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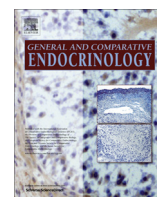
Initial reactivity and magnitude of the acute stress response associated with personality in wild great tits (*Parus major*)

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ABSTRACT

Phenotypic correlations, such as those between functionally distinct behavioral traits, can emerge through the action of selection on individual traits, on trait combinations, and through pleiotropic mechanisms. Steroid hormones are known to have pleiotropic effects on a suite of behavioral and physiological traits, including stable individual differences in coping with stress. Characterizing the stress axis in relation to personality, however, has typically focused on estimating baseline and peak levels of glucocorticoids, principally in captive animals. In contrast, the reactivity of the stress response—how quickly it turns on and persists—may better indicate the ability of an individual to cope with challenges, particularly in free-living animals. Using wild great tits (*Parus major*) we tested the hypothesis that cautious individuals respond to a standardized stressor with a more reactive stress response compared to bolder individuals. Wild birds were captured and tested for exploration behavior in a novel environment—an operational measure of personality in this species—and assessed separately for their glucocorticoid response to a standardized stressor. Slower explorers exhibited a greater elevation in glucocorticoid levels within the first three minutes after capture. Further, slower explorers reached a higher maximum CORT concentration and had higher total exposure to glucocorticoids during the stressor period. These data provide evidence that the temporal reactivity of the endocrine stress response, specifically its speed and magnitude, is associated with stable behavioral traits in free-living animals.

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1. Introduction

Individual animals exhibit behavioral differences that are stable over time and across contexts (Gosling, 2001; Réale et al., 2007). Such behavioral differences are referred to as coping styles or personality, and have been shown to have a genetic (van Oers et al., 2005) and developmental basis (Stamps and Groothuis, 2010), and be under sexual (Schuett et al., 2010) and natural selection (Dingemanse et al., 2004; Quinn et al., 2009; Smith and Blumstein, 2008). The idea that hormones serve a key role in promoting such differences is a sustained hypothesis in animal behavior research (Williams, 2008). Stress hormones in particular are thought to be involved in one of the major axes of personality variation: the shy–bold continuum (Carere et al., 2010; Korte et al., 2005; Øverli et al., 2007).

The endocrine stress response is coordinated by the hypothalamic–pituitary–adrenal (HPA) axis. The HPA axis releases glucocorticoid hormones and one of the critical functions of this system is to cope with the demands of normal life, for example day–night rhythmicity, locomotor activity and metabolism (Landys et al., 2006). Moreover, the HPA axis is essential for coping with unpredictable, 'stressful' events, such as exposure to unfamiliar environments (Lendvai et al., 2011), inclement weather (Breuner and Hahn, 2003), or predators (Cockrem and Silverin, 2002; Eilam et al., 1999). The stress response consists of several components: First, the response is initiated within a few minutes after a stimulus (stressor, e.g., capture) is perceived, as glucocorticoids (and their upstream secretagogues) are secreted above baseline concentrations. Second, levels of glucocorticoids continue to increase in the blood until they reach a peak concentration, typically within 30–60 min. Third, a process of negative feedback reduces circulating glucocorticoid levels, allowing baseline levels to be re-achieved, thus enabling the animal to respond to future challenges. The main glucocorticoid in birds is corticosterone (CORT), and like many steroid hormones CORT can affect diverse regulatory and behavioral processes simultaneously. For example, elevated CORT

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stimulates locomotor activity and foraging when food reserves are low, but can also suppress non-essential and energetically demanding processes such as immune defense, reproductive physiology and behavior (Sapolsky et al., 2000).

Because a single hormone such as CORT can generate co-variation at multiple physiological levels (c.f., hormonal pleiotropy; (Ketterson and Nolan, 1999), and because the HPA axis can influence behavior directly, the pleiotropic effects of CORT might contribute to behavioral co-variation (Baugh et al., 2012; Korte et al., 2005; Øverli et al., 2005). In fact, several of the canonical behavioral traits that characterize personality (e.g., shyness in animals, neuroticism in humans; (Gosling, 2001) may relate to differences in how individuals respond hormonally to stressors (Koolhaas et al., 2007; Martins et al., 2007). Furthermore, there is often remarkable intra-population variation in concentrations of baseline and stress-induced CORT (Pottinger et al., 1992; Tort et al., 2001). Importantly, a portion of this variance has been shown to be repeatable among individual birds (Evans et al., 2006; Pottinger and Moran, 1994; Rensel and Schoech, 2011; Romero and Reed, 2008) and to have a heritable component (Brown and Nestor, 1973; Edens and Siegel, 1975; Evans et al., 2006; Satterlee and Johnson, 1988). On the basis of these established hormone–behavior relationships, glucocorticoid physiology has been hypothesized to underlie animal personality (Korte et al., 2005). Specifically, the HPA axis of shyer individuals is predicted to exhibit greater reactivity (Ellis et al., 2006). Operationally, 'reactivity' of the HPA axis has been used to refer to the magnitude of the stress response, including both the area under the curve (Juster et al., 2012) and the maximum concentration of stress hormones observed during a stress assessment (Wada et al., 2007)—definitions that deemphasize the temporal dimension. Nonetheless, studies probing the relationship between individual differences in behavior and stress physiology have yielded support for this first prediction (Baugh et al., 2012; Cockrem, 2007; Koolhaas et al., 1999; Lendvai et al., 2011; Mackenzie et al., 2009; Øverli et al., 2005). And while much can be gleaned from such studies, many have explored this question using captive or domesticated animals, which might differ in fundamental ways from wild organisms, particularly in their stress physiology (Calisi and Bentley, 2009; Dickens and Romero, 2009).

