

Pros And Cons Of Artificial Insemination In Rabbits Raised Under Current Climate Change Era

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ABSTRACT

Climate change refers to the long-term alterations in weather, including temperatures and humidity patterns. Such deviations can be natural, due to changes in the sun's motion or large volcanic eruptions, or induced due to the current global epidemics and wars. However, investigation into why change in environmental exposure impacts the mammalian reproductive system has increased dramatically in the previous decades. This is because damage to the reproductive system may result in changes in genes, hereditary cancer, or either temporary or permanent infertility. Sperm quality has been declining over the past years due to several factors, including environmental alterations and pollutions. One of the major problems facing rabbit production is heat stress in hot regions of the world, especially during summer season.

Therefore, those responsible for promoting the production process and improving reproduction have come to the idea of artificial insemination for a long time to overcome the problems facing the reproductive and productive processes. They have resorted to looking at the previously mentioned process, through which the safety of the semen used in the process of reproduction and production is examined and verified. The large-scale use of AI in rabbit farming dates back to the late 80s. Artificial insemination involves the collection of semen and examination of sperm quality from a male, usually of superior genetic merit, followed by the transfer of that semen to an asexually receptive female in order to result in fertilization. It has become one of the most important techniques ever devised for the genetic improvement of rabbit's farm. Heat stress adversely affects welfare and semen quality, fertility and economy of production in rabbits farming.

Finally, we can recommend that AI in rabbits under climate change become more economic in rabbits farming compared with natural mating.

Key words: Pros & Cons , Artificial Insemination, In Rabbits Raised, Current Climate Change Era

INTRODUCTION:

Artificial insemination (AI) has been applied on domestic farm animals and poultry, including rabbits long time ago (Ivanoff, 2009). Artificial insemination in rabbits has been employed since 1920s (Adams, 1961) resulting in similar or better pregnancy rates than natural breeding (Harkness and Wagner, 1983). The first person who used AI in rabbit farming was Bonadonna in 1937, which also developed the first artificial vagina for rabbits. In France, AI was used in the 1970s in selection farms, but its use was withdrawn due to the poor results obtained. In the 80s, AI began to be used in field work in Germany and Hungary. Facchin *et al.*,(1987) proposed it's used

coupled with band management, arousing great interest. Therefore, we can say that the systematic use of AI in rabbit breeding began in the late 80s in some countries. In Spain, its use was delayed until the end of the 20th century.

A great effort was performed to introduce artificial insemination (AI) in the Egyptian rabbit industry in both limited usage and wide scale rabbitries to gain more profits (El-Gaafary and Marai, 1994). Consequently, the reliable evaluation of both semen and the fertilizing ability of bucks are of vital importance to the success of using the AI technique. Conception rate in natural mating about 85% (Harkness and Wagner, 1983) but AI permits more controlled management and better planning than conventional means, e.g. in batch parturition and batch weaning (Adams, 1987). In addition, AI offers the same benefits for rabbit breeding as in other species in the control of genetic diversity, rapid upgrading of stock, establishment of pregnancies in females which refuse to mate, and avoidance of the spread of diseases such as *pasteurellosis maltocida*. The number of males needed is reduced compared with natural mating, where one male is required for approximately 8-10 females (Hafez, 1980): an ejaculate from one male can be used to inseminate up to 20 females, depending on the original sperm concentration and required insemination dose. Semen donors from other colonies can be used, provided that proper quarantine procedures are carried out. The latter is particularly important where disease such as rabbit hemorrhagic disease are concerned, since it is not known at present whether this virus can be transmitted in semen (Dr H. Fuller, personal communication).

The introduction of AI in rabbit farming provided the same advantages as in other species but, in this case, it also allowed the development of band management, which facilitates farm work by grouping all reproductive tasks in a few fixed days (Castellini, 1996). It has become one of the most important techniques ever devised for the genetic improvement of farm animals (Chupin and Schuh, 1993).

One of the major problems facing rabbit production is heat stress in hot regions of the world, especially in Egypt through summer season. This is further aggravated by global warming being experienced as a result of climate change (El-Sawy, 2023). El-Sawy, 2023 also showed that heat stress adversely affect welfare and adaptation, feed consumption and utilization, immunity, health status, growth, reproduction, and milk production in rabbits.

Artificial insemination (AI) is a procedure by which one manually deposits a sperm suspension, fresh, cooling, or frozen-thawed, into the female reproductive tract to overcome logistical problems associated with natural mating. The rabbit's production industry is a powerful example of the potential for AI to enhance the genetic health and sustainability of animal populations. Ultimately, successful AI requires sperm of adequate quality and quantity, oocytes that have attained nuclear maturation and cytoplasmic competence, operational gamete transport systems, accurate timing, and proper placement of sperm in the female reproductive tract. Increased understanding of semen collection, evaluation, and preservation techniques, estrus synchronization, and ovulation detection, and insemination instrumentation before AI success rates approach those of the livestock industry.

In the rabbit doe, ovulation does not occur spontaneously, but it has to be induced through a neuro-hormonal reflex, which is initiated during mating. When using AI, in the absence of a male, ovulation has to be induced by artificial hormonal

stimulation. The ovulation inducing method most frequently used is an intramuscular application of GnRH (Quintela *et al.*, 2004).

Pregnancy loss after AI can be attributed to genetic defects in the embryo and/or anatomical, nutritional, or physiological inadequacies of the dam, rather than epigenetic factors caused by exposure of oocytes or embryos to inappropriate *in vitro* conditions. Separation of the sexes for AI eliminates exposure to disease and traumatic injury and is most useful for aged, infirm, or behaviorally incompetent males. Paternity identification is assured if the semen of a single male is inseminated and accurate records are maintained.

