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Effect of Breed, Cage Type, and Reproductive Phase on Fecal Corticosterone Levels in Doe Rabbits

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Fecal corticosterone concentration (FCC) is increasingly being used as a nonin-vasive indicator of stress in assessment of nonhuman animal welfare. The aim of this study was to evaluate effects of breed, cage type, reproductive phase, and their interactions on FCC levels in doe rabbits. A total of 252 doe rabbits were randomly assigned to 2 groups. Does were individually housed in either standard dimension cages (SC) or in cages with a volume more than double that of the SC. Bigger cages (BC) were equipped with a plastic foot mat. Breed, cage type, and reproductive phase significantly affected FCC. New Zealand hybrids showed higher FCCs ($p < .001$) when compared witho the autochthonous breed (27.77 ± 0.47 vs. 24.61 ± 0.36 pg g^{-1}, respectively). Increased cage size coupled with a plastic foot mat resulted in a significant decrease in doe FCC. The highest FCCs were detected at partum (BC: 30.40 ±

0.81 pg g^{-1}; SC: 33.36 ± 0.86 pg g^{-1}; $p \leq .05$), followed by postweaning (BC: 25.09 ± 0.95 pg g^{-1}; SC: 27.63 ± 0.95 pg g^{-1}; $p \leq .05$). These results support the hypothesis that measurement of FCC provides a useful indicator of chronic stress in doe rabbits.

Keywords

animal welfare, cage type, faecal corticosterone, rabbit, reproductive cycle, stress

Rabbit breeding is a "medium-size" nonhuman animal production sector. Italy is the leading country in Europe in this sector, and it is second worldwide following China. Over 76% of total rabbit production in the European Union occurs in Italy, Spain, and France, and family-type farming is still widespread. In Italy, the sector, which employs approximately 10,000 people, is valued at more than 600 million euros per year (Food and Agriculture Organization [FAO], 2012). There are about 10,000 rabbit farms in Italy, with a hundreds of millions of rabbits produced per year, yielding 230,000 tons of meat (Italian Institute of Statistics [ISTAT], 2011). Mean consistency is approximately 1,000 animals per farm. In 2005, the Panel on Animal Health and Animal Welfare of the European Food Safety Authority (EFSA; 2005) documented its opinion on the health and welfare of farmed rabbits, which concluded by recommending that the health management standards should be raised for the benefit of both the animals and the farmers themselves.

The report shows that the mortality of farmed rabbits is considerably higher than it is in any other species of animal on the farm, which is due to respiratory infections and reproductive problems that shorten their lives, often resulting in required annual animal turnover. The medium mortality rate in intensive rabbit farms is 10 to 15% (Dal Bosco,

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2008). Considering the mortality rate and the calling rate, the annual turnover of doe rabbits is about 100% (EFSA, 2005). Concerning the improvement of farmed rabbit health and welfare, the report recommends that cage sizes are increased and stocking density levels are decreased, taking into account the mean rabbit weight for each class, which varies greatly from one country to another according to local traditions and demand. The key problems hindering the development and improvement of farmed rabbit health and welfare result from the lack of research on chronic stress in this sector compared with such research on other farmed species. Evaluation of chronic stress is fundamental for the study of animal welfare because it is known to lead to the depression of both the immune and reproductive systems as well as cause alterations in brain structures and function (Lane, 2006; Moberg, 2000; Wiepkema & Koolhaas, 1993). In order to evaluate stress, scientific indicators must be measured and monitored, taking into account the species' evolution in the natural environment and during the domestication process as well as the species' coping systems toward the stressors. Literature on stress indicators in farmed rabbits is rather scarce; however, physiological indicators identified in other farm animals may be applied (Morisse & Maurice, 1994). Among physio- logical indicators, the concentration of glucocorticoid (GC) is widely used as an indicator of stress, but there is some disagreement regarding the effect of chronic stress on GC levels. A number of studies show elevated GC concentrations when nonhuman primates, heifers, bulls, and pigs are under chronic stress conditions (Mendoza, Capitanio, & Mason, 2000), whereas other authors have reported GC concentrations remain at baseline levels during exposure to chronic stress (Beattie, O'Connell, Kilpatrick, & Moss, 2000; Hasegawa, Nishiwaki, Sugawara, & Ito, 1997; Ladewig & Smidt, 1989). Factors affecting GC levels considered in the studies were mainly social factors (dominance, hierarchy) and housing conditions (tethering, environmental enrichment). Indeed, an important dispute is ongoing regarding how GC should be measured;

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some authors have used absolute GC concentrations after exposure to the stressor (Gupta, Earley, & Crowe, 2007; Harris et al., 2004), and others have used the difference between baseline and poststressor concentrations (Beattie et al., 2000; Janssens, Helmond, & Wiegant, 1994).

