Induced stress and tactile stimulation applied to primiparous does and their consequences on maternal behavior, human-animal relationships, and future offspring´s sexual disorders

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Abstract

The aim of this study was to investigate whether tactile stimulation in rabbits during the gestation phase improve the maternal behavior and human-animal relationships as well as the effects on reproductive behavior of male kits when reached maturity compared to induced stress. A total of 33 primiparous New Zealand does were selected after pregnancy confirmation and allocated in a randomized complete block design. The treatments applied were as follows: (C) animals not stimulated during the experimental period; (TS) animals that received tactile stimulation; and (SS) does which were immobilized. The nest building behavior as well as the weight, sexual behavior, mortality, and semen analysis of the offspring was recorded. In addition, the novel object, flight distance, social isolation, and human-approach tests were conducted. Under the conditions of the present trial, TS animals showed more trust in the unfamiliar observer when compared to the other two treatments. The treatments applied to the females (TS and SS) were sufficient to confirm that the control group presented better values for the number of stillbirths and the proportion of deaths in the first week. Finally, the handling of does reduce the males' ejaculation and sperm presence but not inhibited sexual behavior or impaired semen quality. It is possible to conclude that TS did not impair does welfare or maternal behavior and it improved the human-animal relationship, however there was a negative impact on the litter. More studies that directly assess impact on the future reproductive capacity of the offspring are necessary.

Keywords: Behavior; Demasculinization; Fear level; Maternal stress.

Declarations

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Conflict of Interest Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing, we confirm that we have followed the regulations of our institutions concerning intellectual property.

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Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

Code availability

Not applicable

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Angela C. F. Oliveira, Luiza M. Bernardi, Ana Larissa B. Monteiro, Kassy G.

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Statement of Animal Ethics

This research adhered to the legal requirements of the country in which the work was carried out and all institutional guidelines. The CEUA (Ethics Committee on the Use of Animals) is intended to conduct an ethical review of any and all proposals for scientific or educational activity involving the use of live non-human animals, essentially from vertebrate groups, under the responsibility of the institution, following and promoting national and international normative guidelines for research and teaching involving such animals. This study was approved by the Ethical Committee of Animal Experimentation of Pontifícia Universidade Católica do Paraná - PUCPR, Curitiba, Brazil under protocol number 01070B.

Consent to participate

Not applicable

Consent for publication

Not applicable

1. Introduction

Rabbit breeding has undergone several changes in recent years due to the continual search for technologies that improve productivity, especially in relation to cost and animal welfare (Munari et al. 2020). Housing conditions and management normally impair animal behavior at different levels and therefore developing systems and practices that maintain a high level of animal welfare while preserving productive results is a necessity (Botelho et al. 2020). These factors combine to directly influence animal performance.

The production of kits has the largest profit margin in a rabbitry, since they will be marketed according to the production objective. Therefore, the efficiency of reproductive management is essential for successful results (Krupová et al. 2020). The females collect material (hay from their surroundings and fur from their body) to construct their nest, keeping some traits of wild rabbits, such as social and maternal behaviors (Benedek et al. 2020). When animals are deprived of these innate routines or when they undergo stressful situations in this sensitive phase (gestation), their welfare is impaired (Dalmau et al. 2020). Furthermore, behavioral indicators of acute stress caused by handling practices can directly influence food activity, social behavior, and even maternal behavior (Morisse et al. 1999; Roblero and Mariscal 2018). Similarly, handlers are of vital interest, not only from an animal welfare perspective, but also in relation to the One-Welfare concept (Leon et al. 2020). The One-Welfare concept discusses how animal welfare is interconnected with human well-being, biodiversity, and the environment, at different levels of society (Pinillo et al. 2016). A poor human-animal relationship can result in stress and injury to both animals and handlers and therefore must be improved (Wildridge et al. 2019).

Another important issue that is rarely studied in rabbitry is tactile stimulation that is described as a crucial modulator in the satisfactory development of the organism as a whole (Okabe et al. 2020). Classic studies with rats (Levine 1960) demonstrated the importance of tactile contact, where stimulated animals showed better performance in cognitive tests, earlier development of the endocrine system, more developed motor skills and more active behavior; the animals were also more explorative and more docile. Tactile stimulation also mediates a more intimate relationship with animals and has been explored in several studies on fear reduction and stress management, using different species, such as cows (Ujita et al. 2020), sheep (Nowak and Boivin 2015), lambs (Coulon et al. 2015), pigs (Wang et al. 2020), horses (Ligout et al. 2008), and chickens (Muvhali et al. 2019). However, few studies have shown the relationship between tactile stimulation during the gestation phase and its effects on the offspring (Liu et al. 2000), and there are no known reports on these effects in rabbits. Some authors suggest that stroking, grooming, brushing, and other types of tactile contact may also elicit positive emotions in farm animals (Schmied et al. 2008; Proctor and Carder 2014; Tamioso et al. 2017). Furthermore, tactile stimulation has already been linked to indirect stimulation of the central nervous system and possible influence (improvement) in neuronal plasticity (Smotherman and Robinson 1988; Jenkins et al. 1990).

In relation to the offspring, some authors indicate that during pregnancy and soon after birth, the brains of animals are more sensitive to environmental stimuli (Mabandla et al. 2007; Lupien et al. 2009). During this developmental period, phenomena, such as neurogenesis, glycogenesis, and myelination occur in different regions of the offspring's brains, and it is suggested that stressful experiences, especially during the final third of gestation, have an influence on these processes of brain development (Bánszegi et al. 2015). Over 50 years of research has shown that maternal experiences at this stage have profound and diverse consequences, affecting the behavior of the young in relation to food selection, memory, learning, cognitive ability, psychomotor development, and temperament as well as parental, aggressive, exploratory, and sexual behaviors (Brunton 2013). Studies carried out with rats exposed to stress during pregnancy concluded that the male offspring had impaired sexual behavior, with evidence of under-masculinization in adulthood (Souza et al. 2019; Hernández et al. 2020). However, in rabbits, few articles on this subject matter have been published (Brunton 2013; Bánszegi et al., 2015; Simitzis et al., 2015; Benedek et al., 2020).