Fertility of does under heat stress:

Ahmad Para *et al.*, (2020) reported that heat stress has profound effects on the fertility of both bucks and does of rabbits. Moreover, Garcia and Argente (2017) reported negative effects of heat stress on embryogenesis. When young females were raised up to 16 weeks of age, and ovulation rate was measured, a reduction in the number of ova per ovulation was observed, where the number of ova was 7.4 for does raised at 30°C compared to 9.2 ova observed for those raised at 23°C (Lebas, 2005). Evidently, Verga (1992) indicated that hot climate was the main cause for abnormal maternal and sexual behavior, and postulated that the doe that was capable to produce 10 litters a year may give only 4 to 5 litters in hot climate, which is about 50-60% reduction.

Exposure to high ambient temperature induces rabbits to try to balance the excessive heat load by using different means to dissipate, as much as possible, their latent heat. Above 35°C, rabbits can no longer regulate their internal temperature (Lebas *et al.*, 1986). At high levels of humidity, the event consequences mentioned above occur at lower temperatures than those recorded, due to that the feeling of warmth under hot ambient temperature increases with high relative humidity, although the temperature is interrelated with other climatic factors such as solar radiation, wind, photoperiod, etc. Such a relationship between ambient temperature and humidity (Lphsi, 1990) led to the measurement of the severity of heat stress by using both factors, and this was termed temperature-humidity index (THI). This parameter was modified by Marai *et al.*, (2001) for rabbits as small animals. Protection from cold winds may be required, but it must not be at the expense of adequate ventilation. Apparently, conception rate of the doe rabbit appears to be affected by seasonal variations (Ayyat and Marai, 1996). The later authors found that exposure of adult female rabbit to severe heat stress adversely affects their reproductive performance, ovulation rate, number of implantation sites and number of viable embryos per a doe. Conception rate was lower in hot summer than in winter by 76.8% (Marai *et al.*, 2001). Maya-Soriano *et al.*, (2015), on the other hand, experimented with long period of heat exposure and reported that heat stress had negative effects on prolificacy of female rabbits exposed.

Fertility of bucks and semen quality under heat stress:

El-Sawy *et al.*, (2017) found that testosterone levels were reduced in stressful hot conditions during summer season in compared with winter. The adverse effect of summer season on late of age at maturity, testicular index and testosterone

concentration may be related to decrease on relative of testes weight for rabbits reared under summer season (El-Sawy *et al.*, 2017). High ambient temperatures decreased motility, curvilinear, velocity and metabolic activity of rabbit's spermatozoa incubated at 42°C, compared to 32.5°C (Sabés-Alsina *et al.*, 2016). Sabes-Alsina *et al.*, (2016) found out that acute heat exposure of 3 h at 42 °C compromised sperm functions. Heat stress, whether acute or chronic, has profound and drastic effects on the reproductive efficiencies of both male and female rabbits. Histological studies on the testes of heat-stressed bucks revealed altered testicular cell and membranous structures (Aldemir *et al.*, 2014 and Bharti *et al.*, 2014). Libido of rabbit and sperm density was decreased by exposure to heat stress (Pei *et al.*, 2012). Heat stress causes lowered male reproductive efficiency (Turner and Lysiak 2008). Spermatogenesis is efficiently successful in rabbit bucks when the testicular temperature is kept below body temperature. This becomes impossible under hot climatic conditions. Heat stress causes damage to the spermatogonia and other stages of sperm formation. High temperature lowers sperm quality and viability. The fertilizing capacity and resulting embryos are negatively affected by high environmental temperature (Yaeram *et al.*, 2006).

Testosterone concentration, sperm output, semen quality, and fertility were listed among reproductive traits affected by heat stress in male rabbits (Marai *et al.*, 2002). El-Sobhy (2000) found that the testes of heat exposed animal's revealed significant focal degeneration in both seminiferous tubules and interstitial cells. Baglioicca *et al.*, (1987) reported that male fertility was impaired when ambient temperature was about 28°C and some individuals began to die at 35°C. In addition, Jegou *et al.*, (1984) mentioned that the reduction in testosterone level during summer season resulted from reduction in the ability of leydig and sertoli cells responding to luteinizing hormone. However, Katongola *et al.*, (1974) reported that decline in testosterone level during summer season causing disorders in accessory glands secretion and spermatogenesis. El-Sawy *et al.*, (2017); Lavara *et al.*, (2013) and Safaa *et al.*, (2008) reported that the libido, mating activity and the physical semen quality of NZW rabbit bucks during the winter season were highly significant ($P \leq 0.01$) better than those observed in the summer season. Semen volume increased and motility indexes decreased during summer (Roca *et al.*, 2005). Mate rabbits are extremely sensitive to heat stress therefore a rise in testicular temperature in rabbits leads to reduced spermatogenesis; temporary sterility; decreased sexual desire, ejaculate volume, motility, sperm concentration, and total number of spermatozoa in an ejaculate; and increased sperm abnormalities and dead sperm (Marai *et al.*, 2001). Also, Finzi *et al.*, (1995) reported that the daily exposure of rabbits in a climatic chamber to high ambient temperature (30°C) and humidity (70%) for 21 hr. over a 60 days period increased the number of abnormal spermatozoa.

Bio-stimulation to induce ovulation:

The rabbit's doe is a mating-induced ovulation. Therefore, luteinic phase only takes place when intercourse occurs, while the rest of the time the follicular phase is maintained (Bouvier and Jacquinet, 2008). Historically, two different theories have been postulated: those that propose that does are in permanent heat (Hammond and Marshall, 1925), and those that state that does alternate states of greater or lesser sexual receptivity (Hill and White, 1933). The latter is the accepted one nowadays (Moret,

1980 and Hulot *et al.*, 1988). The alternation of behavior, with greater and lesser sexual receptivity, seems to be due to the fact that in the rabbit ovary there are waves of follicular growth that last 10-12 d, with an overlap of 4-6 d between one cycle and the next (Alvariño, 1993 and Arias-Álvarez *et al.*, 2007). When there are a high number of pre-ovulatory follicles in the ovary, large amounts of 17β -estradiol are produced and the doe is receptive. On the other hand, at the onset of the wave, the concentration of 17β -estradiol is smaller, so the doe shows scarce sexual receptiveness (Bouvier and Jacquinet, 2008). Moreover, the antagonism between prolactin and gonadotropins must be considered, as AI is performed around the peak of lactation of the doe, day's 7-11 postpartum (Rebollar *et al.*, 1992 a; Theau-Clément and Roustan, 1992). Therefore, despite the previously mentioned statement that does are always in heat, their receptiveness and number of available follicles are not constant and there are individual and important variations that have not allowed us to determine the concentration of estradiol from which a doe can be considered receptive (Ubilla and Rebollar, 1995). As a consequence, to improve the outcome of AI, it is recommended to synchronize all does to make the moment of maximum receptivity coincide with the day of AI, to achieve the best result regarding fertility and prolificacy (Maertens *et al.*, 1995).

