Running title: Pace-of-life syndrome in roe deer

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

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Summary statement

Experimental approach on roe deer highlighted a gradient of individual responses to stress and immune challenges, as revealed by co-variation between behavioural profiles and changes over time in glucocorticoids and immunity.

Abstract

The biomedical literature has consistently highlighted that long-term elevation of glucocorticoids might impair immune functions, while short-term elevation might enhance them. In contrast, in wild and free-ranging animals, patterns are less clear. In the present study, we explored the stress-immunity relationship in controlled conditions, taking into account 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 standardised capture protocol and vaccination affected changes in thirteen immune parameters of the innate and adaptive immunity, and 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 over time also exhibited an increase in immunity, for both the innate and adaptive immunity and were characterised by more reactive behavioural profiles (as indicated by low activity levels, docility to manipulation and neophilia). Consequently, our results suggest that immunity of large mammals may be influenced by their stress level, but also by their behavioural profiles, as it is predicted by the pace-of-life syndrome hypothesis. Our analysis therefore highlights the need to consider co-variations between behavioural profiles, immunity and stress hormones in order to gain a better understanding of the unexplained among-

individual variability in the glucocorticoids-immunity relationships observed in wildlife, as they may be underpinned by different life-history strategies.

Introduction

The immune system is one of the most important mechanisms in vertebrates to improve their 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 specific) immunity, each composed of numerous cells and effectors (Demas, 2004). This system, however, is not cost-free (Lochmiller and Deerenberg, 2000; Demas 2004), and there should be trade-offs between immune defences and other functions that use a common resource and contributes to fitness (Sheldon and Verhulst, 1996; Rauw, 2012). Glucocorticoids (such as cortisol and corticosterone) play a major role in regulating the use of energy (Landys et al., 2006; Busch and Hayward, 2009) and may therefore underlie such trade-offs.

In addition to their role as metabolic hormones, 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 (Wingfield, 1998; Romero and Wingfield, 2015). In particular, the activation of the hypothalamic-pituitary-adrenal (HPA) axis that results in the secretion of glucocorticoid hormones helps organisms to cope with stressful situations by making stored energy available (Sapolsky et al., 2000). However, when glucocorticoid hormones are secreted repeatedly or for long periods of time, negative effects on other energetically demanding functions such as reproduction (Boonstra, 2005) and immunity (Martin, 2009) may appear.

Over the past years, several studies have investigated the relationship between stress and immunity, particularly in the biomedical domain where it has been shown that short-term

elevation of glucocorticoids (i.e., few minutes to few hours) enhances immune functions (Bilbo et al., 2002; Viswanathan et al., 2005; Dhabhar, 2014) while chronically elevated glucocorticoid levels are immunosuppressive (Dhabhar and McEwen, 1997; Sapolsky et al., 2000; Dhabhar, 2009). Focusing on long-term elevation of glucocorticoids (i.e., few days to few months), studies on wild animals have shown mixed results, suggesting that chronically elevated glucocorticoid levels may be associated with decrease, increase, or no changes in immune functions (Matson et al., 2006; Delehanty and Boonstra, 2009; Merrill et al., 2012; Brooks and Mateo, 2013). 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 (Bourgeon and Raclot, 2006; Stier et al., 2009). These contrasting patterns indicate that our understanding of the nature of the stress-immunity relationship in the wild remains limited and fragmentary.

Among the factors likely to modulate this relationship and to help to shade light on the discrepancies, the consideration of behavioural profiles has been largely neglected. From a theoretical point of view, close links between physiology and behaviour are expected due to the underlying energetic basis of both traits (Careau et al., 2008). Accordingly, consistent among-individual differences in behaviour, also called personality (Réale et al., 2007), are related to numerous physiological traits including glucocorticoid secretion and immune function (Koolhaas et al., 1999; Koolhaas et al., 2008; Réale et al., 2010). For instance, a study on wild superb fairy-wrens (*Malurus cyaneus*) showed that individuals exhibiting proactive behavioural traits (fast exploration of a novel environment) exhibited lower level of natural antibodies (Jacques-Hamilton et al., 2017). In addition, previous works highlighted that slower explorer or more reactive individuals tend to exhibit higher baseline and stress-induced glucocorticoid levels compared to faster or more proactive ones (Koolhaas et al., 1999; Stöwe et al., 2010;

Montiglio et al., 2014). In addition, a recent study on laying hens (*Gallus gallus domesticus*) highlighted that more reactive individuals exhibited greater stress and immunological (greater swelling in response to phytohemagglutinin injection) responsiveness compared to more proactive ones (Pusch and Navarra, 2018). Such co-variations between behavioural and physiological traits can be interpreted within the pace-of-life syndrome hypothesis described by Denis Réale and his collaborators (2010). 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, it is expected that individuals with slower life history strategies may have more reactive behavioural profiles, higher glucocorticoid levels and higher investment in overall immunity, while those with faster life history strategies may have more proactive behavioural profiles, lower glucocorticoid levels and lower overall investment in immunity.

In the present study, we investigated the relationships between changes in baseline glucocorticoid levels and changes in thirteen immune parameters, 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 adaptive and innate immune functions, before and after a standardised capture stress protocol associated with an immune challenge using anti-rabies vaccination, in a captive roe deer (*Capreolus capreolus*). In addition, we evaluated how these changes may be related to behavioural profiles, as characterised by three commonly used behavioural traits: docility, neophobia, and activity levels.

