World Rabbit Sci. 2021, 29: 247-261
doi:10.4995/wrs.2021.14897
WRSA, UPV, 2003

LEVELS OF TESTOSTERONE, PROGESTERONE AND OESTRADIOL IN PREGNANT-LACTATING DOES IN RELATION TO AGGRESSION DURING GROUP HOUSING

BRACONNIER M.*, GONZÁLEZ-MARISCAL G.†, WAUTERS J.‡, GEBHARDT-HENRICH S.G.*

*Centre for Proper Housing of Poultry and Rabbits (ZTHZ), Division of Animal Welfare, VPH Institute, University of Bern, Burgerweg 22, CH-3052 Zollikofen, Switzerland.
†Centro de Investigación en Reproducción Animal, CINVESTAV-Universidad Autónoma de Tlaxcala, Tlaxcala, Mexico.
‡Division of Reproduction Biology, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany.

Abstract: The neuroendocrine regulation of rabbit maternal behaviour has been explored in detail. However, little is yet known about the hormonal regulation of aggression in concurrently pregnant-lactating does, a reproductive condition that prevails during group housing of rabbits on farms. Therefore, in this study we determined the relation between a) the levels of progesterone, testosterone, and oestradiol during lactation; b) the anogenital distance at artificial insemination; and c) the timing of grouping with the intensity of agonistic behaviour, published previously. We performed four consecutive trials, where three groups of eight does each were artificially inseminated on day 10 postpartum (pp) and grouped on either day 12, 18 or 22 pp. Using *Dipetalogaster maxima*, a reduviid blood-sucking bug, we collected blood samples during the pregnant-lactating phase (days 13, 15, 17, 19, 21, 23 pp) on one or two randomly chosen does per treatment group. Testosterone levels varied little across the pregnant-lactating phase, agreeing with results from pregnant-only rabbits, while progesterone levels increased from day 3 (=13 dpp) to day 7 (=17 dpp) and remained unchanged until day 13 (=23 dpp) of pregnancy. All oestradiol concentrations fell below the limit of detection. Overall, all concentrations were slightly lower in comparison to rabbit studies with pregnant-only does. The agonistic behaviour was not related to the respective hormonal concentrations at grouping. In conclusion, the time point of grouping does after artificial insemination (AI) in the semi-group housing system only had a weak influence on aggression and the hormonal profile did not indicate an optimum time for grouping.

Key Words: Rabbits, agonistic interactions, progesterone, testosterone, oestradiol.

INTRODUCTION

The neuroendocrine regulation of maternal behaviour in rabbits has been investigated before (see recent review in: González-Mariscal *et al.*, 2016). Several studies have determined that specific hormones (e.g., oestradiol, progesterone, testosterone, prolactin) act on particular brain structures (e.g., preoptic region, anterior hypothalamus, lateral septum) to control the expression of nest building (i.e., digging a burrow, carrying straw into it and lining the straw-nest with plucked body hair). Throughout lactation, nursing (inside the nest box) is restricted to a single bout per day, lasting around three min, and displayed with circadian periodicity. This precise temporal regulation relies heavily on the characteristics of the suckling stimulus provided by the kits (González-Mariscal *et al.*, 2013a, 2013b).

However, hardly any studies have focussed on the hormonal basis of aggression in female rabbits. In addition to nest building and nursing, the behavioural repertoire of lactating rabbits also includes agonistic behaviour and nest defence. This has been documented in European rabbits (*Oryctolagus cuniculus*) living under semi-natural conditions (Rödel *et al.*, 2008), in domestic rabbits kept on the farm (Mugnai *et al.*, 2009; Andrist *et al.*, 2012) and also in

Correspondence: M. Braconnier, *braconnierm@yahoo.com.* Received *January 2021* - Accepted *October 2021.*
https://doi.org/10.4995/wrs.2021.14897

the laboratory (Denenberg *et al.*, 1958). Yet, hardly anything is known about the hormonal factors that regulate this maternal aggression in lactating does. This information is important in behavioural neuroendocrinology, as well as in animal welfare and science. In the latter field, rabbit breeders are trying to ensure the well-being of their animals (for review, see González-Mariscal *et al.*, 2017). Accordingly, as rabbits are gregarious by nature, the group housing system is becoming more popular, especially on European farms devoted mainly to meat production (Maertens *et al.*, 2011; Zomeño *et al.*, 2018). In Switzerland, the semi-group housing system consists of separating pregnant does around the time of parturition and grouping them around lactation day 12. This type of housing has been labelled an "especially animal-friendly" housing system (BTS, 2019). It supposedly combines the positive aspects of continuous single housing and continuous group housing, i.e. the does are undisturbed during early lactation and, from day 12 onwards, they have more space for locomotion, can interact with conspecifics and, therefore, they develop less stereotypies compared to animals in continuous single housing (Chu *et al.*, 2004; Dal Bosco *et al.*, 2019). Additionally, compared to continuous group housing, the rate of infanticides, litters of two does in the same nest box and pseudopregnancies are reduced in the semi-group housing system (Andrist *et al.*, 2013; Szendrő *et al.*, 2016). For more details on this housing system as well as other existing ones, please refer to Nielsen *et al.* (2020).

However, recent studies have shown fighting among the does at grouping, with resulting injuries in up to 60% of the animals (Andrist *et al.*, 2013; Buijs *et al.*, 2015). Fights are partially caused by a natural need to reinstate hierarchy, as the replacement and exchange of sick and non-pregnant does are common practice on farms (Andrist *et al.*, 2013; Rommers *et al.*, 2014). Another trigger could be the defence of their kits. As they are only 12 d old at grouping, the does may still react defensively to conspecifics approaching their nest (Rödel *et al.*, 2008). Other factors that might contribute to aggression among lactating does have not been investigated.

The endocrine conditions of concurrently pregnant-lactating does differ from those found in pregnant-only and lactating-only rabbits (Fortun *et al.*, 1993). Specifically, progesterone (P4) concentrations are lower in pregnant-lactating than in pregnant-only does on gestation days 7 and 14, whereas oestradiol (E2) levels are higher in the former on pregnancy days 1 and 21 (González-Mariscal *et al.*, 2009). Testosterone (T) is also produced across gestation and even during lactation, though at lower levels in the latter condition. It promotes particular aspects of nest building (i.e., digging and hair-loosening; González-Mariscal *et al.*, 2003) but its concentration in pregnant-lactating does is unknown.

Little is known about how steroid hormones could influence agonistic behaviour in female does: two studies found an increased aggressive response in ovariectomised, oestradiol-treated does given P4 peripherally (Hoffman *et al.*, 2009) or into the hypothalamus (Palka and Sawyer, 1966). Therefore, the main aim of this study was to determine the concentrations of testosterone, progesterone and oestradiol during the pregnant-lactating phase of does and analyse their relation with the agonistic behaviour shown at grouping which was reported previously (Braconnier *et al.*, 2020). A better understanding of the temporal variation in hormonal secretion during lactation and simultaneous gestation might help us determine an optimal time point for grouping. In addition, we correlated the anogenital distance (AGD) measured at AI (Braconnier *et al.*, 2020) with the hormonal values during pregnancy/lactation because: a) earlier studies suggested that AGD at birth could be useful in selecting less aggressive does for communal breeding (Buijs *et al.*, 2016); b) there is a correlation between the AGD measured at birth and that observed in adult does (Bánszegi *et al.*, 2009). If a correlation was found, AGD could serve as a prediction of the aggressive potential of individual does. This could be used for selecting does during breeding or when assembling groups.

ANIMALS, MATERIALS AND METHODS

Ethical approval

This study was approved by the Cantonal Office of Aargau (No. 30611). It met all federal and cantonal regulations on animal experimentation.

Animals and housing

This experiment was part of a study carried out on a Swiss rabbit breeding farm using 57 multiparous does of the Hycole hybrid. Eight does were randomly chosen as focal animals and sampled for hormonal analyses in this experiment. All the animals were multiparous (>than 2 births).

