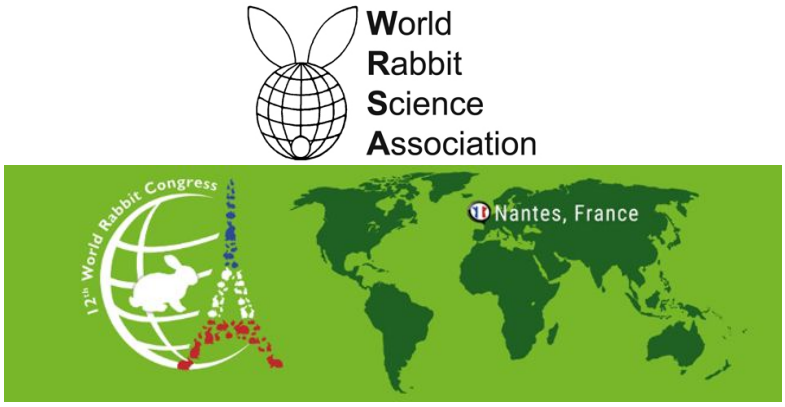

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REPRODUCTION

Villamayor PR., Gullón J., Vilá M., Yáñez U., Aramburu O., Sánchez M., Sánchez-Quinteiro P., Martínez P., Quintela L.

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PRELIMINARY REPORT OF POTENTIAL BIOSTIMULATION METHODS BASED ON CHEMICAL COMMUNICATION IN RABBIT DOE REPRODUCTION

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ABSTRACT

Biostimulation is an animal management practice that helps improving reproductive parameters by modulating animal sensory systems. Chemical signals, mostly known as pheromones, have a great potential on this regard. This study was conducted to determine the influence of short-term female rabbit exposure to different conditions, potentially pheromone-mediated, on reproductive parameters of inseminated does. Groups of 60 females/each were exposed to 1) female-female interaction, 2) female urine, 3) male urine and 4) seminal plasma, just before artificial insemination. Controls of isolated females and Ringer Lactate exposure were, respectively, performed. The following reproductive parameters were analyzed for each group: receptivity (vulva color), fertility (transabdominal palpation), and prolificity and number of born alive and dead kits ⁄ litter. Despite some differences could be noticed in receptivity rate, especially in the 'female-female interaction' group, the results showed no fertility and prolificity significant differences among groups suggesting that all the stimulation methods employed had similar effects. Moreover, similarity between the 'female-female interaction' group –usually performed in rabbit farms– and its control –no animal handling– should be reconsidered to avoid unnecessary animal management and time cost. On the other hand, fertility ranges were lower for animals with white vulva color whereas no differences were noticed among the other three (pink, red, purple), thus suggesting that these three could be grouped together. Overall, despite all groups showed similar effects, it remains to be elucidated how chemical signals released by both urine and seminal plasma can affect reproduction.

Key words: Rabbit, biostimulation, pheromones, reproduction, urine.

INTRODUCTION

Many animals use chemical cues to interchange information among them and with the environment (Wyatt, 2014). These sensory signals, mainly known as pheromones, play a pivotal role at modulating behavioral and physiological responses such as maternal behavior or reproductive strategies, and are released by the animal through biological secretions (i.e. urine, seminal plasma) or exocrine glands (i.e. lacrimal, mammary), which trigger a specific reaction in another individual who detect them.

Due to the difficulty to characterize specific pheromones -only 63 have been well-characterized to date in mammals- (Apps *et al.*, 2015), more effort has been invested on pheromone-mediated behavioral analysis. For instance, female rabbits (does) reproductive performance appears to increase when they are exposed to male (buck) odors just before artificial insemination (AI) (El-Azzazi *et al*., 2017). Similarly, male-female interaction before AI, the so-called 'buck effect', slightly improves does fertility at first lactation but no positive effect has been detected on the reproductive performance of lactating does (Bonanno *et al*., 2003). What specific biological secretions (i.e. urine or seminal plasma) are responsible for such changes remains still to be elucidated. On the other hand, placing together two females before AI has shown 'riding behavior', and despite the studies performed to date

are not conclusive at improving reproductive parameters by employing this procedure (González-Urdiales, 2005), it has become an established routine as a biostimulation method in rabbit farms. Accordingly, we aim to determine whether female-female interaction increases reproductive parameters in female does; we hypothesize that urine and seminal plasma, as a source of pheromones, might have the potential of increasing some reproductive parameters.

