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"Stress and habitat fragmentation in *Eulemur collaris* evaluated by glucocorticoid metabolite levels in faecal samples"

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ABSTRACT

This study investigates the relationship between Madagascar's environment and the stress physiology of the red collared brown lemur, *Eulemur collaris*. Habitat disturbance in Madagascar is pervasive and lemurs possess many unique traits compared with other primates that are primarily attributed to environmental or nutritional stress. Glucocorticoids (GCs) like cortisol are stress indicators. The assessment of welfare in animals is a developing field of study, and stressors in natural habitat can be estimated by measuring GC metabolites (GCM) in faeces. The littoral forests of southeastern Madagascar are among the most endangered habitats in the world and *Eulemur collaris* is well adapted to survive in these forests. The red collared brown lemur is a mostly frugivorous and cathemeral lemur with an extraordinary ecological flexibility that allows it to tolerate a certain degree of habitat changes. The goal of this study was to assess whether the health state measured by GCM levels of animals which living in a degraded habitat is comparatively different than the state of con-specific individuals living in a more pristine area. For this we compared faecal GCM levels in wild adults *Eulemur collaris* in a degraded (Mandena) and an intact littoral forest (Sainte Luce) in South-eastern Madagascar over a one year period (February 2011- January 2012). We analysed how GCM levels varied among reproductive stages and phenological seasons, using forest degradation, rank and sex as covariates. In general, lemurs at Mandena were more stressed than lemurs at Sainte Luce. Lemurs showed higher GCM levels during the mating season, followed by gestation season, lactation season and the non-reproductive season. We found that females at Mandena were more stressed than females at Sainte Luce during the gestation season. In relation to phenological seasons, individuals at Mandena were significantly more stressed than individuals at Sainte Luce, and lemurs at Mandena were more stressed during the lean season than lemurs at Sainte Luce. This study found no rank-related

GCM levels. These results showed, as predicted, that lemurs in a degraded habitat are more stressed and this has important implications for future conservation plans. *Eulemur collaris* is the largest frugivorous primate present in the littoral forest and is an important seed disperser, its presence is of vital importance for the forest *per se*. Due to its role of seed disperser, protecting this lemur species also means helping regeneration of degraded habitats.

CHAPTER 1

INTRODUCTION

1.1 Defining Stress

"Stress" has become an increasingly popular and widely applied term in everyday language, probably owing to the involvement of scientists from different disciplines such as biomedicine, psychology, ethology, and animal welfare (Palme et al. 2005).

The definition of stress is difficult and contentious since the term is often used to indicate a whole range of symptoms and levels of suffering (Lane 2006).

Even among biologists, the word "stress" has many meanings, thus it is generally best to avoid the word stress using instead the more explicit terms "stressors" to refer to the condition provoking a response, and "stress response" to refer to changes in internal state induced by the external condition (Creel 2001). The term "chronic stress" is used to refer to long-term overstimulation of coping response (Romero 2004).

All living organisms maintain a complex dynamic equilibrium, or homeostasis, which is constantly challenged by internal or external adverse effects (Chrousos et al. 1996).

There are several potential stressor stimuli for free-ranging animals, they may be both natural, such as climatic factors, high predation pressure, decreased food accessibility, social aggression or competition, and anthropogenic, such as noise and habitat alteration (Cavigelli 1999; Pride 2005a; Ostner et al. 2008). Social instability due to immigration events or rank reversals may lead to a stress response (Beehner et al. 2005; Bergman et al. 2005). Immigration of adult individuals, in particular, may represent a source of stress, as the newcomer(s) may not only challenge rank positions (Alberts et al. 1992; Bergman et al. 2005)

but, in the most extreme case, may oust the resident individual(s) (Pope 1990; Ostner and Kappeler 2004; Ostner et al. 2008).

Selye (1936) defined stress as "the nonspecific response of the body to any demand made on it", in this case there is a tendency to consider any difficult or energetically demanding situation as a stressor, but recent studies suggest that the 'predictability' or 'controllability' of a stimulus are the most important characteristics that determine the degree of challenge it poses. In other words, difficult conditions that are predictable and/or controllable, including, for example, seasonal decreases in food accessibility or visually prominent predators, often do not provoke a physiological stress response (Cavigelli 1999; Creel 2001). A classic example is the study by Weiss and colleagues (1968), who gave two rats similar electric shocks. One rat was given a lever to press, which terminated the shock for both rats. The rat that controlled the lever had a dramatically lower glucocorticoid response (one characteristic of the vertebrate stress response is the release of glucocorticoid steroid hormones, see paragraph 1.2) than did the second rat. Therefore, the degree of control over the stressor (i.e. the context) determines the degree level of the hormonal response (Romero 2004).

1.2 Physiological Stress

The discovery of metabolic, immunological and neuroendocrine mechanisms make it possible to describe the stress response in physiological terms (Balm 1999).

The stress response is mediated by the stress-system, which is located in both the central nervous system and peripheral organs. There are many hormones that have been identified as playing a role in the vertebrate stress response, two categories of hormones are thought to form the central components of the endocrine response. These are the catecholamines, epinephrine and norepinephrine (also known as adrenalin and noradrenalin) and

glucocorticoids (GCs, a class of steroid hormones). Together, these hormones help to orchestrate the body's stress response (Romero and Butler 2007).

Catecholamine Responses

The adrenal medulla and nerve terminals of the sympathetic nervous system release epinephrine and norepinephrine when a stressor is detected, then they activate organism-level responses via their receptors in target tissues. These hormones, which have been produced beforehand, are already present inside secretory vesicles thus allowing for a very fast release upon detection of a stressor. Catecholamines are responsible for the activation of many responses including: shutting down digestion, decreasing visceral activity, increasing visual acuity, increasing brain blood flow and arousal, increasing gas exchange in the lungs, the breakdown of glycogen in order to release glucose, inducing vasodilation in muscles and vasoconstriction in the periphery, increasing heart rate and inducing piloerection. The responses mediated by epinephrine and norepinephrine are known as the "Fight-or-Flight" response because they immediately increase readiness and activity allowing the animal to survive an acute threat such as an attack by a predator or competitor (Romero and Butler 2007).

Glucocorticoid Responses

The steroid hormones are responsible for a slower endocrine response. The primary hormones released upon detection of a stressor are the glucocorticoids (GCs), cortisol and corticosterone. The specific glucocorticoid released is species-specific (Von Holst 1998; Broom and Johnson 1993). Most species rely primarily upon cortisol (e.g. most mammals

including primates and marine mammals and fishes), while birds, reptiles and amphibians use corticosterone. Some rodents rely upon cortisol, others upon a mixture of the steroids cortisol and corticosterone (Palme et al. 2005).

When released GCs travel in the peripheral circulation primarily bound to corticosteroid binding globulins (CGB). Steroids are highly lypophilic so that most GCs are bound to CBG, however, unbound GCs increase dramatically during a stress response. When they reach the target tissue GCs pass through the cell membrane and bind to the intracellular cytoplasmic receptor. Activated receptors then enter the nucleus and begin acting as transcription factors. Activated receptors bind to short stretches of DNA sequences known as glucocorticoid response elements and act as regulators of gene transcription, so the end result of GCs stimulation is either the production of new proteins or the inhibition of protein production. Some evidence shows that a membrane-bound receptor for GCs exists; this receptor is believed to regulate rapid behavioural effects of GCs. The HPA (hypothalamic-pituitary-adrenal) axis is a multistep process which ensures a time lag between the onset of a stressor and an increase in GCs blood level concentrations (Fig. 1.1).
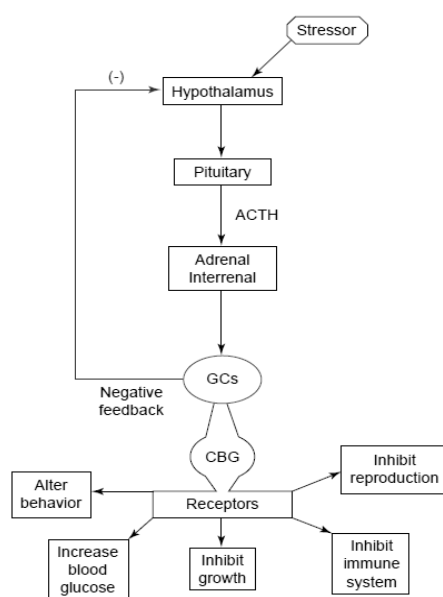
Fig. 1.1: The hypothalamic–pituitary–adrenal (HPA) axis. An acute stressor is detected by the cortex of the brain, which sends a neuronal signal to the hypothalamus. The hypothalamus then sends a hormonal signal to the pituitary, resulting in the pituitary sending a hormonal signal (adrenocorticotropin, or ACTH) to the adrenal or interrenal (depending upon the species) gland to release GCs. GCs have multiple effects that are mediated by blood-borne carrying proteins (corticosteroid-binding globulins, CBG). A negative feedback loop shuts off the HPA pathway leading to GC release. If the stressor persists and GCs remain elevated, negative feedback ceases to function and the deleterious chronic effects of GCs begin (Romero 2004).

Generally increases in GCs concentrations take more than 3-5 min. (for some species even a longer time) to be detected. When coupled with GCs primary effect of altering gene transcription rates, the physiological impact of GCs starts approximately 20-30 min. after stressor onset. If a stressor is interrupted, negative feedback will generally begin to reduce GCs concentrations in 30-60 min., although GCs physiological effects can last considerably longer as the newly produced proteins can continue to function; therefore the catecholamines and the GCs dovetail produce both an immediate and longer-term response to stressors.

Although GCs alter gene transcription rates for a very large number of genes at the organismal level GCs can be classified as having five broad effects (Romero 2004; Romero and Butler 2007) (see Fig 1.1).

1. Increasing glycemia.

The classic effect of GCs is to increase the blood glucose available to tissues involved in responding to a stressor. In fact the name "glucocorticoids" was given to these hormones because of this important role which takes two general forms: first GCs increase blood glucose by converting protein to glycogen and by stimulating the catabolism of protein to form new glucose in a process called gluconeogenesis. Second GCs reduce the blood glucose uptake by target tissues causing higher blood concentrations of glucose available to tissues involved in responding to stress. GCs do this by stimulating the internalization of glucose transport molecules from the surface of the cell of target tissues. Fewer glucose transporters result in less utilization of glucose leading to higher blood glucose concentrations. Tissues which need extra glucose to respond to a stressor (e.g. muscles) compensate for the GCs effect and essentially have a preferential access to the increased pool of blood glucose. The result of these effects is that GCs manage the allocation of energy supplies during either prolonged stressors or after stressors have ended (Dallman et al. 1993).

2. Altering behaviour.

In different environmental contexts, GCs can promote a behavioural strategy of "hiding and waiting out a stressor" or promote a behavioural strategy of "abandoning an area and fleeing the stressor" (Wingfield and Ramenofksy 1997). The mechanism behind how GCs alter behaviour are currently unknown, they may involve membrane-bound G-protein receptor that induce rapid behavioural effects.

3. Inhibiting growth.

GCs inhibit growth by blocking the secretion of growth hormone from the pituitary, decreasing the sensitivity of target cells to growth hormone and inhibiting protein synthesis (Sapolsky 1992a).

4. Inhibiting reproduction.

GCs also inhibit reproduction (Wingfield and Romero 2001). Vertebrate reproduction is regulated by a hormonal cascade that is similar to the HPA axis. Interestingly, the reproductive system can become resistant to inhibition by GCs in some reproductive contexts. For example, if GCs allocate resources away from reproduction and then reduce individual fitness (i.e. successful production of offspring), the benefit of the reproductive system ignoring the GCs signal may outweigh the cost of not responding to the stressor. In semelparous species (those species that breed once and then die) such as some salmon species and several Australian marsupial rodents, death occurs in all individuals (or all individuals of one sex) shortly after breeding. The proximate cause of death is extremely high levels of GCs that catabolise essential proteins (Wingfield and Romero 2001). Reproduction of these animals continues despite elevated GCs levels. GCs do not inhibit reproduction in many short-lived species and in older individuals and in dominant individuals in some species,

where the dominant individual has a limited period of access to mates (Wingfield and Sapolsky 2003). Consequently, susceptibility to GCS-induced inhibition of reproduction is highly specific depending on the importance of the continuation of reproduction in the presence of stress.

5. Modulating the immune system.

GCs have various effects on the immune system including the inhibition of synthesis, release and efficacy of cytokines (immune system proteins): inhibiting antigen presentation through reduced major histocompatibility complex (MHC) expression, reducing the activation and proliferation of T cells, B cells and macrophages, reducing lymphocyte chemotaxis, lowering the circulating levels of lymphocytes , reducing the number of phagocytic cells at inflammation sites, stimulating atrophy of the thymus and triggering the death of immature T and B cells. These different effects lead to immunosuppression especially with long-term GCs exposure. Some evidence shows that GCs may well enhance immune function in the short-term (Dhabhar and Mc Ewen 1999; Dhabhar 2006) Why GCs have such great immunosuppressive effects is not entirely clear but it has been suggested it may be a mechanism to prevent over-activation of the immune system which could very well lead to autoimmune diseases (Palme et al. 2005).

Metabolism and excretion of glucocorticoids

Several studies investigated metabolism and excretion of GCs. Lindner (1972) investigated the excretion of infused ^{14}C-cortisol in sheep. He stated that two-thirds of the radioactivity was subsequently found in the bile. The main metabolites were glucuronides of tetrahydrocortisol, tetrahydrocortisone and cortolones. Also $C_{19}O_3$ steroids (androstanes) were formed. Additionally, enterohepatic circulation of these metabolites occurred and cortisol

metabolites were eliminated *via* the faeces. Palme et al. (1996) infused radioactive cortisol intravenously into sheep, ponies and pigs. They found high interspecies differences regarding the amount of radioactivity excreted via urine and faeces and the time course of the excretion. In faeces the maximum concentration were measured about 12 h (sheep), 24 h (ponies) or 48 h (pigs) later, this reflects the intestinal passage time in these species.

There are also considerable species differences in the metabolites formed (Palme and Möstl 2000). Möstl *et al.* (2002) showed that in ruminants at least 21 cortisol metabolites can be detected in faecal samples using high performance liquid chromatography (HPLC)/mass spectroscopy.

Möstl et al. (1999) compared chromatograms of fecal cortisol metabolites after infusion of ^{14}C-cortisol into ponies and pigs. In ponies, one dominating metabolite was present, whereas in pigs a total of five more prominent radioactive peaks were measured after HPLC. Using HPLC and enzyme immunoassays (EIA), Palme and Möstl (1997) showed that almost no authentic cortisol was excreted in sheep, even after intravenous infusion of 1 g of cortisol. A similar situation was described in ponies and pigs (Möstl et al. 1999). In some other species, cortisol metabolites also occur in cats, primates and hares (Schatz and Palme 2001; Bahr et al. 2000; Teskey-Gerstl et al. 2000). In particular, Bahr and colleagues (2000) have found that low cortisol amount were detected in faeces in the marmoset and it was virtually absent in macaques and chimpanzees.

1.3 Chronic stress effects

The actions of glucocorticoids may become maladaptive when individuals are subjected to chronic (weeks to months) stressors (Selye 1956; Coe and Scheffler 1989; Sapolsky 1994). In this case, many of the short-term benefits become long-term pathologies, including immune

suppression and atrophy of tissue, reproductive suppression, gastric ulcers and muscle wasting (Cameron 1997; Heistermann et al. 2004). Rises in GC cortisol during reproductive periods may co-occur with increased fertility (Bercovitch and Ziegler 2002), cortisol secretion associated with reproduction may increase to higher levels in response to induced stress (Saltzman et al. 1994), and cortisol has been found to inhibit female primates' ability to reproduce (Wasser and Starling 1988; Sapolsky 1992a; Cameron 1997; Bronson 1999). Physiological modifications in response to stress during reproductive stages can be particularly detrimental to reproductive success in slowly reproducing species as primates (Tecot 2013). Because high glucocorticoid levels suggest the presence of environmental threats, and chronically high glucocorticoid levels are themselves associated with health risks (Dhabhar and McEwen 2000), it is believed that high glucocorticoid levels indicate lower individual fitness or population viability (Pride 2005a).

1.4 Measuring Stress

Because of the potentially deleterious effects of chronic stress, there is great interest among different fields of study in measuring animal stress (Millspaugh and Washburn 2004).

There are obvious benefits to conducting studies in the wild. By gathering data from naturally assembled groups in their normal context, one can eliminate concerns about the effects of crowding, constant interaction, imposed group compositions and limited dispersal options. Some of the strength of field endocrinology can be lost by invasive sampling (Creel 2001).

The concentration of cortisol in the blood is widely used as an indicator of stress. Blood samples can be difficult to collect from free-ranging animals and one has to consider that sample collection, which often involves capture and handling, may by itself be stressful and can lead to rapid and significant increases in circulating GC levels, which may compromise

the results (Sapolsky 1982; Astheimer et al. 1994; Wingfield et al. 1994; Cavigelli 1999; Creel 2001; Möstl and Palme 2002).

Stressors in the natural habitat can be estimated by measuring cortisol levels in faeces (Risler et al. 1987; Whitten et al. 1998; Ziegler and Wittwer 2005). Faecal samples can be collected easily and without disturbing the animal, thereby allowing frequent sampling, even over a long time period (Palme et al. 2005), thus they can be used as a powerful non-invasive measure of stress levels in free-ranging animals (Cavigelli 1999; Creel 2001; Pride 2005a; Goymann 2012).

Are faecal GC levels reliable?

Particular sources of error and variability have been linked with the measurement of faecal GC metabolites. The most common method to determine steroid hormone levels is via immunoassay. With this method, the hormone (antigen) in the sample is bound to a specific antibody against this antigen and detected through enzymatic (enzyme-immunoassay) or radioactive labelling (radio-immunoassay). The concentration of the hormone is then determined using a calibrated standard curve of the same hormone (Chard 1995). Potential cross-reactions of the specific antibody with structurally similar antigens are negligible in blood plasma, because the cross-reacting substances (for example structurally similar metabolites of steroid hormone) occur only in low concentrations compared with the active hormone. In contrast, in faeces the metabolites of steroid hormones occur at much higher concentrations than the original and biologically active steroid (Palme et al. 2005); thus if the antibody cross-reacts with metabolites of structurally similar but functionally different hormones, this can have major and distorting impacts on the results of the study (Goymann 2012). It is essential, therefore, that before embarking on any research involving the

measurement of faecal GCs the most appropriate antibody and assay is carefully selected and validation tested to demonstrate the assay system's capability to detect relevant metabolites only (Wasser et al. 2000; Möstl et al. 2005; Palme 2005; Touma and Palme 2005). Thus, immunoassays utilizing antibodies specific to blood glucocorticoids may have relatively little affinity to the faecal GC metabolites and thus may have limited suitability for quantifying faecal glucocorticoid metabolites excretion (Bahr et al. 2000; Wasser et al. 2000; Möstl et al. 2002; Touma and Palme 2005).

 A number of studies have reported that females and males metabolize the same hormone differently and excrete different metabolites of this hormone; thus sex differences in the hormone metabolism suggest that biological and physiological validations of hormone metabolite measurements may need to be performed for each sex separately (Goymann 2012). Faecal GC metabolite (GCM) measurements have been validated with the ACTH challenge test in most primate species (e.g. Heistermann et al. 2006; Fichtel et al. 2007) which consist in a physiological stimulation of the HPA axis for example by administering ACTH and/or anaesthesia, but this kind of test is not always practicable due to ethic reasons, in fact this test is very stressful for the animals. Several studies used "stressful situations" (such as transportation/ translocations and change in housing conditions) known to elicit increased GCs output to test the biological validity of faecal GCM measurements (Heistermann et al. 2006).

Dietary differences may affect the pooling time of steroids and increase metabolite variability (von der Ohe and Servheen 2002). A day to day change in dietary fibre may have a significant effect on gastrointestinal time but, as faecal measurements of GC metabolites tend to be part of long-term studies, a change in time lag of a few hours is unlikely to impact on the accuracy of the final data (Lane 2006). If the faeces are not treated with preservatives or kept in cold storage, naturally occurring bacteria and bacterial enzyme in faeces decompose steroid

metabolites within hours after defecation (Wasser et al. 1988; Möstl et al. 1999; Khan et al. 2002). Ethanol either alone or with sodium azide has been used as a preservative for short-term ambient temperature storage of faecal samples (Wasser et al. 1997; Ziegler et al. 2000) or long term storage for 5 months (Cavigelli 1999) to 3.5 years (Curtis et al. 2000). When faecal measurement of GC metabolites is carried out by a suitable and validated technique it can be an incredibly useful method with very few limiting factors. Faecal sampling is relatively simple and can usually be conducted without disturbing the subject and without interfering with other welfare measures running in parallel, for example behavioural assessment (Lane 2006).

1.5 Lemurs and Stress

GCs may fluctuate both seasonally and daily (Rose et al. 1978; Stavisky et a. 2001; Lane 2006). Seasonal fluctuations may be associated with transitory factors such as reproductive cycle and weight (Reeder and Kramer 2005) or level of mate competition (Lynch et al. 2002). Breeding season in males is accompanied by cortisol level increase, where male competition for female can be both high or low (Lynch et al. 2002). In lemur females basal cortisol levels increase in mid-gestation (Ziegler et al. 2004).

GCs levels and their fluctuations have been associated with seasonal changes in weather, resource distribution and availability, and habitat quality (Huber et al. 2003; Pride 2005a; Chapman et al. 2006). Lemurs possess many unique traits compared with other primates, such as "cathemerality" (activity pattern throughout a 24 hour cycle; Tattersall 1987), synchronized breeding with very short oestrus periods and breeding season (Lancaster and Lee 1965), low basal metabolic rate (Snodgrass 2007) and lack of sexual dimorphism (Wright 1999). These traits are primarily attributed to environmental or nutritional stress. The energy

frugality hypothesis (Wright 1999) proposed that unique lemur traits are a way to conserve energy and maximize food resources. Two environmental factors that make Madagascar distinct from other regions are climate and habitat fragmentation.

