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# The stressed bird in the hand: Influence of sampling design on the physiological stress response in a free-living songbird

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ABSTRACT

Despite the widely used application of standardized capture-handling protocols to collect blood and assess the physiological stress response, the actual sampling design (e.g., timing and the number of blood samples) often differs between studies, and the potential implications for the measured physiological endpoints remain understudied. We, therefore experimentally tested the effects of repeated handling and multiple blood sampling on the stress response in wintering free-living great tits (*Parus major*). We modified a well-established sampling protocol of avian studies by adding either an additional blood sample or a "sham-manipulation" (i.e., handling associated with the blood sampling procedure without venepuncture), to disentangle the effects of handling stress and blood loss. We combined three different stress metrics along the endocrine-immune interface to investigate the acute short-term stress response: total corticosterone concentrations (Cort), the heterophil/lymphocyte ratio (H:L), and the Leucocyte Coping Capacity (LCC). Our study provided three key results: i) no relationship between Cort levels, LCC and H:L, confirming that these three parameters represent different physiological endpoints within the stress response; ii) contrasting dynamics in response to stress by the measured parameters and iii) no difference in physiological stress levels 30 min after capture due to one additional blood sampling or handling event. By optimising the sampling design, our results provide implications for animal welfare and planning experimental procedures on stress physiology in passerine species.

## 1. Introduction

Sampling blood to assess the individual stress responses became a standard procedure in field biology, conservation- and veterinary sciences. In wild, free-ranging animals, however, finding the balance between a reliable representation of physiological conditions by increasing blood sampling units (i.e., the number of blood sampling events and/or the total amount of blood taken) while minimising handling-induced stress and its effects on individual welfare is a challenging task [1-4]. In addition, in small-sized vertebrate species, the amount of blood that can be collected is limited and drawing multiple samples over a short time may induce additional physiological changes. Hence, understanding how multiple blood sampling, repeated handling and restrain-time affect individual physiological response patterns is imperative for the interpretation of physiological target parameters within the physiological stress response and may also have implications to maintain high

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standards of animal welfare [5-7].

Passerine species are a popular model and the current standards of good sampling and handling practice in birds have been widely discussed and follow strict rules, i.e., short handling procedures and blood sampling within 1% of the body-mass [6, 8]. In this context, several studies suggest that taking multiple blood samples from a healthy animal has minor effects on its condition, behaviour and survival [7, 9-13]. Small animals, however, likely perceive the capture and restraint during blood sampling as a life-threatening situation. Given the severity of this acute, short-term stress response, the possible effects of the sampling procedure itself, resulting in a small open wound and the loss of blood, may only marginally affect physiological stress parameters [10, 12-14]. Whereas it is well recognised that stressors (i.e., unpredictable, uncontrollable adverse changes in the immediate environment of the individual) experienced before capture may have a significant impact on the measurements of the individual physiological stress responses, only few studies have shown that even after the onset of the handling-induced stress response additional disturbance (e.g., multiple handling and/or blood sampling) and the accomanying potential increase in perceived stress intensity can alter the physiological response patterns [15-18]. For example, Canoine et al. [19], reported higher stress-associated hormone concentrations (corticosterone; Cort henceforth) in birds exposed to a predator in addition to the blood sampling procedure and thereby demonstrated an unexpectedly high context-specific plasticity in the Cort response [20]. On the contrary, studies which applied different stress metrics, such as the change in total white blood cells (WBC), show that additional handling and blood sampling only has negligible effects [10, 13]. A possible explanation for this discrepancy may be that the different physiological stress systems and their measurable endpoints vary in their sensitivity to stressors and the time point at which each parameters responds within the physiological stress response [21]. These differences potentially impede the interpretation of data, aiming to reveal the effects of multiple blood sampling and handling on physiological measurements of the individual stress response and the potential direct effects of sampling [7, 10, 22].

We, therefore, tested different blood sampling regimes and their impact on the outcome of physiological stress in a free-living passerine species, the great tit (*Parus major*). We used the well-established "capture-handling stress" protocol [23], as the underlying framework and included three stress metrics representing different physiological systems with varying response latencies and sensitivity towards handling-induced stress [5, 21, 24]. The basic protocol is designed to measure the magnitude of activation and reactivity of the hypothalamic-pituitary-adrenal- (HPA) axis and the concomitant changes in circulating Cort concentrations in response to a defined stressor (i.e., capture and handling) by collecting a series of blood samples. Thereby the standardized capture and handling procedure are considered to elicit a maximal HPA axis response, which can be compared between and within individuals in dependence of varying conditions (i.e., external/environmental and internal/individual factors shaping the physiological stress response) [23]. The most commonly applied form of the protocol (we refer to it as "standard protocol") is a reduced version of the pioneering study by Wingfield et al., 1982 [23] and includes the collection of a sample immediately after capture (< 3 min), which is regarded to represent Cort concentrations near the baseline, and 30 min thereafter, considered as the Cort peak response [25, 26].

