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## Co-variation between glucocorticoids, behaviour and immunity supports the pace-of-life syndrome hypothesis: an experimental approach

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1 **Co-variation between glucocorticoids, behaviour and**  
2 **immunity supports the pace-of-life syndrome hypothesis:**  
3 **an experimental approach**

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## 21 **Abstract**

22 The biomedical literature has consistently highlighted that long-term elevation of  
23 glucocorticoids might impair immune functions. In wild animals, patterns are less clear. Here,  
24 we re-explored the stress-immunity relationship considering the potential effects of behavioural  
25 profiles. Thirteen captive roe deer (*Capreolus capreolus*) were monitored over an eight-week  
26 period encompassing two capture events. We assessed how changes in baseline faecal cortisol  
27 metabolite (FCM) concentrations following a standardised capture protocol and vaccination  
28 affected changes in thirteen immune parameters of the innate and adaptive immunity, and  
29 whether behavioural profiles were linked to changes in baseline FCM levels and immune  
30 parameters. We found that individuals showing an increase in baseline FCM levels also  
31 exhibited an increase in immunity and were characterised by more reactive behavioural profiles  
32 (low activity levels, docility to manipulation and neophilia). Our results suggest that immunity  
33 of large mammals may be influenced by glucocorticoids, but also behavioural profiles, as it is  
34 predicted by the pace-of-life syndrome hypothesis. Our results highlight the need to consider  
35 co-variations between behaviour, immunity and glucocorticoids in order to improve our  
36 understanding of the among-individual variability in the stress-immunity relationships observed  
37 in wildlife, as they may be underpinned by different life-history strategies.

38

39 Key words: stress, innate immunity, adaptive immunity, inflammation, coping style, cortisol

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## 43 **Introduction**

44 The immune system is one of the most important mechanisms in vertebrates for improving  
45 survival. This complex system is composed of two complementary arms, the innate (relatively  
46 fast and non-specific) and adaptive (slower at first encounter, but more long-lasting and  
47 specific) immunity, each composed of numerous cells and effectors [1]. This system, however,  
48 is not cost-free [1, 2], suggesting trade-offs between immune defences and other functions that  
49 use a common resource and contributes to fitness [3, 4]. Glucocorticoids (such as cortisol and  
50 corticosterone) are metabolic hormones that play a major role in the regulation of energy use  
51 [5, 6] and may therefore underlie such trade-offs.

52         Glucocorticoids are also one of the main mediators of the stress response. In response  
53 to external or internal stimuli, the activation of behavioural and physiological responses allows  
54 an organism to cope with challenges [7, 8]. In particular, the activation of the hypothalamic-  
55 pituitary-adrenal (HPA) axis that results in the secretion of glucocorticoids helps organisms to  
56 cope with stressful situations by making stored energy available [9]. However, repeated or  
57 chronic elevation of glucocorticoids may have negative effects on other energy-demanding  
58 functions such as reproduction [10] and immunity [11].

59         Over the past years, several studies investigated the relationship between stress and  
60 immunity, particularly in the biomedical domain where it has generally been shown that short-  
61 term elevation of glucocorticoids (i.e., few minutes to few hours) stimulates immune functions  
62 [12, 13], whereas chronically elevated glucocorticoid levels are immunosuppressive [9, 14,].  
63 Focusing on long-term elevation of glucocorticoids (i.e., few days to few months), studies in  
64 wildlife have shown mixed results ranging from decreased, increased, or no change in immune  
65 functions with chronic glucocorticoid elevation [15, 16, 17, 18]. Evidence is also accumulating  
66 that glucocorticoid levels do not affect all aspects of the immune system in the same manner,

67 such that immunoglobulin production may be impaired while other parameters (T-cell  
68 mediated, or constitutive immunity) might not be affected [19, 20].

69 To understand the stress-immunity relationship, little consideration has been given to  
70 the link with behavioural profiles. Close links between physiology and behaviour are expected  
71 due to the underlying energetic basis of both traits [21]. Accordingly, among-individual  
72 differences in behavioural traits are linked to their physiology, including glucocorticoid  
73 secretion and immune functions [22, 23]. For instance, in wild superb fairy-wrens (*Malurus*  
74 *cyaneus*), individuals exhibiting proactive behavioural traits (fast exploration of a novel  
75 environment) had the lowest level of natural antibodies [24]. Conversely, in several species,  
76 slower explorer or more reactive individuals tend to exhibit higher baseline and stress-induced  
77 glucocorticoid levels compared to faster or more proactive ones [22, 25]. In addition, a recent  
78 study on laying hens (*Gallus gallus domesticus*) highlighted that more reactive individuals  
79 exhibited greater stress and immunological (swelling in response to phytohemagglutinin  
80 injection) responsiveness than more proactive ones [26]. Such co-variations between  
81 behavioural and physiological traits can be interpreted within the pace-of-life syndrome  
82 hypothesis formulated by Réale et al. [23]. This hypothesis posits that species, populations or  
83 individuals experiencing different ecological conditions should differ in a suite of behavioural,  
84 physiological and life-history traits that may have co-evolved according to the particular  
85 ecological conditions encountered, leading to differences in life-history strategies. Within this  
86 hypothesis, individuals with slower life history strategies are expected to have more reactive  
87 behavioural profiles, higher glucocorticoid levels and higher investment in overall immunity,  
88 while those with faster life history strategies should have more proactive behavioural profiles,  
89 lower glucocorticoid levels and lower overall investment in immunity. Empirical data is  
90 however lacking to support this hypothesis.

91 In the present study, we investigated the relationships between changes in baseline  
92 glucocorticoid levels and changes in immunity, and how these were related to behavioural  
93 profiles. To do so, we investigated the link between variations in baseline faecal cortisol  
94 metabolite (FCM) levels and variations in thirteen adaptive and innate immune parameters,  
95 before and after a standardised capture stress protocol associated with an immune challenge  
96 using anti-rabies vaccination, in captive roe deer (*Capreolus capreolus*). In addition, we  
97 evaluated how these changes may be related to behavioural profiles, as characterised by three  
98 commonly used behavioural traits: docility, neophobia, and activity levels.

