Non-invasive cortisol measurements as indicators of physiological stress responses in guinea pigs

Matthias Nemeth, Elisabeth Pschernig, Bernard Wallner, Eva Millesi

Non-invasive measurements of glucocorticoid (GC) concentrations, including cortisol and corticosterone, serve as reliable indicators of adrenocortical activities and physiological stress loads in a variety of species. As an alternative to invasive analyses based on plasma, GC concentrations in saliva still represent single-point-of-time measurements, suitable for studying short-term or acute stress responses, whereas fecal GC metabolites (FGMs) reflect overall stress loads and stress responses after a species-specific time frame in the long-term. In our study species, the domestic guinea pig, GC measurements are commonly used to indicate stress responses to different environmental conditions, but the biological relevance of non-invasive measurements is widely unknown. We therefore established an experimental protocol based on the animals' natural stress responses to different environmental conditions and compared GC levels in plasma, saliva, and fecal samples during non-stressful social isolations and stressful two-hour social confrontations with unfamiliar individuals. Plasma and saliva cortisol concentrations were significantly increased directly after the social confrontations, and plasma and saliva cortisol levels were strongly correlated. This demonstrates a high biological relevance of GC measurements in saliva. FGM levels measured 20 hours afterwards, representing the reported mean gut passage time based on physiological validations, revealed that the overall stress load was not affected by the confrontations, but also no relations to plasma cortisol levels were detected. We therefore measured FGMs in two-hour intervals for 24 hours after another social confrontation and detected significantly increased levels after four to twelve hours, reaching peak concentrations already after six hours. Our findings confirm that non-invasive GC measurements in guinea pigs are highly biologically relevant in indicating physiological stress responses compared to circulating levels in plasma in the short- and long-term. Our approach also underlines the importance of detailed investigations on how to use and interpret non-invasive measurements, including the determination of appropriate time points for sample collections.

1 **Non-invasive cortisol measurements as indicators of**

2 **physiological stress responses in guinea pigs**

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Abstract

26   Non-invasive measurements of glucocorticoid (GC) concentrations, including cortisol and

27   corticosterone, serve as reliable indicators of adrenocortical activities and physiological stress

28   loads in a variety of species. As an alternative to invasive analyses based on plasma, GC

29   concentrations in saliva still represent single-point-of-time measurements, suitable for studying

30   short-term or acute stress responses, whereas fecal GC metabolites (FGMs) reflect overall stress

31   loads and stress responses after a species-specific time frame in the long-term. In our study

32   species, the domestic guinea pig, GC measurements are commonly used to indicate stress

33   responses to different environmental conditions, but the biological relevance of non-invasive

34   measurements is widely unknown. We therefore established an experimental protocol based on

35   the animals' natural stress responses to different environmental conditions and compared GC

36   levels in plasma, saliva, and fecal samples during non-stressful social isolations and stressful

37   two-hour social confrontations with unfamiliar individuals. Plasma and saliva cortisol

38   concentrations were significantly increased directly after the social confrontations, and plasma

39   and saliva cortisol levels were strongly correlated. This demonstrates a high biological relevance

40   of GC measurements in saliva. FGM levels measured 20 hours afterwards, representing the

41   reported mean gut passage time based on physiological validations, revealed that the overall

42   stress load was not affected by the confrontations, but also no relations to plasma cortisol levels

43   were detected. We therefore measured FGMs in two-hour intervals for 24 hours after another

44   social confrontation and detected significantly increased levels after four to twelve hours,

45   reaching peak concentrations already after six hours. Our findings confirm that non-invasive GC

46 measurements in guinea pigs are highly biologically relevant in indicating physiological stress

47 responses compared to circulating levels in plasma in the short- and long-term. Our approach

48 also underlines the importance of detailed investigations on how to use and interpret non-

49 invasive measurements, including the determination of appropriate time points for sample

50 collections.

51

52

Introduction

54   Non-invasive measurements of steroid hormones reliably reflect endocrine states and

55 have therefore become common tools in biological and biomedical research (Kersey & Dehnhard

56 2014). Investigations in many species often rely exclusively on non-invasive sampling methods,

57 because commonly applied analyses using plasma would be either not possible or simply

58 inappropriate because their high invasiveness can cause stress responses (Vahl et al. 2005).

59 Especially glucocorticoids (GCs), including cortisol and corticosterone, measured non-invasively

60 in saliva or fecal samples can reliably indicate an individual's hypothalamic-pituitary-adrenal

61 (HPA) axis activity and physiological stress responses (Sheriff et al. 2011; Cook 2012). Beyond

62 the importance of careful physiological and biological validations of non-invasive GC

63 measurement techniques (Goymann 2012; Touma & Palme 2005; Cook 2012), relations to the

64 biologically active circulating concentrations in plasma should additionally be tested to

65 determine their biological relevance. If non-invasively measured GC levels predict and resemble

66 those in plasma, they can be used to reliably indicate the strength of physiological stress

67 responses and related, well-described physiological actions of GCs (Munck, Guyre & Holbrook

68 1984; Blanchard, McKittrick & Blanchard 2001; McEwen 2008).

69   GCs and other circulating steroid hormones are transported unaltered and rapidly into the

70   salivary glands (Riad-Fahmy et al. 1982). Comparable to the circulating levels in plasma, saliva

71   GC concentrations also represent single-point-of-time measurements. Such measurements are

72   suitable for studying short-term or acute stress responses or for assessing the present

73   physiological stress load (Goymann 2005). Measurements in feces are, by contrast, not

74   essentially affected by short-term fluctuations of circulating GC levels, because GCs are

75   metabolized by the liver and excreted via the gut with a species-specific time lag (Palme et al.

76   2005). Fecal GC metabolite (FGM) concentrations therefore reflect long-term stress loads

77   (Goymann 2005). Accordingly, measurements in feces are usually preferred over plasma and

78   saliva to assess an individual's overall stress load especially in wild animals (Touma & Palme

79   2005). Although saliva cortisol and FGM levels indicate physiological stress loads in different

80   contexts, non-invasive GC measurements correlate highly with plasma levels in various species,

81   including horses (Peeters et al. 2011), cattle (Pérez et al. 2004), lemurs (Cavigelli 1999), rats

82   (Thanos et al. 2009), or humans (Hellhammer, Wüst & Kudielka 2009). A biological relevance

83   of non-invasive GC measurements is indicated if they predict plasma cortisol levels equally

84   under basal and stimulated conditions. Moreover, the ratio of the measured concentrations in

85   plasma and non-invasive sources must remain steady under the different conditions.

