Risk-averse personalities have a systemically potentiated neuroendocrine stress axis: a multilevel experiment in *Parus major*

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Running title: HPA axis underlies avian personalities

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Hormonal pleiotropy—the simultaneous influence of a single hormone on multiple traits—has been hypothesized as an important mechanism underlying personality, and circulating glucocorticoids are central to this idea. A major gap in our understanding is the neural basis for this link. Here we examine the stability and structure of behavioral, endocrine and neuroendocrine traits in a population of songbirds (*Parus major*). Upon identifying stable and covarying behavioral and endocrine traits, we test the hypothesis that risk-averse personalities exhibit a neuroendocrine stress axis that is systemically potentiated—characterized by stronger glucocorticoid reactivity and weaker negative feedback. We show high among-individual variation and covariation (i.e. personality) in risk-taking behaviors and demonstrate that four aspects of glucocorticoid physiology (baseline, stress response, negative feedback strength and adrenal sensitivity) are also repeatable and covary. Further, we establish that high expression of mineralocorticoid and low expression of glucocorticoid receptor in the brain are linked with systemically elevated plasma glucocorticoid levels and more risk-averse personalities. Our findings support the hypothesis that steroid hormones can exert pleiotropic effects that organize behavioral phenotypes and provide novel evidence that neuroendocrine factors robustly explain a large fraction of endocrine and personality variation.

Keywords

ACTH, behavioral syndromes, corticosterone, dexamethasone, glucocorticoid receptor, HPA axis, mineralocorticoid receptor, negative feedback, personality, stress.

INTRODUCTION

Upon exposure to a social or environmental challenge, individuals within a population often differ consistently in their behavioral response (reviewed in Reale et al., 2007; Bell et al. 2009; Dall et al. 2012). Moreover, single behaviors (e.g. aggressiveness) are often linked within an individual with other behaviors (e.g. exploration; reviewed in Groothuis and Carere, 2005). These consistent individual differences and trait correlations are the basis for the concept of animal personality (similar to 'coping styles', 'behavioral syndromes'), which has now been demonstrated in a wide variety of species (van Oers and Naguib, 2013). This research highlights the constraints on behavioral flexibility, on the independent evolvability of traits, and suggests that the mechanisms that underlie one particular behavior might subserve other behaviors (Réale et al., 2007).

The hypothesis that hormones serve as mechanisms underpinning animal personality has been the subject of growing interest (Williams, 2008; Koolhaas et al., 2010). Glucocorticoids (hereafter CORT) are proposed to be key steroids involved in one of the major axes of personality: the shy-bold continuum (Øverli et al., 2007; Carere et al., 2010). In part, this hypothesis rests on the pleiotropic nature of steroids—these endocrine products circulate throughout the organism and bind to multiple receptor types across diverse tissues. Hence, a single hormone can simultaneously affect multiple targets, thereby precisely modulating the expression of several behaviors (Ketterson and Nolan, 2009).

As the end products of the hypothalamic-pituitary-adrenal (HPA) axis, CORT facilitate critical functions in vertebrates: coping metabolically with the fluctuating demands of normal life, such as day-night rhythmicity, locomotor activity and predictable daily and life-history events (Landys et al., 2006). Further, the HPA axis is essential for coping with unpredictable,

85 acutely challenging events, such as exposure to unfamiliar environments or objects (Lendvai et
86 al., 2011), inclement weather (Breuner and Hahn, 2003), predators (Cockrem and Silverin,
87 2002), but also sexual behaviors and social victory (Koolhaas et al., 2011). The regulation of the
88 HPA axis consists of several components: First, low baseline concentrations fluctuate according
89 to diel rhythms and metabolic demands and are known to promote feeding behavior (Dallman et
90 al., 1993). Second, within a few minutes after an acute challenge is perceived, CORT (following
91 an elevation of their upstream secretagogues such as adrenocorticotropic hormone, ACTH)
92 becomes elevated and continues to rise in the blood until it reaches a peak, typically within 30–
93 90 min (Baugh et al., 2013; Droste et al., 2011). At these stress-induced concentrations, CORT
94 facilitates a metabolic shift from protein and fat synthesis towards gluconeogenesis by altering
95 transcription in target cells (Gray et al., 1990; Hasselgren, 1999; Sapolsky et al., 2000; Oakley
96 and Cidlowski, 2013). Third, negative feedback reduces circulating levels, allowing baseline
97 concentrations to be re-achieved (Romero, 2004).
98 Regulation of circulating CORT concentrations is made possible by two intracellular
99 receptors in the brain that bind CORT. The mineralocorticoid receptor (MR) has a high affinity
100 and low capacity for CORT and is therefore thought to be principally active at baseline CORT
101 concentrations (Romero, 2004; Landys et al. 2006). In contrast, the low affinity and high
102 capacity glucocorticoid receptor (GR) exhibits increased binding at stress-induced concentrations
103 (de Kloet et al., 1998; Funder, 1997) and is also thought to play a critical role in regulating
104 negative feedback through binding to receptors located in the pituitary and hypothalamus,
105 thereby inhibiting the secretagogues that lead to further elevations in CORT (De Kloet 1991;
106 Ronchi et al. 1998; Romero 2004). Moreover, because of its upstream location in the HPA axis,
107 receptor expression in the brain has the potential to explain intraspecific variation in stress

physiology and behavior. Here we examine MR and GR expression in the hypothalamus and hippocampus, two brain regions known for their involvement in HPA regulation and roles in mediating behavior (Nelson 2005). Higher GR expression in these regions, for example, might result in stronger negative feedback and thus a systemically less potentiated HPA axis (i.e. lower CORT at all post-stressor time-points).

