Do glucocorticoids indicate stress? Meta-analysis reveals a tight association between glucocorticoids and metabolic rate

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ABSTRACT

Glucocorticoid (GC) variation has long been thought to reflect variation in organismal ´stress´, but associations between GCs and Darwinian fitness components are diverse in magnitude, direction, and highly context-dependent. This paradox reveals our poor understanding of the causes of GC variation, contrasting with the detailed knowledge of the functional consequences of GC variation. Amongst an array of effects in many physiological systems, GCs orchestrate energy availability to anticipate and recover from predictable and unpredictable environmental fluctuations and challenges. Although this is mechanistically well-known, the extent to which GC levels are quantitatively explained by energy metabolism is unresolved. We investigated this association through meta-analysis, selecting studies of endotherms in which (1) an experiment was performed that affected metabolic rate and (2) metabolic rate and GC-levels were measured simultaneously. We found that an increase in metabolic rate was associated with an increase in GC levels in 20 out of 21 studies (32 out of 35 effect sizes). More importantly, there was a strong positive correlation between the increases in metabolic rate and GCs ($p=0.003$). This pattern was similar in birds and mammals, and independent of the nature of the experimental treatment. We conclude that metabolic rate is a major driver of GC variation within individuals. Stressors often affect metabolic rate, leading us to question whether GC levels provide information on 'stress' beyond the stressor's effect on metabolic rate.

KEYWORDS

Glucocorticoid, cortisol, corticosterone, metabolism, energy expenditure.

INTRODUCTION

The accepted - but often overlooked - metabolic role of glucocorticoids

Glucocorticoid hormones (GCs; e.g. cortisol, corticosterone) were identified by Hans Selye (1907-1982) as the key molecular mediators of the 'stress reaction', and named in reference to their capacity to increase glucose in blood. Selye highlighted the fundamental role of GCs in the 'general adaptation syndrome' - i.e. "*the physiological mechanisms that help to raise resistance to damage as such, irrespective of the specific nature of the damaging agents*" (Selye 1950). In this view, GCs play a role in 'adapting' to the challenge, by triggering mechanisms that help the organism return to, or maintain, homeostasis after an environmental challenge. Indeed, towards the later stages of his career, Selye's definition of stress was "*the nonspecific response of the body on any demand on it*" (Selye 1976), making the point that very different stimuli (i.e. 'stressors') triggered the same response. However, in the literature this perspective has changed over time, and ´stress´ and, by extension, GCs, have been widely linked to negative outcomes (but see Koolhaas *et al.* 2011; Herman 2022, MacDougall-Shackleton et al. 2019). Consequently, researchers in fields from biomedicine to conservation physiology and animal husbandry have focused on GCs to find proxies of 'physiological and/or psychological stress´ to evaluate physical and/or welfare status. Indeed, GCs have predominated over other traits throughout the stress physiology literature, to the point of being referred to as vertebrate 'stress hormones' (Madliger *et al.* 2015; Madliger & Love 2014; McCormick & Romero 2017). While several authors have argued against this simplified view of GC regulation during the past decades (e.g. Koolhaas *et al.*, 2011; MacDougall-Shackleton *et al.* 2019; Herman 2022; Romero 2004; Landys *et al.* 2006; Bonier *et al.* 2009), such association prevails, in the sense that publications still abound in which GC levels are assumed to provide information on organismal stress.

The focus on GCs to measure organismal stress can be understood from their role as key mediators of organismal responses to challenges, triggering a cascade of effects on many physiological systems (Koolhaas *et al.*, 2011; Sapolsky, Romero & Munck, 2000; Deviche *et al.* 2017; Zimmer *et al.* 2019; Zimmer *et al.* 2020). Furthermore, acute increases in circulating GCs are a defining aspect of the so-called "stress response" (Koolhaas *et al.*, 2011; Sapolsky *et al.* 2000). Consequently, higher GC levels circulating in plasma or deposited in keratinized tissues (i.e. hair or feathers) have traditionally been interpreted as an indication of homeostatic unbalance, poor condition, implicating low fitness prospects (reviewed in Schoenle *et al.* 2021, Romero & Fairhurst 2016). However, the latter assumption is at best poorly supported by the literature, with associations between GCs and fitness (survival / reproduction) being diverse in direction and often non-existent (Schoenle *et al.* 2021; Zimmer *et al.* 2022; Busch & Hayward 2009; Bonier *et al.* 2009; Petrullo *et al.* 2022). This inconsistency raises the question what alternative inference can be made from GC-variation. The urgency of this question is further underlined by the observation that GCs also increase in response to experiences we would not normally qualify as stress; for example, sexual activity induces a GC increase in humans, horses and rodents (Siciliani 2000; Colborn *et al.* 1991; Buwalda *et al.* 2012). We suggest that this question can potentially be resolved through a better understanding of physiological mechanisms and environmental factors driving GC variation.

Selye (1976) emphasised that homeostatic challenges of any kind lead to increases in body demands, loosely defined as "*the rate at which we live at any one moment*". Activation of the Hypothalamus-Pituitary-Adrenal

(HPA) axis, represents a primary hormonal response to homeostatic challenges that, through the release of GCs, mobilises the resources needed to fuel the current or anticipated rise in energy expenditure (current and anticipatory responses) or recover from an immediate threat that induced an unanticipated increase in energy expenditure (reactive response) (Herman *et al.* 2016, McEwen & Wingfield 2003). In this context, GCs are involved in the metabolism of most types of energy reserves, modulating glucose, fat and protein metabolism in liver, skeletal muscle, and other target tissues (Box 1). Allusions to the energetic role of GCs and their tight link to energy expenditure are common in physiological and ecological studies, especially those using approaches which underline the adaptive function of GC responses (McEwen & Wingfield 2003; Landys et al. 2006; Romero *et al.* 2009; Deviche *et al.* 2017), but the extent to which GC variation can be quantitatively explained as facilitator of variation in energy expenditure has rarely been addressed (but see Jimeno *et al.* 2018, Malkoc *et al.* 2021 supplementary information). We here schematically review the role of GCs in energy metabolism (Box 1), and investigate this link quantitatively through meta-analysis.