In this study we extended this line of investigation to test the idea that variation in stress reactivity—including both its onset and magnitude—is associated with behavioral differences in wild birds. Specifically, variation in the initiation phase of the glucocorticoid response might play a role in acute coping behavior, while its magnitude might have longer term consequences, including how effectively an individual can endure a subsequent stressor and which individuals survive stressful natural events (Romero and Wikelski, 2010).

We used wild great tits (Parus major), tested in a standardized way for both exploration behavior and stress responses. Great tits have become a model vertebrate for the study of animal personality, in part because this species has been studied from an ecologically informed perspective. Previous research in this species has shown that exploration behavior in a novel environment (open field test; Verbeek et al., 1996) is a repeatable behavioral trait over long periods of time (Dingemanse et al., 2002), is correlated with mate choice (van Oers et al., 2008), aggression (van Oers et al., 2004; Verbeek et al., 1996), territorial behavior (Amy et al., 2010), learning (Titulaer et al., 2012), reproductive success (Both et al., 2005; Quinn et al., 2009), and survival (Dingemanse et al., 2004). We thus used exploration behavior as an operational measure of great tit personality. Further, heritability studies of wild (Dingemanse et al., 2002; Quinn et al., 2009) and captive great tits (Drent et al., 2003; van Oers et al., 2004) have demonstrated a high degree of heritability in exploration behavior, and a genetic correlation between exploration behavior and stress physiology (Baugh

et al., 2012; Carere et al., 2003). In the present study we tested the hypothesis that slower explorers exhibit a more reactive (earlier elevation and greater magnitude) endocrine stress response.

2. Materials and methods

2.1. Study system

The Westerheide study area near Arnhem, The Netherlands (52° 0′ 38″ N, 5° 50′ 30″ E) is a forest of approximately 100 ha and hosts a large long-term study population of color-ringed personality-typed wild great tits. Since personality has been shown to be a trait that is consistent over time, we did not retest individuals of known personality, but conducted new tests upon first capture of unringed individuals. We carried out behavioral testing and hormone sampling on separate dates to avoid the potential confounding effects that the bleeding experience might have on performance in the behavior assay, and vice versa. Because we could not target the collection of specific individuals, we instead sampled birds opportunistically for hormones and behavior, resulting in a median interval between behavioral testing and hormone sampling of 28 days (mean ± SD: 145 ± 300 d). Our exploration assay has been shown to estimate persistent characteristics of an individual (Carere et al., 2005; Dingemanse et al., 2002), indicating that the scores have a high explanatory power across prolonged periods of time.

2.2. Behavioral testing

Birds were caught with mist nets near feeding stations in Westerheide and transported for approximately 0.5 h in transport boxes to a custom designed housing and behavioural testing facility (Heteren, The Netherlands) where they were kept overnight in individual cages ($0.9 \times 0.5 \times 0.4$ m) in a room that shares a common wall with the test chamber. Exploration behavior was measured separately for each individual following a standardized protocol using a test chamber (2.0×4.0 m, 2.5 m high) with five artificial 'trees' as a novel environment (for details see Dingemanse et al., 2002). On the morning following capture (0800–1200) each bird was released individually from its cage directly into the test chamber without handling, by opening a sliding door on the chamber side of the common wall. After entry into the chamber, we monitored behavior for 2 min and recorded the number of tree visits and hops and flights between and within perches (e.g., branches of the artificial trees, sliding doors, floor). We calculated the exploration scores by summing all hops and flights per individual. Exploration scores are known to vary seasonally (increasing as the breeding season approaches) but remain repeatable at the individual level (Dingemanse et al., 2002). We therefore corrected for 'July date', which is the number of days from 1 July onward (exploration scores from the sample ($n = 86$): mean = 23.09, SD = 9.46, range = 5.7–49.9). All individuals in this study were tested for the first time during their lives, thus precluding any effects of habituation to the testing conditions (Dingemanse et al., 2002). Behavioral testing was conducted blindly and independently of hormone sampling and measurement. Birds were released at their site of capture within a few hours following behavioral testing.

