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**A multifactorial evaluation of different reproductive rhythms and housing systems for improving welfare in rabbit does**

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(Article begins on next page)

1 **A multifactorial evaluation of different reproductive rhythms and**  
2 **housing systems for improving does welfare**

3

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13

14 **ABSTRACT**

15 The most widespread reproductive rhythm in rabbit does rearing is based on artificial insemination  
16 (AI) performed around 10 d after kindling, resulting in high production rates, but with a high yearly  
17 replacement of animals, that are unable to sustain the enormous energy demand. The consequence is  
18 an energy deficit causing a reduction of doe reproductive career. Moreover, housing systems currently  
19 applied do not allow animals to express the typical behavioural pattern of the species, with presence  
20 of abnormal behaviours that contribute to reduce welfare. Thus, the aim of the present study was to

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21 evaluate the best combination between two different housing systems (Standard vs Combi cage) and  
22 three reproductive Rhythms (RRs), to improve does welfare through a multiple indicators  
23 (performance, behaviour and corticosterone assessments). A total of 75 nulliparous rabbit does of  
24 Grimaud hybrid maternal line were randomly housed into 2 different systems, Standard cage (C) vs  
25 Combi cage (CC), within which 3 RRs (Intensive, I, with AI 11 days postpartum, vs Alternating, IE,  
26 with AI Alternated between 11 days postpartum and 30 days postpartum vs Extensive, E, with AI 30  
27 days postpartum) were applied, for six consecutive reproductive cycles, following a multifactorial  
28 balanced experimental plan (2x3), as follows: CI (5 does) vs CIE (5 does) vs CE (5 does) vs CCI (20  
29 does) vs CCIE (20 does) vs CCE (20 does). At main critical phases (AI, kindling and lactation peak)  
30 reproductive and behavioural evaluations were carried out, as well as hormonal level assessment  
31 (salivary corticosterone). RR strongly affected reproductive performances with the highest number  
32 of live-born kits ( $P < 0.02$ ) and weight of weaned litter ( $P < 0.01$ ) in CIE group, together with the  
33 lowest pre-weaning mortality ( $P < 0.04$ ). The housing system also influenced behaviour: C does  
34 showed the highest frequencies of self-grooming ( $P < 0.01$ ), feeding ( $P < 0.001$ ) as well as stereotypes  
35 (sniffing and biting bars,  $P < 0.01$ ), indicative of frustration for the lack of enough stimuli and  
36 consequent boredom. Corticosterone level resulted to be 3 times higher ( $P < 0.01$ ) in the CC system.  
37 From these results, we can assume that AI after kits weaning was the best RR in Combi cage and the  
38 Alternating rhythm in Standard cage, permitting to restore fat deposits in time to sustain energy  
39 requirements, resulting in high productive performances along consecutive cycles. Whereas, from an  
40 ethological point of view, in C cage problems related to the small size of the housing system still  
41 remain.

42

43 **Keywords:**

44 Rabbit does, innovative housing system, reproductive rhythm, behaviour, welfare, corticosterone

## 45 **1. Introduction**

46 In wildlife conditions, rabbit (*Oryctolagus cuniculi* L.) is a gregarious animal and the social  
47 unit consists of an average of 2-9 females, 2-3 bucks and their progeny (SurrIDGE et al., 1999). In  
48 commercial farm conditions, breeding does are individually housed, in standard wire cages (50–60L  
49 × 35-40H cm) with an external nest, which the doe prepares with straw materials provided by the  
50 farmer and hair from her body (Canali et al., 1991; Hudson et al., 2000).

51 The commercial breeding system is well adapted to cycled production, but it does not take  
52 into account neither the welfare (EFSA, 2005) nor the reproductive physiology of the doe, with  
53 reduction of fertility along cycles and high annual replacement of females (Cervera et al., 1993). In  
54 fact, the most common reproductive rhythm is intensive, based on Artificial Insemination (AI)  
55 performed 11 days *postpartum* and on weaning of the litter at about 28–30 days of age.