Separation of the litter to induce ovulation:

This method is based on the existing antagonism between lactation and reproduction (Rebollar *et al.*, 1992 b; Theau-Clément and Roustan, 1992; Fortum-Lamothe and Bolet, 1995). Various experiments carried out in the last decades showed a positive effect (+20 to +40%) on receptiveness with litter separation before AI (Maertens, 1998; Bonanno *et al.*, 1999 a, b; Theau-Clément and Poujardieu, 1999; Virág, 1999; Szendrő *et al.*, 1999 and Bonanno *et al.*, 2000). Even an increase in the litter size was also cited (Maertens, 1998). However, it was verified that a drop in litter weight (-20 to -70 g) occurs, as well as a drop in the individual growth of the kits, although their survival ability is not affected (Maertens, 1998; Alvariño *et al.*, 1998; Bonanno *et al.*, 1999a, b; Theau-Clément and Poujardieu, 1999; Szendrő *et al.*, 1999 and Bonanno *et al.*, 2000).

Artificial insemination takes places when the doe is in lactation, so the suckling stimulus induces a release of prolactin, which inhibits or reduces the synthesis and release of Gonadotropin-releasing hormone (GnRH, LH and FSH). This slows down the follicular growth. Cano *et al.*, (2005) partly demonstrated this hypothesis, as they observed that, after separation of the litter for 48 h, the levels of FSH and LH were higher compared to the group that kept the litter. However, they found virtually no difference in the prolactin levels. Nevertheless, in other studies, it could be shown that there was a decrease in the prolactin levels 24 h after litter separation, but not at 48 h (time of insemination). Coinciding with this decrease, there was also an increase in FSH levels and, at the time of insemination, an increase in estradiol levels (Ubilla *et al.*, 2000 and Rebollar *et al.*, 2006). In short, separation of the litter would initially produce a drop in prolactin levels and unlock FSH release, which would stimulate follicular development, producing a high number of pre-ovulatory follicles that would release large amounts of estrogen, which would increase the receptivity of the doe. This method can be combined with two types of suckling during the rest of the lactation: free suckling (the nest is open and the kits can access their mother whenever

they want), or controlled suckling (the nest remains closed and is only opened once a day for a few minutes; Theau-Clément, 2000; Bonanno *et al.*, 2000). Due to the maternal behavior of the doe, which only nurses once a day for 3-5 min and always at the same hour (González-Mariscal, 2001), independently of having continuous access to her kits, results do not differ between systems (Bonanno *et al.*, 2000).

Feeding programs to induce ovulation:

During lactation, feed intake of the doe rapidly increases. However, this rise is not enough to cover all energy requirements of basic needs and milk production. This causes a situation of negative energy balance that leads to stored fat mobilization (Parigi-Bini *et al.*, 1990 and Fortun-Lamothe, 2006). In primiparous does, this state is even more serious. These females must obtain enough energy to cover basic needs, milk production and also growth, so energy balance in these animals is extremely negative during postpartum (Parigi-Bini and Xiccato, 1993). Over the years, in an attempt to maximize the productivity of this species, the period between kindling and insemination has been reduced, with inseminations currently taking place on day 11 postpartum on most farms in Spain (Rebollar *et al.*, 2009). This means that mating/insemination coincides with the start of lactation, in the middle of a period of negative energy balance. The detrimental effect of negative energy balance on reproduction has been extensively studied in other species and it can be concluded that animals that gain weight postpartum (positive energy balance) are more likely to become pregnant than those that lose weight (negative energy balance) according to Santos *et al.*, (2009). Fortun-Lamothe (2006) indicates that postpartum energy deficit has adverse effects on oocyte production, gestation rates and embryo mortality. In the same vein, Brecchia *et al.*, (2006) found that, in animals in which a negative energy balance was induced prior to insemination by feed deprivation for 48 h, significantly lower LH peaks were observed after administration of a GnRH analogue.

Furthermore, feed deprivation for only 24 h significantly reduced the receptivity and fertility of the does. Based on this, feeding programs have been proposed in order to reduce the negative energy balance in the postpartum period, and thus improve reproductive performance. One of the possibilities explored has been the use of an energy precursor, such as propylene glycol (administered in water at 2%), in the days prior to AI, which improves pregnancy rates (Luzi *et al.*, 2001 and González, 2005). However, perhaps the most widely used method nowadays is feed flushing (Theau-Clément, 2000; Fortun-Lamothe, 2006 and Theau-Clément, 2008). This system consists of increasing energy intake (through feeding) in the days prior to AI. Based on this and considering the feeding system commonly used on farms (daily rationing of 140-150 g/d of feed, in replacement females from 12 wk. of age and in non-lactating does), flushing would consist of removing the restriction and feeding *ad libitum* in the week prior to insemination. For lactating does, rationing is not recommended, and propylene glycol can be used (González, 2005). These feeding programs can achieve fertility results similar to those obtained by administration of 20 IU of eCG (González, 2005). At the beginning of the 21st century, different studies were carried out in breeding rabbits by increasing the fiber content in their diet in order to increase the long-term intake capacity. Arias-Álvarez *et al.*, (2009) and Rebollar *et al.*, (2011) have observed that this type of diet increases intake capacity during rearing and first

gestation, with a tendency to improve fertility at 11 d postpartum. However, intake capacity does not increase during lactation, with reduced leptin concentrations at the time of first AI (16 wk of age) and reduced in vitro embryo survival. Leptin is one of the factors linking metabolism and reproduction in mammals and birds, acting both at the central nervous system and ovarian level. It has been verified that feed restriction reduces leptin levels in plasma in rabbits, with negative consequences on reproduction (Sirotkin *et al.*, 2014).