The aim of this study was to investigate whether tactile stimulation in rabbits during the gestation phase improve the maternal behavior and human-animal relationships as well as the effects on reproductive behavior of male kits when reached maturity compared to induced stress. The hypothesis of the present work is that the tactile stimulation positively influences does' maternal behavior and the reproductive behavior of male kits when reached maturity.

2. METHODS

2.1 Animals and management

This study was approved by the Ethical Committee of Animal Experimentation of Pontifícia Universidade Católica do Paraná - PUCPR, Curitiba, Brazil under protocol number 01070B. The experiment was conducted at Fazenda Experimental Gralha Azul-FEGA/PUCPR in the rabbitry sector (Latitude 25° 39' 29" S; Longitude 49° 17' 17" W) during September 2016 to May 2018. A total of 33 primiparous New Zealand does were selected, weighed, and bred with 15 male rabbits, and consanguinity was avoided. The mating was carried out for three consecutive days, and the male was placed with a single female at a time to ensure that all had at least one copulation (155 ± 2 days of old). Between the 12th and 14th days of gestation, an ultrasound (*Chison Eco Vet 3*, China) exam was conducted to confirm pregnancy. During the experimental period no false pregnancy or pregnancy loss were recorded. When females did not get pregnant, they were not used in the experiment. From the pregnancy confirmation (considered D0 of experimentation), the animals were allocated in a randomized complete block design, with three treatments and eleven rabbits per treatment, with each experimental unit composed of one animal. They were given free access to commercial feed, hay, and water, and were housed individually in suspended wire cages (80 x 60 x 45 cm) with automatic water dispensers, manual feeders, and hay racks.

2.2 Treatments

The treatments groups were: (C) control group: animals did not receive stimulation; (TS) stimulated group: animals received tactile stimulation; and (SS) stress group: animals received a stress treatment. The both treatments were performed by the same student (L. M. B.), and the animals already had previous contact with the evaluator. The control group was not stimulated during the experimental period, their activities were not changed from the farm standard procedures, and they had no intimate human contact. In contrast, the other two treatments were applied daily (between 14:00 and 15:00), over three minutes, from the first day after the pregnancy confirmation (D0-ultrasound exam) until parturition, with an average of 20 ± 2 days under treatment. The TS group consisted of calmly brushing, with one movement per second, the dorsal line of the rabbit with a soft brush, with the animal inside the cage. Tactile stimulation with the brush was a hypothesis chosen by the authors based on previous studies (Schmied et al. 2008; Proctor and Carder 2014; Tamioso et al. 2017). One day before the beginning of the treatment, the brush was presented to the does for a 30minute period, giving the animals the opportunity to investigate the object. The SS group treatment was conducted outside the cage. Does were removed, placed inside a box (dimensions of 34.5 x 18 x 36 cm, with openings of 18 x 4 cm at the top and 2 x 2 cm at the side) during a period of three minutes and, every minute, the box was moved horizontally by three cm to the left and three cm to right. The dark box associated with lateral movements was chosen in order to provide a non-severe stress to the animals, principle of light/dark (LD) test applied to rodents (Arrant et al. 2014). All females received the same standard handled after born and, since they were primiparous, the manipulation occurred only at the beginning of the study, when pregnancy was confirmed (155 ± 2 days of old). The standard management consisted of manipulation for sexing and periodic weighing, monthly, to monitor weight gain.

2.3 Behavioral tests applied

Considering that validated protocols assessing rabbit welfare and behavior are scarce, and that one of the studies in this area was published recently (Botelho et al. 2020), the behavior tests applied in the present study were based on and adapted from Welfare Quality®. Appropriate behavior can be assessed by the combination of social behavior, other behaviors (consisting of animals scratching or biting the cage, or performing repetitive behaviors without an apparent objective), and human-animal relations (Welfare Quality 2009; Botelho et al. 2020). The behavior tests were carried out on the 25th day of gestation for the nest building test and five days after parturition for the novel object, flight distance, social isolation, and human-approach tests. All tests were performed in a single day for each female, consecutively, without removing the animal from the arena and keeping the order described above.

2.3.1 Nest building test

Nest-building performance is directly linked to maternal behavior. In that sense, a wooden nest (measuring 40 × 25 cm, with a frontal opening of 25 cm) was placed in the cage for the does on the 25th day of gestation, and the cages were checked daily. Two nest-building evaluations were obtained daily, until parturition: 1) nest filling percentage (hay and fur), and 2) predominance of material used (hay or fur). The primiparous does were given free access to hay that was provided in hay racks. These nest-building percentages were visually evaluated through the nest opening when the female was not inside the nest. The same trained observer (L. M. B.) conducted all nest evaluations. The observer estimated the filling percentage of hay and fur by adding all the small spots of material in a single large spot. The evaluator determined 100% of filling when the sum of hay and fur filled 100% of the wooden nest, with no visible spaces. An example of 100% filling can be seen at Figure 1.

Fig. 1 (a) photographic record in the wooden nest provided for the females during the experimental period; and (b) example of 100% filling wood nest

2.3.2 Novel-object test (NO)

The NO evaluates exploratory behavior. The observer (L. M. B.) positions himself in front of the cage, opens the cage door, and then proceeds to place a tennis ball (object never seen before by the animals) between the side of the nest and the cage door (Figure 2). The behavioral measures evaluated were in relation to the animal touching the object or not during a five-minute-observation period. The latency for the animal to touch the object (in seconds) was measured in this test.

