Effect of Cage Type on Fecal Corticosterone Concentration in Buck Rabbits During the Reproductive Cycle

Paolo Cornale; Elisabetta Macchi; Manuela Renna; Liviana Prola; Giovanni Perona; Antonio Mimosi. Effect of Cage Type on Fecal Corticosterone Concentration in Buck Rabbits During the Reproductive Cycle. JOURNAL OF APPLIED ANIMAL WELFARE SCIENCE. 19 (1) pp: 90-96. DOI: 10.1080/10888705.2015.1072468

Effect of Cage Type on Fecal Corticosterone Concentration in Buck Rabbits during the Reproductive Cycle

Paolo Cornale,[1] Elisabetta Macchi,[2] Manuela Renna,[1] Liviana Prola,[2] Giovanni Perona,[2] and Antonio Mimosi[1]

[1]*Agriculture, Forest and Food Sciences, University of Torino, Torino, Italy*

[2]*Veterinary Science, University of Torino, Torino, Italy*

Correspondence should be sent to Paolo Cornale, Agriculture, Forest and Food Sciences, University of Torino, largo Paolo Braccini, 2, Grugliasco, Torino 10095 Italy. Email: paolo.cornale@unito.it

Fecal corticosterone concentration (FCC) was measured in 14 buck rabbits individually housed in standard dimension cages (SC) or in bigger cages (BC, with a volume more than double that of SC and equipped with a plastic foot mat) during four consecutive reproductive cycles. Cage type and reproductive phase (estrous synchronization of doe rabbits, artificial insemination, partum, pre-weaning, and post-weaning) were not statistically significant, but tendentially affected FCCs ($0.05 < p < 0.10$). Buck rabbits housed in SC showed higher FCCs than those housed in BC (27.42 pg g^{-1} dried feces and 25.57 pg g^{-1}, respectively; *SEM*: 2.952). The highest FCC values were detected at artificial insemination (BC: 27.91 pg g^{-1}; SC: 30.45 pg g^{-1}; *SEM*: 3.520), highlighting the phase of semen collection could be one of the most critical moments for buck rabbits, although further investigations are needed. These preliminary results suggest that measurement of FCC could be used as an indicator of chronic stress in buck rabbits.

Keywords: animal welfare, buck rabbits, fecal corticosterone, stress, housing conditions

After China, Italy is the second leading country in the nonhuman animal production sector of rabbit farming (Food and Agriculture Organization [FAO], 2012). In intensive farming systems, besides young rabbits (growers or fatteners) reared for meat production, a key role is held by breeding adults. Buck rabbits are adult reproductive males. They are usually individually housed in wire cages, kept separate from females, and reared according to specific housing conditions (Verga, Luzi, & Carenzi, 2007).

Currently there is no species-specific legislation protecting the welfare of farmed rabbits in the European Union, although very serious issues are well known to affect rabbits' welfare in intensive farming conditions (Trocino & Xiccato, 2006). In 2005, besides providing specific recommendations aimed at enhancing the health and welfare of farmed rabbits, the European Food Safety Authority highlighted the need for research on chronic stress in this sector (EFSA, 2005).

Stress has traditionally been investigated through behavioral assessments, but hormone (i.e., steroids and their metabolites) measurements can provide quantitative indexes of animal welfare conditions (Möstl & Palme, 2002). The noninvasive assessment of physiological stress through fecal studies has been increasingly used among animal species (Millspaugh & Washburn, 2004). Particularly, the use of fecal glucocorticoid metabolites has the advantage to provide an accurate assessment of long-term stress since it reveals an estimate of cumulative cortisol secreted over a certain period of time compared to point values detected in blood and plasma samples (Morméde et al., 2007).

Many confounding factors (e.g., circadian rhythm, sex, reproductive events, etc.) have to be taken into account when using fecal samples to determine glucocorticoids concentrations (Palme, 2012). Concerning the effect of sex, von der Ohe and Servheen (2002) indicated that glucocorticoids could vary between males and females for reasons not strictly related to stress sources. Furthermore, the same authors highlighted that the effect of

sex hormones on glucocorticoids can be particularly evident in different moments of the reproductive cycle (e.g., mating, pregnancy, and lactation).

