

## Selection for fast and slow exploration affects baseline and stress-induced corticosterone excretion in Great tit nestlings, *Parus major*

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### ABSTRACT

In nestlings, glucocorticoid (GC) secretion has short-term and long-term fitness consequences. For example, short-time elevations trigger begging activity, whereas chronically elevated GC levels impair body condition, growth and cognitive abilities. Despite a growing body of literature on personality traits, the effects of selection for fast and slow exploration on GC secretion have received little attention. We compared baseline and stress-induced hypothalamic–pituitary–adrenal (HPA) axis activity of hand-reared great tit nestlings of lines selected for fast and slow exploration. Nestling droppings were collected under three conditions: control, test (following handling stress, day 14 after hatching) and the following day. The concentrations of excreted immunoreactive corticosterone metabolites (CM) were determined via an enzyme immunoassay. We also observed nestlings' begging behaviour. CM differed significantly between the lines. Nestlings of the fast line excreted lower CM than slow-line birds. In response to handling stress, nestlings excreted significantly higher concentrations of CM than during the control and on the day after handling. Sex and begging activity were not related to CM levels. Under the control condition, but not after handling, males begged significantly more often than females. In both lines, adults excreted significantly less CM compared to nestlings. Both nestlings and adults of the slow line produced higher baseline CM values than fast-line birds. Fast-line nestlings excreted lower baseline CM than nestlings of a wild population not selected for fast or slow exploration. Slow-line nestlings did not. Our results show that selection on the basis of exploratory behaviour affected HPA axis reactivity.

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### Introduction

Individuals differ in suites of correlated behavioural and physiological characteristics (“personality”, “behavioural syndrome”, “coping style”) leading to a cross-context consistency in the way they deal with challenges (Verbeek et al., 1996, Gosling and John, 1999, Sih et al., 2004, Kralj-Fišer et al., 2007). Behavioural phenotypes are genetically and epigenetically heritable (Dingemanse et al., 2002, Drent et al., 2003, Daisley et al., 2004, van Oers et al., 2004a, Evans et al., 2006).

In great tits, *Parus major*, exploratory behaviour is related to aggressiveness (Verbeek et al., 1996, 1999), risk taking (van Oers et al., 2004b, 2005) and the use of social information (Marchetti and Drent, 2000). Moreover, it affects dispersal (Dingemanse et al., 2003), dominance (Verbeek et al., 1999, Dingemanse and de Goede, 2004), coping with defeat (Carere et al., 2001, 2003), mate choice (Groothuis and Carere, 2005), reproductive success (Both et al., 2005, van Oers

et al., 2008) and survival (Dingemanse et al., 2004). These patterns have been observed in wild great tits as well as in great tits selected for fast and slow exploration (Drent et al., 2003). It is probable that selection on the basis of exploratory behaviour could be related to glucocorticoid (GC) secretion patterns but surprisingly little attention has been paid to the hypothalamic–pituitary–adrenal (HPA) axis reactivity of the birds of these selection lines. Carere et al. (2003) observed that only slow male great tits excreted higher concentrations of immunoreactive corticosterone metabolites (CM) after a social defeat. In a study assessing the effects of social context on hormonal stress-response patterns, females were subjected to handling stress and returned to their home cages either with or without their mate. Females of both lines significantly increased CM excretion in response to handling. Fast females excreted significantly lower CM when with their mate than when they were alone after the stressful event. Slow females showed a trend towards the same result. In addition, slow females sat close to their mate for significantly longer after the handling stress than under the control condition (mates together, no handling stress, Stöwe et al., 2009).

Glucocorticoid secretion during early development has wide-ranging consequences on fitness. In altricial nestlings, short-term GC

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elevation may be associated with begging activity (Kitaysky et al., 2001, Quillfeldt et al., 2006) and increased GC secretion at the end of the nestling phase seems to promote fledging (e.g. Heath, 1997, Sprague and Breuner, 2010). Chronically elevated GC levels may have detrimental consequences on growth, immune defence and body condition (e.g. Sapolsky, 2002, Korte et al., 2005) and impair cognitive abilities (Kitaysky et al., 2003). Body condition at the end of the nestling phase affects survival, dispersal and recruitment and may influence reproductive success during the first breeding (e.g. Tilgar et al., 2009). These fitness consequences not only influence the individual itself but may also have trans-generational effects (Naguib et al., 2006).

HPA axis reactivity shows ample inter- and intra-individual variation. In nestlings, inter-individual and intra-individual differences in both baseline and stress-induced GC secretion are modulated by factors such as genetic background (Evans et al., 2006), maternal effects (Love et al., 2003a, Hayward and Wingfield, 2004, Groothuis et al., 2005, Naguib et al., 2006), body condition (Sockman and Schwabl, 2001, Brewer et al., 2008, Lendvai et al., 2009), age (Starck and Ricklefs, 1998, Schwabl, 1999, Blas et al., 2006 and references therein) and social interactions (aggressive e.g.: Tarlow et al., 2001, Fraud et al., 2008; socio-positive: Stöwe et al., 2008).

