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Research paper

Temporal dynamics of the HPA axis linked to exploratory behavior in a wild European songbird (*Parus major*)

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ABSTRACT

Variation in the reactivity of the endocrine stress axis is thought to underlie aspects of persistent individual differences in behavior (i.e. animal personality). Previous studies, however, have focused largely on estimating baseline or peak levels of glucocorticoids (CORT), often in captive animals. In contrast, the temporal dynamics of the HPA axis—how quickly it turns on and off, for example—may better indicate how an individual copes with stressors. Moreover, these HPA components might be correlated, thereby representing endocrine suites. Using wild-caught great tits (*Parus major*) we tested birds for exploratory behavior using a standardized novel environment assay that serves as a validated proxy for personality. We then re-captured a subset of these birds ($n = 85$) and characterized four components of HPA physiology: baseline, endogenous stress response, a dexamethasone (DEX) challenge to estimate the strength of negative feedback, and an adrenocorticotropic hormone (ACTH) challenge to estimate adrenal capacity. We predicted that these four HPA responses would be positively correlated and that less exploratory birds would have a more rapid onset of the stress response (a CORT elevation during the baseline bleed) and weaker negative feedback (higher CORT after DEX). We found support for the first two predictions but not the third. All four components were positively correlated with each other and less exploratory birds exhibited an elevation in CORT during the baseline bleed (<3 min from capture). Less exploratory birds, however, did not exhibit weaker negative feedback following the DEX challenge, but did exhibit weaker adrenal capacity. Together, our findings provide partial support for the hypothesis that the temporal reactivity of the HPA axis is linked with consistent individual differences in behavior, with more cautious (slower exploring) individuals exhibiting a faster CORT response.

1. Introduction

Individuals within a population often differ in their behavioral responses to challenges, and these differences can be stable over time and across contexts (Gosling, 2001; Sih et al., 2004), Moreover, functionally distinct behaviors (e.g. exploration and aggressiveness) can be correlated at the individual level. Such consistent individual differences in suites of traits serve as the basis for the concept of coping styles or animal personality and in many cases are known to have a genetic basis (van Oers and Mueller, 2010) and fitness consequences (Dingemanse et al., 2004; Quinn et al., 2009; Schuett et al., 2010; Smith and Blumstein, 2008). These limits to behavioral flexibility suggest that the proximate mechanisms that underlie one particular behavior might also influence other behaviors (Réale et al., 2007).

Glucocorticoids (CORT) have been proposed to serve an important role in the shy-bold continuum, a major axis of animal personality (Øverli et al., 2007; Carere et al., 2010; Koolhaas et al., 2010). The pleiotropic nature of steroids—the fact that a single hormone can exert simultaneous actions across diverse tissues—provides a theoretical basis for this idea (Sapolsky et al., 2000). As the end product of the hypothalamic-pituitary-adrenal (HPA) axis, CORT plays a critical role in vertebrates: coping metabolically with the fluctuating demands of normal life, such as predictable daily and life-history events, diel rhythms and locomotor activity (Landys et al., 2006). The HPA axis also plays an essential role in coping with unpredictable, acutely challenging events, such as predation risk (Cockrem and Silverin, 2002a), inclement weather (Breuner

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and Hahn, 2003), but also non-noxious experiences such as sexual behaviors and social victory (Koolhaas et al., 2011). There are multiple regulatory aspects of the HPA axis: First, low baseline concentrations fluctuate according to diel rhythms and metabolic demands. Second, within a few minutes after an acute challenge is perceived, CORT levels (following an elevation in adrenocorticotropic hormone, ACTH; Sumpter et al., 1986) become elevated. Circulating CORT concentrations then continue to increase until they reach a maximum, generally within 30–90 min in many fishes, mammals and birds (Sumpter et al., 1986; Dickens et al., 2009a; Droste et al., 2011; Baugh et al., 2013; Cockrem and Silverin, 2002b). At these high concentrations, CORT enables a metabolic shift towards gluconeogenesis by altering transcription in target cells (Gray et al., 1990; Hasselgren, 1999; Sapolsky et al., 2000; Oakley and Cidlowski, 2013). Lastly, a process of negative feedback regulation (via glucocorticoid receptors in the brain) terminates the stress response, allowing baseline concentrations to be re-achieved (Dallman et al., 1992; Romero, 2004; Baugh et al., 2017).

There are now multiple lines of evidence demonstrating that shyer individuals have, on average, a more potentiated HPA axis, typically including one or more of the following characteristics: a faster onset of the glucocorticoid response, higher peak levels and weaker negative feedback (Satterlee and Johnson, 1988; Korte et al., 1997; Koolhaas et al., 1999; Carere and van Oers, 2004; Ellis et al., 2006; Martins et al., 2007; Cockrem et al., 2010; Van Oers et al., 2011; Baugh et al., 2013, 2017), Furthermore, there is evidence that individuals differ consistently in functional aspects of the HPA axis—the circulating concentrations of CORT and the expression patterns of receptors in behaviorally relevant tissues (e.g. nervous system; Senft et al., 2016; Baugh et al., 2017). For example, the proactive-reactive coping model described by Koolhaas et al. (1999) suggests individual covariation in boldness, environmental sensitivity and endocrine stress responsiveness. Studies in a variety of vertebrate groups now support this HPA-personality link (Jones et al., 1992; Harri et al., 2003; Øverli et al., 2005; Cockrem, 2007; Mackenzie et al., 2009; Lendvai et al., 2011; Baugh et al., 2012; Baugh et al., 2013; Hau et al., 2016; Baugh et al., 2017; Baugh et al., in press).