Beyond single behaviors, the ways in which individuals respond hormonally to stressors may underlie several of the correlated behaviors that often characterize personality (Koolhaas et al., 2007). Further, if individuals vary consistently in functional aspects of the HPA axis—the circulating concentrations of glucocorticoids (CORT) and the expression patterns of receptors in behaviorally relevant tissues (e.g. nervous system)—this could give rise to variation in personality. Indeed, there is often remarkable intra-population variation in concentrations of baseline and stress-induced CORT (Hau et al., 2016). The fraction of this variation that represents among-individual variance has been studied in recent years and has yielded mixed results, reflecting in part the fact that only a subset of these studies used repeated measures designs (Baugh et al., 2014). However, understanding the endocrine basis of animal personality requires repeatedly characterizing behavioral, endocrine and neuroendocrine traits in the same individuals (reviewed in Ball and Balthazart, 2008)—a step that, to our knowledge, has not been undertaken until now.

In the present study we tested for the presence of among-individual variance in both behavioral traits and functional aspects of the HPA axis and then tested the hypothesis that variance in HPA axis function explains behavioral variance. Because environmental context can drive considerable acute variation in plasma glucocorticoids, we sought to control experimentally certain aspects of the environment—nutrition and exposure to conspecifics—but allowed

physical aspects of the environment to vary naturally (e.g. weather). Using semi-natural enclosures, we studied wild-caught great tits (*Parus major*), a species that has been the subject of extensive investigation in animal personality (van Oers and Naguib, 2013) and, more recently, of intra-population variation in glucocorticoid physiology (Hau et al., 2016). We predicted that: (1) risk-taking behaviors expressed in the context of a foraging task will both vary at the among-individual level (i.e. exhibit repeatability) and covary at the among-individual level (i.e. exhibit syndromes); (2) four functional aspects of the HPA axis—baseline CORT, the stress response, negative feedback strength and adrenal sensitivity—will likewise vary and covary at the among-individual level; (3) the expression patterns of MR and GR in two regions of the brain that regulate the HPA axis (hippocampus and hypothalamus) will be correlated with HPA function, with higher GR expression predicted to strengthen negative feedback; and thus GR expression in these regions is predicted to correlate negatively with a systemically potentiated HPA axis (Romero, 2004); and (4) repeatable elements of the behavioral phenotype are correlated with repeatable elements of the endocrine phenotype; specifically, that birds with lower GR expression would express a consistently potentiated HPA stress axis and more risk-averse personalities.

MATERIALS AND METHODS

Animals

We used a repeated measures study design that included behavioral testing (N=27; 15 females), plasma hormone assessment (N=25; 13 females) and neural hormone receptor mRNA quantification (N=25; 13 females; unequal sample sizes reflect the fact that two birds died of unknown causes between behavioral and hormonal assessments; Fig. 1). In 2009, we collected

154 eggs from 14 nests (7 nests had clutch sizes of 1; 1 nest had a clutch size of 2; 6 nests had clutch
155 sizes of 3) from an established nest box population (Westerheide, NL). Eggs were then
156 distributed to unique and random wild foster parents to decouple nestling experience and
157 relatedness among siblings. Because other maternal effects prior to hatching (e.g. yolk
158 hormones) might influence the adult phenotype, we call this a 'nest of origin' effect (hereafter
159 NestID) rather than strictly genetic relatedness. Ten days after hatching, fledglings were
160 transported to the Netherlands Institute for Ecology (NIOO-KNAW, Heteren, NL) and hand-
161 raised in captivity until nutritional independence.
162 In November 2010, the birds were transported by automobile to the Max Planck Institute
163 for Ornithology-Radolfzell, where all experimental and laboratory work was conducted. After
164 two weeks of quarantine, birds were housed singly in large outdoor aviaries (3 x 3 x 2 meters
165 high) in alternating male-female adjacencies (birds had audible but not visible contact). These
166 captive conditions facilitated control of the social and nutritional environments—singly housed
167 birds were fed an *ad libitum* diet and fresh water. Each aviary contained an elevated feeding
168 platform, a nest box, hanging perches and live shrubs. Birds were acclimatized to these housing
169 conditions for three months before testing began. We first characterized behavioral traits using
170 three repeated samplings, and then characterized HPA axis function using two repeated measures
171 sampling events, and lastly we sacrificed the birds to estimate the expression of hormone
172 receptors in the brain (Fig. 1).
173
174 *Behavioral Testing*
175 Twenty-seven birds were tested in a behavioral assay for object neophobia and risk-taking on
176 three repeated occasions (RTA_{1-3}; Fig. 1). Testing order was randomized with the exception that

177 adjacent aviaries were never sampled on the same day and the two sexes were balanced each
178 day. To ensure motivation and to habituate birds to feeding on the ground, each bird was
179 restricted to three mealworms per day in a bowl centered on the floor of the aviary during a
180 three-day window prior to testing. We tested a maximum of 7 birds per day during the morning
181 (7:30-12:00). To habituate birds to the experimental set-up, we placed a camouflaged blind in
182 front of each aviary at a distance of 3 m beginning 24 h prior to testing. The experimenter
183 occupied the blind during the testing.