Each pen contained eight individual cages (each approximately 1.6 m^2). Every cage included a nest box (0.30×0.40 m), a straw bedded platform, a feeder and a nipple drinker. During the grouping phase, the cages were opened at the top and the animals had access to a common floor area (3.20×2.20 m) (Figure 1). Air temperature and relative humidity were continuously recorded during the experiment (HOBO® Datalogger U10-003, Onset Computer Corp., Bourne, MA 02532) and varied between 8.4°C and 20.1°C and 26 to 67% humidity, respectively. There was natural daylight in the barn, supplemented with artificial light during feeding and early working times during winter in a haphazard way. A 16/8 lighting regime was established five days before until two days after AI, as this has been reported to be beneficial for sexual receptivity in does (Theau-Clément *et al.*, 1990). The does were supplied *ad libitum* with hay, water and commercial rabbit pellets (UFA 925, UFA AG, Herzogenbuchsee, Switzerland) containing 10.0 MJ digestible energy, 170 g crude protein, 145 g crude fibre, 35 g crude fat and 95 g crude ash per kg.

Breeding management and grouping

The does were kept in three groups of eight animals/pen on a 41-day reproductive cycle. The isolation period of the semi-group housing started for every group one day before parturition of the kits (day –1). On lactation day 10, a 24 h doe-litter separation was performed to improve fertility rates following artificial insemination (AI; Arias-Álvarez *et al.*, 2010). Does with unsuccessful AI were excluded from the study, but stayed in their groups until the end of a trial to assure a consistent group number. The day of AI was considered day 0 of pregnancy. The time of grouping (=treatment) occurred on different days postpartum (dpp), specifically: 12 (TG12), 18 (TG18), or 22 (TG22). Each trial lasted from day –1 to day 25 pp (weaning of kits) (Figure 2).

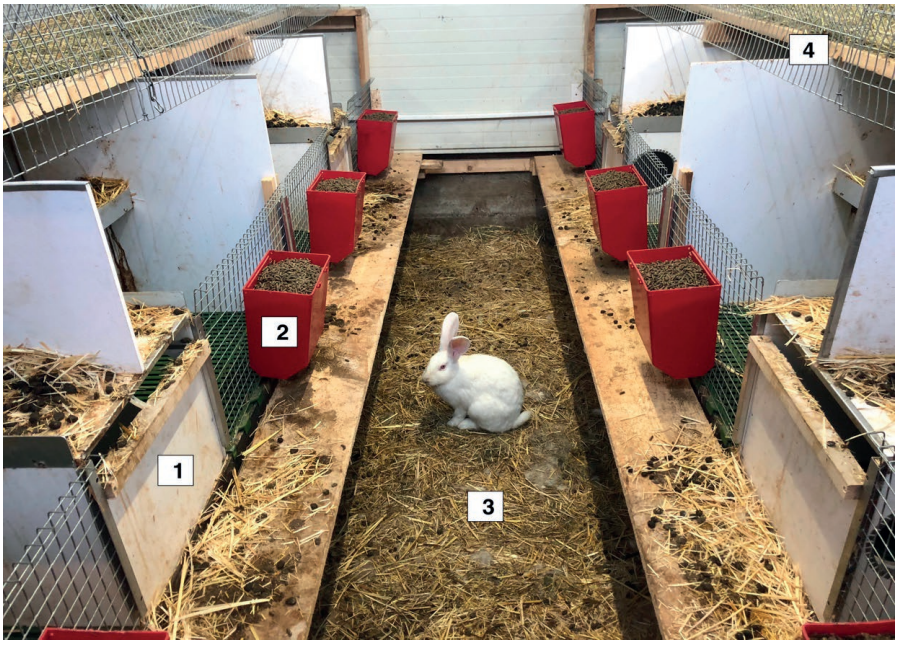


Figure 1: Pen design. (1) Nestbox, (2) Feeder, (3) Common area, (4) Grid for isolation purposes.

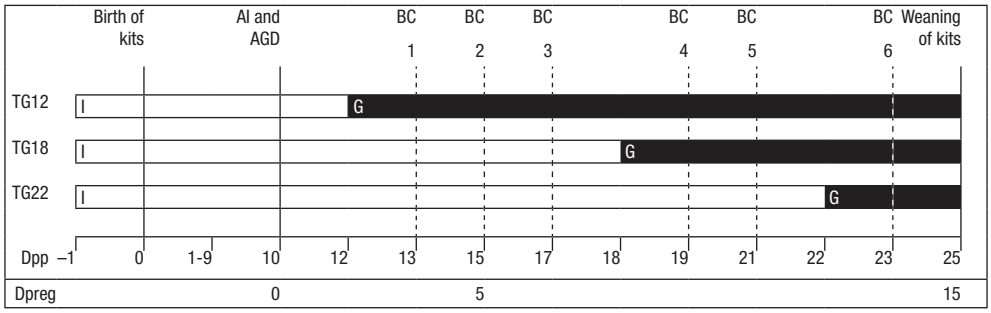


Figure 2: Timeline of a trial.
AGD=Anogenital distance; AI=Artificial insemination; BC=Blood collection; Dpp=Day postpartum; Dpreg=Day of pregnancy; G=Group housing; I=Isolation; TG=Treatment group.

In total, we conducted four trials with one group in each treatment from October 2018 until March 2019. Before each new trial, sick or non-pregnant animals were removed and replaced with pregnant does, as is common practice on farms. Initially, three groups were randomly allocated to one of the three treatments (TG12, TG18 or TG22). After each trial, the groups switched to another treatment in the following trial. All does were individually marked with ear tags and livestock spray. The number of suckling kits was documented per doe and litters were not standardised. See timeline in Figure 2 for clarification of the procedures performed in a trial.

Blood sampling

Background information and bug method

Frequent blood sampling in rabbits —usually from the ear (*Vena auricularis*) or leg (*V. saphena lateralis)* veins— can pose difficulties, due to handling stress or the formation of hematomas (Hein, 2014, 2019). In a group housing system, an indwelling catheter is not an option.

Reduviid blood-sucking bugs (*Dipetalogaster maxima*) are a valid alternative to the conventional blood sampling method in tapirs and rabbits (Voigt *et al.*, 2006; Kosowska *et al.*, 2015). Having a proboscis with a diameter about 10 times smaller than a conventional needle, they cause less mechanical stimulus and pain during the blood extracting process (Vos *et al.*, 2010). Moreover, the metabolism of *D. maxima* does not interfere with the hormonal concentrations determined in rabbit blood for up to eight hours after a blood meal (Voigt *et al.*, 2004). Therefore, *D. maxima* in the 3rd, 4th and 5th nymphal stage (N3, N4, N5) were used as a blood sampling method in this experiment (see details below).

Hormonal profile

To obtain the hormonal profile of pregnant-lactating does, blood samples were collected from 1-2 selected animals per treatment group on days 3, 5, 7, 9, 11 and 13 of pregnancy (dpreg) between 7-11 a.m. (=6 samples/animal). For unknown reasons, three does did not become pregnant after AI. The samples of these lactating-only does were excluded from the hormonal analysis. See Table 1 for details.

Table 1: Doe rabbits used for blood collection and behavioural observations.

Trial	Doe ID	TG	RS	Doe ID	TG	RS	Doe ID	TG	RS	Doe ID	TG	RS	Doe ID	TG	RS
1	Epsilon	12	pl	Delta	18	pl				Beta	22	pl			
2	Beta	12	pl	Epsilon	18	l	Zeta	18	l	Alpha	22	pl	Delta	22	l
3	Gamma	12	pl	Alpha	18	pl				Beta	22	pl			
4	Alpha	12	pl	Eta	18	pl	Theta	18	pl	Gamma	22	pl			

AI=Artificial insemination; l=Lactating-only (not analysed); pl=Pregnant-lactating; TG=Treatment group; RS=Reproductive status after AI.

