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# Novelty Induces Behavioural And Glucocorticoid Responses In A Songbird Artificially Selected For Divergent Personalities

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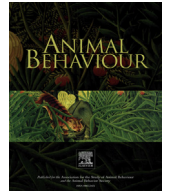
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## Novelty induces behavioural and glucocorticoid responses in a songbird artificially selected for divergent personalities



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Stress physiology is thought to contribute to individual differences in behaviour. In part this reflects the fact that canonical personality measures consist of responses to challenges, including novel objects and environments. Exposure to novelty is typically assumed to induce a moderate increase in glucocorticoids (CORT), although this has rarely been tested. We tested this assumption using great tits, *Parus major*, selected for divergent personalities (bold-fast and shy-slow explorers), predicting that the shy birds would exhibit higher CORT following exposure to a novel object. We also scored behavioural responses to the novel object, predicting that bold birds would more frequently approach the novel object and exhibit more abnormal repetitive behaviours. We found that the presence of a novel object did induce a moderate CORT response, but selection lines did not differ in the magnitude of this response. Furthermore, although both selection lines showed a robust CORT elevation to a subsequent restraint stressor, the CORT response was stronger in bold birds and this effect was specific to novel object exposure. Shy birds showed a strong positive phenotypic correlation between CORT concentrations following the novel object exposure and the subsequent restraint stress. Behaviourally, the selection lines differed in their response during novel object exposure: as predicted, bold birds more frequently approached the novel object and shy birds more strongly decreased overall locomotion during the novel object trial, but birds from both selection lines showed significant and similar frequencies of abnormal repetitive behaviours during novel object exposure. Our findings support the hypothesis that personality emerges as a result of correlated selection on behaviour and underlying endocrine mechanisms and suggest that the relationship between endocrine stress physiology and personality is context dependent.

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Wild animals regularly cope with challenges in their natural environments and benefit from behavioural flexibility. Individuals are not infinitely flexible, however, and constraints on plasticity can generate consistent behavioural differences (i.e. personality), which have been described in a variety of taxa (Gosling, 2001). Personality is often measured using assays of exploratory behaviour (e.g. spatial and object neophobia) with responses that can be described along a shy–bold continuum (Carere, Drent, Privitera,

Koolhaas, & Groothuis, 2005). These personality traits show an established set of correlated physiological and behavioural characteristics that are stable across time and contexts in both free-living (reviewed in Carere, Caramaschi, & Fawcett, 2010) and captive populations (Groothuis & Carere, 2005). Past studies have shown that more shy personalities ('slow explorers') are more sensitive to environmental changes and often perform better in dynamic environments, whereas bolder personalities ('fast explorers') may be better adapted to stable environments (Dingemans, Both, Drent, & Tinbergen, 2004; Drent & Marchetti, 1999; Korte, Koolhaas, Wingfield, & McEwen, 2005; Marchetti & Drent, 2000; Verbeek, Drent, & Wiepkema, 1994). These findings suggest that personalities might also differ in the frequency of

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routine-forming behaviours (i.e. stereotypies; abnormal repetitive behaviours), which are often observed in stable captive populations (Garner, Mason, & Smith, 2003; Keiper, 1969; Mason, 1991), with bolder individuals predicted to exhibit a higher frequency of abnormal repetitive behaviour.

A variety of neural and hormonal adaptations underlie behavioural responses to environmental challenges. A growing body of research on the proximate correlates of animal personality has demonstrated that endocrine phenotypes can be individually consistent and associated with personality (reviewed in Hau, Casagrande, Ouyang, & Baugh, 2016; Koolhaas, de Boer, Buwalda, & van Reenen, 2007; but see Bell, Hankison, & Laskowski, 2009; Pavitt, Walling, Möstl, Pemberton, & Kruuk, 2015). Because of their systemic nature and pleiotropic effects, steroid hormones have been proposed as key mechanisms that organize behavioural traits into correlated suites (Carere et al., 2010; Ketterson, Atwell, & McGlothlin, 2009; Koolhaas et al., 1999). However, the extent to which hormonal mechanisms evolve alongside behavioural mechanisms and are responsible for individual variation in behaviour remains under debate (Carere et al., 2010; Mutzel, Kempenaers, Laucht, Dingemans, & Dale, 2011).

Several of the behaviours that typify personality variation (e.g. shyness in nonhuman animals, neuroticism in humans; Gosling, 2001) are thought to be related, either directly or indirectly, to differences in how animals cope hormonally with stress (Koolhaas et al., 1999). Indeed, the proactive–reactive coping model described in Koolhaas et al. (1999) suggests individual covariation in boldness, environmental sensitivity and endocrine stress responsiveness. Therefore, the hypothalamic–pituitary–adrenal (HPA) axis, which produces glucocorticoids as its main end product, is often studied in this context (Hau et al., 2016). The principal glucocorticoid in birds is corticosterone (CORT), which serves primarily metabolic functions at baseline levels but becomes elevated shortly after the perception of a stressor, and continues to increase until a process of negative feedback permits baseline levels to be re-achieved (i.e. the glucocorticoid stress response; Romero, 2004). This endocrine stress response is conserved across vertebrates (Ellis, Jackson, & Boyce, 2006; Wingfield, 2003) and functions as a critical adaptation that enables organisms to regain homeostasis following acute challenges. Given the speed with which the stress response can be initiated (<3 min; Baugh, van Oers, Naguib, & Hau, 2013; Baugh, Davidson, Hau, & van Oers, 2017; Heading, Nisbet, & Ketterson, 2006; Romero & Reed, 2005; Small et al., 2017), it is also conceivable that the CORT response plays a direct role (through activational effects) in modulating behavioural responses to stressors in addition to the more rapid response mediated by the sympathetic nervous system (reviewed in Axelrod & Reisine, 1984).

Indeed, several lines of evidence suggest 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 (Baugh et al., 2012, 2013, 2017; Baugh, Davidson et al., 2017; Carere & van Oers, 2004; Koolhaas et al., 1999; Korte, Beuving, Ruesink, & Blokhuis, 1997; Martins, Roberts, Giblin, Huxham, & Evans, 2007; Satterlee & Johnson, 1988; van Oers, Buchanan, Thomas, & Drent, 2011), including in humans (reviewed in Ellis et al., 2006). Such studies have also been carried out using individuals derived from artificial selection experiments in both mammals (Harri, Mononen, Ahola, Plyusnina, & Rekilä, 2003) and birds (Cockrem, Candy, Castille, & Satterlee, 2010; Jones, Satterlee, & Ryder, 1992), thus providing an opportunity to identify genetically correlated traits.

Most of our understanding of interindividual variation in stress physiology and its relationship to personality stems from studies

applying a standardized handling-restraint stressor (reviewed in Cockrem, 2007). This method is easy to apply uniformly across studies and species, universally induces a strong and rapid CORT response, and likely reflects the HPA response to one type of potent stressor: interaction with predators (Canoine, Hayden, Rowe, & Goymann, 2002; Cockrem & Silverin, 2002a; Jones, Smith, Bebus, & Schoech, 2016; Wingfield & Ramenofsky, 1999). However, restraint methods are probably not representative of other less potent real-world stressors that often provide opportunities for behavioural coping, thus potentially revealing ethologically relevant individual differences. Interestingly, conventional exploratory assays, which often involve exposure to novel environments or novel objects, are typically assumed to induce a mild stress response, but this is rarely tested in a rigorous manner (but see Cavigelli & McClintock, 2003). These assays more closely resemble natural scenarios in which individuals experience novel physical or social environments, and therefore provide an opportunity to test this broader hypothesis about the link between personality and glucocorticoid phenotypes.

