Increases in glucocorticoids are sufficient but not necessary to increase cooperative burrowing in Damaraland mole-rats

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Highlights

•The underlying physiological mechanisms of cooperation are poorly understood.
•Hormones may be important but experimental evidence is lacking.
•We manipulated levels of glucocorticoids and cooperation in a cooperative breeder.
•Increases in glucocorticoids stimulate cooperative burrowing.
•However, cooperative burrowing can also be increased independently of glucocorticoids.

Abstract

Despite widespread interest in the evolution of cooperative behaviour, the physiological mechanisms shaping their expression remain elusive. We tested the hypothesis that glucocorticoid (GC) hormones affect cooperative behaviour using captive Damaraland mole-rats (*Fukomys damarensis*), a cooperatively breeding mammal. Within groups, individuals routinely contribute to public goods that include foraging tunnels, which provide all group members access to the tubers of desert plants they feed on, communal food stores and nests. We found that experimental increases in glucocorticoid concentration (GCc) in non-breeding female helpers led them to be active for longer and to burrow more while active, raising their daily contributions to burrowing, but not food carrying or nest building. However, experimentally induced increases in burrowing did not lead to elevated GCc in helpers of both sexes. These results suggest that heightened GCc may stimulate some cooperative behaviours that are energetically demanding (a characteristic shared by many types of cooperative activities across species) but that the cooperative behaviours affected by GCc can also be regulated by other mechanisms.

Keywords: Cooperative breeder; Mammal; Mole-rat; Cooperation; Cooperative behaviour; Hormone; Glucocorticoid; Cortisol

1. Introduction

While evolutionary explanations for cooperative behaviour have attracted widespread interest (Hamilton, 1964; West et al., 2007a), the physiological mechanisms shaping their expression in animals are still uncertain (Soares et al., 2010). Cooperative behaviours, which increase the direct fitness of other individuals, and have been selected at least partially because of these effects (West et al., 2007b), are highly developed in cooperative breeders where a majority of individuals do not breed and support a minority of breeders in their reproductive effort (Clutton-Brock, 2016; Koenig and Dickinson, 2016). Cooperative behaviours can take a diversity of forms that include caring for young, defending and maintaining a territory, or acquiring resources (Clutton-Brock, 2016; Koenig and Dickinson, 2016) and can also be expressed in non-cooperative contexts. Elucidating the physiological mechanisms affecting cooperative behaviours is now necessary to determine how such mechanisms are shaped by evolutionary history, *e.g.* behaviours transitioning from being selfish to also benefitting others (Rogers and Bales, 2019; Schradin et al., 2018), and how individuals adjust their contributions to cooperative activities (Bell, 2010; Bergmüller and Taborsky, 2005; Cant, 2005; Clutton-Brock et al., 2002; Russell et al., 2003).

Several hormones may modulate the expression of cooperative behaviours (Montgomery et al., 2018; Schoech et al., 2004; Soares et al., 2010), though experimental support is still scarce (Bender et al., 2008b; Raynaud and Schradin, 2014; Zöttl et al., 2018). The diversity of cooperative activities within and across species makes hormones unlikely to affect all cooperative behaviours in a modular and consistent way. Instead, the effect of hormones on cooperative behaviour is likely to depend on the evolutionary history of the behaviour and its relationship with a hormonal pathway; the relationship between hormones and cooperative behaviours may be affected to a larger degree by the intrinsic characteristics of behaviours rather than their cooperativeness *per se*. For example, it has been suggested that alloparental care, just like parental care (Rilling and Mascaro, 2017; Ziegler, 2000; but see Lynn, 2016) may be increased by oxytocin and prolactin (Carlson et al., 2006b; Madden and Clutton-Brock, 2011; Mota and Sousa, 2000; Schoech et al., 2004) and decreased by testosterone (Schoech et al., 2004; Young et al., 2005).

Glucocorticoids (GC) hormones may have profound effects on cooperative behaviours (Raulo and Dantzer, 2018). Moderate increases in glucocorticoid concentrations (GCc) commonly occur in association with daily-life processes and predictable life history stages, where their effects on activity, feeding behaviour and energy metabolism support individuals in meeting an increase in their energetic needs (Landys et al., 2006). In response to unpredictable life-threatening events, GCc increase even further as a constitutive part of the stress response, where their actions orchestrate the adaptive physiological and behavioural responses to stressors (Sapolsky et al., 2000). Under non-stressful conditions, elevations in GCc may consequently support cooperative behaviours that are energetically demanding and part of behavioural routines, a characteristic shared by many types of cooperative activities across species (Canestrari et al., 2007; Lovegrove, 1989; Price, 1992; Russell et al., 2003; Sanderson et al., 2014; Taborsky and Grantner, 1998). Under stress, the direction of GC effect on cooperative behaviours may depend on whether increased cooperation represents an adaptive stress response. Stress-induced elevations in GCc may stimulate cooperative behaviours that reduce the fitness costs of stressors but suppress cooperative behaviours that increase such costs (McEwen and Wingfield, 2003; Sapolsky et al., 2000; Wingfield et al., 1998).

Correlational studies of cooperative carnivores (Carlson et al., 2006a, Carlson et al., 2006b; Sanderson et al., 2014), rodents (Raynaud and Schradin, 2015), primates (Mota et al., 2006), birds (Rubenstein, 2007), fish (Bender et al., 2008a) and humans (Buchanan and Preston, 2014; von Dawans et al., 2012) have demonstrated positive, negative, or null associations between GCc and contributions to cooperative behaviour. Although they are commonly used to infer the effect of GCc on cooperative behaviours (Carlson et al., 2006a, Carlson et al., 2006b; Raynaud and Schradin, 2015; Sanderson et al., 2014), findings from correlative studies are insufficient to demonstrate causality (Ball and Balthazart, 2008; Vullioud et al., 2013). For example, positive correlations between GCc and cooperative contributions could arise as a consequence of the increased energetic costs imposed by higher cooperative contributions (Lovegrove, 1989; Stranahan et al., 2008; Taborsky and Grantner, 1998) while negative correlations could occur because reduced energy intake and poorer body condition independently increase GCc (Levay et al., 2010; Lynn et al., 2003, Lynn et al., 2010; Schwartz and Seeley, 1997) and decrease cooperative behaviour. As a result, correlational studies do not provide a reliable basis for concluding that variation in GCc affect the contributions of individuals to cooperative activities, complicating the interpretation of inconsistencies in the associations between GCc and cooperative behaviours (Carlson et al., 2006a; Sanderson et al., 2014).