Despite the long-term fitness consequences of GC values during early development, up to date no attention has been paid to the hormonal stress-response patterns of nestlings of the lines selected for fast and slow exploration. We report here an investigation of how selection for fast and slow exploratory behaviour affects HPA axis reactivity in great tit nestlings. We compared the begging behaviour and CM excretion of fast- and slow-line nestlings in response to handling stress. Birds were tested shortly before fledging, at a developmental stage in which HPA axis reactivity leads to measurable GC secretion (Schwabl, 1999, Sims and Holberton, 2000, Wada et al., 2007, Rensel et al., 2010). We also compared nestling baseline CM excretion to that of adults of the same selection lines and to the one of nestlings of a wild population, not selected for fast and slow exploratory behaviour.

## Methods

### *Animals and housing*

The Great Tit is a territorial, non-migratory passerine. Its clutch size ranges from 5–12 eggs and the young fledge at 16–23 days of age and continue to be fed by their parents for another 2–3 weeks (Perrins, 1965, Naef-Daenzer et al., 2001).

In May 2007 we tested great tit nestlings of the fifth generation of lines bidirectionally selected for fast and slow exploration (Drent et al., 2003). The eggs of selection line pairs were exchanged with those of wild great tit pairs breeding in nest boxes in the area of Westerheide and Warnsborn near Arnhem in the Netherlands. Chicks were reared by wild great tit foster parents until day 10 after hatching, when they were transferred to the lab (at the NIOO-KNAW institute in Heteren) to be hand reared. It is quite difficult to hand-raise chicks directly from hatching but survival rates reach 100% when chicks are taken to the lab at the age of 10 days or more (Drent, unpublished data). Three to four birds were kept together in one nest (nests contained siblings and non-siblings or non-siblings only, all of similar age per nest). Except for one nest (with one fast-line and two slow-line birds), all nests contained either slow-line or fast-line nestlings. Birds were fed every 30 min from 7 a.m. to 9 p.m. Food consisted of a beef heart/water mixture supplemented with eggs, cereals and insects (see also Drent et al., 2003).

### *Data collection*

We began the experiment (control day) when nestlings were 13 days old. Thus, nestlings could accustom themselves to the rearing

conditions in the lab for at least 2.5 days before the onset of the experiment. In total, 33 fast (16 males, 17 females) and 16 slow (4 males, 12 females) nestlings were observed, distributed among 15 nests. We monitored CM excretion under three experimental conditions: the day before the handling stress (control), directly after handling stress (test) and the day after the stress (day after). On the control and test days the experimenter (M.S.) additionally observed the nestlings' behaviour, noting whether individuals begged during feedings. We collected every dropping during feedings in the time span from 2.15 p.m. to 6.00 p.m. On the day of the handling stress, test chicks were taken out of their nest and put into a cotton bag. One individual after the other was kept in the hand for 1 min and then put in a wooden box compartment visually and physically isolated from its nest mates. After 15 min of isolation, nestlings were handled again for 1 min and thereafter returned to the nest (data and further details of the procedure are presented in Fucikova et al., 2009). The isolation box (32 × 12.5 × 6 cm) was divided into 12 compartments (ca. 6 × 6 × 6 cm each) and had a wooden cover with holes. We started collecting droppings at the feeding immediately after the handling stress. Fast-line nestlings excreted the maximum concentration of CM 2 h 46 min (median) after the handling stress, slow-line birds after 2 h 44 min. Droppings (number of droppings per nestling per experimental condition: mean ± SD = 2.54 ± 1.0) were frozen within 15 min of collection and stored at –20 °C until analysis. After fledging, birds were sexed by means of molecular markers (Griffiths et al., 1998). We commenced noting whether nestlings begged during the control and the test condition after we had observed that the handling stress seemed to affect begging behaviour during subsequent feedings. Therefore, data on begging activity under the control condition are absent for the first nine nestlings tested and we have excluded these birds from the analysis of begging data. Around day 16 after hatching, nestlings started to jump out of their nests and accumulated in other nests in the incubator. Some of them already left their own nest on the day after the test (24.2% of the fast line, an equal number of males and females, 13.3% of slow-line nestlings, all females, the difference between lines was not significant: Chi<sup>2</sup> test:  $n_{\text{fast}} = 33$ ,  $n_{\text{slow}} = 16$ ,  $df = 1$ ,  $\chi^2 = 0.52$ ,  $p > 0.3$ ). In such cases we stopped collecting droppings, as the higher mobility together with new experiences (gathering with unfamiliar nestlings, altered competition conditions during the feedings) could have affected GC secretion. We thus collected data only from the nestlings that remained in their original nest until the end of the experiment, which led to a reduced number of individuals on the day after the test.

Adult great tits of the same selection line were housed in pairs at the same institute as the nestlings (adult data: Stöwe et al., 2009). Droppings from the adults were collected from May to July 2007. In the absence of data on stress-induced CM levels of adult males we were only able to compare baseline CM data between nestlings and adults.