Revisiting the associations between GCs and metabolic rate. A meta-analytic approach

Although previous evidence supports the link between energy expenditure and GC secretion (e.g. Koolhaas *et al.* 2011; Sapolsky *et al.* 2000; Beerling *et al.* 2011; Buwalda *et al.* 2012; Jimeno *et al.* 2018; Malkoc *et al.* 2021 supplementary information), the qualitative importance of both this association and the underlying processes to explain GC variation remains unexplored; this is however fundamental towards accurately interpreting GC variation. We here test whether changes in energetic demands are associated with variation in GC levels. Specifically, we (i) use a meta-analytic approach to test whether experimental manipulations leading to increases in metabolic rate in endotherms also lead to an increase in GCs (qualitative approach). We included only experimentally-induced increases of energy expenditure to avoid potential masking effects of anticipatory responses or delayed effects of GCs. Because metabolic rate and GCs can fluctuate rapidly, we targeted metabolic rate and GC measurements taken simultaneously or when animals could be assumed to be in the same physiological state (e.g. within the same day and experimental treatment). We further investigated (ii) whether the magnitude of the experimentally induced changes in metabolic rate and GCs were correlated (quantitative approach) through meta-regression. Our predictions are that (i) increases in metabolic rates are associated with increases in plasma GC concentrations, (ii) that changes in GCs are proportional to induced changes in metabolic rate, and (iii) that the association between increases in metabolic rate and GCs is independent of the treatment used to increase the metabolic rate.

BOX 1: Glucocorticoids and energy metabolism

We here consider GC regulation from the perspective of their role in fuelling metabolic rate. When metabolic rate is low, for example during periods of inactivity, circulating GCs are maintained at low levels and glucose release from fuel stores is released in the blood stream at a low rate matching the modest metabolic needs. (*permissive* actions; Sapolsky *et al.* 2000; Fig. 1). An increase in metabolic rate can be anticipated or unanticipated (GCs will exert *preparative* or *stimulating* actions, respectively; Sapolsky *et al.* 2000; Fig. 1), and acute or gradual. Unanticipated but gradual increases in metabolic rate will occur for example when thermoregulatory costs unexpectedly increase.

In both gradual and acute increases in metabolic rate, a main role of GCs is to increase circulating glucose at a rate matching the metabolic requirements through diverse mechanisms. Decreasing plasma glucose levels trigger a series of hormonal changes that promote a switch in energy usage. Together with a decrease of insulin (in mammals, less so in birds), GCs are released into the circulation (Andrews & Walker 1999; Rosmond & Bjorntorp 2000) reducing anabolic insulin actions (Vegiopoulos & Hertzig 2007). Blood glucose level then increases, both by mobilization from existing stores, and by inhibition of further storage. GCs also inhibit glucose uptake and glycogen synthesis in the liver, redirecting resources to gluconeogenesis and glycogenolysis, along with glucagon and catecholamines as part of the most immediate acute response. Catecholamines act quickly and increase within seconds to induce the release of energy needed to fuel the response (Herman *et al.* 2016; Romero & Beattie 2021; Sapolsky *et al.* 2000). The GC response lags in time -as GCs are produced de novo at the adrenal and take minutes to be secreted– and lasts substantially longer, depending on active (feedback signalling) and passive (GC degradation) processes (Herman *et al.*, 2016; Fig. 1), enhancing and prolonging the increase in blood glucose (Nonogaki 2000; Romero & Beattie 2021), or recovering energy stores after a brief burst of activity. In addition, inhibition of peripheral glucose transport and utilization in response to GCs increases the availability for other tissues, such as the brain (reviewed in Sapolsky *et al.* 2000; Herman *et al.* 2016). GCs also act in other substrates, further increasing lipolysis by inducing hormone-sensitive lipase (Slavin *et al.* 1994), and reducing lipoprotein lipase activity in peripheral fat depots. They also promote pre-adipocyte differentiation, pro-lipogenic pathway activity, and cellular hypertrophy in central fat (Vegiopoulos & Hertzig 2007) as well as decreased thermogenesis in brown adipose tissue (Soumano *et al.* 2000). In various muscle types, GCs suppress protein synthesis while promoting protein degradation and amino acid export. When the energetic and substrate requirements of the organism are further increased (e.g. during fasting or illness), muscle tissue (40% of total body mass) becomes a rich source of amino acids, which can be mobilized as substrates for energy generation, gluconeogenesis and protein synthesis (Kuo *et al.* 2013).

Given the existing evidence on the metabolic role of GCs, and the different time scales associated to the kind of response (anticipatory vs perceived) as well as to the hormones and substrates' physiological actions (see above), GC concentrations cannot be expected to always reflect the 'immediate' energy expenditure. However, we would expect changes in energetic demands to always require a GC response/input to meet the derived metabolic needs (Fig. 1). This prediction of a strong association between GCs and metabolic rate, however, is not well researched, and does not necessarily imply that one trait necessarily affects the other per se, as their interplay is likely to be shaped by the environmental or physiological context. Note further that we make no distinction between baseline and stress-induced GC-levels, and thereby in effect assume these to be points in a continuum from a metabolic perspective; a perspective supported by the monotonic effects of GCs on glucose uptake and fat depletion (Kattwinkel & Munck 1966; Dallman *et al.* 1993). Additionally, although we consider GCs to be regulated to meet energetic demands, we are aware GCs have many complex downstream effects at both baseline and stress-induced levels, besides energy-mobilization (Fig. 1).

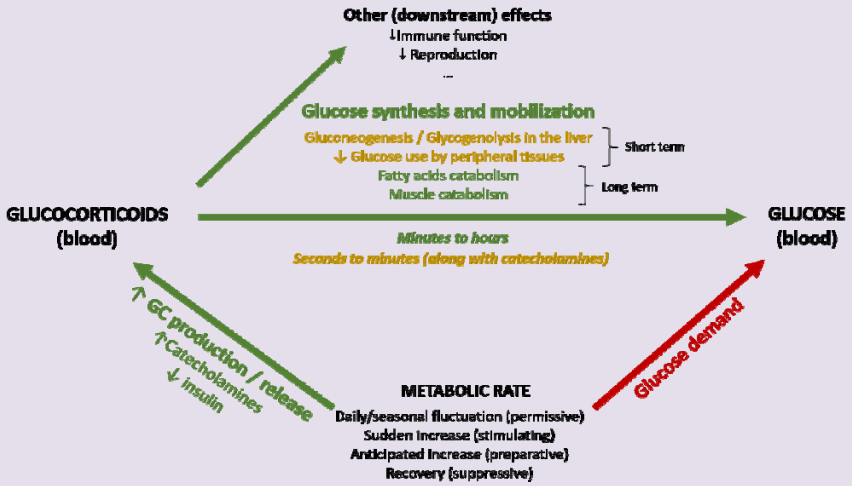

Fig. 1: Schematic representation of the association between metabolic rate and plasma levels of glucocorticoids and glucose. Green arrows represent increasing effects, whereas red arrows represent reducing effects.