Specifically, the aim of the current study is to shed light on 1) the effect of female-female interaction, 2) female urine exposure, 3) male urine exposure and 4) seminal plasma exposure, prior to artificial insemination, on improving the reproductive and productive performances in rabbit does.

MATERIALS AND METHODS

Animals

This study was conducted according to the regulations and general recommendations of the National Board of Agriculture on the use of animals for scientific purposes. All the procedures were carried out under farm conditions in the industrial rabbit farm COGAL S.L. (Pontevedra, Spain). A forced ventilation system was used and the inside temperature was maintained between 18 ºC and 22 ºC using an air conditioned-heater system. All females were 3.5 - 4 kg weight and commercial hybrid (Hyplus strain PS19, Grimaud Frères, Roussay, France), and males were 5 - 7 kg weight and Hyplus strain PS40. Males and females were located in separated farms.

Sample Collection

Urine: Pools of 330 ml of urine from both mature males and females (> 180 days), were obtained by cystocentesis, 24 h before the behavioral experiment was performed, and kept at 4ºC overnight. Pure urine was used in all cases. *Seminal plasma*: Seminal plasma used for does exposure was obtained from an AI Center, 24h prior to the behavioral experiment, from 60 mature males (> 180 days). All seminal doses were mixed together and centrifuged at 3000 rpm, 10 min, to obtain the seminal fluid, which was then kept at 4ºC overnight. Before use, it was diluted 1:3 in Ringer Lactate Solution. To perform the AI, semen were collected and stored at 16 ºC for use within a 24 h period. In all cases, semen were collected using an artificial vagina.

Reproductive Management

All does employed for the behavior experiment were between third and eight number of calving, evenly distributed among the six groups (see below). None of the animals were treated hormonally to synchronize oestrus (eCG). All does were inseminated on day 11 after parturition and were lactating ~10 kits on average. Sexual receptivity was confirmed by determining the color of the vulva (white, pink, red, purple) at the time of AI (Figure 1) (Quintela *et al.*, 2001). Pregnant or lactating does were fed *ad libitum* whereas non-pregnant non-lactating does were restricted to 150 g ⁄ day of commercial food except in the period from day 6 before AI to the day of pregnancy diagnosis, during which they were also fed *ad libitum*. Light intensity was 70 lux, with an artificial lighting programme of 12 h (light) L ⁄ 12 h (dark) D, which was changed to 16 h L ⁄ 8 h D 6 days before does artificial insemination (AI). After parturition, light hours are decreased 1h / day during 4 days until obtaining back the normal programme. Controlled suckling was applied to all does from 0 to10 days post-partum, by keeping the nest door closed and only opening it every 24 h, at 12:00 h for 5 – 10 min, to allow the kits to suck once a day. On the day of AI (day 11 post-partum), suckling was delayed until 5–10 min before performing the AI. This made a 30-h mother–litter separation. From day 12 post-partum (i.e. 1 day after AI) to weaning (30–35 days post-partum), free suckling was allowed by keeping the nest door open. At 11–14 days after AI, all does were diagnosed for pregnancy by transabdominal palpation. Parturitions took place mainly on day 30 post-AI. When all does had completed parturition, the prolificity and number of born alive and dead kits ⁄ litter were recorded. Then, the number of rabbits per litter was adjusted to 10 kits of equal body size.

Note that this experiment will be repeated three times in consecutive inseminations to verify the results obtained. Until now, we have data of trial one and two (from this latter we still do not have neither prolificity rate nor the number of born alive and dead kits ⁄ litter).

Experimental Design

We conducted a behavioral experiment to check how reproductive parameters vary according to several given conditions in does between third and eight number of calving. The six groups (4 experimental conditions and two controls) were composed of 60 lactating does / each in the first trial. We tried to individually monitor the same animals for the two performed trials; in the second one some does could be either non-lactating –if they were not pregnant- or replacing with others of similar 'number of calving' –if they were eliminated for health reasons

Table 1: Experimental design considering the six procedures with their corresponding explanation.

