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

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The biomedical literature has consistently highlighted that long-term elevation of glucocorticoids might impair immune functions. However, patterns are less clear in wild animals. Here, we re-explored the stress–immunity relationship considering the potential effects of behavioural profiles. Thirteen captive roe deer (*Capreolus capreolus*) were monitored over an eight-week period encompassing two capture events. We assessed how changes in baseline faecal cortisol metabolite (FCM) concentrations following a standardized capture protocol and an immune challenge using anti-rabies vaccination affected changes in 13 immune parameters of innate and adaptive immunity, and whether these changes in baseline FCM levels and immune parameters related to behavioural profiles. We found that individuals with increased baseline FCM levels also exhibited increased immunity and were characterized by more reactive behavioural profiles (low activity levels, docility to manipulation and neophilia). Our results suggest that the immunity of large mammals may be influenced by glucocorticoids, but also behavioural profiles, as it is predicted by the pace-of-life syndrome hypothesis. Our results highlight the need to consider covariations between behaviour, immunity and glucocorticoids in order to improve our understanding of the among-individual variability in the stress–immunity relationships observed in wildlife, as they may be underpinned by different life-history strategies.

1. Introduction

The immune system is one of the most important mechanisms in vertebrates for improving survival. This complex system is composed of two complementary arms, innate (relatively fast and non-specific) and adaptive (slower at first encounter, but more long-lasting and specific) immunity, each composed of numerous cells and effectors [1]. This system, however, is not cost-free [1,2], suggesting trade-offs between immune defences and other functions that 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 [5,6] and may therefore underlie these 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, activation of the hypothalamic–pituitary–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 have investigated the relationship between stress and immunity, particularly in the biomedical domain where it has generally been shown that short-term elevation of glucocorticoids (i.e. a few minutes to a few hours) stimulates immune functions [12,13], whereas chronically elevated glucocorticoid levels are immunosuppressive [9,14]. Focusing on long-term elevation of glucocorticoids (i.e. a few days to a few months), studies in wildlife have shown mixed results ranging from decreased, increased or no change in immune functions with chronic glucocorticoid elevation [15–18]. Evidence is also accumulating 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-cell mediated or constitutive immunity) might not be affected [19,20].

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

In the present study, we investigated the relationships between long-term stress 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 metabolite (FCM) levels and variations in thirteen adaptive and innate immune parameters, before and after a standardized capture stress protocol associated with an immune challenge using anti-rabies vaccination, in captive roe deer (*Capreolus capreolus*). Vaccination was performed to provide a standardized immune challenge with good safety, and rabies vaccination was elected to mimic a never-encountered but highly immunogenic antigen, with the possibility of measuring specific antibody production using standardized procedures. In addition, we evaluated how these changes may be related to behavioural profiles, as characterized by three commonly used behavioural traits: docility, neophobia and activity levels.

We expected that (i) changes in baseline FCM levels between the two observation periods (before/after capture) would be negatively linked to changes in innate immune parameters, and (ii) changes in baseline FCM levels should be less related to adaptive than innate immune parameters and inflammatory markers, due to the relatively low cost of adaptive immunity [27]. We also expected that (iii) baseline FCM levels as well as variations in baseline FCM levels should be related to individual behavioural profiles [22,28], with higher baseline levels and higher increase in baseline levels in more docile, less active and more neophilic individuals (i.e. more reactive individuals). Finally, we predicted that (iv) the increase in immune parameters between the two observation periods should be greater for the most reactive individuals, which are expected to invest more in overall immunity [23,26].

2. Material and methods

(a) Study site

The study was conducted on a captive population of roe deer living in the Gardouch research station, located in southwest of France. The station is owned and managed by the French Research Institute for Agriculture, Food and Environment (INRAE). It consists of 12 enclosures of 0.5 ha with meadow, each containing between one and 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 since birth or their first year of life. All showed some degree of habituation to humans but expressed normal behavioural responses (e.g. vigilance and escape) to stressful situations.

