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BIOSTIMULATION AND REPRODUCTIVE PERFORMANCE OF ARTIFICIALLY INSEMINATED RABBIT DOES (*ORYCTOLAGUS CUNICULUS*)

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Abstract: Biostimulation is a non-hormonal and practical technique that has not yet been widely utilised when applied immediately before insemination to improve reproductive efficiency in livestock species. This study was conducted to determine the influence of short-term male biostimulation on behavioural and reproductive performance of inseminated rabbit does. A total of 142 female New Zealand White rabbits were randomly assigned to 3 groups. Females were either exposed to male odour (Odour group) or an adult aproned male (Male group), while the remaining does that were neither exposed to the male odour nor the adult male are considered the control group. All females were inseminated after the 2 h exposure session. Conception rates were determined by abdominal palpation 12 d after insemination. The results showed that conception rate of the male odour group (79.59%) was greater than that of male presence group (76.09%) and that of the control group (68.09%). Moreover, biostimulated does showed significant behavioural activities during the 2 h exposure session compared to the control group. Although no significant differences were recognised, litter size at birth and at weaning was slightly increased in biostimulated compared to control females. Nor were there any significant difference in serum oestradiol concentrations between treated groups. Conclusively, short-term 2 h biostimulation of rabbit does resulted in the appearance of various behavioural responses followed by differences in conception rates between groups after routine artificial insemination.

Key Words: rabbit, reproduction, biostimulation, artificial insemination, behavioural, hormonal.

INTRODUCTION

Animals are daily challenged by myriads of sensory signals from the surrounding environment (Fleischman *et al.,* 2016; Boucaud *et al.,* 2016) that induce specific modulations of behavioural and physiological responses in many species. In particular, chemical cues are thought to be the primary source of information exploited by animals to gauge the ever-changing environmental circumstances, including predation threats (Korgan *et al.,* 2016), food choices (Thibert *et al.,* 2016), habitat selection (Lecchini 2011) and, notably, their reproductive strategies (Baum and Cherry, 2015; Caro *et al.,* 2015; Fujiwara *et al.,* 2016).

Pheromones are chemical elements emitted by one individual and received by another individual from the same species, triggering a broad array of behavioural and endocrine activities (Brennan, 2010, Hegab *et al.* 2015). These responses are reciprocally integrated into the male-female interaction. For example, an introduction of female pheromones elicits significant elevation in male sex hormones that regulate spermatogenesis in male Syrian hamsters (Richardson *et al.,* 2004). Pheromones from male mice also play a significant role in mate choice and neuroendocrine functions in females (Asaba *et al.,* 2014). Remarkably, pregnant female mice that sniffed pheromones of unfamiliar males abort their pregnancies in a phenomenon known as "*Bruce effect*", but they synchronise their oestrous cycles after exposure to the pheromones of a dominant male; this term is recognised as "*Whitten effect*" (de Catanzaro, 2015).

Another striking example of the Whitten effect was demonstrated in domestic animals. Gelez *et al.* (2004) found that anoestrous ewes subjected to ram odour showed endocrine activation and restoration of cyclicity in most females. Yet another confounding situation, slightly similar to the Whitten effect, has been described in rabbits as the "*Buck effect*". The difference between the Whitten and buck effects lies in the fact that the former depends exclusively on the pheromones or odours from a male, while the latter encompasses multiple visual, acoustic and olfactory cues. Several studies have been conducted to illustrate the differences between the female behavioural and endocrine responses to both effects (Ola and Oyegbade, 2012; Ola and Olatunbosun, 2013), with some limitations found in the sole exposure to male odours (Gelez *et al.,* 2004). However, this discrepancy across the literature deserves further investigation.

High reproductive performance in artificially inseminated rabbit does is a multifaceted criterion that depends on the physiological and behavioural repertoire of females at the time of insemination (Castellini, 1996; Theau-Clément, 2007). Receptivity is a key element which greatly affects both productive and reproductive traits of rabbit does, so that poor rates of receptive does during insemination result in a less successful insemination process (Theau-Clément *et al.,* 2015). Sexual biostimulation procedures, which act through sensory signals, have a potential effect on both receptivity and fertility of rabbit does. Indeed, the hormonal regulation of receptivity in rabbit does appears to be strongly linked to oestrogen levels, as it is generally used to assess follicular growth and ovarian functions (Marongiu and Dimauro, 2013). Interestingly, ovariectomised rabbits restored signs of sexual receptivity upon oestrogen treatments (McDonald *et al.,* 1970).

