive effects of stress on assisted reproductive techniques in wild animals [12–14], and stress is considered to be a contributing factor of reproductive failure in wild species maintained in captivity [15–17].

Stress influences female reproductive function [18–20]. Chronic stress suppresses or inhibits reproduction, but it is unclear if or how acute stress or repeated short-term stress may affect it [21]. Stressors may, for instance, affect the ovarian response to FSH administration, reducing the number of oocytes recovered. They may also affect oocyte maturation since it has been found that glucocorticoids would inhibit pig oocyte maturation *in vitro* [22]. Thus, factors generating stress should be minimized whenever possible [20].

A range of drugs is available for the control or reduction of the effects of stress [23] but there is little information on its use during assisted reproductive procedures in wild animals. Certain chemical immobilizing agents and anesthetics have a detrimental effect on or interfere with reproductive function in both males and females. Several agents tested on laboratory or domestic species have been found to delay the onset of estrus [24], suppress the LH surge [25], compromise ovulation [26] or inhibit embryo development [27].

Long acting neuroleptics (LANs) are drugs used to provide long-term tranquilization. Their prolonged activity results from their pharmaceutical formulations, consisting of a fatty acid ester of the basic neuroleptic compound, which has been dissolved in a lipid vehicle leading to a slow release of the active neuroleptic drug. They have been successfully used to reduce the high mortalities and stress-associated problems encountered with translocations of non-domestic ungulates [28]. Perphenazine enanthate is a member of the phenotiazine group of neuroleptic drugs that binds to the dopamine D1 and dopamine D2 receptors and inhibits their activity. It is one of the most extensively used of all of the long acting neuroleptic drugs causing a reduction of the anxiety associated to capture when administered in non-domestic ungulates [28–35]. Perphenazine enanthate has been used previously in Mohor gazelle to reduce intraspecies aggression when new individuals were introduced to an established group [36].

There are few studies examining the effects of LANs on reproductive function. Spanish ibex females tranquilized with perphenazine enanthate or haloperidol chlorohydrate were superovulated with ovine FSH, allowing for the collection of transferable embryos [14]. In mouflons, the use of fluphenazine decanoate resulted in a higher number of follicles on the ovarian surface after a superovulation treatment and an increase in the number of recovered oocytes in comparison to previous trials not employing tranquilizers [37]. To the best of our knowledge, there are no previous reports using perphenazine enanthate or other LANs during assisted reproductive procedures in gazelles. The use of tranquilizers could improve the well being of animals during intensive handling or ameliorate the effects of stress during assisted reproductive procedures, facilitating the recovery of healthy gametes and embryos that could be preserved in genome resource banks.

The aims of the present study were (1) to assess the use of a LAN, perphenazine enanthate, on handling

conditions for gazelles, its effects on ovarian response after FSH stimulation for the recovery of immature oocytes, and subsequent *in vitro* maturation, fertilization and culture, and (2) to examine embryo viability following oviduct transfer of oocytes matured and fertilized *in vitro*.

2. Materials and methods

2.1. Reagents

Chemicals used in the present study were obtained from Sigma Chemical Co. (Madrid, Spain), unless indicated otherwise.

2.2. Animals

All animal procedures were performed following the Spanish Animal Protection Regulation, RD1201/2005 which, in turn, conforms to European Union Regulation 2003/65. Procedures also adhered to the Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences and recommended by the journal.

The study was carried out in December 2005, using 16 healthy mature female Mohor gazelles (aged >2 years) from the breeding herd maintained and managed by the EEZA at the Parque de Rescate de la Fauna Sahariana in Almería, south-eastern Spain. Three separate herds were available for the study. One group ($n = 6$) received no treatment (control). A second group ($n = 6$) (LAN-group) was treated with perphenazine enanthate (Trilafon Enantato; perphenazine enanthate in sesame oil, Schering-Plough, Milan, Italy). The third group ($n = 4$) included females whose estrous cycles were synchronized and were kept as potential recipients for embryo transfer (ET-group).

The animals within each experimental group were housed in the same enclosure without males or young. Their diet consisted of commercial pellets, fresh alfalfa hay and barley grain. Water and mineral salts were available *ad libitum*.

2.3. Handling of animals during stimulation of follicular growth

The study was conducted over a 3-week period during which each animal in the untreated and LAN-treated groups was captured a total of 11 times. The gazelles were trapped with the aid of nets, immobilized and immediately blindfolded. The animals usually calmed down rapidly. The capture method was considered to represent a moderate stress for the animals; trapping of a whole herd often lasted less than 30 min, whereas individual capture and restrain for blood collection and other procedures took only a few minutes for each female.

Estrous cycles were synchronized by insertion of controlled progesterone internal drug release devices (CIDR, type G, 330 mg progesterone; InterAg Hamilton, New Zealand) for 15 days. Insertion of CIDR was considered the day 0. The vaginal devices were replaced on day 10, and removed the day of ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) given in four equal doses administered at 8:00 h and 18:00 h on days 13 and 14 [8].

Every animal in the LAN-group received a single deep intramuscular injection of 3.5 mg perphenazine enanthate per kg of body weight [14] on day 10 (i.e., the day of CIDR replacement). The effect was expected to begin 12–16 h after administration, reaching its peak 3 days after injection [32] and coinciding with the first administration of FSH. Clinical effects were expected to last 7–10 days [32].

Blood samples were taken at the time of each capture. Additional captures for blood sample collection were done 7 and 4 days before CIDR insertion (days -7 and -4) and on days 3 and 6 after CIDR insertion (see Section 3). Before blood collection, females were observed for 5–10 min to estimate flight distance and activity level when approached.

Several physiological variables were monitored to characterize the response of animals to the stress of management during assisted reproductive procedures. Heart rate, respiratory rate and rectal temperature were assessed from day 6 to day 15 of treatment. Before measurements, animals were allowed to calm down for at least 5 min. Heart and respiratory rates were assessed, with the aid of a stethoscope, by the same veterinarian to ensure repeatability of measurements.