(b) Experimental design

The experimental procedure was carried out between mid-September and mid-November 2018 and is summarized in figure 1. During period 1, to assess baseline glucocorticoid level of each individual, we collected faeces every 4 days during four weeks and measured FCM concentrations. Faeces were collected immediately after defecation was observed and kept at +4°C for a maximum of 1 h before being stored at −20°C until steroid analysis. At the end of period 1, each roe deer was subjected to a standardized capture stress protocol involving restrained immobilization [30]. During immobilization, we collected faeces from the rectum, collected blood samples and fitted collars equipped with tri-axial accelerometers (see next section for details). We also injected an inactivated, adjuvanted rabies glycoprotein vaccine (Rabisin, Merial, France, 1 ml) subcutaneously. Collection of faecal samples was then continued every 4 days for an additional four weeks (i.e. period 2), as described above, to access the effect of

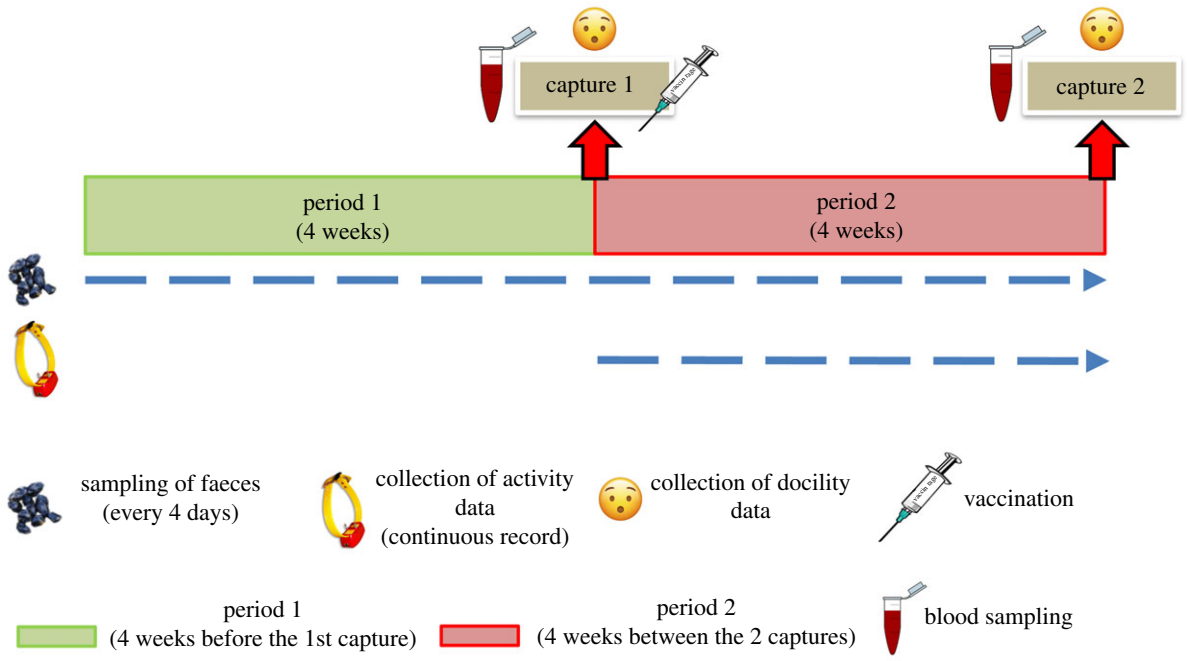

Figure 1. Summary of the experimental design. Data relative to the assessment of neophobia scores were obtained prior to this protocol (in February 2015 for all individuals, except two that were assessed for neophobia in February 2018, following the same protocol; see details in [29]). (Online version in colour.)

capture on baseline glucocorticoid level for each individual. Period 2 started 2 days after capture 1, in order to avoid measuring the acute increase in glucocorticoid level due to capture [31]. At the end of period 2, roe deer were recaptured following the same procedure (capture 2) and faeces and blood were collected again.

(c) Capture protocol and data collection

Roe deer were directed into their hut by slowly approaching them and then pushed through a trap door into a retention box. Once in the box, animals were tranquilized with an intramuscular injection of acepromazine (Calmivet, Vetoquinol, France; targeted dose of 0.075 mg kg^{-1}) [32]. Individuals were weighed with an electronic balance to the nearest 100 g.