To the best of our knowledge, no previous studies have been focused on fecal corticosterone concentration (FCC) in adult buck rabbits as a measurement of chronic stress. Since exposure to stressors, especially sources of chronic stress, may alter males' reproductive activity through the suppression of testosterone, spermatogenesis, and libido (Retana-Márquez, Bonilla-Jaime, Vázquez-Palacios, Martínez-García, & Vázquez-Moctezuma, 2003), the assessment of the welfare status of reproductive males may provide relevant information for the possible related economic consequences at the farm level.

The present study is part of a trial focused on testing FCC as an index of chronic stress in reproductive rabbits. Previous results on the effects of breed, cage type, and phase of reproductive activity on FCC levels in doe rabbits were reported in Prola et al. (2013). Since buck and doe rabbits were housed together, buck data were collected using the same timings applied in the does' trial. The aim of the present study was to evaluate the effects of cage type and stage of reproductive activity on FCC levels in buck rabbits.

MATERIALS AND METHODS

Animals and Housing

The experiment was conducted from March to September on a commercial rabbit breeding farm located in Buttigliera d'Asti, northwest Italy, using 14 sexually mature buck rabbits from the Grigio del Monferrato breed. One week before the beginning of the experimental period, the bucks arrived at the farm together; they were 6 months old and their mean body weight was 3,938.4 ± 284.85 g. Prior to being enrolled in the experiment, the bucks were reared under the same conditions. In the farm of origin, rabbits were housed in a single room building equipped with an automatically controlled natural ventilation system and a photoperiod of 11 to 12 hr of daylight was

maintained. The kits were reared with their dam in standard wire cages until weaning (approximately after 35 days of lactation). At weaning, the growing rabbits were housed in pairs in bicellular standard wire cages.

Rabbits were fed ad libitum with the same pelleted diet (formulated to fulfill the rabbits' requirements according to ages), and water was freely available from one nipple drinker per cage. All animals were vaccinated according to the Italian law prescriptions. At farm entry, after being exposed to the same transport, they were randomly divided into two groups and individually housed in either (a) standard wire cages (SC) sized 60 × 38 × 32 cm (0.07 m^3) or (b) bigger wire cages (BC) sized 90 × 46 × 46 cm (0.19 m^3). Each BC was also equipped with a plastic foot mat covering approximately 25% of the cage floor. The use of the plastic foot mat provided the hind legs a more suitable surface than wire, and it considerably reduced area of mesh opening in the cage floor. The commercial pelleted rabbit chow was simultaneously fed to all the cages by an automatic feeding system.

Since reproduction was managed by artificial insemination, semen was collected from the males using an artificial vagina. Prior to the experimental period, the subjects were trained to serve the artificial vagina (Morrell, 1995), which allowed the evaluation of their libidos and semen characteristics. Semen collection was carried out every 12 days, with two ejaculations per male during the experiment. Semen was always collected from all the buck rabbits involved in the trial to assure that each individual would be subjected to the same handling treatments. The same skilled operators monitored the health statuses of the subjects during each semen collection. No serious heath problems occurred during the experimental trial. A semi-intensive rhythm was used in the farm during the experiment: doe rabbits were artificially inseminated on day 11 postpartum, while weaning occurred after 31 days of lactation.

The animals were housed and managed according to EFSA (2005) guidelines for rabbit breeding. The farm stock was a single-room, thermal-insulated building with a natural ventilation system. Temperatures ranged from 15 to 25 °C during the experimental trial. The daily lighting regime was maintained at 11 to 12 hr light/day; the lighting program was increased to 16 hr light/day 4 days before artificial insemination. The husbandry was managed by the same expert operator during the experimental trial.

Sample Collection

Four consecutive reproductive cycles were considered in the experiment. According to Prola et al. (2013), feces samples were collected at five different phases of the reproductive cycle: (a) before estrous synchronization of doe rabbits; (b) before artificial insemination, (c) before partum, (d) before weaning, and (e) after weaning. In the morning of the day before each phase, the areas below the cages were cleaned to remove old feces. Fine wire meshes were then placed below the cages to collect feces. Thanks to this collection system, urine could pass through the netting without staying in contact with feces.