METHODS

Literature search

We reviewed the literature to identify empirical studies reporting measurements of both metabolic rate and plasma GCs. We compiled studies that met all following criteria: (1) Including an experimental manipulation of any kind leading to increases in metabolic rate which was quantified (i.e. both significant and non-significant increases). Among these, we also included those studies reporting heart rate as a metabolic measure, as heart rate and metabolic rate are strongly correlated (Bevan *et al.* 1994; Bevan *et al.* 1995; Butler *et al.* 2004, Word *et al.* 2022). (2) Including measurements of natural GC concentrations in plasma (i.e. not exogenous or chemically induced –e.g. with ACTH or CRH). (3) measurements of GCs and metabolism had to be on the same individuals and measured in the same physiological state. The latter condition excludes, for example, studies with daily energy expenditure measurements combined with GCs measured at one time-point. Finally, we only included studies on endotherms (birds and mammals), because metabolic regulation differs strongly between endotherms and ectotherms.

We conducted a database search (Web of Science, 20/07/2021) to identify candidate studies, using the following two combinations of search terms: *"energy expenditure" AND (glucocorticoid OR cortisol OR corticosterone)* and *"metabolic rate" AND (glucocorticoid OR cortisol OR corticosterone)*. After the search, we consecutively selected articles after a) abstract review, b) full text review and c) data availability for effect size calculations. Using this approach, we identified a total of 14 studies that met all our criteria (see Table S1 for additional information on the number of studies obtained on each of the search steps). We also systematically checked the reference list of these 14 papers, which yielded an additional 7 papers. Thus, we included a total of 21 papers in our analyses, of which 12 were on birds, and 9 on mammals. Nine of the 22 papers included more than one experimental treatment, yielding a total of 35 effect sizes. For each of these studies, we extracted information on study species or metabolic and GC variables reported, among others (Table S2). Additionally, we recorded variation related to the experimental design, the variables that were quantified, and the type of treatment used: a) Before / after design: whether the experimental manipulation included a time effect (i.e. individuals served as their own control, being measured before and after the experimental manipulation); b) Experiment / control design: Whether the experiment accounted for within-individual variation (i.e. all individuals went through all experimental treatments); c) Whether metabolic rate or heart rate was the metabolic variable; and d) The type of treatment that induced an increase in metabolic rate (see below) (Table S2).

Effect size calculations

To estimate effect sizes of metabolism and GCs, we used the web-based effect size calculator Practical Meta-Analysis Effect Size Calculator (www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-Home.php), following Lipsey & Wilson 2000 and Nakagawa & Cuthill 2007. We calculated standardized mean-difference effect sizes (Cohen's D), which we computed from means and standard deviations (19 studies) or t-test (2 studies). When metrics were presented graphically only, we extracted data from the figure(s) using the GetData Graph Digitizer software (http://getdata-graph-digitizer.com/). See Table S3 for details on data extraction and effect size calculations.

142 For each study, we compared the mean metabolic rate and level of plasma GCs of individuals in the
143 treatment group(s) to that of individuals in the control group. For studies in which treatment was
144 confounded with time, because pre-treatment measurements were used as control and compared with
145 measurements during treatment, the pre-treatment measure was used as control when calculating effect
146 sizes in studies where there was a single treatment. When studies with a before-after design included more
147 than one experimental treatment, the treatment yielding the lowest metabolism was taken as control for
148 the effect size calculations. Thus, confounding time with treatment was avoided whenever possible.

149 **Statistical analyses**

150 We conducted all meta-analyses using the *rma.mv* function from the *metafor* package (Viechtbauer 2010),
151 implemented in R (version 4.0.1, R Core Team 2020). Standard errors were used for the weigh factor. All
152 models contained a random intercept for study identity to account for inclusion of multiple experimental
153 treatments or groups from the same study. Most species were used in a single study, and we therefore did
154 not include species as a random effect in addition to study identity. The number of species was however
155 insufficient to reliably estimate phylogenetic effects, we therefore limited the analysis in this respect with a
156 comparison between birds and mammals (see below). The dependent variable was either the metabolic rate
157 or the GC effect size. One model was fitted with the metabolic rate effect size as a dependent variable, to
158 estimate the average effect on metabolic rate across all studies in the analyses. All other models had the GC
159 effect size as dependent variable, and metabolic rate effect size as a moderator. Distribution of metabolic
160 rate effect sizes was skewed which was resolved by ln-transformation, which yielded a better fit when
161 compared to a model using the linear term (evaluated using AIC, see results for details). Our first GC model
162 contained only the metabolic rate effect size as a fixed independent variable. This model provides a
163 qualitative test of whether GC levels increase when metabolic rate is increased and tests prediction (i) by
164 providing an estimate of the intercept, which represents the average GC effect size because we mean
165 centered the ln-transformed metabolic rate effect size (Schielzeth 2010). The same model tests prediction
166 (ii) whether the GC effect increases with an increasing metabolic rate effect size, which will be expressed in a
167 significant regression coefficient of the metabolic rate effect size.

168 Following the model with which we tested our main predictions, we ran additional models to test for the
169 effects on GC effect sizes of (a) taxa (birds vs. mammals), two design effects, namely (b) before / after effect,
170 and (c) experiment / control effect, (d) metabolic variable measured (metabolic rate or heart rate), (e) type
171 of treatment (categorized in climate, psychological, or other). This last factor tests our prediction iii. We
172 included these variables as modulators in the analysis, as well as the two-way interactions of these factors
173 with the metabolic rate effect size. All factors were coded as 1 (bird / no before – after effect / no
174 experiment-control effect / metabolic rate) or 2 (mammal / before-after effect / experiment-control effect /
175 heart rate), and then mean-centered. Treatment type was categorized as 1 (climate), 2 (psychological), or 3
176 (others). We compared models with vs. without these additional variables using Akaike's Information
177 Criterion, with correction for small sample sizes (AICc, Akaike, 1974)) for which a change in AICc of 2 is
178 considered significant (Burnham, Anderson & Huyvaert 2011). Models within ΔAICc < 2 were considered
179 best fitting models, and we further explored the effects of the main predictors when present in these top
180 models. To rule out publication bias effects (i.e. regression test for funnel plot asymmetry), we included a

weighing variable (square root of the sample size) as moderator in the models, as Egger's test is not a reliable test of funnel plot asymmetry in multilevel models. Variable effects and results remained quantitatively very similar and qualitatively unchanged. Furthermore, because many labs contributed multiple studies, we tested the effect of including Lab (N=16) as random factor in the models. However, this variable had a negligible effect on the models, and we therefore excluded it from the final models.