Sampling and extraction process

The bugs were fasted for five weeks prior to the experiment to ensure stinging (Markvardsen *et al.*, 2012) and kept in an incubator with 60% relative humidity and a temperature between 26-28°C (Stadler *et al.*, 2011). On the farm, one-two bugs were hidden in a specially designed plastic container that was tied around the chest area of the animal (Figure 3). At the bottom of each container was a gauze bandage through which the bugs could pierce the skin of the rabbit. Bugs that had not started sucking after 10 min were replaced. During the sampling process, the rabbit sat in a separate box to prevent disturbance from the rest of the group. Bugs that survived the extraction process were individually marked and re-used again after a 5-wk starvation period, but only on the same animal to avoid eventual transmission of disease. The does were regularly health checked by a trained veterinarian to document eventual occurring allergies or injuries.

Handling of the blood samples

Anticoagulants were not needed, as the bugs' saliva contains Dipetalogastin, a potent thrombin inhibitor (Voigt *et al.*, 2003). The blood was centrifuged at 3000 *g* for 15 min and the plasma was transferred to a microtube and frozen at –20°C until assayed.

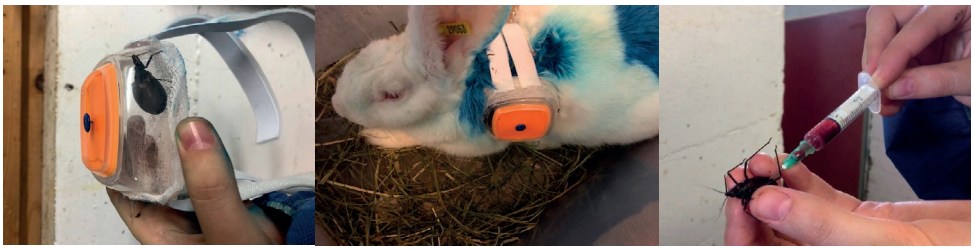
Blood analyses

Testosterone (T)

Blood plasma (0.1 mL) was extracted with 2 mL of butyl t-methyl ether: petroleum ether (30:70) for 30 min. After freezing at –80°C for 10 min, the organic phase was decanted, evaporated at 55°C under N_2 stream and dissolved in 0.2 mL of 100% methanol and 0.3 mL of bide stilled water.

Testosterone was quantified by an enzyme immunoassay (EIA) using polyclonal antibodies (rabbit) against testosterone-3-CMO-BSA and the corresponding 3-CMO-peroxidase as label. The cross-reactivities were as follows: testosterone, 100%; dihydrotestosterone (DHT), 53.85%; 4-androsten-3ß,17ß-diol, 4.6%; 19-nortestosterone, 2.3%; <0,01% for 11ß-hydroxyetiocholanolone, 11-oxo-etiocholanolone, cortisol, corticosterone, 5α-androstan-17-one, androstenedione, androsterone, 5α-androsterone, dihydroepiandrosterone (DHEA), testosterone-glucuronide and epiandrosterone.

The range of our calibration curve (standard testosterone) was 0.2-100 pg/20 µL. The linear range, between B80 and B20, was determined between 0.75 and 10.36 pg/20 µL. All EIA measurements were performed in duplicate with acceptance criteria of a coefficient of variation (CV) below 5%.



A B C

Figure 3: Blood sampling from a rabbit using blood-sucking *Dipetalogaster maxima*: (A) 1-2 bugs were hidden in a specially designed container fixated around the chest area of the rabbit. After finding an underlying vein, the bugs punctured the skin and started sucking. (B) After an average time of 17 min, the bugs had finished their meal and released. The container was removed. (C) The bug was taken out of the container and its lower abdominal area was punctured with a syringe (18-21 G). The blood was withdrawn under slow and steady pressure.

The inter-assay CV (5 assays), based on a low quality control sample (LQC) and high quality control sample (HQC), both fitting the linear range of the curve and run in duplicate, was respectively 9.20 and 7.72%.

The intra-assay CV, determined on two biological samples including low and high concentration (16 repeats in duplicate each), was 9.72 and 4.31%, respectively.

Progesterone (P4)

Blood plasma (0.1 mL) was extracted with 2 mL of petrol ether for 30 min. After freezing at –80°C for 10 min, the organic phase was decanted, evaporated at 55°C under N_2 stream and dissolved in 0.4 mL of 100% methanol and 0.6 mL of bi-distilled water.

Progesterone was quantified by an enzyme immunoassay (EIA) according to (Dehnhard *et al*., 2008) using a commercial progesterone antibody raised in rats (Sigma P1922) and a 4-pregnen-3,20-dione-3-CMO-peroxidase label. The cross-reactivities to other steroids were as follows: 4-pregnen-3,20-dione (progesterone), 100%; 5α-pregnan-3,20-dione (5α-DHP), 76.8%; 5α-pregnan-3ß-ol-20-one (5a), 18.3%; <0.1% for 5-pregnen-3ß-ol-20-one; 5ß-pregnan-3α-ol-20-one, 20α-dihydroprogesterone, pregnandiol, 17α-hydroxyprogesterone, testosterone, oestradiol and cortisol.

The range of the calibration curve (standard progesterone) was 0.2-100 pg/20 µL. The linear range, between B80 and B20, was determined between 1.79 and 18.83 pg/20 µL. All EIA measurements were performed in duplicate with acceptance criteria of a coefficient of variation (CV) below 5%.

The inter-assay CV (4 assays), based on a low quality control sample (LQC) and high quality control sample (HQC), both fitting the linear range of the curve and run in duplicate, was respectively 14.70 and 10.31%.

The intra-assay CV, determined on two biological samples including low and high concentration (16 repeats in duplicate each), was 9.07 and 4.46%, respectively.

Oestradiol (E2)

Blood plasma (0.1 mL) was extracted with 2 mL of butyl t-methyl ether: petroleum ether (30:70) for 30 min. After freezing at –80°C for 10 min., the organic phase was decanted, evaporated at 55°C under N_2 stream and dissolved in 0.1 mL of 100% methanol and 0.15 mL of bide stilled water.

Oestrogens were quantified by an enzyme immunoassay (EIA) according to (Carnaby *et al*., 2012) using a polyclonal antibody raised in rabbits to 1,3,5(10)-estratrien-3,17b- diol-17-HS-BSA and a 1,3,5(10)-estratrien-3,17b-diol-17- HS-peroxidase label. The cross-reactivities to oestrogens were as follows: 1,3,5(10)-estratrien-3,17b-diol (17b-E2), 100%; 1,3,5(10)-estratrien-3,17-one (estrone), 100%; 1,3,5(10)- estratrien-3,17a-diol (17a-E2), 66%; 1,3,5(10)-estratrien- 3,16a,17b-triol (oestriol), 1.5%; and 0.1% for 19-nortesto- sterone, P4 and testosterone.

The range of our calibration curve (standard 17b-E2) was 0.2-100 pg/20 µL. The linear range, between B80 and B20, was determined between 0.79 and 1.05 pg/20 µL. All EIA measurements were performed in duplicate with acceptance criteria of a coefficient of variation (CV) below 5%.

The inter-assay CV (3 assays), based on a low quality control sample (LQC) and high quality control sample (HQC), both fitting the linear range of the curve and run in duplicate, was respectively 4.99 and 7.75%.

The intra-assay CV, determined on two biological samples including low and high concentration (16 repeats in duplicate each), was 7.26 and 3.28%, respectively.

Agonistic behaviour

Agonistic behaviour after grouping of the does was described and published previously (Braconnier *et al*., 2020). In brief, 24-h video recordings were conducted immediately after grouping. They started when the cages were opened at around 7 a.m. All does were observed on the videos, however for this part of the experiment, we only focussed on the eight animals that were sampled for blood (shown in Table 2). The frequency of the coded agonistic behaviour ("aggression score") was assigned to the animal that started the interaction. For the aggression score we used a

modified version of Graf's ethogram (Graf *et al.*, 2011) including behaviours like "boxing", "biting", "threatening", ripping, jumping on each other, nudging, biting, heavy-chasing and "carousel fights".