Based on the literature, we expected that 1) changes in baseline FCM levels between the two observation periods (before/after capture) would co-vary with changes in immune parameters and inflammatory markers; 2) 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 (McDade et al., 2016). In addition, we expected 3) that baseline FCM levels as well as variations in baseline FCM levels should be related to individual behavioural profiles (Koolhaas et al., 1999; Montiglio et al., 2012), 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 4) the increase in immune parameters between the two observation periods should be more important for more reactive individuals, that are supposed to invest more in overall immunity (Réale et al. 2010; Pusch and Navarra, 2018).

Material and methods

Study site

This study was conducted on a captive population of roe deer living in the Gardouch research station, located in south-west of France, approximately 30 km south-east of Toulouse. The station is located on the slopes of a hill, approximately 230 m above sea level. The climate is oceanic with summer droughts. 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, each containing between one to six captive roe deer. Each enclosure is composed of meadow, trees and groves that provide shelter. Each enclosure contains a hut where individuals are supplemented with food pellets (600 g per individual per day) and can obtain a shelter. The current experiment included a total of 13 females, living in these enclosures since several years already, aged from 4 to 13 years old. All roe deer were raised at the station from their birth or their first year of life. In addition, they all had some degree of habituation to humans but were able to express normal behavioural responses (e.g., vigilance, escape) to stressful situations.

Experimental design

The experimental procedure was carried out between mid-September and mid-November 2018 and is summarised in Fig.1. To assess the baseline glucocorticoid levels of each individual, we collected faeces every four days during four weeks to further determine faecal cortisol metabolite concentrations (i.e., period 1). Faeces were collected immediately after defecation was observed by experienced personnel 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 standardised capture stress protocol involving restrained immobilisation (Monestier et al., 2016). This procedure made it possible to collect faeces from the rectum, to draw blood samples, to inject an anti-rabies vaccine, and to fit collars equipped with tri-axial accelerometers (see Capture protocol and data collection for details). Collection of faecal samples was then continued every four days for an additional four weeks, as describe above (i.e., period 2), in order to evaluate the effect of 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 (Dehnhard et al., 2001). At the end of period 2, roe deer were recaptured following the same procedure (capture 2) and faeces and blood were again collected.

Capture protocol and data collection

Roe deer were directed into their hut (one enclosure and one individual at a time) by slowly approaching them. Each individual was then pushed through a trap 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), as recommended by Jordi Montané and his colleagues (2003). Individuals were weighed with an electronic balance to the nearest 100 g.

In addition, for each individual, we characterised three behavioural traits at three different moments. First, we recorded whether animals struggled during handling, and this measure was used as an index of docility, as previously used and described for roe deer (Gervais et al., 2020). A score of 0 was assigned to individuals that did not struggle (docile), while a score of 1 was attributed to those that did struggle (non-docile) during handling. This behavioural trait during handling has been shown to be repeatable over time (r=0.26) with a tendency to be heritable (h=0.17) in roe deer (Gervais et al., 2020). The second behavioural trait consisted in determining spontaneous daily activity (Réale et al., 2007) during four weeks with tri-axial accelerometery technology. To this end, we fitted collars equipped with tri axial accelerometers (Daily Diary tags, Wildbytes Ltd., Swansea University) recording at 20 Hz. Daily activity was measured using the Vectorial Dynamic Body Acceleration (VeDBA) metric (Wilson et al. 2020), a measure of dynamic body acceleration (and hence activity) derived from the 20 Hz tri-axial accelerometer channels – here, using a 2 second smoothing window and standard procedures (Wilson et al. 2020), as implemented in the DDMT software (Wildbytes Ltd., Swansea University). VeDBA values were summed for each individual, date, and hour of the day ('total VeDBA'), from which an average was taken for each individual as the mean daily activity through the four weeks between the two capture events (period 2). The third behavioural trait we considered was neophobia, defined as the avoidance of novel stimuli in the environment, and a widely studied dimension of animal personality (Réale et al., 2007; Greenberg and Mettke-Hofmann 2001). The neophilia-neophobia continuum was assed using the difference in feeding efficiency with and without the presence of a novel object, a measure previously used and validated for this roe deer population (Monestier et al., 2017). The complete protocol for evaluating feeding efficiency has been described in detail by Chloé Monestier and her colleagues (2017) for this roe deer population. Briefly, we calculated the ratio between 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), with and without the presence of a novel object in the hut, near the manger. Measurements were repeated for five days for each condition (with and without novel object), and the difference in the ratio between the two conditions was then evaluated. More neophobic individuals should be less inclined to feed during a given visit when there was a novel object present, resulting in a higher score on the neophilia-neophobia continuum.

Finally, we obtained a functional index of the adaptive immune system activity by injecting a standardized dose of an anti-rabies vaccine (Rabisin®, Merial, France, 1ml) administered subcutaneously during the first capture and by quantifying the circulating specific antibodies at capture 1 and 2 (see below).