Blood samples were collected from the jugular vein and aliquots stored with EDTA and with heparin–lithium. Plasma was recovered by centrifugation, frozen at −20 °C and then stored at −80 °C until assayed. Blood samples stored with EDTA were analyzed for hematological parameters using a veterinary cell analyzer (Medonic CA530, A. Menarini Diagnostics SA, Barcelona, Spain). The following were estimated: numbers of red blood cells (RBC), white blood cells (WBC) and platelets, mean cell volume of red cells (MCV), mean platelet volume (MPV), hemoglobin concentration (Hb),

packed cell volume (PCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and the granulocyte and lymphocyte concentrations. Plasma sub-samples were used for analyses of biochemical parameters using commercial kits (Multipanel Spotchem EZ, A. Menarini Diagnostics SA, Barcelona, Spain). Alanine acetiltransferase (ALT), aspartate acetiltransferase (AST), lactate dehydrogenase (LDH), total proteins (TP), albumin, total cholesterol, blood ureic nitrogen (BUN) and glucose were measured by dry biochemistry according to the instructions provided by the manufacturer.

Plasma cortisol, progesterone and 17β-estradiol concentrations were determined in duplicate by radioimmunoassay (Laboratoire de Dosages Hormonaux, INRA, Tours, France).

For ease of handling, all animals in one group were routinely trapped and treated before those in the other group. Reproductive procedures in the LAN-group and in the control-group were performed with a 24 h difference to avoid overlapping of OPU sessions.

2.4. Estrus synchronization in recipients for embryo transfer

Estrous cycles were synchronized by insertion of CIDRs for a total of 13 days. CIDRs were replaced on day 10 and, on that day, a single intramuscular injection of the synthetic prostaglandin analog cloprostenol sodium (125 μg per female; Estrumate, Schering-Plough, Madrid, Spain) was given to induce luteolysis of possible corpora lutea. Follicular growth was stimulated by the intramuscular administration of 300 IU eCG (Folligon, Intervet SA, Madrid, Spain) on day 13, when CIDR was removed. Ovulation was expected to occur approximately 64 h after the administration of eCG (our unpublished data).

2.5. Oocyte recovery

Cumulus-oocyte complexes (COCs) were recovered using a semi-laparotomy technique. Female donors were anesthesized with a combination of an intramuscular administration of xylazine (0.8 mg/kg body weight; Rompun, Bayer, Barcelona, Spain) and intravenous ketamine chlorohydrate (10 mg/kg body weight; Imalgene 1000, Merial, Lyon, France) and then secured in a cradle in dorsal recumbency. When necessary, surgical anesthesia was maintained using halothane inhalation. The effects of xylazine were reversed by administration of yohimbine chlorohydrate (0.3 mg/kg body weight; Sigma, Madrid, Spain).

Ovum pick-up was carried out following procedures described previously [8,38]. After endoscopic visualization, the ovaries were fixed at their ligament origin and exposed through the laparotomy incision. Follicles present on the ovarian surface were counted and aspirated. *Corpora hemorrhagica* (i.e., early stages in the formation of *corpora lutea*), if any, were also recorded. The COCs were collected in HEPES-buffered TCM-199 supplemented with 5% (v/v) heat-treated fetal calf serum, antibiotics (50 μg/ml streptomycin plus 50 IU/ml penicillin), polyvinyl-alcohol (PVA, 0.1%, w/v) and heparin (15 IU/ml). After OPU, ovaries were washed with warm physiological solution.

2.6. In vitro maturation

The COCs obtained from each female donor were kept separate throughout the *in vitro* procedures. After rinsing 3–4 times in HEPES-buffered TCM-199 with PVA, heparin and antibiotics, the COCs were placed in four-well culture dishes (Nunclon, Nalgene, Nunc International, Roskilde, Denmark) containing 500 μl of the *in vitro* maturation medium (TCM-199 plus 10% (v/v) heat-treated estrus sheep serum, 10 μg ovine FSH/LH/ml, 1 μg estradiol/ml, 100 μM cysteamine and 0.1 mg glutamine/ml), covered with 250–300 μl of mineral oil and cultured at 38.5 °C under 5% CO_2 in air and maximum humidity. One female (in the LAN-treatment group), which yielded a low number of recovered oocytes, had no oocytes maturing *in vitro*; data from this female were removed from this analysis.

2.7. In vitro fertilization

After 24 h in culture, oocytes were inseminated *in vitro* with cryopreserved spermatozoa in Synthetic Oviduct Fluid (SOF) [39] supplemented with heat-inactivated 2% (v/v) estrus sheep serum, 1 μg hypotaurine/ml and 10 μg heparin/ml. To avoid confounding effects due to male-to-male variations, pooled samples from three males were used. In preliminary trials, semen samples from various males were tested and those with good cryosurvival were employed.

Semen was collected by electroejaculation and cryopreserved as described previously [8,40]. Straws were thawed for 30 s at 37 °C in a water bath and the content of each straw was poured into a round-bottom glass tube and assessed for sperm motility and acrosome integrity [40]. A sperm motility index (SMI) was calculated as follows: [sperm % motility + (quality of motility × 20)] × 1/2. Spermatozoa were selected by

using a swim-up procedure. Briefly, spermatozoa in the cryodiluent (0.25 ml) were placed gently in the bottom of a round-bottom glass tube (15 × 75 mm) which contained 1 ml SOF medium with sheep serum, hypotaurine and heparine (as described above) and incubated at 38.5 °C under 5% CO_2 in air for 30 min. At the end of incubation two fractions of 0.3 ml and 0.2 ml (fractions 1 and 2, respectively) were removed, aspirating the sperm suspension at a point 3 mm below the surface of the medium, and sub-samples were taken for evaluation of sperm motility and acrosome integrity.

The final concentration of spermatozoa used during *in vitro* fertilization was 1×10^6 spermatozoa/ml. Gametes were co-incubated in 500 μl of fertilization medium (SOF with sheep serum, hypotaurine and heparine; see above) in four-well culture dishes under mineral oil for 24 h at 38.5 °C under 5% O_2, 5% CO_2 and 90% N_2.

