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González-Mariscal G.

**NEUROENDOCRINOLOGY APPLIED TO RABBIT BREEDING
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NEUROENDOCRINOLOGY APPLIED TO RABBIT BREEDING

González-Mariscal, G.

Centro de Investigación en Reproducción Animal, CINVESTAV-Universidad Autónoma de Tlaxcala. Apdo. Postal 62.
Tlaxcala, Tlax. 90000. México.
gabygmm@gmail.com

ABSTRACT

Successful rabbit production relies heavily on the use of adequate practices that enhance specific aspects of reproduction, such as mating, ovulation, and lactation. Regardless of the type of production unit or strain of rabbits used those processes rely on a complex chain of neuroendocrine steps that include particular hormones, peripheral stimuli, and activation of discrete brain regions. Such is the case, for instance, of reflex ovulation which occurs in response to copulation but is inhibited throughout lactation. Little is known about the mechanisms mediating lactational anestrus and the restoration of estrus following the cancellation of a single suckling episode (biostimulation). The latter procedure (adopted worldwide to accelerate reproduction) has, however, unwanted consequences for the doe and her litter. After successive episodes of biostimulation the former show a loss of fertility and body mass. In the kits alterations are observed in their neuroendocrine response to mildly aversive stimulation in adulthood as well as reductions in sexual behavior. In addition to milk intake a good nest is essential for the normal growth and development of the litter. If this is not available, or if it deteriorates, rabbit caretakers can easily (re) build one from hair sheared off other rabbits or using synthetic material. Lactating does will nurse equally well their own or 'alien' young, placed inside her nest. It is crucial to have a minimum of six suckling kits in the nest as the doe relies on such stimulation to maintain a normal nursing behavior, i.e., only once-a-day throughout lactation. Recent work is revealing the similarities and differences in the responsiveness to mating among estrous, lactating, and biostimulated does. The relevance of these findings for the likelihood of reflex ovulation and the additional contribution of factors contained in the semen warrant deeper investigations. New insights on these issues, essential to reproductive neuroendocrinology, can emerge by fostering a richer interaction between academic laboratories and rabbit production settings worldwide.

Key words: mating, lactation, ovulation, stress, welfare

INTRODUCTION

Rabbit breeding is performed worldwide under a variety of conditions, ranging from small production units associated with human dwellings to highly mechanized farms in which specific processes are automated and continuous measurements of innumerable parameters are constantly made. Moreover, there is no consistent uniformity in the breeds of rabbits, environmental conditions, or feedstuffs used in either the 'low-tech' or the 'high-tech' conditions. Despite such differences rabbit production can be successful *within* each of these two approaches, although the *absolute values* regarding, e.g., fertility, meat production, cost-benefit relationship, etc. can vary widely. Much of this variation stems from the use (or not) of findings derived from scientific research performed on areas as diverse as: Nutrition, Feeding, Pathology, and Reproduction. Such studies have been conducted for many years, in several countries -on specialized farms and academic laboratories- and have provided the backbone for the recommendations made to rabbit breeders to improve production and to treat or prevent disease. Nonetheless, innovation is continuously required to deal with the unexpected, to make a 'traditional' process more efficient, and to correct obsolete or outright inadequate practices. Innovation in the agronomic sciences requires solid scientific research and the latter, in turn, can enrich its inquiries and interpretations by interacting with workers, staff, and technicians who breed animals under a variety of conditions.

I have been studying the Reproductive Neuroendocrinology of mammals for many years within an academic setting. My interaction with the Rabbit Sciences began when I attended a Cuniculture meeting in Mexico many years ago. I was then able to notice that specific issues concerning productivity on the farm were directly related with Reproductive Neuroendocrinology, e.g.: the underpinnings of ‘biostimulation’, the concurrency of pregnancy and lactation, the differences between pregnancy and pseudopregnancy, the likelihood of mating-induced ovulation, etc. These topics are ‘big themes’ for an academic and the possibility that investigating them could also have an impact on rabbit production seemed rather exciting. I, therefore, chose to devote part of my research endeavors to investigate topics of common interest to Neuroendocrinology and to the Rabbit Sciences. It is the purpose of my presentation to show results that illustrate specific examples of this approach.

BIOSTIMULATION: IMPACT ON DOES AND KITS

Doe rabbits show lactational anestrus (Beyer and Rivaud, 1969) despite the fact that –unlike most mammals– suckling bouts are brief (ca. 3 min) and they occur only once a day (González-Mariscal *et al.*, 2016). Lactational anestrus is characterized by a lack of sexual receptivity and ovulation, effects presumably provoked by the neuroendocrine conditions of lactation and maintained by the suckling young (Mc Neilly, 2006). Indeed, estradiol concentrations in blood (Ubilla *et al.*, 2000), sexual receptivity, scent-marking (chinning), and ambulation in an open field are reduced as a consequence of nursing. Interestingly, such behavioral traits are not equally sensitive to the inhibitory effects of suckling: more kits are required to reduce the lordosis quotient (a measure of sexual receptivity) than to antagonize chinning and ambulation (García-Dalmán and González-Mariscal, 2012; Table 1).

Table 1: Decrease in lordosis, chinning frequency, and ambulation provoked by nursing a variable number of kits (lactation day 14; modified from García-Dalmán and González-Mariscal, 2012)

Hrs post-suckling	Chinning (# marks/10 min; median)			Ambulation (# crossings/10 min; median)			Lordosis quotient (median)		
	# suckling kits			# suckling kits			# suckling kits		
	1	5	10	1	5	10	1	5	10
Baseline	32	2	0	35	28	50	100	65	30
0	2	0	0	30	27	23			
10	13	4	0	32	20	22			

Yet, separating lactating does from their litters for 48 hrs (rather than the usual 24 hrs) restores estrus in all studied breeds, kept under a variety of environmental conditions (Alvariño *et al.*, 1998; Bonanno *et al.*, 2002; Theau-Clément and Mercier, 1999). Consequently, this so-called ‘biostimulation’ procedure has been adopted worldwide to accelerate production in rabbit farms. It has drawbacks, however, for both the doe and the kits. After repeated cycles of concurrent pregnancy and lactation does decrease their fertility and lose body mass (Bonanno *et al.*, 2002; Eiben *et al.*, 2008). The metabolic mechanisms underlying this ‘unavoidable’ physical deterioration are not well understood. Clearly, more scientific research is warranted in this area to ensure the welfare of breeding does while simultaneously promoting a profitable productivity on the farm.

From the litter’s point of view the 48-hr fast and separation from the mother has specific neuroendocrine consequences that persist into adulthood. Brecchia *et al.* (2009) clearly documented a massive secretion of corticosterone following a 48-hr mother-litter separation in early lactation. This single, acute response, however, permanently modified the rabbit’s neuroendocrine reactivity to mildly aversive stimulation: in contrast to the ‘non-deprived’ group, *adults* whose mothers were ‘biostimulated’ showed a blunted corticosterone secretion together with more abundant concentrations of glucocorticoid receptors in the dorsomedial hypothalamus. The latter would support a more intense negative feedback by corticosterone.

Recently, we undertook a broader investigation of this issue by exploring the behavioral and neuroendocrine responses of adult male and female rabbits, which were the progeny of ‘biostimulated’ or control mothers (García-Fernández *et al.*, 2019). Regardless of time of day or sex we found no differences between control and ‘deprived’ litters in any of the six behavioral tests used, previously documented in rabbits as indicative of stress/anxiety (Table 2).

Table 2: Responses of control and deprived litters in specific behavioral tests presumably measuring stress-anxiety in rabbits (median \pm iqr; modified from García-Fernández *et al.*, 2019)

	Females		Males	
	Control (n=28)	Deprived (n=24)	Control (n=17)	Deprived (n=25)
Righting reflex (sec)	41.5 \pm (74,19)	39.5 \pm (65,21)	33 \pm (59,17)	40 \pm (95,23)
# Contacts with human	3 \pm (5,2)	2 \pm (5,1)	3 \pm (6,2)	3 \pm (5,1.5)
Latency to exit dark box (sec)	30 \pm (69,17)	27.5 \pm (180,19)	36 \pm (58.5,18)	48 \pm (135,19.5)
Ambulation (# lines crossed/min)	8.2 \pm (9.6,5.7)	6.9 \pm (8.1,5.3)	7.7 \pm (8.7,6.3)	7.6 \pm (9.1,6.4)
Chinning (# marks/min)	1.2 \pm (2,0.2)	1.4 \pm (3.1,0.2)	6.6 \pm (8,4.1)	7.9 \pm (9.5,0.9)

contrast, sexual behavior was altered in both sexes when comparing *non-kin* animals: ‘deprived’ males showed a significantly larger ($p=0.002$) ‘miss rate’ (indicative of a large number of mounts not culminating in ejaculation) and the lordosis quotient of ‘deprived’ females was reduced (this difference, however, did not reach statistical significance; $p=0.21$; Figure 1).