RESULTS

Among the studies selected for inclusion in the analysis, the treatment effect size on metabolic rate (MR) was on average 1.85 ± 0.87 (Fig. S1). In accordance with prediction, effects on GCs were positive in the majority of cases (32/35; Fig. 2), and consequently the overall average effect size deviated significantly from zero, with the average GC effect size estimated at 0.73 ± 0.11 (Table 1). There was a strong association between MR effect sizes and GC effect sizes (Table 1, Fig. 3), thus confirming prediction ii. It is further worth noting that the residual heterogeneity did not exceed the level expected by chance (Table 1). MR Cohen's D was Ln-transformed (see methods) to normalize the distribution (Fig. S2), and AICc of models including Ln MR were significantly lower when compared to models including untransformed MR Cohen's D (AICc = 70.58 vs 74.97, respectively).

The association between MR and GC effect sizes remained statistically significant when adding Taxa, before / after, experiment / control effect, metabolic variable or treatment type one by one to the model. Furthermore, none of these variables had a significant effect on GC effect size, nor did the association between MR and GC effect sizes depend on those factors (i.e. interactions between these variables and MR effect sizes were always non-significant; Table 2, S4). The latter result confirms prediction iii. Given that none of these effects significantly improved the model, the final model when removing all factors was the one including MR effect size as only predictor of GC effect size (Table 1). Despite these modulators being non-significant, the associations were in the expected directions, with studies including within-individual variation (i.e. experiment / control effect), and not including a before / after effect reporting higher GC effect sizes (Table S4, Fig. S3).

Table 1. Meta regression model testing the association between metabolic rate (MR) effect sizes and glucocorticoid effect sizes.

	Estimate	s.e.	Z	P	95% C.I..
Intercept	**0.72**	**0.11**	**6.40**	**<0.0001**	**0.50-0.95**
MR effect size (ln)	**0.31**	**0.10**	**2.94**	**0.003**	**0.10-0.51**
Variance components: Study.ID (Sigma^2)– Estimate = 0.00, sqrt = 0.00, n= 21					
Residual heterogeneity: QE(df = 33) = 28.40, p = 0.70					
Test of moderators: QM(df = 1) = 8.64, p= 0.003					

211 **Table 2.** Table showing the main effects of all variables considered (Metabolic Rate, Taxa, Time effect,
212 Within-individual variation, Metabolic variable, and Treatment Type) to modulate glucocorticoid effect sizes
213 across studies. Full models are shown in Table S4.

Variable	P
MR effect size (ln)	**0.003**
Taxa	0.63
Before / after	0.49
Experiment / control	0.68
Metabolic variable	0.94
Treatment type	
Treat. 2	0.93
Treat. 3	0.60

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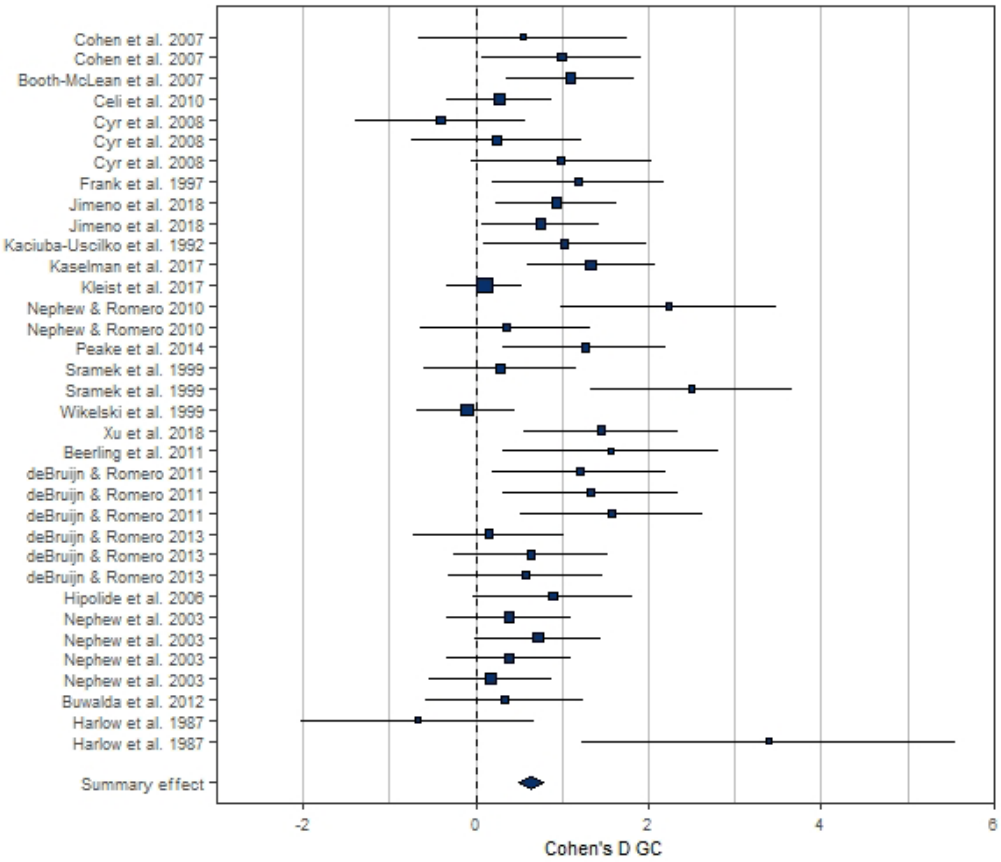

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218 **Fig. 2**: Forest plot showing the glucocorticoid (GC) effect sizes (Cohen's D ± 95% CI) associated to
219 experimental manipulations of metabolic rate, grouped by treatment group and study. Area of squares is
220 proportional to the experiment sample size (1/s.e.).