Lighting programs and ovulation:

Females do not have regular estrous cycle but may show variation in sexual receptivity, linked to waves of follicular growth and regression with the ovary. Seasonal patterns of reproductive behavior have been reported (Farreli *et al.*, 1968), although this may be influenced by the husbandry system under which the animals are kept, such as controlled day length.

Rabbits are classified as induced ovulators, with ovulation occurring 9-13 h after coits, although spontaneous ovulation is also possible. In our colony, spontaneous ovulation occurred at a rate of approximately 3%, as assessed by measurement of progesterone levels in females housed singly for at least 3 weeks before collection of blood samples. The precise mechanism of induction of ovulation is not known in this species but appears to be different from that of other induced ovulators. The inducing stimulus in cats is thought from that mechanical stimulation of the cervix, while the prolonged, sometimes violent, courtship behavior of ferrets may play a role in initiating ovulation (Porter and Brown, 1987).

The use of lighting programs to improve receptivity and fertility in rabbits is based on the existence of a reproductive seasonality of this species in the wild (Hafez, 1993). In our latitudes, the highest percentage of pregnancies occurs between February and early August, with a peak in May (Hammond and Marshall, 1925 and Boyd, 1986). It is evident that the best reproductive results are obtained when the length of daylight hours increases (Theau-Clément *et al.*, 1998). This seasonality is also observed in farm rabbits when light programs are not used, as demonstrated a study by Vega *et al.*, (1999), carried out in north-west Spain, where the best reproductive efficiency was achieved in summer and the worst in autumn. With this physiological basis, over the years several experiments have been carried out on rabbit farms in search of the best relationship between hours of light and darkness, in order to achieve optimum reproductive outcomes. From these studies we can assume that, except for the one carried out by Schüddemage (2000), applying periods of artificial light longer than 14 h per day increases the productivity of the does (Uzcategui and Johnston, 1992 and Theau-Clément and Mercier, 2004). The possible beneficial effect of increased daylight hours in the days prior to insemination has also been studied (Theau-Clément *et al.*, 1990 and Mirabito *et al.*, 1994). However, these studies showed that the weight of the kits at weaning was lower in the group with increased daylight hours (from 8 to 16) in the days prior to insemination (Mirabito *et al.*, 1994). This could indicate that the change in daylight hours could have an adverse effect on the intake of the kits. Quintela *et al.*, (2001) and Gerencsér *et al.*, (2008 a, b), showed that increasing daylight hours in the week prior to AI gave similar results to those obtained with eCG and without significantly affecting growth and mortality of the kits. In summary, the use of a

constant photoperiod of 8 hours of light and 16 h of darkness up to 7-8 d prior to insemination, with an increase to 16 h of light on the days prior to insemination, results in a marked improvement in the productivity of the does (in the absence of eCG administration).

Animal handling and ovulation:

This system consists of changing does from their cage from 48 h until shortly before AI or placing several does in the same cage before AI (Theau-Clément, 2000, 2008). Studies carried out by Lefevre and Moret (1978) postulated that an abrupt change of environment facilitates the onset of estrus in nulliparous rabbits. They explained this as the consequence of a hormonal release due to stress caused by the sudden change of environment. A similar phenomenon had previously been described in other species (Du Mesnil du Buisson *et al.*, 1962). However, shortly afterwards, Verita and Finzi (1980) found that moving the does to another cage caused significant stress to the animals, altered feeding behavior, diminished feed intake for the next three days and reduced movement of the females for more than a week. Based on these studies, numerous researchers tried to use this handling pattern to improve doe productivity. One of the first was Rebollar *et al.*, (1995), who demonstrated that, by cage changing the rabbits 48 h before insemination; it was possible to improve fertility. Subsequently, several experiments (Castellini *et al.*, 1998; Bonanno *et al.*, 1999 a, b; De Lara, 2001 and Gómez-Ramos *et al.*, 2005) confirmed the hypothesis, although with some nuances.

Thus, it was found that the effect was much higher in nulliparous rabbits, while it was practically null in primiparous females. This effect was more evident on the receptivity of the treated rabbits, but less so when considering fertility. Furthermore, it sometimes only manifested itself as an increase in litter size, and its intensity varied greatly between animals and breeds. Frequently, this technique is combined with litter separation and, in most cases, is more effective than cage changing. A recent study (Villamayor *et al.*, 2022 a), shows that, in multiparous does subjected to feed flushing, increased daylight hours prior to insemination and litter separation, the mixing of females prior to insemination improves the receptivity (analyzed by vulva color), but does not significantly affect either fertility or prolificacy, following the same pattern as previous studies.

Considering the practical difficulties in applying this technique (workforce, animal identification, sanitary issues, etc.) and that the results are highly variable and can be improved with simpler techniques, this method is rarely put into practice (Theau-Clément, 2000, 2008 and De Lara, 2001).

