Hormones and cooperative behaviours in the Damaraland mole-rat (*Fukomys damarensis*)

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Summary

Large individual differences in cooperative contributions are common within animal societies such as cooperative breeders, where helpers care for offspring which are not their own. Understanding this variation has been a major focus in behavioural ecology and while evidence has shown that individuals are capable to adaptively adjust their cooperative behaviours, the physiological mechanisms underlying such adjustments remain poorly understood. Steroid hormones are prominent candidates to regulate cooperative behaviours due to their ability to integrate internal physiological state and environmental stimuli to produce an adaptive behavioural response. In this thesis, I investigate the effects of two steroid hormones, Cortisol (CORT) and Testosterone (T), in the regulation of cooperative behaviours in the Damaraland mole-rat (*Fukomys damarensis*). Because these hormones are susceptible to both modulate and be modulated by cooperative contributions, I experimentally tested both sides of this relationship. I show that, despite the absence of correlation between CORT and T and cooperative contributions, experimental increases of cooperative contributions elevate CORT levels, but not T (Chapter 3). Additionally, experimental increases of CORT levels in female helpers raised their cooperative contributions by more than one half demonstrating the regulatory effect of CORT on cooperative behaviours (Chapter 4). As breeding opportunities are likely to affect cooperative contributions and because T is a likely candidate to mediate a trade-off between future reproduction and current cooperation, I tested the effects of experimental increases of T levels in female helpers. I show that such elevations have no measurable effect of aggression, dispersal tendencies (both important to attain a breeding position) or cooperative contributions (Chapter 5). Taken together, the results of this thesis

demonstrate that CORT can both respond to and regulate cooperative behaviours and suggest that this hormone may play a major role in the adaptive regulation of cooperative behaviour.

Preface

This thesis is a result of my own work and contains no work done in collaboration, except where stated otherwise. The text does not exceed 60,000 words. No part of this thesis has been submitted to any other university in application for a higher degree.

Philippe Vullioud

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Chapter 1

General Introduction

1.1 Individual differences in cooperative behaviours: evolutionary explanations

Large individual differences in cooperative contributions are characteristic of animal societies where individuals behave to the benefit of others (Bergmüller et al., 2010; Clutton-Brock, 2016; Komdeur, 2006). Such differences are particularly striking in the context of cooperative breeding (Clutton-Brock et al., 2001a; Hodge, 2007; Zöttl et al., 2016b) where a majority of group members known as helpers delay dispersal, forgo or forfeit reproduction, and help a minority of breeders in raising their young (Clutton-Brock, 2016; Koenig and Dickinson, 2016).

Inclusive fitness theory, which is encapsulated by Hamilton's rule (Bourke, 2011; Hamilton, 1964), has provided the most valuable framework to explain why some helpers cooperate more than others: the expression of cooperative behaviours should be favoured whenever the sum of their lifetime effects on the reproductive success of the performer (C; $C<0$ for altruistic behaviour, $C>0$ for mutually beneficial behaviour) and their positive effects on the reproductive success of the recipient (B) weighted by the genetic relatedness between the two (r) is greater than zero ($rB + C > 0$).

Empirical work has supported these predictions by revealing that cooperative contributions are conditional on individual, social and environmental conditions affecting the direct (C) and indirect (B) fitness components of cooperative behaviours (Bergmüller et al., 2005a; Cant, 2005; Clutton-Brock et al., 2002). Individuals typically adjust their cooperative contributions such as to minimize the negative downstream fitness effects of the short-term energetic costs of cooperative activities (Boland et al., 1997; Canestrari et al., 2007; Grantner and Taborsky, 1998; Hodge, 2007; Lovegrove, 1989; Russell et al., 2003). They cooperate less when they are relatively lighter (Clutton-Brock et al., 2002; Russell et al., 2003), when food resources are scarce (Bell, 2010; Bruintjes et al., 2010; Nichols et al., 2012), when they have been more generous in the past (Russell et al., 2003; Sanderson et al., 2014), or when they approach reproduction (Bell, 2010; Bergmüller et al., 2005a; Cant, 2005; Gilchrist and Russell, 2007; Young et al., 2005). In contrast, they help more when they forage more efficiently (Clutton-Brock et al., 2001a) and when the energetic constraints of cooperative activities are relaxed by supplementary feeding (Boland et al., 1997; Clutton-Brock et al., 2002; Russell et

al., 2003). Helpers also increase their cooperative contributions in the presence of a smaller workforce or when the ratio of helper to offspring is lower (Gilchrist and Russell, 2007; Liebl et al., 2016; Russell et al., 2003), since the fitness benefits of each unit of cooperative contributions to the recipients are likely to be greater.

1.2 Individual differences in cooperative behaviours: physiological explanations

Although individuals adjust their cooperation contributions in an adaptive manner, the physiological mechanisms through which the fitness costs and benefits of cooperative actions are evaluated and the expression of cooperative accordingly fine-tuned, remain largely unknown. Elucidating the neural, genetic, epigenetic and neuroendocrine pathways regulating cooperative behaviours represents an outstanding challenge. Yet, this is needed to develop an integrated understanding of cooperation in nature and move the field beyond the phenotypical gambit that has been long-standing in the field of behavioural ecology (Rubenstein and Hofmann, 2015). Ultimately, a better understanding of the physiological mechanisms regulating cooperative behaviours has the potential to shed some light on potential constraints acting on the expression of cooperative behaviours (Hau, 2007; Ketterson and Nolan, 1999).

Cooperative behaviours ultimately represent a uniform category of behaviours that increase the reproductive success of their recipients (West et al., 2007), but their underlying physiological regulatory mechanisms are likely to vary between the multiple forms they can take (Clutton-Brock, 2016; Koenig and Dickinson, 2016; Soares et al., 2010). While both helpers' territory defence and provisioning of offspring can be viewed as cooperative, the aggressive display advantageous to the expression of the first may be highly inappropriate to the expression of the second.

In cooperative breeders, three major classes of hormones have so far attracted most of the interests: the neurohormones oxytocin and vasopressin, the peptide hormone prolactin and the steroid hormones glucocorticoid (GC) and testosterone (T), both of which are the focus of this thesis. The acknowledged effect of the neurohormones oxytocin and vasopressin on social bonding and parental behaviours raised interest on their role in the regulation of some forms of

alloparental behaviour, relying on close associations with offspring (Bales et al., 2004; Madden and Clutton-Brock, 2011; Olazábal and Young, 2006). The role of the peptide hormone prolactin on the expression of alloparental care has been investigated based on its ubiquitous role in the regulation of parental behaviours (Carlson et al., 2006b; Ziegler, 2000).

1.3 Individual differences in cooperative behaviours: a role for steroid hormones?

Glucocorticoids and T are principally secreted by the gonads and the adrenals respectively, as the end-product of the hypothalamic-pituitary-adrenal (HPA, HP-interrenal axis in fish and amphibians) and hypothalamic-pituitary-gonadal (HPG) axes, also referred to as the stress and reproductive axis (Nelson, 2005). GC and T are essential regulators of development and exert profound and pleiotropic effects on physiology, morphology and behaviours (Adkins-Regan, 2005; Arnold, 2009; Nelson, 2005; Seckl, 2004). The common pathway through which these hormones shape phenotypes is via the formation of a complex with specific intracellular receptors that regulate genes' expression. Through their capacity to modulate the structure and the activity of the nervous system, steroids mediate behaviours via the interaction of effects falling within a continuum, which extremes can be categorized as "organizational" and "activational". Organizational effects are developmental effects that last for the entire life and usually take place during finite sensitive time-windows, often early in life, while activational effects are rapid, reversible and can occur at any developmental stage (Oliveira, 2009; Phoenix et al., 1959; Schulz et al., 2009).

Secretions of both GC and T not only regulate genes' expression but also integrate internal physiological states with behavioural and environmental stimuli, including the ones susceptible to shape the fitness values of cooperative actions. Indeed, GC and T profiles can vary with sex, developmental stage (age), food availability and nutritional status, season, group size, social rank and conditions (Creel et al., 2013; Hau et al., 2016; Oliveira, 2004; Sapolsky, 2005; Wingfield et al., 1990). GC and T are particularly responsive to social conflicts during which their secretions are increased by the expression of aggressive behaviour (Goymann, 2009; Landys et al., 2007; Oliveira, 2004; Ros et al., 2014) and to mating opportunities and behaviours (Harding, 1981).

Glucocorticoids are essential physiological regulators of energy balance (Landys et al., 2006; McEwen and Wingfield, 2003) and may thus be critical to the regulation of energetically costly cooperative behaviours such as the ones frequently expressed by cooperative breeders. Glucocorticoids regulate locomotor activity, foraging behaviours and energy metabolic pathways (Landys et al., 2006) to ultimately allow individuals to meet the energetic demands imposed by both predictable and unpredictable life history events (Landys et al., 2006; McEwen and Wingfield, 2003). The expression of energetically demanding activities increase energetic needs causing an elevation in GC (Hackney and Viru, 1999; Malisch et al., 2008; Stranahan et al., 2008) that facilitate the mobilisation of energy stores and energy production (Landys et al., 2006; Sapolsky et al., 2000). Elevations in GC secretions could thus facilitate the expression of energetically demanding cooperative activities but an increase in the expression of cooperative behaviours could also raise GC secretions.

Elevated T levels have frequently been shown to be detrimental to the expression of parental care (Hirschenhauser and Oliveira, 2006; Peters et al., 2002; Rilling, 2013; Rosvall, 2013; Wingfield et al., 1990; but see: Lynn, 2008) and if the neuroendocrine mechanisms regulating the expression of alloparental care in helpers are derived from parental care, high T levels would be expected to decrease alloparenting. Studies of cooperatively breeding birds have supported this possibility by showing that T levels of helpers often decrease during provisioning stage of the chicks (Khan et al., 2001; Mays et al., 1991; Schoech et al., 2004; Vleck and Brown, 1999). Increased T also favours competitive abilities through heightened aggression, especially within the context of reproduction (Hau, 2007; Wingfield et al., 2006). Thus, T could more generally be hypothesized to mediate the trade-off between cooperation and future breeding, by decreasing investments into costly cooperative activities and by favouring investments into traits susceptible to facilitate future breeding. In cooperative breeders, increased competitive abilities may be essential to obtain and successfully maintain a breeding position as suggested by the period of intense competition among group members (Clarke and Faulkes, 1997; Clutton-Brock et al., 2006; Cooney and Bennett, 2000) and the unusually high levels of T measured in dominant breeders (Creel et al., 1997; Davies et al., 2016; Desjardins et al., 2008).

In cooperative breeders, investigations of direct associations between GC, T and cooperative behaviours are scarce and primarily focused on alloparental care. The relationship between GC and cooperative behaviours has highlighted both positive, null and negative associations. In meerkats (*Suricata suricatta*), plasma GC levels of male helpers were lower at

the beginning of a day spent babysitting newly born pups at the burrow and higher at the end of it as compared to days spent foraging with the rest of the group (Carlson et al., 2006b). Although this suggests that babysitting raises GC levels, individual babysitting contributions throughout the entire baby-sitting period were not correlated with GC levels (Carlson et al., 2006b). Male helpers which had higher plasma GC during the babysitting period provisioned more food items to pups after their emergence from the burrow (Carlson et al., 2006a). In banded mongooses, the relationships between GC and pup-feeding are somewhat opposite to the pattern highlighted in meerkats. Higher levels of faecal GC metabolites prior to the pup-provisioning period predicted lower, not higher, individual contributions to pup feeding (Sanderson et al., 2014). When GC were measured during the period of pup-feeding, the direction of the associations was reversed and individuals that fed pups more throughout the provisioning period had higher GC levels (Sanderson et al., 2014). In striped mice, GC increased with the time female helpers previously spent huddling the pups whereas they decreased in male helpers (Raynaud and Schradin, 2015). In common marmosets (*Callithrix jacchus*) no associations between GC and infant carrying were found (Mota et al., 2006).

Investigations of the associations between T and cooperative contributions have mostly reported null associations. In meerkats, plasma T levels of male helpers during the baby-sitting period did not correlate with their baby-sitting contributions (Carlson et al., 2006b) and did not predict subsequent contribution to pup feeding (Carlson et al., 2006a). However, male helpers conducting extra-territorial forays, during which they seek for mating opportunities, have increased T levels and contribute less to pup care after returning to their group (Young et al., 2005). In striped mice, plasma T levels were not affected by the time helpers previously spent huddling pups in the nest (Raynaud and Schradin, 2015). In the cooperatively breeding cichlid *Neolamprologus pulcher*, breeding females had higher T levels and contributed more to cooperative activities than the other classes of individuals within the social group, and their T levels were positively correlated with their contributions to territory defence and territory maintenance (Desjardins et al., 2008).

The correlative nature of the studies conducted so far in cooperative breeders prevents to draw firm conclusions on whether GC and T regulate cooperative behaviours. Experimental manipulation of GC and T are exceptions and have so far been unsuccessful to manipulate hormone levels within the physiological range of non-experimental individuals, hence requiring a cautious interpretation (Raynaud and Schradin, 2014; Santema and Clutton-Brock, 2012). It is currently difficult to assess the extent to which the lack of consistencies in the

associations highlighted so far originate from genuine differences in the regulation of cooperative behaviour by hormones or from discrepancies in the type (plasma, urine, or faeces) or timing (before, during or after behavioural sampling) of the samples used for hormone measurements.

Although acknowledged, the possibility that cooperative behaviours could influence hormone levels, has rarely been addressed even in the studies in which hormone levels were determined after the quantification of cooperative contributions. This may be because the primary role of GC in the regulation of energy balance has been largely disregarded, which is somewhat surprising considering the energetic costs of cooperative behaviours in cooperative breeders. This omission has certainly contributed to the general lack of clear predictions regarding the expected directions of associations. The integration of the role played by GC in regulating energy homeostasis would certainly facilitate the formulation of clear predictions by forcing researchers to consider the energetic costs of the cooperative activities under investigations and the variables that may affect individuals' ability to cope with such costs.

Overall, the relationships between GC and T and cooperative behaviours remain poorly understood. Experimental manipulations of HPA and HPG axis are essential to elucidate whether cooperative behaviours are regulated by GC and/or T whereas experimental manipulations of cooperative behaviours are required to elucidate whether variation in GC or T can be modulated by cooperative contributions. It is currently unknown whether differences in GC and T can generate variation in cooperative behaviours and for how much of the variation they might account for.

My dissertation aims to advance the understanding of the hormonal mechanisms regulating the expression of cooperative behaviours and contribute to the development of a more integrated understanding of cooperation.

I focus on the short-term and reversible interactions between hormones and cooperative behaviours. I investigate the relationships between GC and T and the expression of cooperative behaviours along two complementary axes. The first axis explores the general hypothesis of a positive association between GC and cooperative contributions while the second explores the hypothesis of negative association between T and cooperative contributions. Each of those two axes is investigated through a combination of correlative and experimental approaches. Specifically, I address two major questions:

1) Do cooperative contributions modulate GC and T levels?
2) Do GC and T levels modulate cooperative contributions?

1.4 The Damaraland mole-rat

I investigate these questions in the Damaraland mole-rat (*Fukomys damarensis*), a cooperatively breeding subterranean rodent which offers unparalleled advantages to investigate the interaction between hormones and individual variation in cooperative behaviours for two reasons.

First, there are large individual differences in cooperative contributions between group members. These differences originally led to the suggestion that helpers may be separated into a "frequent" and an "infrequent" worker castes where slow growing animals were believed to specialize in helping and fast-growing animals in dispersing (Bennett and Faulkes, 2000; Bennett and Jarvis, 1988; Scantlebury et al., 2006). The large individual variation in helpers' cooperative contributions were confirmed in a recent study conducted on a larger sample size of colonies, yet the existence of helpers' working castes was rejected (Zöttl et al., 2016b). Second, colonies can be housed in artificial tunnel systems that approximate natural conditions, where natural behaviours are readily observable (Cooney and Bennett, 2000; Zöttl et al., 2016b). This allows for extremely controlled experiments to be carried out while retaining ecological validity as well as the timely collection of weight and endocrine data.

1.5 Thesis structure

In Chapter 2, I provide general information on the biology of my model system and species, the Damaraland mole-rat, and I detail the methods and conditions in which the data were collected and how they were analysed.

In Chapter 3, I investigate whether differences in CORT and T may arise as a consequence of individual variation in cooperative contributions. I present the associations between CORT and T with individual cooperative contributions using correlative data as well as the effects of experimental manipulations of cooperative contributions on CORT and T levels.

In Chapter 4, I investigate whether CORT modulates the cooperative contributions of female helpers by experimentally elevating their CORT levels.

In Chapter 5, I investigate whether T modulates the cooperative contributions of female helpers by experimentally elevating their T levels. I also investigate whether T modulates females' competitive abilities to assess if increased T may facilitate the transition from helping to breeding.

In Chapter 6, I provide a synthesis of my research and offer a critical discussion on the relevance and weaknesses of my findings and highlight promising areas for future research.

Chapter 2

General Methods

2.1 Study System

2.1.1 Study Site

All the behavioural data and biological samples of my thesis were collected from a breeding population of captive Damaraland mole-rats (*Fukomys damarensis*) at the Kuruman River Reserve (26°58'S, 21°49'E), Northern Cape, South Africa (Figure 2.1).

Figure 2.1 - Map of South Africa showing the location of the study site (green dot).

2.1.2 Study species

Damaraland mole-rats (*Fukomys damarensis*) are part of the Bathyergidae, a family of obligatory subterranean hystricognath rodents that defines the African mole-rats. The phylogeny of the Bathyergidae is still debated (Faulkes et al., 2011; Van Daele et al., 2007) and African mole-rats are composed of at least 30 species from 6 distinct genera. African mole-rat species range on a continuum of social systems from solitary (genera *Heliophobus*, *Bathyergus*, *Georychus*) to highly social like in the cooperatively breeding naked mole-rat (*Heterocephalus glaber*) and Damaraland mole-rat (Faulkes et al., 2013). Damaraland mole-rats are widely distributed in southern Africa, being found mostly in Botswana and Namibia but also in Zambia, Zimbabwe and South Africa, in xeric habitats where rainfall is unpredictable and low (Bennett and Faulkes, 2000)

Damaraland mole-rat colonies typically consist of family groups (Burland et al., 2002) of up to 41 individuals (Jarvis and Bennett, 1993), characterized by an extreme reproductive skew. Within a colony, reproduction is monopolized by a single breeding female (Burland et al., 2004; Young et al., 2010), often referred to as the queen, while the number of males achieving paternity can be higher than one and up to three (Burland et al., 2004). Breeding males and females are unrelated (Burland et al., 2004) and colonies in which breeders of one sex are missing become reproductively quiescent (Bennett et al., 1996; Jacobs et al., 1998; Rickard and Bennett, 1997) suggesting that Damaraland mole-rats are obligate outbreeders. This suggestion has been experimentally supported by showing that the immigration of unrelated individuals causes helpers to attempt breeding (Cooney and Bennett, 2000) and reproductively quiescent colonies to resume breeding (Jacobs et al., 1998; Rickard and Bennett, 1997). Also, pairs of siblings fail to show copulatory behaviours and fail to conceive, in contrast with non-sibling pairs which show copulatory behaviours within minutes of pairing and eventually conceive (Bennett et al., 1996).

In intact colonies, the reproductive axis of female helpers is physiologically suppressed since the development of follicles in the ovaries is incomplete, causing anovulation (Bennett et al., 1994; Molteno and Bennett, 2000). This suppression possibly occurs at the level of the pituitary gland which, in helpers, is less responsive to an exogenous stimulation with Gonadotropin Releasing Hormone (GnRH) (Bennett et al., 1993). GnRH is a peptide hormone that is naturally secreted by the hypothalamus and which stimulates the pituitary to release Luteinizing Hormone (LH) in the blood stream which in turn stimulates the release of Testosterone (T) by the gonads. In agreement with a downregulation of their reproductive axis, female helpers have lower baseline levels of LH (Bennett et al., 1993) and in some cases lower levels of sex hormones such as oestradiol, progesterone and T (Bennett, 1994; Clarke et al., 2001; Lutermann et al., 2013; Rickard and Bennett, 1997). In contrast to females, there is no evidence of a physiological suppression of the reproductive axis of male helpers, whose sperm production and sperm motility is not different from breeding males (Faulkes et al., 1994).

Both the presence of the breeding female and the absence of breeding opportunities have been suggested as two non-mutually exclusive components of the physiological reproductive suppression of female helpers. In the absence of a breeding female, this suppression of helpers is relieved despite a lack of breeding opportunities (Bennett et al., 1996; Molteno and Bennett, 2000), but others have argued otherwise (Clarke et al., 2001). When female helpers face a breeding opportunity their physiological reproductive suppression is

relieved (Clarke et al., 2001; Cooney and Bennett, 2000), even in the presence of a breeding female (Cooney and Bennett, 2000). Furthermore, it has been suggested that when ecological conditions favour breeding opportunities by the relaxation of dispersal constraints, female helpers' suppression is eased (Young et al., 2010). In intact colonies and in the absence of helpers' breeding opportunities, intra-colony levels of aggression are low (Clarke et al., 2001; Cooney and Bennett, 2000; Rickard and Bennett, 1997), raising the question as to whether the breeding female plays an active role in helpers' physiological reproductive suppression.

Helpers' breeding opportunities have dramatic consequences on the social dynamic of colonies. Immigration of unrelated individuals trigger intense aggression among group members (Cooney and Bennett, 2000; Jacobs et al., 1998), that can have lethal consequences (Jacobs et al., 1998) and which can culminate into changes in the breeding hierarchy (Cooney and Bennett, 2000). In the wild, within group reproductive competition and social instability may be favoured by rainfall which facilitates underground dispersal (Young et al., 2010) of adult males and females (Hazell et al., 2000a; Young et al., 2010), by softening the sand substrate and consequently decreasing the energetic demands of burrowing behaviours (Lovegrove, 1989).

Most of the cooperative behaviours expressed by Damaraland mole-rats are related to contributions to common goods from which all group members can benefit. Energetically demanding burrowing activities (Lovegrove, 1989) enable the extension of the burrow system, necessary to locate and expose the underground storage organs of geophytes on which this species feeds (Jarvis et al., 1998) (Figure 2.2). Food items can also be transported to constitute communal food stores (Jarvis et al., 1998), as well as provide vegetal material that is used to build a communal nest (Figure 2.3). In contrast to most other cooperative breeders, direct alloparental care, as interpreted from observations in captive colonies, is rare (Zöttl et al., 2016b). However, young pups that occasionally wander in tunnel system will be retrieved into the nest where they are huddled and groomed by all group members and lactated by the breeding female. The reproductive success of breeders is increased by helpers (Young et al., 2015). Insofar this effect is assumed to be contingent on helpers' behaviours, it supports the cooperative characters of individual contributions to common goods and offspring care (West et al., 2007). However, the respective contribution of these distinct activities on breeders' reproductive success (Young et al., 2015) and the extent to which they benefit helpers indirect and direct reproductive success is unknown.

Figure 2.2 – The underground storage organs of geophytes on which wild Damaraland mole-rats feed, commonly called gemsbok cucumber (*Acanthosicyos naudinianus*). Courtesy of Kyle Finn.

Figure 2.3 – Communal nest of a wild Damaraland mole-rat colony. Courtesy of Kyle Finn.