184 Our neophobia/risk-taking assessment was modified from a procedure previously
185 validated as a measure of personality in this species (van Oers et al., 2005). Briefly, a small
186 platform (30 cm^2) containing live mealworms and a loaded mousetrap was introduced to the
187 aviary floor and monofilament was used to trigger the trap from a distant observation blind (see
188 S2). All birds alighted initially on the aviary floor and then hopped onto the front of the platform
189 near the mealworm dish, at a distance of approximately 20 cm from the mousetrap. No birds
190 were injured during the course of this assay.

191 We recorded (1) *Initial Latency*: time elapsed from the start of the trial until the bird
192 approached the platform and retrieves a mealworm—because this is each bird's first exposure to
193 the platform, we assume this latency reflects a low risk response to novelty (object neophobia);
194 (2) *Reward Latency*: time elapsed since retrieval of the first worm and the return to the platform
195 for a second mealworm (note: all birds flew to a shrub to consume each mealworm). Upon
196 alighting on the platform for this second mealworm the experimenter triggered the trap, startling
197 the bird (all birds flew away); and (3) *Startle Latency*: time elapsed between triggering the trap
198 and the bird returning to the platform and retrieving a mealworm—we assume this latency
199 reflects a high risk response. Trials in which birds did not return within the maximum trial

duration (20 min) were not included in the repeatability, covariance or PCA analyses because assigning a constant value (e.g. 1200 sec) would artificially inflate these estimates. Although, exclusion of these incomplete trials could under-represent the most risk-averse personalities, a pilot study conducted with wild-caught birds showed that extending this time window to longer durations (60 min) resulted in few additional latency data. The number of mealworms on the platform was counted following each trial to ensure that the expected number of worms was retrieved. This testing procedure was repeated three times per bird, with a three-day interval separating repeated trials. Upon completion of the third trial, each bird was measured for tarsus length, flattened wing cord length, body mass, and fat score (Cherry, 1982); body condition was estimated using the scaled mass index method (SMI; Pieg and Green, 2009), and completion of prebasic moult was confirmed.

HPA Assessments

Validation

In March 2012 we validated the HPA assessments following (Dickens et al., 2009a), including the pharmacological dosages, time courses, and the cross reactivity of the pharmacological reagents in the ELISA. We used wild-caught adult great tits—used only for this validation—from a nest box population in Radolfzell, Germany (N=13) (S1).

Blood collection

Twenty-five birds were tested for plasma glucocorticoid dynamics using a repeated measures HPA assessment (Fig. 1). The first assessment (HPA_1) was conducted during a one-week period in late August following the third and final set of behavioral trials to facilitate analysis of

hormone-behavior relationships. The second assessment (HPA_2) was performed in November preceding the brain collection. Sampling was limited to 0800-1100. Samples were collected by puncturing the brachial vein and collecting the blood using a heparinized microcapillary tube. Birds were restrained in small cotton bags during the intervals between sampling time points.

Our method for assessing the HPA axis has been used previously in birds (Dickens et al., 2009a; Hau et al., 2015) and reptiles (Romero and Wikelski, 2010) to simultaneously quantify four aspects of HPA axis function: (1) Baseline CORT (*BaseCORT*): this first blood sample precedes the handling/restraint-induced stress response. *BaseCORT* was collected within 2 min following entry into the aviary to reduce contamination from the stress response (Baugh et al., 2013; Romero and Reed, 2005) and was followed by placing the bird in a small cotton restraint bag for 15 min. (2) Stress response (*StressCORT*): this second blood sample provides an estimate of the early stage of each bird's acute response to handling/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 (Dickens et al., 2009a; Hau et al., 2015); this injection was followed by a 90-min restraint period. (3) Negative feedback strength (*DexCORT*): the CORT concentration here 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 sensitivity (*ActhCORT*): finally, 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, released into their aviary

and monitored for health. 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.

Enzyme Immunoassay

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). The details of our EIA procedure, including its validation, extraction, recoveries, technical repeatability and preparation of standards are reported in (Baugh et al., 2014; Ouyang et al., 2011). The intra- and inter-assay coefficients of variation (CV; 9 plates)—8.1% and 8.2%, respectively. 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.

Neural Receptors Quantification

Following a 14-day recovery from the second HPA assessment, birds (N=25) were captured by hand net and decapitated. Trunk blood was collected and kept on wet ice while whole brains were dissected from the skulls and frozen in aluminum foil on dry ice and maintained at -80 C until cryosectioning. The interval separating entry into the aviary and frozen tissue was < 3 min. We transported brains on dry ice to the Roslin Institute at the University of Edinburgh, mounted them on OCT (TissueTek) and sectioned them coronally at 15 μm onto polysine pretreated slides. Tissue was stored at -80 C. Two slides from each animal (each with six sections) were used for radioactive *in situ* hybridization and a custom Python script and ImageJ were used for silver grain quantification in the paraventricular nucleus of the hypothalamus (PVN) and the

hippocampus (HP)—two regions implicated in HPA axis regulation (Dickens et al., 2009b). Details of our methods are described in (Senft et al., 2016).