Cort concentrations increase within minutes after the onset of a stressor and act on several physiological pathways in a parallel manner. They stimulate essential physiological functions, such as cardiac-, respiratory and brain-activity to enhance immediate survival and energy maintenance to re-establish homoeostasis thereafter [27, 28]. Stress responses also affect the distribution and function of innate immune cells [29, 30]. The physiological stress response activates the glucocorticoid-, α- and β-adrenoceptors of polymorphonuclear granulocytes (PMNLs; i.e., neutrophil granulocytes in mammals and heterophil granulocytes in birds) and an "oxidative burst" is triggered to produce and release superoxide free radicals as the basic molecule for an array of several other reactive oxygen species (ROS; [31, 32]). This reaction can be simulated under experimental conditions and measured in real-time via chemiluminescence from whole blood in mammals and birds [33-36]. The relatively recent technique called Leucocyte Coping Capacity (LCC) is based on the observation that PMNLs obtained from animals during or after a physiological stress response have a diminished capacity to produce ROS in response to a secondary chemical challenge [35]. Hence an increase in stress leads to a decrease in LCC and vice versa [33]. Another consequence of the physiological stress response is the redistribution of immune cells. Thereby, PMNLs diffuse from the periphery into the bloodstream and lymphocytes migrate from the circulation into the peripheral tissues, leading to a shift in the heterophil to lymphocyte ratio (H:L ratio). The increase in H:L ratio is representative for high Cort levels and considered a reliable and frequently used measure for stress in birds and other vertebrates [24, 37]. It further serves in the interpretation of other applied stress metrics and should, for example, be considered to control for a potential mass effect of increased neutrophil numbers on the measured LCC response.

In our experiment we applied modified versions of the "standard protocol" and assigned individual great tits to three sampling regimes: (1) *Standard*, blood samples near the baseline (immediately after capture) and 30 min post-capture; (2) *3-samples*, where we included an additional blood sample 15 min post-capture; (3) *Sham*, handling without venepuncture at 15 min post-capture, in order to separate the potential effects of blood loss from handling. If additional handling and sampling result in higher stress intensity and affect the measured physiological parameters after the initial stressor [19], we expect to observe higher Cort concentrations in individuals that experience higher handling frequency (groups 2 and 3), in comparison to individuals assigned to the *Standard* protocol. Even though heterophil numbers increase in the bloodstream after a stressor, we do not expect to observe a difference in the H:L ratio between the *Standard* and the *Sham* group. Changes in the H:L ratio are considered to be slow in comparison to the other parameters, and become measurable 60–90 min after the onset of the stressor [10]. However, if blood loss and the decrease of blood cells affect these measures, the H:L ratio is expected to decrease in birds in the *3-samples* group. Regarding the LCC dynamics, we predicted that birds undergoing the *Standard* would show an increase in LCC levels (i.e., lower stress levels) with the time of the procedure (i.e., a partial recovery from capture and the first handling in the cloth bag). In contrast, individuals from the *3-samples* and *Sham* protocols will show a decreasing dynamic in LCC levels over the timespan of the experiment, which is expected to be even more pronounced in the *3-samples* group.

## 2. Methods

We caught 57 great tits (*Parus major*) on a winter-feeding site in the Botanical Garden of the University of Debrecen (47°33′24.6″N, 21°37′16.2″E). The artificial feeder was established one week prior to the start of the experiment and regularly maintained. To reach a sufficient sample size, we provided an additional stimulus: we placed a speaker under the feeder, which broadcasted the calls of a mixed-species flock (in addition to great tit, the playback included blue tit, *Cyanistes caeruleus*, long-tailed tit *Aegithalus caudatus* and coal tit *Periparus ater*).