The coder was blind to the identity of each animal. The recorded periods were chosen based on a prior scanning of the videos for the phases with the most agonistic interactions after grouping. Moreover, it has been reported that most agonistic interactions happen immediately after grouping (Graf *et al.*, 2011; Rommers *et al.*, 2011; Andrist *et al.*, 2013) or during early morning. Indeed, many behavioural and endocrine parameters of rabbits show that these animals are crepuscular (for review see: Aguilar-Roblero and González-Mariscal, 2020).

As the animals' aggression scores were highly variable between individuals (from 0 up to 75 attacks during the coded period), we were also able to test for differences in testosterone and progesterone concentrations between aggressive (aggression score: >10) and submissive does (aggression score: 0-1) during grouping.

Anogenital distance

In brief, we measured the AGD on the day of AI using a digital calliper, from the base of the genital papilla to the centre of the anal opening, as described by (Bánszegi *et al.*, 2012). The mean of three consecutive measurements per animal was used to improve accuracy.

STATISTICAL EVALUATION

The data were evaluated using R (*R Core Team*, 2019). Non-parametric statistics were used for all analyses, as the scores obtained were not normally distributed. The differences in the hormonal levels between aggressive and submissive does, treatments and trials, as well as between the daily concentrations of P4 and T during the pregnancy-lactation, were analysed by Kruskal–Wallis analysis of variance followed by Mann–Whitney U tests with Bonferroni adjustment for multiple comparisons. As no significant differences in hormonal concentrations were detected between trials (testosterone: χ^2=1.4679, *P*=0.68, progesterone: χ^2=2.359, *P*-value=0.5), all trials were pooled. All oestradiol concentrations fell below the detection limit and therefore could not be used for a statistical evaluation. Initially, repeated analyses were planned among does to account for individual variation. However, only two does were pregnant-lactating across 3 trials and only one doe was pregnant-lactating across 2 trials (Table 2). As individual does differed in hormonal status as well as in level of aggression across trials, all measurements were considered independent. The correlation between the frequencies of aggressive behaviour and the hormonal levels at grouping was calculated with Spearman rank correlation coefficients for each treatment group. The same test was performed to estimate correlations between the AGD (measured at day 10 pp=AI) and the hormonal concentrations (measured at day 13 pp). All means are given as means±standard deviation. *P*-values below 0.05 were considered significant.

RESULTS

Hormonal measurements

Testosterone (T)

Most (92%) of our samples yielded testosterone concentrations below the linear range, but detectable. Figure 4 shows the concentration of T in pregnant-lactating does. We combined the three treatments because there were no differences in the mean concentrations of T among them (TG12=121.7±9.8 pg/mL, TG18=119±37.1 pg/mL, TG22=137.5±28.1 pg/mL; χ^2=0.33, *P*=0.8, N=12). Thus, when considering all animals, T ranged from 118±25.5 pg/mL on 3 dpreg to 137±35.2 pg/mL on 13 dpreg. The rise in T was not significant (χ^2=4.0, *P*-value=0.55) (Figure 4).

Progesterone (P4)

Figure 4 shows the concentration of P4 in PL from the three treatments combined because there were no differences in the mean concentrations of P4 among the three treatments: TG12=3.5±1.4 ng/mL, TG18=3.7±2.1 ng/mL, TG22=2.8±1.2 ng/mL; χ^2=3.64, *P*=0.2 (N=12).

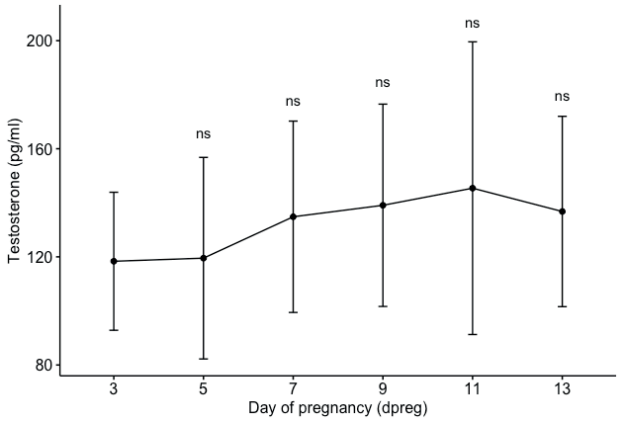


Figure 4: Testosterone concentrations on specific days of concurrent pregnancy-lactation (solid line; mean±standard deviation). No significant differences were found across days.

The values rose from 3 dpreg (1.96±1.35 ng/mL) to 7 dpreg (3.89±0.978; $P<0.01$); from then onwards, no significant increases were observed (Figure 5).

Oestradiol (E2)

For the E2 analyses, the used EIA had a calibration curve starting at 10 pg/mL. Thus, 87% of the samples fell below that concentration, the rest was below the linear range, starting at 39.5 pg/mL. Therefore, we did not draw any conclusions from the results.

Correlation between agonistic behaviour and steroid plasma levels

The frequency of aggressive behaviour (=aggression score) of the does was not correlated with the testosterone levels following grouping or with the progesterone concentrations in any of the treatments. The correlations were all below 0.3 and none of them were significant (*P*-values above 0.48) (Figures 6 and 7).

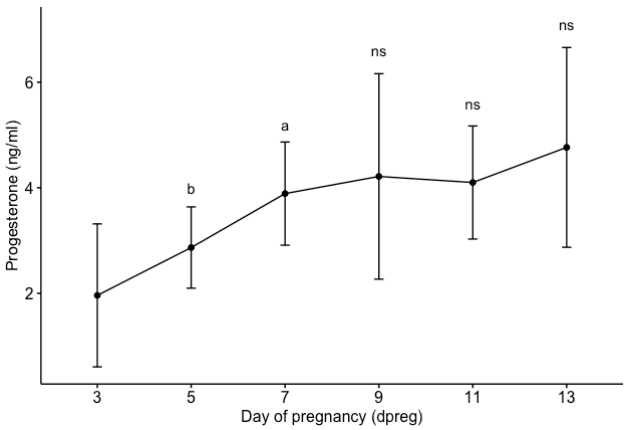


Figure 5: Progesterone concentrations on specific days of concurrent pregnancy-lactation (solid line; mean±standard deviation; a=$P<0.05$, b=$P<0.01$ *vs.* preceding value in the corresponding curve).

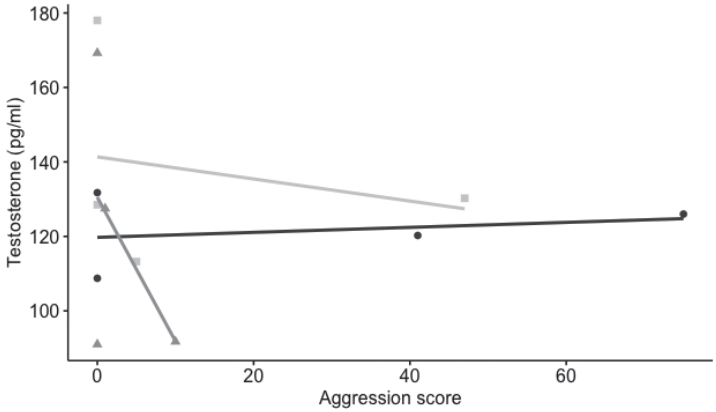


Figure 6: Correlations between the testosterone concentration and the aggression score of the does in the 3 treatment groups (Grouped on day 12 after parturition, TG12: R=0.25, *P*=0.75; Grouped on day 18 after parturition, TG18: R=–0.51, *P*=0.49, Grouped on day 22 after parturition, TG22: R=–0.24, *P*=0.76). Treatment group: —●—12; —▲—18; —■—22.

T and P4 concentrations did not differ between aggressive and submissive does. Mean testosterone concentrations were 122.8±16.9 pg/mL (aggressive does; N=7) and 126.3±35.2 pg/mL (submissive does; N=8); χ^2=0.001, *P*=0.98, N=15. Mean progesterone concentrations were 3.00±1.4 ng/mL (aggressive does; N=7) and 3.6±2.8 ng/mL (submissive does; N=8); χ^2=0.054, *P*=0.82, N=15.