Group / Procedure	Explanation
1. Female-female interaction	Two does were placed in the same cage 15 min before AI
2. Urine female	Urine female exposure
3. Urine male	Urine male exposure
4. Seminal plasma	Seminal plasma exposure
5. Isolated females (control)	Animals kept in their own cages
6. RL (control)	Ringer Lactate Solution exposure

For conditions 2, 3, 4 and 6 (Table 1), the corresponding stimulant was sprayed in the nose area 1 h, 15 min, and 1 min before insemination; specifically 1 ml nasal spray in each exposure per animal, in total 3 ml / individual. Additionally, urine drenched wool was hung in the cages after the first exposure to ensure the contact between the stimulant and the animal –some animals gnawed it–. AI was always performed by the same person to reduce variability in the obtained results. The following reproductive data were collected: 1) sexual receptivity (vulva color); 2) pregnancy diagnostic by trans-abdominal palpation; 3) prolificity and number of born alive and dead kits/litter.

Statistical Analysis

Data on receptivity and kindling rates were analyzed by χ^2 and Kruskal-Wallis tests. Prolificity and number of born and dead were analyzed using the General Linear Model procedure of SPSS 20.0 software (SPSS Inc., Chicago, IL, USA), considering the effects of the treatments. Differences between means were tested by the Fisher F-test and differences were considered statistically significant at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Sexual Receptivity

Regardless the experimental group, most animals showed a consistent similar pattern of vulva color at the time of AI: pink > red > purple > white (55.7%, 33.8%, 8.6%, 2%, respectively) (Figure 1A), excluding the behavior joint group, where the red vulva color overtook the other colors, purple was higher than pink, and no white was present (Figure 1B). Additionally, purple vulva color in such group showed significant greater values than the other experimental conditions ($p < 0.05$). Finally, and as a qualitative estimation, we also found a strong 'riding behavior' in this group when the two does were placed together before AI.

Taken together, the biostimulation methods employed in this study, specifically female-female interaction, significantly influenced female does receptivity, in both the AI performed and in each of them independently ($p < 0.01$).

Fertility

No conception rate differences were found among the six given conditions. Additionally, does with vulva white color showed a significant decrease of fertility (66.7%) compared with the other three (pink (93%), red (91.1%), and purple (92%)) ($p < 0.05$), which displayed similar fertility. These data suggest that fertility varies depending on sexual receptivity (vulva color), but we can only correlate 'white' with 'lower fertility', and 'not-white', which is the same and the sum of pink, red and purple, with 'higher fertility'.

Finally, taken into account that female-female interaction is a commonly used biostimulation method in some farms, we can also argue that since no differences in conception and prolificity rates were noticed between group 1 and group 5, such management should be reconsidered in order to reduce animal handling and a substantial time cost.