99 We expected that 1) changes in baseline FCM levels between the two observation  
100 periods (before/after capture) would co-vary with changes in immune parameters; 2) changes  
101 in baseline FCM levels should be less related to adaptive than innate immune parameters and  
102 inflammatory markers, due to the relatively low cost of adaptive immunity [27]. We also  
103 expected 3) that baseline FCM levels as well as variations in baseline FCM levels should be  
104 related to individual behavioural profiles [22, 28], with higher baseline levels and higher  
105 increase in baseline levels in more docile, less active and more neophilic individuals (i.e., more  
106 reactive individuals). Finally, we predicted 4) that the increase in immune parameters between  
107 the two observation periods should be greater for the most reactive individuals, which are  
108 expected to invest more in overall immunity [23, 26].

## 109 **Material and methods**

### 110 **Study site**

111 The study was conducted on a captive population of roe deer living in the Gardouch research  
112 station, located in south-west of France. The station is owned and managed by the French  
113 Research Institute for Agriculture, Food and Environment (INRAE). It consists of 12 enclosures  
114 of 0.5 ha with meadow, each containing between one to six captive roe deer, supplemented with

115 food pellets. The experiment included 13 females, aged from 4 to 13 years old, and raised at  
116 the station from their birth or their first year of life. All had some degree of habituation to  
117 humans but were able to express normal behavioural responses (e.g., vigilance, escape) to  
118 stressful situations.

## 119 **Experimental design**

120 The experimental procedure was carried out between mid-September and mid-November 2018  
121 and is summarised in figure 1. During period 1, to assess baseline glucocorticoid level of each  
122 individual, we collected faeces every four days during four weeks and measured FCM  
123 concentrations. Faeces were collected immediately after defecation was observed and kept at  
124 +4°C for a maximum of 1 h before being stored at -20°C until steroid analysis. At the end of  
125 period 1, each roe deer was subjected to a standardised capture stress protocol involving  
126 restrained immobilisation [29]. During capture, we collected faeces from the rectum, collected  
127 blood samples, injected an anti-rabies vaccine (Rabisin<sup>®</sup>, Merial, France, 1 ml) subcutaneously,  
128 and fitted collars equipped with tri-axial accelerometers (see Capture protocol and data  
129 collection for details). Collection of faecal samples was then continued every four days for an  
130 additional four weeks, as described above (i.e., period 2), in order to evaluate the effect of  
131 capture on baseline glucocorticoid level for each individual. Period 2 started two days after  
132 capture 1, in order to avoid measuring the acute increase in glucocorticoid level due to capture  
133 [30]. At the end of period 2, roe deer were recaptured following the same procedure (capture 2)  
134 and faeces and blood were again collected.

## 135 **Capture protocol and data collection**

136 Roe deer were directed into their hut by slowly approaching them and then pushed through a  
137 trap door into a retention box. Once in the box, animals were tranquilised with an intramuscular

138 injection of acepromazine (Calmivet®, Vetoquinol, France; targeted dose of 0.075 mg/kg) [31].

139 Individuals were weighed with an electronic balance to the nearest 100 g.

140 In addition, we characterised each individual behavioural profiles using three  
141 behavioural traits, docility to capture, activity level and neophilia, at three different moments.  
142 We point out that, here, behavioural profiles do not refer to personality or behavioural  
143 syndromes, which would require repeated measures of each behavioural traits considered, and  
144 to partition phenotypic (co)variation at the among-individual versus residual levels, which was  
145 not possible to do with our data. First, docility was indexed during handling as follows:  
146 struggling (score of 1), not struggling (score of 0). This has been shown to be repeatable over  
147 time ( $r=0.26$ ) with a tendency to be heritable ( $h=0.17$ ) in roe deer [32]. The second trait,  
148 spontaneous daily activity [33] was measured using accelerometry data recorded at 20 Hz from  
149 tri axial accelerometers (Daily Diary tags, Wildbytes Ltd., Swansea University) mounted on  
150 animal collars. We calculated the Vectorial Dynamic Body Acceleration (VeDBA) metric [34],  
151 using a 2 second smoothing windows and the DDMT software (Wildbytes Ltd., Swansea  
152 University). VeDBA values were summed for each individual, date, and hour of the day ('total  
153 VeDBA') and averaged through the four weeks between the two capture events (period 2) to  
154 index daily activity. The third measured trait, neophobia, was defined as the avoidance of novel  
155 stimuli in the environment [33] and was assed using the difference in feeding efficiency with  
156 and without the presence of a novel object [35]. We calculated the ratio of the number of visits  
157 to the hut that resulted in a successful feeding bout (numerator) and the total number of visits  
158 to the hut (denominator). Measurements were repeated for five days for each condition (with  
159 and without novel object), and the difference in the ratio between the two conditions was  
160 calculated (see [35] for details). More neophobic individuals should be less inclined to feed  
161 during a given visit when a novel object is present, resulting in a higher score on the neophilia-  
162 neophobia continuum.



163 **Immune parameters measurement**

164 Blood samples were taken on EDTA and dry tubes. EDTA blood was preserved at 4°C and  
165 served to measure the total leukocyte concentration (white blood cell [WBC]) with an automat  
166 (Sysmex 2000iV, Sysmex). A differential cell count (neutrophil, basophil, eosinophil,  
167 lymphocyte and monocyte) was performed on the first 100 WBCs on Wright-Giemsa-stained  
168 blood smears [36]. To obtain concentrations of each leukocyte type, the total leukocyte count  
169 was multiplied by the proportion of each cell type. The serum, was obtained after blood  
170 centrifugation (1500 g for 15 min) and was stored at -20°C for subsequent measures of total  
171 proteins, using a refractometer, albumin and alpha-1, alpha-2, beta, and gamma globulins using  
172 electrophoresis on agarose gel. Haptoglobin, an alpha-2-globulin, was also measured by  
173 spectrophotometry (Konelab 30i PLC, Fisher Thermo Scientific) Circulating levels of natural  
174 antibodies (NAbs) were measured by a hemagglutination test (HA), that measures NAbs ability  
175 to agglutinate exogenous cells, while the complement activity was revealed by the ability of  
176 proteins to induce hemolysis (HL) [37, 38]. Finally, we quantified the level of anti-rabies  
177 antibody following the method described by Cliquet et al. [39]. We therefore measured 6  
178 markers of innate immunity (neutrophils, basophils, monocytes, eosinophils, hemagglutination  
179 and hemolysis titers), 4 inflammatory markers (haptoglobin, alpha-1, alpha-2 and beta-2  
180 globulins), and 3 markers of adaptive immunity (lymphocytes, gamma-globulins and anti-  
181 rabies antibodies).