Pheromone communication in reproductive behavior:

The positive effect that males have on female reproduction has long been well-documented in different domestic species (Lishman, 1969; Brooks and Cole, 1970; Kirkwood *et al.*, 1981 and Roelofs *et al.*, 2007). These effects are mainly mediated by pheromones, described by Karlson and Lüscher (1959) as chemical signals transferred between members of the same species that trigger a particular response in the receiver. These chemosensory cues are delivered through physiological secretions like urine and seminal plasma (Mastrogiacomo *et al.*, 2014), as well as exocrine glands like the

lacrimal, mammary, mentonian and harderian (Melo and Gonzalez-Mariscal, 2010). Rabbits are one of the best models for researching pheromone communication in mammals (González-Mariscal *et al.*, 2016), as it is the only animal species for which a mammary pheromone (2-methylbut-2-enal, or MP) has been thoroughly characterized (Schaal *et al.*, 2003). Lactating females produce MP, which awakens newborn rabbits and triggers the nipple-sucking reflex. In rabbits, there is evidence that the presence of males increases the receptivity of females (Lefevre *et al.*, 1976) and their fertility (Berepubo *et al.*, 1993) and also induces sexual maturity in pre-pubertal rabbits (Frank, 1966). More recent studies have determined that female rabbit reproductive performance appears to increase when they are exposed to male odors just before AI (El-Azzazi *et al.*, 2017). Similarly, male-female interaction before AI, the so-called 'buck effect', slightly improves does fertility at first lactation, but no positive effect has been detected on the reproductive performance of lactating does (Bonanno *et al.*, 2003). Previous studies suggested the importance of the chin, lachrymal and harder glands in rabbit reproductive performance (Cerbón *et al.*, 1996 and Melo and González-Mariscal, 2010). Most studies related to pheromone interaction have been based on behavioral analyses of females being exposed to males, but only a few pheromones with farm applications have been fully characterized to date. Such is the case of the male pig pheromone 'Boar Mate' which improves sow (female pig) fertility by triggering immobility reflex; this is commercialized by Kubus SL. More recently, a novel boar pheromone mixture has proved to induce sow estrus behaviors and reproductive success (McGlone *et al.*, 2019); indicating that mixed pheromone compounds might have a greater effect. Regarding rabbits, two different 'pheromone products based on a combination of pheromones' including the rabbit mammary pheromone 2MB2, have been commercialized:

1. Ceva Santé Animale created the first 'Rabbit Appeasing Pheromone' in 2007. According to their results, does were less stressed and technical actions were easier. Moreover, fertility (percentage of parturitions per AI, live-born rabbits per litter and rabbits' viability at birth) improved (Bouvier and Jacquinet 2008).
2. Sibpma and SIGNS laboratories commercialize Secure Rabbit®, a synthetic analogue of the maternal appeasing pheromone (license IRSEA – US Patent 6-077-867, 6-054-481 y 6-169-113), which prevents the negative effects of stress, improves animal production and enhances animal welfare. However, no scientific data have been reported and its efficiency should be considered with caution. In recent years, in order to further our knowledge on the possible use of pheromones to improve reproductive performance in rabbit breeding, not only in terms of estrus synchronization, but also in terms of their possible influence on ovulation and even on the production of seminal doses, we have started to work on this subject through a multidisciplinary approach. This multidisciplinary approach allows us to consider the study from different points of view: reproductive (effect of the presence of males or females on reproduction in both sexes), anatomical (morphological and morpho-functional study of the pheromone releasing organs and receptors) and genetic/biochemical (study of the molecular composition of the pheromones, as well as the receptors involved in their uptake). This approach is essential to understand the functioning of pheromone-mediated communication and to be able to apply the results obtained to animal production practice. On the one hand, we evaluated the

reproductive efficiency of female rabbits (receptivity, fertility, prolificacy, and number of live born and dead kits/litter) when exposed to different conditions: female urine, male urine, seminal plasma and female–female (F–F) separated, just before AI, and female–female interaction. F-F separated referred to females not being exposed to other individuals prior to AI, whereas F-F interaction was the most common practice in the farm analyzed and consisted of placing two females together 10 min before AI. Results from this study indicated that urine does not seem to have a role in pheromone-related reproductive performance, though more studies are needed to discard this fact, considering that only lactating females with high overall fertility rates were considered in this study (Villamayor *et al.*, 2022 a). The main systems responsible for detecting chemical signals are the main olfactory system (MOS) and the accessory olfactory system (AOS) –also called vomeronasal system– (Brennan and Zufall, 2006). As part of the AOS, the vomeronasal organ (VNO) and in particular its vomeronasal receptors have been developed to detect specific ligands, notably intraspecific pheromonal cues, but also a variety of heterospecific cues from sympatric competitors. In contrast, the main olfactory epithelium (MOE) receptors have evolved to detect a wide range of odorants. The dearth of understanding regarding the anatomical and physiological basis of the sensory systems involved in rabbits' chemo-communication is demonstrated by the fact that the receptors responsible for MP detection have yet to be identified.

Therefore, there is a need for structural and morpho-functional studies of the chemosensory systems in rabbits, especially regarding the vomeronasal system, very crucial for reproduction and maternal behavior in such closely related species as rodents (Keverne, 2002). Recent studies from our group have further elucidated the structural and morpho-functional organization of the two major components of the vomeronasal system of the rabbit, the VNO and the accessory olfactory bulb (AOB) determining that the adult rabbit possesses a well differentiated VNO and a sexually dimorphic AOB, featuring many specific particularities at both structural and functional levels (Villamayor *et al.*, 2018, 2020). Additionally, the first comprehensive RNA sequencing study of the rabbit VNO across gender and sexual maturation stages has allowed us to update the number and expression of the two main vomeronasal receptor families, including 128 V1Rs and 67 V2Rs, and determined that several sex hormone-related pathways were consistently enriched in the VNO, highlighting the relevance of this organ in reproduction (Villamayor *et al.*, 2021). Recent transcriptomic analyses have also proven the rabbit VNO plastic capacity and suggested a role of vomeronasal receptors at the onset of puberty (Villamayor *et al.*, 2022 b). These results have contributed towards understanding the genomic basis of behavioral responses mediated by the VNO and open the door to the future use of pheromones to improve reproductive efficiency in rabbit animal production.

Hormonal induced ovulation:

The simplest method to achieve these results was, in the beginning, the use of follicular growth stimulating hormones. Within this group of hormones, we can find eCG (equine Chorionic Gonadotrophin) and follicle stimulating hormone (FSH). The latter has a drawback that makes it unusable on a practical level, i.e., that its half-life is too short, so several administrations must be performed to achieve the desired effect.