Blood samples are commonly used for the analysis of GC concentrations, but it is important to bear in mind that the act of obtaining a blood sample from a rabbit, a relatively fearful species, can itself induce an immediate increase in blood GC concentrations (Boiti & Yalow, 1978; Touma, Palme, & Sachser, 2004). Furthermore, circadian variations and acute stressors can all influence the concentration of blood GC at the moment of withdrawal (Lane, 2006; Szeto et al., 2004). Nevertheless, some of these confounding effects are attenuated when glucocorticoid metabolites (GCM) in fecal samples are assayed instead as a measure of systemic GC because feces sampling is not an invasive procedure and the effect of acute stressors is minimized (Palme, Rettenbacher, Touma, El-Bahr, & Mostl, 2005; Touma et al., 2004). Living space allowance can also influence GC levels in the blood (Barnett et al., 1992; Gupta et al., 2007; Villagra et al., 2009), although this effect may be species-specific (Buijs, Keeling, Rettenbacker, Van Poucke, & Tuyttens, 2009; Moreira, Brown, Moraes, Swanson, & Monteiro, 2007).

The aim of this study was to evaluate effects of breed, cage type, stage of reproductive activity, and all possible interactions among these factors on fecal corticosterone concentration (FCC) levels in doe rabbits. Small (i.e., standard- size) wire cages (83 × 38 × 32 cm) were expected to correlate with higher FCC levels or an altered FCC response to stressful reproductive stages, such as partum and weaning, compared with FCC measured in doe rabbits housed in bigger cages (113 × 46 × 46 cm) equipped with a plastic foot mat—conditions that were expected to alleviate some aspects of chronic stress and thus correlate with lower FCC levels and a smaller response to distressing reproductive stages.

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MATERIALS AND METHODS

Animals and Housing

A total of 252 doe rabbits (126 belonging to the Grigio del Monferrato breed and 126 to a New Zealand hybrid) arrived at the farm altogether (1 week before the beginning of the experimental period). They were 4-months-old at the arrival, and their mean body weight was 3,626.31 ± 457.76 g. They were acquired from the same supplier and were exposed to identical transport conditions, vaccinated, randomly divided into two groups, and individually housed in either standard wire cages (SC) sized 83 × 38 × 32 cm (0.10 m^3) or in bigger wire cages (BC) sized 113 × 46 × 46 cm (0.24 m^3). Nests were included in both cage types. Each BC was also equipped with a plastic foot mat covering approximately 25% of the cage floor. In the surface covered by the foot mat, mesh size was double that of the SC.

Reproduction was managed by artificial insemination (AI) using on-farm bucks. Doe rabbits were submitted to a semi-intensive rhythm (AI on Day 11 post-partum and weaning following 31 days of lactation). Water was available ad libitum from nipples in cages and feed (a commercial pelletted rabbit diet) was administered by an auto-feeder. Animals were housed according to EFSA (2005) guidelines for rabbit breeding.

Sample Collection

Feces were collected by placing fine wire netting boxes underneath the cages. As such, each sample contained feces from all of the animals from each group. Collection took place at the same time each day (between 7:00 a.m. and 9:00 a.m.). Following collection, samples were frozen directly and stored at −20 °C until analysis. Sample collection was performed at five different stages of the reproductive activity: before oestrus synchronisation (by hormonal treatment), before AI, before partum, the day before weaning, and the day after weaning. Four consecutive reproductive cycles were considered in the trial.

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Steroid Hormone Assay: Extraction and Determination of FCC

In order to assess the intra-individual variability of adrenal activity, fecal samples were collected from 20 randomly selected subjects (10 from each group) every day for a week, without stressor or reproductive moment. Subsequently, stored samples were thawed and prepared for hormonal assays. To extract steroids from non-liquid matrices (such as dried solids) feces were subjected to an organic phase extraction using ethanol; the use of ethanol is recommended as a means to completely solubilize the dried steroid since certain steroids have limited aqueous solubility.

Feces were kiln-dried at 55 °C for 24 hr, thoroughly crushed and five aliquots of pulverised feces (0.20 g each) were put into extraction tubes, which were then sealed with a Teflon cap. Next, 1 ml of ethanol (Sigma Aldrich, St. Louis, MO) for every 0.1 g of solid were added to each tube, and the mixture was shaken vigorously for 30 min. Samples were centrifuged at 3,300 × g for 15 min, and the supernatant recovered in a clean tube for evaporation to dryness in a SpeedVac (ThermoFisher Scientific, Waltham, MA). Extracts were stored at −80 °C.

Extracted samples were dissolved into 100 µl ethanol followed by at least 400 µl of kit Assay Buffer (Arbor Assays, Ann Arbor, Michigan), then they were vortexed and rested for 5 min twice to ensure complete steroid solubility. The FCCs were determined using commercial enzyme immunoassay kits (K014; Arbor Assays, Ann Arbor, MI) validated for dried fecal extracts. All analyses were repeated twice. Inter- and intra-assay coefficients of variation were less than 10%. The test's sensitivity was determined by measuring the least amount of hormone standard consistently distinguishable from the zero concentration standard and was calculated to be 15.6 pg ml^{-1}.

