

STUDY OF COPULATORY BEHAVIOUR IN OLD MALE RABBITS

JIMÉNEZ P., CUAMATZI E., GONZÁLEZ-MARISCAL G.

Centro de Investigación en Reproducción Animal CINVESTAV-Universidad Autónoma de Tlaxcala, Mexico.

Abstract: Male rabbit sexual behaviour consists of a single mount followed immediately by ejaculation. In young bucks this pattern changes gradually as they reach sexual satiety in a day and sexual exhaustion after several daily tests. Little is known about the characteristics of sexual behaviour in old rabbits (aged 48-54 mo) within a day and across daily tests leading to sexual exhaustion. By using sexually receptive (young) females, changed within a session to maximise copulation, we found that: a) the inter-ejaculatory interval increased between the first and last days of testing; b) test duration was 3.1 h on day 1 and 0.5 h on day 15; c) the “miss rate” (i.e., mounts not accompanied by ejaculation) significantly increased from the first to the last day of testing, regardless of when this occurred in each individual buck; d) the total number of ejaculations displayed in a session significantly decreased between the first and the last day of testing in all males; e) scent-marking (“chinning”) frequency significantly decreased after copulation to satiety, relative to that quantified at baseline, and was restored the following day. Compared with young bucks our results indicate quantitative, rather than qualitative, differences in sexual behaviour associated with age in rabbits. Specifically, on day 1 old bucks spent a shorter time engaged in copulation and displayed a lower number of ejaculations before reaching satiety than young males. In contrast, the interval between ejaculatory events and the “miss rate” increased across test days in both old and young rabbits. These results merit investigating the neuroendocrine mechanisms underlying the display of such an active sexual behaviour by old rabbit bucks.

Key Words: sexual satiety, copulation, ageing, scent-marking, ejaculation, rabbits.

INTRODUCTION

Free contact between male rabbits and sexually receptive does leads to the display of a single mount with pelvic thrust, followed by ejaculation (Beyer *et al.*, 1980; Rubin and Azrin, 1967). Uninterrupted contact with one or several females leads to sexual satiety in a day and to sexual exhaustion after several days (Fuentes *et al.*, 2005; Melin and Kihlstrom, 1963; Villagran *et al.*, 2003). In our previous study (Jiménez *et al.*, 2012) we reported that young bucks can display as many as 22 ejaculations in a day (with successive rabbit does) and this activity—in turn—provokes a marked, transitory reduction in the frequency of scent-marking (chinning). The number of daily ejaculations gradually declines over repeated tests, as does the total amount of time bucks engage in sexual activity per day. Accordingly, the interval between successive ejaculations and the “miss rate” increases over time.

Male mammals show a progressive decline in their sexual activity during middle or advanced age (Craigie and Bronson, 1982; Leatham, 1977). This has been confirmed in various mammalian species, including rodents and primates (Slonaker, 1935; Minniek *et al.*, 1949; Ratcliffe, 1949; Larsson, 1957, 1958; Bishop, 1970; Leatham, 1977; Huber *et al.*, 1980; Chambers and Phoenix, 1982; Meites *et al.*, 1980). For instance, Spruijt *et al.* (1989) reported, in senescent rats (24 mo old): an increased latency to engage in sexual activity (more than double the time required for a young rat), and fewer visits to an oestrous female. Moreover, the ratio of mounts/intromissions prior to ejaculation was 71 and 36% in young vs. old males, respectively. These changes have been correlated with a decline in serum testosterone and higher levels of androgen receptors in the preoptic area (POA; Wu *et al.*, 2009).

Correspondence: G. González-Mariscal, gabygmm@gmail.com. Received August 2020 - Accepted January 2021.
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Nevertheless, other studies have provided no conclusive results in this regard (Chambers and Phoenix, 1984; Frankel, 1984; Smith *et al.*, 1992).

