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Assessment of Biostimulation Methods based on Chemical Communication in Female Doe Reproduction

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Simple Summary: Biostimulation is a natural technique employed in animal production to enhance reproductive parameters. In this study, we assessed the reproductive efficiency of female rabbits (receptivity, fertility, prolificacy and number of born alive and dead kits / litter) when exposed to different biostimulation conditions, which involved exposure to urine, seminal plasma or social interaction between females, prior to artificial insemination. Overall, despite all groups showed similar reproductive performance, our results indicated that biostimulation methods might be a good practice to improve reproductive management in livestock since it could reduce the use of hormones and enhance animal welfare. Future studies are needed to fully elucidate how chemical signals released through bodily secretions influence reproduction.

Abstract: Biostimulation is an animal management practice that helps improve reproductive parameters by modulating animal sensory systems. Chemical signals, mostly known as pheromones, have a great potential in this regard. This study was conducted to determine the influence of short-term female rabbit exposure to different conditions, mainly pheromone-mediated, on reproductive parameters of inseminated does. Groups of 60 females/each were exposed to 1) female urine, 2) male urine, 3) seminal plasma and 4) female-female interaction, just before artificial insemination, and compared to isolated females controls (female-female separated). The following reproductive parameters were analyzed for each group: receptivity (vulvar color), fertility (calving rate), prolificacy and number of born alive and dead kits / litter. Our results showed that the biostimulation methods employed in this experiment did not significantly improve any of the analyzed parameters. However, female doe exposure to urine, especially to male urine, slightly increased fertility levels when compared to the rest of the experimental conditions. Female-female interaction before artificial insemination, which is a common practice in rabbit farms, did not have any effect, which suggests its removal to avoid unnecessary animal management and time cost. On the other hand, fertility ranges were lower for animals with pale vulvar color whereas no differences were noticed among the other three colours which measure receptivity (pink, red, purple), thus suggesting that these three colours could be grouped together. Additionally, equine chorionic gonadotropin injection could be replaced with various biostimulation methods, therefore reducing or replacing current hormonal treatments, and contributing to animal welfare and to a natural image of animal production.

Keywords: rabbit, biostimulation, reproduction, pheromones, urine, seminal plasma, chemocommunication, olfaction

1. Introduction

Socio-sexual behaviours, such as fighting and mating, are essential for animal reproduction and survival [1,2]. In nature, individuals are continuously exposed to sensory signals from conspecifics and the environment, allowing them to communicate between themselves and modulate their behaviour and reproductive physiology [3]. In high performance livestock, animals are usually kept indoors with less access to natural stimuli. Therefore, implementation of techniques based on interaction with natural cues, has the potential, not only to increase their reproductive efficiency, but also to allow individuals to develop their own natural behaviour, thus enhancing animal welfare.

Biostimulation is a natural technique employed in animal production to enhance reproductive parameters, and is based on modulating external environmental cues (visual, olfactory, pheromone, tactile, auditory, social and nutritional cues – among many others yet to be discovered) which elicit specific behavioural and endocrine responses in conspecifics [4,5]. Despite biostimulation methods usually entail a mix of various external cues [6], pheromone signals play a pivotal role since they can trigger sexual behaviours by influencing reproductive physiology [7-9]. Indeed, the terms biostimulation and pheromone communication have been confusedly interchanged by the literature [6,10]. Pheromones are defined as chemical signals exchanged between organisms of the same species, causing a specific reaction in the receiver [11,12]. For instance, the sex pheromone 'darcin' is released in mice male urine and elicit sexual attraction of females [13]. These chemosensory cues are carried in bodily secretions (i.e. urine, seminal plasma) [14-16] and exocrine glands (i.e. lacrimal, mammary, mentonian, Harderian) [9,17,18].

Pheromone communication together with other visual and auditory cues participate in the biostimulation method called 'male effect', in which females exposed to sexually active males trigger activation of luteinizing hormone (LH) secretion and synchronized ovulation [19]. In rabbits, 'male effect' points to an improvement of doe reproductive performance [20] especially in does at first lactation [21]. However, little is known about the bodily secretions and pheromone cues involved in such behaviour. Similarly, female-female interaction elicits a reproductive response in females after interacting between them [22]. In rabbits, despite studies to date are not conclusive at improving doe reproductive parameters by female-female interaction [23], placing together two females before artificial insemination (AI), has become an established routine as a biostimulation method in rabbit farms. Further studies are needed to validate the actual improvement of rabbit performance when two females are placed together before AI, especially due to the additional animal handling and greater workforce needed for this practice. Other biostimulation techniques such as lighting control [24], feeding control [25] and mother-litter separation [26] are also commonly used in rabbit farms.