Immune parameters measurement

Blood samples were taken from each individual from the left jugular vein (up to 20 mL for a 20 kg roe deer) and divided in two. Part of blood was collected on EDTA, preserved at 4 °C and served to measure total leukocyte concentration (white blood cell [WBC]). WBC were determined at the Ecole Nationale Vétérinaire de Toulouse (ENVT) with an automat (Sysmex 2000iV, Sysmex). To obtain concentrations of each leukocyte type (neutrophil, basophil, eosinophil, lymphocyte and monocyte), the total leukocyte count was multiplied by the proportion of each cell type. The differential cell count was performed on the first 100 WBCs on Wright-Giemsa-stained blood smears by trained technicians (Houwen, 2001). The remaining blood was collected on a dry tube, centrifuged at 1500 g for 15 min, and the serum was stored at -20°C. Serum was used to measure total proteins using a refractometer and to perform electrophoresis on agarose gel that allowed differentiating albumin and alpha-1, alpha-2, beta, and gamma globulins. Haptoglobin that belongs to the alpha-2-globulin fraction was also measured by spectrophotometry (Konelab 30i PLC, Fisher Thermo Scientific) with a non-

species-specific method based on the ability of haptoglobin to bind to haemoglobin. Circulating levels of natural antibodies (NAbs) was 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) (Matson et al., 2005). The HA/HL protocol defined by Matson et al., (2005) in birds has been adapted and previously used in roe deer (Gilot-Fromont et al., 2012). Finally, we quantified the level of anti-rabies antibody following the method described by Florence Cliquet and her colleagues (1998). We therefore measured 10 markers of innate immunity (neutrophils, basophils, monocytes, eosinophils, hemagglutination and hemolysis titers) including 4 inflammatory markers (haptoglobin, alpha-1, alpha-2 and beta-2 globulins), and 3 markers of adaptive immunity (lymphocytes, gamma-globulins and anti-rabies antibodies).

Extraction and quantification of FCMs

FCMs were extracted following a methanol-based procedure and assayed using a group-specific 11-oxoaetiocholanolone enzyme immunoassay (EIA), as previously described (Möstl et al., 2002) and validated for roe deer (Zbyryt et al., 2017). Briefly, each faecal sample was homogenised and 0.5 ± 0.005 g of the homogenate was transferred to a glass tube containing 5 ml of methanol 80%. The suspended samples were vortexed at 1500 rpm for 30 min and centrifuged at 2500 g for 15 min (Palme et al., 2013). An aliquot of the supernatant was further diluted (1:10) with assay buffer prior to EIA analysis. Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively).

Ethical approval

All applicable institutional and/or national guidelines for the care and use of animals were followed. The overall protocol, including captures and blood sampling of roe deer was approved

by the Ethical Committee 115 of Toulouse and was authorized by the French government (APAFIS#14706).

Statistical analyses

Relationship between behaviour and changes in immunity and baseline FCMs

To analyse relationships between change in baseline FCM levels, change in immune parameters and behavioural profiles, we first calculated the difference between the measures obtained during the second capture event and the first one for immune parameters (Δ immunity). Similarly, in order to obtain a measure of change in baseline FCM levels (Δ glucocorticoids), we calculated the difference of averaged baseline FCM levels between period 2 and period 1 for each individual. In addition, behavioural scores from the first capture were used to index docility since values did not differ between the two captures within-individual, except for one individual for which the score passed from 1 to 0. We chose to use values from the first capture rather the second one in order to avoid a potential habituation effect for the only individual that exhibited a change in its docility score.

Then, to test our hypothesis, we used Partial Least Square Path Modelling (PLS-PM) analysis. PLS-PM is a statistical method for studying complex multivariate datasets, in particular when complex individual traits (here, immunity arms and behavioural profiles) have been approached by several observed variables, that are generally correlated. In this case, groups (blocks) of variables describing a character can be summarised by a latent variable defined as a linear combination of observed variables, without imposing any restriction on within-block covariance. Then relationships between blocks (summarised by latent variables) are analysed taking into account previous knowledge on causes and consequences in the studied system. This statistical analysis is particularly recommended when dealing with variables showing high correlation in order to avoid redundancies and high type I error (Sanchez, 2013).

Here we built the following blocks, 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, Table 1).

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. model of relationships among latent variables) that consisted of three latent variables: Δ glucocorticoids, behavioural profile, and Δ of one of the three immune arms (innate, adaptive and 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 were 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 (Demas et al., 2011). This diversity is reflected in the moderate correlation among components of this latent variable.

We then ran PLS-PM analysis to adjust both the structural (Fig. 2) and measurement models, through multiple linear regressions. Lastly, we performed the diagnosis of each model following the recommendations of Gaston Sanchez (2013). For blocks of reflective variables, we checked for unidimentionality using Dillon-Goldstein's Rho and eigenvalues of the correlation matrix, and also checked for cross-loadings, to determine the differentiation among blocks. For all three models, we checked the measurement model using weights and loadings

of observed variables in the latent variable definition, as well as communality (squared loadings, part of variance in common between observed and latent variable) and redundancy (variance of indicators predicted from the corresponding latent variable) (presented in Table S1). 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 ($0 < Gof < 1$).