Additionally, we should not forget that its cost is higher than that of eCG. Different studies aiming to demonstrate the effect of eCG as a synchronization method in rabbits have shown that eCG, in dosage between 20 and 40 IU, administered 48-72 h before AI, allowed a proper degree of synchronization of the does, and that the administration of this hormone could be repeated in several consecutive cycles without having undesired effects (Maertens *et al.*, 1995; Bonanno *et al.*, 1996), although some researchers have observed a certain reduction of its efficiency when used repeatedly (Maertens, 1998; Rebollar *et al.*, 2006). The most commonly used protocol during the early years was the administration of 25 UI of eCG 48 h before AI (Rebollar and Alvaríño, 2002; Milanés *et al.*, 2004 and Rebollar *et al.*, 2006). At the end of the 20th century, in order to maintain a “natural image” of rabbit meat, an important discussion took place about the replacement of hormones by different bio-stimulation methods to boost sexual receptiveness of does at the moment of AI and, consequently, their fertility and prolificacy. The methods proposed are numerous and, in some cases, their effectiveness has not yet been fully demonstrated. However, we will briefly review all of them, with special emphasis on the last one, as it is a topic that is currently being worked on and has certain prospects for the future of AI in rabbits.

Addition of GnRH to the seminal dose to induce ovulation does have certain reproductive characteristics that contrast with those of other animal species. Some of these differences are related to the absence of a defined and regular estrus cycle (Arias-Álvarez *et al.*, 2007). Besides, induced ovulation species, like rabbits, do not have pre-ovulatory peaks of LH in response to high steroid levels, as there is no positive feedback on the pituitary LH (Sawyer and Markee, 1959), in contrast to spontaneous ovulation females (Bakker and Baum, 2000 and Brecchia *et al.*, 2006). Therefore, ovulation occurs as a consequence of the coital stimulus. In the past, it was thought that this stimulus was more physiological than mechanical. Fee and Parkes (1930) observed that anaesthetizing the cervix did not impair ovulation after mating. In addition, Salvetti (2008) determined that mating simulation with two does induced ovulation in the dominated doe. However, Rebollar *et al.*, (2012) indicated that both stimuli may be necessary to induce ovulation. These researchers observed that the mechanical stimulus, without the physiological one, and vice versa, was not enough to induce a LH peak and, consequently, ovulation. On the other hand, the application of both stimuli did induce an increase in LH concentration and ovulation in 75% of does. Nevertheless, similar percentages have been recently achieved by the inducement of ovulation using a short and flexible cannula (Viudes-de-Castro *et al.*, 2017). In short, mating induces complex neuroendocrine processes (Spies *et al.*, 1997; Ramírez and Soufi, 1994 and Bakker and Baum, 2000) that determine a pre-ovulatory LH release 60-120 min afterwards (Rodríguez, 2004 and Brecchia *et al.*, 2006). Ovulation occurs 10-12 hr. after mating (Foote and Carney, 2000 and Brewer, 2006). For this reason, it is mandatory to utilize a system to induce ovulation and obtain a pre-ovulatory LH peak when using AI in the doe. In this regard, ovulation can be induced by several methods and intervening at various levels of the hypothalamus-hypophysis-ovary axis. The simplest way to induce ovulation in this species is by mating. To this end, vasectomized males have been used in AI programs (Khalifa *et al.*, 2000). However, the results were fairly random and generally ineffective (Hulot and Poujardieu, 1976). Additionally, this method requires labor and keeping the males in the farm, so part of

the advantages of AI would be lost. Consequently, this method was cast aside. Another attempt to induce ovulation by non-hormonal practices was proposed by Kishk *et al.*, (2000). It consisted of the injection of copper salts, based on studies that mentioned the existing synergism between copper and gonadotropins (Cheng *et al.*, 1999). Although the results showed an elevation of LH concentration after copper salts administration, it was necessary to inject them intravenously, and the negative consequences on the vessels' integrity needed further research.

Moreover, experiments with human chorionic gonadotrophin (hCG) were also performed to induce ovulation (Bomsel-Helmreich *et al.*, 1989 and Romeu *et al.*, 1995). It was concluded that this hormone was effective to induce ovulation in the doe, but repetitive injections in subsequent reproductive cycles induce immunization and a loss of effectiveness after the 4th or 5th administration, in contrast with GnRH analogues (Adams, 1981). Furthermore, embryonic survival was also inferior (Romeu *et al.*, 1995 and Mehaisen *et al.*, 2006). For these reasons, its use has been strongly restricted. Finally, the most common method to induce ovulation in does is the intramuscular administration of GnRH or synthetic analogues at the moment of AI (Quintela *et al.*, 2004). First, 250 ng/kg of gonadorelin were administered to each doe to achieve ovulation (Kanematsu *et al.*, 1974). Taking this experiment as a basis, different analogues of this molecule, such as buserelin and lecirelin, were used. The former was efficient at 0.8 ng/doe dose (Theau-Clément *et al.*, 1990 and Perrier *et al.*, 2000), while doses of 2-4 ng/doe were necessary for the latter (Zapletal *et al.*, 2003 and Zapletal and Pavlik, 2008). As previously mentioned, intramuscular administration was always performed. In recent decades, several studies have been conducted testing the intra-vaginal administration of different GnRH analogues (buserelin, triptorelin, lecirelin, alarelin, goserelin and leuprolide, (Quintela *et al.*, 2004; Viudes-de-Castro *et al.*, 2007; Ondruska *et al.*, 2008; Quintela *et al.*, 2009 and Gogol, 2016).

These studies shows that it is possible to administer GnRH analogues vaginally, added to the seminal dose, without a decrease in fertility and prolificacy. Some of them even show an improvement in both parameters compared to intramuscular administration (Quintela *et al.*, 2009). The advantages of this new administration route are mainly related to the time needed to perform the AI, fewer sanitary risks and a reduction of hormone administration mistakes. The only drawback, from an economic standpoint, is that the dosage of GnRH analogues must be increased to achieve the desired effect. This increment is probably due to the fact that in the seminal plasma there are proteolytic enzymes that reduce the availability of the hormone added to the semen, along with the status of the vaginal mucosa, the analogue used or the semen characteristics (Vicente *et al.*, 2011 and Dal Bosco *et al.*, 2011). Nowadays, in Spain, a semen diluent that incorporates an analogue of GnRH is commercialized, and its use in farms is becoming more widespread (Quintela *et al.*, 2012). In 2020, the EM Authorized the inclusion on the market of MRAbit®, (Kubus, Spain), a diluent for refrigerated storage of rabbit semen containing alarelin, a GnRH analogue.