The limitations of correlative studies emphasize the need to explore the relationship between GCc and cooperative behaviour experimentally. As yet, experimental investigations on the effects of GC on cooperative behaviours in cooperatively breeding animals have only been performed in wild meerkats (Dantzer et al., 2017; Santema et al., 2013) where they produced mixed results. Substantially increased GCc, raised beyond their physiological range by a single injection of cortisol, had no effect on the contributions of male and female non-breeding helpers to pup-feeding or on the time they spent looking for predators (Santema et al., 2013). In a second study, the oral-provisioning of helpers for 10 days with cortisol reduced the contributions to baby sitting in males and females compared to same-sex individuals provisioned with mifepristone (Dantzer et al., 2017), a drug that antagonizes both progesterone and glucocorticoid receptors (Sitruk-Ware and Spitz, 2003). Cortisol provisioning also led males to give a higher proportion of the food they found to pups but made females less likely to feed pups at all compared to same-sex individuals provisioned with mifepristone (Dantzer et al., 2017).

In this study, we experimentally investigated the relationship between GCc and cooperative behaviours unrelated to alloparental care through independent manipulations of (i) GCc and (ii) cooperative contributions in captive Damaraland mole-rats (*Fukomys damarensis*). Damaraland mole-rats can live in groups of up to 40 individuals (Jarvis and Bennett, 1993) in which reproduction is monopolized by a single breeding female, paternity can be shared between several males, and nonbreeding helpers are commonly the offspring of the breeding female (Burland et al., 2002, Burland et al., 2004). Throughout the day and the night, individuals alternate periods of rest (mostly huddling in the communal nest) and activity ranging from a few minutes to several hours (Oosthuizen and Bennett, 2015). During activity periods, individuals routinely perform energetically demanding burrowing activities (Lovegrove, 1989; Zöttl et al., 2016) to maintain and expand an extensive network of underground galleries that provide access to tubers of desert plants which all group members feed on (Bennett and Faulkes, 2000; Zöttl et al., 2016). They also contribute to communal nests and food stores, though in smaller proportions of their time (Thorley et al., 2018; Zöttl et al., 2016). In captive groups, helpers' cumulative contribution of to these collective activities is positively correlated with how much breeding females rest, eat and gain mass

during gestation, and with their fecundity (Houslay et al., 2020). Individuals that are generally more active and spend more time away from the nest also tend to engage more in burrowing, nest building and food carrying activities (Thorley et al., 2018). This suggests that these three types of cooperative activities may be affected by similar hormonal mechanisms and that hormonal mechanisms increasing general activity, like higher GCc, could make individuals more cooperative. Individuals also defend the group against intruders and predators, huddle, groom and retrieve wandering pups into their birth chamber, but do not directly provision pups or juveniles (Bennett and Faulkes, 2000; Cooney, 2002; Zöttl et al., 2018).

To determine the effects of GC on cooperative behaviours, we experimentally increased GCc of female helpers, inducing GCc that largely overlapped with the range of natural GCc, and compared their contributions to burrowing, nest building and food carrying with a control treatment. To investigate whether cooperative behaviours affect GCc, we experimentally increased the contributions of helpers to burrowing activities by providing their groups with more sand to excavate and compared their GCc to a control treatment. We assumed individuals, fed *ad libitum* throughout the experiments, to be in good condition and have low constitutive GCc and thus predicted that elevations in GCc would stimulate the expression of cooperative behaviours. In turn, we predicted that heightened burrowing contributions would increase energy expenditure, inducing a rise in GCc.

2. Methods

2.1. General information

Groups were individually maintained in standardized artificial tunnel systems allowing behavioural observations (see electronic Supplementary material, Fig. S1). Every morning, groups were provisioned with an excess of food (sweet potatoes and cucumber) and nest material (paper towel), while sand was provided every morning and evening by pouring sand into sand dispensers.

Individuals were identified with RFID microchip and colourful hair dyes. In groups that were transferred from the wild, breeding males were identified by genotyping the males that were present in the group at conception, the breeding females and their offspring at thirteen previously used micro-satellite loci (Burland et al., 2001). In groups that were formed in captivity, by the pairing of two opposite-sex and unrelated individuals, paired males were considered as the sole breeders.

2.2. Experiment 1: manipulation of GCc (cortisol) in female helpers

To determine whether GCc affect cooperative behaviours, we experimentally manipulated cortisol, (the main form of GCc in Damaraland mole-rats (Clarke et al., 2001)) in female helpers and quantified their behaviours. Within seven pairs of females (subject 1 and subject 2) from 7 distinct groups (group-size range: 6 to 21 individuals, mean = 10.71, SD = 5.12), both subjects were exposed to a cortisol and a control treatment. On the first experimental day (day 1), subjects 1 and 2 respectively received a 7-day release pellet containing 0.001 mg (control treatment) and 5 mg of cortisol (cortisol treatment) before 8:00 a.m. Hormone pellets (Innovative Research of America) were inserted subcutaneously in the neck area using a 10-gauge precision trochar (Innovative Research of America) under anaesthesia with isoflurane (Safe Line Pharmaceuticals). Fourteen days after the end of the first treatment week, the same

procedure was repeated but the treatments were reversed. During treatments, food was provided in the form of transportable and non-transportable items in places of the tunnel system that could be reached by digging through sand as well as places that were accessible without digging.

For each experimental treatment, the behaviour of experimental subjects was recorded by observers, who were blind to the treatments that the subjects had received, during two 12-hour scan observation sessions (day 2 and day 5). To standardize digging opportunities across observations, sand dispensers were filled shortly before observations started and every 2 h thereafter.

To determine the effect of the cortisol treatment on cortisol levels, urine samples were collected in the morning of day 3 and day 6 of each treatment. To assess the physiological relevance of treatments, and support ecologically relevant interpretations of the effects of cortisol on cooperative behaviours, we also included in our dataset non-experimental cortisol levels of female helpers of similar body mass to the experimental subjects. We included cortisol levels quantified from female helpers' urine samples collected (i) in the absence of social conflict (n = 29 samples, from 12 individuals), because we assumed those levels to be low and reflect baseline variation in cortisol, and (ii) after their eviction by the dominant female (n = 12 samples, from 11 individuals), because we expected those levels to be higher than baseline cortisol levels (Young et al., 2006). Post-eviction samples were collected up to two days after female helpers were removed from their group, to prevent lethal injuries by the dominant female, and cortisol levels were thus likely to reflect decreasing levels of the recovery phase rather than peak levels of the acute phase of the stress response.