Due to the induced-ovulation nature of female rabbits, various biostimulation methods are generally used in conjunction with hormone treatments to ensure ovulation and reproductive efficiency [27]. Gonadotropin-releasing hormone (GnRH) (or its analogues) is generally used at the time of insemination, either by intramuscular or intravaginal (into the insemination straw) administration [28-30] to induce luteinizing hormone (LH) peak triggering ovulation [31]. Recently, nano-drug delivery systems have emerged as a promising method to reduce GnRH dose in rabbit does [32]. Additionally, equine chorionic gonadotropin (eCG) -intramuscular injection 48-72 h before AI- is also used to synchronize oestrus [33] and it has been proved to increase receptivity, and prolificacy. However, repeated use of this hormone can induce immune response [34] and affect ovary function [35], with the consequent loss of reproductive efficiency [36]. Interestingly, several studies have shown that biostimulation methods (lighting and feeding programs, and/or mother-litter separation) could replace eCG administration [25,37,38], thus demonstrating that biostimulation methods are powerful tools that could potentially replace the use of hormones in rabbit farms.

Accordingly, the current study aimed to gain insights into the role of pheromone communication in rabbit doe reproduction, which could potentially reduce or replace current hormonal treatments in rabbit performance. Specifically, our objective was to

shed light on 1) the effect of female urine exposure, 2) male urine exposure, 3) seminal plasma exposure and 4) female-female (F-F) interaction, prior to AI, on improving the reproductive and productive performances in rabbit does.

2. Materials and Methods

2.1. 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. (Rodeiro, 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 from commercial hybrid (Hyplus strain PS19, Grimaud Frères, Roussay, France), and males were 5 - 7 kg weight from Hyplus strain PS40. Males and females were located in separated farms.

2.2. Sample collection

Urine: Pools of 330 ml of urine were obtained by ultrasound-guided cystocentesis from mature males and females (> 180 days), 24 h before the behavioural experiment was performed, and kept at 4ºC overnight. Pure urine was used in all cases.

Seminal plasma: Obtained from an AI Center, 24h prior to the behavioural experiment, from 60 mature males (> 180 days). All ejaculates were mixed together and centrifuged at 3000 rpm, 10 min, to obtain the seminal plasma, which were then kept at 4ºC overnight. Before use, it was diluted 1:3 in Ringer Lactate Solution.

2.3. Semen processing and artificial insemination

To perform the AI, semen was collected with artificial vagina and stored at 16 ºC before use within a 24 h period. Once the ejaculates were collected, they were pooled and diluted with a commercial extender (MRAbit® (Alarelin); Kubus SA) to a standard concentration of 60×10^6 spermatozoa ∕ ml. Does were vaginally inseminated using disposable plastic pipettes, receiving a dose of 30×10^6 spermatozoa in a volume of 0.5 ml.

2.4. Reproductive managenent

All does employed for the behaviour experiment were between the third and ninth calving and were evenly distributed among the five experimental groups (see 'Experimental Design' in M & M). None of the animals were treated hormonally with eCG to synchronize oestrus. All does were inseminated on day 11 after parturition and were lactating a maximum of 11 kits. Sexual receptivity was confirmed by determining the color of the vulva (pale, pink, red, purple) at the time of AI [25]. Pregnant or lactating does were fed *ad libitum* whereas non-pregnant or non-lactating does were restricted to 150 g ∕ day of commercial food except in the period from 6 days 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 program of 12 h (light) L ∕ 12 h (dark) D, which was changed to 16 h L ∕ 8 h D 6 days before does AI. After AI, light hours were decreased 1h ∕ day during 4 days until coming back to the normal program. Controlled suckling was applied to all does from 0 to 10 days post-partum, by keeping the nest door closed and only opening it every 24 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 6 h 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 completed parturition, the prolificacy and number of born alive and dead kits ∕ litter were recorded. Then, the number of rabbits per litter was adjusted to 11 kits of equal body size.

2.5. Experimental design

We conducted a behavioural experiment in does to determine whether female reproductive parameters (receptivity, calving rate, prolificacy and number of born alive and dead kits ⁄ litter) vary according to four given conditions: 1) Exposure to female urine, 2) Exposure to male urine, 3) Exposure to seminal plasma and 4) Female-female (F-F) interaction regarding controls (F-F separated) (Table 1). The experiment was repeated three times (three time points), every 42 days (in three consecutive inseminations). Each of the five groups were composed of 60 does, and for the first time point all animals were lactating does between third and seventh calving. Note that the same animals were kept during the three time points, and therefore in the second and third time points some does could be non-lactating –if they were not pregnant, also called 'negative does'–; these animals were not considered for the statistical analysis. Additionally, some animals were eliminated during the experiment, mainly due to health reasons during the peripartum, and they were also removed for the statistical analysis. When possible, they were replaced by other animals under the same conditions. A total of 734 animals were evaluated, all of them individually monitored for each of the reproductive parameters analyzed.