1.6 Coping with harsh environments: Madagascar

Madagascar is considered an important global conservation priority because of the high endemicity of its flora and fauna (Mittermeier et al. 1998) and the severe threats to the island's environment. Of Madagascar's original habitat, 90% has been lost largely due to anthropogenic disturbance (Mittermeier et al. 2006), which decreases resource abundance, diversity and seasonal predictability (Tecot 2008). In 1985, 3.8 million hectares of rain forests remained, representing only 50% of the 7.6 million hectares existing in 1950, and 34% of the estimated original extent (11.2 million hectares). Between 1950 and 1985, the rate of deforestation averaged 111 000 hectares per year (Green and Sussman 1990) (Fig 1.2).
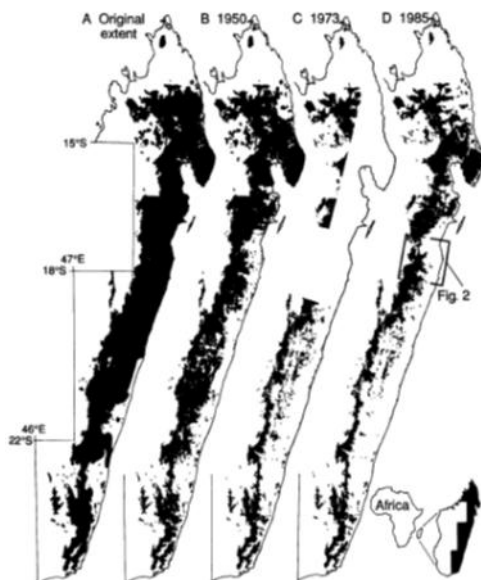

Fig 1.2: Maps of distribution of rain forest in eastern Madagascar through time (Green and Sussman 1990).

Madagascar is suggested to differ from other primate habitats (Wright 1999; Wright et al. 2005) because of extremely unpredictable rainfall patterns which result in unpredictable fruiting patterns, thus frugivorous lemurs seem to be particularly vulnerable (Dewar and Richard 2007). On the other hand, some flexibility has been observed and a number of mainly frugivorous lemurs may switch to lower quality foods during lean periods (Sussman 1974; Gould et al. 1999; Ratsimbasafy et al. 2002), modify their activity and ranging patterns (Pereira et al 1999; Donati et al. 2007; Donati et al. 2009), or use food patches of different size and split into subgroups (Ganzhorn 1988; Balko 1998; Irwin 2007). Thus, adaptation to fluctuating ecological conditions may have been potentially selected for the ability of lemurs to cope with degraded habitats (Donati et al. 2011).

The littoral forests of south-eastern Madagascar

Prior to human occupation, humid and sub-humid littoral forest covered approximately 461 000 ha (0.8 % of the total land surface of Madagascar), of which only about 47 900 ha remain today, for a habitat loss of 89.7 % (Consiglio et al. 2006). Thus, from the original littoral forest, only 10.3 % remains in the form of little forest fragments, and only 1.5 % of these are included within the existing protected areas network (Vincelette et al. 2007). In particular, the littoral forest on sandy soil of south-eastern Madagascar is one of the most threatened

ecosystems on this island. By 2005, the Tolagnaro region (Fig 1.3) littoral forests were heavily fragmented and covered only 3128 ha, distributed in 289 blocks varying in size from 0.06 ha to 252.4 ha (Vincelette et al. 2007).

Fig. 1.3: Anosy region in (red) and Fort Dauphin (Tolagnaro)

The littoral forest ecosystem is restricted to unconsolidated sand in a narrow coastal band, extending along Madagascar's east coast from north of Vohimarina to just southwest of Tolagnaro (Consiglio et al. 2006). This forest has a seasonal rainfall and strongly seasonal patterns in flushing, flowering, and ripe fruiting. Intra-annual differences occur in phenophases leading to periods of abundance and scarcity (Bollen and Donati 2005). In the Fort Dauphin (Tolagnaro) area the remaining littoral forests on sandy soil form three groups of fragments: Petriky, Mandena and Sainte Luce.

QIT Madagascar Minerals (QMM) is a company jointly owned by Rio Tinto (80%) and the Malagasy state (20%). QMM is an ilmenite mining project (ilmenite contains titanium dioxide, used principally as a raw material in the manufacture of white pigment). Since 1985 the company began an extensive exploration program along the eastern coast of Madagascar to locate deposits of heavy mineral sands containing titanium dioxide. Major mineral sediments were found in Mandena, Petriky and Sainte Luce (the largest remaining stands of littoral forest). A series of studies commissioned by QMM demonstrated the importance of the region's biodiversity that require appropriate conservation plans (e.g Ganzhorn et al. 2007). Since the duration of the project is expected to be around 40-50 years, the goal of QMM is to minimize the mine's negative impact on biodiversity and create a model project for investment compatible with biodiversity conservation. Avoiding impacts is the priority of QMM Biodiversity Action Plan, thus were designated avoidance areas (where no mining will take place) called "Conservation Zones", and QMM established three zones (Fig. 1.4):

1. The Mandena Conservation Zone (230 ha), established in 2000;

2. The Sainte Luce Conservation Zone (274 ha), established in 2005, set up with local communities called "dinas";

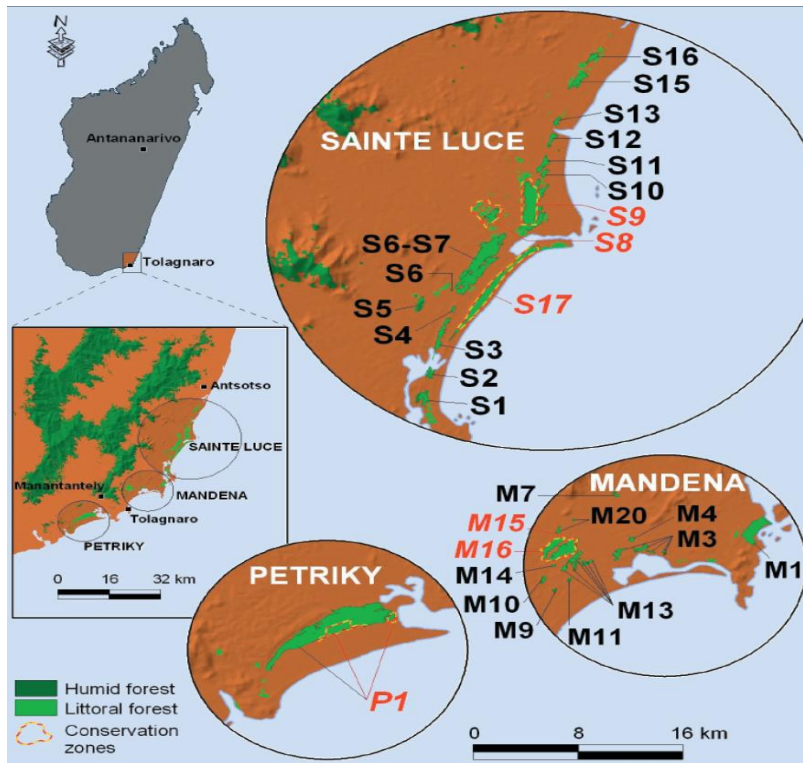3. The Petriky Conservation Zone (120) ha established in 2008;

Fig. 1.4: Maps of the Sainte Luce, Mandena and Petriky areas in South-eastern Madagascar, indicating the location of major littoral forest parcel (light green) including those that comprise the established conservation zones at the three sites (dashes red and yellow lines), (Lowry et al. 2008).

1.7 The red collared brown lemur, *Eulemur collaris.*

The red collared brown lemur, *Eulemur collaris* GEOFFROY 1817, is a strepsirrhine primate, belonging to the family Lemuridae. It was previously classed as one of the six subspecies of *Eulemur fulvus*. Subsequently, Groves (2001) elevated all former *E. fulvus* subspecies *(albifrons, albocollaris, collaris, fulvus, rufus* and *sanfordi)* to full species. *Eulemur collaris* was listed as "Vulnerable" on the " Red List of Threatened Species" (IUCN 2008) but leading conservationists have gathered at a summit meeting organised by the International Union for Conservation of Nature (IUCN) on July 2012 in Madagascar. This commission reviewed the conservation status of the world's 103 lemur species and elevated *Eulemur collaris* from "Vulnerable" to the "Endangered" category (Donati, pers.comm.).

The results of the conference highlighted that many lemur species are on the very brink of extinction and in need of urgent and effective protection measures.

Eulemur collaris is a medium-sized lemur, mean body mass is 2.15±0.25 kg and mean body length is 46.1±2.6 cm (n=11) (Donati et al. 2011). This species is sexually dichromatic. In males the dorsal coat is brownish-gray, the tail is darker, and there is also a dark stripe along the spine. The ventral coat is a paler gray. The muzzle, face and crown are dark gray to black. The creamy to rufous-brown cheeks and beard are thick and bushy, while the creamy to gray-colored eyebrow patches vary in their prominence. In females, the dorsal coat is browner or more rufous than that of the male. The ventral coat is a pale creamy-gray. The face and head are gray. The cheeks are rufous-brown, but less prominent than in males (Fig 1.5). Both sexes have orange-red eyes" (Mittermeier et al. 2010).

The red-collared brown lemur is similar in coloration to *Eulemur cinereiceps* (valid name of *E. albocollaris*, Johnson et al. 2008). Male *E. collaris* can be distinguished from male *E. cinereiceps* by their beards, those of the former being cream-colored or rufous while those of the latter are white. Female *E. collaris* are virtually indistinguishable from female *E. cinereiceps*. Recent genetic analyses support full species status for both taxa (Djletati et al. 1997; Wyner et al. 1999).

Fig 1.5: Octavio (adult male) and Barabarà (collared adult female) of Group AB at Mandena. Photos by Michela Balestri.

The red-collared brown lemur, and brown lemurs in general, have been characterized as highly flexible in their ecology, in accordance with their broad geographic distribution which spans many distinct habitat types and elevation zones (Tattersall and Sussman 1998; Goodman and Ganzhorn 2004).

These lemurs are found in tropical moist lowland and mountain forests in south-eastern Madagascar, from Tolagnaro (Fort Dauphin) north to the Mananara River (Fig. 1.6). The western limits of this range are the forests of the Kalambatritra region, including the Kalambatritra Special Reserve. The Mananara River serves as a boundary between the ranges of *E. collaris* and *E. cinereiceps*, except for an isolated population at Midongy du Sud

National Park (Irwin et al. 2005) and another at Vohipaho, near Vangaindrano (Mittermeier et al. 2010). Even if the typical habitat of *Eulemur collaris* is the mountain rain forest, it can also inhabit the humid littoral forests. In fact, we can find this lemur in both Mandena and Sainte Luce Conservation Zones (Fig. 1.6).

Fig 1.6: distribution of *Eulemur collaris* (in red).

Red-collared lemurs live in multi-male, multi-female groups;. Median group size in intact littoral forest is 7 (range. 2-17; n=13) and in intact rain forest is 5 (range 2-7; n=11) (Donati et al. 2011).

Eulemur collaris is a mainly frugivorous species, even if it can feed on leaves or less nutritios food when fruits are not available (Donati et al. 2011). Malagasy rainforests represent a challenging environment for arboreal frugivorous primates as compared to other tropical

forests (Ganzhorn et al. 1999; Wright 1999; Ganzhorn 2002; Bollen and Donati 2005; Wright et al. 2005). The unpredictable phenology and seasonal lean periods make these forests a hard challenge also for this species, despite its social and ecological flexibility (Donati et al. 2011). Red-collared lemurs are cathemeral (Tattersall 1987), this activity pattern provides temporal flexibility in foraging, optimizing the exploitation of food resources during times of greatest abundance and quality, and low predation risk (Rasmussen 1999). Cathemeral activity seems to be higher during the dry season, while a mostly diurnal activity was observed in the wet season (Donati and Borgognini-Tarli 2006).

1.8 Aim of the study

The main sources of ecological stressors proposed for primates are food availability and habitat quality. Other challenging stimuli may be due to reproductive stages, such as gestation for females and mating season for males, and dominance status for both sexes.

Male sifakas of both rank classes (dominant and subordinate) have higher GCM levels during the reproductive season as compared to the birth season (Fichtel et al. 2007). GCM levels during the mating season are related to generally increased rates of aggression during this time of the year when male-male reproductive competition is most pronounced. Additionally, there was a clear rank effect in GC output in the mating season, with dominant males exhibiting higher faecal GCM levels (Fichtel et al. 2007). Similar findings have been reported for male mouse lemurs (Schilling et al. 1984). Higher GC levels in dominants have been linked with high reproductive skew in several species (Creel 2001). In *Eulemur fulvus rufus* males of both rank classes had similar levels of both testosterone and GC levels, and they were excreted at high levels during the mating season as well as during the short annual birth season, maybe due to the predictable risk of infanticide in this highly seasonal species. No

clear rank related GC output was found for red-fronted lemurs (Ostner et al. 2008). In female ring-tailed lemurs (*Lemur catta*) faecal cortisol levels were relatively high during late gestation and at the end of the dry season, when intensite antipredatory behaviour and estimates of feeding effort were high. In addition, faecal cortisol measures were significantly correlated with dominance index: dominant females had higher cortisol values compared to subordinate females (Cavigelli 1999). Similar finding have been reported also for female dwarf mongooses (*Helogale parvula*; Creel 1992).

In *Eulemur rubriventer* the lowest GC levels occurred between weaning and mating, in this species male competition is low. According to the above authors low prebreeding levels may also be related to high food abundance. This is in contrast with what has been observed in male sifakas, which do not participate in infant care. The highest GC levels were excreted at the convergence of parturition and fruit scarcity. The paper by Tecot (2013) was the first study to compare lemur faecal GC between an intact and a degraded habitat, and contrary to expectations, GC levels were higher in the intact site. This study has suggested that populations with a higher mean value of GC levels may not be more stressed, the attenuation found in the degraded site may reduce the chances of additional pathological stress due to chronic stress and increase chances of survival in difficult environments. Infant mortality was higher in the degraded habitat, thus prolonged attenuation may indicate insufficient coping (Tecot 2013).

A comparative study between group size and phenological season in *Lemur catta* underlined that food scarcity had a direct role in raising cortisol levels during the weaning/breeding seasons where animals face the combined effects of high energy demand and low food supply (Pride 2005b). Faecal GC levels can be useful predictors of individual survival probabilities in wild populations as a population exhibiting high GC levels can be expected to have high mortality rates (Pride 2005a).

The aim of this study is to investigate whether lemurs were ecologically stressed and when they were stressed. This study examined how physiological responses of red collared lemurs varied with reproductive stages, habitat quality, food availability and social status. Physiological responses were investigated by analysing GCM excretion profiles, and these responses were compared among lemur groups in a degraded (Mandena) and in a more intact littoral forest (Sainte Luce) in South-eastern Madagascar. These two sites were ideal for this study because they are subjected to similar climates (average monthly temperature 23°C (range: 18.2-25.9; n= 30) and annual rainfall ranging 1600-2480 mm; Donati et al. 2011) facilitating inter-site comparison without the confounding influence of climate differences. As stated above, this species has social and ecological flexibility. This study may help to identify the extent of their flexibility, if they can compensate adverse environments only by changing their behaviour (activity pattern and diet) or by some physiological response.

<u>Hypotheses and predictions</u>

We predict that:

1) Reproductive stages.

Male will be more stressed as compared to females during the mating season because this period is short in this species. Gestation season, which is coincident with the lean period, could be very challenging for females. Thus, we predict higher GCM levels in females compared to males during gestation.

2) Food availability.

Lean season poses a significant challenge and lemurs require both behavioural and physiological modifications to face this period. Thus, we predict higher GCM levels during

the lean season for both sexes and sites. Additionally, we predict that the lean period represents a more difficult challenge for lemurs living in the more degraded forest of Mandena, where we expect to find higher GCM levels.

3) Habitat quality.

Anthropogenic disturbance creates unpredictable environments. Habitat loss and fragmentation can alter both quantity and quality of food resources available to primates. Logging affects density, size and distribution of plant species in forest fragments (Donati et al. 2011). Populations with higher corticoid levels are assumed to be exposed to greater stress, suggesting that more degraded habitats should induce higher cortisol levels (Tecot 2013). Thus, we predict significantly higher GCM levels in the degraded forest fragment of Mandena for both sexes.

Since GCM levels can reflect animal welfare, we can observe if this species can cope with habitat degradation. Frugivorous primates are important seed disperser and therefore fundamental to catalayze the regeneration of degraded habitats (Donati et al. 2011). *Eulemur collaris* is the largest frugivorous primate present in the two sites, thus improving this species' welfare means also helping habitat regeneration.

4) Rank.

Despite the numerous studies on the relationship between stress physiology and social status in vertebrates, no clear pattern has yet emerged (Creel 2001). Two publications, one on mammals and birds in general (Goymann and Wingfield 2004) and one on primates in particular (Abbott et al. 2003) focused on the intensity of stress in either dominance position, i.e., the cost of acquiring and maintaining a high rank, and the intensity of aggression a subordinate is exposed to. Since *Eulemur collaris* is known to show high social flexibility and

no strict dominance (Pereira and Kappeler, 1997), we assigned similar costs for both rank classes and consequently we predicted no rank related differences in GCM output.

CHAPTER 2

MATERIALS AND METHODS

2.1. Sites

This comparative study was conducted in the littoral forest fragments of Mandena (MAN) and Sainte Luce (STL) near Fort Dauphin in south-eastern Madagascar (Fig 2.1).
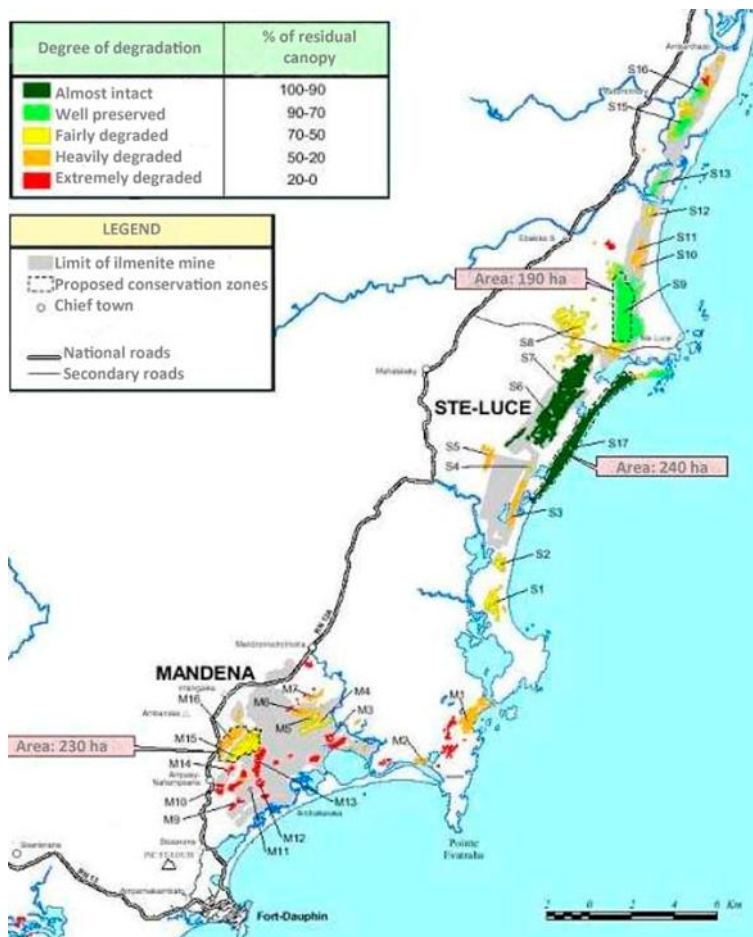

Fig. 2.1: Study site and forest conservation status. Map of littoral forest fragments remaining in 2000 at Sainte Luce and Mandena. Study sites at Mandena (M15 and M16) are more degraded than the study site at Sainte Luce (S9), that is well preserved.

The Conservation Zone of Mandena (24°57' S, 47°0' E), 11 km North-West of Fort Dauphin, is located on sandy soils at an altitude 0–20 m above sea level. Our study was conducted in M15 and M16 fragments (about 148 ha of "fairly degraded" littoral forest), included in the Conservation Zone. The two fragments are surrounded by a swamp (approximately 82 ha), that collared lemurs use for daily activities such as travelling, resting and feeding, but also for drinking during the hottest periods. Thus, we considered the two fragments (M15 and M16) and the swamp as a single area of about 240 ha, included in the Conservation Zone. The average canopy height in M15 and M16 is 8.9 ± 4.4 m (Rabenantoandro et al. 2007). In addition, some groups of *Eulemur collaris* use to live in another smaller fragment not included in the Conservation Zone: M20, that is approximately 6 ha of "heavily degraded" forest, and is located North-East of the other two fragments (see Ganzhorn et al. 2007 for fragments areas). Red-collared lemurs use especially the swamp that links M20 with the other fragments. This swamp (about 40 ha) is out of the Conservation Zone and local people use it to pick up fruits, gather dead woods and cut trees. In addition to *Eulemur collaris*, four nocturnal lemurs (*Microcebus murinus*, *Cheirogaleus medius*, *Cheirogaleus major*, *Avahi meridionalis*), and one cathemeral lemur species (*Hapalemur meridionalis*) are found in this area.

The second study site, the littoral forest of Sainte Luce (24°46'S, 47°10'E), around 30 km North of Fort Dauphin, is among the most intact littoral ecosystems in Madagascar and possesses a very high vegetation diversity (Bollen and Donati 2006). Our study fragment was S9, about 252 ha of "well preserved" littoral forest and swamp, 190 of which are included in the Conservation Zone. *Eulemur collaris* still occur in S17, an "almost intact" fragment of 237 ha, all included in the Conservation Zone. We studied only groups in S9 because in S17 there are no tracks and there is not a biological station as in S9. The average canopy height in S9 is 14.7 ± 4.3 m (Rabenantoandro et al. 2007). In addition to *Eulemur collaris*, four

nocturnal lemurs (*Microcebus murinus*, *Cheirogaleus medius*, *Cheirogaleus major*, *Avahi meridionalis*), are present in S9. *Hapalemur meridionalis* is not present in Sainte Luce because the swamp, that is vital for this critical species, is smaller than in Mandena.

Floristically Mandena and Sainte Luce littoral forests are very similar, suggesting that these two areas were once connected. However, structural differences indicate that at the time of study, the forest of Mandena represents a degraded form of the vegetation type in Sainte Luce. This deduction is also suggested by the disappearance of some tree families known to be logged in Mandena but not in Sainte Luce (Rabenantoandro et al. 2007).

