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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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21 Abstract

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

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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 fast and non-specific) and adaptive (slower at first encounter, but more long-lasting and 46 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 corticosterone) are metabolic hormones that play a major role in the regulation of energy use 50 51 [5, 6] and may therefore underlie such trade-offs.

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

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

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

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

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

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

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

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

119 Experimental design

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

135 Capture protocol and data collection

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

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.

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

163

Immune parameters measurement

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

182

Extraction and quantification of FCMs

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

187 Ethical approval

All applicable institutional and/or national guidelines for the care and use of animals were
followed. The protocol was approved by the Ethical Committee 115 of Toulouse and was
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 (Δ 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 (Δ 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.

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

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

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

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

227

Relationship between baseline FCMs throughout the experiment and behaviour

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

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

The best models of variation in FCM levels were selected based on the second-order 243 Akaike Information Criterion (AICc) [45]. Models with a difference in AICc (Δ AICc) > 2 units 244 from the best model were considered to have less support [45]. In addition, we removed models 245 within two AICc units of the top model that differed from a higher-ranking model by the 246 addition of one or more parameters, as recommended [46]. In addition, we calculated AICc 247 weights (AICcw) to measure the relative likelihood that a given model was the best among the 248 249 set of fitted models. The normality of model residuals was tested (Shapiro-Wilk test) and visually assessed. Goodness-of-fit was assessed by conditional and marginal R² values and 250 standard residual plot techniques [47]. 251

All analyses were carried out with R version 3.6.0 [48], using the lmer function from 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

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

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

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

According to the model selection procedure, the best model describing among-individual 293 differences in baseline FCMs throughout the experiment in relation to individual behavioural 294 profiles included PC1 score and period of the experimental protocol (table S4). Specifically, 295 roe deer that exhibited a more reactive behavioural profile (low daily activity levels, docility 296 and neophilia) also exhibited higher baseline FCM levels throughout the experiment compared 297 298 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 level of the studied roe deer population was lower during period 2 than during period 1. Age 301 302 and Julian date did not increase the fit of the model.

303 **Discussion**

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

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

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

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

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

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

Regarding the association between behavioural profiles and change in immunity, we 365 found an overall similar association for innate, adaptive and inflammatory immunity. Precisely, 366 individuals showing a propensity to be active, non-docile to manipulation and neophobic 367 showed a decreased investment in their immune system following the first capture event. This 368 369 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]. Stronger investment in immunity is thus expected for these individuals as they are more likely 374 375 to face repeated encounter with the same pathogens.

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

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

Finally, considering that all of our results tend to support a co-variation between stress hormones, immunity and behaviour, we recommend that future work goes one step further and 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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413 Author contributions

JC, BRey, EGF and HV conceived and designed the study. JC, HV, MLB, NC, JLR, GLL undertook the fieldwork. LB performed the VeDBA analysis. RP performed the FCMs analysis. MW quantified anti-rabies circulating antibodies. TL, BR and EGF performed the immunological analysis. CM designed and performed the neophobia analysis. JC performed the statistical analysis, wrote the first draft of the paper and then received input from all other coauthors. All authors approved the final version of the manuscript and agree to be held 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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Table 1. Characteristics of the partial least squares path modelling analyses to explain the relationships between behavioural profiles, change in baseline glucocorticoid levels, and change in innate, adaptive and inflammatory markers of immunity. GoF indicates the goodness of fit of the model. SE stands for Standard Error. See text for definition of the observed variables that composed each latent variable. Variables in bold represent endogenous variables.

Parameter	Estimate	SE	t value	P value		
Structural model for innate immunity biomarkers (GoF: 0.32)						
FCMs						
Intercept	2.4 e-17	0.301	7.98 e-17	1.00		
Behavioural profile	0.055	0.301	0.183	0.86		
Innate immunity						
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	Structural model for adaptive immunity biomarkers (GoF: 0.42)					
FCMs						
Intercept	1.15 e-17	0.299	3.84 e-17	1.00		
Behavioural profile	0.127	0.299	0.424	0.68		
Adaptive immunity						
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)						
FCMs						
Intercept	1.41 e-16	0.294	4.79 e-16	1.00		
Behavioural profile	0.226	0.294	0.183	0.46		
Inflammatory markers						
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		

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

Parameter	Weight	Loading	
Outer model for inna	ate immunity biomarkers		
Behavioural profile			
Lack of docility	0.521	0.847	
Neophobia	0.434	0.753	
Activity	0.358	0.648	
Glucocorticoids			
FCMs	1.00	1.00	
Innate immunity			
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 adapt	tive immunity biom	arkers	
Behavioural profile			
Lack of docility	0.551	0.824	
Neophobia	0.637	0.877	
Activity	-0.041	0.306	
Glucocorticoids			
FCMs	1.00	1.00	
Adaptive immunity			
Gamma globulins	0.365	0.581	
Lymphocytes	0.523	0.754	
Anti-rabies antibody	0.543	0.725	
Outer model for inflamm	natory immunity bio	omarkers	
Behavioural profile			
Lack of docility	-0.319	0.115	
Neophobia	0.115	0.215	
Activity	1.051	0.962	

Glucocorticoids		
FCMs	1.00	1.00
Inflammatory immunity		
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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Table 3. Characteristics of the selected linear mixed-effect models for explaining variation in baseline FCM levels (log-transformed) in the roe deer population of Gardouch. The effect of PC1 (behavioural profile ranging from proactive behavioural profiles to reactive behavioural profiles), age of individuals, period of sample collection, and Julian date of sample collection were fitted. Models included individual identity and enclosure number as random effects. R^{2m} and R^{2c} are the marginal and conditional explained variance of the models, respectively. SE stands for Standard Error. See text for definition of model sets.

Parameter	Estimate	SE	t-value	P-value
$(R^{2m}: 0.06; R^{2c}: 0.27)$				
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



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]).
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647 Figure 2. Structural models of relationships among behavioural profiles, change in baseline glucocorticoid levels and change in immunity as determined by the partial least 648 squares path modelling analyses. Arrows indicates the direction of effect and the thickness of 649 arrows indicates the strength of the correlation between latent variables. Absence of p-value (P) 650 indicate the relationship was not significant. 651 652 653 654 655 656 657 658



Figure 3. Relationship between baseline FCMs level (log-transformed) and behavioural 660 profiles. Behavioural profiles' scores correspond to the score for the first axis (PC1) of the PCA 661 662 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, with negative values indicating proactive behavioural profiles (high activity levels, neophobia, 664 and lack of docility), and positive values indicating reactive behavioural profiles (low activity 665 levels, neophilia, and docility). Points represent observed values, lines represent model 666 predictions and dashed lines represent the 95% confidence interval. 667

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Different outcomes following the stress and immune challenges





Gradient of individual response

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