Here we evaluate the assumption that behavioural responses to novelty are directly related to stress physiology in a species that has served as a model for personality studies, the great tit, *Parus major*. In great tits, novel environment exploration and boldness towards novel objects are phenotypically correlated and individually consistent over time, and artificial selection experiments have demonstrated a heritable component (Drent, van Oers, & van Noordwijk, 2003). Furthermore, this heritable combination of behaviours ('fast-bold'/'slow-shy') correlates phenotypically and genetically with other behavioural tendencies, such as risk taking (van Oers, Drent, de Goede, & van Noordwijk, 2004), aggression and dominance (Verbeek, Boon, & Drent, 1996). Earlier work has also demonstrated that great tits selected for a combination of slow exploration and shyness ('slow explorers') show elevated CORT concentrations compared to 'fast explorers' in response to social challenges (faecal CORT: Carere, Groothuis, Möstl, Daan, & Koolhaas, 2003) and handling-restraint stress (plasma CORT: Baugh et al., 2012). These results imply a genetic correlation between behavioural and hormonal traits.

We tested four specific predictions: (1) exposure to a novel object should induce a mild CORT increase; (2) this novelty-induced CORT response should be higher in slow explorers; (3) the elevation in CORT should be further amplified following a subsequent restraint stressor, again with slow explorers experiencing a higher secondary stress response due to a predicted positive correlation between initial and subsequent stress responses; and (4) slow explorers should less frequently approach a novel object and show a lower frequency of routine-forming (route-tracing) behaviour.

## METHODS

### *Subjects, Novel Object Exposure and Hormone Sampling*

At the Netherlands Institute of Ecology (NIOO-KNAW, Wageningen, NL), we tested birds from the fourth generation of lines bidirectionally selected for fast exploration and boldness (fast explorers) and slow exploration and shyness (slow explorers). For details on the parental population and the artificial selection process, including the behavioural screening, see Drent et al. (2003), van Oers, Drent et al. (2004), van Oers, de Jong, Drent, and van Noordwijk (2004) and Verbeek et al. (1994).

Three weeks prior to the start of our experiments, adult birds were transferred from a group-housed aviary to singly housed cages in three rooms of an indoor–outdoor aviary facility, where

they remained until and throughout data collection. Cages ( $0.9 \times 0.4$  m and 0.5 m high) consisted of solid walls except for a wire-mesh front, three perches, and food and water dishes. Rooms were equipped with large open windows that allowed for natural lighting and airflow, maintaining the natural light–dark cycle and ambient temperatures. Birds were provided with ad libitum water, seed mixture and calcium, supplemented daily with mealworms and a mixture of sour milk, ground beef heart, commercial egg product and multivitamins.

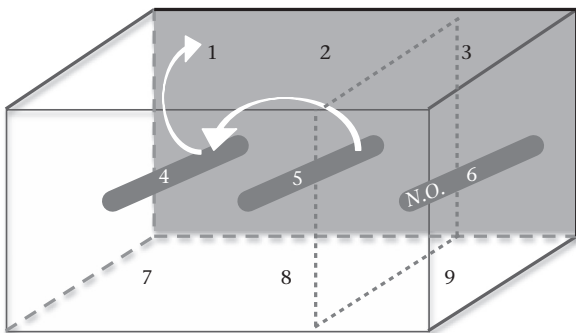
We conducted trials in November 2010 and limited our sampling period to 0800–0900 hours in order to control for diel rhythms in CORT secretion and to provide birds a 45 min period for morning foraging (automated supplemental light period: 0715–1700 hours). All birds had completed their annual prebasic moult. To minimize unintended stress, the tripod and digital camcorder used to monitor behaviour during novel object exposure were placed 1 m in front of the focal bird's cages at 1600 hours on the evening prior to testing so that the birds had an opportunity to habituate to their presence, and the birds were subsequently left undisturbed overnight prior to sampling. We simultaneously sampled two birds per room per day and balanced selection line and sex for each day. Each trial involved the following steps: (1) two researchers simultaneously entered the room; (2) filming was initiated on both camcorders; (3) the rightmost perch was removed from each cage and replaced with either an identical empty perch (control treatment;  $N = 12$  slow explorers, 10 fast explorers), or an identical perch with a bendable Pink Panther figurine (8 cm) attached to it (experimental treatment;  $N = 16$  slow explorers, 22 fast explorers; see Fig. 1); (4) researchers exited the room until 10 min had elapsed and then (5) re-entered the room, captured each bird and collected approximately 30  $\mu$ l of blood from the brachial vein (hereafter 'CORT-10'); hormone levels in this blood sample potentially reflect the cumulative stress of exposure to humans (both treatment groups) and the novel object (experimental treatment); (6) each bird was then immediately restrained in a small cloth bag for 30 min; (7) we then collected a second 30  $\mu$ l of blood from the opposite brachial vein (hereafter 'CORT-30'); hormone levels in this blood sample reflect the potentially cumulative effects of the novel object stressor and the handling/restraint stressor at the CORT-10 time point. Finally (8) we confirmed the ring number and completion of moult, measured body mass and subcutaneous fat (Cherry, 1982), returned the birds to their home cages and

maintained as normal during a 2-day recovery period during which no other birds from that room were tested. Exactly 48 h after the start of each trial (i.e. day 2), we entered each room and collected a blood sample from both birds to estimate baseline CORT, hereafter 'CORT-0'. This baseline CORT sample was collected after and not before the novel object/stress trials to ensure that the birds' behaviour was not affected by a previous noxious experience with the experimenter and because we expected the return to baseline CORT to occur within 48 h (Breuner, Greenberg, & Wingfield, 1998). Birds were again measured for body mass and subcutaneous fat (Cherry, 1982) before they were returned to their home cages.

All blood samples were collected rapidly (<3 min from entry into room or collection from restraint bag until the completion of the bleed; hereafter 'handling time') because CORT becomes significantly elevated at longer durations in this species and many other taxa (Baugh et al., 2013; Baugh, Davidson et al., 2017; Romero & Reed, 2005; Small et al., 2017). Whole-blood samples were maintained on wet ice for <1.5 h before being centrifuged (5000 revolutions/m for 10 min; ca. 1400 g). The plasma fraction was then stored at  $-80^{\circ}\text{C}$  until transfer on dry ice to the Max Planck Institute for Ornithology (Radolfzell, Germany) for hormone measurement. Plasma volumes were too low to quantify for five fast explorers from the experimental treatment (final  $N = 16$  slow explorers (11 females), 17 fast explorers (10 females)) and one slow explorer from the control treatment was accidentally handled during perch replacement and thus omitted (final  $N = 11$  slow explorers (8 females), 10 fast explorers (6 females)).