Statistical Analyses

Behavior: General

We used a repeated measures ANOVA to test for effects of repeat number and latency type, including covariates for sex, SMI, and fat score. This permitted us to evaluate whether birds became habituated to the novel object platform across repeated trials. Effect sizes (partial eta-squared and Cohen's d) were calculated for significant main effects, interactions and pair-wise comparisons.

Among-individual variances (i.e. repeatabilities)

All repeatability and covariance analyses were performed in R 3.0.2. We estimated the within- and among-individual variance components using Bayesian general linear mixed models (GLMMs) with a Gaussian error distribution, and used the variance component estimates to calculate the repeatability of each behavioral and hormonal trait ($\log_{10}$ transformed and z-standardized). Our linear mixed models approach (Sokal and Rohlf, 1995; Nakagawa and Schielzeth, 2010) has important advantages over earlier ANOVA-based methods for repeatability estimation (intra-class correlation coefficients; Lessells and Boag, 1987), including the incorporation of environmental covariates and nested terms (i.e. adjusted repeatabilities), robustness to data heterogeneity (missing values, unbalanced designs), and the ability to estimate uncertainty around the repeatability estimate because variances are estimated directly. These models were constructed in MCMCglmm (Hadfield, 2010). We ran models without fixed effects

292 (i.e. agreement repeatabilities), with individual identity as the sole random effect, and used
293 inverse-Wishart priors. A second set of models were conducted to correct our estimates for nest
294 of origin effects and determine whether the nest of origin explained some of the variation in
295 traits. Therefore, we added NestID as a random effect to the models. We then repeated all the
296 above models, this time accounting for fixed effects (i.e., adjusted repeatabilities), by adding
297 variation in body condition (SMI, scalar) and the fat score as fixed covariates (S4 for adjusted
298 repeatabilities), with a similar prior. Sex was not included as a term in any of the models because
299 behavioral, endocrinological and quantitative genetics studies in *P. major* have shown no
300 evidence for sex-dependent expression of exploratory behavior (Dingemanse et al. 2002; van
301 Oers et al. 2004a; Carere et al. 2005), risk-taking behavior (van Oers et al. 2005), or HPA axis
302 function (Stöwe et al. 2010; Baugh et al. 2014).
303 We used the variance component estimates to calculate effects of individual identity and
304 nest of origin. We ran each model for 1,000,000 iterations, used default sampling, and we ran
305 model diagnostics to confirm that the autocorrelation between subsequent stored iterations was
306 not higher than 0.1. We report the repeatabilities, and variance components calculated as a ratio
307 of the total variance, with 95% credible intervals (95CI).
308
309 *Covariances*
310 We performed bivariate GLMMs to estimate within- and among-individual covariation. These
311 models were constructed in MCMCglmm (Hadfield, 2010), where individual identity was fitted
312 as random effect. Covariances in both the random effect and the residual were allowed to take on
313 any value (for a similar analysis and sample size, see Araya-Ajoy and Dingemanse, 2016). We
314 compared the Deviance Information Criterion (DIC, Spiegelhalter et al., 2002) of these models

315 with one from a model where we fixed the covariance within individuals to zero. A difference of
316 >5 in DIC was considered statistically significant (Spiegelhalter et al., 2002). We ran these
317 models with uninformative priors and ran each model for 2,000,000 iterations. Standard model
318 diagnostics confirmed that autocorrelation among sampled iterations was low.
319
320 *Linking neural receptors, HPA dynamics and behavior*
321 HPA_2 was timed to precede brain collection (Fig. 1) to test the hypothesis that MR and GR
322 expression in the HP and PVN predict HPA axis function. To do this we used the average MR
323 and GR expression in the PVN and HP calculated across four coronal sections per bird (N=25).
324 We constructed general linear models to describe how neural receptor expression predicts CORT
325 concentrations for the four HPA components. We also included two fixed variables in these
326 models to represent body condition, SMI and furcular fat score, that have been shown to be
327 correlated with CORT secretion (Wingfield et al., 1994).
328 To test the broader relationships among receptors, hormones and behavior—and in order
329 to reduce family-wise error rates—we reduced the dimensionality for all three of these
330 phenotypic categories using principal components analysis and then used path analyses (SPSS
331 version 21) to test the strength and direction of relationships among phenotypic levels (Fig. 2).
332 We tested two *a priori* models that minimized the number of paths: (1) Full model: MR and GR
333 directly influence both HPA axis function and behavior and the HPA axis also directly influences
334 behavior; and (2) Reduced model: MR and GR only indirectly influence behavior via the HPA
335 axis. We calculated the fit of both models ($1-\pi(e_{HPA}*e_{Risk})$) and a summary statistic ($Q=(1-Fit_{Full})/(1-Fit_{Reduced})$) and then compared the significance of this quotient with a Chi-squared test
337 of significance ($W = -(N-d)*\log_e(Q)$, where N=sample size and d=the number of dropped paths).

Measurements from RTA_1 and HPA_1 were used and all datasets were log_{10}-transformed and z-standardized prior to component extraction. For the behavioral data, we excluded trials in which the bird did not return to the platform within the maximum window of time (20 min) because assigning maximum values here would artificially inflate the eigenvalues (final $N_{path\ analysis}$=14). All analyses extracted only one component (PC1) with eigenvalues > 1 (which explained 63-82% of the variance) and correlation matrices indicated that all pairwise correlation coefficients varied between 0.1 and 0.9 (S8). Therefore, we performed path analyses using PC1 for each trait category (Fig. 2). Residual error for these analyses did not deviate from Gaussian, visually or statistically (Shapiro-Wilk; all $p > 0.20$) and observed power for the omnibus path model was adequate (power=0.83).

