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Mating behaviour and male refusal during early pregnancy in female alpacas

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CONTENTS

ABSTRACT

With this project we want to study the relation between the "spitting" behavior and the pregnancy status of 6 alpaca females and subsequently to see if there is a possible the correlation among plasma progesterone levels and the previous mentioned spitting behavior.

Based on bibliographic researches, high progesterone levels are a good way to ascertain the pregnancy status of the female and so she should refuse the male presence by spitting at him and trying to escape.

We also tried to find a sequence of actions that could predict the spitting behavior performed by the female towards the male.

The 6 females studied in this project were divided in two groups because the stallions used were just 3 and each of them was presented to the corresponding female 4 times: mating day (T0), first behavioral pregnancy test (T7), second behavioral pregnancy test (T12) and third behavioral pregnancy test (T15). The encounters were filmed with a camera and subsequently the duration of the chosen behaviors was calculated with the exception of kicking and spitting because, being events, we looked at their occurrence and frequency. The day before each presentation, some blood was collected from the female so to have a trend of how the plasma progesterone levels were changing before and during the first days of pregnancy to then compare the hormonal results with the behavioral ones.

The results obtained during this study showed some individual components that did not allowed us to confirm the reliability of the spitting behavior as an indirect method to assess pregnancy in female alpacas, at least during the first 15 days of pregnancy.

1. INTRODUCTION

1.1 GENERAL INFORMATION

The alpaca, *Vicuña pacos*, is an ungulate of the Southern America (Andes Mountain) and is defined as a pseudo-ruminant because it possesses three stomachs. Alpacas belong to the family Camelidae, suborder Tylopoda (they walk on pads), class Mammalia and they have been tamed for the first time by the Inca population more than 6000 years ago (Wheeler, 2012).

Alpacas are only found in captive environments and descend from their wild ancestor Vicuña which is now a rare and protected species; llamas instead are bred from the wild Guanaco which is still found throughout South America (Wheeler, 2012).

Alpacas are frequently confused with the llamas; however, there are two main ways to distinguish these animals: alpacas are smaller than llamas and are mainly kept for their fiber, or *fleece* and then they can also be recognized by looking at the ears: alpacas have straight ears, while llamas' ears are banana-shaped (https://www.greenstyle.it/differenza-lama-alpaca-374631.html).

There are two breeds of alpaca: Suri alpaca and Huacaya alpaca, where the only difference stays on the fleece: Huacaya alpacas have thicker fleece that adapts well to the high altitudes of the Andes and confer them the "poofy" aspect; Suri alpaca's fleece descends towards the lower part of the animal's body like long hair. The first ones need to be sheared every year; the others need time to grow their fleece so they are sheared every two years (Wheeler, 2012).

Although the general and social behavior of alpacas has not been much investigated yet, their vocalizations are well known and are: "*humming*" used to communicate among them; "*orgling*" emitted by the male throughout all the coitus period and it is believed to also help the female to ovulate (Alpacainfo, 2022); "*alarming*" to group up the herd in a defense position and to warn possible dangers; "*clucking*" made by the mothers by clicking the tongue as affection sign towards their crias (Società Italiana Alpaca, 2022).

1.2 ALPACAS REPRODUCTIVE PHYSIOLOGY

Alpaca females reach sexual maturity at 14-18 months of age but usually, because of their small size and the potential for dystocia (E. Wiedner, 2022), they are not allowed to reproduce until they reach 45-50kg; males instead reach sexual maturity from 12 months to 3-5 years of age.

The gestation lasts 11.5 months (+/- 2 weeks), where the 80% of the fetal growth occurs in the last trimester of pregnancy and they give birth to one offspring which is called "cria"; twin births are rare and often results in problems for both the mother and the offspring. Crias are weaned around 6-

7 months depending on the dimensions, maturity of the baby and the health of the mother. After 15-20 days from the delivery the female is ready to be impregnated again (Società Italiana Alpaca, 2022), this condition is known as "post-partum estrus".

In their natural habitat alpacas bred form December to March for food availability and temperature reasons (Sumar, 1996), however they could reproduce throughout all the year since both sexes are constantly fertile and females are induced ovulators, therefore ovulation is induced by mating thanks to the presence of an ovulation induction factor (OIF) in the male's semen (E. Wiedner, 2022) and the rupture of the follicle occurs spontaneously approximately 26h after coital stimulation (San-martin et al., 1967). From 2-3 days after the ovulation, a functional corpus luteum is present; the fertilized oocyte is usually found in the uterus by day 7 after mating and the implantation occurs around the 30th day of gestation (E. Wiedner, 2022).

Alpacas have repetitive cycles (waves) of ovarian follicular growth and regression without ovulation in absence of copulation. In fertile, non-mated females, during each follicular wave there is the recruitment of a follicles' cohort, followed by the synchronous emergence of a group of antral follicles; one of these follicles is selected to become the dominant one, while the others undergo atresia and regress (J. Vaughan, 2004).

The dominant follicle exerts a negative feedback on the rest of the follicles in both ovaries by regulating their number and diameter thanks to the reduction in FSH release because of high estradiol plasma concentrations, locally produced substances such as inhibin and androgens. Before the final regression of the dominant follicle, there is the emergence and growth of a new follicle resulting in the coexistence of regressing and growing follicles over several days and this phenomenon is known as *"overlapping follicular waves".* These overlapping follicular waves maintains blood estradiol concentrations at a sufficient level to maintain sexual receptivity; if asynchronous waves occur, estradiol concentration may drop and cause sexual receptivity to decline leading the females to be indifferent to the male (J. Vaughan, 2004).