2.3. Experiment 2: manipulation of cooperative contributions

To investigate whether increased cooperative contributions affect GCc and assess whether changes in GCc are necessary to regulate cooperative contributions, we experimentally increased individual contributions to burrow maintenance and quantified the cortisol levels of helpers. To increase burrowing contributions, groups were provisioned with more sand (sand treatment) and food was provided in the form of transportable and non-transportable items placed behind the sand dispenser to encourage digging and moving of sand to access food. As a control, the same groups were fed in a similar way but their sand provisioning was not increased (control treatment). For two successive days, the sand providers were filled every hour between 07:00 and 19:00 during the sand treatment and were only filled at 07:00 and 19:00 during the control treatment. For a subset of the tested groups, treatments were extended to seven days and additional procedures were conducted for other purposes (Mendonça et al., 2020). The sequence of treatments was balanced across groups, and seven days separated the end of the first treatment from the beginning of the second treatment.

To determine the effects of sand manipulations on individual cooperative contributions, the behaviour of all group members was recorded during a 4 h (6 groups and 8 solitary individuals, group-size range: 1 to 17 individuals, mean = 7.04, SD = 7.18) or a 12 h (7 groups, group-size range: 9 to 20 individuals, mean = 13.5, SD = 3.28) scan observation session on the second day of each treatment. Twelve-hour scan sessions started between 07:00 and 08:00 and 4 h scan sessions started between 15:30 and 16:30. A urine sample was collected from helpers older than 355 days after the scan session for measurement of cortisol. Samples were collected either in the evening immediately after a 4 h scan session (n = 31

females, *n* = 26 males) or in the morning after a 12 h scan session (n = 31 females, n = 26 males).

2.4. Behavioural observations

Scan observations (for details, see electronic supplementary material) were recorded with android handheld tablets operating the software Pocket Observer (Noldus Information Technology, NL). The behaviour of all group members was recorded every 4 min, leading to the collection of 180 individual scan samples for 12 h sessions and 60 individual scan samples for 4 h sessions. Behaviours were scored following a pre-defined and fixed sequence of individuals based on an ethogram including 17 distinct behaviours (see Table S1). Analyses were restricted to all active behavioural states (*i.e.* excluding resting states), which were grouped into a Total activity category. Total activity was divided into three cooperative and one non-cooperative categories: Burrowing included behaviours used by animals to dig through the sand, move it out of the tunnel system, and use it to seal small openings of the tunnel system; Food carrying consisted in the carrying and deposition of food items in food stores; Nest building referred to the transport of paper towel in the tunnel system and its deposition and reorganisation in the communal nest; Non-cooperative behaviours included the remaining behavioural states expressed by active individuals. For this study, we did not aim to investigate the effects of GCc on alloparental care, like huddling pups in the nest, and pups were only present in 2 groups during Experiment 1.

2.5. Urine samples

Urine was collected from individual urine chambers, immediately transferred into Eppendorf tubes with a disposable Pasteur pipette, and stored at −20 °C until processing. Urination delay (time between placement in the chamber and urination) was recorded to control for a possible stressful effect of urine sampling procedures on cortisol levels in later statistical analyses. Individuals remained a maximum of 180 min in the urine chambers.

2.6. Cortisol quantification

For Experiment 1 and the non-experimental samples used for treatment validation, urinary cortisol was quantified using radioimmunoassay kits (Coat a Count, Diagnostic Products Corporation, Los Angeles, CA) validated for Damaraland mole-rats (Clarke et al., 2001). For Experiment 2, a solid-phase extraction was performed using Isolute C18(EC) cartridges (50 mg/1 cc, Biotage) and urinary cortisol quantification was performed with ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The specific gravity of all urine samples was determined using a digital handheld refractometer (Atago Ltd) to adjust raw cortisol concentrations following Miller et al. (2004) and correct for differences in urine concentrations. Details of the methods followed for sample preparation, analytical procedures, quality control and calculation of intra- and inter-assay coefficient of variation are provided in the electronic supplementary material.

2.7. Statistical analyses

All data analyses were performed with R version 3.6.1 (R Core Team, 2019). The “tidyverse” packages were used for data wrangling and visualization (Wickham et al., 2019). Prior to statistical analyses, we checked for the presence of outliers or collinearity between model covariates. For statistical analyses, we fitted (generalized) linear mixed models (GLMMs) in

glmmTMB (Brooks et al., 2017) in which group and individual identity were specified as nested random effects to account for the dependency structure in the data caused by the repeated measurements of individuals within groups. We specified a gamma error structure with log link when cortisol concentration was set as the response variable, a beta-binomial error structure with logit link when the odds of a behavioural category (the ratio between the count of scan samples that the relevant category was scored and was not scored) was set as the response variable, and a gaussian error structure with identity link when differences in cortisol concentration or body mass across treatment were set as response variables. Odds computed over all individual scan samples (180/12 h session; 60/4 h session) were used to estimate the proportion of time budget dedicated to a behavioural category throughout complete scan sessions, whereas odds computed over active scan samples were used to estimate the proportion of time dedicated to a behavioural category during periods of activity. The choice of model covariates included in our full models was determined by *a priori* hypothesis and/or data exploration, with treatment effects anticipated to vary as a function of subjects' body mass (centred within each sex to facilitate the interpretation of estimated model parameters (Schielzeth, 2010)), day of treatment (Experiment 1), and sex (Experiment 2). In models where cortisol level was set as the response variable, we specified the delay between placement in the urine chamber and urination as an independent covariate because such sampling procedures could be stressful and increase cortisol levels. In case full models of choice suffered from convergence issues, interactions between covariates of lesser relevance to the interpretation of our results were removed until the models converged. Full models were simplified following a stepwise backward deletion of non-significant covariates in which higher order terms and non-significant covariates were sequentially removed until only significant terms remained. Model validation was performed using functions from the DHARMa package (Hartig, 2018). Details on the specification, simplification and output of all models are available in the electronic supplementary materials (see Table S2-S10). Figures show raw data, to which horizontal jitter was in some cases added to facilitate visualization, and lines indicate repeated individual measurements.