Relationship between baseline FCMs throughout the experiment and behaviour

In order to test hypothesis 3 and evaluate the link between among-individual differences in baseline glucocorticoid levels throughout the experiment (period 1 + period 2) and behavioural profiles, while taking into account other factors affecting FCM levels, we analysed baseline FCM levels by performing linear mixed-effects models (LMMs) on the 181 observations of 13 individuals (14 repetitions per individuals with 1 missing value for one individual). FCM values were log transformed to achieve normality of model residuals. As we measured three different behavioural traits, we analysed the overall correlation pattern between docility, neophobia and activity using a normed Principal Component Analysis (PCA). Consequently, we used scores from the first principal component (PC1) which indexed a gradient of behaviour ranging from individuals showing more proactive behavioural profile (non-docile, active and neophobic) to individuals showing a more reactive behavioural profile (docile, less active and neophilic) (Table S2, Fig. S1). 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 sample collection. Individual identity and enclosure identity were included as random effects to avoid pseudo-replication issues (Hurlbert, 1984) and to control for unexplained variance due to among-individual differences and among-enclosure variation.

To select the best models of variation in FCM levels we used a model selection procedure based on the second-order Akaike Information Criterion (AICc, Burnham and Anderson, 2003). Models with a difference in AICc (ΔAICc) > 2 units from the best model were considered to have less support, following Burnham and Anderson (2003). 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. These were rejected as uninformative, as recommended by Todd Arnold (2010) and Shane Richards (2008). 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. Normality of the residuals for the selected models was tested (Shapiro-Wilk test) and visually assessed. Goodness-of-fit was assessed by calculating conditional (i.e. total variance explained by the best supported model) and marginal (i.e. variance explained by fixed effects alone) R^2 values and standard residual plot techniques (Nakagawa and Schielzeth, 2013).

All analyses were carried out with R version 3.6.0 (R Development Core Team 2016), using the lmer function from the lme4 package (Bates et al., 2014) and the plspm function from the plspm package (Sanchez, 2013).

Results

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 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 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. Regarding

behavioural profiles, we found, as predicted, that proactive individuals were characterised by high daily activity levels, lack of docility and neophobia (Table S2). 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 (Fig. S1).

Our analyses showed links between behavioural profiles and changes in the three studied aspects of immunity (Table 2). Precisely, individuals that exhibited more proactive behaviours, expressed by high daily activity levels, lack of docility and neophobia, showed an overall strong decrease in innate ($r = -0.53$; $P < 0.05$; Fig. 2), adaptive ($r = -0.80$; $P < 0.001$; Fig. 2), and inflammatory ($r = -0.77$; $P < 0.005$; Fig. 2) markers of immunity. However, differences appeared among models as for the weights of observed variables in the definition of latent variables. When analysing Δ adaptive immunity, gamma globulins, lymphocytes and anti-rabies antibody contributed approximately equally to the latent variable (weights [w] of 0.37; 0.52 and 0.54 respectively), while behavioural profile was particularly represented by docility and neophobia ($w = 0.65$ and 0.64 respectively) and very little by activity ($w = -0.04$). On the opposite, for Δ inflammatory markers, behavioural profile was largely represented by mean daily activity levels ($w = 1.05$) and weakly by docility ($w = -0.32$) and especially neophobia ($w = 0.12$), while markers of inflammation contributed overall to the same proportion to their latent variable (Table 3). Lastly, for Δ innate immunity, behavioural traits contributed to the same extent to their latent variable while basophils and hemagglutination contributed more strongly to their one (Table 3). In addition, it is important to note that among the innate immune parameters, neutrophils were negatively correlated to other biomarkers of innate immunity (see the negative loading in Table 3), meaning that the negative relationship observed between behavioural profiles and innate immunity only occurred for other biomarkers

of innate immunity, while temporal changes in neutrophil concentrations were actually positively linked to activity, neophobia and lack of docility.

Changes in baseline glucocorticoid levels also appeared to be 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 2; Fig. 2). Precisely, 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 profiles and changes in baseline FCM levels was non-significant for all three models (Table 2).

Co-variation between baseline FCM 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 (Table S3). Specifically, roe deer that exhibited a more reactive behavioural profile (as expressed by low daily activity levels, docility and 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 4; Fig. 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 4), meaning that the mean baseline FCM level of the studied roe deer population was lower during period 2 than during period 1. Age, and Julian date did not increase the fit of the model.

Discussion

In this study, we first aimed 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, at the scale of few weeks (8 weeks). Our results demonstrated a correlation between baseline glucocorticoids and immunity, such that an increase in baseline FCM levels was related to an increase in immune parameters of the innate and adaptive arms, but not inflammation. Second, we tested whether behavioural profiles could underlie the co-variation 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 not related to changes in baseline glucocorticoids between the two periods of the study.

First, an increase in innate immunity was generally linked to an increase in baseline glucocorticoid levels between the two periods, except for neutrophil concentrations, which decreased as glucocorticoid levels increased. The observed negative relationship with neutrophils is consistent with the previous finding of an immunosuppressive effect of long-term elevation of glucocorticoids on immunity (Dhabhar, 2014; Martin 2009), while the overall increase in innate immunity may appear as more surprising, and opposite to our predictions. One of the assumptions underlying 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 functions (Lochmiller and Deerenberg 2000). However, it is important to note that the studied population, while living in large enclosures, is supplemented with food resources. Consequently, resources are not limiting and no unpredictable variations in food resources availability occurs. This particular context may lead to a disappearance of some trade-offs between energy demanding functions and may explain the absence of a negative

association between change in baseline glucocorticoids and immunity. Alternatively, to the dominant energy cost hypothesis, it has also 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 (Martin, 2009).