Pioneering work on Damaraland mole-rats had suggested that helpers could be assigned to a frequent and an infrequent helper classes (Bennett and Faulkes, 2000; Bennett and Jarvis, 1988; Scantlebury et al., 2006) but this possibility was recently rejected (Zöttl et al., 2016b). Although differences in individual cooperative contributions are large, their distribution falls alongside a continuum and is not bimodally distributed as would have been predicted by the existence of distinct helper classes (Zöttl et al., 2016b). Helpers' cooperative contributions are better described by an age-related polyethism in which contributions increase in the first year of life before plateauing (Zöttl et al., 2016b). Also, faster growing helpers have higher cooperative contributions than slower growing helpers, which is opposite to the pattern initially suggested for frequent and infrequent helpers (Bennett and Faulkes, 2000; Bennett and Jarvis, 1988).

2.1.3 Study Population

The captive population used for this thesis is maintained in laboratory facilities of the Kuruman River Reserve, Northern Cape, South Africa. This population originated from 25 wild colonies trapped in the surrounding of the research site between February and October 2013 (242 individuals). Throughout the development of the work presented here, the study population has increased in size due to recruitment in both originally wild caught colonies and colonies that were formed by the pairing of unrelated individuals from opposite sex. Currently, the study population is composed of 72 colonies with group sizes ranging from 1 to 26 animals. This captive population comprised 472 animals, 365 of which were born in the lab.

2.2 Laboratory Settings

2.2.1 Artificial Habitat

Captive colonies were kept individually in standardized artificial tunnel systems of three different sizes with total tunnel lengths of about 4, 8 or 18 meters (Figure 2.4). Artificial tunnel systems were built with PVC pipes which upper part had been cut off and replaced with a transparent plastic film to enable behavioural observations. For the medium and large size tunnel systems, a large plastic box, referred to as the waste box, was connected to one extremity of the tunnel system to enable animals to sweep unwanted material out. Nesting and toilet areas were provided in the form of standardized size transparent plastic boxes which, when used as a nest, were filled by the animals with small pieces of paper towel that were compacted against the boxes' walls. Each tunnel system was equipped with several dead ends PVC pipes which were frequently used as food storage spaces. Fresh Kalahari sand could be supplied to the colony through vertical PVC pipes referred to as sand dispensers, located at the opposite extremity of the tunnel system to the one constituted by the waste box.

The temperature in the animal rooms was controlled with an air-conditioning system set between 20 °C in winter and 23 °C in summer leading the temperature to effectively range between 17 and 26 °C.

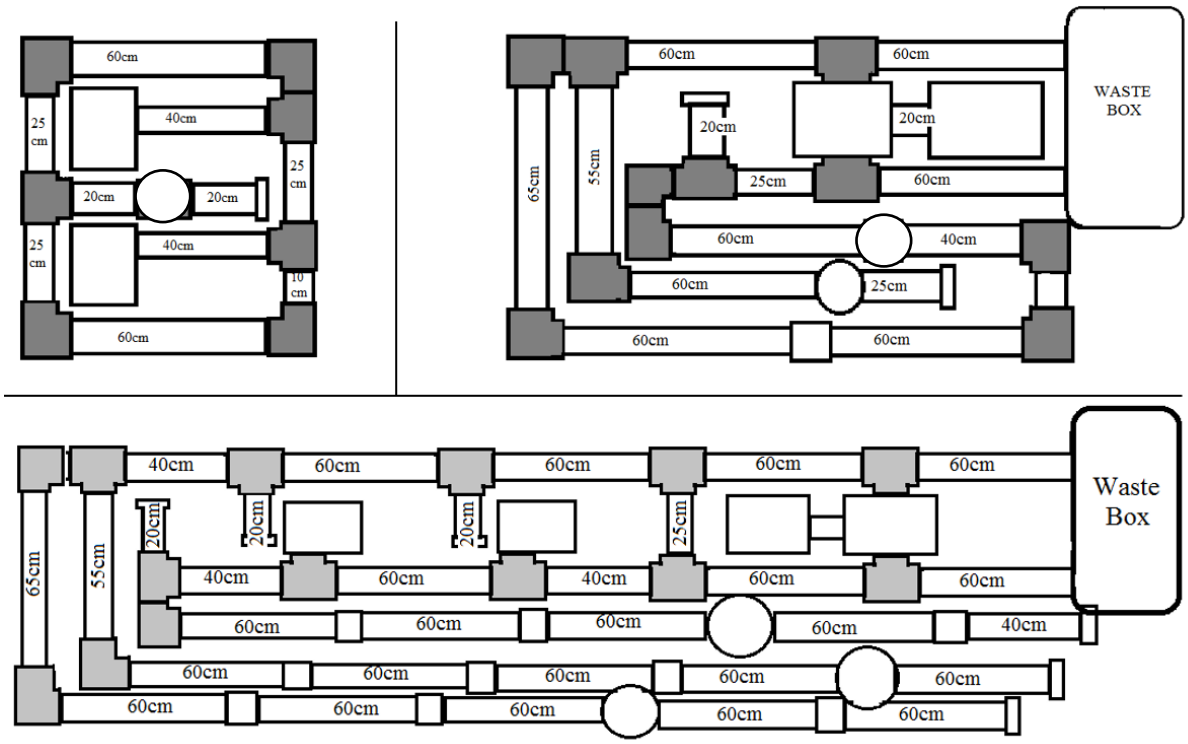

Figure 2.4 – Different sizes of tunnels systems where Damaraland mole-rat colonies were housed. The smallest sized systems (top left) were used for colonies with up to three individuals; medium sized systems (top right) were used for colonies with up to ten individuals and large sized systems (bottom) were used for colonies with more than ten individuals. Rectangles represent transparent plastic boxes used as nesting areas. Circles represent the vertical sand dispensers. Diagrams represent a top view of the tunnel systems.

2.2.2 Husbandry

All tunnel systems were subjected to a daily clean in the morning. Dirty PVC pipes were cleaned with paper and a toilet brush, toilet areas were emptied, rinsed with water and dried, waste boxes were emptied and obstructed sand dispensers, PVC pipes and plastic boxes were unblocked. Nest material was provided away from the nest and consisted of small pieces of paper towel. Sand dispensers were refilled with fresh sand and the animals were fed *ad libitum* a diet of sweet potatoes, cucumber and occasionally apples twice daily. Food items were provided behind the food dispensers to encourage burrowing, although a freely accessible food source was always made available. Additionally, colonies were provided with sand and extra food if necessary every evening.

2.3 Behavioural Data

All the behavioural data of this thesis were obtained using scan and focal observations that were recorded on a handheld Android device operating the software Pocket Observer (Noldus, Wageningen). Each individual within the colonies where observations took place could be readily identifiable by a unique coloured dye mark and/or its natural fur colour pattern. Also, individuals carried a passive implantable transponder for identification which was inserted subcutaneously at the age of 3 months for individuals born in captivity.

A delay of at least 20 minutes after morning cleaning and 1 hour after urine sampling/weighing was respected before the start of a behavioural observation to minimize the effect of possible disturbances. Fresh sand was provided in the sand dispensers at the start of each observation to create burrowing opportunities. During scan observations, fresh sand was added every 2 hours.

2.3.1 Scan observations

The scan observation protocol combined an all individual´s instantaneous and an all occurrences continuous sampling techniques as defined by Altmann (1974). During the instantaneous sampling of a given colony, the behaviour of each group member was determined from a list of 17 defined behaviours (Table 2.1) following a pre-defined and fixed sequence of individuals. Instantaneous samples were separated by 4 minutes. The available time between the end of an instantaneous sample and the beginning of the following one was used to collect *ad libitum* data on social interaction (Table 2.2). Scan observations typically included between 10 and 20 individuals and lasted for 12 or 24 hours, leading respectively to the collection of 180 or 360 instantaneous samples per individual. Observations usually started between 07:00 and 08:00 and were carried out by at least two observers alternating shifts every 2 to 4 hours.

Table 2.1 – Instantaneous sampling ethogram used for scan observations. The left side of the table shows how behaviours were grouped to form variables used in the statistical analyses ([1] - for details on how this behaviour was included in the statistical models please refer to each chapter's methods).

Variables			Behaviour	Description
Activity	Total Cooperation	Food Carrying	Food carry	Transporting of food pieces
		Nest Building	Nest material	Preparing nest material for transport and transporting nest material
		Burrowing	Dig	Excavating sand using incisors and front paws
			Sweep	Moving sand backwards using hind legs
			Kick	Compacting sand against tunnel using nose or hind legs
			Locomotion work	Moving between bouts of the above behaviours
			Pup carry[1]	Grabbing and/or moving a pup using incisors
	Non-Cooperation		Locomotion	Moving unrelated with cooperative behaviours
			Sniff	Investigating objects with the nose
			Eat	Ingesting food
			Self-Groom	Hygiene maintenance behaviours directed to the actor's body
			Social interaction	Any interaction with another individual
			Pump	Repetitive up and down movement of the body
			Other	Any behaviour that cannot be assigned to the described behaviours
			Gnaw[1]	Chewing the plastic tunnels with incisors
Rest			Rest	Sleeping in the nest or tunnels
			Huddle	Resting in the tunnels in physical contact with at least one individual

Table 2.2 – Continuous sampling ethogram used for scan observations.

Behaviour	Description
Pup Carry	Grabbing and/or moving a pup using incisors
Bite	Closing incisors on another individual's body part
Overt aggression	Rapid succession of high intensity aggressive behaviours (spar, bite, chase)
Pull tail	Grabbing the tail of another individual and pulling it, often resulting in dragging the receiver of this behaviour
Pass	Moving past another individual with physical contact
Submissive call	High pitched call co-occurring with small backwards jumps
Pump	Repetitive up and down movement of the body
Gnaw	Chewing the plastic tunnels with incisors
Rest	Sleeping in the nest or tunnels
Huddle	Resting in the tunnels in physical contact with at least one individual
Sniff	Investigating another individual with the nose
Spar	Locking incisors with another individual, pulling and pushing each other
Sex foreplay	Quick succession of social behaviours displayed before a copulation
Chase	Following another individual with accelerated locomotion
Copulation	Mounting another individual attempting sexual intercourse
Allogroom	Grooming directed towards another individual
Food Competition	Pushing away another individual with the hind legs from a food resource
Nest material	Preparing nest material for transport and transporting nest material

2.3.2 Focal subject observations

Focal observations (Altmann, 1974) were opportunistically conducted on active subjects only. The recording of complete activity bouts, starting with the focal subject waking up and leaving the nest and finishing with the return to the nest, were prioritized over incomplete activity bouts. Observations were terminated whenever the focal subject rested, or disappeared in the nest uninterruptedly for 5 minutes, but could otherwise be terminated at any time. During each activity bout, all the behaviours displayed by the focal subject were recorded using a list of 31 behaviours (Table 2.3).

2.3.3 Data preparation

For some statistical analyses, behaviours sharing functional similarities were merged into new variables (Table 2.1, Table 2.3). The Activity variable was obtained by grouping all behaviours, at the exception of resting and huddling. Behaviours that were part of the activity variable were separated into the Total Cooperation and the Non-cooperation variables. All the behaviours which could be defined as beneficial to other group members were assigned to the Total cooperation variable whereas the remaining behaviours were assigned to the Non-cooperation variable. The Total Cooperation variable, which was used to assess individual general cooperative contributions, was sub-divided into the Burrowing, the Food carrying and the Nest building variables. Burrowing included all the behaviours related to tunnel maintenance, sand excavation and transport in the tunnel system (Table 2.1, Table 2.3). Alloparental care estimated through the behaviour Carry pup was part of the Total cooperation variable but did not integrate any of its sub-variables nor was analysed separately due to its extremely low occurrence frequency.

Unless otherwise specified, the behaviour Gnaw was removed from the dataset prior to statistical analyses for which any cooperative and non-cooperative variables were used as response variables. This was justified by the undefined cooperative character of gnawing which could be assimilated to digging as well as to a displacement behaviour. Furthermore, gnaw was significantly increased by the administration of cortisol and there was a positive effect of CORT treatment on its expression (CORT treatment effect: estimate=0.475, SE=0.199, Z=2.39, p=0.017), which together could have influenced my conclusions.

For statistical analyses of scan data, the total number of scans recorded, after the exclusion of gnaw when it applied, the sum of scans a subject had been observed displaying the activity under investigation (success), and the difference between the two (failure) were computed. Focal data were pooled over each observation day or treatment week for each subject, the total duration of the activity under investigation and the total daily or weekly duration of observation, after exclusion of gnawing when it applied, were computed.

Table 2.3 – Focal observation ethogram. The left side of the table shows how behaviours were grouped to form variables used in the statistical analyses. S.E. denotes state events; P.E. denotes point events

Variables		Behaviour	Description	Event Type
Total Cooperation	Food Carrying	Food carry	Transporting of food pieces	S.E.
	Nest Building	Carry nest material	Transporting nest material	S.E.
		Nest Building	Shredding nest material in small piece by chewing it with incisors	S.E.
	Burrowing	Dig	Excavating sand using incisors and front paws	S.E.
		Sweep	Moving sand backwards using hind legs	S.E.
		Kick	Compacting sand against tunnel using nose or hind legs	S.E.
		Pup carry	Grabbing and/or moving a pup using incisors	P.E.
Aggressive behaviour		Displace	Pushing away another individual with the hind legs from a space or resource	S.E.
		Bite	Closing incisors on another individual's body part	P.E.
		Overt aggression	Rapid succession of high intensity aggressive behaviours (spar, bite, chase)	P.E.
		Pull tail	Grabbing the tail of another individual and pulling it, often resulting in dragging the receiver of this behaviour	P.E.
		Pass	Moving past another individual with physical contact	P.E.
		Submissive call	High pitched call co-occurring with small backwards jumps	P.E.
		Beg call	Pup vocalization to another individual to gain access to a space or resource	P.E.
		Unknown call	Any vocalization that cannot be classified as submissive or beg	P.E.
		Eat	Ingesting food	S.E.

Table 2.3 – *(Continuation)*

Behaviour	Description	Event Type
Self-Groom	Hygiene maintenance behaviours directed to the actor's body	S.E.
Pump	Repetitive up and down movement of the body	P.E.
Gnaw	Chewing the plastic tunnels with incisors	S.E.
Rest	Sleeping in the nest or tunnels	S.E.
Huddle	Resting in the tunnels in physical contact with at least one individual	S.E.
Sniff	Investigating another individual with the nose	P.E.
Spar	Locking incisors with another individual, pulling and pushing each other	S.E.
Back spar	Sparing with another individual while turned on its back	P.E.
Sex foreplay	Quick succession of social behaviours displayed before a copulation	S.E.
Chase	Following another individual with accelerated locomotion	S.E.
Copulation	Mounting another individual attempting sexual intercourse	P.E.
Allogroom	Grooming directed towards another individual	P.E.
Retreat	Quickly moving away from another individual	P.E.
Drop food	Binging a piece of food to a food storage place	P.E.
Miscellaneous	Any behaviour that cannot be assigned to the described behaviours	S.E.

2.4 Endocrine Data

2.4.1 Urine samples collection

Individuals were removed from their original tunnel system and placed in individual urine chambers between 07:00 and 08:00, where a food item was provided. Individuals were kept in the chambers until 0.4 ml of urine had been collected which generally occurred after a single urination, shortly after the transfer into the chamber. If no urination had occurred within 180 minutes, individuals were placed back in their original colony. Urine samples were kept in a -20 ºC freezer until hormone analysis. Both the time of urination and the delay to urination were recorded to allow the control of their effects on endocrine values.

2.4.2 Blood samples collection

Individuals were removed from their tunnel systems and anaesthetized with 5% Isoflurane (Isofor, Safe Line Pharmaceuticals, Johannesburg, South Africa) mixed with oxygen gas, delivered through a vaporizer at a rate of 1 l/min through a cone which was gently fitted on the subject's snout. Once fully sedated, the isoflurane dose was reduced to 2% until the completion of the blood sampling procedure. Blood was collected into 0.5 ml Lithium-Heparin Minicollect tubes with micro haematocrit capillary tubes (Sodium-heparinised 80 IU/ml) after piercing a foot vein with a sterile needle. Blood samples were immediately centrifuged for 5 minutes at 2000 G, and the plasma separated from the cell pellet and stored at -20 ºC until hormone analyses. The completion of blood collection usually occurred within 5 minutes (mean=270 s, SD=105 s, for the samples used in this thesis). After blood samples, subjects were kept in a bucket until full mobility was regained and then placed back in their original tunnel system.

2.4.3 Hormones Analyses

Cortisol (CORT) and Testosterone (T) determinations were carried out using two different analytical methods. The endocrine data presented in Chapter 2 were obtained by the

analyses of urine samples at the University of Pretoria in collaboration with Professor Nigel C. Bennett using radio-immunoassay (RIA). All other endocrine data presented in this thesis were obtained by the analyses of urine and plasma samples at the Neuchâtel Platform of Analytical Chemistry (NPAC) in collaboration with Dr. Gaëtan Glauser using Ultra-High Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC-MS/MS).

To assess the reproducibility of sample preparation and analysis, multiple urine samples from captive males and females Damaraland mole-rats were pooled into a male and a female control samples (MC and FC respectively). Aliquots of 1 ml of MC or FC were then stored at -20 °C until hormone analyses.

Radioimmunoassays

The RIAs were conducted using commercially available coated tubes assay kits (Coat a Count, Diagnostic Products Corporation, Los Angeles, CA) validated for both CORT and T determinations in Damaraland mole-rats (Clarke et al., 2001). All, samples were analysed in duplicates and at least two MC and a FC samples were analysed at the beginning and at the end of each samples batch (minimum of 4 control samples/batch) for the calculation of intra and inter-assay variation.

For CORT analyses, the procedures described by the kit supplier were followed. Standard solutions of known CORT concentrations provided by the supplier were used to establish a reference standard calibration curve. Briefly, for each sample duplicate, 25 µl of urine were added into a polypropylene tube coated with anti-CORT antibodies. One ml of tracer solution containing iodinated (^{125}I) CORT was then added to the tubes to enable the CORT contained in the urine and the radiolabelled CORT from the tracer solution to compete for the antibodies' binding sites. After 45 minutes of incubation at 37 ºC, the tubes were emptied and radioactivity was measured with a gamma counter. Cortisol concentrations were determined using the standard calibration curve drawn from the radioactivity measured in tubes of known CORT concentrations. The limit of detection (LOD) varied across batches and ranged from 0.023 to 0.425 ng/dl. The intra-assay variation determined by averaging the coefficient of variability (CV) between duplicates, after the exclusion of all samples which concentration fell below the quantification limit (LOQ) of the kit (0.7 ng/dl), was of 10.98%. When determined

using the average CV of the control samples the intra-assay variation was of 5.48%. The inter-assay variation was of 7.6% when calculated with the control samples.

For Testosterone, a similar procedure was followed at the difference than the tubes were coated with antibodies anti-testosterone and that 50 µl of urine were used in each duplicate. The tracer consisted of iodinated (^{125}I) T and the incubation time was of 3 hours at 37 ºC. The intra-assay variation determined by averaging the CV between duplicates, after the exclusion of all samples which concentration fell below the LOQ of the kit (20 ng/dl) was of 11.11%. When determined using the average CV of the control samples the intra-assay variation was of 9.67%. The inter-assay variation was of 12.59% when calculated with the control samples.

Ultra-High Performance Liquid Chromatography – Tandem Mass Spectrometry (UHPLC-MS/MS)

For UHPLC-MS/MS analyses, 100 µl of urine were added to 410 µl of a solution containing 400 µl of sodium phosphate buffer (0.1M, pH7) and 10 µl of methanol containing isotopically labelled internal standards at 80, 40 and 800 ng/ml for cortisol-D4, testosterone-D3 and dehydroepiandrosterone-D5, respectively (Toronto Research Chemicals). Spiking labelled internal standards enabled to accurately account for variations resulting from steroid loss during sample preparation and from matrix effects and sensitivity variation in the mass spectrometer over time (Stokvis et al., 2005). Differing from RIA, the glucuronated forms of steroids excreted in the urine were deconjugated by adding 2.5 µl of beta-glucuronidase from *Escherichia Coli* (Roche chemicals) to each sample and allowing 1 hour incubation at 50 ºC. A solid phase extraction (SPE) using Isolute C18(EC) cartridges (50 mg/1cc, Biotage, Sweden) was then performed. Briefly, the cartridges were conditioned with 1 ml of methanol 100%, equilibrated with 1 ml of methanol 5%, the samples were passed through the cartridges which were then washed with 1 ml of methanol 5% followed by 1 ml of hexane. Steroids were recovered by eluting the cartridges with 1 ml of ethylacetate which was evaporated in a centrifugal evaporator (Labconco) at 35 ºC and reconstituted in 100 µl of methanol 50%.

Samples were injected in an Acquity UPLC^{TM} coupled to a Xevo TQ-S triple quadrupole (Waters, Milford, MA, USA) with all aspects of the system optimized for steroid analyses. Calibration solutions containing Cortisol, Testosterone and DHEA at 0.1, 1, 20, 100

and 250 ng/ml as well as internal standards were prepared in methanol 50%. For DHEA, additional concentrations at 500, 1500 and 3000 ng/ml were used.

For CORT and T quantifications, the mass-spectrometer peaks were integrated using the program Quanlynx^{TM} and normalized to those of the internal standards following an automated method developed at the NPAC. The peak integration was visually controlled for each hormone and each sample. Different calibration equations were applied to each batch of samples by selecting the most appropriate model (linear, quadratic or cubic) and weighting factor (in most cases 1/x). All CORT and T concentrations measured in urine samples fell well above the LOQ of the method which was set at a signal to noise ratio of 8 corresponding to 0.7 ng/ml of CORT and 0.09 ng/ml of T. Inter-day coefficient of variation, calculated from control samples prepared independently for each new series of samples analysis, was of 5.22% for CORT and 2.06% for T.

Testosterone levels in plasma samples were only used in Chapter 5. The LOD and LOQ of the method were set at a ratio signal to noise of 3 and 8 corresponding to a plasma concentration of 0.008 and 0.021 ng/ml of T, respectively. All the plasma samples had T concentrations higher than the LOD, however 5 of the 80 samples analysed returned a T value below the LOQ and were kept for statistical analyses. All plasma T samples were analysed on a single sample batch and therefore inter-day variation cannot be provided.

All raw hormone concentrations determined with RIA and UHPLC-MS/MS were corrected for variation in urine dilution by the determination of urine specific gravity (SG) using a digital hand-held pen refractometer (Atago, Ltd). Correction of hormone concentration with SG has been shown to be reliable and arguably more accurate than creatinine correction (Miller et al., 2004). For each sample, triplicate SG values were determined with 10 µl of urine, at the few exceptions of insufficient urine volume availability where only one value was measured. For each urine sample, SG values were averaged and hormone concentrations were obtained following Miller and colleagues (Miller et al., 2004) formula:

$$[\text{Corrected Hormone}] = [\text{Raw Hormone}] \times (SG_{\text{Population}} - 1) / (SG_{\text{Target Sample}} - 1),$$

where $SG_{\text{Population}}$ represents the population average of SG values and $SG_{\text{Target Sample}}$ represents the SG value of the sample which hormone concentration is to be corrected.

2.5 Statistical Analyses

All the data exploration, analyses and plotting were conducted using the software R version 3.1.2 (R Development Core Team, 2011).

Prior to statistical analyses, a data exploration procedure largely inspired by Zuur et al. (2010) was conducted to check for the presence of outliers and zeros. Also, the variance inflating factor (VIF) of the explanatory variables used in the statistical models were calculated using the R corvif function (Ieno and Zuur, 2015) to assess collinearity. If explanatory variables returned a VIF higher than 3, suggesting a high degree of collinearity, they were sequentially excluded from the maximal model until all explanatory variables returned a value smaller than 3.