A key aspect of the relationship between the stress axis and personality might lie in the temporal dynamics of the endocrine stress response, which is poorly understood in general (but see Sarabdjitsingh et al., 2010) and largely unexplored in free-living organisms (but see Baugh et al., 2013). Given the speed with which the stress response can be initiated (<3 min; Romero and Reed, 2005; Heidinger et al., 2006; Baugh et al., 2013; Small et al., 2017), it is possible that the CORT response plays a direct role in modulating behavioral responses to stressors in addition to the more rapid response mediated by the sympathetic nervous system (reviewed in Axelrod and Reisine, 1984). Specifically, variation in the initiation stage of the glucocorticoid response might play a role in immediate coping behavior, while the duration of the stress response might affect the fitness consequences of elevated stress hormones (Romero and Wikelski, 2010), how quickly the organism returns to a non-stressed state (Baugh et al., 2013), and how an individual responds to a subsequent stressor (Baugh et al., in press).

In the present study, we tested wild great tits (*Parus major*) using standardized and validated methods for assessing personality (exploratory behavior in a novel environment) and HPA axis reactivity (HPA assessments), a model for the study of animal personality in which previous research has validated that exploratory 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 a variety of other functionally distinct behaviors (Van Oers et al., 2008, 2004; Verbeek et al., 1996; Amy et al., 2010; Titulaer et al., 2012), and appears to have consequences for reproductive success (Both et al., 2005) and survival (Dingemanse et al., 2004). Here we tested the hypothesis that more cautious birds (i.e., slower explorers) will exhibit a more potentiated HPA axis, with higher reactivity (earlier onset; higher adrenal capacity) and a more enduring endocrine stress response (weaker negative feedback). We also predicted that four functional aspects of the HPA axis—baseline CORT, the endogenous stress response, negative feedback strength and adrenal capacity—will exhibit positive phenotypic correlations with each other, particularly sequential components.

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 deciduous forest of approximately 100 ha that hosts a large long-term study population of ringed wild great tits. In the present study we captured 85 ringed adult birds, each on two occasions—once to test them for exploratory behavior and then subsequently for the HPA assessments. By separating the behavior and hormone sampling we precluded the potentially confounding effect that the bleeding experience would have on performance in the behavior assay, and vice versa. Because we could not target the collection of specific individuals at a controlled date prior to bleeding, we instead sampled birds opportunistically, which resulted in a median interval between behavioral testing and hormone sampling of 35 days (mean ± SD: 113.5 ± 151.2 d).

Our exploration assay has been shown to estimate persistent characteristics of an individual (repeatability range: 0.27–0.66; heritability range: 0.22–0.61; Dingemanse et al., 2002), indicating that the scores have a high explanatory power across prolonged periods of time (>1 year; Dingemanse et al., 2002). Nevertheless, we tested the assumption that the interval (days) between captures for behavior and hormone measurement contributed to the observed patterns by including this covariate in our analyses.

2.2. Behavioral testing

Between July and September (2011 & 2012), we measured exploratory behavior for each individual following a standardized protocol using an indoor 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). Birds were caught with mist nets adjacent to feeding stations in Westerheide and transported for approximately 0.5 h in custom boxes to a specially designed housing and behavioral testing facility (Netherlands Institute of Ecology, Heteren, The Netherlands) where they were kept overnight in individual cages (0.9 × 0.5 × 0.4 m) adjacent to the test chamber. On the following morning (0800–1200) each bird was permitted to exit its cage directly into the test chamber without handling. 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 (i.e., branches of the artificial trees, sliding doors, floor). We calculated exploration scores by summing all hops and flights per individual. Exploratory behavior is known to vary seasonally (increasing as the breeding season approaches) but remains repeatable at the individual level (Dingemanse et al., 2002). We therefore corrected the exploratory scores for date of capture (range: 1–365 days), based on empirically characterized within-individual changes in behavior with capture date (for details see Dingemanse et al., 2002). All individuals were assayed for the first time during their lives, thus eliminating any habituation effects (Dingemanse et al., 2002). Behavioral testing was conducted blindly and indepen-

dently of hormone measurement. All birds were released at their capture site within a few hours following behavioral testing.

The exploratory assay is standardized in order to eliminate confounding effects of the individual's ecological context at the time of testing (e.g. territory quality, interaction with conspecifics). We therefore used this novel environment assay as an operational measure of great tit personality (reviewed in Groothuis and Carere, 2005; van Oers and Naguib, 2013). 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 exploratory behavior and a genetic correlation between exploratory behavior and stress physiology (Carere et al., 2003; Baugh et al., 2012; Baugh et al., in press).

