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ORIGINAL ARTICLE

Long-term effects of repeated handling and bleeding in wild caught Great Tits *Parus major*

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Abstract Handling and bleeding are frequently used procedures in avian research and several studies show that they can exert short-term effects, such as elevation in corticosterone levels. However, the long-term effects of exposure to such manipulations are largely unknown, but could have important implications, especially for much of the long-term research on birds and experiments that involve longitudinal assessments. In this study, we evaluated the effect of handling and bleeding on some physiological and behavioural parameters. Hand-reared Great Tits *Parus major* originating from wild nests were used in two different experiments for other purposes. In these experiments, the birds were exposed to different frequencies of bleeding and handling events across a period of 45 days. The "high stress" group experienced a total of seven times handling and five times bleeding, while a "low stress" group was handled three times and bled only once. Thirty days after the experiments, when caught and handled from a cage, individuals of the high stress group were easier to catch, displayed significantly higher breath rates, and were more docile than individuals of the low stress group. No differences in body mass were detected. These results indicate that repeated manipulations cause evident long-term changes in coping with such procedures, which are likely due to learning effects, and provide empirical evidence that the past experimental history of an animal has to be taken into account in subsequent experiments.

Keywords Breath rate · Coping · Handling · Learning · Long-term stress

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Introduction

In many bird studies, individuals are routinely and repeatedly handled and bled for several kinds of investigations, such as ringing, morphometric measurements, assessment of peripheral hormone levels, metabolic and immunological markers, and molecular sexing. Handling and bleeding may trigger the glucocorticoid levels to rise, which is commonly used as an indication of short-term stress (e.g. Wingfield et al. 1992; LeMaho et al. 1992; Cockrem and Silverin 2002; Müller et al. 2006). In fact, standardised handling protocols have been widely used to assess the hypothalamic–pituitary–adrenal axis response. In most species, the rise in glucocorticoid occurs within 3 min following handling, with a return to baseline occurring within a few hours (Silverin 1998). High glucocorticoid levels are often also associated with a rise in body temperature (Cabanac and Aizawa 2000; Cabanac and Guillemette 2001), It is, however, usually assumed that bleeding and handling will have no long-term consequences (e.g. Hoysak and Weatherhead 1991).

The emotional and stress response are controlled, at least in part, by the autonomic nervous system. Therefore, cardiovascular parameters, such as tachycardia and breath rate, are well-established indices of the emotional and stress response (Koolhaas et al. 1997). Several studies

show that tachycardia and breath rate are reliable indicators of acute stress in birds. Birds, like other animals, react to life threatening situations with behavioural and physiological responses. Following handling, Eider Ducks *Somateria mollissima* display an elevated heart rate for 2–3 min without any visible motor response (Cabanac and Guillemette 2001). In Great Tits *Parus major*, breath rate has been measured to look at short-term stress (Carere et al. 2001; Carere and Van Oers 2004). When birds were caught during the activity phase (day time), breath rate was shown to be higher in a measurement immediately after capture, compared to a second measurement after birds had been kept in a bag for 5 min. When birds were caught during the inactive phase (night time), no difference between the first and second measurement was found. This suggests that during the active phase the first measurement represents a stress-induced rise while the decrease observed after 5 min represents the tendency to return to more normal levels (Carere et al. 2001).

In this paper we use breath rate to verify the long-term consequences of repeated handling and bleeding. For this purpose, we compare two groups of Great Tits that were part of two different experiments about a month prior to the study, thereby looking specifically for effects at a group, not an individual level. A "low stress" group was thereby handled twice and bled once, whereas the "high stress" group was handled six times and bled five times. One month after this repeated series of stressful events, the groups were compared in their breath rate response upon catching and handling. In addition, we measured body mass and two behavioural responses (the time to be caught from the home cage and the number of attempts to escape while handled; a measure of docility), which are known to be affected by long-term stress as a consequence of unpredictable events in rodents (Van Dijken et al. 1992) and by short-term handling stress in birds (Kitaysky et al. 1999; Cabanac and Aizawa 2000).

