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Sexual Preference of Crossbreed Ewes for Rams Medicated with Naloxone

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ABSTRACT

Ewe sexual preference depends on several factors; ram attractiveness is important. This work was carried out in in the first week of September 2020 and repeated in November 2021. For the first trial two rams were treated with 0.5 mg naloxone im for 15 days, two control rams were treated with saline injections. Sexual preference was studied with synchronized ewes (n 0 12) displaying estrus at the end of naloxone treatment of the rams. Ewe's proceptive and receptive behavior were noted. Plasma testosterone levels were monitored through the duration of the experiment in treated and control rams. For the second trial 4 rams and ewes were treated same as trial 1, and the number of ewes was increased to 24. It was observed that on the first week of treatment, testosterone levels were not significantly different between treated and control rams; but at the beginning of the second week of treatment testosterone plasma levels in naloxone treated rams increased until values reached levels of 15 ng/mL compared with 6 ng/mL in control rams. When ewes were exposed to treated and untreated rams during the first trial, 11 ewes showed high proceptivity and receptivity for the naloxone treated ram ($p<0.01$) and they were mated, for the control ram only one ewe showed complete preference. In the second trial it was observed that 19 ewes also showed high proceptivity and receptivity for naloxone treated rams ($p < .001$). Hormone levels behaved following the same pattern as that observed during the first trial. It was concluded that naloxone treatment facilitated the secretion of testosterone in rams and the high concentration of this androgen motivated ewes to prefer mating with the naloxone treated ram.

KEYWORDS: Ewe, Sexual Preferences, Rams Naloxone

INTRODUCTION

The ewe is a promiscuous species, they do not develop a pair bonding relationship. In the open field the ewe accepts copulation from different rams and when in estrus ram seeking is commonly observed. It appears that the soliciting behavior of the ewe is not a major determinant of her sexual "attractiveness" (Tilbrook and Lindsay, 1987**)**

Three aspects of sexual behavior in the ewe are considered:

Attractivity refers to the female's value as a sexual stimulus whereas proceptivity consists of appetitive activities shown by females.

Receptivity includes the behaviors exhibited by the female that allow mounting and intravaginal ejaculation (Tilbrook et al. 1990).

The level of Proceptive behavior clearly influences the chances of the ewe being mated, and the number of ewes that are mated will influence the fertility of the flock (Tilbrook et al. 1990). In ewes, Proceptive and receptive behaviors are only expressed for a short period during the estrous cycle, around the time of ovulation (Tilbrook et al. 1990).

In feral Soay sheep males are predominantly important and they focus their mating activity and siring success towards heavier females with higher inclusive fitness (Preston et al., 2005). Also psychosocial stress inhibits LH secretion and the ability of ewes to attract rams (attractivity) and the motivation of ewes to seek rams and initiate mating (proceptivity) (Pierce et al., 2004). Proceptive behavior of the ewe includes afilliative (tendency to approach and remain with rams) and physical contact (appetitive responses in the Mating Test) responses **(**Tilbrook et al., 1990). The behavior of the ram reflects the level of Proceptive and receptive behavior of the ewe, emphasizing the importance of the interaction between both sexes. Furthermore, the level of Proceptive behavior of the ewe clearly influences the amount of attention that she receives from the ram and her chances of being mated (Tilbrook et al., 1990)**.**

In most species, females are more attracted and interested by the male odor around the estrous period: rats, dogs, pigs, elephants, rams male presence or its odor does not induce drastic behavioral changes (Gelez and Fabre-Nts, 2006).

Males do not elicit strong attraction but seem to focus the attention of ewes and induce urine emission (Gelez and Fabre-Nys, 2006). Also the age of the ram influences ewe proceptivity, they tend to choose younger rams for mating, probably due to scent and hormone cues (Ramos and Ungerfeld, 2006).

Testosterone levels and age in rams can be a factor of attractiveness for ewes (Roselli et al., 2002; Ramos and Ungerfeld, 2006). In earlier work it was reported by Perkins et al. (1992) that rams with higher testosterone levels were more sexually active as compared with rams with low androgen levels.