Effect of cancelling one nursing episode in early lactation on the sexual behavior of *non-kin* adult male and female rabbits

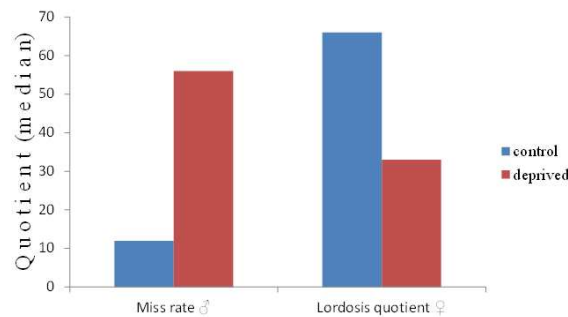


Figure 1: Reductions in male and female sexual behavior of ‘deprived’ vs control adult non-kin rabbits. (Modified from García Fernández *et al.*, 2019).

The injection of i.m. saline in adulthood increased (relative to baseline) the secretion of corticosterone and cortisol in all animals. However, the magnitude of these responses was modified by maternal deprivation, time of day, and sex (Figure 2).

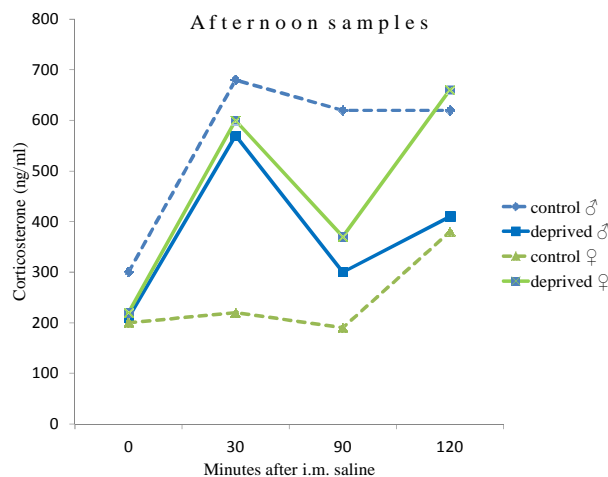


Figure 2: Reactive corticosterone secretion of control and ‘deprived’ adult male and female rabbits, sampled in the afternoon (Modified from: García Fernández *et al.*, 2019).

Taken together our results show that the massive corticosteroid secretion that occurs in suckling rabbits as a consequence of a 48-hr fast and a brief maternal separation has profound, long-lasting effects on reproductive behavior and reactive corticosteroid secretion in adulthood. Is there any relevance of these findings for the way rabbits are raised under ‘high-tech’ or ‘low-tech’ conditions? Before attempting an answer I believe one must first reflect on issues like: how is fertilization of does

achieved (i.e., natural mounts or artificial insemination)? At what time of day (i.e., morning or afternoon) are animals commonly subjected to potentially stressful procedures (e.g., tattooing, transportation to slaughterhouse, medication)? Is any sex preferred for a particular use (e.g., shearing, shipping to local market, sacrificing *in situ*)? Discussing these points, with consideration to different rearing conditions, strains of rabbits, and interests of the farmers is essential for a constructive, bi-directional collaboration between neuroendocrinology and livestock science.

NURSING: ONCE A DAY IS BEST

Rabbits have evolved a unique mothering system in which the direct contact between nursing does and kits is restricted to a brief, single, daily nursing bout per day (González-Mariscal *et al.*, 2016). In contrast, does compensate for such ‘absentee’ mothering style by building an elaborate maternal nest (of straw and body hair) from early pregnancy to parturition (González-Mariscal *et al.*, 1994). Such nest acts like a ‘proxy’ of the mother as it provides thermal insulation and protection for the litter and it also guides the kits’ feeding preferences after weaning (Altbäcker *et al.*, 1995). Thus, a low-quality nest leads to death of littermates and alterations in the mother’s nursing behavior. We have found that the number of suckling young plays a major role in determining the circadian periodicity of nursing (González-Mariscal *et al.*, 2013). While does suckling six kits display a single nursing bout (at around 0300 hrs) females given one, two, or four young enter the nest box many times a day (Figure 3).

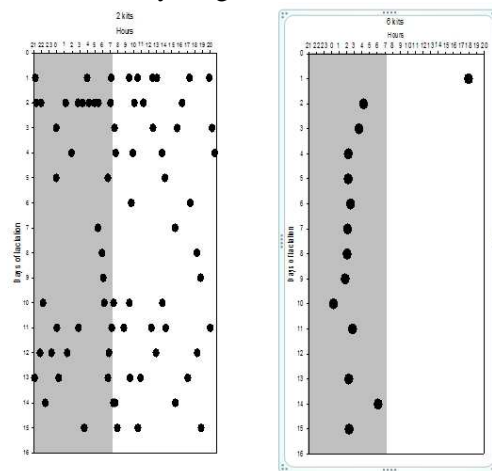


Figure 3: Nursing bouts displayed across lactation days 1-15 by does suckling two (left panel) or six (right panel) kits. Shaded area indicates darkness. (Modified from González-Mariscal *et al.*, 2013).

An insufficient suckling stimulation, that provokes multiple entrances into the nest box, also leads to accidental hurting of the young and to a gradual loss of nursing behavior (González-Mariscal *et al.*, 2013; Figure 4).

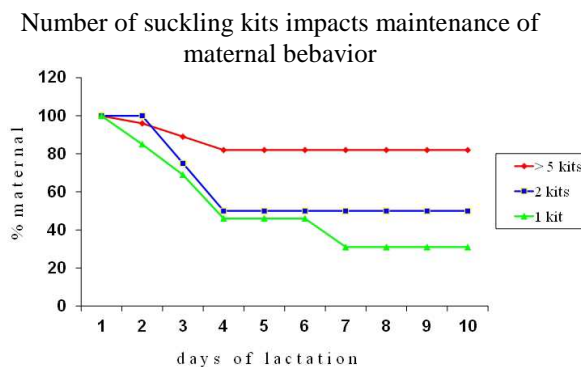


Figure 4: Gradual loss of nursing behavior in does suckling small litters; more than 5 kits are required to maintain maternal behavior throughout lactation

These findings emphasize the need to ensure that: a) a good quality nest is maintained, especially in early lactation; b) nursing does always have at least six kits to suckle. Depending on the type of setup used and the environmental characteristics surrounding the farm maternal nests can deteriorate over time; additionally, some does (especially primiparous ones) do not always build good nests. These inconveniences can be easily overcome by adding to the female's home cage straw or hay that she can then carry into the nest box. Additionally, hair of different types (e.g., synthetic, or shaved from other female or male rabbits) can also be placed in a container and the doe will collect it and line the straw nest with it (González-Mariscal *et al.*, 1998).

Still, for a number of reasons (e.g., delivery outside the nest box, low birth weight, insufficient milk intake) kits can die in the first days postpartum. To maintain a minimal litter size of six suckling kits can be exchanged among does without compromising the doe's maternal behavior. Rabbits do not show exclusive nursing; rather, they will nurse any kit inside the nest box (provided it is of a similar age as her own; González-Mariscal and Gallegos, 2007; Table 3).