#### Enzyme Immunoassay for Corticosterone

To estimate plasma CORT concentrations, we used a commercial enzyme immunoassay (EIA) kit (Enzo Life Sciences, Cat. No. ADI 900-097; Donkey anti-Sheep IgG). The details of our EIA procedure, including its validation and preparation of standards are reported in Ouyang, Hau, and Bonier (2011) and Baugh, van Oers, Dingemans, and Hau (2014). Briefly, concentrations were determined following a double diethyl-ether extraction of a 10  $\mu$ l plasma volume. After drying extracts under a stream of  $\text{N}_2$  gas, samples were diluted at a 1:30 dilution using Tris-buffered saline (provided by kit) and samples were allowed to equilibrate overnight at  $4^{\circ}\text{C}$ . Samples were then randomly assigned to wells along with blanks and five standards (0.032–20 ng/ml CORT). Plates were read on a VERSA<sub>max</sub> microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 405 nm. All samples, standards and controls were assayed in duplicate and any sample that exceeded a coefficient of variation between duplicates of 15% was reassayed until meeting that criterion. Average recovery, which we determined previously using individual samples spiked with radioactively labelled CORT, was uniformly high (mean  $\pm$  SD =  $85 \pm 2.7\%$ ;  $N = 9$ ) and therefore we did not correct for it. The intra- and interassay coefficients of variation (CV), 7.7% and 7.9%, respectively, were determined by distributing a minimum of three duplicate samples of stripped chicken plasma (using activated charcoal and dextran) that were spiked with commercial CORT (supplied by kit) to a concentration of 20 ng/ml across each of the seven plates. We previously reported a high degree of 'technical repeatability' ( $r$ ) for this assay, which provides a cumulative estimate of measurement error for nonassay sources of variation, including extraction, pipetting, and freeze-thaw ( $r \pm$  SEM =  $0.962 \pm 0.015$ ,  $N = 23$  birds sampled twice each,  $P = 0.0001$ ; see Baugh et al., 2014). The assay has a lower detection limit of 27 pg/ml. The cross-reactivity of the antiserum is 100% for corticosterone, 28.6% for deoxycorticosterone and 1.7% for progesterone.



**Figure 1.** Diagram of individual cage and example flight path. To sequence flight paths, we assigned nine invisible and equally spaced zones. Each home cage was identical and contained three perches (zones 4–6). The novel object (N.O.) was always located at the near end of perch 6 ('novel object perch'). The 'novel object zone' comprised the rightmost one-third of the cage (right of the dotted line), including zones 3, 6 and 9. The arrows drawn on this diagram represent an example of a flight path involving two transitions (triad 5–4–1) that did not involve entry into the novel object zone.



## Behaviour

We manually scored behaviour from the 10 min video files to estimate total locomotor activity and route tracing, a form of abnormal repetitive behaviour often observed in caged birds (Garner et al., 2003; Keiper, 1969). We limited this analysis to the experimental condition for three reasons: (1) we were principally interested in estimating individual variation and comparing the behaviour of the two selection lines in the presence of a novel object; (2) previous studies in these selection line birds indicated that the control manipulation (perch replacement alone) does not influence behaviour (van Oers, n.d.); and (3) estimating route tracing is extremely labour intensive. We analysed movement from a total of 38 birds ( $N = 16$  slow explorers (12 females), 22 fast explorers (13 females)).

Start times began when the replacement perch was installed. We subsampled each continuous 10 min video file by sampling behaviour during five evenly spaced minutes (0–1, 2.25–3.25, 4.5–5.5, 6.75–7.75 and 9–10 min; hereafter referred to as Min1, Min3, Min5, Min7 and Min9, respectively). This sampling scheme sought to reduce the time-consuming nature of our continuous scoring of every movement and position for each bird (ca. 400 h). We verified that this sampling method resulted in representative estimates for individuals through a sensitivity analysis that involved scoring five randomly selected trials for the full 10 min and for this sample of 5 min (slope = 1.74,  $r = 0.97$ ).

Lastly, two judges that were blind to the study observed the video trials for a subset of eight randomly selected subjects and scored them for each of the behavioural measurements included in this study (e.g. number of transitions, number of triads, sequential dependency scores, etc.). Interjudge agreement was estimated using linear regressions and was high for all behaviours scored in this study ( $R^2 = 0.82–0.97$ ,  $F_{1,6} = 27.6–206.8$ , all  $P < 0.002$ ).

### Behaviour toward the novel object

Each trial video was observed at 0.5× speed and a template of the cage was divided vertically into equal thirds (a perch was placed in the middle of each one-third). The rightmost one-third contained the ‘novel object perch’ and thus constituted the ‘novel object zone’ (Fig. 1). We quantified the frequency with which each bird transitioned onto the novel object perch and into the other areas of the novel object zone (e.g. floor, cage walls and ceiling but not the novel object perch), while controlling for the total number of transitions.

### Total locomotor activity

We began by describing each bird’s movement pattern. We hand-traced the bird’s movement path on printed templates, using arrows to denote direction among nine major, evenly spaced stopping zones. Based on these locations, movement paths were transcribed into numerical sequences for each sampled minute (Fig. 1). One coder drew these flight patterns, and another coder transcribed these drawings into sequences. All coders were blind to each bird’s selection line status, hormone results and sex.

We counted the number of flight transitions for each minute of observation. A transition was defined as any abrupt stop in movement, including two sequential stops made in the same location (e.g. hopping and landing on a single perch). These sequences allowed us (1) to compare the two selection lines for the total number of transitions and the change in the number of transitions from the first to the last sampled minute (Min1 – Min9) and (2) to control for the total number of transitions in our analysis of abnormal repetitive behaviour, because a bird with a greater number of transitions might otherwise appear to have more route tracing and more often approach the novel object.

### Abnormal repetitive behaviour: triad analysis

To obtain a basic understanding of repetitive movement patterns, we counted the number of times a sequence of three numbers (triads; e.g. 3-5-6; 1-1-7) was repeated using a custom Python script (version 2.7.10, [www.python.org](http://www.python.org)). We examined triads because repetitive three-step sequences capture both longer and shorter repetitive sequences and have been validated previously as measures of repetitive behaviour (Asher, Davies, Bertenshaw, Cox, & Bateson, 2009), and because it allowed us to look at changes in route-tracing behaviours over time.

To establish operational thresholds for repetitive sequences, we did the following: in a given sampling minute, a particular sequence of three positions had to occur at least two times in order to be considered a ‘repetitive triad’. We then summed the number of times each ‘repetitive triad’ occurred in each sampling minute and divided by the total number of transitions made during that minute. This standardization is needed to examine repetitive sequences per se, unconfounded by the fact that an individual who moves more will, by chance alone, tend to accumulate more repeated sequences of movement. To optimize our estimates of repetition, we repeated this process for thresholds of at least four repetitions and at least eight repetitions of single triads. This triad analysis permitted us to estimate changes in repetitive behaviour during the course of the 10 min period.

### Abnormal repetitive behaviour: sequential dependencies

The triad analysis is constrained by the length of sequences in a repeated route. Therefore, we also conducted a Markov chain analysis of sequential dependency, a sequence length-independent (i.e. generalizable) measure previously validated for quantifying route-tracing behaviour, following Asher et al. (2009) and Brilot, Asher, Feenders, and Bateson (2009). We used a modified chi-square test statistic for first- versus second-order dependency to find all transitional probabilities, or the probability that a bird transitioned from state  $i$  to state  $j$ , for each of nine states (zones). This analysis results in a sequential dependency score (SDS) that can be interpreted as the degree of repetitive route-tracing behaviour, with a higher score indicating a more repetitive bird.