2.8. In vitro culture

After 24 h of gamete co-culture, presumptive zygotes were washed in culture medium to remove spermatozoa and cell debris. They were transferred to 500 μl of SOF medium supplemented with 0.4% (w/v) BSA, nonessential and essential amino acids at oviduct concentrations [41] under mineral oil, and cultured at 38.5 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for several days. Cleaving embryos were separated from 1-cell presumptive zygotes after 36 h of culture. Embryo development was monitored every 24 h until it was arrested.

2.9. Evaluation of meiosis progression

The resumption and progression through meiosis process was evaluated retrospectively after *in vitro* fertilization as described previously [8]. Uncleaved oocytes and arrested embryos were stained with Hoechst 33342 and propidium iodide (10 μg/ml each) and examined using epifluorescence to determine maturation status and/or fertilization as deduced from the presence of ≥2 pronuclei or decondensed sperm heads. Fluorescence of Hoechst and propidium iodide was observed simultaneously using an Hg excitation beam and a filter set (UV-2A Nikon) consisting of a UV330-380 excitation filter, a DM400 chromatic beam splitter and a 420 barrier filter.

2.10. Embryo transfer

Embryo transfer was performed on day 4 after CIDR removal in recipient females. Response to synchronization and follicular stimulation was evaluated by endoscopy prior to embryo transfer while the animals were in dorsal recumbency under general anesthesia. The ovary containing the *corpus hemorrhagicum* and ipsilateral oviduct were exposed by midventral laparotomy and surgical transfer was performed with the aid of a fine catheter (umbilical catheter 8888-160341, 38 cm/1.7 mm; Sherwood Medical Argyle, Tyco Healthcare Italia SA, Segrete, Italy) attached to a 20 μl tip fitted to an automatic micropipette. The catheter was passed through the infundibulum into the lumen of the oviduct to deliver the embryos.

Embryos were transferred in 20 μl sterile physiological solution [0.9% (w/v) NaCl] supplemented with 20% (v/v) heat-inactivated fetal calf serum. After embryo transfer, ovaries and exposed reproductive tract were washed with warm physiological solution.

Embryos were pooled and three synchronized gazelles were used as recipients. Four 2–4 cell stage embryos were transferred into each recipient female. Pregnancy was assessed by quantifying plasma levels of estrone sulfate on day 128 after embryo transfer. Three non-pregnant gazelles were used as negative controls for quantification of estrone sulfate levels. Estrone sulfate levels in plasma were determined in duplicate by radioimmunoassay (Laboratoire de Dosages Hormonaux, INRA, Tours, France).

2.11. Statistics

Variables (percentages) which did not have a normal distribution were arcsin-transformed. To avoid pseudoreplication percentages of oocytes recovered, oocytes reaching MII, fertilized or cleaving were calculated for each female and then averaged for either the control or the LAN-treated group. Differences between the two groups were analyzed by one-way analysis of variance (ANOVA) or Mann–Whitney *U*-test using the exact probability adjusted for small samples. The nonparametric alternative to the one-way repeated measures analysis of variance, Friedman ANOVA test, was used to analyze differences in variables within each group thoughout treatments. Possible relations between hormone levels (cortisol, progesterone, estradiol), oocyte harvesting parameters (recovery rate, number of follicles aspirated, number of COCs recovered) and *in vitro* culture-dependent variables (maturation, fertilization and cleavage rates) were examined. Regression analysis was done between ovarian response and culture variables. Results are expressed as mean ± S.E.M. Probability values of less than 0.05 were considered significant. Statistical

analyses were performed using Statistica (Statsoft, Inc. 2001, STATISTICA, data analysis software system, version 6; www.statsoft.com).

3. Results

3.1. Effect of the long acting neuroleptic

There were no observable adverse reactions at injection sites of perphenazine and no observed extrapyramidal signs, the most commonly reported side effects of the use of LANs in animals. Behavioral observations revealed that LAN-treated animals spent more time lying, although they were attentive to their surroundings. None of the gazelles was removed from the experiment due to handling stress and there were no clinical cases of capture myopathy.

On the days of FSH administration (days 13 and 14) and OPU (day 15) LAN-treated animals were easier to capture than untreated controls, to the extent that some were captured by hand. It was obvious to observers that they were calmer and showed no signs of fear when approached by humans. Two days after OPU (8 days after perphenazine administration), animals in both groups seemed to behave similarly.

3.2. Physiological, hematological and biochemical parameters

Heart rates of females in the LAN-treated group showed no significant differences throughout the experiment (Fig. 1A). On the other hand, females in the control group showed decreasing heart rates over time (Friedman test from day 10 to 15; $\chi^2 = 21.1$, $p < 0.001$). Significant differences were observed between the LAN-treated group and the control from day 14 onwards (day 14 am, Mann–Whitney U-test; $Z = 2.0$, $p < 0.05$; day 14 pm; Mann–Whitney U-test; $Z = 2.3$, $p < 0.05$; day 15; Mann–Whitney U-test; $Z = 2.4$, $p < 0.05$), with mean heart rate being lower in the control group.

There were significant differences within groups in respiratory rates over time from day 10 onwards, with a steady decrease in both groups (Fig. 1B). In the LAN-treated group, respiratory rates decreased from 33.3 ± 1.3 breaths/min on day 10 (day of LAN administration) to 26.7 ± 1.3 breaths/min on day 15, before OPU (Friedman test, from day 10 to 15, $\chi^2 = 12.3$; $p < 0.05$). In the control group, respiratory rates decreased from 41.3 ± 2.9 breaths/min on day 10 to 32.7 ± 3.3 breaths/min on day 15 (Friedman test, from day 10 to 15, $\chi^2 = 12.6$; $p < 0.05$). A larger variation between subjects was found in control animals than in the LAN-treated group. Significant differences between groups were observed on day 10 (Mann–Whitney U-test; $Z = -2.3$; $p < 0.05$), and in the mornings of day 13 (Mann–Whitney U-test; $Z = -2.7$, $p < 0.01$) and day 14 (morning, Mann–Whitney U-test; $Z = -2.7$; $p < 0.01$; afternoon, Mann–Whitney U-test: $Z = -1.96$; $p = 0.05$) (Fig. 1B).