Table 3: Does nurse equally well their own and alien kits, even in different types of nests (modified from: González-Mariscal and Gallegos, 2007)

Time (min; mean±s.e.) spent nursing own or alien young, placed within nests made of straw and different types of hair, across early lactation				
Type of litter	Own	Type of nest		
		Other doe	Synthetic hair	Male hair
Own	3.0±1.0	3.0±1.0	4.0±1.0	3.0±1.0
Alien	3.0±0.1	4.0±0.3	4.0±0.4	4.0±0.3

REFLEX OVULATION: NOT AN EASY MATTER

Most estrous rabbits ovulate in response to mating. Despite its brief duration (Contreras and Beyer, 1979) copulation provides the tactile, olfactory, acoustic, and visual stimuli necessary and sufficient to induce the release of hypothalamic GnRH (Yang *et al.*, 1996) followed by a massive secretion of pituitary LH (Ramírez and Beyer, 1988). In addition, following copulation slow waves appear in the EEG of the cerebral cortex, hypothalamus, and hippocampus (Sawyer and Kawakami, 1959) and scent-marking plus lordosis are immediately inhibited (González-Mariscal *et al.*, 1997; Figure 5).

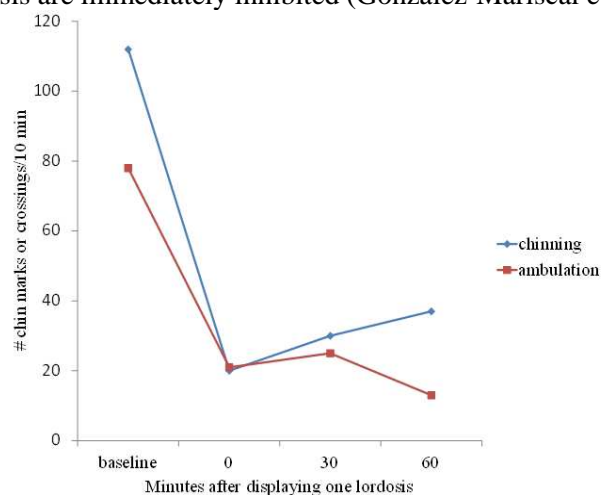


Figure 5: Mating-induced inhibition of chinning and ambulation (Modified from González-Mariscal *et al.*, 1997).

However, the adequate, predictable operation of this neuroendocrine reflex in estrous rabbits (plus its associated behavioral consequences) requires that does have an adequate concentration of estrogens in

blood (Hilliard and Eaton, 1971), a sufficient food intake (Brecchia et al., 2006), and a daily photoperiod of 12-14 hrs (Hudson et al., 1994). In contrast, suckling does show lactational anestrus, presumably as a consequence of their lower estradiol concentration in blood together with suckling stimulation *per se* (García-Dalmán and González-Mariscal, 2012). Nonetheless, as described above, lactational anestrus is abolished by cancelling a single nursing bout (biostimulation). To further understand the similarities and differences in the responsiveness to mating among estrous, lactating, and biostimulated does, we recently compared their expression of the c-FOS protein in specific brain regions known to play a crucial role in rabbit reproduction (González-Mariscal *et al.*, 2015). The c-FOS protein, detected by means of immunohistochemistry, has been reliably used as a proxy for neuronal activation. We found that in the preoptic area (POA; rich in GnRH neurons) and the paraventricular nucleus (PVN; rich in oxytocin-producing cells) of estrous does copulation markedly increased the number of c-FOS-immunoreactive cells, relative to unmated rabbits. This rise, however, was not seen in the POA of lactating or biostimulated females. Moreover, in the PVN of these groups mating reduced c-FOS expression (Figure 6).

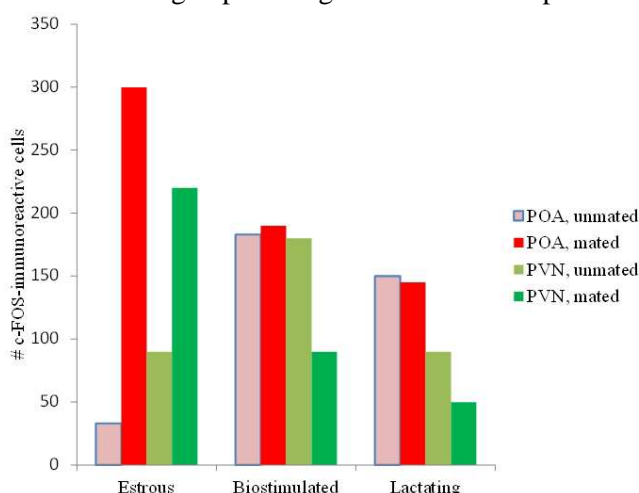


Figure 6: Differential expression of the c-FOS-protein in the POA and PVN of estrous vs biostimulated vs lactating rabbits. (Modified from González-Mariscal *et al.*, 2015).

These findings are beginning to reveal that the brain's differential reactivity to the stimulus of mating is finely modulated by as yet undetermined factors associated with the doe's reproductive stage. Future studies are warranted to establish the degree to which the probability of reflex ovulation following copulation depends upon such differences.

In addition to the above, recent evidence obtained by a multinational team (Maranesi *et al.*, 2018), has revealed that the intravaginal deposition of semen is an important additional factor that promotes ovulation. Such complex biological fluid has, of course, a myriad proteins but we found evidence that one of them (nerve-growth factor, NGF), by acting on its cognate receptors in the ovary, facilitates ovulation. Additionally, NGF -synthesized by the uterus- may act on its pituitary receptors to promote LH secretion.

CONCLUSION

Taken together, the results shown in the preceding sections are opening new avenues where the academic setting of neuroendocrinology laboratories can meet findings coming from the animal sciences. We hope that a greater collaboration between these two spheres of endeavor will ultimately lead to a better handling of animals, improved procedures to enhance reproduction, and a reconciliation between profitability and care for the environment.

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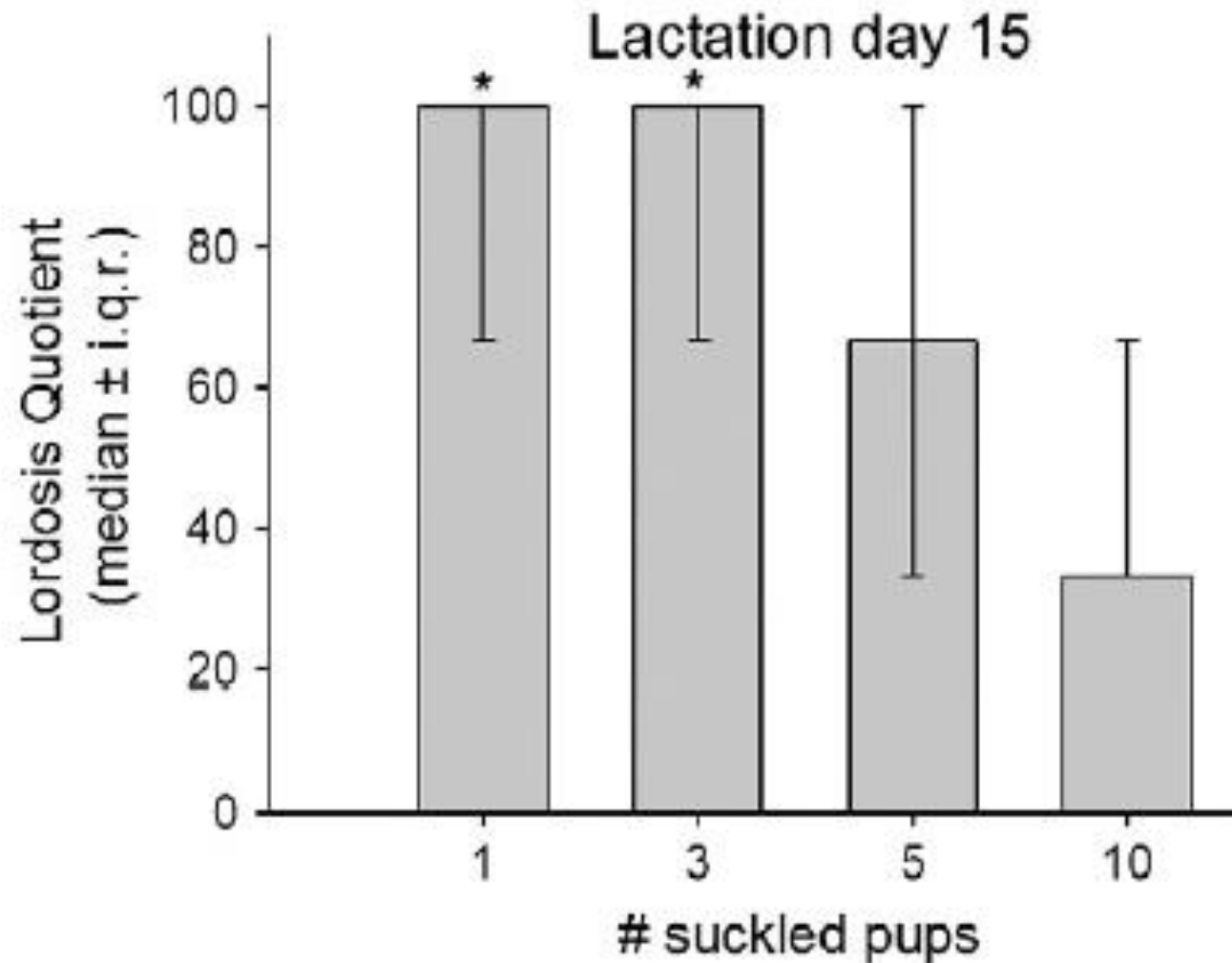
NEUROENDOCRINOLOGY APPLIED TO RABBIT BREEDING