Whenever necessary and possible, I used a mixed effect modelling approach to account for the dependency structure in the data caused by the repeated measurements of individuals within colonies. Mixed models enable the specification of random effect terms which estimate the variance between and within clusters of non-independent data. Whenever the data structure did not comply with the mixed model assumptions, I conducted non-parametric statistical tests, which are detailed in each relevant chapter.

For continuous response variables, I fitted Linear Mixed Models (LMMs) with a normal error structure and identity link function to the data. In cases where the model validation plots (see further section) did not comply with the model assumptions, the response variables were transformed by their natural logarithm before being specified in the model. In the few cases where the data set did not consist of repeated measurements, Linear Models (LMs) were fitted instead. LMs are similar to LMMs, at the exception that no random effect term is specified. In cases where response variables were continuous and strictly positive, they were either transformed by their natural logarithm and specified in LMMs.

When the response variables were count data, GLMMs with a negative binomial error structure and logit link function (negative binomial GLMMs) were fitted to the data.

When the response variables were proportions, such as for the analyses of the scan data, GLMMs with a beta-binomial error structure and logit link function were fitted to the data (beta-binomial GLMMs). Beta-binomial GLMMs were preferred over conventional binomial

GLMMs because data from the scan data were always overdispersed which can lead to biased parameter estimates and standard errors. When beta-binomial GLMMs failed to converge, GLMMs with a binomial error structure and logit link functions were fitted, and an Observation-level Random Effect term added into the model (OLRE binomial GLMMs). An OLRE enables to capture any important pattern in the response variable that cannot be explained by the other terms specified in the model (Zuur et al., 2013). In both OLRE binomial and beta-binomial GLMM, the count of scan during which an individual was observed displaying the behaviour under investigation was used as a response variable and, unless specified otherwise, the total number of scans conducted during an observation session was used as the binomial total.

All models were fitted using the R package lme4 (Bates et al., 2015) except the beta-binomial GLMMs which were fitted in the glmmADMB package (Fournier et al., 2012).

The choice of covariates included in our maximal models was driven by *a priori* hypothesis and/or based on the data exploration procedures. All continuous explanatory variables that were specified within interaction or polynomials terms were centred and scaled in order to enable the independent interpretation of their main effects (Schielzeth, 2010). Maximal models were simplified following a stepwise backward deletion of non-significant terms in order of descending interactions least significant terms until only significant terms remained. The p-values for each term were computed by conducting a likelihood ratio test between two nested models, one of which contained and one of which did not contain the term under investigation. P-values lower than 0.05 indicate that the removal of the term from the model significantly decrease its explanatory power. Terms that were dropped during model simplification process are reported with the model estimates, standard errors, and test-statistic with which they were last included in the model selection process.

The validation of minimal models relied on the visual assessment of homogeneity of variance between the model residuals and its fitted values as well as the normality of its residuals. When count or proportion data were used as a response variable, I checked that the data were not overdispersed. To this purpose, the ratio between the model residual sum of square from the original data and the residual sum of square from a hundred data sets generated from the minimal model using parametric bootstrapping were computed. The confidence interval of those 100 ratios was then computed and overdispersion was assumed whenever the lower limit of the 95% confidence interval was greater than 1.

For all graphical representation using box and whiskers figures, lower and upper hinges of the boxplot display the 25^{th} and 75^{th} quartile (inter-quartile range, IQR) respectively, while whiskers extend to lower and higher value laying within 1.5 * IQR of the lower and upper hinges.

Chapter 3

Effect of individual cooperative contributions on cortisol and testosterone levels

Abstract

Unravelling the physiological mechanisms underlying cooperative behaviours represents one of the remaining challenges in the study of cooperation. Empirical studies have shown that the expression of cooperative behaviours varies with individual and social characteristics but the physiological mechanisms modulating this variation are still poorly understood. In cooperative breeders, where a majority of group members does not breed and cares for the breeders' offspring, it has been suggested that cooperative behaviours could be modulated by steroid sex hormones, like testosterone (T), and glucocorticoid stress hormones (GC). However, previous correlative studies cannot tell whether differences in GC and T levels causally modulate individual cooperative contributions. Since GC and T secretions are responsive to behavioural output, in this chapter I investigate the alternative possibility that differences in GC and T levels can arise due to variations in cooperative behaviours. I show that GC levels, but not T, were close to being significantly increased by an experimental treatment in which individual cooperative contributions were more than doubled. However, variations in cooperative contributions generally failed to explain variations in GC levels, suggesting that additional factors may have contributed to the rise of GC levels.

3.1 Introduction

Large differences in individual cooperative contributions are common within cooperative societies (Clutton-Brock et al., 2001b, 2000; Field et al., 2006; Robinson, 1992; Zöttl et al., 2016b) where a majority of group members forfeit or forgo their own reproduction, care for offspring which are not their own (Clutton-Brock, 2016; Koenig and Dickinson, 2004), or maintain foraging tunnels and food stores (Bennett and Faulkes, 2000). Research on cooperative breeders has shown that part of this variation can be explained by individual adjustments of cooperative activities to individual, environmental and social cues (Clutton-Brock et al., 2002; Russell et al., 2003; Wright and Dingemanse, 1999; Young et al., 2005), but the physiological mechanisms modulating these adjustments remain poorly understood.

It has been suggested that steroid glucocorticoid (GC) stress and sex hormones, like testosterone (T), modulate cooperative behaviours in cooperative breeders (Carlson et al., 2006a, 2006b; Sanderson et al., 2014; Young et al., 2005). Increased GC levels facilitate energy production (Landys et al., 2006; Sapolsky et al., 2000) and may support the expression of the energetically demanding forms of cooperative activities (Clutton-Brock et al., 1998; Grantner and Taborsky, 1998; Russell et al., 2003), leading to positive correlations between GC and cooperative behaviours (Sanderson et al., 2014). Conversely, increased T levels have been shown to reduce parental care (Lonstein and De Vries, 2000) suggesting that they may have similar effects on the expression of alloparental care (Vleck and Brown, 1999; Young et al., 2005).

Rises in GC levels may occur as a direct response to the increased energetic demands associated with the expression of cooperative activities expressed by cooperative breeders (Grantner and Taborsky, 1998; Russell et al., 2003). Indeed, elevations in GC levels represent a common response to increased physical activity (Hackney and Viru, 1999; Malisch et al., 2008; Stranahan et al., 2008) where the positive effects of GC on energy production (Landys et al., 2006; Sapolsky et al., 2000) represent a physiological adaptation to exercise-induced elevated energetic demands (Malisch et al., 2008; Stranahan et al., 2008; Viru and Viru, 2004). Physical activity has also been shown to modulate T levels (Copeland et al., 2002; Daly et al., 2005). Since GC decrease T secretions (Cumming et al., 1983; Moore et al., 1991), it has been suggested that exercise induced decreases in baseline T could be caused by post-exercise elevations in CORT (Daly et al., 2005). However, the correlational nature of the studies has

precluded firm conclusions on whether GC and T causally modulate the expression of cooperative activities (Carlson et al., 2006a, 2006b; Young et al., 2005) and increased GC and T levels could also arise as a consequence of individual differences in cooperative activities.

To determine whether variation in GC or T levels can be generated by differences in cooperative behaviours, experimental manipulations of individual cooperative contributions are required. While such manipulations have already been carried out in cooperative breeders (Bergmüller and Taborsky, 2005; Liebl et al., 2016; Russell et al., 2003), their effects on GC or T profiles have not been investigated. I combined a correlative and an experimental approach to investigate whether individual variation in cooperative contributions could generate variations in GC and T levels in captive Damaraland mole-rat (*Fukomys damarensis*). I first determined whether helpers' cooperative contributions, measured as the expression of burrowing, nest building and food carrying activities, were correlated with cortisol (CORT), the GC measured in Damaraland mole-rats, and T under non-experimental conditions. Subsequently, individual cooperative contributions were manipulated during two successive days to test whether experimentally induced increases in cooperative contributions would affect CORT or T levels. Since energetically demanding activities can increase CORT levels and increased CORT can down-regulate T, I investigated whether: i) CORT would be positively and T negatively correlated with cooperative contributions, ii) experimentally induced increases in individual cooperative contributions would raise CORT levels and decrease T levels.

3.2 Methods

3.2.1 Colony maintenance and husbandry

For details of colony maintenance and general animal husbandry refer to Chapter 2.

3.2.2 Experimental design and procedures

Study 1: Correlation between CORT, T and individual cooperative contributions

The correlation between CORT, T and individual cooperative contributions was investigated by determining CORT and T concentrations in urine samples collected the morning following a 12 hours scan observation (n=40 observation sessions). CORT and T were measured in non-breeding females (20 individuals; weight range: 71-176 g, mean=115.86 g) and males (21 individuals; weight range: 92-215 g, mean=153.9 g) born in captivity from 20 distinct colonies (colony size range: 3-17 individuals, mean=9.43 individuals) and older than 350 days. Each individual returned 1 to 3 combined behavioural and endocrine measurements which led to a total sample size of 70 data points.

Experiment 1: Effect of cooperative contribution manipulations on CORT and T levels

To investigate whether individual cooperative contributions modulate CORT and T, cooperative contributions were experimentally manipulated by changing the sand provisioning regime. Five originally wild-caught colonies (colony size range: 9-14 individuals, mean=12.2, SD=1.77) were subjected to an increased sand provisioning treatment (the sand treatment) and to a control treatment. During the sand treatment, sand dispensers (see Chapter 2, Figure 2.4) were refilled every hour for 12 successive hours starting at 07:00, while during the control treatment they were filled once in the morning and once in the evening. The control treatment corresponded to a decrease and the sand treatment to an increase in the sand provisioning as compared to Study 1, in which sand was provided every two hours. Each treatment lasted 7

days, although only the first two days are relevant for the data presented here. The two treatments were separated by 7 days, and their sequence was balanced across colonies.

A 12 hours scan observation session was conducted on the second day of each treatment and a urine sample was collected the following morning for the subsequent determination of CORT and T levels. Urine samples of all adult individuals, excluding breeding females (undetermined age but older than 2 years; females weight range: 86-163 g, mean=129 g; males weight range: 92-214 g; mean=165 g), were analysed resulting in a sample size of 88 samples (21 females and 23 males). In the absence of parentage analyses, I was unable to identify the breeding males. Since multiple males can mate with each breeding female, excluding all males which had been observed mating would have dramatically reduced the sample size and I therefore decided to retain all male data. Additional animal procedures (blood and sperm samples collection) were conducted one day before and one day after each treatment for other purposes (Mendonça et al., in preparation).

3.2.3 Hormone analyses

All hormone analyses were conducted at the Neuchatel Platform of Analytical Chemistry using ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Details of the analytical procedures are presented in Chapter 2. All raw hormone concentrations were corrected for urine specific gravity as detailed in Chapter 2, to control for variation in urine concentration.

3.2.4 Data management and statistical analyses

A mixed modelling approach was followed to account for the non-independence in the data caused by the repeated measurements of individuals. Individual and colony (when colony number >6) identities were specified as random factors. Model simplification and validation followed the procedures described in Chapter 2. In models where hormone concentration was specified as a response variable, its natural logarithm was used and urination delay was specified as an independent covariate to control for the effects of the time spent in the urine chamber on hormones levels. This was especially relevant for CORT since data exploration revealed that CORT levels increased with urination delay (LM; urination delay effect:

estimate=0.20, χ^2=22.98, p<0.001). This pattern is more likely to result from lengthening in urination delay to increase CORT rather than the opposite since the urination delay of female helpers administered with a 5 mg CORT implant (Chapter 4) did not differ from when they were administered with a control implant (gamma GLMM; Treatment effect: estimate=-0.017, SE=0.053, χ^2=0.102, p=0.750). Whenever the data did not comply with the linear mixed model assumptions, non-parametric statistical tests were used instead.

Behavioural variables represent grouped behaviours, following the procedures detailed in Chapter 2.

Study 1: Correlation between CORT, T and individual cooperative contributions

To determine whether CORT and T levels correlated with individual cooperative contributions, I specified the CORT and T levels as response variables in two distinct LMMs. I hypothesized that the effect of cooperative contributions on CORT and T could be dependent on individual sex and weight and therefore specified the proportion of Total Cooperation, sex (female, male), weight and all possible interactions between them as covariates. Age and group size were specified as two independent covariates to control for potential effects of developmental stage and of social environment on hormone values, respectively. Testosterone levels of two females were unusually high and were excluded from the T model.

Experiment 1: Effect of cooperative contribution manipulations on CORT and T levels

I investigated whether the behavioural and hormonal responses to treatment were dependent on subjects' weight and sex. Therefore, treatment (control, sand), sex (female, male), weight, and all possible interactions between them were specified as covariates. To control for the effects of potentially stressful animal procedures conducted prior and after each treatment week, I specified treatment week (week 1, week 2) as an independent covariate.

First, I investigated whether the sand treatment fulfilled its goal of increasing individual cooperative contributions. Total Cooperation was specified as a response variable in an OLRE binomial GLMM. Additionally, I determined whether the effect of treatment was shared across the different types of cooperative activities: Burrowing was tested with an OLRE binomial

GLMM while a non-parametric Wilcoxon sign ranked test was used for Food Carrying and Nest Building.

Secondly, I tested the effect of treatment on non-cooperative behaviours, activity and social behaviours, as these are susceptible to influence CORT and T levels (Creel et al., 2013). I specified Non-cooperation and Activity as response variables in two distinct OLRE binomial GLMMs. The number of Passes was specified as a response variable in a negative binomial GLMM to test the effect of treatment on interferences between individuals and potential opportunities for social interaction. I tested the effect of treatment on aggressive interactions by specifying the number of individuals involved in Overt-aggression, or Chase or Food Competition bouts using a chi-squared test.

Third, I tested the effect of treatment on subjects' body condition. I specified the weight difference between the first and the third day of treatment as a response variable in a LMM. Treatment, sex and the interaction between the two were specified as covariates. To control for the fact that heavier animals have more weight to lose (regression to the mean effects: Kelly and Price, 2005), weight at the beginning of treatment was fitted as a covariate. I then tested whether differences in weight changes may be explained by an effect of treatment on feeding behaviour by specifying Eat as a response variable in an OLRE binomial GLMM.

Finally, I investigated the effect of treatment on hormonal levels, by specifying CORT and T levels as response variables in two distinct LMMs. Since treatment had a nearly significant effect on CORT levels (see results section), I investigated whether the differences in Burrowing (the cooperative activity that was increased by the sand treatment) between treatments explained the changes in CORT levels. For this, I specified the difference in the log transformed CORT levels between sand and control treatments as a response variable in a linear model and the difference in Burrowing between treatments, as a covariate. Log transformed CORT levels during the control treatment were specified as an additional independent covariate, to control for a potentially more pronounced effect of the sand treatment on lower CORT levels. To account for differences in CORT that may be caused by differences in the urine collection delay between treatments, I specified this difference as an independent covariate. I then investigated whether Burrowing would predict CORT levels when data of both treatments were pooled together. To test this possibility, I specified a LMM similar to the one used to test the effect of treatment on CORT levels, the only difference being that the covariate treatment was replaced by the proportion Burrowing.

3.3 Results

Study 1: Correlation between CORT, T and individual cooperative contributions

Cortisol (Table 3.4, Model 1, Figure 3.5) and testosterone (Table 3.4, Model 2, Figure 3.6) levels were not affected by individual cooperative contributions. Cortisol levels significantly increased with age and urination delay. Testosterone levels were significantly higher in males than in females and increased with weight.

Experiment 1: Effect of cooperative contribution manipulations on CORT and T levels

The sand treatment significantly increased individual Total Cooperation, from 7.2 to 16.7 percent (Table 3.2, Model 1, Figure 3.7). This increase was driven by the significant effect of treatment on Burrowing (Table 3.2, Model 2), as Nest Building (paired Wilcoxon signed-rank test; females: n=21, V=22, p=0.608; males: n=23, V=45, p=1) and Food Carrying (paired Wilcoxon signed-rank test; females: n=21, V=38.5, p=0.627; males: n=23, V=62, p=0.550) were unaffected.

The effect of treatment on Non-cooperation was dependent on weight: non-cooperative behaviours increased with weight during the control treatment and decreased with weight in the sand treatment (Table 3.6, Model 1). However, within this interaction, the effect of treatment was not significant. The combined effects of treatment on the expression of cooperative and non-cooperative behaviours led to a significant increase in Activity, which was close to being significantly affected by weight and sex (Table 3.6, Model 2). Indeed, heavier individuals increased their activity to a lesser extent and males increased their activity to a greater extent than females. Treatment also affected social conditions: number of Passes (Table 3.7) and number of individuals involved in aggressive interactions (Chi-square test: χ^2=5.572, df=1, p=0.018) were both significantly higher in the sand treatment.

Treatment significantly affected the pattern of weight changes: individuals had a stable weight during the sand treatment and lost weight during the control treatment (Table 3.5), despite eating significantly more (Table 3.6).

Testosterone levels were not modulated by treatment, and similarly to the results of Study 1, T was significantly higher in males and increased with weight (Table 3.10, Model 1). Individual CORT levels were increased by the sand treatment to a degree that fell short of significance and significantly increased with urination delay (Table 3.10, Model 2, Figure 3.8). However, individual changes in CORT levels across treatments were not predicted by the changes in burrowing but there was a significant negative correlation with the CORT levels during the control week (Table 3.11, Figure 3.9). When data from both treatments were pooled, individual burrowing contributions did not explain CORT levels (Table 3.12).

Table 3.4 – Predictors of CORT (*Model 1*; n=70 urine samples from 20 females and 21 males) and T (*Model 2*; n=68 urine samples from 19 females and 21 males) levels. The response variables were ln-transformed and the data were analysed in LMMs. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled; [b] indicates variables centred and scaled by sex.

Covariates	Estimate	SE	test statistic	p-value
Model 1. Predictors of CORT levels				
Intercept	**1.504**	**0.198**	**7.584**	
Urination delay	**0.015**	**0.002**	**6.224**	**<0.001**
Age [a]	**0.177**	**0.089**	**1.993**	**0.047**
Sex	-0.198	0.185	-1.069	0.261
Total Cooperation [a]	-0.039	0.092	-0.421	0.788
Total Cooperation [a] x Sex	0.368	0.197	1.872	0.105
Group size [a]	0.171	0.108	1.582	0.096
Weight [b]	0.014	0.127	0.110	0.930
Total Cooperation [a] x Weight [b]	0.076	0.103	0.743	0.384
Sex x Weight [b]	0.106	0.185	0.576	0.523
Total Cooperation [a] x Sex x Weight [b]	0.166	0.261	0.635	0.524
Model 2. Predictors of T levels				
Intercept	**2.040**	**0.211**	**9.664**	
Weight [b]	**0.281**	**0.110**	**2.560**	**0.013**
Sex	**1.350**	**0.170**	**10.269**	**<0.001**
Urination Delay	0.004	0.002	1.783	0.071
Age [a]	-0.100	0.094	-1.066	0.299
Group size [a]	-0.050	0.128	-0.388	0.793
Total Cooperation [a]	0.029	0.098	0.298	0.791
Sex x Weight [b]	-0.316	0.200	-1.577	0.227
Total Cooperation [a] x Sex	-0.331	0.208	-1.593	0.122
Total Cooperation [a] x Weight [b]	-0.024	0.109	-0.221	0.810
Total Cooperation [a] x Sex x Weight [b]	-0.447	0.268	-1.666	0.076

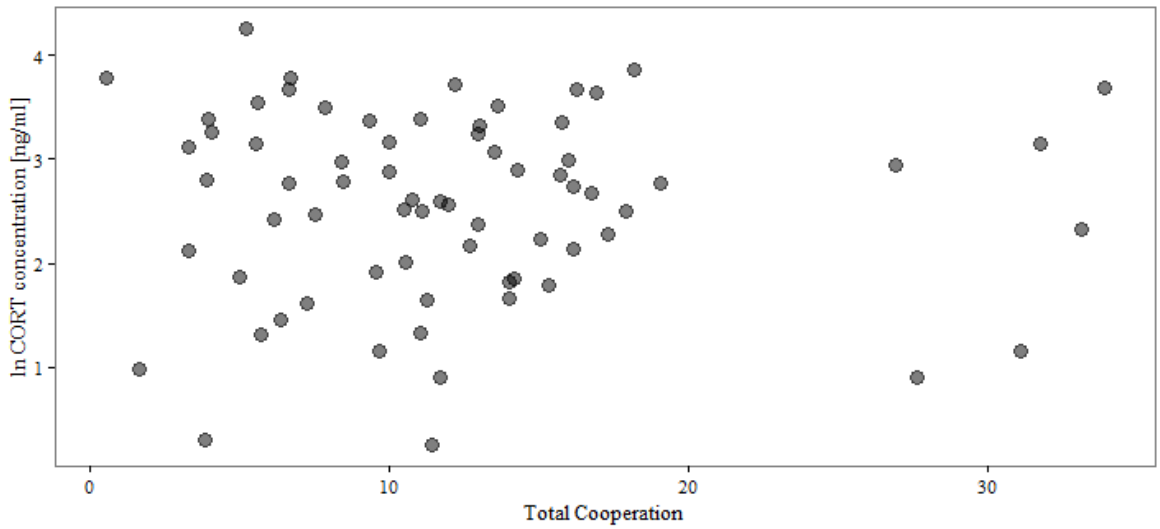

Figure 3.5 - Correlation between ln-transformed urinary CORT levels and Total Cooperation. Total Cooperation is expressed as percentage of scans displayed during scan observation sessions. Each dot represents a distinct measurement, although measurements are not all independent from one another as 1-3 repeated measurements were collected from each individual.

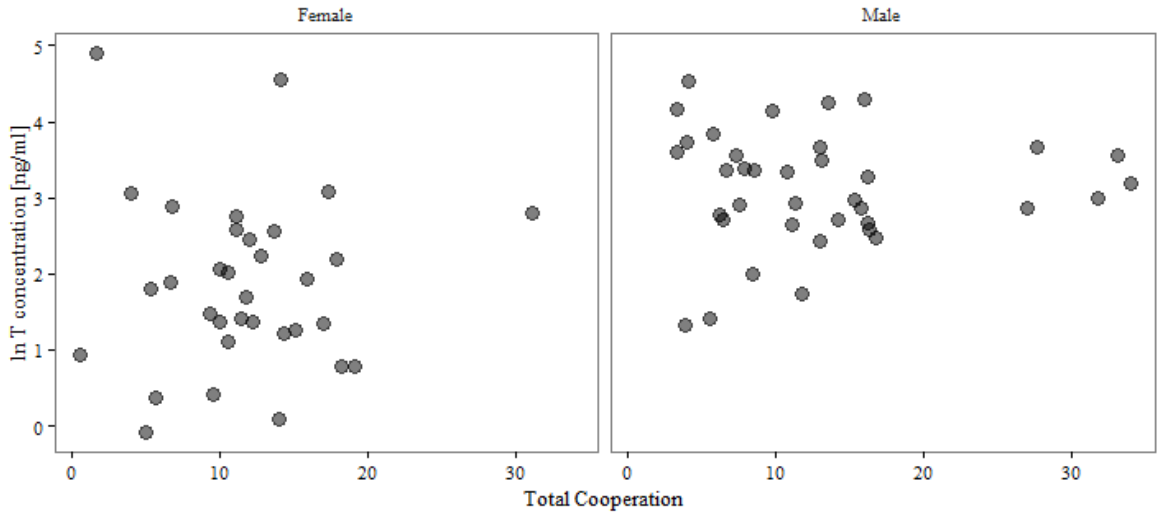

Figure 3.6 - Correlations between ln-transformed urinary T levels and Total Cooperation in females (left) and males (right). Total Cooperation is expressed as percentage of scan displayed during scan observation sessions. Each dot represents a distinct measurement, although measurements are not all independent from one another as 1-3 repeated measurements were collected from each individual.