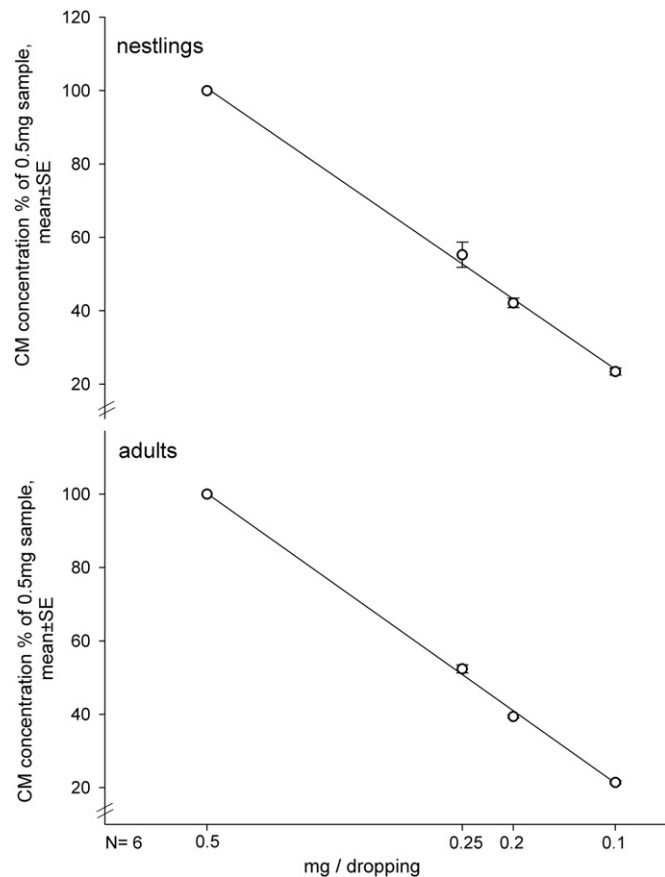
During the breeding seasons in 2008 and 2009 we collected droppings from great tit nestlings of nestbox populations in the Viennese woods (in collaboration with Hans Winkler of the Konrad Lorenz Institute for Ethology, Austrian Academy of Sciences). These nestlings were raised by their parents and ringed at the age of 11–12 days, when droppings were collected (Stöwe et al., in preparation). We compared CM values of the hand-raised nestlings of the selection lines to those of the nestlings raised by their parents in the Viennese woods.

All studies adhered to the NIH standards (DHEW publication 80–23, revised 1985) and to the relevant laws of the Netherlands and Austria.

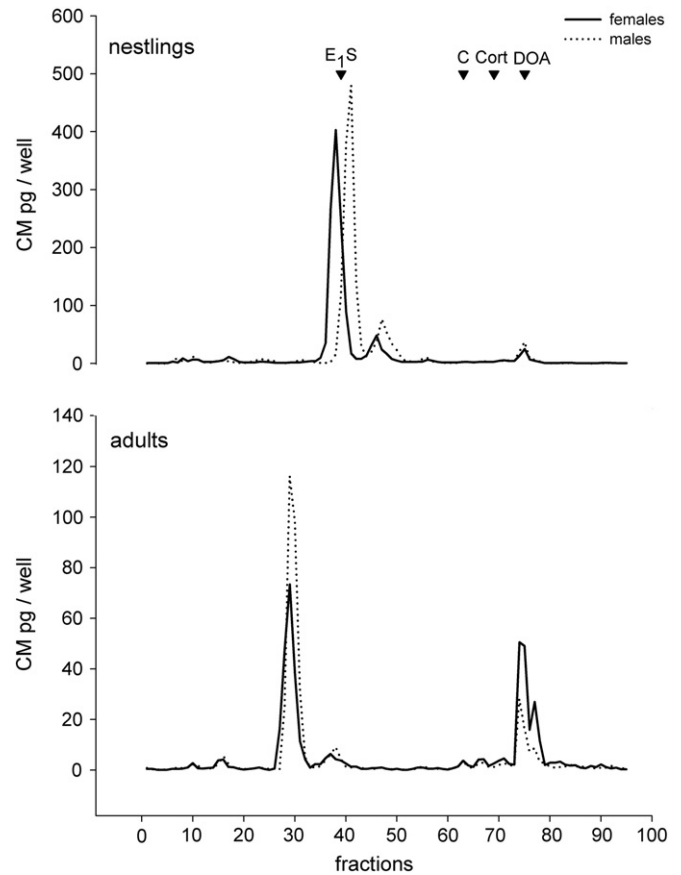
### *Determining the concentrations of excreted immunoreactive corticosterone metabolites*