182 **Extraction and quantification of FCMs**

183 FCMs were extracted following a methanol-based procedure and assayed using a group-specific  
184 11-oxoetiocholanolone enzyme immunoassay (EIA), as previously described [40] and  
185 validated for roe deer [41]. Measurements were carried out in duplicate (intra- and inter-assay  
186 coefficients of all samples were less than 10% and 15%, respectively).

## 187 **Ethical approval**

188 All applicable institutional and/or national guidelines for the care and use of animals were  
189 followed. The protocol was approved by the Ethical Committee 115 of Toulouse and was  
190 authorized by the French government (APAFIS#14706 – 12-11-2018).

## 191 **Statistical analyses**

### 192 **Relationship between behaviour and changes in immunity and baseline FCMs**

193 Changes in immunity ( $\Delta$  immunity) were calculated for each parameter as the difference  
194 between the measurements obtained at the two capture events. Similarly, changes in baseline  
195 FCM levels ( $\Delta$  glucocorticoids), were calculated as the difference of averaged baseline FCM  
196 levels between period 2 and period 1 for each individual. In addition, we used the behavioural  
197 scores at capture as an index of docility. Values did not differ between the two captures within-  
198 individual, except for one individual for which the score passed from 1 to 0. We chose to use  
199 values from the first capture in order to avoid a potential habituation effect for this individual.

200 Then, to test our hypotheses, we used Partial Least Square Path Modelling (PLS-PM)  
201 analysis [42]. This statistical analysis is particularly recommended when dealing with variables  
202 showing high correlation in order to avoid redundancies and high type I error [42]. Here we  
203 built the following blocks of variables, each being summarised by a latent variable:  $\Delta$   
204 glucocorticoids (1 variable),  $\Delta$  innate immunity (6 variables),  $\Delta$  adaptive immunity (3  
205 variables),  $\Delta$  inflammatory markers (4 variables), and behavioural profile (3 variables,  
206 electronic supplementary material table S1).

207 We then ran 3 PLS-PM analyses, each one evaluating the relationships between  
208 behavioural profile, change in baseline glucocorticoids, and change in 1) innate immunity, 2)  
209 adaptive immunity, and 3) inflammatory markers. For each of the three analyses, we built a

210 structural model (or inner model, i.e., describing relationships among latent variables) that  
211 consisted of three latent variables:  $\Delta$  glucocorticoids, behavioural profile, and  $\Delta$  immunity  
212 (innate, adaptive or inflammatory). The statement for the structural models was as follows:  
213 change in immunity depends on both behavioural profile and change in baseline  
214 glucocorticoids, which also depends on behavioural profile. Finally, in the measurement model  
215 (or outer model, i.e., relationships between observed and latent variables), the observed  
216 variables were considered as reflecting the corresponding latent variable (reflective mode),  
217 except for innate immunity where observed variables were considered as constituting the latent  
218 variable (formative mode). This option was chosen due to the high number of biomarkers used  
219 and the complexity and diversity of the biological actions of the innate immune system [43].  
220 This diversity is reflected in the moderate correlation among components of this latent variable.

221 We then ran PLS-PM analysis to adjust both the structural (figure 2) and measurement  
222 models, through multiple linear regressions. Lastly, we performed the diagnosis of each model  
223 (communality and redundancy are presented in table S2) following the recommendations of  
224 Gaston Sanchez [42]. The structural models were checked using  $R^2$ , redundancy index (ability  
225 to predict) and Goodness-of-fit index, a pseudo GoF measure that reflects the overall prediction  
226 power of the model ( $0 < \text{Gof} < 1$ ).

### 227 **Relationship between baseline FCMs throughout the experiment and behaviour**

228 In order to test the hypothesis that baseline FCM levels throughout the experiment (period 1 +  
229 period 2) should be higher in more docile, less active and more neophilic individuals (i.e., more  
230 reactive individuals), while controlling for other factors affecting FCM levels, we performed  
231 linear mixed-effects models (LMMs) on the 181 observations of FCM levels from 13  
232 individuals (14 repetitions per individuals with 1 missing value for one individual). FCM values  
233 were log transformed to achieve normality of model residuals. We analysed the overall

234 correlation pattern between docility, neophobia and activity using a normed Principal  
235 Component Analysis (PCA) and used scores from the first principal component (PC1) which  
236 indexed individual proactive-reactive gradient of behaviour (electronic supplementary material  
237 table S3, figure S3). We then built a reference model that included all biologically relevant  
238 variables to explain baseline glucocorticoids levels, and compared this model with all its sub-  
239 models. The reference model included: PC1, age of individuals, and Julian date of sampling.  
240 Individual identity and enclosure identity were included as random effects to avoid pseudo-  
241 replication issues [44] and to control for unexplained variance due to among-individual  
242 differences and among-enclosure variation.

243 The best models of variation in FCM levels were selected based on the second-order  
244 Akaike Information Criterion (AICc) [45]. Models with a difference in AICc ( $\Delta\text{AICc}$ ) > 2 units  
245 from the best model were considered to have less support [45]. In addition, we removed models  
246 within two AICc units of the top model that differed from a higher-ranking model by the  
247 addition of one or more parameters, as recommended [46]. In addition, we calculated AICc  
248 weights (AICcw) to measure the relative likelihood that a given model was the best among the  
249 set of fitted models. The normality of model residuals was tested (Shapiro-Wilk test) and  
250 visually assessed. Goodness-of-fit was assessed by conditional and marginal  $R^2$  values and  
251 standard residual plot techniques [47].

252 All analyses were carried out with R version 3.6.0 [48], using the lmer function from  
253 the lme4 package [49] and the plspm function from the plspm package [42].