2.2 Subjects

Individuals of red-collared brown lemurs presently living in the Mandena forest fragments have been translocated in M15 and M16 between 2000 and 2001 (Donati et al. 2007a) from other littoral forest fragments (M3 and M4), threatened by charcoal makers (Fig. 2.2). M15 and M16 in the meantime became an effectively protected area.
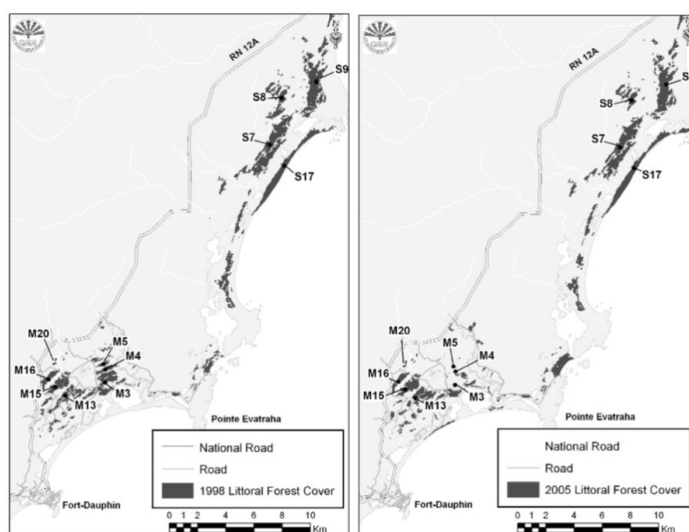

Fig. 2.2: Forest fragments in Mandena and Sainte Luce and extent of littoral forest reduction between 1998 and 2005 (Donati et al., 2007b).

At the moment of translocation the animals were 28, resulting in a density of 12 individuals per Km^2 (Donati et al. 2007b). During our study period the population of red collared brown lemurs at Mandena was about 12-17 animals, resulting in a density of 4.8-6.8 individuals per km^2. The cause of this reduction of population density is still unknown. It may be due to predation, scarcity of food resources, hunting for food by local people or to animal migration towards other forest fragments out of the Conservation Zone.

In this study we analysed hormonal data collected from adults (n=19) in four groups: two groups at Mandena (group AB and group C), and two groups at Sainte Luce (group A and group B). To ensure continuos observations of the groups, four animals (one for each group) were captured in order to install radio-tracking collars, to be monitored by the use of a SIKA receiver (Biotrack). The adult individuals were captured by caging them, using banana slices as a bait, and rapidly anesthetized with Zoletil 100 (5 mg/kg of tiletamine hydrochloride) to prevent trauma. Morphometric measurements were taken and then thermo-sensitive collars TW-3 (medium mammal tag) were fixed. All animals recovered from anaesthesia within 1.5 hours and were not moved from the capture area nor kept in a cage, but were followed until regaining full mobility. There were no injuries as a consequence of the captures. Individuals were identified by observing radio-collars and some characteristics of the animals such as age, sex, size, canine length, tail shape, fur colour, and distinctive traits.

Group composition changed over time (Table 2.1): unfortunately, on February 2011 a female infant of group A died. In group B one adult male and one adult female (Fotsy and Rano) left the group in September, we did not know if they died (maybe for hunting or predation), or if they left the group probably due to overcrowding. Also in group C one sub-adult female (Tina) left the group in September. Tina was collared and we found her with a male, so in this case we were sure that she was alive. Probably, it is not by chance that she left the group a short time before the birth season and after the mating season. One adult male (Eddy) chased

away from group AB on June (after a battle with the dominant male of the group, Abelardo), joined group C in September after a period spent alone. Infants were born between the end of September at Mandena (Barabarà, the female of the group AB, gave birth to the first infant, Dodò, on September 22^d, while another infant in Group C, Olivia, was born on October 4th). At Sainte Luce infants were born at the beginning of October (since we were not present we can only argue that all infants were born between September 31st and October 10th). Every adult female in our groups gave birth to at least one infant, and two females in group A gave birth to two twins (Volana one male and one female, Ala two females).

Group ID	# adult females	# adult males	# Infants And juveniles	# Immigration (adults)	# emigration	# Deaths	# Faecal samples
A	3	4	6	0	0	1	84
B	3	3	5	0	2[1]	0	69
AB	1	2	1	0	1	0	69
C	1	2	2	1	1	0	57
Total	8	11	14	1	2-4	1-3	279

[1] Emigrated or died

Table 2.1: Group Composition. Demography for groups in Sainte Luce (Group A and B) and Mandena (Group AB and C) throughout the study period (February 2011-January 2012).

2.3 Behavioral observations

The study period was from February 2011 to January 2012. From February 2011 to July 2011 data were collected by Marta Barresi and Valentina Serra. I collected data with Marco Campera, and on July 2011 data was collected by the two team pairs to evaluate inter-observer differences.

Habituated groups from each site were followed four days per month and one group per site was followed one night per month. Overall, 962 diurnal observation hours at Mandena were compared with 1118 hours at Sainte Luce, while the amount of nocturnal observations was 260 hours, 134 h at Mandena and 126 h at Ste Luce. Group C observations started from June 2011 due to the impossibility to follow this group into the swamp during wet periods, because of the high water level of the marsh. The groups were followed from 6:00 to 18:00 during each observation day and from 18:00 to 6:00 during each observation night. We recorded behavioural data concerning animal activity: resting, moving, feeding, social interactions and anti-predator strategies. Social interactions were separated in three categories: associative, affiliative, and agonistic behaviours. On the bases of social interactions the dominance relationships among animals were evaluated. The result of this analysis were used to calculate animal ranks (see table 2.3, paragraph 2.9).

During nocturnal observations we followed the general activity of the group using a very basic ethogram, with the only voices of resting, moving and feeding. We also recorded resting/huddling postures. Feeding and resting trees were identified and the diameter at breast height (DBH) was measured. Every half hour the position of the animals was recorded by GPS (Garmin eTrex Legend HCx). Data on feeding ecology, time budgets, thermoregulation, sociality and home ranges were analysed by Marco Campera and Valentina Serra in their MS dissertations.

2.4 Faecal samples collection

We collected faecal samples from each site over the whole study period. A total of 537 faecal samples were collected in parallel with behavioural observations (Fig. 2.3). Due to the mainly frugivouros diet of *Eulemur collaris* most of the faecal samples contained seeds. For the first

set of faecal samples (from February 2011 to July 2011), we did not take to account the fact that the faecal weights were compromised by the presence of different amounts of seeds.

Fig. 2.3 : Faecal samples collected at both sites.

The hormone concentrations are related to the dry faecal weight. If the latter is influenced by variable amounts (and thus weights) of seeds, this will affect the faecal hormone concentration per faecal weight in an uncontrollable manner and, if seed weight is high, also to a great extent. Principally, the margin of error depends on the relative proportion that seed weight has on the total faecal weight and this may obscure real hormone levels and may lead to wrong interpretations of the hormonal data. For this reason we decided not to consider the first set of sample, even if we had the possibility to analyse 30 samples of the first set (we removed seeds by tweezers when faeces were completely dry and then we determined the real faeces weight; we did the same process with all the samples of the second set, from August 2011 to January 2012).

A total of 279 faecal samples (Table 2.2; from 2 to 25 per animal; Rano, a female of group B, was excluded as we did not have her faecal samples), were used for the statistical analysis as

we had the real faeces weight. The faecal samples were collected immediately after defecation: site, group, date, time, and identify of the donor were recorded. Faecal samples were preserved in 10 ml tubes with 96% ethanol, until hormonal analysis. Ethanol was added at the end of diurnal observations. Faecal samples were stored at room temperature for one year until processing, we therefore performed the storage effect test (see paragraph 2.8).

Animal ID	Group ID	# Samples gestation season	# Samples Lactation season	# Samples Mating season	# Samples non-reproductive season	# Samples lean season	# Samples abundance season	# Samples total
Ala	A	4	7	1	2	8	6	14
Chefo be	A	3	5	0	0	5	3	8
Fana	A	7	6	0	3	9	7	16
Masoandro	A	6	7	2	1	12	4	16
Rivotra	A	2	5	2	1	6	4	10
Sotro	A	5	4	0	2	6	5	11
Volana	A	3	4	0	2	4	5	9
Alina	B	2	1	1	1	4	1	5
Fotsy	B	2	0	0	0	2	0	2
Kintana	B	4	5	0	1	5	5	10
Lanitra	B	8	8	0	1	12	5	17
Nify	B	9	7	0	1	12	5	17
Omby	B	1	6	0	0	3	4	7
Shielo	B	5	5	0	1	7	4	11
Abelardo	AB	14	6	1	3	18	6	24
Barabarà	AB	11	10	0	4	16	9	25
Octavio	AB	9	7	1	3	13	7	20
Apollo	C	6	5	2	2	11	4	15
Eddy	C	2	4	0	1	3	4	7
Filippo	C	6	7	1	2	11	5	16
Mamiska	C	5	7	1	2	9	6	15
Tina	C	3	0	1	0	4	0	4
Total	C	117	116	13	33	200	99	279

Table 2.2 number of faecal samples for each individual in reproductive and phenological seasons

I personally previously processed and extracted all faecal samples using the protocol (see Appendix I) described by Heistermann et al. (1995). Briefly, each faecal sample was extracted with 3 ml of 80% ethanol by vortexing for 15 minutes. Following centrifugation of the faecal suspension, the supernatant was recovered and stored at -20°C until a hormone analysis was performed. Faecal extracts were then sent to the "Department of Reproductive Biology" of the "German Primate Center" (Göttingen, Germany) where Dr. Michael Heistermann and his staff performed the second part of the procedure to obtain GCM concentrations (as described in paragraph 2.8).

2.5 Validation of faecal glucocorticoids measurement

Since for *Eulemur collaris* no method was available for assessing adrenocortical function using faecal hormone analysis, we determined the validity of four glucocorticoid assays, which have recently applied successfully to monitor glucocorticoid output in several other primate species (Heistermann et al. 2006; Fichtel et al. 2007; Ostner et al. 2008). The assays were a cortisol assay, a corticosterone assay (CCST) and two assays measuring groups of major cortisol metabolites (3a,11ß-dihydroxy-CM and 3a, 11oxo-CM). The latter two group-specific assays were used most successfully for monitoring stress hormone output in many primate taxa, ranging from lemurs to great apes (see Heistermann et al. 2006).

A biological validation was performed on three captive animals (one adult male, one adult female, and one sub-adult) at the "Cotswold wildlife park & gardens" in Burford (UK). The stress event consisted in following the animals for ten minutes and catching them with a net. During the pre-event period (five days) the zoo-staff collected one samples per day per animal. During the 3 days after the event they collected one sample per animal in the morning

and one in the afternoon. During the 4th and 5th day after the event the staff collected one sample per animal per day.

2.6 HPLC analysis

HPLC analysis was performed on faecal extracts of a male and female red-collared lemur as described in detail by Heistermann et al. (2006) to obtain information on the characteristics of metabolites measured in the $3\alpha,11$oxo-CM assay and in the $3a,11\beta$-dihydroxy-CM assay to help towards a decision which of the two group-specific GC assays we should use for analysis of our samples.

2.7 Glucocorticoid metatabolites (GCM) analysis

For measurement of $3a,11$ oxo-CM levels, faecal extracts were diluted (1:20 to 1:600 depending on concentration) with assay buffer (0.04 M PBS, pH 7.2) and duplicate 50 µl aliquots were measured by microtiterplate EIA along with 50 µl aliquots of reference standard in doubling dilutions over the range of 1.02-125 pg (Heistermann et al. 2006). Briefly, following incubation of the plates overnight at 4 °C, the plates were washed three times and incubated with 150 µl streptavidin–peroxidase (HRP) for 30 min in the dark at room temperature after which (following a second washing step) 150 µl of HRP-substrate solution was added to each well. Following substrate incubation (45 min), the enzyme reaction was stopped with 50 µl 2 M H_2SO_4 to each well and absorbance was measured at 450 nm (reference 630 nm) on a plate reader. Sensitivity of the assay was 3 pg. Specificity data (cross-reactivities) of the assay are reported in Möstl et al. (2002). Intra-assay coefficients of variation (CV) for low- and high value quality controls were 7.7% and 6.9%, respectively.

Respective figures for inter-assay CV values were 10.3% and 14.5%. All hormone levels reported are expressed as ng/g dry faecal mass.

2.8 Evaluation of a possible Storage effect

To evaluate if the storage of red-collared brown lemur faecal samples on ethanol potentially affected GC concentrations as demonstrated for other species (Khan et al. 2002; Hunt and Wasser 2003), we collected other 12 faecal samples and divided each samples into three aliquots and stored them in ethanol as described above. Aliquots were kept at field station at ambient temperature before being transported to the laboratory for hormone analysis. The first aliquot of the samples was extracted after 3 months, while the other two aliquots were extracted after 6 and 12 months respectively (relative to their time of collection) because time period to test should cover the maximum period that samples were now stored in ethanol. After each extraction, extracts were stored at -20°C before all extracts were analysed at the end of the experiment.

2.9 Data analysis

For each individual we calculated average values for each reproductive stage (Gestation season: from July to September; Lactation season: from October to December; Non reproductive season: from January to April; Mating season: from May to June) and for each phenological season (Lean season: from May to October; Abundance season: from November to April). We made the distinction between lean and abundance periods on the basis of previous long term studies in our study sites (Bollen and Donati 2005; Randriahispara, unpub. data). We considered differences between sites, sexes and ranks. Ranks were analysed by

Valentina Serra (Serra unpub. data; Table 2.3). Mamiska (female of group C) was considered high ranking, in spite of the fact that David's score (DS; Gammel et al. 2003) in this case gave a low value (Mamiska resulted the highest ranking female by analysing attack frequencies). Statistical comparisons were made by repeated measures ANOVA, with Tukey's HSD post hoc analysis. We tested for normality (Kolmogorov-Smirnov test), sphericity (Mauchly's test) and equality of variances (Levene's test) as underlying assumptions. All analyses were performed using STATISTICA for Windows, version 8.0, and we considered $p<0.05$ as the significant level.

ANIMAL	SEX	DS	RANK	H/L
Group A (STL)				
Sotro	Male	2,7	7	H
Ala	Female	2,5	6	H
Chefo Be	Male	2	5	H
Rivotra	Male	1,93	4	H
Volana	Female	0,37	3	H
Fana	Female	-4,71	2	L
Masoandro	Male	-4,77	1	L
Group B (STL)				
Fotsy	Male	8,35	8	H
Lanitra	Female	7,75	7	H
Nify	Male	5,25	6	H
Alina	Female	1	5	H
Rano	Female	-2,75	4	L
Omby	Male	-2,5	3	L
Kintana	Female	-7	2	L
Shielo	Male	-10,1	1	L
Group AB (MAN)				
Barbarà	Female	2,32	4	H
Abelardo	Male	2,16	3	H
Eddy	Male	0	2	L
Octavio	Male	-4,48	1	L
Group C (MAN)				
Filippo	Male	5	5	H
Mamiska	Female	0	3	H
Tina	Female	0	3	L
Eddy	Male	0	3	L
Apollo	Male	-5	1	L

Table 2.3 : individual ranks for each adults in study groups at Mandena (MAN) and Sainte Luce (STL). Ranks obtained by David Score (DS) evaluated by data collected during the study period.

CHAPTER 3

RESULTS

3.1 Validation of faecal glucocorticoid measurements

In each assay (Figure 3.1; 3.2; 3.3), most post-stress GCM concentrations were in the same range as the pre-stress GCM levels (indicated in the graphs as red dots with error bars representing means plus/minus SD) with no clear elevation seen. The only clear elevations found were in the first post-stress sample in the female (Figure 3.1) and the juvenile male (Figure 3.3).
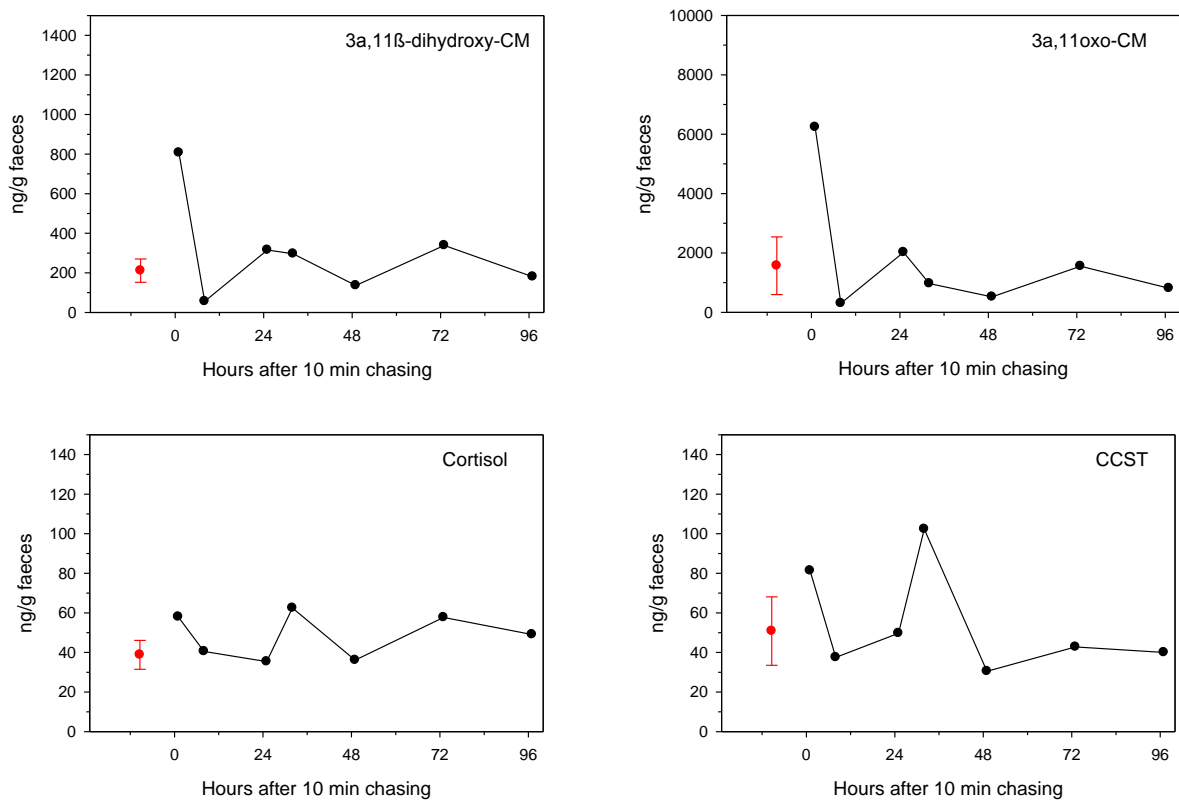

Fig. 3.1: Faecal GCM concentrations in response to stressful chasing in an individual female *Eulemur collaris*. Red dots with error bars represent pre-stress GCM levels (mean±SD).
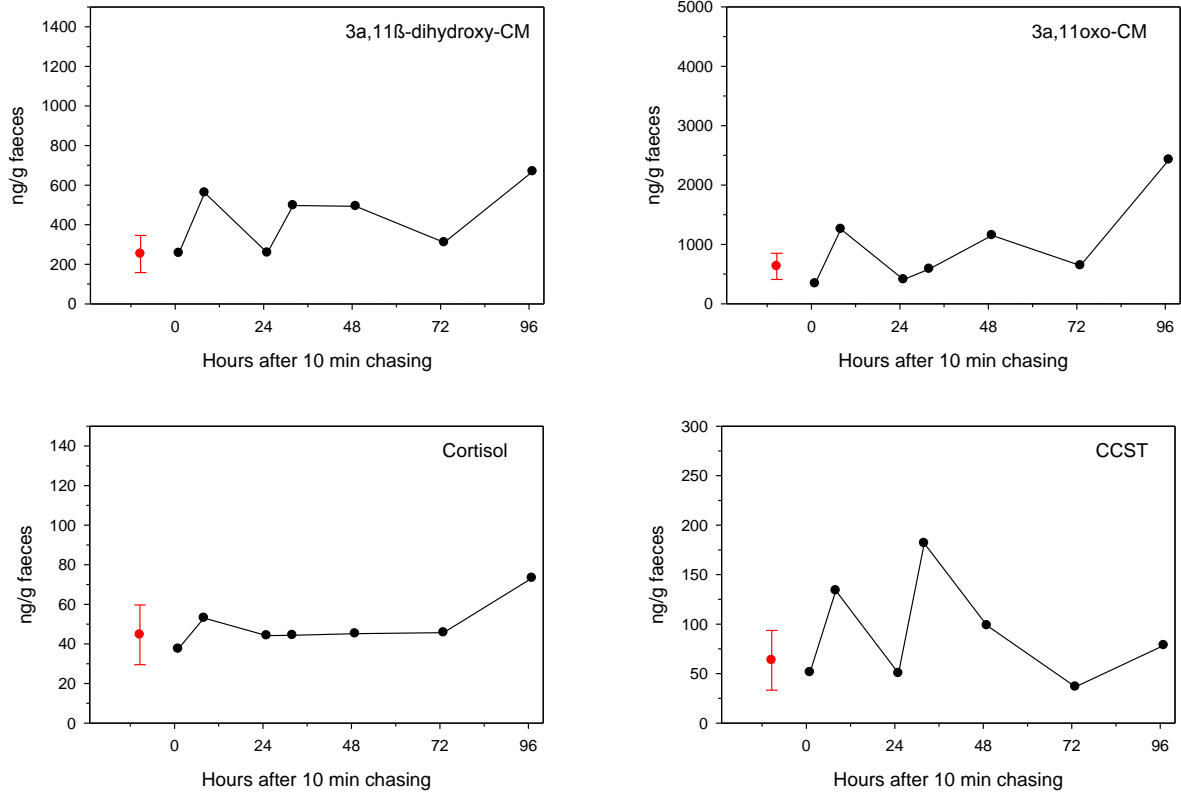
Fig. 3.2: Faecal GCM concentrations in response to stressful chasing in an individual male *Eulemur collaris.* Red dots with error bars represent pre-stress GCM levels (mean±SD).
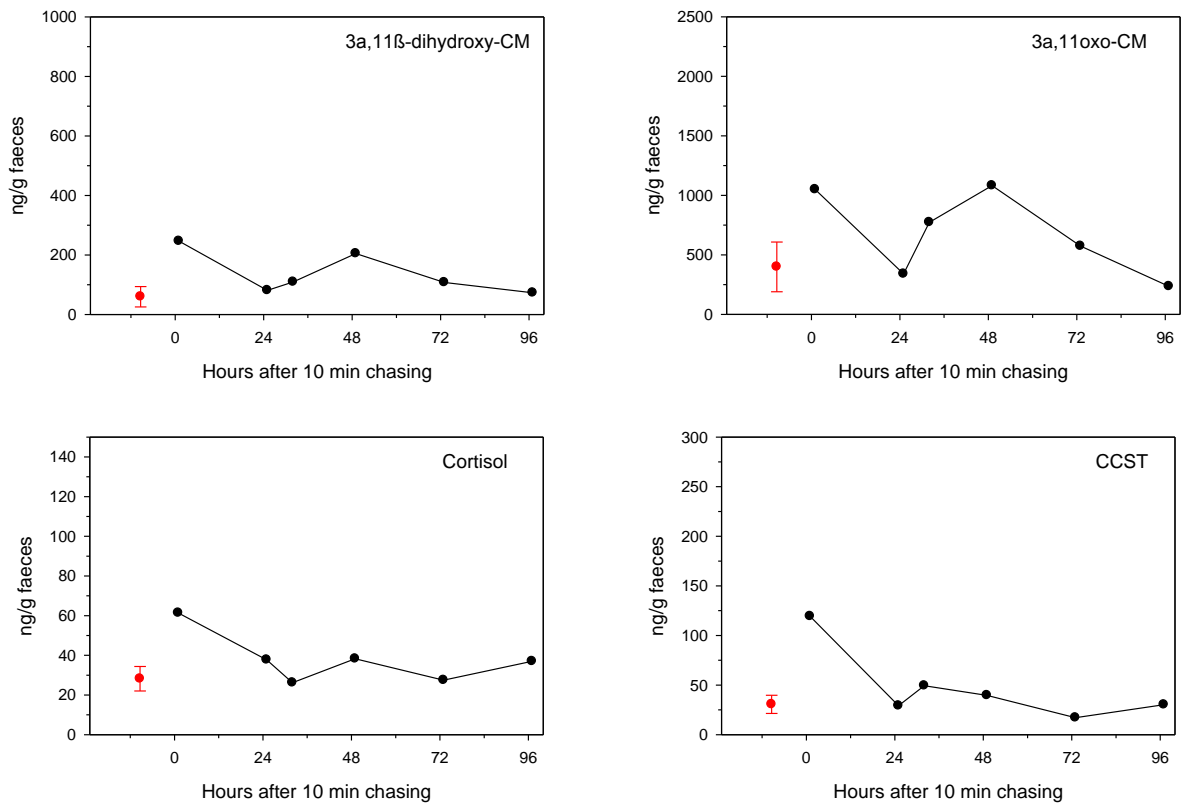