Fig. 2 Novel-object test. In the figure, the contact between the animal and the object is observed

2.3.3 Flight distance test (FD)

This test leads to a better understanding of the human-rabbit relationship when the animal is in a familiar environment, in this case, the cage. It measures the distance an observer can approach the animal until it reacts. To apply the test, if the doe is inside the nest, the observer (L. M. B.) made noise across the wire netting, to ensure that the rabbit is awake and aware of observer presence. The observer stayed still in the aisle in front of the cage, opened the cage door, and then proceeded immediately to put their hand through the opening with the intention of touching the animal around the scapula. The test was stopped when the animal showed an escape response. When the animal presented an escape response, the test was interrupted. If this occurred when the hand was out of the cage, the maximum distance is considered 60 cm, which is the size of the cage. In contrast, if the doe does not escape and she is touched in the scapula by the assessor, the score is considered to be 0 cm.

2.3.4 Social isolation and human-approach tests

Each doe was individually subjected to the same series of three behavioral tests, within a five minutes duration (detailed below). In contrast to the FD test explained above, the human-approach tests were conducted when the animal was in an unfamiliar environment (arena test), five days after parturition, with a familiar (F - L. M. B.) and an unfamiliar (UN – A. L. B. M.) observer. Both, the F and UN observer, was veterinary student and the same experimenter for all the does. The F student was taller than the UN and both observers wore a white coat and white gloves for handling the females.

All three behavioral tests were performed sequentially in an empty open-field arena (95 cm × 95 cm), divided into nine equal 28.3 × 28.3 cm zones, and surrounded by a wall of 60 cm height. Each zone was sequentially numbered, as demonstrated in Figure 3. Zone 6 had a wall opening (50 x 32 cm), where the rabbits were introduced in the test arena. It also represents the human initial position (symbolized as "A" in Figure 3). Zone 5 represents the starting position of the rabbit (symbolized as "B" in Figure 3).

A sub-division of the zones was done in order to represent the position of the animal during the social isolation test and voluntary human-approach test (test descriptions following). All doe and human activity during the tests were video-recorded with a standard camera (HD 1080 P 16 M 16x Zoom Digital Video Camcorder Camera).

Fig. 3 Diagrammatic representation of the open-field / human-approach test arena, divided into 9 zones (28.3 x 28.3 cm). A - position of the test-people; B Starting position of the doe. Zones 3,6 and 9 (highlighted with a red color) represents the maximum animal-human approach

Social isolation-test (SIT)

The most commonly used test to assess fearfulness in rabbits is the open-field test (Buijs and Tuyttens 2015). The animal was positioned in the test arena (in zone 5), and the opening located in zone 6 was closed. The test lasted for one minute, and the animal was left alone. The movement in the zones and escape behaviors (animal stayed on its hind limbs with the forelimbs on the wall - response designed to move away from or eliminate an already present aversive stimulus) were recorded by a continuous behavioral observation method (Altmann 1974; Welfare Quality 2009; Andersson et al 2014; Botelho et al. 2020). To calculate the movement between the zones of the arena, some criteria were stipulated. For the doe to be considered as inside a numbered zone, at least one of its front legs and its head had to be located in the zone. Animals that distributed their time in a larger number of zones were considered more explorative.

Voluntary approach-test (VAT)

This test began after the social isolation test and lasted for two minutes. An observer (F/UN) was seated, positioned in the zone 6 opening, did not move, and avoided eye contact with the animal. The test was performed first with an F observer and then with a UN, in the sequence. The number of standing behaviors and movement between zones were recorded; however, for this test, the zones had two possibilities of classification. First classification was: maximum human-approach (when the rabbit was in zones 3, 6, and 9); close (when the rabbit was in zones 2, 5, and 8); and far (when the rabbit was in zones

1, 4, and 7). Second classification was: the zones were classified as perimeter (sum of time spent in zones 1, 2, 3, 4, 6, 7, 8, and 9) and central (sum of time spent in zone 5). This classification was based on Trocino et al. (2012) who affirmed that stressed animals continuously walk in circles when they are afraid of new environments. These two methodologies were also applied for the other behavioral tests.

Forced approach-test (FAT)

This test began after the voluntary approach-test, and lasted for two minutes. An observer (F/UN) looked at the rabbit and tried to touch it. The test was also performed first with an F observer and then with a UN. The number of touches achieved was recorded. We considered that an animal was touched when the examiner's hand touched the rabbit's scapula, disregarding any other locations.

2.4 Mortality and offspring weights

The period from copulation to kits' birth had an average of 31.48 ± 1.70 days. The kits from each treatment (7 ± 2 kits per litter – mean ± SD) were weighed biweekly from 15 days of age until 90 days of age. Although only males were selected to continue the study, the initial litter weighing was carried out in groups (male and female together). Such management was adopted to follow the standards of the farm. Thus, sexing was performed only after 15 days of life. When they were 30 days old, after the kits were weaned and sexed, weighing was carried out individually. In addition, data related to the offspring mortality rate (number of stillborn, and number dying in the 1st, 2nd, 3rd, 4th weeks, and post-weaning) were recorded. After weaning, the animals were kept in a group, respecting the individuals of the same litter, until males reach reproductive age (150 ± 2 days of old).

2.5 Offspring sexual behavior and semen analysis

Of the 33 mated does (11 for each treatment), with an average duration of pregnancy was 31.48 ± 1.70 days (mean ± SD), a total of 46 male were selected to sexual behavior and semen analysis. The males came from 17 different litters, with the following distribution: 7 litters for the C group, 5 litters for the TS group and 5 litters for the SS group. The final distribution of males per treatment was 23 male kits for the C group, 12 for the TS group and 11 for the SS group. In addition to losses during the postpartum weeks, 15 does had 100% stillbirths and 1 doe had a litter of females. This evaluation was carried out in three consecutive months, on the 5th, 6^{th,} and 7th months after birth, corresponding to the sexual maturity of male offspring.