2.3. Hormone sampling

In the autumn of 2010 we captured birds for the measurement of plasma CORT using a standardized handling-restraint protocol to examine initial and stress-induced concentrations (Romero et al., 1997). By sampling plasma CORT during the non-breeding season, when hormone levels fluctuate less (Romero and Wingfield, 1998), and by sampling during a restricted time of day

(0800–1200), we aimed to minimize variation in hormone concentrations resulting from seasonal and diel fluctuations (Astheimer et al., 1994; Breuner et al., 1999; Romero, 2002). We captured birds by erecting mist nets adjacent to feeding stations at five locations. We observed mist nets from a distance of ca. 20 m behind vegetation and initiated a digital timer the instant a bird intercepted the net.

We collected approximately 40 μL of blood at each sampling time point by puncturing the brachial vein using a 30-gauge needle. The first blood sample is referred to as initial CORT ('CORT0') and was collected in less than 3 min following entry into the net—this duration of elapsed time is referred to as 'handling time'. Most passerine species exhibit a detectable increase in plasma CORT approximately 3 min after the onset of an acute stressor (Romero and Reed, 2005; Romero and Romero, 2002). After the initial bleed, birds were held in cloth bags until 30 min after capture, when they were re-bled ('CORT30'). From these 86 birds we randomly sampled a subset ($n = 16$) at one additional time point: 90 min after capture ('CORT90'). This second stress-induced time point provided us with an opportunity to examine the relationship between behavioral scores and the total (area under the curve) and maximum CORT exposure (highest value reached for each bird) during the stressor.

Following the final bleed for each bird we measured tarsus length (Ecotone mechanical callipers, ±0.1 mm), body mass (30 g Pesola scale, ±0.3%), and furcular fat score (0–5; Cherry, 1982). Although most birds had completed a pre-basic molt, a minority of birds were in the final stages and therefore we also included molt score. Lastly, we recorded the air temperature at capture (Royal Netherlands Meteorological Institute, Deelen Station) and ring number. We fitted unringed birds with a uniquely numbered aluminium ring (Vogeltrek Station, NIOO). Birds were then immediately released at the site of capture.

Blood samples were kept on wet ice for less than 3 h before being centrifuged (5000 rpm for 10 min; ca. 1400 g). The plasma fraction was stored at −80 °C until November 2010 when samples were transferred on dry ice to the Max Planck Institute for Ornithology (Radolfzell, Germany) for hormone measurement.

2.4. Enzyme immunoassay for corticosterone

Plasma CORT concentrations were measured using standard enzyme immunoassay techniques (Enzo Life Sciences, Cat. No. ADI 900-097). Details on the validation (parallelism and precision) of our EIA protocol have been reported elsewhere (Ouyang et al., 2011). Briefly, concentrations were determined following a diethyl-ether extraction of a 5–10 μL sample volume. After drying extracts under a stream of N_2 gas, samples were diluted at a 1:30 dilution using Tris-buffered saline (supplied by kit) and samples were allowed to equilibrate overnight at 4 °C. Samples were then assayed in duplicate along with blanks and five standards (0.032–20 ng mL^{-1} CORT), and values were corrected for average recovery loss, which we determined previously using individual samples spiked with radioactively labelled CORT (mean ± SD; ca. 85% ± 2.7). The intra- and inter-assay coefficients of variation (CV)—9.03% and 9.51%, respectively—were determined by including a minimum of two duplicate samples of stripped chicken plasma spiked with commercial corticosterone (supplied by kit) at a concentration of 20 ng mL^{-1} on each of the nineteen plates. All samples were processed during a two-week period and any sample exceeding a 15% coefficient of variation between duplicates was re-analyzed until CV values met this criterion. The assay has a detection limit of 27 pg mL^{-1}. The cross-reactivity of the antiserum is 100% for corticosterone, 28.6% for deoxycorticosterone, and 1.7% for progesterone.

2.5. Statistics

Following log$_{10}$ transformation of CORT values, the residuals from the general linear model were tested for normality by visual inspection of Q–Q plots and Kolmogorov–Smirnov tests, and homogeneity of variances were examined with Levene's test. The assumptions of all test statistics were met after we excluded four extreme outlying CORT0 samples (>3 SD above mean), along with their associated CORT30 samples. These four CORT0 samples exceeded the average CORT30 concentrations and therefore likely represent animals that had been stressed prior to capture. Using t-tests we tested the assumption that our subset of birds sampled at three time points was a representative subsample of our larger two time point dataset for both exploration scores and CORT values.