Table 3.5 – Predictors of Total Cooperation (*Model 1*) and Burrowing (*Model 2*) during the sand provisioning experiment (control: n=44 individuals; sand: n=44 individuals; 21 females, 23 males). The data were analysed in OLRE binomial GLMMs with a logit link function. All variables shown in bold were retained in the minimal model. [b] indicates variables centred and scaled by sex.

Covariates	Estimate	SE	test statistic	p-value
Model 1. Predictors of Total Cooperation				
Intercept	**-0.280**	**0.137**	**-20.412**	
Treatment	**0.999**	**0.144**	**6.955**	**<0.001**
Sex	0.304	0.219	1.390	0.166
Treatment Week	0.113	0.143	0.792	0.431
Weight [b]	0.053	0.110	0.477	0.633
Treatment x Weight [b]	-0.190	0.142	-1.338	0.187
Weight [b] x Sex	0.146	0.221	0.659	0.510
Treatment x Sex	0.092	0.278	0.331	0.742
Treatment x Weight [b] x Sex	-0.460	0.275	-1.674	0.100
Model 2. Predictors of Burrowing				
Intercept	**-2.931**	**0.142**	**-20.580**	
Treatment	**1.076**	**0.146**	**7.380**	**<0.001**
Sex	0.306	0.230	1.329	0.189
Treatment Week	0.091	0.145	0.625	0.535
Weight [b]	0.061	0.115	0.532	0.596
Treatment x Weight [b]	-0.182	0.144	-1.266	0.212
Weight [b] x Sex	0.133	0.230	0.578	0.563
Treatment x Sex	0.103	0.284	0.364	0.720
Treatment x Weight [b] x Sex	-0.461	0.280	-1.647	0.105

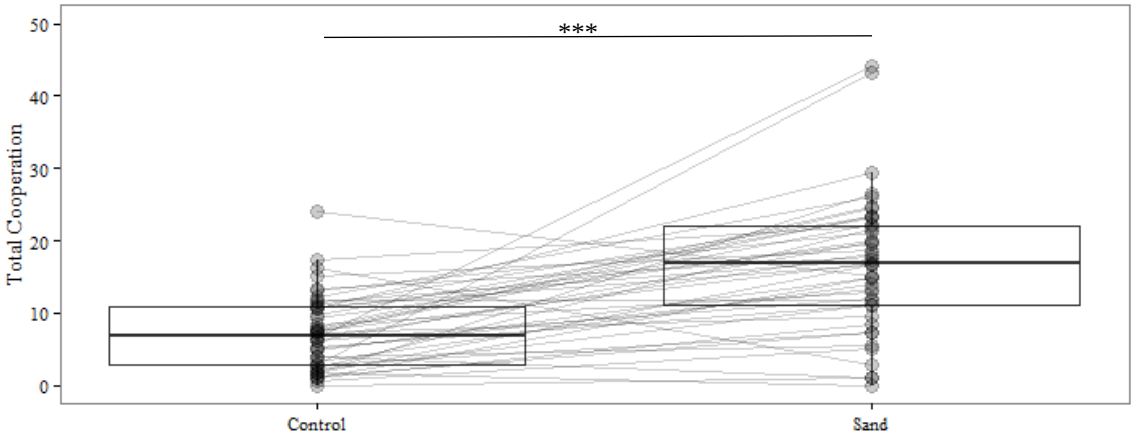

Figure 3.7 - Effect of sand provisioning experiment on individual Total Cooperation, expressed as the percentage of scan displayed during the 12 hours scan sessions of the control (left) and the sand (right) treatments. Lines between points illustrate the repeated measurements of same individuals. ***: $p<0.001$).

Table 3.6 - Predictors of Non-cooperation (*Model 1*) and Activity (*Model 2*) during the sand provisioning experiment (control: n=44 individuals; sand: n=44 individuals; 21 females, 23 males). The data were analysed in OLRE binomial GLMMs with a logit link function. All variables shown in bold were retained in the minimal model. [b] indicates variables centred and scaled by sex. [c] indicates p-values returned by the lmerTest package.

Covariates	Estimate	SE	test statistic	p-value
Model 1. Predictors of Non-cooperation				
Intercept	**-1.359**	**0.051**	**-26.861**	
Treatment	**-0.019**	**0.050**	**-0.379**	**0.705** [c]
Weight [b]	**0.055**	**0.050**	**1.093**	**0.275** [c]
Treatment x Weight [b]	**-0.124**	**0.052**	**-2.405**	**0.021**
Sex	-0.065	0.087	-0.746	0.457
Treatment Week	-0.010	0.051	-0.198	0.843
Treatment x Sex	0.165	0.098	1.682	0.098
Weight [b] x Sex	-0.024	0.088	-0.271	0.787
Treatment x Weight [b] x Sex	-0.056	0.100	-0.560	0.567
Model 2. Predictors of Activity				
Intercept	**-0.091**	**0.076**	**-11.951**	
Treatment	**0.416**	**0.084**	**4.921**	**<0.001**
Treatment Week	0.080	0.084	0.957	0.341
Sex	0.090	0.125	0.719	0.473
Weight [b]	-0.008	0.063	-0.121	0.904
Treatment x Weight [b]	-0.162	0.081	1.999	0.052
Treatment x Sex	0.303	0.153	1.979	0.052
Weight [b] x Sex	0.009	0.126	0.068	0.946
Treatment x Weight [b] x Sex	-0.253	0.151	-1.680	0.099

Table 3.7 – Predictors of Passes during the sand provisioning experiment (control: n=44 individuals; sand: n=44 individuals; 21 females, 23 males). The data were analysed in negative binomial GLMMs with a logit link function. All variables shown in bold were included in the minimal model. [b] indicates variables centred and scaled by sex. [c] indicates p-values returned by the lmerTest package.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**0.826**	**0.119**	**15.327**	
Treatment	**0.494**	**0.122**	**4.054**	**<0.001** [c]
Weight [b]	**0.057**	**0.117**	**0.489**	**0.625** [c]
Treatment x Weight [b]	**-0.389**	**0.129**	**-3.011**	**0.002**
Sex	-0.337	0.185	-1.815	0.074
Treatment Week	-0.049	0.122	-0.401	0.689
Weight [b] x Sex	0.187	0.189	0.988	0.321
Treatment x Sex	0.200	0.244	0.821	0.412
Treatment x Weight [b] x Sex	0.122	0.258	0.472	0.637

Table 3.8 – Predictors of weight changes during the sand provisioning experiment (control: n=44 individuals; sand: n=44 individuals; 21 females, 23 males). The data were analysed in LMMs. All variables shown in bold were retained in the minimal model.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-1.591**	**0.630**	**-2.526**	
Treatment	**1.818**	**0.882**	**2.061**	**0.041**
Sex	0.197	1.116	0.177	0.861
Weight	-0.018	0.015	-1.183	0.230
Treatment x Sex	-0.338	1.773	-0.190	0.845

Table 3.9 – Predictors of Eat (control: n=44 individuals; sand: n=44 individuals; 21 females, 23 males). The data were analysed in OLRE binomial GLMM with a logit link function. All variables shown in bold were retained in the minimal model. [b] indicates variables centred and scaled by sex.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-2.804**	**0.049**	**-5.732**	
Treatment	**-0.211**	**0.072**	**-2.930**	**0.003**
Weight [b]	0.066	0.037	1.790	0.072
Treatment Week	-0.012	0.072	-0.160	0.873
Sex	0.009	0.072	0.124	0.902
Treatment x Sex	0.080	0.144	0.555	0.579
Weight [b] x Sex	-0.019	0.073	-0.255	0.798
Treatment x Weight [b]	-0.022	0.074	-0.030	0.976
Treatment x Weight [b] x Sex	0.092	0.148	0.623	0.533

Table 3.10 – Predictors of T (*Model 1*) and CORT (*Model 2*) levels during the sand provisioning experiment (control: n=44 individuals; sand: n=44 individuals; 21 females, 23 males). The data were analysed in LMMs. All variables shown in bold were retained in the minimal model. [b] indicates variables centred and scaled by sex.

Covariates	Estimate	SE	test statistic	p-value
Model 1. Predictors of T				
Intercept	**2.113**	**0.187**	**11.311**	
Weight [b]	**0.309**	**0.127**	**2.441**	**0.016**
Sex	**1.437**	**0.258**	**5.562**	**<0.001**
Treatment Week	-0.091	0.107	0.856	0.387
Urine collection delay	0.002	0.003	0.961	0.354
Treatment	0.024	0.117	0.206	0.837
Weight [b] x Sex	-0.376	0.257	-1.464	0.128
Treatment x Sex	0.043	0.214	0.199	0.835
Treatment x Weight [b]	-0.020	0.110	-0.183	0.846
Treatment x Weight [b] x Sex	-0.046	0.229	-0.203	0.835
Model 2. Predictors of CORT				
Intercept	**1.855**	**0.156**	**11.867**	
Urine collection delay	**0.013**	**0.002**	**5.952**	**<0.001**
Treatment	0.290	0.154	1.883	0.058
Treatment Week	0.199	0.150	1.325	0.177
Weight [b]	0.059	0.075	0.785	0.420
Sex	-0.056	0.153	-0.365	0.706
Treatment x Sex	0.453	0.298	1.520	0.116
Weight [b] x Sex	-0.221	0.152	-1.461	0.128
Treatment x Weight [b]	-0.054	0.150	-0.361	0.704
Treatment x Weight [b] x Sex	0.069	0.304	0.227	0.810

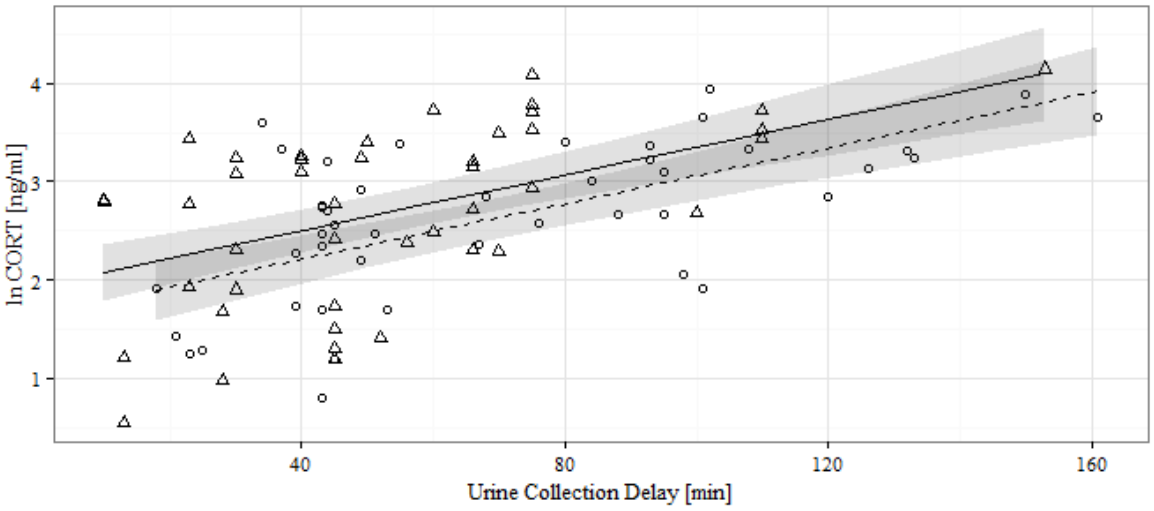

Figure 3.8 – Effect of the sand provisioning experiment on CORT levels. After controlling for the significant effect of urination delay on CORT levels, the sand treatment led to an increase in CORT levels (p=0.057). CORT levels are expressed after their ln transformation in ng/ml of urine. Urination delay is given in minutes from transfer into the urine chamber until urination. Circles and triangles represent the raw data for the control and the sand treatment respectively. Dashed and solid line represent the LMM predictions for the control and sand treatment, respectively. The 95 % confidence intervals are depicted in grey.

Table 3.11 – Predictors of changes in Burrowing between the sand and the control treatments (n=44 individuals). The data were analysed in a LM. All variables shown in bold were included in the minimal model. [a] indicates variables that were transformed by their natural logarithm.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**25.504**	**8.206**	**3.108**	
Urine collection delay difference	**0.224**	**0.080**	**2.806**	**0.005**
CORT [a]	**-7.347**	**3.205**	**-2.293**	**0.021**
Burrowing difference	0.124	0.135	0.922	0.336

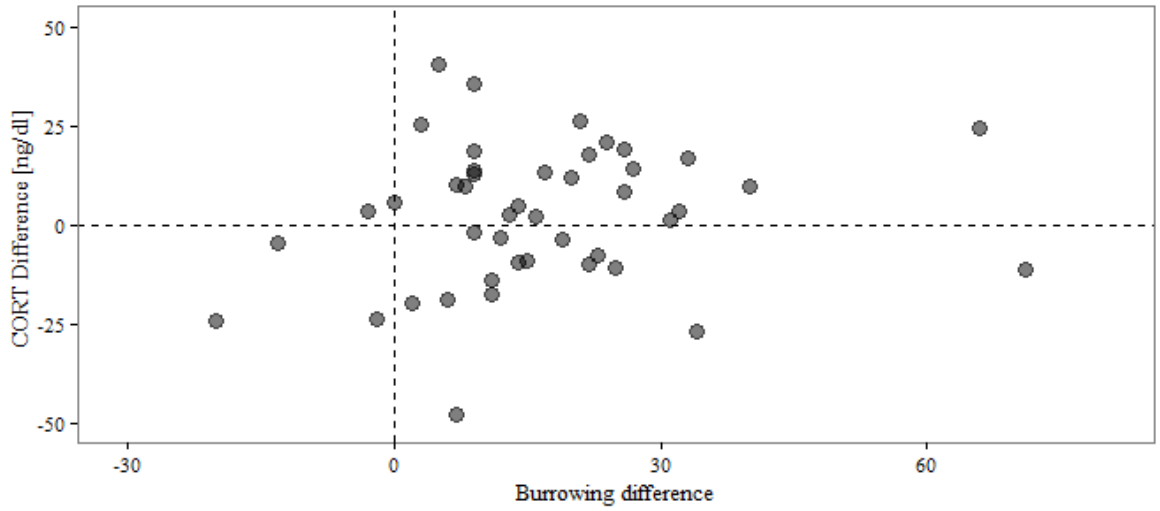

Figure 3.9 - Correlation between differences in urinary CORT and differences in number of Burrowing scans between the control and sand treatment.

Table 3.12 – Predictors of CORT levels during the sand provisioning experiment (control: n=44 individuals; sand: n=44 individuals; 21 females, 23 males). Data of the two treatments were pooled and analysed in a LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled; [b] indicates variables centred and scaled by sex.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**1.854**	**0.156**	**11.867**	
Urine collection delay	**0.013**	**0.002**	**5.952**	**<0.001**
Treatment Week	0.230	0.150	1.518	0.125
Weight [b]	0.059	0.076	0.773	0.430
Burrowing [a]	0.050	0.077	0.650	0.504
Sex	-0.087	0.157	-0.553	0.567
Weight [b] x Sex	-0.212	0.154	-1.373	0.155
Burrowing [a] x Sex	0.044	0.163	0.269	0.778
Burrowing [a] x Weight [b]	0.005	0.089	0.055	0.954
Burrowing [a] x Weight [b] x Sex	-0.065	0.188	-0.346	0.713

3.4 Discussion

My results show that when Damaraland mole-rats were provided with more sand, individuals more than doubled their cooperative contributions, raising their CORT levels by 25%, although this fell short of significance. Burrowing was the only cooperative activity elevated by the treatment, suggesting it could be responsible for the increase in CORT. Therefore, individual burrowing contributions would have been expected to positively correlate with CORT levels, yet my results show this is not the case. First, individual differences in burrowing contributions across the two treatments did not correlate with the variations in CORT levels. Second, when the low burrowing contributions of the control and the high burrowing contributions of the sand treatments were pooled, they still failed to predict CORT levels, paralleling the results of the non-experimental data.

The lack of correlation between burrowing and CORT levels does not exclude the possibility that increased burrowing raised CORT levels. Following the evening interruption of the increased sand provisioning, it is likely that burrowing contributions rapidly returned to their usual levels. Since GC profiles closely match recent levels of activity (Girard and Garland, 2002; Hackney and Viru, 1999; Malisch et al., 2008), the overnight delay left until urine sampling may have been sufficient for elevated CORT levels to fade (Smith and French, 1997). In laboratory rodents, differences in GC levels induced by experimental increase in physical activity can persist beyond a period of rest, but this may require a longer treatment duration than the one used in this experiment (Naylor, 2005). Overall the effect of increased cooperative activities on CORT levels may have been more pronounced if samples had been collected in the evening immediately after the interruption of the sand provisioning and/or after a longer period of treatment.

Increased sand provisioning also influenced social interactions and feeding, which could lead to the reported effects of treatment on CORT levels. Investigations of the effects of voluntary exercise on GC profile have mostly been conducted on animals housed in social isolation (Fediuc et al., 2006; Girard and Garland, 2002; Lancel et al., 2003; Makatsori et al., 2003), allowing to dismiss the effect of social influences on the modulation of GC levels (Creel et al., 2013). In highly social species such as cooperative breeders, changes in the expression of cooperative behaviours may be associated with changes in social conditions. My results highlight that such confounding social influences should be considered when investigating the

effect of cooperative contributions on GC levels. When colonies were provided with more sand, individuals became more active, they encountered other group members more frequently and more of them were involved in aggressive interactions. Although colonies were fed *ad libitum*, individuals were observed eating less during the sand treatment. However, individuals did not suffer a loss of body condition, suggesting that they ate more at night, when no sand was provided. This advocates a neutral energetic balance, making it unlikely that changes in feeding may have contributed to the increases in CORT.

Candidate explanations for a modulation of CORT – cooperative activities, social conditions and feeding - could act in concert and are challenging to tease apart. Both a decrease in energy intake (Levay et al., 2010; Lynn et al., 2010, 2003) and social isolation (Sapolsky et al., 1997; Weiss et al., 2004) could explain the increase in GC levels which is characteristic of babysitting meerkats (Carlson et al., 2006b). Similarly, both increased energetic demands and decreased food intake caused, respectively, by acquisition and provisioning of food items for pups may explain the positive association between GC and pup feeding in banded mongooses (Sanderson et al., 2014). Clearly, future studies aiming to unravel the effects of cooperative behaviours on hormone levels will have to rely on carefully designed experiments in which the effects of these varied influences can be distinguished.

Neither the correlative nor the experimental results suggest that cooperative contributions affect T levels. These results indicate that the elevation of CORT levels was insufficient to induce a decrease in T, a hormone that generally favours competitive abilities and reproductive behaviours (Hau, 2007). This does not support the hypothesis that higher cooperative contributions may lead to hormonal changes contributing to helpers' lower competitiveness and reproductive investment.

In summary, regardless of the factors that may modulate CORT levels, life in an environment where individual cooperative contributions are elevated can lead to increased stress levels. Further experimental manipulations are now needed to test whether stress hormones could also modulate individual cooperative contributions.

Chapter 4

Effects of Cortisol manipulation on the cooperative contributions in female helpers

Abstract

Individual cooperative contributions are seldom equally distributed within animal societies partly because individuals adjust their level of cooperation to perceived individual and environmental characteristics. The physiological mechanisms controlling these adjustments remain poorly understood but glucocorticoid (GC) stress hormones may represent a general mechanism underlying adaptive plasticity in cooperative contributions. Studies of cooperative breeders have demonstrated associations between GC and the expression of cooperative behaviours but have not yet demonstrated a causal link between the two. In Chapter 3, I provided evidence that GC can be increased by cooperative behaviours. In this chapter, I test the reverse, yet not mutually exclusive possibility, that GC causally modulate cooperative contributions. I show that experimental elevation of GC in non-breeding female helpers increased their cooperative contributions, indicating that GC facilitates the expression of energetically demanding cooperative activities. These findings offer the first experimental evidence in a cooperative breeder that GC modulates cooperative contributions.

4.1 Introduction

Individual cooperative contributions are highly variable and are conditional on individual, social and environmental characteristics affecting the fitness costs and benefits of cooperation (Clutton-Brock et al., 2002; Russell et al., 2003). While the adaptive character of individual abilities to alter their level of cooperation in a context-dependent fashion has received considerable attention (Cant, 2005; Griffin and West, 2002), the physiological basis of these changes remain largely unknown (Soares et al., 2010). Elucidating the physiological mechanisms modulating cooperative behaviours represent one of the remaining challenges in the study of cooperation.

Glucocorticoid (GC) stress hormones may play an important role in modulating the expression of energetically costly cooperative behaviours as a result of their profound effect on the regulation of energy metabolism, feeding behaviour and locomotor activity (Landys et al., 2006). Costly cooperative behaviours are commonly displayed in cooperative breeders, where so called helpers forego their own reproduction and care for offspring which are not their own (Clutton-Brock, 2016; Koenig and Dickinson, 2004). The energetic costs of cooperative activities arise because of increased energy expenditure when defending a territory, decreased energy intake when forgoing feeding to babysit pups at the burrow, or a combination of the two when digging for a prey that is subsequently provisioned to a young. Glucocorticoids may promote the expression of these cooperative activities since increased GC release promotes energy production (Landys et al., 2006; Sapolsky et al., 2000). Alternatively, increased GC levels may reflect poor body condition (Levay et al., 2010; Lynn et al., 2010, 2003), promote food consumption (Landys et al., 2006) and lead to a decrease in costly cooperative contributions. Previous correlational studies have indeed suggested that GC affect both baby-sitting and pup feeding (Carlson et al., 2006a, 2006b; Sanderson et al., 2014) but were unable to demonstrate GC causal effect on the modulation of these activities. Experimental manipulations of GC neuroendocrine pathways are required to unequivocally determine whether GC modulate cooperative activities.

Using captive Damaraland mole-rat (*Fukomys damarensis*) as a model system, I investigated whether cortisol (CORT), the form of GC secreted by this species (Clarke et al., 2001), modulates cooperative contributions. In contrast to other cooperative breeders, alloparental care is rarely observed and cooperative activities mostly consist of excavating

activities necessary to maintain and expand underground foraging tunnels to locate food sources and of building and maintaining a communal nest and food stores (Bennett, 1990; Zöttl et al., 2016b). These behaviours can be defined as cooperative as they contribute to common goods from which all group members can benefit. Furthermore, the possibility that helpers' cooperative activities may have been selected through their positive effects on other group members' reproductive success has recently been supported by a field study showing that helpers increase offspring recruitment (Young et al., 2015). I experimentally increased female helpers' CORT levels within its physiological range during a week. Elevations of GC levels are known to increase locomotor activity and energy production (Landys et al., 2006). Since cooperative behaviours in the Damaraland mole-rat are energetically demanding (Lovegrove, 1989), I investigated whether experimentally increases in CORT levels raised helpers' cooperative contributions. Since increased CORT levels can downregulate the secretions of the sex steroid hormone testosterone (T) (Viau, 2002), which may conflict with the expression of cooperative behaviours, I also measured T levels to assess the possibility that CORT modulates cooperative activities via its effect on T release.