Birds were captured using mist nets between December 2018 and February 2019. All samples were collected between 09:00–12:00. We collected a blood sample immediately after the capture by puncturing the brachial vein using a sterile 26 G needle. The initial blood sample (~50 μl) was drawn into heparinised micro capillary-tubes immediately after a bird hit the net, (average time including all treatment groups: 155 ± 51 (SD) *sec.*). The handling time was not related to any of the physiological variables measured at the S1 level (all $p > 0.16$). Including handling times into the models did not improve model fit in any case. Immediately after the sampling, the birds received a numbered

aluminium ring and were transferred into a cotton bag. Individuals were randomly assigned to the three treatment groups after the first sample was drawn. In the first group (*Standard*), after initial sampling, the birds ($n = 18$) remained in a cloth bag until the second sample was drawn (after 30 or 15 min). In the second group (*3-samples,* $n = 18$), we collected blood samples, 15 min and 30 min after the capture. In the third experimental group (*Sham,* $n = 21$), we took a second blood samples after 30 min, as in the *Standard* group, but in order to separate the effects of handling from blood loss, 15 min after the capture we performed the entire handling procedure (removing the bird from the bag, opening and preparing the wing for blood sampling) without the actual venepuncture.

Immediately after each sampling event, a small drop of blood was used to prepare the blood smears using the two-slide wedge technique. In addition, we aliquoted 20 μl whole blood for the LCC analyses, and the remaining sample was centrifuged for 5 min at 2500 g to separate plasma from red blood cells. We removed the plasma with a Hamilton syringe and stored the samples in a –20 °C freezer until assayed for Cort. During all subsequent laboratory procedures researchers were blind towards the treatment groups.

### 2.1. Leucocyte coping capacity

Immediately after blood collection, 20 μl of heparinised whole blood was transferred into a silicon anti-reflective tube (Lumivial, EG & G Berthold, Germany), containing 180 μl of $10^{-4}$ mol $l^{-1}$ lucigenin (bis-N-methylacridinium nitrate; Sigma Aldrich, Vienna, Austria) dissolved in dimethyl sulfoxide (DMSO; VWR International, Stockholm, Sweden) and diluted in Phosphate Buffered Saline (PBS, pH 7.4). Lucigenin produces chemiluminescence when combined with an oxidising agent (i.e., superoxide anion) and was used to quantify the production of extracellular ROS production of heterophil granulocytes in real-time [38, 39]. After that, the sample was mixed gently and aliquoted into two anti-reflective tubes. In one tube, 10 μl of PBS (pH 7.4) was added in order to measure unstimulated blood chemiluminescence levels, providing information on individual baseline concentrations of superoxide anion and acts as a control. In the second tube, we added 10 μl of $10^{-5}$ mol $l^{-1}$ phorbol-myristate-acetate (PMA; Sigma Aldrich, Vienna, Austria) to assess full blood chemiluminescence in response to this secondary (the first natural challenge occurs in vivo) chemical challenge [40]. Immediately after sampling (4 −10 min after a sample was drawn), blood chemiluminescence (expressed in relative light units (RLU); arbitrary scale reflecting photon count divided by 10) for each tube was measured for 30 s every 10 min over 80 min by using two portable high sensitivity chemiluminometers (Junior LB 9509, EG & G Berthold, Germany). All measurements were carried out in the laboratory with temperatures between 20 °C and 25 °C. The tubes were kept at 40 °C in a glass beaker with metal beads placed in a water bath (40 °C) to simulate in vivo temperature conditions slightly below the average active body temperature and were gently swivelled from time to time to avoid pelleting of the blood cells [41]. Samples were not centrifuged as the texture and adhesiveness of the cell microenvironment is essential for the in vivo determination of cell reactivity [42]. In order to correct for background noise, we subtracted the values of the control sample from that of the challenged sample measured at the same time point. From the resulting LCC response curve reflecting the PMA induced production of PMNL ROS in real-time, we extracted the LCC peak as a variable, i.e., the maximum in ROS production within the 80 min measure period. To account for individual differences in the number of heterophils and thus a potential mass effect on LCC, we used the residuals of a general additive model between the LCC peak and the number of heterophils (Fig. S1), hereafter 'corrected LCC' as our response variable in the analyses (see *Statistical analyses*).

### 2.2. Leucocyte cell count

Blood smears were air-dried, fixed with ethanol and dyed with Wright-Giemsa Quick stain following previous protocols established for songbirds [e.g., 43]. Briefly, smears were examined at 1000 × magnification, and a minimum of 50 leukocytes was counted per slide while keeping track of the number of view fields and the number of erythrocytes. The number of each leucocyte type, i.e., lymphocytes, heterophils, eosinophils, monocytes and basophils, was expressed per 10,000 erythrocytes. In addition, the total number of white blood cells (the sum of total lymphocytes, heterophils, eosinophils, monocytes and basophils) and the heterophil to lymphocyte ratio (H:L) was calculated (ratio between the total numbers of heterophils and lymphocytes as described in [44]. All the cell counts were performed by the same observer (E.Z. Szarka), and a random subset of smears ($n = 15$) was analysed in duplicates showing a moderately to high repeatability between counts for the main cellular types, lymphocytes ($R = 0.722$; 95% CI [0.349, 0.891]; $p < 0.001$), heterophils ($R = 0.492$; 95% CI [0.029, 0.817]; $p = 0.028$) and the H:L ratio ($R = 0.84$; 95% CI[0.592, 0.942]; $p < 0.001$).