Steroid analysis was undertaken according to the protocol described in Stöwe et al. (2008) with slight modifications. Wet

droppings (0.05 g) were shaken in a mixture of methanol (0.3 ml) and distilled water (0.2 ml); 0.1 ml of this extract was evaporated and the sediment dissolved in 0.1 ml Na-acetate buffer and 0.2  $\mu$ l  $\beta$ -glucuronidase-arylsulfatase (Merck 1.04114.0002) and hydrolyzed at 38 °C for 18 h. The 11-oxoetiocholanolone enzyme immunoassay used has been validated for adult great tit droppings (Carere et al., 2003). It measures GC metabolites with a 5 $\beta$ -3 $\alpha$ -ol-11one structure (detailed description in Möstl et al., 2002, Ganswindt et al., 2003). Because challenging nestlings with ACTH when they are only 10–13 days of age could have negative short and long-term consequences, we tested the applicability of the assay for nestling dropping in a different way. Before sample analysis we made dilution curves of 6 adult and 6 nestling samples (Fig. 1). In addition, we separated sample pools of male and female nestlings and adults via reversed-phase high-performance liquid chromatography (HPLC, linear water/methanol gradient 20%–100%) to compare the patterns of excreted metabolites. We measured the metabolite contents of each individual fraction by means of the assay used by Carere et al. (2003). The metabolite pattern of adults differed from that of nestlings but it should be noted that the assay reacts to metabolites that eluted at fractions 27–30 (adults) and to those of the nestlings that eluted at fractions 36–43 (Fig. 2). The second peak (fractions 73–77) is in the same fractions in droppings from nestlings and adults. Thus the same assay may be used for adults and nestlings. The sensitivity of the assay is 3 pg/well (Ganswindt et al., 2003), which in our case correlates to 16.5 ng/g dropping. Measured CM values ranged from 72 ng/g dropping (fast line, control) to 2057 ng/g dropping (slow line, day after).



**Fig. 1.** Dilution curve of nestling and adult samples. We did not use a pool sample but six different samples for each age class. For comparison we therefore expressed the measured concentrations of excreted immunoreactive corticosterone metabolites (CM) as percentage of the 0.5 mg sample (y-axis). Dilution concentrations are indicated on the x-axis (mg/dropping). Circles present mean and standard error values. We also show the linear regression line.



**Fig. 2.** Immunograms of corticosterone metabolite excretion patterns of great tit nestlings and adults. The dotted lines show the excretion patterns of males, the continuous lines the ones of the females. The elution pattern of the following steroids are indicated on top: E<sub>1</sub>S: oestrone sulphate as a marker for the polarity, the assay does not measure E<sub>1</sub>S, thus the metabolite we measured in this position is not E<sub>1</sub>S, C: cortisol, DOA: 11,17-dioxoandrostanone.

All samples (of adults and juveniles) were analysed together in the same lab and extraction and CM quantification followed the same protocol. Droppings of nestlings in the Viennese woods were treated in the same way. Samples were assayed in duplicate. Overall intra-assay variation was 10.0%, and inter-assay variations were 10.4% for the low-level pool and 6.1% for the high-level pool.

#### Data analysis

The CM excretion data were analysed by general linear mixed models (GLMM, SAS 8.2). Mean CM values per individual were calculated under each set of experimental conditions. The resulting mean CM values were log<sub>10</sub> transformed to satisfy the criteria of normal distribution. A repeated measure approach was employed, in which the individuals were the subjects and the experimental conditions the repeated measure factor. Nest was a random factor in the analysis. The initial model also included line and sex as fixed factors and the line  $\times$  experimental condition and sex  $\times$  experimental condition interactions. Residuals of the models were normally distributed. The Satterthwaite approximation of the degrees of freedom was applied and a backward stepwise model selection, first removing the least significant interaction and then the least significant main effect if they were not part of a significant interaction. The non-significant results presented are p-values immediately before the variable was removed from the model (Table 1).

To determine the effects of sex, line and CM on begging activity, generalized linear mixed models (GLIMMIX macro in SAS 8.2.) were used with binomial error and logit link. Begging activity was defined

**Table 1**

Results of general linear mixed models testing the effects of line and sex on CM excretion under the experimental conditions: control, test, day after. The interaction which was dropped first during backward model selection is at the top. Main effects retained in the final model are indicated in bold. Non-significant results presented are the *p*-values just before the interactions/main effects were omitted from the model.

Variable	F	df	<i>p</i>
Sex × condition	0.08	2,111	0.92
Line × condition	2.48	2,114	0.09
Sex	0.45	1,123	0.50
<b>Line</b>	<b>6.65</b>	<b>1,21.8</b>	<b>0.02</b>
<b>Condition</b>	<b>12.87</b>	<b>2,116</b>	<b>&lt;0.001</b>

as the proportion of feeding events during which nestlings begged. Data for the control and the test condition were analysed separately and sex, line, standardized CM (see below) and the two-way interactions of these variables were included as fixed factors. Nest was included as a random factor. As CM levels differed between lines (see results), CM was standardized by group (same sex, same line, same test condition) to avoid the problem of colinearity, i.e. the difference between the individual's CM level and the group mean was divided by the group mean.

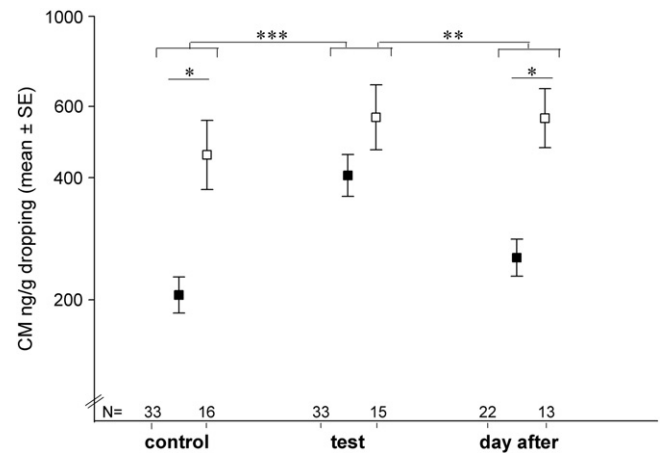
Nestling mean baseline CM were compared to those of adults by means of linear mixed models (GLMM, SAS 8.2.) using nest/cage as random factor. The initial model included age, line and sex as fixed factors as well as the interactions of these factors. Again, a backward stepwise model selection was applied.