RESULTS

Behavior: General

Sex (F_1=0.674, p=0.443), SMI (F_1=0.044, p=0.840) and fat score (F_3=0.503, p=0.694) did not explain significant variance in behavior. The repeated measures ANOVA therefore only included the within-subjects factors (latency type and repeat number; S2). Latencies were generally high for *Initial Latency* and *Startle Latency* (mean ± SD, sec: 347.8 ± 279.9; 283.3 ± 232.0, respectively)—indicating that this assay predictably elicited a neophobic and startle response, respectively—but low for *Reward Latency* (143.5 ± 163.8), suggesting that the first mealworm acted as a food reward. There was a significant main effect of latency type ($F_{2,26}$=22.82, $p<0.001$, partial η^2=0.74). There was no main effect of repeat number ($F_{2,26}$=1.54, p=0.233), but there was an interaction between latency type and repeat number ($F_{4,52}$=3.08, p=0.024, partial η^2=0.73) due to a marginal reduction in the *Initial Latency* between RTA_1 and RTA_2 (pairwise comparison:

p=0.01, cohen's d=0.92 (95CI=0.19-1.64); Fig. 1). No other pairwise comparison was significant (S2), suggesting that the novelty of the platform diminishes over repeated exposures.

Behavioral variances

Repeatabilities for all three latencies were high (0.41–0.53; see Bell et al. 2009) and statistically significant (i.e. with 95CI that were not zero-bound; Table 1; S5(a–c)). NestID did not explain significant variance in the three behaviors (Table 1), suggesting that risk-taking phenotypes may not be strongly explained by relatedness or pre-hatching maternal effects (e.g. yolk hormones) or both. However, the statistical power to detect NestID effects was not particularly high because half of the nests did not have siblings, so we cannot exclude this possibility. Moreover, results stayed qualitatively the same when accounting for fat score and SMI (S4 for adjusted repeatabilities).

Behavioral covariances

There was a significant positive among-individual covariance between *Initial* and *Startle Latencies* (Table 3; Fig. 3a; S5(d–m)), demonstrating that birds that are chronically neophobic are chronically more risk-averse (i.e. personality). There was a similar trend between *Initial* and *Reward Latencies* (Table 3; Fig. 3b). The DIC of the model in which the covariance between *Initial Latency* and *Reward Latency* was fixed to zero did not fit the data better than a model in which the covariance was allowed to take on any value (ΔDIC = 13.6). This was also true for the model including covariances between *Initial Latency* and *Startle Latency* (ΔDIC = 23.8). In both models the covariance within-individuals was positive and statistically significant (95CI do not

383 overlap zero), demonstrating that at a given moment in time a bird exhibiting more neophobic
384 behavior will also predictably exhibit more risk-averse behavior (Table 3).
385
386 *Hormones: General*
387 Our validation study indicated that our drug dosages and timeline induced predictable variation
388 in CORT concentrations, with low *BaseCORT*, moderately high *StressCORT*, low *DexCORT* and
389 high *ActhCORT* (S1). In our experimental study we observed a main effect of HPA component
390 ($F_{3,72}$=186.8, p<0.001, partial η^2=0.97), again with low *BaseCORT* and *DexCORT*, and high
391 *StressCORT* and *ActhCORT* (S3). There was also a main effect of season, with higher CORT
392 concentrations in November (HPA_2) compared to August (HPA_1) ($F_{1,24}$=24.29, p<0.001, partial
393 η^2=0.51; S3). Lastly, there was an interaction effect between season and HPA component
394 ($F_{3,72}$=25.06, p<0.001, partial η^2=0.71), driven by weakened negative feedback (higher
395 *DexCORT*) in November and a concomitant increase in *ActhCORT* (S3). The between-subject
396 factors of sex ($F_{1,23}$=3.46, p=0.08), SMI ($F_{1,23}$=2.5, p=0.12), and fat score ($F_{1,23}$=1.99, p=0.17)
397 did not significantly explain variance in the CORT variables and therefore were not included in
398 the repeated measures ANOVAs.
399
400 *Hormonal variances*
401 One bird had a very low CORT concentration for the first *BaseCORT* sample. We ran models
402 that included and excluded this statistical outlier, and the results did not differ qualitatively
403 (direction and proportionality of estimates), thus we report the inclusive results. For all four
404 traits, repeatabilities were statistically significant (i.e. 95CIs were not zero-bound). *DexCORT*
405 and *ActhCORT* exhibited qualitatively higher repeatabilities than *BaseCORT* and *StressCORT,*