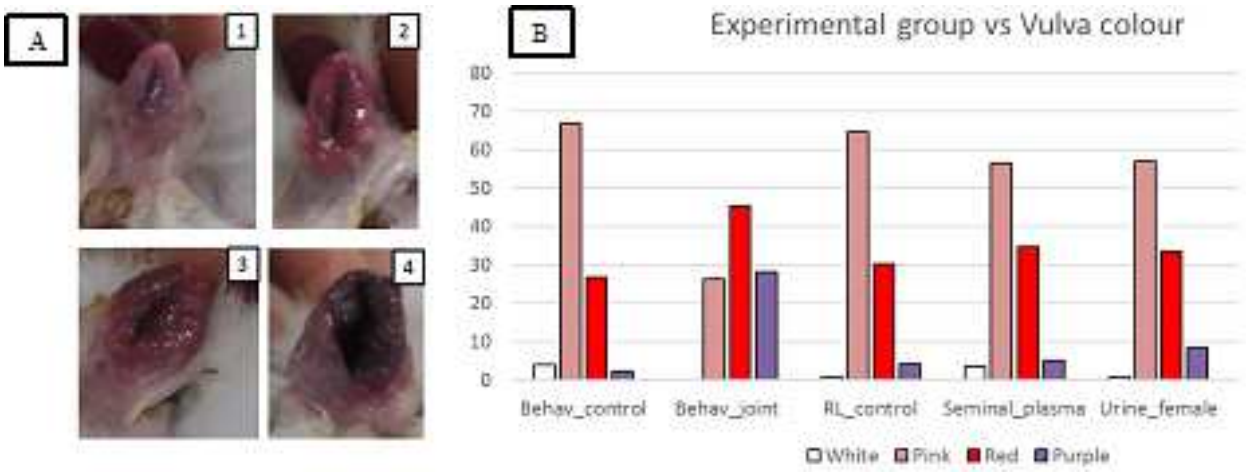

Figure 1: A. Does vulva color just before insemination. 1 white; 2 pink; 3 red; 4 purple. **B**. Graph of the vulva color of the different experimental groups.

Prolificity and Number of Born alive and dead kits/litter

We only collected data from the first trial, and we found the following prolificity rate per group: Female-female interaction: 10,92 ± 7,01; female urine: 10,78 ± 5,50; male urine: 11,68 ± 5,48; seminal plasma: 10,32 ± 7,12; females isolated (control): 10,89 ± 6,06; RL (control): 12,017 ± 6,10;. Accordingly, no significant differences were noticed among the six groups, which is consistent with the rest of the reproductive parameters analyzed.

CONCLUSIONS

The biostimulation methods employed did not improve any of the analyzed parameters. Hence, placing together two females before AI –a routine commonly used in some rabbit farms, does not improve fertility or prolificity rates. Considering that the average conception rate overtakes 90% in the employed farm, which is considerably high, this experiment might offer better results in a farm with lower fertility, where there is a higher possibility for improvement.

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Preliminary report of potential **biostimulation methods** based on **chemical communication** in rabbit doe reproduction

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12th WRC, 03-05/11/2021, Nantes (France)

BACKGROUND

BIOSTIMULATION METHODS

Lighting control

Feeding control

Suckling control

CHEMICAL COMMUNICATION

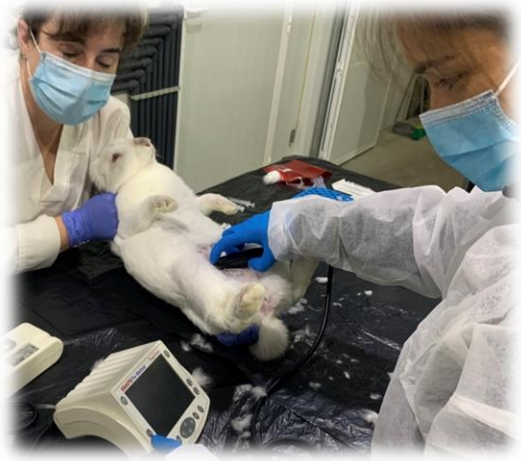
- *Pheromones (urine, seminal fluid, etc.)*
- *Animal behaviour and reproduction*
- *Likely mediates doe exposure effect (males) and buck effect (females, pimiparous)*
- *Female-female interaction prior to artificial insemination (AI)*

OBJECTIVES

1. To check whether **female-female interaction prior to AI** has an effect on their reproductive parameters: receptivity (vulvar color), fertility (calving rate), prolificity and number of born alive and dead kits / litter, and therefore can be used as a biostimulation method.

2. To determine whether **exposure of urine / seminal fluid** (as a source of pheromones) **to female does** has an effect on their reproductive parameters: receptivity (vulvar color), fertility (calving rate), and prolificity and number of born alive and dead kits / litter.

