

1 Conditioned place avoidance using encapsulated calcium propionate as an appetite suppressant  
2 for broiler breeders

3 Conditioned place avoidance and calcium propionate

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8 ¶Aitor Arrazola designed the experiment, did the analyses, and wrote the paper. Stephanie  
9 Torrey wrote the grant application, provided input into experimental design and edited the paper.

## 10 **Abstract** (<300)

11 Broiler breeders, the parent stock of meat chicks, are feed-restricted throughout rearing to avoid  
12 obesity-related problems in their health and reproductive performance. Broiler breeders often  
13 show signs of chronic hunger, lack of satiety and feeding frustration, and the development of  
14 alternative feeding strategies has investigated the inclusion of calcium propionate (CaP) as an  
15 appetite suppressant. However, the mechanisms involved in the reduction of voluntary feed  
16 intake are unknown, but are thought to be due to low palatability, gastrointestinal discomfort, or  
17 both. The objective of this experiment was to examine the effect of CaP as an appetite  
18 suppressant on the experience of a negative affective state, using a conditioned place preference  
19 test. Twenty four broiler breeders were trained to associate the consumption of CaP or a placebo  
20 pill with a red or blue place, depending on inherent colour preference. Pullets consumed two pills

21 followed by 20 g feed allotment. The CaP pill contained 160 mg of CaP and the placebo pill had  
22 160 mg of feed. Conditioning lasted for 90 min/pullet/day over 8 consecutive days at 7 and 9  
23 weeks of age, and pullets' choice was tested in a T-maze twice on two consecutive days at both 8  
24 and 10 weeks of age. Data were analysed using a linear mixed regression model, with pen nested  
25 in the model and age as a repeated measure. Pullets were less likely to choose the place  
26 conditioned with the consumption of CaP ( $P<0.05$ ) and the preference of the placebo linearly  
27 increased with training sessions ( $P<0.05$ ). These results suggest that calcium propionate as an  
28 appetite suppressant can induce a negative affective state, with the lower feed intake resulting  
29 from a conditioned response to the negative effect of calcium propionate rather than to satiety.

30 **Introduction**

31 Calcium propionate (CaP) is an organic acidifier commonly used in human nutrition as a feed  
32 preservative (e.g., bakery [1], in fermented dairy products [e.g., 2]) in animal nutrition as a feed  
33 preservative [e.g., 3-6], as a growth promoter [6,7], as an intestinal microbiota enhancer [6,8], and  
34 as an appetite suppressant [9-12]. The effect of dietary CaP been researched in poultry because of  
35 its role as appetite suppressant. Previous research reported that increasing concentration of CaP  
36 (1-3%) linearly decreased feed intake in broiler chickens (i.e., meat chickens) [9], and high  
37 inclusion rates of CaP (5-9%) has been proven to lower feed intake in broiler breeders [10-12].  
38 Broiler breeders are the parent stock of broiler chickens and both share the same genetic  
39 predisposition for fast growth rate, feed efficiency and high feed intake [13]. Feeding broiler  
40 breeders to satiety is associated with obesity-related problems such as low liveability and negative  
41 consequences in reproductive performance [14,15]. Therefore, broiler breeder pullets are feed-  
42 restricted throughout their production cycle up to 43% of *ad libitum* feed intake for the same broiler

43 body weight, leading to chronic hunger, lack of satiety and frustration [16]. The experience of  
44 negative affective states is an ethical concern in animal welfare, and the development of alternative  
45 diets has considered the inclusion of CaP as an appetite suppressant to reduce voluntary feed intake  
46 and feeding motivation [11,17,18].

47 Previous experiments have indicated the negative consequences of dietary CaP in humans (e.g.,  
48 behavioural disturbance [19] and seizures [20]), rats [21], and poultry [12,17]. Daily calcium  
49 propionate intake in humans is estimated about 0.1% of food intake [22], although health  
50 recommendations allow an inclusion rate up to 0.2% for daily human consumption (e.g., [1,19]).

51 In medical studies, propionic acidemia can result in seizures independent of metabolic acidosis  
52 [20] and a controlled trial with children indicated that short-term high inclusion of CaP (at 2% in  
53 a bakery product) can lead to irritability, restlessness and sleep disturbance [19]. In the case of  
54 poultry, much higher inclusion rates are added in poultry diets compared to human nutrition and  
55 previous research noted that chickens avoided consuming the CaP diet if possible [23,24]. For  
56 example, Savory *et al.* [23] indicated that the feed intake of broiler breeders (i.e, naïve to CaP) was  
57 four times higher with a standard diet compared to a standard diet with CaP. Torrey *et al.* [24] also  
58 reported that feed intake of an alternative diet (40% soybean hulls and 3-5% CaP) was low in  
59 chickens given free access to both a standard and an alternative diet in their home pen. Afterwards,  
60 authors tested the preference for the alternative and the standard diet in a Y maze, and 50% of the  
61 non-feed restricted pullets consistently chose the standard diet, with only 6.9% pullets  
62 preferentially choosing that alternative diet [24]. Tolkamp *et al.* [12] also observed a high number  
63 of oral lesions, and morphological changes in the crop and gastric lesions in broiler breeder fed  
64 alternative diets *ad libitum* at 6-9% CaP, while broiler breeders on the standard diet had no lesions.  
65 The effect of CaP as an appetite suppressant in poultry has been associated with low palatability,