For conditions 1, 2 and 3, the corresponding stimulant was sprayed in the nose area 1 h, 15 min, and 1 min before insemination; specifically, 1 ml nasal spray was used 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 permanent exposition of the animal to the stimulant –some animals gnawed it–. AI and the corresponding handling were always performed by the same farm workers to reduce statistical noise.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Data on receptivity and kindling rates were analyzed by χ^2 and prolificacy and number of born and dead were analyzed using analyses of variance (Anova procedure), considering the distributions of the variables.

A binary logistic regression was performed with calving rate (yes/no) as dependent variable, while univariate general linear models (GLM) were performed with total born, alive and dead kits/litter as dependent variables, in both analyses, taking the number of insemination (1st, 2nd and 3rd), the number of calving (1st, 2nd, etc.), the experimental group (urine female, urine male, etc.), and receptivity (vulva colour) as independent variables. These analyses aimed to determine the factors that influence calving rate (fertility) and prolificacy, respectively. For the logistic regression, the most predictable variables were tested by using the method "Backward conditional" Hosmer and Lemeshow test (*p*-value > 0.5).

In all cases, differences were considered statistically significant at $p < 0.05$ level.

3. Results

We estimated the reproductive parameters of a total of 734 female does (receptivity, fertility, prolificacy and number of born alive and dead kits ⁄ litter) when they were exposed to different biostimulation conditions: exposure to either female or male urine and seminal plasma as potential source of pheromones, and also physical interaction (F-F interaction, during the 15 minutes before AI) as a source of chemical communication between individuals taking a group of F-F separated females as control.

3.1. Fertility

When assessing fertility (calving rate) (Table 1), we found no significant differences between experimental groups (Fig. 1). However, females exposed to urine, especially those exposed to male urine, showed slightly higher calving rates, although not significant (p value > 0.05).

In the third insemination, female does showed significantly higher calving rate (p value < 0.01) than in the first and second inseminations (Table 1). It should be noted that in all cases calving rate was above 90%, and a range between 85-98% lies within the usual rate of this farm depending on different reasons (i.e. animal management, diet, environmental and external factors, etc.).

Table 1. Fertility (calving rate), prolificacy (total born) and number of born alive and dead kits / litter considering experimental group, insemination number, vulvar colour and calving number. N: number of positive/total animals. SD: standard deviation. *Different letter in the same column indicates p-value < 0.05.*

| Experimental Group	N (fertility)	Fertility%	N (prolificacy)	Prolificacy ±SD	Alive ±SD	Dead ±SD
Urine_female	138/146	94.5	138	12.76 ± 3.2	11.83 ± 4.15	0.93 ± 2.38
Urine_male	144/151	95.4	144	13.18 ± 3.38	12.09 ± 4.18	1.09 ± 2.79
Seminal_plasma	127/137	92.7	127	13.53 ± 3.36	12.87 ± 3.41	0.67 ± 1.99
F-F interaction	137/148	92.6	137	13.29 ± 3.65	12.33 ± 4.02	0.96 ± 2.35
F-F separated	140/152	92.1	140	13.6 ± 2.99	12.68 ± 3.4	0.92 ± 2.49
Insemination Number						
1	234/259	90.3a	234	13.46 ± 3.73[abc]	12.35 ± 4.2	1.11 ± 2.39
2	224/244	91.8a	224	13.55 ± 2.97[ab]	12.72 ± 3.44	0.83 ± 2.15
3	228/231	98.7b	228	12.78 ± 3.19[ac]	11.98 ± 3.88	0.81 ± 2.7
Vulvar Colour						
Pale	09/10	90	9	9 ± 3.77[a]	8.56 ± 4.3[ac]	0.44 ± 1.01
Pink	352/377	93.4	352	13.49 ± 3.18[b]	12.63 ± 3.58[b]	0.87 ± 2.38
Red	256/276	92.8	256	13.19 ± 3.34[b]	12.16 ± 4.09[b]	1.03 ± 2.58
Purple	69/71	97.2	69	12.95 ± 3.61[b]	12.12 ± 4.08[c]	0.84 ± 2.18
Calving Number						
3	52/56	92.9	52	14.13 ± 4.17	13.15 ± 4.32	0.98 ± 1.84
4	144/157	91.7	144	13.34 ± 3.27	12.58 ± 3.88	0.76 ± 2.26
5	126/138	91.3	126	13.53 ± 3.13	12.94 ± 3.46	0.6 ± 1.8
6	120/130	92.3	120	13.4 ± 3.48	11.89 ± 4.42	1.51 ± 3.29
7	120/128	93.8	120	12.7 ± 3.4	11.77 ± 3.78	0.93 ± 2.27

