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Shyness–Boldness, but not Exploration, Predicts Glucocorticoid Stress Response in Richardson's Ground Squirrels (*Urocitellus richardsonii*)

Dawson Clary*, Lindsay J. Skyner†, Calen P. Ryan†, Laura E. Gardiner†, W. Gary Anderson† & James F. Hare†

* Department of Psychology, University of Manitoba, Winnipeg, MB, Canada
† Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada

Correspondence
James F. Hare, Department of Biological Sciences, 222 Biological Sciences Building, 50 Sifton Road, University of Manitoba, Winnipeg MB R3T 2N2, Canada.
E-mail: james.hare@ad.umanitoba.ca

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Abstract

The relationship between stress and personality has often been studied using captive animals in a laboratory context, yet less often in wild populations. Wild populations, however, may reveal aspects of the personality–stress relationship that laboratory-based studies cannot. Here, we assessed the personality and stress hormone response of adult females within a free-living population of Richardson's ground squirrels (*Urocitellus richardsonii*). Personality was assessed by quantifying individual responses to a novel object, and physiological stress was measured from faecal glucocorticoid metabolites. Principal component and principal component regression analyses were performed to determine whether the behavioural and endocrine measures were related. Based on these analyses, shyness–boldness was found to best predict glucocorticoid levels, in that individuals expressing the greatest vigilance in response to the novel object also had the highest measured concentrations of faecal glucocorticoids. Exploration, however, was independent of measured glucocorticoid levels, consistent with a multidimensional interpretation of non-human animal personality.

Introduction

Stable behavioural traits, often referred to as personality, are now considered common in non-human animals (Sih et al. 2004; Réale et al. 2007). An animal's tendency to escape or explore novel objects and spaces is often used to assess its personality (Verbeek et al. 1994; Fox et al. 2009; Ruiz-Gomez et al. 2010), allowing individuals to be classified according to their behavioural response (shy/bold: Wilson et al. 1993; proactive/reactive: Koolhaas et al. 1999; see Réale et al. 2007 for a suggested integrated nomenclature). Such variability along the personality spectrum, its adaptive value and fitness implications, has garnered considerable empirical interest in biomedical (Koolhaas et al. 2010), agricultural (Lawrence et al. 1991) and ecological research (Wilson et al. 1993; Réale et al. 2007).

Personality traits have proved to be at least partially heritable (Dingemanse et al. 2002; van Oers et al. 2005), so contemporary research has turned to identifying the physiological mechanisms underlying personality variation. The hypothalamic–pituitary–adrenal (HPA) axis, which regulates stress responses, thereby promoting allostasis (an organism's response to maintain homeostasis after a disturbance, McEwan & Wingfield 2003), represents one potential endocrine system modulating personality (Carere et al. 2010). The hypothalamic release of corticotropin-releasing factor stimulates the release of adrenocorticotropic hormone from the anterior pituitary into the bloodstream, which acts on the adrenal cortex to promote the release of glucocorticoids (cortisol and/or corticosterone). In the context of personality, shy/reactive individuals have been shown to have a greater HPA axis response, indicated by increased glucocorticoid levels, compared with bold/proactive individuals (Ellis et al. 2006; Carere et al. 2010). Further, HPA responsiveness, like personality, is partially heritable (Satterlee & Johnson 1988; Odeh et al. 2003). Indeed, correlations between glucocorticoids and personality traits across vertebrate taxa have prompted researchers to suggest that glucocorticoids commonly serve as mediators of personality (Koolhaas et al. 1999; Korte et al. 2005; Carere et al. 2010; Costantini et al. 2012).