We calculated a modified chi-square test statistic ( $C_y$ ; an unstandardized SDS) for each bird ( $y$ ) for every possible triad (XYZ) using following equation:

$$C_y = \sum_X \sum_Z \frac{(N_{XYZ} - N_{XY}P_{YZ})^2}{N_{XY}P_{YZ}} \quad (1)$$

$N_{XYZ}$  is the number of times that a given triad (XYZ) is repeated.  $N_{XY}$  is the number of times the bird went from location X to location Y.  $P_{YZ}$  is the transitional probability that a bird moves from location Y to location Z ( $P_{YZ} = N_{YZ}/N_Y$ ). Equation (1) follows the standard formula for the chi-square test statistic. Here our observed value is  $N_{XYZ}$  and the expected value is  $N_{XY}P_{YZ}$ .  $C_y$  is the summation of chi-square test statistics for all 729 possible triads (permutations with replacement:  $9^3$ ) for bird  $y$ . We wrote a Python script (version 2.7.10) to generate these counts and then we calculated a test statistic for every possible triad and summed these together to calculate  $C_y$  for each bird. To calculate degrees of freedom ( $df$ ) we used the following equation:

$$df = (A - N_v - 1) (A - 1) \quad (2)$$

In equation (1), we sum over X and Z, not Y, which is why we have two terms in our  $df$  calculation. Here  $A$  equals all possible locations and  $N_v$  is all starting locations in the cage that the bird never visited. The first term ( $A - N_v - 1$ ) differed for each bird. If a given bird never occupied a particular zone, then all transitions

from that zone were impossible. For this study, the second term ( $A - 1$ ) was always equal to  $(9 - 1)$ , because it was technically possible for a bird to go to any location from all starting locations. After calculating  $df$  we standardized our  $\chi^2$  test statistic using the following equation:

$$Z = \frac{C_y - df}{\sqrt{2df}} \quad (3)$$

$Z$  represents our standardized SDS, which now exhibits an approximately normal distribution with a mean of 0 and a standard deviation of 1 (Canal, 2005). A higher  $Z$  corresponds to a more predictable (repetitive) locomotor sequence. We calculated a  $P$  value for each bird's  $Z$  score by comparing the observed  $Z$  score to the normal distribution (one-tailed area  $\geq Z$ ). This  $P$  value provides a significance test of whether each bird's sequence of contiguous events are more repetitive than expected chance. Additionally, to account for potential differences over time, we binned the first two and last two sampled minutes, omitting the middle sampled minute, and calculated  $Z$  for each of those two collapsed time windows.

#### Ethical Note

This study was carried out in accordance with the animal ethical committee of the Royal Dutch Academy of Sciences (DEC-KNAW) under protocol NIOO 10.06 (K.V.O.). The great tit is not an endangered or threatened species. The cumulative time spent in trials and under handling-restraint was kept to the minimum necessary for blood sampling and assessments of behaviour and stress reactivity. Components of these protocols have been employed previously in this species (Baugh et al., 2012, 2013, 2014; Baugh, Davidson et al., 2017; van Oers de Jong et al., 2004) and our methods adhered to the ASAB/ABS Guidelines for the treatment of animals in research (2016). We did not detect any adverse health effects in the sampled birds (see Results), and all birds remained in captivity for the duration of their natural lives.

#### Statistical Analyses

To test the first prediction that exposure to a novel object induces a mild CORT increase, we used a repeated measures ANOVA with treatment group as a between-subjects factor and the three CORT time points as the within-subjects response variables followed by post hoc comparisons. To evaluate the second and third predictions, that slow explorers would exhibit a stronger CORT response to novelty and experience a higher secondary stress response to restraint, we used separate repeated measures ANOVAs and post hoc comparisons for the experimental and control groups with selection line as a between-subjects factor. In addition, we used ANCOVAs to evaluate whether accounting for a bird's state following novelty (CORT-10) aided in explaining variation in the subsequent CORT-30 measurement between selection lines. Moreover, we used comparison of slopes tests to determine whether the selection lines differed in the relationship between CORT-10 and CORT-30, which might indicate differential HPA constraints for the two selection lines. We used Pearson's correlations and  $t$  tests to evaluate whether handling times correlated positively with CORT concentrations and whether selection lines differed in average handling times for the three bleeds, respectively. Lastly, we calculated the area under the CORT curve for each bird in two ways: (1) total CORT (area under the three time-point curve) and (2) response CORT (CORT-30 minus CORT-10).

To test the fourth prediction that slow explorers would approach the novel object less, we used  $t$  tests. To evaluate selection line differences for general locomotor behaviour (total transitions)

across sampling time points as well as differences in the frequency of repetitive sequences (triads) corrected for total transitions, we used repeated measures ANOVAs. Lastly, we used a Markov chain analysis to estimate each individual bird's route-tracing repetitiveness followed by  $t$  tests and Fisher's exact tests to assess selection line differences.

We used SPSS (version 21, IBM, Armonk, NY, U.S.A.) for all statistical analysis. We included handling time at each time point, body condition index (residuals from length versus mass) on the novel object day and on the baseline day and sex as between-subjects factors in the initial hormone analyses but dropped them from the final models due to lack of explanatory value (see [Supplementary Tables S1 and S2](#)). We have shown previously in this species that sex does not explain variance in the behavioural or CORT traits that we report on here (Baugh et al., 2013, 2014; Baugh, Davidson et al., 2017; Carere et al., 2005; Stöwe, Rosivall, Drent, & Möstl, 2010). Hormone data were square-root transformed and tests were two tailed where relevant. We checked graphically whether error variances met assumptions for normality, homogeneity and sphericity. We used Welch's  $t$  test corrections when variances between the two lines were unequal. Bonferroni corrections were applied to  $P$  value calculations for post hoc multiple comparisons.

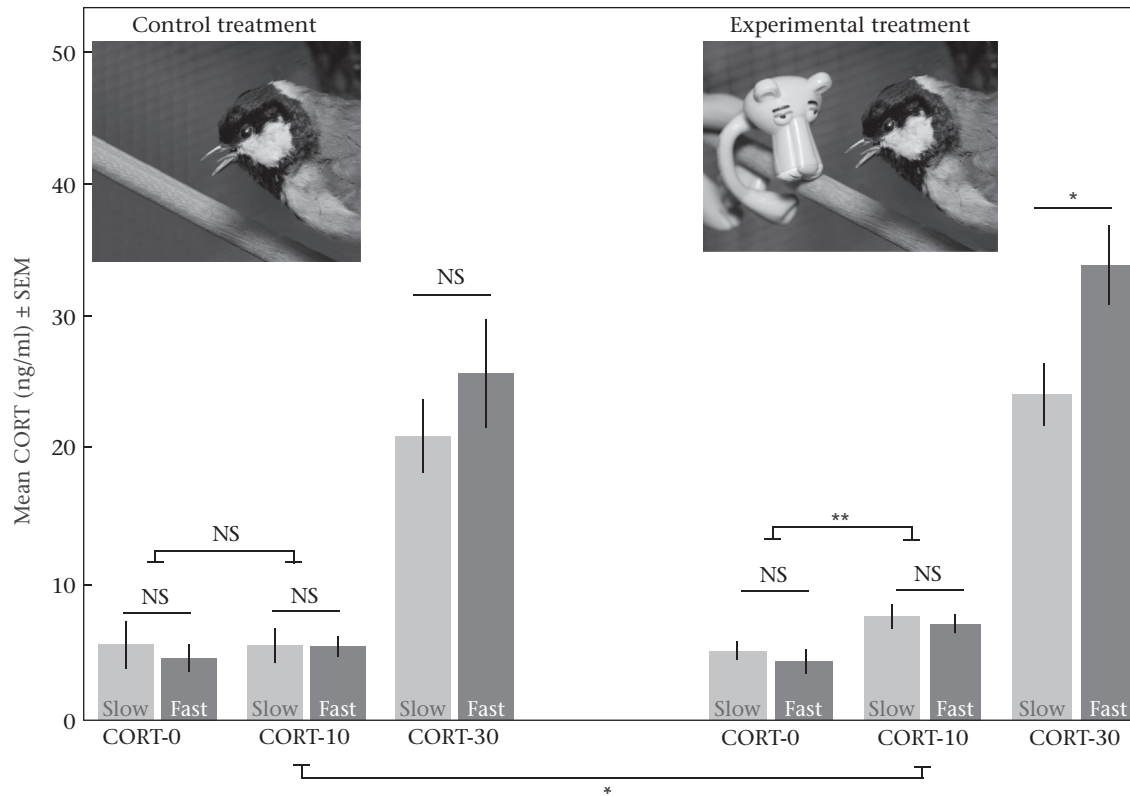
## RESULTS

### Corticosterone

A repeated measures ANOVA demonstrated a significant interaction between time point and treatment ( $F_{2,51} = 3.0$ ,  $P = 0.05$ , partial  $\eta^2 = 0.11$ ). Post hoc tests revealed that CORT-10 was higher in the experimental treatment than in the control treatment ( $P = 0.02$ ) while the treatment groups did not differ in CORT-0 ( $P = 0.71$ ) or CORT-30 ( $P = 0.12$ ; [Fig. 2](#)). There were no significant correlations between any of the behaviours measured during novel object exposure and any of the CORT measures (see [Supplementary Tables S1 and S2](#)). Below we report the separate hormone analyses within each treatment group.