Ovulation occurs 1 to 2 days after mating, but plasma progesterone levels do not significantly rise until 4 days after copulation and the peak occurs 7 days from the moment of mating, while it decreases rapidly in nonpregnant females. During the first 3-4 days after mating, when the corpus luteum is growing and progesterone levels are still low, some females may appear receptive to the male.

After a successful mating there is the formation of a corpus luteum, which remains functional throughout the entire gestational period and it is responsible for the production of progesterone necessary for the maintenance of pregnancy. In the alpacas the CL reaches the maximum diameter (14mm) on the 8th/9th day post copulation; in absence of pregnancy, it starts decreasing from 12 days after copulation and the complete regression happens on the 18th day.

The concentration of progesterone follows a similar pathway reaching the apex in the 8th day and declining until the 18th day after copulation. In pregnant females happens something alike until the 8th day post copulation, it starts then declining close to the 13th day and then the values increase again between the 18th and 23rd day (Baca et al., 1970a; Aba et al., 1995).

Almost all alpaca fetuses are found in the left uterine horn (based on the conceptus site and site of umbilical attachment), even though ovulation occurs from both ovaries with equal frequency (Sumar, 1996). Thus, the embryos that originate in the right side, migrate to the left horn for the attachment, but the reason for this migration is still not well known. One explanation could be the differential luteolytic effect between the left and right uterine horns: the right one effect luteolysis via a local pathway, whereas the left one effects luteolysis through both systemic and local pathways (Baca et al., 1979).

1.2.1 FEMALE REPRODUCTIVE BEHAVIOR

Non-pregnant females appear receptive to males on most occasions as plasma progesterone levels remain low (Baca, 1993), but they become non-receptive in the presence of a corpus luteum and circulating plasma progesterone levels (Pollard et al., 1994) greater than 1–2 ng/mL (3.2–6.4 nmol/L) (Sumar et al., 1988; Aba et al., 1995). When females are pregnant, they strongly reject the male if placed in a yard together and may run away or spit, kick and/or scream (Pollard et al., 1994).

"Spitting-off" is the most used indirect method to diagnose pregnancy in alpaca females and it is under the influence of the high levels of hormone progesterone that are produced during pregnancy and make the female unreceptive to the male. Three positive "spit-offs" a week apart, beginning a week after the mating, are a good sign that the mating has held. The theory is that the first spit is a sign that the female has ovulated, the second that she has conceived and the third that she held.

After 30 days is useful to perform an ultrasound scanning of the abdomen to help assess whether the female is pregnant or not, but it has to be kept in mind that up until 60 days of pregnancy there is a high re-absorption rate in this species (10-20%) so late checking is recommended (S. Greenhalgh et. al., 2010).

1.2.2 PREGNANCY TESTS

There are different means through which we can confirm or deny pregnancy in alpacas and llamas. We can have indirect methods such as the use of a "teaser" male to assess sexual receptivity, or measurement of progesterone in milk and blood; or we can use direct methods which involve

palpation, ballottement, and ultrasonography of the conceptus and associated fluid (P. Adams et. al., 2016).

1.2.3 PREGNANCY LOSS

Pregnancy loss is a common complaint in camelids and could be divided based on the period of the lost: early embryonic death (before 40 days), early fetal loss (between 40 and 120 days) and late fetal loss (120 days to term). The best evidence of pregnancy loss is the presence of abnormal discharge from the vagina or the expulsion of the fetus and the fetal membranes; during pregnancy examination, evidences of disrupted fetal membranes, fetal heart rate or abnormal ultrasound appearance of uterine content may suggest a non-viable pregnancy (issuu.com/camelidconnections/docs/camelid_connections_issue_11_web/s/10264469).

It is useful to consider sexual behavior together with systemic concentrations of progesterone as indicators of pregnancy status because there is a relationship between the two, that is, females under high levels of progesterone are non-receptive to male sexual advances.

1.3 OBJECTIVES

We decided to focus on reproductive behavior of alpaca females because this field is still poorly studied; we also took advantage of the availability of the females' blood samples to better understand the correlation between progesterone plasma levels and the rejection of the male.

The specific behavior that will be analyzed is called "spitting" which is an easy indirect method used to evaluate pregnancy status.

From a behavioral point of view, we expect that at the presentation of the male to the female after 7, 12, and 15 days from the day of mating (T0), non-receptive females will spit to the male and try to escape; while from a physiological point of view, we expect that the progesterone levels of pregnant females will be greater than 1-2ng/mL (Sumar et al., 1988; Aba et al., 1995).

2. ANIMALS, MATERIALS AND METHODS

2.1 ANIMALS

The animals that we use for this study are raised in the "A&Alpaca" farm owned by Alberto Baruffato, located in Monteviale (VI), Italy. This farm includes 23 Huacaya alpacas: 15 males, 6 females and 2 crias born at the end of February. They are used for the production of fiber and the males are involved in touristic activities because Alberto, the owner, organizes hikes during the weekends. Males and females are kept in distinct pens due to their reproductive physiology and the two crias stay together with their mothers up to 6/7 months old in the female paddock.