Recent research, however, has suggested that the relationship between the stress response and personality traits may not be so straightforward (Koolhaas et al. 2010; Montiglio et al. 2012; Ferrari et al. 2013). For instance, Van Reenen et al. (2005) found that plasma cortisol of calves was related to behavioural indices of fearfulness but not to locomotion during an open-field test. Ferrari et al. (2013) found no link between cortisol and personality indices such as activity and impulsivity in a wild population of alpine marmots. In some cases then, the cortisol stress response may act only on certain dimensions of personality or be independent of personality variation altogether. Ferrari et al. (2013) emphasized the need to study coping styles (i.e. personality) in wild populations because the results of laboratory studies may be attributable to selection for individuals representing opposite extremes of the personality spectrum. Although a laboratory-based approach affords the advantages of readily elucidating the genetic basis of personality traits and providing more stringent environmental control, studying wild populations facilitates the investigation of natural variation in personality and stress profiles, and hence, promotes the identification of their underlying mechanisms (Archard & Braithwaite 2010). Furthermore, an individual's personality in captivity may not be representative of its personality in the wild (Réale et al. 2000). Thus, supplementing laboratory studies with studies of wild populations is necessary to robustly document the relationship between stress and personality.

In this study, we examined the exploratory and escape responses of free-living adult female Richardson's ground squirrels (*Urocitellus richardsonii*) to a novel object to assess personality variation and quantified faecal glucocorticoid metabolite levels of those individuals as an index of their stress response. Ground squirrels are ideal for examining personality as they are diurnal, large enough to be marked individually and habituate readily to human observers (Michener & Koeppl 1985). Furthermore, the complexity of their social systems may select for the evolution and maintenance of behavioural polymorphisms (Hare & Murie 2007). We analysed behavioural and glucocorticoid measures using an exploratory principal components approach to identify how many personality dimensions are present, and whether variation in the underlying behavioural variables and faecal glucocorticoid metabolite concentration were explained by a shared component. We also conducted a principal components regression to examine which combinations of behavioural measurements best explained variation in the stress response. If personality and the glucocorticoid stress response are related, we predicted that their indices would load on a common component and that each would explain a significant proportion of the other's variance.

Methods

Field Site and Study Animals

Observations were made of 12 adult female Richardson's ground squirrels (*Urocitellus richardsonii*) between April and July 2009 that were members of a free-living population on the grounds of the Assiniboine Park Zoo (49°52′N, 97°14′W) in Winnipeg, Manitoba, that has been the focus of ongoing research since 2003. Our present research focused on adult females because female philopatry and male dispersal in Richardson's ground squirrels (Davis & Murie 1985) results in the formation of matrilineal kin clusters (Michener 1983), with relatively few adult males surviving extreme male competition for mates during the spring breeding season (Michener & Locklear 1990).

Capture Protocol

Ground squirrels were live-trapped using National or Tomahawk live traps baited with peanut butter. Each individual was marked for permanent identification with a metal ear tag (Monel #1; National Band and Tag Co., Newport, KY, USA) affixed to the right pinna and marked for identification in the course of observations with a unique pattern of hair dye (Clairol Hydrience, No. 52 Pearl Black, Stamford, CT, USA) applied to their dorsal pelage. After capture for tagging and marking, individual ground squirrels were released and left undisturbed for 7 d before commencing novel object trials. Ground squirrels commonly defecate when live-trapped, so faeces were collected from the bottom of the trap from each individual within 1 h of excretion. While live-trapping resumed during novel object testing on non-test days, faecal samples were not collected for glucocorticoid analysis for at least 48 h after handling. These samples provide a non-invasive means of assessing circulating glucocorticoids approximately 10–24 h prior to defecation (Palme et al. 2005; Dantzer et al. 2010) and are not as subject to potential biases owing to differential responsiveness to capture and handling as plasma-derived glucocorticoid samples (Hare et al. 2014). All faecal samples were collected between 1200 and 1500 h CDT and stored in 20-ml polycarbonate vials with a unique numeric label so that they could be cross-referenced with the date, time of collection and animal identity from field notes. Vials were stored in a −4°C freezer for up to 7 d at the Assiniboine Park Zoo and then transferred to a −20°C freezer at the University of Manitoba until samples were extracted and measured by radioimmunoassay. All procedures involving animal subjects were approved under protocol F08-012 of the University of Manitoba, Fort Garry Campus Protocol Management and Review Committee and adhered to guidelines of the Canadian Council on Animal Care.