### Experimental treatment

A repeated measures ANOVA demonstrated a significant main effect of time point ( $F_{2,62} = 186.2$ ,  $P < 1 \times 10^{-7}$ , partial  $\eta^2 = 0.92$ ) driven by increases in CORT in both selection lines among all three time points ([Fig. 2](#)). There was no main effect of selection line ( $F_{1,31} = 0.734$ ,  $P = 0.398$ ), but there was an interaction between time point and selection line ( $F_{2,31} = 9.775$ ,  $P = 0.004$ , partial  $\eta^2 = 0.28$ ), with fast explorers having higher CORT-30 concentrations ( $P = 0.014$ ). Pairwise comparisons showed that all three time points differed from each other within each selection line (slow explorers: CORT-0 versus CORT-10:  $P = 0.044$ ; CORT-10 versus CORT-30:  $P < 1 \times 10^{-7}$ ; fast explorers: CORT-0 versus CORT-10:  $P = 0.005$ ; CORT-10 versus CORT-30:  $P < 1 \times 10^{-7}$ ). Selection lines did not differ in CORT-0 ( $P = 0.283$ ), and in opposition to our prediction, slow explorers did not exhibit higher CORT-10 ( $P = 0.655$ ). Similarly, an omnibus ANCOVA model (response variable: CORT-30; covariate: CORT-10; between-subjects factor: selection line;  $F_{3,29} = 5.93$ ,  $P = 0.003$ , model fit  $R^2 = 0.38$ ) showed a main effect of selection line ( $F_{1,29} = 7.84$ ,  $P = 0.009$ ), significant covariation between CORT-10 and CORT-30 ( $F_{1,29} = 4.30$ ,  $P = 0.047$ ) and a significant interaction between selection line and CORT-10 ( $F_{1,29} = 4.80$ ,  $P = 0.037$ ). This result reflects the fact that there was a strong positive phenotypic correlation between CORT-10 and CORT-30 in the experimental treatment, but only in slow explorers (slow explorers:  $r = 0.74$ ,  $R^2 = 0.54$ ,  $F_{1,14} = 16.42$ ,  $P = 0.001$ ; fast explorers:  $r = 0.02$ ,  $R^2 = 0.0003$ ,  $F_{1,15} = 0.005$ ,  $P = 0.947$ ). A comparison of



**Figure 2.** Mean corticosterone concentrations in control ( $N = 11$  slow explorers, 10 fast explorers) and experimental ( $N = 16$  slow explorers, 17 fast explorers) treatments. CORT-0 (i.e. 'baseline') samples were collected on day 2 and CORT-10 (novel object stress) and CORT-30 samples (novel object + restraint stress) were collected on day 0. Control and experimental treatments differed in one respect: the replacement perch in the experimental treatment had a flexible plastic Pink Panther figurine attached to it. Statistical significance was estimated from a repeated measures ANOVA (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

slopes test showed that the regression coefficient ( $B$ ) for the correlation between CORT-10 and CORT-30 differed between the selection lines ( $B_{\text{slow explorers}} - B_{\text{fast explorers}} = -1.162$ ;  $F_{1,29} = 4.797$ ,  $P = 0.037$ ; Supplementary Fig. S1). This result was unaffected by inclusion/exclusion of an outlier (a fast explorer with low CORT-10 ( $\Delta R^2_{\text{exclusion-inclusion}} = 0.05$ ; Supplementary Fig. S1)).

We then compared the selection lines for the two measures of area under the curve using an ANOVA. The selection lines did not differ in total CORT ( $F_{1,31} = 2.675$ ,  $P = 0.112$ ) but did differ in response CORT (mean  $\pm$  SD (ng/ml): slow explorers:  $231.3 \pm 155.6$ ; fast explorers:  $340.1 \pm 116.8$ ;  $F_{1,31} = 9.80$ ,  $P = 0.004$ ), again driven by the higher CORT-30 values in fast explorers.

All blood samples were collected within 3 min from the moment of stressor onset (mean  $\pm$  SD handling time =  $108.4 \pm 51.1$  s, range 37–179 s). Handling time did not correlate with CORT concentrations at any time point (all  $P > 0.24$ ) or differ between selection lines for CORT-10 or CORT-30 (all  $P > 0.62$ ); however, handling times were higher at the CORT-0 time point in slow explorers (mean  $\pm$  SD: slow explorers:  $118.3 \pm 39.9$  s; fast explorers:  $82.7 \pm 34.4$  s;  $t_{19} = 2.7$ ,  $P = 0.01$ ). Selection lines did not differ in tarsus length, subcutaneous fat, body mass or body condition index on either the novel object test day (day 0) or baseline bleed day (day 2) (all  $P > 0.05$ ) and there was no change in body condition index from day 0 to day 2 (repeated measures ANOVA:  $F_{1,30} = 1.05$ ,  $P = 0.315$ ) or interaction with selection line ( $F_{1,30} = 3.18$ ,  $P = 0.084$ ).

#### Control treatment

A repeated measures ANOVA demonstrated a main effect of time point ( $F_{2,38} = 84.9$ ,  $P < 1 \times 10^{-7}$ , partial  $\eta^2 = 0.86$ ). This was driven

by the increase in CORT in both selection lines between CORT-10 and CORT-30 (Fig. 2). There was no main effect of selection line ( $F_{1,19} = 0.114$ ,  $P = 0.739$ ) or interaction between time point and selection line ( $F_{2,19} = 1.02$ ,  $P = 0.325$ ). Pairwise comparisons showed that CORT-30 differed from CORT-0 and CORT-10 ( $P < 1 \times 10^{-7}$ , pooled selection lines) and that CORT-0 did not differ from CORT-10 when selection lines were pooled ( $P = 0.249$ ). Similarly, an omnibus ANCOVA (response variable: CORT-30; covariate: CORT-10; between-subjects factor: selection line) model was not significant ( $F_{3,17} = 2.07$ ,  $P = 0.14$ ). This result reflects the fact that there was no phenotypic correlation between CORT-10 and CORT-30 in the control treatment (pooled selection lines:  $r = 0.28$ ,  $R^2 = 0.08$ ,  $F_{1,31} = 2.64$ ,  $P = 0.114$ ; slow explorers:  $r = 0.16$ ,  $R^2 = 0.03$ ,  $F_{1,9} = 0.234$ ,  $P = 0.64$ ; fast explorers:  $r = 0.60$ ,  $R^2 = 0.36$ ,  $F_{1,8} = 4.52$ ,  $P = 0.07$ ). A comparison of slopes test showed that the regression coefficients ( $B$ ) for CORT-10 versus CORT-30 between the two selection lines did not differ ( $B_{\text{slow explorers}} - B_{\text{fast explorers}} = -1.316$ ;  $F_{1,17} = 2.751$ ,  $P = 0.116$ ). Lastly, the two selection lines did not differ in total CORT ( $F_{1,19} = 0.318$ ,  $P = 0.580$ ) or response CORT ( $F_{1,19} = 0.856$ ,  $P = 0.367$ ; Greenhouse–Geisser correction for violation of sphericity).