To check the validity of our cortisol treatment, we tested whether cortisol concentrations of female helpers were increased during the cortisol treatment in comparison to the control treatment. We anticipated that release of cortisol from the implant may not be constant across time and that the degree to which cortisol concentrations were affected by the cortisol treatment may depend on body mass and thus specified treatment, treatment day and body mass and all interactions between them as model covariates. To support an ecologically relevant interpretation of our results, we compared the cortisol concentrations induced by the cortisol treatment to cortisol concentrations of non-experimental female helpers living in environments assumed to be non-stressful (absence of social conflict) and stressful (within 48 h of eviction). We specified the condition under which urine samples were collected as model covariate but ignored body-mass, as our selection of non-experimental helpers included individuals of similar range of body mass than experimental female helpers.

To determine whether higher GCc increased the daily contributions of female helpers to cooperative activities, we investigated whether the proportion of individual time budget dedicated to burrowing, nest building and food carrying over 12 h scan sessions were increased by the cortisol treatment. To investigate whether the significant effects of cortisol were specific or whether they resulted from a general effect of cortisol on activity, we tested the effect of treatment on daily activity and non-cooperative behaviours, and on the proportion of time dedicated to relevant cooperative behaviours during activity bouts. We anticipated that the effect of the cortisol treatment could vary as a function of the release of

cortisol by the implants and of the behaviours expressed over the preceding days and thus specified treatment, treatment day and an interaction between the two as model covariates. We also expected that the effect of treatment could have a different effect on heavier than on lighter individuals and specified body mass and its interaction with treatment as additional covariates.

To support an ecologically relevant interpretation of the effect of cortisol on cooperative behaviour, we investigated whether the cooperative behaviour which daily contributions were significantly affected by the cortisol treatment were differently affected by lower and higher elevations in cortisol. We divided the cortisol concentrations induced by the cortisol treatment by their median and assigned them into a "low" and a "high" cortisol category. These two categories were specified as a three-level covariate that also included the control treatment. In a separate model, we tested whether changes in daily cooperative contributions correlated with increases in cortisol induced by the cortisol treatment. For each individual and treatment day, we subtracted the daily cooperative contributions (proportion of daily time budget) of the cortisol treatment to that of the control treatment and specified this difference as response variable. Since we had no *a priori* reasons to anticipate whether absolute (cortisol treatment – control treatment) or relative increases (cortisol treatment/control treatment) in cortisol concentrations were more likely to affect cooperative contributions, each was individually specified as covariate in two separate models. To account for the possibility that changes in cooperative contributions may be affected by cooperative contributions during the control treatment (individuals with high cooperative contributions may have less scope to increase their cooperative contributions), the proportion of cooperative contributions during the control treatment was specified as a covariate in both models.

To check the validity of the sand treatment, we investigated whether individual burrowing contributions, the proportion of time budget dedicated to burrowing over entire scan sessions, were increased by the sand treatment. To assess the specificity of effects of the sand treatment on behaviours, we determined whether the sand treatment also affected the proportion of time budget dedicated to food carrying, nest building and non-cooperative behaviours. We anticipated that the effect of treatment on behaviours could depend on sex and body mass and thus specified treatment, sex and body mass and all possible interactions between them as covariates.

To determine whether higher burrowing contributions were associated with increased cortisol, we investigated whether cortisol concentrations were higher after the scan session (12 h or 4 h sessions) in the sand treatment as they were in the control treatment. In addition to the effect of treatment, we anticipated that cortisol concentrations could also differ between males and females and between the morning and the evening. Accordingly, we specified treatment, sex, period of urine sample collection (morning the day after 12 h scan session or evening immediately after 4 h scan session) and all possible interactions between them as model covariates.

To assess whether our experimental treatments were associated with loss of body-condition, we determined for both experiments the effect of treatment on changes in body mass. We specified the difference in body mass between the end and the beginning of each treatment as response variables. We anticipated that changes in body mass could vary with the body mass at the start of treatment (heavier individuals may be subjected to larger loss of body mass) and the period of body mass measurements (morning or evening; Experiment 2 only). Thus,

we specified treatment, body mass at the start of treatment, period of body mass measurements and all possible interactions between them as model covariates.

3. Results

3.1. Experiment 1: manipulation of GCc in female helpers

The cortisol treatment caused a 3-fold increase in urinary cortisol of female helpers in comparison to the control treatment ($p < 0.001$; Fig. 1a, Table S2). During the cortisol treatment, female helpers had higher cortisol levels than non-experimental female helpers that lived in an environment assumed to be non-stressful due to the absence of social conflict ($p < 0.001$, Fig. 1b, Table S3). During the cortisol treatment, female helpers had cortisol levels that did not significantly differ from those of non-experimental female helpers that had been evicted from their natal group within the last 48 h ($p = 0.132$, Fig. 1b, Table S3). Despite these differences, the range of cortisol levels induced by the cortisol treatment largely overlapped with the upper half of cortisol levels in the control treatment and in the absence of conflict, and with cortisol levels post-eviction (Fig. 1b). After eviction, cortisol levels were higher than in the absence of conflict, supporting that eviction is stressful and that post-eviction cortisol levels reflect stress-induced cortisol levels ($p = 0.03$, Fig. 1b, Table S3).