We examined the relationship between CORT concentrations and exploration scores using general linear models. In addition to exploration scores, we included a set of variables that have been shown previously to modulate the HPA axis in wild birds: air temperature at capture (Romero et al., 2000), molt score (Cornelius et al., 2011; Romero et al., 2005), handling time (Heidinger et al., 2006; Romero and Reed, 2005), fat score and body condition (Romero et al., 2000). We estimated body condition using the Scaled Mass Index (SMI) (Peig and Green, 2009). The SMI improves on previous methods of calculating body condition from mass/length data (e.g., residuals from an ordinary least squares regression) by incorporating an allometric scaling principle. To calculate SMI, a regression between lnTarus and lnMass was made to calculate the value of the exponent in the SMI calculation. To maximize the accuracy of our formula, we used a linear regression based on a larger sample of birds captured concurrently with this study and measured for mass and tarsus ($n = 188$), and not just the subsample of birds for which we had both hormone and behavior data ($n = 86$).

For the CORT0 model, we included the interaction between handling time and exploration score in order to test the hypothesis that individual differences in exploration score are reflected in the early elevation of CORT levels. We included capture order (whether behavioral testing or hormone sampling came first or second) and intercapture interval (number of days between behavior and hormone sampling) as a factor and covariate, respectively. For the sixteen birds with three time points we calculated the area under the curve ('total integrated CORT') using Prism 4.0 (GraphPad, La Jolla, USA). In our general linear models we used a backwards elimination process and excluded variables with a $p > 0.2$. To avoid over-parameterization in this sample and to corroborate the results from the GLMs we performed partial correlations controlling only for SMI and molt score. Lastly we calculated the difference between CORT concentrations at 30 min and 90 min to investigate recovery magnitude. We used SPSS (version 16.0, SPSS Inc., Chicago, IL, USA) for all statistical analyses.

3. Results

We found no effect of testing order (behavioral testing versus stress series; all $p > 0.4$), testing interval (days separating behavior and stress series characterizations; all $p > 0.2$), or air temperature at capture (all $p > 0.4$) as either main effects or interaction effects with exploration behavior, and therefore these variables were not included in our final models. The subset of birds sampled at three time points (0, 30, 90 min) did not differ from those birds sampled at only two time points (0, 30 min) in exploration scores (mean ± SD, all two-tailed: two time points: 22.1 ± 9.2; three time points: 26.2 ± 9.6; $t_{80} = 1.6$, $p = 0.12$), CORT0 concentrations (two time points: 4.5 ± 3.2 ng mL^{-1}; three time points:

Table 1
Effects of independent variables on $\log_{10}$ transformed concentrations of corticosterone (ng mL⁻¹) at initial (CORT0) and stress-induced time points (CORT30, CORT90), and the area under the curve (total CORT). (Independent variables with $p < 0.2$ were included in the final analyses and significant effects are indicated in bold; b, unstandardized regression coefficients from an analysis of untransformed CORT (ng mL⁻¹); partial eta squared values are presented as estimates of effect size. Significant testing was conducted on $\log_{10}$ transformed data.).

	CORT0 (n = 82)				CORT30 (n = 82)				CORT90 (n = 16)				Total CORT (n = 16)			
	F (d.f.)	b	η_p^2	p	F (d.f.)	b	η_p^2	p	F (d.f.)	b	η_p^2	p	F (d.f.)	b	η_p^2	p
Model (omnibus)	**6.37 (8,73)**	n/a	0.411	**3×10^{-6}**	1.16 (6,75)	n/a	0.085	0.338	**3.91 (6,9)**	n/a	n/a	**0.033**	**6.125 (6,9)**	n/a	n/a	**0.008**
Exploration score	**9.70 (1,73)**	0.536	0.117	**0.003**	0.11 (1,75)	0.063	0.001	0.742	**8.92 (1,9)**	−0.417	0.498	**0.015**	**12.03 (1,9)**	−24.66	0.572	**0.007**
Molt score	**6.46 (1,73)**	−0.251	0.081	**0.013**	1.96 (1,75)	−0.504	0.026	0.165	2.89 (1,9)	−1.676	0.243	0.123	**9.52 (1,9)**	−164.9	0.514	**0.013**
SMI	3.24 (1,73)	−0.528	0.042	0.076	0.95 (1,75)	−1.587	0.013	0.332	4.44 (1,9)	−2.448	0.331	0.064	3.66 (1,9)	−185.6	0.289	0.088
Fat score (factor)	1.87 (3,73)	−0.448	0.072	0.142	0.97 (3,75)	−2.248	0.037	0.411	3.44 (3,9)	n/a	n/a	0.065	**4.87 (3,9)**	n/a	n/a	**0.028**
Handling time	**19.6 (1,73)**	0.120	0.211	**3×10^{-5}**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Explor $*$ handl time	**8.59 (1,73)**	−0.004	0.105	**0.005**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