Fig. 3.3: Faecal GCM concentrations in response to stressful chasing in an individual juvenile male *Eulemur collaris.* Red dots with error bars represent pre-stress GCM levels (means±SD).   39

We found no significant difference between the pre-stressor and the post-stressor GCM levels in the three individuals by using the four assays (Fig. 3.4).
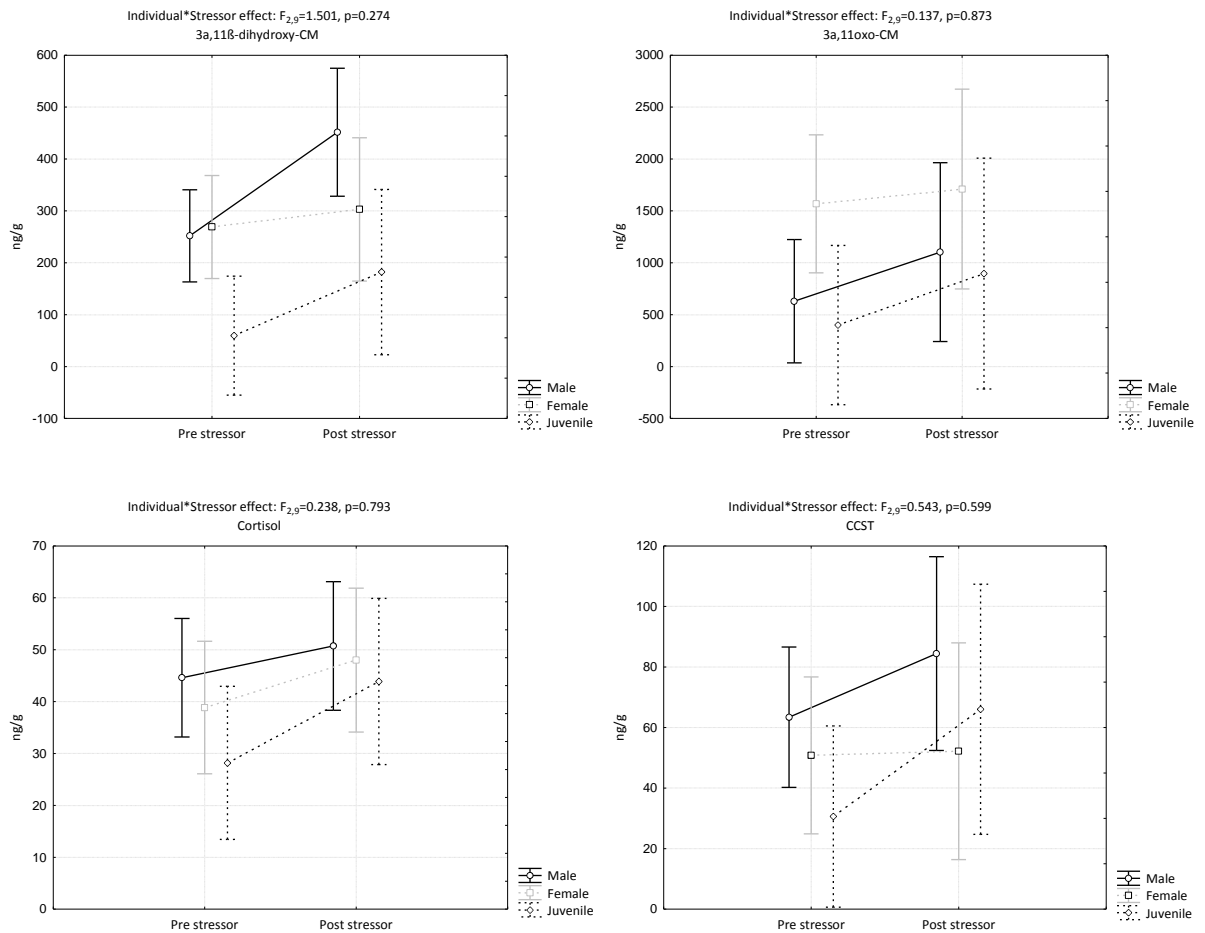

Fig. 3.4: Pre and post stressor GCM levels (means and standard deviations) for each individuals evaluated by "3a,11β dihydroxy-CM", "3a,11 oxo-CM", "Cortisol" and "CCST" assays. Results of repeated measures ANOVA are shown for each assay.

We found differences in GC levels between individuals only using the 3a,11β dihydroxy-CM assay (Figure 3.5). In this case we found that the GCM levels of the juvenile male was lower as compared to the GC levels of both the adult male and female (Table 3.1), even though the difference with the adult female was not significant.
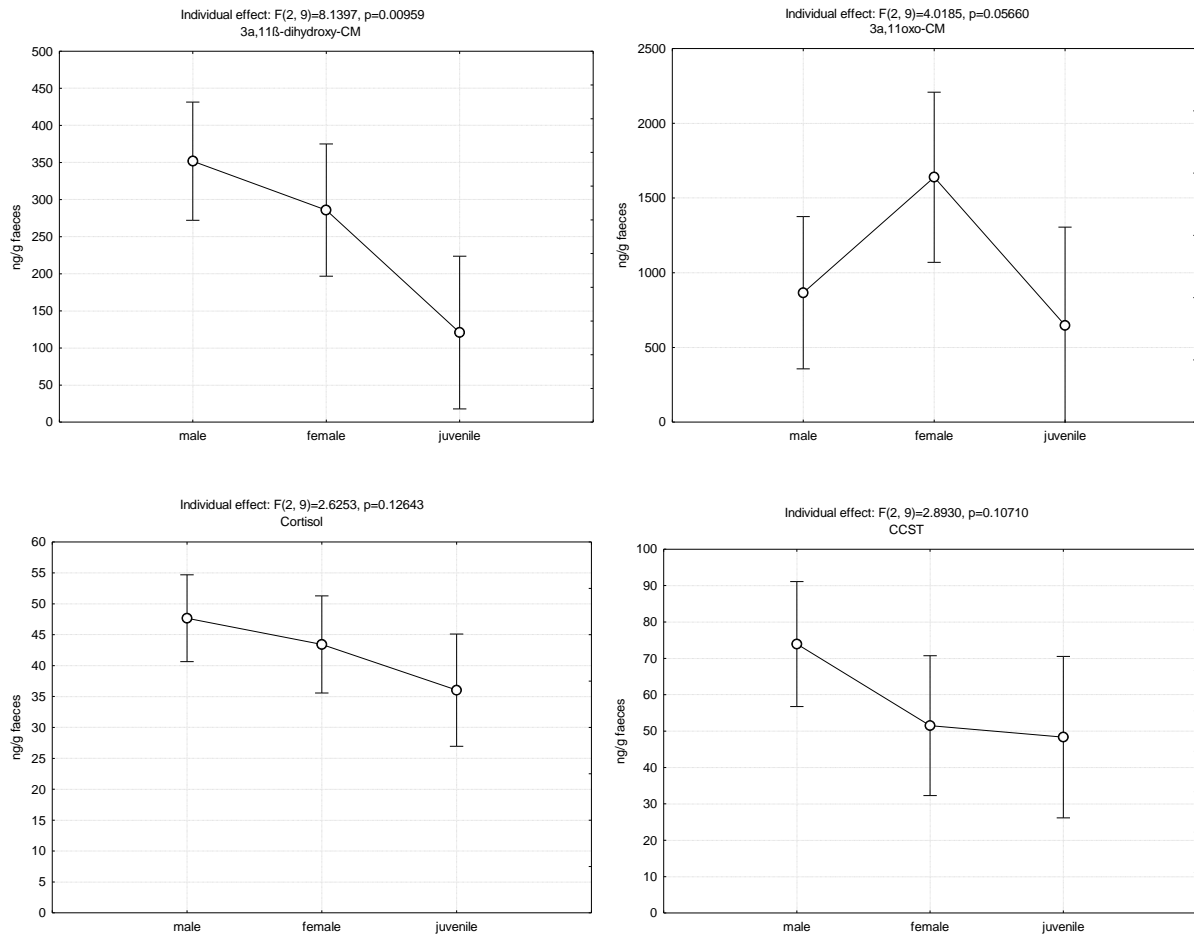
Fig. 3.5: GCM levels (means and standard deviations) for each individual evaluated by "3a,11β dihydroxy-CM", "3a,11 oxo-CM", "Cortisol" and "CCST" assays. Results of repeated measures ANOVA are shown for each assay.

Individual	Male	Female	Juvenile
Male	-	-	-
Female	0.457	-	-
Juvenile	**0.008**	0.054	-

Table 3.1: P values of pair-wise comparison of mean differences in GCM levels across different individuals

3.2 HPLC analysis

HPLC profiles (Fig. 3.6) suggest that the 3a,11oxo-CM assay might be potentially better suited for monitoring GC output in *Eulemur collaris*. In fact, the data show that the 3a,11oxo-

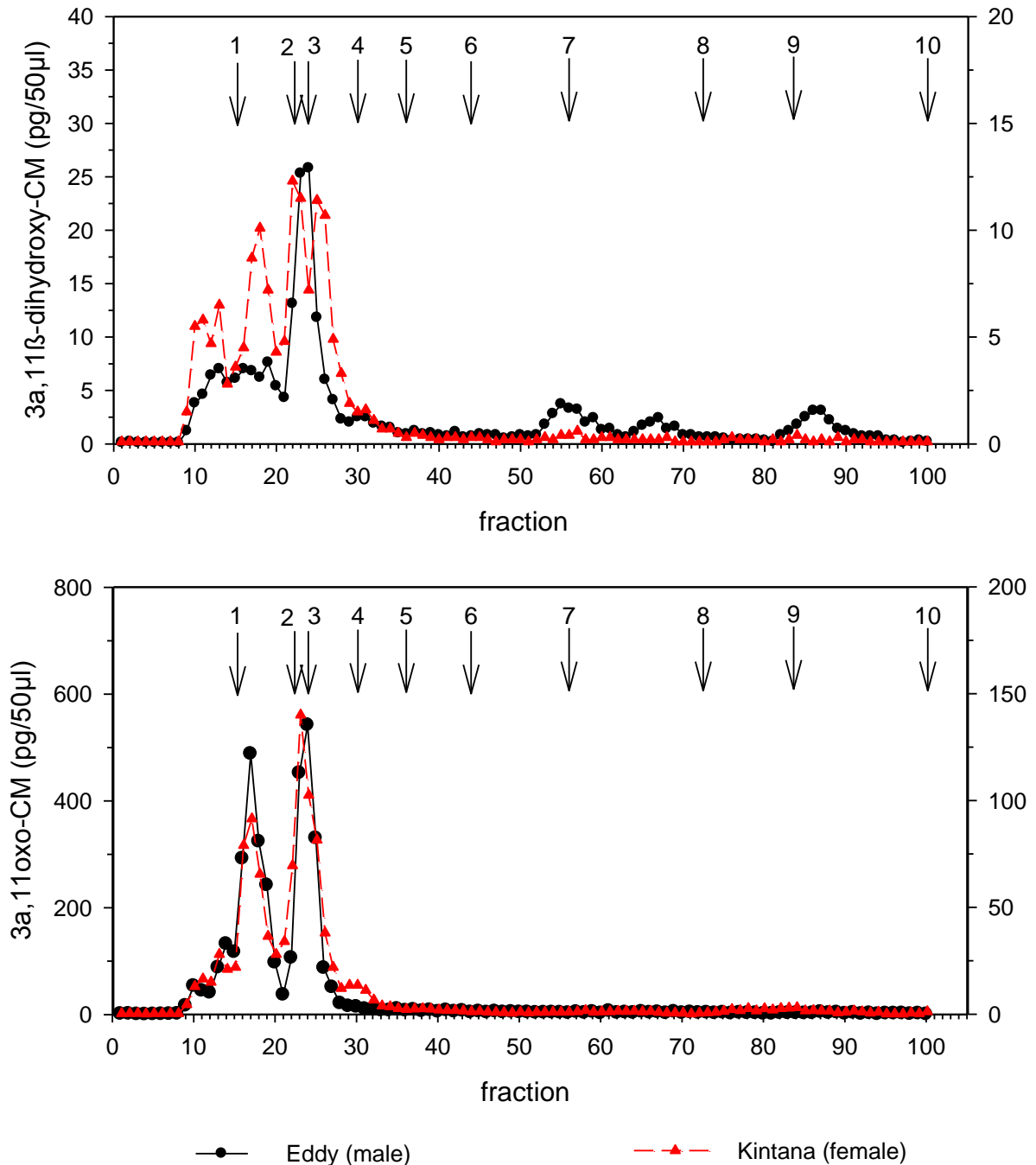CM assay shows higher levels of immunoreactivity compared to the 3a,11ß-dihydroxy-CM assay.

Fig. 3.6: HPLC profiles of immunoreactivity detected with the 3a,11oxo-CM assay (above) and the 3a,11ß-dihydroxy-CM assay (below). Arrows indicate elution positions of reference standards (1) cortisol (fraction 15), (2) corticosterone (22), (3) 11β-hydroxyetiocholanolone (23/24), (4) 11-oxoetiocholanolone (29), (5) βandostrane-3,11,17-trione (36), (6) testosterone (44), (7) androstendione, dehydroepiandrosterone (55/56), (8) epiandrosterone, 5β-DHT, 5β-androstrane-3β-ol-17-one (72), (9) 5β-androstrane-3α-ol-17-one (82/83), (10) androsterone (100).

3.3 Storage effects on faecal GCM levels

For the glucocorticoid measurement we did not found a significant effect of storage duration on faecal hormone levels both using the 3a,11oxo-CM essay (Figure 3.7) and using the 3a,11ß-dihydroxy-CM essay (Figure 3.8).
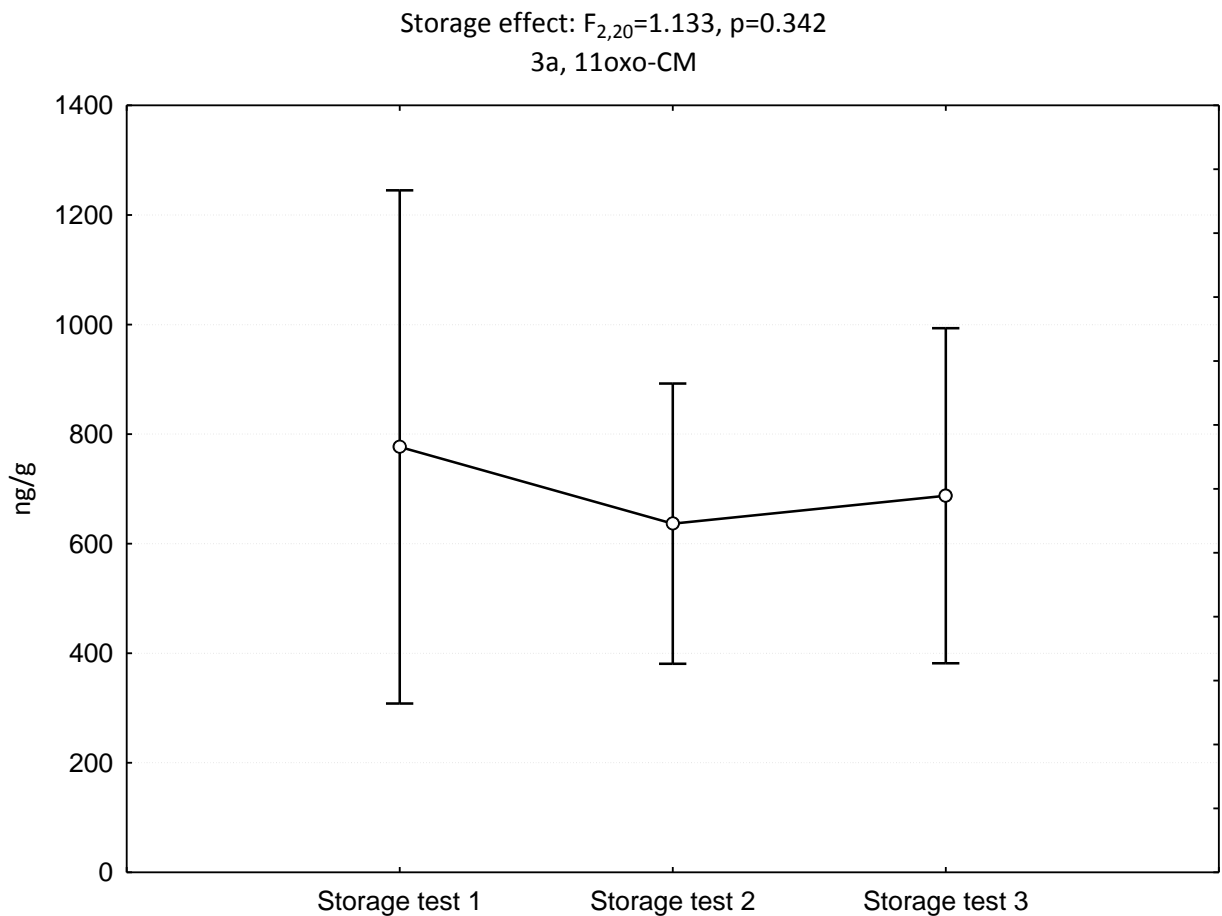

Figure 3.7: Effect of storage of faecal samples in ethanol on GCM levels evaluated by the 3a,11oxo-CM essay. Values are in means and standard deviations. Above: results of repeated measures ANOVA.
Storage test 1: after 3 months; Storage test 2: after 6 months; Storage test 3: after 12 months.

Figure 3.8: Effect of storage of faecal samples in ethanol on GCM levels evaluated by the 3a,11ß-dihydroxy-CM assay. Values are in means and standard deviations. Above: results of repeated measures ANOVA.

