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Positive association between experimental cortisol increases and cage-measures of feeding behavior in wild-caught gerbils

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Supplementary material

Material S1

Validation and measurement of cortisol in *G. a. allenbyi* plasma samples

For detection of both exogenous and endogenous cortisol in blood plasma samples, we used ELISA kits from Cayman Chemical Company, Item no. 500360, and lot no. 0448270. The ELISA assay was conducted according to manufacturer's recommendations. According to the manufacturer, the kit sensitivity is 35pg/mL (0.035 ng/mL) and the linearity range is 30-800 pg/mL (0.03-0.8 ng/mL).

Samples

G. a.allenbyi plasma samples (n = 30) from first baseline sampling were pooled together. The kit suggests extraction of steroids from samples, but from our experience it is usually not needed. In order to examine the necessity of an extraction step, 50 µl of pooled GA plasma was diluted with 100 µl H_2O and extracted with 1 mL ethyl acetate for60seconds. Samples were then centrifuged at 13,000 rpm for 10 minutes. The steroid-containing fraction in the upper phase was then separated and evaporated by a stream of nitrogen. The extract was compared with a second set of samples that did not undergo extraction. Both were tested on the same ELISA plate. Analysis of the results showed that extracted vs. non-extracted gave similar results ($n = 30$; $t = 0.17$; $p = 0.08$), both were linear, and thus justified omitting the extraction before running samples through the ELISA kit.

Sample dilution

Samples were diluted 100 fold with water, except for several samples that had low cortisol levels (below the limit of detection) and were re-diluted 1:10.

GA pool

It contained baseline levels before pellet implantation. Average pool concentration was 595.5 pg/mL (0.596 ng/mL).

Assay validations

Serial dilutions of the pool provided a linearity range between 40pg/mL-700pg/mL (0.04 – 0.7 ng/mL).

Parallelism

The slopes of the standard reference and the serum sample curves were 1.14 and 0.79, respectively.

Sensitivity

The lowest concentration detected by the assay was 3.5 pg/mL (0.0035ng/mL).
Coefficient of variation Intra-assay repeatability was determined using 6 duplicates of the pool on the same ELISA plate. The coefficient of variation calculated was 5.5%.
Inter-assay precision was determined by running duplicates of the pool on 5 different days.

The coefficient of variation ranged between 8.5-15%, depending on its location on the plate.

Material S2

Cortisol is major glucocorticoid in *Gerbillus andersoni allenbyi.*

In order to determine the major glucocorticoid in gerbil circulation we quantified cortisol and corticosterone in 16 gerbil samples using LC-MS/MS (Liquid chromatography–mass spectrometry). Plasma samples were prepared according to Koren et al., 2012. The samples were analyzed using an AB SCIEX QTRAP® 5500 mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source and an Agilent 1200 liquid chromatograph (LC) system. Forty microlitres of sample was loaded onto a Phenomenex Kinetex 2.6 µ C18, 100 × 3.00 mm column maintained at 45˚C. Mobile Phase A consisted of water and Mobile Phase B consisted of methanol. For the analysis, the elution gradient was 10% B for 0.50 min, 10-40% B from 0.50-1.50 min, 40-70% B from 1.50-5.50 min, 70-80% B from 5.50-8.50 min, 80-95% B from 8.50-8.70 min, 95-95% B from 8.70-11.50 min, 95-10% B from 11.50 to 11.70 min, and held at 10% B from 11.70-15.00 min at a flow rate of 0.550 mL/min. A scheduled MRM (Multiple Reaction Monitoring) scan was performed in positive mode with a 30 second detection window. The mass spectrometer conditions and methods are as in Koren et al., 2012. Nitrogen was used as the source, nebulizer, and collision gasses. Mass resolution in Q1

and Q3 was set to unit resolution. Two transitions were monitored for each analyte, a quantifier transition (-1) and a qualifier transition (-2).

Table S1. Resulting hormonal levels for handling stress and corticosterone injection.

Sample name	Treatment	Cortisol (ng/ml)	Corticosterone (ng/ml)
GA 1	Control	46.34	8.42
GA 2	Control	< LOQ	2.20
GA 3	Control	2.45	1.68
GA 4	Control	< LOQ	0.41
GA 5	Control	1.02	1.15
GA 6	Control	< LOQ	0.60
GA 7	Handling stress	406.05	87.31
GA 8	Handling stress	288.13	54.29
GA 9	Handling stress	199.71	32.09
GA 10	Handling stress	187.55	71.74
GA 11	Handling stress	288.00	50.09
GA 12	Corticosterone injection	< LOQ	254.92
GA 13	Corticosterone injection	1.31	308.90
GA 14	Corticosterone injection	< LOQ	136.98
GA 15	Corticosterone injection	< LOD	274.48
GA 16	Corticosterone injection	< LOD	26.30

LOQ - limit of quantitation

LOQ = 1 ng/mL for cortisol

LOD - limit of detection

There is no correlation between cortisol and corticosterone concentration as obtained from OLS (Ordinary least squares) regression ($p = 0.606$). It is because both cortisol and corticosterone signal independently; nonetheless both of their reflected values to stress should be included in integrated endocrine models of stress responses (Koren et al., 2012). General linear model with hormone (cortisol and corticosterone) quantity (ng/ml) as dependent variable showed that the treatments (handling stress, and injecting corticosterone) affect cortisol levels differently than they affect corticosterone levels for the effect of Treatment ($MS = 72{,}433.622$, $F_{2,26} = 21.168$, $p < 0.001$) and Treatment X Hormone Type interactions ($MS = 107{,}558.639$, $F_{2,26} = 31.433$, $p < 0.001$). Multivariate test statistics using MANOVA indicated the same ($p < 0.001$) on resulting cortisol levels counting for within individual effects statistically.