Sexual behavior is controlled by hormonal and neurochemical actions in the brain (Pfaus et al. 2012). Reproduction is an event that requires the coordination of peripheral organs with the nervous system to ensure that the internal and external environments are optimal for successful procreation of the species. This is accomplished by the hypothalamic-pituitary-gonadal axis that coordinates reproductive behavior with ovulation (Christensen wt al. 2012). Bilkis et al. (2012) reported that testosterone concentration is correlated with libido, and if GnRH is inactivated by active immunization sexual behavior is diminished, It appears that Endogenous opioid peptides (EOPs) play an important role in modulating several aspects of pulsatile GnRH release during the ovine estrous cycle (Goodman et al., 1995; Goodman et al.2002). Opioid antagonists seem to increase the secretion of GnRH in the hypothalamus which then causes an increase of the pulsatile release of LH in the pituitary and consequently testosterone levels are increased (Tenhola et al., 2012). Naloxone has been used as an opioid antagonist with a wide range of affinity to all opioid receptors: μ, δ and κ, decreasing affinity in that order, high doses of naloxone interact with different receptors and the lowest dose probably react with μ receptors only (Sciorsci et al. 2000).

Small doses of naloxone induce changes in plasma testosterone levels in rams and bucks (Fuentes et al., 1997, 1998) and facilitate sexual behavior and duration of estrus in the ewe (Fuentes et al., 1998). Similar observations were also reported in rabbits (Fuentes et Al. 2012). Considering that small doses of naloxone facilitate sexual behavior and increases Lh and testosterone blood levels. It was considered of interest to study sexual preference of ewes with induced estrus for rams treated with small doses of naloxone.

MATERIALS AND METHODS

Experimental Animals

For the first trial, an intensive sheep farm with 120 ewes and five serving rams, 12 three-year-old Creole ewes and four three years old Suffolk rams were selected at random. Ewes were sexually experienced after two parturitions. Rams were sexually experienced after one mating season. The experiment was initiated on the first day of September 2020. Ewes were painted with large numbers in their flanks and housed together in a barn under natural light and temperature conditions during the transition to fall (August to October, n =12). Ewes and rams were fed with four kg of corn hay, and 300 grams of a pelleted ration in individual troughs; fresh water was continuously available for both ewes and rams. Rams were housed at least 100 meters apart from ewes to avoid biostimulation. Care was taken also to separate treated from control rams. Animal care was in compliance with the Ethical Scientific Committee for Animal Experimentation in this University Center.

For the second trial 4 rams were selected, together with 24 barren ewes with an average age of 2.5 years. Treatment for the second trial was identical as that described for the first trial.

Naloxone Treatment

For the first trial, two of the four rams, selected at random were injected im at 12 hour intervals with 0.5 mg Naloxone HCl (Nx) dissolved in saline solution. Injections were synchronized with the intravaginal insertion of progesterone impregnated sponges on the ewes used for this study. Naloxone injections in experimental rams continued for 15 days and treatment was discontinued when estrus would be present in the ewes. Control rams were treated with saline injections. Blood samples were taken every other day from all four rams to measure testosterone levels by RIA. Care was taken to separate treated from control rams for at least 100 meters, to avoid biostimulation. For sexual preference trials, one ram (treated and control) was chosen at random to be presented to the estrous ewes. Treatment and handling of rams and ewes for the second trial was identical as described for the first trial

Behavioral Tests

Ewes received an intravaginal sponge with 65 mg of Medroxi Progesterone Acetate (MAP) for 14 days, on sponge withdrawal they were treated with 250 I.U. of equine Chorionic Gonadotropin (eCG).

To test ewes ram preference we followed the method described by Ramos and Ungerfeld (2006) with some modifications. In each test, two rams, one treated and one control, were tied in a 20 X 20 m paddock at equal distances from the point of entrance of the ewes. Maximum mobility for each ram was 3 m. On the following day after sponge withdrawal (first trial); ewes were allowed to enter at the opposite end of the paddock where the chosen rams were tied. When ewes directed her attention to a ram and was mounted and mated it was withdrawn to allow the ram to mate another approaching ewe; procedure repeated twice a day at 8:00 am and 6:00 pm. Note was taken of the mounted ewe and the ram chosen for mating. This procedure was initiated the day after sponge withdrawal and continued until estrus was not evident or ewes were not interested in the rams.