406 but the 95CI did overlap so the estimates are not statistically significantly different from each
407 other (Table 2). When accounting for NestID, the amount of variance explained by differences
408 among individuals was qualitatively lower, and a larger part of the variance, especially for
409 *BaseCORT*, was explained by NestID, but again, 95CIs overlapped (Table 2; S6(a–d)). Results
410 stayed qualitatively the same when accounting for fat score and SMI (S4 for adjusted
411 repeatabilities).
412
413 *Hormonal covariances*
414 The difference in DIC between the bivariate model of *BaseCORT* and the *StressCORT* where
415 covariances were fixed to zero and where they were allowed to take on any value was large
416 (ΔDIC = 27), thus we assumed that covariance modelling better explained the data. There were
417 no statistically significant covariances at the among-individual level (Table 3; 95CI overlap
418 zero), despite a trend for some trait pairs, including high *BaseCORT* being linked with a high
419 *StressCORT* (Fig. 3c), and weak negative feedback (high *DexCORT*) linked to strong adrenal
420 sensitivity (high *ActhCORT*) (Fig. 3d). The covariances within individuals for all trait pairs
421 examined were statistically significant and positive (Table 3; S6(h,l,p)). Similarly, the model that
422 included covariance between the *StressCORT* and *DexCORT* was statistically significant (ΔDIC
423 = 19.5) and the covariance within individuals was statistically significant and positive, while the
424 covariance among individuals was not different from zero (Table 3; S6(e–p)). Similarly, the
425 covariances within individuals were statistically significant and positive for the pairs *DexCORT*
426 versus *ActhCORT*, and *StressCORT* versus *ActhCORT*. Again, the differences in DICs confirmed
427 that the models including covariance better explained the data ($\Delta DIC_{Dex\ vs\ Acth} = 26$ and ΔDIC_{Stress}
428 $_{vs\ Acth} = 16$).

Linking neural receptors, HPA dynamics and behavior

Expression of GR was higher in the PVN than in the HP, and MR exhibited the opposite pattern. Expression of MR was positively correlated across the PVN and the HP and there was a trend for a positive correlation for GR across the two nuclei (Fig. 2 and Senft et al., 2016). In contrast, there were no correlations between the two receptor types (MR versus GR) for any combination of nuclei or PC (all $p>0.15$).

Given the widespread covariance among trait items (e.g. *Initial* and *Startle Latencies*), the principal component analyses allowed us to reduce our dataset into four trait categories (MR phenotype; GR phenotype; HPA potentiation phenotype; risk-aversion phenotype; Fig. 2; see S8 for PCA details; Budaev 2010) and minimize family-wise error rates. Using these trait categories, our path analyses indicated that higher MR and lower GR predict a more potentiated HPA axis (higher CORT) and more risk-averse (longer latencies) personalities (Fig. 2). Model fit was significantly higher for the full model (0.785) compared to the reduced model (0.539) with two dropped paths ($\chi^2=9.12$, df=2, $p<0.05$; Fig. 2), indicating support for a direct and an indirect (i.e., via HPA axis) influence of MR and GR on risk aversion. These results were robust to the structural details of the path model—the qualitative outcome did not change with inclusion/exclusion of items in principal components and the relationships among these higher-level trait dimensions mirrored patterns detected at lower levels of analysis. For example, variation in *ActhCORT* positively predicted startle latencies ($R^2=0.41$, $F_{1,12}=8.36$, $p=0.014$); GR expression in the hippocampus negatively predicted startle latencies ($R^2=0.39$, $F_{1,12}=7.76$, $p=0.016$); GR expression in the PVN negatively predicted *ActhCORT* ($R^2=0.24$, $F_{1,23}=6.51$,

p=0.019); and MR expression in the hippocampus positively predicted *StressCORT* (R^2=0.20, $F_{1,23}$=5.82, p=0.024; see also S7, S8, S9).

DISCUSSION

We found support for the hypothesis that avian personality is correlated with individual differences in HPA axis function. Our repeated measures design allowed us to partition variance in behavioral, endocrine and neuroendocrine traits in the same individuals under semi-natural conditions, providing an integrative picture of trait lability and interaction—to our knowledge this is the first such study to integrate across these levels of organization. The results support our prediction that individuals with consistently more potentiated HPA axes exhibit risk-averse personalities. These results help to unify findings from previous research examining the HPA axis and personality in vertebrates (Ellis et al., 2006; Koolhaas et al., 1999) including great tits (Hau et al., 2016) and provide an important test of the assumption that pharmacological challenges provide a window into upstream neural receptor phenotypes.

Personality

Risk-taking behaviors were repeatable—approximately 30-50% of the variation in each can be attributed to individual differences, a relatively high fraction for behavioral traits (Bell et al., 2009). And in contrast to the HPA components, nest of origin explained only a small fraction of the behavioral variation. This finding extends previous work that indicates that these types of risk-taking behaviors are components of a more general personality suite that includes exploration and boldness (van Oers et al., 2004b, 2004c; van Oers et al., 2005; Hall et al. 2015).

Moreover, these behaviors are phenotypically correlated, owing to the joint contributions of positive among- and within-individual correlations. The within-individual correlation implies that either dynamic internal (e.g., circadian state) or external variables (e.g., temperature) or both varied across observations of the same individual and that these variables modulated the expression of both traits simultaneously (see Baugh et al., 2014). Given that we controlled the social and nutritional environments, this within-individual correlation further indicates that these two behaviors are codependent on a variety of influences beyond nutritional state and social context (see Drosmann et al. 2014). This within-individual correlation could emerge as a consequence of ultradian cycles (Droste et al., 2011); for example, baseline CORT and stress-induced CORT will both be higher following the periodic and pulsatile release of ACTH. The among-individual correlation between these two behaviors, however, provides evidence for their joint contribution to personality and is only possible given the among-individual variance (i.e. repeatability) of both behaviors (Baugh et al., 2014). This correlation means that birds that are on average more neophobic are also on average more risk-averse, and vice versa. This is consistent with the finding in this species that risk-taking behavior is genetically linked with general aspects of the shy-bold continuum, including spatial and object neophobia (van Oers et al., 2005).

