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Variation in susceptibility to parasite infection: patterns, determinants and consequences in red-fronted lemurs

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Dagmar Clough

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Referentin/Referent: Prof. Dr. Peter M. Kappeler

Korreferentin/Korreferent: Prof. Dr. Eckhard W. Heymann

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1 Variation in susceptibility to parasite infection: General Introduction

The abundance and magnitude of parasite infections in the wild is characterized by patterns of aggregation (Anderson and May 1978; Shaw and Dobson 1995). Such distributions are determined by both the variability in host exposure to infective parasite stages and host susceptibility once an infectious agent has been encountered (Wilson *et al.* 2002), and can emerge at an individual, population and/or species level. Tightly linked with the degree of parasite aggregation across a host population, is the regulatory effect of parasites on host populations, for example, by inducing mortality, altering outcomes of intra- and interspecific competition or compromising host fitness (Anderson and May 1978; May and Anderson 1978; Schall 1983; Scott 1987; Endler and Lyles 1989; Møller *et al.* 1990a; Hudson *et al.* 1998; Coltman *et al.* 1999). Particular attention has been paid to parasite infection as a driver of mate choice in models of sexual selection as one sex may advertise parasite resistance via elaborate secondary selected traits (Hamilton and Zuk 1982; Folstad and Karter 1992; Able 1996). Many aspects of primate sociobiology and socioecology have been studied in great detail (e.g. Crook and Gartlan 1966; Wrangham 1980; Terborgh and Janson 1986; Dunbar 1988; Smuts and Smuts 1993; Kappeler and Heymann 1996; van Schaik 1989; 1996; Kappeler and van Schaik 2004a), but in comparison to other factors, the importance of parasites in primate socioecology has received remarkably little attention (Nunn and Altizer 2006a). In this thesis, I focus on gastro-intestinal parasite infection in a wild primate species using an inter-disciplinary, longitudinal approach that allows a comprehensive and simultaneous investigation of patterns, determinants and potential consequences of individual variability in parasite infection susceptibility.

Determinants of parasite infection susceptibility

The probability of encountering parasites is largely determined by habitat preferences of both host and parasite, parasite population dynamics, environmental seasonality and host behaviour and diet (Hudson *et al.* 2002; Nunn and Altizer 2006b). However, once a parasite or disease agent is encountered, the probability of infection requires that a host is susceptible to the parasite in question. Individual differences in susceptibility are caused by a multitude of factors, broadly

categorized as genetic, endocrine, and environmental factors (Hudson and Dobson 1995; Nunn and Altizer 2006b; Sorci *et al.* 2009), which may determine differences in susceptibility between animals of different sex, age and social rank or that live in different social organisations.

The genetic component underlying variation in susceptibility to parasites mainly affects the ability to recognize a variety of parasitic antigens and to elicit the associated response (Wakelin and Apanius 1997). In this respect, genetic loci associated with the major histocompatibility complex (MHC) are primarily discussed as key factors for vertebrates. Empirical studies have shown that individuals that are heterozygous at the MHC loci have a selection advantage and are more capable of combating diverse infections than MHC homozygotes (Harf and Sommer 2005; Knapp 2005; Schad *et al.* 2005). In terms of parasite resistance, heterozygosity in general may be advantageous, i.e. beyond the MHC, as suggested by data collected on various model systems, (e.g. in seas lions, *Zalophus californianus*: Acevedo-Whitehouse *et al.* 2003; mountain white-crowned sparrows, *Zonotrichia leucophrys oriantha:* MacDougall-Shackleton *et al.* 2005; roe deer, *Capreolus capreolus*: Da Silva *et al.* 2009). Recent evidence also suggests that any genetic variation in parasite resistance could be associated with specific alleles of candidate-genes rather than heterozygosity per se (Hill 2006). Several of these candidate genes have been identified as cytokine genes that are directly involved in the cellular response by stimulating the production of immune globulins and T helper cells such as Interleukin-4 (Lawrence *et al.* 1998; Anthony *et al.* 2007; Fumagalli *et al.* 2009).

The effectiveness of the immune response is not only determined by genetic factors but is also dependent on the endocrine function of the host. Steroid hormones, such as glucocorticoids and androgens, play a major role in immune regulation, as changes in hormone levels following stressful events or reproductive periods, for example, can result in altered parasite susceptibility (Alexander and Stimson 1988; Khansari *et al.* 1990). Glucocorticoids, released from the adrenal cortex that prepare the body to cope with a crisis, have differential regulatory effects on the immune system by suppressing antibody production, decreasing killer-cell activity or affecting cytokine production (Khansari *et al.* 1990). Testosterone affects the immune system mainly by binding to specific receptors in the thymus region, known to be involved in T-cell maturation and immune regulation, resulting in a decline in both cell-mediated and humoral immune responses (Alexander and Stimson 1988). An immune-suppressive effect associated with steroid hormones has often been invoked to explain differences in parasite infections between hosts of different social rank (Hausfater and Watson 1976; Müller-Graf *et al.* 1996) or different sexes (Klein 2004; Schalk and Forbes 1997). The concept of androgen-dependent immune-suppression as also been employed to help explain patterns of parasite-mediated sexual selection (see below).