In this research we analyzed 6 alpaca females: Kate, Riba, Nocciolina, Princess, Pandorra and Heidi, where Heidi and Nocciolina are primiparous and Kate and Pandorra are the mothers of the two crias. The males used as stallion are three: Colin (mated with Princess and Pandorra), Chechi (mated with Riba and Kate) and Mirko (mated with Nocciolina and Heidi).

The females were divided in two groups because the mounts were done using only three males: the first group comprehends Princess, Nocciolina and Riba; while in the second group is composed by Heidi, Pandorra and Kate.

	Females	**Males**
Group 1	Nocciolina	Mirko
	Riba	Chechi
	Princess	Colin
Group 2	Heidi	Mirko
	Pandorra	Colin
	Kate	Chechi

Tab. 1: Couples used in breeding

Each female was weighted the first day of blood sampling: Riba weighed 52kg (BCS 2.5 because she just finished milking); Nocciolina weighed 49.6kg (BCS 3/3.5); Princess weighed 71.5kg (BCS 3.5); Pandorra weighed 70kg (BCS 4); Heidi weighed 53kg (BCS 3.5/4); Kate weighed 76.8kg (BCS 3/3.5).

Females	**Weight**	**BCS**
Nocciolina	49.6 kg	3/3.5
Riba	52 kg	2.5
Princess	71.5 kg	3.5
Heidi	53 kg	3.5/4
Pandorra	70 kg	4
Kate	76.8 kg	3/3.5

Tab. 2: Females' weights and Body Condition Score the day before the first presentation

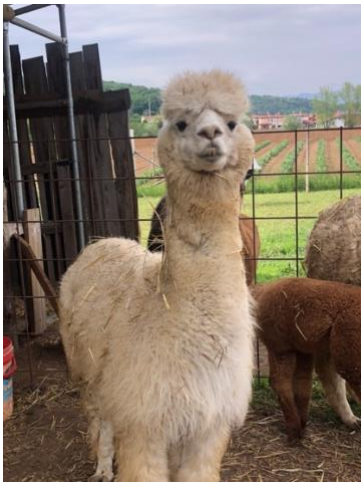


Fig. 1: Riba

Fig. 2: Nocciolina

Fig. 3: Princess

Fig .4: Pandorra

Fig. 5: Heidi

Fig. 6: Kate

2.2 EXPERIMENTAL DESIGNS AND WORKING ETHOGRAM

This study consists in analyzing the female behavior towards the male by looking at some videos taken with a GoPro4. Each video started right after the male was introduced in the female paddock, where the female was already present, and it ended when the male was caught to be moved away from the pen. For the analysis of these videos, I previously had to create a working ethogram with all the behaviors I was interested in.

The males used were three and each of them was presented to two females four different times: T0 (mating day), T7 (first behavioral pregnancy test), T12 (second behavioral confirmation of pregnancy) and T15 (last behavioral test). The first presentations happened on the 15/03/2022 (first group) and 16/03 (second group) and after 30 (14/04/2022) and 60 (19/05/2022) days from the mating day we proceeded with a trans-abdominal and trans-rectal echography with a Mindray DP-30Vet ultrasound as confirmation or not of the pregnancy.

Since Alberto, the owner, wanted all the females to be pregnant, on the same day of the second echography test (19/05/2022), the two individuals (Heidi and Nocciolina) which did not result pregnant within the 15 days settled for this study were mated with a different male respect to the initially assigned one: Colin instead of Mirko.

Every time, the day before the presentation of the male, blood samples (10ml) were collected from the females to be able to assess the plasma progesterone levels. The animals were restrained by a person while the veterinarian was collecting the blood from the jugular vein with a 10ml syringe; the blood then was transferred into an EDTA vacutainer, centrifuged at 2000r/min for 10 minutes to divide the plasma from the cellular part of the blood; the plasma was then transferred into micro centrifuge tubes with a pipette and refrigerated at -22°C.

We decided to add another day of blood sampling (T20) because following the literature, between the 18th and 23rd day the progesterone levels of pregnant females should increase again, after being decreased close to the 13th day (Baca et al., 1970a; Aba et al., 1995).

WORKING ETHOGRAM

Mating

FEMALE

- Kushing: the female lies on her belly with all four legs under the abdomen, lowering the front legs before and then the posterior ones after that the male mounts her
- Sniffing: during the coitus the female turns her head back towards the male's neck and legs and draws in air through her nose

MALE

- Mounting: the male assumes an erected position putting his weight on the hindlegs and elevating the anterior ones with the intention of putting them around the female's back to start the coitus; the ears are directed backward
- Coitus: the male is on the female's back while she is laying on the ground. The male abdomen is placed over the female back; his front legs are on each side of the female's back and are used to maintain equilibrium; the hind legs are bent under him. The male is performing an undulatory movement with the pelvis; he is emitting a guttural sound with the nostrils that open and close; his ears are pointed backward
- Investigating: at the end of the coitus the male stands up on his four legs and puts his nose near the back of the female

Non-mating

FEMALE

- Running away: the female starts moving one leg at time in a quite fast pattern to move away from the male. The neck is erected and the ears can be both erected or pointed backward
- Kicking: while running away the female quickly extends backward one or both of her hindlegs, with the intention of pushing away the male
- Spitting: the female chews a small quantity of bolus, without having eaten before, with a lateral movement of the jaw; she raises her head, while lowering the ears and ejects a small amount of bolus, saliva and air from the oral duct

MALE

- Chasing: while the female is running away the male follows her from behind with the intention of mounting the female. His ears are pointed backwards and he is emitting a guttural sound through the nostrils

Other

- Ears backward: when the female and/or the male rotates the ears in order to have the tip that points towards the tail and the head gets an overall elongated appearance
- Out of sight: when the animal is completely not visible because it's hidden by the other individual, it's behind the structure within the paddock, it's out of the camera visual field or when we cannot establish whether the ears are pointed backwards or not

Tab. 3: Working ethogram listing the behaviors looked at in the videos.