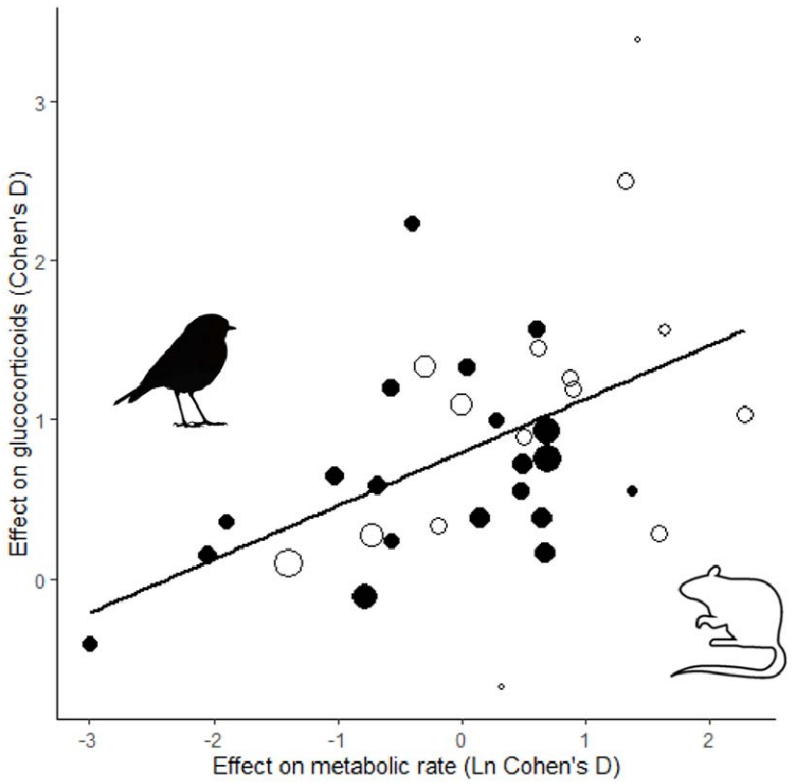

Fig 3. Glucocorticoid effect size (Cohen´s D) increases with increasing metabolic rate effect size similarly in studies of mammals (open circles) and birds (closed circles). Area of dots is proportional to the experiment sample size (i.e. square root of the number of individuals in which GCs were measured).

DISCUSSION

Finding a consistent functional interpretation of GC variation has proven challenging, and to this end we presented a simplified framework focusing on the interplay between energy metabolism and GCs (Box 1). Based on this framework, we made three predictions that we tested through a meta-analysis of studies in endotherms in which metabolic rate was manipulated and GCs were measured at the same time. The analysis confirmed our predictions, showing that experimental manipulations that increased metabolic rate induced a proportional increase in GCs (Fig.3), and our interpretation of this effect is that GC secretion facilitated increases in MR. This association indicates that fluctuations in energy turnover are a key factor driving variation in GC levels. From this perspective, the many downstream effects of GCs (e.g. down regulation of immune function and reproduction; McEwen & Wingfield 2003; Sapolsky *et al.* 2000) may be interpreted as allocation adjustments to the metabolic level at which organisms operate. Specifically, within-individual blood GC variation signals the metabolic rate at which the organism is functioning to all systems in the body. In this light, downstream effects of GCs can be interpreted as evolved responses to metabolic rate fluctuations, reallocating resources in the face of shifting demands on the whole organism level.

The effect of metabolic rate on GC levels was independent of the type of manipulation used to increase metabolic rate, confirming our third prediction. Note however that confirmation of this prediction relied on

242 the absence of a significant effect, and absence of evidence is not evidence of absence. However, the
243 residual heterogeneity of our final model did not deviate from a level expected due to sampling variance,
244 providing additional support for our third prediction.

245 Note that in the context of our analysis we made no distinction between 'baseline' and 'stress-induced GC-
246 levels (Box 1). Firstly, because these concepts are not operationally well defined – baseline GC-levels are
247 usually no better defined than 'not stress-induced'. Secondly, when considering the facilitation of metabolic
248 rate as primary driver of GC regulation, there does not appear a need to invoke different classes of GC-levels
249 instead of the more parsimonious treatment as continuum. This is not to say that this also applies to the
250 functional consequences of GC-level variation: it is well known that receptor types differ in sensitivity to GCs
251 (Landys *et al.* 2006; Sapolsky *et al.* 2000; Romero 2004), thereby potentially generating step functions in the
252 response to an increase in GC-levels.

253 We restricted the meta-analysis to experimental studies, and expect the association between MR and GCs to
254 be less evident in a more natural context. Associations between GCs and MR will be most evident when
255 animals are maintained at different but stable levels of metabolic rate, because then the rate at which
256 tissues are fuelled is likely to be in equilibrium with the metabolic needs. While equilibrium conditions can
257 be created in laboratory studies, conditions will usually be more variable in the wild. When metabolic rate
258 fluctuates, e.g. due to short term variation in activity, GC variation will track metabolic rate fluctuations, but
259 with a time lag (Box 1), thus adding complexity to the MR / GC association and its detectability. Furthermore,
260 experiments yield estimates of associations within the average individual in the study, while data collected in
261 a natural context usually rely on variation between individuals (but see Malkoc *et al.* 2022). Associations
262 between individuals will be less strong than associations within individuals due to individual variation in GC
263 levels and GC reactivity (e.g. Liu *et al.* 1997; Weaver *et al.* 2004; Yehuda *et al.* 2014; Taff et al 2018, 2022).
264 Between subject variation is likely to be even larger on an interspecific level, and in line with this expectation
265 the comparative evidence on the MR / GC association is mixed. While a strong positive MR / GC correlation
266 was reported for mammals (Haase et al 2016), Francis et al (2018) found no consistent GC / MR association
267 in birds and other tetrapod taxa. However, recent comparative analyses showed avian GC variation to be
268 positively correlated with estimated thermoregulatory costs (Rubalcaba & Jimeno 2022a), and GC variation
269 in lizards to be positively correlated with body temperature, which directly influences metabolic rate in
270 ectotherms (Rubalcaba & Jimeno 2022b). The contrast between the findings may be due to the MR and GC
271 data not always being collected on individuals in a comparable state. We emphasize therefore the
272 importance of measuring metabolic rate and GCs when animals are in the same state, preferably by
273 measuring both variables at the same time.

