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# Changes in cortisol and glucose concentrations in rabbits transported to the slaughterhouse

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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<sup>2</sup>/rabbit), medium (MSA, 373 cm<sup>2</sup>/rabbit) and low space allowance (LSA, 475 cm<sup>2</sup>/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

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

## 52 53 54 **2. Materials and methods**

### 55 56 *2.1. Animals and farm*

57  
58 364 meat rabbits (Hycote commercial hybrid) of both sexes were used, approximately weighted 2.50–  
59 3.00 kg and aged 80–100 days. The animals were bred in a home-managed farm with the complete pro-  
60 duction cycle (reproduction and growth/fat) composed of about 500–700 breeding does and about 20,000  
61 to 30,000 fattened rabbits per year. The space available for the animal was about 600 cm<sup>2</sup>/head; the  
62 mortality varied from 5% to 7%. The mean temperature in the rabbits crates was 18.7 °C in winter and  
63 27.8 °C in summer. The farm was located approximately 220 km from the slaughterhouse.

### 64 65 *2.2. Transport*

66  
67 Rabbits were loaded in groups corresponding to three different stocking densities: high space allowance  
68 (HSA), mean space allowance (MSA) and low space allowance (LSA). The rabbits were randomly as-  
69 signed to three groups and the animals transported together always came from the same crate on the farm.

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

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

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

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

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

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

### 86 87 *2.3. Slaughtering*

88  
89 Rabbits were slaughtered in summer (n = 148) and in winter (n = 177). In particular, in summer  
90 were slaughtered 50 rabbits for MSA group and 49 rabbits both in HAS and in LSA group, whereas in  
91 winter were slaughtered 59 rabbits in each treatment.

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

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

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

#### 106 **2.4. Blood sampling and analysis**

107  
108 Blood samples were obtained from the jugular vein wound made at the time of slaughtering in all  
109 groups (C, HSA, MSA, LSA groups).

110 All blood samples were collected in tubes without anticoagulant, thus using serum for analysis. Serum  
111 was divided into aliquots, immediately stored at  $-20^{\circ}\text{C}$  until determination of cortisol and glucose.

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

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

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

121 Cortisol serum concentration was determined as described by Tamanini et al. (1983). Analysis was  
122 performed in duplicate. 100  $\mu\text{L}$  of 3H-cortisol (specific activity 100 Ci/mmol, amount 30 pg/tube vial,  
123  $\approx 12,771$  dpm/100  $\mu\text{L}$ ) and 100  $\mu\text{L}$  of an anti-cortisol antibody (dilution 1:20,000) were added to 100  
124  $\mu\text{L}$  of the solution obtained with the serum extraction. After incubation at  $+4^{\circ}\text{C}$  for 18 h, the free  
125 steroid was separated with the addition of 1 mL of 1% charcoal solution (Sigma Chemical Co.) +  
126 0.025% dextran (Sigma Chemical Co.), and incubation at  $+4^{\circ}\text{C}$  for 15 min followed by centrifugation  
127 (4000g) for 4 min at  $+4^{\circ}\text{C}$ . The supernatant containing the hormone bound to its antibody was  
128 decanted into scintillation vials and measured in a liquid scintillation  $\beta$ -counter (Perkin-Elmer Life  
129 Science Inc.).

130 Parameters for the analysis validation were: sensibility 0.78 pg/mg, assay variability 6.8%, variability  
131 between assays 9.3%, specificity (%): cortisol 100, corticosterone 9.5,  $11\alpha$ -hydroxy-progesterone 8.3,  
132 cortisone 5.3,  $11\alpha$ -desossicortisol 5.0, progesterone 0.6, desossicorticosterone 0.5,  $20\alpha$ -  
133 dihydrocortisone 0.4, testosterone 0.3, aldosterone 0.1, dehydroepiandrosterone,  $5\beta$ -pregnenolone,  
134  $17\beta$ -estradiolo, cholesterol  $< 0.0001$ .

135 Readings of radioactive counting by liquid phase  $\beta$ counter were treated to construct linear standard  
136 curves and eventually processed to calculate corresponding concentrations using an ad hoc designed soft-

137 ware program.

138 Glucose levels in serum were determined by enzymatic colorimetric glucose oxidase/peroxidase assay.

139 The principle is:  $\text{glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{gluconate} + \text{H}_2\text{O}_2$ . The last step is a reaction  
140 between

141  $\text{H}_2\text{O}_2 + \text{o-dianisidine}$  with peroxidase to produce a detectable brown colour. Samples' absorbance was  
142 measured in a spectrophotometer (Biotek instrument Inc. - mod. ELX 808 IU) at a wavelength of 450 nm.