No significant differences were identified in body temperature over time in the LAN-treated group (Friedman test from day 10 to 15; $\chi^2 = 4.4$, $p > 0.05$)

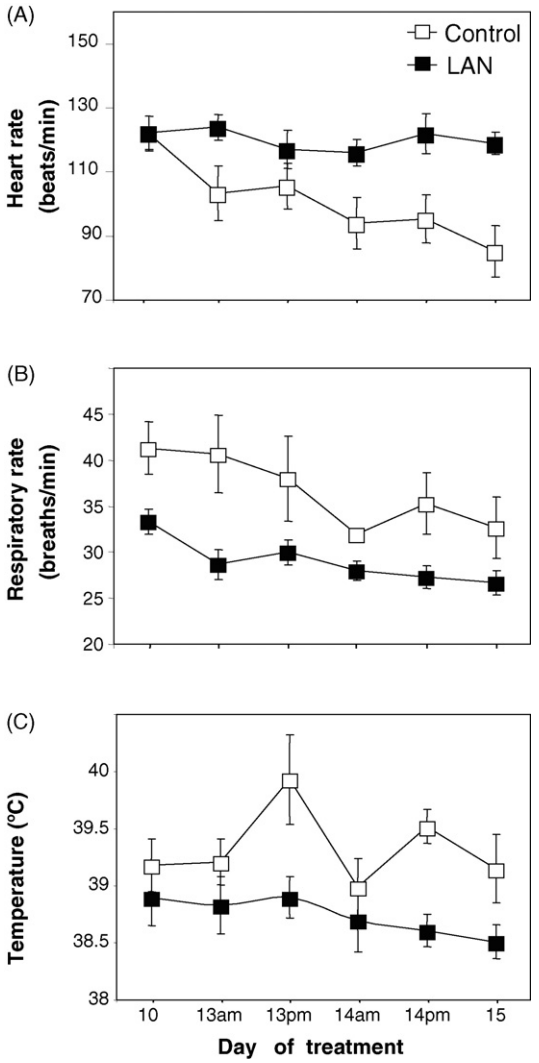

Fig. 1. Heart (A) and respiratory (B) rates, and body temperature (C) during days 10–15 of synchronization and ovarian stimulation in Mohor gazelles (*Gazella dama mhorr*) treated with the long acting neuroleptic (LAN) perphenazine enanthate or untreated controls. Day 10: replacement of CIDR and perphenazine administration in the LAN group; days 13 and 14: administration of oFSH; day 15: ovum pick-up (OPU). Data are means ± S.E.M.

(Fig. 1C), whereas in the control group, temperature differed between days (Friedman test from day 10 to 15; $\chi^2 = 13.4$, $p < 0.05$). Between groups, significant differences were only observed in the afternoon of day 14 (Mann–Whitney *U*-test; $Z = -2.9$, $p < 0.01$).

Several hematological and biochemical parameters were quantified. Clear and significant changes were seen over time in some hematological and biochemical parameters within both groups (Table 1) although no differences were found between the control and the LAN-treated groups. A general decrease ($p < 0.05$) in RBC (Table 1), PCV and Hb (not shown) was observed in both groups from day 10 to 15 (with the exception of RBC in the LAN group which remained unchanged). Increasing numbers of WBC and lymphocytes were observed in control and tranquilized animals over time ($p < 0.05$) (Table 1). Glucose and TP did not change throughout treatment in the LAN-group ($p > 0.05$), but showed significant variation over time in the control group ($p < 0.05$) (not shown). Plasma ALT and AST activities increased ($p < 0.01$) in both treated and control groups from day 10 to day 15 (Table 1). Plasma LDH activity increased ($p < 0.001$) in the LAN-group, whereas changes were not observed in control animals (Table 1).

3.3. Oocyte harvesting, maturation, fertilization and embryo culture

In LAN-treated females, a total of 82 follicles were aspirated (13.7 ± 1.9 follicles per female) and 52 COCs were recovered (8.7 ± 1.6 oocytes per female), resulting in an overall recovery rate of 63% (61.7 ± 4.5% recovery per female). In the untreated control group, a total of 115 follicles were aspirated (23.0 ± 5.2 follicles per female) and 75 COCs were recovered (15.0 ± 3.8 oocytes per female), resulting in an overall recovery rate of 65% (62.4 ± 5.3% recovery per female) (Table 2). Thus, the numbers of punctured follicles and recovered COCs were higher in the untreated group, although differences did not reach statistical significance (number of follicles: Mann–Whitney *U*-test: $Z = -1.6$; $p > 0.05$; number of recovered COCs, Mann–Whitney *U*-test: $Z = -1.29$; $p > 0.05$). The recovery rate, calculated as the percentage of COCs recovered in relation to the follicles aspirated from the ovarian surface, was not different between groups ($F_{1,9} = 0.01$, $p > 0.05$).

Comparison of the proportions of oocytes matured *in vitro* revealed a higher percentage of oocytes in MII per female in the LAN-treated group (64.8 ± 6.0%) than in the untreated controls (43.0 ±5.3%), and the difference was statistically significant ($F_{1,8} = 7.45$, $p = 0.026$) (Table 2). This result not only indicates that the use of the neuroleptic had no negative effect on oocyte maturation but, more importantly, it shows that there was an actual improvement in the percentage of maturation *in vitro*.