Sexual behavior was assessed during semen collection using a binary questionnaire, with yes or no answers. The females were held by hand under the evaluator's arm, and an artificial vagina was used with the open end pointed in the caudal direction. As the male begins to rise, the device is positioned more posteriorly and inferiorly to allow the male rabbit to penetrate the artificial vagina (Naughton et al. 2003). At the moment when the female was introduced, the following points were recorded from the research: if the male exhibited copulatory behavior, if the male completed the copulatory behavior and ejaculated, and if there was the semen present in the ejaculate. Quantities below 10 μL could not proceed for further evaluations.

To continue the analysis and assess the physical characteristics of semen, the following were documented: presence or absence of the gel fraction (yes or no answers), and semen color (white or yellowish). Immediately after collection and before rabbit sperm evaluation, the gel fraction was removed. Sperm motility, vigor, total sperm, and viable sperm were evaluated under a microscope at a magnification of 40 x according to Lavara et al. (2005).

2.6 Statistical analysis

All statistical analyses were performed using IBM SPSS (version 24) - IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.

2.6.1 Doe variables

Data from nest fill percentage and flight distance test were compared by analysis of variance (ANOVA-Type III) followed by Tukey's test when homogeneity of variance was observed (Levene's test). As the data did not show normality (Shapiro-Wilk), nor homogeneity of variance (Levene), for the novel object test, a Wilcoxon test was conducted followed by a Student's *t*-test. The results are presented as the mean and standard error. The behavior tests (SIT, VAT, and FAT) were analyzed considering the following variables: number of attempts to escape and number of zones covered in arena-test; time spent in zones 3, 6, and 9 representing the maximum human approach (considering only VAT); and number of allowed human touches (for FAT only). The significant factors were included in the model according to the generalized linear models (GLM) process using stepwise forward regression (Poisson distribution). The factors used were the treatments (TS, SS, and C), the type of person (F or UN), and all possible interactions. The goodness of fit was tested by R-squared (adjusted for degrees of freedom) and the correlation of the variables was verified by Durbin-Watson (Durbin and Watson, 1950). Non-significant factors were excluded (*P*>0.05). The residual maximum likelihood was used as the estimation method. The data were tested for normality using the Shapiro-Wilk test, and the medians were compared using the Kruskal-Wallis and Mann-Whitney-Wilcoxon test.

2.6.2 Kits and male variables

Fisher's exact test was done in order to compare the type of kits mortality between treatments (stillborn, dead 1st week, 2nd week, 3rd week, 4th week and dead after weaning). Quantitative data from semen analysis (volume, progressive motility, total sperm, and viable sperm) were compared by analysis of variance followed by Tukey's test when homogeneity of variance was observed (Levene's test). The significant factors were included in the model according to generalized linear models (GLM) process using stepwise forward regression. The factors used were the treatments (TS, SS, and C), time (5th, 6th, and 7th months), and all possible interactions. The results are presented as the mean and standard error. For offspring sexual behavior and spermiogenesis, binary and multinomial logistic regressions were performed (represented by the below model), considering the experimental months (5th, 6th, and 7th) and the treatment (TS, SS, and C) for mounting and ejaculating, presence or absence of semen, gel fraction, color, and vigor analysis.

$$\log(p/1\text{-}p) = b0 + b1treat\_0 + b2treat\_1 + b3time\_0 + b4\_time\_1 + b5treat\_0{*}time\_0 + b6treat\_0{*}time\_1 + b7treat\_1{*}time\_0 + b8treat\_01{*}time\_1$$

In the model, *p* indicates the success probability, b0 to b8 are the fitted parameters of the regression, treat 0 and treat 1 are absence and presence (mounting and ejaculating, presence or absence of semen, gel fraction and color analysis), respectively, and time represents the 5th, 6th, and 7th months. The multinomial model is similar.

3. RESULTS

3.1 Nest building test

The amount of material deposited in the nest (hay or fur) was equal between treatments ($P = 0.516$). However, the TS and C groups showed a gradual increase in nest material deposition over time, whereas the SS group concentrated their deposition at the final phase of pregnancy (Table 1). The does from all treatments deposited more nest material on the day of parturition than five days before ($P < 0.05$). However, for treatments C and TS, the similar percentage of nest material was deposited on day -4 until day 0.

Table 1. Percentage (± SE) of nest material deposition (hay and fur) according to treatments and days before parturition.

Day	Treatments[1]			Mean
	C	TS	SS	
-5	18.18 ± 9.56 ^{a}	16.82 ± 9.25 ^{a}	10.00 ± 6.57 ^{a}	15.00 ± 4.83 ^{a}
-4	24.09 ± 11.62 ^{ab}	28.18 ± 12.22 ^{ab}	14.09 ± 7.71 ^{a}	22.12 ± 6.07 ^{ab}
-3	30.00 ± 12.38 ^{ab}	36.36 ± 12.60 ^{ab}	16.36 ± 8.31 ^{a}	27.58 ± 6.47 ^{ab}
-2	29.55 ± 11.69 ^{ab}	38.18 ± 11.60 ^{ab}	25.91 ± 10.61 ^{a}	31.21 ± 6.39 ^{ab}
-1	36.82 ± 11.08 ^{ab}	47.73 ± 13.44 ^{ab}	37.73 ± 11.63 ^{ab}	40.76 ± 6.82 ^{b}
0	70.45 ± 11.35 ^{b}	67.73 ± 11.59 ^{b}	72.27 ± 10.65 ^{b}	70.15 ± 0.08 ^{c}
P-value	0.0297	0.047	0.0001	<0.0001

[1]C: control group (not-stimulated); TS: tactile stimulated group; SS: stressed group; Mean: average of nest material deposition, of all treatments, per day.