While the above hypothesis 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 (Demas et al. 2011). In addition, eosinophils are known to be a specific component that bridges innate and adaptive immunity (Demas et al. 2011). Considering these differences in the functions of the different biomarkers we used, it is likely that they may not all be linked in the same manner with glucocorticoids, which could explain the difference we observed. Regarding basophils, which represent the major component of the latent variable “innate immunity” alongside hemagglutination, they have been shown to be particularly secreted in presence of ticks in order to develop an acquired resistance to this ectoparasite (Wada et al., 2010; Karasuyama et al., 2018). Roe deer living in this experimental facility are particularly exposed to ticks each year (unpublished data), and investment in production and maintenance of basophils may therefore be important in order to decrease the costs associated with ticks’ parasitism and improve their fitness, which could be another explanation to the absence of a trade-off and positive relationship between changes in glucocorticoid levels and innate immunity.

With respect to a change in the adaptive arm of the immune system, our results did not support the finding of the immunosuppressive effect of long-term elevation of glucocorticoids, but instead showed that adaptive immune parameters increased in individuals that showed an

increase in baseline FCM levels. A first possible explanation of this result could be linked to the transient increase in glucocorticoids that occurred during the first capture, where vaccination was done. Previous work showed that a short-term elevation in glucocorticoids at the time of vaccination reinforces the efficacy of vaccination (Dhabhar, 2014). A similar scenario may have 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 may partly explain our result if there is a correlation between the change observed in baseline FCM levels and the stress-induced increase in glucocorticoids. Another possible explanation is that the energetic cost of mounting an antibody response is relatively low (McDade et al., 2016), and a trade-off between antibody levels and other functions was therefore not necessarily expected. The positive relationship we observed in our study also supports the pace-of-life syndrome hypothesis, where individuals exhibiting higher baseline glucocorticoid levels should show stronger investment in immunity than those exhibiting lower baseline glucocorticoid levels (Réale et al. 2010).

To summarise the immunity-glucocorticoids relationship found during our experiment, individuals that experienced an increase in baseline glucocorticoid levels showed an investment towards a strong immune system, while individuals that experienced a decrease in baseline glucocorticoid levels showed a down-regulation of their immunity. Consequently, it appears that in our study, individuals that underwent a decrease in baseline glucocorticoids may have switched their investment from the immune system, possibly toward another unstudied function, according to their changes in baseline stress levels. We suggest that this possible switch could be underlain 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 supporting either long-term survival or current reproduction. 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 (Réale et al. 2010).

Regarding the association between behavioural profiles and change in immunity, we found an overall similar association for the three components of the immune system assessed in our study, namely, innate, adaptive and inflammatory immunity. Precisely, individuals showing a propensity to be active, non-docile to manipulation and repulsed by novelty showed a decreased investment in their immune system following the first capture event. This result is in accordance with our predictions and supports the hypothesis that individuals with a fast pace-of-life should exhibit proactive behavioural profile and should invest less in immune functions than individuals with a slow pace-of-life, as they should prioritise investment in current reproduction du to their relatively short longevity, and functions supporting aggressive behaviours, high activity or elevated heart rate for instance, which characterised these behavioural profiles (Réale et al. 2010). In this manner, the low investment in immunity showed by fast-living individuals would support energy savings in order to invest in the functions cited above rather than into mechanisms enhancing survival, such as immunity. 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 (Réale et al., 2010), hence a stronger investment in immunity as they are expected to frequently encounter pathogens throughout their life, and most likely to face repeated encounter with 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 8-week period. Our results did not provide any support for the latter, but instead supported 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 (Carbillet et al., 2019) and captive (Monestier et al., 2016) roe deer. It is also in accordance with the coping style framework (Koolhaas et al., 1999) and the pace-of-life syndrome hypothesis (Réale et al., 2010) which states that behavioural and physiological responses to stressful situations are correlated. During our experiment, we therefore observed a different relationship between behavioural profiles and baseline glucocorticoids throughout the experiment, and between behavioural profiles and the change of hormones between two specific periods. The difference we observed in our results may 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 (Koolhaas et al., 1999). Precisely, only activity of the HPA axis may be associated with behavioural profiles, or at least more strongly associated with behavioural profiles than reactivity of the HPA axis.

Overall, our results indicate that individual roe deer reacted differently to our protocol, with part of the individuals showing an increase in baseline glucocorticoid levels, while others showed a decrease. In addition, our results suggest that the increase in baseline glucocorticoid levels may be associated with a re-allocation of energetic resources towards innate and adaptive immunity, but also that this re-allocation towards immunity might be associated with a gradient of behavioural profiles, such that individuals showing more reactive behaviours showed increased investment in immunity and higher baseline glucocorticoid levels (see Fig. 4 for a summary of the outcomes of the protocol). Immune functions are known to be costly in terms of energy (Lochmiller and Deerenberg, 2000; McDade et al., 2016), and glucocorticoids are metabolic hormones that regulate the use of energy (Palme, 2019). We therefore suggest that the observed association between immunity and baseline glucocorticoid levels may be

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 (Réale et al., 2010).