| 8 | 85/86 | 98.8 | 85 | 13.03 ± 2.69 | 12 ± 3.47 | 1.04 ± 2.86 |
| 9 | 39/39 | 100 | 39 | 12.82 ± 3.33 | 12.44 ± 3.36 | 0.38 ± 1.13 |
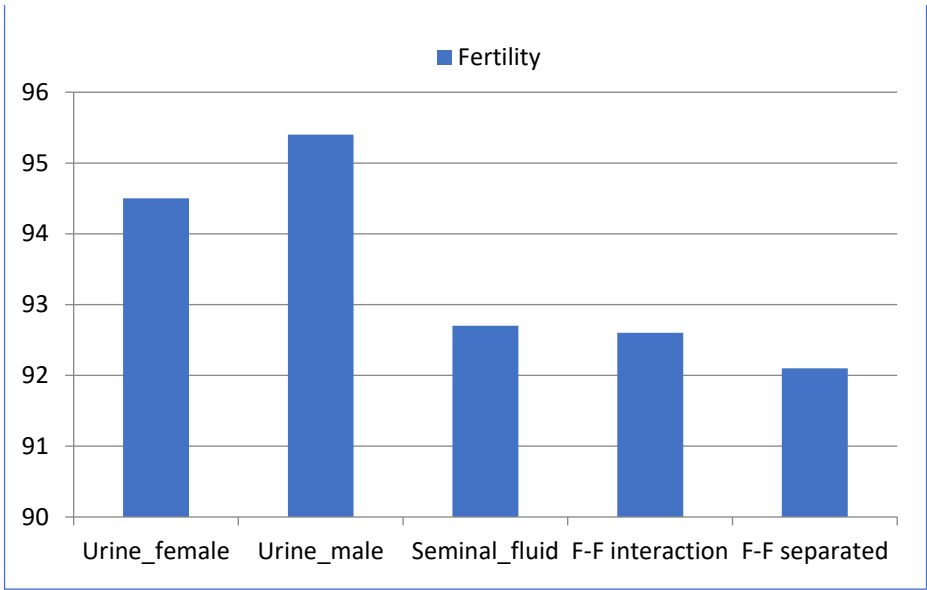
Figure 1. Fertility (calving rate) in each of the different experimental groups.

On the other hand, receptivity rate measured by vulvar colour (Fig. 2) did not show a significant impact on fertility (p value > 0.05), even though our results point to higher calving rate in females with purple vulvar colour (97.2%) than those with pale vulvar colour (90%) (Table 1). Finally, calving rate seems to be not influenced by calving number, despite females in their 8th and 9th calving showed higher calving rate. This could be an artifact, especially because a significant fewer number of animals were considered for these two calving. Of note, in the first trial there were only animals from 3rd to 7th calving, and only in the second and third trials animals of 8th and 9th calving, respectively were employed.
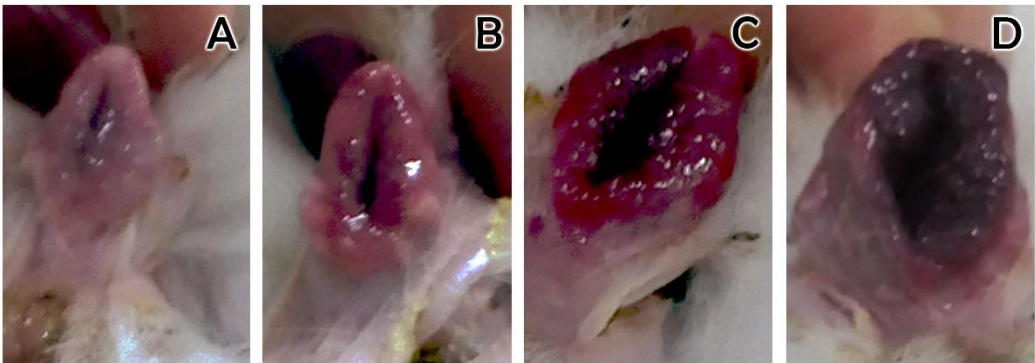