86   Our study species, the domestic guinea pig, is a classical laboratory model species, which

87   is extensively used in behavioral and physiological studies, for example regarding social

88   relationships and stress management (Sachser, Dürschlag & Hirzel 1998; Hennessy et al. 2006),

89   nutritional influences (Bauer, Dittami & Huber 2009; Weiler et al. 2012), or fatty acid and lipid

90   metabolism (Fu & Sinclair 2000; Fernandez & Volek 2006). Knowledge on the HPA-axis

91   reactivity and validity of non-invasive GC measurements are crucial for several research topics

92   conducted using this species. Non-invasive measurements of cortisol, which constitutes the

93   major GC in guinea pigs (Malinowska & Nathanielsz 1974), have been validated physiologically

94   via ACTH-challenges (injection of adrenocorticotropic hormone to induce the adrenal glands to

95   release GCs) for saliva cortisol levels (Fenske 1996; Fenske 1997) and FGMs (Bauer et al.

96   2008). Under the stressful conditions of the ACTH-challenge, a strong correlation between GC

97   levels in plasma and saliva was found (Fenske 1996), and a gut passage time with peak

98   concentrations of FGMs18-20 hours post injection was reported (Bauer et al. 2008). Nonetheless,

99   the power of the biological relevance of non-invasive GC measurements under different

100 conditions and different physiological stress loads, indicated by circulating cortisol levels in

101 plasma, remains unclear.

102   Changes in environmental conditions can evoke physiological stress responses and

103 increased GC secretion rates (Wingfield et al. 1998; Romero 2002). Especially changes in the

104 social environment can strongly affect physiological stress loads and can easily be achieved via

105 social confrontations with unfamiliar conspecifics (Goymann et al. 1999; Franceschini et al.

106 2007; Kuo, Jong & Lai 2011). In order to demonstrate the biological relevance of non-invasive

107 GC measurements in guinea pigs, we analyzed and compared GC levels in plasma, saliva, and

108 fecal samples, as well as their ratios and relations during social isolations, a non-stressful

109 situation (Machatschke et al. 2011; Nemeth et al. 2015), and after two-hour social confrontations

110 with unfamiliar individuals in a novel environment, which also constitutes a highly stressful

111 situation in our study species (Wallner & Dittami 2003; Nemeth et al. 2014). We hypothesize

112 that non-invasive GC measurements reflect plasma GC levels under basal and stressful

113 conditions in guinea pigs. We expect elevated plasma and saliva GC levels shortly after social

114 confrontations compared to the social isolation phase and, considering the gut passage time, a

115 delayed increase in FGM concentrations in response to the social stressor.

116

117

Methods

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Animals and housing conditions

120

121   A total of 30 sexually intact male domestic guinea pigs (*Cavia aperea f. porcellus*), bred

122 at the Department of Behavioural Biology, University of Vienna, Austria, were used in this

123 study. The animals were 18.1 ± 3.6 (mean $\pm$ SD) months old and weighed 992.8 ± 88.3 g (mean

124 $\pm$ SD). All animals could be individually identified by natural fur marks. Prior to the

125 experiments, animals were kept in socially established single-sexed groups of ten individuals.

126 Each groups' enclosure (2 m × 1.6 m) was environmentally enriched and the floor covered with

127 standard woodchip bedding material. The daily provided food consisted of guinea pig pellets

128 (ssniff V2233, ssniff Spezialdiäten GmbH, Soest, Germany) and some hay to promote tooth

129 abrasion. Tap water was provided in several drinking bottles. Pellets and water were always

130 available *ad libitum*. Animals were kept under constant conditions, including a light-dark cycle

131 of 12/12 h, with lights on at 07:00 a.m., a temperature of 22 ± 2 °C, and a humidity of 50 ± 5 %.

132   Housing conditions and the experimental procedure were in line with EU Directive

133 2010/63/EU and the Austrian laws for animal experiments and animal keeping. The study was

134 checked and approved by the internal board on animal ethics and experimentation of the Faculty

135 of Life Sciences, University of Vienna, Austria (# 2014-005), and permitted by the Austrian

136 Federal Ministry of Science and Research (BMWF-66.006/0024-II/3b/2013).

137

Experimental procedure

138

139   The study consisted of two experiments, which were carried out on a total of 30 animals

140 (20 animals for experiment 1 and ten animals for experiment 2). Experiment 1 was conducted to

141 analyze and compare cortisol concentrations in plasma, saliva, and fecal samples, as well as their

142 ratios and relations repeatedly during social isolation and after social confrontations with

143 unfamiliar individuals in a novel environment. This approach reliably elicits physiological stress

144 responses in all involved individuals, as it represents an equally challenging situation for all

145 animals with no advantages of being a resource holder or of already established social

146 hierarchies between single individuals, which can affect GC levels as well. The situation is

147 therefore unbiased by previous behavioral and environmental factors and usually constitutes a

148 strong stressor in all involved animals (Wallner & Dittami 2003; Nemeth et al. 2014), while

149 social isolations can be considered as a non-stressful situation in this species (Machatschke et al.

150 2011; Nemeth et al. 2015). As physiological stress loads were not reflected in feces in the first

151 experiment, another experiment was performed to monitor FGM levels at regular intervals

152 during social isolation and after a social confrontation. This enabled monitoring the diurnal

153 variation, the gut passage time, and peak concentrations in response to the social stressor. Both

154 experiments, including the different conditions (social isolation and social confrontations), were

155 carried out in a separate test room under the same ambient conditions. For the experiments,

156 animals were transferred from their social groups to single cages, so they were visually and

157 socially isolated from each other. Although auditory and olfactory contact with the other isolated

158 animals in the test room was still possible, social isolations prevented direct social interactions

159 between the individuals, which usually results in low physiological stress loads (Nemeth et al.