In old mice (25 mo old), Huber *et al.* (1980) reported that some of the males showed a sexual behaviour pattern similar in all aspects to that of young males. Other males, however, showed a specific loss of one or more of the components of copulatory behaviour (i.e., mounts, intromissions or ejaculations), while a third set of old males showed the entire copulatory sequence, but performed it less frequently than young animals (1.4 vs. 2.4, respectively). Specific variations in the capacity of aged males to experience arousal accounts for up to 67% of the variation in their total sexual performance (i.e., total number of mounts and total number of ejaculations with 4 females provided in sequence; Craigen and Bronson, 1982). As in rats, the decline in the sexual activity of old mice is correlated with low levels of testosterone and it is restored by the administration of polypeptides that enhance androgen synthesis (Zang *et al.*, 2015, 2016).

To the best of our knowledge, there are no studies of sexual behaviour in old rabbits (i.e., aged more than 30 mo). Therefore, the main aim of this experiment was to describe and quantify their daily copulatory pattern up to sexual satiety and the eventual manifestation of sexual exhaustion after several days. Additionally, we determined whether mating to satiety reduced chinning frequency on every test day.

MATERIAL AND METHODS

Animals and housing

Ten old (48-54 mo old), sexually experienced, New Zealand white male rabbits, born in our colony, were used. Seven adult ovariectomised does injected subcutaneously with estradiol benzoate (5 µg/d in 0.1 mL sesame oil; Sigma, St. Louis Missouri, USA) were used as stimulus females. All animals were housed individually in wire-mesh cages (52 cm long×42 cm wide×41 cm high) and given water and feed (Purina™ rabbit pellets) *ad libitum*. They were kept under controlled light (14 h light:10 h dark) and natural temperature (18-23°C) conditions. Throughout this work, animal care and handling complied with the Law for the Protection of Animals (Mexico) and with international guidelines regarding animal welfare (González-Mariscal *et al.*, 2017).

Experimental design

The general outline of the experimental design used to evaluate daily scent-marking and sexual behaviour is depicted in Figure 1.

Quantification of scent-marking and sexual behaviour. Chinning frequency was determined twice daily: before copulation (*baseline*) and at the end of the sexual satiety test (*post-copula*). To this end, we used the same method described to quantify scent-marking in laboratory buck rabbits (González-Mariscal *et al.*, 1993, 1997). Briefly, the male was placed inside a round wire-mesh arena (1 m in diameter×43 cm high) that contained three terracotta brick

DAY 1:

Baseline chinning test (10 min)	Remove bricks, introduce ♀ #1	Allow copulation <i>ad libitum</i> until no activity in 30 min	Change ♀ for new one, allow copulation <i>ad libitum</i> until no activity in 30 min	Repeat until no activity in 2 h: sexual satiety	Remove ♀, introduce bricks: <i>post copula</i> chinning test (10 min)
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↑ DAY 2, DAY 3, DAY 4..... Repeat as above until no sexual activity is displayed at all: sexual exhaustion.

Figure 1: Timeline showing the order in which scent-marking and copulatory behaviour were quantified daily until sexual satiety was reached on such day. Sexual exhaustion was achieved in all bucks after repeating this procedure for a variable number of days.

piles upon which the rabbit could rub its chin. To determine chinning frequency, we counted the number of chin marks made on any of the brick piles across 10 min. At the end of this test, the bricks were removed and the first stimulus female was introduced into the arena to initiate the sexual behaviour test.

The buck was allowed to mate *ad libitum* until no interest in the female was shown for 30 min. At this point the female was removed and replaced by another one, and so on, until the male showed no interest in any female for a period of two hours (criterion used to establish *sexual satiety* in our earlier study; Jiménez *et al.*, 2012). This change of stimulus females was performed to maximally enhance copulatory behaviour in apparently satiated males (the so-called "Coolidge effect"; Wilson *et al.*, 1963). At the end of this test, the doe was removed and the three brick piles were re-introduced to quantify chinning *post-copula*. This whole procedure was repeated daily until the male showed no sexual behaviour at all on a given day (criterion used to determine sexual exhaustion). The number of days required to reach sexual exhaustion (and, consequently, the total number of daily tests performed) varied among bucks (see below). Behavioural tests were started daily at 09:00 h.