Novel Object Trials

Trials involving the presentation of a novel object were conducted before 1200 h CDT when trials could be conducted reliably without disturbance from members of the public. The experimenter (LJS) initiated each trial by approaching a prospective subject until it retreated into a burrow. The distance from the experimenter at which the ground squirrel escaped would then be recorded (EscapeD). Next, a tan Biltmore hat (32.5 × 19.5 cm brim × 13 cm high) was placed 1 m outside the escape burrow and served as the novel object. Once the hat was placed, the experimenter started a stopwatch and retreated to a position 15 m from the burrow entrance to observe the ground squirrel's behaviour. The latency for the ground squirrel's head to reappear from the burrow was measured (Emerge), at which point the ground squirrel's behaviour was video-recorded (SONY Hi 8 Camcorder CCD-TR700; Oradell, NJ, USA) for 10 min. From the video, we coded the latency for each individual ground squirrel's entire body to emerge from the burrow (FullEmerge), latency to approach the novel object (Approach), the number of subterranean escapes made to the burrow after emerging (SubEscapes), total time the ground squirrel remained visible (Visible) and the proportion of time the ground squirrel spent in a vigilant posture (Vigilant). Thus, a total of seven behavioural measures were documented and used for analysis. For latency measures, if a behaviour did not occur within the 10-min trial, it was given a maximum value of 600 s. Four trials, each separated by 16–29 d so as to coincide with late gestation, late lactation, early post-juvenile emergence and pre-hibernation, were conducted and used in the analysis for each subject thus exploring potential changes in response over the female reproductive cycle. For one individual, however, only a single trial was used owing to infrequent sightings of the subject in question. Further, we chose to conduct repeated trials to obtain a more reliable estimate of each ground squirrels' response to the object, as the field setting did not afford the environmental control of a laboratory setting and other environmental factors could influence the ground squirrels' behaviours (e.g. presence of humans, weather conditions, recent encounters with conspecifics or allospecifics). Thus, subsequent trials were not true 'novel' object trials (Carter 2013), but quantified average response over a series of presentations allowing an equal probability of habituation among subjects, while avoiding confounds associated with differential response to different novel objects (Heyser & Chemero 2012; Oliveira & Galhardo 2013).

Sample Extraction and Assay

Procedures were based on Mateo & Cavigelli (2005), but modified for *U. richardsonii* (Ryan et al. 2012; Hare et al. 2014). Faecal samples were dried overnight at 60°C before approximately 0.2 g of the faeces was combined with 1.5 ml 95% ethanol. Samples were vortexed, then centrifuged at 4°C for 10 min at 13 000 ***g***. The resulting supernatant was stored at −80°C until used in the radioimmunoassay. At this point, 100 μl of supernatant was removed and the ethanol was evaporated using a sample concentrator (Savant SpeedVac; Thermo Scientific, Waltham, MA, USA).

The resultant pellet was then resuspended and vortexed in a 0.1 M phosphate buffer, 0.9% NaCl [w/v] and 0.5% bovine serum albumin [w/v]. Seventeen to 23 faecal samples obtained from each subject had glucocorticoids extracted, except for the individual that participated only in one novel object trial, for which seven faecal samples had glucocorticoids extracted.