2.3. HPA assessments

In September and October of 2012, we re-captured 85 of the adult great tits that we tested for exploratory behavior for the measurement of plasma CORT. We restricted our capture dates and times (0830–1100) to minimize the variation caused by seasonal and circadian rhythms (Breuner et al., 1999). Moreover, autumn represents a relatively stable life history period for this population (e.g. prebasic molt was complete for 82 of the 85 birds), and plasma CORT tends to fluctuate less than during the breeding season (Romero and Wingfield, 1998).

We observed mist nets erected near one of five feeding stations from a distance of ca. 20 m behind vegetation and initiated a digital timer the instant a bird intercepted the net. Samples were collected by puncturing the brachial vein and collecting the blood (ca. 50 μl) using a heparinized microcapillary tube. Our method for assessing the HPA axis has been used previously in birds (Dickens et al., 2009b; Hau et al., 2015) and reptiles (Romero and Wikelski, 2010). For details on the validation of this protocol in great tits see Baugh et al. (2017). This repeated measures assessment sequentially quantifies four aspects of HPA axis function: (1) Baseline CORT (*BaseCORT*): this first blood sample precedes the experimentally-induced stress response and is assumed to reflect a non-stressed state. *BaseCORT* was collected <3 min following entry into the mist net to minimize contamination from the stress response (Baugh et al., 2013; Romero and Reed, 2005); birds were then placed in a small cotton restraint bag for 15 min. (2) Endogenous stress response (*StressCORT*): this second blood sample immediately following the 15-min restraint period provides an estimate of the early stage of each bird's acute response to restraint and was immediately followed by an intramuscular injection of dexamethasone (DEX; 1000 μg kg^{-1}; diluted to 50 μL in PBS), which stimulates strong negative feedback of the HPA axis, thereby down-regulating subsequent CORT secretion (Baugh et al., 2017); this injection was followed by a 90-min restraint period. (3) Negative feedback strength (*DexCORT*): the CORT concentration following the 90-min restraint reflects the strength of negative feedback following the DEX injection (higher CORT here indicates weaker negative feedback); this was followed immediately by an intramuscular injection of adrenocorticotropic hormone (ACTH; Sigma #A6603; 100 IU kg^{-1} diluted to 50 μL in PBS), followed by a 15-min restraint period. (4) Adrenal capacity (*ActhCORT*): after this final 15-min restraint, birds were bled a fourth time to estimate the capacity of the adrenal glands to produce CORT upon pharmacological stimulation of the HPA axis by the injected secretagogue. Birds were then immediately measured for biometrics, including tarsus length (Ecotone mechanical callipers, ±0.1 mm), body mass (30-g Pesola scale, ±0.3%), furcular fat score (0–5; Cherry, 1982), and pre-basic molt score. We also recorded the air temperature at capture (Royal Netherlands Meteorological Institute, Deelen Station) and ring number. Birds were then immediately released at the site of capture. Blood samples were kept on wet ice during sample collection and then centrifuged (1400*g* for 10 min). The plasma fraction was frozen at -80 C until all samples were assayed simultaneously.

2.4. Enzyme immunoassay for corticosterone

In July 2013 we estimated plasma CORT concentrations using a commercial enzyme immunoassay kit (Enzo Life Sciences, Cat. No. ADI 900-097; Donkey anti-Sheep IgG) at the Max Planck Institute for Ornithology (Radolfzell). The details of our EIA procedure, including its validation, extraction, recoveries, technical repeatability and preparation of standards are reported in Ouyang et al. (2011) and Baugh et al. (2014). Concentrations were determined following a double diethyl-ether extraction of a 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 (provided by kit) and samples were allowed to equilibrate overnight at 4° C. Samples were then randomly assigned to wells and assayed in duplicate along with blanks and five standards (0.032–20 ng mL^{-1} CORT). The average percent recovery efficiency, which we determined previously using individual samples spiked with radioactively labelled CORT, was uniformly high (mean ± SD; ca. 85% ± 2.7; $n = 9$). Here we report CORT concentrations that have been corrected for this average recovery efficiency. The intra- and inter-assay coefficients of variation (CV)—9.5% and 8.3%, respectively—were determined by including three duplicate samples of stripped great tit plasma spiked with commercial corticosterone (supplied by kit) at a concentration of 20 ng mL^{-1} on each of the 13 plates. All samples were processed during a one-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 met the assumption of normality.

We examined the relationship between exploration scores and CORT concentrations using repeated measures general linear models and linear regressions. In addition to exploration scores, we included a set of variables that can impact HPA axis function in wild birds, including air temperature at capture (Romero et al., 2000; Wingfield et al., 1983), handling time (Heidinger et al., 2006; Romero and Reed, 2005), fat score (Romero et al., 2000), sex (Astheimer et al., 1994) and residuals from length versus mass (Romero et al., 2000). We included inter-capture interval (number of days between behavior and hormone sampling) as a covariate. For the BaseCORT 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 onset of CORT secretion (Baugh et al., 2013).