Methods

Subjects and housing

We carried out the experiments on a pool of 94 juvenile Great Tits in December 1998. Birds were collected from a wild population at the age of 10 days after hatching in May–June of the same year and hand reared under standard conditions until independence (for details, see Verbeek et al. 1994; Drent et al. 2003; Van Oers et al. 2004). From independence on day 25–30 after hatching, birds were housed individually in standard cages of 0.9 × 0.4 × 0.5 m with a wooden bottom, top, sides and rear walls, a wire-mesh front and three perches. Birds were kept under natural light conditions and had auditory and visual contact with other individuals. During the stress procedure, birds were housed in semi-open aviaries of 4 × 2 × 2 m with a wire-mesh front and top, six branches and six nest boxes. We fed the birds with a protein-rich mixture, and a commercial seed mixture, supplemented daily with mealworms (*Tenebrio molitor*) or sunflower seeds, while water was provided ad libitum. During the period between the stress procedure and the final testing, birds were housed again in individual cages as described above. Birds were sexed with molecular markers, according to the method of Griffiths (1998).

Stress procedure

For a time scheme of the stress procedure the birds were exposed to during the earlier experiments see Table 1. On day zero, all birds were caught from their individual home cage and blood samples of 10 µl were taken from the wing vein for sex determination. Directly after taking the samples they were put back in their home cage. Twenty-four hours later, all birds were caught from their home cage and housed in 22 uni-sex flocks of four and 1 flock of six individuals in aviaries. Each flock consisted of birds that received the same treatment.

Sixty birds from 15 aviaries (high stress group) were caught group-wise every 7 days and a 5-µl blood sample was taken for other purposes. After catching and bleeding, all birds of one aviary were released into their aviary simultaneously (about 30 min after initial capture). This procedure was carried out every 7 days on four occasions. Thirty-four birds from eight aviaries (low stress group; one flock consisted of six individuals) remained in their aviaries for 4 weeks without being caught or bled. The birds

Table 1 Period of the stress and the test procedure

	High stress group		Low stress group	
Stress procedure				
Day 0	H, BL, W	In cages	H, BL, W	In cages
Day 1	H	In aviaries	H	In aviaries
Day 8	H, BL			
Day 15	H, BL			
Day 22	H, BL			
Day 29	H, BL			
Day 45	H, W	Back to cages	H, W	Back to cages
Test procedure				
Day 75	H, BR		H, BR	

H Handling, *BL* bleeding, *W* weighing, *BR* breath rate measurement

Day 0 is the start of the experiment. The test procedure was carried out 30 days after the last handling event (day 75)

of both the high stress and low stress group were caught from the aviaries, weighed and transported to their individual home cage 16 days after the high stress group was caught and bled for the last time. Consequently, the individuals from the two groups differed in the frequency of these stress episodes they were subjected to: the birds from the high stress group were caught and bled four times more than birds from the low stress group in a 4-week period. We measured body mass with an electronic balance to the nearest 0.1 g and tarsus with sliding callipers to the nearest 0.1 mm twice, at days 0 and 45 (last manipulation of the stress procedure; Table 1).

Test procedure and parameters measured

All parameters were measured 1 month after the last manipulation. To avoid time of day effects, all measurements were taken from 0900–1200 hours. In the test procedure, we caught the subjects from their home cage without knowing to which experimental group a bird belonged. We measured the time from the moment the cage was entered with the right hand until the bird was actually caught ("catch latency"). Subsequently, we held the birds gently in one hand while counting the number of breast movements (breath rate, B1) during 60 s. During the same period, we counted the number of times a bird attempted to escape (docility, E1). After taking these measurements birds were then kept in a cotton catching bag for 5 min. Immediately after taking the bird out of the bag, the breath rate (B2) and the number of escape attempts (E2) were counted again. Thereafter, we released the bird back in its home cage. For the breath rate measurement we used the same procedure of earlier studies conducted on the same species (Carere et al. 2001; Carere and Van Oers 2004).

