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ABSTRACT

The effect of transport on Cortisol and Glucose serum concentrations were assessed in rabbits during summer and winter. Animals were divided into high (HSA, 307 cm^2/rabbit), medium (MSA, 373 cm^2/rabbit) and low space allowance (LSA, 475 cm^2/rabbit) transport groups, and rabbits slaughtered directly in the farm were used as control group (C). During summer, cortisol and glucose concentrations were significantly higher in HSA (high space allowance), MSA and LSA than in C rabbits ($P < 0.01$). LSA cortisol concentrations were significantly lower than MSA and HSA samples and MSA rabbits' glycaemia was significantly higher ($P < 0.01$) compared with HSA animals. During winter, cortisol concentrations in group C were significantly lower than in MSA and HSA groups. Glycaemia in group C was lower than in LSA ($P < 0.01$) and HSA ($P < 0.02$) groups. Cortisol and glucose levels in summer were significantly higher than in winter. Our data clearly show that some stress-related physiological parameters are significantly modified by transport, in particular in the case of overcrowded transport crates.

Keywords: Rabbits transport Space allowance Cortisol Glycaemia Animal welfare

1. Introduction

In the last two decades, public awareness regarding farm animal welfare has spread all over the world (Maria, 2006; Blokhuis et al., 2008; You et al., 2014) and is now considered a central legislative goal by the Organization of the United Nations FAO (2010). Such interest is guiding and influencing trade trends, not only to guarantee animal welfare but also to ensure food quality. Animal welfare in breeding and slaughtering influences the characteristics, quality, commercial lifespan of the final product and sanitary features of food, making the different tissues variably sensitive to microbial attack (Dalle Zotte et al., 1995; Jolley, 1990; Lambertini et al., 2004).

Transport represents a considerable stressor for animals and in particular stocking density condition, trip duration and waiting time at the slaughterhouse are the most effective factors that increase stress (Jolley, 1990; De La Fuente et al., 2004; Corese, 2005). Transport space allowance could also have an important impact on animal conditions when arriving to the slaughterhouse and consequently on the quality of the carcasses and meat with possible economic damages (Weeks et al., 1997).

To date, European laws (Council Regulation (EC) No 1/2005) do not establish space allowance limitations for rabbit transport, while for other species the optimal, or good conditions for animal space allowance are dictated. Therefore, for rabbit transportation, it is necessary to refer to laws concerning transport conditions of other species. (De La Fuente et al., 2004).

Few studies concerning rabbit welfare during transport are available in literature. Many Authors considered this subject, but they focused on carcass quality rather than trying to state which densities could be better to guarantee transport welfare (Coppings et al., 1989; Jolley, 1990; Kola et al., 1994; Batchelor

and Giddins, 1995; Trocino et al., 2003; Corese, 2005). The aim of the present study was to identify parameters that could represent an objective measurement of the rabbits physiological response to different transport conditions. We decided to focus on cortisol and glucose blood concentration, as they represent the most indicative and easiest parameters to be studied for the assessment of stress levels (Fordham et al., 1989; Broom et al., 1996; Broom, 2000; Ibañez et al., 2002; Teke et al., 2014).

2. Materials and methods

2.1. Animals and farm

364 meat rabbits (Hycole commercial hybrid) of both sexes were used, approximately weighted 2.50–3.00 kg and aged 80–100 days. The animals were bred in a home-managed farm with the complete production cycle (reproduction and growth/fat) composed of about 500–700 breeding does and about 20,000 to 30,000 fattened rabbits per year. The space available for the animal was about 600 cm^2/head; the mortality varied from 5% to 7%. The mean temperature in the rabbits crates was 18.7 °C in winter and 27.8 °C in summer. The farm was located approximately 220 km from the slaughterhouse.

2.2. Transport

Rabbits were loaded in groups corresponding to three different stocking densities: high space allowance (HSA), mean space allowance (MSA) and low space allowance (LSA). The rabbits were randomly assigned to three groups and the animals transported together always came from the same crate on the farm.