Figure 2. Vulvar colour of rabbit doe. A: pale; B: pink; C: red; D: purple.
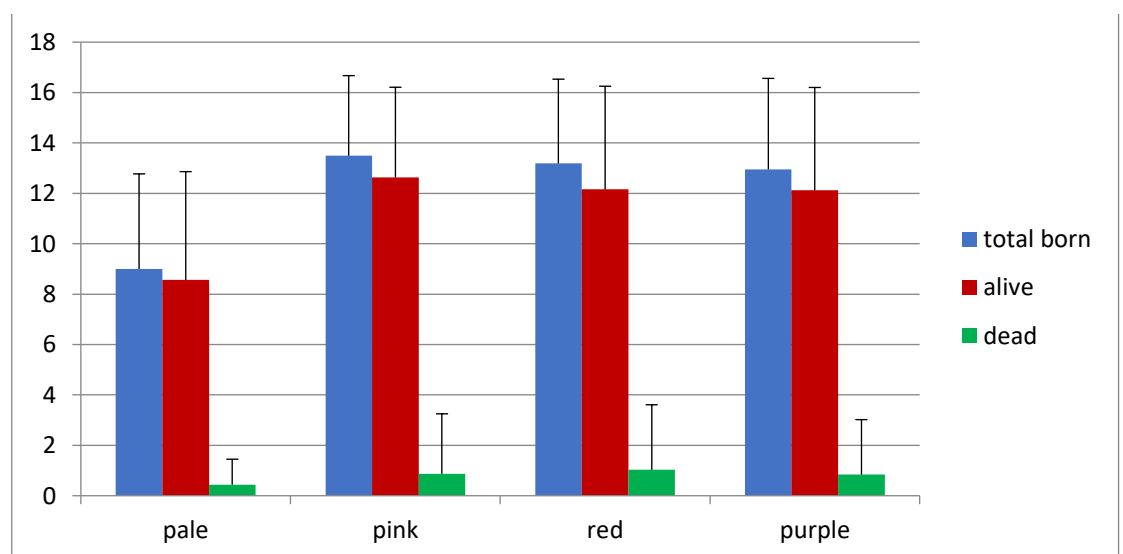
3.2 Prolificacy

We found no prolificacy differences between the five experimental groups (Fig. 3). The mean of the total born animals considering all the conditions presented in Table 1 (experimental group, insemination number, vulvar colour, calving number) was 13,04 ± 3,36 (12.16 ± 3.86 alive and 0.88 ± 2.25 dead). This indicates that female exposure to urine of both females and males does not have an impact in prolificacy. Similarly, F-F interaction and the control group (F-F separated) showed similar results. Prolificacy was sig-

nificantly different between second and third insemination (p value > 0.05), which highlights the importance of considering different insemination times due to physiological reasons or farm conditions.
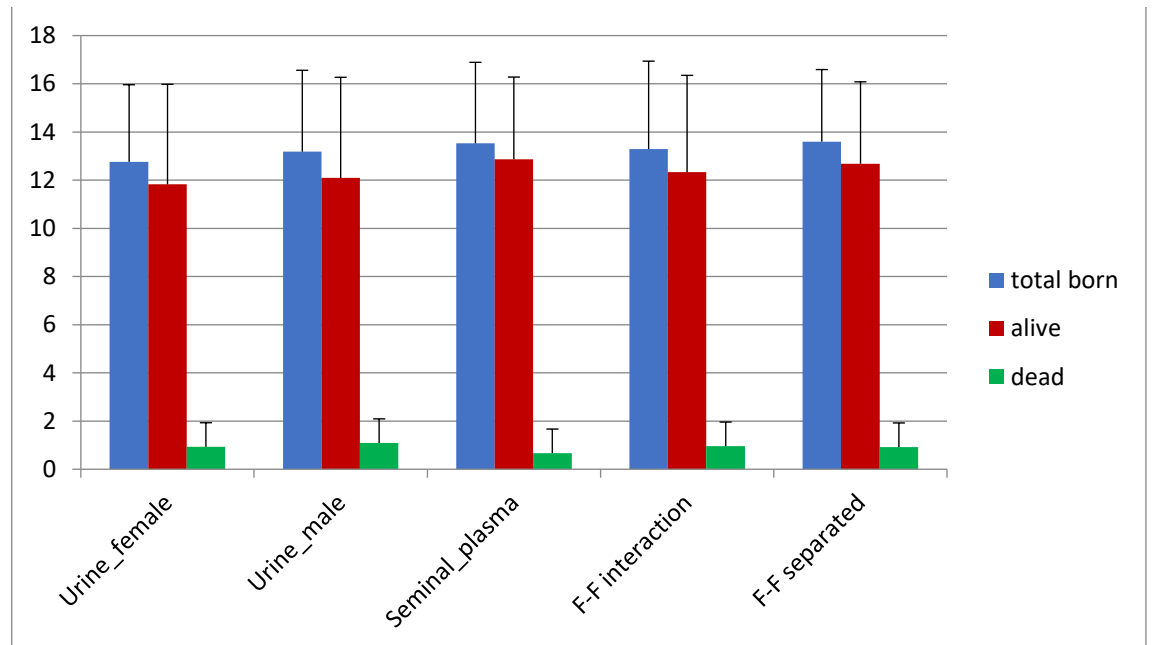
**Figure 3. Prolificacy rate (number of born alive / dead animals) in each of the different experimental groups**.