56 Regarding housing system, the European Food Safety Authority (EFSA) published a scientific  
57 opinion on the impact of housing systems on health and welfare of farmed rabbits (EFSA, 2005),  
58 highlighting several problems due to space restriction and housing practice. Several solutions have  
59 been proposed for breeding group growing rabbits (Dal Bosco et al., 2000, 2002; Trocino et al., 2004;  
60 Mugnai 2008, 2014); whereas only a few studies have been carried out on does and kits (breeding  
61 group pen, Stauffacher, 1992; double height cage and two-floor cage, Finzi et al., 1996, Berthelsen,  
62 2000, Mirabito, 2003, 2004; colony cage, Dal Bosco et al., 2004; Mugnai et al., 2009; Dal Bosco et  
63 al., 2019) or on the semi-group housing where does are put together for a specified amount of time  
64 during the reproductive cycle (Andrist et al., 2012). Unfortunately, even if group-housing would  
65 permit to better satisfy animals needs, some critical aspects (aggression and a high kit mortality due  
66 to nest box competition among females) negatively affected productivity (Rommers et al., 2006).  
67 Moreover, Dal Bosco et al. (2019) reported a lower fat depot in does housed in colony cages compared

68 to standard cage, due to a higher energetic output thanks to a greater possibility of movement that  
69 further negatively affected reproductive performance of does.

70 Currently, housing systems have to be consistent with animal welfare regulations proposing  
71 higher space allowance (EFSA, 2005), but considering that cycled production of rabbits is efficient  
72 thanks to low space availability that permits to save energy for performance. It appears obvious that  
73 improving space allowance and consequently motor activity, energy balance, that is already a critical  
74 point in doe cycled conduction (Feugier et al., 2005), will become even more the limiting factor of  
75 the reproductive performance of does if not properly supported with a new reproductive protocol.

76 For all these reasons, our research aimed at evaluating the best combination between housing  
77 system (innovative (Combi) vs conventional cage) and reproductive rhythm (intensive vs alternating  
78 vs extensive), through a multifactorial evaluation (physiological, behavioural, reproductive  
79 parameters), to determine the best management practice for breeding does.

80

## 81 **2. Materials and Methods**

### 82 *2.1. Animals and experimental design*

83 The study was performed at Cascina Campora commercial farm in Buttigliera d'Asti, Italy,  
84 from October 2018 to November 2019. All animals were handled in accordance with the Turin  
85 University Bioethics Committee recommendations (Prot. N 256053 of 4/07/2017).

86 A total of 109 77-days old rabbit does from Grimaud hybrid maternal line, homogenous for genetic  
87 strain and age, were randomly divided into two different housing systems:

88 - 60 nulliparous does in Combi cage (WRSA, Combi 6®, CC), bought from a commercial  
89 company (Meneghin S.r.l, Povegliano TV, Italy), where the main characteristics of the cage were:  
90 the dimensions (97.5L x 113H cm), the plastic platform and the absence of a top cover; mobile walls,

91 which can allow the group housing of 4 does with their kits. The CC, was considered as an  
92 autonomous and independent production unit (Figure 1); in our trial we decided to test the CC with  
93 mobile walls in.

94 - 15 nulliparous does in Conventional cage (C), with dimensions of 60L x 35H cm and an  
95 external nest box. The single cage was considered as an autonomous and independent production unit.

96 - the remaining 34 were used as replacement.

97 Nulliparous does were weekly weighted, from their arrival until 133<sup>th</sup> day, to control weight gain to  
98 the first artificial insemination (AI at live weight of  $4060.61 \pm 334.15$  g at 19<sup>th</sup> week).

99 At second AI, both CC and C does were divided into three different experimental subgroups,

100 according to 3 reproductive rhythms:

101 - Intensive (I): AI 11 days *postpartum*;

102 - Alternating (IE): AI 11 days *postpartum*, alternated with AI after weaning (30 days  
103 *postpartum*);

104 - Extensive (E): AI performed after weaning (30 days *postpartum*).

105 The corresponding balanced (2x3) experimental plan, was composed by:

106 - CCI group = 20 CC does submitted to an Intensive rhythm

107 - CCIE group = 20 CC does submitted to an Alternating rhythm

108 - CCE group = 20 CC does submitted to an Extensive rhythm

109 - CI = 5 C does with an Intensive rhythm

110 - CIE = 5 C does with an Alternating rhythm (second AI 11 days *postpartum*, third AI  
111 insemination after weaning (30 days *postpartum*))

112 - CE = 5 C does with an extensive rhythm.