MSA group arrangement was obtained from Biscotto et al. (2007) and it consisted in 350 cm^2/rabbit (for fat rabbits with an average weight of 2.6–2.9 kg) as the minimum limit to safeguard the animals welfare during transport. Transport crates were 5225 cm^2 in area. Crates for MSA group were loaded with 14 rabbits each (373 cm^2/ rabbit). To obtain the HSA and LSA stocking densities, crates were loaded with a difference of 3 animals each (17 rabbits, 307 cm^2/rabbit, HSA; 11 rabbits, 475 cm^2/rabbit, LSA). In summer (July) and winter (January) trials, transport was carried out in the same manner.

Rabbits were transferred from breeding to transport crates manually by the farm staff.

Transport crates were made of rigid plastic with a rectangular base (96.40 × 54.20 × 25.00 cm) and rounded corners; a plastic grid would allow for proper air circulation from floor, ceiling and walls. During transport, crates were arranged in columns of 10 each in the central part of the lorry.

For each journey, the same lorry driven by the same operator was used and the procedures of charge, transport and discharge were identical. Crates charge and discharge were carried out by the lorry driver.

Animals were transported during the night (between 1:00 and 2:00 a.m.) and they reached the slaughterhouse in about 3 h. The travel from farm to slaughterhouse was carried out on normal roads, avoiding highways and rabbits did not have access to food or water.

The ambient temperature at the arrival to the slaughterhouse was 17 °C in summer and 1 °C in winter.

2.3. Slaughtering

Rabbits were slaughtered in summer (n = 148) and in winter (n = 177). In particular, in summer were slaughtered 50 rabbits for MSA group and 49 rabbits both in HAS and in LSA group, whereas in winter were slaughtered 59 rabbits in each treatment.

The slaughtering of rabbits was performed with standardized procedures as described by Regulations ((EC) N° 852/2004, N° 853/2004, N° 854/2004) (hygiene of foodstuffs) and N° 1099/2009 (protection of animals at the time of killing). Animals were manually extracted from the crates and stunned with specific electric rabbit stunner by trained personnel. The current intensity applied to each rabbit was of 150 mA with a voltage of 250 Hz and the time of application was 0,3 s. Subsequently, rabbits were hung on the slaughter line and jugular ex-sanguination performed. Afterwards, rabbits were skinned and eviscerated and the health inspection was carried out by the Veterinary Officer. Subsequently, carcasses were transferred to the local refrigerator for cooling.

Before they were killed rabbits waited in the slaughter from 5 a.m. to 6:30 a.m. and the slaughtering process ended 1 h later; during this period environmental conditions did not change.

On the same day of slaughtering, between 01:00 and 02:00 a.m., 39 rabbits (19 in summer and 20 in winter – control group, C) were slaughtered directly in the farm following the same slaughtering procedures used in the slaughterhouse.

2.4. Blood sampling and analysis

Blood samples were obtained from the jugular vein wound made at the time of slaughtering in all groups (C, HSA, MSA, LSA groups).

All blood samples were collected in tubes without anticoagulant, thus using serum for analysis. Serum was divided into aliquots, immediately stored at −20 °C until determination of cortisol and glucose.

C rabbit blood samples were used as reference values to evaluate changes in cortisol and glucose blood concentrations.

Cortisol serum concentration was determined by radioimmunoassay (RIA), glucose was quantified by enzyme-colorimetric methods using a commercial kit (Far, Verona, Italy).

Cortisol extraction from serum was performed as described by Tamanini et al. (1983): briefly, 100 µL of serum were added with 5 mL of diethyl ether (BDH Italia, Milan, Italy). Samples were placed on a multivortex for 30 min, then centrifuged at 2000*g* for 4 min at 4 °C. The ethereal part, separated from the serum component by suction, was evaporated under aspiration hood in current of air at 37 °C. The dry residue containing the steroid hormone was dissolved in 0.5 mL of 0.05 M phosphate buffer (PBS).