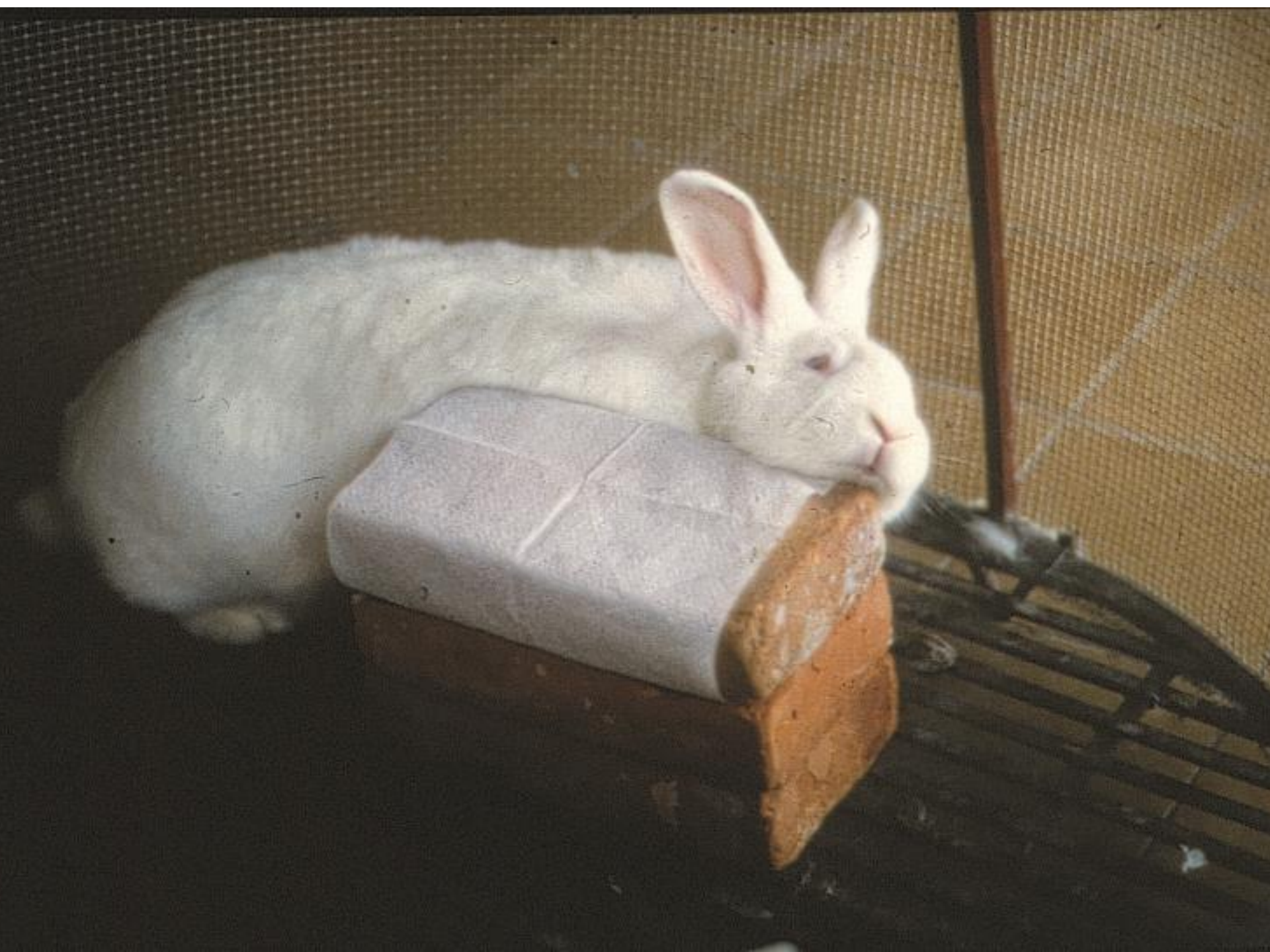
Gabriela González-Mariscal

*Centro de Investigación en Reproducción
Animal, CINVESTAV-Universidad Autónoma
de Tlaxcala, México*

BIOSTIMULATION: IMPACT ON DOES AND KITS

Suckling 10 kits (but not 1, 3 or 5) reduces sexual receptivity (García-Dalmán and González-Mariscal, 2012)





Suckling 5 or 10 kits severely reduces, or abolishes, baseline chinning frequency. Suckling even a single kit provokes a short-lasting inhibition of chinning

(García-Dalmán and González-Mariscal, 2012)

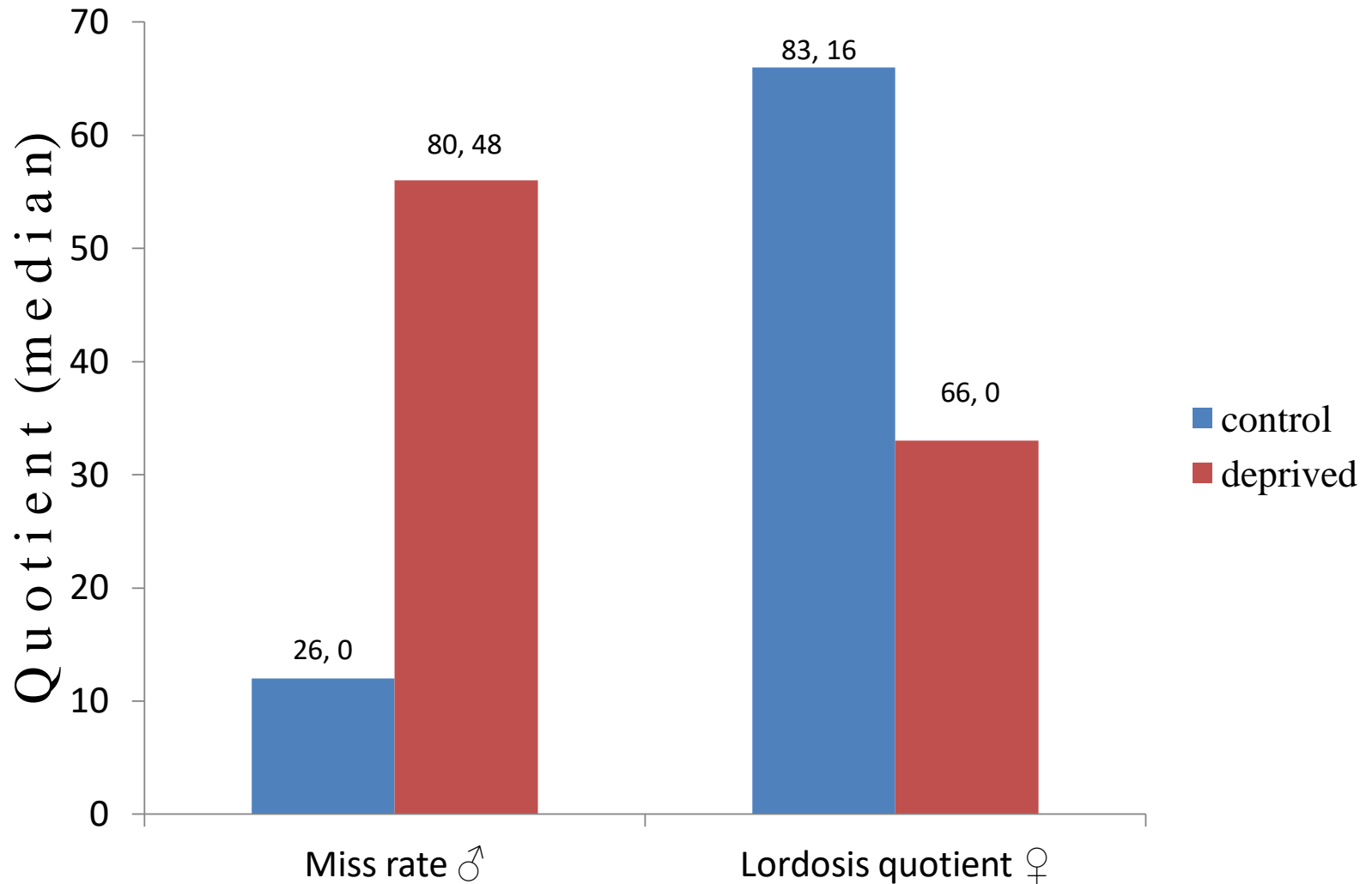
Hrs post-suckling	Chin marks/10 min (median±iqr): lactation day 14		
	# suckling kits		
	1	5	10
Baseline	32 (23, 29)	2 (2,1)	0 (0,0)
0	2 (13,2)	0 (0,0)	0 (0,0)
10	13 (13,14)	0 (0,0)	0 (0,0)