We compared CM values of the hand-reared slow- and fast-line nestlings to those of parent-reared nestlings using a Kruskal–Wallis and Mann–Whitney *U* test (SPSS 17.0.3, PASW Statistics) to see whether nestlings reared by parents had lower control levels than the hand-reared, slow-line nestlings. To test for line differences in fledging date, we used a Chi<sup>2</sup> test calculated manually according to Siegel and Castellan (1988).

## Results

### Hormonal stress response

Concentrations of excreted immunoreactive corticosterone metabolites (CM) differed significantly among experimental conditions and between selection lines. Nestlings of the fast line excreted lower CM than birds of the slow line (Table 1, Fig. 3). The sex of the nestlings was not related to CM levels (Table 1). The selection line × experimental condition interaction tended to influence CM levels (Table 1) with fast-line nestlings showing a steeper increase in CM excretion under the test condition (CM concentrations in test-day droppings were 261 ± 222% of those on the control day) and a sharper decline thereafter (day after 65 ± 48% of test-day CM) than slow-line nestlings (159 ± 97% as opposed to 105 ± 61%, Fig. 3). Taking all nestlings together in a pair-wise comparison of experimental conditions, when controlled for line, birds excreted significantly lower levels of CM under the control condition and the day after handling than under the test condition (control–test: *df* = 1,80.3, *F* = 28.57, *p* < 0.0001; test–day after: *df* = 1,70.6, *F* = 9.70, *p* = 0.003). They tended to have higher CM levels on the day after than on the control day (*df* = 1,77.7, *F* = 3.09, *p* = 0.08). Calculating the post hoc test for each line separately, fast-line nestlings excreted significantly more CM under the test condition than under the control condition and on the day after handling (control–test: *df* = 1,54.9, *F* = 30.76, *p* < 0.0001; test–day after: *df* = 1,46.1, *F* = 14.83, *p* = 0.0004) and tended to have higher CM levels on the day after than on the control day (*df* = 1,48.2, *F* = 3.24, *p* = 0.08). In contrast, the CM secretion of slow-line nestlings did not differ significantly under different experimental conditions (control–test: *df* = 1,24.1, *F* = 2.23,

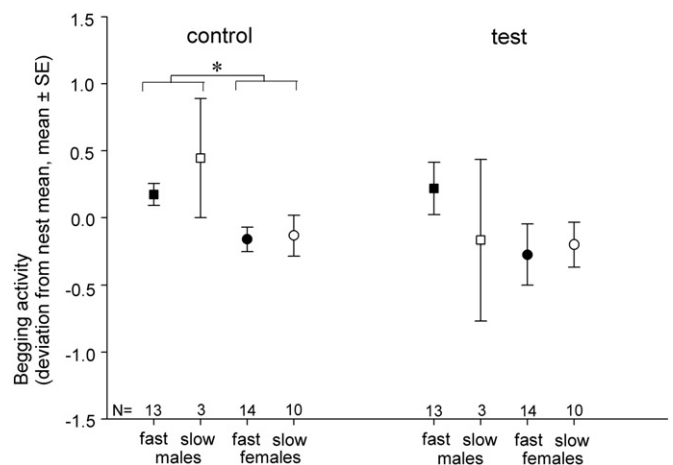


**Fig. 3.** Concentrations of excreted immunoreactive corticosterone metabolites (CM) of selection line nestlings under three test conditions. Filled squares stand for fast-line nestlings, open squares for slow-line nestlings. Squares represent mean CM and bars standard error values. Asterisks indicate significant between line and among condition differences, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. The y-axis is log<sub>10</sub> scaled. *N* varies between conditions, because it was impossible to get droppings of each individual in all three test conditions (see Methods, *N*<sub>max</sub>: fast line = 33, slow line = 16).

*p* = 0.15; test–day after: *df* = 1,22.6, *F* = 0.01, *p* = 0.92; control–day after: *df* = 1,23.2, *F* = 0.50, *p* = 0.49). The sex × experimental condition interaction was not significant (Table 1).