The corticosterone antibody used to quantify fecal hormone cross-reacted to 100% of corticosterone, 12.30% of desoxycorticosterone, 0.38% of cortisol, and less than 0.08% of

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cortisone. Serial dilutions (1:4, 1:8, 1:16, and 1:32) of fecal samples from three Grigio del Monferrato rabbits and three New Zealand hybrid rabbits were assayed to test for parallelism against the standard curve ($p < .05$ for all assays). The mean recovery rate of corticosterone added to dried faeces was 94% ($n = 6$).

Statistical Analysis

Statistical analysis of the data was performed using SPSS software, version 16.0 for Windows (SPSS Inc., Chicago, IL; SPSS, 2007). The Kolmogorov-Smirnov test for normality was used to check whether residuals followed a Gaussian distribution. The assumption of equal variances was assessed using the Levene's homogeneity of variance test. The data were submitted to ANOVA according to the following model:

$$X_{ijkl} = \mu + CT_i + B_j + P_k + (CT \times B)_{ij} + (CT \times P)_{ik} + (B \times P)_{jk} + (CT \times B \times P)_{ijk} + \varepsilon_{ijkl}$$

where X_{ijkl} = observation; μ = overall mean; CT_i = fixed effect of cage type (1, standard cage; 2, bigger cage); B_j = fixed effect of breed (1, New Zealand hybrid; 2, Grigio del Monferrato); P_k = fixed effect of phase of the reproductive cycle (1, synchronisation; 2, artificial insemination; 3, partum; 4, pre-weaning; 5, post-weaning); $(CT \times B)_{ij}$ = interaction between cage type and breed; $(CT \times P)_{ik}$ = interaction between cage type and phase of the reproductive cycle; $(CT \times B \times P)_{ijk}$ = interaction between cage type, breed, and phase of the reproductive cycle; and ε_{ijkl} = residual error.

Because the assumption of equal variances did not hold, the Brown-Forsythe statistic was performed to test for the equality of group means instead of the *F* test. Tamhane's T2 pairwise multiple comparisons were performed to test for differences between each pair of means. Significance was declared at $p \leq .05$.

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RESULTS

FCCs were higher ($p \leq .001$) in the New Zealand hybrid than in Grigio del Monferrato breed (27.77 vs. 24.61 pg g^{-1}, respectively). FCCs were significantly higher ($p \leq .001$) in doe rabbits housed in SC than in BC (27.47 vs. 24.91 pg g^{-1}, respectively). Different stages of reproductive activity had different FCC levels ($p \leq .001$). In particular, the highest FCC level was observed at partum (31.88 pg g^{-1}), followed by post-weaning (26.36 pg g^{-1}). The lowest FCC levels were observed for sincronization and pre-weaning (23.77 and 24.37 pg g^{-1}, respectively). AI was at an intermediate level (24.57 pg g^{-1}). Effects of cage type, breed, and interactions among these factors are reported in Table 1.

Table 1 about here

For both groups, the highest FCCs were observed during pre-partum, reflecting the physiological corticosterone peak triggering the partum (McLean & Smith, 2001; Kindahl et al., 2002). A second peak was also noted during post-weaning, and it was probably related to the stress caused to does by early weaning—a procedure typical of intensive rabbit farming. Early weaning increases anxiety and neuroendocrine stress responses in both the mother and litter, and this condition may induce the onset of disease or decrease the production performance or cause behavioral changes (Kikusui, Nakamura, & Mori, 2008; Glavin & Pare, 1985; Kikusui, Nakamura, & Mori, 2009; Waran, Clarke, & Farnworth, 2008; Greenberg & Ackerman, 1986). The AI lead to increases in FCC levels, but the levels were not comparable to those found during partum.

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DISCUSSION

The significantly higher FCCs observed in rabbits housed in small cages suggest that small living allowances exacerbate the corticosteroid response to stress via a mechanism similar to that observed by Buijs, Keeling, Rettenbacker, Maertens, and Tuyttens (2011) where the stress factor was transport. It would be interesting to see whether even larger cages bring about further reductions in FCCs in order to identify the living space allowance that causes the least stress to intensively farmed doe rabbits.

During the pre-weaning period, the space available for does is decreased due to the presence of rabbit kits, but this factor does not affect corticosterone levels. The lack of effect could be due to the fact that animal density during this period increased gradually (i.e., with the kits' growth).