Behavioural measurements

The first copulatory series was distinguished from the rest, as it provides information on the buck's behaviour in the *absence* of immediately previous sexual activity (Jiménez *et al.*, 2012). We quantified: a) duration of the test with the first female, i.e., time elapsed between the doe's introduction into the arena and the last mount performed by the male with that female; b) total number of ejaculations displayed towards that female; c) interval between copulatory events (i.e., between two successive mounts, two successive ejaculations, or a mount and an ejaculation); d) "miss rate": total number of mounts that did not culminate in ejaculation, calculated by the formula: $[(\text{number of mounts}/\text{number of mounts} + \text{number of ejaculations})] \times 100$. If the buck showed no interest in the doe for 30 min, a new one was introduced. From then until the end of testing on a given day, the following parameters were quantified: a) total duration of the test, i.e., time elapsed between the introduction of the first female and the last mount performed by the male on the last doe; b) total number of mounts and ejaculations in the test, i.e., number of mounts that culminated in ejaculation and number of mounts that did not; c) interval between copulatory events (see above); d) interval between ejaculations, i.e., time elapsed between one ejaculation and the next one; e) "miss rate" (see above). Given that the tests lasted several hours for each buck and many details of sexual behaviour were recorded during each session (see Results), no more than two animals were tested per day. As in our earlier work in young bucks we found no evidence that the copulatory activity of a male influenced the behaviour of the next one, tested inside the same arena (Jiménez *et al.*, 2012), we did not clean it between sessions of two animals tested successively in the same day.

Statistical analysis

Interval between ejaculations towards the first female. An unpaired t-test was used to compare the first and last ejaculatory intervals.

Duration of the test. Data from the first and last days of testing were compared using a paired samples t-test.

A one-way ANOVA, followed by an unpaired t-test, was used to compare the data of days 1 vs. last for the following parameters: a) interval between copulatory events; b) interval between ejaculations; c) miss rate; d) number of copulatory events; e) number of ejaculations. Baseline chinning frequency was compared with that shown post-copula also with a one-way ANOVA, followed by an unpaired t-test.

RESULTS

Interval between ejaculations with the first female. The time elapsed between two successive ejaculations, displayed towards the first female, significantly increased from the first (2.3 ± 0.6 min) to the second one (4.23 ± 1.5 min; $P=0.025$) and remained unchanged until the sixth ejaculation (Figure 2A). Only one male displayed a seventh ejaculation with the same doe and most stopped copulating with the same female after five ejaculations. Moreover, the time required to reach ejaculation significantly increased from the first day to the last one (Figure 2B). Furthermore, the number of females required to reach sexual satiety within a day markedly decreased from the first to the last day

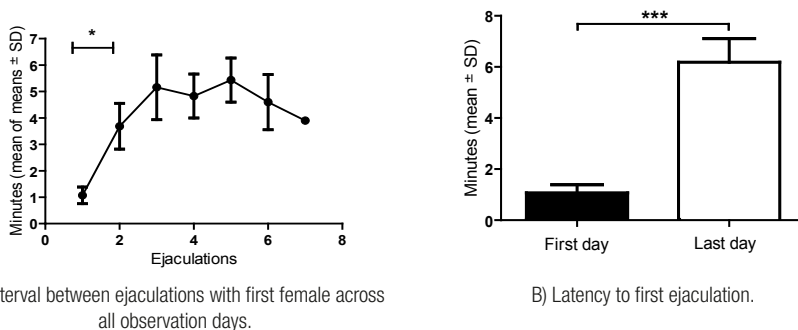


Figure 2: A. The following parameters were determined after introducing the first female used on a given day: i) latency to the first ejaculation (i.e., time elapsed between the introduction of the female and occurrence of the first ejaculation); ii. time elapsed between two successive ejaculations (e.g., 1st to 2^d; 2^d to 3^d, etc.). These measures were determined daily until each male reached sexual exhaustion. Such time intervals between the same pair of successive ejaculations were then averaged for each individual across all test days. A mean of means was then calculated from the values of all ten bucks for: latency to first ejaculation and five intervals between two successive ejaculations. Only one male displayed a seventh ejaculation. ****P*=0.025 B. Latency to the first ejaculation, determined on the first and last days of testing.