Storage test 1: after 3 months; Storage test 2: after 6 months; Storage test 3: after 12 months.
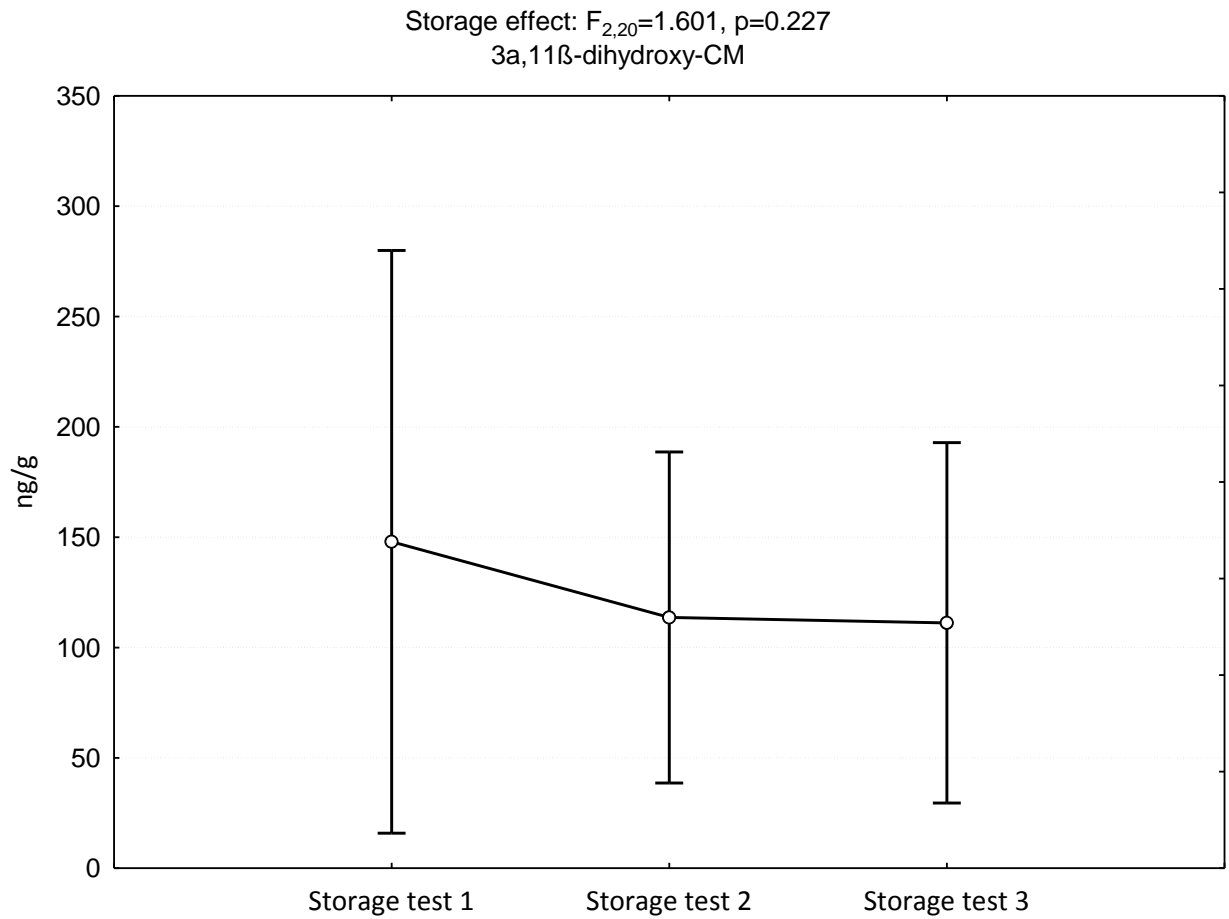
In Figure 3.9 we see that there was no significant variation between GCM levels of the three aliquots during one year.
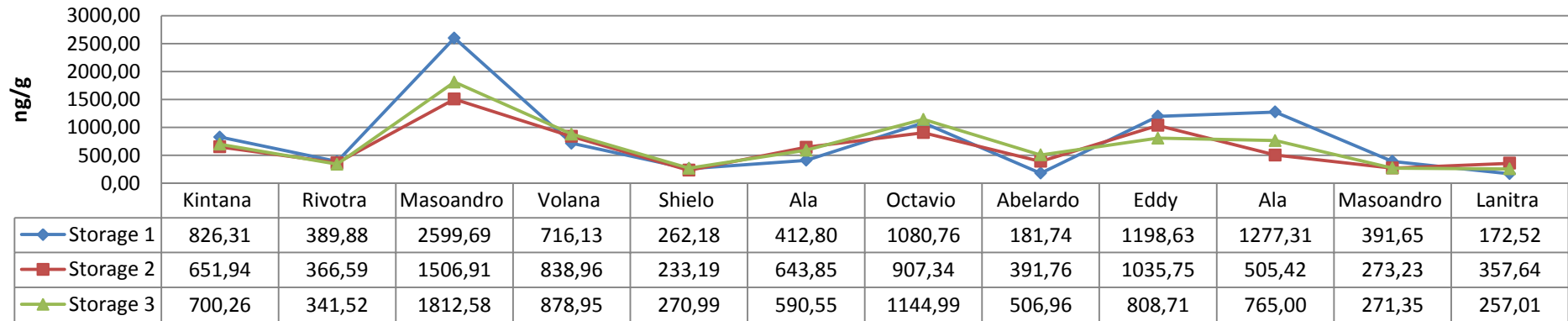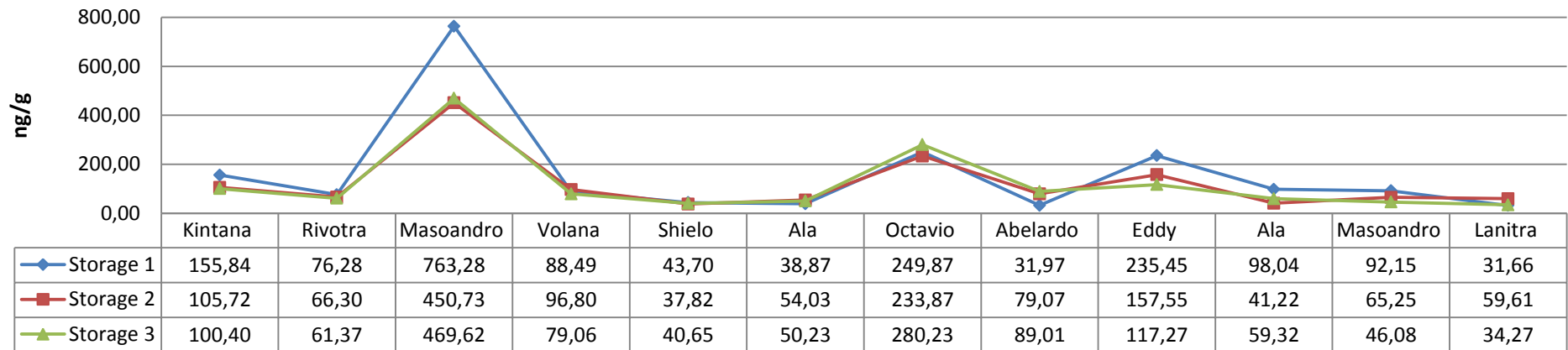
3a, 11oxo-CM

	Kintana	Rivotra	Masoandro	Volana	Shielo	Ala	Octavio	Abelardo	Eddy	Ala	Masoandro	Lanitra
Storage 1	826,31	389,88	2599,69	716,13	262,18	412,80	1080,76	181,74	1198,63	1277,31	391,65	172,52
Storage 2	651,94	366,59	1506,91	838,96	233,19	643,85	907,34	391,76	1035,75	505,42	273,23	357,64
Storage 3	700,26	341,52	1812,58	878,95	270,99	590,55	1144,99	506,96	808,71	765,00	271,35	257,01

Fig 3.9: Storage effect evaluated by variations of GCM levels (ng/g) measured by " 3a, 11oxo-CM" and "3a, 11β-dihydroxy-CM" assays, for each individuals after 3 months (Storage 1), 6 months (storage 2) and 12 months (storage 3).

3a, 11β-dihydroxy-CM

	Kintana	Rivotra	Masoandro	Volana	Shielo	Ala	Octavio	Abelardo	Eddy	Ala	Masoandro	Lanitra
Storage 1	155,84	76,28	763,28	88,49	43,70	38,87	249,87	31,97	235,45	98,04	92,15	31,66
Storage 2	105,72	66,30	450,73	96,80	37,82	54,03	233,87	79,07	157,55	41,22	65,25	59,61
Storage 3	100,40	61,37	469,62	79,06	40,65	50,23	280,23	89,01	117,27	59,32	46,08	34,27

3.4 Faecal GCM levels in relation to reproductive stages

GCM levels (Table 3.2) were not significantly affected by site after having controlled the four reproductive stages (Site effect: $F_{1,6}=2.860$, p=0.142), even though lemurs at MAN were more stressed (1250.40 ± 882.59 ng/g) than lemurs at STL (861.33 ± 520.30 ng/g). This difference was significant if we exclude the mating season (Site effect: $F_{1,14}=11.533$, p=0.004).

GCM levels differed between reproductive stages both including all the four reproductive stages (Reproductive stage effect: $F_{3,18}=6.502$, p=0.004) and excluding the mating season (Reproductive stage effect: $F_{2,28}=12.153$, p<0.001).

	Sainte Luce	Mandena
Gestation	N=14	N=8
Mean (±SD)	580.35 (±219.46)	1640.05 (±600.68)
Range	293.79-1122.28	875.03-2540.41
Lactation	N=13	N=7
Mean (±SD)	868.62 (±287.81)	766.38 (±199.84)
Range	440.50-1287.37	530.79-1168.75
Non reproductive	N=11	N=7
Mean (±SD)	830.26 (±511.57)	682.34 (±403.48)
Range	201.25-2089.07	152.28-1474.70
Mating	N=4	N=7
Mean (±SD)	1906.54 (±677.14)	1857.17 (±1315.17)
Range	1110.20-2515.21	676.18-3965.96

Table 3.2: GCM levels at Mandena and Sainte Luce during different reproductive stages. Values are in means, standard deviation and range. N=number of individuals with faecal samples available.

Post hoc analyses (Table 3.3) showed that this difference was between the mating and the lactation season, and also between the mating and non-reproductive season. On the whole, during the mating season, lemurs had the highest GCM levels (1875.12 ± 1084.43 ng/g),

followed by gestation (965.69±649.86 ng/g), lactation (832.84±259.67 ng/g) and non-reproductive season (772.73±465.73 ng/g).

Season	Gestation	Lactation	Non-reproductive	Mating
Gestation	-	-	-	-
Lactation	0.349	-	-	-
Non-reproductive	0.252	0.996	-	-
Mating	0.117	**0.004**	**0.002**	-

Table 3.3 P values of pair-wise comparisons on mean differences in GCM levels across different reproductive stages

When excluding the mating season, post hoc test suggested that the main difference was between gestation and non-reproductive season (Table 3.4).

Season	Gestation	Lactation	Non-reproductive
Gestation	-	-	-
Lactation	0.125	-	-
Non-reproductive	0.066	0.945	-

Table 3.4 P values of pair-wise comparisons of mean differences in GCM levels across different reproductive stages (mating season excluded)

Lemurs living in different sites do not had different GCM levels among reproductive stages (Figure 3.10) considering all of them (Reproductive stage* Site effect: $F_{3,18}=2.184$, $p=0.125$), while they had different GCM levels among reproductive stages excluding the mating season (Reproductive stage* Site effect: $F_{2,28}=24.548$, $p<0.001$). Post hoc analyses showed lemurs living at MAN had different GCM levels during the gestation season as compared to those of lemurs living at MAN during the lactation and the non-reproductive seasons, and to those of lemurs living at STL during gestation, lactation, and non-reproductive season (Table 3.5).
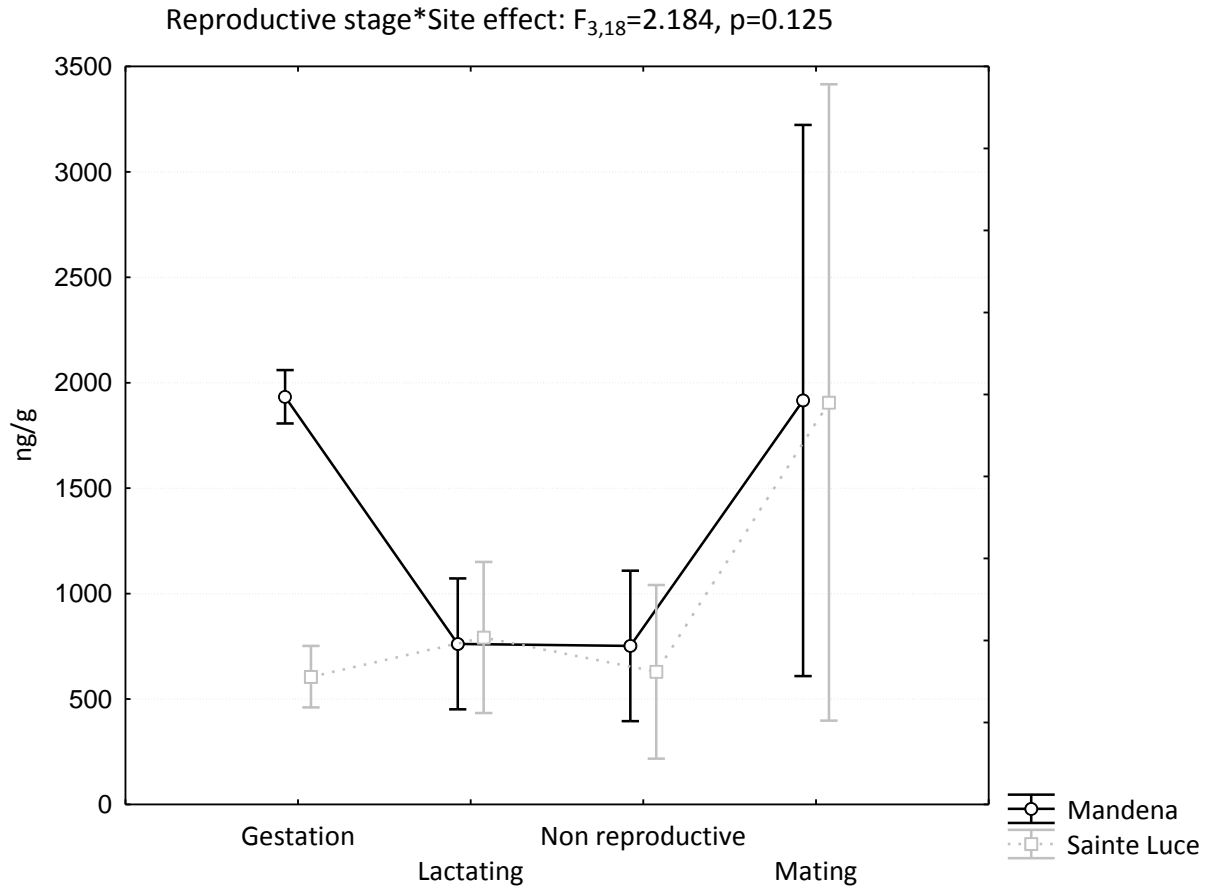
Figure 3.10: Site differences in GCM levels (ng/g) during the four reproductive stages. Values are in means and standard deviations. Above: results of repeated measures ANOVA.

Season	MAN Gestation	MAN Lactation	MAN Non-reproductive	STL Gestation	STL Lactation	STL Non-reproductive
MAN Gestation	-	-	-	-	-	-
MAN Lactation	**<0.001**	-	-	-	-	-
MAN Non-reproductive	**<0.001**	0.995	-	-	-	-
STL Gestation	**<0.001**	0.952	0.999	-	-	-
STL Lactation	**<0.001**	0.998	0.932	0.654	-	-
STL Non-reproductive	**<0.001**	0.999	0.933	0.657	1.000	-

Table 3.5: P values of pair-wise comparisons of mean differences in GCM levels across different reproductive stages (mating season excluded) at Mandena and Sainte Luce.

GCM levels were not significantly different between sexes (Table 3.6) considering the four reproductive stages (Sex effect: $F_{1,6}=0.098$, p=0.764), while, excluding the mating season, females were more stressed than males (Sex effect: $F_{1,14}=9.691$, p=0.008). Overall, females had higher GCM levels (1107.23±656.21 ng/g) than males (956.60±752.09 ng/g).

Males had not different GCM levels as compared to females lemurs among reproductive stages (Figure 3.11) considering all of them (Reproductive stage* Sex effect: $F_{3,18}=1.013$, p=0.410), while males had different GCM levels as compared to females among reproductive stage excluding the mating season (Reproductive stage* Sex effect: $F_{2,28}=7.228$, p=0.003). Post hoc analyses showed that females in gestation had higher GCM levels as compared to males during the non-reproductive season (Table 3.6).

	Males	Females
Gestation	N=13	N=9
Mean (±SD)	753.09 (±448.47)	1272.78 (±792.07)
Range	293.79-1484.73	559.98-2540.41
Lactation	N=12	N=8
Mean (±SD)	872.11 (±243.83)	773.91 (±288.07)
Range	530.79-1245.68	440.50-1287.37
Non reproductive	N=10	N=8
Mean (±SD)	611.22 (±399.89)	974.63 (±487.16)
Range	152.28-1474.70	552.16-2089.07
Mating	N=6	N=5
Mean (±SD)	2142.13 (±1254.53)	1554.71 (±858.70)
Range	769.46-3965.96	676.18-2515.22

Table 3.6: GCM levels between males and females during different reproductive stages. Values are in means, standard deviation and range. N=number of individuals with faecal samples available.
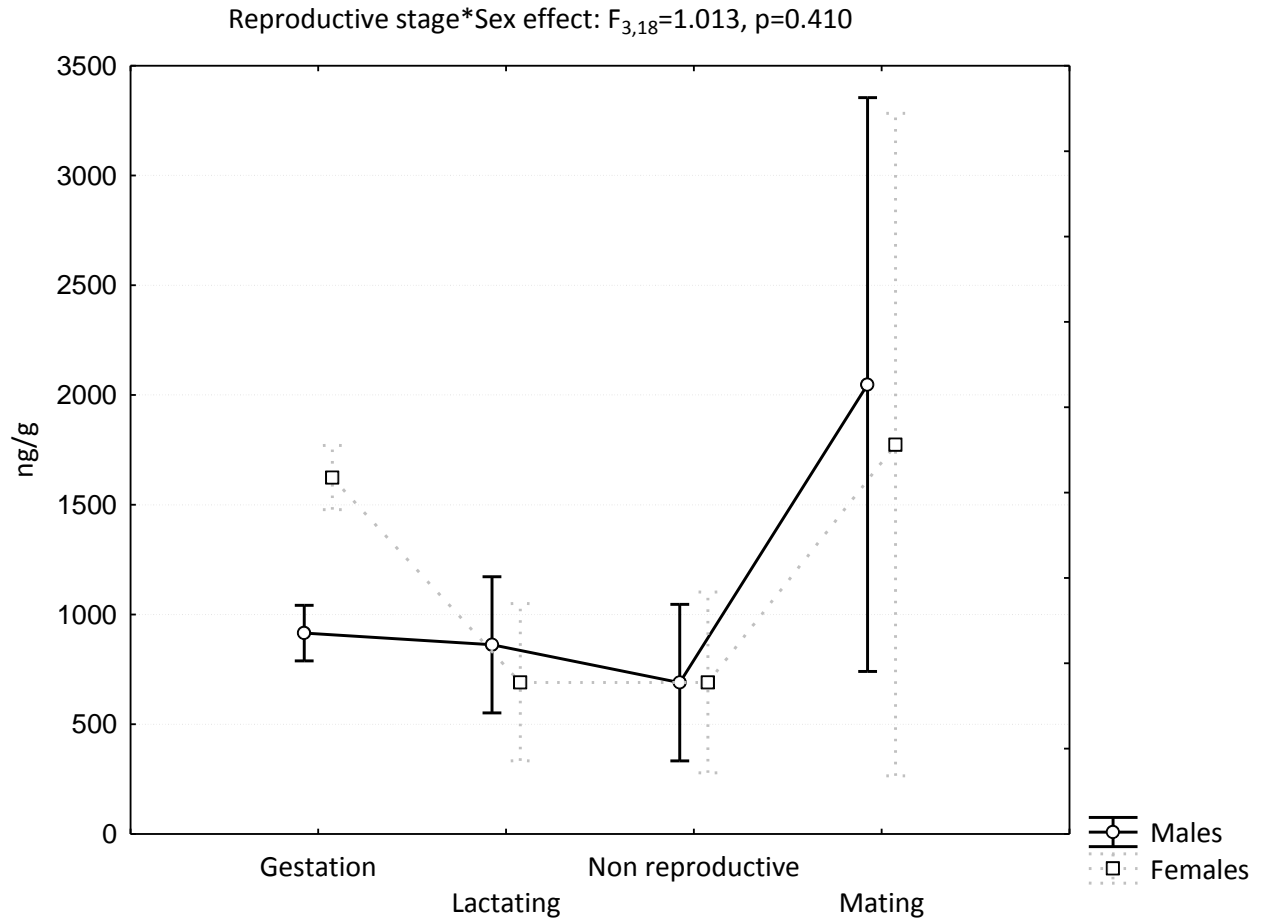
Fig 3.11: Sex differences in GCM levels (ng/g) during the four reproductive stages. Values are in means and standard deviations. Above: results of repeated measures ANOVA.

Season	Males Gestation	Males Lactation	Males Non-reproductive	Females Gestation	Females Lactation	Females Non-reproductive
Males Gestation	-	-	-	-	-	-
Males Lactation	1.000	-	-	-	-	-
Males Non-reproductive	0.482	0.610	-	-	-	-
Females Gestation	0.235	0.165	**0.005**	-	-	-
Females Lactation	0.993	0.999	0.895	0.086	-	-
Females Non-reproductive	0.975	0.936	0.193	0.664	0.783	-

Table 3.7: P values of pair-wise comparisons of mean differences in GC levels between males and females across different reproductive stages (mating season excluded).

Lemurs of both sexes, living in MAN, had not different GCM levels among the four reproductive stages as compared to lemurs of both sexes living in STL (Table 3.8) (Reproductive stage*Site*Sex effect: $F_{3,18}=0.915$, p=0.453) (Fig. 3.12).

SAINTE LUCE	Males	Females
Gestation	N=8	N=6
Mean (±SD)	436.17 (±94.69)	772.58 (±187.28)
Range	293.79-587.19	559.98-1122.28
Lactation	N=7	N=6
Mean (±SD)	938.34 (±239.47)	787.27 (±339.47)
Range	624.10-1245.68	440.50-1287.37
Non reproductive	N=5	N=6
Mean (±SD)	544.91 (±330.26)	1068.05 (±535.61)
Range	201.25-1053.12	552.16-2089.07
Mating	N=2	N=2
Mean (±SD)	1765.43 (±926.64)	2047.64 (±661.25)
Range	1110.20-2420.67	1580.06-2515.22
MANDENA	Males	Females
Gestation	N=5	N=3
Mean (±SD)	1260.16 (±255.73)	2273.18 (±412.31)
Range	875.03-1484.73	1798.33-2540.41
Lactation	N=5	N=2
Mean (±SD)	779.40 (±243.03)	773.85 (±20.20)
Range	530.79-1168.75	719.60-748.13
Non reproductive	N=5	N=2
Mean (±SD)	677.52 (±489.64)	694.37 (±131.85)
Range	152.28-1474.70	601.13-787.60
Mating	N=4	N=3
Mean (±SD)	2330.48 (±1481.54)	1226.09 (±922.57)
Range	769.47-3965.96	676.18-2291.19

Table 3.8: GCM levels between males and females during different reproductive stages at Mandena and Sainte Luce. Values are in means, standard deviation and range. N=number of individuals with faecal samples available.
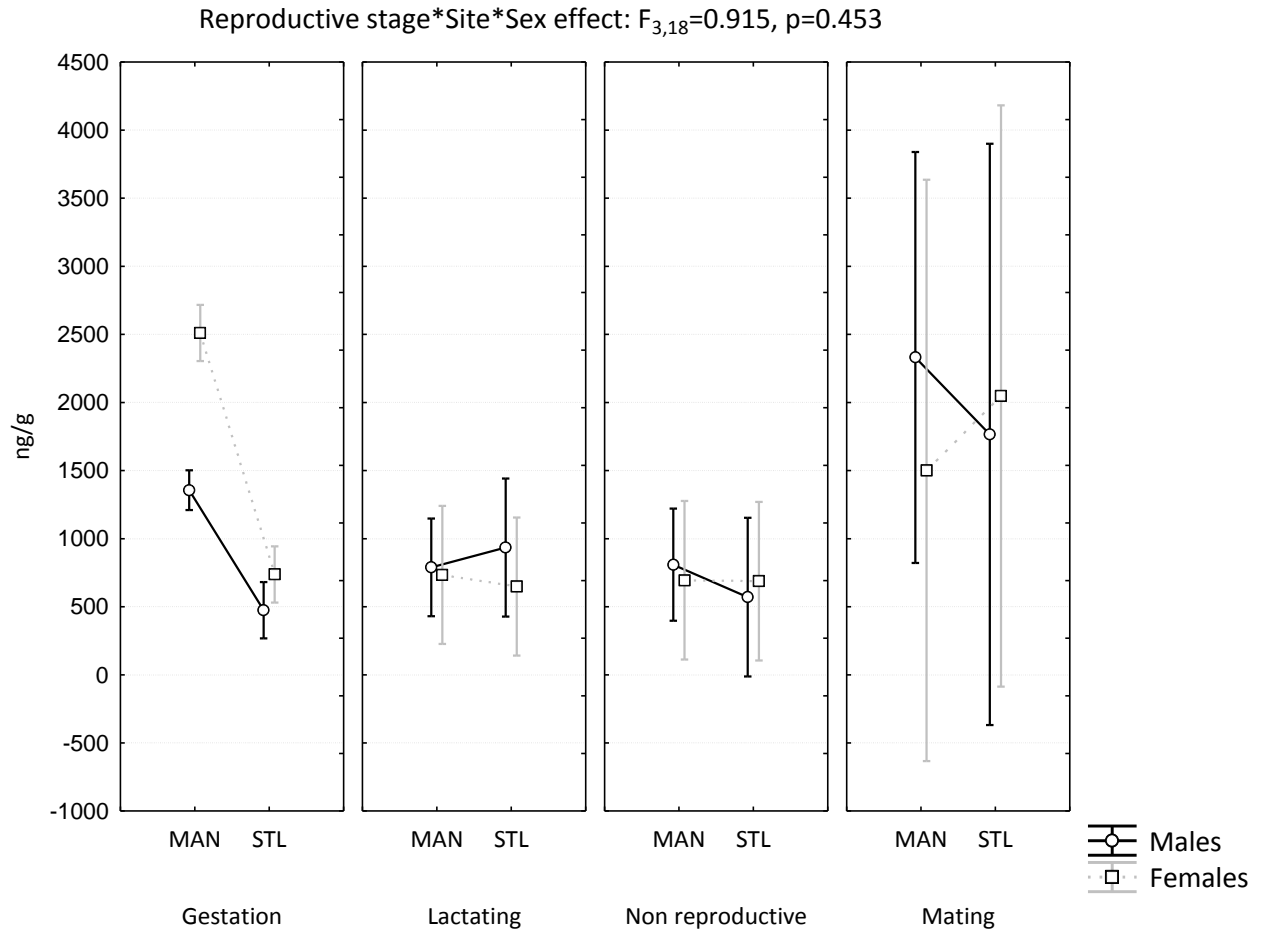
Fig 3.12: : Differences in GCM levels (ng/g) between males and females at Mandena and Sainte Luce during the four reproductive stages. Values are in means and standard deviations. Above: results of repeated measures ANOVA.

