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1 **Object and food novelty induce distinct patterns of c-fos immunoreactivity in amygdala**  
2 **and striatum in domestic male chicks (*Gallus gallus domesticus*).**

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14

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20 Declarations of interest: none

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23

24 **Abstract**

25 Avoidance of novelty, termed neophobia, protects animals from potential dangers but can also  
26 impair their adaptation to novel environments or food resources. This behaviour is particularly  
27 well described in birds but the neurobiological correlates remain unexplored. Here, we  
28 measured neuronal activity in the amygdala and the striatum, two brain regions believed to be  
29 involved in novelty detection, by labelling the early gene c-fos following chicks exposure to a  
30 novel food (NF), a novel object (NO) or a familiar food (FF). NF and NO chicks showed  
31 significantly longer latencies to touch the food, less time eating and emitted more fear-  
32 vocalizations than control chicks. Latency to touch the food was also longer for NO than for  
33 NF chicks. Significantly higher densities of c-fos positive cells were present in all the nuclei  
34 of the arcopallium/amygdala of NF and NO chicks compared to FF chicks. Also, NO chicks  
35 showed higher positive cell densities than NF chicks in the posterior amygdaloid, the  
36 intermediate and the medial arcopallium. Exposure to novel food or object induced a similar  
37 increase in c-fos expression in the nucleus accumbens and the medial striatum. Our data  
38 provide evidence activation of the arcopallium/amygdala is specific of the type of novelty.  
39 The activation of striatum may be more related to novelty seeking.

40

41 **Keywords:** Neophobia, Arcopallium/amygdala, Striatum, c-fos, Emotions

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## 47 **1. Introduction**

48 A large range of avian species exhibit neophobia, a reluctance to approach novelty. This  
49 behaviour brings about both positive and negative consequences on their survival. It allows  
50 animals to adapt their reactions to changes or potential threats in their environment [1]. When  
51 strongly expressed, neophobia can also impair foraging flexibility, innovations, and reduce  
52 home range size or species' niche breadth [1-5]. On farm, it can be a cause for concern in  
53 relation to bird welfare as it can impair the capacity of fowl to adapt to changes in their  
54 feeding regime [6]. Neophobia is also considered a trait to estimate behavioural syndromes or  
55 copying styles within populations, that is to say birds' capacity to cope behaviourally and  
56 physiologically with stressful environmental challenges [3, 4, 7-9].

57 Although response to novelty has been shown to be important for bird's adaptation and  
58 survival, few studies investigated its neuro-physiological bases. Japanese quail (*Coturnix*  
59 *coturnix japonica*) exposed repeatedly to the sudden introduction of a novel object in their  
60 home cage showed a significant rise in plasma corticosterone levels [13]. This endocrine  
61 response induced by a novel object was also found in great tits (*Parus major*) exposed to a  
62 novel perch in their home cage [14]. These results indicate exposure to novelty induces fear-  
63 related responses in birds that are likely underpinned by the activation of brain regions  
64 involved in fear or emotions. In line with this, previous studies demonstrated that rats with  
65 amygdala lesions, a brain region involved in the processing of emotional cues and fear  
66 leaning, show attenuated response to novelty [10-13]. In birds, wild black-capped chickadees  
67 from Alaska that showed less of a neophobic response to a novel object than their Kansas  
68 counterpart [14], had a larger arcopallium volume, a brain region that **some authors**  
69 **considered** as the homolog of the mammalian amygdala [15]. This study highlighted that the  
70 birds from Alaska had a higher density of neurons in the lateral striatum, believed to be the  
71 homolog of the mammalian caudate nucleus and putamen. The mammalian striatum has been

72 repeatedly shown to be involved in novelty seeking, motivation and reward processing [16-  
73 20]. These findings indicate that neophobia in birds is not only a fear response but also related  
74 to the processing of motivation triggering cues and goal directed behaviours. However, a clear  
75 relationship between neophobia and amygdala/arcopallium or striatum subnuclei activation  
76 still need to be investigated in birds.

77

78 To better characterize the neural circuit activated by novelty in birds, we labelled chicks brain  
79 with the early gene c-fos following exposure to novel food and novel object. In birds and  
80 mammals, c-fos cellular expression is widely used to reliably map the brain activity  
81 underlying behaviours such as fear-related behaviours, decision-making or learning [21-23].  
82 This study was conducted in the domestic chicken (*Gallus gallus domesticus*) which is  
83 particularly appropriate for such a study. Food and object neophobia are easily observed in  
84 young chicks in experimental conditions. A brain atlas as well as immunohistochemical  
85 procedures for studying c-fos expression are well established [24-27]. We studied c-fos  
86 expression following exposure to both novel food and object because both types of neophobia  
87 have been described in chicks and can have an impact on animal adaptation. Moreover, we  
88 previously observed that responses to novel food did not correlate with responses to a novel  
89 object and were milder [28]. This may suggest both types of neophobia may be associated  
90 with specific and distinct brain activations. We investigated c-fos expression in different  
91 subnuclei of both the arcopallium/amygdala and striatum, as these brain structures has  
92 previously been found to be engaged in novelty detection [11, 15].

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## 96 **2. Method**

### 97 **2.1 Ethics statement**

98 All birds were maintained at the Experimental Unit PEAT of INRA (Nouzilly, France, license  
99 number B-37-175-1). All experiments were approved by the Ethics Committee for Animal  
100 Experimentation of Val de Loire, CEEA Vdl (reference number 2012-10-6) and were  
101 performed in accordance with the European Communities Council Directive 2010/63/UE. The  
102 remaining chicks were sold at the end of the experiment.