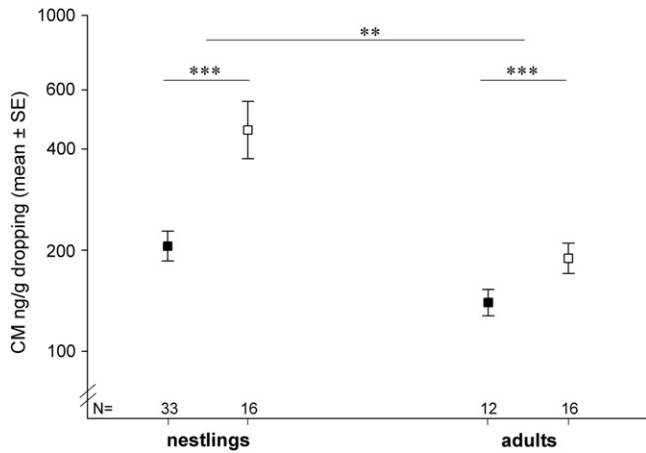
### Behaviour

There was no difference between the lines in begging activity, either under the control condition (*df* = 1,9.14, *F* = 1.73, *p* = 0.22) or on the test day (*df* = 1,17.4, *F* = 0.2, *p* = 0.66). On control days males begged significantly more often than females but on test days this difference disappeared (control: *df* = 1,29.1, *F* = 5.92, *p* = 0.02; test: *df* = 1,30.5, *F* = 2.06, *p* = 0.16, Fig. 4). On control days the sex × line interaction was not significant, whereas on test days it showed a slight tendency (control: *df* = 1,27.9, *F* = 1.41, *p* = 0.25; test: *df* = 1,33.1, *F* = 3.57, *p* = 0.07). CM excretion was not related to begging activity (control: *df* = 1,35, *F* = 0.06, *p* = 0.81; test: *df* = 1,35.8, *F* = 0.92, *p* = 0.35).



**Fig. 4.** Begging activity of male and female nestlings of both lines under the control and the test condition. Squares represent male nestlings, circles female nestlings. Filled symbols indicate fast-line birds, open symbols slow-line individuals. Symbols show mean begging activity (expressed as deviation from nest mean) and bars standard errors. The asterisk indicates significant difference \**p* < 0.05.





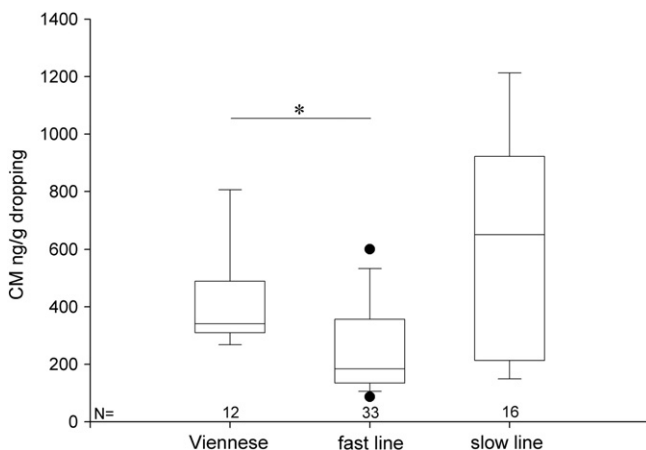
**Fig. 5.** Concentrations of excreted immunoreactive corticosterone metabolites (CM) of nestlings and adults of selection lines for fast and slow exploration. Filled squares stand for fast-line birds, open squares for slow-line individuals. Squares represent mean CM and bars standard error values. Asterisks show significant between line and between age class differences, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . The y-axis is log<sub>10</sub> scaled.

#### Comparison of nestlings and adults

In both age classes, nestlings and adults, fast-line birds excreted lower baseline CM compared to slow-line birds ( $df = 1,73$ ,  $F = 13.38$ ,  $p < 0.0005$ , Fig. 5). Nestlings had significantly higher baseline CM values than adults ( $df = 1,73$ ,  $F = 18.89$ ,  $p < 0.0001$ , Fig. 5). Sex did not significantly affect CM excretion ( $df = 1,72$ ,  $F = 0.02$ ,  $p = 0.88$ ). None of the interactions reached significance level (all  $p > 0.15$ ).

#### Hormone secretion in hand-reared and parent-reared nestlings

CM control values of parent-reared great tit nestlings from the Viennese woods did not differ significantly from the slow-line nestlings' CM values but were significantly higher than CM values of the fast-line birds ( $n_{\text{fast line}} = 33$ ,  $n_{\text{slow line}} = 16$ ,  $n_{\text{vienna}} = 12$ ; Kruskal–Wallis:  $\chi^2 = 16.22$ ,  $df = 2$ ,  $p < 0.001$ ; Mann–Whitney  $U$  test: fast-line – Viennese nestlings:  $z = -3.16$ ,  $p = 0.002$ , considering conservative Bonferroni correction ( $p < 0.017$ ) still significant, slow-line – Viennese nestlings  $z = -0.60$ ,  $p = 0.55$ , Fig. 6).



**Fig. 6.** Concentrations of excreted immunoreactive corticosterone metabolites (CM) of great tit nestlings of the selection lines and of parent-raised nestlings from the Viennese woods.  $N$  = number of birds, box plots show the median and the interquartile range from the 25th to the 75th percentile. Whiskers above and below the box indicate the 10th and the 90th percentiles. The asterisk marks the significant between group difference as determined by Kruskal–Wallis and Mann–Whitney  $U$  tests (\* $p < 0.05$ ).

