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Published in:
Journal of Experimental Biology

DOI:
10.1242/jeb.166124

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Strong association between corticosterone levels and temperature-dependent metabolic rate in individual zebra finches

Blanca Jimeno1,2,*, Michaela Hau2,3 and Simon Verhulst1

ABSTRACT

Glucocorticoid hormones (GCs) are often assumed to be indicators of stress. At the same time, one of their fundamental roles is to facilitate metabolic processes to accommodate changes in energetic demands. Although the metabolic function of GCs is thought to be ubiquitous across vertebrates, we are not aware of experiments which tested this directly, i.e. in which metabolic rate was manipulated and measured together with GCs. We therefore tested for a relationship between plasma corticosterone (CORT; In transformed) and metabolic rate (MR; measured using indirect calorimetry) in a between- and within-individual design in captive zebra finches (Taeniopygia guttata) of both sexes. In each individual, CORT and MR were measured at two different temperature levels: ‘warm’ (22°C) and ‘cold’ (12°C). CORT and MR were both increased in colder compared with warmer conditions within individuals, but also across individuals. At the between-individual level, we found a positive relationship between CORT and MR, with an accelerating slope towards higher MR and CORT values. In contrast, the within-individual changes in CORT and MR in response to colder conditions were linearly correlated between individuals. The CORT–MR relationship did not differ between the sexes. Our results illustrate the importance of including variation at different levels to better understand physiological modulation. Furthermore, our findings support the interpretation of CORT variation as an indicator of metabolic needs.

KEY WORDS: Corticosterone, Taeniopygia guttata, Glucocorticoid, Metabolic rate

INTRODUCTION

Glucocorticoid hormones (GCs; e.g. cortisol, corticosterone) are often quantified to assess whether individuals or populations are ‘stressed’ (reviewed in Dantzer et al., 2014; Koolhaas et al., 2011). However, circulating GC concentrations can also increase during non-stressful situations, for example with the regular daily increases in energy demands that individuals routinely experience (McEwen and Wingfield, 2003; Landys et al., 2006; Romero et al., 2009; Beering et al., 2011). This is in line with one of the primary functions of GCs, which is to interface with metabolism in a variety of ways. GCs have been named for their function to convert stored energy into glucose, and are therefore predicted to fluctuate in concert with metabolic demands. However, although this basic prediction underlies many concepts of GC regulation and function (McEwen and Wingfield, 2003; Romero et al., 2009), the existence and nature of the relationship between metabolic rate (MR) and GCs is still surprisingly unresolved (Holtmann et al., 2017; reviewed in Romero and Wingfield, 2015).

Multiple lines of evidence suggest that GCs and metabolism may be linked, both at the inter- and intra-specific level. Perhaps the most convincing evidence available comes from a recent comparative study on mammals, which found that both baseline and stress-induced cortisol levels correlated positively with mass-specific MR (Haase et al., 2016). At the intraspecific level, GC levels have been shown to be associated with energy expenditure (Welcker et al., 2015), and with factors that presumably affected energy expenditure. For example, baseline GC concentrations are generally higher with increased workload, resource limitations, reproductive investment, immune responses or thermoregulatory demands (Romero et al., 2009; Bonier et al., 2011; Miller et al., 2009; Bauch et al., 2016; Guymann et al., 2017; Merkling et al., 2017; Ouyang et al., 2013). Likewise, stress-induced concentrations (increases following exposure to acute stressors) can be affected by energetically-demanding processes such as molt (Cyr et al., 2008; Bauer et al., 2011; de Bruijn and Romero, 2013), climatic conditions (de Bruijn and Romero, 2011) or reproductive behaviour (Buwalda et al., 2012; Ouyang et al., 2013). In contrast, other studies have not detected a covariation between GCs and metabolism (e.g. MR, daily energy expenditure), perhaps because GCs and metabolism were not measured at the same time (e.g. Buehler et al., 2012; Wecker et al., 2009). A number of studies have employed exogenous GC administration to test for effects on metabolism (Preest and Cree, 2008; Miles et al., 2007; Wack et al., 2012; Buttemer et al., 1991; Wikelski et al., 1999; Spencer and Verhulst, 2008). However, results have been inconsistent, especially among endotherm species (Buttemer et al., 1991; Wikelski et al., 1999; Spencer and Verhulst, 2008), perhaps because GC-induced increases in blood glucose levels may be required to maintain a high MR, but they may not necessarily cause a high MR.