274 GCs increased in the studies included in our meta-analysis in response to an induced increase in MR, but GCs
275 can also increase in response to an *anticipated* increase in MR (Box 1). For example, the early morning GC
276 increase in humans, known as the 'cortisol awakening response' (Fries et al 2009) can be interpreted as
277 preparation for an increase in MR – indeed human "early birds" show higher GC-levels than "night owls" in
278 the hour after awakening (Kudielka et al 2006). Likewise, GC levels increase in athletes preceding
279 competition (van Paridon et al 2017), although separating effects of psychological stress from anticipated
280 metabolic needs is difficult in this context. Experiments in which animals are trained to anticipate an

281 increase in MR to investigate whether this generates an anticipatory increase in GCs would be an interesting
282 additional test of the framework laid out in Box 1.

283 Given that GC levels are often assumed to provide information on organismal ´stress´ and welfare, the
284 question arises whether the observed pattern can be the consequence of effects on psychological stress
285 instead of metabolic rate. This question arises because manipulations of energy expenditure are always
286 'indirect', in that an external treatment is used to induce an increase in metabolic rate, as opposed to a
287 direct manipulation of metabolic rate, and this leaves room for other factors to cause the observed effects.
288 While we acknowledge that it is not possible to demonstrate conclusively that a process is not happening,
289 we consider it unlikely that 'stress effects' explain our findings. Firstly, because the way metabolic rate was
290 manipulated varied widely between studies but manipulation type had no discernible effect on the MR / GC
291 association. This was also the conclusion of one of the studies included in our meta-analysis, designed
292 specifically to compare MR / GC associations between two MR increasing treatments, ambient temperature
293 and noise as psychological stressor (Jimeno et al 2018). Secondly, the finding that the GC increase was
294 proportional to the increase in MR can only be explained by psychological stress when the induced
295 psychological stress was proportional to the induced MR. Thirdly, the pattern is consistent with what is
296 known of the functional consequences of GC variation in relation to metabolic needs (Box 1). Lastly, diverse
297 non-injurious psychological stressors increase metabolic rate in humans (Sawai *et al.* 2007, Balanos *et al.*
298 2010, Carroll *et al.* 2009), mammals (Harris *et al.* 2006; mild and unpredictable chronic stress; García-Díaz *et*
299 *al.* 2007) and birds (Jimeno *et al.* 2018), explaining why GCs generally increase in response to a stressor. We
300 conclude therefore that while a causal link between MR and GCs is not the only possible explanation of our
301 findings, we argue it to be the most parsimonious explanation. Direct manipulations of MR could confirm or
302 reject this explanation, and may for example be achieved using thyroid hormones, which have been shown
303 to affect MR (Moreno *et al.* 2002, Kim 2008).

304 We selected studies in which experimental treatments affected MR, leading us to conclude that the most
305 parsimonious explanation of our finding is that GC levels were causally related to MR. Suppose however that
306 instead we reported a correlation between MR and GCs, using for example unmanipulated individuals. The
307 question would then be justified whether changes in GCs affected MR or vice versa. Direct effects of GCs
308 could be studied using pharmacological manipulations. However, while many studies show that GC
309 administration induces a cascade of effects, when the function of GCs is to *facilitate* a level of MR, as
310 opposed to *regulate* variation in MR, we do not anticipate such manipulations to induce an increase in MR
311 (Box 1). On the other hand, when MR is experimentally increased in conjunction with pharmacological
312 manipulations that *supress* the expected GC-increase (an experiment that to our best knowledge has not yet
313 been done), we would predict that the increase in MR can be maintained less well compared to the same
314 MR treatment in the absence of the pharmaceutical manipulation. This result, we would interpret to
315 demonstrate that maintaining a particular level of MR may be dependent on GCs as facilitator, but it would
316 be misleading to interpret this pattern to indicate that GCs regulate MR, as is sometimes proposed.
317 Additionally, it would be informative to investigate whether energy turnover immediately before blood
318 sampling is a predictor of GC levels, as we would predict on the basis of the interpretation of our findings.
319 Increasing the use of devices and techniques that monitor energy expenditure or its proxies (e.g.
320 accelerometers) may be a way to increase our understanding of the generality of the GC-MR association.

Authors that assumed GC levels to be a proxy of physiological stress have struggled with the interpretation of findings such as the mixed results with respect to fitness consequences of GC-variation. Our findings offer a way to interpret such variation: GCs are regulated with respect to their role in facilitating energy metabolism, and we encourage researchers to approach and interpret findings from this perspective. For example, a positive association between GCs and reproductive success may indicate that individuals that are able to sustain high metabolic rates attain higher fitness (e.g. Bauch et al, 2016), while a negative association indicates the opposite effect (e.g. Ouyang et al 2013; see Atema *et al.*, 2021, for a more general discussion of this specific contrast). Given that GCs have many other downstream effects (Sapolsky *et al*. 2000), for example supressing immune function (Cain & Cidlowski 2017), and growth (Allen 1996), this may seem an overly simplistic approach. However, in this framework, downstream effects of GCs may be understood as responses to a system level readout of the current level of energy metabolism, with high levels affecting the allocation of energy to different energy demanding processes. In this view, the link between GCs and energy demanding processes is asymmetric, in the sense that GCs affect energy allocation to e.g. growth, but there is no direct feedback from growth to GC levels. In conclusion, whereas GCs are widely seen as 'stress hormones', we offer a different interpretation and question whether GC variation reveals any physiological stress beyond fluctuations in energy expenditure.

FUNDING

BJ was funded by grant FJC2019-039748-I of MCIN/AEI /10.130 39/501100011033.

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589 **SUPPLEMENTARY INFORMATION**

590 # Do glucocorticoids indicate stress? Meta-analysis reveals a tight association
591 # between glucocorticoids and metabolic rate

592 **Blanca Jimeno & Simon Verhulst**

593

594 **Table S1.** Study selection steps and number of studies found

"energy expenditure" AND (glucocorticoid OR cortisol OR corticosterone) (20/07/2021)			
Total	**After abstract**	**After text**	**After effect size calculations**
707	108	43	9
"metabolic rate" AND (glucocorticoid OR cortisol OR corticosterone) (20/07/2021)			
Total	**After abstract**	**After text**	**After effect size calculations**
447	71 (64*)	28	5
Selected papers' reference check			
Total (excl. previously included)	**After abstract**	**After text**	**After effect size calculations**
20	15	9	7

Text step requires paper selection criteria to be met. Effect size calculation step requires enough data reported to calculate effect sizes by experimental treatment.
Main reasons for not including articles after abstract / text step: i) Experimental treatment had no effect on metabolic rate, ii) chemical or drug induction of metabolic rate increase, iii) GCs were measured in other tissues than plasma, iv) no experimental manipulation of metabolic rate.
**Number of studies excluding those overlapping with previous search, which were not further considered.*

595

596

597 **Table S2:** Table with the information extracted from each study included in the meta analysis (excel sheet available).