The evaluator determined 100% of filling when the sum of hay and fur filled 100% of the wooden nest, with no visible spaces.

$^{a-b-c}$ different letters in the column compare time and represent differences between means by ANOVA followed by Tukey-test ($P < 0.05$).

SE: standard error.

3.2 Flight-distance and novel object-test

At Table 2 it is possible to observe the values for the flight-distance and novel-object tests. The variable used in the flight-distance test represents the percentage of animals that allowed to be touched by the observer (0 cm). The C group had the highest flight distance, being statistically ($P < 0.05$) different from the TS group (Table 2). In the novel-object test, the does of all treatments achieved similar latencies for touching the object (*P > 0.05*).

Table 2. Percentage of females that allowed touch (%) and manipulation time for the flight-distance and novel-object tests (mean ±SE), respectively.

Treatment[1]	C	TS	SS	*P-value*
Flight-distance test (%)	27.3[b]	90.0[a]	54.5[ab]	0.021
Novel-object test (sec)	87.45 ± 96.42	93.10 ± 114.30	98.60 ± 111.33	0.990

[1]C: control group (not-stimulated); TS: tactile stimulated group; SS: stressed group.

[a-b] Different letters on the row for flight-distance test represent differences between treatments by ANOVA followed by Tukey-test (*P < 0.05*). Novel-object test did not show means differences between treatments by ANOVA followed by Tukey-test (*P > 0.05*).

SE: standard error.

3.3 Social isolation and human-approach tests

Regarding the performed SIT and FAT tests, no statistically significant differences were found between treatments (*P = 0.367*), or in the case of FAT, between the treatment (*P = 0.828*) and observer (*P = 0.647*) for all considered variables (Table 3). However, in VAT, the number of attempts to escape when a UN person was present was greater (*P = 0.031*) in the C than in the TS group. The animals in the SS showed an intermediate number of attempts to escape between the other treatments but did not differ statistically (*P = 0.051*). For the variable "time stayed close," which represents the time that the does stayed close to the observer, the females of the C *(P = 0.015)* and SS *(P = 0.037)* treatments showed more time close to the F observer than the UN observer, and the opposite was observed for the TS group (*P = 0.026*). Furthermore, the C group remained close to the F observer longer than those of the TS (*P = 0.048*) and SS treatments (*P = 0.049*), which were significantly the same (*P = 0.449*). In contrast, the animals in the TS treatment were closer to the UN than the C animals (*P = 0.049*), which in turn were closer to those in the SS group (*P = 0.049*). The number of quadrants visited by the animals in the three treatments did not differ (*P = 0.186*). In each treatment, there was also no difference between the number of quadrants for the F and UN observers (*P = 0.292*).

Table 3. Median (minimum - maximum) of social isolation and human-approach tests performed in the arena according to treatment and observer.

Test	Variable	Observer	C	TS	SS
SIT	Attempts to escape (n)	-	2.0 (0-4)	2.0 (0-5)	1.0 (0-7)
	Zones covered (n)	-	14.0 (5-30)	12.0 (3-19)	13.5 (6-22)
VAT	Attempts to escape (n)	F	5.0 (0-10)	5.0 (0-14)	4.5 (0-8)
		UN	5.0 (0-7)^{a}	2.0 (0-5)^{b}	4.0 (1-9)^{ab}
	Zones covered (n)	F	25.0 (1-46)	17.0 (3-34)	17.0 (2-33)
		UN	15.0 (5-33)	16.5 (1-36)	13.5 (4-28)
	Time stayed close (sec)	F	45.0 (29-120)^{aA}	37.5 (0-113)^{bB}	38.5 (0-107)^{bA}
		UN	31.0 (19-99)^{bB}	51.5 (12-120)^{aA}	26.0 (0-61)^{cB}
FAT	Attempts to escape (n)	F	0.0 (0-7)	0.0 (0-6)	0.0 (0-1)
		UN	0.0 (0-1)	0.5 (0-6)	0.0 (0-2)
	Achieved touches (n)	F	56.0 (43-82)	56.0 (30-80)	41.5 (32-67)
		UN	53.0 (39-72)	50.5 (28-81)	53.0 (44-86)

C: control group (not-stimulated); TS: tactile stimulated group; SS: stressed group.

SIT: Social isolation test; VAT: Voluntary approach test; FAT: forced approach test;

F: Familiar observer; UN: Unfamiliar observer;

$^{a-b,}$ Different letters on the row represent differences between treatments by Mann-Whitney-Wilcoxon ($P < 0.05$);

$^{A-B}$ Different letters on the column represent differences between familiar and unfamiliar person, by Mann-Whitney-Wilcoxon ($P < 0.05$);

3.4 Mortality and offspring weights

Data on mortality and offspring weights are presented in Table 4. The number of live and stillbirths was the same between treatments. For the proportion of deaths in the first week, the treated groups (SS and TS) had the highest values when compared to the control group. From the second week onwards, mortality was equal among the three treatments.

The kits' weight increased significantly every 15 days in each of the three treatments. At 15 days, there was a difference between the three groups. The kits from group C were lighter than those from the TS and SS groups, which did not differ statistically from each other. At 30 days, the TS group was the same as the other two, but the C continued to show a lighter average weight than the SS group. From 75 days onwards, the three groups had equal mean weights (Table 4).

Table 4. Zootechnical data (mortality, sex ratio and weights) over time of kits as a result of treatments.