During the following 24-hr after the wire meshes were placed, all the feces were collected by the same skilled operators at approximately 8-hr intervals and immediately refrigerated at the farm. If feces were contaminated with urine, they were excluded from the sampling. The next morning, between 7:00 a.m. and 9:00 a.m., the third feces collection took place in order to cover the 24-hr period since feces started to be collected the previous day. The same operators pooled all the collected feces within a treatment and obtained a pooled fecal sample for the laboratory analysis. Therefore, each sample consisted of 24-hr feces from all the buck rabbits of each group. After being collected, the samples were immediately refrigerated, transported to the laboratory, frozen, and then stored at −20 °C until analysis.

Steroid Hormone Assay: Extraction and Determination of FCC

Prior to the start of the experiment, in order to assess the intraindividual variability of adrenal activity, fecal samples were collected from the 14 subjects every day for a week. Extraction and determination of corticosteroids in the fecal samples were carried out as previously reported by Prola et al. (2013). Briefly, samples were kiln-dried at 55 °C for 24 hr and homogenized. Five aliquots of pulverized feces (0.20 g each) were put into extraction tubes, where 1 ml of ethanol (Sigma Aldrich, St. Louis, MO) was added for every 0.1 g of solid. After centrifugation (15 min at 3,300 g) the supernatant was recovered in a clean tube for evaporation to dryness. Obtained extracts were stored at −80 °C until analysis with the enzyme immunoassay. Extracted samples were dissolved into 100 µl ethanol with 400 µl of kit Assay Buffer (Arbor Assays, Ann Arbor, MI), vortexed, and rested to ensure complete steroid solubility.

The immunoreactivity of fecal samples was determined using commercial enzyme immunoassay kits (K014; Arbor Assays, Ann Arbor, MI) validated for dried fecal extracts. All analyses were repeated twice. The corticosterone antibody used to quantify fecal hormone cross-reacted to corticosterone (100%), desoxycorticosterone (12.30%), cortisol (0.38%), and cortisone (< 0.08%). All fecal samples were analyzed at multiple dilutions (from 1:4 to 1:32) and were found to be parallel to the standard curve ($p < 0.05$ for all assays). The mean recovery rate of corticosterone added to dried feces was 94% ($n = 6$). The results are reported as pg of corticosterone on g of dried feces.

Statistical Analysis

Statistical analysis of the data was performed using SAS 9.1.3 (SAS Institute Inc., 2008). The data were submitted to ANOVA (PROC GLM) according to the following model:

$$X_{ijk} = \mu + CT_i + P_j + (CT \times P)_{ij} + \varepsilon_{ijk}$$

where X_{ijk} = observation; μ = overall mean; CT_i = fixed effect of cage type (1, standard cage; 2, bigger cage); P_j = fixed effect of phase of the reproductive cycle (1, synchronization; 2, artificial insemination; 3, partum; 4, pre-weaning; 5, post-weaning); $(CT \times P)_{ij}$ = interaction between cage type and phase of the reproductive cycle; and ε_{ijk} = residual error.

Since the assumption of equal variances did not hold, the Brown-Forsythe statistic (HOVTEST=BF) was performed to test for the equality of group means instead of the *F* test. Significance was declared at $p \leq 0.05$, and tendencies are discussed at $p < 0.10$.

RESULTS

The interaction between cage type and phase of reproductive activity was not statistically significant. Although the effect of cage type was over the declared significance threshold, the FCC of buck rabbits housed in SC was tendentially higher ($p = 0.08$) compared to that of the animals housed in BC (27.42 pg g^{-1} and 25.57 pg g^{-1}, respectively; *SEM*: 2.952). Similarly, the effect of reproductive phase on FCCs was not significant but showed a tendency ($p = 0.09$; *SEM*: 3.227). In particular, the absolute highest FCC value was detected at artificial insemination (29.18 pg g^{-1}), followed by the concentration detected at post-weaning (27.05 pg g^{-1}). Synchronization, pre-weaning, and partum showed quite similar FCC levels: 25.67, 25.62, and 25.04 pg g^{-1}, respectively.