In addition, we characterized the behavioural profiles of each individual using three behavioural traits, docility during capture, activity level and neophilia, at three different times. We point out that, here, behavioural profiles do not refer to personality or behavioural syndromes, which would require repeated measures of each behavioural traits considered, and to partition phenotypic (co)variation at the among-individual versus residual levels, which was 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 time ($r = 0.26$) with a tendency to be heritable ($h = 0.17$) in roe deer [33]. The second trait, spontaneous daily activity [34] was measured using accelerometery data recorded at 20 Hz from tri-axial accelerometers (Daily Diary tags, Wildbytes, Swansea University) mounted on animal collars. We calculated the vectorial dynamic body acceleration (VeDBA) metric [35], using a 2 s smoothing windows and the DDMT software (Wildbytes, Swansea University). VeDBA values were summed for each individual, date and hour of the day (total VeDBA) and averaged through the four weeks between the two capture events (period 2) to index daily activity. The third measured trait, neophobia, was defined as the avoidance of novel stimuli in the environment [34] and was assessed using the difference in feeding efficiency with and without the presence of a novel object [36]. We calculated the ratio of the number of visits to the hut that resulted in a successful feeding bout (numerator) and the total number of visits to the hut (denominator). Measurements were randomized and repeated for 5 days for each condition (with and without novel object), and the difference in the ratio between the two conditions was calculated. Novel objects were polystyrene geometric shapes (circle, diamond, square and triangle) painted with contrasting colours, since roe deer are more sensitive to shapes and contrasts than to colours (see [36] for details). More neophobic individuals should be less inclined to feed on a given visit when a novel object is present, resulting in a higher score on the neophilia–neophobia continuum.

(i) Immune parameters measurement

Blood samples were taken on EDTA and dry tubes. EDTA blood was preserved at 4°C and served to measure the total leucocyte concentration (white blood cell (WBC)) with an automat (Sysmex 2000iV, Sysmex). A differential cell count (neutrophil, basophil, eosinophil, lymphocyte and monocyte) was performed on the first 100 WBCs on Wright-Giemsa-stained blood smears [37]. To obtain concentrations of each leucocyte type, the total leucocyte count was multiplied by the proportion of each cell type. The serum was obtained after blood 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 electrophoresis on agarose gel. Haptoglobin, an alpha-2-globulin, was also measured by spectrophotometry (Konelab 30i PLC, Fisher Thermo Scientific). Circulating levels of NAbs were measured by a hemagglutination test (HA), that measures NAbs ability to agglutinate exogenous cells, while the complement activity was revealed by the ability of proteins to induce hemolysis (HL) [38,39]. Finally, we quantified the level of anti-rabies antibody following the method described by Cliquet and her colleagues [40]. We therefore measured six markers of innate immunity (neutrophils, basophils, monocytes, eosinophils, hemagglutination and hemolysis titers), four inflammatory markers (haptoglobin, alpha-1, alpha-2 and beta-2 globulins) and three markers of adaptive immunity (lymphocytes, gamma-globulins and anti-rabies antibodies).