Because CORT concentrations across the HPA assessment time points are likely correlated (Baugh et al., 2014; Baugh et al., 2017), we reduced the dimensionality of the four-component HPA assessment using principal components analysis. We used SPSS (version 21) for all statistical analyses.

3. Results

3.1. HPA assessments

We found no effect of testing interval (days separating behavior and HPA assessments) as either a main effect or interaction effect

with any of the HPA components (all $p > 0.08$). Nevertheless, we report the model results with and without this covariate below. Likewise, there were no effects of fat score (all $p > 0.41$), temperature at capture (all $p > 0.16$), sex (all $p > 0.5$) or residual body mass (all $p > 0.25$) in any of the models. Therefore these variables were omitted from our final models.

BaseCORT concentrations were low (Fig. 1) and similar to other reported 'baseline' values from wild and captive great tits from this population (Baugh et al., 2012, 2014), indicating that our capture procedure was effective at yielding initially unstressed birds. All 85 birds experienced an increase in CORT concentrations above their *BaseCORT* values following the 15-min restraint period (ng mL^{-1}: mean: +14.98, SD: 10.08; range: 0.51–40.24). Most birds (66 of 85; 78%) exhibited a decrease in CORT below their *StressCORT* concentrations following DEX injection (ng mL^{-1}: mean: −8.04; SD: 13.18; range: −39.12–31.76), and all birds but one (84 of 85) exhibited an increase in CORT above *DexCORT* concentrations following ACTH injection (ng mL^{-1}; mean: +30.20; SD: 11.39; range: −1.80–66.02). The repeated measures ANOVA demonstrated a significant main effect of time point ($F_{3,252} = 290.0$, $p < 0.0001$), and all time points differed from each other (all $p < 0.0001$; Bonferonni correction; Fig. 1).

As predicted, there were positive phenotypic correlations between all CORT time points (all $p < 0.05$; R^2 range: 0.05–0.5; Fig. 2). These correlations were stronger when the time interval separating the two blood samples was brief and sequential (e.g. *BaseCORT* versus *StressCORT*).

3.2. CORT and exploration scores

As predicted, there was a significant interaction between handling time and exploration score on *BaseCORT*, with slower explorers experiencing an increase in *BaseCORT* during the <3 min handling period while faster explorers did not (testing interval excluded: $F_{1,83} = 13.7$, $p = 0.0004$; Fig. 3a; testing interval included: $F_{1,82} = 13.6$, $p = 0.0004$, main effect of testing interval: $F_{1,82} = 1.1$, $p = 0.30$). This interaction might have occurred if, for instance, it took longer to obtain a blood sample in slower explorers. However we found no correlation between handling time and exploration score ($r^2 = 0.01$, $F_{1,83} = 0.73$, $p = 0.40$; Fig. 3b). Furthermore, the main effect of exploration score in the *BaseCORT* model does not remain significant when the interaction term with handling time in the model is removed ($F_{1,82} = 1.63$, $p = 0.21$; see also Fig. 3a inset), indicating that it is the interaction between exploration scores and handling time that explains variation in the *BaseCORT* concentrations.

As we have shown previously, there was no correlation between *StressCORT* and exploration ($F_{1,83} = 2.20$, $p = 0.14$; $R^2 = 0.03$). We found no support for our prediction of a negative correlation between *DexCORT* and exploration scores ($F_{1,83} = 1.64$, $p = 0.20$; $R^2 = 0.02$). We did find, however, a significant but weak positive correlation between *ActhCORT* and exploration score—more exploratory birds exhibited higher CORT following the ACTH challenge ($R^2 = 0.051$, $p = 0.04$; Fig. 4) and this result was unaffected by inclusion of testing interval as a covariate ($R^2 = 0.052$, $p = 0.03$). This same relationship was present when all four HPA time points were reduced to a single PC (PC1 explained 56.9% variance; $R^2 = 0.05$, $p = 0.04$; Kaiser-Meyer-Olkin = 0.64; Bartlett's Sphericity p = 0.0001), driven mostly by the *ActhCORT* measure (communalities: *BaseCORT* = 0.37; *StressCORT* = 0.55; *DexCORT* = 0.66; *ActhCORT* = 0.69).