The effect of cage type on FCCs at each stage of the reproductive cycle is reported in Table 1. No statistically significant differences were detected between FCCs of buck rabbits housed in BC and SC during the considered reproductive phases. For both groups, the highest FCCs were observed at artificial insemination and a quite high FCC level was also noted at post-weaning, suggesting these two moments are the most critical phases for buck rabbits welfare.

TABLE 1

Mean Values of FCCs (pg g^{-1}) in Buck Rabbits

According to Cage Type and Reproductive Phase

Stage	BC	SC	*SEM*	*p*-value
Synchronization	24.51	26.64	3.503	0.94
Artificial insemination	27.91	30.45	3.520	0.71
Partum	23.37	26.71	2.071	0.56
Pre-weaning	25.23	26.01	3.903	0.68
Post-weaning	26.82	27.28	1.968	0.59

BC = bigger cage; SC = standard cage.

DISCUSSION

The data analyzed in the present study are part of an experiment aimed at verifying if FCC may be used as a useful indicator of chronic stress in reproductive rabbits. Previously (Prola et al., 2013), we presented the results obtained on doe rabbits. The timings used for collecting doe and buck rabbits' data were chosen by selecting phases or moments that, at the same time, were potentially critical for the animals (i.e., cause of stress) and already required the rabbits' manipulation by the farmers. In fact, the study was designed to minimize experimental procedures at the farm and, consequently, to keep at a minimum any external stressful stimuli for rabbits.

Furthermore, since females and males were housed together, a potential influence of females on males' physiology cannot be excluded. In fact, Schneider et al. (2010) demonstrated that the presence of pregnant and estrous female tammar wallabies (*Macropus eugenii*) increased the plasma testosterone in tammar males. They hypothesized that this increase of testosterone in males could be explained through the release of an odor signal (i.e., pheromone) by females, as was previously described occurring in rats (Purvis &

Haynes, 1978). Rodríguez-De Lara et al. (2010) proposed doe rabbits' exposure is a biostimulation method to improve buck rabbits' reproductive performance, demonstrating that the females' presence is beneficial to sexual drive and to sperm production and quality in male rabbits.

During the same four consecutive reproductive cycles of the current study, the results for rabbit females (Prola et al., 2013) showed that cage type and reproductive phase significantly affected FCCs. Particularly, increasing cage size coupled with the use of a plastic foot mat led to a significant reduction in FCC levels of doe rabbits. Contrariwise to females, the FCC difference between buck rabbits housed in standard and bigger cages was not clearly significant. However, males housed in BC showed a tendency towards lower FCC levels than subjects housed in SC.

The effect of a chronic stress on glucocorticoids levels is still controversial, and Mormède et al. (2007) suggested that glucocorticoids metabolites in feces could be effective in detecting the small changes in glucocorticoids concentrations resulting from chronic stress. Even when considering the biological relevance of an acute stress on glucocorticoids metabolites, there are contrasting results. While snowshoe hares (*Lepus americanus*) increased their fecal cortisol metabolites concentrations 175% compared with baseline values when they were exposed to a dog (Sheriff et al., 2009), wild hares (*Lepus europaeus*) after being roused three times (Teskey-Gerstl, Bamberg, Steineck, & Palme, 2000) and rabbits after a 30-min transport (Buijs, Keeling, Rettenbacker, Maertens, & Tuyttens, 2011) obtained significant results, but the glucocorticoids' differences before and after the acute stress were less than 2- or 3-fold.

The results obtained in the present study support those of Buijs et al. (2011) who showed the utility of fecal glucocorticoid metabolites in a long stress assessment of growing

rabbits. They suggested that environmental enrichments might reduce rabbits' stress due to housing conditions.