66 gastrointestinal discomfort/malaise or the combination of both [17,23]. Independent of its  
67 palatability, whether CaP causes a negative affective state post-ingestion remains unclear.

68 The affective states of animals cannot be measured directly, but the study of animals' choices is  
69 an indirect approach to assess their preferences [25]. For example, behavioural tests that looked at  
70 the affective states and the preferences of broiler breeders include operant learning/response, state  
71 dependent learning and conditioned place preference/avoidance [26-31]. Conditioned place  
72 preference/avoid (CPP/CPA) tests are often used to analyse the possible positive (i.e., rewarding)  
73 or negative (i.e., punishing) effects of pharmaceutical drugs or dietary additives [32]. During  
74 CPP/CPA tests, animals are trained to associate given characteristics of its environment with  
75 affective states induced by the treatment, and this environmental cue can become a conditioned  
76 stimulus associated with the given affective state [32,33]. Once the condition is created, animals  
77 are hypothesized to prefer the environment associated with the relative higher positive affective  
78 state when they are tested in a T-maze [32,33]. Indeed, Phillips and Strojan [34] showed that  
79 broilers were able to discriminate three pairs of conditioned stimuli for CPP after 7-days  
80 conditioning period per each pair. Previous research in broiler breeders has examined the  
81 preference between qualitative restriction with CaP and quantitative feed restriction using a CPP  
82 [28]. Results in that experiment suggested that chickens failed to learn the CPP due to severe feed  
83 restriction [27,30]. However, a lower feed restriction level could facilitate conditioning learning  
84 during the training sessions [35]. The objective of this experiment was to examine the causal effect  
85 of CaP as an appetite suppressant on a negative affective state by using a conditioned place  
86 preference (CPP) test in broiler breeder pullets. Pullets were hypothesized to avoid the place  
87 conditioned with the consumption of CaP if the CaP induces a negative affective state.

## 88 **Materials and Methods**

89 A total of 24 Ross 308 broiler breeder pullets were donated at one day of age, courtesy of Aviagen  
90 (via Horizon Poultry, Hanover, Ontario, Canada). All the procedures used in this experiment were  
91 approved by the University of Guelph's Animal Care Committee (AUP # 3141) and were in  
92 accordance with the guidelines outlined by the Canadian Council for Animal Care.

## 93 **Housing and management**

94 At the hatchery, chicks were infrared beak treated and vaccinated based on local recommendations,  
95 and chicks were raised at the OMAFRA Arkell Poultry Research Station (Guelph, ON, Canada).  
96 Chicks were distributed upon arrival to cages (10.3 chicks/m<sup>2</sup>) and were relocated at 22 days of  
97 age to six floor pens (4 pullets/pen at 1.1 pullets/m<sup>2</sup>). Floor pens (1.7 m wide x 2.0 m deep x 1.2  
98 m high) had wood shavings as bedding and water was available *ad libitum* from a 5-nipple drinker.  
99 Chicks were selected based on target body weight and were individually wing tagged.

100 Pullets were managed based on breeding company's guidelines [36]. Room temperature started at  
101 32 °C at 1 day old, and temperature gradually decreased to 29 °C by 2 weeks of age. After transfer  
102 to floor pens, room temperature was reduced to 24 °C at 4 weeks of age and to 22 °C at 6 weeks of  
103 age. Relative humidity remained around 26% during rearing. Light program was 23D:1D at 100  
104 lux at 1 day old, 12L:12D at 30 lux at 4 days of age until 14 days of age, and 10L:14D at 40 lux  
105 until the end of the experiment. Lights came on at 9:00 and pullets were fed simultaneously. Pullets  
106 were hand-fed daily using a hanging cylinder feeder with 15 cm/pullet feeder space, and pullets  
107 were feed restricted following feed allotment recommendations by breeding company (Table 1;

108 [37]) using standard broiler breeder pullet diet in Starter (0 to 5 weeks of age) and Grower (6 to  
109 10 weeks of age) according to nutrition specifications [38].