Ewe sexual behavior

Estrus behavior was quantified for each female individually using proceptivity and receptivity tests previously used by Fabre-Nys and Vénier (1987).

Receptivity was measured when the females were introduced into the test paddock. Receptivity index (RI) was calculated as the percentage of immobilizations (characteristic of estrus) shown by the female in response to the courtship of the male over the total number of courtship attempts by the male. The number and latency of mounts and ejaculations, and the percentage of immobilizations in response to mounting were also recorded. A ewe was considered receptive when the RI was > 50% or highly receptive when the RI was >85% *(*Fabre-Nys and Vénier, 1989).

Hormone Measurements

Testosterone was quantified only on the first trial, using a commercial kit (Medidores Industriales, Mexico). Interassay and intraassay coefficients of variation were 9.6% and 6.5%, respectively. The lower limit of detectability, defined as the first point on the standard curve lying outside of 3 standard deviations from the counts per minute in tubes containing unlabeled hormone, was 3.2 pg/tube.

Statistical Analysis

Hormone data were analyzed by use of one-way ANOVA. Significant overall effects were noted among naloxone and control treated rams and differences among means were evaluated by the Newman-Keuls multiple range test. Behavior data were analyzed by the Wilcoxon signed rank test. Simple linear correlation coefficients were calculated to examine within-ram interrelationships between serum Testosterone concentrations. Probability values of $P < 0.05$ were considered statistically significant.

RESULTS

During the first trial it was observed that on the first exposure of ewes (first trial, day one after sponge withdrawal) to rams, estrus was not evident and ewes were interested for both rams, looks and sniffs were the main behavioral traits observed. On the second exposure to rams (second trial, day one after sponge withdrawal) looks, sniffs, tail fanning and non firm standing was observed in all ewes with a particular interest for the naloxone treated ram. (Table 1).

On the following day (3rd Trial), after two days of sponge withdrawal, 11 ewes grouped around the naloxone treated ram and showed high degree of receptivity with behavioral traits such as head turning, soliciting and firm standing when the ram mounted and ejaculated. Proceptivity and receptivity for the control ram was observed in one ewe. After ewes were mounted and ejaculated were withdrawn from the test field to avoid interference with receptive ewes. Once all ewes were mounted they were returned to their paddock. (Table 1).

On the afternoon trial of day two after sponge withdrawal, the same pattern of selectivity was observed in the same ewes of the first trial of this day, and they grouped preferably with the naloxone treated ram, and the same ewe that was mounted by the control ram. (Table 1).

On the third day after sponge withdrawal, during the morning testing trial, only 7/11 ewes were mounted and copulated by the naloxone treated ram. No ewe showed interest for the control ram. (Table 1).

On the afternoon trial of the third day after sponge withdrawal, no proceptive or receptive behavior was observed in all ewes (trial 6). (Table 1).

Testosterone plasma levels in Nx treated and control rams were similar during the first week of treatment. At the beginning of the second week, plasma testosterone levels in Nx treated rams increased steadily to reach a maximum on the second week of treatment (Figure 1). Testosterone levels in rams treated during November 2010 are similar to the ones treated in September 2010 and are not displayed.

Testosterone levels of Nx treated rams during the first trial (September 2010) remained above control rams levels when the experiment was terminated.

When the experiment was repeated on November 2021 using 4 rams and 24 ewes, it was observed that on the first day (first trial, day one after sponge withdrawal) there was no significant signs of sexual behavior.

On the second exposure to rams (second trial, day one after sponge withdrawal) (November 2021) behavior of ewes and rams was similar to that observed during the September 2021 trial. Looks, sniffs, tail fanning and non firm standing was observed in all ewes and similarly, ewes were particularly interested for the naloxone treated rams. (Table 2).