Concerning the effect of reproductive phase, doe rabbits showed two FCC peaks (Prola et al., 2013): the first one occurred immediately after the partum (reflecting the physiological glucocorticoids peak triggering the partum), and the second peak occurred at post-weaning, probably related to the stress caused by early weaning adopted in intensive rabbit farming systems. Otherwise, buck rabbits showed a peak, although statistically nonsignificant ($p < 0.10$), at artificial insemination. This result was expected since the handling procedure occurring at artificial insemination involved the manipulation of buck rabbits for semen collection (Boiti et al., 2005).

Contrasting results of sex effect on fecal glucocorticoid metabolites are reported in the literature for various animal species. Cabezas, Blas, Marchant, and Moreno (2007) did not find differences in fecal glucocorticoid metabolites between male and female wild rabbits, even after a quarantine period. More recently, Sheriff, Krebs, and Boonstra (2010) did not reveal any significant differences in fecal cortisol metabolites between male and female snowshoe hares (*Lepus americanus*). On the contrary, higher proportions of glucocorticoid metabolites were measured in male compared to female mice (*Mus musculus* f. *domesticus*) by Touma, Sachser, Möstl, and Palme (2003), as well as in starlings (*Sturnus vulgaris*) by Romero and Remage-Healey (2000).

It is important to note that the limited sample size of the present study might have affected the statistical significance of cage type effect on FCCs in buck rabbits. However, cage type and reproductive phase seem to affect the FCCs more in doe rabbits than in buck rabbits. The reason of the less pronounced FCCs response of buck rabbits may be two-fold First of all, it is worth mentioning that buck rabbits are usually less manipulated than doe rabbits. During each reproductive cycle, farmers handled buck rabbits just for semen

collection, while doe rabbits underwent many handling procedures such as estrous synchronization, artificial insemination, litter equalization, and kits' weaning. Rabbits are generally considered very sensitive to external stimuli, as they are easily frightened: disturbances, especially subordination stress and fear, induce a rapid activation of the hypothalamic–pituitary–adrenal axis (Cabezas et al., 2007).

Secondly, Hansen and Berthesen (2000) found that female rabbits performed more "escaping" behavior and more stereotypic behavior of gnawing cage bars than males, concluding that females are more affected by environment than males. Therefore, it could be hypothesized that such predisposition of females coupled with the multiple handling procedures may exacerbate the stressful effect of environment (e.g., cage type) on the welfare status of doe rabbits.

CONCLUSION

These preliminary results suggest that FCC could be used as an indicator of chronic stress in buck rabbits. However, further investigations are needed to reveal possible correlations among FCC levels and other welfare measurements (e.g., behavior) or reproductive parameters (e.g., semen assessment) of buck rabbits.

ACKNOWLEDGEMENTS

The authors wish to gratefully thank Dr. Paolo Novara for his help and for allowing us to perform our experiments in his rabbit stable. The research was funded by Regione Piemonte (Direzione Regionale Agricoltura – Settore Sviluppo delle Produzioni Zootecniche).

REFERENCES

Boiti, C., Castellini, C., Besenfelder, U., Theau-Clément, M., Liguori, L., Renieri, T., & Pizzi, F. (2005). Guidelines for the handling of rabbit bucks and semen. *World Rabbit Science, 13,* 71–91.

Buijs, S., Keeling, L. J., Rettenbacker, S., Maertens, L., & Tuyttens, F. A. M. (2011). Glucocorticoid metabolites in rabbit faeces – Influence of environmental enrichment and cage size. *Physiology & Behavior*, *104*, 469–473.

Cabezas, S., Blas, J., Marchant, T. A., & Moreno, S. (2007). Physiological stress levels predict survival probabilities in wild rabbits. *Hormones and Behavior*, *51*, 313–320.

European Food Safety Authority. (2005). The impact of current housing and husbandry systems on the health and welfare of farmed domestic rabbits. *The EFSA Journal, 267*, 1–31.

Food and Agriculture Organization. (2012). *Sito delle statistiche dell'Organizzazione delle Nazioni Unite per l'Alimentazione e l'Agricoltura*. [Statistics site of Food and Agriculture Organization of the United Nations]. Retrieved from http://faostat.fao.org/default.aspx.