Lemurs of both sexes, living in MAN, had different GCM levels among reproductive stages, excluding the mating season, as compared to lemurs of both sexes living in STL (Reproductive stage*Site*Sex effect: $F_{2,28}=5.209$, p=0.012). Results of post hoc analyses are shown in Table (3.9). Males at MDN during the gestation season had higher GCM levels as compared to males at STL during the gestation season, and lower GCM levels as compared to females at MDN during the gestation season. Females at MDN during the gestation season had higher GCM levels as compared to both sexes at STL among the three reproductive stages, and as compared to both sexes at MDN during lactation and non-reproductive season.

Sex	Site	Season	M MAN GE	M MAN LA	M MAN NR	M STL GE	M STL LA	M STL NR	F MAN GE	F MAN LA	F MAN NR	F STL GE	F STL LA	F STL NR
M	MAN	GE	-	-	-	-	-	-	-	-	-	-	-	-
M	MAN	LA	0.402	-	-	-	-	-	-	-	-	-	-	-
M	MAN	NR	0.167	1.000	-	-	-	-	-	-	-	-	-	-
M	STL	GE	**0.016**	0.914	0.995	-	-	-	-	-	-	-	-	-
M	STL	LA	0.787	1.000	0.997	0.585	-	-	-	-	-	-	-	-
M	STL	NR	0.047	0.990	1.000	1.000	0.840	-	-	-	-	-	-	-
F	MAN	GE	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	-	-	-	-	-
F	MAN	LA	0.727	1.000	1.000	0.997	1.000	1.000	**<0.001**	-	-	-	-	-
F	MAN	NR	0.634	1.000	1.000	0.999	1.000	1.000	**<0.001**	1.000	-	-	-	-
F	STL	GE	0.377	1.000	1.000	0.902	1.000	0.989	**<0.001**	1.000	1.000	-	-	-
F	STL	LA	0.422	1.000	1.000	0.872	1.000	0.983	**<0.001**	1.000	1.000	1.000	-	-
F	STL	NR	0.997	0.940	0.697	0.117	0.998	0.279	**<0.001**	0.980	0.954	0.873	0.904	-

Table 3.9: P values of pair-wise comparisons of mean differences in GCM levels between males and females at Mandena and Sainte Luce across different reproductive stages (mating season excluded). Sex: M=males, F=females; Site: MAN= Mandena, STL=Sainte Luce; Season: GE=gestation, LA=lactating, NR=non-reproductive.

Dominant lemurs had not different GCM levels as compared to subordinate lemurs (Table 3.10) both considering the four reproductive stages (Rank effect: $F_{1,8}=0.049$, p=0.831) and excluding the mating season (Rank effect: $F_{1,16}=0.267$, p=0.613). GCM levels were 1044.37±718.92 ng/g in high ranking and 983.20±713.26 ng/g in low ranking lemurs.

	High rank	Low rank
Gestation	N=13	N=9
Mean (±SD)	981.68 (±746.16)	942.60 (±521.96)
Range	401.04-2540.41	293.79-1798.33
Lactation	N=12	N=8
Mean (±SD)	747.66 (±231.36)	960.60(±260.42)
Range	440.50-1158.81	552.85-1287.37
Non reproductive	N=11	N=7
Mean (±SD)	885.09 (±502.81)	596.17 (±365.64)
Range	328.74-2089.07	152.28-1117.93
Mating	N=7	N=4
Mean (±SD)	1919.75 (±927.24)	1797.03 (±1479.05)
Range	710.89-3150.74	676.18-3965.96

Table 3.10: GCM levels between high and low ranks during different reproductive stages. Values are in means, standard deviation and range. N=number of individuals with faecal samples available.

3.5 Faecal GCM levels in relation to phenological seasons

GCM levels (Table 3.11) were higher in lemurs at MAN (1108.20±406.48 ng/g) as compared to lemurs at STL (806.61±267.53 ng/g) (Site effect: $F_{1,16}=13.209$, p=0.002). GCM levels were higher during the lean season (1040.15±398.45 ng/g) as compared to the abundance season (775.37±227.67 ng/g) (Phenological season effect: $F_{1,16}=16.100$, p=0.001)

Table 3.11: GCM levels (ng/g) at Mandena and Sainte Luce during different phenological seasons. Values are in mean, standard deviation and range. N=number of individuals with faecal samples available.

	Sainte Luce	Mandena
Lean	N=14	N=8
Mean (±SD)	866.71(±278.80)	1343.67 (±408.04)
Range	401.04-1230.04	671.08-1915.95
Abundance	N=13	N=7
Mean (±SD)	741.06 (±248.99)	839.09 (±181.27)
Range	281.48-1282.63	675.49-1187.35

Lemurs living at MAN had different GCM levels as compared to lemurs living at STL during the two phenological seasons (Figure 3.13) (Phenological season* Site effect: $F_{1,16}$=5.762, p=0.029. Post hoc analyses showed that lemurs at MAN during the lean season had higher GCM levels as compared to lemurs at MAN during the abundance season and to lemurs at STL during both the lean and the abundance season (Table 3.12)
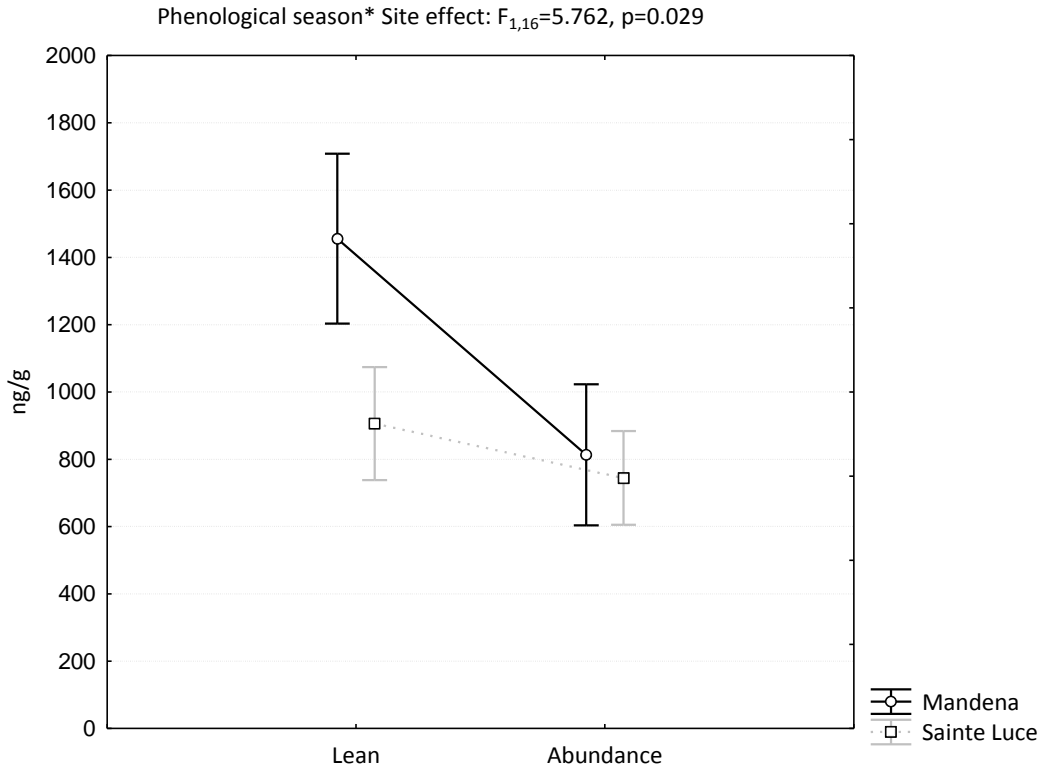

Fig 3.13: Differences in GCM levels (ng/g) at Mandena and Sainte Luce during the two phenological seasons. Values are in means and standard deviations. Above: results of repeated measures ANOVA.

Season	Lean MAN	Abundance MDN	Lean STL	Abundance STL
Lean MAN	-	-	-	-
Abundance MAN	**0.027**	-	-	-
Lean STL	**0.010**	0.954	-	-
Abundance STL	**<0.001**	0.854	0.483	-

Table 3.12: P values of pair-wise comparisons of mean differences in GCM levels (ng/g) at Mandena and Sainte Luce across different the two phenological seasons.

We found only a different trend in GCM levels between sexes (Table 3.13) considering the phenological seasons (Sex effect: $F_{1,16}=4.264$, p=0.056). Overall, females had higher GCM levels (1001.74±396.70 ng/g) than males (854.45±310.64 ng/g).

	Males	Females
Lean	N=13	N=9
Mean (±SD)	931.04 (±357.66)	1197.76 (±421.40)
Range	401.04-1491.90	691.44-1915.95
Abundance	N=12	N=8
Mean (±SD)	771.47 (±237.79)	781.21 (±227.53)
Range	281.48-1187.35	539.79-1282.63

Table 3.13: GCM levels (ng/g) between males and females during different phenological seasons. Values are in means, standard deviation and range. N=number of individuals with faecal samples available.

Males had not significantly different GCM levels as compared to females during the two phenological seasons (Figure 3.14) (Phenological season* Sex effect: F1,16=3.588, p=0.076). Post hoc analyses showed that females during the lean period had higher GCM level as compared to males during the abundance period (p=0.015).
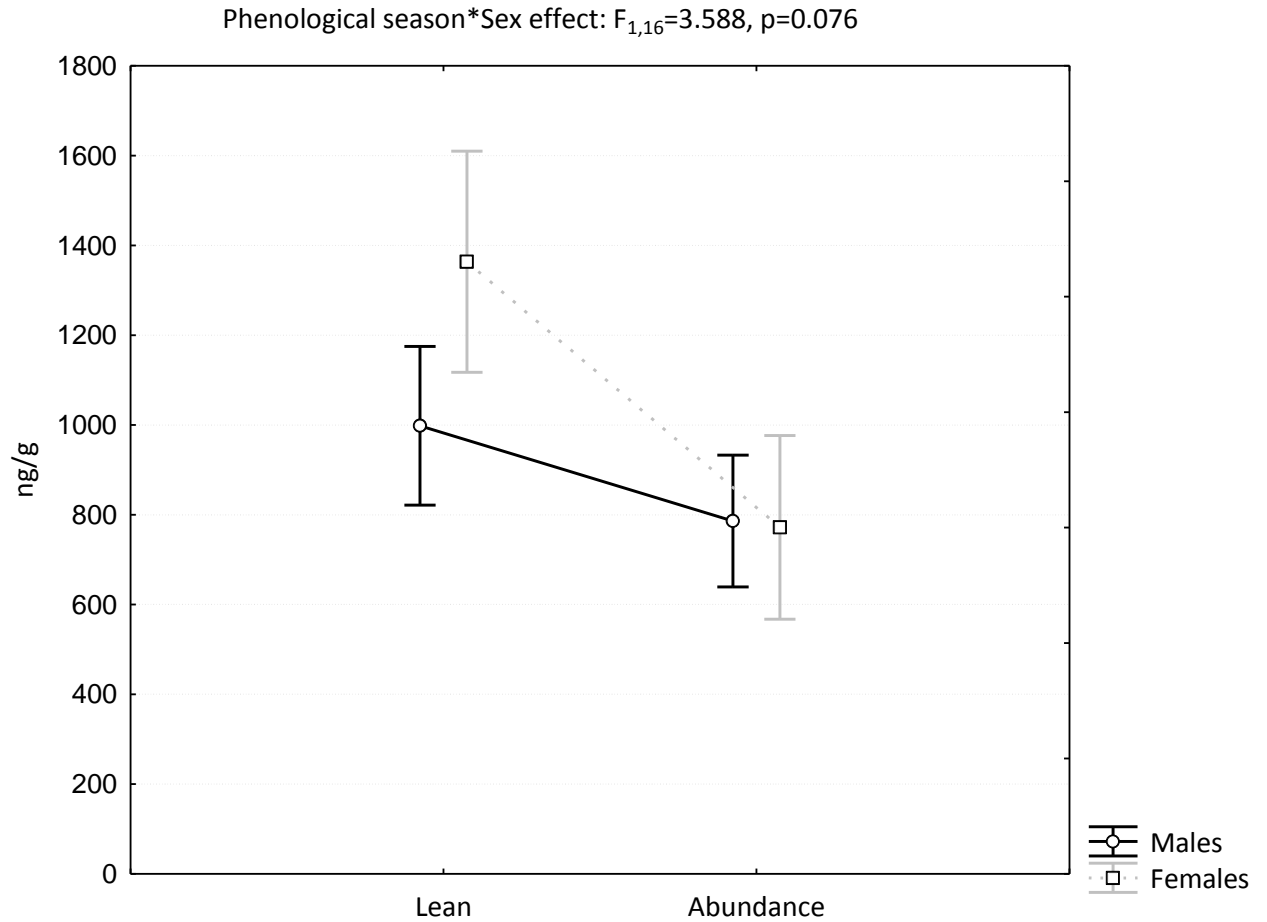
Fig.3.14: Differences in GCM levels (ng/g) between males and females during the two phenological seasons. Values are in means and standard deviations. Above: results of repeated measures ANOVA.

Lemurs of both sexes, living in MAN, had not different GCM levels between the two phenological seasons as compared to lemurs of both sexes living in STL (Table 3.14) (Phenological seasons*Site*Sex effect: $F_{1,16}=3.588$, p=0.076) (Fig. 3.15).
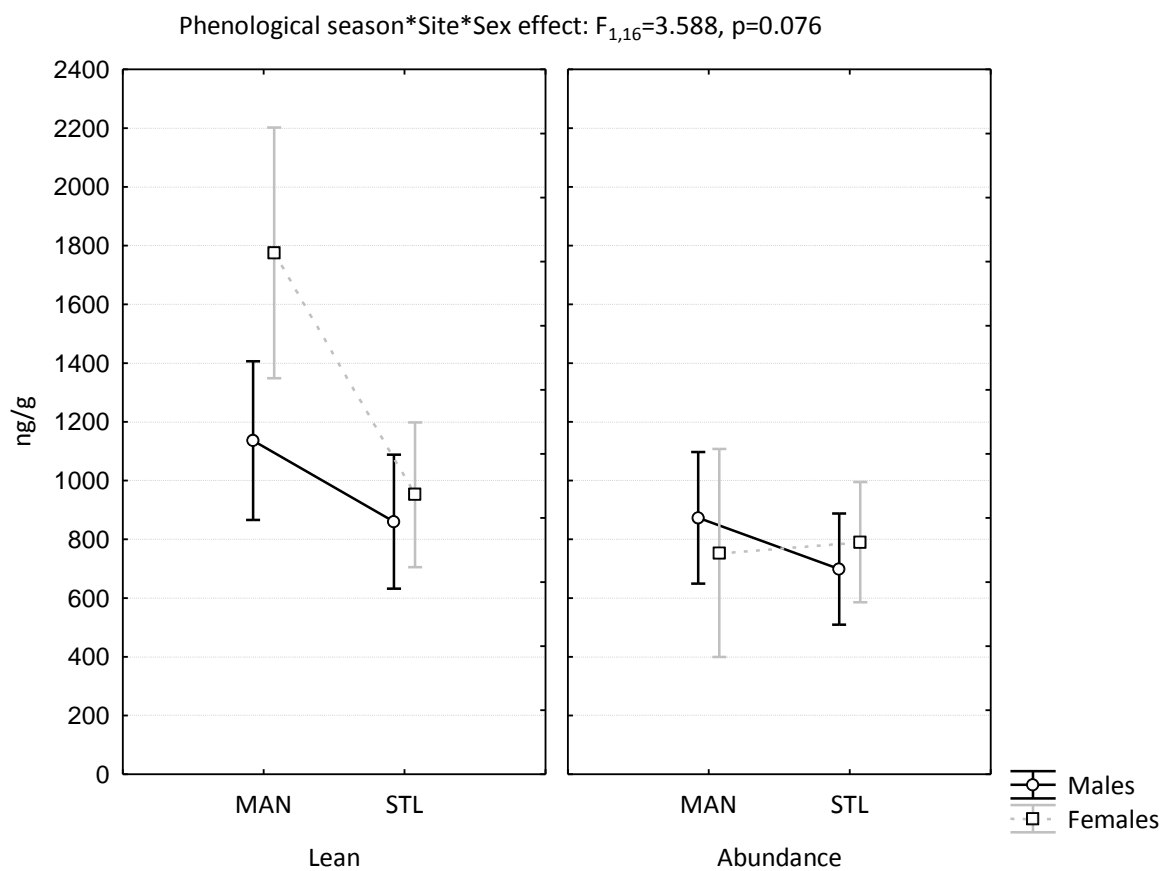
Table 3.14: GCM levels (ng/g) between males and females at Mandena and Sainte Luce during different phenological seasons. Values are in means, standard deviation and range. N=number of individuals with faecal samples available.

SAINTE LUCE	Males	Females
Lean	N=8	N=6
Mean (±SD)	436.17 (±94.69)	951.86 (±222.95)
Range	293.79-587.19	691.44-1229.10
Abundance	N=7	N=6
Mean (±SD)	938.34 (±239.47)	790.41 (±263.88)
Range	624.10-1245.68	539.79-1282.63

Table 3.14 (Continued)

MANDENA	Males	Females
Lean	N=5	N=3
Mean (±SD)	1136.14 (±356.19)	1689.55 (±204.63)
Range	671.08-1491.90	1517.79-1915.95
Abundance	N=5	N=2
Mean (±SD)	873.26 (±202.79)	753.66 (±110.55)
Range	689.04-1187.35	675.49-831.83

Fig 3.15: Differences in GCM levels (ng/g) between males and females at Mandena and Sainte Luce during the two phenological seasons. Values are in means and standard deviations. Above: results of repeated measures ANOVA.

Post hoc test (Table 3.15) showed that females at MAN during the lean season had higher GCM levels as compared to both males and females at STL during the two phenological seasons, and to males and females at MAN during the abundance season.

Sex	Site	Season	M MAN AB	M MAN LE	M STL AB	M STL LE	F MAN AB	F MAN LE	F STL AB	F STL LE
M	MAN	AB	-	-	-	-	-	-	-	-
M	MAN	LE	0.810	-	-	-	-	-	-	-
M	STL	AB	0.943	0.118	-	-	-	-	-	-
M	STL	LE	1.000	0.624	0.954	-	-	-	-	-
F	MAN	AB	0.999	0.658	1.000	1.000	-	-	-	-
F	MAN	LE	**0.006**	0.103	**<0.001**	**0.003**	**0.037**	-	-	-
F	STL	AB	0.999	0.389	0.998	1.000	1.00	**0.002**	-	-
F	STL	LE	1.000	0.936	0.663	0.998	0.981	**0.011**	0.970	-

Table 3.15: P values of pair-wise comparisons of mean differences in GCM levels between males and females at Mandena and Sainte Luce across between the two phenological season.
Sex: M=males, F=females; Site: MAN=Mandena, STL= Sainte Luce; Season: LE=lean, AB=abundance.

Dominant lemurs had not different GCM levels as compared to subordinate lemurs (Table 3.16) considering the phenological seasons (Rank effect: $F_{1,18}=0.130$, p=0.723). GCM levels were similar in high ranking (905.30±378.02 ng/g) than in low ranking lemurs (926.95±318.11 ng/g).

	High	Low
Lean	N=13	N=9
Mean (±SD)	1003.89 (±452.64)	1092.53 (±322.81)
Range	401.04-1915.95	530.88-1517.79
Abundance	N=12	N=8
Mean (±SD)	798.51 (±253.64)	740.67(±193.13)
Range	281.48-1282.63	539.79-1187.35

Table 3.16: GCM levels (ng/g) between high and low ranks at Mandena and Sainte Luce during different phenological seasons. Values are in means, standard deviation and range. N=number of individuals with faecal samples available.