Responses of control and “deprived” litters in specific tests presumably measuring stress/anxiety (medians±iqr)

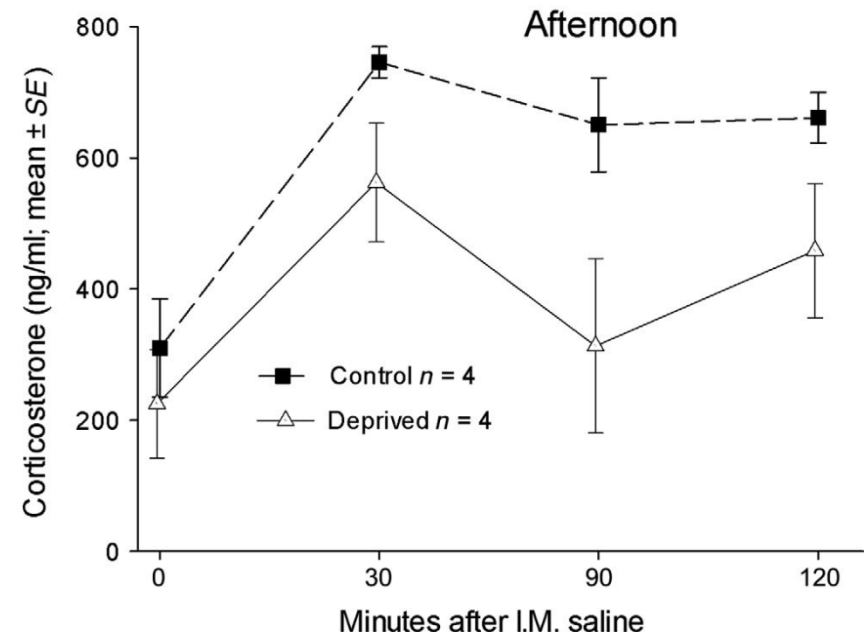
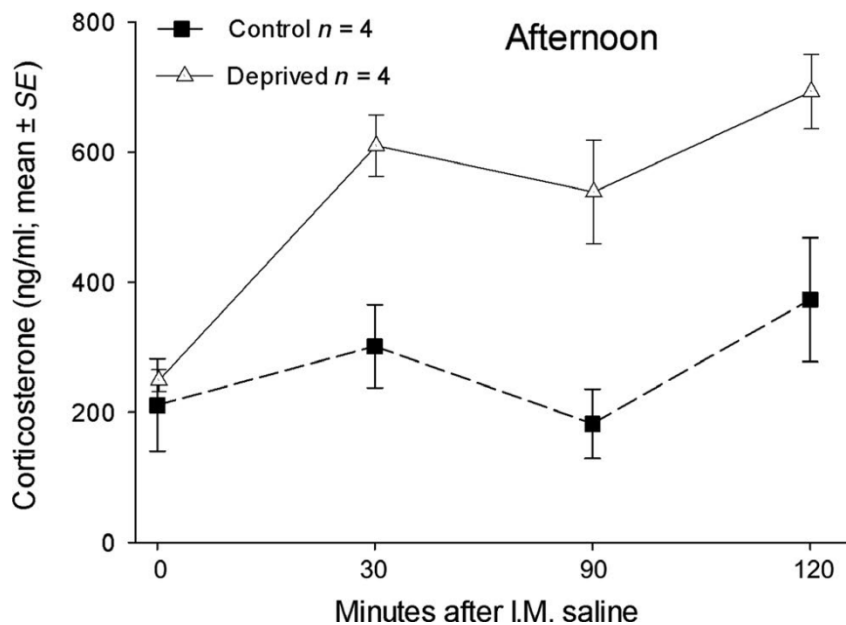
(García Fernández et al., 2019)

Test	FEMALES		Test	MALES	
	control	deprived		control	deprived
Righting reflex	41 (74,19)	39 (65,21)	Righting reflex	33 (59,17)	40 (95,23)
Contacts with human	3 (5,2)	2 (5,1)	Contacts with human	3 (6,2)	3 (5,5)
Latency to exit dark box	30 (69,17)	27 (180,19)	Latency to exit dark box	36 (58,18)	48 (135,19)
Ambulation	8 (10,6)	7 (8,5)	Ambulation	8 (9,6)	8 (9,6)
Chinning frequency	1 (2, 0.2)	1 (3,0.2)	Chinning frequency	7 (8,4)	8 (9,0.9)

Effect of cancelling one nursing episode in early lactation on the sexual behavior of *non-kin* adult male and female rabbits



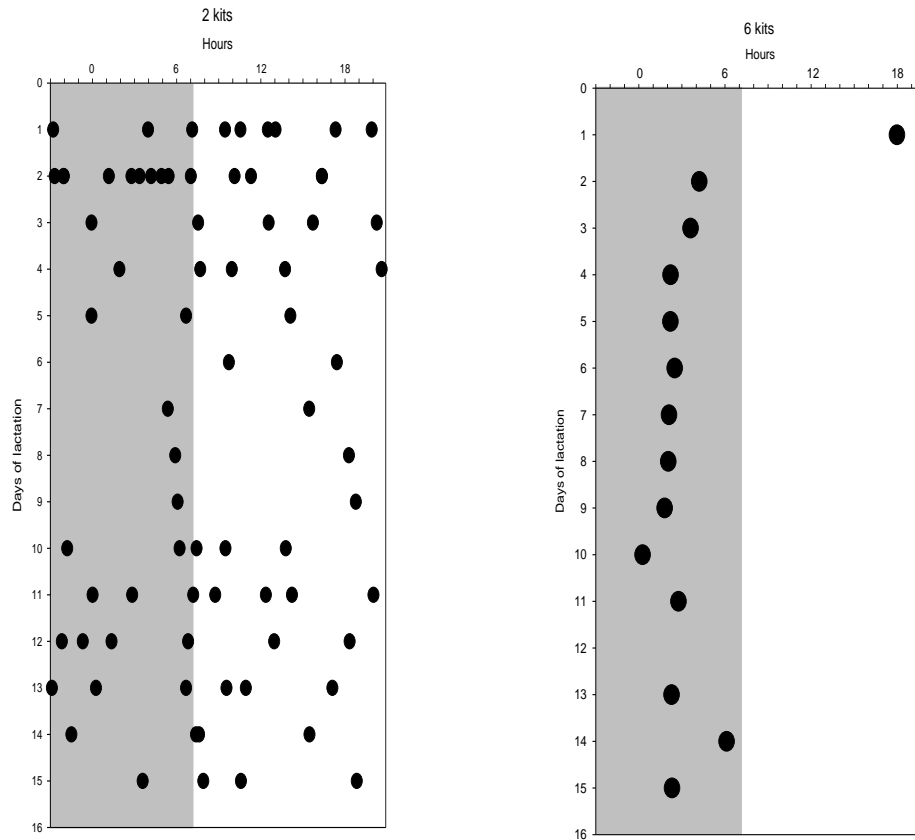
Adult does (left), deprived of a single nursing bout in early lactation, show a *higher* reactive corticosterone secretion. In adult bucks (right) the opposite occurs. (García Fernández et al., 2019)