(Table 1). Thus, on the first day, 6/10 bucks needed three does to reach satiety and two males copulated with eight does before becoming satiated. In contrast, on the last day 8/10 males were satiated after copulating with just one doe and only one buck copulated with four females.

Duration of test. The test duration was 3.1±0.78 h on day 1 and only 0.5 h on day 15 (n=1). However, the number of males that engaged in copulation decreased from day 7 onwards: 70% of bucks (n=7) were sexually active then and by day 13 this proportion decreased to 10% (n=1; Figure 3A). Such individual continued to copulate for two more days. When comparing the first and last days of sexual activity (regardless of when the latter occurred in each male), the test lasted 3.1±0.78 vs. 1.8±1 h, respectively (Figure 3B).

Interval between copulatory events. The interval between the first and last copulatory events became longer across each test, i.e., as the number of successive matings increased. This pattern was similar between the first and last days, although it was clearer on the first day of testing than on the last (Figure 4).

Interval between ejaculations. The inter-ejaculatory interval significantly increased between the first and last days (Figure 5 A, B); mean of means was 11.03±4.7 and 17.09± 6.48 min, respectively; *P*=0.0029.

Miss rate. Mounts not followed by ejaculation occurred daily and gradually increased across tests. Consequently, the miss rate significantly increased from the first to the last day of testing (Figure 6A). However, the specific days on which this occurred varied among individuals. Therefore, a day-to-day comparison did not reveal significant increases in the miss rate (Figure 6B).

Number of mounts alone and with ejaculation. The total number of copulatory events (i.e., mounts+ejaculations) decreased between the first and last days of testing. This difference, however, was not significant. Yet, when

Table 1: Variation, among the 10 bucks (♂) used, in the number of does (♀) required to reach sexual satiety on:

First day		Last day	
♂	♀ needed	♂	♀ needed
		8	1
		1	2
6	3		
2	4		
2	8	1	4

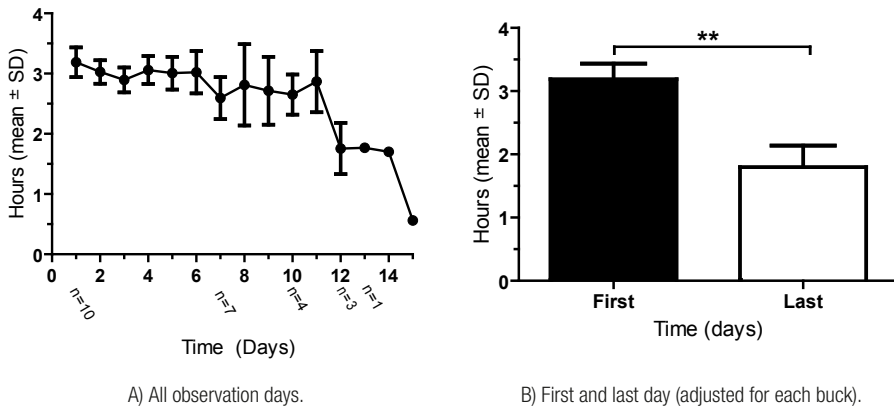


Figure 3: A. Duration of each sexual satiety test: time elapsed from the introduction of the first doe to the arena to the last mount performed by the male on the last doe of that day. The number of bucks displaying sexual behaviour on a given day gradually decreased from $n=10$ (days 1-6), to $n=7$ (days 7-9), to $n=4$ (days 10-12) to $n=1$ (days 13-15). B. Duration of the first and the last day of testing. The latter, adjusted for each individual, refers to the day when sexual exhaustion was reached. $**P<0.01$.

considering only the total number of ejaculations displayed on the first vs. the last days of testing, a significant reduction was evident ($P\leq 0.001$; Figure 7).