143

### 144 *2.5. Statistical analysis*

145

146 Normal distribution was tested using the Kolmogorov–Smirnov test. Data were subjected to ANOVA  
147 by GLM using space allowance, season and interaction in the model. The following model was adopted:

148  $Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$  where:  $Y_{ij}$  = elements,  $\mu$  = overall mean,  $\alpha_j$  = treatment (HSA, MSA and LSA),  $\beta_j$  =  
149 season (summer and winter),  $(\alpha\beta)_{ij}$  = interaction,  $\varepsilon_{ij}$  = experimental error.

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

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

154

## 155 **3. Results**

156

157 Serum cortisol concentrations were significantly modified by the different space allowance ( $F_{3357}$   
158 = 18.12,  $P < 0.01$ ) and season ( $F_{1357} = 30.47$ ,  $P < 0.01$ ). During summer, cortisol concentrations in  
159 trans-ported animals were significantly higher ( $P < 0.01$ ) than those observed in group C. Cortisol  
160 concentrations detected in LSA rabbits were significantly lower ( $P < 0.01$ ) when compared to those of  
161 MSA and HSA groups, while the differences between MSA and HSA groups were not significant (Fig. 1).  
162 In winter, cortisol levels in group C rabbits were significantly lower ( $P < 0.02$ ) than those observed in  
163 MSA and HSA animals (Fig. 1). Whereas seasonality modulates cortisol secretion, to compare results  
164 between seasons (summer vs winter), variations of this hormone were compared to the C group value  
165 (considering C = 100%). The comparison between cortisol concentration in percentage (referred to the C  
166 group concentrations) showed that summer levels were significantly ( $P < 0.01$ ) higher than winter ones in  
167 all the groups: LSA 667.91% vs 160.75%; MSA 1336.57% vs 198.26%; HSA 1041.79% vs  
168 202.14% (Fig. 2).

169 Serum glucose concentrations were significantly influenced by the different space allowance ( $F_{3357}$   
170 = 31.11,  $P < 0.01$ ), season ( $F_{1357} = 31.42$ ,  $P < 0.01$ ) and by the interaction on two main factors  
171 ( $F_{3357} = 18.50$ ,  $P < 0.01$ ). Glycemia during summer varied depending on the different stocking  
172 density condition: HSA, MSA, LSA conditions influenced serum glucose values ( $P < 0.01$ ) from  
173 those of the C group. In addition, rabbits glycemia in the MSA group was significantly higher ( $P < 0.01$ )  
174 than that of HSA animals, but no significant difference was noticed between the LSA group compared  
175 respectively to the MSA and HSA group (Fig. 3). In winter, C group glucose concentration was  
176 significantly lower than LSA and HSA groups, while no differences were evident with the MSA group  
177 (Fig. 3). As with cortisol, comparing the percentage changes of the glucose concentration in summer and  
178 winter in the C group (set 100%) to those in all the other groups, it resulted that HSA, MSA and LSA group  
179 values in summer were significantly higher ( $P < 0.01$ ) than those detected in winter (LSA 173.70% vs  
180 122.47%; MSA 198.01% vs 106.51%; HSA 160.12% vs 115.34%)(Fig. 4).

181

182 **4. Discussion**

183

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

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

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

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

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

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

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

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

240

## 241 **5. Conclusions**

242

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

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

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

258

## 259 **Disclosure statement**

260 No potential conflict of interest was reported by the authors.

261

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264

## 265 **Ethical approval**

266 All applicable international, national, and/or institutional guidelines for the care and use of animals  
267 were followed

268

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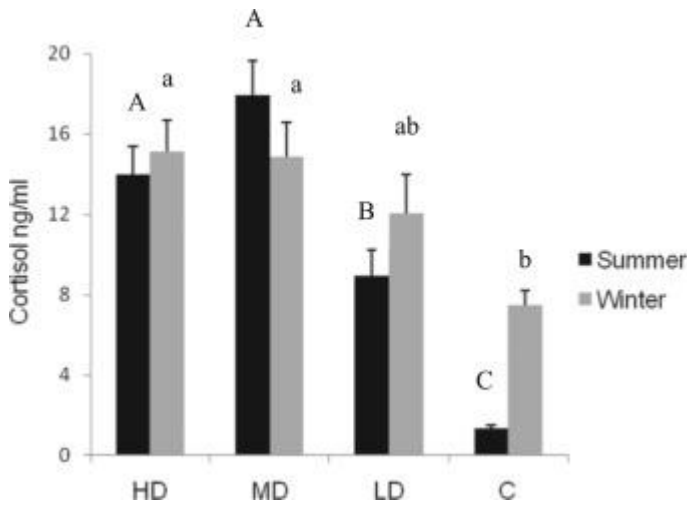


Fig. 1. Serum cortisol concentration (mean  $\pm$  SE) in control rabbits (C) and in animals transported at different densities: HD (high density), MD (mean density) and LD (low density) during summer (July) and winter (January). A, B =  $P < 0.01$ ; a, b =  $P < 0.05$  between groups in the same season.