160 2014). The floor of each cage (100 cm × 60 cm × 45 cm) was covered with woodchip bedding

161 material and a shelter was provided. Guinea pig pellets and water were available *ad libitum*

162 throughout the experiments, and a daily ration of 5 g of hay was provided to each animal.

163

Experiment 1

165   Experiment 1 was carried out in two consecutive runs, each starting at 12:00 p.m., with

166 ten animals being tested per run. After the animals were transferred singly to cages, they were

167 left undisturbed for the first 24 hours. On the following three days (days 'Iso1', 'Iso2', 'Iso3'),

168 animals remained socially isolated in their cages and baseline blood, saliva, and fecal samples

169 were collected during this social isolation period. On each of the next three days (days 'Soc1',

170 'Soc2', 'Soc3'), all animals of the current run were socially confronted with each other at 10:00

171 a.m. Here, they were transferred to a squared arena (1.6 m × 1.6 m) placed in the same room.

172 The walls of the arena were built of laminated fiberboard and the floor was covered with

173 woodchip bedding material. Animals remained together within the arena until 12:00 p.m. for

174 social interactions. After these daily two-hour social confrontations, they were returned to their

175 cages. Blood, saliva, and fecal samples were collected after social confrontations in order to

176 measure cortisol concentrations due to the stressful condition. To factor out the circadian rhythm

177 of circulating cortisol levels (Garris 1979; Fujieda et al. 1982), blood and saliva samples were

178 always collected at 12:00 p.m., directly after each other, during social isolation and after social

179 confrontations. The whole procedure lasted less than three minutes per animal. Fecal samples

180 were collected daily at 08:00 a.m., starting 20 hours after the first collection of blood and saliva

181 samples (referred to as '+20h' samples in the following). This time span was previously shown

182 to reflect peak concentrations of fecal cortisol metabolites in guinea pigs under stressful

183 conditions using an ACTH-challenge test (Bauer et al. 2008). After the last collection of fecal

184   samples, animals were returned to their single-sexed groups.

185

186   **Experiment 2**

187   According to the procedure in experiment 1, also in experiment 2 animals were

188   transferred singly to cages at 12:00 p.m. and left undisturbed for the first 24 hours. For the next

189   24 hours, animals remained in their cages and baseline saliva and fecal samples were collected

190   during isolation. A single saliva sample was collected at 12:00 p.m., fecal samples were collected

191   at 12:00 p.m. and at 18:00 p.m. on the same day, and at 06:00 a.m. on the following day (these

192   saliva and fecal samples are referred to as 'Base' samples in the following). Afterwards, at 10:00

193   a.m., all animals were transferred to a squared arena for two hours, as described in experiment 1,

194   and then returned to their cages. After the social confrontation, saliva samples were collected at

195   12:00 p.m. (referred to as 'postSoc' sample in the following) as a control for the acute HPA-axis

196   reactivity to the social stressor. Starting at 14:00 p.m., fecal samples were collected in two-hour

197   intervals until 12:00 p.m. on the following day, 24 hours after the end of the social confrontation

198   (referred to as samples 'postSoc2', 'postSoc4', … , 'postSoc24' in the following). Each sample

199   included all the feces that were defecated during the preceding two hours. After the last

200   collection of fecal samples (24 hours after the end of the social confrontation) saliva samples

201   were collected again (referred to as 'postSoc24' sample in the following). Throughout

202   experiment 2, a red light was placed in the test room during the night time (19:00 p.m. until

203   07:00 a.m.), which could not been perceived by the animals but allowed an unrestricted

204   collection of fecal samples. After the final fecal and saliva samples, animals were returned to

205   their single-sexed groups.

206

Sample collections

208   Saliva samples were collected following Fenske (1997) by inserting a standard cotton bud

209   into the animal's cheek pouch for 30 seconds. Cotton buds with saliva were stored in 1.5 ml

210   tubes containing previously prepared brackets made of 1 ml pipette tips: a tip was cut into four

211   pieces of the same length; the two outer pieces were discarded; the two inner pieces were slotted

212   together and put into the tube so that the conical inner piece of the former tip could hold the

213   cotton bud. Saliva was extracted from the cotton buds by centrifugation (10 minutes, 14000 rpm,

214   17968 × g). Brackets and dry cotton buds were removed and pure saliva was stored at -20 °C

215   until further analysis.

216   Blood samples were collected via ear veins based on Sachser & Pröve (1984) with few

217   modifications. Immediately after gently squeezing one of the ears to accumulate blood in the

218   veins, a prominent ear vein was punctured with a sterile lancet and approximately 300 µl of the

219   flowing blood was collected with heparinized (5000 units) micropipettes and released into 1.5 ml

220   tubes. Plasma was separated by centrifugation (10 minutes, 14000 rpm, 17968 × g) in two

221   consecutive steps, to obtain pure plasma, which was then transferred to a new 1.5 ml tube and

222   stored at -20 °C until further analysis.

223   Fecal samples were collected directly from the bedding material. Only those samples

224   were collected that were definitely not contaminated with urine. Fecal samples were stored at -20

225   °C until further analysis.

226

Hormone analyses

228   All hormonal analyses, using biotin-strepdavidin enzyme-linked immunoassays, and

229   preceding hormone extractions from plasma and fecal samples followed Palme & Möstl (1993;

230 1997). Enzymes and antibodies were purchased from the University of Veterinary Medicine,

231 Vienna, Austria. All analyses were run in duplicates; the confidence criterion for the samples

232 was set at a coefficient of variance (CV) of ≤ 15 % for duplicates. The CV for sample duplicates

233 was calculated as the percentage of the standard deviation of the duplicates on the mean of the

234 duplicates. This serves as a control of the sample and analysis quality (in CVs > 15 %, samples

235 would have to be reanalyzed or excluded from further analyses).