The aim of the current study is to shed some light on the effect of male odour or male presence prior to artificial insemination on improving the reproductive and productive performances in rabbit does. The dearth of literature comparing the 2 effects renders this point worthy of research. In the current experiment, we hypothesise that presence of a male rabbit or its odour will elicit robust behavioural, endocrine and reproductive changes in rabbit does.

MATERIALS AND METHODS

Animals and Management

This work was conducted on a rabbitry section of the farm of the Faculty of Agriculture, Suez Canal University, Ismailia, Egypt between September and December 2016. One hundred and forty-two mature (3.421±0.070 kg) New Zealand White rabbit does were used in this experiment. All does were housed individually in a wired cage measuring 40×30×20 cm provided with a kindling box (30×15×20 cm). Animals were kept on a constant 12L:12D light cycle at 21-23°C. All animals were fed *ad-libitum* isonitrogenous and isocaloric commercial diet. The diet contained 18% crude protein, 2.8% fat, 10% crude fibre and 2600 kcal digestible energy/kg diet, which meets the requirements according to NRC (1977). The animals were kept under the same environmental and managerial conditions throughout the experiment. Wood shavings and shredded papers were supplied as bedding materials in the kindling box 5 d prior to parturition. All procedures were in compliance with the Institutional Animal Care and Use Committee of the Faculty of Agriculture, Suez Canal University (Ismailia, Egypt).

Testing apparatus

A wooden box (2.5×2.5×1 m) was used to assess the behavioural responses of rabbit does to different treatments. The floor of the testing arena was divided into small-square (20×20 cm) areas marked with black lines. The testing apparatus was mounted with a video network camera (Panasonic® WV-NS202AE) for recording and scoring of behaviours. Rabbit does were transferred from the cages to the testing room by a familiar person. A computer was linked to the recording cameras and was located outside the testing room.

Odour source

Rabbit male odour was obtained by placing a towel in a cage beneath an adult male for approximately 3 d prior to the experiment. Control towels were clean towels stored in plastic bags prior to use. The towels were transported to the experimental room just before testing (Masini *et al.,* 2005).

Experimental procedures

All rabbit does were handled for 5 d prior to the start of the exposure session. Handling included lifting does, catching them for 1 min, freeing them again into cages, and then conveying them to the testing room without introducing them to the test apparatus. Does were handled with rubber gloves. The test was divided into 2 parts: adaptation and testing sessions. At the beginning of the experiment, each doe underwent a familiarisation time on 2 successive days before the experiment, throughout which they were placed in a test apparatus for 10 min with neither odour nor male present.

Animals were randomly allocated into 3 groups; the first group served as Control group (n=47) where animals were exposed to a clean towel, the second group (Odour group; n=49): animals exposed to the towel contains the male odour and the final group (Male group; n=46): animals exposed to a clean towel in the presence of an aproned (diaper-wearing) male. Both clean and odour-containing towels were fixed on the central squares of the arena. The testing session lasted for 2 h after each session, faeces and other dirt were removed and the testing arena was wiped out with 5% alcohol.

Semen collection and artificial insemination

Prior to the end of the exposure session, semen from 10 healthy mature (1-yr-old) rabbit bucks was collected using an artificial vagina. Only visually and microscopically-passed sound semen samples were pooled and extended with Tris buffer extender just before the insemination process. Semen diluents were composed of 300 mM Tris (hydroxymethyl) amino methane, 94.7 mM citric acid, 27.75 mM fructose, 20% egg yolk and antibiotics (Penicillin 50000 IU and streptomycin 50000 µg per 100 mL extender). After the testing session, all females were artificially inseminated by the method described by Quintela *et al.* (2004). Freshly diluted semen was deposited in rabbit does using a sterilised glass tube. Each doe received 0.5 mL, containing approximately 5×10^7 spermatozoa. All does were intramuscularly injected with 0.2 mL GnRH Analogue (Receptal®, Hoechst Marion Roussel, S.A., Madrid, Spain) immediately after the insemination process for induction of ovulation (Morrell, 1995).