## 254 **Results**

### 255 **Co-variation between behaviour and changes in immunity and baseline FCMs**

256 Among the 13 individuals considered in our study, 9 showed a decrease in baseline FCM levels  
257 during period 2 compared to period 1 (ranging from -786 to -8 ng/g of wet faeces), while 4  
258 showed an increase (ranging from 93 to 346 ng/g). In addition, for each individual, vaccination  
259 increased the level of anti-rabies antibody, but large among-individual differences were  
260 observed, with values ranging from +0.60 to +41.50 IU, with a median of +10.39. Proactive  
261 individuals were characterised by high daily activity levels, lack of docility and neophobia  
262 (table S3). In addition, the 13 individuals appeared to be homogeneously distributed along the  
263 gradient ranging from proactive to reactive behavioural profiles as showed by the PC1 axis  
264 scores ranging from -1.92 to 2.66, with a median value of 0.20 (figure S3).

265 Our analyses revealed links between behavioural profiles and changes in the three  
266 studied aspects of immunity (table 1). Individuals that exhibited more proactive behaviour,  
267 expressed by high daily activity levels, lack of docility and neophobia, showed an overall strong  
268 decrease in innate ( $r = -0.53$ ;  $P < 0.05$ ; figure 2), adaptive ( $r = -0.80$ ;  $P < 0.001$ ; figure 2), and  
269 inflammatory ( $r = -0.77$ ;  $P < 0.005$ ; figure 2) markers of immunity. However, the weights of  
270 observed variables in the definition of latent variables differed according to the analysis. When  
271 analysing  $\Delta$  adaptive immunity, gamma globulins, lymphocytes and anti-rabies antibodies  
272 contributed similarly to the latent variable (weights [w] of 0.37; 0.52 and 0.54 respectively),  
273 while behavioural profile was essentially represented by docility and neophobia ( $w = 0.65$  and  
274 0.64 respectively). On the opposite, for  $\Delta$  inflammatory markers, behavioural profile was  
275 largely represented by mean daily activity levels ( $w = 1.05$ ) and less by docility ( $w = -0.32$ ) and  
276 neophobia ( $w = 0.12$ ), while markers of inflammation contributed overall to the same  
277 proportion to their latent variable (table 2). Lastly, for  $\Delta$  innate immunity, behavioural traits  
278 contributed to the same extent to their latent variable (table 2). It is also important to note that  
279 among innate immune parameters, neutrophils were correlated negatively to other biomarkers  
280 (see the negative loading in table 2), thus the negative relationship between behavioural profiles

281 and innate immunity only occurred for these markers, while temporal changes in neutrophil  
282 concentrations were actually positively linked to activity, neophobia and lack of docility.

283 Changes in baseline glucocorticoid levels were associated with changes in innate ( $r =$   
284  $0.61$ ;  $P < 0.05$ ) and adaptive immunity (tendency:  $r = 0.37$ ;  $P < 0.06$ ), but not inflammatory  
285 markers (table 1; figure 2). Individuals that underwent an increase in baseline FCM levels  
286 between periods also exhibited an increase in both innate and adaptive immunity. However, as  
287 pointed out above, this positive relationship means that individuals exhibiting an increase in  
288 baseline FCM levels actually had a decrease in neutrophil concentration.

289 Finally, the relationship between behavioural profile and change in baseline FCM levels  
290 was non-significant for all three models (table 1).

### 291 **Co-variation between baseline FCM levels throughout the experiment and** 292 **behaviour**

293 According to the model selection procedure, the best model describing among-individual  
294 differences in baseline FCMs throughout the experiment in relation to individual behavioural  
295 profiles included PC1 score and period of the experimental protocol (table S4). Specifically,  
296 roe deer that exhibited a more reactive behavioural profile (low daily activity levels, docility  
297 and neophilia) also exhibited higher baseline FCM levels throughout the experiment compared  
298 to roe deer exhibiting a more proactive behavioural profile ( $0.103$ ;  $P < 0.005$ ; table 3; figure 3).  
299 In addition, baseline FCM levels decreased during the second part of the experimental protocol  
300 compared to the first one ( $-0.1663$ ;  $P < 0.05$ ; table 3), meaning that the mean baseline FCM  
301 level of the studied roe deer population was lower during period 2 than during period 1. Age  
302 and Julian date did not increase the fit of the model.

## 303 **Discussion**

304 In this study, we used an experimental approach to gain a better understanding on how changes  
305 in baseline glucocorticoid levels may affect simultaneous changes in immune parameters of the  
306 innate, adaptive and inflammatory markers of immunity, at the scale of few weeks (8 weeks).  
307 Our results demonstrated that an increase in baseline FCM levels was associated with an  
308 increase in immune parameters of the innate and adaptive arms, but not in inflammation.  
309 Secondly, we tested whether behavioural profiles could influence the co-variation between  
310 changes in immune parameters and baseline glucocorticoid levels. As predicted, behavioural  
311 profiles appeared to be strongly linked to changes in overall immunity, but also to baseline  
312 glucocorticoid levels throughout the experiment, while there were not related to changes in  
313 baseline glucocorticoids between the two periods of the study.

314 An increase in baseline glucocorticoid levels between the two periods of the study was  
315 generally related to an increase in innate immunity, except for neutrophil concentrations, which  
316 decreased as glucocorticoid levels increased. The observed negative relationship with  
317 neutrophils is consistent with the previous finding of an immunosuppressive effect of long-term  
318 elevation of glucocorticoids on immunity [11, 13]. However, the overall increase in innate  
319 immunity was unexpected and contrary to our predictions. One of the assumptions underlying  
320 the hypothesis of a negative relationship between immunity and glucocorticoids is the energy  
321 cost of immunity, which should subsequently trade-off against other energy demanding  
322 functions [2]. However, it is important to note that in our studied population, resources are not  
323 limiting and roe deer are not exposed to unpredictable variations in food resources. This  
324 particular context may diminish our ability to detect trade-offs between energy demanding  
325 functions. Alternatively, it has been proposed that, as the main function of the stress response  
326 is to recover from stressors, a decrease in immunity should not necessarily occur when  
327 glucocorticoids increase, as it could improve survival [11].