Fig. 7: Princess on the right and Colin on the left. Behavioral pregnancy test on T15 where spitting-off behavior is visible.

2.3 CONTINUOUS FOCAL SAMPLING

After the preliminary observation, the individuals' behavior was recorded using a continuous focal sampling method. Duration of behaviors were considered with the exception of kicking and spitting in which, being events, we considered the number of occurrences of the behaviors and their frequency.

The videos in which the female showed to be receptive to the male lasted 60 minutes (± 10 min), but only the first half hour (± 5 min) was analyzed because we saw that after that period of time, they stopped interacting with each other and spent the time grazing by their own in the pen. The videos where the female rejected the male lasted 5 minutes (± 2 min) and they were entirely analyzed.

2.4 PROGESTERONE ANALYSIS

Progesterone (P4) was extracted from serum by petroleum ether. Serum samples were thawed and 0.2 mL P4 were placed in Pyrex extraction tubes with 8 mL of solvent and mixed vigorously for 10 min at room temperature. Tubes were centrifuged (2500 rpm, 4°C) for 10 min and the aqueous phase was frozen at -20°C for 1 h. The organic phase was transferred to fresh tubes and evaporated under nitrogen current. The dry extracts were carefully dissolved in a 0.2 mL RIA buffer.

Plasma P4 concentrations were measured by the specific microtitre radioimmunoassay described by Battocchio et al. (1999). Samples were analyzed in duplicate, the sensitivity of the method defined as the dose of hormone measured at 90% binding (B/Bo) was 3.1 pg/well.

2.5 STATISTICAL ANALYSIS

Descriptive analyses of all results were done. Correlations were investigated between P4 levels and behavioral data by means of a Pearson correlation test (for ear backwards duration) and Spearman correlation tests (all other behaviors).

A strong negative correlation (Spearman Correlation Coefficient = -0.81; $p<0.001$) was found between P4 levels and kushing duration, and a weaker, still negative, one between P4 levels and sniffing duration (Spearman Correlation Coefficient = -0.59; $p=0.002$). A positive correlation was found between P4 levels and running away duration (Spearman Correlation Coefficient = 0.74; $p<0.001$).

3. RESULTS

3.1 BEHAVIORAL DATA

Name	Day 0 (T0)	Day 7 (T7)	Day 12 (T12)	Day 15 (T15)
Heidi	Lays down	Lays down	Lays down	Runs away, spits, lays down
Kate	Lays down but spits first	Lays down	Lays down but spits first	Runs away and spits
Nocciolina	Lays down	Runs away and spits	Lays down, but first kicks and runs away	The first 3 minutes she runs away and spits, then she lays down
Pandorra	Lays down	Runs away and spits	Runs away, kicks and spits	Runs away and kicks
Princess	Lays down but also spits and kicks	Runs away and spits	Runs away	Runs away and spits
Riba	Lays down but firstly spits	Runs away and spits	Runs away and spits	Runs away

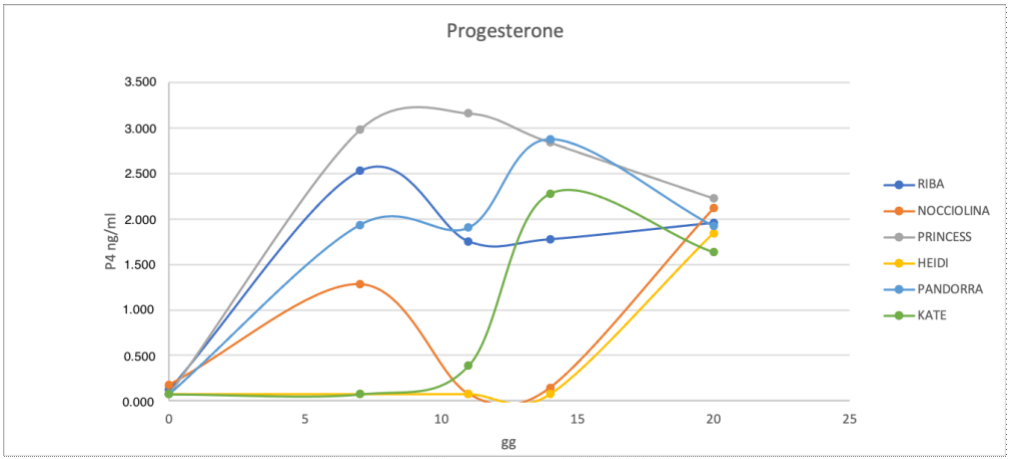
Tab. 4: Female response to male presentation.

The detailed results of the behavioral observations by animal within sex and by day of observation are shown in Appendix A.