MATERIAL & METHODS

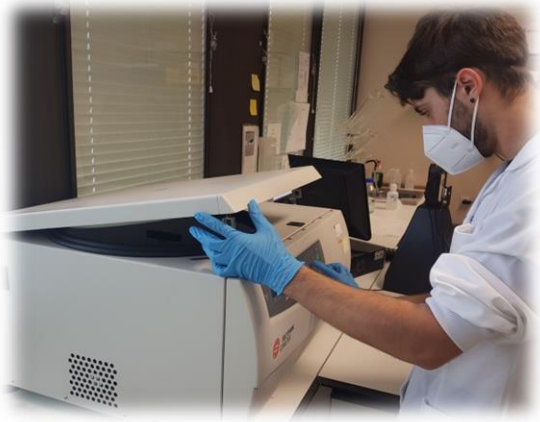


1. Urine and seminal fluid (SF) collection and preparation

24 h, 4ºC
Urine: pure
SF: centrifugation; 1:3 in serum

2. Other biostimulation methods

✗ eCG



MATERIAL & METHODS

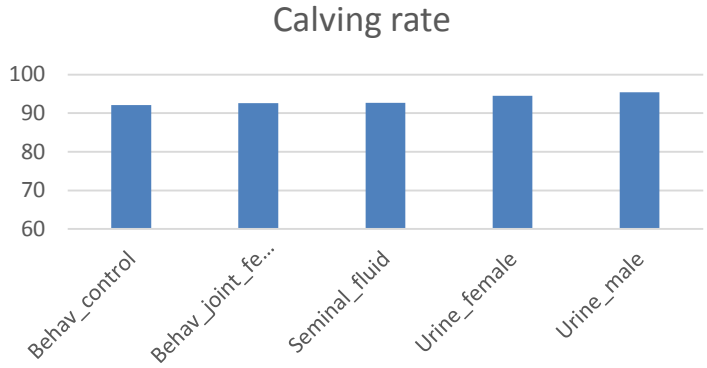
3. Experimental design

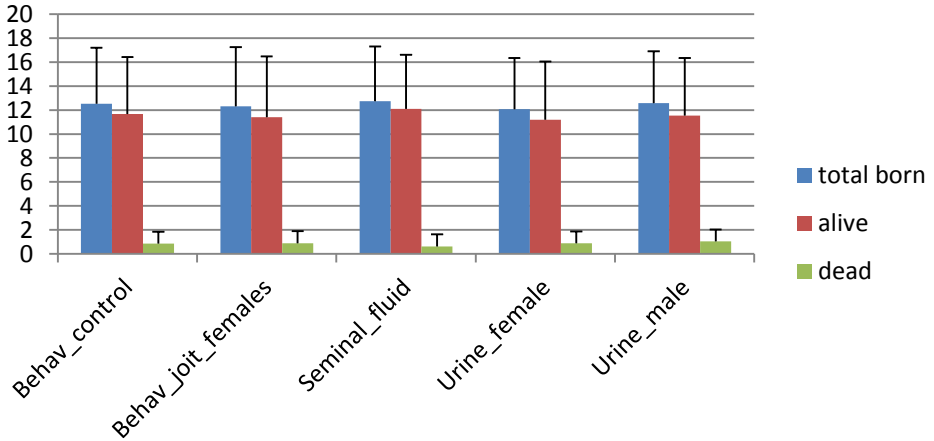
Group / Procedure	*Explanation*
1.Female-female interaction	Two does were placed in the same cage 15 min before AI
2. Urine female	Urine female exposure
3. Urine male	Urine male exposure
4. Seminal fluid	Seminal fluid exposure
5. Isolated females (control)	Animals kept in their own cages

- 60 lactating does / group (between 3rd and 9th calves)
- Exposure to fluids (spray) 1h, 10 min and 1 min before AI (1ml/each)
- Repeated 3 consecutive AI cycles
- Measurement of reproductive parameters: **receptivity** (vulvar color), **fertility** (calving rate), **prolificity** and **number of born alive and dead kits / litter were** checked

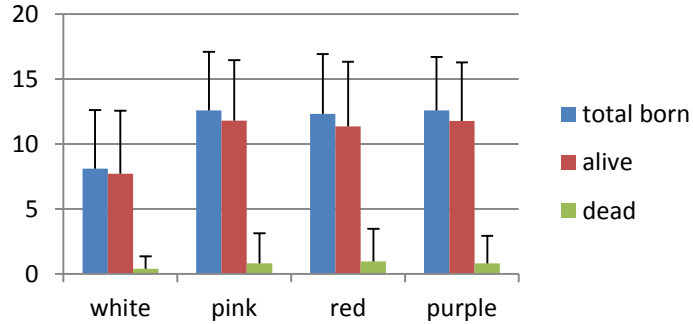
RESULTS

1. **No** significant differences in any reproductive parameter were found between experimental groups