113 All not pregnant animals were re-inseminated after manual palpation (12 days after AI) following the  
114 corresponding initial rhythm.

115 Rabbits were raised with daily control of the environmental temperature (15-18 °C) and relative  
116 humidity (60-75%). The barn was artificially ventilated (0.3 m/sec) and animals were submitted to  
117 natural photoperiod. The animals received a commercial diet containing 16.9% crude protein, 14.2%  
118 crude fibre, and 3.5% ether extract.

119 The colony effect was not investigated because we decided to first compare the greater availability  
120 of space of the Combi system, compared to the smaller control cage, focusing on the evaluation of  
121 different reproductive rhythms combined with the two type of housing.

122

## 123 *2.2. Reproductive and productive performances*

124 AI was performed in the morning by inseminating 0,5 mL of diluted fresh semen, containing  
125 about 10 million spermatozoa (Castellini and Lattaioli, 1999). No estrus synchronization was done.  
126 Ovulation was induced by inoculating 0,2 mL/doe of intramuscular lecirelin (Dalmarelin, Fatro®,  
127 Italy). Manual palpation to assess pregnancy was performed 12 days after AI. The following  
128 reproductive/productive indexes were recorded: does weight at AI, sexual receptivity (SR: vulva color  
129 and turgescence: a doe was deemed receptive when its vulva was red or purple and turgid), fertility  
130 rate (F: kindling/inseminations x 100); at kindling we recorded number of live born (LK) and dead  
131 kits/litter (DK), weight of weaned litter and individual weight at weaning (weaned kits total weight  
132 /total number of weaned kits) and pre-weaning mortality (dead kits/number of weaned kits).

133 The indexes of efficiency were calculated in terms of: overall productivity (number and weight of  
134 rabbits sold/year/doe), production losses (difference between actual and theoretical production

135 considering fertility rate = 100, mortality of the young rabbits = 0 and kindling interval = 60) and  
136 efficiency of the system (Castellini et al., 2005).

137 Controlled nursing was performed until 16 day after birth by permitting the does access to the nest  
138 only once a day for 15 min (Castellini et al., 2003).

139

### 140 *2.3. Body Condition Score*

141 Body Condition Score (BCS) was attributed at each AI. The scoring was based on feel by  
142 hand loin and rump regions. The loin was felt for vertical bone protrusions (spinous process) and  
143 fullness of muscle over and around the vertebrae, and the rump for bone protrusions and fullness of  
144 muscle. The loin was subjectively evaluated according to poor, intermediate or wide level, whereas  
145 the rump for poor or wide level. The body condition was scored “0” if loin was poor; “1” if loin was  
146 intermediate and rump was poor; “2” if loin was intermediate or wide and rump was wide (Bonanno  
147 et al., 2008).

148

### 149 *2.5. Behavioral recordings*

150 Behavioral patterns of 39 rabbit does (10/rhythm in CC system and 3/rhythm for C system)  
151 were recorded through direct observations by two experienced operators who had been previously  
152 trained together, in order to guarantee an accurate inter-observer agreement (Cohen,1960), following  
153 the Focal Animal Scan Sampling Method (Martin and Bateson, 1986) during all reproductive cycles,  
154 in three significant moments: 2 days after artificial inseminations, 2 days after birth and 2 days after  
155 lactation peak for a total of 6 days/cycle/group. Data were reported on a designed table. Before each  
156 observation, 5 minutes were allowed to the animals for adaptation to the presence of operators. To



157 establish the end of a performed behaviour, 10 sec were allowed to determine if the same behaviour  
158 was repeated; after this time, a new behaviour was recorded (Bornett et al., 2000).

159 To develop the ethogram (Table 1), the following behaviours were recorded: static activities  
160 (crouching, staying, sitting up, lying down), moving features (running, exploring, jumping), self-  
161 grooming, alert (standing-up), presence of stereotypies (sniffing and biting bars), maternal attitude  
162 (quality of the nest), feeding, drinking, use of the elevated platform (only for Combi cages).

163 The inter-observer reliability index (Cohen's kappa) was assessed at 0.97. For all the experimental  
164 groups, does were observed for a total of 4212 min (117 min/day x 6 days of observations x 6  
165 reproductive cycles). For each animal the percentage of a particular behavior was calculated as the  
166 number of times it occurred divided by the total number of observations and multiplied by 100.