After two days of sponge withdrawal, 19 ewes grouped around the naloxone treated ram and showed high degree of receptivity with behavioral traits such as those observed on the experiment of September 2010. Ewe's behavior showed head turning, soliciting and firm standing when the ram mounted and ejaculated. Proceptivity and receptivity for the control ram was observed in five ewes. (Table 2)

DISCUSSION

Previous findings using low doses of naloxone in bucks were corroborated (Fuentes et al., 1998), testosterone plasma concentration did not change during the first week of treatment with low doses of naloxone, but at the beginning of the second week of treatment plasma testosterone levels increased steadily to reach a maximum of 15 ng/mL as compared with plasma testosterone levels of 6 ng/mL observed in control rams, for the duration of the experiment. Furthermore, testosterone levels in Nx treated rams, remained above control levels several days after treatment

was terminated. The latter observation gives way to postulate that naloxone is exerting a durable effect when administered in low doses. Effect that shows that naloxone is producing a modification on the GS coupling mechanism of the μ receptor (Wang and Burns, 2009) thus explaining the effect of the opioid antagonist when administered in low doses. Observations that demonstrate that naloxone can have opposite effects depending on the dose used, therefore it might function as a partial agonist when administered at high doses (1mg/kg)

The time of exposure to estrous ewes was coincident with high testosterone plasma concentrations in the naloxone treated rams, this would explain why ewes were attracted to the Naloxone treated rams, observation that is in agreement with Roselli et al. (2002) and Resko et al. (1996). It is possible to postulate that the high concentration of testosterone in the naloxone treated rams increased male secretions (pheromones?) to attract female ewes, because ewes follow olfactory cues to choose their preferred mate (Gelez and Fabre Niz, 2006). There is evidence that EOP also interact with the expression of sexual behavior and regulation of the hypothalamic pituitary gonadal axis (Tenhola wt al., 2012).

There is one observation that must be discussed and this is the dose of the opioid antagonist naloxone. In this work and in previous reports, single doses of 0.5 mg of naloxone were used, while the dose used in many reports is 1 mg/kg. This latter dose follows research of the 60s and 80s, when using LH for a response parameter, there are reports of fatalities both in human and animals after the administration of 1 mg/kg, experimental animals showed signs of discomfort including death after the administration of opioid antagonists (Andre, 1980; Cohen et al., 1983; Yang et al., 1988; Kamerling et al., 1990; Sathe et al., 2001). With doses of 1 mg/kg, naloxone also binds to δ and κ opioid receptors. This probably explains why in some reports using greater doses of naloxone, no significant changes in both behavior and hormone concentrations were observed (Ebling and Lincoln, 1985).

Proceptive and receptive behavior of ewes used in this experiment is similar to other reports (Tilbrook et al. 1990). Age and sexual experience in this work is not a factor to consider (Ramos and Ungerfeld, 2008), care was taken to choose ewes of the same age and sexual experience. It is possible to argue that in this experiment further expression of ewe behavior was not permitted because once mated the ewe was withdrawn with no further contact with rams.

CONCLUSIONS

It was concluded that the administration of intermittent low doses of the opioid antagonist naloxone facilitated the secretion of testosterone in the treated rams, effect that enhanced sexual preference of ewes induced to estrus. This observation give further evidence of the importance of endogenous opioids as regulators of sexual behavior and hormone secretions. The use of small intermittent doses of the opioid antagonist naloxone should be subjected to further research.

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Table 1.- Proceptive, receptive behavior and sexual preference of creole ewes for rams treated with naloxone (Nx) on September 2010

	PROCEPTIVE BEHAVIOR		RECEPTIVE BEHAVIOR		
	Ram + Nx	Ram + Saline	Ram + Nx		Ram + Saline
Trial 1	12/12	12/12			
Trial 2	10/12	02/12	03/12		
Trial 3	11/12	01/12	11/12	(P<0.001)	01/12
Trial 4	11/12	01/12	11/12	(P<0.001)	01/12
Trial 5	07/12	00/12	07/12	(P<0.001)	00/12
Trial 6	00/12	00/12	00/12		00/12

For time of Trials see text.