Moreover, we did find significant differences in the prolificacy rate depending on the receptivity (Fig. 4). Four vulvar colours have been previously described to assess receptivity rate: pale, pink, red and purple (Fig. 1), showing increasing levels of receptivity [25]. We saw that females showing pale vulvar colour at the time of AI had a significant reduced number of total born kits when compared to females with pink and red vulvar colours (p value < 0.01). Our data confirmed that prolificacy depends on sexual receptivity, but only when considering 'pale' with 'lower prolificacy' vs 'not-pale or the sum of pink, red and purple', with 'higher prolificacy.

Figure 4. Prolificacy rate depending on the receptivity (vulvar colour).

3.2 Receptivity

When looking at the receptivity rate (vulvar colour), the experimental group 'F-F interaction' showed the highest purple vulvar colour (Fig. 5), but importantly, this did not affect fertility and prolificacy parameters, as previously explained. As a qualitative estimation, we also found a strong 'riding behaviour' in this group when the two does were placed together before AI. On the other hand, the group that showed the highest percentage of pale vulvar colour was the control 'F-F separated' (3.3% of all 'F-F separated') (Fig 4). This percentage is quite low and overall, only 10 out of the 734 (1.3%) individuals used for the analysis showed pale vulvar colour, which indicates successful levels of female estrus synchronization in the farm.
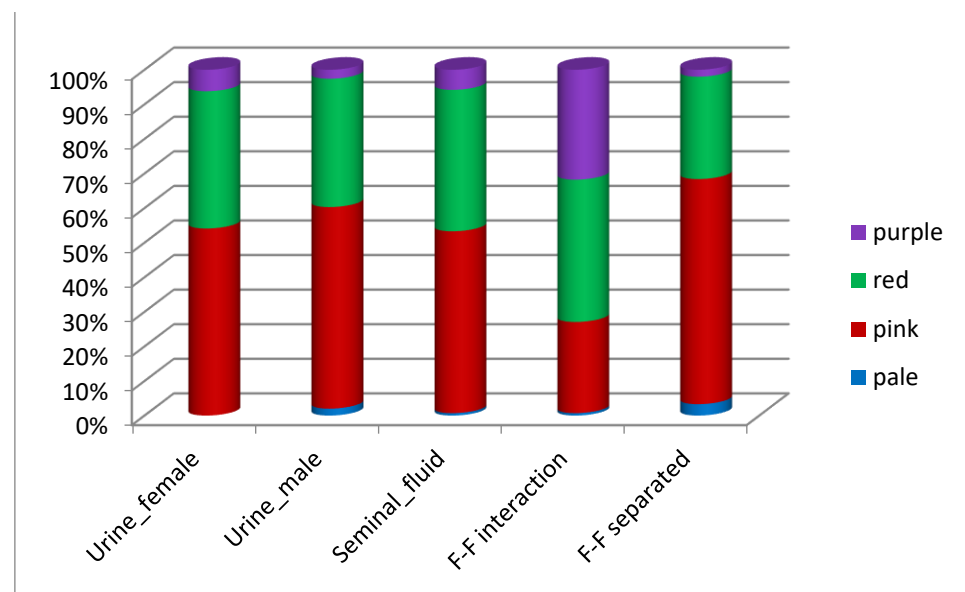
Figure 5. Receptivity rate (vulvar colour) depending on the experimental group. Among variables statistical comparisons.

We also performed a binary logistic regression to estimate the relationship between fertility levels and the experimental group, number of insemination, vulvar colour and number of calving. The results were similar to those obtained with the chi-squared test. The experimental group did not influence fertility, and the only significant independent variable was the insemination number (Table 2). Note that vulvar colour did not significantly influence calving rate in this model likely due to the low number of females presented with pale vulvar colour - no statistical power.

Table 2. Final model of the binary logistic regression. Dependent variable: calving rate. The only predictable independent variable was the number of insemination (p-value = 0.03).

Variable	Values	OR (ods ratio)	Confidential interval for OR	P-value
Insemination	first	reference		
	second	1.2	0.646-2.215	0.568
	third	8.12	2.418-27.266	0.001

Finally, significant results (p value < 0.05) were obtained in the univariate general lineal model (GLM) analysis when only considering 'prolificacy / number of born alive and dead kits / litter' as dependent variables. Considering as dependent variable 'born dead kits', we found significant influence with the interaction between 'experimental group', 'vulvar colour' and 'number of calving'. Considering as dependent variable 'born

alive kits', we found significant influence only with 'vulvar colour'. Considering as dependent variable 'total born', we found significant influence with 'vulvar colour' and also with the interaction between 'insemination number', 'experimental group' and 'vulvar colour'. Accordingly, the GLM analysis confirmed a significant influence between prolificacy levels and receptivity rate (vulvar colour), insemination number and experimental group.