Anogenital distance

The AGD of all focal animals was around 10.3±1.1 mm (see Table 2 for details). The AGD was not correlated with the T levels (r=0.36, N=15, *P*=0.26) or the P4 levels (r=0.26, N=15, *P*=0.43).

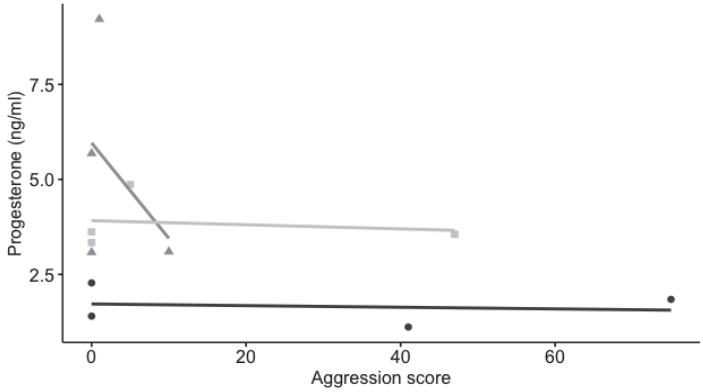


Figure 7: Correlations between the progesterone concentration and the aggression score of the does in the 3 treatment groups (TG12: R=-0.15, *P*=0.85; TG18: R=–0.42, *P*=0.58; TG22: R=–0.18, *P*=0.82). Treatment group: —●—12; —▲—18; —■—22.

Table 2: Mean±standard deviation of anogenital distance (AGD) (in mm) over 4 trials.

Trial	Mean	SD	N
1	11,34	1,14	3
2	9,94	1,08	5
3	9,75	0,81	3
4	9,75	0,79	4

Bug method for blood sampling

The average sucking time of the bugs was 17 min (min. 8 min, max. 43 min). On average, 0.5 mL of blood was collected with N3, 1.3 mL with N4 and 2.1 mL with N5. A total of 101 bugs were used for 90 blood samples (TG12, n=4, samples=24; TG18, n=6, samples=36; TG22, n=5, samples=30). After attaching the container, 84.4% of the bugs immediately stung and started sucking. None of the rabbits showed any swelling or hematoma at the sucking site of the bugs. In addition, no allergic reactions were documented after repeated blood sampling on the same animal over the course of two weeks. None of the samples was lost due to clotting. Seven samples had to be discarded due to severe haemolysis.

DISCUSSION

To our knowledge, this study is the first to determine the concentrations of oestradiol, testosterone and progesterone during the pregnant-lactating phase of does and analyse their relation with the agonistic behaviour shown in a previous publication (Braconnier *et al.*, 2020), by the animals used in the three experimental groups. To increase external validity, the sample of does included different seasons (=trials). As there was no indication that trials or treatments affected hormonal levels and sample sizes were small, trials and treatments were pooled in the analyses. This might have led to a low power of this study but at the same time it avoided spurious results, difficult to replicate on commercial farms.

Hormonal measurements

Testosterone (T)

In comparison to values reported in pregnant-only does of around 250 pg/mL (10 dpreg, with little variation until the end of pregnancy; González-Mariscal *et al.*, 1994), the values of pregnant-lactating does in this study were lower with 145±54.2 pg/mL on 11 dpreg.

Progesterone (P4)

The concentrations of progesterone in pregnant-lactating does were similar to results reported in past studies (González-Mariscal *et al.*, 2009). In both works, P4 increased from 1 to 14 dpreg in PL rabbits and non-significant changes until day 28.

Oestradiol (E2)

In general, E2 concentrations have been reported to be extremely low in lactating does in the past. González-Mariscal *et al.* (1994) reported mostly stable E2 levels of 60 pg/mL from day 10 to day 25 of pregnancy in pregnant-only rabbits, with great variation among individuals. In pregnant-lactating does values around 30 pg/mL have been measured (González-Mariscal *et al.*, 2009). This matches the few values that we were able to detect (data not shown).

Agonistic behaviour and steroid plasma levels

While the aggressive behaviour during grouping was variable among trials (Braconnier *et al.*, 2020) and is known to depend on the season (Andrist *et al.*, 2013), the T levels showed little or no variation during the entire pregnant-lactating phase in all three treatments, trials and groups, as has been shown in pregnant-only does (González-Mariscal *et al.*, 1994). On the other hand, P4 levels were increasing over the course of the pregnant-lactating phase, but this did not mirror increased aggressive behaviour at grouping: TG18 and TG22 did not show an increased aggressive behaviour compared to the TG12 group (Braconnier *et al.*, 2020). In domestic European rabbits living under semi-natural conditions, does react defensively to an approach to their burrow for up to 20 d of lactation (Rödel *et al.*, 2008). In contrast, Zomeño *et al.* (2017) stated that late lactation and kits leaving the nest boxes may decrease intra-female aggression, as has also been shown in rodent model studies (Squire, 2009). However, the time point of grouping did not affect aggression in does in two experimental studies (Braconnier *et al.*, 2020; Bill *et al.*, 2020). However, as weaning naturally occurs around 20-28 d postpartum, depending on the current pregnancy state of the does (González-Mariscal and Gallegos, 2014; Hudson *et al.*, 1996, 2000), the later time points of grouping might still have been too early to reduce aggression. Nevertheless, progressing gestation, similar to early lactation, could cause an increase in aggression compared to mid- or early gestation, as has been described in mice (Mann and Svare, 1982). Additionally, we cannot exclude that the relatively small sample size and the high individual variation within treatment groups may have masked possible effects.

Overall, the amount of aggression was highly variable between individuals (Braconnier *et al.*, 2020). This is supported by other studies on domesticated rabbit does (Rommers *et al.*, 2011) as well as in wild living rabbits (Holst *et al.*, 2002; Rödel *et al.*, 2006). However, the mean testosterone and progesterone concentrations did not differ between aggressive and submissive does in our study. This has also been shown in other studies in baboons and lemurs, where aggressiveness was not related to physiological T levels measured across females (Beehner *et al.*, 2005; von Engelhard *et al.*, 2000). Nonetheless, another study on reproducing female ibexes found an association between aggression and testosterone levels (Shargal *et al.*, 2008). For progesterone, the first experiment of Mann *et al.* (1984) showed no positive relationship between the P plasma levels of untreated mice and their individual display of fighting. Past results in rabbits reporting a possible role of P on aggression cannot be related to our present findings, as both of those works (Hoffman *et al.*, 2009; Palka and Sawyer, 1966) used ovariectomised, oestrogen-primed does, whereas in our study pregnant-lactating animals had almost undetectable oestradiol levels. Thus, the possibility that P may promote aggression in the absence of oestradiol, but in the presence of T, needs further investigation.

Indeed, testosterone as well as progesterone concentrations underlie dynamic changes triggered during social interactions i.e., in response to winning a fight during the day (Wingfield *et al.*, 1990; Davis and Marler, 2003; Trainor *et al.*, 2004) and they cannot be assessed by blood sampling only once a day. Another complication is that testosterone is converted to dihydrotestosterone or oestradiol within the brain (Simpson, 2002) and there is growing evidence that this regulates behaviour as well, including aggression (Takahashi *et al.*, 2018; Trainor *et al.*, 2006).

Anogenital distance

Although the repeatability for the AGD was good (Braconnier *et al.*, 2020), we could not find a correlation between AGD and P4 or T levels. Possibly, our sample size was again too low to create stable estimates for AGD (Schönbrodt and Perugini, 2013). In studies on AGD in mice, sample sizes were higher: 89 females used in Drickamer (1996) or 205 females in Palanza *et al.* (1995).

In the past, it has been shown that higher testosterone exposure *in utero* causes greater AGD in mice (Albert *et al.*, 1990) and in rabbits (Bánszegi *et al.*, 2012). More recently, a cross-sectional study in women (Mira-Escolano *et al.*, 2014) found that a correlation between these two parameters still existed in adulthood: AGD was positively associated with serum testosterone levels, but not with any other reproductive hormones. However, a study on Holstein cows (Gobikrushanth *et al.*, 2017) was not able to find a similar, significant relationship between these two parameters when measured in adulthood.