4. Discussion

We show that exploratory behavior—a validated measure of avian personality in wild great tits—is linked with variation in HPA axis function. Our results provide partial support for the prediction that individuals with a more potentiated HPA axis exhibit more cautious personalities. As predicted, less exploratory birds exhibited a faster onset of the glucocorticoid response. This result replicates an earlier field-based study in the same population (but different individuals), which found a nearly identical pattern of

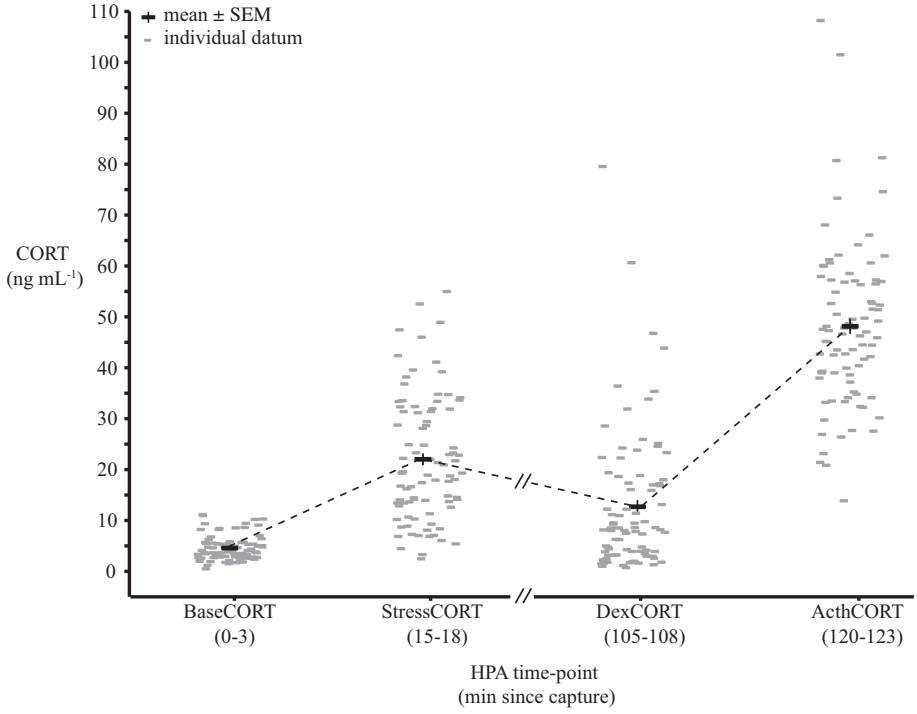

Fig. 1. Corticosterone concentrations for each of the four HPA components. Grey dashes represent individual birds and black dashes connected to dashed line indicate mean (±SEM) values. For each HPA component, the spread of points on the abscissa is strictly for clarity of illustration. Untransformed values that have been corrected for average recovery efficiency are depicted.