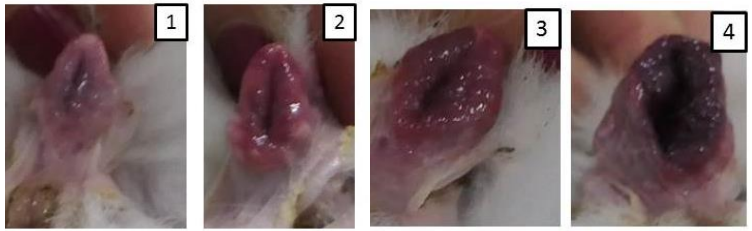




2. Does presented with **white vulvar colour** have a reduced number of total born individuals

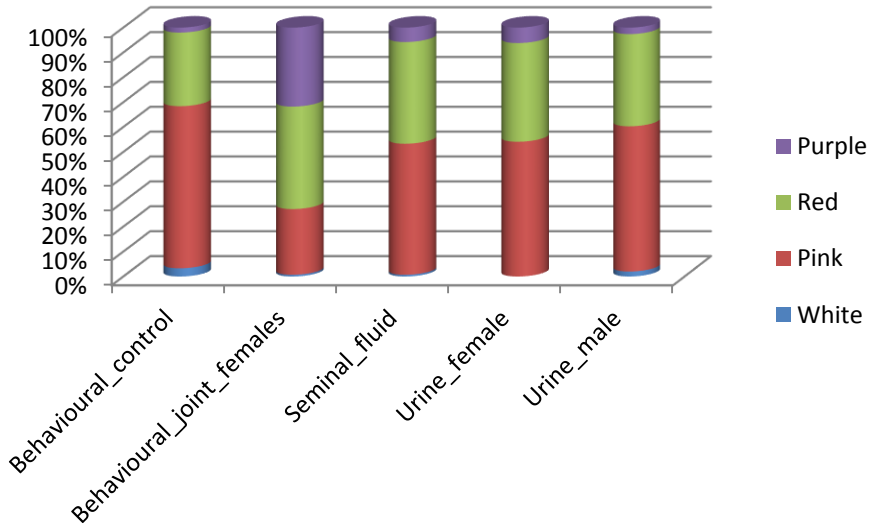


Receptivity was checked by vulvar colour



RESULTS

3. Behavioural joint group have the highest percentage of purple vulvar colour individuals whereas behavioural control group (BCG) have the highest percentage of white vulvar colour individuals (no significative)



Of note:

- Vulvar colour does not correlate with fertility and prolificity rates: pink, red and purple could be joint and defined as 'non-white'
- Only 1.3% of all individuals have white vulvar colour (good synchronization rate)
- Despite there is slightly higher percentage of white vulvar colour in the BCG (3.3%), this is still very low, suggesting that **female-female interaction prior to AI is NOT an efficient biostimulation method**

CONCLUSIONS

1. Urine and seminal fluid do not influence any of the analyzed reproductive parameters
2. Female-female interaction prior to AI is not an efficient biostimulation method
3. No differences in any reproductive parameter can be detected between the experimental groups
4. Receptivity assessment should be done by considering ‘white’ and ‘non-white (pink, red, purple)’ vulvar colour analysis
5. There is no need of using eCG in female synchronization when other biostimulation methods are used

FURTHER EXPERIMENTS

1. Primiparous does
2. Farms with lower female reproductive performance (60-70% calving rate)
3. Exocrine glands as a source of pheromones
4. Pheromone biostimulation methods in male performance (sexual drive and sperm production and quality)

TAKE HOME MESSAGE

Even though little research has been done in the field, biostimulation methods based on chemical communication are a potential powerful tool to improve animal production and welfare

THANK YOU!