Items	C	TS	SS	*P-value*
Live*	50 (4.00 ± 3.35)	28 (2.36 ± 2.80)	32 (2.55 ± 3.11)	0.408
Stillbirths *	14 (1.27 ± 1.62)	21 (1.91 ± 2.51)	21(1.91 ± 2.62)	0.3833
Sex (Day 15)				
Female kits (n)	24	12	18	-
Male kits (n)	26	16	14	-
Mortality (%)				
Dead (1st week)	5.0 ± 0.69^{a}	24.0 ± 3.09^{b}	20.0 ± 2.56^{b}	0.002
Dead (2nd week)	1.0 ± 0.30	0.0 ± 0.0	0.0 ± 0.0	0.379
Dead (3rd week)	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.30	0.380
Dead (4th week)	2.0 ± 0.40	0.0 ± 0.0	0.0 ± 0.0	0.126
Dead after weaning	6.0 ± 0.52	2.0 ± 0.40	4.0 ± 0.92	0.439
Weights (kg)				
Day 15	0.23 ± 0.003^{A}	0.26 ± 0.005^{B}	0.25 ± 0.004^{B}	<0.0001
Day 30	0.52 ± 0.01^{A}	0.57 ± 0.02^{AB}	0.62 ± 0.02^{B}	<0.0001
Day 45	1.12 ± 0.03^{A}	1.26 ± 0.03^{B}	1.28 ± 0.03^{B}	<0.0001
Day 60	1.73 ± 0.03^{A}	1.84 ± 0.04^{AB}	1.88 ± 0.04^{B}	<0.001
Day 75	2.26 ± 0.04	2.36 ± 0.05	2.42 ± 0.05	0.510
Day 90	2.86 ± 0.05	2.96 ± 0.07	2.97 ± 0.06	0.151

C: control group (not-stimulated); TS: tactile stimulated group; SS: stressed group.

* Total number of animals (mean ± SD).

[a-b] Different letters on the row represent differences between treatments by Exact Fisher´s test (P < 0,05), for stillbirths and mortality.

A-B Different letters on the row represent differences between treatments by ANOVA test ($P < 0{,}05$) followed by Bonferroni test, for weights.

3.5 Offspring sexual behavior and semen analysis

The results and logistic regression models across treatments and time regarding the offspring sexual behavior and semen analysis are shown in Tables 5 and 6. Comparing sexual behavior and semen between treatments, the litters from the TS and SS groups ejaculated less than the control ($P = 0.021$). However, the TS and SS groups did not differ statistically from each other. In addition, males from the TS group showed a greater absence of sperm in the ejaculates when compared to the control ($P = 0.037$). The SS and C groups were statistically equal. With regard to mounting behavior, gel fraction, color, vigor, motility, volume, total sperm, and viable sperm parameters, no statistical differences were found between the treatments applied.

Comparing the mounting behavior across time (global average of the three groups), the number of males that copulated was greater in month 6 than in month 5 ($P = 0.026$). In addition, the percentage in the 7th month was statistically equal to the 5th and 6th month. No statistical differences across time were found in ejaculation behavior, gel fraction, color, vigor, motility, volume, total sperm, and viable sperm parameters.

Table 5. Offspring sexual behavior and semen analysis (%) across treatments and time.

		Treatment			Months*		
		C	TS	SS	5	6	7
Mounting behavior	Yes	97.1	93.8	87.9	84.8^{A}	100^{B}	97.7^{AB}
Ejaculation behavior	Yes	95.7^{a}	65.6^{b}	78.8^{b}	76.1	88.6	88.6
Sperm	Presence	91.9^{a}	66.7^{b}	77.4^{ab}	70.5	87.8	89.5
Gel fraction	No	84.2	95.0	91.7	80.6	91.7	91.2
Color	White	91.2	75.0	91.7	93.5	88.9	82.4
	Score 1	10.5	10.0	8.3	9.7	8.3	11.8
	Score 2	7.0	20.0	12.5	16.1	8.3	8.8
Vigor	Score 3	29.8	20.0	8.3	19.4	22.2	26.5
	Score 4	33.3	25.0	29.2	25.8	38.9	26.5
	Score 5	19.3	25.0	41.7	29.0	22.2	26.5

C: control group (not-stimulated); TS: tactile stimulated group; SS: stressed group.

*Global average of the three groups across time: 5 - fifth month; 6 - sixth month; 7 - seventh month.

[a-b] Different letters on the row represent differences between treatments by Binary and a Multinomial Logistic Regression (*P* < *0,05*).

[A- B] Different letters on the row represent differences between months by Binary and a Multinomial Logistic Regression (*P* < *0,05*).

Table 6. Analysis of the semen across treatments and time.

Item	Treat	Month		
		5th	6th	7th
Volume (ul)	C	0.55 ± 0.32	0.54 ± 0.29	0.59 ± 0.45
	TS	0.62 ± 0.38	0.43 ± 0.3	0.45 ± 0.35
	SS	0.61 ± 0.35	0.59 ± 0.43	0.74 ± 0.52
	P-value	0.414	0.205	0.346
Progressive motility (%)	C	79.44 ± 31.17	77.95 ± 19.71	77.06 ± 35.6
	TS	64.17 ± 37.02	76.67 ± 42.76	77.5 ± 40.41
	SS	77.86 ± 40.89	73.75 ± 38.02	78.89 ± 33.87
	P-value	0.249	0.412	0.952
Total Sperm (10^8 ul)	C	8.21 ± 5.52	8.81 ± 4.95	6.86 ± 4.18
	TS	4.81 ± 2.84	7.34 ± 4.62	6.02 ± 3.59
	SS	6.44 ± 4.52	6.99 ± 4.51	1.02 ± 5.07
	P-value	0.120	0.059	0.084
Viable sperm (x 10^8 ul)	C	6.88 ± 4.72	6.95 ± 4.27	5.33 ± 3.33
	TS	3.34 ± 2.34	6.27 ± 4.32	4.87 ± 2.98
	SS	5.23 ± 3.83	5.57 ± 3.90	7.83 ± 3.78
	P - Value	0.110	0.136	0.174

C: control group (not-stimulated); TS: tactile stimulated group; SS: stressed group.