5.2 ± 2.2 ng mL⁻¹; $t_{80} = 1.4$, $p = 0.17$) or CORT30 (two time points: 20.0 ± 13.7 ng mL⁻¹; three time points: 18.7 ± 9.9 ng mL⁻¹; $t_{80} = 0.15$, $p = 0.88$), indicating that this subset of birds is representative of our larger dataset. We found significant effects of molt status and fat score in the CORT0 and total integrated CORT models (Table 1). Prebasic molt score was associated with lower CORT0 values (Table 1); birds that had completed molt ($n = 67$) tended to have lower CORT0 values compared to birds that were nearly complete ($n = 15$). Excluding molt status did not change the statistical significance of the analyses. Finally, higher fat scores were associated with lower total integrated CORT values (Table 1).

3.1. Initial CORT

There was a significant main effect of handling time on initial CORT concentrations (Table 1). On average, individuals sampled closer to the 3 min mark had higher CORT0 values compared to those sampled more quickly (e.g., <2 min). As predicted there was a significant interaction between handling time and exploration scores on the CORT0 levels, and the direction of the effect indicated that slower explorers experienced a more positive elevation in initial CORT concentrations during the first 3 min after capture compared to faster explorers (GLM: exploration score $*$ handling time: $F_{1,73} = 8.59$, $p = 0.005$, $n = 82$; Table 1). In Fig. 1a we illustrate this interaction by dividing our sample of birds into three groups with respect to exploration scores (fastest quartile ($n = 21$), slowest quartile ($n = 21$), intermediate quartiles ($n = 40$). This is for graphical purposes only—exploration scores are a continuous variable and were included as such in the model above. This interaction did not occur because it took longer to obtain a blood sample in slower explorers, as we found no correlation between handling time and exploration score ($r^2 < 0.001$, $F_{1,80} = 0.003$, $p = 0.958$, $n = 82$; Fig. 1b). Likewise, from a related study involving repeated captures and bleeds, we found no evidence that handling time or the volume of blood collected was related to bird identity (Baugh, unpublished). Further, the main effect of exploration score in the CORT0 model did not remain significant when the interaction term with handling time in the model is removed ($F_{1,74} = 1.47$, $p = 0.23$; see also Fig. 1a inset), indicating that it is the interaction between exploration scores and handling time that explains variation in the CORT0 concentrations, and not differences in true 'baseline' concentrations. Likewise, there is no main effect of exploration score when CORT0 values are 'corrected' for handling time (i.e., using predicted values from the regression of handling time on CORT0)—again suggesting no bias in the distribution of handling times across personalities. Handling time remains significant with or without the interaction term in the model (all $p < 0.001$).