236   Cortisol was extracted from plasma samples by adding 2 ml diethyl ether (100 %) to 100

237 µl of plasma. After vortexing the samples four times for 15 minutes each, with 15minute

238 intermissions in between, they were frozen overnight at -20 °C and evaporated at 30 °C

239 afterwards. Samples were then diluted 1:400 and analyzed using a cortisol-specific antibody

240 (Palme & Möstl 1997). Analysis of saliva cortisol concentrations did not require any preceding

241 extraction procedures. Saliva was diluted 1:50 and analyzed using the same cortisol-specific

242 antibody as used for plasma cortisol concentrations. Fecal samples were dehumidified at 60 °C,

243 crushed, and 0.1 g of the samples was suspended in 2 ml methanol (80 %). After centrifugation

244 (15 minutes, 3000 rpm, 825 × g), samples were diluted 1:20 and analyzed using an 11-

245 oxoetiocholanolone antibody measuring FGMs (Palme & Möstl 1997). Intra- and inter-CVs

246 based on pooled control samples were as follow: plasma 6.9 % (intra) and 4.2 % (inter), saliva

247 11.3 % (intra) and 8.6 % (inter), feces 5.3 % (intra) and 7.5 % (inter).

248

249 Calculations of plasma cortisol : saliva cortisol and plasma cortisol :

250 FGM ratios

251   To further assess of the biological relevance of non-invasive GC measurements in

252 experiment 1, the plasma cortisol : saliva cortisol ratio and the plasma cortisol : FGM ratio were

253 calculated for each single animal and sampling point in time. Cortisol levels in plasma served as

254 reference values because they represent the actual and biologically relevant stress load, but

255 especially GC measurements in feces appeared to be higher in approximately half of the

256 samplings. The calculations were therefore performed by using a formula, which linearizes the

257 ratios across positive and negative values:

258
$$x = e^{\left| \ln\left(\frac{a}{b}\right) \right|} * \frac{a - b}{|a - b|}$$

259 By exponentiating Euler's number with the absolute value of the natural logarithm of the

260 quotient of two values (in this case: a = plasma cortisol levels, b = saliva cortisol or FGM

261 levels), the absolute positive factorial difference between the two values was calculated, meaning

262 that $x > 1$ for each calculation. Multiplication with the quotient of the difference between the two

263 values a and b, and the absolute value of the difference, resulted in a positive value ($x > 1$) if $a >$

264 b or a negative value ($x < 1$) if $a < b$, given that $a \neq b$. Considering two positive and unequal

265 values for a and b, dividing a by b will yield a value of $+ x$, while dividing b by a will yield a

266 value of $- x$; the mean of a/b and b/a would be 0. In the present data, in which ratios were

267 mostly undirected throughout the sampling points in time, this formula was most qualified for

268 further statistical analyses of the ratios.

269

Statistical analysis

271 All statistics were performed using the statistical package R version 3.1.0 (R

272 Development Core Team, 2013) and additional packages 'nlme' (Pinheiro et al. 2013), to

273 perform linear mixed models (LMEs) due to repeated measurement analyses, and 'phia' (De

274 Rosario-Martinez 2013) for post-hoc interaction analyses of significant effects.

275   Plasma, saliva, and FGM concentrations, as well as the plasma cortisol : saliva cortisol

276   ratio and the plasma cortisol : FGM ratio in experiment 1 were analyzed separately by

277   performing LMEs on the time course across the experiment with 'days' ('day1', 'day2', 'day3'),

278   'condition' ('isolation', 'social confrontation'), and their interaction as fixed effects and

279   individual ID as random effect to correct for the repeated measurements. To determine the

280   degree to which saliva cortisol and FGM concentrations predict plasma cortisol levels under non-

281   stressed and stressful conditions, an LME was performed with 'saliva cortisol', 'fecal cortisol',

282   and 'condition' ('isolation', 'social confrontation'), including interactions of condition with

283   saliva cortisol and fecal cortisol as fixed effects, and individual ID as random effect. To assess

284   the immunoassay's quality, an LME was performed on the sample duplicates' CVs, including the

285   'sampling type' ('plasma', 'saliva', 'feces') and the 'actual cortisol concentration' as fixed

286   effects, and individual ID and sampling type as random effect, to control for repeated measures

287   within single individuals and across the different sampling types. Significant factorial effects

288   were further analyzed by applying post-hoc interaction analyses with Bonferroni corrections.

289   Saliva cortisol and FGM concentrations in experiment 2 were analyzed by applying

290   LMEs with 'sampling time' (saliva cortisol: 'Base', 'postSoc', 'postSoc24'; fecal cortisol:

291   'Base', 'postSoc2', 'postSoc4', … , 'postSoc24') as fixed factor and individual ID as random

292   effect due to the repeated measurements. Another LME was calculated with saliva cortisol as

293   response variable, FGM levels as fixed effect, and individual ID as random effect, to analyze

294   whether fecal cortisol levels predict saliva cortisol levels.

295   Model assumptions (linearity, normal distribution, and homogeneity of variance) were

296   checked by visually inspecting of residual and fitted value plots as well as by performing

297   Shapiro-Wilk normality tests and Levene's tests for homogeneity of variance. Some of the data

298 had to be transformed by applying the natural logarithm or by taking the second or third root. To

299 ease interpretation, non-transformed data were used for visualizations whenever this was

300 appropriate. Significance was set at a level of $p \leq 0.05$. All values represent means ± standard

301 errors of the mean.

302

303

Results

305

Experiment 1

Plasma cortisol, saliva cortisol, and FGM concentrations

308   Plasma cortisol concentrations differed significantly between and within the experimental

309 conditions (days: $F_{2,95} = 0.370$, $p = 0.692$; condition: $F_{1,95} = 62.234$, $p < 0.001$; days:condition:

310 $F_{2,95} = 5.077$, $p = 0.008$) (Fig. 1A) and were generally higher after social confrontations

311 compared to the social isolation period. While no differences were detected between the single

312 days of social isolation ($\chi^2 = 0.739$, $p = 1$), plasma cortisol concentrations were significantly

313 higher after the first social confrontation compared to the third (day1-day2: $\chi^2 = 2.670$, $p =$

314 0.614; day1-day3: $\chi^2 = 16.854$, $p < 0.001$; day2-day3: $\chi^2 = 6.108$, $p = 0.081$).

315   Saliva cortisol concentrations were exclusively affected by the experimental conditions,

316 with no day-related effects (days: $F_{2,95} = 0.872$, $p = 0.422$; condition: $F_{1,95} = 19.401$, $p < 0.001$;

317 days:condition: $F_{2,95} = 1.750$, $p = 0.179$) (Fig. 1B). Corresponding to plasma cortisol, saliva

318 cortisol levels were significantly increased after social confrontations.