5: fifth month; 6: sixth month; 7: seventh month

The evaluated parameters do not differ significantly from each other by Tukey-test (*P* > *0,05*).

4. DISCUSSION

4.1 Nest building test

The preparation of high-quality nests at the appropriate time, which has been referred to as "maternal nest", is considered essential to ensure the well-being and survival of the hairless kits (González-Mariscal et al. 1996). In the present study, the time of initiation of nest-building behavior occurred on days 25–26 of pregnancy and finished a day before parturition, considered a normal nest building behavior. At about day 26 of gestation rabbits start preparing nests from straw and other available materials. Two days before parturition they start plucking fur from their bodies and further prepare the nest for the coming kits (Negatu and McNitt 2002; González-Redondo 2010). In cases where the timing of nest building may be abnormal, rabbits could fail to prepare the maternal nest before parturition and they are not successful in raising their kits (Zarrow et al. 1963).

In the present study, the SS group concentrated their deposition in the final phase of pregnancy.

Studies in the domestic rabbit revealed that different elements of the behavioral sequence, such as digging, collection and carrying of nest material are under hormonal control (Zarrow et al.1963; González-Mariscal et al., 2016). Furthermore, physiological state, genetic factors and individual's social environment, could conceivably alter the females'burrow and nest building behavior, modifying the timing of the hormonal signals involved (González-Redondo 2010; Benedek et al. 2021).

In a study conducted by Seltmann et al. (2017), the authrs observed in 18% of cases that females constructed both the nursery burrow and nest during the last 24 h before parturition. This behavioral pattern was associated with a period of stress and intra-sexual competition that does went through, since the females of the social group gave birth concurrently. The occurrence of perinatal mortality within a litter increased by 24% (and by 33% when only considering cases of nest mortality of at least 50% of pups) with mothers that displayed such late burrow and nest construction.

According to Benedek et al. (2021), progesterone is one of the most dominant in the process of nest construction. The authors demonstrated that its level is altered by the level of cortisol elevation in the animal, which potentially influence the preparation of the nest for the newborn kittens. Based on the present results, it can be concluded that the stress applied to the animals was not enough to cause a significant impact and inhibit the expression of nest building behavior, but it influenced the pattern of execution and delayed the process. Further studies are needed to assess the impact of stress on female progesterone and individual variance in cortisol levels

4.2 Flight-distance and novel object-test

These tests lead to a better understanding of the human-rabbit relationship (flight distance) and general fearfulness (NO). In this way, the effect of early handling has been of special interest because there is accumulating evidence that the handler might be considered a predator by adult animals (Suarez and Gallup 1982), resulting in an undesirable level of fear in experimental studies that use direct observation. Although the study by Crowell-Davis (2007) indicates that rabbits may be afraid of humans due to painful or frightening experiences; in the present study, there was no statistical difference between TS and SS. A possible explanation for this could be the level of intensity of stress applied to the females, which was not strong enough to induce statistical differences. It is noteworthy that behaviors such as freezing, bent over with the ears glued to the body, being excessively bouncy and vigilant (bulging eyes), being aggressive towards people or other rabbits, were not observed during the applied tests, excluding the possibility of touching due to immobility and extreme fear. Furthermore, the C group was the one that presented the highest percentage of females that did not allow touching, which suggests that human contact stimulation is more beneficial in general, and not just during routine handling. According to Csata´di et al. (2005), in kits, even minimal human contact is effective in reducing rejection of the caretaker, so handling could be a useful tool to reduce stress and improve welfare. Kersten et al. (1989) found that early handling seems most effective in reducing emotionality if applied after the 10th day of life, while Jezierski and Konecka (1996) recorded higher growth rates and higher activity levels in rabbits handled from day 10 to 10 week of age.

According to precursor studies in the area, handling could affect fear-related emotionality. An example is of handled rats (infant animals) that display more activity and less defecation than non-handled controls, when exposed to an open field test (Levine et al. 1960; Kersten 1989). The authors reinforced the idea that enhanced human contact at early life could be beneficial, since this difference in response was interpreted as a reduction in fear due to handling.

About the possible effects of handling on animal cognition and exploratory behavior, Denenberg et al. (1973) found that handling rabbits at infancy result in more active animals, that interacted with new stimuli more often and spent more time exploring new situations. However, in the present study, no differences were found, since all treatments achieved similar latencies ($P > 0.05$) for touching the object (Table 2). The intensity of stress applied to the females, and the calmly brushing of the animal inside the cage may not have been strong enough stimuli to increase or impair the exploratory behavior.

Another hypothesis is that the object used did not have the expected novelty impact on the females. As shown in Figure 2, the animals were housed in wire maternity cages, which allowed visual contact with the cages on the side. Since the tests were conducted in the same location, the doe may have observed the tennis ball in the neighboring cage, which may have mitigated the exploratory behavior. According to Denenberg et al. (1973), objects lose their novelty over the time and frequency with which they are presented to the animals. One possibility for future studies would be to develop the test outside the maternity cages, not allowing visual contact with other animals.

4.3 Social isolation and human-approach tests

The most common test to evaluate fearfulness in rabbits is the open-field test (during which an animal is placed in social isolation in a novel arena). Originally, it was assumed that greater locomotion reflects decreased fearfulness (Hall 1934). However, the interpretation of this test is complex and species-specific. The increase in movement, in this study, evaluated by the number of zones covered, may also indicate a stronger motivation to explore the new environment, or the frightened attempt to reinstate contact with conspecifics (Buijs and Tuyttens 2015).