Cortisol serum concentration was determined as described by Tamanini et al. (1983). Analysis was performed in duplicate. 100 µL of 3H-cortisol (specific activity 100 Ci/mmol, amount 30 pg/tube vial, ≈ 12,771 dpm/100 µL) and 100 µL of an anti-cortisol antibody (dilution 1:20,000) were added to 100 µL of the solution obtained with the serum extraction. After incubation at + 4 °C for 18 h, the free steroid was separated with the addition of 1 mL of 1% charcoal solution (Sigma Chemical Co.) + 0.025% dextran (Sigma Chemical Co.), and incubation at + 4 °C for 15 min followed by centrifugation (4000*g*) for 4 min at + 4 °C. The supernatant containing the hormone bound to its antibody was decanted into scintillation vials and measured in a liquid scintillation β-counter (Perkin–Elmer Life Science Inc.).

Parameters for the analysis validation were: sensibility 0.78 pg/mg, assay variability 6.8%, variability between assays 9.3%, specificity (%): cortisol 100, corticosterone 9.5, 11α-hydrossi-progesterone 8.3, cortisone 5.3, 11α-desossicortisol 5.0, progesterone 0.6, desossicorticosterone 0.5, 20α-dihydrocortisone 0.4, testosterone 0.3, aldosterone 0.1, dehydroepiandrosterone, 5β-pregnenolone, 17β-estradiolo, cholesterol < 0.0001.

Readings of radioactive counting by liquid phase βcounter were treated to construct linear standard curves and eventually processed to calculate corresponding concentrations using an ad hoc designed soft-

ware program.

Glucose levels in serum were determined by enzymatic colorimetric glucose oxidase/peroxidase assay. The principle is: glucose + O2 + H2O $\xrightarrow{\text{glucose oxidase}}$ ~~gluconat~~e + H2O2. The last step is a reaction between H2O2 + o-dianisidine with peroxidase to produce a detectable brown colour. Samples' absorbance was measured in a spectrophotometer (Biotek instrument Inc. - mod. ELX 808 IU) at a wavelength of 450 nm.

2.5. Statistical analysis

Normal distribution was tested using the Kolmogorov–Smirnov test. Data were subjected to ANOVA by GLM using space allowance, season and interaction in the model. The following model was adopted: $Y_{ij}\ \mu\ a_I\ \beta_j\ (a*b)_{ij}\ \varepsilon_{ij}$ where: Y_{ij} = elements, μ = overall mean, α_j = treatment (HSA, MSA and LSA), β_j = season (summer and winter), $(\alpha*\beta)_{ij}$ = interaction, εij =experimental error.

Since the ANOVA test revealed a significant effect, Tukey post-hoc test was performed to identify differences between groups.

The differences were considered significant with $P \leq 0.05$ ($\alpha = P \leq 0.05$). All results are expressed as means ± SE.

3. Results

Serum cortisol concentrations were significantly modified by the different space allowance ($F_{3357} = 18.12$, $P < 0.01$) and season ($F_{1357} = 30.47$, $P < 0.01$). During summer, cortisol concentrations in trans- ported animals were significantly higher ($P < 0.01$) than those observed in group C. Cortisol concentrations detected in LSA rabbits were significantly lower ($P < 0.01$) when compared to those of MSA and HSA groups, while the differences between MSA and HSA groups were not significant (Fig. 1). In winter, cortisol levels in group C rabbits were significantly lower ($P < 0.02$) than those observed in MSA and HSA animals (Fig. 1). Whereas seasonality modulates cortisol secretion, to compare results between seasons (summer vs winter), variations of this hormone were compared to the C group value (considering C = 100%). The comparison between cortisol concentration in percentage (referred to the C group concentrations) showed that summer levels were significantly ($P < 0.01$) higher than winter ones in all the groups: LSA 667.91% vs 160.75%; MSA 1336.57% vs 198.26%; HSA 1041.79% vs 202.14% (Fig. 2).