110 Table 1. Daily feed allotment and feed reward per pullet depending on age and weekly  
111 experimental schedule.

Age (day)	Daily feed allotment <sup>z</sup> (g)	Reward <sup>y</sup> (g)	Experimental schedule
21	32	-	Arrival
26 - 28	33	-	Habituation
28 - 34	35	20	Pill consumption training
43 - 44	41	-	Colour preference test
47 - 54	43	20	CPP training
56 - 57	48	-	Testing
60 - 67	49	20	CPP training
70 - 71	53	-	Testing
72	<i>Ad libitum</i>	-	End

<sup>z</sup>Based on Aviagen [37]

<sup>y</sup>Extra feed allotment per day

112

113 **Experimental design**

114 The experiment lasted from 3 to 10 weeks of age, and pullets were trained to associate the effect  
115 of consumption of CaP with a given place accounting for inherent colour preference. Pullets were  
116 fed two identical pills (white lock-ring gelatine pill, size 3, CapsulCN): both either CaP or placebo  
117 during the CPP training, and pill consumption was rewarded with 20 g of their standard (mash)  
118 diet. The CaP pill contained an inclusion of 160 mg of CaP and the placebo pill had 160 mg of  
119 their standard diet. In combination with the feed reward, CaP was fed at 1.6% inclusion rate,  
120 similar to the inclusion rate of CaP in broiler breeder alternative diets [18]. Placebo pills were used  
121 for the voluntarily pill consumption training, the conditioned place preference (CPP) training and  
122 for T-maze choice (one pill per place); CaP pills were only used during the CPP training depending  
123 on training schedule (Table 2).

124 There were six pens, and the four pullets per pen were trained simultaneously. Two pullets per pen  
125 were trained to associate the CaP pill with the blue place and remaining two pullets per pen were  
126 trained to the CaP pill with the red place (Table 2). During the CPP training, pullets alternated pills  
127 every two consecutive days for 8 days. Two pullets per pen started the training with the CaP pills,  
128 and the other two pullets started with the placebo pills (Table 2). On the two consecutive days with  
129 the same pill in two apparatuses (Fig 1), pullets were trained in both sides of the T-maze for the  
130 same place colour. For example, if a pullet was trained in the left side the first day with blue walls,  
131 the following day, the same pullet was trained on the right side with blue walls, in the other  
132 apparatus (Fig 1). The reverse order was used for the other pullets from the same pen (Table 2).

133 Table 2 Experimental design and individual training protocol for the development of conditioned  
134 place preference according to pullets' age. The same design was applied to all pens.

Age (week)	Pullet id	Colour conditioned with the CaP pill <sup>z</sup>	First pill <sup>y</sup>	First colour <sup>y</sup>	First side <sup>x</sup>	T-maze <sup>x</sup>
6	1	Blue	CaP	Blue	Left	1
	2	Red	CaP	Red	Right	1
	3	Blue	Placebo	Red	Left	2
	4	Red	Placebo	Blue	Right	2
8	1	Blue	Placebo	Red	Left	2
	2	Red	Placebo	Blue	Right	2
	3	Blue	CaP	Red	Right	1
	4	Red	CaP	Blue	Left	1

<sup>z</sup>Colour was selected based on the initial preference before training started

<sup>y</sup>Pullets remained on this pill/colour for two consecutive days

<sup>x</sup>Alternated every other day

135 **Fig 1. Diagram of the T-maze used for the inherent colour preference, for the conditioned**  
136 **place preference and the choice test.** Pullets were trained in each arm for conditioned place  
137 preference, visually isolated from each other during the CPP training. Two mirror-image

138 apparatuses were used, with colour location differing between arms (left versus right side).

139 Diagram is drawn to scale (1:8.5).

140 The choice and preference for the placebo was tested in the T-maze (Fig 1). Fig 1 represents the  
141 apparatus used for the CPP and the T-maze of the choice test. Two apparatuses were used in the  
142 experiment and each one was located inside a pen similar to pullet's home pen within the same  
143 room. Both apparatuses had the same dimensions (145 cm wide x 132.1 cm deep x 48.3 cm height)  
144 but differed in the location of the blue and red arms (right versus left side). Each pullet per pen  
145 was trained to associate the effect of the consumption of CaP with one of the colours, controlling  
146 for side. On testing days, one placebo pill was offered in each arm of the T-maze, but pullets could  
147 only consume one of them in each testing event. The operant response in this experiment was the  
148 consecutive consumption of two pills and pullets were positively rewarded afterwards; however,  
149 pill consumption was not rewarded on testing days due to only one pill being accessible in each  
150 testing event.