3.2PROGESTERONE LEVELS AND ECOSCAN

	Day 0	Day 6	Day 11	Day 14	Day 20	Eco day 30	Eco day 60
Riba	0.125	2.525	1.754	1.777	1.958	YES	YES
Nocciolina	0.177	1.283	0.075	0.146	2.116	NO	NO
Princess	0.075	2.976	3.159	2.839	2.226	NA	NA
Heidi	0.075	0.075	0.075	0.075	1.842	NO	NA
Pandorra	0.075	1.931	1.902	2.874	1.918	NO	YES
Kate	0.075	0.075	0.387	2.276	1.632	NA	YES

Tab. 13: Progesterone levels results in ng/mL. Progesterone concentrations below 0.075ng/mL were not possible to measure because they were lower than the method sensitivity (0.075ng/mL).
Pregnancy results 30 and 60 days after the first mount: YES (pregnant), NO (not pregnant), NA (not available)



Tab. 14: Graph of progesterone levels trends

By analyzing female behavior while being offered to the male, all females showed receptivity towards the males at day 0 performing the kushing behavior for a mean of 23.15 minutes (Appendix A, Tab. 5); spitting events also occurred in three cases with a total of 5 spits (Riba, Princess and Kate), as well as a kicking event (Princess) (Appendix A, Tab. 5). Princess and Heidi also ran away for a mean of 0.14 seconds (Appendix A, Tab.5)

Plasma progesterone analysis from blood collected at T6 indicate a sensible variation (rising) for 4 of the females: Riba, Nocciolina, Princess e Pandorra (Tab. 13); while the other 2 females (Heidi and Kate) progesterone levels did not show any change from the first sampling (Tab.13).

At the first behavioral pregnancy test (T7) only 2 of the females, Heidi and Kate, showed receptivity for mating performing the kushing for a mean of 23.21 minutes (Appendix A, Tab. 7). Spits events were recorded for a total of 18 times from 4 females: Nocciolina, Riba, Princess and Pandorra (Appendix A, Tab.7) and the same females ran away from the male for a mean of 2.04 minutes (Appendix A, Tab.7).

Progesterone levels recorded at T11 changed in 5 out of 6 females: 3 of them (Riba, Nocciolina, Pandorra) showed a decreased in hormonal concentration, while the other 2 (Princess and Kate) had an increase in the P4 trend (Tab.13).

At the second behavioral pregnancy test (T12) Heidi and Nocciolina showed a receptive behavior performing the kushing for a mean of just 7.49 minutes (Appendix A, Tab. 9). Kicking and spitting events occurred during this presentation with and occurrence of 4 and 12 times respectively; Nocciolina as well kicked twice before kushing (Appendix A, Tab. 9). Running away behavior was performed for a mean of 1.06 minutes by 4 out of 6 females (Nocciolina, Riba, Princess and Pandorra) (Appendix A, Tab. 9).

At T14 progesterone levels changed in 5 out of 6 females: in 4 of them (Riba, Nocciolina, Pandorra and Kate) increased, while in the other one (Princess) decreased (Tab. 13).

At the end, at the last behavioral pregnancy test (T15) kushing behavior was performed for a mean of 6.29 minutes by Nocciolina and Heidi (Appendix A, Tab. 11). Kicking and spitting occurred in five cases with a total of 3 kicks (Pandorra) and 9 spits (Nocciolina, Princess, Heidi and Kate); 5 out of 6 females (Nocciolina, Riba, Princess, Pandorra and Kate) ran away from the male for a mean of 0.29 seconds (Appendix A, Tab. 11).

On the last day of progesterone sampling (T20) all females showed a change in their hormonal concentration: in 3 of them (Riba, Nocciolina, Heidi) P4 increased, while in the remaining 3 (Princess, Pandorra and Kate) it decreased (Tab. 13).

On the first day of echography test (Eco day 30) only in one female was confirmed to be pregnant (Riba), 3 of them seemed to be still empty (Nocciolina, Heidi and Pandorra) and for 2 of them (Princess and Kate) no results were available (Tab. 13).

On the second day of echography test (Eco day 60) 3 females were certified to be pregnant (Riba, Pandorra and Kate), one female resulted empty (Nocciolina) and for 2 females (Princess and Heidi) no results were available (Tab. 13).

4. DISCUSSION

We found different correlations with the sampled progesterone levels, where the strongest one is the negative relationship among the hormonal levels and the kushing behavior: high P4 levels are linked to the absence of kushing time showing that the female is pregnant. Another negative correlation is found between P4 levels and sniffing behavior which is done only during the mating: the female, while in kushing position, turns her head back and sniffs the male's neck and legs, but being the progesterone levels high the female does not kush and so the sniffing behavior is not performed. There are then two positive correlations between the hormonal levels and two behaviors analyzed in this study: high P4 levels go along with the "running away" behavior which is one of the ways in which the female refuses the male indicating that the she is pregnant; secondly, high mean progesterone levels coincide with a high frequency in spitting behavior (Tab. 15).