### 2.3. Hormone assays

We quantified the total Cort, using direct radioimmunoassay [45]. Before the assays, we extracted the Cort from the plasma samples, using diethyl-ether. Then, the extracts were reconstituted in PBS. After overnight incubation at 4 °C, we added ~10 K dpm of 3H—Cort (Catalogue number: NET399250UC, lot number: B00025; Perkin Elmer, Waltham, MA, USA), antiserum (MP Biomedicals 07–120,016, lot number: 3R3-PB-20E2) and PBS. After another incubation overnight at 4 °C, the dextran-coated charcoal was added to separate Corticosterone bound to antibodies. The radioactivity of the bound fraction was counted in a liquid scintillation counter (QuantaSmart). We processed all samples in one assay (intra-assay CV = 4.17%).

### 2.4. Statistical analysis

The physiological short-term stress response was analysed from 57 individuals. All statistical analyses were conducted using R version 3.6.2 [46]. Physiological variables were analysed in linear mixed-effects models using treatment group and sampling time points (baseline, 15 and 30 min) as fixed effects (factors) and individual identity as a random intercept. The models also included the group × sampling point two-way interaction, as this was part of the experimental design. We report F and p values for main effects and their interaction, and we also report t-values associated with parameter estimates to show the detailed differences between treatment groups at 30 min (time × treatment, *Standard* group as the reference level). Degrees of freedom were determined using Satterthwaite's approximation and significance tests were obtained as implemented in the 'lmerTest' R package [47]. Assumptions of the models were assessed by visual inspection of the residuals. H:L was arc-sine square-root transformed. Cort and LCC values were not transformed. To test the relationship between the variables recorded to represent different aspects of the individual short-term stress response, we conducted principal component analyses (PCA) using the 'prcomp' function which calculates a singular value decomposition of the centred and scaled data matrix of H:L, LCC, and Cort concentrations separately for baseline and stress-induced concentrations.

## 3. Results

### 3.1. Relationship between the response variables

At the baseline level, principal component analyses showed that Cort, LCC and H:L were not strongly related. The PCA had three components, with Eigenvalues PC1 = 1.09, PC2 = 1.05, PC3 = 0.84. The variance explained by each component was similar (PC1 = 39.4%, PC2