HPA function

Our HPA assessments yielded the predicted results (i.e. low BaseCORT, stress-induced increases, decrease after DEX- and increase following ACTH-injection). This is similar to what has been documented in some other species (Romero and Wikelski, 2010; Schmidt et al., 2012; MacDougall-Shackleton et al., 2013; Hau et al., 2015), but differs from the lack of ACTH sensitivity reported in chukar partridge (*Alectoris chuckar*; Dickens et al. 2009a). The higher

CORT in November compared August suggests a seasonal pattern in DEX sensitivity, with weaker sensitivity in November. There was also a seasonal increase in *ActhCORT*, but this is likely due to the positive correlation between *DexCORT* and *ActhCORT*. Although our birds had recently completed prebasic molt at the time of the August assessment, the enduring physiological consequences of a recent molt or other seasonally variable inputs to the HPA axis might have downregulated axis sensitivity (Romero 2006). Despite this seasonality, all four HPA measures exhibited significant repeatability.

Baseline CORT exhibited the lowest repeatability, consistent with previous studies in great tits (Baugh et al., 2014) and other species (Hau et al., 2015; Ouyang et al., 2011). The other three HPA components contained moderate (*StressCORT*) to high (*DexCORT, ActhCORT*) amounts of repeatability, consistent with the only other report that has estimated repeatabilities in a subset of these traits (Hau et al., 2015). The repeatabilities of *DexCORT* and *ActhCORT* might be interesting to examine in future functional studies. Variation in negative feedback, for example, has been shown to correlate with nutritional state and mortality in Galapagos marine iguanas (*Amblyrhynchus cristatus*; Romero and Wikelski, 2010).

We also demonstrated that there are positive phenotypic correlations between several of these HPA components. Most of the covariance between traits is at the within-individual level. In other words, this suggests that a bird with high baseline CORT at a particular moment will have high stress-induced CORT minutes later; but that same bird a month later might have moderate initial CORT and moderate stress-induced CORT. This hypothetical bird is a relatively high-CORT individual (i.e. there is repeatability in both components), but dynamic variables, not stable individual differences, are driving the correlation between the HPA traits (Baugh et al., 2014). The interdependence of these HPA measures has implications for coping with repeated

stressors; for example, a positive trend between *DexCORT* and *ActhCORT* suggests that birds with stronger negative feedback are less able to mount a strong secondary stress response, owing perhaps to a refractory state. To our knowledge, these are the first estimates of combined phenotypic, within- and among-individual covariances in these HPA axis traits. Given trends indicating among-individual correlations between *BaseCORT* and *StressCORT* and between *DexCORT* and *ActhCORT*, we suggest future research examine this question further. However, because *BaseCORT* and *StressCORT* concentrations are likely regulated by separate receptor populations, thereby potentially decoupling them, we would not predict strong correlations between these measures (de Kloet et al., 1993; Romero, 2004).

Linking neural receptors, HPA dynamics and behavior

By estimating the expression of MR and GR in two brain regions known to regulate the HPA axis—the PVN and hippocampus—we provide support for the hypothesis that functional aspects of HPA axis dynamics can be predicted on the basis of receptor expression. Overall, we showed that higher MR levels predicted a more potentiated HPA axis (higher CORT) and, more importantly, higher GR levels predicted a less potentiated axis. Previously we showed that the expression of hippocampal MR is positively correlated with the expression of MR in the PVN, and a similar trend was observed for GR across these two nuclei (Senft et al., 2016). This within-individual correlation across nuclei is similar to what has been shown across diverse tissues in songbirds (Lattin et al., 2015) and suggests a neuroendocrine suite that constrains the flexibility of HPA axis function, which might explain individual differences in the HPA components measured in this study. Future research should also quantify receptor protein expression, as transcript and protein levels have been shown to be uncorrelated in house sparrow (*Passer*

543 *domesticus*) brains (Medina et al., 2013), and transcript expression might yield different results.
544 For example, in vivo studies in rodents have demonstrated that GR under-expression, which
545 leads to reduced negative feedback (i.e. more potentiated HPA axis) can be compensated for by
546 MR over-expression (Harris et al., 2013), suggesting that higher MR densities in rodent brains
547 might be associated with increased negative feedback (i.e. less potentiated HPA axis). The
548 classic model that MR strictly controls basal HPA drive whereas GR controls negative feedback
549 is being revisited (Kolber et al., 2009), and there is empirical evidence that it is the ratio of MR
550 and GR that might be critical to understanding HPA axis function (Harris et al., 2013). Lastly,
551 because MR and GR are widely distributed throughout the brain, including in *P. major* (Senft et
552 al., 2016), links between receptor expression and behavior need to be more thoroughly explored.
553 We found support for the hypothesis that variation in the neuroendocrine stress axis is
554 correlated with personality. Specifically, more neophobic and risk-averse birds exhibited a more
555 potentiated stress axis. This finding was confirmed with data reduction methods showing that the
556 three behavioral measures were largely explained by a single principal component, and likewise
557 for the four HPA components. PC1 for the behavioral measures might represent behavioral
558 inhibition under conditions of risk and PC1 for the HPA components might represent endocrine
559 potentiation. These two principal components were significantly and positively correlated: birds
560 exhibiting more risk-averse personalities also exhibited more potentiated HPA axes. It is
561 remarkable that the expression patterns of two receptors in the brain can explain such a large
562 amount of variation in HPA axis function and animal personality. We agree with Ball and
563 Balthazart (2008) that identifying functional interrelationships between behavior and hormones
564 at the individual level, which has occasionally failed (Crews 1998; Adkins-Regan 2005), is
565 greatly facilitated by inclusion of target tissue variables such as neural receptor expression. We