151 **Methodology**

152 This research was divided in four phases: 1) habituation, 2) individual colour preference, 3) CPP  
153 training, and 4) choice test. Habituation to the T-maze, training for pill consumption, place  
154 preference test, conditioned place training, and testing started after all pullets consumed their daily  
155 feed allowance. The preference and choice tests started one hour after feeding, and the conditioned  
156 place training started one hour after feeding for the first pen to seven hours and half for the last  
157 pen. The average body weight ( $\pm$  SD) was  $366.7 \pm 34.8$  g,  $1075.0 \pm 114.9$  g and  $1408.5 \pm 144.9$  g  
158 at 3, 8 and 10 weeks of age, correspondently.

159 **Habituation**

160 Pullets were trained for voluntary and consecutive consumption of two placebo pills during the  
161 first week of habituation. On the first day, pills are manually fed and consumption of two pills was  
162 rewarded with 20 g of the standard diet. Afterwards, two placebo pills were simultaneously offered  
163 per pullet per day for seven days, until pullets voluntarily consumed both pills when offered.

164 Pullets were rewarded with 20 g of feed if pills were manually fed or voluntarily consumed.

165 Pullets were placed inside the testing pens where the T-maze was located, and pullets could explore  
166 the T-maze for 2 hours to habituate them to training and testing conditions. Habituation to the T-  
167 maze started at 4 weeks of age and lasted for 10 days. Initially, the four pullets per pen were  
168 grouped within the same testing pen and the T-maze. After the second exposure, habituation was  
169 done in pairs. At the end of the first week, pullets were individually placed in the testing pen for 2  
170 hours to habituate them to isolation. Once pullets were individually isolated, two placebo pills  
171 were provided, and pill consumption was rewarded with 20 g of the standard diet.

172 **Inherent bias test (Pre-CPP)**

173 After habituation and training, each pullet was tested on two consecutive days and twice per day  
174 to assess individual preference for side and colour at 34 days of age. During the preference tests,  
175 pullets saw both T-maze arms (coloured) for the first time. The order of pullets in this test was  
176 randomly chosen and pullets were retested after all pullets were tested once on the same day (no  
177 one was tested consecutively). Another random pattern was generated for the following testing  
178 day. Pullets were placed into the starting box for 30 sec, and then the door to the maze was opened.  
179 An observer registered pullets' inherent choice, defined as the whole body of the pullet inside one

180 of the sides of the T-maze for more than 5 seconds. Pullets had 5 minutes to choose either left or  
181 right, otherwise they were registered as having no choice (assuming no colour preference). Colour  
182 and side preference was set for pullets that chose one colour or side at least three times out of four  
183 trials ( $\geq 75\%$ ). All pullets were tested on the same day in both apparatuses and the order of the  
184 starting apparatus was reversed for the second day of testing.

185 **CPP training**

186 The CPP training lasted for eight consecutive days per training session. All pens were trained daily  
187 and the four pullets per pen were training simultaneously, one in each conditioned place. Two  
188 pullets per pen were trained to associate the placebo pills with the blue walls and the CaP pills  
189 with the red walls, and vice versa for the other two. Pullets that had an inherent bias for a colour  
190 before the CPP training started received the placebo pills in the opposite colour to their preference  
191 (except for two pullets for which three pullets from the same pen showed preference for the same  
192 colour). The CPP training sessions were repeated twice at 7 and 9 weeks of age, and pullets were  
193 tested for preference choice for the placebo place at the end of each training session.

194 Each pullet was individually allocated in the conditioned place for 90 min. Pullets were trained to  
195 consume two pills in 30 seconds and were rewarded with 20 g of the standard diet after the  
196 consumption of the second pill. Pullets had *ad libitum* access to nipple drinkers in the apparatus  
197 during the conditioned phase and were visually isolated from the other conditioned place.

198 **Choice test**

199 Pullets' choice was tested individually on two consecutive days using the same protocol for the  
200 inherent bias test (i.e., same testing order and methodology) at 8 and 10 weeks of age one day after

201 the last day of each training session. During the choice test, one placebo pill was left in each T-  
202 maze arm and each pill was set in the middle of the conditioned place (equidistant and visible from  
203 the fork). An observer recorded the place from which pullets consumed the pill. Pill consumption  
204 was not feed-rewarded and pullets were returned to their home pen after consuming one pill. Data  
205 were collected according to pullet's choice and preference for the placebo pills. Choice for the  
206 placebo was defined as the percentage of times that pullets consumed the pill from the placebo  
207 place. Preference for the placebo was defined as the percentage of pullets that chose the placebo  
208 place consistently ( $\geq 75\%$ ) divided by the total number of pullets tested.

209 Individual body weight was recorded on the last testing day at 8 and 10 weeks of age. After all  
210 pullets were tested at 10 weeks of age, pullets were individually presented with four pills (two  
211 placebo pills and two CaP pills) to examine whether pill consumption would stop after the  
212 consumption of the second pill and whether pullets could differentiate between both pills. Both  
213 pills were visually identical and had the same weight, but pills might have differed for pullets'  
214 perception. In addition, pullets were fed *ad libitum* afterwards in their home pen and remaining  
215 placebo pills were offered in the feeders.