It is likely that these contradictory results may be caused by variation in AGD during the female cycle: one study (Dušek and Bartoš, 2012) found AGD to be variant during a single oestrus cycle in mice. In our earlier study, we

found a general decrease in AGD for all rabbit does during winter (for more details see: Braconnier *et al.*, 2020). There, we discussed a possible decrease in receptivity (smaller, non-swollen vulva) during the winter due to a shorter photoperiod. However, as we were unable to obtain reliable results for oestradiol levels that would cause this effect (Lebas, 1997; O'Malley, 2005), we cannot confirm this possibility here. One existing study from Ubilla and Rebollar, (1995) found no correlation between plasma E2 levels and turgidity or colour of the vulva.

Bug method

The bug method using *Dipetalogaster maxima* performed well for our purposes. The animals showed no allergic or pain reaction, similar to what has been reported in other studies (Thomsen and Voigt, 2006; Markvardsen *et al.*, 2012). Blood extraction from the bug should be done carefully and slowly, as haemolysis otherwise occurs due to an excessive shearing force. We would recommend the bug method as a valid alternative for regular blood withdrawal in rabbits. However, while it has already been validated for hormonal measurements in rabbits (Voigt *et al.*, 2004), significant differences in some clinical and haematological parameters in comparison to the conventional method have been found. For more details, see (Markvardsen *et al.*, 2012).

CONCLUSION

Testosterone levels showed little variation across the pregnant-lactating phase, corresponding to results from pregnant-only rabbit studies. Progesterone levels increased from day 3 to day 7 and then remain unchanged until day 13 of pregnancy. They were slightly lower in comparison to pregnant-only rabbit studies. The agonistic behaviour was not related to the concentrations of either testosterone or progesterone. However, sample sizes used were low and the study was performed on one commercial farm, only. More studies are needed to determine a possible influence of steroid and non-steroid hormones on the aggressive behaviour in pregnant-lactating female does. In conclusion, the hormonal profile, which was not related to the level of aggression in our preliminary study, did not indicate an optimal time for grouping.

Acknowledgements: We thank the Federal Food Safety and Veterinary Office (BLV, Project No. 2.18.04) for the financial support of the project. We would also like to thank Felix Näf, Frans Köhlen and Abdulsatar Abdel Rahman for the professional assistance during the data collection for this study. Furthermore, many thanks to Prof. Dr. Günter Schaub from the University of Bochum for his help and expertise in breeding the bugs used in this project. Finally, we would like to thank Mareen Albrecht and Katrin Paschmionka from the Leibniz-Institut für Zoo- und Wildtierforschung (IZW) for the blood sample analysis.

The authors declare no conflict of interest.

REFERENCES

Aguilar-Roblero R., González-Mariscal G. 2020. Behavioral, neuroendocrine and physiological indicators of the circadian biology of male and female rabbits. *Eur. J. Neurosci., 51: 429-453. https://doi.org/10.1111/ejn.14265*

Albert D.J., Jonik R.H., Walsh M.L. 1990. Hormone-Dependent Aggression in Female Rats: Testosterone Implants Attenuate the Decline in Aggression Following Ovariectomy I. *Physiol. Behav., 47, 659-664. https://doi.org/10.1016/0031-9384(90)90074-E*

Andrist C.A., Bigler L.M., Würbel H., Roth B.A. 2012. Effects of group stability on aggression, stress and injuries in breeding rabbits. *Appl. Anim. Behav. Sci., 142: 182-188. https://doi.org/10.1016/j.applanim.2012.10.017*

Andrist C.A., van den Borne B.H.P., Bigler L.M., Buchwalder T., Roth B.A. 2013. Epidemiologic survey in Swiss group-housed breeding rabbits: Extent of lesions and potential risk factors. *Prev. Vet. Med., 108: 218-224. https://doi.org/10.1016/j.prevetmed.2012.07.015*

Arias-Álvarez M., García-García R.M., Torres-Rovira L., González-Bulnes A., Rebollar P.G., Lorenzo P.L. 2010. Influence of hormonal and nonhormonal estrus synchronization methods on follicular and oocyte quality in primiparous lactating does at early postpartum period. *Theriogenology, 73: 26-35. https://doi.org/10.1016/j.theriogenology.2009.07.017*

Bánszegi O., Altbäcker V., Bilkó Á. 2009. Intrauterine position influences anatomy and behavior in domestic rabbits. *Physiol. Behav., 98: 258-262. https://doi.org/10.1016/j.physbeh.2009.05.016*

Bánszegi O., Szenczi P., Dombay K., Bilkó Á., Altbäcker V. 2012. Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. *Physiol. Behav., 105: 1226-1230. https://doi.org/10.1016/j.physbeh.2012.01.002*

Beehner J.C., Phillips-Conroy J.E., Whitten P.L. 2005. Female testosterone, dominance rank, and aggression in an Ethiopian population of hybrid baboons. *Am. J. Primatol., 67, 101-119. https://doi.org/10.1002/ajp.20172*

Bill J., Rauterberg S., Herbrandt S., Ligges U., Kemper N., Fels M., Agonistic behavior and social hierarchy in female domestic rabbits kept in semi-groups. *J. Vet. Behav., 38: 21-31. https://doi.org/10.1016/j.jveb.2020.03.004*

Braconnier M., Gómez Y., Gebhardt-Henrich S.G. 2020. Different regrouping schedules in semi group-housed rabbit does: Effects on agonistic behaviour, stress and lesions. *Appl. Anim. Behav. Sci., 228: 105024. https://doi.org/10.1016/j.applanim.2020.105024*

BTS. 2019. https://www.blw.admin.ch/blw/de/home/instrumente/direktzahlungen/produktionssystembeitraege/tierwohlbeitraege.html.

Buijs S., Maertens L., Hermans K., Vangeyte J., André F., Tuyttens M. 2015. Behaviour, wounds, weight loss and adrenal weight of rabbit does as affected by semi-group housing. *Appl. Anim. Behav. Sci., 172: 44-51. https://doi.org/10.1016/j.applanim.2015.09.003*

Buijs S., Vangeyte J., Tuyttens F.A.M. 2016. Effects of communal rearing and group size on breeding rabbits' post-grouping behaviour and its relation to ano-genital distance. *Appl. Anim. Behav. Sci., 182: 53-60. https://doi.org/10.1016/j.applanim.2016.06.005*

Carnaby K., Painer J., Söderberg A., Gavier-Widèn D., Göritz F., Dehnhard M., Jewgenow K. 2012. Histological and endocrine characterization of the annual luteal activity in Eurasian Lynx (*Lynx lynx*). *Reproduction, 10.1530 / REP-12-0166 144: 477-483. https://doi.org/10.1530/REP-12-0166*

Chu L.R., Garner J.P., Mench J.A., 2004. A behavioral comparison of New Zealand White rabbits (*Oryctolagus cuniculus*) housed individually or in pairs in conventional laboratory cages. *Appl. Anim. Behav. Sci., 85: 121-139. https://doi.org/10.1016/j.applanim.2003.09.011*

Dal Bosco A., Mugnai C., Martino M., Szendrő Z., Mattioli S., Cambiotti V., Cartoni Mancinelli A., Moscati L., Castellini C. 2019. Housing Rabbit Does in a Combi System with Removable Walls: Effect on Behaviour and Reproductive Performance. *Animals, 9: 528-528. https://doi.org/10.3390/ani9080528*

Davis E.S., Marler C.A. 2003. The progesterone challenge: steroid hormone changes following a simulated territorial intrusion in female *Peromyscus californicus. Horm. Behav., Special Issue on Aggressive and Violent Behavior, 44: 185-198. https://doi.org/10.1016/S0018-506X(03)00128-4*

Dehnhard M., Naidenko S., Frank A., Braun B., Göritz F., Jewgenow K. 2008. Non-invasive Monitoring of Hormones: A Tool to Improve Reproduction in Captive Breeding of the Eurasian Lynx. *Reprod. Domest. Anim., 43: 74-82. https://doi.org/10.1111/j.1439-0531.2008.01145.x*