## Discussion

We observed significant differences in CM excretion between the two selection lines. Nestlings of the slow line excreted higher concentrations of CM than fast-line birds. These results are the first evidence that selection on the basis of exploratory behaviour affects both baseline glucocorticoid concentrations and stress-induced HPA axis reactivity. Previous studies have suggested that reactive coping (slow line) is associated with higher GC baseline levels and a higher HPA axis reactivity (Koolhaas et al., 1999, 2007, Carere et al., 2003). The first finding is in accordance with our results (both slow-line nestlings and adults had higher baseline CM and nestlings also higher stress-induced CM) but they do not clarify the issue of which line has higher HPA axis reactivity, as fast-line birds showed a steeper increase in CM concentrations in response to handling stress and a steeper decline thereafter than did slow-line nestlings, while slow-line birds had higher baseline and stress-induced CM values. Because a notable number of nestlings left their nest starting from the day after the test, we do not know whether CM levels of slow nestlings would have subsequently declined, although a later decline would have interfered with an increase in GC secretion towards fledging (Heath, 1997, Schwabl, 1999, Corbel and Groscolas, 2008, Sprague and Breuner, 2010). This increase towards fledging also seems to explain why fast-line nestlings tended to excrete higher concentrations of CM on the day after the test compared to the control day. Three fast-line males remained sitting in their nest until three days after the stress test and increased their CM excretion steadily. This suggests that an increase in CM towards fledging, rather than prolonged effects of the handling stress, is the explanation for the difference in CM measured between the control and the day after condition.

Slow-line nestlings had significantly higher control CM levels and might therefore have not been able to increase GC secretion as could fast-line nestlings in response to the handling stress. It is possible that the difference in baseline CM levels between fast and slow nestlings is an artefact because, unlike fast-line nestlings, slow-line birds had not yet habituated to the conditions of captivity before the onset of the experiment. A further possibility is that being reared by hand was generally more stressful for slow-line birds. To assess these possibilities it would be necessary to compare the baseline CM levels of hand-reared slow-line nestlings to those of slow-line nestlings raised in the wild. Because we do not have data on parent-raised slow-line nestlings in the wild, we compared the CM baseline levels of hand-reared slow-line nestlings to those of wild, unselected nestlings from the Viennese woods. We did not find any difference in CM levels, suggesting that it is unlikely that the differences between slow-line and fast-line birds are artifactual. Without the comparison between fast-line birds and Viennese nestlings, we would argue that like in adults (see above) reactive coping is related to higher GC baseline levels from an early age on (nestlings) compared to the baseline levels in the proactive coping line. The fact that fast-line nestlings excreted significantly lower CM than the Viennese nestlings indicates that selection for exploratory behaviour has modulated GC secretion in fast-line birds, that have reduced baseline CM levels. It remains to be tested whether fast-line individuals show increased sympathetic activity as has been described for other proactive coping lines (e.g. Koolhaas et al., 1999). GC secretion could be a mechanism behind the expression of the trait, exploratory behaviour, that has been selected, or it might be related to another trait that has changed together with exploratory behaviour without representing the focus of the selection project. Further investigation will be required to distinguish between these possibilities.

Nestling baseline and stress-induced GC excretion seems to be an indicator of future fitness (survival, reproduction: Blas et al., 2007; early start of egg-laying: Wada et al., 2008). Thus, focussing on GC excretion only, one would predict that the reduced baseline and stress-induced CM excretion would provide fast-line birds with

fitness advantages over slow-line nestlings. However, feeding conditions and social environment (see Introduction, Stöwe et al., 2009) have an important modulating impact. For example, either assortatively or disassortatively mated pairs had more recruits in a breeding season depending on food availability in the preceding winter (Both et al., 2005). Hence, to determine the fitness consequences of GC secretion during early development it would be necessary to observe reproductive success, survival, etc., of individuals with high or low GC values during the nestling phase for each line separately rather than presuming that slow-line birds have fitness disadvantages due to their higher levels of CM as nestlings.

Under the control condition, males begged significantly more often than females did. This may reflect metabolic differences between the sexes and a correspondingly higher nutritional need of developing males (Clutton-Brock et al., 1985, Tschirren et al., 2003, Fletcher and Hamer, 2004 but see Oddie, 2000, Råberg et al., 2005), which gain mass faster than females (Rosivall et al., 2009) and accordingly may be provided with food by their parents differently than female siblings (Krebs et al., 1999, Dickens et al., 2008). On test days, begging activity was reduced and there were no longer any significant differences between the sexes. This could be because nests were taken out of the incubator immediately before feeding, but also directly before nestlings were subjected to the handling stress. During the feedings following the handling, many nestlings crouched down in the nest showing a kind of predator avoidance behaviour instead of begging, presumably afraid of being handled again. As nestlings were always fed ad libitum, also males could probably “afford” to feed less during some feedings following the handling stress and this may be why we did not observe sex differences in begging activity on test days.

According to a previous study, increased secretion of corticosterone is linked to augmented begging activity (Kitaysky et al., 2003). In our experiment, however, CM excretion did not relate to begging activity, neither under control nor under test conditions. Possibly due to ad libitum feeding, nutritional stress was damped and sibling competition reduced. Furthermore, our measurement of begging activity was only approximate: we recorded simply whether a nestling begged during a feeding. A more precise assessment of begging effort might conceivably have revealed a connection to CM excretion.