Pregnancy diagnosis was performed by abdominal palpation 12 d after insemination. Pregnant and non-pregnant does were recorded to calculate fertility percentages. At parturition, the total number of young born was recorded to calculate the prolificacy. Conception rates (%) and litter sizes at birth and at weaning were recorded.

Measurement of behavioural activities

The following behavioural activities were considered in this experiment: (1) Contact: the does make direct tactile contact with the towel, including chewing (Hegab *et al.,* 2014); (2) Chasing: One rabbit chasing the other around the spot; (3) Resting: does lie on the ground with stretched body (Prebble *et al.,* 2015); (4) Locomotion: any movement from one marked section to another commenced by line crossing (Dielenberg and McGregor, 2001); (5) Others: including grooming, rearing, freezing, sniffing, etc. Scan sampling technique was used at 1 min intervals during the recording session (Paul and Patrick, 2007) and the number of animals that displayed each behavioural element was recorded. Later, these numbers were expressed as a percentage of individuals in each behavioural category. Each testing session was analysed using the Behavioural Observation Research Interactive Software (BORIS, v. 2.95, University of Torino, Torino, Italy).

Measurement of serum oestradiol levels

Prior to insemination, blood samples (3 mL) were withdrawn into non-heparinised tubes from the marginal ear vein of 10 does from each group. Immediately after collection, the blood samples were centrifuged at 3000 rpm for 20 min and the collected serum was stored at −20°C prior to the assessment of serum oestradiol (E2) concentration using the ELISA kit (BioCheck®, Foster City, USA), according to the manufacturer's instructions. The assay sensitivity was <10 pg/mL. The intra- and inter-assay coefficients variations were 5.5 and 4.8%, respectively.
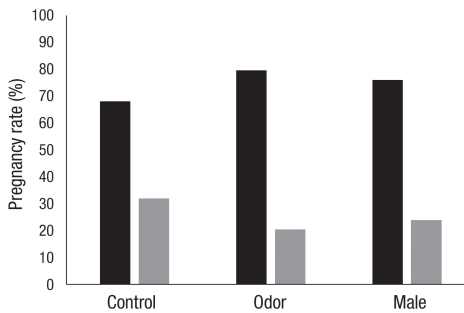
Figure 1: Percentage (%) of pregnant and non-pregnant rabbit does in different treatment groups after artificial insemination. ■ Pregnant; ▪ Non-pregnant.

Statistical analysis

SPSS 22.00 software was used for all analyses. Percentages of animals that performed each behavioural category were analysed between treatment groups using the Kruskal-Wallis test [X^2(df)], as the values did not show any normality of distribution. Pearson Chi-square test was used to compare the number of pregnant and non-pregnant females between treatment groups. One-way analysis of variance (One-way ANOVA) was used with treatment groups as fixed variable and litter size (Birth & Weaning) and serum oestradiol level as dependent variables. The level of significance at which the null hypothesis was rejected was α=0.05.

RESULTS

The results in Figure 1 showed that biostimulated rabbit does from either odour or male presence groups displayed higher percentages of conception rates than that observed in the control group. However, the outcome of the Pearson Chi-Square test performed between the 3 treatment groups (Control *vs.* Odour *vs.* Male Presence) was χ^2=1.75, P=0.44, which demonstrated that there was no statistically significant association between the 3 treatments in pregnancy rate.

As shown in Figure 2, litter size at birth was slightly increased in biostimulated groups compared to control group. The average mean values of litter size at birth were 6.29±0.43, 6.79±0.36 and 6.57±0.38 for Control, Odour and Male groups, respectively. Likewise, litter size at weaning was still higher in treated groups (6.52±0.51 and 6.31±0.46 for odour and male groups, respectively) than that in Control group (5.55±0.48). Nevertheless, there was no statistically significant difference between treatment groups as determined by one-way ANOVA in litter size either at birth (F= 0.43; P=0.65) or at weaning (F= 1.08; P=0.35).