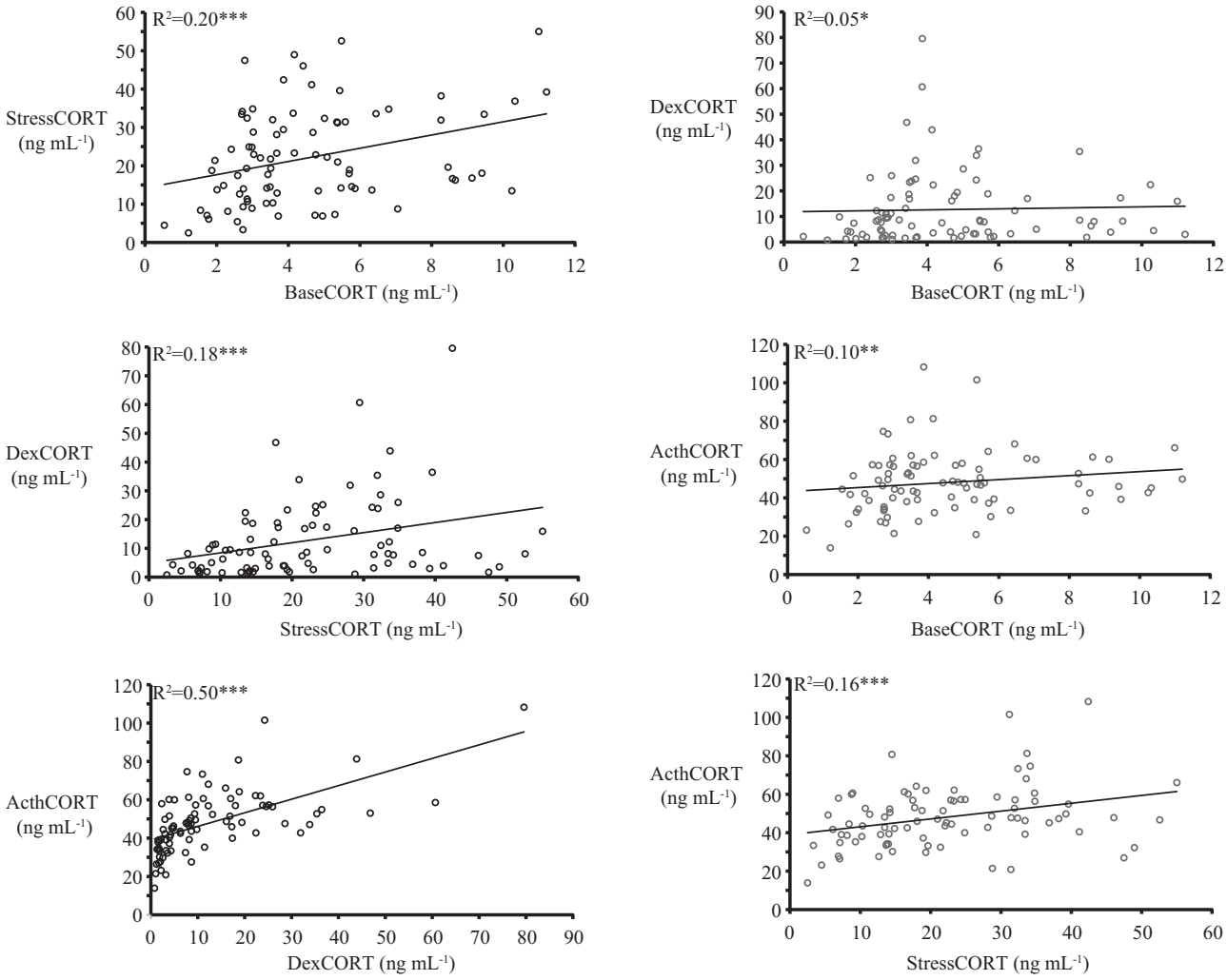

Fig. 2. CORT concentrations were positively correlated between all HPA component pairs, with stronger correlations between sequential HPA components (open black circles) compared to non-sequential ones (open grey circles). Untransformed values that have been corrected for average recovery efficiency are depicted.

onset of stress-induced CORT increases among exploratory phenotypes (Baugh et al., 2013) as well as earlier studies demonstrating no main effect of baseline CORT and personality in this species (Baugh et al., 2012, 2013; Carere et al., 2003). Importantly, this onset pattern was evident despite all samples being collected in less than 3 min, which has been reported in a few other bird species (Heidinger et al., 2006; Romero and Reed, 2005). Here we independently replicate the finding that slow explorers exhibit an elevation in less than 3 min while faster explorers do not (Baugh et al., 2013), which is consistent with a the general pattern of greater HPA reactivity in more cautious phenotypes (Baugh et al., 2017; Erhard et al., 1999; Korte et al., 1992; Korte et al., 1997). Additional support for this personality-dependent CORT onset hypothesis is needed and would ideally involve multiple precisely timed blood draws during the first few minutes after capture of an individual. This would permit the analysis of individual differences in the inflection point for stress-induced CORT onset and would likely require a larger species that can be catheterized (e.g. chickens). Moreover, still missing from this line of research is an experiment that uncovers the mechanistic basis and directionality of the link between personality and HPA axis function. The experience of a novel object or environment—which are thought to be mildly stressful (Baugh et al., in press)—are conducted over a minimum of two minutes, and therefore the relatively rapid individual differentiation in CORT onset times proposed here means that it is conceivable that the behavioral variation is caused in part by variation in the very early phase of the stress response (e.g. via membrane bound receptors; Breuner and Orchinik, 2001). Note, however, that the capture/restraint stressors used to characterize the HPA axis are may be perceived as inescapable and potent compared to the stressors experienced during a novel environment test, which permit behavioral coping which may attenuate or potentially accentuate individual differences in the endocrine stress response. For example, in a study of captive great tits, Baugh et al. (in press) demonstrated that exposure to a novel object induces a significant but small elevation in CORT (ca. 36% increase above baseline) compared to the increase observed following a similar duration of restraint (ca. 200% increase above baseline; Cockrem and Silverin, 2002b; Baugh et al., 2012).

The challenge ahead is to identify methods that permit testing these acute mechanistic hypotheses without dysregulating the HPA axis—if the fine temporal dynamics of HPA axis activity are key to understanding stress-related behavioral variation, then conventional hormone implant approaches might not be physiologically relevant. This is particularly challenging yet important to evaluate in free-living animals, which may differ in fundamental ways from captive animals (e.g., artificially selected for tameness or for extreme, non-continuous behavioral traits), particularly in their stress physiology (Calisi and Bentley, 2009; Dickens and Romero, 2009; Dickens et al., 2009a).