Serum glucose concentrations were significantly influenced by the different space allowance ($F_{3357} = 31.11$, $P < 0.01$), season ($F_{1357} = 31.42$, $P < 0.01$) and by the interaction on two main factors ($F_{3357} = 18.50$, $P < 0.01$). Glycemia during summer varied depending on the different stocking density condition: HSA, MSA, LSA conditions influenced serum glucose values ($P < 0.01$) from those of the C group. In addition, rabbits glycemia in the MSA group was significantly higher ($P < 0.01$) than that of HSA animals, but no significant difference was noticed between the LSA group compared respectively to the MSA and HSA group (Fig. 3). In winter, C group glucose concentration was significantly lower than LSA and HSA groups, while no differences were evident with the MSA group (Fig. 3). As with cortisol, comparing the percentage changes of the glucose concentration in summer and winter in the C group (set 100%) to those in all the other groups, it resulted that HSA, MSA and LSA group values in summer were significantly higher ($P < 0.01$) than those detected in winter (LSA 173.70% vs 122.47%; MSA 198.01% vs 106.51%; HSA 160.12% vs 115,34%)(Fig. 4).

4. Discussion

Transport represents a considerable stressor for animals; in particular, stocking density condition, trip duration and waiting time at the slaughterhouse are considered among the most effective factors that increase stress and consequently influence carcasses and meat quality (Jolley, 1990; Weeks et al., 1997, De La Fuente et al., 2004; Corese, 2005).

Animal welfare is also an important topic, because it should be taken into account when assessing law requirements. Moreover, the sensibility of the public opinion on animal rights is increasing. It is a specific due for the slaughterhouse-holder as well as for the official veterinary to verify rabbits welfare condition after travel.

There are a few publications and articles that discuss common practices for the transportation of research animals (National Research Council, 2006), including the *AATA Manual for the Transportation of Live Animals* (AATA, 2001), the *IATA Live Animals Regulations* (IATA, 2005), and a Report of the Transport Working Group Established by the Laboratory Animal Science Association (Swallow et al., 2005). Very few studies concern rabbit transport and the available literature focus on carcass quality after slaughtering instead of animal well-being during transport (Coppings et al., 1989; Jolley, 1990; Kola et al., 1994; Batchelor and Giddins, 1995; Trocino et al., 2003; Corese, 2005).

The present study was carried out to clarify density at transport effects in rabbits after the transport by measuring cortisol and glucose serum concentrations. Results demonstrate that stocking density can induce significant differences in the cortisol and glucose response. In fact, both in summer and winter, the two parameters studied, were significantly higher in rabbits transported with a lower area avail- ability.

HSA group rabbits, in spite of the predictability, presented similar cortisol levels as compared to those in MSA group, both in summer and winter. These results seem to contradict the hypothesis that the increase of stress level (measured by animals space allowance) induces an in- crease in serum cortisol concentrations. It is possible to argue that the animal adaptation to stressors is possible into certain limits: outside these physiological limits, the animal does not have the resources to contrast further requests (vibration, shock, noises, temperature, fasting, dehydration, etc.). Therefore, we could hypothesize that there is a “maximal load level” beyond which the organism uses up its resources and is not able to produce more cortisol and thereby mobilize glucose. De la Fuente et al. (2004) studied the effects of different stocking density in rabbits of an average age of 60 days and an average weight of 1.85 kg. Those animals were transported in normal crates at different densities (515 cm^2/rabbit or 340 cm^2/rabbit) and with different temperatures (winter, 12 °C, summer 27 °C). Those authors evaluated some haematic parameters and/or activity (cortisol, creatinin-kinase, lactdehydrogenase, lactate, glucose, osmolarity, albumins and globulins) but they found no differences related with stocking density condition or temperature. The discrepancy between our results and those data could be found in the relative short travel time in De la Fuente's work (1 h.20 min) as well as to the different weight of the rabbits and the space allowance per kg of body weight, which were way higher in this study. Furthermore, our results confirm this trend, as there is no significant difference between HSA and MSA group.

Cortisol and glucose serum concentrations of all the experimental groups (HSA, MSA, LSA) were similar in their trends, but presented a lower variation (increase) in winter rather than in summer when compared with the C group. These results agree with those by De la Fuente et al. (2004): they found that plasma concentration of cortisol and glucose was significantly higher in rabbits transported in summer than those transported in winter. These seasonal differences in cortisol and glucose serum concentrations probably depend on the higher transport temperature in summer. This could also suggest that besides stocking density, in particular when the external temperature is high, rabbit welfare during transport should

be achieved with other strategies, such as good ventilation or air conditioning. In fact, as the outside and inside lorry temperatures correlated highly (Lambooy, 1988), an adequate ventilation can maintain the internal temperature close to the thermo-neutral zone (Collins, 1993).