			P4 (ng/ml)		Spitting frequency (% time)		Kicking frequency (% time)		Individual behavioral response (N)		
Diagnosis	**N**	**Day**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Spitting**	**Kicking**	**Spit & Kick**
Pregnant	4	0	0.09	0.03	0.036	0.028	0.007	0.014	3	1	3
		7	1.88	1.28	0.863	0.787	0.000	0.000	3	0	3
		12	1.80	1.13	0.368	0.457	0.172	0.345	3	1	3
		15	2.44	0.52	0.267	0.365	0.273	0.545	2	1	3
Not Pregnant	2	0	0.13	0.07	0.000	0.000	0.000	0.000	0	0	0
		7	0.68	0.85	0.495	0.699	0.000	0.000	1	0	1
		12	0.08	0.00	0.000	0.000	0.029	0.041	0	1	1
		15	0.11	0.05	0.531	0.711	0.000	0.000	2	0	2

Tab. 15: Daily statistical parameters of female pregnancy status

As we can see from Tab.13, on the second and last echography test 3 females were confirmed to be pregnant (Riba, Pandorra and Kate), one resulted to be empty (Nocciolina) and for 2 females (Princess and Heidi) the diagnosis was not available.

The details of the results obtained will be discussed accordingly to the different cases that have been detected during the revision of the behavioral and hormonal analysis.

4.1 PRINCESS

Before beginning with the mounts, Alberto noticed that Princess was mounting her daughter (Nocciolina) and this behavior was manifested also the previous years but it stopped once she got pregnant. This time as well, she stopped this behavior after the first mount, so we assumed that she became pregnant after the first mount. Comparing the results of the videos (Tab. 4) and those of the

progesterone levels (Tab. 13) we can surely assume that the mounting behavior performed on her daughter is manifested when this female is empty and stops once she is pregnant.
By literature, alpaca females mount other females for two main reasons: sexual arousal and establishment of dominance (G. Gaines 2021). By looking at Princess behavior this explanation could work with both reasons since she was mounting her daughter (dominance) and she was performing it while she was still empty and ready to be mated (sexual arousal).
On both echography day tests (Tab. 13) we were not able to get the results for Princess because she was too nervous to stay still and she was continuously spitting, but this behavior might be indicator of positive pregnancy status of this female.

4.2 KATE

Based on what Alberto noticed the previous years, Kate shows a receptive behavior every time a male is presented to her and this situation happened also during this experience. From what we can see, analyzing the behavior together with the progesterone levels, she behaved accordingly to what expected from literature (Pollard et al., 1994): on the 11th day her progesterone levels were increasing (0.387 ng/mL) (Tab. 13) and at the presentation of the male she spit but laid down (Tab. 4); while at the following presentation (T15) she showed a rejecting behavior towards the male (Appendix A, Tab. 11) and it is possible to see that her progesterone level of 2.276 ng/mL (Tab. 13) confirmed this situation.
This could be explained by the fact that the copulation did not work the first two times (while Princess, the other female that was mated with the same male, became pregnant on the first try) because of an "ovulation failure" which is primarily caused by the bad timing of breeding in relationship to the follicular development (S. Rodriguez et. al., 2017).
Another explanation that could be taken into consideration while talking about this condition is a "continuous receptivity" by the female, which is a lack of period of rejection following mating with a proven male. In some instances, females may show a receptive or submissive behavior despite the presence of a corpus luteum or even of a pregnancy (S. Rodriguez et. al., 2017). This is a highly improbable situation, since her P4 levels and behavioral tests agree with each other, but it is an eventuality that, in the light of the actual knowledge about these animals, could not be completely excluded.
During the first echography test (Day 30) no results were available for Kate (Tab.13) because she sat during the visit, not allowing the veterinarian to use the ultrasound; while during the second

pregnancy test (Day 60), through the use of the trans-rectal ultrasound, the corpus luteum was found, confirming the pregnancy status of this female.

4.3 RIBA AND PANDORRA

Analyzing the results obtained by the progesterone (Tab. 13), we can say that Riba and Pandorra were already pregnant after the first mount and this agree with the behavior observed during the analysis of the videos since both of them laid down only at the first mounting day (T0) and by T7 (first behavioral pregnancy test) they rejected the male running away, kicking and spitting towards him (Tab. 4).

Riba was confirmed to be pregnant already at the first echography day test (Day 30); while Pandorra had a negative result at the first echography test, but she was confirmed to be pregnant during the second examination at Day 60 (Tab.13).

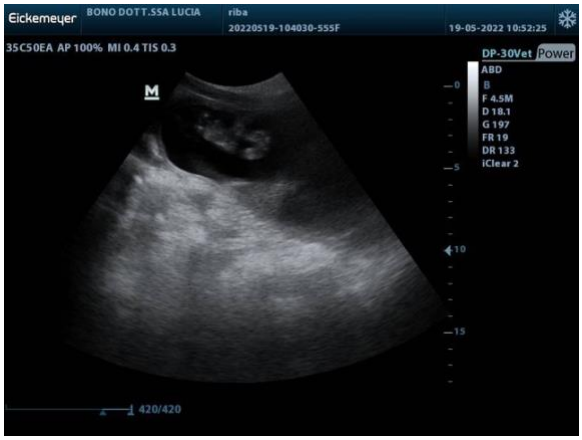


Fig. 8: Riba's trans-abdominal ultrasound 60 days after mating where the fetus is visible

4.4 NOCCIOLINA AND HEIDI

On T7 (first behavioral pregnancy test) Nocciolina looks like she ovulated by looking at both the behavioral and the progesterone results (Tab. 4 and Appendix A, Tab 7), but then for some reason she did not hold or could be that the embryo was reabsorbed: she rejected the male running away from him and the hormonal level was 1.283 ng/mL (Tab. 13), which by literature is sufficient to confirm pregnancy (Sumar et al., 1988; Aba et al., 1995).