Statistical analysis

We used Generalized Linear Models (GLM) with "catch latency" as dependent variable and sex and treatment (high stress and low stress) as factors. For the analysis of the breath rate measurements we used Repeated Measures ANOVA's, incorporating the variation within (trial, two levels: B1 versus B2) and between (treatment and sex) individuals. We included "catch latency", condition and body mass as covariates in these models. Condition was calculated by taking the residuals of a linear regression from body mass on tarsus. Since males are larger and heavier than females, we calculated condition for sexes separately. We used a GLM with Poisson errors to analyse the difference in attempts to escape in relation to the breath rate measurements, and included individual and sex as fixed factors. We applied a Mann–Whitney U test for analyzing the number of escape attempts per se. Since male Great Tits are in general about 1.0 g heavier than females (Van Balen 1967), we corrected for sex differences on body mass by including sex in all models containing body mass. Since the standard deviations of catch latency time were proportional to their means, we log-transformed this variable (Zar 1999). In all cases where catch latency is given, this is the back-transformed time.

We used backwards procedures in all analyses, starting with a full model including all two- and three-way interactions and subsequently removed the less significant term, starting with the highest interaction, until only significant terms were left (final model). Two- and three-way interactions in all analyses were non-significant unless reported differently. We used the software packages R 2.4.0 (Ihaka and Gentleman 1996) and GLIM4 (Crawley 1993) for statistical analyses.

Results

Three birds in the high stress group and one bird in the low stress group died during the period of the experiment, and therefore we used data of 57 and 33 individuals respectively for the analysis.

Catch latency

The time it took us to catch birds from their home cage ranged from 1 to 120 s (mean ± SEM, 9.8 ± 2.3). It took significantly less time to catch a bird from the high stress group, than to catch one from the low stress group ($F_{1,87} = 5.1$, $P = 0.030$; Table 2) after controlling for sex effects. Overall, females (mean ± SEM: 11.3 ± 1.4 s) were

Table 2 The mean time (± SEM) in seconds (s) needed to catch male and female Great Tits *Parus major* of the high stress and the low stress groups

Treatment	*n*	Sex	Catch latency (s)
High stress	27	Male	13.1 ± 2.1
High stress	30	Female	10.3 ± 1.5
Low stress	13	Male	29.3 ± 10.0
Low stress	20	Female	13.7 ± 3.1
Statistics	Treatment	$P = 0.032$	
	Sex	$P = 0.030$	
	Sex × treatment	$P = 0.416$	

Significance levels (P) for treatment, sex and their interaction are given in the bottom rows

N sample size

significantly ($F_{1,87} = 5.5$, $P = 0.032$) easier to catch than males (mean ± SEM, 17.6 ± 3.3 s).

The time to catch a bird did not influence the breath rate measurement shortly after catching (B1; $F_{1,88} = 0.45$, $P = 0.50$) or after the bird was kept in a cotton bag for 5 min (B2; ANOVA, $F_{1,88} = 0.38$, $P = 0.54$). Nor did it influence the difference between B2 and B1 (repeated measures ANOVA, $F_{1,88} = 0.001$, $P = 0.97$). Furthermore, we did not find any significant correlation between catch latency and the number of escape attempts in either the first (E1; $R_s = -0.04$, $n = 90$, $P = 0.72$) or the second measurement (E2; $R_s = -0.05$, $n = 90$, $P = 0.67$).

Body mass and condition

The experimental groups did not change differentially in condition (repeated measures ANOVA, $F_{1,80} = 0.45$, $P = 0.50$) or body mass (repeated measures ANOVA, $F_{1,80} = 0.112$, $P = 0.74$) from the beginning compared to 2 weeks after the stress procedure. This was not the case for absolute body mass (ANOVA, $F_{1,80} = 0.02$, $P = 0.89$) nor for condition ($t = 1.13$, $df = 79$, $P = 0.26$) measured after the stress procedure.