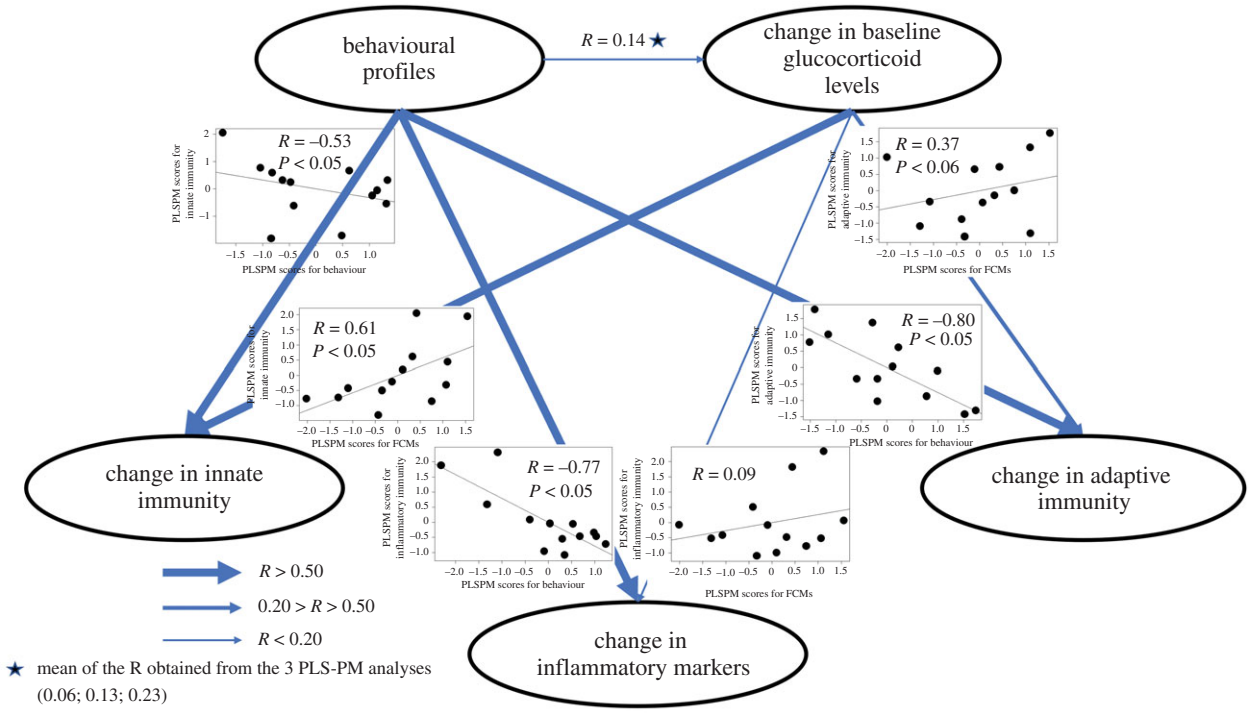

Figure 2. Structural models of relationships among behavioural profiles, change in baseline glucocorticoid levels and change in immunity as determined by the partial least-squares path modelling analyses. Arrows indicates the direction of effect and the thickness of arrows indicates the strength of the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

(ii) Extraction and quantification of faecal cortisol metabolites

FCMs were extracted following a methanol-based procedure and assayed using a group-specific 11-oxoaetiocholanolone enzyme immunoassay (EIA), as previously described [29] and validated for roe deer [41]. Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively).

(d) Statistical analyses

(i) Preliminary analyses

As the number of individuals varied between enclosures, we performed preliminary analyses to evaluate if group size could influence behavioural traits. Behavioural profiles, indexed by PC1 (see details below), were not influenced by the number of accompanying roe deer in the enclosure ($F_{1,11} = 0.55$, $p = 0.48$), and the same applied when considering each behavioural trait separately, docility ($F_{1,11} = 0.04$, $p = 0.84$), neophobia ($F_{1,11} = 4.20$, $p = 0.07$) and activity ($F_{1,11} = 1.25$, $p = 0.29$). While we observed a trend for neophobia to decrease when group size increased, group size effect is also included as part of the random effect of enclosure identity (see below), given that group size remained constant for each enclosure throughout the study. Consequently, we did not include the number of accompanying roe deer as a covariate in the following linear mixed-effects models (LMMs), or as a latent variable in partial least square path models.

(ii) Relationship between behaviour and changes in immunity and baseline faecal cortisol metabolites

Changes in immunity (Δ immunity) were calculated for each parameter as the difference between the measurements obtained at the two capture events. Similarly, changes in baseline FCM levels (Δ glucocorticoids), were calculated as the difference of averaged baseline FCM levels between period 2 and period 1 for each individual. In addition, we used the behavioural scores at capture as an index of docility. Values did not differ between the two captures within-individual, except for four individuals for which the score passed from 1 to 0 (for 2 of them) or 0 to 1 (for 2 others). We chose to use values from the first capture to avoid a potential habituation effect and for consistency.

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 a high correlation in order to avoid redundancies and high type I error [42]. Here we built the following blocks of variables, each being summarized by a latent variable: Δ glucocorticoids (one variable), Δ innate immunity (six variables), Δ adaptive immunity (three variables), Δ inflammatory markers (four variables) and behavioural profile (three variables, electronic supplementary material, table S1).

We then ran three PLS-PM analyses, each one evaluating the relationships between behavioural profile, change in baseline glucocorticoids, and change in (i) innate immunity, (ii) adaptive immunity and (iii) inflammatory markers. For each of the three analyses, we built a structural model (or inner model, i.e. describing relationships among latent variables) that 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: change in immunity depends on both behavioural profile and change in baseline glucocorticoids, which also depends on behavioural profile. Finally, in the measurement model (or outer model, i.e. relationships between observed and latent variables), the observed variables were considered as reflecting the corresponding latent variable (reflective mode), except for innate immunity where observed variables were considered as constituting the latent variable (formative mode). This option was chosen due to the high number of biomarkers used 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 (electronic supplementary material, table S2).