328           While the above hypothesis may partly explain the link between change in innate  
329 immunity and change in baseline glucocorticoids, it does not explain the difference observed  
330 between neutrophils and other biomarkers of innate immunity. Neutrophils are part of the  
331 cellular immunity and reflect acute inflammatory response while monocytes reflect chronic  
332 inflammatory response, and hemagglutination and hemolysis are both part of the humoral innate  
333 response [43]. Basophils are particularly secreted in presence of ticks [50] that are frequently  
334 encountered in the experimental facility (unpublished data). Finally, eosinophils are known to  
335 specifically bridge innate and adaptive immunity [43]. Considering the differences in the  
336 functions of these biomarkers, it is likely that they are not all linked to glucocorticoids in the  
337 same way. This could explain that we did not detect any trade-off, and that positive relationship  
338 between changes in glucocorticoid levels and innate immunity may occur (e.g., for basophils in  
339 the presence of ticks).

340           With respect to a change in the adaptive arm of the immune system, our results did not  
341 support the finding of the immunosuppressive effect of long-term elevation of glucocorticoids,  
342 but instead showed that adaptive immune parameters increased in individuals that showed an  
343 increase in baseline FCM levels. A first possible explanation of this result could be linked to  
344 the transient increase in glucocorticoids that occurred during the first capture, where  
345 vaccination was done. Previous work showed that a short-term elevation in glucocorticoids at  
346 the time of vaccination reinforces the efficacy of vaccination [13]. A similar scenario may have  
347 occurred in our study, and the stress of capture, resulting in a sudden increase in circulating  
348 glucocorticoids, would have strengthened the immune response to anti-rabies vaccine. This  
349 would explain our result, at least in part, if there is a correlation between the change observed  
350 in baseline FCM levels and the stress-induced increase in glucocorticoids. Another possible  
351 explanation is that the energy cost of mounting an antibody response is too low [27] for a trade-  
352 off between antibody expression and other functions to be detectable. The positive relationship



353 we observed in our study also supports the pace-of-life syndrome hypothesis, according to  
354 which individuals with higher baseline glucocorticoid levels should show stronger investment  
355 in immunity than those with lower baseline glucocorticoid levels [23].

356 Overall, individuals that underwent a decrease in baseline glucocorticoids may have  
357 switched their investment away from the immune system, possibly toward another energy  
358 demanding function. We suggest that such a switch could be underpinned by a plastic response  
359 to the stressful event of capture, leading to an adjustment of the individuals' life history  
360 strategies and change in investment between functions supporting either long-term survival or  
361 current reproduction. This would be in accordance with the pace-of-life syndrome hypothesis,  
362 where a positive association is expected between glucocorticoid levels and immunity, with  
363 higher levels in individuals favouring their long-term survival, while lower levels are expected  
364 in individuals favouring reproduction and growth [23].

365 Regarding the association between behavioural profiles and change in immunity, we  
366 found an overall similar association for innate, adaptive and inflammatory immunity. Precisely,  
367 individuals showing a propensity to be active, non-docile to manipulation and neophobic  
368 showed a decreased investment in their immune system following the first capture event. This  
369 result is consistent with our predictions and supports the hypothesis that fast-living individuals  
370 should have a proactive behavioural profile and a low investment in immune functions that  
371 would allow them to favour immediate reproduction over survival [23]. On the opposite,  
372 individuals showing more reactive behavioural profiles are supposed to have a slower pace-of-  
373 life and are expected to favour functions enhancing survival and future reproduction [23].  
374 Stronger investment in immunity is thus expected for these individuals as they are more likely  
375 to face repeated encounter with the same pathogens.

376 Finally, we investigated whether behavioural profiles could be associated with among-  
377 individual variations in baseline glucocorticoid levels throughout the experiment, and also with  
378 changes in baseline glucocorticoid levels over an 8-week period. Our results did not provide  
379 any support for the latter, but instead supported the former. Specifically, individuals exhibiting  
380 proactive behavioural profiles also showed lower baseline glucocorticoid levels throughout the  
381 experiment compared to individuals exhibiting more reactive behavioural profiles. This result  
382 supports previous studies on wild [51] and captive [29] roe deer. It is also in accordance with  
383 the coping style framework [22] and the pace-of-life syndrome hypothesis [23], which states  
384 that behavioural and physiological responses to stressful situations are correlated. The  
385 difference we observed in our results may thus suggest that activity (reflected by baseline  
386 levels) and reactivity (reflected by the changes following the first capture event) of the HPA  
387 axis may not be associated in the same manner with among-individual variations in behaviour  
388 [22].

389 Overall, individual roe deer responded differently to our protocol, with some individuals  
390 showing an increase in baseline glucocorticoid levels, while others showed a decrease. In  
391 addition, our results suggest that increased baseline glucocorticoid levels are associated with a  
392 re-allocation of energy resources to innate and adaptive immunity in individuals with more  
393 reactive behaviours (see figure 4 for a summary of the outcomes of the protocol). We suggest  
394 that the observed association between immunity and baseline glucocorticoid levels is associated  
395 with different life-history strategies and underpinned by energetic trade-offs between functions  
396 enhancing survival, reproduction and growth, which would be congruent with the pace-of-life  
397 syndrome hypothesis [23].

398 Finally, considering that all of our results tend to support a co-variation between stress  
399 hormones, immunity and behaviour, we recommend that future work goes one step further and  
400 investigates how among-individual variations in behaviour could modulate the variation of

401 glucocorticoid levels, as well as the relationship between glucocorticoid hormones and  
402 immunity. The extent to which co-variations between these traits are influenced by different  
403 life history strategies in the wild also warrants further investigations as it may help to  
404 understand the large and unexplained among-individual variability in the glucocorticoids-  
405 immunity relationship observed in wildlife studies.

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412 experimental station.

## 413 **Author contributions**

414 JC, BRey, EGF and HV conceived and designed the study. JC, HV, MLB, NC, JLR, GLL  
415 undertook the fieldwork. LB performed the VeDBA analysis. RP performed the FCMs analysis.  
416 MW quantified anti-rabies circulating antibodies. TL, BR and EGF performed the  
417 immunological analysis. CM designed and performed the neophobia analysis. JC performed the  
418 statistical analysis, wrote the first draft of the paper and then received input from all other co-  
419 authors. All authors approved the final version of the manuscript and agree to be held  
420 accountable for the content therein.