167 At kindling, the quality of the nest was evaluated, through a qualitative analysis (Blumetto et  
168 al., 2010), in which were assessed: level of mixing (1: no evidence of mixing between the material  
169 and the doe's hair; 2: an important level of mixing between them; 3: almost the totality of the material  
170 was mixed), hair (1: there is no hair in the nest; 2: it is observed that more than 50% of the nest has  
171 the material visible; 3: it is observed that more than 50% of the nest has the material invisible; 4: only  
172 hair can be seen) and material, in which the preservation of the original material was assessed (1: less  
173 than 30% of the original material is kept; 2: between 30% and 60% of the original material is kept;  
174 3: more than 60% of the original material is kept).

175

## 176 2.6. Tonic immobility test

177 The tonic immobility test was performed on 39 rabbit does (10/rhythm in CC system and  
178 3/rhythm for C system) and repeated on the same animals in three moments of each reproductive  
179 cycle: insemination, kindling and peak of lactation. The operator took the rabbit out of the cage and

180 induced immobility by turning the animal on its back and onto his arms. The immobile rabbit was  
181 laid down on a plastic support (Ferrante et al., 1992). A maximum of three attempts were carried out  
182 to induce immobility and animals were left in the immobility condition for not more than 10 minutes.  
183 The number of attempts necessary to induce immobility and the total duration of the condition were  
184 recorded for each doe.

185

### 186 *2.7. Corticosterone samples collection and hormonal evaluation*

187 To assess animal welfare, physiological indicators as saliva corticosterone level evaluation  
188 was performed. Before the start of the experimentation, two collection days were carried out to have  
189 baseline hormone levels. Saliva was collected using saliva collectors ® (Sarstedt SRL – Verona,  
190 Italy).

191 During the trial, samples were collected for 6 consecutive cycles in the same does that were submitted  
192 to TI, at three different phases: 1 day after AI, 1 day after kindling and 1 day after lactation peak (16  
193 d after birth). After being collected, samples were immediately frozen and stored at – 80 °C until  
194 laboratory analysis. Subsequently, stored samples were thawed and prepared for hormonal assays:  
195 were centrifuged for 2 minutes at 1000 x g and, after centrifugation, recover saliva was diluted 1:4  
196 with Assay Buffer (Arbor Assays, Ann Arbor, US) prior running in the assay. The corticosterone  
197 present in saliva samples was determined using a multispecies commercial enzyme immunoassay kits  
198 (K014; Arbor Assays, Ann Arbor, MI) validated for saliva and other biological substrates. Inter- and  
199 intra-assay coefficients of variation were <10%. According to the manufacturer, the corticosterone  
200 kit presents the following cross reactivity: 100% with corticosterone, 18.9% with 1-  
201 dehydrocorticosterone, 12.3% with Desoxycorticosterone and 0,38 with cortisol. Serial dilutions  
202 (1:4,1:8,1:16, and 1:32) of saliva samples were assayed to test for parallelism against the standard

203 curve ( $P < 0.05$  for all assays). The mean recovery rate of corticosterone added to saliva samples ( $n=6$ )  
204 was 96.8%. The results are reported as nanogram (ng) of corticosterone on ml of saliva.

205

206

## 207 *2.8. Statistical analysis*

208 Statistical analysis were conducted using SPSS 16.0 software package, using two-way  
209 analysis of variance (ANOVA), considering the effects of housing system, reproductive rhythms and  
210 their interactions. Since no differences were found among the three physiological phases and cycles,  
211 all the data were pooled to obtain a mean value. Data are presented as mean  $\pm$  SE. Multiple  
212 comparisons of the means were carried out with LSD using Duncan test. Statistical significance was  
213 assessed as significant when  $P < 0.05$ .

214

215

## 216 **3. Results**

### 217 *3.1. Reproductive performances*

218 No significant differences were recorded regarding animals' weight, BCS and sexual  
219 receptivity (Table 2). Alternating and Extensive C cage showed the highest fertility rate, as well as  
220 Extensive CC ( $P < 0.04$ ). The number of weaned kits resulted to be better ( $P < 0.04$ ) in does reared in  
221 conventional cages with Intensive and Alternating reproductive rhythms, which reflected in a higher  
222 ( $P < 0.01$ ) weight of the litter at weaning and consequently better ( $P < 0.03$ ) individual weight of each  
223 kit. Moreover, regarding pre-weaning mortality, CC group showed the worst ( $P < 0.04$ ) results.