### 103 **2.2 Chicks and housing conditions**

104 The layer breeder Novogen (SCEA JEGOU, France) provided eggs from White Leghorn  
105 layers. The eggs were incubated at 37.8 °C and 56% relative humidity with automatic and  
106 continuous turning. Three days before hatching, the rotation was stopped and the temperature  
107 was reduced to 37.6 °C. The sex was determined at hatching (21st day of incubation) and we  
108 kept 60 chicks (30 males and 30 females). Each chick was identified with a numbered ring on  
109 its leg the day of hatching. The 60 chicks were placed in pairs (one male and one female) in  
110 wire-covered plastic tubs (50 cm × 40 cm × 30 cm; length × width × height) with wood  
111 shavings on the floor. Chicks were maintained in an 11 h light/13 h dark cycle, with water and  
112 food available ad libitum. The chicks were fed with a conventional starter mash (PEAT,  
113 INRA Val de Loire, France). The ambient temperature was maintained at  $33 \pm 1$  °C from  
114 hatching until the chicks were 8 days old after which it was decreased. The chicks were  
115 weighted when they were 5 days old.

### 116 **2.3 Behavioural characterization**

117 Three independent groups of chicks were constituted with weight controlled across groups: a  
118 control group of chicks exposed to the familiar food (FF, N = 10 pairs of chicks); a group of  
119 chicks exposed to novel food (NF, N = 10 pairs of chicks) and a group of chicks exposed to a

120 novel object (NO, N = 10 pairs of chicks; weight of chicks: FF chicks:  $48.87 \pm 0.93$  g; NF  
121 chicks:  $47.94 \pm 0.68$  g;  $49.28 \pm 0.56$  g;  $F_{2,45} = 0.85$ ,  $P = 0.43$ ).

122 Behavioural testing occurred in a room different from where the animals were housed but in  
123 an experimental cage that had the same features as the home cage (Fig. 1). As chicks become  
124 distressed when they are socially isolated, we tested cage mates together (N = 30 pairs). The  
125 day before the testing session the pairs of chicks were familiarized with the test cage and  
126 handling procedure. Pairs were transported to the test room in a box and were placed in an  
127 opaque enclosure within the test cage, opposite to the feeding trough (their home cage feeding  
128 trough filled with their usual food). After 30 seconds, the enclosure was removed and the  
129 chicks could freely explore the test cage for 4 minutes. After exposure to the test-cage, the  
130 chicks were placed back in their home cage.

131 For all testing sessions, chicks were tested 90 minutes after the feeder had been removed from  
132 the home cage. FF chicks were exposed to a familiar food for 4 minutes following the  
133 procedure described for habituation. Novel food and object tests followed protocols  
134 previously validated [27, 28]. The procedures were similar to the habituation procedure  
135 except that, for NF chicks, the feeder was filled with millet seeds and for the novel object test  
136 (NO chicks), the familiar feeder was replaced by an unfamiliar coloured feeder (green and  
137 yellow plastic instead of grey metal) containing their familiar food. To reduce potential sex  
138 effects within a small sample size, we choose to follow and analyze male chicks only. An  
139 observer hidden behind a curtain recorded the behaviour of male chicks within each pair  
140 (tagged with a blue mark on the head the day before). Latency to touch the food with the  
141 beak, time spent eating, and the number of fear-trills vocalizations were recorded. After  
142 testing, the pairs of chicks were placed back in their home cage. The testing sessions were  
143 conducted between 7 and 10 days of age. Each day the treatments (familiar food, novel food,

144 novel object) were tested alternatively and all observations were conducted between 9 am and  
145 12 am.

## 146 **2.4 Histology**

147 The brains of 30 male chicks (10 FF, 10 NF and 10 NO) were collected 90 minutes after each  
148 testing session. Chicks were deeply anaesthetized by intraperitoneal injection of pentobarbital,  
149 perfused transcardially via the left ventricle with 15 mL of 1% sodium nitrite in phosphate  
150 buffer saline, then with 70 mL of ice-cold fixative (4% paraformaldehyde solution in 0.1 M  
151 phosphate buffer, pH 7.4). The brain was removed from the skull, post-fixed by immersion  
152 for one hour in the same fixative and then cryoprotected in 20% sucrose for two days [29].  
153 Brains were frozen in isopentane at -40°C and stored at -80°C before being cut into 20 µm  
154 coronal sections on a cryostat, in a caudal to rostral direction. Four series of alternate sections  
155 (with an equal distance of 200 µm to one another) were collected onto SuperFrost® glass  
156 slides. One series was stained with Cresyl Violet to provide accurate anatomical localization.  
157 The other three series were stored at -80°C for immunohistochemistry analysis.