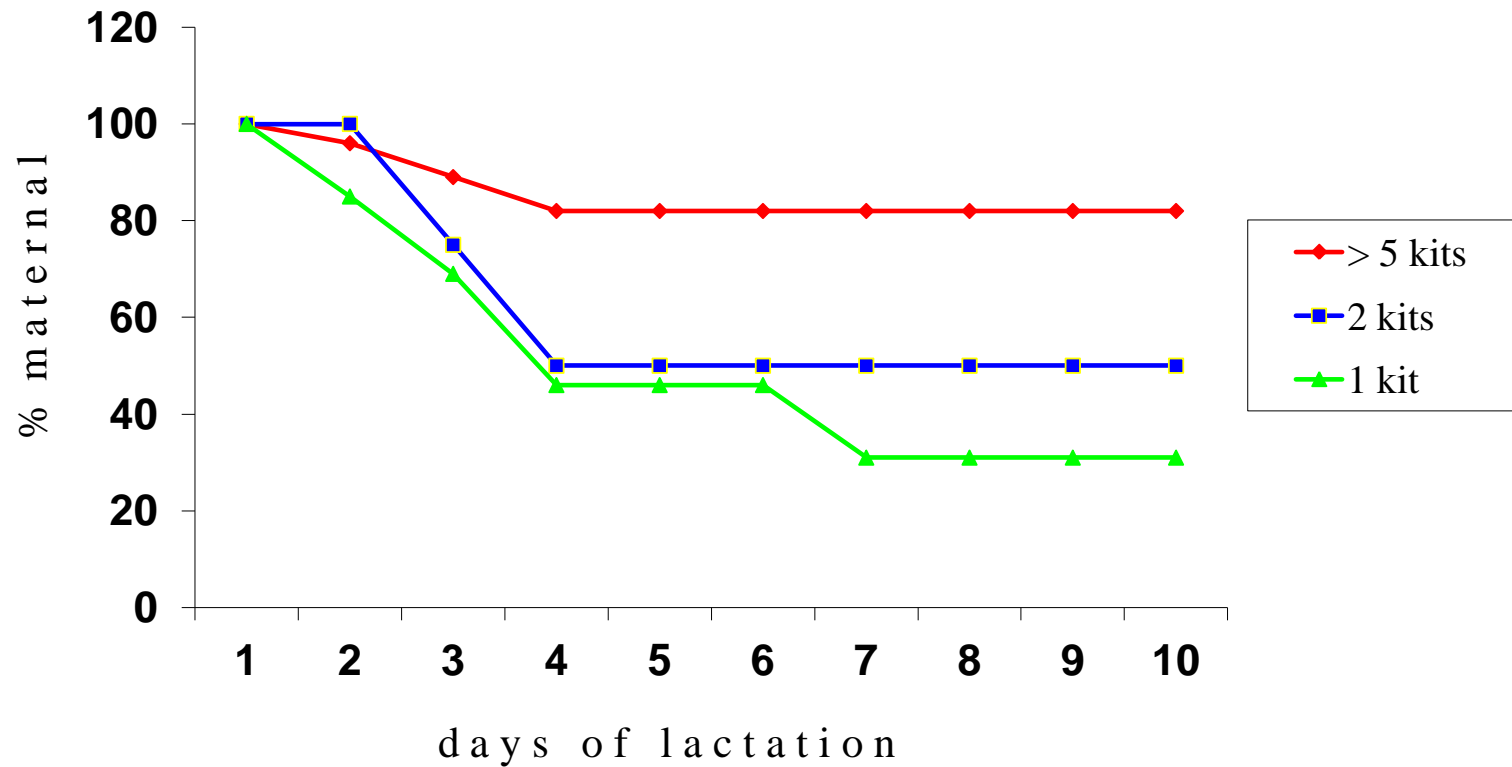
NURSING: ONCE A DAY IS BEST

Nursing bouts displayed across lactation days 1-15 by does suckling two (left panel) or six (right panel) kits

(González-Mariscal et al., 2013)