224 The indexes of global productivity (Table 3) showed that C group under the intensive reproductive  
225 rhythm produced the highest number and weight of rabbits sold/year/doe ( $P < 0.05$ ), with the lowest  
226 production losses (kg) among groups, but with the highest doe replacement rate ( $P < 0.05$ ). On  
227 contrary, CC does submitted to an extensive rhythm showed high productive performances with the  
228 lower incidence of animals replaced but less kindling/year as a consequence of greater kindling  
229 interval due to the applied reproductive rhythm ( $P < 0.05$ ).

### 230 3.2. Behavioural patterns

231 In Table 4 are presented behavioural welfare results. Static activities were the most frequent behaviors  
232 performed, with higher values in CC does for crouching ( $P < 0.04$ ) with erect ears, while lying down  
233 ( $P < 0.03$ ) with ears down was more performed in C housing. CI does showed the higher percentages  
234 in terms of feeding ( $P < 0.001$ ) and drinking ( $P < 0.002$ ) as well as the worst results in terms of biting  
235 ( $P < 0.01$ ) and sniffing bars ( $P < 0.01$ ). Standing up with ears down resulted to be performed only in  
236 conventional cages ( $P < 0.02$ ), with higher values in intensive standard cage. Moving features were  
237 not affected neither by housing system nor by reproductive rhythm, except for jumping ( $P < 0.04$ )  
238 only performed in alternative cages due to presence of the platform. Self-grooming was performed  
239 significantly more in CI group ( $P < 0.01$ ).

240 The tonic immobility test resulted not significant between CC and C groups. Behaviours on platform  
241 were only performed in CC cages, due to the absence of this structure in conventional housing.

242 The quality of the nest resulted to be significant only for the level of mixing between the hair and the  
243 material supplied by the farmer for both group ( $P < 0.006$ ) and rhythm effect ( $P < 0.05$ ), resulting in  
244 greater values within the CCIE does.

245

246

247 *3.3. Salivary corticosterone*

248 Effects of housing system, reproductive rhythm and their interactions are displayed in Table 5. No  
249 significant differences were recorded in corticosterone level (ng/ml) regarding the rhythm effect and  
250 interactions, while the group strongly affected ( $P < 0.007$ ) hormone levels with values approximately  
251 3 times higher in does caged in CC housing.

252

253 **4. Discussion**

254 Reproductive performances of females are influenced by sexual receptivity, lactation stage and order  
255 of birth (Theau-Clément, 2007). Lactation requires a great amount of disposable energy and it's  
256 closely dependent from some variables, such as fecundity. In particular, nulliparous does seems to be  
257 more affected by energy deficit than multiparous, as they have to support also growth till adult age  
258 (Pascal et al., 2013). In our trial, reproductive performances were not affected by cage type and  
259 reproductive rhythm concerning body weight, Body Condition Score and sexual receptivity. While,  
260 as expected, the extensive rhythm improved fertility rate of rabbit does, resulting in higher values for  
261 both housing systems, as reported in a previous work of Theau-Clément et al. (2016), where females  
262 subjected to a less intensive rhythm showed the best results in terms of fertility, litter size and number  
263 of live born kits, so inseminating after kits weaning can be a possibility to improve the percentage of  
264 pregnancies, thanks to an improvement of the body status with a consequent longer reproductive  
265 career and also to a peak of sexual receptivity physiologically showed after weaning of the litter  
266 (Theau-Clément et al. (1990). Inseminating does after weaning seems to eliminate the hormonal and  
267 energetic antagonism between lactation and pregnancy and animals compensate for the lower  
268 production efficiency with a higher fertility rate than conventional intensive rhythm (79.0% vs.  
269 60.2%) (Castellini et al., 2003). Body conditions of does has a fundamental role, being them