In the glucocorticoid radioimmunoassay, 100 μl of a corticosteroid-specific antibody (1:16 000 dilution; Fitzgerald Industries, North Acton, MA, USA) was combined with 5000 disintegrations per min/100 ml (dpm) of tritiated cortisol (GE Healthcare, Piscataway, NJ, USA) and 100 μl of sample or a known concentration of cortisol (Steraloids, Newport, RI, USA) in an assay tube. The assay was incubated at room temperature for 1 h, then at 4°C overnight. The reaction was terminated by adding dextran-coated (0.5% w/v) charcoal (5% w/v) (100 μl) to the solution. The assay tubes were placed on ice for 15 min, then centrifuged for 30 min at 4°C (2500 ***g***), with the resulting supernatant decanted into a 7-ml scintillation vial, to which 4 ml of Ultima Gold scintillation fluid (Perkin Elmer) was added. Radioactivity was measured using a liquid scintillation counter (LS6500; Beckman Coulter, Brea, CA, USA). Cortisol concentrations (ng/g of dry mass of faecal matter) were determined by interpolating from a standard curve of known concentration of cold cortisol. Interassay and intra-assay variations were 10.9 ± 6% and 15.3%, respectively, with minimum detectable levels of 0.051 ng/ml. Samples demonstrated good parallelism, and extraction efficiency was 90%. Radioimmunoassay methods for the measurement of glucocorticoid metabolites from the faecal matter of Richardson's ground squirrels employed in the present study are validated in Hare et al. (2014).

Statistical Analysis

Initial exploration of the data revealed that many of the variables were moderately to strongly correlated with one another (r = 0.4–0.9). Due to this multicollinearity, principal component analysis (PCA) and principal component regression (PCR) were chosen for the analysis, as these methods are designed to reveal patterns in data based on the correlative relationship among variables (Tabachnick & Fidell 2007). Three of the variables (Emerge, FullEmerge and SubEscapes) were found to have non-normal distributions. These variables were $\log_{10}$-transformed to improve normality. A constant (K = 1) was added to SubEscapes before the transformation, as this variable had some zero values. Although PCA makes no explicit distributional assumptions, non-normal variables may diminish linearity among variables and the ability of the PCA to efficiently extract components (Quinn & Keough 2002). Transformations can improve the linearity between variables (Tabachnick & Fidell 2007), and in this case, while transformations did not entirely normalize the variables, linearity was improved. Thus, all analyses were completed using the transformed variables.

Two principal component analyses were conducted. The first (All Variables PCA) included our measure of faecal glucocorticoid metabolite concentrations (FGMs) in addition to the seven behavioural measures (EscapeD, Emerge, FullEmerge, Approach, SubEscapes, Visible and Vigilant). The purpose of this analysis was to ascertain whether the glucocorticoid measure would load onto the same component as the behavioural measures, indicating that both the glucocorticoid and behavioural variables are reflective of the same underlying phenomenon (i.e. personality). The second principal component analysis included only the behavioural measures (Behaviour Only PCA). This was undertaken to identify the number of components present within the behavioural measures, so that these components could then be used as predictor variables for a PCR analysis treating faecal glucocorticoid concentration as the response variable. Variables were measured in different units (e.g. time, distance and discrete measures); therefore, the PCA was based on a correlation matrix rather than a covariance matrix (Quinn & Keough 2002). Additional rotations were achieved through a Varimax orthogonal rotation to improve simple structure. A variable was considered to have loaded strongly onto a component if it produced a loading of greater than 0.4 or less than −0.4 (Ferrari et al. 2013). One-way repeated-measures ANOVAs were conducted on each behavioural measure to examine whether the ground squirrels responded to the object differently over trials. Paired samples t-tests with a Holm adjustment were used to detect *post hoc* differences among trials. For all significance tests, alpha was set at 0.05.