598

599 **Table S3:** Effect size calculations. The document includes one sheet per study with the data extracted, the part of the
600 article it was extracted from, and the effect size calculations and results further included in the meta analysis and Table
601 S2 (excel sheets available).

602

603 **Table S4.** Meta regression model (quantitative approach) testing the effect of (a) Taxa, (b) Before / after effect, (c)
604 Experiment / control effect, (d) Use of Metabolic Rate or Heart Rate as metabolic variable and (e) Treatment type, on
605 the association between metabolic rate (MR) and glucocorticoid effect sizes across studies.

(a)	Estimate	s.e.	Z	P	95% C.I.
Intercept	0.70	0.12	6.00	<0.0001	0.47-0.93
MR effect size (ln)	**0.29**	**0.11**	**2.70**	**0.007**	**0.08-0.50**
Taxa (mammal)	0.12	0.24	0.49	0.63	-0.35-0.58
MR : Taxa	0.17	0.22	0.80	0.42	-0.25-0.60
Variance components: Study.ID (Sigma^2)– Estimate = 0.00, sqrt = 0.00, n= 21					
Residual heterogeneity: QE(df = 31) = 27.44, p = 0.65					
Test of moderators: QM(df = 3) = 9.60, p= 0.022					
(b)	**Estimate**	**s.e.**	**Z**	**P**	**95% C.I.**
Intercept	0.73	0.11	6.42	<0.0001	0.51-0.95
MR effect size (ln)	**0.31**	**0.11**	**2.95**	**0.003**	**0.10-0.52**
Before / after effect (yes)	-0.26	0.38	-0.70	0.49	-0.99-0.48
MR : Time effect	0.02	0.31	0.05	0.96	-0.59-0.62
Variance components: Study.ID (Sigma^2)– Estimate = 0.00, sqrt = 0.00, n= 21					
Residual heterogeneity: QE(df = 31) = 27.91, p = 0.62					
Test of moderators: QM(df = 3) = 9.13, p= 0.028					
(c)	**Estimate**	**s.e.**	**Z**	**P**	**95% C.I.**
Intercept	0.73	0.11	6.51	<0.0001	0.51-0.96
MR effect size (ln)	**0.31**	**0.10**	**2.97**	**0.003**	**0.11-0.51**
Exp./ control effect (yes)	0.05	0.31	0.16	0.87	-0.56-0.65
MR : Exp. / control effect	-0.06	0.30	-0.22	0.83	-0.64-0.51
Variance components: Study.ID (Sigma^2)– Estimate = 0.00, sqrt = 0.00, n= 21					
Residual heterogeneity: QE(df = 31) = 28.18, p = 0.61					
Test of moderators: QM(df = 3) = 8.94, p= 0.03					
(d)	**Estimate**	**s.e.**	**Z**	**P**	**95% C.I.**
Intercept	0.73	0.12	6.11	<0.0001	0.50-0.97
MR effect size (ln)	**0.33**	**0.11**	**2.97**	**0.003**	**0.11-0.54**
Met. variable (HR)	0.14	0.24	0.57	0.57	-0.33-0.60
MR : Met. variable	-0.02	0.22	-0.07	0.94	-0.45-0.42
Variance components: Study.ID (Sigma^2)– Estimate = 0.01, sqrt = 0.09, n= 21					
Residual heterogeneity: QE(df = 31) = 28.09, p = 0.62					
Test of moderators: QM(df = 3) = 8.67, p= 0.022					
e)	**Estimate**	**s.e.**	**Z**	**P**	**95% C.I.**
Intercept	0.78	0.21	3.79	0.0002	0.38-1.19
MR effect size (ln)	**0.27**	**0.19**	**1.39**	**0.166**	**-0.11-0.64**
Treat. Type 2	-0.03	0.27	-0.09	0.93	-0.56-0.51
Treat. Type 3	-0.16	0.31	-0.52	0.60	-0.78-0.45
MR : Treat. Type 2	0.05	0.25	0.22	0.83	-0.44-0.55
MR : Treat. Type 3	0.08	0.29	0.27	0.79	-0.48-0.64
Variance components: Study.ID (Sigma^2)– Estimate = 0.02, sqrt = 0.14, n= 21					
Residual heterogeneity: QE(df = 29) = 27.99, p = 0.52					
Test of moderators: QM(df = 5) = 8.86, p= 0.115					