158

## 159 **2.5 C-Fos immunohistochemistry**

### 160 **2.5.1 C-Fos protein staining**

161 Due to perfusion failure in 6 chicks, immunohistochemistry was performed on 24 animals (10  
162 FF, 7 NF, 7 NO chicks). All the incubations in bathes were made under gentle agitation. The  
163 slides were first immersed for 15mn in acetone bath at -20°C, dried and rehydrated in TBS.  
164 They were then immersed for 15 mn in TBS at 4°C containing 0.1% hydrogen peroxide to  
165 inactivate endogenous peroxidase activity and rinsed in TBS for 15 mn at room temperature,  
166 followed by an incubation for 1h at room temperature in TBS containing 0.3% saponin, 0.1%  
167 sodium azide and 1% bovine serum albumin (BSA; Roche Diagnostics, USA). Sections were  
168 then incubated at 4°C for 24h with the same buffer containing the c-fos primary rabbit



169 polyclonal antibody (ref K25, Santa Cruz, USA) diluted at 1:1000. They were rinsed four  
170 times for 5 mn and then incubated at 4°C for 3h in a sheep anti-rabbit gamma-globulin serum  
171 diluted in TBS-saponin-BSA. Sections were rinsed again with TBS and incubated overnight at  
172 4°C with rabbit peroxidase-antiperoxidase complex (PAP, Jackson, USA) diluted at 1:1000 in  
173 TBS-saponine-BSA. The following day, the sections were rinsed twice in TBS and three  
174 times in Tris-HCl (50mM, pH7.6) for 5mn each time. The PAP complex was finally  
175 visualized using a metal-enhanced diaminobenzidine hydrochloride solution (0.04% DAB,  
176 0.3% nickel ammonium sulphate, 0.003% H<sub>2</sub>O<sub>2</sub>) in Tris-HCl. Finally the sections were  
177 rinsed three times for 5mn in Tris-HCl, dried, dehydrated through graded alcohol, cleared in  
178 toluene and cover-slipped with Depex (BHD Laboratory Supplies, Poole, UK).

179

## 180 **2.5.2 Staining quantification**

181

182 The density of c-fos positive cells (c-fos + cells) was assessed using a motorized microscope  
183 (Axioskope 2; Zeiss, Germany) connected to the image analysis software Mercator®  
184 (Exploranova, La Rochelle, France) through a camera. We analyzed the expression of c-fos in  
185 the following subnuclei of the arcopallium/amygdala: intermediate (IA), medial (MA), dorsal  
186 (DA), posterior (PoA) and nucleus taeniae (TnA). In the striatal complex, we analyzed the  
187 expression of c-fos in the medial striatum (MSt), the lateral striatum (LSt) and the nucleus  
188 accumbens (Ac) [30]. The brain regions were identified in accordance with the stereotaxic  
189 atlas of the chick brain [31] and its revised nomenclature [26] (Fig. 2). With the help of the  
190 Cresyl Violet the areas of interest were outlined on a magnification of 2.5x for the  
191 arcopallium/amygdala nuclei and nucleus accumbens. The location of the nucleus accumbens  
192 in chick illustrated in the atlas of Kuenzel and Masson (1988) [30] has been modified in  
193 accordance with recent studies [31-33]. These studies provided evidence the nucleus

194 accumbens (or former lobus parolfactorius), at caudal level, is instead the bed nucleus of the  
195 stria terminalis pars lateralis (BSTl; [33]). To limit this, c-fos expression was counted only on  
196 rostral sections. Moreover, although it is not possible to totally exclude that c-fos expression  
197 was also partly counted in the BSTl, the BSTl represents only a small proportion of the total  
198 area considered for counting c-fos expression in the nucleus accumbens. The precise limits of  
199 the different nuclei of the striatum being difficult to determine, we choose to draw a  
200 standardized counting frame in the lateral striatum (350  $\mu\text{m}$  X 700 $\mu\text{m}$ ) and in the medial  
201 striatum (900  $\mu\text{m}$  X 700  $\mu\text{m}$ ).

202 Then c-fos- immunoreactive cells were semi-automatically counted on a magnification of  
203 20x, according to a predetermined grey scale, as well as size criteria : only dark and round or  
204 ovoid stains were counted as positive nuclei. Before each series of counting a reference slice  
205 containing a known number of immunoreactive cells was analyzed to verify the settings of the  
206 microscope and of the software. For each section, the density of positive cells (cells/mm<sup>2</sup>)  
207 was calculated as the total number of cells divided by the total area. Depending on the extent  
208 of the region, a mean of  $9.3 \pm 0.1$  sections per bird and per subnucleus of the  
209 arcopallium/amygdala were analyzed and a mean of  $7.5 \pm 0.1$  sections for the striatum. One  
210 experimenter blind to experimental conditions counted the right and left side of each section  
211 for all the structures.

212

## 213 **2.6 Statistics**

214 We used Kolmogorov–Smirnov tests to determine whether the data sets were normally  
215 distributed and Bartlett tests to assess homogeneity of variances. For behavioural data, the  
216 requirements for parametric statistics were not obtained despite transformation of data.  
217 Therefore, we used the function aovp of the lmPerm package in R 3.4.2, to conduct an  
218 analysis of variance with permutation tests (method = Exact) with the Treatment (FF, NF,