Hansen, L. T., & Berthelsen, H. (2000). The effect of environmental enrichment on the behaviour of cage rabbits (*Oryctolagus cuniculus*). *Applied Animal Behaviour Science, 68*, 163–178.

Millspaugh, J. J., & Washburn, B. E. (2004). Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and Comparative Endocrinology*, *138*, 189–199.

Morméde, P., Andanson, S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., … Veissier, I. (2007). Exploration of the hypothalamic–pituitary–adrenal function as a tool to evaluate animal welfare. *Physiology & Behavior*, *92*, 317–339.

Morrell, J. M. (1995). Artificial insemination in rabbits. *British Veterinary Journal, 151,* 477–488.

Möstl, E., & Palme, R. (2002). Hormones as indicators of stress. *Domestic Animal Endocrinology*, *23*, 67–74.

Palme, R. (2012). Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. *Animal Welfare*, *21*, 331–337.

Purvis, K., & Haynes, N.B. (1978). Effect of the odour of female rat urine on plasma testosterone concentration in male rats. *Journal of Reproduction and Fertility, 53*, 63–65.

Prola, L., Cornale, P., Renna, M., Macchi, E., Perona, G., & Mimosi, A. (2013). Effect of breed, cage type, and reproductive phase on fecal corticosterone levels in doe rabbits. *Journal of Applied Animal Welfare Science*, *16(2)*, 140–149.

Retana-Márquez, S., Bonilla-Jaime, H., Vázquez-Palacios, G., Martínez-García, R., & Vázquez-Moctezuma, J. (2003). Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. *Hormones and Behavior*, *44*, 327–337.

Rodríguez-DeLara, R., Noguez-Estrada, J., Rangel-Santos, R., García- Muñiz, J. G., Martínez-Hernández, P. A., Fallas-Lopez, M., & Maldonado-Siman, E. (2010). Controlled doe exposure as bioestimulation of buck rabbits. *Animal Reproduction Science, 122,* 270–275.

Romero, L. M., & Remage-Healey, J. (2000). Daily and seasonal variation in response to stress in captive starlings (*Sturnus vulgaris*): Corticosterone. *General and Comparative Endocrinology*, *119*, 52–59.

SAS Institute Inc. (2008). *SAS/STAT User's Guide. Release 9.1.3.* [Computer software]. Cary, NC: Author.

Schneider, N. Y., Fletcher, T. P., Shaw, G., & Renfree, M. B. (2010). The effect of pregnant and oestrous females on male testosterone and behaviour in the tammar wallaby. *Hormones and Behavior, 58*, 378–384.

Sheriff, M. J., Bosson, C. U., Krebs, C. J., & Boonstra, R. (2009). A non-invasive technique for analyzing fecal cortisol metabolites in snowshoe hares (*Lepus americanus*). *Journal of Comparative Physiology B*, *179*, 305–313.

Sheriff, M. J., Krebs, C. J., & Boonstra, R. (2010). Assessing stress in animal populations: Do fecal and plasma glucocorticoids tell the same story? *General and Comparative Endocrinology*, *166*, 614–619.

Teskey-Gerstl, A., Bamberg, E., Steineck, T., & Palme, R. (2000). Excretion of corticosteroids in urine and faeces of hares (*Lepus europaeus*). *Journal of Comparative Physiology B, 170,* 163–168.

Touma, C., Sachser, N., Möstl, E., & Palme, R. (2003). Effects of sex and time of day on metabolism and excrection of corticosterone in urine and feces of mice. *General and Comparative Endocrinology*, *130*, 267–278.

Trocino, A., & Xiccato, G. (2006). Animal welfare in reared rabbits: a review with emphasis on housing systems. *World Rabbit Science*, *14*, 77–93.

Verga, M., Luzi, F., & Carenzi, C. (2007). Effects of husbandry and management systems on physiology and behavior of farmed and laboratory rabbits. *Hormones and Behavior, 52*, 122–129.

von der Ohe, C., & Servheen, C. (2002). Measuring stress in mammals using fecal glucocorticoids: Opportunities and challenges. *Wildlife Society Bulletin, 30*, 1215–1225.