Despite the wealth of circumstantial evidence for a GC–MR association, we are not aware of studies in which MR was simultaneously manipulated and measured in conjunction with GC measurements. The latter is an important addition, because measured effects on MR are more convincing than assumed effects. Furthermore, direct measurements are necessary for direct quantification of the GC–MR association, and individual variation in MR can otherwise not be incorporated in the analyses. We therefore tested for an association between manipulated MR and endogenous corticosterone (CORT; the main GC in birds) in captive zebra finches (Taeniopygia guttata), using both between- and within-individual approaches. For each individual, we measured CORT and...
MR (oxygen consumption) in ‘warm’ (room temperature; 22°C) and ‘cold’ (12°C) conditions. Both temperatures are below the thermoneutral zone of zebra finches and differ strongly in the imposed thermoregulatory demands, i.e. energy expenditure (Calder, 1964; Briga and Verhulst, 2017). Based on the hypothesis that CORT variation reflects metabolic needs, we predicted that each individual will increase CORT when exposed to the cold compared to the room-temperature treatment insofar as the cold treatment induces an increase in MR (within-individual approach). We tested for the same association between individuals, but have less of a prediction at this level because there may be individual variation in the CORT–MR association, leading to weak or no correlation at the between-individual level (e.g. Goymann and Dávila, 2017). Finally, we compared the CORT–MR association between the sexes, because we previously found that natural variation in ambient temperature was related to CORT in females but not in males, and this contrast can potentially be explained by sex differences in the CORT–MR association (Jimeno et al., 2017).

MATERIALS AND METHODS

Subjects
A total of 36 birds (18 males and 18 females; *Taeniopygia guttata* Reichenbach 1862) were used in this study. They were reared as part of a larger population in our facilities at the University of Groningen, The Netherlands, in outdoor aviaries (L×H×W: 310×210×150 cm) containing 12 pairs each, with free access to food and water. After reaching independence, birds were moved to big single-sex outdoor aviaries (L×H×W: 310×210×300 cm). One month before the experiment started, birds were moved to four separate single-sex outdoor aviaries (L×H×W: 310×210×150 cm) with 10 birds each. Food and water were provided *ad libitum*. To avoid potential age effects, all birds were of similar age (8–13 months) when the experiment started, and born during the breeding season of 2014.

Blood sampling and experimental treatments
The experiment was carried out during April and May 2015. Each bird went through four respirometry sessions (with a minimum time of 2 weeks of recovery time between sessions) of 3 h each, and was subjected to four different treatments in random order, two of which were the warm and cold treatment mentioned above (the treatments in the other two sessions – 15 min noise stress applied either early or late during the 3 h measurement session – fall outside the scope of the present paper). The identity of the bird to be sampled was predetermined and target birds were previously marked with colour rings to facilitate their individual identification when catching. In the ‘warm’ treatment, the ambient temperature was kept at 22°C for the entire session (3 h). In the ‘cold’ treatment, the ambient temperature was decreased to 12°C after 1.5 h, and kept low for the remaining 1.5 h. Average temperature in the outdoor aviaries during sampling hours was 14.38±0.38°C (mean±s.e.m.). Respirometry measurements were conducted either in the morning (9:00–13:00 h) or in the afternoon (14:00–18:00 h). In each respirometry session, two birds (one male and one female) were measured simultaneously, and the sets of two birds remained the same throughout all trials.

Birds were captured from the outdoors aviaries (one bird per aviary per day) and transported indoors into the respirometer room in separate cages (L×W×H: 40×40×15 cm) with access to food. See Fig. 1 for a schematic overview of the measurement procedure. The birds were left undisturbed in the respirometer room for 1 h to acclimate to room temperature (22°C), after which we took the first blood sample for CORT analysis (CORTstart). Birds were weighed to the nearest 0.1 g before going into the metabolic chambers. The door of the respirometer room was then closed, and MR measurements started. During the following 1.5 h, the birds remained undisturbed to further acclimatize to the metabolic chambers. After this time, for the remaining 1.5 h, the temperature was either decreased to 12°C in the cold treatment (taking 15–20 min; temperature was changed without entering the room), or kept at 22°C for the warm treatment. After this time, the MR was at an approximately stable level (Fig. 2), birds were taken out and a second blood sample was taken (CORTend). Afterwards, they were put into a cage (L×W×H: 40×40×15 cm) with food and water to recover before being returned to their aviary.