4.2 Methods

4.2.1 Colony maintenance and husbandry

For details of colony maintenance and general animal husbandry refer to Chapter 2.

4.2.2 Experimental design and procedures

Seven pairs of non-reproductive adult females (subject 1 and subject 2), originally from 7 originally wild-caught colonies (group-size range: 6 to 21 individuals, mean=10.71, SD=5.12) were used in this experiment. Both subjects were subjected to a cortisol and a control treatment. On day 1, subject 1 and 2 respectively received a 7 days' release pellet containing 0.001 mg (control) and 5 mg (cortisol) of CORT before 8:00. Hormone pellets (Innovative Research of America) were inserted subcutaneously in the neck area using a 10-gauge precision trochar (Innovative Research of America), while subjects were kept under anaesthesia with a constant flow of Isoflurane. Procedures did not exceed 15 minutes and subjects were placed back in their tunnel system as soon as full mobility was regained. Two weeks later, the same procedure was repeated at the only difference that treatments were reversed between the two experimental subjects.

To determine the effect of treatment on subjects' endocrine profile, a urine sample was collected on day 3 and day 6 of each treatment week.

Treatment validation followed a two-step process. First, I assessed whether the cortisol treatment successfully elevated CORT levels. Second, I assessed the physiological validity of the cortisol treatment by comparing the CORT values measured during this treatment with those measured in two distinct social situations: baseline and eviction. Baseline condition was quantified in samples collected from non-experimental adult female helpers of similar weight range to the experimental subjects, living in stable social environment (baseline condition: n=29, 13 individuals). Samples collected within 2 days following the eviction of adult female helpers from their original colony were expected to reflect stress induced CORT levels (eviction condition: n=12 samples, 11 individuals).

During each treatment week, behavioural observations consisted of two 12 hours scan sessions conducted on day 2 and day 5 and focal observations conducted on days 1,3,4,6 and 7 (observation count/individual: range: 10-24, mean=17.14, SD=4.27; observation time in hour/individual: range: 5.7-17.35 mean=11.59, SD=3.36). All behavioural observations were carried out by a total of three observers blind to treatment.

Subjects were weighed on implantation day (day 1) and on the morning after the end of the treatment (day 8).

4.2.3 Hormone analyses

All hormone analyses were conducted at the University of Pretoria, in collaboration with Professor Nigel Bennett using radioimmunoassay. Details of the analytical procedures are available in Chapter 2. Before statistical analyses, all raw hormone concentrations were corrected for urine specific gravity as detailed in Chapter 2, to control for variation in urine concentration across samples.

4.2.4 Data management and statistical analyses

Treatment effects on endocrine values

On the first step of the validation process, I specified CORT concentrations transformed by their natural logarithm (ln transformed) as a response variable in a LMM. I anticipated that implants' CORT release may not be constant throughout the week of treatment and that changes in CORT levels could vary as a function of individual weight. For these reasons, treatment (control, cortisol), day of treatment (day 3, day 6), subject weight on implantation day and all possible interactions between them were specified as covariates. Because CORT concentrations could increase with the time spent in the urine chamber, I also specified urination delay as an independent covariate. Two outliers from different subjects, one on day 3 and one on day 6, during the cortisol treatment were excluded from the analyses leading to a total of 54 CORT concentrations (n=14 individuals, 2 urine samples per treatment).

On the second step, to evaluate the physiological validity of the cortisol treatment, ln transformed CORT values were specified as a response variable in a LMM, where individual condition (baseline, cortisol, eviction), individual weight and urination delay were specified as independent covariates.

To assess if the effects of treatment on cooperative contributions may be mediated via a CORT induced decreases of T secretions, I questioned whether treatment affected T levels. Generally, T concentrations measured were low and fell in the lower part of the assay standard curve. In the 28 samples analysed, 15 had one and 8 had both their duplicates below the assay detection limit, and only 3 samples had a concentration that was higher than the lowest standard curve concentration resulting in a high coefficient of variation between duplicates. Testosterone concentrations were ln transformed and specified as a response variable in a LMM where only treatment was specified as a covariate because of the small sample size. Data from one subject which returned undetectable T concentrations for both treatments were excluded from the analyses as the effect of treatment on T values could not be determined (n=26 data points, 13 subjects). All other undetectable T concentrations were assigned a concentration equal to the assay LOD and kept in the dataset. A similar model was run a second time after the removal of a subject returning an unusually high T concentration during the control treatment.

Treatment effects on behaviour and body-condition

For the analyses described below, I investigated whether treatment effects on behaviour varied as a function of subjects' weight and treatment day. The former, because weight differences may reflect variation associated with differences in the stress axis; the latter, because CORT effects may vary through treatment, due to a non-constant release of the hormone from the pellets. Unless stated otherwise, I specified treatment (control, cortisol), treatment day (day 2, day 5), weight on implantation day, an interaction between treatment and treatment day and an interaction between treatment and subjects' weight as covariates. If not detailed differently, analyses were conducted on scan data using beta-binomial GLMMs (see Chapter 2). Behaviour variables used for statistical analyses represent grouped behaviours, following the procedures described in Chapter 2.

I first tested whether treatment affected individual cooperative contributions by specifying Total Cooperation as a response variable. Because the cortisol treatment induced CORT levels close to those measured during a stressful situation (see results), I asked whether treatment effect would be retained when only data referring to CORT levels closer to baseline were considered. To answer this question, I repeated the previous analyses: a) after excluding all the cortisol treatment behavioural data collected on day 2, because CORT levels were highest earlier in the treatment week (see results), and b) that were associated with CORT levels higher than the treatment median. For these two latter models, only treatment, subjects' weight and their interaction were fitted as covariates.

I subsequently investigated whether changes in cooperative contributions were predicted by the changes in CORT levels between the cortisol and the control treatments. To answer this question, I specified the difference in Total Cooperation (day 2 and day 5) between the two treatments as a response variable in a LMM. I had no *a priori* prediction as to whether changes in cooperative contributions are more likely to be mediated by the absolute difference in CORT levels between treatments or its relative change as compared to the concentration determined during the control treatment. I consequently specified both the absolute and the relative changes in CORT as independent covariates, which was possible because data exploration procedures revealed an absence of collinearity between the two. To control for the possibility that treatment effects on cooperative contributions could be more pronounced in subjects that cooperated less during the control week, Total Cooperation measured during the control treatment was fitted as an additional independent covariate. Covariates accounting for non-linear effects of changes in CORT on Total Cooperation were omitted since a Generalized Additive Model (GAM) conducted as part of the data exploration procedures confirmed the absence of such effects.

To determine whether treatment effect on cooperative contributions were the consequence of a specific effect of CORT on cooperative behaviours or a more general effect on activity, I first tested the effect of treatment on non-cooperative behaviours by specifying Non-Cooperation as a response variable. After, I asked whether in combination with CORT effects on Total Cooperation, this would translate into a general treatment effect on subjects' activity by specifying Activity as a response variable. I then investigated the specificity of treatment effect on cooperative contributions by asking whether Total Cooperation during activity bouts was influenced by CORT. Similar to previous models, Total Cooperation was

specified as a response variable, at the only difference that the number of scans during which subjects were active, not the total number of scans, was used as the binomial total.

To investigate whether treatment effect on individual cooperative contributions was restricted to some of the cooperative activities merged into Total Cooperation, I specified Burrowing, Food Carrying (binomial GLMM) and Nest Building (OLRE binomial GLMM) as distinct response variables in three separate models. For Food Carrying and Nest Building, data from the two scan days were pooled to limit the presence of zeros and treatment, weight and an interaction between the two were specified as covariates. Due to the low frequency at which these two activities were expressed, treatment effects were also investigated using the focal data to complement the analyses of the scan data. The daily focal total duration of Food Carrying was log transformed and specified as a response variable in a Gaussian LMM where subjects' weight, treatment, and their interactions were specified as covariates. Nine out of 141 observations were excluded from the analyses as they returned a zero. The duration of Nest Building over the entire week of treatment was log transformed and specified as response variable in a Gaussian LMM in which treatment, weight, and an interaction between the two were specified as covariates. In both LMMs, the daily (Food Carrying) and weekly (Nest Building) total focal observation duration was fitted as independent covariate in their respective models, to control for effects due to variation in observation duration.

Finally, I questioned whether CORT treatment could be costly by determining whether treatment affected subjects' weight change over each treatment week. Weight difference between day 1 and day 8 of each week, was specified as response variable with treatment being as a covariate. To control for regression to the mean effects (Kelly and Price, 2005), subjects' weight on day 1 was fitted as an independent covariate.

4.3 Results

Treatment effects on endocrine values

Cortisol treatment caused a 3-fold increase in urinary CORT concentrations (n=28 samples, mean=10.63 ng/dl, SD=9.15 ng/dl), in comparison to concentrations measured during the control week (n=28 samples, mean=3.16 ng/dl, SD=2.35 ng/dl). The effect of the cortisol treatment on urinary CORT levels was significant and there was a trend for significance on an interaction between treatment and treatment day (Table 4.13). The latter was caused by the decrease in CORT concentrations from day 3 to day 6 during the cortisol treatment, but not in the control treatment. This suggests that more CORT was released from the implant at the beginning of the treatment and/or subjects' endogenous CORT secretions decreased as the treatment progressed (Figure 4.10).

A visual inspection of CORT concentrations showed that the CORT levels induced during the cortisol treatment fell within the physiological range of those measured during the control treatment and in non-experimental adult female helpers (Figure 4.11). CORT levels during the cortisol treatment were significantly higher than in female helpers experiencing a stable social environment (baseline), but not significantly different than in evicted female helpers (Table 4.14).

Testosterone concentrations were significantly decreased during the cortisol treatment (LMM; treatment effect: estimate=-1.110, SE=0.390, χ^2=7.190, p=0.007). This effect remained after the removal of an individual data returning an unusually high T concentration during the control treatment (LMM; treatment effect: Estimate=-0.903, SE= 0.358, χ^2=5.472, p=0.019).

Treatment effects on behaviour and body-condition

The cortisol treatment significantly increased the expression of Total Cooperation by more than one half (Table 4.15; Figure 4.12). This effect was maintained when the higher CORT levels were removed from the dataset: Total Cooperation was still significantly higher after the exclusion of all the data collected on day 2 of the cortisol treatment (beta binomial GLMM: treatment effect: estimate=0.493; SE=0.221; χ^2=4.530; p=0.033), and nearly

significantly increased after the exclusion of all the data associated with CORT levels higher than the cortisol treatment median (beta binomial GLMM: treatment effect: estimate=0.450; SE=0.224; χ^2=3.824; p=0.051).

Changes in levels of Total Cooperation were independent on the magnitude of the changes in CORT levels between treatments. Neither the absolute nor the relative increase in CORT levels were correlated to the difference in Total Cooperation between the cortisol and the control treatments (Table 4.16; Figure 4.13). Also, treatment effect on Total Cooperation was more pronounced in subjects that cooperated less in the control treatment: the changes in Total Cooperation between treatments were significantly and negatively correlated with Total Cooperation expressed during the control treatment (Table 4.16).

The upregulation of individual cooperative contributions arose as a combination of a general effect of treatment on individual activity and an upregulation of cooperative contributions during activity bouts. There was a trend for the cortisol treatment to increase the expression of non-cooperative behaviours (Table 4.17). Together with the increase of Total Cooperation by the cortisol treatment, this translated into a significant and positive effect on individual activity (Table 4.18). Because CORT induced increases of Total Cooperation were more pronounced than Non-cooperation, the proportion of Total Cooperation expressed during activity bouts was significantly increased during the cortisol treatment (Table 4.19; Figure 4.14).

The cortisol treatment induced increase in individual cooperative contributions was the consequence of a significant elevation of the expression of Burrowing (Table 4.20; Figure 4.15). Cortisol treatment also caused a significant increase in Food Carrying (Binomial GLMM: treatment effect: estimate=0.354, SE=0.171, χ^2=4.240, p=0.040), but this effect became highly insignificant after the removal of a single outlier (Binomial GLMM: treatment effect: estimate=0.122, SE=0.184, χ^2=0.428, p=0.513; Figure 4.16). Treatment did not affect Nest Building (OLRE binomial GLMM: treatment effect: estimate=0.217, SE=0.372, χ^2=0.342, p=0.559; Figure 4.17). The absence of treatment effect on both Food Carrying and Nest Building was supported by the analyses of focal data (Gaussian LMMs: Food Carrying: treatment effect: estimate=-0.084, SE=0.169, χ^2=0.243, p=0.622; Nest Building: treatment effect: estimate=-0.270, SE=0.314, χ^2=0.776, p=0.379).

Despite the large increase in cooperative contributions and activity, treatment did not affect subjects' weight changes (LMM; treatment effect: estimate=1.214, SE=0.928, χ^2=1.727, p=0.189).

Table 4.13 - Predictors of CORT levels on the first step of the treatment validation process. The response variable was ln-transformed and the data analysed in LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**0.859**	**0.146**	**5.886**	
Treatment	**1.189**	**0.182**	**6.548**	**<0.001**
Day	-0.203	0.181	-1.120	0.252
Weight [a]	-0.029	0.120	-0.242	0.788
Urination delay	-0.027	0.098	-0.277	0.846
Treatment x Day	-0.635	0.370	-1.718	0.072
Weight [a] x Day	0.075	0.186	0.401	0.672
Treatment x Weight [a]	0.028	0.189	0.146	0.876
Treatment x Weight [a] x Day	0.219	0.381	0.573	0.531

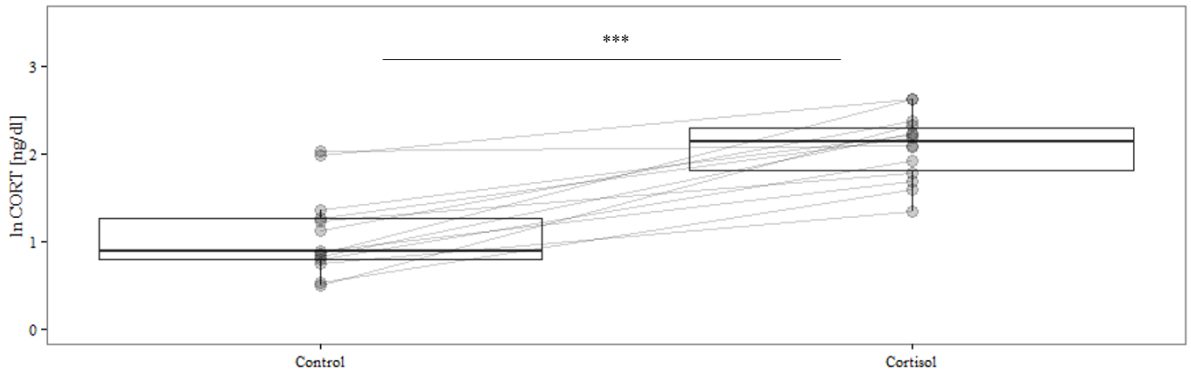

Figure 4.10 - Effect of the cortisol manipulation experiment on CORT levels measured during the control (left) and cortisol (right) treatments. CORT values have been ln transformed. Lines between points illustrate the repeated measurements of same individuals. ***: p<0.001).

Table 4.14 - Predictors of CORT levels on the second step of the treatment validation process. The response variable was ln-transformed and the data analysed in LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled. [b] indicates p-values returned by the lmerTest package.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**1.834**	**0.166**	**11.025**	
Condition				**< 0.001**
- Cortisol	**0.000**	**0.000**	**0.000**	
- Baseline	**-0.961**	**0.189**	**-5.099**	**< 0.001** [b]
- Eviction	**-0.370**	**0.234**	**-1.580**	**0.190** [b]
Urination delay	0.005	0.002	2.047	0.038
Weight [a]	0.091	0.084	1.084	0.262

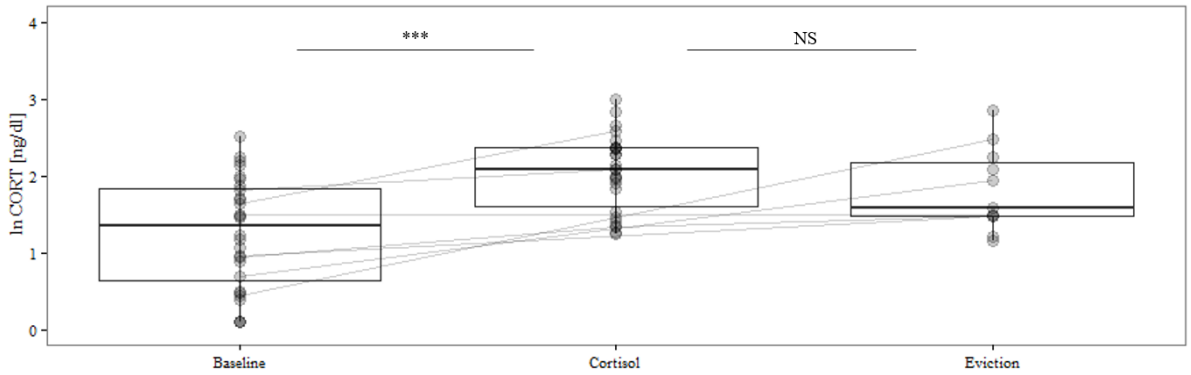

Figure 4.11 – Comparison of the CORT levels measured during the cortisol treatment (middle) with those measured in two distinct social situations: baseline (left) and eviction (right). CORT values have been ln transformed. NS: p>0.05 (non-significant); ***: p<0.001.

Table 4.15 – Predictors of Total Cooperation during the cortisol manipulation experiment. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-2.155**	**0.189**	**-11.400**	
Treatment	**0.576**	**0.170**	**3.400**	**0.001**
Day	-0.219	0.162	-1.350	0.184
Weight [a]	-0.132	0.151	-0.870	0.400
Treatment x Weight [a]	0.234	0.172	0.136	0.173
Treatment x Day	0.252	0.324	0.780	0.437

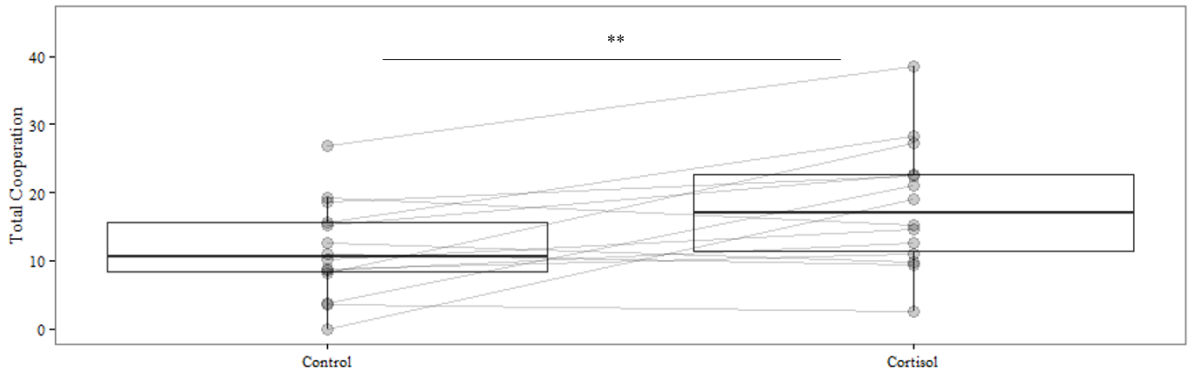

Figure 4.12 - Effect of the cortisol manipulation experiment on individual Total Cooperation, expressed as the percentage of scan displayed during the 12 hours scan sessions (averaged over day 2 and day 5) of the control (left) and the cortisol (right) treatments. Lines between points illustrate the repeated measurements of same individuals. **: p=0.001).

Table 4.16 – Predictors of differences in Total Cooperation between the cortisol and the control treatments, during the cortisol manipulation experiment. The data were analysed in LMM. All variables shown in bold were retained in the minimal model.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**19.030**	**5.274**	**3.608**	
Total Cooperation control week	**-0.421**	**0.147**	**-2.877**	**0.008**
Relative CORT difference	0.059	0.178	0.333	0.740
Absolute CORT difference	-0.118	0.227	-0.521	0.593

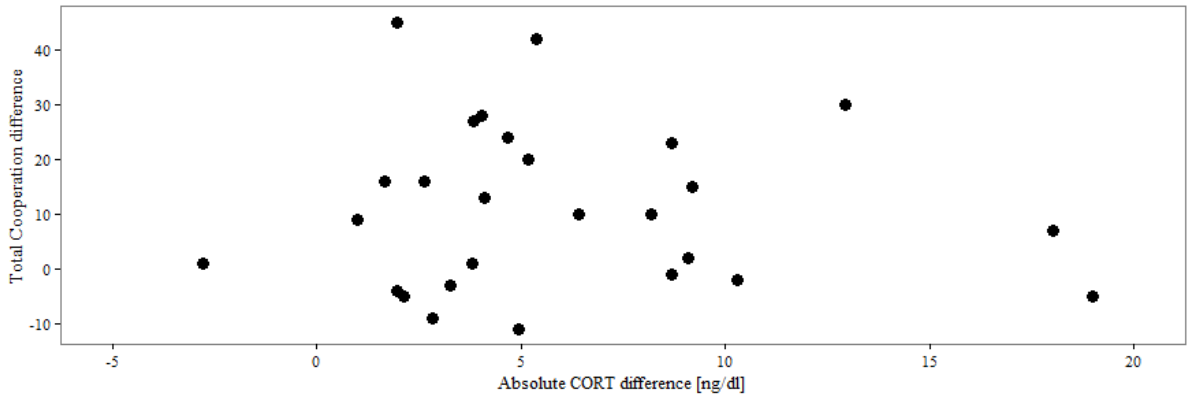

Figure 4.13 - Correlation between changes in Total Cooperation and absolute changes in CORT levels during the cortisol manipulation experiment. Changes in Total cooperation are given as the difference in the number of scans between the cortisol and the control treatment. Absolute changes in CORT levels correspond to the difference in urinary CORT levels between the cortisol and the control treatment. Data points are not all independent from one another as 2 differences are displayed from each individual (day 2 and day 5).