Even without differences between the degree of exploration and movement of the does in the test arena, the present data show that animals who preferred to stay close to the familiar observer belonged to groups with a negative stimulus (SS) or null stimulus (C). Furthermore, it is possible to hypothesize that females from the TS group were more optimistic or curious, or less fearful, since they stayed longer in quadrants close to the unfamiliar observer. As mentioned before, freezing behavior were not observed during the applied tests. Thus, the authors did not work with the possibility of touching due to immobility/freezing caused by extreme fear. However, further studies are needed to evaluate optimism, pessimism, and judgment bias in rabbits.

4.4 Mortality and offspring weights

Adverse experiences, including maternal exposure to stress during pregnancy, can lead to persistent changes in several physiological systems and behaviors. The majority of studies investigating these effects on the offspring have been carried out in rodents (Brunton 2013). Prenatal stress is associated with negative pregnancy outcomes, such as low birth weight, reduced litter sizes, and lower survival rates (de Catanzaro 1988; Pratt and Lisk 1991; Brunton and Russell 2010; Paris et al. 2011). In a pioneering study conducted by Barlow et al. (1978), stress at any stage of pregnancy led to a significant decrease in offspring body weight, which persisted for up to 6 weeks of age and delayed the appearance of certain developmental landmarks such as ear opening, auditory startle, and cliff avoidance responses. According to the present data, the proportion of deaths in the first week was the same between the SS and TS groups, which was greater than of the control group. From the second week onwards, mortality was equal among the three treatments. According to González-Mariscal et al. (1998), preweaning kit mortalities were significantly higher in primiparous than in multiparous does. As in primiparous domestic does, mother-kit contact at birth is a crucial factor in establishing the maternal response and the experience does gain from raising a previous litter therefore enables them to retain their maternal response (González-Mariscal et al. 1998).

In C group there were more kits (lower mortality) and the amount of milk available between them may have kept their weight lower. From the second week and after 75 days, it was possible to observe that mortality and weights were virtually the same between the three treatments. In this case, the severity of stress was considerably mild and was applied during the middle and late gestation periods. The treatments started after the pregnancy confirmation (ultrasound), which was only possible after the 12th day.

4.5 Offspring sexual behavior and semen analysis

In prepubertal male pigs, stress during their mother's pregnancy is associated with a significant reduction in the circulating levels of hormones, such as testosterone and estradiol. When it comes to rats exposed to prenatal stress, the reduced circulating testosterone levels in male fetuses during late gestation (Ward and Weisz 1980) results in the demasculinization and feminization of sexual behaviors in adulthood (Ward 1972). However, few studies have directly assessed the effects of social stress exposure during pregnancy on the future reproductive capacity of the offspring in lagomorphs (Brunton 2013). In the present study, the only variables affected by the treatments applied were ejaculation behavior and the presence of semen in the ejaculate. As expected, the control group showed positive results when compared to the handled group. Manipulating the rabbit, regardless of whether it is a positive or negative stimulus, appears to reduce the sexual performance of males. The breeding behavior occurred normally, but the number of animals that ejaculated with sperm was reduced. No variables related to semen analysis were changed. Knowing that testosterone levels decrease when the female goes through a period of stress (Ward and Weisz 1980), a hypothesis is that the treatment applied was able to influence the ejaculation but was not severe enough to inhibit the behavior or impair the semen quality (volume, total sperm, viable sperm). The role of circulating testosterone, the major androgen in males, is linked to normal spermatogenesis and the expression of secondary sexual characteristics (Souza 2011). The concentration in seminal plasma correlates with sperm concentration, percentage of motile spermatozoa, and other sperm characteristics (Laudat et al. 1998). Bánszegi et al. (2015) found significant results in reduced anogenital distance and chin-gland size, reduced chin-marking activity, and greater timidity at six months of age, when rabbits can be considered sexually mature. However, the stress to which the females were subjected was more invasive than that used in the present study. The treatment consisted of taking rabbits from their cages and injecting them with a sesame oil vehicle on seven consecutive days during late pregnancy. In addition, according to Moore and Power (1986), the sexual behavior of males can also be affected by maternal behavior. In the study reported by those authors, social crowding during the last week of rat's pregnancy leads to reduced mother-pup interactions. This was reflected by reduced anogenital licking of the pups by the dam and, generally, male kits receiving more anogenital licking than females, an effect that is mediated by testosterone dependent cues. In this case, both prenatal stress and decreased maternal licking of kits are associated with deficiencies in male sexual behavior in later life (Ward 1972; Brunton 2013). More studies that directly assess the effects of social stress exposure during pregnancy on the future reproductive capacity of the offspring are necessary.

5. **CONCLUSION**

Under the conditions of the present trial, TS allows the maximum human approximation in the flight distance test when compared to the other two treatments that present the highest fear level (C group: higher flight distance and attempts to escape number in VAT) and incomplete behavioral patterns (SS group: delayed nest building). Furthermore, the treatments applied to the females (TS and SS) were enough to cause a significant impact on the offspring and confirm that the control group presented better values between treatments for number of stillbirths and proportion of deaths in the first week. Finally, handling was able to negatively influence the ejaculation and sperm presence, but was not intense enough to inhibit the behavior or impair the semen quality (volume, total sperm, and viable sperm). By assessing the combination of social behavior, human-animal relationships, and other behaviors, it can be concluded that tactile stimulation did not impact the welfare or maternal behavior and improved the human-animal relationship; however, the impact on the litter was negative. More studies that directly assess the effects of social stress exposure during pregnancy on the future reproductive capacity of the offspring are necessary.

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