Rabbit does displayed various behavioural activities in presence of the rabbit buck or its odour (Figure 3.). During the 2 h testing session, significant numbers of animals tested with the buck odour displayed more contact [χ^2(2)=20.746; P=0.001] and resting [χ^2(2)=43.427; P=0.001]. However, buck presence induced a significant proportion of individuals to display locomotion [χ^2(2)=13.052; P=0.001] and chasing [χ^2(2)=6.326; P=0.042] behaviours. "Other" behavioural activities in control group were significantly higher [χ^2(2)=59.400; P=0.001] compared to the "Odour" or "Male" groups.
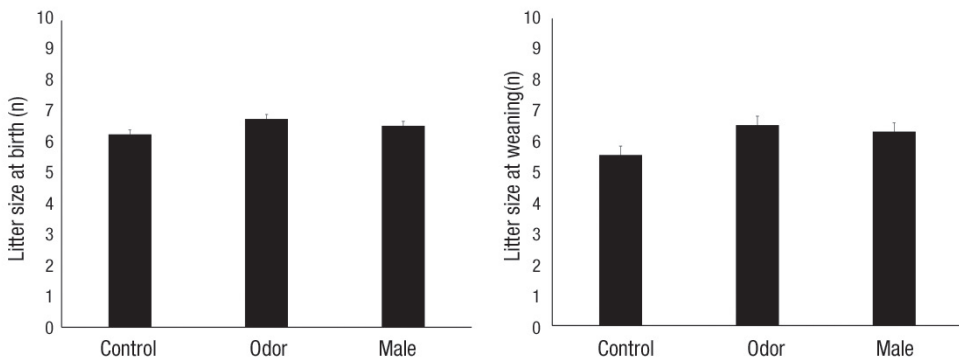

Figure 2: Effect of treatment on litter size at birth and weaning in artificially inseminated rabbit does. Error bars: standard error.

Figure 4 represented serum oestradiol concentrations between treated groups. One-way ANOVA showed that there was no statistically significant difference in serum oestradiol concentrations (pg/mL) in rabbit does subjected to different treatments ($F=0.744$; $P=0.485$).

DISCUSSION

The results of our experiment investigated whether biostimulation either by male presence or male odour had higher mean values of fertility and prolificacy of artificially inseminated rabbit does in comparison to control group. In the same vein, various behavioural responses toward odour and male presence clearly verified the assumption of the general effect of biostimulation on rabbit does.
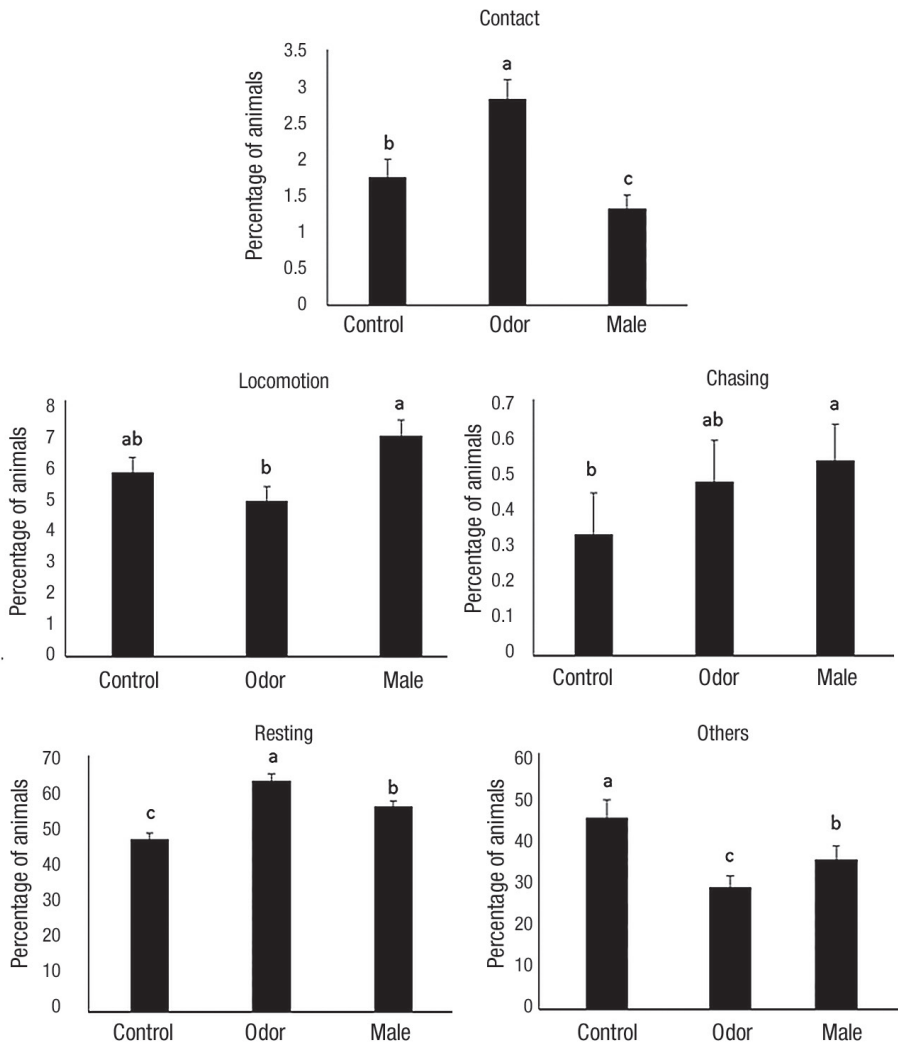