## 216 **Statistical analysis**

217 The choice for the CPP was statistically analysed using a generalized mixed linear model and pen  
218 was nested in the model as the independent research unit. The statistical analysis was performed  
219 using SAS Ver. 9.4 (SAS Institute, Cary, NC) with a glimmix procedure and the degree of  
220 significance was set for p-values lower than 0.05.

221 Age was included as a fixed factor in the model for the choice and preference for the placebo.  
222 Initial observations (i.e, 3 weeks of age) for the choice and the preference for the placebo were  
223 considered as independent observation rather than a covariate to achieve desirable degrees of  
224 freedom as well as for orthogonal regression analysis for age. The effect of the last training day  
225 was included as a covariate. Similarly, the effect of body weight was included as a covariate within  
226 age due to collinearity. The assumptions to the analysis of variance were assessed using  
227 scatterplots of studentized residuals, linear predictor for linearity, and a Shapiro-Wilk test for  
228 normality. Outliers were defined as observations with absolute studentized residuals higher than  
229 3.4. Individual differences, pen, and pen location within the room were included in the covariance  
230 structure as random effects. Age was fit into a repeated structure for placebo choice with pen as  
231 the subject with age as the group. Orthogonal regressions analysed the effect of age into linear and  
232 quadratic response. Significance differences between multiple mean comparisons were corrected  
233 using Tukey-test adjustment.

## 234 **Results**

235 One pullet was excluded from the dataset due to unsuccessful training to voluntarily pill  
236 consumption. Pullets were 62.9% and 32.8% heavier than the performance objectives for broiler  
237 breeders at 8 and 10 weeks of age, respectively [37] due to feed rewards. Pullets were trained to  
238 associate the effect of CaP with the colour they inherently preferred. However, all pullets from one  
239 pen preferred the red colour and for two of these pullets, the placebo remained in the colour they  
240 preferred due to consistency with the training protocols. Hence, the initial choice for the placebo  
241 was higher than 0% (Fig 2). Data is presented using estimated mean values followed by the  
242 standard error of the mean.

243

244 **Fig 2. The effect of conditioned place preference training in broiler breeder pullets over**  
245 **time on the choice (A) and preference (B) for the placebo pill compared to the calcium**  
246 **propionate pill (means  $\pm$  SE).** The choice of the placebo indicates the proportion of times that  
247 the placebo place was chosen (A). The preference for the placebo represents the proportion of  
248 hens that consistently chose the placebo place divided by the total number of hens (B). Pullets  
249 were tested on day 42 for initial choice, on day 56 after the first training session and on day 70 at  
250 the end of the second training session. Repetitive training sessions linearly increased the choice  
251 ( $F_{1,18}=60.90$ ,  $P<0.001$ ) and preference ( $F_{1,18}=38.27$ ,  $P<0.001$ ) for the place conditioned with  
252 placebo pill.

253

## 254 **Individual colour and side preference**

255 Pullets showed a significant preference (choosing  $\geq 75\%$  of times) for colour and side at 6 weeks  
256 of age. Half of the pullets (12 out of 24) preferred the red place over the blue place ( $t=4.49$ ,  
257  $P<0.001$ ) and only one pullet out of 24 preferred the blue place over red. Three pullets showed  
258 lateralization during the choice preference test ( $t=4.89$ ,  $P<0.0001$ ); two pullets preferred the right  
259 side and the one preferred the left side. Moreover, four pullets out of 24 chose no place during the  
260 initial preference test.

## 261 **Choice test**

262 All pullets chose a place at each time they were tested at 8 and 10 weeks of age. During the choice  
263 test, 17.4% (4 pullets out of 23) and 13.1% (3 pullets out of 23) of pullets showed consistent

264 lateralization (choosing either left or right the four times) at 8 and 10 weeks of age, respectively;  
265 and only one pullet was consistent toward the left side at both ages (trend not observed during the  
266 initial side preference). The choice for the placebo was influenced by body weight at 70 days of  
267 age ( $F_{1,22}=5.32$ ,  $P=0.03$ ) and body weight had a quadratic effect on the choice of the placebo at 10  
268 weeks of age (Fig 3). The pullets that preferred the placebo place ranged from 1242 g to 1432 g at  
269 10 weeks of age whereas pullets outside this threshold showed no preference. Between the place  
270 conditioned with the consumption of the placebo or the CaP pill, pullets only showed preference  
271 for the placebo place. Individual variability did not affect the choice for the placebo ( $Z=1.00$ ,  
272  $P=0.16$ ). As well, the last training day before the T-maze choice did not influence the choice  
273 ( $F_{1,44}=0.74$ ,  $P=0.40$ ) nor the preference ( $F_{1,44}=2.20$ ,  $P=0.16$ ) for the placebo during the T-maze  
274 tests.