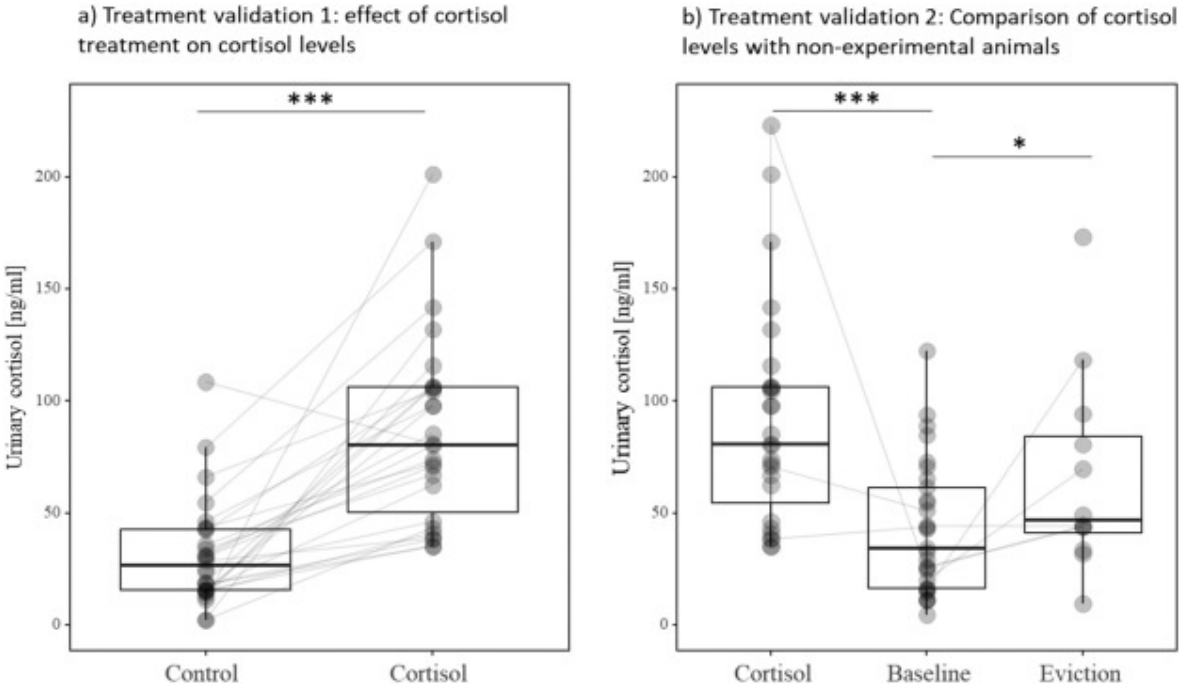

Fig. 1. Effects of cortisol treatment on cortisol levels in female Damaraland mole-rat helpers: (a) Urinary cortisol concentrations (in ng/ml) quantified on day 3 and day 6 of the control and cortisol treatments (b) Urinary cortisol concentrations (in ng/ml) quantified during the cortisol treatment and in female helpers outside of experimental contexts, in the absence of overt social conflict (Baseline) and within 48 h of their eviction from the group (Eviction). Lines between points show repeated measurements of individuals. * indicates $p < 0.05$, *** indicates $p < 0.001$.

In response to the cortisol treatment and compared to the control treatment, female helpers increased their activity from 35.1 to 45.0% of their daily time budget (estimated through 12 hour scan observation; $p = 0.001$; Table S4a). This increase in activity was driven by an

increase in non-cooperative behaviours from 24.0 to 28.1% ($p = 0.032$; Table S4f, Fig. 2d) and burrowing from 9.2 to 14.5% of female helpers' daily time budget ($p = 0.002$; Table S4b; Fig. 2a). Compared to burrowing, the contributions of female helpers to food carrying and nest building were low (<1.8% of daily time budget) and remained unaffected by the cortisol treatment (both $p > 0.132$; Table S4d, e; Fig. 2b, c). The cortisol-induced increase in daily burrowing contributions resulted from female helpers being active for longer and increasing the time they burrowed while active from 23.1 to 30.7% of their active time budget in comparison to the control treatment ($p = 0.014$, Table S5a). Despite the increases in daily burrowing contributions and non-cooperative activities, female helpers did not lose more body mass during the cortisol treatment than they did during the control treatment ($p = 0.189$, Table S6).

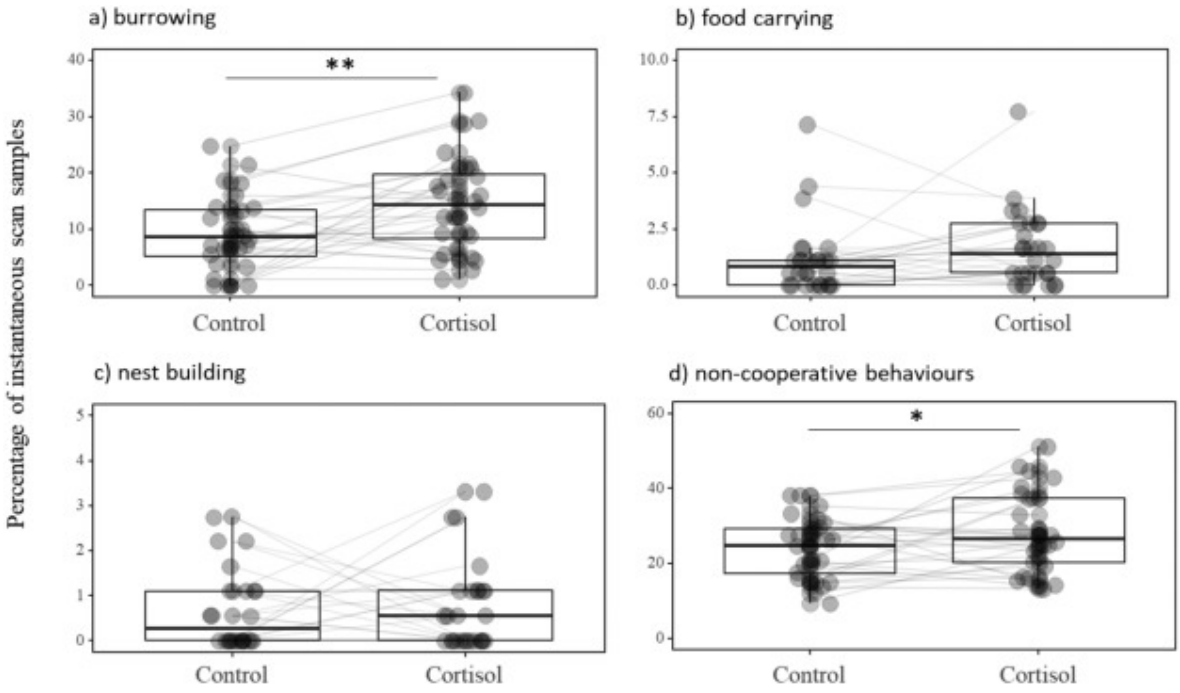

Fig. 2. Effects of cortisol treatment on the behaviours of female Damaraland mole-rat helpers: Percentage of instantaneous scan samples that female and male helpers were scored a) burrowing, b) food carrying, c) nest building and d) being non-cooperative during 12 h observation scan sessions, performed on day 2 and day 5 of the control and cortisol treatment ($n = 14$ per treatment day for each treatment). Lines between points show repeated measurements of individuals. * indicates $p < 0.05$, ** indicates $p < 0.01$.