We then ran PLS-PM analysis to adjust both the structural (figure 2) and measurement models, through multiple linear

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. s.e. stands for standard error. See text for definition of the observed variables that composed each latent variable. Variables in bold represent latent variables.

parameter	estimate	s.e.	*t*-value	*p*-value
structural model for innate immunity biomarkers (GoF: 0.32)				
FCMs				
behavioural profile	0.055	0.301	0.183	0.86
innate immunity				
behavioural profile	−0.53	0.197	−2.70	0.02
FCMs	0.61	0.197	3.08	0.02
structural model for adaptive immunity biomarkers (GoF: 0.42)				
FCMs				
behavioural profile	0.127	0.299	0.424	0.68
adaptive immunity				
behavioural profile	−0.801	0.173	−4.63	0.002
FCMs	0.373	0.173	2.16	0.057
structural model for inflammatory biomarkers (GoF: 0.39)				
FCMs				
behavioural profile	0.226	0.294	0.183	0.46
inflammatory markers				
behavioural profile	−0.770	0.197	−3.91	0.006
FCMs	0.089	0.197	0.454	0.660

regressions. As the tests were not independent and in order to control for type-1 error, we applied false discovery rate to the *p*-values using the p.adjust function in R (see below). Lastly, we performed the diagnosis of each model following the recommendations of Gaston Sanchez [42]. We examined communality and redundancy (electronic supplementary material, table S3). The structural models were checked using R^2, redundancy index (ability to predict) and goodness-of-fit (GoF) index, a pseudo-GoF measure that reflects the overall prediction power of the model ($0 < \text{GoF} < 1$).

(iii) Relationship between baseline faecal cortisol metabolites 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 random intercept – constant slopes LMMs on the 181 observations of FCM levels from 13 individuals (14 repetitions per individuals with one missing value for one individual). FCM values were log-transformed to achieve normality and homoscedasticity of model residuals and residuals degrees of freedom were estimated using the Satterthwaite method. In order to characterize behaviour through a limited set of uncorrelated variables, we analysed the overall correlation pattern between docility, neophobia and activity using a normed principal component analysis (PCA) and used scores from the first principal component (PC1) which indexed individual proactive-reactive gradient of behaviour (electronic supplementary material, table S4 and figure S4). We then built a reference model that included all biologically relevant variables to explain baseline glucocorticoids levels and compared this model with all its sub-models. The reference model included PC1, age of individuals and Julian date of sampling. Individual identity was nested within enclosure identity and included as nested random effects to avoid pseudo-replication issues [44] and to control for unexplained variance due to among-individual differences and among-enclosure variation.

The best models of variation in FCM levels were selected based on the second-order Akaike information criterion (AICc) [45]. Models with a difference in AICc (ΔAICc) greater than 2 units from the best model were considered to have less support [45]. In addition, we removed models within two AICc units of the top model that differed from a higher-ranking model by the addition of one or more parameters, as recommended [46]. In addition, we calculated AICc weights (AICcw) to measure the relative likelihood that a given model was the best among the set of fitted models. The normality of model residuals was tested (Shapiro–Wilk test) and visually assessed. Goodness-of-fit and deviation from homoscedasticity were assessed by conditional and marginal R^2 values and standard residual plot techniques [47].

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].

3. Results

(a) Covariation between behaviour and changes in immunity and baseline faecal cortisol metabolites

Among the 13 individuals considered in our study, nine showed a decrease in baseline FCM levels during period 2 compared to period 1 (ranging from −786 to −8 ng g^{-1} of wet faeces), while four showed an increase (ranging from 93 to 346 ng g^{-1}). In addition, for each individual, vaccination increased the level of anti-rabies antibody, but large

among-individual differences were observed, with values ranging from +0.60 to +41.50 IU, with a median of +10.39. Proactive individuals were characterized by high daily activity levels, lack of docility and neophobia (electronic supplementary material, table S4). In addition, the 13 individuals appeared to be homogeneously distributed along the 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 (electronic supplementary material, figure S4).