Number of suckling kits impacts maintenance of maternal behavior

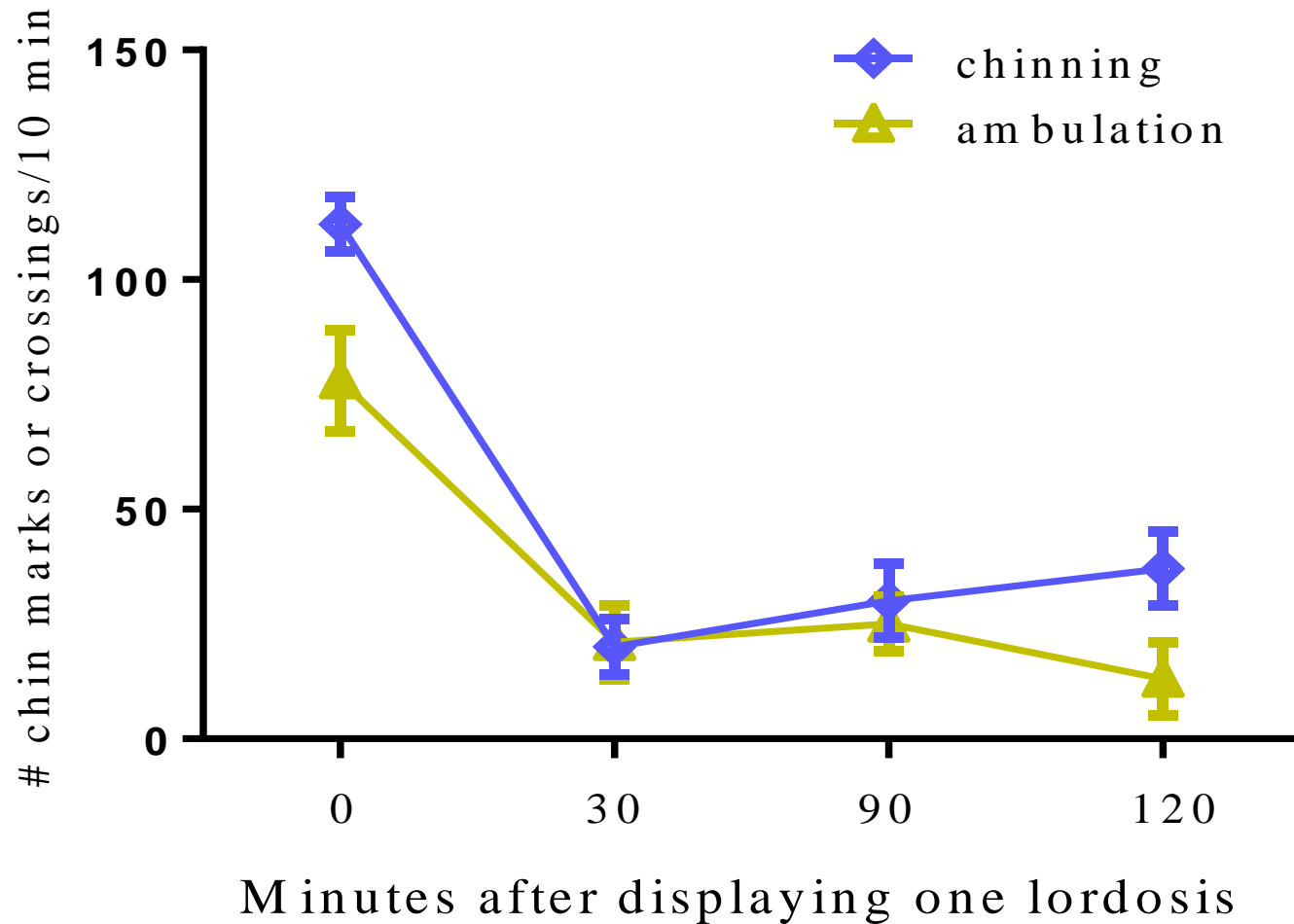


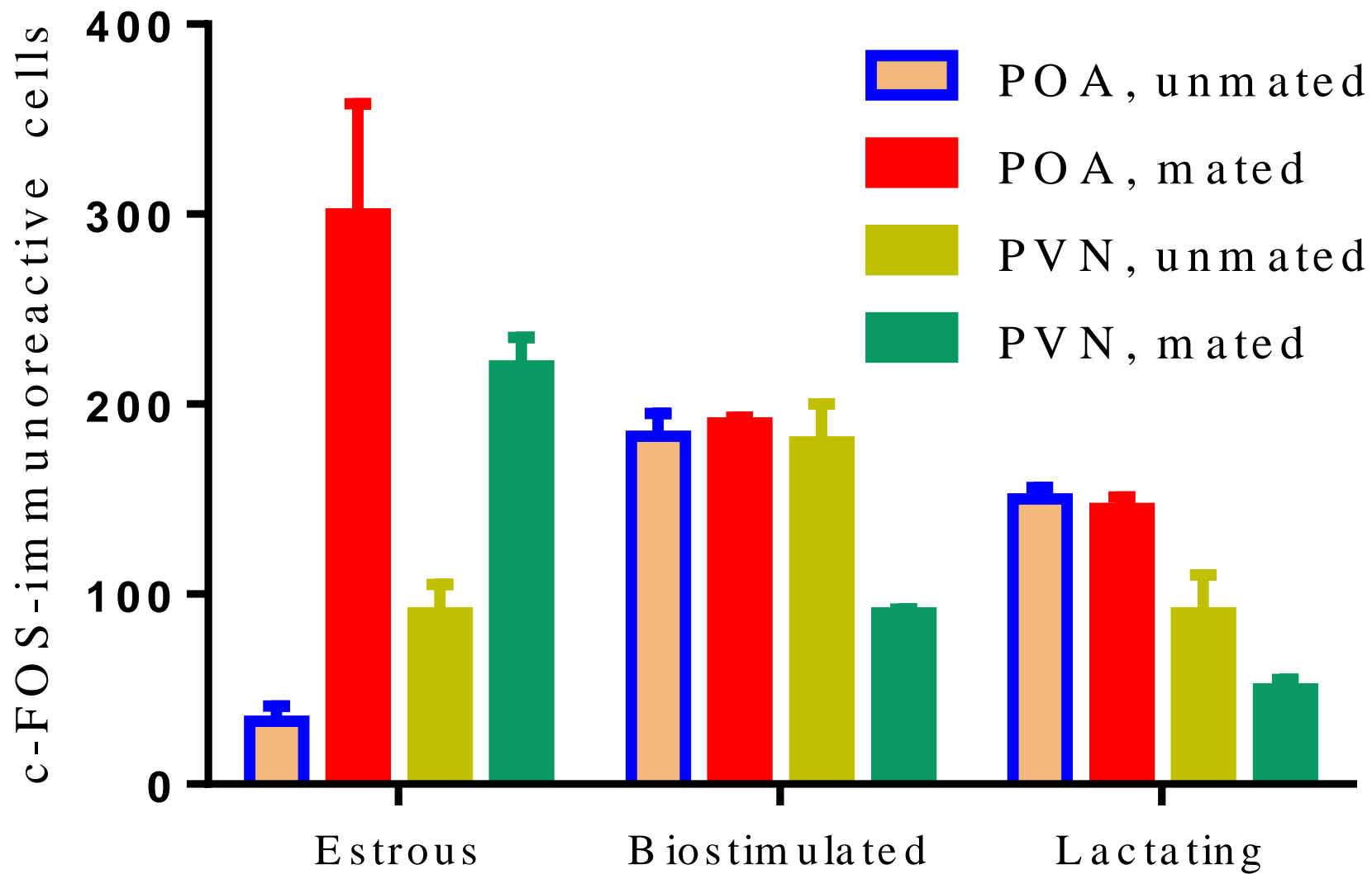
Does nurse equally well their own and alien kits, even in different types of nests (González-Mariscal and Gallegos, 2007)

Time (min; means \pm s.e.) spent nursing				
Type of litter	Type of nest			
	own	other doe	synthetic hair	male hair
own	3 \pm 1	3 \pm 1	4 \pm 1	3 \pm 1
alien	3 \pm 1	4 \pm 0.3	4 \pm 0.4	4 \pm 0.3

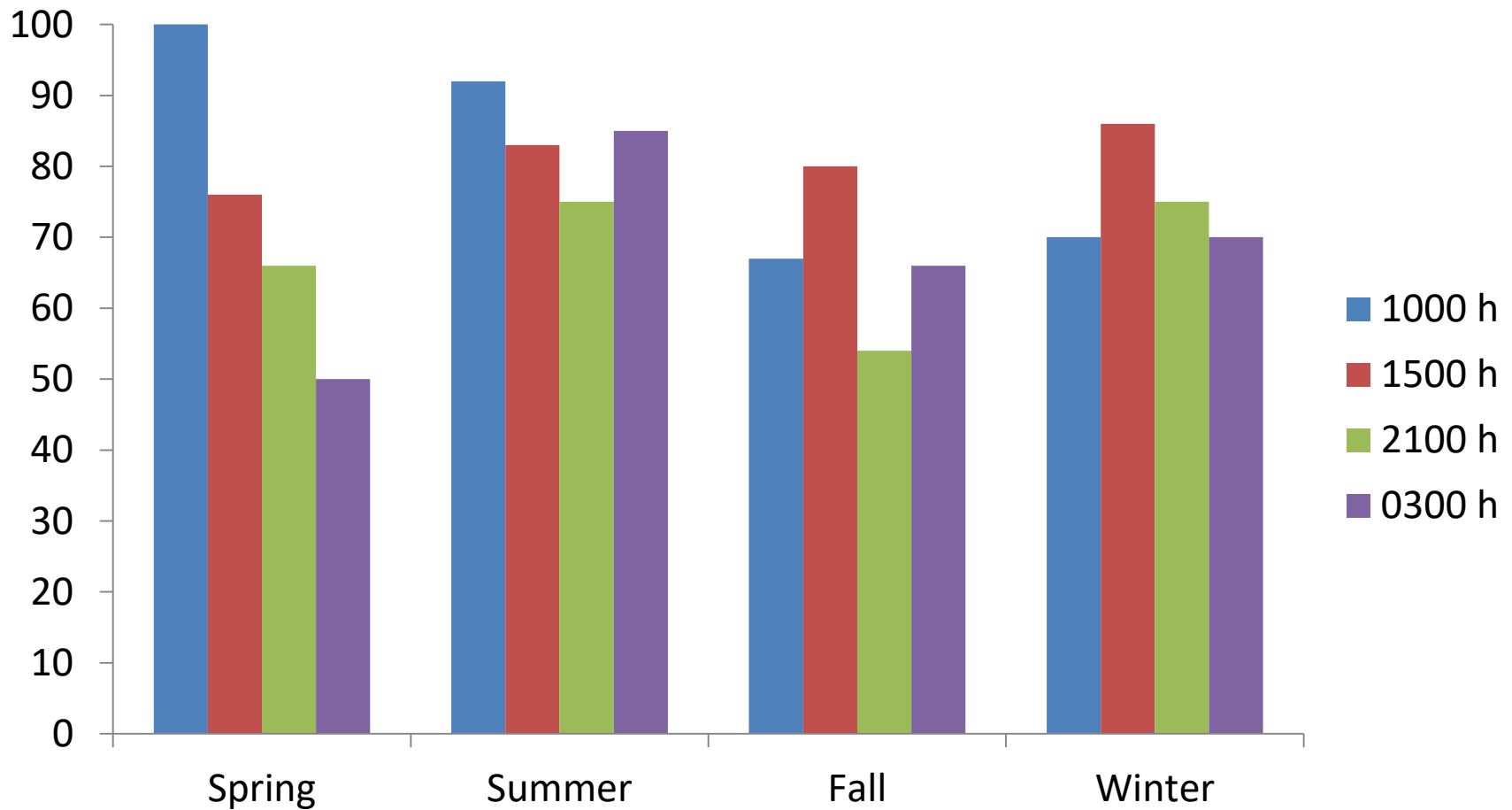
REFLEX OVULATION: NOT AN EASY MATTER

Mating inhibits chinning and ambulation (means \pm s.e.)