Table 4.17 – Predictors of Non-cooperation during the cortisol manipulation experiment. Data were analysed in beta-binomial GLMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-1.340**	**0.118**	**-11.360**	
Treatment	0.160	0.088	1.810	0.076
Weight [a]	-0.058	0.090	-0.640	0.516
Day	-0.039	0.088	-0.440	0.660
Treatment x Day	-0.242	0.172	-1.410	0.164
Treatment x Weight [a]	0.113	0.086	1.310	0.194

Table 4.18 – Predictors of Activity during the cortisol manipulation experiment. Data were analysed in beta-binomial GLMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-0.649**	**0.179**	**-3.630**	
Treatment	**0.433**	**0.126**	**3.440**	**0.001**
Day	-0.110	0.124	-0.890	0.377
Weight [a]	-0.115	0.146	-0.790	0.436
Treatment x Weight [a]	0.169	0.126	1.350	0.181
Treatment x Day	-0.083	0.245	-0.340	0.733

Table 4.19 – Predictors of the ratio of Total Cooperation expressed during activity bouts, during the cortisol manipulation experiment. Data were analysed in beta-binomial GLMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-0.779**	**0.144**	**-5.420**	
Treatment	**0.410**	**0.135**	**3.040**	**0.003**
Day	-0.191	0.130	-1.470	0.146
Weight [a]	-0.143	0.114	-1.250	0.229
Treatment x Day	0.418	0.252	1.660	0.106
Treatment x Weight [a]	0.128	0.129	0.990	0.318

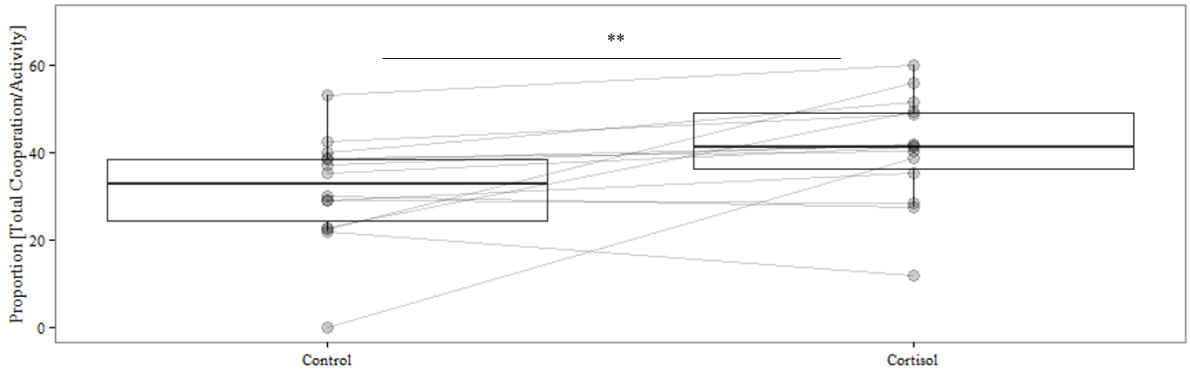

Figure 4.14 – Effect of the cortisol manipulation experiment on the proportion of Total cooperation expressed during activity bouts, in the control (left) and cortisol (right) treatments. Lines between points illustrate the repeated measurements of same individuals. **: $p<0.01$.

Table 4.20 – Predictors of Burrowing expressed during activity bouts, during the cortisol manipulation experiment. Data were analysed in beta-binomial GLMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-2.315**	**0.202**	**-11.460**	
Treatment	**0.638**	**0.184**	**3.470**	**<0.001**
Day	-0.200	0.176	-1.140	0.260
Weight [a]	-0.167	0.156	-1.070	0.301
Treatment x Weight [a]	0.245	0.189	1.300	0.191
Treatment x Day	0.198	0.356	0.56	0.578

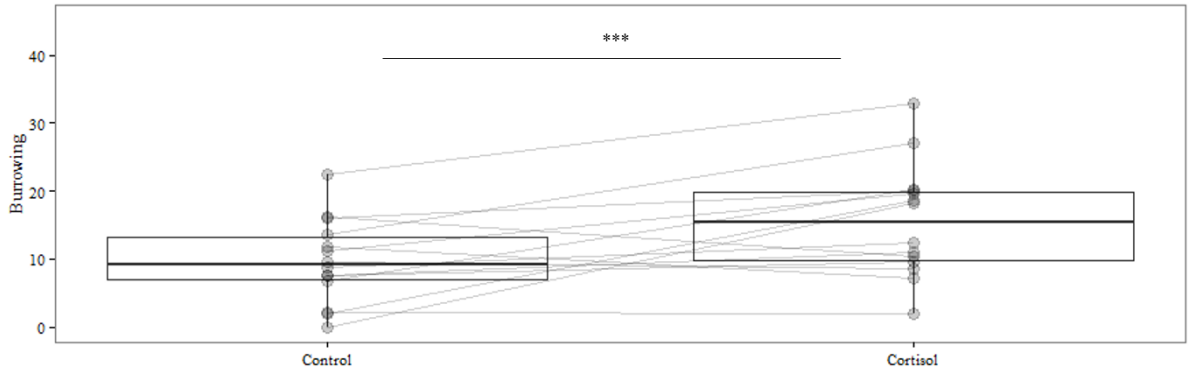

Figure 4.15 - Effect of the cortisol manipulation experiment on Burrowing during the control (left) and cortisol (right) treatments. Lines between points illustrate the repeated measurements of same individuals. ***: $p<0.001$.

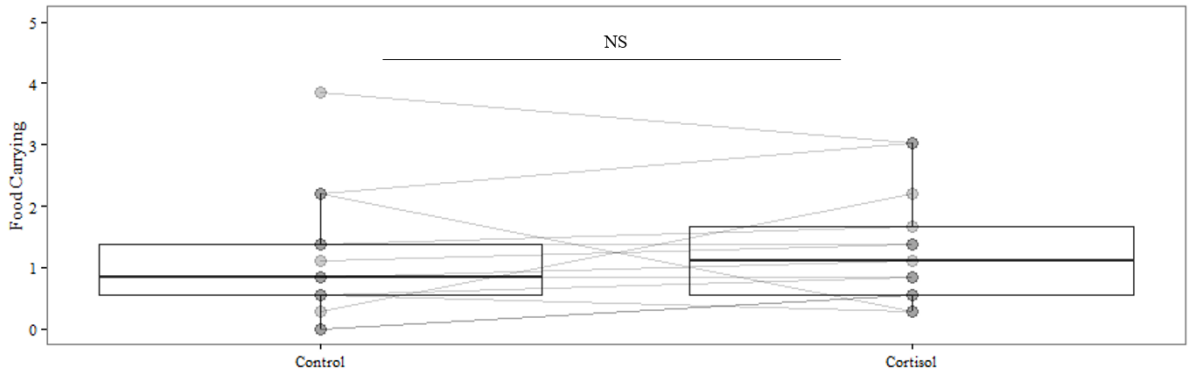

Figure 4.16 – Effect of the cortisol manipulation experiment on Food Carrying (after exclusion of an outlier) during the control (left) and cortisol (right) treatments. Lines between points illustrate the repeated measurements of same individuals. NS: $p>0.05$ (non-significant).

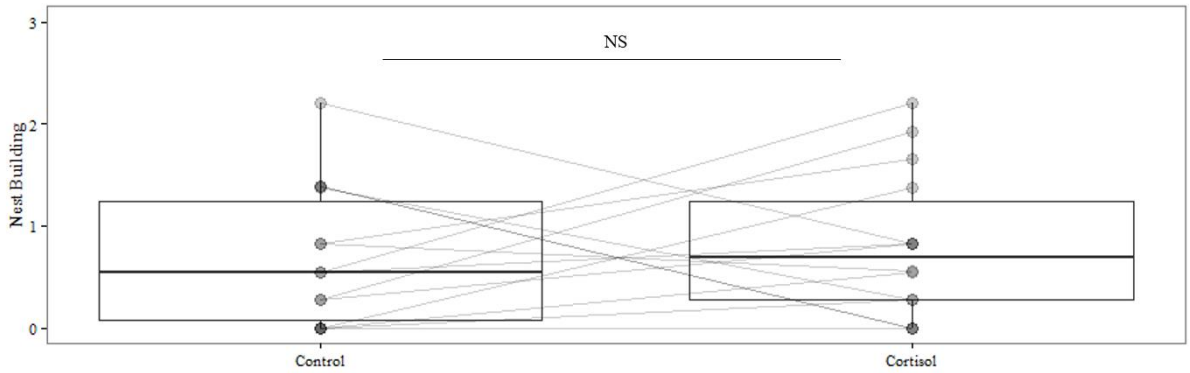

Figure 4.17 - Effect of the cortisol manipulation experiment on Nest Building during the control (left) and cortisol (right) treatments. Lines between points illustrate the repeated measurements of same individuals. NS: $p>0.05$ (non-significant).

4.4 Discussion

The results presented here offer the first experimental demonstration that GC can increase cooperative contributions in cooperative breeders. Experimentally induced increases in CORT led female helpers to increase their cooperative contributions by more than one half, from to 11 to 18% of their time budget. This increase was not a mere consequence of CORT induced increase in helpers' general activity since the ratio between cooperative and non-cooperative behaviours was increased during activity bouts. Hence, CORT induced increases in cooperative contributions arose via a combination of general effects on individual activity and specific effects on cooperative behaviours during activity bouts.

My results suggest that CORT effects on cooperative behaviours were permissive, in the sense that CORT administration, rather than the amplitude of the CORT increases, facilitated their expression. The downregulation in T secretions associated with the cortisol treatment should be interpreted with caution due to low T concentrations measured and their associated high coefficients of variation. But it is possible that the modulation of cooperative contributions may have occurred through reduction of T. The effects of variation in T on cooperative behaviours were further investigated in Chapter 5.

There was no indication that female helpers suffered an energetic cost from increases in cooperative contributions after being treated with CORT, possibly as a result of the *ad libitum* food regime under which the experimental colonies were maintained. Helpers' energetic demands were most probably increased during the cortisol treatment, but available food resources must have been sufficient to maintain a positive energy balance and prevent weight loss. This pattern is in agreement with evidence that in other cooperative breeders more generous helpers do not lose weight when the energetic constraints acting upon cooperative actions are relieved by supplementary feeding (Canestrari et al., 2008; Clutton-Brock et al., 2002, 2001a, 2000), but do so when resources are limited (Russell et al., 2003; Sanderson et al., 2014)

I am confident that the effects of CORT on cooperative contributions reported here are meaningful as the physiological validity of the cortisol treatment was supported by two lines of evidence. First, CORT concentrations during the cortisol treatment overlapped with the concentrations measured during the control treatment and in non-experimental female helpers

of similar weight range. Second, CORT concentrations measured during the cortisol treatment did not significantly differ from concentrations measured in females that had just been evicted from their colonies. The latter suggests that the cortisol treatment simulated CORT concentrations typically encountered in threatening situations such as eviction, which was shown to cause an increase CORT in female meerkats (Young et al., 2006). CORT induced increases in individual cooperative contributions were maintained even after the exclusion of data associated with higher CORT levels, suggesting that CORT effects on cooperative contributions may not be restricted to stress related CORT concentrations. This is relevant since cooperative activities in captive Damaraland mole-rats are expressed as part of the daily-life routine, in the apparent absence of stimuli that may cause CORT to rise above baseline concentrations.

The finding that increased CORT increases female helpers' cooperative contributions contrasts with studies of meerkats, where experimentally induced CORT increase in male helpers failed to modulate cooperative contributions, measured in term of sentinel duty and pup feeding (Santema et al., 2013). Pup feeding is energetically costly (Russell et al., 2003; Sanderson et al., 2014), and it was anticipated that CORT increases pup feeding due to its positive effect on energy production. A possible explanation to the lack of CORT effect on pup feeding is that the short-term observation schedule applied by the authors may have prevented the detection of slower acting genomic effects of CORT on behaviours. It is also possible that the experimentally induced CORT may have fallen above the range of concentrations under which pup-feeding and sentinel behaviours are usually expressed, thereby hindering ecologically relevant conclusions (Crossin et al., 2016). Alternatively, the physiological control mechanisms of distinct forms of cooperative behaviours in meerkat (alloparental care) and in Damaraland mole-rat (contribution to a common goods), although both energetically demanding, may not be shared. More detailed analyses of this chapter's data revealed that this could occur across cooperative activities displayed by Damaraland mole-rats, as the cortisol treatment caused an increase in burrowing but not in food carrying nor nest building activities. Considering the multiple forms of cooperative behaviours displayed in nature, a major challenge will be to identify and explain why some forms of cooperative activities are under the control of similar versus different physiological mechanisms.

My results suggest that it is possible that breeding females may strategically adjust helpers GC levels to increase their cooperative contributions. This suggestion was originally proposed in naked mole-rats more than twenty years ago (Reeve, 1992) but has received little

support since then (Jacobs and Jarvis, 1996; Santema and Clutton-Brock, 2012). Because CORT secretions are increased in response to received aggression and social stress (Goymann and Wingfield, 2004; Romero and Butler, 2007; Wittig et al., 2015) and increase helpers' cooperative contributions (this study), CORT may represent a general physiological mechanism through which cooperative contributions of helpers could be controlled. Using experimental manipulation of breeders' aggression and of helpers' cooperative contributions, I aim to test this possibility in the Damaraland mole-rats. Considering the potential benefit breeders may gain from increased helpers' cooperative contributions (Creel and Creel, 2002; Russell et al., 2007; Young et al., 2015), the mechanisms which may have prevented such breeders' strategy to evolve should be identified.

Chapter 5

Effects of testosterone on cooperative and social behaviour in subordinate females

ABSTRACT

In cooperative breeders, individual differences in breeding opportunities may affect cooperative contributions. Such contributions could compromise the future reproductive success of an individual; hence, as they become more likely to breed, non-breeding female helpers decrease their contributions to cooperative activities. The hormonal mechanisms controlling this life history trade-off between current cooperative contributions and future direct fitness are unknown but testosterone represents a prominent candidate. Indeed, while increased T levels usually support individual reproductive effort by promoting dominance and aggression, which may be essential attributes for helpers to secure breeding opportunities, it has also been suggested to decrease the expression of cooperative behaviours. I investigated the effects of changes in testosterone levels on cooperative behaviour, aggression and dispersal tendencies in captive Damaraland mole-rats by experimentally increasing T levels in female helpers. My results show that experimental increases in T levels had no measurable effect on any of the parameters measured, thereby offering no support that T mediates a trade-off between helpers' current cooperation and future reproduction. The apparent behavioural insensitivity of female helpers to T raises questions on the behavioural consequences of the differences in T levels observed between breeders and non-breeders in the Damaraland mole-rat as well as other cooperative breeding species.

5.1 Introduction

Large individual differences in cooperative contributions are characteristic of animal societies where individuals behave to the benefit of others (Clutton-Brock et al., 2001b, 2000; Field et al., 2006; Robinson, 1992; Zöttl et al., 2016b). Research on cooperative breeders has shown that helpers, which support breeders' reproductive effort (Clutton-Brock, 2016; Koenig and Dickinson, 2016), adjust their cooperative contributions in relation to their own breeding opportunities (Bergmüller et al., 2005a; Field et al., 2006; Young et al., 2005). This may be because helpers differentially solve a trade-off between current cooperation and future reproduction as a function of their proximity to breeding (Cant, 2005; Cant and Field, 2001). Helpers that are closer to breeding often decrease their cooperative contributions (Bergmüller et al., 2005a; Clutton-Brock et al., 2002; Gilchrist and Russell, 2007; Young et al., 2005; Zöttl et al., 2013), an explanation being that their future breeding may be more profoundly affected by the short term energetic costs of cooperative behaviours (Bell, 2010). Such costs may compromise extra-territorial forays, dispersal and competitive abilities which are essential traits for helpers to secure breeding (Cant et al., 2006; Clarke and Faulkes, 1997; Clutton-Brock et al., 2006; Cooney and Bennett, 2000; Young et al., 2007).

The physiological mediator of a trade-off between current cooperative behaviours and future direct reproduction have not been investigated but the sex hormone testosterone (T) represents a prominent candidate (Hau, 2007). In cooperative breeders, competition over breeding opportunities can be particularly intense and there is suggestive evidence that increased T supports breeding. Breeders commonly have higher T levels than same-sex helpers and these differences have been interpreted as a physiological explanation to their dominance and heightened aggression (Clutton-Brock et al., 2006; Creel et al., 1997; Davies et al., 2016; Desjardins et al., 2008; Schoech et al., 2004), ultimately allowing them to largely monopolize reproduction. A positive effect of T on aggression may also be beneficial for helpers seeking reproduction within (Clarke and Faulkes, 1997) or away from their group (Young, 2003). In males, the latter could be further facilitated by a potential positive effect of increased T levels on extra-territorial foray (Young et al., 2005) and dispersal (Raynaud and Schradin, 2014). Increased T levels have also been suggested to decrease alloparental care in several species of birds and mammals (Clark and Galef, 1999; Schoech et al., 2004; Vleck and Brown, 1999; Young et al., 2005), paralleling the correlative and experimental evidence of T effect on

parental care (Hirschenhauser and Oliveira, 2006; but see: Lynn, 2008; Peters et al., 2002; Rilling, 2013; Rosvall, 2013; Wingfield et al., 1990). Due to the correlational nature of most of the studies conducted so far, the role of T in the regulation of cooperative and aggressive behaviours in cooperative breeders remains elusive. It remains unknown whether increased T could support helpers' transitioning to breeding and whether this may cause a simultaneous increase in aggressive and decrease in cooperative behaviours.

I investigated whether experimental changes in T affected female helpers' aggression, dispersal and cooperative behaviours in captive Damaraland mole-rat (*Fukomys damarensis*). In this species, reproduction is monopolized by a single reproductive female (Burland et al., 2004; Young et al., 2010) and cooperative behaviours mostly consist of energetically demanding burrowing activities (Lovegrove, 1989), to locate food sources, as well as building and maintaining a communal nest and food stores (Jarvis et al., 1998; Zöttl et al., 2016b). The reproductive axis of female helpers is physiologically suppressed in the presence of the breeding female (Bennett, 1994; Bennett et al., 1993; Molteno and Bennett, 2000). However, there are indications that this suppression is relieved in the presence, or possibly in anticipation, of breeding opportunities (Cooney and Bennett, 2000; Young et al., 2010), arising as a result of male immigration or female emigration into a foreign group (Hazell et al., 2000b; Jacobs et al., 1998; Young et al., 2010). Also, the outcome of the intense conflicts triggered by such opportunities (Cooney and Bennett, 2000) could be mediated by T since increased T levels have been suggested to support female competitive abilities (Lutermann et al., 2013).

I determined the behavioural effects of T increases on female helpers following the steps leading to their emigration into a foreign colony. Female helpers were observed in their original colony, where they could interact with all other group members, and in standardized behavioural tests, to examine traits that could not be assessed in the usual conditions of captivity. I tested the effects of T on aggressive interactions with the breeding dominant female by enforcing physical proximity between them. I measured the effects of T on behavioural and physiological indices likely to be related to dispersal in a novel environment test (NET) and in a social isolation test (SIT). Finally, I determined T effects on the willingness to engage into aggressive interactions with a foreign breeding female in a test simulating their emigration into a foreign group. I predicted that experimental increases in T levels would increase female helpers' levels of aggression, support dispersal behavioural indices and decrease the physiological stress response to social isolation, and lower cooperative contributions.

5.2 Methods

5.2.1 Colony maintenance and husbandry

For details of colony maintenance and general animal husbandry refer to Chapter 2.

5.2.2 Experimental design

To investigate whether T increases aggressive, dominance and dispersal tendencies but reduces cooperative contributions in Damaraland mole-rats, I evaluated the behavioural and endocrine effects of short term experimental increases of T on captive female helpers. Experimental subjects were treated with intramuscular injections of control (0.1 ml of castor oil), low dose (T_{low}: 0.04 mg of T propionate in 0.1 ml of castor oil) and high dose (T_{high}: 0.1 mg of T propionate in 0.1 ml of castor oil) of testosterone. The T_{low} treatment aimed to elevate female helper's T concentration around the highest T levels measured in wild female helpers while the T_{high} aimed to elevate T within the range of wild breeding females (Lutermann et al., 2013). Seven pairs of adult female helpers living in six originally wild-caught colonies (group-size range: 7-16 individuals, mean=12.14 individuals, SD=2.85 individuals) were used for this experiment.

Behavioural effects of the treatments were assessed with focal observations (Chapter 2; Table 2.3). I also used a set of four tests to examine the effects of T on traits that could not be assessed in the usual conditions of captivity: i) a novel environment test (NET); ii) a social isolation test (SIT); iii) an encounter with a familiar breeding female test and iv) an encounter with a foreign breeding female test.

Given the high number of tests, the experiment was divided in two parts, separated by at least 7 days. All behavioural observations were carried out by a total of three observers.

On the first part of the experiment, both females from each experimental pair (weight range: 108-173 g, mean=138.8 g) were subjected to the control, the T_{low} and the T_{high} treatments. For each subject, fourteen days were allowed between each of the 3 treatments. There were 6 possible different sequences of treatments that allow the subjects within a pair to

never receive the same treatment simultaneously. Each sequence was randomly assigned to 6 pairs and one of the sequences was randomly selected and repeated on the 7th experimental pair. Three days before the start of the experiment a urine sample was collected from each subject. On the first day of each treatment (day 1), both subjects were anaesthetized, treated, weighed and placed back in their original tunnel system at around 13:00. Twenty minutes were allowed before focal observations of subjects in their group started and these were interrupted 6 hours later. On day 2, focal observations were resumed at 7:00 and were terminated 6 hours later, leading to a total observation effort of 12 hours and an average total time of nearly 4 hours per subject, per treatment (mean=226.40 min, SD=88.06 min). A blood sample was collected immediately after the end of the focal observations to determine treatment effects on plasmatic T concentration. During the afternoon of day 2, each subject was individually tested in the NET (see test description below), allowing at least 3 hours after the blood sample. In the evening of day 2, at around 19:00, subjects were individually isolated from their colony (SIT; see test description below). A urine sample was collected on the following morning (day 3), 12 hours after the start of the social isolation.

On the second part of the experiment, subjects from each experimental pair (weight range: 105-178 g, mean=137.8 g) received only the control and the T_{high} treatments. This time subjects were treated three days apart from each other to avoid the repeated daily testing of the familiar breeding female. Within subjects seven days were allowed between treatments. Five of the pairs were tested once and 2 pairs were tested twice (5 months apart). On day 1, treatment was administered as described on the first part of the experiment. At around 08:00 of day 2 the experimental females were exposed to a familiar breeding female test (see test description below) and then returned to their original colony. At around 12:00 of day 2 the experimental females were exposed to a foreign breeding female test (see test description below). Twenty minutes after the end of this last test a blood sample was collected.

The novel environment test (NET)

The NET setup consisted of a plastic box (length=75 cm, width=44 cm, height=40 cm) divided in 3 parallel visible areas of equal surface (Numbers 2, 5 and 8 in Figure 5.18), each containing two parallel 20 cm PVC pipes (Numbers 3, 4, 6, 7, 9 and 10 in Figure 5.18). The plastic box contained an opening in one of its corners that enabled the connection of a PVC pipe on the outer side of the box. Active subjects were transferred to the NET setup by gently

disconnecting a targeted part of their tunnel system while they were inside of it, referred to as the familiar area (Number 1 in Figure 5.18), and connecting it to the plastic box. A 10 min focal observation was carried out to continuously monitor the location of the subject in one of the 10 distinct areas of the box. As soon as the observation was terminated, the tested subjects were replaced in their original colony together with the familiar area.

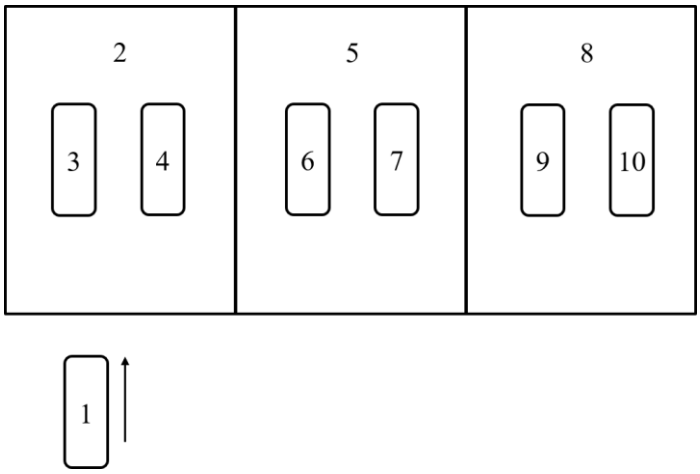

Figure 5.18 - The novel environment test (NET) setup. Refer to the description on the text for more details. Image represents a view from the top of the box.

The social isolation test (SIT)

Subjects were individually isolated from the rest of their colony in an open and clean plastic box containing a thin layer of fresh sand, food and some paper serving nest material.