319   Concentrations of FGMs measured in the +20h samples showed significant differences

320   between and within the experimental conditions (days: $F_{2,95} = 3.671$, $p = 0.029$; condition: $F_{1,95} =$

321   9.454, $p = 0.003$; days:condition: $F_{2,95} = 3.541$, $p = 0.033$) (Fig. 1C) and were generally lower

322   during the three days of social confrontations compared to the social isolation period. While an

323   almost significant difference was detected between the single days throughout the social isolation

324   period ($\chi^2 = 7.342$, $p = 0.051$), where animals showed slightly increased levels on the first day,

325   no differences between the single days were detected after social confrontations ($\chi^2 = 1.779$, $p =$

326   0.822).

327   Only saliva cortisol concentrations proved successful in predicting plasma cortisol levels

328   ($F_{1,98} = 78.154$, $p < 0.001$; $R^2 = 0.402$) (Fig. 2), which was not the case for FGM levels ($F_{1,98} =$

329   2.128, $p = 0.148$; $R^2 < 0.001$). These effects did not differ between experimental conditions

330   (saliva:condition $F_{1,95} = 1.599$, $p = 0.209$, feces:condition $F_{1,95} = 3.705$, $p = 0.057$).

331

332   Plasma cortisol : saliva cortisol and plasma cortisol : FGM ratios

333   The logarithmized ratio of plasma and saliva cortisol showed no significant changes

334   throughout the experiment (days: $F_{2,95} = 0.531$, $p = 0.590$; condition: $F_{1,95} = 0.620$, $p = 0.433$;

335   days:condition: $F_{2,95} = 0.491$, $p = 0.613$). The mean logarithmized ratio was 2.328 (9.944 after

336   back transformation).

337   For the logarithmized ratio of plasma cortisol and FGM levels, significant differences

338   were found between and within the experimental conditions (days: $F_{2,95} = 2.920$, $p = 0.059$;

339   condition: $F_{1,95} = 51.880$, $p < 0.001$; days:condition: $F_{2,95} = 6.148$, $p = 0.003$), with values < 0

340   during isolation and > 0 during social confrontations. No differences were determined between

341   the single days during the social isolation period ($\chi^2 = 5.840$, $p = 0.108$), but a significantly

342   higher ratio was detected after the first social confrontation compared to the third (day1-day2: χ^2

343     $= 0.890$, $p = 1$; day1-day3: $\chi^2 = 8.016$, $p = 0.028$; day2-day3: $\chi^2 = 3.563$, $p = 0.354$).

344

345   **Coefficients of variance (CV) for duplicates of plasma cortisol, saliva cortisol,**

346   **and FGM levels**

347   The CVs for duplicate sample analyses, serving as a quality control for the hormonal

348   analyses, differed significantly between the sampling types but showed no relations to the actual

349   cortisol concentrations (sample: $F_{2,38} = 7.021$, $p < 0.001$; cortisol: $F_{1,297} = 2.564$, $p = 0.110$;

350   sample:cortisol: $F_{2,297} = 1.702$, $p = 0.184$). The mean CV was significantly lowest for FGMs,

351   highest for saliva cortisol, and intermediate for plasma cortisol (plasma-saliva: $\chi^2 = 8.193$, $p =$

352   0.013; plasma-feces: $\chi^2 = 6.480$, $p = 0.033$; saliva-feces: $\chi^2 = 15.718$, $p < 0.001$) (Fig. 3).

353

354   # Experiment 2

355   **Saliva cortisol concentrations**

356   Saliva cortisol concentrations measured throughout experiment 2 showed significant

357   differences across the sampling points ($F_{2,18} = 49.142$, $p < 0.001$). Highest levels were measured

358   directly after the social confrontation, with significantly lower levels 24 hours before and 24

359   hours after social confrontation (Base: 12.34 ± 1.88 ng/ml, postSoc: 71.85 ± 14.07 ng/ml,

360   postSoc24: 6.55 ± 1.01 ng/ml; Base-postSoc: $\chi^2 = 49.704$, $p < 0.001$; Base-postSoc24: $\chi^2 =$

361   6.306, $p = 0.036$; postSoc-postSoc24: $\chi^2 = 91.416$, $p < 0.001$).

362

363   **FGM concentrations**

364   Baseline FGM concentrations measured three times within 24 hours before the social

365   confrontation at 12:00 p.m., 18:00 p.m., and 06:00 a.m. did not differ from each other ($F_{2,18} =$

366   1.584, $p = 0.235$; 12:00 p.m.: 319.25 ± 54.71 ng/g, 18:00 p.m.: 316.82 ± 41.07 ng/g, 06:00 a.m.:

367   411.64 ± 83.12 ng/g). The mean of these samples was therefore calculated and served as an

368   overall baseline value for the analysis of FGM levels measured in two-hour intervals after the

369   social confrontation.

370   The time course of FGM levels, including the mean baseline value measured on the day

371   before the social confrontation and values measured in two-hour intervals until 24 hours after the

372   social confrontation, was highly significant ($F_{12,108} = 13.8247$, $p < 0.001$) (Fig. 4). Significantly

373   increased FGM levels were detected from four to twelve hours after the end of the social

374   confrontation compared to mean baseline values, with peak concentrations occurring six hours

375   after the social confrontation (2h: $t = 1.642$, $p = 0.104$; 4h: $t = 4.439$, $p < 0.001$; 6h: $t = 8.619$, p

376   < 0.001; 8h: $t = 5.972$, $p < 0.001$; 10h: $t = 3.535$, $p = 0.001$; 12h: $t = 2.698$, $p = 0.008$; 14h: $t =$

377   0.258, $p = 0.797$; 16h: $t = 0.176$, $p = 0.861$; 18h: $t = 1.507$, $p = 0.135$; 20h: $t = 1.015$, $p = 0.312$;

378   22h: $t = 1.102$, $p = 0.273$; 24h: $t = 0.395$, $p = 0.693$).

379   It also proved successful to predict saliva cortisol concentrations by FGM levels

380   measured 6 hours afterwards ($F_{1,9} = 13.473$, $p = 0.005$; $R^2 = 0.428$) (Fig. 5).