Our analyses revealed links between behavioural profiles and changes in the three studied aspects of immunity (table 1). Individuals that exhibited more proactive behaviour, expressed by high daily activity levels, lack of docility and neophobia, showed an overall strong decrease in innate ($r = -0.53$; $p < 0.05$; figure 2), adaptive ($r = -0.80$; $p < 0.001$; figure 2) and inflammatory ($r = -0.77$; $p < 0.005$; figure 2) markers of immunity. However, the weights of observed variables in the definition of latent variables differed according to the analysis. When 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), while behavioural profile was essentially represented by docility and neophobia ($w =$ 0.65 and 0.64, respectively). On the opposite, for Δ inflammatory markers, behavioural profile was largely represented by mean daily activity levels ($w = 1.0$) and less by docility ($w = -0.32$) and neophobia ($w = 0.12$), while markers of inflammation contributed overall to the same proportion to their latent variable (table 2). Lastly, for Δ innate immunity, behavioural traits contributed to the same extent to their latent variable (table 2). It is also important to note that 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 and innate immunity only occurred for these markers, while temporal changes in neutrophil concentrations were actually positively linked to activity, neophobia and lack of docility.

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

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

(b) Covariation between baseline faecal cortisol metabolite levels throughout the experiment and behaviour

According to the model selection procedure, the best model describing among-individual differences in baseline FCMs throughout the experiment in relation to individual behavioural profiles included PC1 score and period of the experimental protocol (electronic supplementary material, table S5). Specifically, roe deer that exhibited a more reactive behavioural profile (low daily activity levels, docility and

Table 2. Characteristics of the observed variables that composed each latent variable in the three 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 immunity. Weight represents the contribution of the variable to the latent variable, and loadings indicate the direction of the correlation between the observed variables and their latent variable. Communality indicates the amount of variability in an observed variable that is captured by its latent variable and particularly applies for reflective latent variables. Redundancy indicates the ability to predict for a given observed variable and particularly applies for formative latent variables. See text for definition of the observed variables composing each latent variable. Variables in bold represent endogenous variables.

parameter	weight	loading
outer model for innate 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 adaptive immunity biomarkers		
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 inflammatory immunity biomarkers		
behavioural profile		
lack of docility	−0.319	0.115
neophobia	0.115	0.215
activity	1.00	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

Table 3. Characteristics of the selected LMMs 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. $R^{2\,m}$ and R^{2c} are the marginal and conditional explained variance of the models, respectively. s.e. stands for standard error. See text for definition of model sets.

parameter	estimate	s.e.	d.f.	*t*-value	*p*-value
(R^{2m}: 0.06; R^{2c}: 0.27)					
intercept	6.807	0.180	14.88	37.67	<0.001
behavioural profile (PC1)	−0.106	0.036	180.9	−2.920	<0.005
period (2)	−0.166	0.080	175.9	−2.092	<0.05

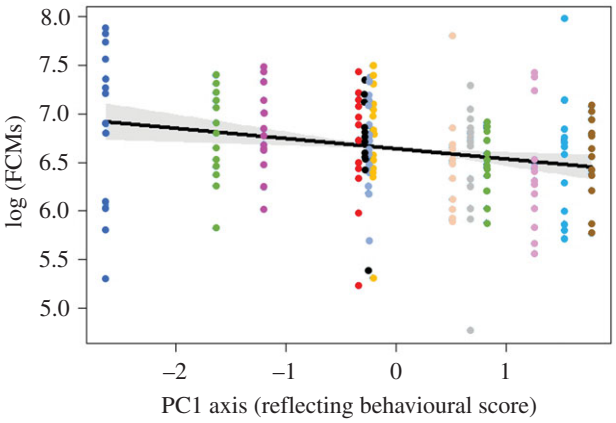

Figure 3. Relationship between baseline FCMs level (log-transformed) and behavioural profiles. Behavioural profiles' scores correspond to the score for the first axis (PC1) of the PCA conducted using docility, activity and neophilia as covariables. The three variables were all positively correlated with PC1. Thus, this axis represents a gradient of behavioural profiles, with negative values indicating reactive behavioural profiles (low activity levels, neophilia and docility), and positive values indicating proactive behavioural profiles (high activity levels, neophobia and lack of docility). Points represent observed values, lines represent model predictions and grey area represents the 95% confidence interval. Each colour corresponds to one of the 13 individuals. (Online version in colour.)

neophilia) also exhibited higher baseline FCM levels throughout the experiment compared to roe deer exhibiting a more proactive behavioural profile (0.103; $p < 0.005$; table 3 and figure 3). In addition, baseline FCM levels decreased during the second part of the experimental protocol compared to the first one (−0.1663; $p < 0.05$; table 3). Age and Julian date did not increase the fit of the model. However, it is worth noting that most of the variability in FCM levels was explained by random effects (R^2c-R^2m = 0.21) compared to fixed effects (R^2m = 0.06), meaning that individual and enclosure identity strongly contributed to among-individual variability in FCM levels.