219 NO) as a fixed factor and the Day of testing (7, 8, 9, 10) as a random factor. We used the  
220 function `pairwise.perm.t.test` for post-hoc comparisons with holm correction for multiple  
221 comparisons. For the density of c-fos positive cells, we analysed the differences in each brain  
222 area according to the Treatment (FF, NF, NO), the Side of the brain (right or left), the  
223 interaction Treatment\*Side, the Day of testing (7, 8, 9, 10) and the Identity of the bird with  
224 Generalized Linear Mixed Models (GLMMs) fitted by maximum likelihood. We assumed a  
225 Poisson error distribution and, in all models, Treatment, Side, Treatment\*Side were entered as  
226 fixed effects while Day and Identity were considered as random factors. We used the function  
227 “`glmmPQL`” from the package MASS in R 3.4.2. The normal distribution and homogeneity of  
228 residuals were checked using “`plotresid`” function from “RVAideMemoire” package. We used  
229 the “`pairwise.wilcox.test`” function from “stats” package (non-paired data) for post-hoc  
230 comparisons between groups of chicks with holm correction for multiple comparisons. The  
231 “`cor.test`” function from the “stats” package was used to perform Pearson correlations tests  
232 independent of the treatment to estimate the correlations between the mean latency to touch  
233 the food and mean c-fos positive cells density in each brain area. Throughout the text,  
234 significance was accepted at  $P < 0.05$ .

### 235 **3. Results**

#### 236 **3.1 Behavioural characterization**

237 We found a significant effect of the treatment on the latency to touch the food ( $F_{2,20} = 32.6$ ,  $P$   
238  $< 0.01$ ; Fig. 3). Post-hoc analyses showed that NF and NO chicks had longer latencies to  
239 touch the food than control FF chicks ( $P < 0.01$  for both comparisons) and, that NO chicks  
240 had longer latencies than NF chicks ( $P = 0.05$ ; Fig. 3). The time spent eating during testing  
241 differed significantly between treatments ( $F_{2,20} = 24.02$ ,  $P < 0.01$ ; Fig. 3). Post-hoc analyses  
242 showed that the time spent eating was lower in NF and NO chicks than in FF chicks ( $P < 0.01$

243 for both comparisons) whereas NF and NO chicks did not differ significantly ( $P = 0.99$ ; Fig. 3). There was a significant effect of the treatment on the number of fear-trills expressed  
244 3). There was a significant effect of the treatment on the number of fear-trills expressed  
245 during testing ( $F_{2,20} = 10.61$ ,  $P = 0.01$ ). Post-hoc analyses showed NF and NO chicks  
246 expressed a significantly higher number of fear-trills than FF chicks (NF vs. FF chicks,  $4.5 \pm$   
247  $1$  trills vs.  $0$ ,  $P < 0.01$ ; NO vs. FF chicks,  $7.1 \pm 3$  trills vs.  $0$ ,  $P < 0.01$ ). NF and NO chicks did  
248 not differ significantly ( $P = 0.55$ ). The random factor Day had no significant effect on any of  
249 the parameters ( $P > 0.05$ ).

## 250 3.2 Immunohistochemistry: c-fos expression

### 251 3.2.1 Arcopallium/amygdala complex

252 The density of c-fos positive cells differed significantly between groups in all the sub-nuclei  
253 of the arcopallium/amygdala observed (Table 1, Fig. 4 and Fig. 6). Post-hoc analyses showed  
254 that the density of positive cells was significantly higher in NF and NO chicks than in the  
255 control FF chicks in all the subnuclei (Fig. 4). In the intermediate arcopallium (IA), the  
256 medial arcopallium (MA), and the posterior amygdala (PoA) the density of positive cells was  
257 also significantly higher in NO than in NF chicks (Fig. 4). A trend in the same direction was  
258 observed in the nucleus taeniae (TnA). No significant Side and Treatment\*Side effects on cell  
259 density were observed (Table 1). The random factors Day and Identity had no significant  
260 effect on any of the parameters ( $P > 0.05$ ).

### 261 3.2.2 Striatum complex

262 The density of c-fos positive cells differed significantly between groups in the medial striatum  
263 (Mst) and the nucleus accumbens (Ac) but not in the lateral striatum (Lst) (Table 1, Fig. 5 and  
264 Fig. 6). In the medial striatum (Mst), post-hoc analyses failed to show significant differences  
265 between groups. In the nucleus accumbens (Ac), the density of positive cells was  
266 significantly higher in NF and NO chicks than in the control FF chicks and, the density did

267 not differ significantly between NF and NO chicks (Fig. 5). No significant effects of the side  
268 or the interaction Side\*Treatment were found (Table 1). The random factors Day and Identity  
269 had no significant effect on any of the parameters ( $P > 0.05$ ).

### 270 3.2.3 Correlations

271 A significant positive correlation was found between the latency to touch the food and c-fos  
272 positive cell density in the posterior amygdala PoA (cor = 0.49,  $P = 0.01$ ) and in the nucleus  
273 accumbens (Ac) (cor = 0.41,  $P = 0.04$ ). Non significant trends in the same direction were  
274 found with the others nuclei of the arcopallium/amygdala (IA: cor = 0.35,  $P = 0.08$ ; MA: cor  
275 = 0.29,  $P = 0.14$ ; DA: cor = 0.33,  $P = 0.10$ ; TnA: cor = 0.33,  $P = 0.09$ ). No significant  
276 correlations were observed in the striatum (Lst: cor = -0.01,  $P = 0.92$ ; Mst: cor = 0.09,  $P =$   
277 0.64).