Apart from stress, potentially important environmental conditions can affect the development and functioning of the immune system (Sorci *et al.* 2009). Setting up, maintaining and developing specific immune responses are energetically costly. Strong nutritional constraints can therefore negatively impair the ability of a host to respond to parasite attacks and, thus, increase susceptibility to infections (Coop and Holmes 1996; Steketee 2003). Some nutrients such as dietary proteins and carotenoids can even directly affect the immunity of hosts (Chew and Park 2004; Coop and Holmes 1996). Food availability may also have an effect, as has been shown for bird chicks, where food availability explained changes in humoral immune responses (Gasparini *et al.* 2006). Additionally, conditions under which young individuals are reared, and the associated differences in exposure to parasites, will shape the state of the immune system (Sorci *et al.* 2009) since the long-term immunological memory established by the acquired immune response contributes to heterogeneity in susceptibility to parasite infection between individuals, populations and species.

Fitness consequences of parasite infection

Long-term behavioural field studies provide direct and indirect evidence that parasite infection in wild primate populations can potentially reduce reproductive success (Cheney *et al.* 1988), induce or contribute to death (Brain and Bohrmann 1992; Milton 1996), and that population declines are associated with epidemics (as summarized in Nunn and Altizer 2006a). On an individual level, parasite infections are also assumed to play an important role in sexual selection processes (Hamilton and Zuk 1982; Møller 1990a; Clayton 1991; Folstad and Karter 1992).

The theory of sexual selection has been primarily developed to explain the existence and evolution of conspicuous male traits, such as bright colours or large antlers that did not appear to promote survival, and, hence, could not be explained by the theory of natural selection (Darwin 1859; Darwin 1871). Darwin suggested that although these traits pose a serious risk to a male's survival, these costs should be outweighed by the advantage the male gains when competing with other males or attracting females (Darwin 1871). Two main mechanisms have been identified that drive the evolution of extra-ordinary morphological traits in one sex; the competition for mating partners (intrasexual competition) and mate choice (intersexual competition) (Darwin 1871; 1876; Møller 1988; Andersson 1994; Andersson and Iwasa 1996; Kappeler and van Schaik 2004a; Clutton-Brock 2007). In relation to mate choice, males can increase their reproductive success by mating with more than one female, while females, on the other hand, are limited by the resources required for gestation, lactation and offspring care and,

therefore, cannot increase the number of offspring produced by mating with more than one male (Trivers 1972). Thus, females are typically the more discriminatory sex in mate choice and can gain direct (resources, parental care, protection) and indirect (good or compatible genes) benefits from choosing the right male (Fisher 1930; Trivers 1972; Zahavi 1975; Andersson 1994; Penn and Potts 1998). Hamilton and Zuk advanced the idea of the indirect benefits of shopping for good genes by integrating genetic resistance to pathogens and parasites into the model and introduced the theory of *parasite-mediated sexual selection* (PMSS, Hamilton and Zuk 1982).

Three main models of PMSS can be distinguished, of which the first two models highlight direct benefits that females may gain from mating with little-parasitized males, and the third model, the Hamilton-Zuk model, emphasizes the indirect benefits of increased genetic resistance to parasites. The *contagious-indicator hypothesis* or parasite avoidance hypothesis, is the most generally applicable model of PMSS (Price *et al.* 1993; Loehle 1995; 1997; Able 1996). In species with internal fertilization, mating involves close body contact and facilitates direct transmission of contagious parasites. This applies to the transmission of ectoparasites in particular but also to sexually transmitted diseases (STDs) and directly transmitted helminth parasites (Able 1996). Avoiding mating with obviously parasitized males prevents transmission and would thus provide a direct benefit to females. The expression of condition-dependent secondary sexual traits should provide a key to infection status and help females to identify healthy mating partners (Freeland 1976; Borgia and Collis 1990; Able 1996; Hillgarth 1996; Loehle 1997; Walther *et al.* 1999).

The second direct benefit females may gain from mating with little-parasitized males is the maximisation of *parental care quality* (Hamilton 1990; Milinski and Bakker 1990; Price *et al.* 1993). Males that are impaired by parasite infection might not provide sufficient parental care and, therefore, the choice for males with fewer parasites might be more adaptive (Hillgarth and Wingfield 1997).