CHAPTER 4

DISCUSSION

4.1 Validation of faecal GCM measurements

As expected, the two assays measuring groups of cortisol metabolites had the highest concentrations while concentrations measured by the two more specific assays (Cortisol and CCST) were markedly lower. None of the measures showed a clear response to the stressful event (10 min chasing of the animals). Thus, in each assay, most post-stress concentrations were in the same range as the pre-stress levels with no clear elevation seen. The only clear elevations found were in the first post-stress sample in the female and the juvenile male. However, these samples were collected approximately one hour after chasing and thus unlikely to reflect a response to the stressor given that faecal excretion lag times should be fairly longer. In fact, time lags associated with faecal measurements are longer and variable, both between and within species (Hodge and Heistermann 2011). In most large-bodied species for which data are available, steroids are excreted in faeces 36-48 h after appearance in circulation (Shideler et al. 1993), although there are exceptions (e.g. 22 h for testosterone and cortisol in the chimpanzee; Mohle et al. 2002; Bahr et al. 2000). In contrast, passage time is quicker in smaller animals (e.g. 4-8 h for testosterone and cortisol in the common marmoset, Mohle et al., 2002; Bahr et al., 2000). Faecal GCM levels, particularly those of 3a,11dihydroxy-CM, were overall higher in the two adult animals as compared to the juvenile animal, differences between adults and juveniles in faecal GCM levels are known also for other species (Dufty and Belthoff 1997; Mashburn and Atkinson 2004; Millspaugh and Washburn 2004).

We were strongly in doubt that none all four assays were not suitable for monitoring GCM output in *Eulemur collaris* because each one of the assays has been used for all previous primate species examined so far. The most acceptable reason for the lack of a clear faecal GCM response might be that the stressor event was presumably too mild. Probably the cortisol response, was too weak and/or not long enough to be seen in the faeces given that faecal hormones represent an integrated measure over several hours. Thus, if there was a rather short-term elevation in blood circulating cortisol, it was just not visible in the faecal measure. Another reason might be that not all samples defecated by the animals were collected, thus we may have missed the elevated GCM concentrations in response to the stressful event. Hence, the validation test results were inconclusive.

4.2 HPLC Analysis

The validation test results were not clear. However, the two group-specific assays (3a,11ß-dihydroxy-CM and 3a, 11oxo-CM assays) are known to be the most successful for monitoring stress hormone output in many primate taxa (Heistermann et al. 2006). Thus, we decided to perform high-performance liquid chromatography (HPLC) on two faecal extracts (one from a male and one from a female) to help towards a decision which of the two group-specific GCM assays we should use to analyse our faecal samples. Almost all immunoreactivity eluted between fractions 9 and 31, at positions at which in other primate species major cortisol metabolites are detected (Heistermann et al. 2006; Ostner et al. 2008). The data showed that the 3a,11oxo-CM assay, the one used also in *Eulemur fulvus rufus* (Ostner et al. 2008), had higher levels of immunoreactivity (that were associated with the presence of several peaks) compared to the 3a,11ß-dihydroxy-CM assay. The 3a,11oxo-CM assay showed a similar pattern for the male and the female, suggesting that identical metabolites were measured in

both sexes. The 3a,11ß-dihydroxy-CM assay, contrary to 3a,11oxo-CM assay, showed substantial immunoreactivity after fraction 31 where it is known that certain potentially cross-reacting androgen metabolites usually elute (Ganswindt et al. 2003; Heistermann et al. 2006; Pirovino et al. 2011). Overall, the 3a,11oxo-CM profile was also quite similar to the one found for *Eulemur fulvus rufus* (Ostner et al. 2008). For these reasons we decided to perform the 3a,11oxo-CM assay for the GCM measurements. The similarity between HPLC-GCM-immunoreactivity profiles derived from our samples and the findings on *Eulemur fulvus rufus,* suggested (although indirectly) that the 3a,11oxo-CM assay reliably detected GCM output in red collared brown lemurs.

4.3 Storage test

We showed that storing faecal samples in ethanol for a period of 12 months did not substantially affect GCM levels, this finding was in contrast to other species (Khan et al. 2002; Hunt and Wasser 2003) but similar to what found in *Propithecus verreauxi* (Fichtel et al. 2007) and in *Eulemur fulvus rufus* (Ostner et al. 2008). Ethanolic storage of faecal samples permits long-term preservation of samples collected in field. Since all the faecal samples were extracted after the same period of storage (one year), we can argue that differences in GCM concentrations found in our analysis were much greater than the time-related effects of storage. Thus we are confident that our data are reliable.

4.4 Faecal GCM levels in relation to reproductive stages

We compared all the reproductive stages both considering and excluding the mating season. We found a very high variability inside the mating season and it might be due to many reasons. We had a very little sample size (only 13 samples from 11 individuals were available) thus, we did not have an

average value for each individual, and this could entail an higher variability. Additionally, the mating season is a very short period (almost two weeks; Lancaster and Lee 1965), and we might have included samples belonging to another reproductive stage. This high variability might also be due to the fact that individuals might have different reproductive skew in association with their dominance (Creel and Sands 2003).

The highest faecal GCM levels were during the mating season, followed by the gestation season. The lactation and the non-reproductive season showed the lowest levels. This support the model where, during the mating season, we can find higher GC levels also in prosimians species, due to high rate of stressors, such as high level of aggression associated with reproductive competition (Abbott et al. 2003). This is in accordance with the findings on male mouse lemurs (Schilling et al. 1984), male sifakas (Fichtel et al. 2007), male ring-tailed lemurs (Pride 2005b), and male red-fronted lemurs (Ostner et al. 2008).

Elevation in GC outputs have been associated to increase rates of aggression in several other primate species, such as baboons (Sapolsky 1992b; Bergman et al. 2005) and chimpanzees (Muller and Wrangham 2004). Low cortisol levels have been observed in species where mate competition is low, such as male muriquis (*Brachyteles arachnoides*; Strier et al. 1999), male Tufted Capuchin monkeys (*Cebus apella nigritus*; Lynch et al. 2002), and male red-bellied lemurs (*Eulemur rubriventer*; Tecot 2013). In this study, lemurs showed higher aggression rates during the mating season as compared to the other reproductive stages (Campera 2012; Serra 2012). Thus, higher GCM levels found during the mating season were probably related to generally increased aggression rates. Additionally, we found higher GCM levels also in females during the mating season, suggesting that this period was stressful also for them. This is in accordance with the findings on other primates (Schiml et al.1996; 1999) and other mammal species (Lindburg et al. 2001; Owen et al. 1005) where the elevation have been attributed to the effects of ovarian cycling and general anxiousness.

As expected, females had high faecal GCM levels also during the gestation season. This is in accordance with the studies on female dwarf mongooses (Creel et al. 1992) and on female ring tailed lemurs (Cavigelli 1999). In particular, we found high GCM levels in females at MAN during the gestation season, which represent a serious challenge for them. One of the reasons can be that the gestation season overlaps with the lean season, thus females may be more stressed in a degraded habitat when they have a higher energetic demand (see below). Moreover, males at MAN during the gestation season showed higher GCM levels as compared to males at STL during the same season, suggesting that food availability could be the main stressful factor.

4.5 Faecal GCM levels in relation to phenological seasons

Ecological pressures, such as reduced food availability, predation risk, or climatic factors, have been reported to affect GCs output in a variety of species (Muller and Wrangham 2004; Weingrill et al. 2004; Ostner et al. 2008). High cortisol levels are typically assumed to indicate a poorer quality food and a more stressful habitat when populations are compared (Wingfield and Romero 2001). As expected, in this study we found higher GCM levels during the lean season, when food accessibility was low. Martínez-Mota et al. (2007) reported that populations of black howlers in disturbed areas show higher faecal GCM concentrations than those from undisturbed forests, probably due to stress. In females free-ranging ring-tailed lemurs, GCs were higher at the end of the dry season, with increased predation-risk and decreased food accessibility (Cavigelli 1999). Comparing the two sexes, we found that females showed higher GCM levels during the lean season. The lean season may be a very stressful period for females because it also overlaps with the gestation season. As expected, GCM levels were higher in lemurs at Mandena as compared to lemurs at Sainte Luce. The only previous study which compared lemurs faecal GCs in disturbed and undisturbed habitats (Tecot 2013) showed opposite results. In fact, contrary to her expectations, lemurs in the undisturbed habitats showed higher faecal GCs levels compared to lemurs in the disturbed habitats during the lean period. The author

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argued that the results were due to the presence of *Psidium cattleianum* (where lemurs ate unripe fruits) in the disturbed habitats, and feeding on *Psidium cattleianum* may allow lemurs to cope with fruit scarcity. The attenuation of GCs levels in the disturbed habitats may be a result of excessive stress, thus attenuation may enhance chances to survive in harsh environments by reducing pathological stress due to chronic stress, and more sensitive individuals might be selected out of the population. The results showed also a higher infant mortality in the disturbed habitats, suggesting that a prolonged attenuated response may also indicate insufficient coping. The lower corticoid levels in populations living in more disturbed sites than populations living in relatively undisturbed sites may also be due to "acclimation" to the stressor. In fact, with acclimation, the animal no longer responds in the same robust manner to repeated or chronic stressors because it no longer perceives the stressor as noxious (Romero and Wikelski 2002; Homan et al. 2003; Romero 2004).

In our study the highest GCM levels were found in females at MAN during the lean season, followed by males at MAN during the same period. Thus, we can argue that habitat degradation and food availability were the most important factor in shaping GCM levels. On the bases of the findings on *Eulemur rubriventer* (Tecot 2013), we can hypotesize that habitat degradation at Mandena was not as severe as to induce an attenuated response by *Eulemur collaris*.

4.6 Faecal GCM levels in relation to rank

As expected we found no rank- related GCM pattern between reproductive stages and phenological seasons. Individuals of both ranks had similar GCM levels, in accordance with the social flexibility of this species and also with what found in the closely related species *Eulemur fulvus rufus* (Ostner et al. 2008). Ostner and colleagues (2008) found opposite results (in one lemur group the dominant male had higher GCs levels whereas the opposite was found in another group) thus they proposed social instability as the main predictor of GC levels in individuals. In other studies, GC levels of dominants and subordinates did not differ detectably, such as in long-tailed macaques (van Schaik et al. 1991), in male dwarf mongooses (Creel et al. 1992), in mountain gorillas (Robbins and Czekala 1997), and in

tufted capuchins (Lynch et al. 2002). A rank related effect was found for male mouse lemurs, female dwarfs mongooses, female ring-tailed lemurs and sifakas (Schilling et al. 1984; Creel et al. 1992; Cavigelli 1999; Fichtel et al. 2007). In male sifakas a clear rank effect was found in GCs output during the reproductive season, maybe due to high reproductive skew in favour of dominant males (in fact no rank related GCM levels were found during the birth season; Fichtel et al. 2007).

CONCLUSIONS

In this study we provide a non- invasive method to assess adrenocortical activity in *Eulemur collaris*. By comparing faecal GCM levels in lemurs living in a degraded (Mandena) and in a more intact forest fragment (Sainte Luce) we found that higher GCM levels occur in lemurs which live in the degraded habitat, which appears to be a stressful environment. This is also in accordance with what found in the two studies of Marco Campera and Valentina Serra. They found that lemurs living at Mandena showed a smaller group size, had low daily ranges and distance travelled, had higher time spent resting during the day and used more energy saving postures while resting, as compared to lemurs living at Sainte Luce. Moreover, Lemurs at Mandena integrated their diet with "bad" fruits that are considered marginal in a more intact forest and assumed also a high quantity of non-digested fibres and secondary compounds.

Higher GCM levels at Mandena may also suggest that lemurs were not able to cope with the harsh environment only by changing their behaviour. This study underline the importance of conservation plans, because habitat loss can be a detrimental factor for animal welfare. What found in this study is in contrast with the only other study which compares lemurs living in different habitats (Tecot 2013). Because of the lack of studies which compare lemurs living in different environments, we need other comparative studies to support our findings and to provide a broader perspective.

Appendix I

Endocrinology Lab of the German Primate Center

Protocol for extraction of faecal samples stored in alcohol

- Number 30 ml or 50 ml conical polypropylene tubes and determine their individual weight (without the cap) with an accuracy of 4 decimals. Whether you use 30ml or 50ml tube depends on the volume of faeces and alcohol you have, probably 30 ml tubes are fine.

- Homogenize faeces mechanically (e.g. using a metal stick or metal or wooden spatula) in its original alcoholic solvent. This is important if the faeces is not broken up already in small pieces but is present as a compact bolus.

- Decant the suspension into the pre-weighed 30ml or 50 ml tube(s).

- Rinse the original tube with 2 ml 80% ethanol in water (shake the ethanol in the tube). Rinse also the stick or spatula (if it is re-used) with 1 ml of ethanol and add the rinse into the tube containing the faecal suspension.

- Extract the hormones from the faecal sample by vortexing on a multitube vortex for 15 minutes. For an efficient and consistent extraction it is important that the faeces in the tube is swirling very well.

- Following vortexing, centrifuge the sample at 3000 rpm for 10 min.

- Decant the supernatant carefully into a 15 ml tube with gradation which is pre-numbered with sample ID (i.e. carries the same number as the original extraction tubes).

- Determine the volume of the extract as accurate as possible using the check marks on the tube.

- Fill about 1.5ml of the extract into a 2 ml polypropylene tube which should be properly labelled with sample ID etc. (do not fill the tube fully to the top). For labelling preferably use a sticky freeze-resistant label (e.g. tough tag).

- Put parafilm around the micro tube to reduce the risk of evaporation (critical!!) and store the tube with the extract at -20°C.

- Dry the faecal pellet(s) to a constant weight by putting the tube(s) under the fume cabinet for 2 days (this helps to evaporating the ethanol in the faecal pellet) and subsequently placing the tube(s) in an oven (with or without vacuum) at 50°C until the pellet is completely dried (tube weight remains constant).

- Determine the dry faecal weight by weighing the tube (with dried faecal pellet, but without cap, see above) and substracting from this weight the net weight of the empty tube (see above). The dry weight is used for calculation of the faecal hormone concentration as it control for variation in water content of the samples.

- The rest of the extract can be discarded or a second aliquot of it can be stored frozen for safety reasons (e.g. in case samples are getting lost during shipment).

- Prepare an excel file with all necessary information (e.g. sample ID, extract volume, dry faecal weight etc.).

REFERENCE

Abbott, D.H., Keverne, E.B., Bercovitch, F.B., Shively, C.A., Mendoza, S.P., Salzman, W., Snowdon, C.T., Ziegler, T.E., Banjevic, M., Garland, T.J., Sapolsky, R.M., (2003). Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. *Horm. Behav*. 43, 67–82.

Alberts, S.C., Sapolsky, R.M., Altmann, J., (1992). Behavioral, endocrine, and immunological correlates of immigration by an aggressive male into a natural primate group. *Horm. Behav.* 26, 167–78.

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C. (1994). Gender and seasonal differences in the adrenocortical response to ACTH challenge in an arctic passerine, *Zonotrichia leucophrys gambelli*. *Gen. Comp. Endocr*. 94, 33–43.

Bahr, N.I., Palme, U., Möhle, J.K., Heistermann, M., (2000). Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. *Gen. Comp. Endocr.* 117, 427-38.

Balm, P.H.M., (1999). Preface. In: Balm, P.H.M, editor. *Stress physiology in animals*. Sheffield: Academic Press.

Balko, E.A., (1998). The behavioral plasticity of *Varecia variegata* in Ranomafana National Park, Madagascar. PhD dissertation. Syracuse, NY: SUNY-College of Environmental Science and Forestry.

Beehner, J., Bergman, T., Cheney, D., Seyfarth, R., Whitten, P., (2005). The effect of new alpha males on female stress in free-ranging baboons. *Anim. Behav*. 69, 1211–21.

Bercovitch, F.B., Ziegler, T.E., (2002). Current topics in primate socioendocrinology. *Annu. Rev. Anthropol.* 31, 45-67.

Bergman, T., Beehner, J., Cheney, D., Seyfarth, R., Whitten, P., (2005). Correlates of stress in free-ranging male chacma baboons, Papio hamadryas ursinus. *Anim. Behav.* 70, 703–13.

Bollen, A., Donati, G., (2005). Phenology of the littoral forest of Sainte Luce, southeast Madagascar. *Biotropica* 37, 32-43.

Bollen, A., Donati, G., (2006). Conservation status of the littoral forest of southeastern Madagascar: a review. *Oryx* 40, 57-66.

Broom, D.M, Johnson, K.G., (1993). *Stress and Animal Welfare: Animal Behaviour Series*. London: Chapman & Hall.

Bronson, F.H., (1999). Puberty and energy reserves: a walk on the wild side. In: Wallen, K., Schneider, J.E., editors. *Reproduction in Context*. Cambridge: MIT Press. 15-34.

Cameron, J.L., (1997). Stress and behaviorally induced reproductive dysfunction in primates. *Semin. Reprod. Endocr*. 15, 37-45.

Campera, M., (2012). Eco-ethology of the red collared brown lemur (*Eulemur collaris*): comparison between groups living in well preserved and degraded littoral forest fragments, in South-eastern Madagascar. MS dissertation, University of Pisa.

Cavigelli, S.A., (1999). Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. *Anim. Behav*. 43, 166-79.

Chapman, C.A., Wasserman, M.D., Gillespie, T.R., Speirs, M.L., Lawes, M.L., Saj, T.L., Ziegler, T.E., (2006). Do food availability, parasitism, and stress have synergistic effects on Red Colobus populations living in forest fragments? *Am. J. Phys. Anthropol.* 131, 525-34.

Chard, T., (1995). *An Introduction to Radioimmunoassay and Related Techniques*, 5th edn. Amsterdam: Elsevier.

Chrousos G.P, Loriaux, D.L., Gold, P.W., (1996). Mechanisms and Clinical Implications. *Ann. NY. Acad. Sci*. Vol. 771.

Coe, C.L., Scheffler, J., (1989). Utility of immune measures for evaluating psychological well-being in nonhuman primates. *Zoo Biol*. 1, 89-99.

Consiglio, T., Schatz, G.E., McPherson, G., Lowry II, P.P., Rabenantoandro, J., Rogers, Z.S. and Rabehevitra, D., (2006). Deforestation and plant diversity of Madagascar's littoral forests. *Conserv. Biol.* 20, 1799-803.

Creel, S., Creel, N.M., Wildt, D.E., Monfort, S.L., (1992). Behavioral and endocrine mechanisms of reproductive suppression in Serengeti dwarf mongooses. *Anim. Behav*. 43, 231–45.

Creel, S., (2001). Social dominance and stress hormones. *Trends. Ecol. Evol.* 16, 491-97.

Creel, S., Sands, J.L., (2003). Is social stress a consequence of subordination or a cost of dominance? In: De Waal, F.B.M., Tyack, P.L., editors. *Animal Social Complexity. Intelligence, Culture, and Individualized Societies*. Cambridge, Massachusetts, London: Harvard University Press. 153–69.

Curtis, D.J., Zaramody, A., Green, D.I., Pickard, A.R., (2000). Non-invasive monitoring of reproductive status in wild mongoose lemurs (*Eulemur mongoz*). *Reprod. Fert. Develop.* 12, 21–29.

Dallman, M.F., Strack, A.M., Akana, S.F., Bradbury, M.J., Hanson, E.S., Scribner, K.A., Smith, M., (1993). Feast and famine: Critical role of glucocorticoids with insulin in daily energy flow. *Front. Neuroendocrinol.* 14, 303-47.

Dewar, R.E., Richard, A.F., (2007). Evolution in the hypervariable environment of Madagascar. *P. Natl. Acad. Sci. U.S.A.* 104, 3-27.

Dhabhar, F.S., McEwen, B.S., (1999). Enhancing versus suppressive effects of stress hormones on skin immune function. *P. Natl. Acad. Sci. U.S.A.* 96, 1059-64.

Dhabhar, F., McEwen, B., (2000). Bidirectional effects of stress and glucocorticoid hormones on immune function: possible explanations for paradoxical results. In: Ader, R., Felton, D., Cohen, N., editors. *Psychoneuroimmunology*. New York: Academic Press. 301–38.

Dhabhar, F.S., (2006). Stress-induced changes in immune cell distribution and trafficking: Implications for immunoprotection versus immunopathology. Welsh, C.J., Meagher, M., Sternberg, E., editors. *Neural and neuroendocrine mechanisms in host defense and autoimmunity*. 7-25.

Djletati, R., Brun, B., Rumpler, Y., (1997). Meiotic study of hybrids in the genus *Eulemur* and taxonomic considerations. *Am. J. Primatol.* 42, 235-45.

Donati, G., Borgognini-Tarli, S.M., (2006). Influence of abiotic factors on cathemeral activity: the case of *Eulemur fulvus collaris* in the littoral forest of Madagascar. *Folia Primatol.* 77, 104-22.

Donati, G., Bollen, A., Borgognini-Tarli, S.M., Ganzhorn, J.U., (2007a). Feeding over the 24-h cycle: dietary flexibility of cathemeral collared lemurs (*Eulemur collaris*). *Behav. Ecol. Sociobiol.* 61, 1237-51.

Donati, G., Ramanamanjato, J.B., Ravoahangy, A.M., Vincelette, M., (2007b). Translocation as a conservation measure for a threatened species: the case of *Eulemur collaris* in the Mandena littoral forest, south-eastern Madagascar. In: Ganzhorn, J.U., Goodman, S.M., Vincelette, M., editors. *Biodiversity, ecology and conservation of the littoral ecosystems of South-eastern Madagascar.* Washington DC: Smithsonian Institution Press, 237–43.

Donati, G., Baldi, N., Morelli, V., Ganzhorn, J.U., Borgognini-Tarli, S.M., (2009). Proximate and ultimate determinants of cathemeral activity in brown lemurs. *Anim. Behav.* 77, 317-25.

Donati, G., Kesch, K., Ndremifidy, K., Schmidt, S.L., Ramanamanjato, J.B., (2011). Better few than hungry: flexible feeding ecology of collared lemurs *Eulemur collaris* in littoral forest fragments. *Plos One* 6 (5).

Dufty Jr., A.M., Belthoff, J.R., (1997). Corticosterone and the stress response in young western screech-owls: effects of captivity, gender, and activity period. *Physiol. Zool.* 70, 143–9.

Fichtel, C., Kraus, C., Ganswindt, A., Heistermann, M. (2007). Influence of reproductive season and rank on fecal glucocorticoid levels in free-ranging male Verreaux's sifakas (*Propithecus verreauxi*). *Horm. Behav*. 51, 640-48.