An opposite situation appears instead from the results obtained on the 12th day of presentation: her P4 concentration fell below 0.075 ng/mL (minimum detectable P4 concentration due to method sensitivity) (Tab.13) and she showed receptivity to the male, after kicking and running away (Appendix A, Tab. 9) revealing that she is not pregnant. The same situation is visible by the comparison of the results on the 15th day which show the same contradictions: Nocciolina, soon after

the introduction of the male in the paddock, starts running and spitting at him but at the end she lays down (Tab. 4), even though the low progesterone (Tab. 13) does not explain the initial rejecting behavior.

Lastly, her progesterone concentration at D20 increased again to the level of 2.116 ng/mL (Tab. 13) which is compatible with an ovulation occurred after the last behavioral pregnancy test (T15). This late impregnation could be the cause of the negative result obtained during the first echography test (which in her case would not be after 30 days from the mount, but just 16 days from the possible ovulation). If she would have held the embryo, during the second echography (D46 instead of D60, calculating the late ovulation) a corpus luteum should have been detectable, but this is not the case since also at the second ultrasound examination she resulted to be empty (Tab. 13).

Her controversial behavior could be affected by the P4 which influenced the hypothalamus for a very short period and even after the regression of the corpus luteum the neuroendocrinal message could have been misinterpreted leading to these mixed behavioral signals (W. Brown, 1999).

Since Nocciolina was not able to become pregnant during the established period for this study and the owner wanted to impregnate all the individuals, on the same day of the second echography test Nocciolina was mated with a male (Colin) different from the initially assigned one (Mirko) from which it resulted a successful conception.

As well as Nocciolina, also Heidi showed some difficulties in becoming pregnant and some controversial behaviors: on the 15th day she rejected the male by spitting at him to then kush down for 0.27 seconds (Appendix A, Tab. 11), even if her progesterone levels were too low for her to show the initial non-receptive response (Tab. 13).

As happened with Nocciolina, Heidi's progesterone levels increased at D20 reaching a concentration of 1.842 ng/mL (Tab.13) which by literature is sufficient to confirm pregnancy (Sumar et al., 1988; Aba et al., 1995), signaling a possible ovulation after the last behavioral pregnancy test (which for her will count as first mating day) and potentially bringing out the reason why at the first echography test she got a negative result (Tab. 13).

An opposite situation happened instead during the second pregnancy test at Day 60 because she was too nervous to stay still and it has not been possible performing the examination (Tab. 13). Since Heidi is usually a very calm and cooperative individual, her behavior during this second pregnancy test was very strange, so we tried to present her a male to double-check through the behavioral pregnancy test and, in the light of the fact that she rejected him by spitting and running away (no official data were recorded), a positive pregnancy status was supposed.

Trying to find a feasible explanation to the struggling encountered by these two females in being pregnant, we could talk about the "ovulation failure" which may be caused by the inadequate

luteinizing hormone (LH) release after copulation. This situation is primarily caused by the bad timing of breeding in relationship with follicular development, so breeding during the regression or before the follicle has acquired the ability to respond to LH could result in difficult impregnation (S. Rodriguez et. al., 2017).

Another aspect that could have played a role in this situation is the use of the male: both Nocciolina and Heidi were mated with the same male (Mirko) and can happen that some males with low fertility may have a lower concentration or potency of the ovulation inducing factor (OIF) which is fundamental, together with the guttural sound emitted by the male (Alpacainfo, 2022), in helping the female ovulating (S. Rodriguez et. al., 2017).

The last explanation stands in the copulation time, because has been seen that short copulation time may also be implicated in the lack of ovulation (S. Rodriguez et. al., 2017), but we do not know what is to consider “too short” because copulation generally lasts from 3 to 65 minutes, with an average time of 20 to 30 minutes (A. Aba, 2017).

4. CONCLUSION

Female alpacas are induced ovulators, therefore the ovulation is induced by mating, this implies that they are receptive to the male throughout all the year (E. Wiedener, 2022) as plasma progesterone levels remain low (Fernandez-Baca, 1993), but they become non-receptive in the presence of a corpus luteum and circulating plasma progesterone levels (Pollard et al., 1994) greater than 1–2 ng/mL (3.2–6.4 nmol/L) (Sumar et al., 1988; Aba et al., 1995).

The presence of the "spitting-off" behavior performed by the females is induced by the high levels of progesterone and has been identified as a reliable indirect method to assess the pregnancy status of these animals (S. Greenhalgh et. al., 2010).

However, the results of this study do not confirm the use of a "teaser" male to cause the spitting-off in the female as a reliable indirect method to confirm pregnancy at least in the first 15 days because, as we can see from Tab. 15, mean spitting frequency is higher than 0.00 also at T0 (when no female was pregnant) and there was no correlation among spitting behavior and P4 levels.

Following the literature, the concentration of progesterone in non-pregnant females should reach the apex in the 8th day and decline until the 18th day after copulation. In pregnant females instead, happens something alike until the 8th day post copulation, but then it starts declining close to the 13th day to lastly increase again between the 18th and 23rd day (Baca et al., 1970a; Aba et al., 1995).