Three males were selected as sperm donors based on preliminary tests of survival during sperm cryopreservation and postthaw incubation at 37 °C for 2 h. Cryopreserved spermatozoa thawed and recovered after swim-up for 30 min, revealed an increase in the proportion of motile spermatozoa (75.7 ± 6.1%), quality of motility (3.1 ± 0.2) and SMI (68.5 ± 4.7) in the upper fraction (fraction 1) when compared with values obtained postthaw (63.3 ± 1.8; 2.8 ± 0.1; 59.7 ± 1.6, respectively) (Mann–Whitney *U*-test; $p < 0.05$). There were no differences between values in the lower fraction (fraction 2) (% motility, 70.3 ± 5.1; quality, 3.0 ± 0.1; SMI, 65.2 ± 3.3) and postthaw spermatozoa (see above). The proportion of sperm cells with an intact acrosome was higher in the upper fraction (77.3 ± 1.6%) than in the lower fraction (69.7 ± 2.3%) or after thawing (58.2 ± 1.5%) (Mann–Whitney *U*-test; $p < 0.05$). Accordingly, the percentage of spermatozoa with a damaged acrosome decreased from thawed spermatozoa (39.6 ± 1.5%) to the lower (27.7 ± 2.1%) and upper fraction (20.9 ± 1.5%) (Mann–Whitney *U*-test; $p < 0.05$). Sperm concentration was lower in both fractions compared to that in thawed spermatozoa (Mann–Whitney *U*-test; $p < 0.05$). Out of an average of 271.6 ± 20.7 × 10^6 spermatozoa/ml in the thawed straws, an average of 0.97 ± 0.3 × 10^6 and 0.84 ± 0.2 × 10^6 spermatozoa/ml were recovered in the upper and lower fractions after swim-up.

Fertilization rate, calculated in relation to the number of matured oocytes (and expressed as percentage of fertilized oocytes per female), was similar between groups (83.4 ± 10.5% in the LAN-treated group *vs.* 81.8 ± 11.0% in the control group, $F_{1,9} = 0.24$, $p > 0.05$) (Table 2). None of the fertilized (1-cell) oocytes showed evidence of polyspermy. These results indicate that the use of perphenazine enanthate did not affect fertilization of oocytes collected from females treated with the neuroleptic during ovarian stimulation.

The average percentage of cleaving embryos per female in relation to matured oocytes (Table 2) was higher in the LAN-treated group but differences between this and the control group did not reach statistical significance (44.4 ± 19.3% cleavage in the LAN-treated group *vs.* 35.8 ± 18.6% cleavage in the control group; $F_{1,9} = 0.002$, $p > 0.05$). The average

Table 1
Hematological and biochemical parameters in Mohor gazelle (*Gazella dama mhorr*) from days 10 to 15 of ovarian stimulation and OPU in control and long acting neuroleptic (LAN)-treated animals[a]

Parameter	Treatment	Day of treatment						ANOVA Friedman test
		10	13 am	13 pm	14 am	14 pm	15	
RBC (10^6/μl)	Control	11.3 ± 0.4	11.3 ± 0.2	10.5 ± 0.5	9.1 ± 0.2	10.3 ± 0.5	9.5 ± 0.2	14.4 (*)
	LAN	10.2 ± 0.2	11.1 ± 0.3	10.9 ± 0.5	10.5 ± 0.5	11.1 ± 0.2	9.5 ± 0.3	ns
WBC (10^3/μl)	Control	4.7 ± 0.8	4.9 ± 0.7	5.8 ± 0.7	7.1 ± 0.7	5.5 ± 0.6	7.2 ± 1.1	21.1 (***)
	LAN	6.9 ± 0.7	5.6 ± 1.2	10.3 ± 4.4	6.9 ± 1.6	9.2 ± 1.8	8.2 ± 1.2	11.2 (*)
Lymphocytes (10^3/μl)	Control	1.6 ± 0.3	3.1 ± 0.3	3.4 ± 0.3	4.7 ± 0.5	3.2 ± 0.3	4.1 ± 0.7	23.2 (***)
	LAN	2.0 ±0.2	2.3 ± 0.6	5.8 ± 2.6	5.3 ±1.4	5.7 ± 1.2	4.4 ± 0.7	21.19 (***)
ALT U/l	Control	25.5 ± 2.0	22.2 ± 1.1	27.0 ± 4.2	25.2 ± 2.5	51.7 ± 5.3	53.6 ± 5.5	18.7 (**)
	LAN	20.7 ± 1.1	25.2 ± 2.5	34.7 ± 2.9	38.3 ± 6.4	47.5 ± 5.6	42.5 ± 8.7	20.3 (**)
AST U/l	Control	194.7 ± 86.7	144.5 ± 41.5	162.3 ± 44.5	181.8 ± 35.8	263.2 ± 34.6	294.0 ± 30.4	16.6 (**)
	LAN	107.0 ± 6.7	118.7 ± 9.7	163.3 ± 26.1	192.8 ± 41.1	203.5 ± 36.3	261.8 ± 38.5	28.1 (***)
LDH U/l	Control	1026.5 ± 506.5	765.7 ± 333.1	1025.8 ± 353.8	1192.0 ± 313.8	1548.0 ± 226.9	1476.6 ± 222.9	ns
	LAN	541.0 ± 53.8	508.8 ± 38.9	1016.5 ± 408.2	1128.2 ± 370.6	1069.3 ± 318.5	1811.5 ± 426.8	23.7 (***)

[a] Day 10 is the day of CIDR replacement in both groups and perphenazine enanthate administration in the LAN group; days 13 am, 13 pm, 14 am and 14 pm are days of FSH injection; day 15 is the day of ovum pick-up (OPU). Data are means ± S.E.M. Data were analyzed using an ANOVA Friedman test to examine changes in variables within groups over time; *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$. Differences between groups on the same day of treatment were analyzed using a Mann–Whitney *U*-test. There were no significant differences between control and the LAN group ($p > 0.05$). RBC, red blood cells; WBC, white blood cells; ALT, alanine acetiltransferase; AST, aspartate acetiltransferase; LDH, lactate dehidrogenase; ns, not significant.