Finally, considering that all of our results tend to support a co-variation between stress hormones, immunity and behaviour, it might seem important that future work goes one step further and investigates how among-individual variations in behaviour could modulate the variation of glucocorticoid levels, as well as the relationship between glucocorticoid hormones and immunity. The extent to which co-variations between these traits are influenced by different life history strategies in the wild also warrants further investigations as it may help to understand the large and unexplained among-individual variability in the glucocorticoids-immunity relationship observed in wildlife studies.

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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, BRannou 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 co-authors. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing interests

The authors declare that they have no conflict of interest.

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Table 1. Summary of the latent variables used in the present study. Observed variables are presented in blocks reflecting their associated latent variable.

Latent variables	Observed variables	Average values (min ; max)
Δ baseline glucocorticoids	Δ FCMs (ng/g of wet faeces)	-146 (-786 ; 346)
Δ innate immunity	Δ Neutrophils (10^3 cells/mL)	-1.23 (-4.03 ; 1.5)
	Δ Eosinophils (10^3 cells/mL)	0.01 (-0.29 ; 0.38)
	Δ Basophils (10^3 cells/mL)	0.06 (-0.36 ; 0.46)
	Δ Monocytes (10^3 cells/mL)	-0.05 (-0.37 ; 0.09)
	Δ Hemagglutination (titer)	-0.04 (-1 ; 1.5)
	Δ Hemolysis (titer)	-0.07 (-1 ; 1.5)
Δ adaptive immunity	Δ Lymphocytes (10^3 cells/mL)	0.76 (-0.80 ; 2.23)
	Δ Anti-rabies antibody (IU)	12.30 (0.60 ; 41.5)
	Δ Gamma globulins (mg/mL)	0.10 (-4 ; 3.50)
Δ inflammatory markers	Δ Alpha-1 globulins (mg/mL)	0.10 (-0.20 ; 0.80)
	Δ Alpha-2 globulins (mg/mL)	-0.06 (-0.90 ; 2.90)
	Δ Beta-2 globulins (mg/mL)	0.13 (-0.40 ; 0.50)
	Δ Haptoglobin (mg/mL)	0.13 (-0.26 ; 1.19)
Behavioural profiles	Docility score	0.5 (0 ; 1)
	Neophobia score	0.20 (0.01 ; 0.40)
	Mean daily activity	228.8 (183.6 ; 259.9)

Table 2. 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 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 3. 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 apply for reflective latent variables. Redundancy indicates the ability to predict for a given observed variable, and particularly apply 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	1.051	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

Table 4. 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

Fig. 1. Summary of the experimental design used in the present study. Data relative to the assessment of neophobia scores were obtained prior to this protocol. Precisely, data were collected in February 2015 for all the individuals, except for two that were assessed for neophobia in February 2018, following the same protocol (see details in Monestier et al. 2017).