In the study by Buijs et al. (2011), the authors detected lower baseline FCCs in enriched, but not in larger, cages. The same observation has also been made in mink (Hansen, Malmkvist, Palme, & Damgaard, 2007). The EFSA reccomendation for cage size is 34 to 48 cm wide and 65 to 75 cm long, resulting in an available surface between 0.221 m^2 and 0.360 m^2. In our study, SC were at the lower limit (0.228 m^2), and BC were higher than the EFSA (2005) reccomendation.

Because the aim of our experiment was to obtain a measurement of FCC in housed rabbits using non-invasive means, fecal samples were collected from underneath the wire floor. Collected samples could not therefore be linked to individuals, and they contained feces from both subordinate and dominant animals. These two factors might have been influenced differently by cage type, thus adding to individual differences in FCC. An effect of stocking density on FCC has previously been described for individually sampled mice (Nicholson et al., 2009). Moreover, the lack of effect of cage size in the study by Buijs et al. (2011) could be due to the fact that all cage sizes tested induced an increase in FCC.

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On the other hand, in the study by Buijs et al. (2011), the use of relatively large group sizes (8 rabbits per pen) might have decreased the effects of space allowance, as larger groups have more opportunities to share space (McGlone & Newby, 1994). Small cages and high stocking densities are also reported to limit locomotion, social and resting behaviors, and increase fearfulness, aggression, redirected grooming, and cage manipulation (Verga, Luzi, & Carenzi, 2007). It therefore follows that limited living space allowances might also be expected to exert influence on GC levels, but little is currently known about this possibility.

Basal plasma corticosterone concentrations were reported to be elevated in rabbits housed in groups of five compared to those housed in smaller groups (Onbasilar & Onbasilar, 2007). No difference was observed, however, between rabbits housed individually or in groups of eight individuals when stocked at the same density (Whary, Peper, Borkowski, Lawrence, & Ferguson, 1993), supporting the notion that stocking density is a key factor.

Until more is known about the effects of chronic stress on the neuroendocrine cascade, it remains important to determine baseline concentrations as well as levels following exposure to novel stressors. In a recent study of the influence of environmental enrichment and cage size on GCM concentrations in rabbits exposed to a novel stressor (transport), cage enrichment was found to lead to a decrease in GCM levels, while increasing cage size had no effect (Buijs et al., 2011).

CONCLUSION

In the last 3 decades, rabbit meat production has evolved from more or less traditional production systems to more intensive ones due to relevant advances in genetic selection, reproductive management, and feeding systems (Pascual, 2010). Requirements of

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reproductive rabbits increased considerably in recent years, perhaps compromising body condition, lifespan, and general health on the farm (Pascual, 2010). In some species, selection for exclusively productive criteria is frequently observed to have negative associated effects, such as higher disease incidence (Dourmad, Etienne, Prunier, & Noblet, 1994). One of the problems acknowledged by EFSA is the need to improve health management and conditions for farmed rabbits. The EFSA (2005) states that the mortality of farmed rabbits is considerably higher than in other species of farmed animals, which could be due to a lack of knowledge on welfare conditions and rabbit physiology. Moreover, the evaluation of welfare in the field will only be feasible through the provision of relatively easy, cheap, and fast tools.

This paper provides evidence in support of the measurement of FCC as such a tool. Breed, cage type, and reproductive phase significantly affected FCC, while none of the tested interactions was statistically significant. The New Zealand hybrid showed significantly higher FCC when compared to the autochthonous breed. Increasing cage size coupled with the use of a plastic foot mat lead to a significant reduction in doe FCC. Considering the different reproductive stages, the highest FCC were detected at partum, followed by post-weaning. No differences were found in FCCs among synchronization, AI, and pre-weaning. Such results support the hypothesis that measurements of FCCs provide a useful indicator of chronic stress in doe rabbits.

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TABLE 1

Mean Values of FCCs (pg g^{-1}) in Doe Rabbits According to Cage Type and Breed

	BC		SC		*SEM*	*p*-value		
	NZ	GM	NZ	GM		CT	B	CT×B
Synchronization	23.78[a,b]	21.33[b]	27.26[a]	22.71[b]	3.165	0.07	*	ns
Artificial insemination	26.18[a]	19.64[b]	29.36[a]	23.08[b]	2.396	*	***	ns
Partum	31.48[ab]	29.33[b]	33.89[a]	32.83[a]	2.827	*	ns	ns
Pre-weaning	24.51	22.64	26.41	23.93	3.289	ns	ns	ns
Post-weaning	26.05[a,b]	24.14[b]	28.79[a]	26.46[a,b]	1.873	*	*	ns

Note. BC = bigger cage ; SC = standard cage; NZ = New Zealand genotype; GM = Grigio del Monferrato breed; CT = cage type; B = breed; ns = not significant.

[a,b]Different letters in the same row indicate differences in FCC levels for stage of reproductive activity ($p < 0.05$).

*$p \leq 0.05$. ***$p \leq 0.001$.

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