In this regard, experiments have recently been carried out by adding different proteolytic enzyme inhibitors to the diluent or by protecting the GnRH analogue, encapsulating it with nanoparticles, as is already done in human medicine (Casares-Crespo, 2020). In these studies, researchers observed that the use of unspecific inhibitors negatively affected prolificacy. However, the addition of amino-peptidase

specific inhibitors (best at in and ethylene-di-amine-tetra acetic acid) did not affect either fertility or prolificacy. Moreover, the use of chitosan and dextran sulfate nanoparticles, combined or not with these inhibitors, did not affect reproductive performance, and allowed the reduction of buserelin dosage. Although these are preliminary studies and further investigation is needed, they open the way to the reduction of the amount of GnRH analogue added to the semen. Ratto *et al.*, and Adams *et al.*, (2005) stated that the intramuscular administration of seminal plasma of alpaca and llama induced ovulation in more than 90% of the treated alpacas and llamas. Later, different researchers verified that rabbit seminal plasma induced ovulation in llamas (Silva *et al.*, 2011), 100% when using rabbit' seminal plasma. Kershaw-Young *et al.*, (2012) identified the factor present in the seminal plasma of llamas that was responsible for the induction of ovulation, *i.e.* β -Nerve Growth Factor, a protein of approximately 14 KDa. This protein is able to induce a LH peak and ovulation in 90% of llamas, after intrauterine administration, in absence of mating (Berland *et al.*, 2013 and Silva *et al.*, 2015). The presence of this protein in the seminal plasma of different species (hamster, rabbit, boar and bull) has been known since the 80s (Castellini *et al.*, 2020), but its effect on the female as not yet been studied. Following the discoveries in alpacas and llamas, the interest in β -Nerve Growth Factor as a possible ovulation inducer in rabbits grew exponentially. As a result, numerous studies have been published in recent years (Sánchez-Rodríguez, 2019; García-García *et al.*, 2020 and Mattioli *et al.*, 2021). However, the results obtained so far are not as expected. The administration of rabbit seminal plasma to does, as previously performed by Ratto *et al.*, (2005) and Adams *et al.*, (2005) in llamas, does not induce an increase in LH concentration or ovulation (Silva *et al.*, 2011 and Masdeu *et al.*, 2012). On the other hand, recombinant β -Nerve Growth Factor vaginal administration, added to the seminal dose, is able to induce ovulation, but in a smaller percentage of animals compared to intra-muscular-gonadorelin administration (60% vs. 100%, Sánchez-Rodríguez *et al.*, 2019), which leads us again to the idea that both mechanical and physiological stimuli are necessary to induce ovulation in the doe.

Artificial insemination technique

Dimitrova *et al.*, (2009) compared two schemes of hormonal stimulation. First scheme included application of PMSG and GnRH. In the second it was applied only GnRH. As a result in first case we obtain 28.57% fertility rate and in second – 62.50% fertility rate. White New Zealand does show higher level of fertility than Californian does. They showed that the second scheme of hormonal stimulation, using only GnRH application was more effective. Similar results reported Rodriguez De Lara *et al.*, (2000). Some authors have reported efficiency by stimulation with PMSG in follicular growth (Bonanno *et al.*, 1990) and consequently increase prolificacy (Maertens *et al.*, 1995), but followed by higher stillborn birth mortality. The results obtained in our experiment did not show difference in both prolificacy between two compared schemes of hormonal stimulation 7.5 vs. 7.6 average numbers of litter size and in birth mortality rate. Nulliparous does generally exhibit high fertility rate, whereas the reproductive performance of multiparous does is lower, because of the intensive reproductive rhythms (Castellini *et al.*, 2005). In our case we used extensive reproductive system (insemination two days after winning) and this is why in this

experiment we obtained higher or equal fertility rate for multiparous does and nulliparous does. Maertens and Bousselmi (1999) reported fertility rate of nulliparous does 78.7%. Lower fertility rate of Californian does in comparison with White New Zealand ones was observed from other authors too (Rodriguez De Lara *et al.*, 2000 and Gomes *et al.*, 2004).

The reproductive performance of rabbit does are affected not only by parity, but also by other factors as body condition, rhythm of insemination, lactating status, sexual receptivity and season (El-Gaafary and Marai, 1994 and Brecchia *et al.*, 2008). It was shown that season affected both natural mating and artificial insemination. Our experiments were carried out in August and September, the part of the year which was reported as a period with lowest conception rate (Szendro *et al.*, 1992) and it is possible reason for very low results of animals of Californian breed.

Semen evaluation:

In fresh semen, count of total sperm output, pH, semen color per buck visually assessed and grade from one to three scores, semen density, semen movement grade from 0 to 5 scores, mass motility, a drop of fresh semen put on a clean warmed microscopic slide (37°C) without a cover slip, wave motion record with low power lens (10×), and grade from 0 to 5 scores (from total immobility to rapid wave motion) according to Chemineau *et al.*, (1999). For the determination of sperm progressive (advanced) motility (%), the percentage of motile spermatozoa measure according to Seleem (2005) by placing a small drop of semen on a clean warmed microscope slide, and then diluted with drops of a 2.9% of sodium citrate solution (a dissolve 2.9 g of sodium citrate in a 100 ml of sterile distilled water). Then they carefully mixed together and covered with a warmed cover slip. Assessments were made with high power lens (40×) using a scale graded from 0 to 5 scores (from no displacement of sperm cells to straight and rapid displacement of spermatozoa) according to Chemineau *et al.*, (1999). The sperm-cell concentration per ejaculate calculates with the aid of a hemocytometer according to Loskutoff and Crichton (2001). The number of sperms per millimeter calculates as follows:

$$\text{Concentration/ml} = (\text{Dilution factor}=200) \times (\text{Count in the five squares}) \times (0.05 \times 10^6).$$