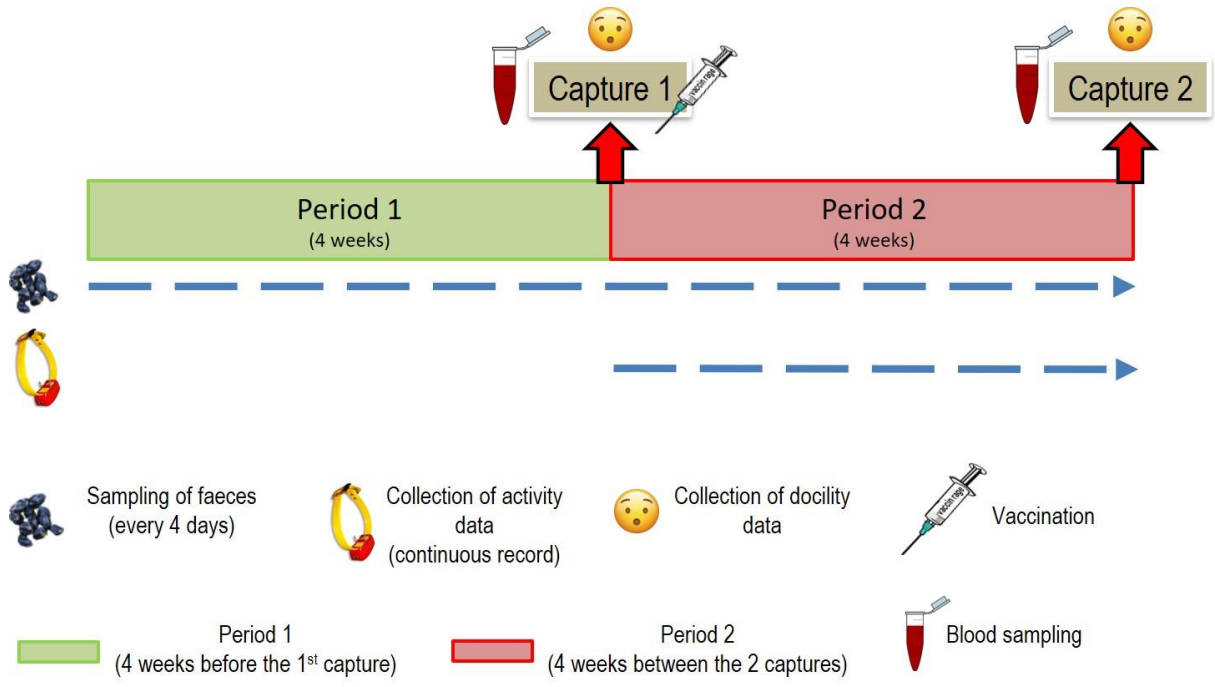

Fig. 2. Structural models of relationships among behavioural profiles, change in baseline glucocorticoid levels and change in immunity as determined by the partial least squares path modelling analyses. Arrows indicates the direction of effect, and the shape of arrows indicate the strength of the observed correlation among the latent variables. Stars indicate the degree of significance (see Table 2 for details), and absence of star indicate the relationship was not significant.

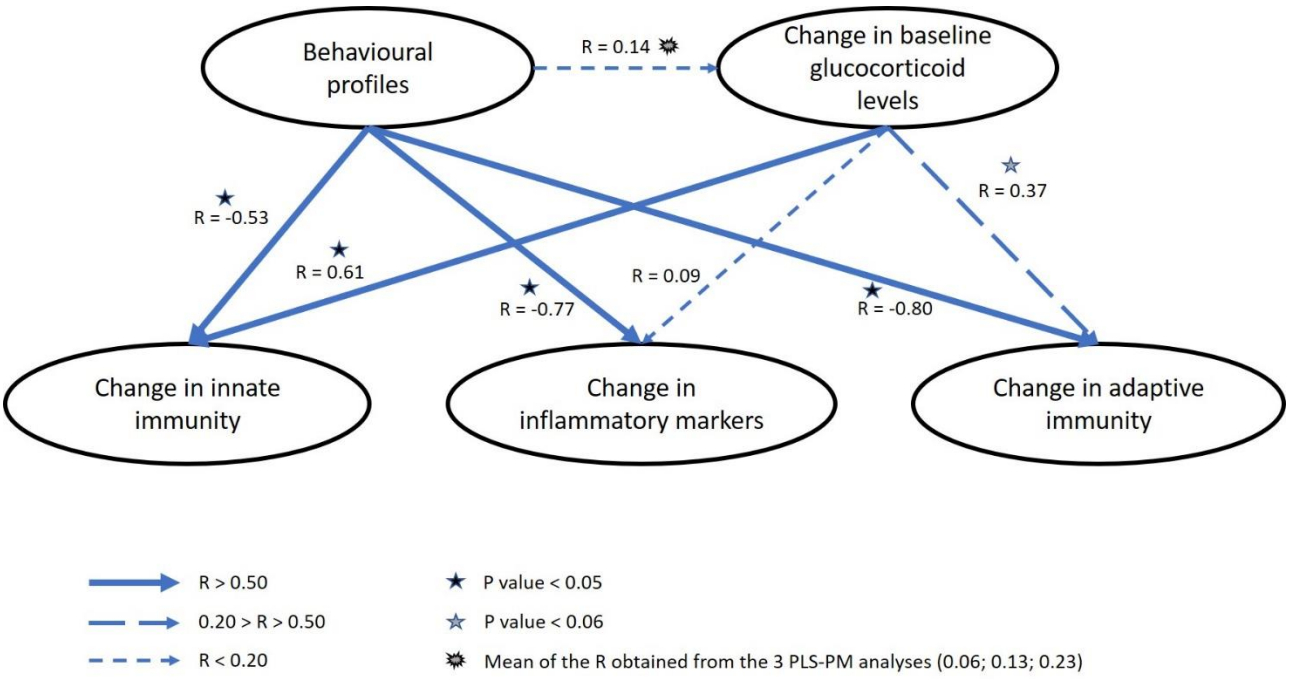

Fig. 3. Relationship between baseline FCMs level (log-transformed) and behavioural profiles. Behavioural profiles' scores correspond to the score for the first axis (PC1) of the PCA conducted using docility, activity and neophilia as co-variables. The three variables were all negatively correlated with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