4. Discussion

We did not find any significant improvement in female rabbit performance when they were exposed to female urine, male urine or seminal plasma. Similarly, F-F interaction prior to AI did not show higher fertility or prolificacy levels when compared to the control group (F-F separated), or with any other experimental group.

4.1. Social interaction seems not influencing reproductive physiology in farm female doe

In nature, individuals interact among them influencing their reproductive physiology [39]. The biostimulation method 'male effect' has been a valuable management tool exploited in small ruminants [19,40,41] and swine [42] husbandry to stimulate the onset of puberty and to reduce the postpartum period. In cattle, 'male effect' has received little ductive activity [43], the literature is not consistent [6,42], and therefore this practice has not yet been implemented as a common farm routine. In rabbits, 'male effect' appears to slightly improve doe reproductive performance [20]. However, significant effects were only found in does at first lactation [21] and published data have been contradictory [44,45], hampering consistent conclusions.

Females also elicit a reproductive response to female-female interaction, and female chemical signals play important roles in sexual attraction [46]. Specifically, reproductive response to F-F interaction has been shown in goat [47], wild boar [48], human [49] and beef cow [50]. In rabbits, we found an increase in receptivity rate (vulvar colour) in the experimental group F-F interaction -highest number of females with purple vulvar colour (Fig. 4)- but importantly, this did not affect fertility and prolificacy parameters. Additionally, we also found strong 'riding behaviour' in the F-F interaction group (qualitative estimation) which could confuse operators, who might associate such behaviour to higher fertility and prolificacy rates. Considering that F-F interaction is used as a common biostimulation method in rabbit farms, we argue that since no differences in fertility and prolificacy rates were noticed between experimental groups in our study, such management should be reconsidered in order to reduce animal handling and a substantial time cost. Likely this management might offer better results in farms with lower fertility, where there is higher room for improvement.

4.2. Urine as a potential source of sex pheromones in female doe reproduction

Despite no significant differences were found between experimental groups, females under male and female urine exposure before AI reached the highest levels of fertility, especially when exposed to male urine (95.4% with male urine and 94.5% with female urine, compared to 92% in the rest of the groups).

Urinary pheromones have been largely studied in mice and are known to influence sexual behaviour [13,51]. Indeed, the 'Whitten effect (1958)' [52] refers to female estrus synchronization when they are exposed to male urine [53]. In farm animals, urine has been shown to accelerate puberty in cattle [54], whereas in goats did not improve reproductive parameters [55]. Interestingly, females have preference towards urinary pheromones of dominant mice, but not towards subordinate ones [56]. In our experiment, we did not consider differences between dominant and subordinate males since urine from both was pooled. Further studies should consider only urine from dominant males.

4.3 Seminal plasma might arise as a new source of pheromones

Although not as widely known as urine, seminal plasma might also be a reliable source of pheromones. In rabbits, a lipocalin was found in seminal plasma, showing significant similarity with 'urinary' and 'salivary' pheromone carriers [14]. More recently, [15] Scott et al. (2019) identified a sex pheromone in seminal plasma of sea lamprey. We did not find significant improvement of female doe reproduction when exposed to seminal plasma before AI. However, we should consider that seminal plasma was diluted 1:3 and higher concentration might render better results. Similarly, it might be the case where only dominant males release these molecules, and therefore pheromone power has got diluted by pooling seminal plasma from all males.

Interestingly, seminal plasma is known to contain an ovulation-inducing factor (OIF) in several species [57,58] including rabbits [59]. OIF has been identified as a β neurotrophin (β-NGF) [57], which modulates ovulation [60], and has been suggested to have direct action on GnRH neurons outside the blood barrier [58]. Intramuscular injection of seminal plasma containing β-NGF showed a positive effect in llama but not in rabbit ovulation [59]. However, adding β-NGF to seminal dose has been proposed to replace GnRH in rabbit reproduction [61,62]. Despite their site and mechanism of action are unknown, β-NGF action appears to involve hypothalamic kisspeptin neurons [60]. Since male odors detected through the vomeronasal organ (main pheromone-receiver organ) are known to activate kisspeptin neurons in female mice [63], we argue that β-NGF might act as a pheromone or pheromone carrier, which triggers activation of kisspeptin neurons in the central nervous system and modulate ovulation. Further studies should be performed to determine whether female nasal exposure to OIF activates vomeronasal and hypothalamic kisspeptin neurons, and ultimately influences ovulation. Importantly, understanding the female response to seminal plasma will eventually shed new light on human infertility and pregnancy disorders [64].