3.2. Stress-induced CORT

Our standardized capture-restraint stress protocol resulted in an increase in CORT levels between the initial and 30 min time points (paired t-test, two-tailed: $t_{81} = 18.16$, $p < 0.001$; Fig. 2). We found no evidence for a relationship between stress-induced CORT concentrations at 30 min and exploration scores or the other covariates (Table 1). Concentrations of CORT at 90 min were highly variable among birds but were on average equal or higher compared to the 30-min time point (paired t-test, two-tailed: $t_{15} = 2.08$, $p = 0.06$). The majority of birds (11 of 16) reached their maximum CORT value at the 90 min time point and slower explorers had higher CORT concentrations at this time point (GLM: $F_{1,9} = 8.92$, $p = 0.015$, $n = 16$; partial correlation: $r = -0.572$, $p = 0.032$, $n = 16$; Table 1, Fig. 3). Likewise, slower explorers reached higher maximum CORT concentrations (GLM: $F_{1,9} = 8.19$, $p = 0.019$, $n = 16$; partial correlation: $r = -0.562$, $p = 0.037$, $n = 16$; Supplementary Fig. 1). Lastly, slower explorers experienced
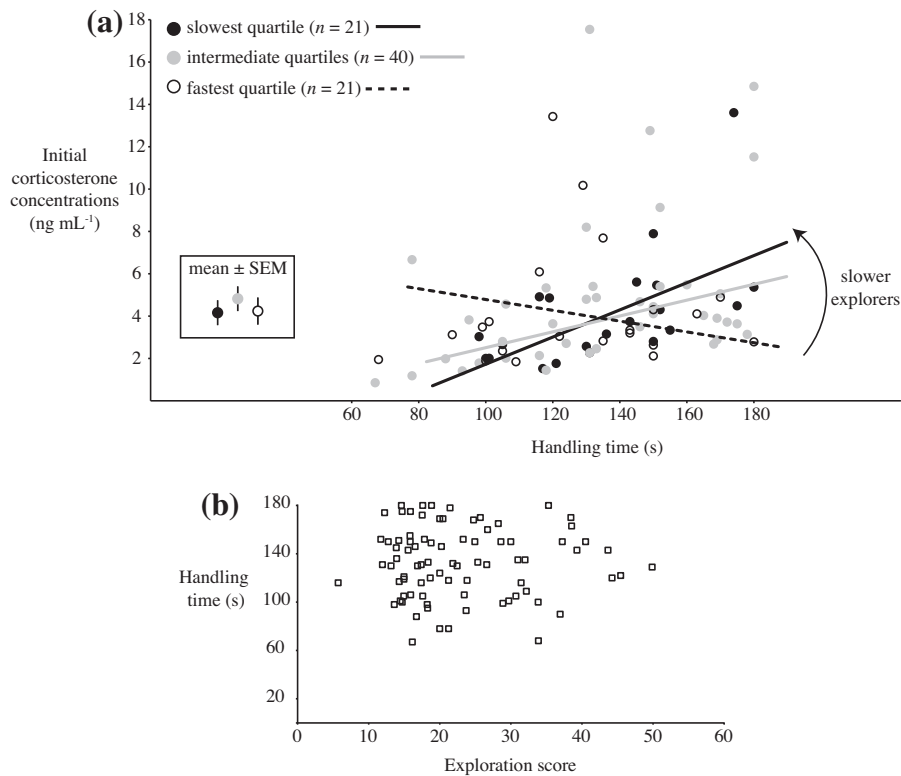
Fig. 1.. (a) Depiction of the interaction between handling time (duration of time elapsed between when each bird intercepted the mist net and the completion of blood collection) and exploration score on initial corticosterone levels. In order to illustrate the interaction between exploration score (a continuous variable) and handling time on CORT0 concentrations, birds are divided into three groups: (i) fastest quartile ($n = 21$); (ii) intermediate quartiles ($n = 40$); and (iii) slowest quartile ($n = 21$). Plotted values are raw initial concentrations ($n = 82$). Fitted lines were calculated from the general linear model for each group. Inset depicts average CORT0 concentrations for each group. (b) Handling time and exploration score were uncorrelated.
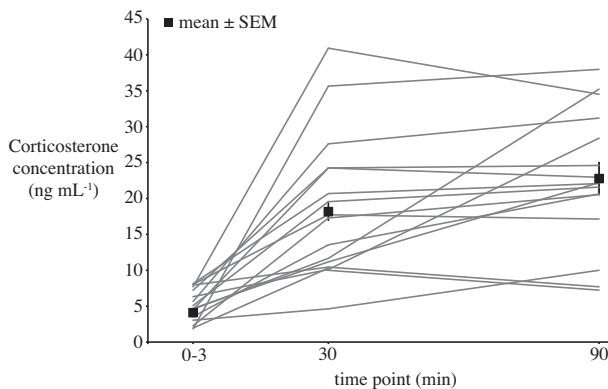

Fig. 2.. Mean ($\pm$SEM) raw corticosterone concentrations at capture ($n = 82$), 30 min ($n = 82$) and 90 min ($n = 16$) post-capture. Grey lines represent the individual curves for the sixteen birds with three time points, whose values are also incorporated into estimates of the mean and SEM

significantly higher total integrated CORT during the stressor period (GLM: $F_{1,9} = 12.03$, $p = 0.007$, $n = 16$; partial correlation: $r = -0.592$, $p = 0.026$, $n = 16$; Supplementary Fig. 2). We found no relationship between exploration score and the change in CORT concentrations between 30 and 90 min ($F_{1,9} = 0.06$, $p = 0.75$, $n = 16$; partial correlation: $r = -0.082$, $p = 0.78$, $n = 16$).
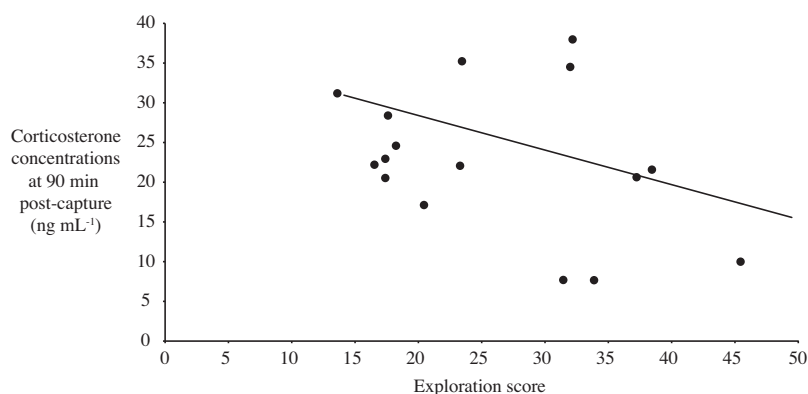
4. Discussion