Cortisol is a hyperglycemic inductor but, unlike glucagon, it acts not only on hepatic glycogen, but also on muscular glycogen, accelerating protein catabolism. Its final effect lies in a loss of protein, particularly from muscle, to give hepatic cells some substrates for glucose synthesis (gluconeogenesis). In fact, it should be remembered that glycogen stores last for a short period that, in human, is almost 15 h. Rabbits were fasted for 12 h before slaughtering, leading to two different con- sequences: the effective possibility of the exhaustion of the body re- serves, as hypothesized in our work, and a reduction in muscular protein quality, in particular due to the meat acidification level that could be reached as a consequence of biochemical post mortem phenomenons (Jolley, 1990; Hulot and Ouhayoun, 1999). Another cause of reduction in hepatic and muscular glycogen content could be the in- crease of energy demand in stressful situations (Hulot and Ouhayoun, 1999; Warriss et al., 1999).

5. Conclusions

These observations lead to link transport welfare, as an ethic responsibility, to investigations on meat quality and durability. Furthermore, the increase in stress levels evidenced by objective measurement of blood parameters, demonstrates that animals welfare condition decreases with space availability. Considering that we studied only 3 different space allowance levels, it is not possible to state which is the limit not to be exceeded to avoid a state of distress in the animals. In medium space allowance level group (MSA group), animals had an increment in cortisol and glucose (except for glucose during winter) that seems to indicate that this level is beyond the limit of adaptation of the rabbits.

These results endorse those of a previous work of our group (Biscotto et al., 2007) on data reported in animal welfare laws and results from other investigations.

In conclusion, our data indicate that different space allowance levels correspond to different stress levels: a higher space availability reduces stress levels, that tend to increase with the space reduction. The adaptation to the different conditions increases until a maximum level is reached; over this limit the animal does not react because of the exhaustion of all resources. Finally, even if our data are not exhaustive, we suggest not reducing the space availability for rabbits of 2,6-2,7 kg under 350 cm^2/rabbit for travels lasting more than 2 h.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding information

This study was supported by Università di Bologna (Galeati 2015–2016) (RFO).

Ethical approval

All applicable international, national, and/or institutional guide- lines for the care and use of animals were followed

Acknowledgements

We are grateful to Danilo Matteuzzi and Antonio Volgarino for their support in biological sample analysis.
We are grateful to Eleonora Petrulli for the English language check.

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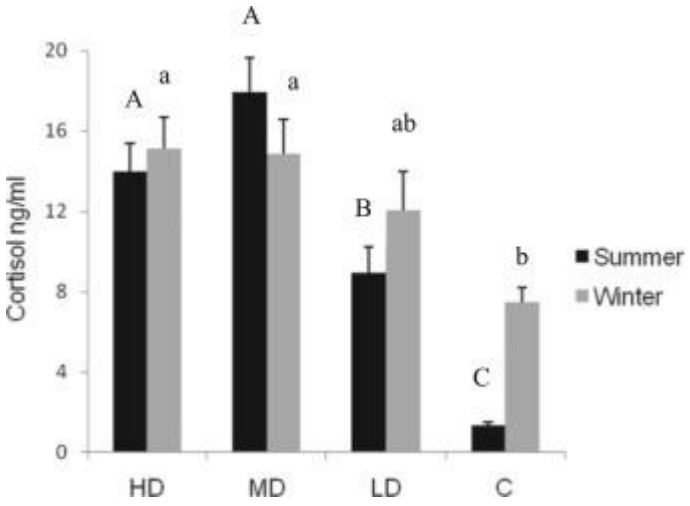

Fig. 1. Serum cortisol concentration (mean ± SE) in control rabbits (C) and in animals transported at different densities: HD (high density), MD (mean density) and LD (low density) during summer (July) and winter (January). A, B = P < 0.01; a, b = P < 0.05 between groups in the same season.