Chinning. Chin marking frequency was markedly decreased after copulation, compared to that quantified at baseline ($P\leq 0.0001$). Next day, baseline chinning frequency was restored. This pattern remained unchanged across successive tests up to day 10. In the few bucks that continued to display sexual activity from then until day 12, baseline chinning frequency increased and was reduced post-copulation (Figure 8).

DISCUSSION

The present study demonstrates for the first time that old bucks show intense copulatory behaviour when presented with a succession of sexually receptive young does. Nonetheless, their sexual performance is weaker, compared to younger males. For instance, old bucks needed 3-8 females to reach sexual satiety on the *first* day of testing, while

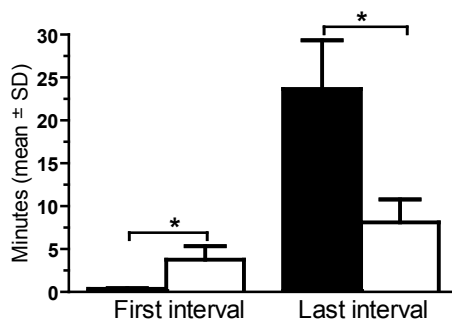


Figure 4: The interval between copulatory events refers to the time elapsed between: two successive mounts, two successive ejaculations, or a mount and an ejaculation. The first and the last intervals on a given test day are shown for the first and the last days of testing. $*P<0.05$. SD: standard deviation. ■ First day; □ Last day.

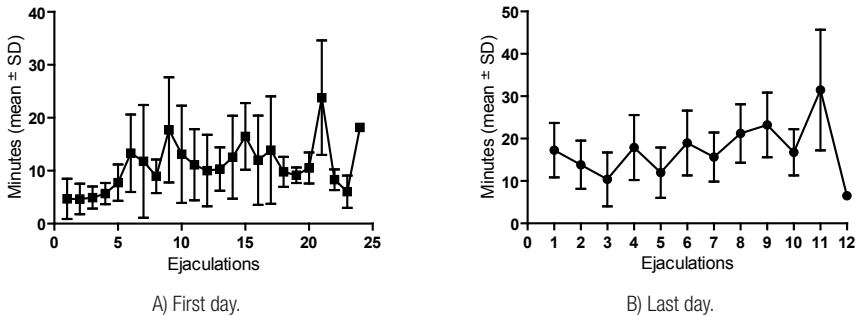


Figure 5: The interval between ejaculations indicates the time elapsed between one ejaculation and the next. Specific values are shown for the first (A) and the last (B) days of testing. Note that: i) the total number of ejaculations displayed in the test is twice as large in A than in B; ii) the last point in both panels lacks an standard deviation (SD), as only one buck displayed 24 ejaculations on the first day and 12 ejaculations on the last one.

young ones needed 1 to 3 (Jiménez *et al.*, 2012). On such first day, old males engaged in sexual activity with a *second* female after 2.45-14.2 min, i.e., sooner than young bucks did (7 to 52 min; Jiménez *et al.*, 2012).

By using the so-called “Coolidge effect” (Rodríguez-Manzo, 1999; Wilson *et al.*, 1963), we were able to keep males copulating for an average of 3.1 h on the first day of testing (maximal value=4.5 h). These values are lower than those we reported for young bucks, i.e.: the test duration on day 1 was, on average, 4.3 h and the maximal value was 6.6 h. Moreover, on the first day of testing the number of ejaculations displayed before becoming sexually satiated was also lower in old bucks, compared to our report in young males (35 vs. 43, respectively; Jiménez *et al.*, 2012), and coinciding with results found in mature/old rabbits (14-20 mo old; Fuentes *et al.*, 2005).

After reaching sexual satiety on day 1, old males were able to re-engage in sexual activity again on the next day. True sexual exhaustion happened after 5 to 15 d of daily activity, though with a great individual variability, as reported in young animals (2-15 d; Jiménez *et al.*, 2012). Despite such individual variability, particular parameters changed in a similar way in all bucks, specifically: a) the duration of the test, which decreased across successive days; b) the interval between copulatory events, the interval between ejaculatory events, and the “miss rate”, all of which increased across test days. These changes, indicative of the approach of sexual exhaustion, are very similar to those reported in young male rabbits (Jiménez *et al.*, 2012).