Table 2
Summary of results of ovarian stimulation, ovum pick-up and *in vitro* culture of oocytes from Mohor gazelle (*Gazella dama mhorr*) treated with the long acting neuroleptic (LAN) perphenazine enanthate or untreated controls

Parameters	Group	
	Control	LAN
Total no. of follicles	115	82
Total no. of COCs recovered	75	52
Mean no. of COCs recovered per female[a]	15.0 ± 3.8^{a}	8.7 ± 1.6^{a}
Mean % oocytes recovered per female	62.4 ± 5.3^{a}	61.7 ± 4.5^{a}
Total no. of oocytes cultured (IVM)	67	42
Total no. of oocytes reaching MII	28	24
Mean % oocytes in MII per female[b,*]	43.0 ± 5.3^{a}	64.8 ± 6.0^{b}
Total no. of fertilized oocytes	20	20
Mean % fertilized oocytes per female[c]	81.8 ± 11.0^{a}	83.4 ± 10.5^{a}
Total no. of matured oocytes cleaving	7	12
Mean % cleaved oocytes per female[d]	35.8 ± 18.6^{a}	44.4 ± 19.3^{a}
Total no. of fertilized zygotes cleaving[e]	7	12
Mean % of fertilized zygotes cleaving per female	38.7 ± 19.0^{a}	47.7 ± 18.4^{a}

Results are means ± S.E.M.
[a] Recovery rate: number of COCs recovered/number of punctured follicles.
[b] Maturation rate: number of matured oocytes/number of COCs included in culture.
[c] Fertilization rate: number of oocytes fertilized/number of matured oocytes.
[d] Overall cleavage rate: number of cleaving embryos/number of matured oocytes.
[e] Development rate: number of cleaving embryos/number of fertilized oocytes.
[*] Excludes one female with limited response and no oocyte maturation *in vitro* (for details see Section 2).[a,b]Different superscripts between columns indicate significant differences (ANOVA, $p < 0.05$).

percentage of cleavage per female calculated in relation to the number of fertilized oocytes was also higher in the LAN-treated group but, again, it was not statistically different from the control group ($47.7 \pm 18.4\%$ cleavage in the LAN-treated group *vs.* $38.7 \pm 19.0\%$ cleavage in the control group; $F_{1,8} = 0.14$, $p > 0.05$). Thus, there was a trend for a higher rate of development of embryos derived from LAN-treated females, although a lack of statistical significance precludes us to conclude that there was a positive effect at this stage. Nevertheless, the results indicate that embryo production was not negatively affected in females treated with the LAN.

Twelve oocytes cleaved after *in vitro* fertilization in the LAN-treated group, yielding eight 2-cell embryos, three 4-cell embryos and one 8–10-cell embryo. From these, 10 embryos were transferred to recipient females. None of the non-transferred embryos maintained in culture developed to the blastocyst stage. In the control group, seven oocytes cleaved after *in vitro* fertilization, yielding six 2-cell embryos and one 4-cell embryo and only two embryos were transferred. None of the remaining embryos of this group that were maintained in culture developed to the blastocyst stage.

3.4. Embryo transfer

A total of 12 embryos at the 2–4-cell stage were transferred to three synchronized recipient females, with four embryos being transferred into each recipient. Embryos were pooled to maximize the possibility of achieving success with embryo transfer and because scarcity of embryos precluded a proper comparison of embryos obtained in each group. Thus, embryo transfer was used in order to establish the feasibility of the technique to overcome the *in vitro* culture block detected in our earlier study. At the moment of transfer, embryos were mainly from females in the LAN group with only 2 embryos from the control group. Embryo transfer was performed in the oviduct ipsilateral to the ovary with a *corpus hemorrhagicum*, with the exception of one female which received the embryos in the contralateral oviduct.

The ovaries of the fourth female in this group were also examined to verify if ovulation had occurred. All the gazelles in this group presented a *corpus hemorrhagicum,* with very similar morphological appearance and with an estimated age of about 48 h, suggesting that ovulation took place at the expected time (about 64 h

after CIDR removal) and that there was a good synchronization of recipient females.

None of the females was found to be pregnant following embryo transfer. On day 128 after transfer, plasma estrone sulfate levels were found to be 0.1 ng/ml in all recipients and in non-pregnant control females. In earlier studies, pregnant Mohor gazelles were found to have higher plasma levels of estrone sulfate (0.5–0.7 ng/ml) from day 90 onwards after artificial insemination (unpublished data).

3.5. Hormone levels during synchronization and FSH stimulation

The results of hormone assays showed that plasma progesterone levels were very similar between groups (Mann–Whitney U-test; $p > 0.05$) (Fig. 2A). In both control and LAN-treated groups, progesterone did not increase after the day when CIDR was inserted or replaced. Levels of progesterone remained at a concentration below 1 ng/ml.

Plasma estradiol increased from the time of the first FSH injection to the day of OPU, reaching a maximum of 4.23 ± 1.15 pg/ml in the LAN-group whereas the peak value was 8.35 ± 1.39 pg/ml in the control group (Fig. 2B). Concentrations of estradiol were already high before FSH injection. There were significant differences in estrogen levels between days 10 to 15 of treatment in the LAN-treated group (Friedman test, $\chi^2 = 12.5$, $p = 0.028$) whereas they did not differ in the control group (Friedman test, $\chi^2 = 6.7$, n.s.). Comparisons between groups revealed differences on day 14 at the time of the third FSH injection (Mann–Whitney U-test; $Z = -2.2$, $p = 0.026$).

Plasma cortisol remained constant throughout most of the early phases of treatment but increased after day 13 when FSH injections began (Fig. 3). Cortisol levels thus rose during the phase of more intense handling of animals in both groups and this increase was more pronounced in the control group (Friedman test from day 10 to 15, $\chi^2 = 23.9$, $p < 0.001$) than in the LAN-treated group (Friedman test from day 10 to 15, $\chi^2 = 11.5$, $p = 0.04$) (Fig. 3). Levels of cortisol in the afternoon of day 14 (the time of the fourth FSH injection) were nearly two-fold higher in the untreated group (104.6 ± 11.7 ng/ml) than in the LAN-treated group (62.1 ± 3.1 ng/ml) and differences were statistically significant (Mann–Whitney U-test; $Z = -2.9$, $p < 0.01$).