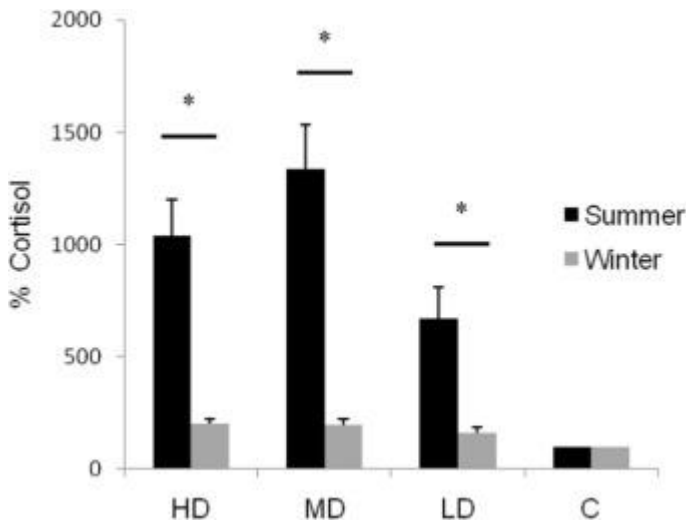


Fig. 2. Percentage changes of serum cortisol concentrations (means  $\pm$  SE) between control rabbits (C) (set equal to 100%) and those transported to slaughter at different densities (high density (HD), mean density (MD) and low density (LD)) during summer (July) and winter (January). \* =  $P < 0.01$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

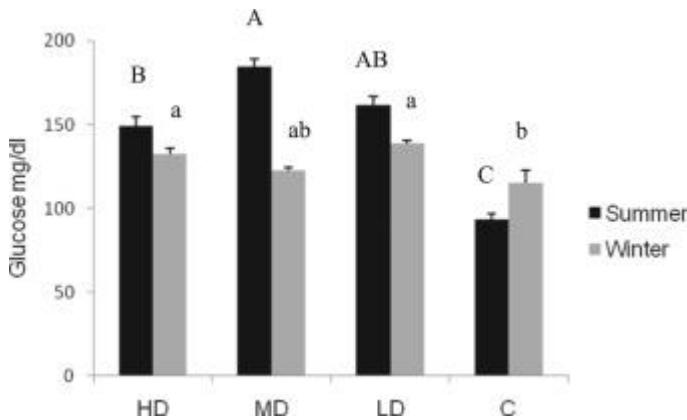


Fig. 3. Serum glucose concentration (mean  $\pm$  SE) in control rabbits (C) and in animals transported at different densities: HD (high density), MD (mean density) and LD (low density) during summer (July) and winter (January). A, B =  $P < 0.01$ ; a, b =  $P < 0.05$  between groups in the same season.

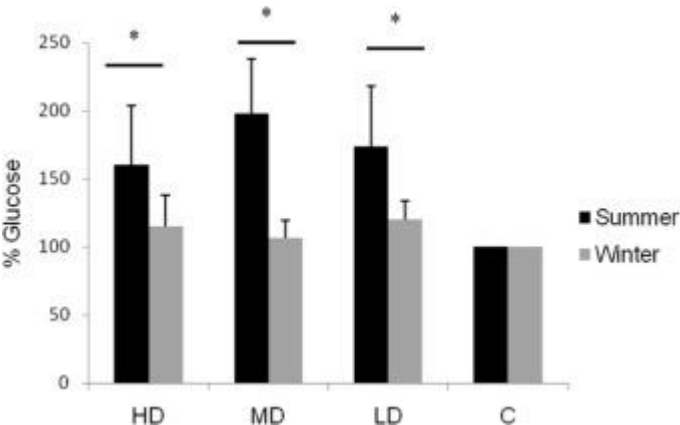


Fig. 4. Percentage changes of serum glucose concentrations (means  $\pm$  SE) between control rabbits (C) (set equal to 100%) and those transported to slaughter at different densities (high density (HD), mean density (MD) and low density (LD)) during summer (July) and winter (January). \* =  $P < 0.01$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