Table 2.- Proceptive, receptive behavior and sexual preference of creole ewes for rams treated with naloxone (Nx) on November 2010

	PROCEPTIVE BEHAVIOR		RECEPTIVE BEHAVIOR		
	Ram + Nx	Ram + Saline	Ram + Nx		Ram + Saline
Trial 1	24/24	24/24			
Trial 2	18/24	06/24	06/12		
Trial 3	19/24	05/24	19/24	(P<0.001)	05/24
Trial 4	21/24	03/24	18/24	(P<0.001)	06/24
Trial 5	18/24	00/24	019/24	(P<0.001)	00/24
Trial 6	00/24	00/24	00/24		00/24

For time of Trials see text.

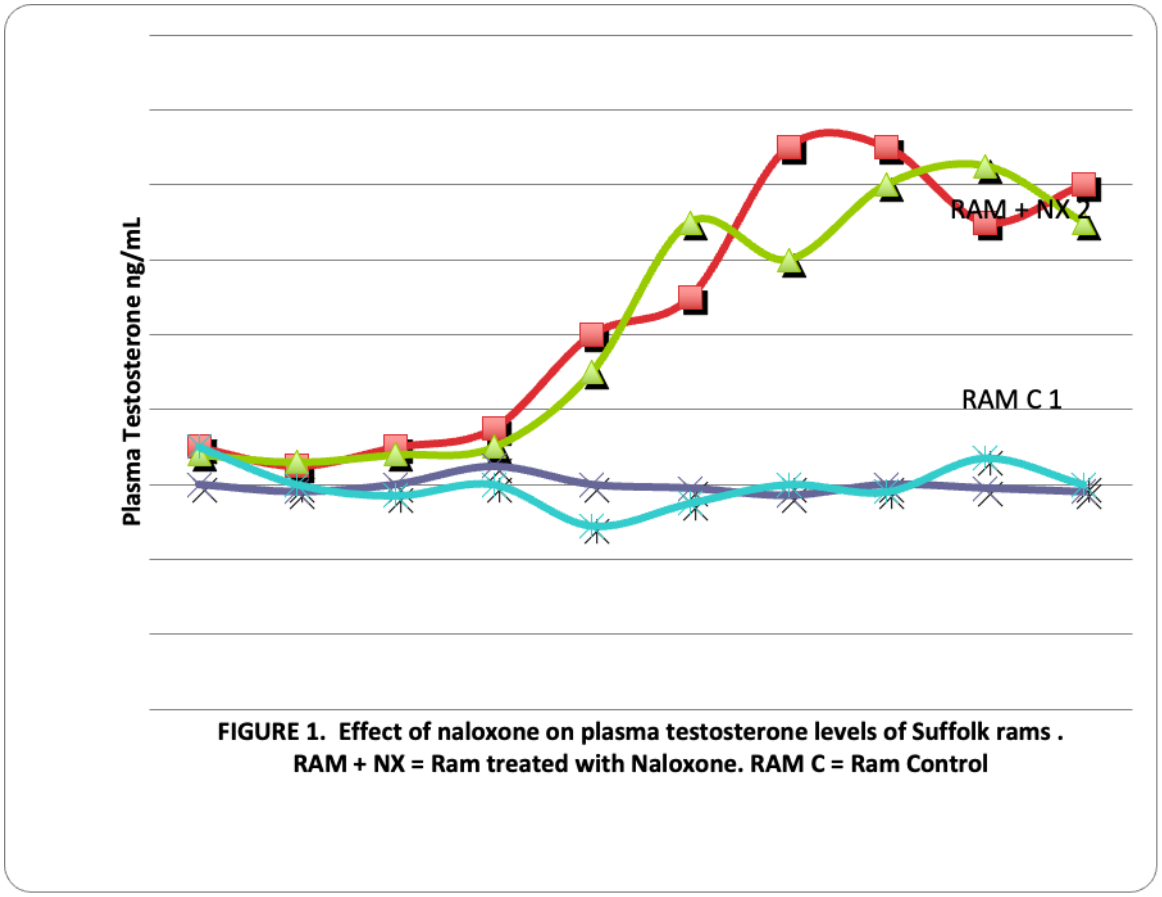

FIGURE 1. Effect of naloxone on plasma testosterone levels of Suffolk rams . RAM + NX = Ram treated with Naloxone. RAM C = Ram Control