Figure 3: Behavioural activity of rabbit does during 2 h exposure to different treatments. The different letters marked over the bars are significant ($P<0.05$) between bars. Error bars: standard error.
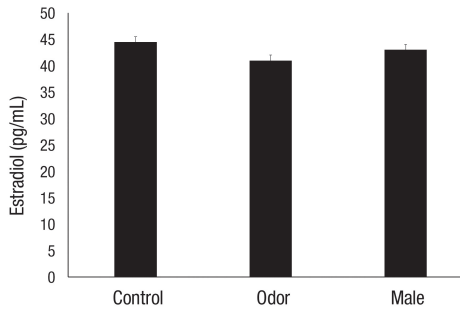
Figure 4: Mean serum oestradiol concentration (pg/mL±standard error) in rabbit does after 2 h exposure to different treatments and before artificial insemination. There were no significant differences between the different treatments (P>0.05).

Rabbit does exposed to male odour exhibited a significant increase in contact time to the odour source compared to other treatment groups. The odour source in the experiment collectively bears fur odour and other secretions from the rabbit buck and therefore acts as a source of attraction to rabbit does. In similar studies, females exposed to male odours showed a strong preference toward the odour source, suggesting that the olfactory signals might be responsible for this preference (Taylor *et al.,* 1982). Specifically, male urine possesses some alluring characteristics due to the presence of many major urinary proteins that can act as pheromones and enable sexual attraction and stimulation to females (Roberts *et al.,* 2010). Similarly, buck odour provokes a significant increase in resting time in rabbit does. Kiyokawa *et al.* (2004) found that pheromones from a donor male decreased significantly resting time in recipient male rats. Hence, it could be intuitively assumed that male pheromones trigger a "calming effect" on rabbit does. This supposition needs further inspection. Male presence also evoked a significant increase in ambulatory behaviour, including locomotion and chasing in females. This could be explained by the male's multiple attempts to mount females, which induced a general state of excitement amongst rabbit does inside the testing arena.