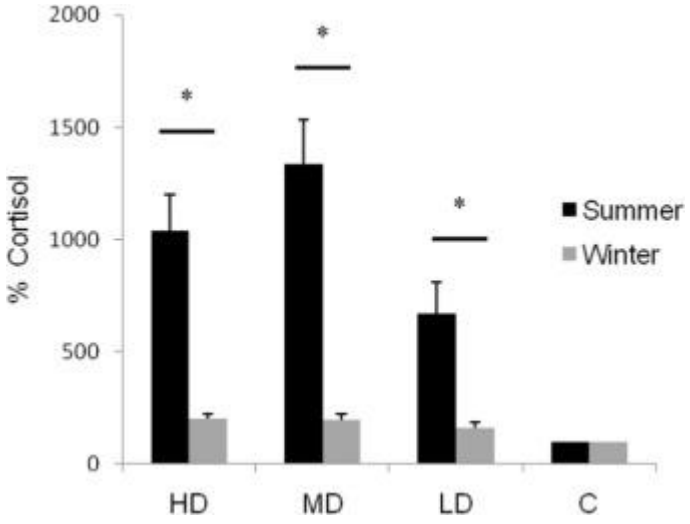

Fig. 2. Percentage changes of serum cortisol concentrations (means ± SE) between control rabbits (C) (set equal to 100%) and those transported to slaughter at different densities (high density (HD), mean density (MD) and low density (LD)) during summer (July) and winter (January). * = P < 0.01.

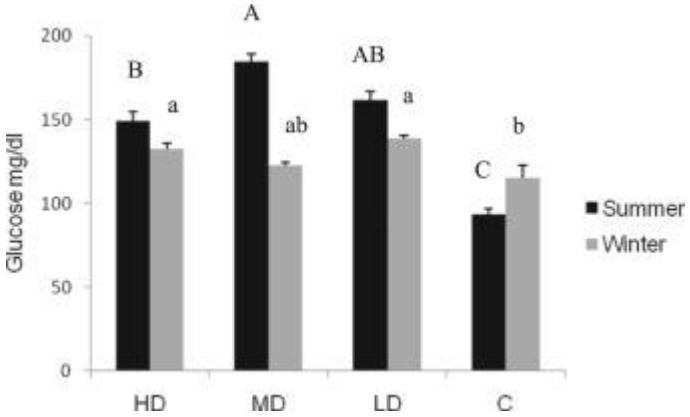

Fig. 3. Serum glucose concentration (mean ± SE) in control rabbits (C) and in animals transported at different densities: HD (high density), MD (mean density) and LD (low density) during summer (July) and winter (January). A, B = P < 0.01; a, b = P < 0.05 between groups in the same season.

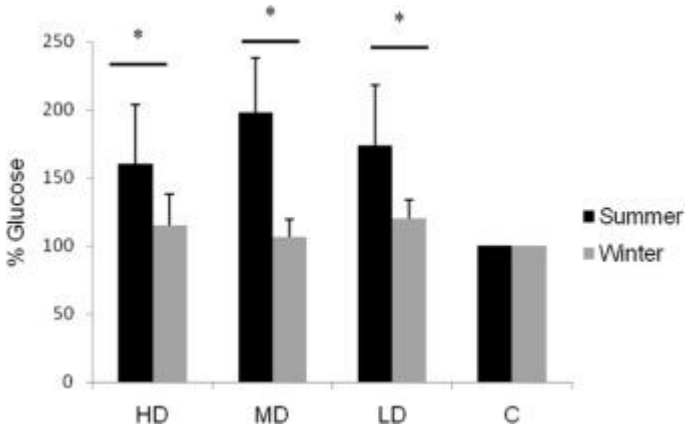

Fig. 4. Percentage changes of serum glucose concentrations (means ± SE) between control rabbits (C) (set equal to 100%) and those transported to slaughter at different densities (high density (HD), mean density (MD) and low density (LD)) during summer (July) and winter (January). * = P < 0.01.