381

382

Discussion

384   The present study revealed a high biological relevance of non-invasive GC measurements

385   in saliva and fecal samples of domestic guinea pigs. Saliva cortisol and FGM levels measured in

386   samples adjusted to the appropriate gut passage time were both significantly increased in

387   response to the social confrontations and were highly correlated to the actual circulating cortisol

388 levels in plasma. In addition to previously conducted physiological validation studies, our

389 findings indicate that non-invasive GC measurements go beyond indicating the presence of a

390 physiological stress response to also showing the strength of such a response to a prevalent

391 stressor.

392   Subjecting guinea pigs to social confrontations with unfamiliar individuals in a novel

393 environment for two hours significantly increased plasma and saliva cortisol concentrations in

394 experiment 1. Similar responses were measured on all three days of social confrontations

395 compared to the isolation period. This confirms our experimental approach of reliably evoking

396 physiological stress responses by subjecting the animals to this challenging situation, as shown

397 previously (Nemeth et al. 2014), without any pharmacological stimuli such as ACTH injections.

398 FGM levels measured in the +20h fecal samples throughout experiment 1, however, remained

399 relatively constant. This indicates that the overall stress load in experiment 1 was not affected by

400 the two-hour social confrontations, otherwise elevated levels throughout the period with daily

401 applied social stress would be expected, but also that physiological stress loads are not reflected

402 in feces 20 hours after a stressful situation. Importantly, as +20h FGM levels were not affected,

403 we conclude that plasma and saliva cortisol levels both reflected the acute stress response to the

404 applied social stressor and that the animals were not long-lastingly stressed. This is of major

405 interest because numerous guinea pig studies investigate short-term or acute stress responses due

406 to different types of stressors or environmental conditions, and these have mainly involved

407 plasma cortisol measurements (Machatschke et al. 2004; Zipser, Kaiser & Sachser 2013). Plasma

408 cortisol levels could be highly predicted by saliva cortisol measurements in this study, supporting

409 previous findings based on physiological validations in guinea pigs (Fenske 1996; Fenske 1997).

410 Our results further confirm the use of saliva samples for measuring physiological stress loads

411   because the same relations were found during non-stressful and stressful conditions. We

412   therefore suggest that GC measurements in saliva can be reliably used in future studies to assess

413   short-term physiological stress responses in guinea pigs. As saliva can be easily sampled with

414   much less disturbance than blood sampling procedures, repeated saliva GC measurements can

415   also be used to detect long-term stress loads (Nemeth et al. 2014). As the saliva sampling

416   procedure lasts approximately one minute in guinea pigs, a time span in which the HPA-axis

417   reactivity plays no role (Reeder & Kramer 2005), the physiological stress load remains

418   unaffected. Other sampling procedures, such as catheters, may also overcome possible handling

419   effects, but their higher degree of invasiveness may be undesired in different research fields.

420   Non-invasive or at least "mildly invasive" methods are therefore preferred and have also been

421   developed in guinea pigs (Bauer et al. 2008; Keckeis et al. 2012).

422   Dissociations between plasma and saliva GC levels can be explained by the conversion to

423   cortisone by the salivary glands and natural fluctuations (Hellhammer, Wüst & Kudielka 2009).

424   Concentrations in plasma are therefore usually ten times higher than in saliva. That ratio was also

425   found here: the mean plasma cortisol : saliva cortisol ratio was 9.944 after back transformation.

426   Moreover, the ratio did not change throughout the experiment and remained constant during non-

427   stressful and stressful conditions, further underlining the strong correlation between plasma and

428   saliva GC levels. GC concentrations in saliva represent the free and active form only, in contrast

429   to plasma where most GCs are bound to globulines and are therefore inactive. Accordingly,

430   measuring GC concentrations in saliva was therefore even suggested to constitute a more reliable

431   assessment of an individual's stress load than measurements in plasma (Levine et al. 2007;

432   Hellhammer, Wüst & Kudielka 2009). Although saliva sampling may be problematic in some

433   animals (Sheriff et al. 2011), but the same clearly holds true for blood sampling procedures, the

434   high biological relevance of saliva GC measurements would definitely argue for the non-invasive

435   method.

436   The applied social confrontation approach in experiment 2 also demonstrated the

437   biological relevance of GC measurements in fecal samples to assess physiological stress

438   responses in guinea pigs. FGM levels peaked six hours after the end of the two-hour social

439   confrontation and these measurements showed high relations to saliva cortisol concentrations

440   measured six hours earlier, directly after the social confrontation. Baseline measurements from

441   the day before show that FGM levels were not elevated at this time point. The detected peak

442   therefore clearly represents the physiological stress response to the social confrontation. FGM

443   levels were also monitored in experiment 1, but these +20h measurements failed to predict

444   plasma cortisol levels throughout this experiment. As previously validated physiologically via an

445   ACTH-challenge, a 20-hour time span constituted the mean gut passage time for GCs in guinea

446   pigs, with peak concentrations and maximum excretion rates of GCs in feces after the induced

447   stress (Bauer et al. 2008). Nonetheless, no measurable responses to the repeated social

448   confrontations or relations to circulating cortisol concentrations could be detected in the +20h

449   FGM measurements. The results of experiment 2 rather suggest a much faster mean gut passage

450   time, as FGM peak levels were detected already six hours after the end, respectively eight hours

451   after the onset of the social stressor. This time lag would correspond to more recent findings in

452   which the gut passage time for injected radioactively marked cortisol was eight hours in guinea

453   pigs (Keckeis et al. 2012), although these measurements were carried out under non-stressful

454   conditions. Bauer et al. (2008) also reported FGM levels to be significantly increased already

455   eight hours after ACTH-injections, although peak concentrations occurred much later at 18 hours

456   post injection, potentially reflecting the heavy physiological intervention caused by the ACTH-

457 challenge.

458   FGMs do not represent single-point-of-time measurement, but display the integrated

459 physiological stress load and GC secretion rates over a certain time span (Dehnhard et al. 2003;

460 Goymann 2005). Heavier stressors and related physiological stress responses can lead to longer-

461 lasting excretions of FGMs in feces and/or higher FGM peak levels after a certain time span, as

462 shown in rats by applying ACTH-challenges (Touma, Palme & Sachser 2004; Lepschy et al.