The familiar breeding female test

Each experimental subject and the breeding female belonging to the same colony were placed in a transparent plastic box attached to the tunnel system in a U shape, referred to as the arena. The arena enabled the isolation of specific group members from the rest of the colony

into a confined area by operating removable gates through the tunnels. At the introduction of the breeding female into the arena, a 10 minute focal observation (Chapter 2; Table 2.3) of the experimental subject was conducted, after which the gates were removed and both animals could freely access the rest of their tunnel system.

The foreign breeding female test

Each experimental subject was placed in the arena of a foreign colony, where it was exposed to that colony's foreign breeding female. Subjects were exposed to a matched-size foreign breeding female that remained consistent across the two treatments (n=13 distinct resident breeding females). Differently to the encounters with the familiar breeding female, the arena was physically divided in two equal parts by a metal grid to avoid physical injuries. Although the grid prevented the expression of some social behaviours, the opponents frequently sniffed each other and sparred through the grid. Also, the size of the arena was sufficiently large which allowed both the animals to stay away from the grid and avoid interactions. Once the resident breeding female was introduced into the arena, on the opposite side of the grid to the subject, a 10 minutes focal observation was started. The foreign breeding female and the grid were then removed from the arena allowing the subject to freely patrol the arena for 3 minutes, after which it was placed back in its original colony.

5.2.3 Treatment Validation

Treatment validation followed a two-step process. First, I determined whether treatment successfully increased T levels of the experimental subjects using: a) the plasma samples collected after focal observations; and b) the urine samples collected before the experiment and after the SIT. As a second step, I assessed the physiological realism of the treatments by comparing the T levels they induced with: c) the plasma samples collected following a social conflict (the foreign breeding female test) of control treatments; d) plasma samples collected after an injection of gonadotropin-releasing hormone (GnRH; see description below), and e) plasma and urine samples of breeding females.

Samples collected following a social conflict and a GnRH injection enabled comparisons with T levels predicted to reflect subjects' ability to secrete T following,

respectively, a natural and an exogenous stimulation of T release. Samples from breeding females were used to determine whether treatment fulfilled the initial aim of inducing T levels that were similar to those of breeding females.

GnRH injections

Five of the seven experimental pairs of helpers were administered with GnRH. Each pair was tested once at the exception of one pair which was tested twice. The injection of GnRH occurred at least 7 days after the termination of the first part of the experiment and 7 days before the beginning of the second.

Between 12:00 and 14:00, both subjects of a pair were successively removed from their tunnel system, anaesthetized and injected subcutaneously with 200 µl of a saline solution containing 2 µg of GnRH. Such treatment had previously been shown to successfully raise the secretion of luteinizing hormone (LH), which stimulates T release, in female Damaraland mole-rat (Bennett et al., 1993; Young et al., 2010). Following GnRH injection, the subjects were placed in a covered plastic box containing a thin layer of sand and provided with food and nest material. Forty minutes after injection, subjects were anaesthetized again and blood sampled following the procedures detailed in Chapter 2.

Plasma and urine samples of breeding females

Five plasma samples and 246 urine samples from breeding females, most of which were collected during pregnancy, were collected following the procedures detailed in Chapter 2.

5.2.4 Hormone analyses

All plasma and urine samples collected were analysed at the Neuchatel Platform of Analytical Chemistry (NPAC) using ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). For the data presented in this chapter, I used T concentrations from plasma samples and CORT and T concentrations from urine samples. Details of the analytical procedures are presented in Chapter 2.

5.2.5 Data handling and statistical analyses

I used a mixed modelling approach to account for the dependency structure in the data caused by the repeated measurements of individuals and, unless stated otherwise, I specified individual and colony identity as nested random effects. For model simplification and validation, I followed the steps described in Chapter 2. Generalized linear hypotheses testing conducted with the glht function (p-values adjusted for multiple comparisons by the single-step method) from the multcomp package, were used for post-hoc pairwise comparisons of means between levels of categorical covariates (Hothorn et al., 2008).

Treatment validation

All analyses were conducted with LMMs in which T levels were ln-transformed and specified as a response variable.

To determine whether the exogenous administration of T successfully increased T concentrations, I used the plasma T levels determined after the focal observations as response variable. I specified weight, treatment (control, T_{low}, T_{high}) and an interaction between the two as covariates.

In a similar model, the urinary T levels measured before the experiment (baseline) and after the SIT (control, T_{low}, T_{high}) were specified as a response variable. A four levels categorical covariate (condition: baseline, control, T_{low}, T_{high}) was specified. The delay to urination was specified as an additional independent covariate. Data of a single individual that returned several outliers were removed from the analyses, which led to a total of 13 baseline, 12 control, 12 T_{low} and 13 T_{high} data points, after accounting for missing samples.

For the comparisons of plasma T levels induced by treatment with the T levels of breeding females and of subjects which T secretions had been stimulated, the data from the T_{high} were excluded from the analyses. This exclusion relied on data exploration showing that T levels in the T_{low} treatment were already substantially higher than in all other conditions and aimed to limit the number of post-hoc pairwise comparisons. To test the effect of sampling condition and to control for the effect of weight differences between subjects and breeding female on T levels, condition (control=14 samples, T_{low}=14 samples, GnRH=11 samples, social

conflict=10 samples, breeding female=5 samples) and individual weight were specified as two independent covariates.

The differences between urinary T levels of experimental subjects and breeding females were investigated using a similar model at the difference that condition (baseline=13 samples, T_{low}=12 samples, T_{high}=13 samples, breeding female=246 samples from 33 individuals) and urination delay were specified as covariates.

Effects of T on aggressive interactions

I used the focal data collected on the first part of the experiment to determine whether T affected agonistic interactions between the subjects and other group members. For all the analyses using the focal data, I extracted subjects' interactions with female group members collected during focal observation and excluded all interactions occurring with juveniles. I used the data from the familiar and the foreign breeding female tests to examine whether T affected agonistic interactions with the familiar and foreign breeding females. Behavioural variables represent grouped behaviours, following the procedures detailed in Chapter 2.

To investigate the effect of treatment on the expression of aggressive behaviours, I used the focal data and specified the count of Aggressive behaviours (see Chapter 2; Table 2.3) given by subjects during the 12 hours window as a response variable in a negative binomial GLMM. To control for the effect of differences in individual tendency to approach or avoid social partners and the number of available social partners on the expression of Aggressive behaviours, both the number of Passes and the number of social partners present in the group were specified as covariates. To investigate whether T changed the perception of subjects by their social partners a similar model was used, with the only difference that the count of Aggressive behaviours received by subjects was specified as a response variable in a negative binominal GLMM. Finally, the number of Aggressive behaviours given by the subject was specified as a response variable in a separate beta-binominal GLMM, where the sum of Aggressive behaviours given and received was specified as the binomial total.

Data from the familiar breeding female test was used to determine the effects of T on the aggressive behaviours directed to and received from the familiar breeding female with a non-parametric Wilcoxon signed-rank test.

Data from the foreign breeding female test was used to determine whether T affects the tendency of subjects to engage in aggressive interactions with a foreign breeding female. I first tested the effect of treatment on the duration spent sparring through the grid with the foreign breeding female. Because this measurement was dependent on the proximity of the opponent to the grid, I also tested the treatment effect on the duration subjects spent interacting aggressively with the grid (sparring and gnawing). I ln-transformed both durations and specified them as response variables in two distinct LMMs where subject and foreign breeding female identities were specified as random effects. Treatment, weight difference between the subject and the foreign breeding female and its quadratic were specified as covariates.

I used the focal data to determine whether T affected subjects' submissive behaviour to other group members and vice-versa. The sum of bouts of Submissive calls given and received by the subjects during the 12 hours observation window were fitted as response variables in two distinct negative binomial GLMMs in which the colony and subjects' identities were specified as nested random effects. To control for the effect of variation in subjects' activity and in aggressive behaviours on the response variable, both observation duration and the number of aggressive behaviours received (for the analyses of Submissive calls given) or given (for the analyses of Submissive calls given) were specified as two independent covariates.

I used the data from the familiar breeding female test to determine whether T affected subjects' submission towards the familiar breeding female with a non-parametric Wilcoxon signed-rank test. Four subjects received submissive calls from the breeding female and never gave submissive calls, suggesting that these subjects were perceived as dominant by the breeding female. Therefore, the model was repeated after the exclusion of these 4 females from the dataset.

Effects of T on traits related to dispersal

Using the data from the NET, I investigated T effects on subjects' willingness to leave a familiar environment and explore an unfamiliar area. I used the ln-transformed latency to leave the familiar area, the total time spent outside of the familiar area, and the total number of unfamiliar areas visited by the subjects as response variables in three different LMMs. An interaction between treatment (control, T_{low} and T_{high}) and subjects' weight was fitted as a covariate. To account for the possibility that subjects habituated to the NET setup throughout

the experiment, the test repetition number (1 to 3) was added as an independent covariate. The data of two tests in which subjects never left the familiar area were excluded from the analyses of the total time spent outside the familiar area.

Using the data from the SIT, I investigated whether T modulated CORT secretions in response to social isolation by specifying the ln-transformed CORT levels as a response variable in a LMM. To test the differences in CORT levels measured before the beginning of the experiment and after the SIT of each treatment, I specified a 4 levels covariate (condition: baseline, control, T_{low} and T_{high}). I controlled for the effect of subjects' weight and urination delay on CORT levels by specifying each factor as independent covariates. To account for the possibility that subjects may have habituated to SIT throughout the experiment, the repetition number (1 to 3) was fitted as an independent covariate.

Effects of T on individual cooperative contributions

For the investigation of T influences on cooperative contributions, I used the focal data collected on the first part of the experiment. Unless specified otherwise, I summed the duration of the different cooperative activities under investigation (see Chapter 2; Table 2.3) over the two days of treatment and transformed them by their natural logarithm before specifying them as response variables in LMMs. Treatment, weight and an interaction between the two were specified as covariates in all LMMs to investigate whether treatment effects would vary with subjects' weight.

I first tested whether T affected subjects' cooperative contributions relative to the time they were active. I specified Total Cooperation as a response variable and the total observation duration excluding Rest duration (i.e. time Active) as a covariate, to control for the effect of differences in activity levels on Total Cooperation. A quadratic effect for this covariate was also added since data exploration revealed a non-linear and mostly quadratic effect of duration of activity on Total Cooperation. I then investigated whether T affected subjects' absolute cooperative contributions. The model was similar to the one described above, at the only difference that the linear and quadratic covariates controlling for the effect of differences in activity on the duration of cooperative contributions were not specified as covariates.

In a similar way, I determined whether T effects could vary across distinct cooperative activities, by specifying Burrowing, Food Carrying and Nest Building durations as response variables in 3 distinct LMMs.

5.3 Results

Treatment validation

The exogenous administration of T significantly increased plasma testosterone levels, as T_{low} and T_{high} levels were on average 5 and 10 times higher than the control treatment, respectively (Table 5.21; Figure 5.18). Also, plasma T levels in the T_{high} treatment were significantly higher than in the T_{low} treatment (Table 5.21, Figure 5.19). The treatment effects on plasma T levels were significantly dependent on individual weight, as T levels increased with weight in the control treatment but less so in the T_{low} and T_{high} treatments (Table 5.21).

Testosterone levels were still increased after the SIT since urinary T levels measured after this test were significantly higher in the T_{high} treatment compared to baseline, whereas the difference was nearly significant for the T_{low} treatment (Table 5.22). Testosterone levels in the T_{high} treatment were significantly higher than in the control treatment (Table 5.22). The absence of differences in T between baseline and the control treatment suggests that social isolation has no effect on T levels, legitimating the use of T values measured after the SIT for treatment validation purposes (Table 5.22).

Plasma T levels of female helpers in the T_{low} treatment were significantly higher than: (i) those following the encounter with a foreign breeding female, (ii) those after T secretions had been stimulated by the administration of GnRH and (iii) those found in breeding females (Table 5.23; Figure 5.20). Comparisons with T levels in the control treatment otherwise showed that T levels of female helpers were significantly increased by 2.2 times after the GnRH challenge but were not increased following the encounter with a foreign breeding female (Table 5.23).

Urinary T levels of female helpers were higher in the T_{low} and T_{high} treatments compared to breeding females (Table 5.24). Finally, there was no difference between urinary T levels of female helpers before the start of the experiment (baseline) and breeders (Table 5.24).

Effects of T on aggressive behaviour

The amount of aggressive behaviours given (negative binomial GLMM; treatment effect: χ^2=0.367, p=0.834) or received (negative binomial GLMM; treatment effect: χ^2=1.338, p=0.512) by subjects when they could interact with all other group members was not affected by T. Also, the proportion of aggressive behaviours given by subjects was not affected by T (beta-binomial GLMM: treatment effect: χ^2=0.912, p=0.634).

Similar results were obtained when subjects could only interact with the familiar breeding female since both aggressive behaviours given to (Wilcoxon signed-rank test: V=26, p=0.324) and received from the familiar breeding female (Wilcoxon signed-rank test: V=41.5, p=0.305) were not affected by T.

Subjects' tendency to behave aggressively towards a foreign breeding female was not affected by T: neither the time subjects spent sparring with the breeding female (LMM: χ^2=0.062, p=0.803) nor the time they spent interacting aggressively with the grid were affected by treatment (LMM: χ^2=0.494, p=0.482).

Testosterone did not affect the number of submissive bouts given (negative binomial GLMM; treatment effect: χ^2=1.990; p=0.370) nor the number of submissive bouts received by other group members (negative binomial GLMM; treatment effect: χ^2=2.060; p=0.357). When subjects could only interact with the familiar breeding female, T had no effect on the number of submissive bouts given (Wilcoxon signed-rank test: V=28, p=0.233; after exclusion of subjects perceived as dominant by the breeding female: V=21, p=0.305).

Effects of T on traits related to dispersal

Testosterone did not affect subjects' latency to leave the familiar area (LMM; treatment effect: χ^2=0.409, p=0.815), nor the time spent outside of the familiar area (LMM; treatment effect: χ^2=2.291, p=0.318) nor the total number of unfamiliar areas visited (LMM; treatment effect: χ^2=2.348, p=0.309).

CORT levels were increased in socially isolated subjects, but CORT levels measured after the SIT were not affected by T (Table 5.25).

Effects of T on individual cooperative contributions

Testosterone had no effect on individual relative cooperative contributions when differences in subjects' activity levels were controlled for (Table 5.26, Model 1), and had no effect on the total time individuals cooperated during the 12 hours observation time window (Table 5.26, Model 2).

When investigated in isolation, neither Burrowing (LMM; treatment effect: χ^2=0.637, p=0.727), nor Food Carrying (LMM; treatment effect: χ^2=1.192, p=0.551), nor Nest Building (LMM; treatment effect: χ^2=0.500, p=0.779) were affected by treatment.

Table 5.21 – Predictors of T levels in plasma samples, used on the first step of the treatment validation process. The response variable was ln-transformed and the data analysed in LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled. All model p-values were computed with lmerTest except those labelled with [b] which were calculated with the likelihood ratio test.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-3.450**	**0.195**	**-17.659**	
Treatment				
- Control	**0.000**	**0.000**		
- T_{low}	**1.743**	**0.140**	**12.409**	**<0.001**
- T_{high}	**2.445**	**0.140**	**17.411**	**<0.001**
Weight [a]	**0.520**	**0.155**	**3.343**	**0.002**
Weight [a] x Treatment			**11.110**	**0.004 [b]**
- Control	**0.000**	**0.000**		
- T_{low}	**-0.371**	**0.146**	**-2.535**	**0.010**
- T_{high}	**-0.449**	**0.139**	**-3.234**	**0.002**
Generalized Linear Hypotheses				
$T_{low} = T_{high}$	0.209	0.038	5.452	**<0.001**

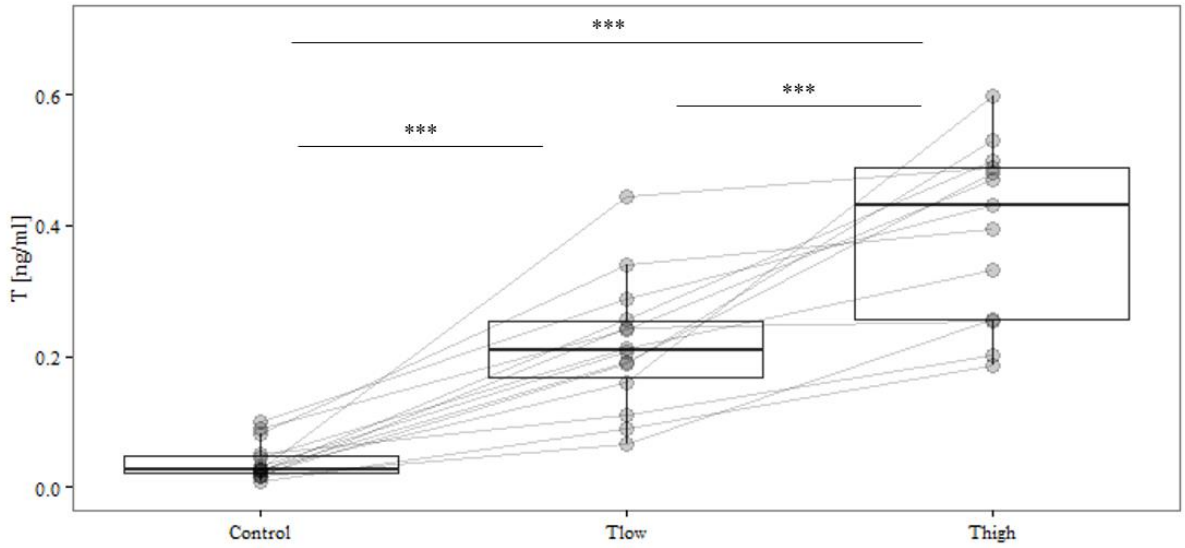

Figure 5.19 - Effect of the T manipulation experiment on T plasma levels measured during the control (left) and T_{low} (middle) and T_{high}(right) treatments. Lines between points illustrate the repeated measurements of same individuals. ***: p<0.001.

Table 5.22 - Predictors of T levels in urine samples, used on the first step of the treatment validation process. The response variable was ln-transformed and the data analysed in LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled. [b] indicates p-values computed with lmerTest.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**1.849**	**0.123**	**15.004**	
Condition			**31.349**	**<0.001**
- Baseline	**0.000**	**0.000**		
- Control	**0.144**	**0.141**	**1.019**	**0.315** [b]
- T_{low}	**0.276**	**0.141**	**1.960**	**0.058** [b]
- T_{high}	**0.863**	**8.138**	**6.276**	**<0.001** [b]
Weight [a]	-0.042	0.093	-0.452	0.504
Urination delay	0.000	0.001	-0.364	0.723
Weight [a] x Condition			1.508	0.680
Generalized Linear Hypotheses				
T_{low} = Control	0.132	0.144	0.919	0.554
T_{high} = Control	0.719	0.141	5.107	**<0.001**

Table 5.23 - Predictors of T levels in plasma samples, used on the second step of the treatment validation process. The response variable was ln-transformed and the data analysed in LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled. [b] indicates p-values computed with lmerTest.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**-1.677**	**0.172**	**-9.745**	
Condition			**61.967**	**<0.001**
- T_{low}	**0.000**	**0.000**		
- Control	**-1.752**	**0.178**	**-9.830**	**<0.001** [b]
- GnRH	**-1.240**	**0.196**	**-6.319**	**<0.001** [b]
- Social Conflict	**-1.566**	**0.197**	**-7.941**	**<0.001** [b]
- Breeding Female	**-1.345**	**0.299**	**-4.491**	**<0.001** [b]
Weight [a]	**0.326**	**0.103**	**3.164**	**0.004**
Generalized Linear Hypotheses				
Control = GnRH	-0.512	0.197	-2.604	**0.027**
Control = Social Conflict	-0.186	0.198	-0.940	0.701
Control = Breeding Female	-0.407	0.300	-1.359	0.417

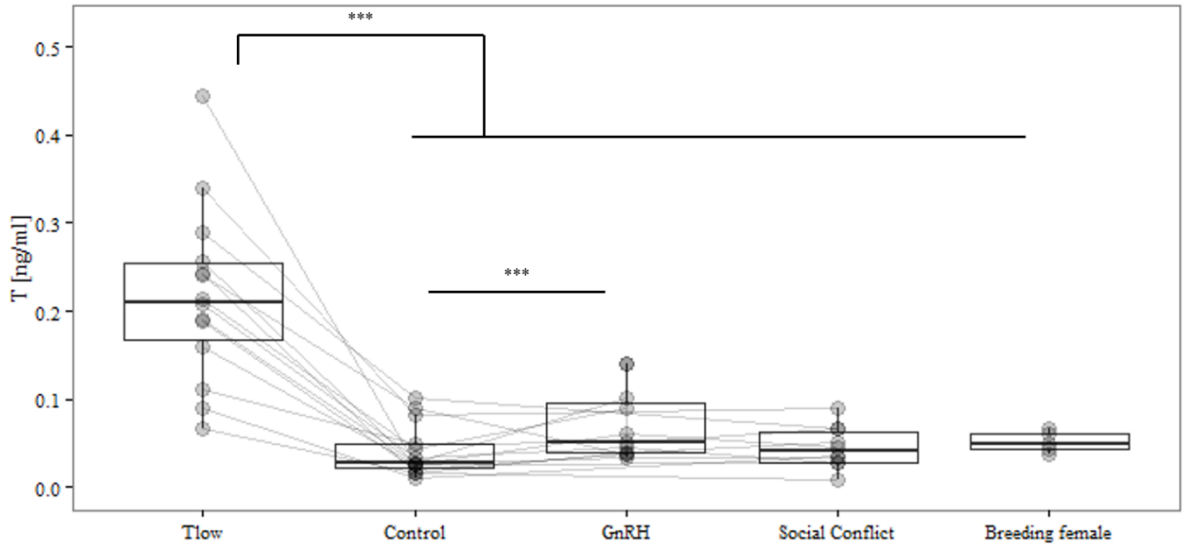

Figure 5.20 - Comparisons of plasma T levels induced by the T_{low} and Control treatments with the T levels measured after exogenous (GnRH) and natural (Social Conflict) stimulations of T release and with T levels measured in breeding females. Lines between points illustrate the repeated measurements of same individuals. ***: p<0.001.

Table 5.24 - Predictors of T levels in urine samples, used on the second step of the treatment validation process. The response variable was ln-transformed and the data analysed in LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled. [b] indicates p-values computed with lmerTest.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**1.197**	**0.148**	**8.119**	
Condition			**16.106**	**0.001**
- Breeding Female	**0.000**	**0.000**		
- Baseline	**0.492**	**0.301**	**1.599**	**0.113** [b]
- T_{low}	**7.678**	**0.316**	**2.431**	**0.017** [b]
- T_{high}	**1.241**	**0.312**	**3.976**	**<0.001** [b]
Weight [a]	**0.267**	**0.081**	**3.300**	**0.003**
Urination delay	**0.003**	**0.000**	**2.384**	**0.022**

Table 5.25 – Predictors of urinary CORT levels to the social isolation test. The response variable was ln-transformed and the data analysed in LMM. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled. All model p-values were computed with likelihood ratio test except those labelled with [b] which were calculated with the lmerTest. [b] indicates p-values computed with lmerTest.