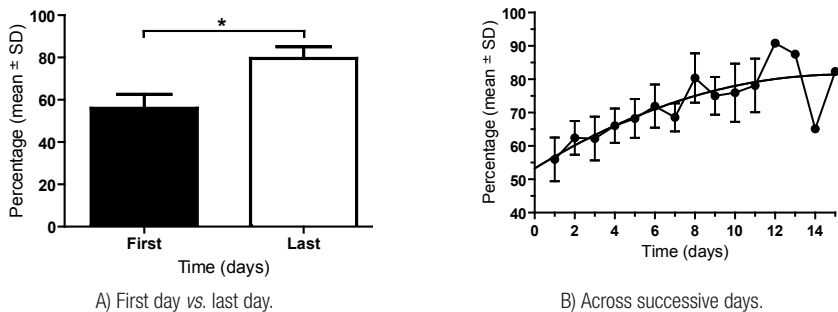


Figure 6: The “miss rate” refers to the total number of mounts that did not culminate in ejaculation, calculated by the formula: $[(\text{number of mounts}/\text{number of mounts}+\text{number of ejaculations})]\times 100$. This parameter significantly increased from the first to the last days of testing (panel A; $*P=0.0029$). On day 12 n=3; on days 13, 14, 15 n=1. SD: standard deviation.

SEXUAL BEHAVIOUR IN OLD BUCKS

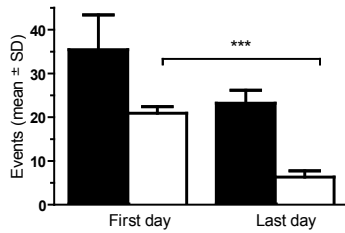


Figure 7: The total number of copulatory events (i.e., mounts that culminated in ejaculation plus those that did not) was larger on the first than on the last day of testing. This decline, however, was not statistically significant. In contrast, the number of ejaculations significantly declined from the first test day to the last ($***P<0.001$). ■ Total; □ Ejaculations. SD: standard deviation.

In contrast, other mammals show profound changes in sexual behaviour during old age. For instance, young rats show a series of mounts, with or without intromission, that culminate in ejaculation (Larsson, 1979). Usually, a second copulatory series starts after 4-5 min, and if given enough time, a male can reach sexual exhaustion after as many as 12 ejaculations. Old rats show longer latencies to engage in sexual activity (almost the double compared to young animals) and fewer mounts and intromissions precede ejaculation (Larsson, 1979). Additionally, in old rats only 36% of the mounts are followed by intromissions (Spruijt *et al.*, 1989). Old mice exposed to sexually active females exhibit a large individual variation in copulatory behaviour. In our study, while some males displayed a behaviour similar in all regards to that of young males, others showed a specific loss in the number of mounts, intromissions or ejaculations performed in the test. Other old individuals displayed the entire copulatory sequence, but with less frequency than young animals (Huber *et al.*, 1980). Old male mice also show a deterioration in their capacity for sexual arousal (Craigie and Bronson, 1982). The latter is also true of old male rhesus macaques: they display less sexual behaviour than young and middle-aged animals and a change in female partner or a change in environment does not lead to an increased sexual performance. Only when old macaques were paired with empirically selected preferred females was their sexual behaviour increased to levels similar to those seen in young males (Phoenix and Chambers, 1986). Interestingly, treatment with testosterone did not increase their sexual activity (Phoenix and Chambers, 1990).

After copulation *ad libitum*, chinning is reduced by approximately 75% in young bucks (González-Mariscal *et al.*, 1997; Jiménez *et al.*, 2012). This was also observed in old rabbits, regardless of the number of copulatory events or the duration of the test among animals. Moreover, baseline chinning levels were rather constant across days, indicating that the reduction in chinning frequency detected at the end of each sexual activity test, lasted less than 24 h.