4. Discussion

In this study, we used an experimental approach to gain a better understanding on how changes in baseline glucocorticoid levels may affect simultaneous changes in immune parameters of the innate, adaptive and inflammatory markers of immunity, on the scale of eight weeks. Our results demonstrated that an increase in baseline FCM levels was associated with an increase in immune parameters of the innate and adaptive arms, but not in inflammation. Second, we tested whether behavioural profiles could influence the covariation between 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 glucocorticoid levels throughout the experiment, while they were not related to changes in baseline glucocorticoids between the two periods of the study. Altogether, our results provide an empirical support to the pace-of-life syndrome hypothesis, with covariations between immunity, glucocorticoids and behaviour.

An increase in baseline glucocorticoid levels between the two study periods was generally related to an increase in innate immunity, except for neutrophil concentrations, which decreased as glucocorticoid levels increased. The negative relationship observed with 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 immunity was unexpected under the hypothesis of energetic trade-off between immunity and other energy-demanding functions [2]. In our captive population, such trade-off may be relaxed because resources are not limiting. Alternatively, it has been proposed that, as the main function of the stress response is to recover from stressors, a decrease in immunity should not necessarily occur when glucocorticoids increase, as it could improve survival [11].

While the above hypothesis of an energetic trade-off may partly explain the link between change in innate 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 cellular immunity and reflect acute inflammatory response while monocytes reflect chronic inflammatory response, and hemagglutination and hemolysis are both part of the humoral innate response [43]. Basophils are particularly secreted in presence of ticks [50], which are frequently encountered in the experimental facility. Finally, eosinophils are known to specifically bridge innate and adaptive immunity [43]. Considering the differences in the functions of these biomarkers, it is likely that they are not all linked to glucocorticoids in the 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 the presence of ticks).

With respect to a change in the adaptive arm of the immune system, our results did not support the prediction of the immunosuppressive effect of long-term elevation of glucocorticoids: instead, adaptive immune parameters increased in individuals that showed an increase in baseline FCM levels. A first possible

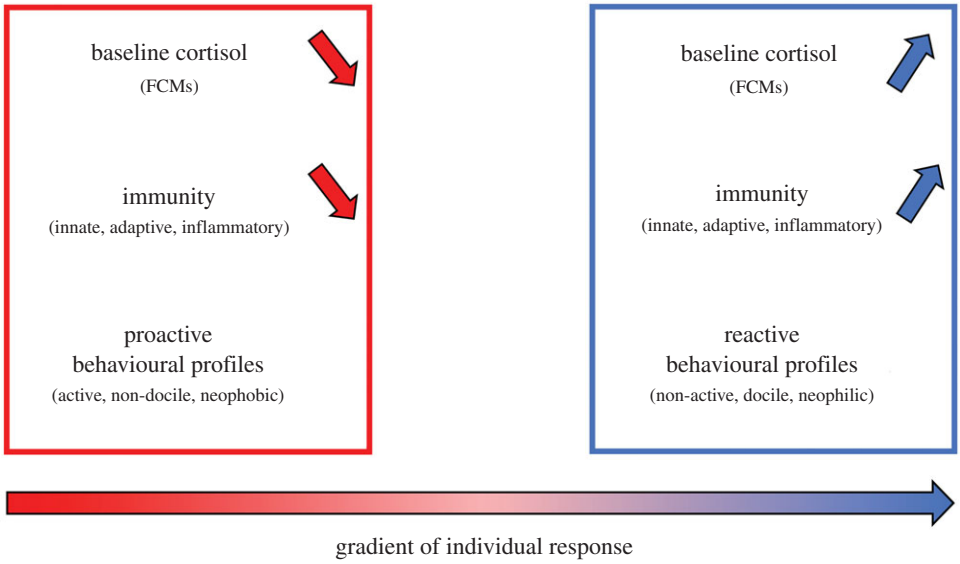

Figure 4. Summary of the observed outcomes and relationships between the latent variables considered in our study. Our protocol (see details in the main text) resulted in different outcomes, with part of the individuals showing an increase in baseline cortisol between the two study periods (indicated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