275 **Fig 3. The effect of body weight on the choice for the place conditioned with the placebo**  
276 **pills at 70 days of age.** Body weight had a quadratic effect on the choice for the placebo at 10  
277 weeks of age ( $F_{1,22}=5.32$ ,  $P=0.03$ ).

278 Fig 2 illustrates the effect of training on the choice ( $F_{2,18}=36.07$ ;  $P<0.0001$ ) and the preference  
279 ( $F_{2,18}=4.19$ ;  $P=0.03$ ) of pullets toward the placebo pill. Repetitive training sessions linearly  
280 increased the probability for the pullets to choose the place conditioned with the placebo pill  
281 ( $F_{1,18}=60.90$ ,  $P<0.001$ ). The probability for the pullets to choose the placebo increased from  $8.0 \pm$   
282  $6.1\%$  at 6 weeks of age (which was artificially manipulated to be as close to 0% as possible) to  
283  $47.9 \pm 7.6\%$  at 8 weeks of age ( $t_{18}=4.68$ ,  $P=0.0005$ ), and to  $58.7 \pm 7.5\%$  at 10 weeks of age  
284 ( $t_{18}=6.06$ ,  $P<0.001$ ). Pullets preferred the place conditioned with the placebo pills after the first  
285 training session at 8 weeks of age ( $25.0 \pm 8.6\%$  [6/23];  $t_{18}=2.91$ ,  $P=0.01$ ) and at 10 weeks of age  
286 ( $38.9 \pm 8.6\%$  [9/23];  $t_{18}=4.53$ ,  $P<0.001$ ). The preference for the placebo linearly increased over

287 time ( $F_{1,18}=38.27$ ,  $P<0.001$ ), and the preference for the placebo increased by  $30.56 \pm 10.57\%$  from  
288 6 to 10 weeks of age after two training sessions ( $t_{18}=2.89$ ,  $P=0.03$ ). Moreover, all pullets  
289 indiscriminately consumed the four pills in less than 30 seconds at the end of the choice test at  
290 week 10 and pullets still ate the placebo pills after *ad libitum* feeding.

291 **Discussion**

292 The present study was designed to determine the causal effect of the CaP as an appetite suppressant  
293 on a negative affective state. A CPP test was conducted using encapsulated CaP at a similar  
294 inclusion rate as in alternative diets for broiler breeders [18]. Pullets were expected to avoid the  
295 place conditioned with the consumption of CaP if CaP induces a negative affective state, and our  
296 results indicate that pullets avoided the place conditioned with the consumption of CaP and  
297 developed preference for the placebo place over time. This study demonstrates the causal effect of  
298 CaP on inducing a negative affective state at an inclusion rate proven to decrease voluntary feed  
299 intake in poultry. Preference was only shown toward the placebo after CPP training and 9 out of  
300 23 pullets that showed a preference chose the placebo place at the end of the experiment (as  
301 illustrated in Fig 3). Savory *et al.* [23] examined the dietary preference for CaP at 5% inclusion in  
302 broiler breeder pullets, and the feed intake among diets differed based on pullets' previous  
303 experience with CaP. In their research, pullets reared on the standard diet consumed more of the  
304 standard diet (81% standard diet vs 19% CaP diet) compared to pullets already on the CaP diet  
305 (65% standard diet vs 35% CaP diet). The authors concluded that the preference for the diet at 5%  
306 CaP was low, although pullets reared at a fixed CaP inclusion rate might have been habituated  
307 [23]. Therefore, the effect of CaP related to a post-ingestive and dose-dependent mechanism, not  
308 acidity, to which pullets can habituate [23]. Although the decrease in feed intake may be due to a

309 lower palatability of the diet with CaP [23,24,39], CaP was encapsulated in our research to avoid  
310 the confound between palatability and a post-ingestive mechanism. Similar results on the  
311 aversiveness of CaP were reported in rats in Ossenkopp *et al.* [21]. The authors infused CaP (at  
312 0.05%) to avoid the confound of palatability, and rats spent less time in the place conditioned with  
313 the CaP, and showed more escape attempts and hyperactivity, indicators of aversiveness, during  
314 conditioning training. In agreement with our results, CaP can induce a negative affective state  
315 regardless of its palatability, although CaP may have an additional effect at reducing voluntary  
316 feed intake due to low palatability [21]. Therefore, the effect of CaP as an appetite suppressant can  
317 relate to a conditioned response to the causation of a negative affective state instead of satiety.