All blood samples were collected within 3 min of handling time (mean  $\pm$  SD =  $90.5 \pm 28.1$  s, range 40–165 s). Handling times did not correlate with CORT concentrations at any time point (all  $P > 0.11$ ) or differ between selection lines for CORT-0 or CORT-30 (all  $P > 0.05$ ). However, handling times at the CORT-10 time point were longer for slow explorers than for fast explorers (mean  $\pm$  SD: slow explorers:  $107.6 \pm 18.04$  s; fast explorers:  $82.9 \pm 20.5$  s;  $t_{19} = 2.9$ ,  $P = 0.009$ ). Selection lines did not differ in tarsus length, subcutaneous fat, body mass or body condition index on either day 0 or



day 2 (all  $P > 0.07$ ) and there was no change in body condition index from day 0 to day 2 (repeated measures ANOVA:  $F_{1,19} = 0.01$ ,  $P = 0.923$ ) or interaction with selection line ( $F_{1,19} = 4.215$ ,  $P = 0.06$ ).

## Behaviour

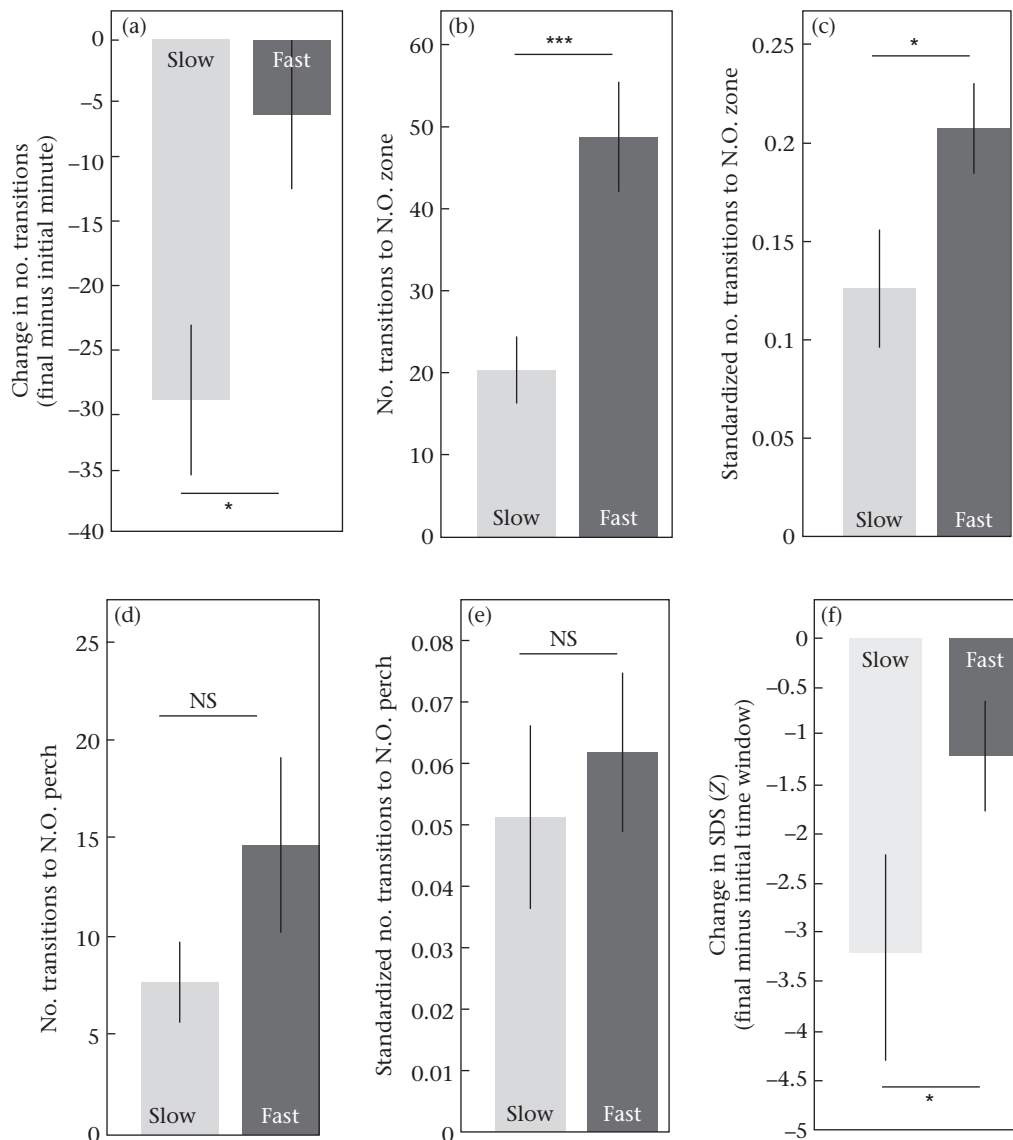
### Behaviour toward the novel object

The selection lines differed in the number of transitions into the novel object zone (mean  $\pm$  SEM: slow explorers:  $20.4 \pm 4.0$ ; fast explorers:  $48.8 \pm 6.7$ ;  $t_{33,08} = 3.62$ ,  $P = 0.001$ , corrected for unequal variances). This effect was still present after accounting for differences in total locomotor activity (mean  $\pm$  SEM: slow explorers:  $0.127 \pm 0.029$ ; fast explorers:  $0.210 \pm 0.023$ ;  $t_{36} = 2.18$ ,  $P = 0.036$ ; Fig. 3, Supplementary Table S3). Slow and fast explorers did not differ in the number of transitions to the novel object perch (mean  $\pm$  SEM: slow explorers:  $7.75 \pm 2.05$ ; fast explorers:  $14.72 \pm 4.46$ ;  $t_{36} = 1.26$ ,  $P = 0.21$ ), which was confirmed after standardization of the number of transitions to the novel object

perch for total activity (mean  $\pm$  SEM: slow explorers:  $0.05 \pm 0.015$ ; fast explorers:  $0.06 \pm 0.013$ ;  $t_{36} = 0.47$ ,  $P = 0.64$ ; Fig. 3, Supplementary Table S3). Three birds (two fast explorers) made occasional physical contact with the novel object by pecking at it. Although this might suggest that the novel object was perceived as an intruder, previous validation studies in this population have shown similar responses (including occasional directed pecking) towards other novel objects that are more neutral and do not have eyespots (e.g. penlight battery; see Verbeek, 1998; Verbeek et al., 1994), which is in stark contrast to their aggressive behaviour towards conspecific intruders (Verbeek et al., 1996).