Denenberg V.H., Sawin P.B., Frommer G.P., Ross S. 1958. Genetic, physiological and behavioral background of reproduction in the rabbit: IV. An analysis of maternal behavior at successive parturitions. *Behaviour 13: 131-142. https://doi.org/10.1163/156853958X00073*

Drickamer L.C. 1996. Intra-uterine position and anogenital distance in house mice: consequences under field conditions. *Anim. Behav., 51: 925-934. https://doi.org/10.1006/anbe.1996.0096*

Dušek A., Bartoš L. 2012. Variation in Ano-Genital Distance in Spontaneously Cycling Female Mice. *Reprod. Domest. Anim., 47: 984-987. https://doi.org/10.1111/j.1439-0531.2012.02003.x*

Fortun L., Prunier A., Lebas F. 1993. Effects of lactation on fetal survival and development in rabbit does mated shortly after parturition. *J. Anim. Sci., 71: 1882-1886. https://doi.org/10.2527/1993.7171882x*

Gobikrushanth M., Bruinjé T.C., Colazo M.G., Butler S.T., Ambrose D.J. 2017. Characterization of anogenital distance and its relationship to fertility in lactating Holstein cows. *J. Dairy Sci., 100: 9815-9823. https://doi.org/10.3168/jds.2017-13033*

González-Mariscal G., Díaz-Sánchez V., Melo A.I., Beyer C., Rosenblatt J.S. 1994. Maternal behavior in New Zealand white rabbits: Quantification of somatic events, motor patterns, and steroid plasma levels. *Physiol. Behav., 55: 1081-1089. https://doi.org/10.1016/0031-9384(94)90391-3*

González-Mariscal G., Jiménez P., Beyer C., Rosenblatt J.S. 2003. Androgens stimulate specific aspects of maternal nest-building and reduce food intake in rabbits. *Horm. Behav., 43: 312-317. https://doi.org/10.1016/S0018-506X(02)00046-6*

González-Mariscal G., Gallegos J.A., Sierra-Ramirez A., Flores J.G. 2009. Impact of concurrent pregnancy and lactation on maternal nest- building, estradiol and progesterone concentrations in rabbits. *World Rabbit Sci., 17: 145-152. https://doi.org/10.4995/wrs.2009.654*

González-Mariscal G., Lemus A.C., Vega-González A., Aguilar-Roblero A. 2013a. Litter size determines circadian periodicity of nursing in rabbits. *Chronobiol. Int., 30: 711-718. https://doi.org/10.3109/07420528.2013.784769*

González-Mariscal G., Toribio A., Gallegos-Huicochea J.A., Serrano-Meneses M.A. 2013b. The characteristics of suckling stimulation determine the daily duration of mother-young contact and milk output in rabbits. *Dev. Psychobiol. 55: 809-817. https://doi.org/10.1002/dev.21071*

González-Mariscal G., Gallegos J.A. 2014. The maintenance and termination of maternal behavior in rabbits: Involvement of suckling and progesterone. *Physiol. Behav., 124: 72-76. https://doi.org/10.1016/j.physbeh.2013.10.031*

González-Mariscal G., Caba M., Martínez-Gómez M., Bautista A., Hudson R. 2016. Mothers and offspring: The rabbit as a model system in the study of mammalian maternal behavior and sibling interactions. *Horm. Behav., 77: 30-41. https://doi.org/10.1016/j.yhbeh.2015.05.011*

González-Mariscal G., Sisto Burt A., Nowak R. 2017. Behavioral and neuroendocrine indicators of well-being in farm and laboratory mammals. *Pfaff D. & Joëlls M., eds. "Hormones, Brain and Behavior" 3a ed. Elsevier, 454-485. https://doi.org/10.1016/B978-0-12-803592-4.00017-1*

Graf S., Bigler L., Failing K., Würbel H., Buchwalder T. 2011. Regrouping rabbit does in a familiar or novel pen: Effects on agonistic behaviour, injuries and core body temperature. *Appl. Anim. Behav. Sci., 135: 121-127. https://doi.org/10.1016/j.applanim.2011.10.009*

Hein D.J. 2019. Labordiagnostik bei Kleinsäugern: Präanalytik und tierartspezifische Befundung. *Schlütersche.*

Hein J. 2014. Blutentnahme und Venenverweilkatheter beim Kaninchen. *kleintier konkret 17: 23-25. https://doi.org/10.1055/s-0034-1384446*

Hoffman K.L., Martínez-Alvarez E., Rueda-Morales R.I. 2009. The inhibition of female rabbit sexual behavior by progesterone: Progesterone receptor-dependent and-independent effects. *Horm. Behav., 55: 84-92. https://doi.org/10.1016/j.yhbeh.2008.08.011*

Holst D. von, Hutzelmeyer H., Kaetzke P., Khaschei M., Rödel H.G. 2002. Social rank, fecundity and lifetime reproductive success in wild European rabbits (*Oryctolagus cuniculus*). *Behav. Ecol. Sociobiol., 51, 245–254. https://doi.org/10.1007/s00265-001-0427-1*

Hudson R., Shaal B., Bilkó Á., Altbäcker V. 1996. Just three minutes a day: The behaviour of young rabbits viewed in the context of limited maternal care. *In Proc.: 6th World Rabbit Congress, July 1996, Toulouse, France, 2: 395-403.*

Hudson R., Shaal B., Martínez-Gómez M., Distel H. 2000. Mother–young relations in the European rabbit: physiological and behavioural locks and keys. *World Rabbit Science 8: 85-90. https://doi.org/10.4995/wrs.2000.424*

Kosowska B., Strzała T., Moska M., Ratajszczak R., Dobosz T. 2015. Cytogenetic Examination of South American Tapirs, *Tapirus Terrestris* (Perissodactyla, Tapiridae), from the Wroclaw Zoological Garden. *Vestnik Zoologii, 49: 529-536.*

Lebas F., Coudert P.,. de Rochambeau H.,. Thebault R.G. 1997. The rabbit - Husbandry, health and production. *FAO Animal Production and Health Series No. 21.*

Maertens L., Rommers J., Jacquet M. 2011. Le logement des lapins en parcs, une alternative pour les cages classiques dans un système "duo"? [Combi-park housing of females, an alternative for the classical cages when using the "duo" management system?]. *14èmes Journées de la Recherche Cunicole, 85-88.*

Mann M.A., Svare B. 1982. Factors influencing pregnancy-induced aggression in mice. *Behav. Neural Biol., 36: 242-258. https://doi.org/10.1016/S0163-1047(82)90867-6*

Mann M.A., Konen C., Svare B. 1984. The role of progesterone in pregnancy-induced aggression in mice. *Horm. Behav., 18: 140-160. https://doi.org/10.1016/0018-506X(84)90039-4*

Markvardsen S.N., Kjelgaard-Hansen M., Ritz C., SØrensen D.B. 2012. Less invasive blood sampling in the animal laboratory: Clinical chemistry and haematology of blood obtained by the triatominae bug *Dipetalogaster maximus. Lab. Anim., 46: 136-141. https://doi.org/10.1258/la.2011.011063*

Mira-Escolano M.P., Mendiola J., Mínguez-Alarcón L., Melgarejo M., Cutillas-Tolín A., Roca M., López-Espín J.J., Noguera-Velasco J.A., Torres-Cantero A.M. 2014. Longer anogenital distance is associated with higher testosterone levels in women: a cross-sectional study. *BJOG, 121: 1359-1364. https://doi.org/10.1111/1471-0528.12627*

Mugnai C., Dal Bosco A., Castellini C. 2009. Effect of different rearing systems and pre-kindling handling on behaviour and performance of rabbit does. *Appl. Anim. Behav. Sci., 118: 91-100. https://doi.org/10.1016/j.applanim.2009.02.007*