Sperm abnormalities (%) estimates using a magnification of 500× with a differential interference contrast microscope. Abnormalities can occur in the head, neck, mid-piece, tail, or any combination of these parts of the sperm cell (Salisbury *et al.*, 1978). Non-vital sperms calculate as dead spermatozoa (%). The recorded number of stained dead sperms could be determined the percentage of dead spermatozoa (Evans and Maxwell, 1987). Acrosome damages (%) or percentages of unreacted acrosome of spermatozoa with an abnormal apical ridge determine using a Giemsa stain procedure as described by Watson (1975).

Economics of artificial insemination:

When following the artificial or natural insemination system in a rabbit farm with strength of 100 females, the cost of insemination from one male will be 2.32 LE in artificial insemination compared to 13.95 LE in natural insemination, which means a decrease of 83.3% in cost in favor of artificial insemination (Table 1). Motedayen *et al.*, (2007) showed that the conception rate is similar in both groups (natural mating or

artificial insemination) and differences are not statistically significant. Also, the productive power of mothers increases by 10-12% in the case of artificial insemination compared to natural insemination, with the number of cages on the farm, service costs, and the rest of the fixed costs in general being constant (Table 2). Motedayen *et al.*, (2007) indicated that a good result of AI in rabbit and have implication to reduce the

Table 1: Economical comparison between the cost of AI and NM of rabbits*

Item	Natural mating	AI
Price of buck/L.E.	500	500
Feeding cost/buck/year/L.E.	1369	1369
Managerial cost/buck/year/L.E.	64	64
Total	1933	1933
No. of ejaculates/buck/week	4	4
volume of ejaculates/buck/week/ml	2	2
Semen produced/buck/year/ml	2*52=104	2*52=104
Diluted semen/buck/year/ml	--	416
No. of inseminating females/buck/year	104 or 208	832
Cost of female inseminating/buck	18.6 or 9.29	2.32

- AI= Artificial insemination, NM= Natural mating.
- Personal observations and calculations.
- Feeding cost/buck/year = 0.250 g feed * 365 day * 15 L.E/ kg feed.
- Managerial cost/buck/year = 24 L.E. (vaccinations) + 20 L.E. Drugs + 20 L.E. housing and cervices.

Table 2: Comparison between the additive productions of AI vs. natural mating of rabbits

Item	Natural mating	AI
Males no./100 females	20	8-10
Females Adding	00	10-12%
Maximizing of production power	00	10-12%

number of bucks, decrease the failure of ovulation in does and increase the fertility rate.

Dimitrova *et al.*, (2009) reported that Artificial insemination (AI) is a powerful tool for genetic improvement of animals, which also offers better sanitary guarantee. It allows better work organization as well as decreased manpower costs. The success of artificial insemination technique in rabbits depends mainly of the parity number, the physiological status and the sexual receptivity at the moment of insemination (Castellini, 1996). In European commercial farms, the most common reproductive rhythm is based on AI of the does around 11 days after kindling and on weaning of the young rabbits at 28–30 days of age (Castelliin *et al.*, 2006).

From the above, it can be said that artificial insemination is considered the best economically in rabbit farms compared to natural insemination, especially under the current climate change conditions.

Conclusively, we can recommend that AI in rabbits under climate change become more economic in rabbits farming compared with natural mating.

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إيجابيات وسلبيات التلقيح الاصطناعي للأرانب المرباه تحت ظروف التغير المناخي الحالي

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تغير المناخ يشير للتغيرات طويلة المدى في الطقس، ويشمل أنماط درجات الحرارة والرطوبة. يمكن أن تكون هذه التغيرات طبيعية بسبب التغيرات في حركة الشمس أو الانفجارات البركانية الكبيرة أو ناجمة عن الأوبئة والحروب العالمية الحالية. ومع ذلك فقد زاد البحث في سبب تأثير التغير في البيئية على الجهاز التناسلي للثدييات بشكل كبير في العقود السابقة. وقد إتضح أن الأضرار التي تصيب الجهاز التناسلي قد تؤدي إلى تغيرات في الجينات أو الإصابة بالسرطان الوراثي أو العقم المؤقت أو الدائم. وقد تدهورت جودة الحيوانات المنوية على مدى السنوات الماضية بسبب عدة عوامل منها التغيرات البيئية والتلوث. ومن أهم المشاكل التي تواجه إنتاج الأرانب الإجهاد الحراري في المناطق الحارة من العالم وخاصة خلال فصل الصيف. لذلك فإن القائمين على تحسين عملية الإنتاج والتكاثر قد توصلوا منذ فترة طويلة إلى فكرة التلقيح الاصطناعي للتغلب على المشاكل التي تواجه العمليات التناسلية والإنتاجية في الأرانب، والتي يتم من خلالها الفحص والتحقق من جودة صفات السائل المنوي المستخدم في عملية التلقيح والإنتاج. وقد بدأ استخدام التلقيح الاصطناعي على نطاق واسع في تربية الأرانب في أواخر الثمانينيات. ويتضمن التلقيح الاصطناعي جمع السائل المنوي وفحص جودة الحيوانات المنوية، وعادة ما يكون الذكر ذا قيمة وراثية متفوقة، ثم نقل هذا السائل المنوي إلى أنثى قابليتها جيدة من أجل الإخصاب. **التوصية:** وقد أصبح التلقيح الاصطناعي من أهم التقنيات التي تم ابتكارها على الإطلاق للتحسين الوراثي في مزارع الأرانب. يؤثر الإجهاد الحراري سلبيًا على رفاة الأرانب وجودة السائل المنوي والخصوبة واقتصاديات الإنتاج في تربية الأرانب.