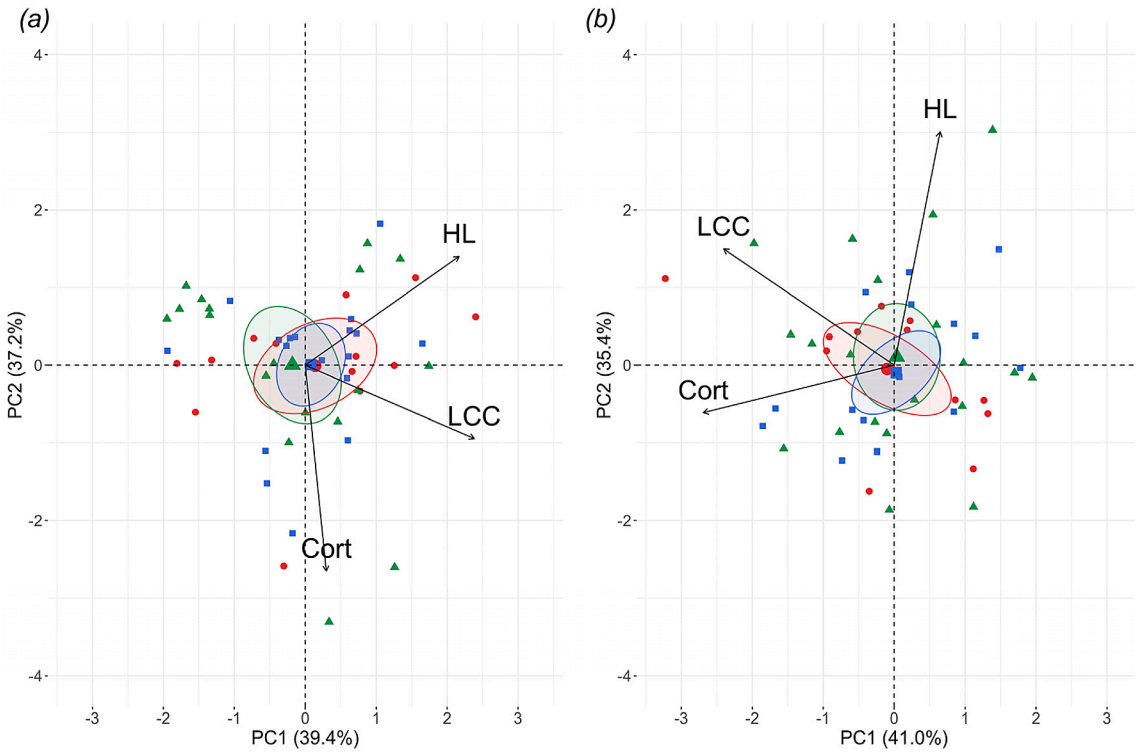


**Fig. 1.** Principal component analysis of the three physiological stress parameters: total Cort, LCC (corrected for the number of heterophils ) and H:L-ratio (HL) measured (a) at baseline or (b) after 30 min. In the course of the stress response, the three response variables were re-organised but remained largely independent from one another, showing that these stress indicators represent different physiological aspects within the stress response. Likewise, the variation explained by each component was similar to the baseline case. Small points represent each individual's sampling point while the larger symbols indicate the bivariate median response with the 95% CI ellipse. *Standard* (red circles), *Sham* (green triangles) and *3-samples* (blue squares).

= 37.2%, PC3 = 23.5%; Fig. 1a). While all variables loaded positively in PC1 (Table S1), LCC and Cort seemed to represented an independent axis to H:L in the second component, although the three variables remain independent from each other (Fig. 1a, PC2). Short-term stress (i.e., capture and subsequent handling and constraint for 30 min) did not modify the relationship between the three response variables and they remained largely independent from one another although the loading across axis differed (Fig. 1b, Table S1). Eigenvalues of the PCA were: PC1 = 1.11, PC2 = 1.03, PC3 = 0.85. The variance explained by each component was similar to the baseline case: (PC1 = 41.0%, PC2 = 35.4%, PC3 = 23.8%). These results were corroborated by the lack of significant correlations between Cort, LCC and H:L ratio either at the initial or at the final stress-induced measurements (Fig. S2., Table S 2.).

### 3.2. *Effects of the blood sampling regime on the three stress response variables*

Baseline Cort concentrations did not differ between the three sampling regimes ($F_{2,63} = 0.79$, $p = 0.456$). Sampling time point indicates a significant increase of Cort ($F_{2,69} = 54.61$, $p < 0.001$), both at 15 min ($t = 4.34$, $p < 0.001$) and at 30 min ($t = 6.24$, $p < 0.001$) post capture. However, at 30 min there was no difference between the three experimental groups ($F_{2,69} = 0.44$, $p = 0.645$): Cort concentrations in the *Standard* group did not differ from either the *Sham* ($t = -0.79$, $p = 0.432$) or the *3-samples* ($t = -0.86$, $p = 0.391$, Fig. 2a, Fig. 3a).

LCC baseline levels did not differ between the three groups ($F_{2,56} = 0.10$, $p = 0.902$). Capture and handling induced a drop in LCC values ($F_{2,66} = 5.96$, $p = 0.004$), but only after 30 min ($t = -3.04$, $p = 0.003$), not at 15 min ($t = 0.50$, $p = 0.620$) post capture. The experimental

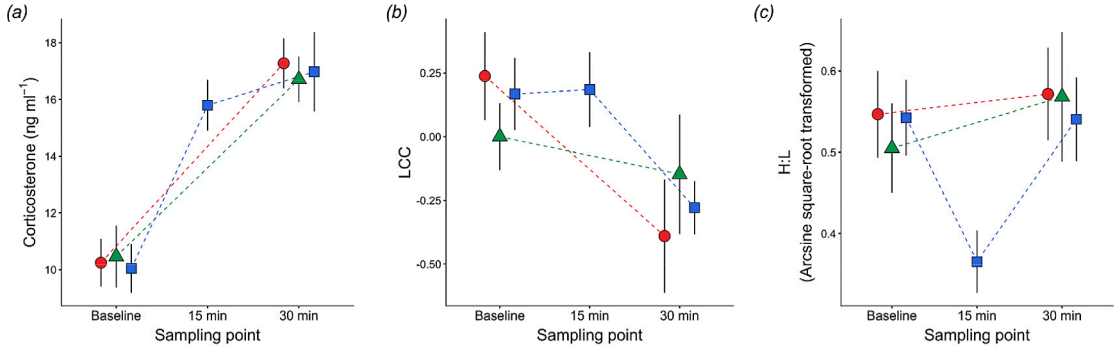


**Fig. 2.** Physiological stress response patterns of great tits exposed to three different handling regimes, *Standard* (red circles), *Sham* (green triangles) and *3-samples* (blue squares). (a) Total Cort concentrations; (b) LCC(corrected for the number of heterophils) and (c) H:L ratio (arcsine square-root transformed). Symbols represent means ± standard error of the means at baseline, 15 min and 30 min after capture.