explanation could be linked to the transient increase in glucocorticoids that occurred during the first capture, where vaccination was done, and which may have reinforced the efficacy of the vaccination [13]. Another possible explanation is that the energy cost of mounting an antibody response is too low [27] for a trade-off between antibody expression and other functions to be detectable. The positive relationship we observed 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 shifted their investment away from the immune system, possibly toward another energy-demanding function. We suggest that such a shift could be underpinned by a plastic response to the stressful event of capture, leading to an adjustment of the individuals' life-history strategies and change in investment between functions. The change may go toward supporting either long-term survival (for individuals increasing baseline glucocorticoids and immunity) or current reproduction (for those reacting by a decrease in baseline glucocorticoids and immunity). This would be in accordance with the pace-of-life syndrome hypothesis, where a positive association is expected between glucocorticoid levels and immunity, with higher levels in individuals favouring their long-term survival, while lower levels are expected in individuals favouring reproduction and growth [23]. Precisely, individuals showing a propensity to be active, non-docile to manipulation and neophobic showed a decreased investment in their immune system following the first capture event. This result is consistent with our predictions and supports the hypothesis that fast-living individuals should have a proactive behavioural profile and a low investment in immune functions that would allow them to favour immediate reproduction over survival [23]. On the opposite, individuals showing more reactive behavioural profiles are supposed to have a slower pace-of-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 to be repeatedly exposed to the same pathogens.

Finally, we investigated whether behavioural profiles could be associated with among-individual variations in baseline glucocorticoid levels throughout the experiment, and also with changes in baseline glucocorticoid levels over an eight-week period. Our results did not support the latter hypothesis, but rather the former. Specifically, individuals exhibiting proactive behavioural profiles also showed lower baseline glucocorticoid levels throughout the experiment compared to individuals exhibiting more reactive behavioural profiles. This result supports previous studies on wild [51] and captive [30] roe deer. It is also in accordance with the coping style framework [22] and the pace-of-life syndrome hypothesis [23], which states that behavioural and physiological responses to stressful situations are correlated. The difference we observed in the results of our two analyses may thus suggest that activity (reflected by baseline 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 [22].

Overall, individual roe deer responded differently to our protocol, with some individuals showing an increase in baseline glucocorticoid levels, while others showed a decrease. In addition, our results suggest that increased baseline glucocorticoid levels are associated with a re-allocation of energy resources to innate and adaptive immunity in individuals with more reactive behaviours (figure 4 for a summary of the outcomes of the protocol). We suggest that the observed association between immunity and baseline glucocorticoid levels is associated 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 syndrome hypothesis [23].

Finally, considering that all our results tend to support a covariation between stress hormones, immunity and behaviour, we recommend that future work should go further and investigate how among-individual variations in behaviour modulate the variation of glucocorticoid levels, as well as the relationship between glucocorticoid hormones and immunity. This could be done by using a larger sample size and performing repeated behavioural measures to assess individual behavioural syndromes. Because behavioural and physiological traits might not be independent from each other, and because selection could act in opposite or same directions on these traits, further investigations on the extent to which covariations between these traits are influenced by life-history strategies may help to understand the evolutionary potential of wild populations.

Ethics. 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).

Data accessibility. All data and code used in this analysis are provided in the electronic supplementary material [52].

Authors' contributions. J.C.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization and writing—original draft; B.R.: conceptualization, funding acquisition, validation and writing—review and editing; R.P.: resources and writing—review and editing; C.M.: methodology and writing—review and editing; L.B.: resources and writing—review and editing; T.L.: resources and writing—review and editing; M.-L.M.: investigation and writing—review and editing; N.C.: investigation and writing—review and editing; J.-L.R.: investigation and writing—review and editing; G.L.L.: investigation and writing—review and editing; M.W.: resources and writing—review and editing; B.R.: resources and writing—review and editing; E.G.-F.: conceptualization, funding acquisition, project administration, supervision, validation and writing—review and editing; H.V.: conceptualization, funding acquisition, investigation, project administration, supervision, validation and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors declare that they have no conflict of interest.

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