Nielsen S.S., Alvarez J., Bicout D.J., Calistri P., Depner K., Drewe J.A., Garin-bastuji B., Luis J., Rojas G., Michel V., Angel M., Chueca M., Roberts H.C., Sihvonen L.H., Spoolder H., Stahl K., Calvo A.V., Viltrop A., Buijs S., Edwards S., Candiani D., Mosbach-schulz O. 2020. *Health and welfare of rabbits farmed in different production systems, EFSA J., 18: 1-96. https://doi.org/10.2903/j.efsa.2020.5944*

O'Malley B. 2005. Rabbits. *In: O'Malley B. (Ed.), Clinical Anatomy and Physiology of Exotic Species. Elsevier, New York, pp. 173-195. https://doi.org/10.1016/B978-070202782-6.50011-9*

Palanza P., Parmigiani S., Vom Saal F.S. 1995. Urine marking and maternal aggression of wild female mice in relation to anogenital distance at birth. *Physiol. Behav., 58: 827-835. https://doi.org/10.1016/0031-9384(95)00107-T*

Palka Y.S., Sawyer C.H. 1966. The effects of hypothalamic implants of ovarian steroids on oestrous behaviour in rabbits. *J. Physiol., 185: 251-269. https://doi.org/10.1113/jphysiol.1966.sp007986*

R Core Team, 2019., A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/*

Rödel H.G., Monclús R., von Holst D. 2006. Behavioral styles in European rabbits: Social interactions and responses to experimental stressors. *Physiol. Behav., 89: 180-188. https://doi.org/10.1016/j.physbeh.2006.05.042*

Rödel H.G., Starkloff A., Bautista A., Friedrich A.C., Von Holst D. 2008. Infanticide and maternal offspring defence in European rabbits under natural breeding conditions. *Ethology, 114: 22-31. https://doi.org/10.1111/j.1439-0310.2007.01447.x*

Rommers J.M., Gunnink H., Klop A., De Jong I.C. 2011. Dynamics in aggressive behaviour of rabbit does in a group housing system: a descriptive study. *In: 17th International Symposium on Housing and Diseases of Rabbits, Fur Providing Animals and Pet Animals, May 11-12. Celle, Germany, pp. 75-85.*

Rommers J.M., Reuvekamp B.J.F., Gunnink H., de Jong I.C. 2014. Effect of hiding places, straw and territory on aggression in group-housed rabbit does. *Appl. Anim. Behav. Sci., 157: 117-126. https://doi.org/10.1016/j.applanim.2014.05.011*

Schönbrodt F.D., Perugini M. 2013. At what sample size do correlations stabilize? *J. Res. Pers., 47: 609-612. https://doi.org/10.1016/j.jrp.2013.05.009*

Shargal D., Shore L., Roteri N., Terkel A., Zorovsky Y., Shemesh M., Steinberger Y. 2008. Fecal testosterone is elevated in high ranking female ibexes (*Capra nubiana*) and associated with increased aggression and a preponderance of male offspring. *Theriogenology 69: 673-680. https://doi.org/10.1016/j.theriogenology.2007.11.017*

Simpson E.R. 2002. Aromatization of androgens in women: current concepts and findings. *Fertil. Steril., 77: 6-10. https://doi.org/10.1016/S0015-0282(02)02984-9*

Squire L.R. 2009. Encyclopedia of Neuroscience, Volume 1. *Academic Press. https://doi.org/10.1093/acprof:oso/9780195380101.003.0001*

Stadler A., Meiser C.K., Schaub G. a. 2011. "Living Syringes": use of Haematophagous Bugs as Blood Samplers from Small and Wild Animals. *Nature Helps... 243-272. https://doi.org/10.1007/978-3-642-19382-8*

Szendrő Z., McNitt J.I., Matics Z., Mikó A., Gerencsér Z. 2016. Alternative and enriched housing systems for breeding does: A review. *World Rabbit Sci., 24: 1-14. https://doi.org/10.4995/wrs.2016.3801*

Takahashi, Kayo, Hosoya T., Onoe K., Takashima T., Tanaka M., Ishii A., Nakatomi Y., Tazawa S., Takahashi, Kazuhiro, Doi H., Wada Y., Watanabe Y. 2018. Association between aromatase in human brains and personality traits. *Sci. Rep., 8: 1-9. https://doi.org/10.1038/s41598-018-35065-4*

Theau-Clément M., Poujardien B., Bellereaud J. 1990. Influence des traitements lumineux, modes de reproduction et états physiologiques sur la productivité de lapines multipares. *In: 5èmes Journées de La Recherche Cunicole, Dec 1990, Paris, France.*

Thomsen R., Voigt C.C. 2006. Non-invasive blood sampling from primates using laboratory-bred blood-sucking bugs (*Dipetalogaster maximus*; Reduviidae, Heteroptera). *Primates, 47: 397-400. https://doi.org/10.1007/s10329-006-0194-8*

Trainor B.C., Bird I.M., Marler C.A. 2004. Opposing hormonal mechanisms of aggression revealed through short-lived testosterone manipulations and multiple winning experiences. *Horm. Behav., 45: 115-121. https://doi.org/10.1016/j.yhbeh.2003.09.006*

Trainor B.C., Kyomen H.H., Marler C.A. 2006. Estrogenic encounters: How interactions between aromatase and the environment modulate aggression. *Front. Neuroendocrinol., 27: 170-179. https://doi.org/10.1016/j.yfrne.2005.11.001*

Ubilla E., Rebollar P.G. 1995. Influence of the postpartum day on plasma estradiol-17 β levels, sexual behaviour, and conception rate, in artificially inseminated lactating rabbits. *Anim. Reprod. Sci., 38: 337-344. https://doi.org/10.1016/0378-4320(94)01366-T*

Voigt C.C., Von Helversen O., Michener R.H., Kunz T.H. 2003. Validation of a Non-Invasive Blood-Sampling Technique for Doubly-Labelled Water Experiments. *J. Exp. Zool. A Comp. Exp. Biol., 296: 87-97. https://doi.org/10.1002/jez.a.10121*

Voigt C.C., Faßbender M., Dehnhard M., Wibbelt G., Jewgenow K., Hofer H., Schaub G.A. 2004. Validation of a minimally invasive blood-sampling technique for the analysis of hormones in domestic rabbits, *Oryctolagus cuniculus* (Lagomorpha). *Gen. Comp. Endocrinol., 135: 100-107. https://doi.org/10.1016/j.ygcen.2003.08.005*

Voigt C.C., Peschel U., Wibbelt G., Frölich K. 2006. An Alternative, Less Invasive Blood Sample Collection Technique for Serologic Studies Utilizing Triatomine Bugs (Heteroptera; Insecta). *J. Wildl. Dis., 42: 466-469. https://doi.org/10.7589/0090-3558-42.2.466*

von Engelhard N., Kappeler P.M., Heistermann M. 2000. Androgen levels and female social dominance in *Lemur catta. In Proc.: R. Soc. Lond. B, 267: 1533-1539. https://doi.org/10.1098/rspb.2000.1175*

Vos A.C., Müller T., Neubert L., Voigt C.C. 2010. Validation of a Less Invasive Blood Sampling Technique in Rabies Serology Using Reduviid Bugs (Triatominae, Hemiptera). *J. Zoo. Wildl. Med., 41: 63-68. https://doi.org/10.1638/2009-0103.1*

Wingfield J.C., Hegner R.E., Dufty, Alfred M., Ball G.F. 1990. The "Challenge Hypothesis": Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. *Am. Nat., 136: 829-846. https://doi.org/10.1086/285134*

Zomeño C., Birolo M., Zu A., Xiccato G., Trocino A. 2017. Aggressiveness in group-housed rabbit does: Influence of group size and pen characteristics. *Appl. Anim. Behav. Sci., 194: 79-85. https://doi.org/10.1016/j.applanim.2017.05.016*

Zomeño C., Birolo M., Gratta F., Zuffellato A., Xiccato G., Trocino A. 2018. Effects of group housing system, pen floor type, and lactation management on performance and behaviour in rabbit does. *Appl. Anim. Behav. Sci., 203: 55-63. https://doi.org/10.1016/j.applanim.2018.03.002*