Gammell, M.P., de Vries, H., Jennings, D.J., Carlin, C.M., Hayden, T.J., (2003). David's score: a more appropriate dominance ranking method than Clutton-Brock et al.'s index. *Anim. Behav.* 66, 601-05.

Ganswindt, A., Palme, R., Heistermann, M., Borragan, S., Hodges, J.K., (2003). Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. *Gen. Comp. Endocrinol.* 134, 156-66.

Ganzhorn, J.U., (1988). Food partitioning among Malagasy primates. *Oecologia* 75, 436–50.

Ganzhorn, J.U., Fietz, J., Rakotovao, E., Schwa, D., Zinner, D., (1999). Lemurs and the regeneration of dry deciduous forest in Madagascar. *Conserv. Biol*. 13, 794-804.

Ganzhorn, J.U., Goodman, S.M., Vincelette, M., (2007). *Biodiversity, ecology and conservation of the littoral ecosystems of South-eastern Madagascar*. Washington DC: Smithsonian Institution Press.

Ganzhorn, J.U., (2002). Distribution of a folivorous lemur in relation to seasonally varying food resourcs: integrating quantitative and qualitative aspects of food characteristics. *Oecologia* 131, 427-35.

Goodman, S.M., Ganzhorn, J.U., (2004). Elevational ranges of lemurs in the humid forests of Madagascar. *Int. J. Primatol.* 25, 331-50.

Gould, L., Sussman, R.W., Sauther, M.L., (1999). Natural disasters and primate populations: The effects of a 2-year drought on a naturally occurring population of ring-tailed lemurs (*Lemur catta*) in southwestern Madagascar. *Int. J. Primatol.* 20, 69-84.

Goymann, W., (2012). On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. *Method. Ecol. Evol*. 3, 757-65.

Goymann, W., Wingfield, J., (2004). Allostatic load, social status and stress hormones: The costs of social status matter. *An. Behav*. 67, 591–602.

Green, G.M., Sussman, R.W., (1990). Deforestation history of the eastern rain forests of Madagascar from satellite images. *Science* 248, 212-15.

Groves, C.P., (2001). *Primate Taxonomy*. Washington DC: Smithsonian Institution Press.

Heistermann, M., Finke, M., Hodges, J.K., (1995). Assessment of female reproductive status in captive-housed Hanuman langurs (*Presbytis entellus*) by measurement of urinary and fecal steroid excretion patterns. *Am. J. Primatol.* 37, 275–84.

Heistermann, M., Ademmer, C., Kaumanns, W., (2004). Ovarian cycle and effect of social chenges on adrenal and ovarian function in *Pygatherix nemaeus. Int. J. Primatol.* 25, 689-708.

Heistermann, M., Palme, R., Ganswindt, A., (2006). Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis. *Am. J. Primatol*. 68, 257-73.

Hodge, J.K., Heistermann, M., (2011). Field endocrinology: monitoring hormonal changes in free-ranging primates. Setchell, J.M., Curtis, D.J. editors. *Field and Laboratory Methods in Primatology: A Practical Guide*. Cambridge: University Press.

Homan, R.N., Regosin, J.V., Rodrigues, D.M., Reed, J.M., Windmiller, B.S., Romero, L.M., (2003). Impacts of varying habitat quality on the physiological stress of spotted salamanders *(Ambystoma maculatum). Anim. Cons*. 6, 11-18.

Huber, S., Palme, R., Arnold, W., (2003). Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). *Gen. Comp. Endocrinol*. 130, 48-54.

Hunt, K., Wasser, S., (2003). Effect of long-term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant. *Physiol. Biochem. Zool*. 76, 918–28.

Irwin, M.T., Jhonson, S.E., Wright, P.C., (2005). The state of lemur conservation on southeastern Madagascar: population assessments for diurnal lemurs using surveys, satellite imagery and GIS. *Orix* 39, 1-15.

Irwin, M.T., (2007). Living in forest fragments reduces group cohesion in Diademed Sifakas (*Propithecus diadema*) in Eastern Madagascar by reducing food patch size. *Am. J. Primatol*. 69, 434-47.

Johnson, S.E., Lei, R., Martin, S.K., Irwin, M.T. and Louis Jr., E.E. (2008). Does *Eulemur cinereiceps* exist? Premilinary evidence from genetics and ground surveys in southeastern Madagascar. *Am. J. Primatol.* 70, 372-85.

Khan, M.Z., Altmann, J., Isani, S.S., Yu, J., (2002). A matter of time: evaluating the storage of fecal samples for steroid analysis. *Gen. Comp. Endocrinol*. 128, 57–64.

Lancaster, J.B., Lee, R.B., (1965). The annual reproductive cycle in monkeys and apes. In: I. DeVore, editor. *Primate Behavior: Field Studies of Monkeys and Apes.* New York: Holt, Rinehart and Winston.

Lane, J., (2006). Can non-invasive glucocorticoid measures be used as reliable indicators of stress in animals? *Anim. Welfare.* 15, 331-42.

Lynch, J.W., Ziegler, T.E., Strier, K.B., (2002). Individual and seasonal variation in fecal testosterone and cortisol levels of wild male Tufted Capuchin monkeys, *Cebus apella nigritus*. Horm. Behav. 41, 275-87.

Lindner, H.R., (1972). Enterohepatic circulation and patterns of urinary excretion of cortisol metabolites in the ewe. *J. Endocrinol*. 52, 19-20.

Lindburg, G., Czekalaa, N.M., Swaisgood, R., (2001). Hormonal and behavioral relationships during estrus in the giant panda. *Zoo Biol.* 20, 537-43.

Lowry II, P.P., Randriatafika, F., Rabenantoandro, J., (2008). Conservation status of vascular plant species from the QMM/Rio Tinto mining area at Mandena, Tolagnaro (Fort Dauphin) region, southeast Madagascar. Madagascar Conservation and Development 3, 55-63.

Mashburn, K.L., Atkinson, S., (2004). Evaluation of adrenal function in serum and feces of Steller sea lions (*Eumetopias jubatus*): influences of molt, gender, sample storage, and age on glucocorticoid metabolism. *Gen. Comp. Endocrinol*. 136, 371–81.

Millspaugh, J.J., Washburn, B.E., (2004). Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen. Comp. End.* 138, 189-99.

Mittermeier, R.A., Konstant, W.R., Hawkins, F., Louis Jr., E.E., Langrand, O., Ratsimbazafy, J., Rasoloarison, R., Ganzhorn, J.U., (2006). Lemurs of Madagascar Illustrated by Nash, S.D. (2nd Eds.). *Conserv. Intern.*

Mittermeier, R.A., Louis Jr., E.E., Richardson, M., Schwitzer, C., Lagrand, O., Rylands, A.B, Hawkins, F., Rajaobelina, S., Ratsimbazafy, J., Rasoloarison, R., Roos, C., Kappeler, P.M.,

Mackinnon, J., (2010). Lemurs of Madagascar Illustrated by Nash, S.D. (3rd Eds.). *Conserv. Intern.*

Martínez-Mota, R., Valdespino, C., Sánchez-Ramos, M.A., Serio-Silva, J.C., (2007). Effects of forest fragmentation on the physiological stress response of black howler monkeys. *Anim. Cons.* 10, 374–79.

Mohle, U., Heistermann, M., Palme, R., Hodges, J.K., (2002). Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. *Gen. Comp. Endocrinol.* 129, 135-45.

Möstl, E., Messmann S., Bagu, E., Robia, C., Palme R., (1999). Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. *J. Vet. Med. A.* 46, 621–32.

Möstl, E., Maggs, J.L., Schrötter, G., Besenfelder, U., Palme, R., (2002). Measurement of cortisol metabolites in faeces of ruminants. *Vet. Res. Commun.* 26, 127-39.

Möstl, E., Palme, R., (2002). Hormones as indicators of stress. *Domest. Anim. Endocrinol.* 23, 67-74.

Möstl, E.S., Rettenbacher, C., Palme, C., (2005). Measurement of corticosterone metabolites in birds' droppings: an analytical approach. *Ann. N.Y. Acad. Sci.* 1046, 17-34.

Muller, M., Wrangham, R., (2004). Dominance, cortisol and stress in wild chimpanzees (*Pan troglodites schweinfurthii*). *Behav. Ecol. Sociobiol.* 55, 332-40.

Ostner, J., Kappeler, P., Heistermann, M., (2008). Androgen and glucocorticoid levels reflect seasonally occurring social challenges in male redfronted lemurs (*Eulemur fulvus rufus*). *Behav. Ecol. Sociobiol.* 62, 627-38.

Ostner, J., Kappeler, P., (2004). Male life history and the unusual adult sex ratio in redfronted lemurs (*Eulemur fulvus rufus*) groups. *Anim. Behav.* 67, 249-59.

Owen, M.A., Czekalaa, N.M., Swaisgood, R., Karen, S., Lindburg, G., (2005). Seasonal and diurnal dynamics glucocorticoids and behavior in giant pandas. *Ursus* 16, 208-21.

Palme, R., Möstl, E., (1997). Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *Int. J. Mammal. Biol.* 62, 192-7.

Palme, R., Fisher, P., Schildorfer, H., Ismail, MN., (1996). Excretion of infused ^{14}C-steroid hormones *via* faeces and urine in domestic livestock. *Anim. Reprod. Sci.* 43, 43-63.

Palme, R., Möstl, E., (2000). Determination of faecal cortisol metabolites in domestic livestock for non-invasive monitoring of disturbances. In: Aktuelle Arbeiten zur artgemäßen Tierhaltung. Kuratorium für Technik und Bauwesen in der Landwirtschaft eV (KTBL), Darmstadt, Germany, KTPL-Schrift. 403, 3–17.

Palme, R., (2005). Measuring fecal steroids: guidelines for the practical application. *Ann. N. Y. Acad. Sci.* 1046, 75-80.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., (2005). Stress hormones in mammals and birds: Comparative aspects regarding metabolism, excretion and noninvasive measurement in fecal samples. *Ann. N. Y. Acad. Sci.* 1040, 162-71.

Pereira, M.E., Kappeler, P.M., (1997). Divergent systems of agonistic behaviour in lemurid primates. *Behaviour* 134, 225–74.

Pereira, M.E., Strohecker, R.A., Cavigelli, S.A., Hughes, C.L., Pearson, D.D., (1999). Metabolic strategy and social behavior in Lemuridae. In: Rakotosamimanana, B., Rasamimanana, H., Ganzhorn, J.U., Goodman, S.M. editors. *New directions in lemur studies*. New York: Plenum Press. 93-118.

Pirovino, M., Heistermann, M., Zimmermann, N. Zingg, R., Clauss, M., Codron, D., Kaup, F.J., Hanspeter, W.S., (2011). Fecal glucocorticoid measurements and their relation to rearing, behavior, and environmental factors in the population of pileated gibbons (*Hylobates pileatus*). *Int. J. Primatol.* 32, 1161-78.

Pope, T., (1990). The reproductive consequences of male cooperation in the red howler monkey: paternity exclusion in multi-male and single-male troops using genetic markers. *Behav. Ecol. Sociobiol.* 27, 439–446.

Pride, E.R., (2005a). High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (*Lemur catta*). *Biol. Lett.* 1, 60-3.

Pride, E.R, (2005b). Optimal group size and seasonal stress in ring-tailed lemurs (*Lemur catta*). *Behav. Ecol.* 16, 550-60.

Rabenantoandro, J., Randriatafika, F., Lowry II, P.P., (2007). Floristic and structural characteristics of remnant littoral forest sites in the Tolagnaro area. In: Ganzhorn, J.U., Goodman, S.M., Vincelette, M., editors. Biodiversity, ecology and conservation of the littoral ecosystems of South-eastern Madagascar. Washington DC: Smithsonian Institution Press. 65-77.

Ratsimbazafy, J.H., Ramarosandratana, H.V., Zaonarivelo, R.J., (2002). How do black-and-white ruffed lemurs still survive in a highly disturbed habitat? *Lemur News* 7, 7–10.

Rasmussen, M.A., (1999). *Ecological Influences on Activity Cycle in Two Cathemeral Primates, the Mongoose Lemur* (*Eulemur mongoz*) *and the Common Brown Lemur* (*Eulemur fulvus fulvus*). Phd Dissertation. Durham, North Carolina: Duke University.

Reeder, D.M., Kramer, K.M., (2005). Stress in free-ranging mammals: Integrating physiology, ecology, and natural history. *J. Mammal.* 86, 225-35.

Risler, L., Wasser, S.K., Sackett, G.P., (1987). Measurement of excreted steroids in *Macaca nemestrina*. *Am. J. Primatol.* 12, 91-100.

Robbins, M.M., Czekala, N.M., (1997). A preliminary investigation of urinary testosterone and cortisol levels in wild male mountain gorillas. *Am. J. Primatol.* 43, 51-64.

Romero, L., (2004). Physiological stress in ecology: lessons from biomedical research. *Trends Ecol. Evol.* 19, 249-55.

Romero, L., Butler, K., (2007). Endocrinology of stress. *Int. J. Comp. Psychol.* 20, 89-95.

Romero, L.M., Wikelski, M., (2002). Exposure to tourism reduces stress-induced corticosterone levels in Gala´pagos marine iguanas. *Biol.* Conserv. 108, 371–374.

Rose, R.M., Gordon, T., Bernstein, I.S., (1978). Diurnal variation in plasma testosterone and cortisol in rhesus monkeys living in social groups. *J. Endocrinol.* 76, 67-74.

Saltzman, W., Schultz-Darken, N.J., Scheffler, G., Wegner, F.H., Abbott, D.H., (1994). Social and reproductive influences on plasma cortisol in female marmoset monkeys. *Physiol. Behav.* 56, 801-10.

Sapolsky, R.M., (1982). The endocrine stress-response and social status in the wild baboon. Horm. Behav. 16, 279–92.

Sapolsky, R.M., (1992a). *Stress, the aging brain, and the mechanisms of neuron death*. Cambridge, MA: MIT Press.

Sapolsky, R.M., (1992b). Cortisol concentrations and the social significance of rank instability among wild baboons. *Psychoneuroendocrino*. 17, 701–709.

Sapolsky, R.M., (1994). *Why Zebras Don't Get Ulcers: A Guide to Stress, Stress-Related Diseases, and Coping*. New York: W.H. Freeman.

Schatz, S., Palme, R., (2001). Measurement of faecal cortisol metabolites in cats and dogs: a noninvasive method for evaluating adrenocortical function. *Vet. Res. Commun.* 25, 271-87.

Schilling, A., Perret, M., Predine, J., (1984). Sexual inhibition in a prosimian primate. A pheromone-like effect. *J. Endocrinol.* 102, 143–51.

Schiml, P.A., Mendoza, S.P., Saltzman, W., Lyons, D.M., Mason, W.A., (1996). Seasonality in squirrel monkeys (*Saimiri sciureus*). *Physiol. Behav.* 60, 1105–113.

Schiml, P.A., Mendoza, S.P., Saltzman, W., Lyons, D.M., Mason, W.A., (1999). Annual physiological changes in individually housed squirrel monkeys (*Saimiri sciureus*). *Am. J. Primatol.* 4, 93–103.

Selye, H., (1936). A syndrome produced by diverse nucuos agents. *Nature* 138, 32.

Selye, H., (1956). *The Stress of Life*. New York: McGraw-Hill.

Serra, V., (2012). Behavioural ecology of the red collared brown lemur (*Eulemur collaris*): comparison between group living in well preserved and degraded littoral forest fragments, in South-eastern Madagascar. MS dissertation, University of Pisa.

Shideler, S.E., Ortuno, A.M., Moran, F.M., Moorman, E.A., Lasley, B.L., (1993). Simple extraction and enzyme immunoassays for estrogen and progesterone metabolites in the feces of *Macaca fascicularis* during nonconceptive and conceptive ovarian cycles. *Biol. Reprod.* 48, 1290-98.

Snodgrass, J.J., Leonard, W.R., Robertson, M.M., (2007). Primate bioenergetics: an evolutionary perspective. Ragosa, M.J., Dagosto, M., editors. *Primate Origins: Adaptations and Evolution.*New York: Springer. 703-37.

Stavisky, R.C., Adams, M.R., Watson, S.L., Kaplan, J.R., (2001). Dominance, cortisol, and behavior in small groups of female cynomolgus monkeys (*Macaca fascicularis*). *Hormones and Behavior* 39(3), 232-38.

Strier, K.B., Ziegler, T.E., Wittwer, D.J., (1999). Seasonal and social correlates of fecal testosterone and cortisol levels in wild male muriquis (*Brachyteles arachnoides*). *Horm. Behav.* 35, 125–34.

Sussman, R.W., (1974). Ecological distinctions between two species of lemurs. In: Martin, R.D., Doyle, G.A., Walker, A.C., editors. *Prosimian Biology*. London: Duckworth. 75-108.

Tattersall, I., (1982). *The primates of Madagascar*. New York: Columbia University Press.

Tattersall, I., (1987). Cathemeral activity in primates - a definition. *Folia Primatol.*. 49, 200-02.

Tattersall, I., Sussman, R.W., (1998). 'Little brown lemurs' of Northern Madagascar. *Folia Primatol.* 69, 379-88.

Tecot, S., (2008). Seasonality and predictability: the hormonal and behavioral responses of the redbellied lemur, *Eulemur rubriventer*, in Southeastern Madagascar. PhD dissertation. University of Texas, Austin.

Tecot, S., (2013). Variable energetic strategies in disturbed and undisturbed rain forest habitats: fecal cortisol levels in southeastern Madagascar. In: J. Masters, M. Gamba, F. Génin, R. Tuttle, editors. *Leaping Ahead: Advances in Prosimian Biology (Developments in Primatology: Progress and Prospects).* New York: Springer. 185-95.

Teskey-Gerstl, A., Steineck, T., Bamberg, E., Palme, R., (2000). Excretion of corticosteroids in urine and faeces of hares (*Lepus europaeus*). *J. Comp. Physio.l B*. 170, 163–8.

Touma, C., Palme, R., (2005). Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Ann. N.Y. Acad. Sci.* 1046, 54-74.

van Schaik, C.P., van Noordwijk, M.A., van Bragt, T., Blankenstein, M.A., (1991). A pilot study of the social correlates of levels of urinary cortisol, prolactin, and testosterone in wild longtailed macaques (*Macaca fascicularis*). *Primates* 32, 345-56.

Vincelette, M., Théberge, M., Randrihasipara, L., (2007). Evaluations of forest cover at regional and local levels in the Tolagnaro region since 1950. In: Ganzhorn, J.U, Goodman, S.M., Vincelette, M., editors. Biodiversity, Ecology and Conservation of Littoral Forest Ecosystems in Southeastern Madagascar, Tolagnaro (Fort Dauphin). Washington DC: Smithsonian Institution Press. 49-58.

von der Ohe, C.G., Servheen, C., (2002). Measuring stress in mammals using fecal glucocorticoids: opportunities and challenges. *Wildlife Soc. B.* 30, 1215-25.

von Holst, D., (1998). The concept of stress and its relevance for animal behavior. *Adv. Study Behav.* 27, 1–131.

Wasser, S.K., Starling, A.K., (1988). Proximate and ultimate causes of reproductive suppression among female yellow baboons at Mikumi National Park, Tanzania. *Am. J. Primatol.* 16, 97-121.

Wasser, S., Bevis, K., King, G., Hanson, E., (1997). Non-invasive physiological measures of disturbance in the northern spotted owl. *Cons. Biol.* 11, 1019–22.

Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crockett, C.M., Bechert, U., Millspaugh, J.J., Larson S., Montfort, S.L., (2000). A generalised fecal glucocorticoid assay for use in a

diverse array of non-domestic mammalian and avian species. *Gen. Comp. Endocrinol.* 120, 260-75.

Weingrill, T., Gray, D., Barrestt, L., Henzi, S., (2004). Faecal cortisol levels in female free ranging chacma baboons: relationship to dominance, reproductive state and environmentals factors. *Horm. Behav.* 45, 259.

Weiss, J.M., (1968). Effects of coping responses on stress. *J. Comp. Physiol. Psychol.* 65, 251–60.

Wingfield, J.C., Deviche, P., Sharbaugh, S., Astheimer, L.B., Holberton, R., Suydam, R., Hunt, K., (1994). Seasonal changes of the adrenocortical responses to stress in redpolls, *Acanthis flammea*, in Alaska. *J. Exp. Zool.* 270, 372–80.

Wingfield, J.C., Ramenofsky, M., (1997). Corticosterone and facultative dispersal in response to unpredictable events. *Ardea* 85, 155-66.

Wingfield, J.C., Romero, L.M., (2001). Adrenocortical responses to stress and their modulation in free-living vertebrates. In McEwen, B.S., Goodman, H.M., editors. *Handbook of physiology*; section 7: The endocrine system; volume IV: Coping with the environment: Neural and endocrine mechanisms. New York: Oxford Univ. Press. 211-34.

Wingfield, J.C., Sapolsky, R.M., (2003). Reproduction and resistance to stress: When and how. *Journal of Neuroendocrinology,* 15, 711.

Whitten, P.L., Brockman, D.K., Stavisky, R.C., (1998). Recent advances in non-invasive techniques to monitor hormone–behavior interactions. *Yearb. Phys. Anthropol.* 41, 1–23.

Wright, P.C., (1999). Lemur traits and Madagascar ecology: Coping with an island environment. *Yearb. Phys. Anthropol..* 42, 31-72.

Wright, P.C., Razafindratsita, V.R., Pochron, S.T., Jernvall, J., (2005). The key to Madagascar frugivores. In: Dew, J.L., Boubli, J.P., editors. *In Tropical Fruit and Frugivores: The Search for Strong Interactors*. Dordrecht: Springer Press, 121-38.

Wyner, Y.M., Absher, R., Amato, G., Sterling, E., Stumpf, R., Rumpler, Y., DeSalle, R., (1999). Species concepts and the determination of historic gene flow patterns in the *Eulemur fulvus* (brown lemur) complex. *Biol. J. Linn. Soc.* 66, 39-56.

Ziegler, T., Hodges, K., Winkler, P., Heistermann, M., (2000). Hormonal correlates of reproductive seasonality in wild female Hanuman langurs (*Presbytis entellus*). *Am. J. Primatol.* 51, 119–34.

Ziegler, T.E., Washabaugh, K.F., Snowdon, C.T., (2004). Responsiveness of expectant male cotton-top tamarins, Saguinus oedipus, to mate's pregnancy. *Horm. Behav.* 45, 84-92.

Ziegler, T.E., Wittwer, D.J., (2005). Fecal steroid research in the field and laboratory: Improved methods for storage, transport, processing, and analysis. *Am. J. Primatol.* 67, 159-74.

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