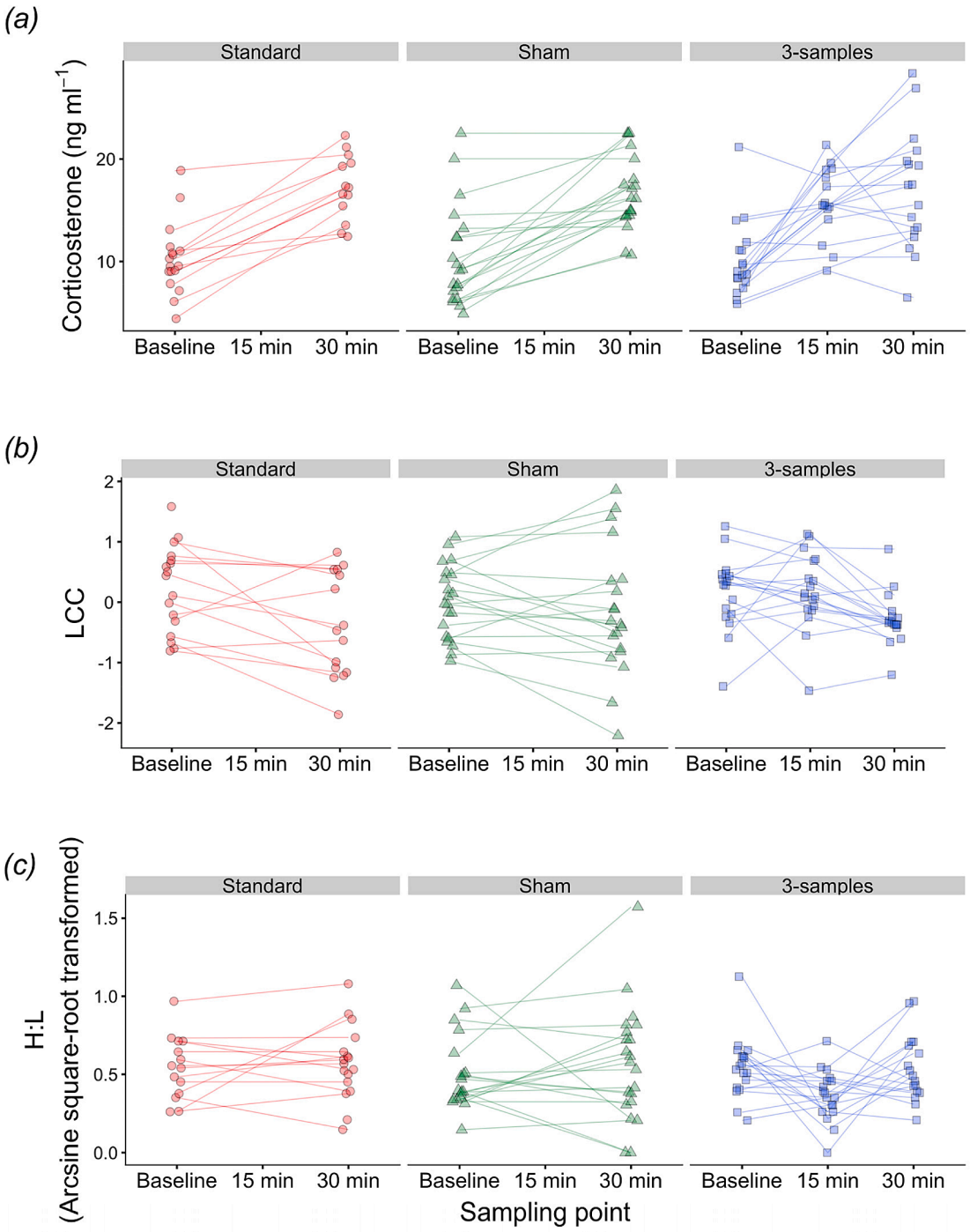


**Fig. 3.** Individual physiological responses of wild great tits exposed to the three different handling regimes, *Standard* (red circles), *Sham* (green triangles) and *3-samples* (blue squares). (a) Total Cort; (b) LCC (corrected for the number of heterophils) and (c) H:L ratio (arcsine square-root transformed). Symbols represent each individual and lines connect the measurements of the same individual at each time point: baseline, 15 min and 30 min after capture.

groups did not differ in their LCC levels at 30 min ($F_{2,67} = 0.69$, $p = 0.505$, Fig. 2b, Fig. 3b).