Results

Repeated Measures

Significant differences among trials were detected for EscapeD ($F_{3,30}$ = 4.973, p = 0.006), with ground squirrels showing a greater escape distance during trial 4 than in previous trials (trials 1–4: p = 0.034; trials 2–4: p = 0.049; trials 3–4: p = 0.019). No significant differences were found for Visible ($F_{3,30}$ = 0.721, p = 0.547), Emerge ($F_{3,30}$ = 2.738, p = 0.061), FullEmerge ($F_{3,30}$ = 2.904, p = 0.051), Approach ($F_{3,30}$ = 1.558, p = 0.220), SubEscapes ($F_{3,21}$ = 0.379, p = 0.769) and Vigilant ($F_{3,18}$ = 0.642, p = 0.598), suggesting that individual ground squirrels responded similarly to the object across all trials, with virtually no evidence of habituation, nor changes in response as subjects progressed from late gestation through parturition and weaning their young.

All Variables PCA

Three components produced eigenvalues of one or greater (4.2, 1.7, 1.0) and were extracted from the

PCA (Norman & Streiner 1994; Tabachnick & Fidell 2007) for an additional orthogonal rotation. These three components explained 85% of the total variance (52%, 21% and 12% for components 1, 2 and 3, respectively). A Varimax orthogonal rotation improved the simple structure (i.e. variables had stronger loadings on a single component) and was used for all subsequent interpretation (Table 1). The first component was characterized by high loadings of LogFullEmerge and LogSubEscapes. The second component had high loadings of FGMs and Vigilant. The third component had high loadings of Visible and Approach. LogEmerge and EscapeD did not load strongly onto any of the three components (Table 1). FGMs did not load onto a common component with the majority of the behavioural variables; therefore, only the behavioural measures were used for a second PCA to determine components for a PCR.

Behaviour Only PCA

Two components produced eigenvalues of one or greater (4.0, 1.2); however, a subsequent Varimax rotation with three components retained produced better simple structure than the two component result. Additionally, the third component's eigenvalue approached one (0.9). Thus, three components were extracted and interpreted from the PCA (Table 2). These three components accounted for 87% of the total variance (57%, 17% and 13% for components 1, 2 and 3, respectively). The first component had high loadings of Visible and Approach. These variables relate well to the typical exploration measures used to assess personality (i.e. willingness to explore a novel situation); therefore, the component was designated Explore. The second component had high loadings of LogFullEmerge and Vigilant. In full body emergence from the burrow, ground squirrels typically assumed a vigilant posture, and therefore, this component was designated Vigilance. The third component consisted of a high loading of LogSubEscapesK. Because only one variable loaded onto this component, it was difficult to interpret, although in that ground squirrels may have returned to the burrow for a number of reasons (not necessarily to escape from the novel object), this component was given the neutral designation, Home. Again, LogEmerge and EscapeD did not load strongly onto any of the three components (Table 2).

A multiple regression using the three behavioural components as predictors of FGMs did not resolve a statistically significant relationship ($F_{3,8}$ = 3.176, p = 0.085; Table 3). The overall model produced an adjusted R^2 of 0.372. Of the components supplied to the model, Vigilance was the strongest predictor in that it approached statistical significance (B = 2.109, p = 0.066; Fig. 1).

Discussion

The repeated-measures analysis showed that no habituation occurred over the four trials and the squirrels did not respond to the object differently during different stages of the reproductive cycle, supporting the notion that the object was perceived as 'novel' during each trial. Subsequent PCA and PCR analysis were consistent in revealing a relationship between the glucocorticoid stress response and vigilance behaviours. From the initial All Variables PCA, it became apparent that the behavioural measures were not indicative of a single component of personality, and that only 'Vigilant' loaded onto the same component as our measured faecal glucocorticoid metabolite concentration. Similarly, the PCR revealed that the Vigilance component was the strongest predictor of FGMs. Vigilance behaviours can be interpreted as shyness–boldness (Réale et al. 2007), as ground squirrels assess risk in their environment through this behaviour (Verdolin & Slobodchikoff 2002). In this context, we found that the shy–bold continuum is positively related to stress, in that individuals that were more

Table 1: Loadings of each variable on each component for the All Variables PCA

	Component 1	Component 2	Component 3
Glucocorticoids		**0.628**	−0.246
Visible	0.119		**0.537**
EscapeD	−0.373	0.133	−0.193
LogEmerge	−0.377	0.128	−0.249
LogFullEmerge	**−0.406**	−0.367	
Approach			**−0.604**
LogSubEscapesK	**−0.722**		0.326
Vigilant	−0.136	**0.656**	0.270

Bold values indicate strong loadings.