All CORT samples were taken within 3 min after entering the respirometer chamber to minimize disturbance effects on CORT values.

Metabolic rate
MR was measured using an open-flow respirometer situated in a temperature-controlled room. Each individual was transferred to a 1.5 l metabolic chamber, without food or water. For detailed information about the technique, see Bouwhuis et al. (2011). In brief, the air flow through the metabolic chambers was kept at 22.1 l h⁻¹ by mass-flow controllers (58505S; Brooks, Rijswijk, The Netherlands) calibrated with a bubble flowmeter. The air was dried using a molecular sieve (3 Å; Merck, Darmstadt, Germany) and analysed using a paramagnetic oxygen analyser (Servomex Xentra 4100, Crowborough, UK). During the measurements, each metabolic chamber or reference outdoor air was sampled for 60 s every 3 min. In each sampling, we measured O₂ and CO₂ concentration and oxygen consumption was calculated using the equation of Hill (1972). An energy equivalent of 19.7 kJ l⁻¹ oxygen consumed was used to calculate energy expenditure in watt (W). When analysing the data, we took the average MR during the last 10 min of the 3 h session as a measure of MR (MRend).

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**Fig. 1. Timeline of the experiment.**
variables had a significant effect on CORTend (Table S1), so we did not include them in further analyses.

For the within-individual approach, we used the change in CORT between the two treatments [calculated as (CORTend in cold) − (CORTend in warm)] as the dependent variable and change in MR [as (MRend in cold) − (MRend in warm)] and sex as predictors. We did not consider CORTstart a proper control for the treatment effect because the experience of the animal prior to sampling (capture and handling) was very different from the experience prior to the sample after treatment. Assay plate was included as a random factor.

While building the two models described above, we used backward elimination of least significant terms. After model selection, the Akaike information criterion (AIC; Akaike, 1973) was also considered to confirm that the final models had the lowest AIC values. All statistical analyses were performed using R version 3.3.2 (http://www.R-project.org/) with the function ‘lmer’ of the R package lme4 (Bates et al., 2015). $R^2$ was calculated using the function ‘r.squaredGLMM’ of the R package MuMIn (https://CRAN.R-project.org/package=MuMIn). Logarithmic transformations were performed to normalize CORT. CORT change was calculated as ln(CORTcold)−ln(CORTwarm). Residuals of the final models showed a normal distribution. While building the models, one individual male was excluded from the between-individual analyses because it was a clear statistical outlier (this data point was 2.75 times the s.d. of the model residuals). That was not the case in the within-individual analyses, where its residuals were within 1 s.d. (0.25) of the model residuals.

**Hormone analyses**

Plasma CORT concentrations were measured using an enzyme immunoassay kit (cat. no. ADI-900-097, ENZO Life Sciences, Lausen, Switzerland), following previously established protocols (Ouyang et al., 2015). Samples taken from one individual were placed in neighbouring wells but, in other respects, samples were randomly distributed. Briefly, aliquots of 10 μl plasma along with a buffer blank and two positive controls (at 20 ng ml$^{-1}$) were extracted with diethyl ether. After evaporation, samples were re-dissolved in 280 μl assay buffer. On the next day, two 100 μl duplicates of each sample were added to an assay plate and taken through the assay. Buffer blanks were at or below the assay’s lower detection limit (27 pg ml$^{-1}$). Intra-plate coefficient of variation (CV; mean±s.e.m.) was 10.76±2.77% and inter-plate CV was 8.2% (n=11 plates; note that plate identity was included as a random effect in the statistical analyses). Samples with CV >20% were re-assayed when there was sufficient plasma. Final CORT concentrations were corrected for average loss of sample during extraction, which is 15% in our laboratory (Baugh et al., 2014).