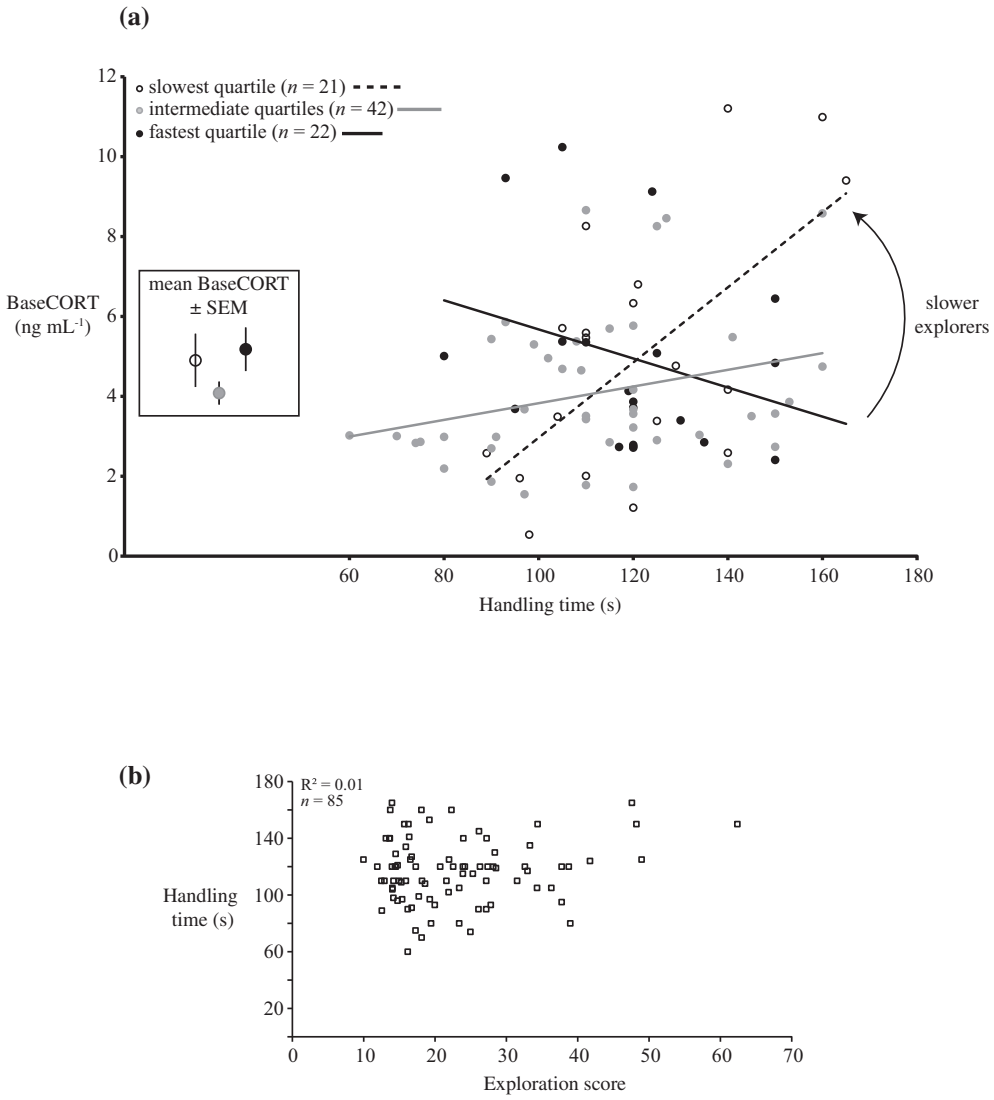

Fig. 3. (a) Depiction of the interaction between handling time (duration of time elapsed between when each bird intercepted the mist net and the completion of *BaseCORT* blood collection) and exploration score on *BaseCORT* levels. In order to illustrate the interaction between exploration score (a continuous variable) and handling time on CORT0 concentrations, birds are divided into three groups: (i) fastest quartile (n = 21); (ii) intermediate quartiles (n = 42); and (iii) slowest quartile (n = 22). This serves merely a graphical purpose—exploration scores are a continuous variable and thus the statistical result of interest is the interaction effect in the model. Plotted values are raw *BaseCORT* (n = 85). Fitted lines were calculated from the general linear model for each group. Inset depicts mean *BaseCORT* concentrations for each group. (b) Handling time and exploration score were uncorrelated. Untransformed values that have been corrected for average recovery efficiency are depicted.

In contrast to the CORT onset results, we found a correlation between adrenal capacity and exploratory scores, with more exploratory birds exhibiting stronger CORT responses following the ACTH challenge. This is despite the fact that there was no relationship between exploratory scores and CORT following the DEX challenge (the preceding time point). This is important because CORT concentrations in these last two time points were strongly and positively correlated: birds with weaker negative feedback (higher CORT after DEX challenge) attained higher CORT concentrations after ACTH injection. Thus, if there had been a positive correlation between *DexCORT* and exploration, we would expect to find a positive correlation between *ActhCORT* and exploration, which could be due strictly to a carry-over effect of the preceding time point. However, there was clearly no such correlation at the preceding time point, suggesting that the positive but weak relationship between adrenal capacity and exploration is likely robust. Thus it appears that the natural onset of the endocrine stress response ("reactivity") is dissociated from adrenal capacity in their relationship to personality. In partial support of this idea, in Japanese quail artificially selected for low and high fearfulness, lines differed in sensitivity to corticotropin releasing hormone but not ACTH or AVT (Hazard et al., 2007). As an alternative mechanism, variation in rapid signalling systems (e.g., serotonin, dopamine and norepinephrine) could be correlated with variation in HPA reactivity, as has been shown in rainbow trout (*Oncorhynchus mykiss*) (Øverli et al., 2005). Future work will need to characterize in greater detail individual variation in adrenal capacity and its relationship to reactivity and behavior.