Table 2: Loadings of each variable on each component for the Behaviour Only PCA

	Component 1	Component 2	Component 3
Visible	**0.562**		
EscapeD	−0.291	0.127	−0.351
LogEmerge	−0.329		−0.355
LogFullEmerge		**−0.471**	−0.372
Approach	**−0.654**		
LogSubEscapesK			**−0.752**
Vigilant		**0.860**	−0.191

Bold values indicate strong loadings.

risk aware, either in terms of devoting a greater proportion of time to vigilance, or by entering a vigilant posture sooner, showed higher faecal glucocorticoid metabolite concentrations. Mateo (2007) reports a similar finding for Belding's ground squirrels.

In addition to vigilance, a second personality component was derived from exploration-related behaviours. Both the All Variables PCA and the Behaviour Only PCA indicated that the exploration aspects of personality (i.e. willingness to approach or explore novelty; Réale et al. 2007) loaded onto a shared component; however, these variables loaded onto a separate component from that related to FGMs. Thus, it appears that in Richardson's ground squirrels, the myriad factors associated with the glucocorticoid stress response do not underlie the exploration dimension of personality. This finding is contrary to the results of previous research, which has indicated that personality, as expressed by exploration-related behaviours, is related to HPA activity (Carere et al. 2003; Stöwe et al. 2010; Costantini et al. 2012; Baugh et al. 2013).

Our results are partially consistent with two-tier models of personality and stress (Van Reenen et al. 2005; Koolhaas et al. 2010). In these models, behavioural activity, also referred to as coping style, is placed on one axis and physiological stress response on another axis. Accordingly, our faecal glucocorticoid metabolite measure was independent of activity towards the novel object (i.e. Explore component). Our findings, however, suggest that multiple personality dimensions are present, with the dimension indicative of shyness–boldness regulated directly by glucocorticoids (i.e. along the same axis). Thus, our results are more compatible with a multidimensional approach to non-human animal personality (Réale et al. 2007) wherein fearfulness and coping style constitute separate personality dimensions (Van Reenen et al. 2005; Koolhaas et al. 2007). In fact, our analysis revealed similar personality dimensions (Explore, Vigilance and Home) as reported for a wild population of eastern chipmunks (exploration, vigilance and centrality: Montiglio et al. 2012).

The fact that our faecal glucocorticoid metabolite measure was not related with measures reflecting general exploration is also consistent with suggestions that novel object and novel environment tasks may be measuring independent aspects of personality (Martins et al. 2007; Fox et al. 2009). Dominance has previously been associated with exploration during novel environment tasks (Verbeek et al. 1996; Fox et al. 2009), whereas novel object tasks seem to measure fearfulness (Van Reenen et al. 2005; Fox et al. 2009). In this context, our use of a novel object task, although untraditional given repeated presentation of that object, would explain why we found a link between vigilance (i.e. fearfulness) and FGMs.

Our results provide additional insight into the relationship between glucocorticoids and personality. Carere et al. (2010) highlighted the need to identify the proximate mechanisms mediating the relationship between glucocorticoids and animal personality and proposed three possibilities: (1) stress physiology determines behaviour, (2) behaviour determines stress physiology or (3) additional factors jointly determine

Table 3: Results of multiple regression analyses with faecal glucocorticoid metabolite concentration as the response variable and the three extracted components from the Behaviour Only PCA as the predictor variables

	Beta	SE	t-value	p-Value
Intercept	16.7204	1.0771	15.524	<0.001
Explore	−0.7608	0.5371	−1.417	0.1943
Vigilance	2.1089	0.9894	2.132	0.0656
Home	1.9683	1.1406	1.726	0.1227