4.4 Practical considerations when assessing biostimulation methods

This study was performed in female rabbits between 3rd and 9th calving. Nulliparous and primiparous females were excluded due to their unstable reproductive parameters -nulliparous usually have better results that the average whereas primiparous tend to have worse results-. Interestingly, previous studies have shown that primiparous does are the only group to significant respond to 'male effect' [21]. This might indicate that biostimulation methods become efficient in animals with lower conception rates. Further studies should consider only primiparous females, where either 'male effect' or F-F interaction might help improve their reproductive rates.

The effectiveness of biostimulation methods or hormonal treatments depends on the basic performance –physiological, health and behavioural states– of does at the time of AI [65]. Previous reports on rabbit reproduction management have shown that biostimulation or hormonal methods improve reproductive performance in females with 50-60% of average conception rate. However, as the percentage of conception rates increased, the methods employed became less efficient, and with average conception rates of 75-80%, the difference between the control and treated groups (either biostimulation or hormonal methods) was non-significant [66].

It should be taken into account that the farm employed for our experiment has considerably overall high fertility (usually higher than 90%) and prolificacy levels, becoming difficult to achieve significant improvement of any reproductive parameter. Our experimental framework emphasizes the importance of large sample size and biological replications to obtain reliable data that allows the detection of statistical differences between the treatments and control. To test biostimulation efficiency, further studies should consider the same experimental approach in farms with lower fertility rates (50-60%).

4.5 Biostimulation methods could reduce or replace hormonal treatments

Female rabbit ovulation does not occur spontaneously, and even though ovulation triggered mechanism is unknown, coitus-related stimulus results in a rise in circulating

luteinizing hormone (LH) that causes ovulation [31]. Therefore, in rabbit farms, this hormonal state has to be artificially induced. Currently, the most frequently used method is either intramuscular or intravaginal (into the insemination straw) administration of GnRH (or its analogues) at the time of AI [28-30], and recently new nano-drug delivery systems have been proposed as a new method for reducing hormone dose and improving AI efficiency [32]. However, even though human exposure to GnRH after rabbit meat consumption is negligible (EMA/CVMP/156095/2017) [67], a report on minimum standards for the protection of farm rabbits (2016/2077(INI)) [68] highlights the importance of meeting high standards of animal health and welfare, where natural biostimulation techniques are encouraged to be implemented to reduce and potentially replace the use of exogenous hormones.

Similarly, eCG injection has probed to improve productivity [33] but biostimulation methods such as mother-litter separation could replace its use [37]. In the farm where we performed our experiment, eCG injection is used as a common routine, which implies animal handling and higher workforce. Despite none of our experimental groups were injected with any hormone, we still obtained considerably high (> 90%) reproductive parameters, comparable to those of the rest of the farm where eCG was injected (data not shown). This indicates that the biostimulation methods employed were sufficient to obtain high fertility levels without using eCG. Of note, several additional biostimulation methods –feeding restriction, mother-litter separation, and lighting control– were also employed in all experimental groups as a common farm routine, and a mix of all of them was likely responsible for the high reproductive efficiency obtained. In any case, we highlight that eCG could be replaced by other biostimulation methods in farms with high reproductive performance. Further studies considering farms with lower reproductive parameters should aim at determining whether eCG could also be replaced by biostimulation methods.

All in all, biostimulation methods have arisen as an alternative way of employing exogenous gonadotropins to improve sexual receptivity and, consequently, the overall productivity of rabbit farms [21]. This is especially important in the context of a society where consumers are contrary to the use of hormones in animal production [66] wishing to buy products labeled as produced with no added hormones [69]. Future research in the field should aim at replacing hormones with different biostimulation methods, thus enhancing animal welfare and greatly improving the image of our industries in society.

5. Conclusion

The biostimulation methods employed in this experiment did not improve any of the analyzed parameters. However, female doe exposure to either male or female urine slightly increased fertility levels when compared to the rest of the experimental conditions. Additionally, F-F interaction before AI did not increase fertility and prolificacy levels, and eCG injection could be replaced with various biostimulation methods. Therefore, both F-F interaction and eCG injection could be removed in high performance farms. Future studies should approach primiparous females (lower reproductive levels) and farms with lower fertility rates (50-60%), where there is more room for improvement. Also, biological fluids used as a source of pheromones should be especially obtained from dominant males. Finally, biostimulation methods based on chemical communication could potentially reduce or replace current hormonal treatments, and therefore contribute to animal welfare and to a natural image of animal production.

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