Covariates	Estimate	SE	test statistic	p-value
Intercept	**2.852**	**0.204**	**13.957**	
Condition			**21.425**	**<0.001**
- Baseline	**0.000**	**0.000**		
- Control	**0.924**	**0.229**	**4.027**	**<0.001** [b]
- T_{low}	**1.019**	**0.229**	**4.442**	**<0.001** [b]
- T_{high}	**0.864**	**0.224**	**3.860**	**<0.001** [b]
Weight [a]	-0.201	0.134	-1.503	0.096
Urination delay	0.002	0.002	0.983	0.275
Test number	-0.019	0.078	-0.249	0.893
Generalized Linear Hypotheses				
Control = T_{low}	-0.095	0.234	-0.406	0.913
Control = T_{high}	0.059	0.229	0.258	0.964
T_{high} = T_{low}	0.154	0.229	-0.673	0.779

Table 5.26 – Predictors of Total Cooperation during the T manipulation experiment. All variables shown in bold were retained in the minimal model. [a] indicates variables centred and scaled. [b] indicates p-values computed with lmerTest.

Covariates	Estimate	SE	test statistic	p-value
Model 1. Predictors of relative Total Cooperation				
Intercept	**3.665**	**0.192**	**19.114**	
Activity duration [a]	**1.049**	**0.103**	**10.204**	**<0.001**
(Activity duration)^2 [a]	**-0.269**	**0.070**	**-3.867**	**<0.001**
Treatment			1.330	0.514
- Control	0.000	0.000		
- T_{high}	-0.177	0.166	-1.063	0.298 [b]
- T_{low}	-0.115	0.164	-0.703	0.489 [b]
Weight [a]	0.066	0.156	0.425	0.655
Treatment x Weight [a]			2.649	0.266
Model 2. Predictors of absolute Total Cooperation				
Intercept	3.379	0.408	8.277	
Treatment			1.142	0.565
- Control	0.000	0.000		
- T_{high}	0.092	0.330	0.280	0.781 [b]
- T_{low}	-0.240	0.330	-0.728	0.473 [b]
Weight [a]	-0.060	0.323	-0.196	0.916
Treatment x Weight [a]			1.336	0.513

5.4 Discussion

Research on cooperative breeders has suggested that helpers may face a life history trade-off between investments in current cooperation and future reproduction (Bergmüller et al., 2005b; Cant, 2005; Field et al., 2006; Young et al., 2005). This study offers no support to the suggestion that T mediates this trade-off: Damaraland mole-rat female helpers did not become more aggressive, or more dominant, or more prone to leave a familiar environment and they did not decrease their cooperative contributions when their T levels were experimentally elevated.

This study suggests that, in Damaraland mole-rats, increases in T levels may be irrelevant for females to transition from helping to breeding. The chances of female helpers to secure a breeding position may heavily rely on their fighting abilities (Cooney and Bennett, 2000; Jacobs et al., 1998). My results suggest that this might be independent of T. Female helpers did not become more aggressive and/or less submissive when their T levels were elevated to values similar to the ones found in wild breeding females (T_{low}= 0.22 ng/ml, T_{high}=0.42 ng/ml; wild breeding female in the summer =1.1 nmol/l = 0.32ng/ml; maximum T levels measured in wild breeding females = 3.7 nmol/l = 1.07 ng/ml; values for wild animals were estimated from Lutermann et al., 2013). Helpers did not receive less aggression or more submissive calls when they had received T suggesting that they were not perceived as more threatening when their T levels were increased. Finally, none of the other investigated phenotypical traits that were hypothesized to facilitate the breeding of female helpers away from their colony were influenced by T – the tendency to leave a familiar environment, to spend time in, and explore, a novel environment, the stress response to social isolation, and the motivation to fight a breeding female when intruding in a foreign colony.

The contributions of female helpers to cooperative activities like burrowing, food carrying and nest building were not regulated by T. This absence of T effect on cooperative behaviour is consistent with the findings from Chapter 3, which show that individual variations in cooperative contributions were not associated with differences in T levels. It also supports that the modulation of burrowing behaviours highlighted in Chapter 4 was the consequence of the experimental changes in CORT levels rather than the consequence of their associated changes in T levels. Earlier findings on the regulation of cooperative activities by T have been inconsistent and showed both positive (Desjardins et al., 2008), negative (Young et al., 2005)

or no associations (Bender et al., 2008; Raynaud and Schradin, 2015) between T and cooperative behaviours. Most of these investigations focused on the expression of direct alloparental care (but see: Desjardins et al., 2008) which were never observed over the course of the current study and which fitness relevance to both breeders and offspring in Damaraland mole-rats is questionable. Therefore, parallels between this study and others may not be straightforward. The current study has only been preceded by a single experimental investigation on the regulatory effects of T on cooperative behaviours in a cooperative breeder (huddling in striped mice Raynaud and Schradin, 2014) and more studies of this kind are necessary to better understand the relationship between T and cooperative behaviours.

My results raise questions on the hormonal basis of dominance in female Damaraland mole-rats and other cooperative breeders. Breeding females are dominant over same sex-helpers but while dominance is accompanied by increased T levels in a variety of species (Davies et al., 2016; Desjardins et al., 2008), this is not the case in captive Damaraland mole-rats (this study; Clarke et al., 2001; Voigt et al., 2014). This highlights that differences in T levels between female breeders and helpers should not be interpreted as an indication that increased T levels causally support dominance, a suggestion that still needs formal testing. In Damaraland mole-rats, female dominance may still be modulated through androgen-dependent neuroendocrine pathways since the expression of androgen and oestrogen receptors in several brain areas regulating aggressive behaviours is increased in breeding females (Voigt et al., 2014). Also, my results suggest that differences in breeding output (Damaraland mole-rat female helpers never breed in the presence of an established breeding female) may not necessarily explain the differences in T levels between breeders and non-breeders.

The absence of differences in T profiles between female breeders and helpers in captivity contrasts with the finding of a study conducted in natural population (Lutermann et al., 2013) suggesting that the relationship between T and dominance vary with environmental contexts. In the wild, male immigration and female emigration into established colonies generate breeding opportunities for female helpers (Bennett et al., 1996; Cooney and Bennett, 2000; Jacobs et al., 1998; Rickard and Bennett, 1997) which could cause intense reproductive conflicts (Cooney and Bennett, 2000; Jacobs et al., 1998). Accordingly, breeding females had higher T levels compared to helpers only in the wet season (Lutermann et al., 2013), when constraints on dispersal are relaxed (Young et al., 2010), suggesting that increased T levels may allow them to successfully defend their status when threatened by helpers (Cooney and Bennett, 2000; Lutermann et al., 2013). In my study, dispersal was strictly constrained by the

conditions of captivity and T levels were low, raising the possibility that T may support aggression only in conflictual situations. The context dependency of T's actions on aggression is well acknowledged (Albert et al., 1989; Soma et al., 2008) and future experiments should investigate whether female helpers' behavioural insensitivity to T is maintained under social instability.

Chapter 6

General Discussion

Overview

Large individual differences in contribution to cooperative activities are characteristics of cooperative breeding societies (Clutton-Brock, 2016; Clutton-Brock et al., 2001a; Hodge, 2007; Zöttl et al., 2016b). While the evolutionary and ecological causes of these differences are increasingly well understood (Cant, 2005; Clutton-Brock et al., 2002), the physiological mechanisms which allow individuals to integrate their internal physiological state and environmental stimuli into an adaptive adjustment of their cooperative contributions remain largely unknown (Schoech et al., 2004; Soares et al., 2010). Previous investigations of the hormonal mechanisms regulating cooperative behaviour have focussed principally on the effects of the glucocorticoid (GC) stress hormones (cortisol and corticosterone) and sex hormones (especially testosterone) in controlling cooperative behaviours and suggestions regarding the directions of these associations have been inconsistent (Bender et al., 2008; Carlson et al., 2006a, 2006b; Desjardins et al., 2008; Raynaud and Schradin, 2015, 2014; Sanderson et al., 2014; Schoech et al., 2004; Young et al., 2005). Most of these studies have been based on correlations between variation in cooperative behaviour and variation in hormone levels, which prevents inferring causalities. Thus, it remains unknown whether GC and T directly regulates cooperative behaviours or if they are modulated as a response of changes in cooperative contributions.

In this thesis, I explored the role of variation in the GC stress hormone cortisol (CORT) and the sex hormone testosterone (T) in controlling cooperative behaviour in Damaraland mole-rats, using a combination of correlational and experimental methods. In this species, individuals' main cooperative behaviour consists of burrowing activities which allow them to maintain and expand the tunnel system to locate food sources subsequently shared between

group-members. I ran three main experiments during which (i) burrowing contributions (Chapter 3), (ii) CORT levels (Chapter 4), and (iii) T levels (Chapter 5) were manipulated.

Overall, my investigations showed that variation in CORT levels can be both a consequence and a cause of variation in cooperative behaviours. In Chapter 3, I showed that, despite the lack of correlation between CORT and individual cooperative contributions, CORT levels tended to be higher in groups where burrowing was experimentally increased, suggesting that changes in cooperative contributions can elevate CORT levels. Subsequently, in Chapter 4, I showed that experimental increases in CORT levels raised the cooperative contributions of female helpers. Together, these findings support the view that CORT may be an essential modulator of within individual plasticity in cooperative behaviours.

In contrast, my thesis provides no evidence for a modulatory role of T on cooperative behaviours. Indeed, in Chapter 3, I showed that T levels were not correlated with individual cooperative contributions and that experimental increases in burrowing did not alter T levels while in Chapter 5, I showed that increasing T levels did not affect cooperative behaviours. Furthermore, I showed that experimental increases of T levels did not affect investments in future reproduction, either within the group of residence (competition for dominance) or outside (dispersal and competition). These results provide no support for the hypothesis that testosterone mediates a life history trade-off in female helpers, with higher T levels supporting investment towards future reproduction at the expense of immediate cooperation.

In sum, I experimentally showed that increases in individual cooperative contributions can raise CORT levels and suggested that elevations in CORT levels can increase individual cooperative contributions suggesting a positive reciprocal feedback between the two. In the following, I discuss in more depth the implication of each of these results in our understanding of the role CORT plays in underlying individual differences in cooperative tendencies in

Damaraland mole-rats and cooperative breeders in general. I then discuss how CORT levels could explain naturally-occurring interindividual differences in cooperative tendencies depending on the developmental and ecological contexts experienced.

Modulation of cooperative contributions by stress hormones

Previous studies have shown positive correlations between cortisol levels and cooperative behaviour, which have been interpreted as suggestive evidence for a causal role for cortisol in the control of cooperative behaviour (Carlson et al., 2006a; Sanderson et al., 2014). My finding that increases in GC raised female helpers' burrowing contributions confirms the hypothesis that increased GC levels may facilitate the expression of energetically demanding cooperative behaviours in Damaraland mole-rats by supporting energy production (Landys et al., 2006; Sapolsky et al., 2000). Contrasting with its effect in the regulation of burrowing behaviours, CORT had no effect on food carrying and nest building. Although this suggests that different cooperative activities may be under the control of different hormonal regulatory mechanisms, it is not obvious why food carrying and nest building would not also be modulated by CORT. A possibility would be that a modulation of nest building by CORT may be non-adaptive since this may cause excessive disturbances to the communal nest where most colony members are resting. The alternative explanation that the standard conditions of captivity in which the behavioural observations were conducted failed to reveal an effect of CORT cannot be rejected. A larger volume of observations and/or the carrying out of behavioural tests designed to increase the usually low frequency of nest building and food carrying, may be required to reliably test a regulatory role of CORT. This also apply to pup carrying which expression was too scarce to be part of any analyses conducted in this thesis.

A positive effect of increased GC on parental chick provisioning has recently been experimentally supported in macaroni penguins (*Eudyptes chrysolophus*, Crossin et al., 2012), but whether similar effects may apply in alloparents in cooperatively breeding birds is unknown. In mammals, none of the only two experimental manipulations of cooperative breeders' hypothalamic–pituitary–adrenal (HPA) axis has supported the hypothesis that increased GC favours the expression of alloparental care (Santema et al., 2013, Dantzer et al. in prep.). In this species, experimentally induced short-term increases of GC levels in male helpers did not increase their cooperative behaviours (pup feeding, sentinel) (Santema et al., 2013). A subsequent experiment using a treatment with mifepristone, a glucocorticoid receptor antagonist, led to an increase in cooperative behaviours (babysitting, pup feeding), suggesting that increased GC has an antagonistic effect on cooperative tendencies (Dantzer et al., in preparation).

The causes of differences in results obtained between these two species of cooperative breeding mammals might be varied but one possibility is that the disparity comes from a fundamental difference in the form of cooperative activities. In meerkats, most cooperative activities induce a period of food deprivation through the forgoing of foraging activities (babysitting, sentinel) or food sharing (pup feeding) which sharply contrasts with mole-rats in which burrowing increases the probability of finding food. Since the release of CORT has a positive effect on food intake (Koch et al., 2002; Landys et al., 2006; Strack et al., 1995), increases in CORT may have led to a shift in activity towards food acquisition causing a decrease in cooperation in meerkat and an increase in mole-rats. The same mechanism could also explain the lack of CORT effect on food carrying and nest building (see above), since these activities are not directly linked to food acquisition.

Other explanation for the disparity in the results obtained between meerkats and mole-rats might come from methodological issues. Firstly, since mifepristone is a potent antagonist

of the progesterone receptors (Spitz and Bardin, 1993), the decrease in pup-feeding in response to the mifepristone treatment reported in Dantzer's study could be the consequence of a modulation of pup feeding by progesterone, not GC. Also, given that in Santema and colleagues' work (2013), the CORT levels were only raised for a few hours and reaching levels beyond those normally encountered in this species, it appears premature to reject a potential positive role of CORT in the modulation of meerkat cooperative contributions.

My thesis offers no support for a regulatory effect of T on cooperative contributions. So far, and to my knowledge, only one study has experimentally tested the effect of T on alloparental care and found no effect of increased T on huddling in cooperatively breeding striped mice (*Rhabdomys pumilio*) (Raynaud and Schradin, 2014). This contrasted with a previous study in Mongolian gerbils (*Meriones unguiculatus*) where increased T decreased huddling, although the breeding status (parents or alloparents) of treated subjects was unclear (Clark and Galef, 1999). Assuming that increased aggression disrupts cooperation, one would expect increased T to exert a detrimental effect on cooperative behaviours in species where T supports aggression, but my results suggest this is not the case in Damaraland mole-rats.

More neuroendocrine manipulations must be conducted to elucidate the physiological mechanisms regulating cooperative behaviours and explain their differences and similarities between species and across different types of cooperative activities. These manipulations should simulate physiologically relevant hormone levels and be of sufficient duration to confidently interpret an absence of effects as truly negative results. This remark applies to the experiment in which I experimentally manipulated the T levels of female helpers (Chapter 5) and where the absence of an effect of T on the short term does not exclude an effect of T on the longer term.

Modulation of stress hormone levels by cooperative contributions

The finding that experimental increases in cooperative contributions levels raised CORT levels strongly suggest that cooperative behaviours can modulate CORT levels and emphasize the weaknesses of correlative studies I raised in Chapter 1. The capacity of cooperative behaviours to increase CORT levels suggest that variation in cooperative contributions alone may have generated the positive correlations between CORT and cooperative contributions highlighted in previous studies (Carlson et al., 2006a, 2006b; Sanderson et al., 2014). This point is furthered by the fact that I found no positive correlation between CORT levels and burrowing contributions, despite the experimental evidence of their association.

Inferring the direct causality of energetically demanding cooperative contributions on CORT levels may be complicated by the fact that changes in cooperative behaviours can have repercussions on social behaviours and the relationship between CORT and behaviours is reciprocal (Landys et al., 2007; Mikics et al., 2004; Summers, 2002). Although almost all group members increased their burrowing contributions when their colony was provided with more sand, these changes were also accompanied by changes in the social conditions that may have contributed to the increases in CORT. Whether direct or not, the effect of cooperative contributions on CORT raises the possibility that differences in CORT levels between individuals of different social ranks (Creel, 2001) could be caused, at least partially, by differences in cooperative contributions.

Beyond these methodological issues, the finding that increases in CORT levels raised burrowing contributions whilst increases in burrowing contributions also raised CORT levels leads to the interesting hypothesis that CORT and individual cooperative contributions are part

of a positive feedback loop. The functional relevance of such a feedback loop is unknown but the possibility that it may enable individuals to better adjust their cooperative contributions is worth considering. To test this hypothesis, one would need to demonstrate that experimentally induced increases in CORT approximating the increases observed when the sand supply was manipulated, would raise individual burrowing behaviours. This cannot be inferred from the results presented in this thesis since CORT levels were increased by 25% when the colony sand supply was manipulated (Chapter 3) and by over 200% when individuals were treated with a CORT implant (Chapter 4; it should be noted that CORT determination in chapter 3 and 4 followed two distinct analytical methods). The experimental demonstration of a positive effect of burrowing contributions on CORT in which the effect of social circumstances could be excluded, would lend further support to this hypothesis. Finally, it is unclear whether the elevation of CORT induced by the increased sand provisioning was necessary for individuals to sustain their higher burrowing contributions. An elegant way to test this possibility would be to combine manipulations of the colony sand supply and of individual HPA axis. One would predict individuals to increase their cooperative contributions when provided with more sand, but not if their CORT signalling pathway is disrupted. Treatment with mineralocorticoid or glucocorticoids receptors (MR and GR) antagonists would allow to test this possibility and determine whether cooperative contributions are regulated via the MR and/or GR pathways.

Hormones and consistent individual differences in cooperative behaviours

Although individuals flexibly adjust their cooperative behaviours to internal and environmental conditions, there are consistent individual differences in cooperative contributions. The existence of cooperative ‘personalities’ has been described in two species

of cooperative breeders, the Kalahari meerkats and the banded mongooses, but their causes and adaptive character remain elusive (Carter et al., 2014; English et al., 2010; Sanderson et al., 2015).

There is increasing evidence that the environment is a source of epigenetic variation, which can have profound and long-lasting effects on offspring phenotypes (social competence:Arnold and Taborsky, 2010; maternal behaviour: Champagne, 2008; growth: Dantzer et al., 2013; dispersal: Höner et al., 2010; reproductive and aggressive behaviours: Kaiser and Sachser, 2005). Such effects are often mediated by the developmental effects of steroid hormones such as CORT and T (Dantzer et al., 2013; Eising et al., 2006), but whether and through which developmental mechanisms cooperative personalities may be generated is unknown.

In species, such as the Damaraland mole-rat, where within individual flexibility in cooperative contributions is regulated by CORT, variation in early-life experiences organizing the HPA axis may generate cooperative personalities. Multiple aspects of the HPA axis are profoundly affected by endocrine conditions, and the environmental circumstances that modulate them, during critical periods of development such as the early-life and the adolescence (Liu, 2001). The developmental programming of the HPA axis is particularly sensitive to food resources availability and social experiences and variation across individuals may generate life-long lasting differences in their HPA axis (Champagne, 2008), and in turn of their tendency to cooperate. In Damaraland mole-rat, higher GC levels raise burrowing contributions (Chapter 4), leading to the prediction that early environmental conditions increasing baseline CORT release and/or the sensitivity to it, may cause individuals to behave more cooperatively. Early-life variation in cooperative contributions may contribute to this process since increased burrowing may raise CORT and the process further reinforced through the hypothesized positive feedback between CORT and burrowing.

Interactions between early-life environment conditions, growth and steroid hormones may also be relevant since growth can be socially modulated (Huchard et al., 2016) and is associated with variation in cooperative contributions (Clutton-Brock et al., 2002). Several correlative studies in both the Damaraland and the naked mole-rat (*Heterocephalus glaber*) have suggested that offspring born into small colonies grow faster and in some cases larger than pups born into larger colonies (Bennett and Navarro, 1997; O'Riain and Jarvis, 1998; Young et al., 2015; Zöttl et al., 2016a), even in the presence of unlimited food resources (Bennett and Navarro, 1997; O'Riain and Jarvis, 1998; Zöttl et al., 2016a). In turn, faster growing individuals contribute more to cooperative activities in the Damaraland mole-rat while the pattern is opposite in the naked mole-rat (Bennett and Faulkes, 2000; Zöttl et al., 2016b).While growth can be influenced by steroid hormones (Dantzer et al., 2013), whether such hormones could exert a pleiotropic effect on growth and cooperative behaviours should be determined.

Towards a more ecologically relevant understanding of the regulatory mechanisms of cooperative behaviours

Developing an understanding of how individuals integrate internal and environmental stimuli into an adapted adjustment of their cooperative contributions represents a major challenge that relies on the embracement of a more integrated approach than the one I followed in this thesis. Once the regulatory actions of a hormone on cooperative behaviours is experimentally demonstrated, these actions should be further investigated beyond the context in which they were originally revealed to determine their context dependency. CORT and T releases being modulated upon the continuous integration of a multitude of cues, it is obvious that the great deal of real-world situations modulating their release in the same directions may

require distinct adapted behavioural responses. This highlights that predicting behavioural effects of a hormone based on conclusions drawn from its experimental manipulation in a very specific context is likely to be unsuccessful or incomplete.

In Damaraland mole-rats, future research should focus on the manipulation of the factors shaping CORT release to investigate whether changes in both CORT and burrowing contributions support the conclusions from the experimental manipulation of CORT levels (Chapter 4). I showed that, in socially stable conditions in which colonies were fed ad libitum, the experimental elevation of CORT levels increased burrowing contributions. Therefore, internal and environmental factors causing a rise in CORT levels could be predicted to increase burrowing contributions. The observation that experimental increases in burrowing contributions conducted raised CORT (Chapter 3) suggested that burrowing could be such a factor through a positive feedback loop with CORT. I also hypothesized that breeding females could behaviourally "manipulate" helpers CORT levels to strategically adjust their cooperative contributions. However, even though helpers' CORT levels may be increased by aggression from the breeding female, this may not enforce helpers to increase their cooperative contribution. Therefore, experimental manipulations of breeding female's aggressions should be used to specifically test this hypothesis.

Food availability may represent a crucial environmental cue susceptible to affect cooperative contributions through its effect of CORT release and the investigation of these interactions may be invaluable to improve the understanding of the physiology of cooperation. The relationship between CORT release and the control of individual energy balance through the regulation of appetite, foraging behaviours and individual activity are intertwined (Landys et al., 2006; Levay et al., 2010; Sanderson et al., 2014; Sapolsky et al., 2000; Strack et al., 1995; Stranahan et al., 2009). Elucidating how these intricate relationships affect cooperative contribution through carefully designed experiment is especially relevant since examples of

cooperative activities which are tightly linked to foraging decisions and individual energetic state are ubiquitous in nature. The Bluestreak cleaner wrasse (*Labroides dimidiatus*) cooperates by feeding against their preferences to remove their clients' ectoparasites (Grutter and Bshary, 2003), vampire bats (*Desmodus rotundus*) donate food resources that they could have kept for themselves (Wilkinson, 1984). Cooperative breeders forgo their own feeding at the expense of dependent pups that they provision and babysit (Clutton-Brock et al., 2001a, 1998), but are more likely to do so when they are in good body conditions (Clutton-Brock et al., 2002).

Building upon the finding that CORT is an essential regulator of individual burrowing contributions, Damaraland mole-rats may represent an ideal study system to test the interactions between CORT, food availability and cooperative behaviours. The combination of food and HPA axis and/or sand provisioning manipulations could determine whether the effect or CORT on burrowing and the effect of increased burrowing on CORT are maintained when food resources are limited.

The combined manipulations of environmental context and neuroendocrine pathways regulating behaviours generally represents a very promising approach to advance the understanding of the physiology of cooperation. The integration of neuro-genomic approaches to such experimental paradigm may prove invaluable as it may facilitate the investigation of the mechanisms underlying the context dependent effect of apparently uniform release of hormone such as CORT and T.

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