## 278 4. Discussion

279 Our aim was to deepen our understanding of the underlying mechanisms of neophobia in  
280 birds. Clear behavioral differences were observed when chicks are exposed to novel food or  
281 object compared to the control condition. Chicks exposed to either novel food or object  
282 showed higher latencies to touch the food, lower time spent eating and a higher number of  
283 fear-trill vocalizations compared to controls. In addition, NO chicks showed a higher latency  
284 to touch food than NF chicks. At the brain level, exposure to novel food or object was  
285 associated with a significant increase in c-fos expression in all the subnuclei of the  
286 arcopallium/amygdala complex and two nuclei of the striatum, the Mst and Ac. Mirroring the  
287 behavioral data, c-fos expression was also higher in NO chicks in comparison to NF chicks in  
288 specific nuclei of the arcopallium/amygdala complex, the IA, MA and PoA nuclei.

289 Latency to touch food is the most classical parameter used to estimate neophobia in avian  
290 species. When birds get in contact with novel food or with the familiar food close to a novel

291 object, it is assumed that they have overcome their neophobia [2, 34, 35]. Here, the higher  
292 latencies to touch the food in the novel food and novel object tests in comparison to the  
293 control condition show that the birds perceived novelty. In conjunction with higher numbers  
294 of fear-trill vocalizations during novel food and novel object tests than in the control  
295 condition, the data support the hypothesis of an emotional-induced response related to fear. In  
296 our testing conditions, and as previously observed [28], the time taken to overcome neophobia  
297 was higher during the novel object test than during the novel food test. These findings  
298 confirm that the latency to touch food is a suitable behavioral marker of neophobia. Our  
299 results also show that chicks were more fearful towards the novel feeder than toward the  
300 novel food.

301 Our data support the hypothesis that exposure to novelty (food or object) is associated with  
302 activation of the arcopallium/amygdala system in birds. We found higher density of c-fos  
303 positive cells following exposure to novelty than in the control condition in all the subnuclei  
304 of the arcopallium/amygdala: the intermediate, medial, dorsal, and posterior amygdaloid and  
305 nucleus taeniae. We also found a positive correlation between the latency to touch food and  
306 the activation of the PoA. Although non significant, the same tendency was observed for the  
307 other nuclei of the amygdala complex. Within the striatum, a correlation was only observed  
308 between the latency and c-fos expression in the nucleus accumbens. Our results highlight an  
309 important and overall involvement of the arcopallium/PoA complex in neophobia. A previous  
310 study using lesions of the arcopallium/amygdala suggested that this structure was not  
311 involved in the avoidance of novel objects since chicks, lesioned or not, did not differ in their  
312 avoidance behaviour [36]. In this previous study, tested chicks were isolated from their cage  
313 mates and importantly the novel object was presented in a novel environment (open-field).  
314 The processing of spatial cues has been shown to require the hippocampus both in mammals  
315 and birds [13, 37-40]. It is thus possible to postulate the arcopallium/amygdala may be

316 specialized in object or food neophobia but not required for spatial novelty. To test this  
317 possibility further experiment are required. In birds, several functional studies have  
318 demonstrated that the arcopallium/PoA complex is engaged in the expression of fear-related  
319 behaviours [21, 41-43]. Activation of the arcopallium/PoA complex may thus be related to  
320 the fear related components of food and object novelty.

321 Our results showed c-fos expression was different according to the type of novelty (food or  
322 object) in different nuclei of the arcopallium. Indeed c-fos expression was higher in the IA,  
323 MA, PoA nuclei of the arcopallium/amygdala complex in chicks exposed to novel object  
324 compared to those exposed to novel food. This suggests the arcopallium is engaged in the  
325 processing or appraisal of the situation. Numerous studies in mammals reported that the  
326 amygdala complex is not solely involved in the expression of fear behaviors but also in the  
327 processing of emotional cues [44-46]. Commonly considered as a temperament trait,  
328 neophobia is rarely tested using both novel objects and novel foods despite suggestions that  
329 they may represent two independent behavioral traits in adult or young birds [7, 28]. Different  
330 cognitive appraisal processes may thus be involved when assessing the threatening properties  
331 of novel foods or objects. Our findings support this idea and show that amygdala activation is  
332 also differentially modulated by food and object neophobia in bird.

333 It is important to consider that the functional role of the arcopallium is still under debate. It  
334 has been compared to different cortical and subcortical areas. Arcopallium (or archistriatum)  
335 has been shown to be involve in sensori-motor control [47], such as orofacial control [48]. In  
336 line with this, lesion of the intermediate arcopallium alters the capacity of pigeons to hold  
337 grains with their beaks [47]. It is also involved in working memory [49]. Recent studies  
338 provided solid evidence for a role of this region in reward anticipation, time and/or energetic  
339 cost for food consumption [50, 51] and the social facilitation of foraging [52]. It is thus not  
340 impossible to exclude that under our condition, exposure to novel food or to familiar food in a

341 novel feeder is associated with cost / benefit processes that involve the arcopallium.  
342 Functional and anatomical studies converge to indicate the arcopallium may be analogous to  
343 the mammalian prefrontal cortex, and more specifically the anterior cingulate cortex.  
344 However, lesion, electrical stimulation studies showed also an involvement of the arcopallium  
345 in fear or anxiety related processing or responses [21, 36, 43, 53-56]. Together with  
346 anatomical [29] and neurochemical [57] considerations these findings point also toward a  
347 similarity between arcopallium and the mammalian basolateral amygdala. Further studies will  
348 be thus necessary to elucidate the neocortical or the amygdala part of the arcopallium.