There was no indication that increases in daily burrowing induced by the cortisol treatment were dependent on cortisol levels or the magnitude of their increases. When data from the cortisol treatment were split in two by their median cortisol levels, lower experimental increases (two-fold) in cortisol, which induced cortisol levels that fully overlapped with cortisol levels measured in the control treatment and in conditions assumed to be non-stressful, still increased daily burrowing contributions compared to controls ($p = 0.004$, Table S4c). This increase was not weaker than the one caused by higher experimental increases (five-fold) in cortisol levels ($p = 0.428$, Table S4c). In addition, daily contributions to burrowing were not more pronounced at the beginning of the cortisol treatment ($p = 0.599$, Table S4b), when cortisol levels tended to be higher than they were later in treatments ($p = 0.07$, Table S2), nor were they more pronounced in lighter female helpers that received more cortisol relative to their body mass, compared to heavier females that received relatively

lower doses ($p = 0.137$, Table S4b). Finally, neither absolute ($p = 0.632$, Table S7a) nor relative ($p = 0.981$, Table S7b) changes in daily contributions to burrowing activities in response to the cortisol treatment were correlated with the degree of increase in cortisol levels.

3.2. Experiment 2: manipulation of cooperative contributions

When groups were supplied with more sand, male and female helpers increased their contributions to burrowing from 4.5 to 16.0% of their time budget (Table S8a; Fig. 3a). The behavioural effects of the sand treatment on active behaviour were restricted to burrowing, since contributions to food carrying ($p = 0.958$; Table S8b; Fig. 3b), nest building ($p = 0.119$; Table S8c; Fig. 3c) or non-cooperative activities ($p = 0.778$; Table S8d; Fig. 3d) were not affected by the sand treatment. Although the contributions of helpers to burrowing activities were more than tripled during the sand treatment, their cortisol levels remained unchanged ($p = 0.444$; Table S9; Fig. 4) and they lost less body mass in comparison to the control treatment ($p < 0.001$; Table S10). There was no evidence of a circadian variation in cortisol as morning and evening cortisol levels did not significantly differ ($p = 0.067$; Table S9).

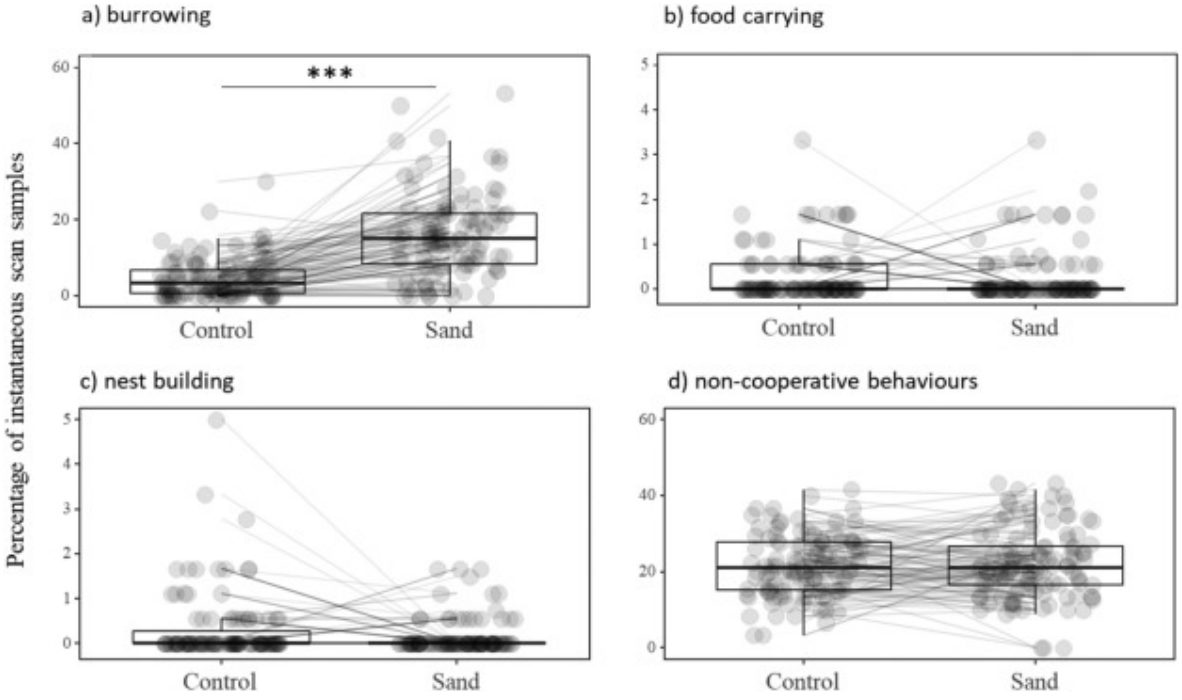

Fig. 3. Effect of increased sand provisioning on the behaviours of Damaraland mole-rat helpers: Percentage of instantaneous scan samples that female and male helpers were scored a) burrowing, b) food carrying, c) nest building and d) being non-cooperative during 4 h or 12 h observation scan sessions performed on the second day of the control and the sand treatment**.** Lines between points show repeated measurements of individuals. *** indicates $p < 0.001$.