There were no significant relations between levels of progesterone, estradiol and cortisol. Similarly, there

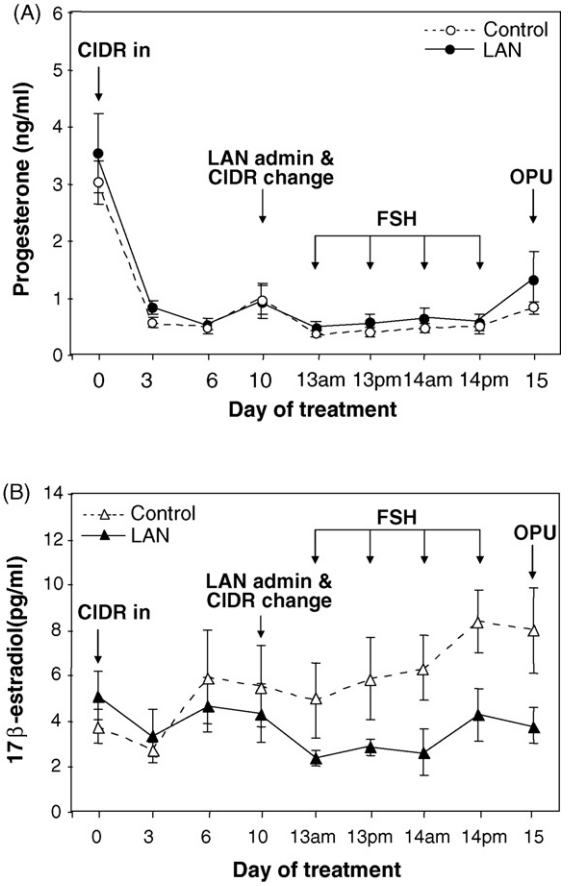

Fig. 2. Plasma progesterone (A) and 17β-estradiol (B) in Mohor gazelle (*Gazella dama mhorr*) females before and during ovarian stimulation without or with administration of the long acting neuroleptic (LAN) perphenazine enanthate. Day 0: insertion of CIDR; day 10: replacement of CIDR and perphenazine administration in the LAN group; days 13 and 14: oFSH administration; day 15: ovum pick-up (OPU). Data are means ± S.E.M.

was no relation between cortisol and the estrogen/progesterone ratio. Hormone concentrations were not related to any of the culture variables analyzed. The number of recovered COCs were positively related to the levels of estradiol on different days of FSH treatment up to the time of OPU (day 13, 8.00 h: $R = 0.63$, $p = 0.05$; day 13, 18:00 h: $R = 0.77$, $p < 0.05$; day 14, 8:00 h: $R = 0.65$, $p < 0.05$; day 14, 18:00 h: $R = 0.73$, $p < 0.05$). Cortisol levels were not related to results of oocyte recovery.

Stepwise regression analyses showed that the percentage of recovery was influenced by the age of animals ($R^2 = 0.45$; $F_{1,8} = 6.4$; $p = 0.035$). None of the other variables of ovarian response, recovery or *in vitro* culture were influenced by age or weight of animals.

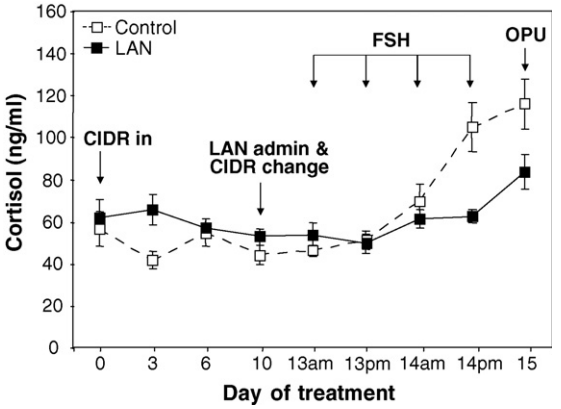

Fig. 3. Plasma cortisol in Mohor gazelle (*Gazella dama mhorr*) females before and during ovarian stimulation without or with treatment with the long acting neuroleptic (LAN) perphenazine enanthate. Day 0: insertion of CIDR; day 10: replacement of CIDR and perphenazine administration in the LAN group; days 13 and 14: administration of oFSH; day 15: ovum pick-up (OPU). Data are means ± S.E.M.

3.6. Fertility of females after treatments

Donor females were allocated to breeding groups 4 months after the end of the experiment, whereas recipients were bred a few months after their expected delivery dates. One donor female was translocated to another institution (and was included in a breeding group there) and another one died. Out of a total of 15 gazelles, 9 (60%) became pregnant and carried their pregnancies to term, indicating that handling and surgical procedures did not negatively affect their reproduction.

4. Discussion

The results of the present study show that the long-acting neuroleptic (LAN) perphenazine enanthate did not have negative effects on maturation, fertilization and embryo development *in vitro*. Furthermore, there was a higher average percentage of oocytes maturing *in vitro* per female in the LAN-treated group and a trend for a higher proportion of embryos developing *in vitro*. Gazelles treated with the LAN were easier to handle and, during the period of intensive handling, just before oocyte collection, cortisol levels were significantly lower in tranquilized animals than in the controls. These results are important for future efforts of collection and banking of germplasm from endangered species.

The effectiveness of several compounds in controlling the multiorganic response to stressful stimuli differs according to their mechanisms of action [23]. In previous studies, animals treated with tranquilizers were found to show lower body temperature, lower heart rate variability, less hemoconcentration (lower erythrocyte count, hemoglobin concentration and PCV) and lower serum ALT, AST, CPK and LDH activities than untreated animals [33,42,43]. Since multiple organs are involved in the adaptive stress response, several physiological variables were measured in the present study to better characterize the stress of handling during a superovulatory treatment in Mohor gazelle.

Some significant differences were found in physiological parameters between the untreated and the LAN-treated group, with clear differences in heart rate, body temperature, and cortisol levels over time.

There were several clear hematological and biochemical changes over time in both groups. Animals showed less hemoconcentration (blood analysis revealed a significant lower red blood cell count and hemoglobin concentration), suggesting that animals were habituating to handling. However, leukocytosis with lymphocytosis was observed in animals throughout the period of study characteristic of the first phase of the catecholamine-dependent stress response. Plasma AST, ALT and LDH activities increased over time in both groups, suggesting skeletal muscle cell damage due to management and restraint, supporting the idea of increasing stress effects throughout the superovulation treatment. However, LAN administration was not able to prevent the hematological and biochemical changes associated with the stress response when compared to the control group.