270 responsible for their reproductive efficiency especially in terms of fertility and productivity (Naturil-  
271 Alfonso et al., 2017). This statement is also supported by the higher productivity (rabbit/sold/year/  
272 doe and live weight sold/year/doe) reached in CCE rabbit does, with the minor replacement of animals  
273 among groups (20% vs 130% of intensive rhythm in C group), without however reaching values of  
274 the intensive rhythm in conventional cage. The CE group showed worst productive performances,  
275 probably due to an increase of stressors for the smaller space available in the cage. The only  
276 disadvantage of inseminating after weaning could be that the prolongation of the fertilization-  
277 conception interval could cause an excessive increase in weight, with a consequent lower conception  
278 rate with the progression of reproductive career (Cerolini et al., 2015). CC cages showed the worst  
279 results in terms of pre-weaning mortality and weight of weaned litter, in accordance with Mikó et al.  
280 (2012) positive correlation of cage enlargement and greater height with mortality of rabbits weaned  
281 at around 30 days of age. CC cages showed different reproductive performances, but all the three  
282 groups analyzed did not reach the productivity of intensive rabbit housing. Regarding the  
283 reproductive rhythm effect on reproductive performances among groups, it should be noted that the  
284 alternate type of *postpartum* in C cage, in comparison with the fixed intensive insemination at 11  
285 days after kindling, seems to be more adapted to the doe physiology, with a fertility rate close to 90%  
286 and reflection in the highest number of live born kits and weight of weaned litter, as well as the lowest  
287 pre-weaning mortality compared to all the experimental groups. This evidence is supported by a  
288 previous work of Castellini et al. (2003) where an improvement of reproductive parameters were  
289 recorded with a semi-intensive reproductive rhythm (alternating AI at 1 day postpartum with AI  
290 performed after kits weaning), reducing energy deficit of females of 6,7% compared to standard 11  
291 days post kindling AI and the replacement rate of about 10%. Extensive rhythm in both housing  
292 systems showed intermediate results, with higher fertility rates but lower number of weaned litters.  
293 The combination intensive/alternating plus CC housing resulted in the lowest reproductive  
294 performances.

295 Regarding the behavioural repertoire expressed, in accordance with Gunn and Morton (1993), static  
296 activities were the most common activities performed in all 6 experimental groups, probably because  
297 direct observations were recorded only during the light period, between 8.00 a.m. and 16.00 p.m.,  
298 during which they are usually less active being crepuscular animals (Jilge and Hudson, 2001). No  
299 significant differences were observed for behavioural pattern between the different reproductive  
300 rhythms of the CC system, while regarding the 3 C groups, the intensive one showed higher  
301 frequencies in terms of standing up, self-grooming, stereotypies, feeding and drinking. Regarding  
302 stereotypies, CI cage showed the highest percentages in terms of biting and sniffing bars, instead in  
303 CC system there was a lower incidence of abnormal behaviours, confirmed by Verga et al. (2007)  
304 which observed more stereotypies in individually housed rabbit does. These disorders are indicators  
305 of anxiety (Poderscek et al., 1991) and usually substitute the normal behaviours which are inhibited  
306 by the lack of eliciting stimuli and enough space (Gunn-Dore and Morton, 1993). According to Gunn-  
307 Dore (1997), the extreme boredom induces animals to eat a greater amount of feed as observed in our  
308 trial for the intensive control cage. The use of enrichment material, such as the platform available in  
309 the innovative system, can lead to distraction from this abnormal behaviour. Trocino et al. (2019)  
310 showed that the use of an elevated platform allows rabbits to move more, to rest in a more comfortable  
311 position and to increase the exploratory behavior, without modifying the production performances.  
312 However, there is often a simultaneous increase in injured rabbits which may discourage their use.  
313 Similarly, an improvement of rabbit welfare conditions was found using enrichment materials such  
314 as wooden bars to gnaw or PVC tubes (Mañia et al., 2005). Our results showed that when a platform  
315 is available, rabbit does jumped on it and spent around 20% of their time resting there. Additionally,  
316 in a previous work, Hansen and Berthelsen (2000), demonstrated that rabbit does in conventional  
317 cages are more bored, showing higher comfort activities and bar biting percentages compared to does  
318 reared in innovative cages with presence of an elevated platform.