Our data on free-living great tits support the hypothesis that exploration behavior as an operational measure of avian personality is related to stress reactivity. This was demonstrated in two ways. First, CORT levels increased significantly during the first few minutes after stressor onset in slower compared to faster explorers—that is, the immediate reaction to a stressor appears to be more rapid in these individuals (c.f. Schoech et al., 1999). Secondly, slower explorers had higher total integrated CORT (area under the curve) and reached higher maximum CORT concentrations during the stressor period, two conventional measures of stress reactivity (Juster et al., 2012; Wada et al., 2007). Importantly, exploration score does not merely have a statistically significant effect—it also has considerable explanatory value: approximately half of the variance in these estimates of stress reactivity is explained by this behavioral measure (see Table 1). Together, these measures of immediate and late stress reactivity indicate that slower individuals have a more potentiated stress response.

4.1. Initial HPA axis reactivity and exploration

We found a significant effect of handling time on CORT0 despite the fact that every sample was collected in less than 3 min from capture. Increases in CORT within 3 min have been reported for a few bird species (Heidinger et al., 2006; Romero and Reed, 2005). However, variation among individuals in the elevation of the CORT

Fig. 3.. Correlation between exploration score and raw corticosterone levels at 90 min ($n = 16$). Fitted line was calculated from the general linear model.

response during the first few minutes after stressor onset and a relationship to functional traits like exploration behavior has to our knowledge not been reported. For the slowest explorers, the endocrine stress response began in less than 3 min, whereas the fastest explorers exhibited no detectable increase by 3 min. If 'reactivity' of the HPA axis is defined as the area under the curve (Juster et al., 2012) or the maximum concentration of stress hormones reached following a stressor (Wada et al., 2007), our results are consistent with the idea that behaviorally cautious individuals have a more reactive stress axis, as has been shown in some domesticated species (Erhard et al., 1999; Korte et al., 1992; Korte et al., 1997). This is the first study to our knowledge, however, that extends this observation to include the initial temporal reactivity of the HPA axis—i.e., the speed of the HPA axis response to a stressor. Methodologically, this result implies that the standard application of the "3 min rule" might result in an upwardly biased (i.e., stress-contaminated) estimate of 'baseline' CORT in more reactive individuals (see Romero and Reed, 2005 for a discussion of this topic as it applies interspecifically). While examining the repeatability of this CORT onset time was beyond the scope of the current study, future research could test this by rapidly sampling individuals (e.g., <1 min) and then re-sampling at early fixed intervals (e.g., 2–3 min, 4–5 min). Although challenging to conduct under field conditions, such a study would permit direct inferences about the timing of CORT secretion onset and sources of variation therein.

Mechanistically, variation in the onset of elevated plasma CORT could be due to variation in one or more components of the HPA axis (Williams, 2008). There are several non-mutually exclusive possibilities: (1) Slower birds might perceive stressors more quickly or more intensely due to greater perceptual (sensory) sensitivity to their environment—an interpretation consistent with the idea that shyer individuals are generally more sensitive to external stimuli (Ellis et al., 2006). However, given the intensity and abruptness of the stressor used in the current study (capture and handling), this seems an implausible explanation. (2) Slower birds might have a greater number of corticotropin-releasing hormone (CRH) producing cells in the hypothalamus or greater CRH synthesis, resulting in a more robust secretion of CRH to the pituitary and a concomitantly rapid elevation in hormones at subsequent stages of the HPA axis cascade. This could be tested by measuring CRH concentrations in the hypophyseal portal following a standardized stressor, the CRH content and the number of CRH positive neurons in the paraventricular nucleus. (3) Variation in the precursor or enzyme content, cell number and receptor number for components of the HPA axis in the anterior pituitary and adrenal cortex could be contributing to differences in reactivity. Variation in CRH sensitiv-

ity was shown, for example, in Japanese quail bidirectionally selected for fearfulness, whereas lines did not differ in sensitivity to either adrenocorticotropic hormone (ACTH) or arginine vasotocin (AVT) Hazard et al., 2007. (4) Variation in more rapidly acting signalling systems (e.g., monoamine neurotransmitters such as serotonin, dopamine and norepinephrine) could be correlated with variation in HPA axis reactivity, as has been shown in rainbow trout (*Oncorhynchus mykiss*) (Øverli et al., 2005).