## 421 **Data accessibility**

422 All data and code used in this analysis are available as electronic supplementary material S5.

## 423 **Competing interests**

424 The authors declare that they have no conflict of interest.

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579 **Table 1. Characteristics of the partial least squares path modelling analyses to explain the**  
 580 **relationships between behavioural profiles, change in baseline glucocorticoid levels, and**  
 581 **change in innate, adaptive and inflammatory markers of immunity.** GoF indicates the  
 582 goodness of fit of the model. SE stands for Standard Error. See text for definition of the  
 583 observed variables that composed each latent variable. Variables in bold represent endogenous  
 584 variables.

Parameter	Estimate	SE	t value	P value
Structural model for innate immunity biomarkers (GoF: 0.32)				
<b>FCMs</b>				
Intercept	2.4 e-17	0.301	7.98 e-17	1.00
Behavioural profile	0.055	0.301	0.183	0.86
<b>Innate immunity</b>				
Intercept	-4.56 e-17	0.197	-2.32 e-16	1.00
Behavioural profile	-0.53	0.197	-2.70	0.02
FCMs	0.61	0.197	3.08	0.01
Structural model for adaptive immunity biomarkers (GoF: 0.42)				
<b>FCMs</b>				
Intercept	1.15 e-17	0.299	3.84 e-17	1.00
Behavioural profile	0.127	0.299	0.424	0.68
<b>Adaptive immunity</b>				
Intercept	3.43 e-17	0.172	2.00 e-16	1.00
Behavioural profile	-0.801	0.173	-4.63	0.001
FCMs	0.373	0.173	2.16	0.057
Structural model for inflammatory biomarkers (GoF: 0.39)				
<b>FCMs</b>				
Intercept	1.41 e-16	0.294	4.79 e-16	1.00
Behavioural profile	0.226	0.294	0.183	0.46
<b>Inflammatory markers</b>				
Intercept	3.64 e-16	0.192	1.90 e-15	1.00
Behavioural profile	-0.770	0.197	-3.91	0.003
FCMs	0.089	0.197	0.454	0.660

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587 **Table 2. Characteristics of the observed variables that composed each latent variable in**  
 588 **the three partial least squares path modelling analyses to explain the relationships**  
 589 **between behavioural profiles, change in baseline glucocorticoid levels, and change in**  
 590 **innate, adaptive and inflammatory immunity.** Weight represents the contribution of the  
 591 variable to the latent variable, and loadings indicate the direction of the correlation between the  
 592 observed variables and their latent variable. Communality indicates the amount of variability  
 593 in an observed variable that is captured by its latent variable, and particularly apply for  
 594 reflective latent variables. Redundancy indicates the ability to predict for a given observed  
 595 variable, and particularly apply for formative latent variables. See text for definition of the  
 596 observed variables composing each latent variable. Variables in bold represent endogenous  
 597 variables.

<b>Parameter</b>	<b>Weight</b>	<b>Loading</b>
Outer model for innate immunity biomarkers		
<b>Behavioural profile</b>		
Lack of docility	0.521	0.847
Neophobia	0.434	0.753
Activity	0.358	0.648
<b>Glucocorticoids</b>		
FCMs	1.00	1.00
<b>Innate immunity</b>		
Neutrophils	-0.324	-0.686
Eosinophils	0.396	0.495
Monocytes	-0.533	0.274
Basophils	0.843	0.416
Hemagglutination	0.662	0.435
Hemolysis	0.245	0.367
Outer model for adaptive immunity biomarkers		
<b>Behavioural profile</b>		
Lack of docility	0.551	0.824
Neophobia	0.637	0.877
Activity	-0.041	0.306
<b>Glucocorticoids</b>		
FCMs	1.00	1.00
<b>Adaptive immunity</b>		
Gamma globulins	0.365	0.581
Lymphocytes	0.523	0.754
Anti-rabies antibody	0.543	0.725
Outer model for inflammatory immunity biomarkers		
<b>Behavioural profile</b>		
Lack of docility	-0.319	0.115
Neophobia	0.115	0.215
Activity	1.051	0.962

<b>Glucocorticoids</b>		
FCMs	1.00	1.00
<b>Inflammatory immunity</b>		
Alpha-1 globulins	0.360	0.936
Alpha-2 globulins	0.279	0.618
Beta-globulins	0.217	0.456
Haptoglobin	0.456	0.859

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614 **Table 3. Characteristics of the selected linear mixed-effect models for explaining variation**  
615 **in baseline FCM levels (log-transformed) in the roe deer population of Gardouch.** The  
616 effect of PC1 (behavioural profile ranging from proactive behavioural profiles to reactive  
617 behavioural profiles), age of individuals, period of sample collection, and Julian date of sample  
618 collection were fitted. Models included individual identity and enclosure number as random  
619 effects.  $R^{2m}$  and  $R^{2c}$  are the marginal and conditional explained variance of the models,  
620 respectively. SE stands for Standard Error. See text for definition of model sets.

<b>Parameter</b>	<b>Estimate</b>	<b>SE</b>	<b>t-value</b>	<b>P-value</b>
( $R^{2m}$ : 0.06 ; $R^{2c}$ : 0.27)				
Intercept	6.638	0.141	47.10	< 0.001
Behavioural profile (PC1)	0.103	0.036	2.849	< 0.005
Period (2)	-0.163	0.080	-2.045	< 0.05