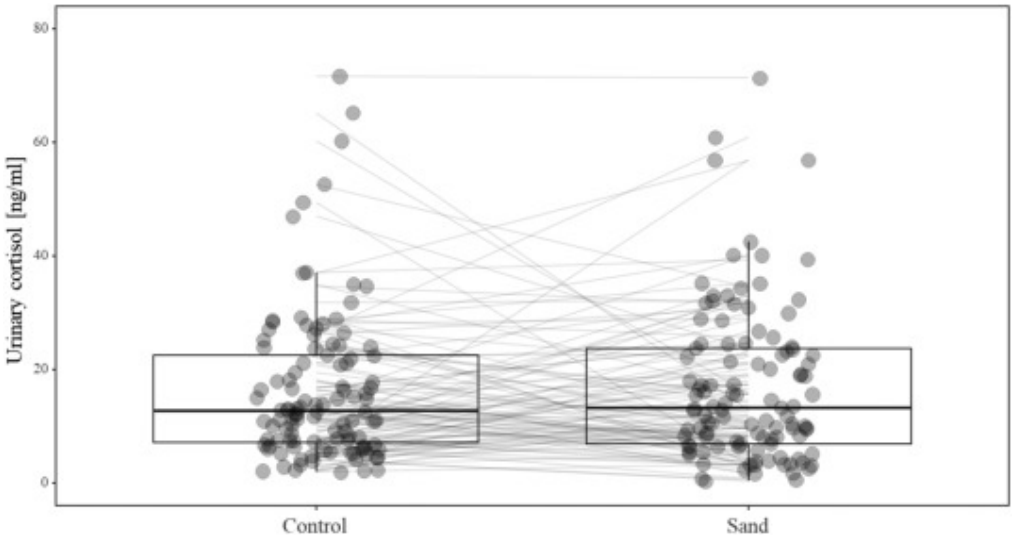

Fig. 4. Effect of increased sand provisioning on the cortisol levels of Damaraland mole-rat helpers. Urinary cortisol concentrations of female and male helpers determined from samples collected in the evening immediately after treatment or the following morning after a night of exposed to non-experimental conditions during the control and the sand treatments. Lines between points show repeated measurements of individuals.

4. Discussion

Our study on captive Damaraland mole-rats provides strong evidence that GC can affect some forms of cooperative behaviour. We show that female helpers were active for longer and burrowed proportionally more while active when their GCc were experimentally increased within GCc natural range, leading to an increase in their daily contributions to burrowing activities. In the wild, these energetically demanding and cooperative activities (Houslay et al., 2020; Lovegrove, 1989) are necessary to maintain and expand the extensive systems of underground tunnels that provide access to tubers of desert plant all group members can feed on. The effect GC on burrowing is unlikely to be explained by hunger or perceived negative energy balance that may have been induced by chronic elevations in GCc (Dallman et al., 1993) and may have caused animals to continuously seek for food. If that was the case, female helpers would have fed on food items that were continuously accessible without the need to burrow during the GC treatment, consequently reducing their burrowing contributions and possibly gaining body-mass. We also show that helpers of both sexes increased their contributions to burrowing in response to experimental manipulations of the physical environment, though their GCc remained unchanged. This demonstrates that increases in GCc, a physiological marker of stress (Sapolsky et al., 1986), are neither necessary to increase burrowing contributions nor a consequence of the increases in energetic demands imposed by higher levels of burrowing. Our findings suggest that the stimulatory actions of higher GCc (Landys et al., 2006) may promote energetically demanding forms of cooperation, which are common among cooperative breeders (Canestrari et al., 2007; Lovegrove, 1989; Price, 1992; Russell et al., 2003; Sanderson et al., 2014; Taborsky and Grantner, 1998), but that the expression of cooperative behaviours affected by GCc can also be adjusted by other mechanisms that do not involve changes in GCc.

In contrast to our predictions, the effect of experimental increases in GCc on cooperative activities were restricted to burrowing and did not increase female helpers' contributions to food carrying and nest building. Since experimentally increased GCc led female helpers to

rest less and to be generally more active, it is not clear why such increases in activity did not translate into higher daily contributions to food carrying or nest building. One possibility is that such absence of GC effect occurred because nest material and transportable pieces of food were only provisioned once a day during treatments. Indeed, had groups been provided with more opportunities to express nest building and food carrying, those cooperative activities may have been increased during the cortisol treatment as well. Similarly, experimental increases in GCc might not have affected daily burrowing contributions if sand had been provisioned only once daily.

The evidence that heightened GCc increase burrowing contributions seems consistent with some of the results of previous experimental studies on the effect of GCc on other energetically demanding forms of cooperative behaviour (Clutton-Brock et al., 1998; Russell et al., 2003). For example, provisioning meerkat helpers with GC for 10 days made males give a higher proportion of the food items they found to pups, in comparison to same-sex helpers provisioned with mifepristone (Dantzer et al., 2017), a GC and progesterone receptor antagonist (Sitruk-Ware and Spitz, 2003). However, they do not seem consistent with others: for example, female and male meerkat helpers injected with GC did not provision pups more than same-sex controls injected with saline (Santema et al., 2013) and GC-provisioned female meerkat helpers were less likely to feed pups compared to same-sex helpers provisioned with mifepristone (Dantzer et al., 2017). In addition, GC-provisioned male and female meerkat helpers decreased their contributions to baby-sitting compared to same-sex helpers provisioned with mifepristone (Dantzer et al., 2017). Though these comparisons could suggest that there are differences in the effects of GCc on cooperative behaviours between mole-rats and meerkats, methodological differences between studies in the two species complicates comparisons of their results. The effect of mifepristone on progesterone receptors (Sitruk-Ware and Spitz, 2003) combined with the absence of differences in cooperative behaviours between GC- and saline-provisioned helpers (Dantzer et al., 2017), suggests that progesterone may have contributed to changes in cooperative behaviours in meerkats. Furthermore, GC injections raised meerkat GCc above its natural range (Santema et al., 2013), compromising interpretations of the effect of GC on cooperative behaviours in non-experimental animals (Crossin et al., 2016).

The GCc of female helpers during the cortisol treatment largely overlapped with the upper half of the ranges of GCc during the control treatment and in the absence of social conflict, and with the range of GCc measured after eviction. This suggests that natural GCc may stimulate burrowing over a wide range of concentrations spanning higher baseline and stress-induced GCc. Although post-eviction GCc are unlikely to reflect GCc at the peak of a stress response (see methods section), we found no evidence suggesting that the effect of higher baseline and stress induced GCc could differ; the cortisol treatment increased burrowing independently of the height of GCc or of the magnitude of GCc increases it induced. This further suggests that the dose-relationship between GCc and burrowing may not follow a graded function but rather a step-function in which increases in GCc beyond a critical threshold, which may vary across individuals, elevate burrowing (Adkins-Regan, 2005). This suggestion could be formally investigated by testing the effect of several doses of GC on burrowing and including both lower and higher doses of GC than the ones used in our study.