Docility

We found a significant difference in docility between the first and the second measurement (Fig. 1b; GLM: $F_{1,88} = 4.8$, $P = 0.031$). The high stress and the low stress group did not differ in the mean number of escape attempts, directly after catching (E1; Mann–Whitney U test: $z = -0.90$, $P = 0.37$). However, after a 5-min resting period in a cotton bag, individuals from the low stress group tried to escape more often than individuals from the high stress group. (E2; Mann–Whitney U test: $z = -3.44$, $P = 0.001$, Fig. 1b). No overall sex difference or interaction with treatment emerged ($P > 0.35$).

Breath rate

None of the two breath rate measurements correlated with the number of escape attempts (B1: $R_s = 0.10$, $P = 0.34$; B2: $R_s = -0.06$, $P = 0.56$). Therefore, breath rate and escape attempts were analysed independently from each other. Additionally, none of the breath rate measurements were dependent on tarsus (ANOVA, B1: $F_{1,86} = 0.077$, $P = 0.78$; B2: $F_{1,86} = 0.576$, $P = 0.45$) or weight (ANOVA, B1: $F_{1,86} = 0.173$, $P = 0.68$; B2: $F_{1,86} = 0.073$, $P = 0.89$).

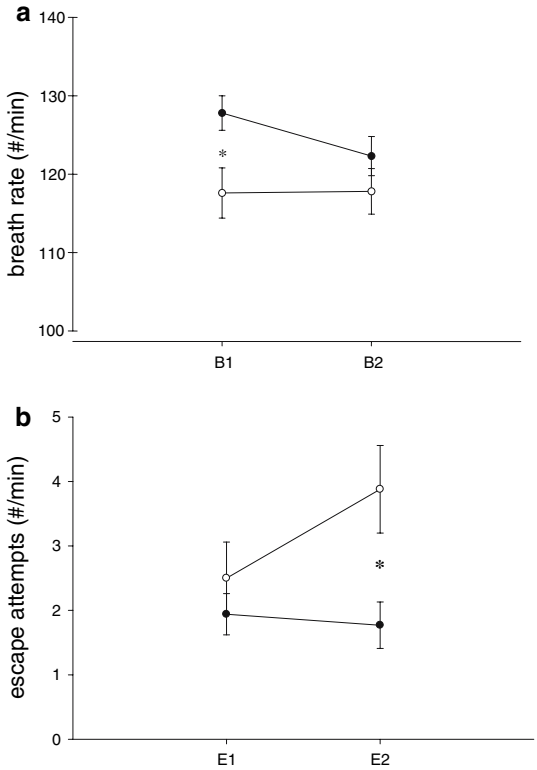

Fig. 1 The mean (±SEM) breath rate (**a**) and escape attempts (**b**) immediately after catching (B1 and E1) and 5 min after capture (B2 and E2). *Open circles* low stress group, *closed circles* high stress group. Significant differences are indicated with an *asterix*

We found no overall differences in breath rate between the high stress and the low stress group (t test: $F_{178} = 2.002$, $P = 0.16$; Fig. 1a). The groups, however, differed in the change in breath rate over the two trials (treatment × trial: $F_{1,88} = 6.5$, $P = 0.013$; Fig. 1a), independent of sex ($F_{1,88} = 0.50$, $P = 0.48$). This effect was due to a higher breath rate shortly after catching compared to the second measurement (paired t test: $t_{63} = 2.16$, $P = 0.035$) in the high stress group. Birds from the low stress group showed no difference between the two measurements (paired t test: $t_{25} = -0.81$, $P = 0.93$). No overall sex difference ($F_{1,88} = 0.58$, $P = 0.49$) or interaction with treatment ($F_{1,86} = 3.05$, $P = 0.07$) emerged.