Similarly to the other two parameters, we observed no difference in the baseline H:L ($F_{2,55} = 0.05$, $p = 0.956$) between the three protocols. In the *3-samples* group, handling induced a significant drop in H:L ($F_{2,59} = 3.83$, $p = 0.027$) after 15 min ($t = -2.39$, $p = 0.02$), but H:L values returned to the baseline after 30 min, and thus did not differ from the *Standard* group ($t = -0.29$, $p = 0.772$). Also, H:L in the *Sham* group did not differ from the *Standard* group after 30 min ($t = 0.27$, $p = 0.791$, Fig. 2c, Fig. 3c).

## 4. Discussion

Our study provides three key results. First, in line with previous findings, we reveal only a weak relationship between Cort, H:L and LCC (Fig. 1), confirming that these three parameters represent different physiological aspects within an acute, short-term stress response [21, 24, 48, 49]. Second, we found that these three physiological parameters showed different kinetic dynamics over the time course of the short-term stress response, reflecting different response latencies (Fig. 2) [21]. Cort concentrations increased markedly in most individuals, but the timing of the peak response underlies substantial between-individuals variation

(Fig. 3a). On the contrary, the average LCC response decreased steadily, with no noticeable differences regarding the timing of the blood sample (Fig. 2b, Fig. 3b). However, our results also revealed a sudden drop of the H:L ratio 15 min post-capture with a subsequent recovery to baseline 30 min post-capture (Fig. 2c, Fig. 3c). Finally, we showed that the differences between sampling regimes applied in this study did not lead to significant differences in the Cort, H:L ratio and LCC response 30 min after capture.

Based on the idea, that even after the initiation of a physiological stress response, the individual Cort response can underlie certain plasticity depending on stress intensity (e.g., handling frequency), we predicted a stronger HPA axis activation with higher Cort in birds that experienced additional handling stress and underwent three sampling procedures [20]. This effect, however, did not become apparent in our study which is supported by findings in other avian and non-avian species [10, 12, 13]. Bonnet and colleagues, for example, recently reported, that blood sampling *per se* did not affect Cort concentrations in dice snakes (*Natrix tessellata*) [13]. Whereas individual condition (i.e., health or nutritional status) and previously experienced stress events can change the stress responsiveness and the overall HPA axis response, additional handling after the onset of the initial stress stimulus may have only little effects on Cort concentrations. Circulating Cort concentrations increased in almost all birds between baseline and 15 min (Fig. 3a), with some individuals showing a weak further increase or even a decrease after the second bleeding, possibly as the result of the effective negative feedback of Cort within the HPA axis. These results are corroborated by previous work on songbirds [50], including recent studies on migrating garden warblers (*Sylvia borin*) and great tits, suggesting that in some species measuring Cort at 15 min (or 10 min) post-capture may be sufficient (and potentially preferable) to study the magnitude of HPA axis activation towards standardised acute, short-term stress [15, 34].

We observed a similar pattern when exploring the effects of the experimental protocol on the shift in H:L ratio [26, 51]. The activation of the HPA axis and the consecutive increase of circulating Cort is leading to a redistribution of innate immune cells into specific target tissues and to a reduction of circulating lymphocytes while simultaneously increasing the number of circulating heterophils [43, 52-54]. This relationship was previously demonstrated in Cort supplementation experiments, but also capture and restraint stress was shown to be sufficient to effect similar responses as is corroborated by a growing number of studies on vertebrates [37, 43, 52]. The H:L ratio, however, is considered to mainly reflect long-term, rather than an immediate, short-term physiological stress responses [21, 53, 55] and there is a strong indication that an increase after capture is species-specific and becomes significant 30 to 60 min post-capture [10, 24]. Remarkably, our data show an unexpectedly fast decrease in the H:L ratio at 15 min after capture, followed by rapid recovery to baseline 30 min post-capture. One possible explanation is the acute "*fight-or-flight*" response and the concomitant increase in noradrenaline, causing the observed transient lymphocytosis shortly after capture [56, 57]. Furthermore, severe stress may cause the exhaustion of mature cell pools and the release of immature cells from the bone marrow, which can result in transient heteropenia [26, 52, 53]. Hence, the rapid drop and the subsequent recovery of the H:L ratio measured in our samples may be due to short-term physiological compensatory mechanisms of the organism in response to severe handling stress and possibly also the sudden loss of blood. However, we can only speculate about the underlying physiological mechanisms and future, additional studies are required to investigate the observed pattern.