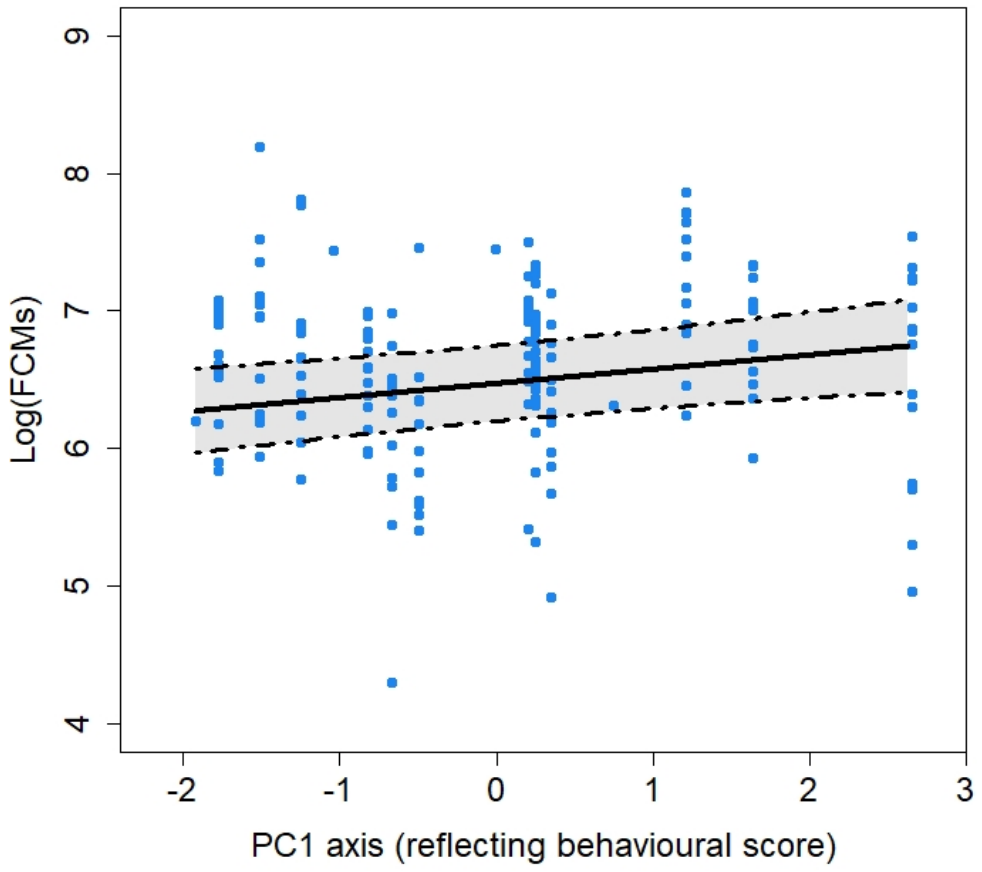
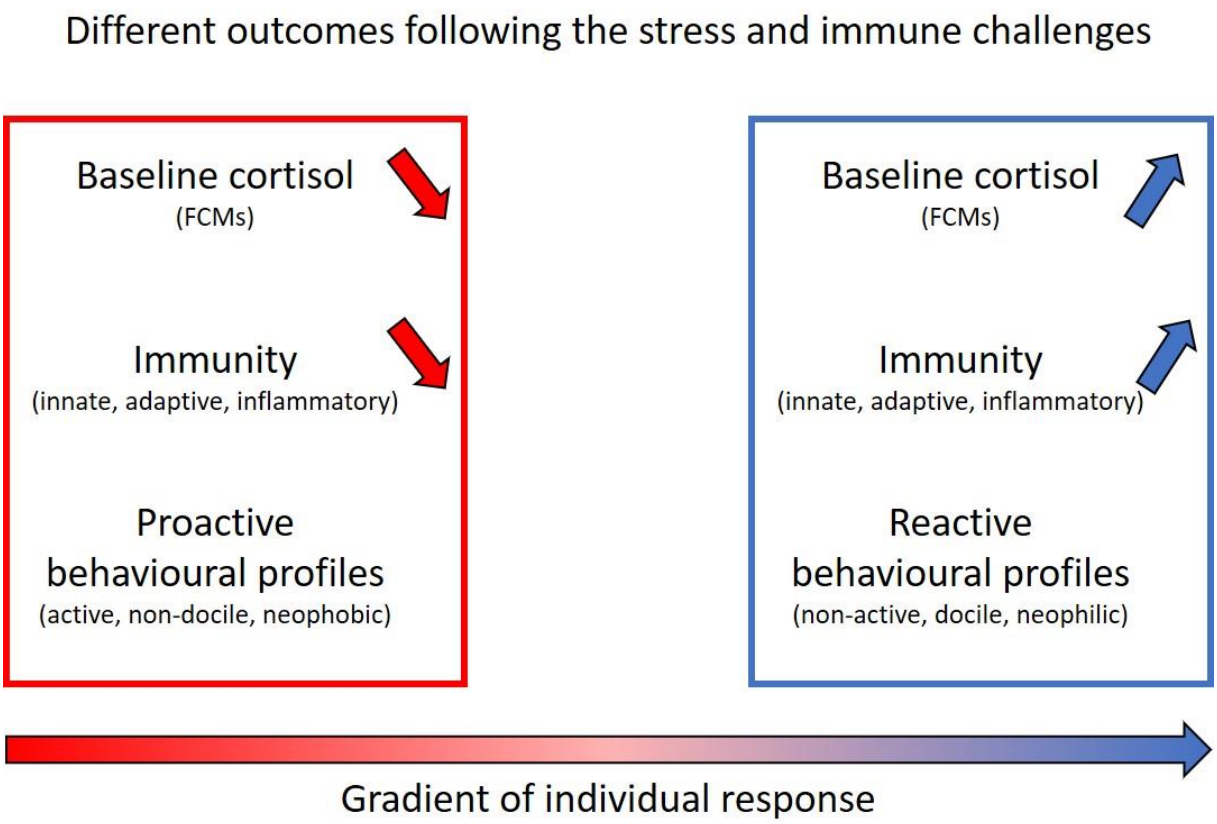
Fig. 4. Summary of the observed outcomes and relationships between the latent variables considered in our study. Our protocol (see details in the main text) resulted in different outcomes, with part of the individuals showing an increase in baseline cortisol between the two study periods (indicated by 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.