The maintenance of the effect of GC treatment on burrowing at lower concentrations GC that fully overlapped with the ones of non-stressed female helpers is ecologically relevant. Indeed, burrowing is an important part of helpers' daily-life routine (Thorley et al., 2018; Zöttl et al., 2016) that may provide both direct and indirect fitness benefits (Burland et al., 2004; Houslay

et al., 2020) in the absence of apparent stressors, when GCc are expected to be relatively low (Landys et al., 2006). GC-induced increases in burrowing activities may also be adaptive in some stressful situations – for example in response to unpredictable rainfall that dampen the sand and reduce the energetic costs of expanding underground tunnels (Jarvis et al., 1998; Lovegrove, 1989; Young et al., 2010). Whether GC always elevates burrowing or do so only in some contexts is still unclear. In particular, we would not expect increases in GCc to have a different effect on burrowing expressed outside of cooperative contexts due to the conserved role of GC on energy production and activity (Landys et al., 2006; Sapolsky et al., 2000; Stranahan et al., 2008). Yet, it is conceivable that increases in GCc induced by poor body-condition may reduce burrowing contributions if the energetic demands of burrowing further compromise body-condition and survival.

It has been proposed that dominant breeders may use aggression to elevate the contributions of subordinate helpers to cooperative activities (Cant, 2011; Clutton-Brock and Parker, 1995), and our results suggest that this may be regulated by aggression-induced increases in GCc (Goymann et al., 2001; Louch and Higginbotham, 1967; Surbeck et al., 2012). As yet, there is no firm evidence of enforcement of cooperative behaviours in cooperatively breeding vertebrates (Clutton-Brock, 2016), but for species like the naked mole-rat (*Heterocephalus glaber*) (Reeve, 1992; but see Jacobs and Jarvis, 1996) and the cichlid *Neolamprologus pulcher* (Fischer et al., 2014) where some support is available, the role of GC remains unknown. In Damaraland mole-rats, future studies should determine whether breeders become more aggressive when the need for burrowing is higher, and whether increases in received aggression coincide with an elevation of helpers' GCc and burrowing contributions.

Large individual differences in cooperative contributions are common in cooperative societies (Bergmüller et al., 2010; Clutton-Brock et al., 2001; Komdeur, 2006; Zöttl et al., 2016) but their underlying physiological basis is unclear. Our findings suggest that variation in hormones, such as GC, can generate differences in cooperative behaviours both within and between individuals. This possibility has received some support in wild meerkats where consistent inter-individual differences in GCc correlate with differences in cooperative contributions (Dantzer et al., 2019). However, not all cooperative behaviours may be affected by GCc (Bender et al., 2008a; Mota et al., 2006) and the expression of cooperative behaviours that are affected by GCc, like burrowing, can be uncoupled from GCc. Together, GCc-dependent and independent regulation of cooperative behaviours may support adaptive adjustments in cooperative contributions: GCc integrate individual and environmental conditions (Creel et al., 2013; Hau et al., 2016) and can thus mediate the effect of such conditions on the expression of cooperative behaviours. However, cooperative contributions are not constrained by GCc and can be increased when GCc remain low but the need for cooperation is high.

How Damaraland mole-rat helpers could meet an increase in burrowing demands imposed by their physical environment in the absence of increases in GCc is unknown. A possibility is that the expression of high-GC-affinity mineralocorticoid receptors (MR) and/or the low-GC-affinity glucocorticoid receptors (GR) (Landys et al., 2006; Reul and De Kloet, 1985) may be increased, leading to an elevation in GC signalling without raises in GCc. The administration of MR (Lainscak et al., 2015) and/or GR (Solomon et al., 2014) antagonists combined with an increase in sand provisioning would allow to test this hypothesis by examining whether increases in burrowing activities are still possible when GCc signal is blocked. Although increases in GC receptors may support the sustained expression of heightened cooperative contributions, a non-mutually exclusive explanation is that increases in cooperative

behaviours are hardwired, *i.e.* individuals are fully equipped to rapidly respond to increases in the demand for cooperative behaviours without the need of prior neuroendocrine adjustments.

Our study provides insights that are relevant for interpreting the results of correlational investigations of the relationship between GCc and cooperative behaviour in other species. The absence of increases in GCc in response to experimentally increased cooperative activities in captive Damaraland mole-rats contrasts with results from correlative studies in wild banded mongooses and meerkats showing that GCc measured during the period of pup-feeding and after baby-sitting bouts respectively, were positively correlated with these cooperative activities (Carlson et al., 2006b; Sanderson et al., 2014). A possible explanation for this contrast is that elevations in GCc, which favour the mobilization of internal energy stores (Landys et al., 2006; Sapolsky et al., 2000), may be unnecessary for helpers to cooperate more when food resources are unlimited and enough energy can be extracted from the environment. This explanation is consistent with our finding that helpers, who were provisioned food *ad libitum*, did not suffer body mass loss when their contributions to burrowing activities were experimentally increased. In the wild, where food resources are often limited, the energetic costs of heightened levels of cooperation may induce an elevation in GCc. This physiological response may in turn support the expression of cooperative behaviours, as suggested by the increases in burrowing in response to experimental increases in GCc. However, the possible uncoupling between GCc and the cooperative behaviour they affect may conceal the effect of GCc on cooperative behaviour because large variation in cooperative contributions can be generated independently of GCc. Thus, null associations between GCc and cooperative behaviour, such as the ones occurring in cooperatively breeding cichlids and primates (Bender et al., 2008a; Mota et al., 2006), should not be interpreted as an evidence of absence of GC effects on cooperative behaviours.

Our study advances the understanding of the physiological mechanisms controlling the expression of cooperative behaviours, providing experimental support to the suggestion that higher GCc stimulate energetically demanding cooperative behaviours. As more experimental studies address the generality of this hypothesis and the action of other neuroendocrine pathways on cooperative behaviours, a major challenge will be to integrate their findings within an ecologically relevant framework. Elucidating when and how these control mechanisms mediate the effect of environmental and individual characteristics on the expression of cooperative behaviours is necessary to unravel how individuals adjust their cooperative contributions to optimize inclusive fitness.

CRediT authorship contribution statement

TCB initiated and organised the project; PV and RM designed the experiments. PV, RM, RF, RL, RK and NK collected the data. PV, GG and NB conducted the hormone analysis. PV analysed the data. PV and TCB wrote the manuscript. PV, RM and MZ contributed to manage the research site.

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