463 2007). Different experimental validation procedures generally seem to lead to several possible

464 interpretations of mean gut passage times and the time span to maximum GC excretion rates.

465 Studies in rats, for example, revealed FGM peak concentrations of 14.8 hours (Lepschy et al.

466 2007) and 16.7 hours (Bamberg, Palme & Meingassner 2001) after injection of radioactive

467 corticosterone. Monitoring circadian patterns of FGM levels, however, suggested a much faster

468 gut passage time of approximately six to nine hours (Cavigelli et al. 2005). Different

469 experimental approaches may yield different physiological stress responses, which may be

470 indicated by dissociations in the mean gut passage times and/or peak concentrations of FGMs.

471 Especially ACTH-challenges may constitute much more challenging situations than biological

472 procedures, as an ACTH-challenge acts directly on the HPA-axis and does not have to be

473 perceived as a stressor initially (Smith et al. 2012). As physiological stress loads might influence

474 gut functions (Trevisi et al. 2007), also the mean gut passage time for FGMs may be affected.

475 Considering these findings, the mean gut passage time for GC metabolites in guinea pigs can be

476 assumed to be six to eight hours, as indicated by the findings of Keckeis et al. (2012) and

477 experiment 2 of this study, rather than the reported 18 to 20 hours by Bauer et al. (2008).

478 Misinterpretations of detected FGM peaks can be excluded because mammals do not show a

479 double peak of high FGM excretion rates as birds do due to the combined excretion of urine and

480 feces via the cloaca (Hirschenhauser et al. 2012). GC metabolites are more rapidly excreted via

481 urine in birds and mammals (Hirschenhauser et al. 2012; Touma et al. 2003), but if urine

482 contamination of fecal samples can be excluded, as in this study, then FGM peak levels in feces

483 after a stressor reliably reflect the mean gut passage time.

484   Circulating GC levels fluctuate across the diurnal cycle (Atkinson & Waddell 1997;

485 Banjanin, Kapoor & Matthews 2004), with peak levels usually occurring before the onset of

486 activity, which makes it necessary to time sample collections appropriately. These diurnal

487 fluctuations are also reflected in FGM levels of rats for example (Touma, Palme & Sachser 2004;

488 Lepschy et al. 2007). Findings in guinea pigs, however, remain controversial but suggest an

489 absence of such diurnal variations (Bauer et al. 2008). FGM measurements in experiment 2,

490 including baseline measurements before the social confrontation and the measurements in two-

491 hour intervals to detect peak concentrations after the social confrontation, further indicate that

492 guinea pigs show no diurnal variation in FGM levels. This is a strong argument for measuring

493 physiological stress responses in fecal samples, as sample collections and GC measurements

494 would remain unaffected by the time of day (Bauer et al. 2008). Rodents defecate very

495 frequently, making knowledge on their gut passage time and on FGM diurnal cycles crucial.

496 Compared to rodents, larger mammals usually defecate in less frequent intervals and show

497 lengthier gut passage times (Goymann et al. 1999; Wasser et al. 2000). This may reflect the

498 generally different metabolic rates in different-sized animals (Hulbert et al. 2007). We were able

499 to collect fecal samples for each animal in each of the two-hour sampling intervals in experiment

500 2 of this study. Rarely did an animal defecate only once per sampling interval, defecation rates

501 and the amounts of excreted feces were rather very high. We can therefore exclude a

502 misinterpretation of the mean gut passage time for FGMs due to diurnal patterns in FGM levels,

503   possible effects of physiological stress responses on gut functions, or to low defecation rates in

504   general.

505   Hormonal analyses using enzyme-linked immunoassays are usually carried out in

506   duplicates. This includes calculating a CV for each sample, as a criterion of sample and analysis

507   quality. The confidence criterion of $\leq 15\,\%$ was reached for each sample, although statistical

508   analyses revealed that the analysis of FGM levels using the 11-oxoetiocholanolone antibody

509   worked best: the mean CV was significantly lower here than in plasma and saliva samples using

510   the cortisol-specific antibody. This substantiates the accuracy and validity of the adapted

511   antibody for FGM measurements (Palme & Möstl 1997; Möstl et al. 2002; Palme 2005). Saliva

512   cortisol measurements showed the highest CV, which may be due to possible contaminations

513   with food items that can influence the sensitivity of the assay. However, the average CVs for all

514   three types of measurements were far below the confidence criterion of 15%, which argues for

515   the reliability of all measurements per se.

516   Although physiological validations have already been carried out in guinea pigs, the

517   current study proved the biological relevance of these non-invasive measurements by monitoring

518   GC levels in regard to the animal's natural responses to different environmental and social

519   conditions. The experimental protocol revealed the strengths of non-invasive GC measurements

520   in saliva and fecal samples but also underlined the importance of detailed investigations on their

521   biological relevance. Especially measurements of FGM levels should be timed appropriately

522   because gut passage times for GCs are species-specific and also seem to be dependent on the

523   type of stressor and the corresponding stress response. By demonstrating the biological relevance

524   of non-invasive GC measurements in saliva and fecal samples, we conclude that saliva cortisol

525   and FGM levels go beyond indicating physiological stress responses to revealing the strength of

526 such a response. This opens new opportunities to draw conclusions about the physiological and

527 metabolic actions of GCs via non-invasive measurements.

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Glucocorticoid levels during social isolation and after social confrontations in experiment 1.

(A) Plasma cortisol concentrations, (B) saliva cortisol concentrations, and (C) fecal glucocorticoid metabolite (FGM) concentrations (+20h samples as control of the overall stress load) on six consecutive days, including three days of social isolation (Iso1, Iso2, Iso3) and three days of two-hour social confrontations (Soc1, Soc2, Soc3). ** $p \leq 0.01$, *** $p \leq 0.001$ comparing both experimental conditions (social isolation and social confrontation); ### $p \leq 0.001$ comparing experimental days Soc1 and Soc3.