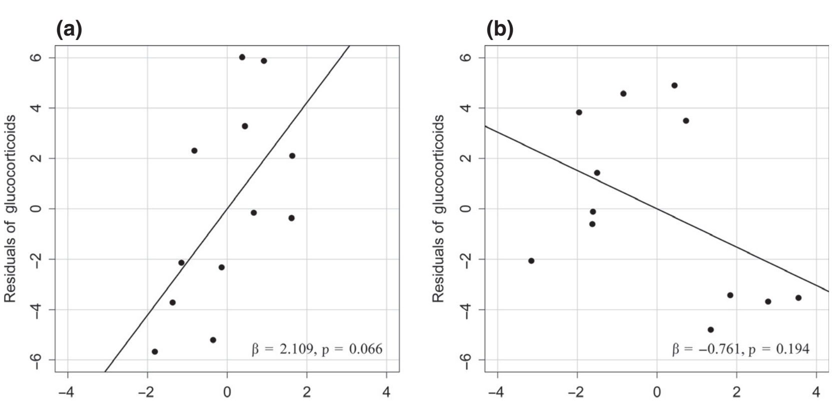

Fig. 1: Partial regression plots showing the relationship between the residuals of faecal glucocorticoid metabolite (FGM) concentration and (a) the Vigilance component or (b) the Explore component. Points represent averages within each female.

stress physiology and behaviour. Our results reveal that stress hormones and shyness–boldness are intimately related. Although we cannot attribute the direction of causation from this experiment, past research suggests glucocorticoids may be the causal factor (Breuner et al. 1998). Our exploration variables, however, loaded onto an orthogonal (i.e. independent) component to FGMs, and thus fail to refute the third possibility proposed by Carere et al. (2010) for this personality dimension. That is, although the glucocorticoid response and exploratory behaviours are correlated, they may be indicative of two separate, albeit highly integrated, processes. This view is consistent with findings that personality characteristics can be flexible under some conditions (Dugatkin & Ohlsen 1990; Carere et al. 2005; Morrell et al. 2005; Frost et al. 2007) and independent of cortisol levels (Ruiz-Gomez et al. 2008; Ferrari et al. 2013).

Similarly, Van Reenen et al. (2005) have suggested that personality reflects the interaction between the stress response and other underlying factors mediating activity. One possible factor that could jointly influence the interaction between exploration and stress could be learning ability and its neurobiological correlates (Koolhaas et al. 2007; Mateo 2008). Indeed, learning ability has been related to variation in exploration style. For example, black-capped chickadees that are fast explorers (i.e. bold) were reported to acquire an operant learning task more quickly than slow explorers (i.e. shy; Guillette et al. 2009), but are slower in responding to contingency changes (Guillette et al. 2011). Therefore, learning history could regulate how individuals explore their environment, and hence, the number and type of stressors they encounter. Furthermore, the type of environment could differentially influence the allostatic load of individuals with different learning strategies: bold individuals are adapted for stable environments, whereas shy individuals are adapted for dynamic environments (habitat-dependent hypothesis: Réale et al. 2007). The dependency of personality and stress on environmental context highlights the importance of studying wild populations from different environments.

Wild populations are underrepresented in the study of non-human animal personality (Archard & Braithwaite 2010). Our findings corroborate the conclusions of other studies involving wild mammal populations (Montiglio et al. 2012; Ferrari et al. 2013), suggesting exploration aspects of personality in wild populations may not be tied directly to the stress response. Our approach of using faecal sampling, a technique that avoids bias owing to the stress of capture, in a wild population reveals aspects of the personality–stress relationship that traditional laboratory studies have not. Future studies of wild populations will prove useful in determining whether the dissociation between exploration and the glucocorticoid stress response is characteristic of free-living animals.

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