Our results also proved that biostimulation might contribute in some way to modifying reproductive and productive traits in artificially inseminated does. Biostimulated rabbit does yielded higher pregnancy rates and litter size at birth and at weaning compared to untreated does. Different biostimulation techniques have been used effectively in initiating and enhancing the sexual behaviour and reproductive cycles in rabbits (Eiben *et al.,* 2007; Rodríguez-de Lara *et al.,* 2010; González-Mariscal *et al.,* 2015). The buck (López-Sebastian *et al.,* 2008) and Whitten (Pallares and Gonzalez-Bulnes, 2009) effects have been proven to be a practical method to induce sexual activity in anoestral females. Bonanno *et al.* (2003) reported that presence of the buck increased the fertility of does. However, the present results suggested that the Whitten effect is superior to the buck effect in inducing induce higher pregnancy rates in artificially inseminated females. This might be attributed to the presence of concentrated major urinary pheromones (Roberts *et al.,* 2010) that had been accumulated for a long duration in the odour source. Conversely, in the male group, the design of the apron made the urine of the rabbit male inaccessible to females during the exposure session. In contrast to our results, Kustos *et al.* (2000) found that the presence of males in contiguous cages, for 3 or 4 d before artificial insemination did not influence the reproductive performance of rabbit does. We propose that caged does in the previous study did not receive enough stimuli from the neighbouring males due to the lack of direct contact between the 2 sexes.

In the present results, litter size at birth was slightly higher in treated does compared to control and this might be related to the positive influence of biostimulation on ovulation rates at insemination time (Khanh *et al.,* 2012). Concurrently, the larger litter size of treated does suggests that biostimulation around insemination may have exerted an improvement on sperm transport (Rodríguez-de Lara *et al.,* 2003). Furthermore, the number of offspring produced in a litter is the result of the interaction of several variables including both maternal and male effects (Schmidova *et al.,* 2016). Several underlying factors could be intertwined, including a failure of fertilisation or implantation and/ or intra-uterine death of embryos (Finn, 1964), which might explain the non-significant difference between treated and control groups.

The triad of productivity traits, sexual receptivity and hormonal influence has been well documented (Theau-Clément, 2008a). For example, the pregnancy rates of artificially inseminated receptive does is 3 to 4 times higher than non-receptive ones (Theau-Clément, 2008b). Likewise, sexual receptivity is well known to be strongly linked to oestrogen levels (Ubilla and Rebollar, 1995; Ubilla *et al.,* 2000; Rebollar *et al.,* 2006), as ovariectomised rabbit does injected

with different doses of oestradiol benzoate restored signs of sexual receptivity (McDonald *et al.,* 1970). Therefore, it could be concluded that higher feminine oestradiol levels are essential for the success of the insemination process. Amazingly, neither biostimulated nor control rabbit does in our study showed any significant variation in serum oestradiol levels immediately after the exposure session, although the productive traits in biostimulated individuals were higher compared to control ones. This contradiction could be discussed via multiple approaches. First, the sensory cues (tactile, olfactory or visual) perceived by females either in odour or male groups around insemination might induce a greater sensitivity of the pituitary gland to exogenous GnRH, thus subsequently enhancing follicular development and the ovulation process. Second, it is also possible that visual cues during male presence and olfactory cues (pheromone stimulation) during the odour presentation at exposure sessions may have acted in a cumulative way to cause the positive stimulatory response in rabbits (Rodríguez-de Lara *et al.,* 2003). Colloquially, those cues may initially "prime" the endocrine glands for the effect of GnRH injections and this growing effect may not reveal itself instantaneously by an increase in oestrogen concentration after the exposure session, although it might have unveiled itself some time later. Finally, and in contrast to some studies, Beyer *et al.* (2007) indicated that sex steroids may not be the sole activator of receptivity, citing some other fundamental pathways that initiate sexual behaviour in females. All the previous reasons might help to clarify the non-significant difference in oestradiol levels between the stimulated and control groups.

CONCLUSION

Taken together, our findings suggest that short-term (2-h) exposure of rabbit does to a male or its odour results in the appearance of various behavioural responses concomitantly with the increase in productive performance after procedural artificial insemination. Our results also support the notion that biostimulation protocols might enhance or strengthen the results of routine GnRH injection on ovulation and consequently pregnancy rates. Finally, prolificacy obtained higher numerical values both in male presence and male odour. Although both approaches proved to be efficient, odour utilisation may be more applicable in rabbit farms before the insemination process. The results presented may expand our understanding of using biostimulation techniques and their potential usefulness in production strategies for the rabbit industry.

All authors equally contributed to this work.

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