### Locomotor analysis

The repeated measures ANOVA on the number of transitions (i.e. general locomotor activity) across the five sampled minutes demonstrated a main effect of time ( $F_{4,144} = 7.7$ ,  $P < 0.0001$ ), no main effect of selection line ( $F_{1,36} = 0.74$ ,  $P = 0.40$ ) and an interaction between time and selection line ( $F_{4,144} = 2.4$ ,  $P = 0.05$ ). This



**Figure 3.** Comparison between slow ( $N = 16$ ) and fast ( $N = 22$ ) explorer selection lines (mean  $\pm$  SEM) in (a) number of movements ('transitions') in the final minute compared to the initial minute of the novel object trial, (b) number of movements into the novel object (N.O.) zone, (c) number of movements into the novel object zone after correcting for (dividing by) the number of total movements for each bird, (d, e) number of movements onto the novel object perch before and after standardizing by total movements, respectively, and (f) change in sequential dependency scores (SDS;  $Z$ ) during the final 2 min compared to the initial 2 min. \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

interaction was driven by a 50% decrease in movement by slow explorers in Min9 compared to the other four time bins, whereas fast explorers exhibited only an 8% decrease (Supplementary Table S3), which represented a significant change for slow explorers (pairwise comparisons: all  $P < 0.05$ ) but not for fast explorers (all  $P > 0.1$ ). Likewise, fast explorers and slow explorers differed in the change in the number of transitions between the first and last minute ( $\Delta$  mean  $\pm$  SEM: slow explorers:  $-29.31 \pm 6.12$ ; fast explorers:  $-6.09 \pm 6.06$ ;  $t_{36} = 2.63$ ,  $P = 0.012$ ; Fig. 3, Supplementary Table S3).

#### Abnormal repetitive behaviour: triad analysis

The repeated measures ANOVA for the threshold criterion of two or more repeats demonstrated a main effect of time ( $F_{4,144} = 4.43$ ,  $P = 0.002$ ) but no main effect of selection line ( $F_{1,36} = 1.32$ ,  $P = 0.26$ ) and a trend for an interaction between time and selection line ( $F_{4,144} = 2.11$ ,  $P = 0.08$ ). The main effect of time was driven by a decrease in repeated triads towards the end of the trial: Min9 differed from the other four time bins (all  $P < 0.05$ ); when we parsed this by selection line, the effect was present for slow explorers (all  $P < 0.05$ ) but not for fast explorers (all  $P > 0.1$ ). Using a threshold of at least four repeats to define a repetitive sequence, the pattern of results was qualitatively repeated, and with a threshold criterion of at least eight repeats, the results were similar with the exception that the decrement in triads in the final bin was no longer significant (all  $P > 0.05$ ; sphericity assumption was violated for  $\geq 8$  repeats).

#### Abnormal repetitive behaviour: sequential dependency

The Markov chain analysis of sequential dependencies showed that 92.1% of birds had sequences that were significantly repetitive when all five time bins were included (SDS mean: 7.73; range 0.28–18.44). The selection lines neither differed in their average SDS (mean  $\pm$  SD: slow explorers:  $7.8 \pm 4.4$ ; fast explorers:  $7.7 \pm 4.8$ ;  $t_{36} = 0.11$ ,  $P = 0.91$ ) nor in whether they were significantly repetitive (binary: Fisher's exact test:  $P = 0.999$ ). In the initial time window (Min1 and Min3), there were 13 birds (35.1%; selection lines pooled) with significantly repetitive sequences, and in the final time window (Min7 and Min9), there were five birds (14.7%; selection lines pooled). Both selection lines experienced a significant decrease in SDS from the initial to the final time window (paired  $t$  test:  $t_{32} = 3.6$ ,  $P = 0.001$ ), with slow explorers exhibiting a larger decrement than fast explorers ( $t_{31} = 2.36$ ,  $P = 0.03$ ; Fig. 3, Supplementary Table S3).

#### Summary

We found the following five main results. (1) Birds from both selection lines exhibited a mild CORT elevation in response to the novel object. This effect was seen within individuals (i.e. from CORT-0 to CORT-10) and among individuals in comparison with control birds that had not been tested with a novel object (at CORT-10) (Fig. 2). (2) This elevation (at CORT-30) was not higher in slow explorers, as predicted. (3) Fast explorers showed a stronger response to a subsequent restraint stressor (CORT-30) following novel object exposure (Fig. 2). (4) Responses to novelty (CORT-10 in the experimental treatment) were correlated with CORT responses to restraint (CORT-30) exclusively in the slow explorers (Supplementary Fig. S1). (5) As predicted, fast explorers approached the novel objects more frequently. The selection lines did not differ in general locomotor activity or abnormal repetitive behaviours, but slow explorers showed a decrease in movement during novel object exposure (Fig. 3, Supplementary Table S3).

## DISCUSSION

We demonstrated that exposure to a novel object induces a moderate elevation in plasma CORT concentrations. Although this hypothesis has been tested previously (Apfelbeck & Raess, 2008; Galhardo, Vitorino, & Oliveira, 2012; Lendvai, Bókonyi, & Chastel, 2011; Mettke-Hofmann, Rowe, Hayden, & Canoine, 2006; Nephew, Kahn, & Romero, 2003; Richard et al., 2008), the mixed findings to date likely reflect important differences in study design. Critically, in the present study we measured both baseline CORT and CORT levels in response to a control manipulation, one or both of which are often missing in earlier studies (but see: Cavigelli & McClintock, 2003). In doing so, we found support for a key aspect of the hypothesis that emotional-behavioural reactivity to novelty has an underlying HPA axis component. Although this does not necessarily indicate that novel objects are experienced as noxious, it suggests that they are appraised as unpredictable as the HPA axis enters a preparative stage (Faustino, Oliveira, & Oliveira, 2015; Koolhaas et al., 2011; Sapolsky, Romero, & Munck, 2000). Consistent with this idea, the increases in CORT in response to the novel object found in this study were much lower than the elevations observed in *P. major* after 10 min of a potent stressor such as handling-restraint found in a previous study ( $\Delta$  mean  $\pm$  SD =  $+1.8 \pm 4.02$  ng/ml; ca. 36% increase above baseline, handling stress: ca. +15 ng/ml, ca. 200% increase above baseline; Cockrem & Silverin, 2002b). This moderate CORT increase, which is within the baseline range, was similar between the selection lines, indicating that these divergent personalities do not differ in their immediate HPA reactivity to novelty. It is unknown what, if any, behavioural effects might arise from such a moderate increase because few studies have experimentally and acutely elevated circulating CORT using noninvasive methods, thus permitting the simultaneous measurement of behavioural changes. An experimental study by Breuner et al. (1998), however, in which the authors elevated circulating CORT in adult white crowned sparrows, *Zonotrichia leucophrys gambelii*, using spiked mealworms, demonstrated that an approximately 100% increase in circulating CORT (ca. 15 ng/ml) resulted in significant increases in locomotor activity (perch hopping). We also know that small but chronic elevations in baseline CORT can influence avian behaviour in ways that impact fitness (Ouyang, Muturi, Quetting, & Hau, 2013). Future studies should employ noninvasive glucocorticoid manipulations to test the dynamic range of CORT changes relevant for behaviour, including experimental tests of personality–CORT relationships.

Furthermore, the selection lines did not differ in their baseline CORT concentrations; this is important because baseline CORT is often positively correlated with stress-induced CORT (Baugh et al., 2014), and therefore baseline differences could otherwise have complicated the interpretation of differences in stress-induced concentrations. We did find, however, that fast explorers experienced a stronger CORT elevation in response to a restraint stressor compared to slow explorers when the restraint stressor followed exposure to the novel object (i.e. not in the control condition). This finding appears to be in contrast with an earlier study that demonstrated that slow explorers exhibit a stronger stress response to 30 min of restraint (Baugh et al., 2012). However, these two studies, besides being conducted in different years and on different individuals, also differ in a few methodological ways. First, in the earlier study, restraint was not preceded by exposure to an experimental stimulus (perch change at 0 min). Moreover, the timeline and number of bleeds differ between the two studies. Namely, in the present study the CORT-30 time point was preceded by only one handling/restraint bleed (the CORT-10 time point), whereas birds in the Baugh et al. (2012) study were bled at two time points prior to the CORT-30 bleed (CORT-0 and CORT-15).