349 Within the striatal complex, c-fos expression was higher in the medial striatum and the  
350 nucleus accumbens in birds exposed to novelty in comparison with controls. Anatomical  
351 studies have reported the existence of different sub regions for the chicken accumbens, the  
352 core and shell subregions [33, 58]. These findings point to the view the nucleus accumbens in  
353 chicken is, as in mammals, a complex brain region. This subdivision was not included in our  
354 study. Indeed, c-fos staining did not allow the possibility to easily separate these subdivisions.  
355 For this, a double staining approach (with c-Fos and a specific marker such as DARPP-32)  
356 would need to have been used. Moreover, the choice of counting only c-fos positive cells on  
357 very anterior planes limits the possibility to analyse the core and shell division of the nucleus  
358 accumbens. Indeed, the subdivision of the nucleus accumbens into a core and shell part was  
359 reported on planes located more caudally than those analysed in our study. Further studies  
360 will be thus required to test whether different types of neophobic responses may be associated  
361 with different involvement of the different subdivisions of the nucleus accumbens. Finally, it  
362 is not possible to totally exclude c-fos expression in the nucleus accumbens also partly  
363 includes c-fos expression in the BSTl, a very thin sub-region that represents a very limited  
364 surface of the area considered for the nucleus accumbens. Our results are in line with a  
365 previous study indicating the striatum could be engaged in novelty detection in birds [15].



366 They also support findings indicating a general role of the striatum in novelty seeking [59].

367 **However, the role of the striatum is not limited to the detection of novelty.**

368 **Electrophysiological and lesion studies provided evidence that the chick's ventral striatum**

369 **was engaged in decision-making and impulsivity [51, 60, 61]. These findings indicate the**

370 **ventral striatum and more specifically the nucleus accumbens is involved in guiding the**

371 **chick's behaviour to approach food resources. C-fos expression in the nucleus accumbens**

372 **following exposure to novel food or to a novel feeder may thus reflect neural correlates**

373 **underlying goal-directed behaviors.**

374 Neophobia is a behavior well observed from invertebrates to vertebrates including humans

375 [62]. In mammals, the amygdala and the nucleus accumbens were also found to be involved in

376 the processing and expression of food neophobia [63-65]. Our data provide additional

377 evidence of convergence between mammals and avian species by revealing comparable

378 patterns of brain activation during neophobia. Additional work is still needed to understand

379 how these different regions of the brain interact and produce cognitive responses in birds.

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391 **6. References**

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559



560 **Figure captions**

561 **Figure 1: time schedule of the experiment.** Pairs of chicks composed of one female and one  
562 male (N=30 pairs) were habituated to a test-cage for 4 minutes when they were between 6-9  
563 days of age. The test-cage was similar to their home cage. It contains a trough similar to the  
564 one placed in their home cage, filled with their usual food. The day following habituation,  
565 pairs of chicks were placed back in the same test cage for 4 minutes. Depending on the group,  
566 this test cage contains the familiar trough filled with familiar food (FF, N=10 pairs), the  
567 familiar trough filled with novel food (NF, N= 10 pairs) or a new trough filled with familiar  
568 food (NO, N = 10 pairs). Ninety minutes following the test, brains of male chicks were  
569 collected.

570

571 **Figure 2. Illustration of the boundaries of the different subregions analyzed in the**  
572 **striatum and the arcopallium amygdala.** Cresyl Violet of chick brain sections illustrating the  
573 analyzed areas at the level of the striatum (a) and arcopallium / amygdala (b) subdivisions.  
574 Abbreviations : AC : nucleus accumbens ; MSt : medial striatum ; LSt : lateral striatum ; DA :  
575 dorsal Arcopallium ; MA : medial Arcopallium ; IA : intermediate Arcopallium ; PoA :  
576 posterior pallial amygdala ; TnA : nucleus taeniae ; V : lateral ventricle. Scale bar = 1500  $\mu$ m.

577

578 **Figure 3. Behavioural characterization.** Mean  $\pm$  SE latency to touch the food, time spent  
579 eating during testing and number of vocalizations of control chicks exposed to their familiar  
580 food (FF, N = 10 pairs), chicks exposed to novel food (NF, N = 10 pairs) and chicks exposed  
581 to novel feeder (novel object, NO, N = 10 pairs) filled with their familiar food and post-hoc  
582 analyses outcomes (*P*-value corrected for multiple comparisons). \*\**P* < 0.01, \**P* < 0.05.

583 **Figure 4. Effect of exposure to novelty on Fos expression in the arcopallium/amygdala**  
584 **complex.** Expression of immediate early gene c-fos (mean  $\pm$  SE positive cell density) in the  
585 nuclei of the arcopallium/amygdala of FF (familiar food, N = 10), NF (novel food, N = 7) and  
586 NO (novel object, N = 7) chicks and post-hoc analyses outcomes (*P*-value corrected for  
587 multiple comparisons). \*\**P* < 0.01, \**P* < 0.05, # 0.05 < *P* > 0.1.

588

589 **Figure 5. Effect of exposure to novelty on Fos expression in the striatum.** Expression of  
590 immediate early gene c-fos (mean  $\pm$  SE positive cell density) in the nuclei of the striatum of  
591 FF (familiar food, N = 10), NF (novel food, N = 7) and NO (novel object, N = 7) chicks and  
592 post-hoc analyses outcomes (*P*-value corrected for multiple comparisons). \*\* *P* < 0.01; NS *P*  
593 > 0.05.