606

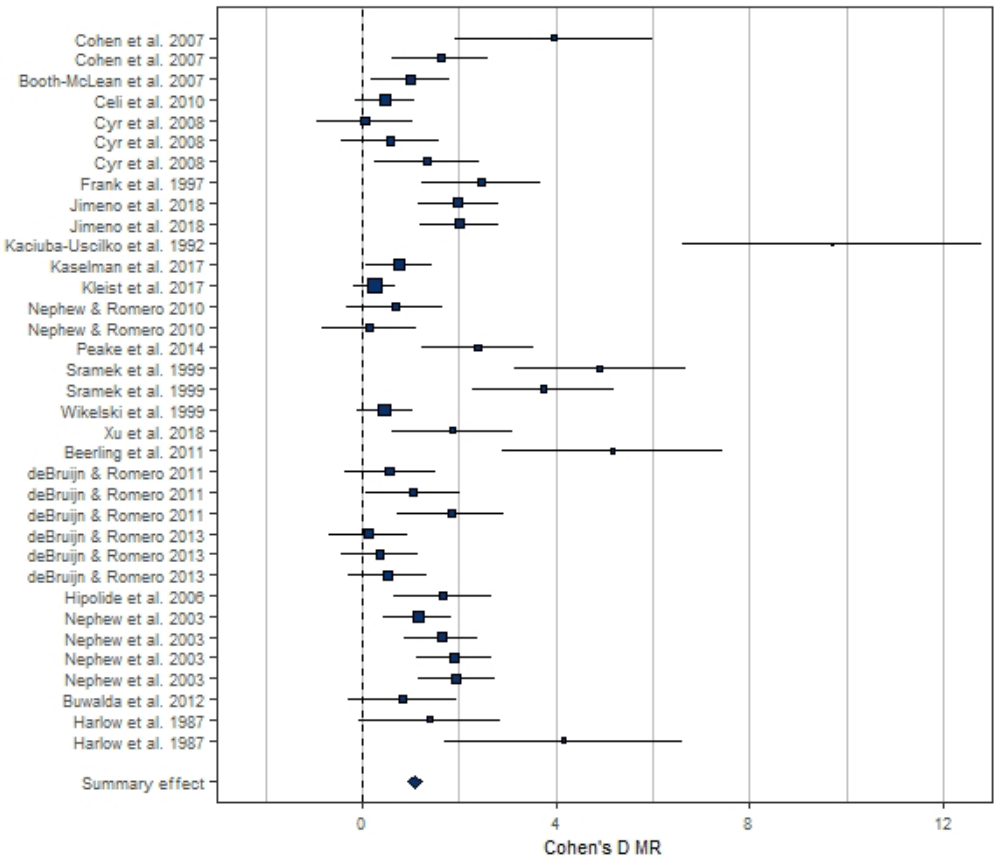

Fig S1: Forest plot showing the metabolic rate (MR) effect sizes (Cohen's D ± 95% CI) associated to experimental manipulations of metabolic rate, grouped by treatment group and study. Area of squares is proportional to the experiment sample size (1/s.e.).

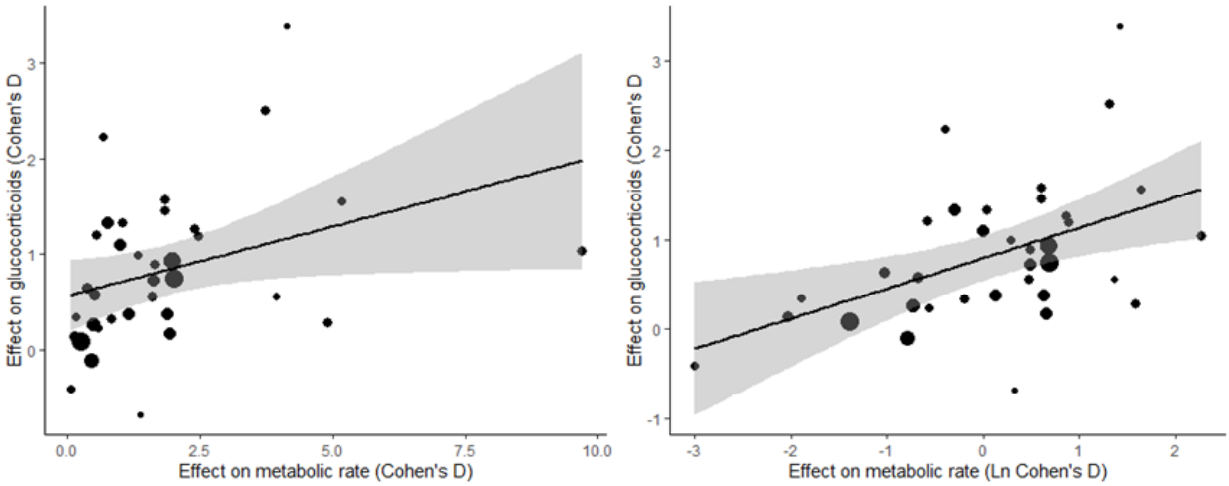

Fig S2. Relationship between metabolic rate and glucocorticoid effect sizes (Cohen's D) across studies. Panels show the association without ln-transforming metabolic rate effect sizes (left panel) and when ln-transforming them (right panel). Size of dots is proportional to the experiment sample size (i.e. square root of the number of individuals in which glucocorticoids were measured). Shaded areas represent 95% C.I. Note that the number of data points in the graph is higher than the number of studies, as some of the studies included multiple experimental treatments (Study ID was included as random factor in statistical analyses).

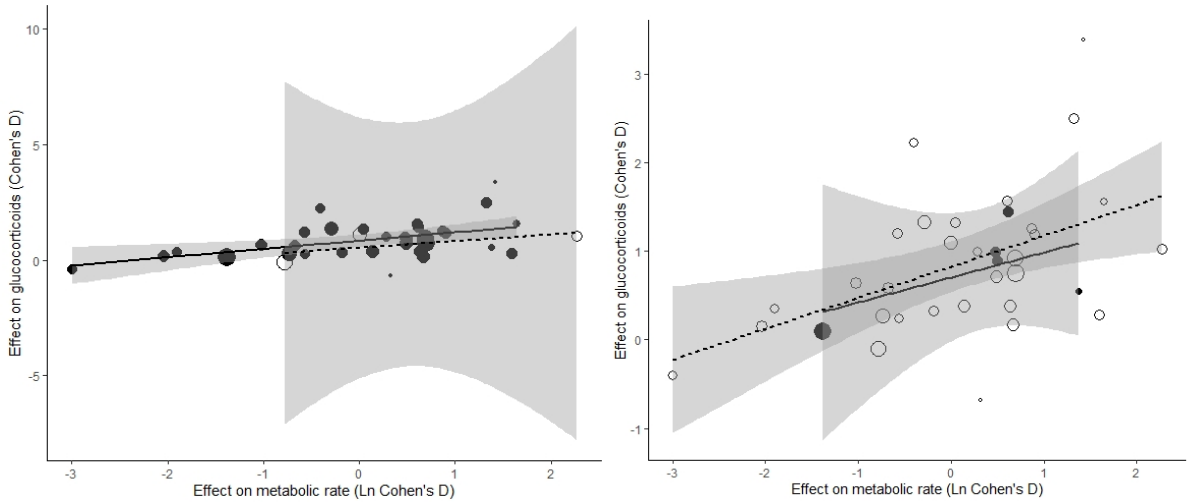

Fig S3. Relationship between metabolic rate and glucocorticoid effect sizes (Cohen's D) across studies as a function of a) Before / after effect (open circles and dashed line for studies including a time effect; closed circles and continuous line for studies not including a time effect; see methods) and b) Experiment / control effect (open circles and dashed line for studies including within-individual variation; closed circles and continuous line for studies not including within-individual variation). Size of dots is proportional to the experiment sample size (i.e. square root of the number of individuals in which glucocorticoids were measured). Shaded areas represent 95% C.I. Note that the number of data points in the graph is higher than the number of studies, as some of the studies included multiple experimental treatments (Study ID was included as random factor in statistical analyses). Before / after effect and Experiment / control effect results should be interpreted with caution, as most studies do include within individual variation and do not include time effect.