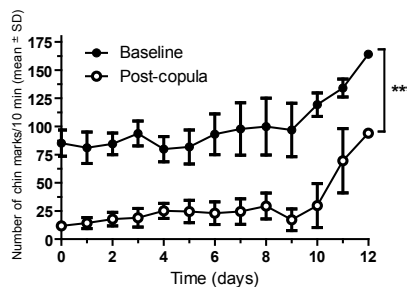


Figure 8: The number of chin rubs made onto the bricks placed inside the arena across 10 min, was determined before copulation (baseline) and at the end of the daily sexual satiety test (post-copula). Baseline chinning frequency was unmodified across test days 1-10 and it was markedly decreased post-copula.

$***P<0.001$, baseline vs. post-copula, all days. ●: Baseline; ○: Post-copula. SD: standard deviation.

Changes in physical, physiological and behavioural characteristics have been reported in old bucks. For instance, in New Zealand white rabbits there is a decrease in the viscoelastic properties of the chondrocytes in the knee articular cartilage (Wei *et al.*, 2009). Also, the physiological characteristics of motor cortex neurons change with age: during spontaneous movements of extensor carpi radialis, the level of neuronal activity in young rabbits (1 yr old), was twice as high as that of aged ones (6-7 yr old; Mednikova and Kopytova, 1994). The relevance of these changes for the control of sexual behaviour in old rabbits remains to be determined.

There are a great number of studies about sexual exhaustion and its regulation, performed mainly in rats (for recent review see: Hull and Rodriguez-Manzo, 2019). These studies have provided evidence for the participation of several neurotransmitter systems (i.e., serotonin, noradrenaline, opiates, GABA) in the regulation of sexual satiety (Miller and Baum, 1987; Fernandez-Guasti and Rodriguez-Manzo, 1997; Pfau and Gorzalka, 1987; Rodriguez-Manzo *et al.*, 2002). In contrast, in rabbits only one study in this regard is reported (Fuentes *et al.*, 2005), where naloxone administration increased sexual behaviour in sexually satiated young and mature bucks.

Since previous works have shown that GABA, opioids, and dopamine play an important role in the expression of (young) male rabbit sexual behaviour (Agmo *et al.*, 1991, 1994, 1996) more studies are clearly needed to reveal the role of such neurotransmitters in the establishment of sexual exhaustion in rabbits of different ages. From the perspective of rabbit breeding, the viability and fertilising capacity of sperm have been studied in relation to semen collection frequency rather than to age. A semi-intensive collection rhythm (i.e., two ejaculates per day on two consecutive days per week) from young adult bucks was found to produce the highest number of weekly doses without compromising an effective ejaculate volume (Nizza *et al.*, 2002). Moreover, daily collection of semen for over two months in young adult rabbits led to the lowest ejaculate volumes and concentration of spermatozoa, as well as to the highest ratios of droplets in the latter. Conversely, a once weekly collection rhythm led to the highest output of spermatozoa (Castellini *et al.*, 2006). In contrast, the effects of age on semen characteristics and fertilising capacity of sperm have not, to the best of our knowledge, been formally investigated in rabbits. However, in rats there is evidence of an age-associated increase in the number of spermatozoa with abnormal flagellar midpieces and in the proportion of spermatozoa with decreased mobility obtained from the cauda epididymis (Syntin and Robaire, 2001). In addition, semen obtained from the female rat after ejaculation of old males revealed an increased percentage of immobile spermatozoa and a reduction in their speed of mobility (Lucio *et al.*, 2013). Similar reductions in sperm motility and increases in structural abnormalities have been reported for old dogs (Bhanmeechao *et al.*, 2018; Brito *et al.*, 2018).

Together, the above evidence indicates that: i. in all mammals studied, specific semen characteristics show a deterioration in sperm fertilising capacity during old age; ii. a high frequency of semen collection in young adult rabbits reduces sperm count and ejaculate volume, thus compromising fertility. Therefore, we must conclude from our present behavioural results that, although old bucks retain a robust capacity to engage in sexual behaviour, they are not recommended as sires to be used on the farm because they can induce pseudopregnancy following natural copulation (due to vaginocervical stimulation to the doe) and their semen is likely to have a reduced fertilising capacity, due to age and successive ejaculations.

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