A

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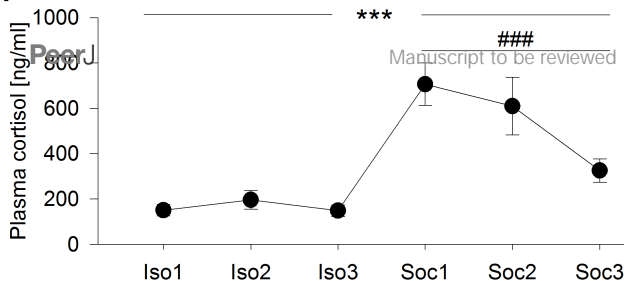
B

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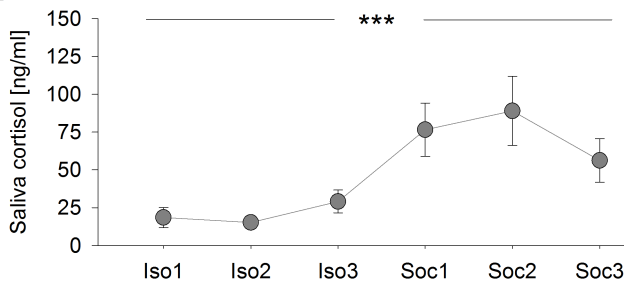
C

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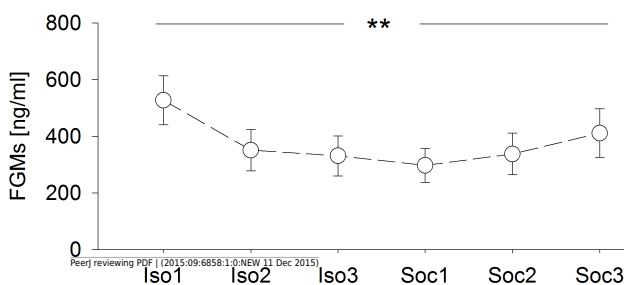
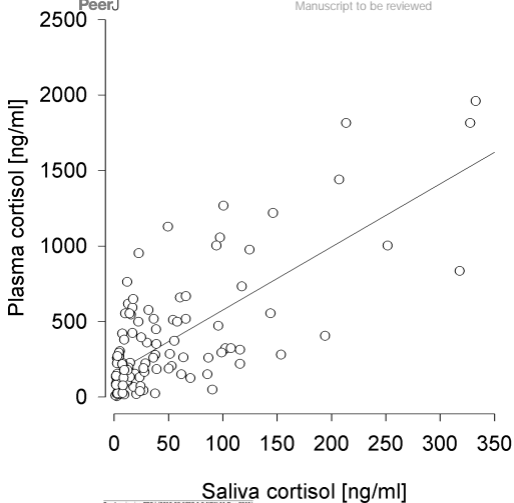
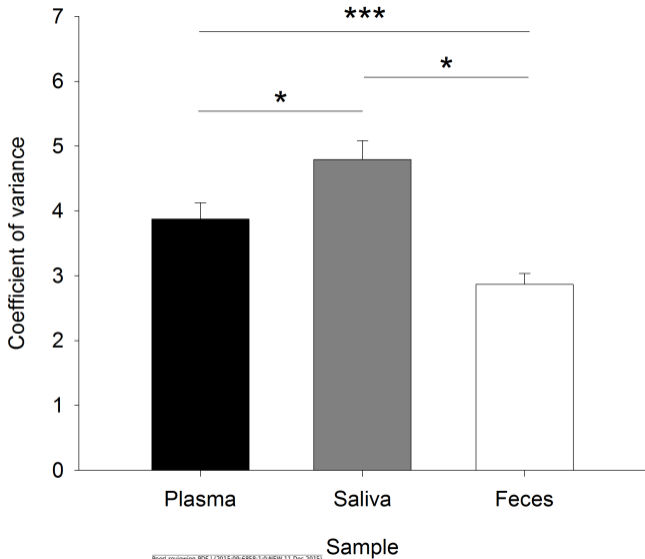
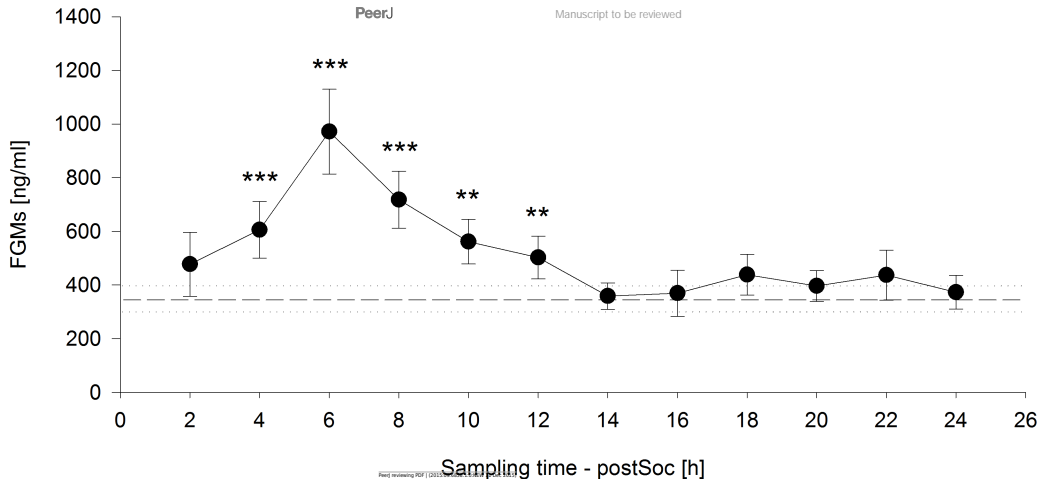

Experimental day

Prediction of plasma cortisol concentrations by saliva cortisol concentrations.

$R^2 = 0.402$.

Coefficients of variance for the duplicate measurements of glucocorticoids in plasma, saliva, and fecal samples as a control of the sample and analysis quality.

The coefficient of variance is the percentage of the standard deviation of the duplicates on the mean of the duplicates. * $p \leq 0.05$, *** $p \leq 0.001$.

Fecal glucocorticoid metabolites (FGMs) measured in two-hour intervals after social confrontation (postSoc).

The dashed line represents the mean baseline value (dotted lines: ± standard error of the mean) based on three measurements on the day before the social confrontation. ** $p \leq 0.01$, *** $p \leq 0.001$ compared to baseline FGM measurements.

Prediction of saliva cortisol concentrations by fecal glucocorticoid metabolite (FGM) concentrations measured six hours afterwards.

$R^2 = 0.428$.