Comparing nestling baseline CM values to those of adults, fast- and slow-line nestlings were found to excrete significantly higher concentrations of CM than adults of the corresponding selection line. Both nestlings and adults of the fast line had lower CM values than slow-line birds. It is important to note that in a previous publication on adult great tits (Stöwe et al., 2009) the data were analysed using non-parametric statistics, which only detect robust patterns, and between-line differences in CM did not reach significance level. Using a more sensitive method for data analysis enabled differences in CM between fast- and slow-line adults to be detected.

The comparison of nestlings and adults should be treated with caution, as the metabolites excreted differ between age classes (Fig. 2). The assay cross-reacts with metabolites excreted by both nestlings and adults but the extent of cross-reactions of these substances might differ, potentially resulting in exaggerated differences in CM values. We used 11-oxoetiocholanolone as standard, so metabolite concentrations measured with our assay are expressed as 11-oxoetiocholanolone equivalents. We determined dilution curves and also examined the metabolite patterns more precisely. Judging from the dilution curves (Fig. 1), there are no differences between nestlings and adults. But comparing the immunograms, differences in metabolite patterns are visible. Our data clearly show that a validation via dilution curves may be insufficient. Moreover, our data imply the need to validate immunoassays for nestlings and adults of each species separately to enable stress-response patterns to be examined properly. Thus, in addition to species (Palme, 2005), season and kind and amount of food (Goymann et al., 2006), the age-class also has to be taken into account.

The pattern we observed in the present study, with altricial nestlings showing significantly higher CM levels than adults (to be seen with caution, as mentioned above), has been found in all previous studies that determined GC secretion via CM in droppings (nestling-subadults: Stöwe et al., 2008 ravens, *Corvus corax*, Stöwe unpubl. jackdaws, *Corvus monedula*, nestling-adult: Lobato et al., 2008 blue tits, *Cyanistes caeruleus* and pied flycatchers, *Ficedula hypoleuca*).

The finding that baseline CM was higher in nestlings than in adults is in contrast to predictions of the developmental hypothesis (Starck and Ricklefs, 1998). The nestlings studied were rather at the end of their nestling phase, so one would expect an adult-like stress response but not higher baseline CM levels than in adults. It is possible that differences in hormone values between nestling and adult seem more pronounced than they are because of differing patterns of excreted metabolites between age classes, potentially leading to different cross-reactions in the assay. Nevertheless, the values measured in nestlings were surprisingly high. It is still unclear how nestlings deal with such high levels of free corticosterone and whether reduced receptor density is used to mitigate the negative effects of elevated GC levels.

When GC secretion was determined by means of total GC in blood, nestlings of several species showed lower baseline and stress-induced GC levels than adults (e.g. Blas et al., 2006: white storks, *Ciconia ciconia*; Quillfeldt et al., 2009: thin-billed prions, *Pachyptila belcheri*; Sims and Holberton, 2000: northern mockingbirds, *Mimus polyglottos*; Rensel et al., 2010: Florida scrub-jays, *Aphelocoma coerulescens*; Tilgar et al., 2009: pied flycatchers). Other studies reported the opposite pattern (Love et al., 2003b: American kestrels, *Falco sparverius*; Wada et al., 2008: zebra finches, *Taeniopygia guttata*). Finally, Raouf et al. (2006) found similar baseline GC levels in nestling and adult cliff swallows, *Petrochelidon pyrrhonota*. Before speculating whether species-specific differences might lie behind these patterns, it would be important to carefully control for factors that influence GC secretion, such as differences in latencies from approach to the nest until blood sampling (Fridinger et al., 2007), the sequence of nestlings handled (Gil et al., 2008), nestling age (e.g. Sockman and Schwabl, 2001, Love et al., 2003b, Blas et al., 2006), hatching order (Love et al., 2003a), repeated sampling of the same individual (Dufty, 2008), brood size, food abundance and body condition (e.g. Kitaysky et al., 1999, Sockman and Schwabl 2001, Brewer et al., 2008, Jenni-Eiermann et al., 2008, Lendvai et al., 2009). Examining GC secretion via droppings, results are not affected by the time until capture, by handling sequence or by repeated sampling. In addition, apart from the difference in time scales (immediate GC response in blood, detection delayed in droppings), measuring CM in droppings provides different physiological information than measuring total GC in blood. The concentration of total corticosterone in blood is the sum of free corticosterone and corticosterone associated with corticosteroid binding globulin (CBG) or albumin, whereas concentrations of CM in droppings relate to free corticosterone (Haack et al., 1979, Palme et al., 2005). Only unbound corticosterone is thought to be involved in the stress-response cascade (the free hormone hypothesis, Rosner, 1990). Due to these methodological differences, comparisons between studies should be made with caution.

In conclusion, we have shown that selection on the basis of exploratory behaviour indeed affects both baseline and stress-induced HPA axis reactivity in nestlings. It is remarkable that selection for fast and slow exploration leads to significant behavioural and hormonal changes as early as the fifth generation.

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