4.2. Stress response magnitude and exploration

There was no relationship between exploration behavior and CORT30 values—a common sampling time point in stress studies. This result is consistent with a previous study of wild-origin great tits, which also found no relationship between personality and plasma CORT at 30 min following capture, handling and restraint (Baugh et al., 2012). At the 90 min time point, however, slower explorers exhibited higher CORT levels—the time point that largely captured the maximum CORT values in this stress series. As a result of high concentrations at this late time point, slower birds experienced more total CORT exposure during the stressor. This result might explain why a previous study that examined faecal CORT metabolites, which presumably reflect levels of CORT integrated over periods of minutes to hours, found elevated CORT values in slower explorers after exposure to a social stressor (Carere et al., 2003). Total glucocorticoid exposure has been shown previously to be related to fitness-relevant measures such as stress-related disease (McEwen, 1991; Olff et al., 1993) and cellular aging (Haussmann et al., 2011). If the longer duration of the stress response in slower birds is due to weaker negative feedback (Dallman et al., 1992; Romero, 2004), this might be due to a lower number of glucocorticoid receptors in the brain (Dickens et al., 2009). Consistent with this idea, threespine sticklebacks (*Gasterosteus aculeatus*) with a bolder and more aggressive personality exhibited elevated glucocorticoid receptor (GR1 and GR2) levels in whole brain homogenates (Aubin-Horth et al., 2012). We stress that negative feedback strength was not tested directly in the present study but could be examined in future research by injecting a standardized dose of a synthetic glucocorticoid (e.g., Dexamethasone) and measuring how quickly levels of CORT decline, in addition to determining the distribution and density of glucocorticoid receptors and their binding capacity in the brain. We caution, however, that our study, like many others using a capture-handling-restraint protocol, examined CORT levels following a intense, enduring stressor, which likely does not reflect certain aspects of natural stressors which are often shorter lived. Our finding of covariation between consistent behavioral differences and

immediate stress reactivity further supports the idea that future studies along these lines should explore stress responses to more transient and naturalistic stressors.

4.3. HPA axis-behavior relationships

The observed relationship between individual differences in behavior and the onset of this physiological response presents the possibility that glucocorticoid physiology plays a direct role in shaping the behavioral coping styles that animals use to respond to challenges. For example, differences in locomotor activity may contribute to variation in exploration behavior during personality testing, which in turn might be a product of heritable differences in HPA axis physiology (Breuner et al., 1998). We think this is an unlikely possibility because (1) the brief testing period used in the present study would favor the contribution of more rapidly acting systems such as catecholamines, which are known to have behaviorally relevant actions (Cannon, 1929); and (2) exploration behavior in hand-reared great tits is unrelated to locomotor activity in home cages (Verbeek et al., 1994).

An alternative, which is not mutually exclusive, is that differential programming of HPA axis reactivity during development (i.e., organizational effects) might underlie personality even in the absence of a causal role for hormone concentrations during behavioral expression (i.e., activational effects). Factors that influence the trajectory of personality development, for instance, might simultaneously direct the maturation of set points for HPA axis reactivity (Ellis et al., 2006).

Extensive studies of aggression in rats and mice suggest a causal role for the hypothalamic–pituitary–gonadal (HPG) axis, serotonin and vasopressin systems as biological substrates for individual differences in aggressiveness. In contrast, variation in the HPA axis in rats appears to be a consequence rather than a cause of behavioral differentiation, potentially due to the related differentiation in cardiovascular and metabolic demands (Koolhaas et al., 2010). Selection line studies in zebra finches (*Taeniopygia guttata*) (Martins et al., 2007) and great tits (Baugh et al., 2012) support the idea of correlated selection (in both directions) for exploration behavior and HPA axis reactivity. It is therefore possible, though presently untested, that the HPA axis-behavior relationship is bidirectional in avian species. Examples of such bidirectionality in HPG axis-behavior relationships have been demonstrated previously, including the extensive body of research on the "challenge hypothesis" (Wingfield et al., 1990) and a seminal study in ring doves (*Streptopelia risoria*), which showed that female vocal behavior is essential for self-stimulation of the endocrine changes necessary to initiate reproductive readiness (Cheng, 2003). Apparent bidirectionality in the HPA axis-behavior correlation in great tits could arise as the product of the indirect effects of one or more latent variables (e.g., HPG axis; Schoech et al., 1999) inadvertently under selection in selection line studies (van Oers et al., 2011) and underlying the observed co-variation in our wild population.

5. Conclusions

Individual differences in behavior, although historically understudied (Bolnick et al., 2003) compared to individual differences in morphology (Lande and Arnold, 1983), have received considerable attention in the past two decades. Our results, in combination with previous work, support the idea that steroid hormones such as glucocorticoids might exert pleiotropic actions, organizing distinct behaviors into suites (Koolhaas et al., 2010). In great tits, individual differences in the slow-fast (shy–bold) continuum appear to be linked to the reactivity of the glucocorticoid stress response. Future studies should address (1) the generality of these findings in

other species; (2) how such co-variation in stress physiology and behavior arises—e.g., the HPA axis could play a direct (activational) or indirect (organizational) role in shaping behavioral differences; and (3) what the potential fitness consequences of such (co)variation might be (Blas et al., 2007; MacDougall-Shackleton et al., 2009; Romero and Wikelski, 2010).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.04.030.

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