594

595 **Figure 6. Neurobiological characterization.** Representative photomicrographs of c-fos  
596 immunoreactivity for the three groups (FF, NF and NO) in the areas shown on Figure 2. V :  
597 lateral ventricle, \* : artefacts. Scale bar = 50  $\mu$ m.

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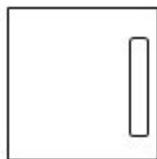
602 **Tables**

603 **Table 1. Results from the linear mixed effect models.** The fixed effects of treatment, side, and treatment\*side on positive c-fos cells density  
 604 (cells/mm<sup>2</sup>) were estimated in the arcopallium/amygdala and the striatum of FF (familiar food, N =10), NF (novel food, N=7) and NO (novel  
 605 object, N = 7) chicks.

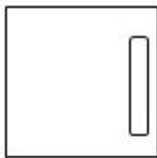
<b>Brain areas</b>	<b>Treatment</b>			<b>Side</b>			<b>Treatment*Side</b>		
	<i>Chisq</i>	<i>df</i>	<i>P value</i>	<i>Chisq</i>	<i>df</i>	<i>P value</i>	<i>Chisq</i>	<i>df</i>	<i>P value</i>
<b><i>arcopallium/amygdala</i></b>									
IA	46.57	2	< 0.01	0.28	1	0.59	3.53	2	0.17
MA	28.42	2	< 0.01	0.72	1	0.39	0.98	2	0.61
DA	22.61	2	< 0.01	1.06	1	0.30	1.76	2	0.41
PoA	65.41	2	< 0.01	0.01	1	0.92	5.62	2	0.06
TnA	30.73	2	< 0.01	0.28	1	0.59	3.52	2	0.17
<b><i>striatum</i></b>									
MSt	6.24	2	0.04	0.07	1	0.78	0.14	2	0.93
LSt	2.20	2	0.33	0.01	1	0.92	2.20	2	0.33
Ac	72.96	2	< 0.01	0.73	1	0.39	0.23	2	0.88

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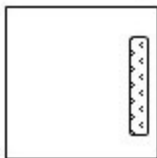
Familiarization



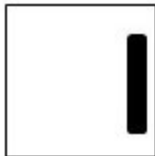
Familiar Food (FF)

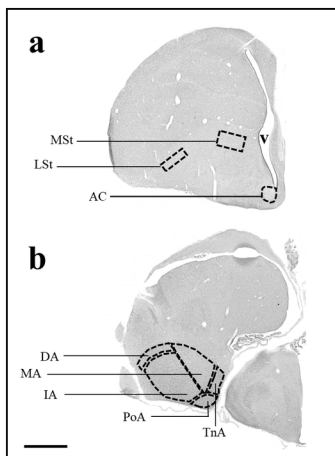


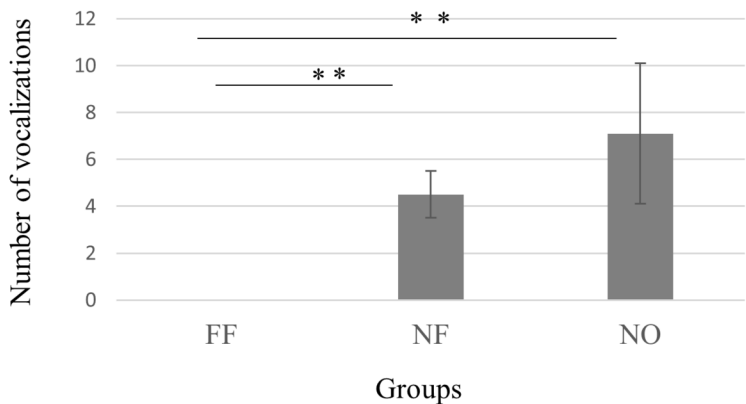
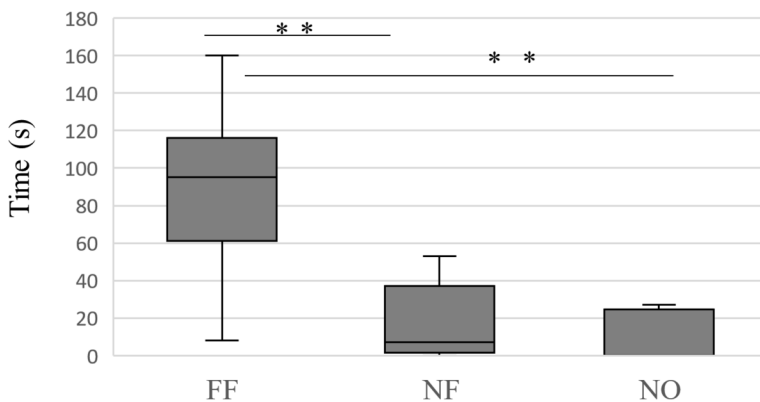
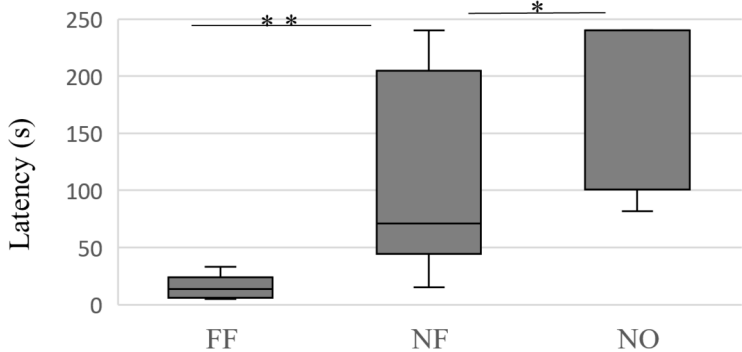
Novel Food (NF)