Comparing the stress-induced CORT concentrations while controlling for the number of preceding acute stressors (i.e. comparing CORT-30 in the present study to CORT-15 in the 2012 study), yields a similar pattern both in absolute concentrations (ca. 25 ng/ml at both the CORT-30 and the CORT-15 time points) and in the lack of a difference between the selection lines.

The relevant question to ask here is: why do fast explorers exhibit a stronger CORT response to a restraint stressor following exposure to a novel object compared to slow explorers? It is possible that fast explorers, which approached the novel object zone more frequently and exhibited higher locomotor activity at the end of the 10 min novel object trial, were more stimulated than slow explorers and this heightened arousal translated into increased HPA activity at the end of this cumulative 40 min period. However, we did not uncover any correlation between CORT levels and our locomotor measures, despite the fact that experimental elevations of CORT are known to increase general locomotor activity in songbirds (Breuner et al., 1998), and natural CORT variation correlates with risk-taking behaviour in fish (Martins et al., 2011). Moreover, there was no difference in CORT-30 concentrations between the control and experimental treatments. This suggests that other mechanisms (e.g. sympathetic responses) underlie the behaviour observed between selection lines in the present study (but see Fischer, Franco, & Romero, 2016; Nephew et al., 2003). Lastly, we observed that in both treatments, collecting blood from the slow explorers occasionally required more time (ca. 30 s; see Results). This is potentially relevant because slow explorers are known to initiate a stress response faster than fast explorers (Baugh et al., 2013; Baugh, Davidson et al., 2017) and longer handling times might result in artificially elevated baseline estimates. In the present study, we did not find differences between selection lines or treatments in baseline CORT concentrations or any correlation with handling time, but handling time remains an important consideration in studies of HPA reactivity (Small et al., 2017).

Our basic prediction that fast explorers would exhibit less neophobia, as measured by more frequent transitions towards the novel object, were supported and are consistent with other methods of estimating neophobia in this species (Verbeek et al., 1994). Likewise, the finding that the selection lines did not differ in general locomotor activity (total transitions) is not surprising given that previous studies with hand-reared great tits have shown that locomotor activity in the absence of a novel object does not differ between selection lines (Verbeek, 1998; Verbeek et al., 1994). In contrast, our prediction that fast explorers would exhibit more repetitive behaviour was not supported. This might suggest that these divergent personalities express similar sensitivities to captivity stress given that abnormal repetitive behaviours are commonly linked with captive living (Mason, 1991; Schumann, Günther, Jewgenow, & Trillmich, 2014). Selection lines did differ, however, in the timeline of activity, with slow explorers exhibiting a decrease in the frequency of transitions and repetitive sequences during the final sampling time point during the novel object trial, perhaps indicating differential habituation between the selection lines (Martin-Iverson, Pisa, Chan, & Fibiger, 1982). One possible explanation might be that slow explorers more quickly assess the novel object as nonthreatening. Hence, in both the hormone and behaviour data sets, we observed that the selection lines diverged from each other over time. This finding provides some support for the hypothesis, tested across several taxa, that personality types often differ in their flexibility (Dingemanse, Kazem, Réale, & Wright, 2010; Faustino et al., 2015). Slow personalities have been shown to be more responsive to changes in the environment and adjust their behaviour to a greater extent (Dingemanse et al., 2004; Exnerova, Svadova, Fucikova, Drent, & Stys, 2009; Guillelte, Reddon, Hoeschele, & Sturdy, 2010). This difference in flexibility

is often interpreted as evidence of adaptive strategies in response to environmental heterogeneity (Mathot, Wright, Kempnaers, & Dingemanse, 2012). And recent work suggests that the timeline of habituation to novelty, termed the 'temporal activity pattern' (Montiglio, Garant, Thomas, & Réale, 2010), might be an important personality component linked with stress coping styles (Carere et al., 2005; Koolhaas et al., 1999). Here we show that flexibility may not be restricted to behavioural traits, and that plasticity in endocrine systems might be relevant to personality variation (Baugh et al., 2013; Baugh, Davidson et al., 2017). Although selection lines did not differ in CORT reactivity to novelty, the CORT-30 levels were tightly linked with CORT-10 in slow explorers but not in fast explorers. This coupling suggests that slow explorers exhibit greater state dependence in their HPA reactivity; i.e. slow explorers with low initial CORT concentrations exhibited low subsequent CORT concentrations and vice versa, whereas fast explorers show no such temporal coupling. Interestingly, it is also the slower explorers in this species that exhibit a more rapid and enduring HPA response to restraint stress (Baugh et al., 2013; Baugh, Davidson et al., 2017). Together, these observations suggest that an initial stressor has both greater immediate valence (resulting in a more rapid CORT onset) and synergizes to a greater extent with the animal's current state (resulting in correlations across time points) in slow-exploring personalities. Alternatively, because this correlation was present only in the experimental condition, it is possible that a psychological stressor induces a tighter coupling in the stress response. We think personality-dependent constraints on endocrine flexibility and their underlying mechanisms should be tested in future studies.

Avian studies of endocrine stress responses typically use a standardized restraint stressor (but see Canoine et al., 2002; Cockrem & Silverin, 2002a; Jones et al., 2016). In addition to physical restraint stress, in the present study we aimed to measure behavioural and endocrine responses to a psychological stimulus that the animals had an opportunity to cope with behaviourally. This is important because typical restraint protocols depart from naturalistic stressors: they are physical and highly potent stressors that do not permit animals to employ the behavioural coping mechanisms that likely represent the initial response to many challenges. A notable exception is a recent study by Jones et al. (2016) in which the authors demonstrated that starlings (*Sturnus vulgaris*) that witness a very brief live raptor attack experience a CORT response equivalent in magnitude to the conventional capture-handling-restraint stressor; interestingly, although the mean CORT responses were equal for these two stressors, there was considerably greater variance in the raptor witness treatment, suggesting perhaps that naturalistic stressors better reveal the scope of individual differences in HPA reactivity.

We occasionally get a glimpse of how animals respond to repeated stressors (Cyr & Romero, 2009; Dickens & Romero, 2013; Hau et al., 2015; Lynn, Prince, & Phillips, 2009; Nephew et al., 2003; Taff & Vitousek, 2016), and examining that question at the level of the individual has demonstrated that sequential measurements of CORT concentrations in the same bird over a short period are often correlated at the within-individual level (Baugh et al., 2014; Dingemanse, Dochtermann, & Nakagawa, 2012). In other words, in a particular instance, an individual's maximum CORT level in response to a stressor can often be predicted based on its early stress response or its baseline level. In the present study, this phenotypic correlation was present only in the experimental treatment of slow explorers: CORT concentrations following exposure to the novel object were positively correlated with levels following the 30 min of subsequent restraint stress. Given the scarcity of studies quantifying endocrine reaction norms and their stability over time, the extent to which there are heritable or



developmentally induced differences in hormonal flexibility is presently unknown, as well as what, if any, relationship these profiles have to personality (Hau et al., 2016). Our findings suggest that it will be important in future studies to examine the interactions between HPA plasticity and personality to consider testing multiple ecologically relevant contexts and timelines (Dingemanse et al., 2010; Lendvai, Giraudeau, Bókony, Angelier, & Chastel, 2015).

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## Supplementary material

Supplementary material associated with this article is available, in the online version, at <http://dx.doi.org/10.1016/j.anbehav.2017.06.028>.

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