318 Our results illustrate that training had a linear effect on the consistency of instantaneous choices,  
319 suggesting that feed-restricted pullets may require more and longer training for the condition to  
320 happen. Dixon *et al.* [30] concluded that broiler breeders under commercial feed restriction were  
321 unsuccessful in learning CPP or CPA tests, and chronic feed restriction can limit cognitive  
322 potential. Previous research indicated that chronic stressful conditions such as feed restriction  
323 could limit associative learning [27,40]. Certainly, broiler breeders display behavioural and  
324 physiological signs of chronic feed restriction [16], and commercial feed restriction can be  
325 distressful for broiler breeders [15]. Buckley and colleagues [40] examined whether broiler  
326 breeders can learn a discrimination feed intake test (*i.e.*, pullets were trained to associate the arm  
327 colour with the amount of feed reward) depending on feed restriction level (commercial feed  
328 restriction, 40% and 80% of *ad libitum* feed intake). Buckley *et al.* [40] indicated that the latency  
329 to perform the choice test decreased as the feed restriction level increased, and a lower proportion  
330 of pullets learned the CPP tests on the highest feed restriction level (*i.e.*, commercial feed  
331 restriction level). The deficient performance of the pullets under commercial feed restriction can

332 be explained by their inability to learn during the CPP training or arousal during the choice test  
333 due to light body weight, high feeding motivation and chronic stress due to severe feed restriction.  
334 Additionally, Buckley *et al.* [28] examined the preference of broiler breeders under commercial  
335 feed restriction between qualitative restriction (at 3-9% CaP) and quantitative feed restriction, but  
336 the authors did not observe differences between both treatments probably due to lack of preference  
337 or inability to learn under such severe feed restriction [28]. In agreement with our results, Buckley  
338 *et al.* [28] and Dixon *et al.* [30] also suggested that broiler breeders might require numerous and  
339 long training sessions to facilitate associative learning and to control for high feeding motivation  
340 under commercial feed restriction. For this reason, the feed restriction level was lowered in our  
341 experiment and pullets in this research exceeded target body weight because of multiple feed  
342 rewards during training for pill consumption and for the conditioned place learning.

343 Body weight varied widely in our research at 10 weeks of age, and lighter and heavier pullets were  
344 less likely to choose the placebo place compared to pullets of average body weight. Previous  
345 research also reported that lighter broiler breeders performed poorly in a learning task [28,30,40].  
346 Results in Buckley *et al.* [40] pinpoint that (light) pullets under commercial feed restriction  
347 performed poorly in a learning task compared to (heavy) pullets reared on a lower feed restriction  
348 level. In our experiment, pullets were more likely to choose the place conditioned with the placebo  
349 at a body weight between 1200 g and 1450 g at 10 weeks of age, which is approximately 100-350  
350 g heavier than target body weight. Feed restriction was the same among pen mates in our  
351 experiment, but pullets were group housed and fed via a communal feeder. Variation in body  
352 weight among pen mates may reflect previous experience in their home pen [41] as well as current  
353 feeding motivation [39], resulting in a high arousal during behavioural tests [40-42]. Lindholm *et*  
354 *al.* [41] reported that pullets at light body weights were more active in a tonic immobility test. In

355 Buckley and colleagues [40], pullets under commercial feed restriction had shorter latencies than  
356 pullets fed at 80% of *ad libitum* feed intake in a T-maze choice test. Previous research with broiler  
357 breeders also noted that fearfulness linearly decreased as feed restriction increased [14,15] and  
358 during off-feed days under non-daily feed restriction [43]. For this reason, light broiler breeders  
359 under feed restriction conditions display high arousal as suggested by short latency and low  
360 fearfulness in behavioural tests [40-42], probably being less consistent in short-term choices due  
361 to previous experiences and current feeding motivation. On the other side, heavy pullets were not  
362 as likely to choose the placebo place as average weight pullets in our experiment, and one of the  
363 heaviest pullets was the only one to show consistent lateralization at 8 and 10 weeks of age.  
364 Buckley *et al.* [40] noted that broiler breeders displayed lateralization in their decision-making  
365 behaviour during a choice test depending on the feed restriction level. All 12 pullets raised under  
366 commercial feed restriction showed lateralization compared to only 6 of 12 pullets at 80% of *ad*  
367 *libitum* feed intake. Buckley *et al.* [40] indicated that lateralization can relate to hunger stress, with  
368 hungrier pullets being more resistant to change behaviour (lower behavioural plasticity). In  
369 Arrazola [39], we also observed that heavy pullets were more highly feed motivated compared to  
370 lighter pen mates at the same age and feed restriction. For this reason, heavy (and dominant) pullets  
371 may be more highly motivated compared to pullets of average body weight, contributing to arousal  
372 and inconsistency during a choice test. However, the role of feeding motivation, body weight and  
373 individual variation in decision-making behaviour is unknown, despite its potential to improve  
374 body weight uniformity under commercial conditions in broiler breeder houses. Anecdotally,  
375 pullets showed short latencies during the choice test, and consumed both pills rapidly during the  
376 CPP training. The choice test in our research was not performed under extinction conditions,  
377 although the T-maze choice was not rewarded. Thus, visual cues (i.e., the pill in our research) can