One important result in this study relates to significant differences in cortisol levels between females with no treatment and those receiving tranquilizer. Cortisol levels rose in all animals after day 13 and during days 14 and 15, the time of more intensive handling for twice-daily FSH administration, but the rise in cortisol was considerably higher in untreated animals than in those receiving tranquilizer. Since this period corresponds to the phase of follicular growth, high cortisol levels may affect the future competence of oocytes. It is worth emphasizing that cortisol levels remained low before this final phase of handling for superovulation, which suggests that prior moderate handling (once every 3 days until day 13) may have not generated a great deal of stress to the animals.

In our previous study on ovum pick-up and *in vitro* maturation, fertilization and culture in Mohor gazelle [8] follicular development was poor after FSH stimulation (about 9 follicles/female) and this was found to be a limiting factor in *in vitro* embryo production. Possible

explanations for these results were a poor response to FSH and the potential negative effects of handling stress. In the present study, a higher number of follicles developed in females in both control and LAN-treated groups (23 and 14 follicles/female, respectively) and no significant differences existed between such groups with recovery rates being also similar between groups. Importantly, a higher proportion of oocytes per female progressed through meiosis in the LAN-treated group compared to non-treated animals, indicating a clear benefit of the use of the neuroleptic.

Fertilization rates achieved were high in both groups of animals (about 80% fertilized oocytes per female). These results represent a considerable improvement over fertilization rates obtained in our previous study where a mean of 54% fertilized oocytes per female were obtained [8]. One possible explanation for this improvement is that a pool of spermatozoa from three males (as opposed to a single male) was employed. In addition, the proportion of sperm with intact acrosomes was much higher at the beginning of gamete co-incubation in the present study. Fertilization rates were very similar between LAN-treated and control groups; importantly, the tranquillizer did not lead to an increase in the occurrence of abnormal fertilizations.

In our previous study [8] embryos did not progress to the blastocyst stage during *in vitro* culture, suggesting the possible existence of an *in vitro* developmental block. A mean of about 32% matured oocytes cleaved after fertilization in the previous work [8]. Slightly better results were obtained in the present study (36-44%) but, again, none of the embryos developed *in vitro* to the morula or blastocyst stage. When cleavage was analyzed in relation to the number of fertilized oocytes, results in this study (39–48%) were slightly lower than those in our previous work (58%). Analyses of cleavage rates between control and LAN-treated groups revealed better results in the latter, both in relation to the number of matured oocytes and the number of fertilized oocytes. The differences did not, however, reach statistical significance, probably due to the low number of animals and the large variation in each group.

To overcome limitations due to poor embryo culture conditions we carried out early embryo transfers. It has been shown that the physiological environment in the oviduct favors the development of cleaved *in vitro* produced embryos [9]. In deer, 2–4-cell embryos from *in vitro* matured and fertilized oocytes were capable of establishing pregnancies more efficiently than those transferred at the 8-cell stage [11]. In the cow, successful pregnancies after laparoscopic oviduct catheterization and transfer of *in vitro* matured and fertilized embryos have been reported [10]. In the present study, none of the recipient gazelles gave birth to offspring after embryo transfer. To assess whether pregnancies were established, estrone sulfate levels were quantified early in the second half of putative pregnancies; no evidence of an ongoing pregnancy was found at 128 days after embryo transfer. Ultrasound was discarded as an option to assess pregnancy at an earlier stage since it has been argued that trapping animals for early pregnancy diagnosis by ultrasound can lead to pregnancy loss [44]. Thus, it could not be elucidated whether any of the embryos progressed after transfer to the oviduct of recipients. The failure to conceive after transfer could be related to an inappropriate oviduct or uterine environment created with the hormonal treatments we used for estrus synchronization.

Females used in the study were subsequently examined for their fertility. We found that a majority of females (60%) conceived and carried their pregnancies to term. This is an important result because it demonstrates that animals submitted to assisted reproductive techniques, involving superovulation and surgical procedures with ovarian manipulation, did not have a diminished reproductive capacity, a key factor in captive breeding programmes of endangered species that rely on a few individuals.

Since higher levels of cortisol (in the control group) were associated to lower rates of *in vitro* oocyte maturation, it is worth considering possible underlying mechanisms that may affect follicular development and, potentially, acquisition of oocyte competence. The ovary is susceptible to the action of glucocorticoids either indirectly via the adrenal-hypothalamus-pituitary axis or directly via the glucocorticoid receptors present in ovarian cells. Evidence for a direct negative effect of glucocorticoids on the *in vitro* maturation of pig oocytes has been presented [22,45]. Our previous work on Mohor gazelle revealed a low response to a super-ovulatory treatment [8] probably due to stress, and in fact one of the females had to be removed from the experiment due to handling stress. Stress induced in wild ungulates during assisted reproductive procedures may also explain the poor responses obtained in earlier studies [12–14]. Therefore, there is a need to study further the possible direct links between cortisol and oocyte maturation, and the potential implications for fertilization and early development.

In conclusion, our results indicate that the use of the long-acting neuroleptic perphenazine enanthate may be useful in reducing the effect of stress during ovarian stimulation and ovum-pick up in Mohor gazelles without any apparent negative effect on *in vitro*

maturation and fertilization ability of the recovered oocytes. Moreover, results revealed an increase in the average proportion of oocytes reaching metaphase II in treated animals in relation to controls, coincident with significantly lower plasma cortisol in these animals during the last day of FSH treatment and prior to the day of oocyte recovery. Since it is essential to avoid or, at least, reduce the animals stress during their management, the use of LANs may become very useful for animal handling in assisted reproduction. Thus, more studies are needed to increase our knowledge of the effect of neuroleptics on reproduction and the influence of glucocorticoids on the success of assisted reproductive procedures in wildlife.

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