In a previous study in great tits, we showed that each of the HPA assessment components exhibits significant repeatability (Baugh et al., 2017). Here again we showed that each of the time points differed from one another, suggesting that each one captured unique attributes of HPA function. That said, the positive phenotypic correlations observed between each of these components—especially *DexCORT* versus *ActhCORT*—suggests that these components are positively inter-dependent. This result replicates a previous finding that showed positive correlations among the four components of the HPA assessment. Using captive great tits

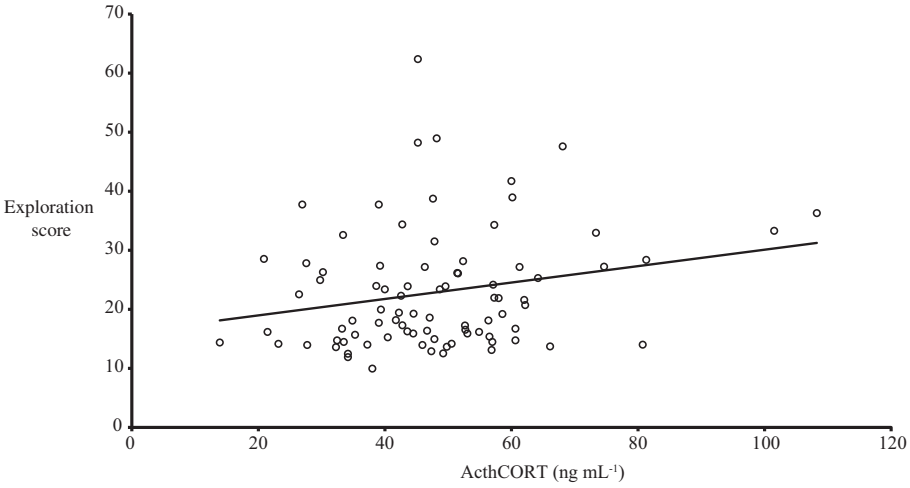

Fig. 4. The correlation between *ActhCORT* and exploration score—more exploratory birds exhibited higher CORT following the ACTH challenge. Untransformed values that have been corrected for average recovery efficiency are depicted.

Baugh et al. (2017) demonstrated positive phenotypic correlations among each of the HPA time points, again with stronger correlations observed between more proximal time points. That repeated measures study decomposed the phenotypic correlations and showed that they were principally driven by within-individual correlations. This means that a bird with high baseline CORT at a particular moment, for example, will have high stress-induced CORT a few minutes later; that same bird a month later, however, might have moderate baseline CORT and moderate stress-induced CORT, implying that dynamic environmental variables, not stable individual differences, are likely driving the correlation between the HPA traits (Baugh et al., 2014). The correlations among these HPA measures have implications for coping with repeated stressors; for example, the strong positive correlation between *DexCORT* and *ActhCORT* observed in the present study and an earlier one (Baugh et al., 2017) suggests that birds with stronger negative feedback are less able to mount a strong secondary stress response, due perhaps to a dexamethasone-induced refractory period or variance in desensitization from the previous disturbances, as has been demonstrated in fishes (reviewed in Barton, 2002).

In our 2010 autumn field study from the same population (Baugh et al., 2013), we showed that less exploratory birds had relatively elevated CORT following 90 min of restraint, suggesting—but not demonstrating—that these individuals had weaker negative feedback. Here we tested this hypothesis pharmacologically using a synthetic glucocorticoid (DEX) and found that CORT concentrations following DEX injection were uncorrelated with exploratory scores. Our results therefore do not support the prediction that less exploratory phenotypes exhibit weaker negative feedback. Importantly, our DEX injections did result in CORT decreases in the majority (78%) of birds, and our validation study (Baugh et al., 2017) showed that our dosage and timeline for the DEX treatment was effective at eliciting a negative feedback response in wild great tits. Given that the past and present studies examined the same population of great tits (though different individuals) at the same time of year (September–October) and yet differed in sample size for this negative feedback aspect ($n = 16$ and $n = 85$, respectively), the lack of agreement between these two studies could arise due to sampling error or have a biological basis. The latter could come about because individual variation in endogenous negative feedback differs from exogenously-induced negative feedback. The former might better reflect individual differences in natural negative feedback while the latter might provide an estimate of asymptotic negative feedback strength. To test this, it will be necessary to conduct both long-duration stress series (e.g. 90 min) and DEX challenges in the same individuals across multiple repeated measures.

By employing HPA assessments rather than more conventional handling/restraint stress series (Baugh et al., 2012, 2013; Juster et al., 2012; Wada et al., 2007), the present study confirms and extends previous work examining the relationships between aspects of HPA axis function and animal personality. In conclusion, we have shown that a single snapshot of endocrine function is probably insufficient to capture individual variation. Moreover, assessing single HPA measurements may not reflect the level of variation that might be most relevant for the expressed personality traits. We found that the temporal dynamics of the stress response has more relevance for individual differences in behavior. The idea that fine-scale temporal dynamics in the HPA axis underlie individual variation in behavior has recently been demonstrated in humans (Booij et al., 2016) and rodents (Sarabdjitsingh et al., 2010) and we believe this line of research would benefit from experimental approaches using free-living animal models for personality.

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