378 be an external factor that may have triggered arousal in pullet's choice, especially in light and  
379 heavy pullets at 10 weeks of age. Elevated feeding motivation (either in light and heavy pullets)  
380 can interfere in decision making behaviour and compromise the performance during the choice  
381 test of broiler breeders [27,30,40,41], which may explain the effect of body weight in our research.  
382 For this reason, previous and current experiences should be considered in the interpretation of  
383 results about animals' preference because high feeding motivation and arousal state can be  
384 translated into inconsistency or lack of preference in their choice. The effect of body weight can  
385 be alternatively explained by pullets' inability to learn the CPP due to chronic stress (35).  
386 However, behaviour was not recorded during the conditioning phase or during the choice test in  
387 this experiment to assess whether this hypothesis holds true for lighter and heavier pullets. But,  
388 further research looking at whether the poor performance in learning task relates to learning  
389 inability or arousal by feeding motivation is necessary to properly understand results from  
390 preference tests in feed-restricted animals.

391 T-mazes are used in behavioural tests to examine the animals' preference between two options  
392 [25]. However, a choice may represent something to be avoided rather than something to be  
393 desired, such as in our preference test. In this case, both choices were feed restriction with or  
394 without the inclusion of CaP. Therefore, these results indicate that the inclusion of CaP (i.e.,  
395 qualitative restriction) is avoided, instead of commercial feed restriction (i.e., quantitative feed  
396 restriction) being preferred. Additionally, results from preference tests rely on previous  
397 experiences and training protocol [25]. Dixon *et al.* [30] highlighted that the side of the T-maze  
398 was a main driver in the preference of feed-restricted broiler breeders and pullets preferred the  
399 place they were not previously housed. The side of the T-maze was considered in the experimental  
400 design, and the effect of the last training day on the choice or the preference during the choice test

401 was not significant in our experiment. Inherent colour preference was also considered in our  
402 experimental design because colour preference in avian species has been previously reported by  
403 Ham and Osorio [44]. Broiler breeders showed an inherent colour preference toward the red colour  
404 over blue at six weeks of age as previously described Taylor *et al.* [45] and Fischer *et al.* [46].  
405 However, this preference switched to the place conditioned with the consumption of the placebo  
406 pill after two training sessions regardless of inherent colour preference. Therefore, lowering feed  
407 restriction (compared to commercial feed restriction) during training and controlling for inherent  
408 colour preference may have facilitated the avoidance response toward the place conditioned with  
409 the CaP during the choice test.

410 These results highlight the negative welfare consequences of CaP as an appetite suppressant in  
411 alternative diets for broiler breeders, but also as a feed additive in poultry diets. CaP is often used  
412 as a growth promoter and feed preservative [6] at concentrations from 0.25% to 0.6% CaP in  
413 standard diets [3-5]. Paul and colleagues [4] reported a lower feed intake at 0.5% CaP in one-  
414 month-old broilers that resulted in an improvement in the feed efficiency, and similar results were  
415 reported by Bonos *et al.* [5] with a standard diet at 0.1% CaP. However, the effect of CaP on feed  
416 and intestinal microbial count (Coliforms, *E. coli*, *Clostridium spp.*, and *Aspergillus spp.*) is not as  
417 evident at such inclusion rates [4]. The fungicidal properties of CaP have been previously indicated  
418 at 1% inclusion rate, a concentration at which CaP can inhibit the germination and proliferation of  
419 *Aspergillus sp.* [47]. For this reason, a similar inclusion rate of CaP to the one used in the previous  
420 experiment might be required to achieve the antimicrobial properties of CaP in standard poultry  
421 diets. However, our results suggest that the effect of CaP as an appetite suppressant can induce a  
422 negative affective state at 1.6% inclusion rate, making its use in poultry, animal and human  
423 nutrition controversial from a welfare perspective.

## 424 Conclusion

425 Pullets were more likely to avoid the place conditioned with the consumption of calcium  
426 propionate and pullets developed an increasing preference for the placebo place over time. These  
427 results indicate that the inclusion of calcium propionate as an appetite suppressant (at 1.6%) can  
428 cause a negative affective state, and this aversive effect can underlie the reduction in feed intake  
429 in diets supplemented with calcium propionate. As well, the effect of feeding motivation on arousal  
430 and on learning ability on the performance of broiler breeders in learning task or choice test should  
431 be considered in the interpretation of animals' preferences.

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