Discussion

Our study on wild born Great Tits shows that repeated stressful events have influences on physiological and behavioural measured 1 month after the birds had been exposed to these recurring stressful procedures. The only difference between the birds of the two treatments consisted of an extra four times handling and bleeding. One month later, birds of the high stress group were easier to

catch, displayed a higher breath rate and attempted to escape less frequently during handling compared to birds exposed to a lower number of stressful episodes (low stress group). These effects are likely to be related to the emotional response and suggest that learning processes can play a role in it.

Body mass and condition were not affected. Body mass is known to decrease following acute stress (Koolhaas et al. 1997; Ruis et al. 1999). We found no relation between the amount of stress received and loss of body mass in our study. A possible reason for this is that the handling stress episodes could have induced more episodic corticosterone peaks, which by stimulating more foraging could have buffered the possible effect on body mass (Belthoff and Dufty 1998; Saldanha et al. 2000). Alternatively, since especially in a captive situation where food is ad lib available animals do not have to put any effort in foraging, body mass losses could be compensated for.

Several studies have shown short-term effects of stress due to handling alone, and this has been used as a standard measurement to identify differences in stress response (e.g. Wingfield et al. 1992; Silverin 1997). Because we always had to both catch and handle the birds to bleed them in this experiment, we are not able to separate the effects of these three sources of stress. These are, however, unavoidably associated in almost all experimental procedures. We can therefore not identify whether or to which part our results are caused by the effects of catching, handling or bleeding independently. Moreover, the effects found seem to be a cocktail of changes that can be demonstrated on a group level but may fall apart on an individual level.

Are these responses a sign of maladaptation or rather of habituation and adaptive learning and coping? The effect of handling and bleeding stress on breath rate is possibly caused by sensitisation of the adrenergic system (Stam et al. 2000). The increased response of breath rate to a stressful stimulus could be seen as a pro-adaptive response, and it is likely that it represents an attempt to cope with stressful demands (Stam et al. 2000). The fact that the two groups did not differ in their breath rate after a period of rest may indicate that the difference in the stress response is not caused by a change in their general physiological state, as we assume this measurement to be closer to the basal level. But the lack of sensitisation in escape attempts in the high stress group shows that this group is not adapting to the stressful situation, which could be seen as an indicator of a depression-state (Koolhaas et al. 1997). These birds were easier to catch, which indicates they habituated to the catching and probably to the handling act. This is likely to be caused by the repeated catching and not by bleeding as habituation is known to be stressor-specific (Kant et al. 1985). Similar results have been found in other studies. Stress responses were altered due to neonatal handling in an Amazon parrot (Collette et al. 2000) in such a way that they were more willing to perch on a finger and differed in corticosterone levels compared to non-handled birds. In rabbits handled after nursing time, repeated, but minimal, human contact was found to be sufficient to decrease sensitivity to human contact (Bilko and Altbacker 2000; Csátadi et al. 2005).

Since sex is thought to be a major factor conferring differential stress susceptibility (Heinsbroek et al. 1991; Blanchard et al. 1991, 1998; Handa et al. 1994), males and females might also be differentially affected by long-term effects (Adamec et al. 2006). We found that females, especially those of the low stress group, were easier to catch than males, although this was not reflected in any sex difference in the other parameters measured. Females might have been "better learners" than males, but this could explain either easy or difficult catchability. The presence of humans, but to a greater extent the physical contact to humans is likely to be perceived as contact with a predator (Müller et al. 2006). Hence, females may have adopted a different anti-predator strategy in respect of males having different locomotor performance during fleeing. Support for this hypothesis has been found in several snake species, in which females express different escape behaviours from males, most likely caused by differences in reproductive burdens (Winne and Hopkins 2006).

In conclusion, this study provides evidence that repeated brief, but intense, stressful events can cause long-term behavioural and physiological effects. The amount of stress experienced in past experiments must therefore be taken into account in research on stress responses on the same individuals, and might be a cause for substantial inter- and intra-individual difference in coping responses.

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