319 Jumping was visible only in CC cages due to a greater availability of space, while in the C system it  
320 was not impossible due to the lack of proper dimensions: this situation usually leads to a frustration  
321 state, confirmed by the higher presence of sniffing and bars biting in this type of housing (Gunn-  
322 Dore, 1994). As reported by Morton et al. (1993), it is fundamental to use sufficiently high cages (75  
323 cm) to allow the rabbit to sit upright without its ears touching the top of the cage. In our trial, high  
324 frequencies of standing up with ears down due to an insufficient cage height (35 cm) were observed  
325 in C cages. However, the relevance of this behaviour as indicator of wide or poor welfare can be  
326 questionable in commercial farm conditions, due to the lack of predators (Princz et al. 2008).  
327 According to the same author, the commonly height utilized in rearing fattening rabbits (35-40 cm)  
328 is satisfactory. In our study, animals caged in C system and CC system (113 cm) spent a percentage  
329 of time in standing alert significantly different, with rabbit does housed in conventional housing most  
330 frequently ( $P < 0.02$ ) observed in this position. The evaluation of the nest showed significant  
331 differences ( $P < 0.05$ ) as regards the level of mixing. This maternal behavior was observed with a  
332 greater frequency in CC rabbit does, especially in those subjected to an alternating reproductive  
333 rhythm: this result is in agreement with several studies that state that high levels of stress cause  
334 alterations of the maternal behavior with consequent acts of cannibalism or, as in the present study,  
335 poor or non-construction and care of the nest (Verga et al., 1978; Gonzáles-Redondo and Zamora-  
336 Lozano, 2008). All behavioral results were found to be extremely accurate, confirmed by Cohen's  
337 kappa reliability index that reached a value of 0.97, in a range where 0 indicates no accuracy and 100  
338 the maximum.

339 The tonic immobility test showed no significant differences among groups. This could be due to the  
340 usual manipulation of rabbits by the farmer during the management "routine", in accordance with  
341 several studies where the state of fear resulted strongly decreased when the animals got used to the  
342 presence and contact with human beings, thus improving their general welfare with positive effects  
343 on production performances and health status (Kersten et al., 1989; Jiezierski and Koneca, 1996).



344 Corticosterone salivary concentration was strongly affected by the housing system, with much higher  
345 values (almost 3 times more) in CC cages. Although this results are in disagreement with most of the  
346 previous literature (Koolhaas et al., 1993; Koob and Heinrichs, 1999; Cornale et al., 2016), this could  
347 be explained by the presence of a greater quantity of stimuli in the innovative system: the greater  
348 space available in CC housing allows a higher body exposure of animals due to the lower stocking  
349 density and this can lead breeding does to react with an anti-predator reaction (physiological stress  
350 response), feeling themselves more vulnerable than in smaller conventional cages (Monclus et al.,  
351 2006), with a consequent raising of corticosterone concentrations. Under stress, the activation of the  
352 HPA axis, together with the activation of the sympathetic-adrenomedullary system, contributes to the  
353 mobilization of energy necessary to cope with the stressful situation (Boissy 1995; von Holst 1998),  
354 with negative consequences on the ability of supporting lactation and pregnancies requirements.  
355 Moreover, Szendro et al. (2012) reported results similar to ours with higher faecal corticosterone in  
356 rabbit does housed in colony with wider space available compared to does in single housing. In our  
357 trial, the greater levels of salivary corticosterone measured in females reared in CC cages may signal  
358 the possibility of more stressful conditions for rabbits housed in wider systems, with observation of  
359 lower reproductive performances. Another factor to take into consideration is that saliva samples  
360 collection was performed only during the daylight following behavioural recordings, so values may  
361 not completely reflect the behaviour of the entire 24h. Anyway, whether this stress condition comes  
362 from the housing system per se (Combi system vs conventional cages) or could be attributed to other  
363 external factors needs to be further elucidated.

#### 364 **Conclusions**

365 Given the results, we can indicate that AI after kits weaning resulted to be the best reproductive  
366 protocol in Combi cages and the Alternating rhythm in standard cage. In fact, these reproductive

367 protocols, thanks to an improvement of the body status, better match the compromise between the  
368 productivity and the physiological welfare of the rabbit doe.

369 From an ethological point of view, standard housing reduces animal behavioural freedom and also  
370 constantly leads to stereotypes, but despite this animal physiological stress seems to be lower.

371 Further studies are required to understand properly what combination of reproductive rhythm and  
372 housing system can completely satisfy both ethological and physiological needs, in any case keeping  
373 the farm remunerative in terms of production and efficiency.

374

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380

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