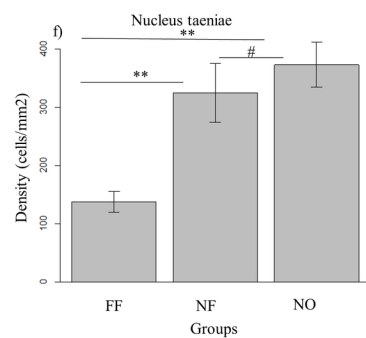
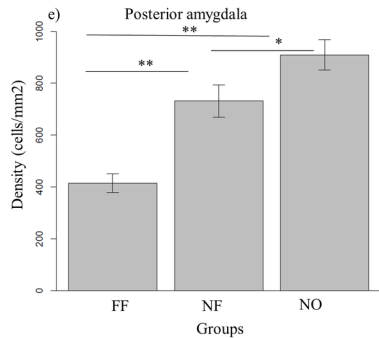
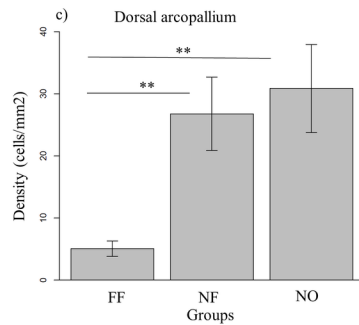
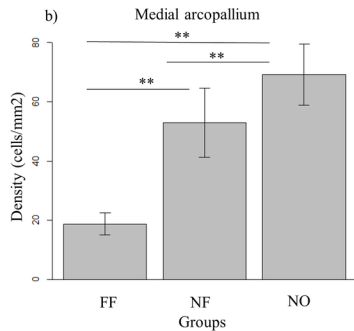
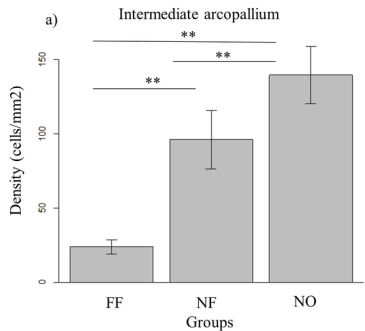


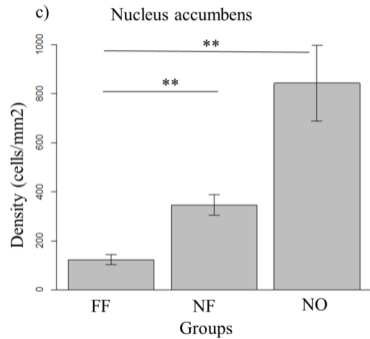
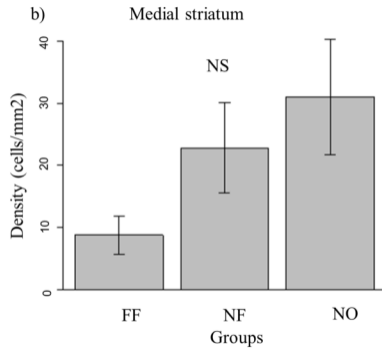
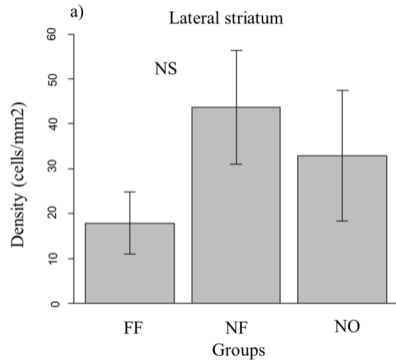
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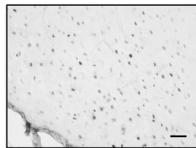
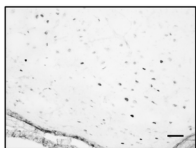
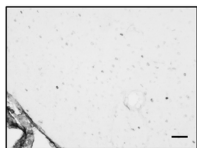
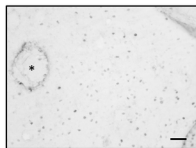
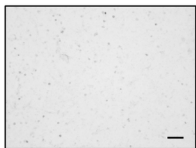
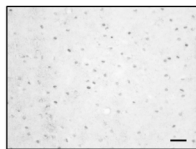
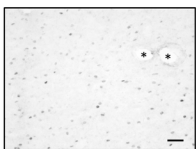
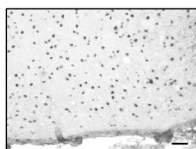
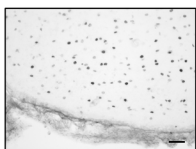
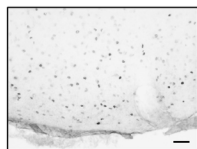
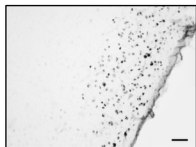
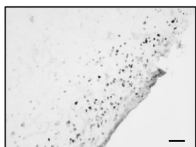
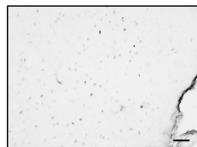










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