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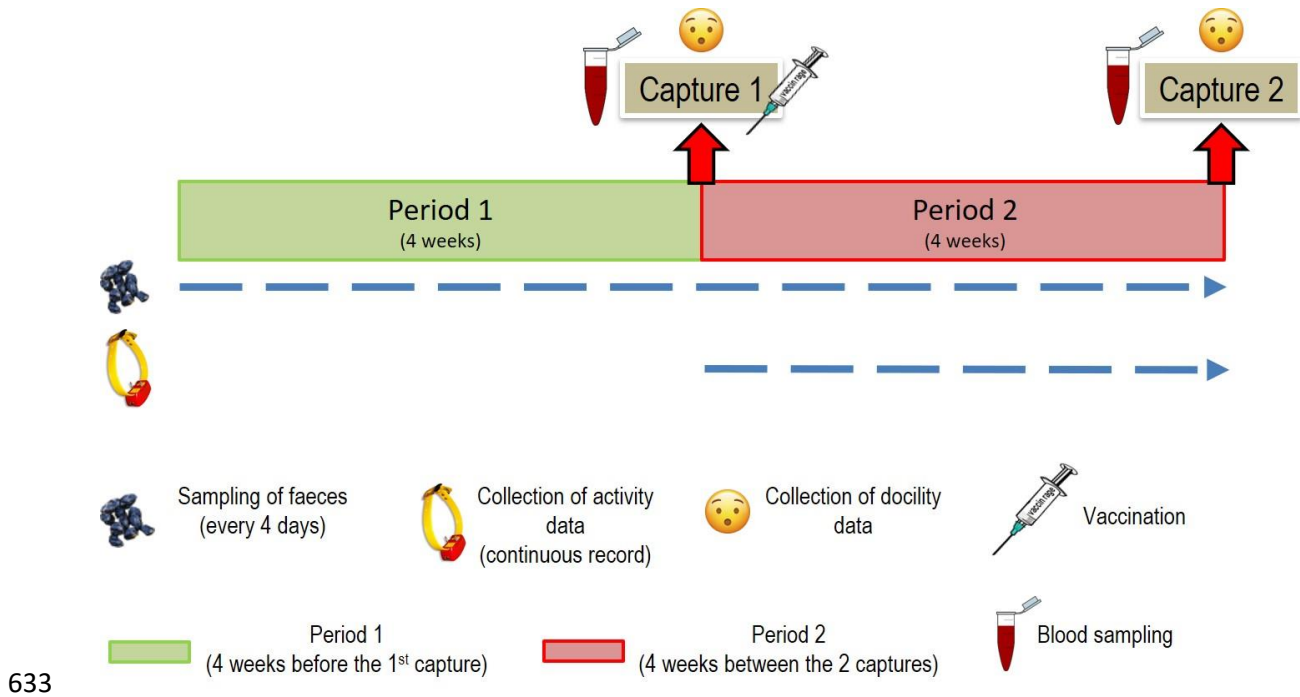
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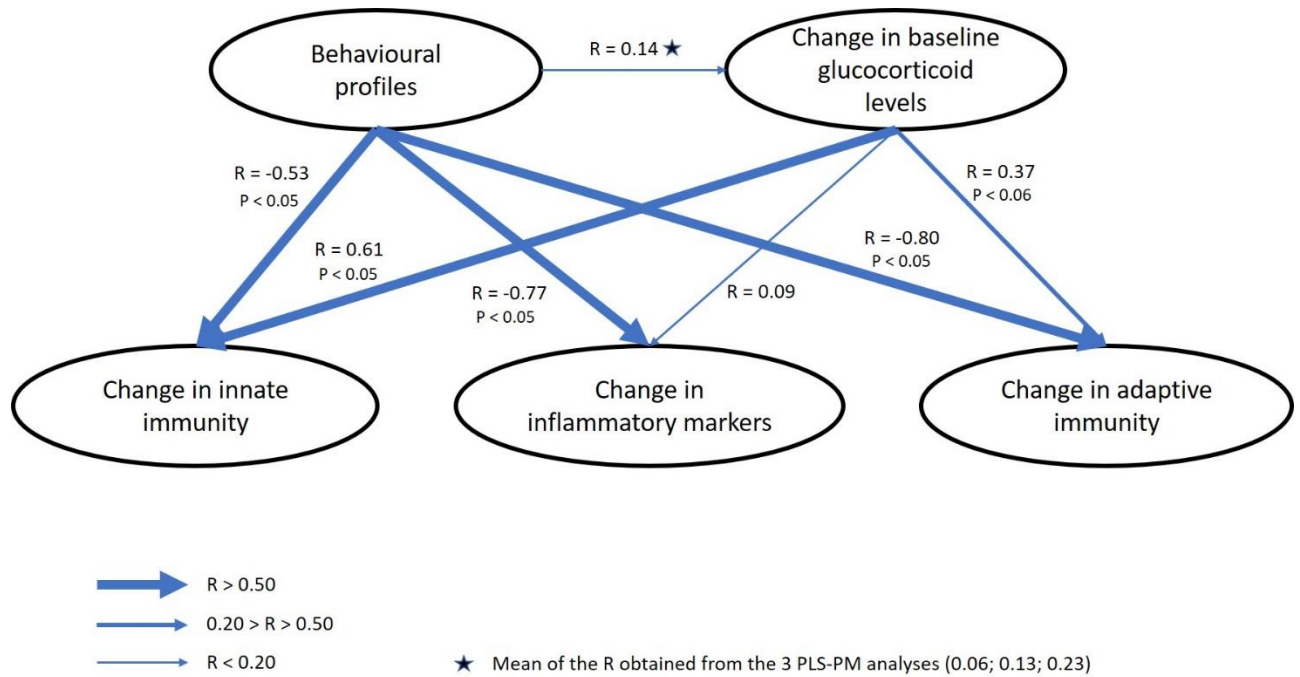
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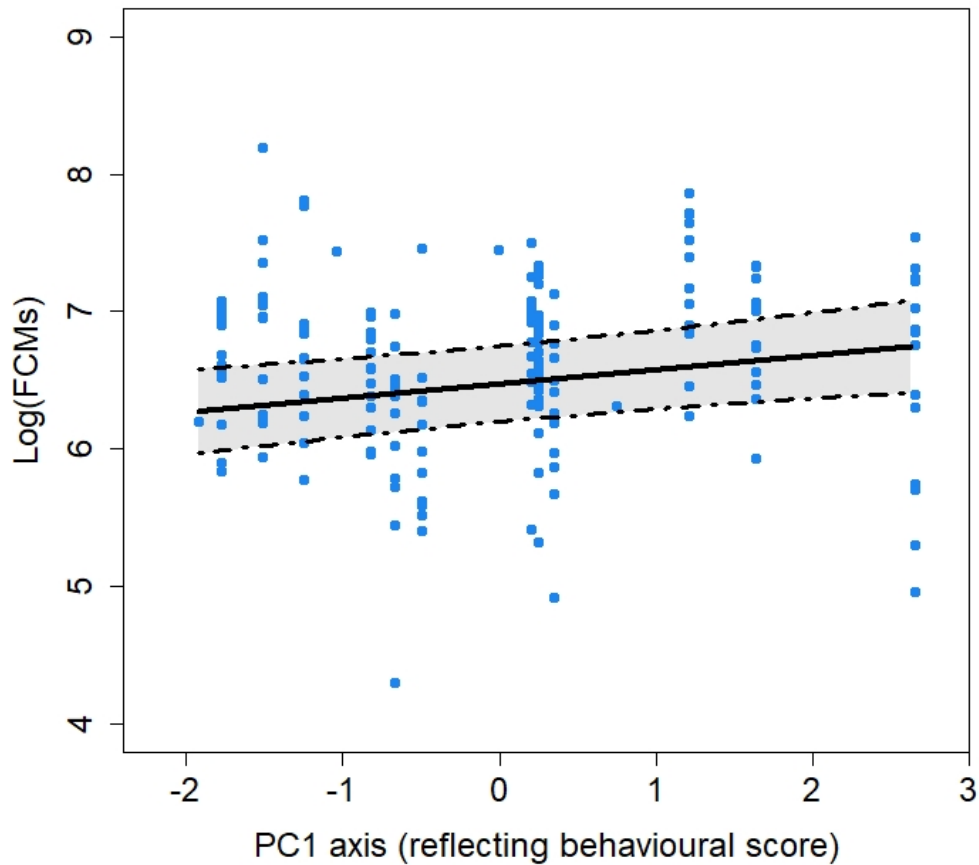