**Statistical analyses**

We used paired t-test to test for the effect of temperature treatments on both MR and CORT. To assess the CORT–MR association, we constructed general linear mixed models for both the between-individual and within-individual approaches. For the between-individual approach, we used CORTend values as the dependent variable and MRen, sex, body mass (as the average between the two measurements taken before and after going into the respirometer) and treatment (warm or cold) as predictors. Individual identity and assay plate (CORT analyses) were included as random factors. Visual inspection of the data suggested the relationship between MR and CORT to be non-linear, so we tested for a quadratic effect of MR on CORT in the analysis. We also tested for potential effects of sampling variables on CORTend variation: sampling round (morning/afternoon), sampling order within the pair (first or second) and whether or not it was the first time the individual was placed into the respirometer. However, none of these variables had a significant effect on CORTend (Table S1), so we did not include them in further analyses.

![Fig. 2. Metabolic rate (MR) throughout the entire trial (3 h). The MRs for the warm and cold treatments are plotted (means±s.e.m.) for each 15 min interval. The arrow indicates the point at which the temperature was decreased in the cold treatment.](image-url)
Within-individual approach

Within-individual changes in MR induced by cold treatment were positively correlated with the associated changes induced by ambient temperature in CORT (Table 2, Fig. 5). Thus, higher temperature-induced increases in MR were associated with higher increases in CORT. This association did not differ between the sexes ($F_{1,24.34}=0.003, P=0.96$).

### DISCUSSION

The generally accepted association between GCs and MR is supported by a wealth of circumstantial evidence (see Introduction), but we are not aware of previous direct measurements combined with manipulations of MR in relation to CORT. We therefore manipulated MR through temperature change and found a strong positive CORT–MR association. Speculating on the mechanism causing the observed association falls outside the scope of the present paper but, on a functional level, our interpretation of this finding is that CORT ensured increased fuel (e.g. glucose, fatty acids) supply to match higher energetic needs, although we recognise that the evidence for such a relationship is mixed (Remage-Healey and Romero, 2001; Landys et al., 2004; Deviche et al., 2014). Our results are in line with previous studies finding a negative association between CORT and ambient temperature (Beaulieu, 2016; Jenni-Eiermann et al., 2008; Lendvai et al., 2009; de Bruijn and Romero, 2011; Jimeno et al., 2017), which generally has a strong effect on MR when below the thermoneutral zone (Briga and Verhulst, 2017). We found this association to be consistent both within and between individuals, and independent of temperature treatment, implying that variation in MR between individuals was associated with CORT in the same way as the temperature-induced variation. Furthermore, the CORT–MR

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<th>Table 1. Between-individual analyses of corticosterone (CORT) concentrations (ln transformed) in relation to body mass and metabolic rate (MR)</th>
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<td>Body mass</td>
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<td>Rejected terms</td>
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<td>Treatment (cold)</td>
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Main model: marginal $R^2=0.346$; conditional $R^2=0.519$.  

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<th>Table 2. Within-individual changes in CORT concentrations in relation to within-individual changes in MR</th>
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<td>Estimate</td>
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Main model: marginal $R^2=0.177$; conditional $R^2=0.399$. Changes in CORT concentrations were calculated as (lnCORTend in cold)−(lnCORTend in warm); changes in MR were calculated as (MRend in cold)−(MRend in warm); ‘end’ refers to the measurement taken at the end of the experiment.
association held across a broad CORT range, including baseline and stress-induced levels (as established in an earlier study on the same study population; Jimeno et al., 2017). The latter finding is in agreement with the comparative study of Haase et al. (2016), who found the GC–MR association to be similar for baseline and stress-induced GC levels.

To the best of our knowledge, MR has not previously been simultaneously manipulated and measured in conjunction with CORT measurements. However, de Bruijn and Romero (2011, 2013) used heart rate as a proxy of MR in conjunction with CORT measurements to investigate effects of experimentally changed climatic conditions in captive European starlings (Sturnus vulgaris). To compare our findings with the results of de Bruijn and Romero (2011, 2013), we plotted average values of heart rate versus CORT for each of their treatment groups (the authors did not report the associations between the two traits). We find that the magnitude of the treatment effects on CORT and MR were strongly correlated between the different treatments in all three groups of experiments (Fig. S1). Thus, we conclude that the results of de Bruijn and Romero (2011, 2013) are in close agreement with the conclusions of the present study that there is a strong association between MR and CORT.