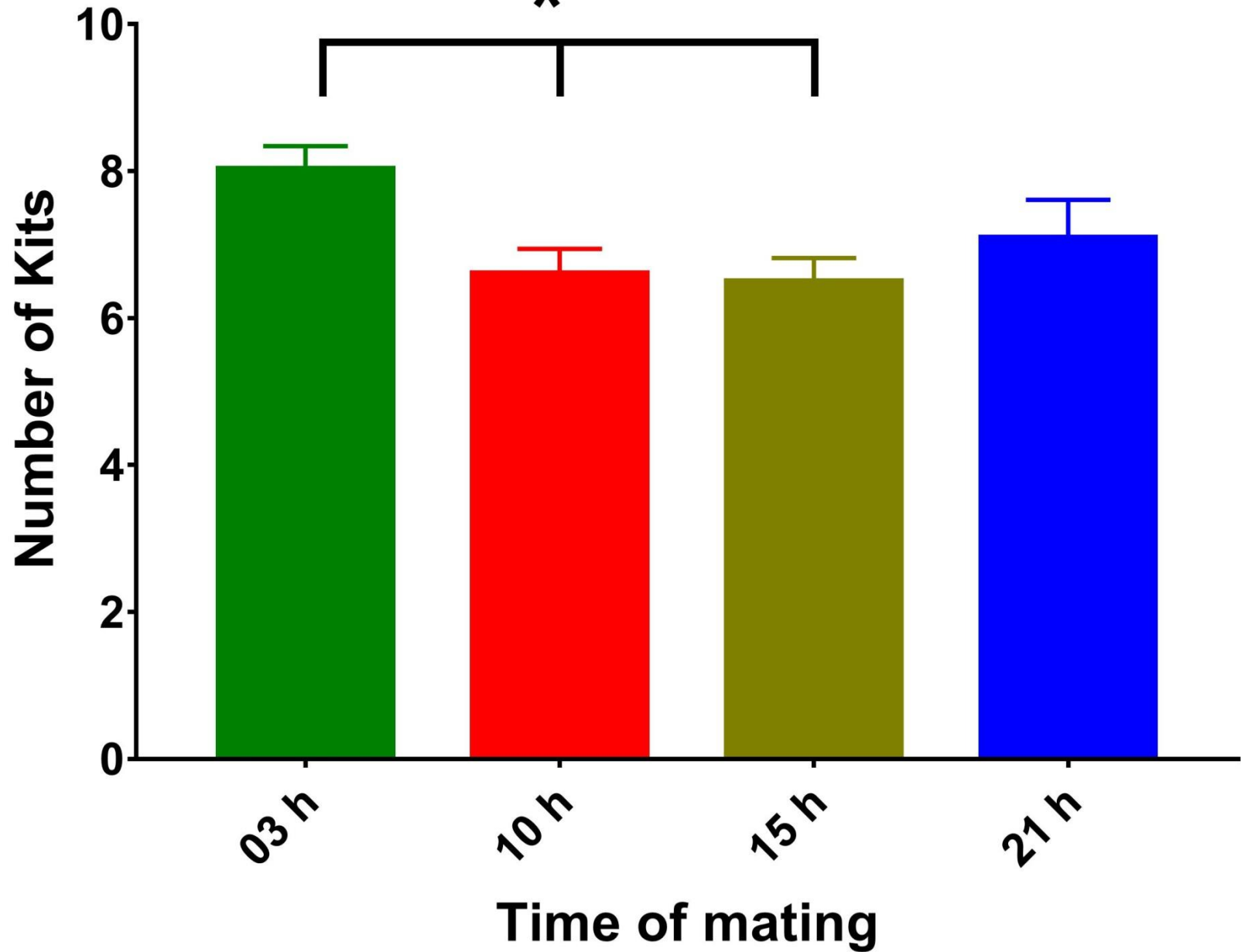


Percent fertility of does mated at different times of day across seasons (# receptive does/# parturient does)

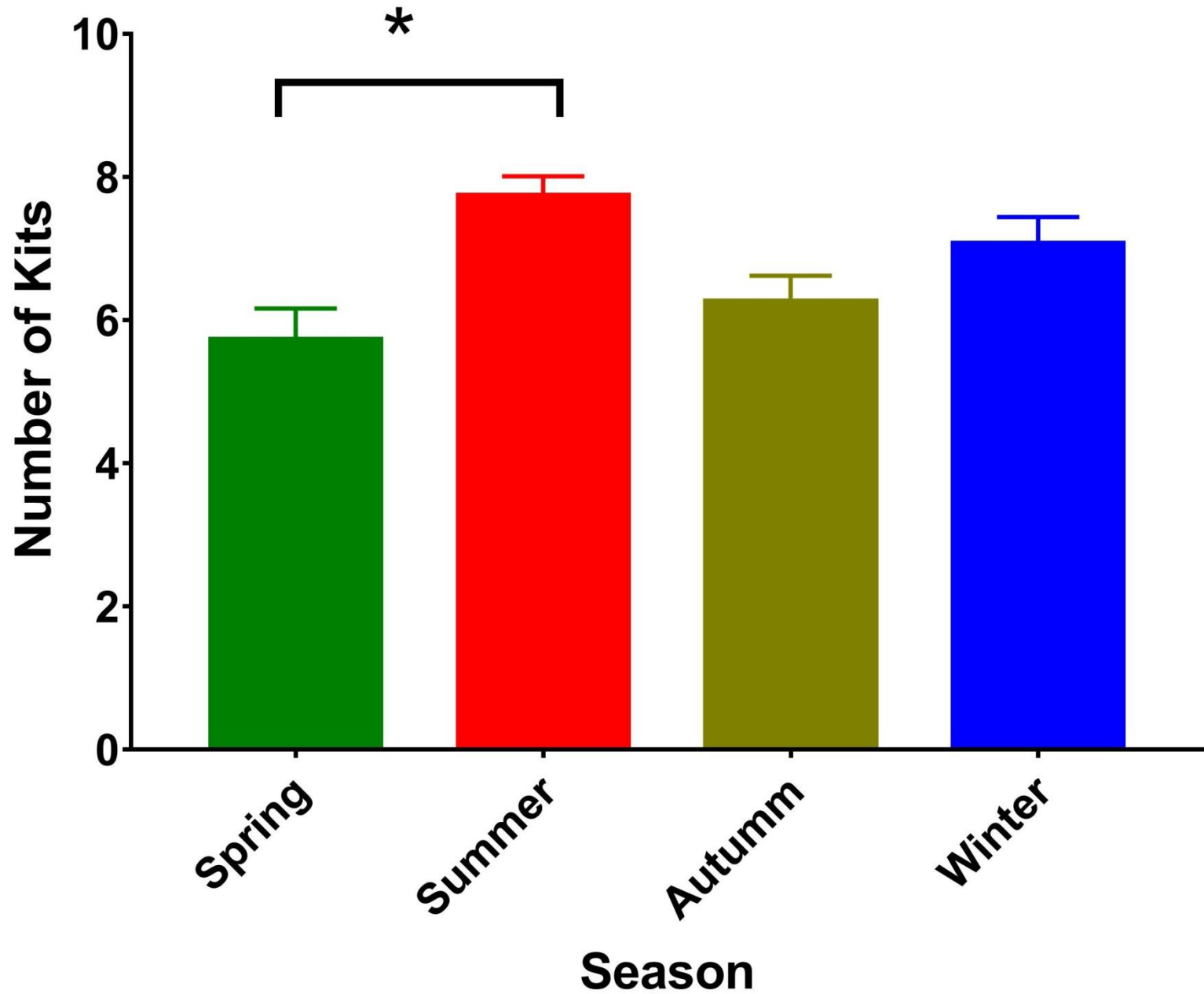


Daily variations

*



Seasonal Variations



Time vs Seasonal Variations