**Figure 1. Summary of the experimental design.** Data relative to the assessment of neophobia scores were obtained prior to this protocol (in February 2015 for all individuals, except two that were assessed for neophobia in February 2018, following the same protocol, see details in [40]).



**Figure 2. Structural models of relationships among behavioural profiles, change in baseline glucocorticoid levels and change in immunity as determined by the partial least squares path modelling analyses.** Arrows indicates the direction of effect and the thickness of arrows indicates the strength of the correlation between latent variables. Absence of p-value (P) indicate the relationship was not significant.





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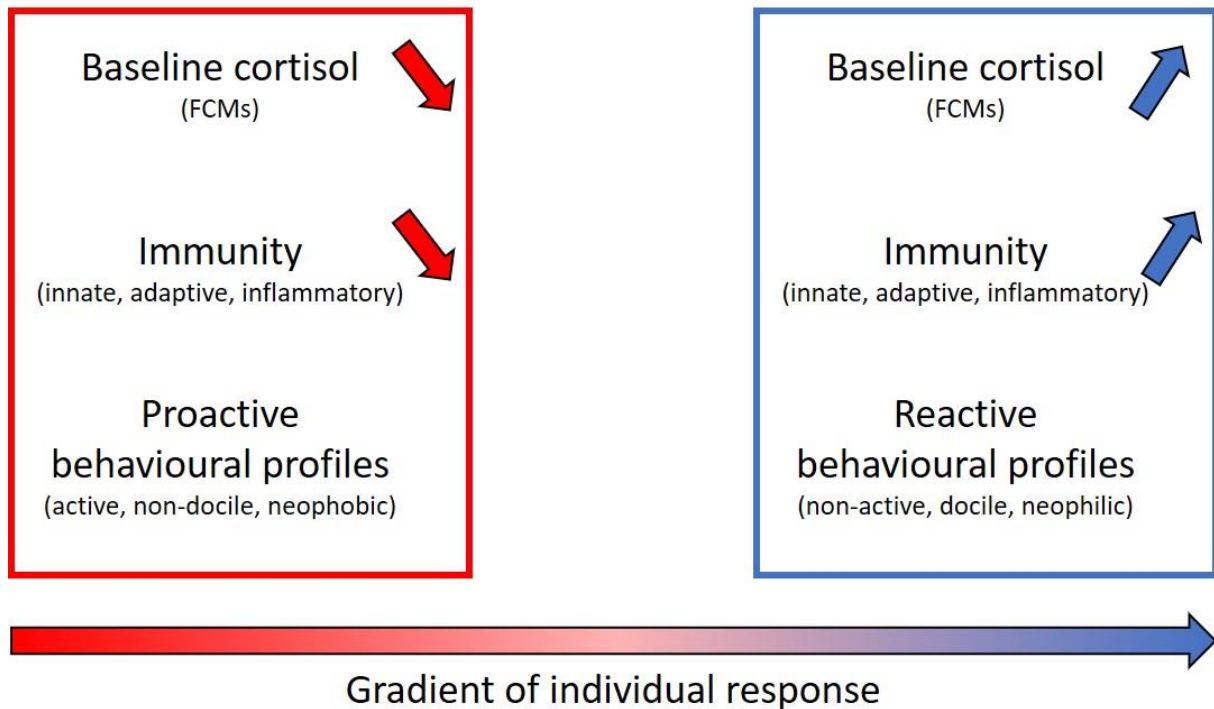
660 **Figure 3. Relationship between baseline FCMs level (log-transformed) and behavioural**  
661 **profiles.** Behavioural profiles' scores correspond to the score for the first axis (PC1) of the PCA  
662 conducted using docility, activity and neophilia as co-variables. The three variables were all  
663 negatively correlated with PC1. Thus, this axis represents a gradient of behavioural profiles,  
664 with negative values indicating proactive behavioural profiles (high activity levels, neophobia,  
665 and lack of docility), and positive values indicating reactive behavioural profiles (low activity  
666 levels, neophilia, and docility). Points represent observed values, lines represent model  
667 predictions and dashed lines represent the 95% confidence interval.

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## Different outcomes following the stress and immune challenges



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672 **Figure 4. Summary of the observed outcomes and relationships between the latent**  
673 **variables considered in our study.** Our protocol (see details in the main text) resulted in  
674 different outcomes, with part of the individuals showing an increase in baseline cortisol between  
675 the two study periods (indicated by an increase in FCMs), while others showed a decrease.  
676 Individuals that showed more reactive behavioural profiles (indicated by low activity levels,  
677 docility to manipulation, and neophilia) also exhibited an increase in baseline cortisol levels,  
678 and an increase in immunity (both innate and adaptive immunity), while the opposite occurred  
679 for individuals that showed more proactive behavioural profile.