At the time that we took the blood sample for CORT (CORTend), the MR had been stable for some time (Fig. 2). We can therefore assume that energy turnover and fuel supply (glucose) were reasonably in balance at the time of measurement, and this may have contributed to our finding that CORT and MR were strongly correlated. Conversely, we would expect such a correlation to be weaker or absent in the extreme case of CORT measurements immediately after an acute increase in energy expenditure (Beerling et al., 2011), while homeostasis is still in disequilibrium. However, such a correlation would likely become stronger with CORT levels measured after an as-yet-undefined time lag, when homeostasis is being restored. Thus, the temporal profile of energy expenditure and the relative timing of the CORT measurement may be crucial when investigating the CORT–MR association, and this may explain the absence of such an association in studies in which CORT and MR were not measured at the same time (e.g. Buehler et al., 2012).

MR and CORT were strongly correlated both at the between- and within-individual levels. However, the shape of the relationship differed between the two levels, being quadratic between individuals and linear within individuals (but note that CORT was logarithmically transformed prior to analysis). The explanation for this discrepancy can either be statistical or biological. A smaller range of variation within individuals when compared with the variation between individuals (see distributions along the x-axis in Figs 4 and 5) may have impeded the detection of a non-linear pattern within individuals. It is possible, therefore, that there also exists an accelerating pattern within individuals that we cannot detect with the present data. Experiments using a wider MR range would be needed to test this explanation. Alternatively, individuals with high CORT may have a lower sensitivity to CORT (which would be the reason for having high CORT), and hence they would need to up their CORT more to achieve the same physiological response as birds with higher CORT sensitivity (and hence low CORT). Such variation in CORT sensitivity of target cells could arise at any step in the causal chain from CORT to glucose and/or other plasma metabolites in the blood stream (see Bamberger et al., 1996) and could lead to associations within individuals being linear while accelerating (as before) upwards among individuals.

The present study was in part inspired by our previous finding in zebra finches of a strong relationship between ambient temperature and CORT in females housed outdoors, whereas this relationship was flat in males (Jimeno et al., 2017). A potential explanation for this finding was that the CORT–MR association is sex dependent, being flatter or absent in males when compared with females. However, the present study falsifies this hypothesis, because the MR and CORT responses to a decrease in ambient temperature were indistinguishable between males and females. However, this may be different in the outdoor aviaries where the birds are housed in groups. The sexes may, for example, differ in their huddling behaviour when subjected to natural variation in ambient temperature.

MR variation can arise in many different ways besides the effect of temperature that we employed. It remains to be tested, therefore, whether other short-term MR-modulating factors (e.g. psychological stressors) will cause MR to associate with CORT in the same way. However, some considerations lead us to believe that our finding of a strong CORT–MR relationship may apply more generally, regardless of context. First, our best-fitting models did not include treatment, which implies that the CORT–MR relationship we observed was independent of temperature context. Second, we find that the results of de Bruijn and Romero (2011, 2013), where MR was manipulated using different climatic variables in addition to temperature, are in complete agreement with our finding (Fig. S1). Last, Buwalda et al. (2012) previously showed that rewarding (sex) and aversive (defeat) social stimuli and habituation to these stimuli affected both CORT and heart rate in a very similar way. Nevertheless, further research is needed to determine to what extent the CORT–MR association we observed is consistent across contexts. To this end, studies are needed in which MR is manipulated in different ways and measured directly in conjunction with CORT measurements.

Acknowledgements
We thank Sabine Jörg for expertly running all assays and Gerard Overkamp for keeping the respirometry set up in good shape. We also thank Michael Briga and Renee Bensdorp for their help during the sampling sessions and the preparation of the experiment.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.J., M.H., S.V.; Methodology: B.J., M.H., S.V.; Formal analysis: B.J., S.V.; Investigation: B.J., M.H., S.V.; Resources: S.V., M.H.; Data curation: B.J.; Writing - original draft: B.J.; Writing - review & editing: B.J., M.H., S.V.; Visualization: B.J., M.H., S.V.; Supervision: M.H., S.V.; Project administration: M.H., S.V.; Funding acquisition: M.H., S.V.
Funding
B.J. was funded by the University of Groningen (Rijksuniversiteit Groningen) and the Max Planck Institute for Ornithology (Max-Planck-Gesellschaft). The Max Planck Society also funded the hormone assays.

Supplementary information
Supplementary information available online at http://jeb.biologists.org/lookup doi:10.1242/jeb.166124.supplemental

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