566 would further propose that understanding the behavior and hormonal sides of the equation are
567 greatly enhanced by multilevel approaches, including the characterization of syndromes. Lastly,
568 because recent work has demonstrated links between immune function and risk-taking behavior
569 (Jacques-Hamilton et al. 2017) and because glucocorticoids are known to suppress the immune
570 system, it will be important for future work to integrate among all three of these phenotypic
571 categories in wild animals.

572 These patterns expand on earlier studies in this species; we previously demonstrated a
573 genetic correlation between spatial/object neophobia and glucocorticoid reactivity, with shy
574 selection line birds exhibiting stronger stress responses (Baugh et al., 2012). Likewise, in a study
575 of wild birds we showed that slower explorers exhibited faster and more enduring glucocorticoid
576 responses (Baugh et al., 2013). The present study, which used pharmacological challenges in
577 addition to the more conventional stress series, confirms that the more enduring stress response
578 in more risk-averse personalities is due to weaker negative feedback *per se*. These results are
579 generally consistent with studies of stress and personality in other vertebrates (Baugh et al.,
580 2012). What is still unclear, however, is the directionality and causality of these hormone-
581 behavior relationships (Koolhaas et al., 2010). Further study is needed to examine the
582 developmental-organizational programming of HPA and behavior phenotypes (Schmidt et al.,
583 2013; Marasco et al., 2016) and new techniques are needed to acutely manipulate endocrine
584 function in a physiologically relevant manner.

585

586 CONCLUSIONS

587 Our multilevel and integrative approach—from neural receptor expression to plasma hormone
588 dynamics and behavior—provides new insight into the network of trait (co)variances that are

589 associated with animal personality. Our results support the hypothesis that two hormone
590 receptors in the brain play an integral role in HPA axis function, which is in turn associated with
591 predictable variation in personality. Further, our findings support the hypothesis that pleiotropic
592 effects of steroid hormones can act as proximate mechanisms that integrate behavioral traits into
593 personality suites. Overall, these results unify earlier research documenting the relationship
594 between endocrine stress reactivity and the shy-bold continuum in songbirds. Lastly, if the
595 widespread trait covariances shown here are the consequences of genetic correlations, as has
596 been demonstrated previously for specific HPA-behavior trait pairs (Baugh et al., 2012), this
597 would imply that selection targeting any one of these levels might affect the evolution of suites
598 of concerted traits (Ketterson and Nolan, 1999; McGlothlin and Ketterson, 2008).
599
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Figure Legends

Figure 1. Experimental timeline. Birds were tested on a risk-taking assay on three occasions (RTA_{1-3}) with 6-day intervals. Following a 9-day recovery period, they were sampled using a four component HPA assessment (A-D), once in August (HPA_1) and again in November (HPA_2) with a 52 day interval separating these two assessments. Following a 14-day recovery period, brains and trunk blood were harvested.

885 **Figure 2.** Relationships among traits depicted in a path model. For each level of organization
886 (neuroendocrine: MR and GR expression; endocrine: HPA_1 components; behavior: Initial and
887 Startle Latencies), traits were reduced to the first principal component (PC1). For traits measured
888 repeatedly per bird, agreement repeatabilities (R; subject as random factor) are indicated inside
889 circular arrows. Estimates of covariance between traits within each category are indicated with
890 bi-directional black arrows at the among- (Cov-A) and the within-individual levels (Cov-W).
891 The phenotypic correlations (Pearson's r) for MR and GR across the two nuclei are indicated
892 with bi-directional grey arrows. The path model yields beta coefficients (ß) describing the
893 direction and magnitude of effect of independent variables (GR, MR, HPA) on dependent
894 variables (HPA, Risk-taking). Error estimates for dependent variables in the path model are
895 indicated in grey elipses. The model indicates that higher MR expression in the hippocampus and
896 hypothalamus and lower GR expression in these two brain areas predicts a more potentiated
897 HPA axis (higher CORT) and more risk-averse personalities (higher latencies). Inset: the
898 reduced model has two dropped paths. * denotes statistical significance ($p<0.05$) for repeatability
899 (R), covariance (Cov) and correlation (r) estimates.
900
901 **Figure 3.** Graphical representations of the among-individual correlations for the three
902 behavioural traits (a,b) and four HPA components (c,d). All values are log_{10}-transformed and
903 plotted as standardized (z) scores and best fit lines are linear regressions. Plots show the
904 correlation between the average (per bird) values of the three behavioral trails (a,b) and two HPA
905 assessments (c,d). A statistically significant positive correlation in (a) is graphical evidence of
906 among-individual correlation (i.e. syndrome) between Initial Latency and Startle Latency (i.e.
907 consistently neophilic birds are also consistently more risk-taking). The other trait pairs (b-d)

were not statistically significant but positive trends here suggest the possiblity of among-individual correlations.