The study does not allow us to confirm this statement because only one female (Riba) out of 6 had her progesterone levels following this trend (Tab. 14); Princess hormonal peak happened at the 11th day, while Pandorra peak was on the 14th day after mating; for what concerns Kate, it looks like she was pregnant during the second behavioral pregnancy test (T12) and following her P4 trend (Tab. 14) the hormonal peak happened just three days after mating, because at T20 the curve was already decreased. At the end, talking about Nocciolina and Heidi, the two females who did not get pregnant within the established 15 days for this study, we do not know how their hormonal levels developed.

Nevertheless, these results should be investigated on a larger number of animals so to have more reliable statistical analysis and the collection of these data should continue to further years to be able to create a database and to increase the knowledge about this topic, since the literature available for this field is still quite scarce.

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Appendix A: detailed behavioral observations' results

ID	Ears backwards	Kicking	Spitting	Kushing	Sniffing	Running away	Out of video
Nocciolina	13.44			16.11	2.14	0.00	0.11
Riba	25.22		2 times	29.20	0.28	0.00	5.14
Princess	28.25	1 time	1 time	18.48	0.00	0.26	0.24
Heidi	10.42			27.26	1.09	0.02	0.00
Pandorra	23.00			31.33	2.52	0.00	7.53
Kate	22.36		2 times	17.55	0.00	0.00	4.38

Tab. 5: Females results T0 in minutes and occurrence of kicking and spitting (mating day)

ID	Ears backwards	Investigating	Mounting	Coitus	Chasing	Out of video
Mirko	2.17	0.16	9.37	14.19	0.00	0.05
Chechi	29.02	0.26	0.39	28.00	0.00	0.45
Colin	20.21	0.05	2.36	18.39	0.00	1.02
Mirko	30.47	0.13	1.41	26.16	0.00	0.00
Colin	30.25	0.37	0.26	27.24	0.26	0.00
Chechi	17.33	0.14	0.03	16.48	0.00	2.29

Tab. 6: Males results T0 in minutes

ID	Ears backwards	Kicking	Spitting	Kushing	Sniffing	Running away	Out of video
Nocciolina	2.59		3 times	0.00	0.00	2.05	0.01
Riba	4.07		4 times	0.00	0.00	0.32	2.30
Princess	3.23		6 times	0.00	0.00	3.42	0.02
Heidi	0.44			31.07	0.29	0.00	1.30
Pandorra	1.02		5 times	0.00	0.00	1.59	0.02
Kate	17.27			14.56	0.00	0.00	13.36

Tab. 7: Females results T7 in minutes and occurrence of kicking and spitting (first behavioral pregnancy test)

ID	Ears backwards	Investigating	Mounting	Coitus	Chasing	Out of video
Mirko	3.02	0.00	1.15	0.00	1.41	0.00
Chechi	0.38	0.00	0.03	0.00	0.29	1.23
Colin	3.47	0.00	0.22	0.00	3.31	0.00
Mirko	32.53	0.37	1.38	27.40	0.00	0.00
Colin	3.11	0.00	0.58	0.00	1.58	0.00
Chechi	14.28	0.27	0.00	12.28	0.00	7.44

Tab. 8: Males results T7 in minutes

ID	Ears backwards	Kicking	Spitting	Kushing	Sniffing	Running away	Out of video
Nocciolina	14.34	2 times		10.21	1.20	0.41	2.19
Riba	4.03		1 time	0.00	0.00	0.19	0.00
Princess	1.40			0.00	0.00	1.20	0.21
Heidi	11.55			5.17	0.40	0.00	2.26
Pandorra	0.21	2 times	3 times	0.00	0.00	1.26	0.08
Kate	20.06		8 times	14.40	0.00	0.00	8.40

Tab. 9: Females results T12 in minutes and occurrence of kicking and spitting (second behavioral pregnancy test)

ID	Ears backwards	Investigating	Mounting	Coitus	Chasing	Out of video
Mirko	21.14	0.00	1.28	17.00	0.00	1.07
Chechi	0.15	0.00	0.00	0.00	0.16	0.02
Colin	1.33	0.00	0.00	0.00	1.19	0.00
Mirko	17.34	0.00	0.33	16.15	0.00	2.22
Colin	2.24	0.00	0.47	0.00	1.14	0.00
Chechi	14.10	0.00	0.00	13.06	0.00	2.54

Tab. 10: Males results T12 in minutes

ID	Ears backwards	Kicking	Spitting	Kushing	Sniffing	Running away	Out of video
Nocciolina	5.34		1 time	13.32	1.24	0.10	15.19
Riba	3.04			0.00	0.00	0.03	0.00
Princess	1.33		1 time	0.00	0.00	0.45	0.00
Heidi	2.34		3 times	0.27	0.02	0.00	0.00
Pandorra	0.11	3 times		0.00	0.00	1.28	0.00
Kate	4.50		4 times	0.00	0.00	0.01	0.00

Tab. 11: Females results T15 in minutes and occurrence of kicking and spitting (third behavioral pregnancy test)

ID	Ears backwards	Investigating	Mounting	Coitus	Chasing	Out of video
Mirko	23.44	0.00	5.38	15.45	0.03	1.06
Chechi	0.34	0.00	0.00	0.00	0.02	0.00
Colin	0.41	0.00	0.00	0.00	0.46	0.00
Mirko	2.36	0.00	0.42	0.15	0.00	0.00
Colin	2.11	0.00	0.41	0.00	1.20	0.00
Chechi	0.15	0.00	0.00	0.00	0.01	1.18

Tab. 12: Males results T15 in minutes