Interestingly, LCC levels did not vary significantly between sampling regimes and decreased in all groups equally in the course of the experiment. LCC has been suggested as a reliable and sensitive tool to measure stress in mammals and birds and has been successfully applied to assess psychological stress in humans [40, 58]. In respect to the existing literature, a decrease in the LCC response, or a lack of recovery, is an indicator for high stress levels and a reduced capacity to cope with and/or recover from a stress event [33, 35]. For instance, in captive house sparrows (*Passer domesticus*), leaving the birds undisturbed for 30 min in a cotton bag was sufficient to allow individuals to recover from capture stress in winter, reflecting in an increase of the measured LCC response [48]. Based on these findings, we expected a more definite decline in the LCC response in birds that underwent multiple sampling and handling and have hence experienced a disrupted recovery period. Our results, however, show that LCC decreased in all three experimental groups and neither the additional handling nor the third blood sample resulted in a different LCC response between the experimental groups 30 min post-capture. However, the captive house sparrows in Huber et al. [48] had *ad libitum* access to food and were habituated to human presence. These conditions may have positively contributed to the recovery of the LCC response and a faster re-establishment of homoeostasis after capture and handling [49]. In addition to having never experienced capture and handling previously, wintering great tits face harsh weather conditions and limited access to food, possibly reducing the capacity to recover from the capture/handling stress. We therefore suggest that the conditions prior sampling and previous experience with capture/handling might affect LCC response in free living avian species [59]. Our results also suggest that the actual blood sampling had no effect on the LCC levels, because the patterns recorded in the *3-samples* group at 30 min are not different from the *Sham* or the *Standard* group. We controlled for a potential effect of changes in white blood cell composition on LCC by correcting this measurement for the individual number of heterophils (corrected LCC), which might occur due to a loss of blood volume after sampling or the stress induced shift in the abundance of heterophils in the circulation. The number of studies on LCC in passerines is limited [34, 48], and the observed patterns might be species-specific and underlie seasonal variation. Furthermore, Gormally et al. [49] have demonstrated that HPA axis regulation, immune function, antioxidant concentrations, DNA damage and stress-related alterations in behaviour change on distinct timescales in response to repeated stressors or differences in recovery time between the stress events [21]. We would, therefore, like to emphasise that we only recorded data over 30 min and encourage follow-up studies to measure the recovery from capture and handling induced stress over a more extended period. Moreover, additional measures for stress, representing different physiological aspects within the stress response such as oxidative stress, should be applied and tested in future studies [60, 61].

The sharp drop of the H:L ratio and more importantly, the fast recovery to baseline levels 30 min post-capture might provide an interesting new perspective for future research questions. Our results also revealed a decrease of the LCC response after 30 min post-capture, in all groups. In agreement with the existing literature, this indicates that birds did not recover from the initial capture stress after 15 min, and their condition in terms of LCC deteriorated until 30 min after capture [36, 62, 63]. However, the timing and the intensity of the physiological stress response is context dependant and species-specific [15, 26, 32]. Therefore, our results and the observed patterns, including Cort concentrations, LCC and the H:L ratio, are representative for overwintering, free-living great tits but may not apply to other bird species in different life history stages.

Shortening the period of human presence and restraint and reducing handling frequency, may minimise additional stress imposed on the animals. This is of particular importance during specific life-history stages, such as the reproductive period, moulting or migration, when animals may be more vulnerable towards stressors. We acknowledge that not all experimental procedures allow fast processing of the animals and require extended periods of capture and handling. However, we advocate the investigation of shorter sampling regimes and their preferential use in standard procedures if they appear to be appropriate for the experimental design [64, 65].

## 5. Author contributions

*Conceptualization*: N.H, K.M., A.Z.L.; *Methodology:* K.M., Z.T., E.Z.S., Y.U.C., N.H.; *Formal analysis*: A.Z.L., P.S.; *Writing - original draft*: K.M., N. H.; *Review & editing*: P.S., Z.T., E.Z.S., Y.U.C., A.Z.L.

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## 7. Ethics

We followed all applicable international, national, and institutional guidelines for the use of animals. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and approved by the institutional animal care and use committee.

## 8. Data availability

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher upon request.

## Declaration of Competing Interest

The authors declare no competing or financial interests.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2021.113488.

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