Finally, the PMSS model that has received most attention is the *Hamilton-Zuk model* (Hamilton and Zuk 1982). The authors proposed that females choose their mates on the basis of variable male secondary sexual characters that honestly signal a male's heritable parasite resistance. Only mates that are resistant to the deleterious effects of parasites will be able to optimally display the signal. Therefore, benefits for females are gained indirectly by mating with parasite-resistant males, allowing females to pass on resistance traits to their offspring. Predictions of this model have been tested empirically by exploring the detrimental effects of parasites on host fitness, heritability of parasite resistance, condition-dependent expression of a secondary sexual character and female preference for elaborate characters, and support for these

predictions have been found in various animal taxa (see Read 1988; Møller 1990a for reviews). Folstad and Karter (1992) further developed the Hamilton-Zuk model by providing a physiological framework for the parasite-ornament interplay. They identified testosterone as a major component of a model which is now referred to as the immunocompetence model (Folstad and Karter 1992). The main assumption of this model is that testosterone acts, on the one hand, as a potential immune-suppressant (see above and Grossman 1985; Alexander and Stimson 1988) which negatively affects parasite resistance, and, on the other hand, as a stimulant for the optimal expression of a sexually-selected signal (Balthazart 1983). The resulting trade-off provides an honest-signalling mechanism through which males that have good immune genes overcome the deleterious effect of testosterone on the immune system while expressing their secondary sexual character optimally. The applicability of the immunocompetence model to natural systems has been hotly debated as empirical tests of the hypothesis provided mixed results (Getty 2002; Roberts *et al.* 2004). Moreover, an immunosuppressive effect of testosterone could only be confirmed in certain taxa and for certain measures of immunocompetence (see Hillgarth and Wingfield 1997; Roberts *et al.* 2004 for reviews).

Red-fronted lemurs as study species

I studied different aspects of variation in parasite infection susceptibility in parasite infection in a free-ranging population of red-fronted lemurs (*Eulemur fulvus rufus,* Lemuridae, Primates) at Kirindy Forest, western Madagascar. The population has been subject to long-term demographic and behavioural studies (e.g. Ostner *et al.* 2002; Wimmer and Kappeler 2002; Kappeler and Erkert 2003; Ostner and Kappeler 2004; Scholz and Kappeler 2004; Kappeler and Port 2008), which provides a good body of knowledge about life history traits, habitat use and mating patterns for this species. Information on parasite infection in wild red-fronted lemurs is scarce (Junge and Louis 2005a) but reports of nematode worms in the faeces of red-fronted lemurs in Kirindy suggested that red-fronted-lemurs are highly infected with parasites (Kappeler P.M., Fichtel C., pers. comm.). In addition, their semi-arboreal habit, which involves frequent change in strata level during a day (Sussman 1974), facilitates direct contact with potentially contaminated food and substrates (Loudon *et al.* 2007). Red-fronted lemurs live in small multi-male/female groups of 5-12 individuals with an even or slightly male-biased sex ratio (Kappeler 2000; Ostner and Kappeler 2004). Reproduction in red-fronted lemurs is highly seasonal with only one mating period per year and during this three-to four-week period, females are in oestrus for approximately one day and mate promiscuously with several males (Ostner and Kappeler 1999; Overdorff 1998). Reproduction is highly skewed towards the dominant male, however,

this male is not able to fully monopolize reproduction as about one third of all paternities are attributed to subordinate males (Kappeler and Port 2008). Male rank is not reflected in differences in steroid hormones but androgen and glucocorticoid levels of all males increase significantly during the mating season (Ostner *et al.* 2002). Males and females differ with regard to pelage colouration with males exhibiting a striking rufous facial colouration (see Figure 1 in Chapter 5, Mittermeier *et al.* 2006). Results of an experimental study suggested that *Eulemur fulvus* sp. females spend significantly more time looking at images of males with bright red colourations than paler males; however mechanisms or the outcome of mate choice has not been investigated in this study (Cooper and Hosey 2003).

Objectives of this thesis

The overall aim of this thesis is to investigate patterns and determinants of parasite infection in a wild primate species, using a comprehensive set of individual parasitological, genetic, endocrine and socio-biological information collected over two field seasons in consecutive years. Additionally, using genetic paternity data as well as data on male colouration, I will investigate a potential consequence of parasite infection in red-fronted lemurs, focussing on the functional role of parasites in sexual selection processes. By studying the interplay of these factors, I hope to contribute to an increased understanding of the effect of parasite infections on their hosts. All interactions that will be examined in this thesis are depicted in Figure 1 and explained in more detail below.

Details on parasite infection in red-fronted lemurs are very limited (Junge and Louis 2005a) and partially anecdotal. Therefore, I first explored the gastro-intestinal fauna of red-fronted lemurs (**Chapter 2)** in order to get an overview of the prevalence of gastro-intestinal parasite infections that could be discerned via faecal samples. To avoid caveats and sampling biases often associated with parasitological studies conducted in the wild, I use a longitudinal study design involving regular sampling over two study seasons in two consecutive years as well as a standardized methodology during laboratory analyses (Gillespie 2006; Filipiak *et al.* 2009).

I will then test whether variation in individual susceptibility to parasite infection is related to genetic variation (**Chapter 3**). I focused on the cytokine Interleukin-4 as an immune genetic factor that is known to play a central role in the humoral immune defence against parasite infections, inducing an IgE switch and regulating worm expulsion from the intestines (Urban *et al.* 1991; Lawrence *et al.* 1998; Finkelman *et al.* 2004; Anthony *et al.* 2007), Thus, Interleukin-4 is a promising candidate for analysing individual differences in parasite susceptibility, but has not been used until now as a genetic marker for parasite susceptibility in field studies. Using

long-term population analyses, results of this study will provide important information on the heritability of parasite susceptibly.

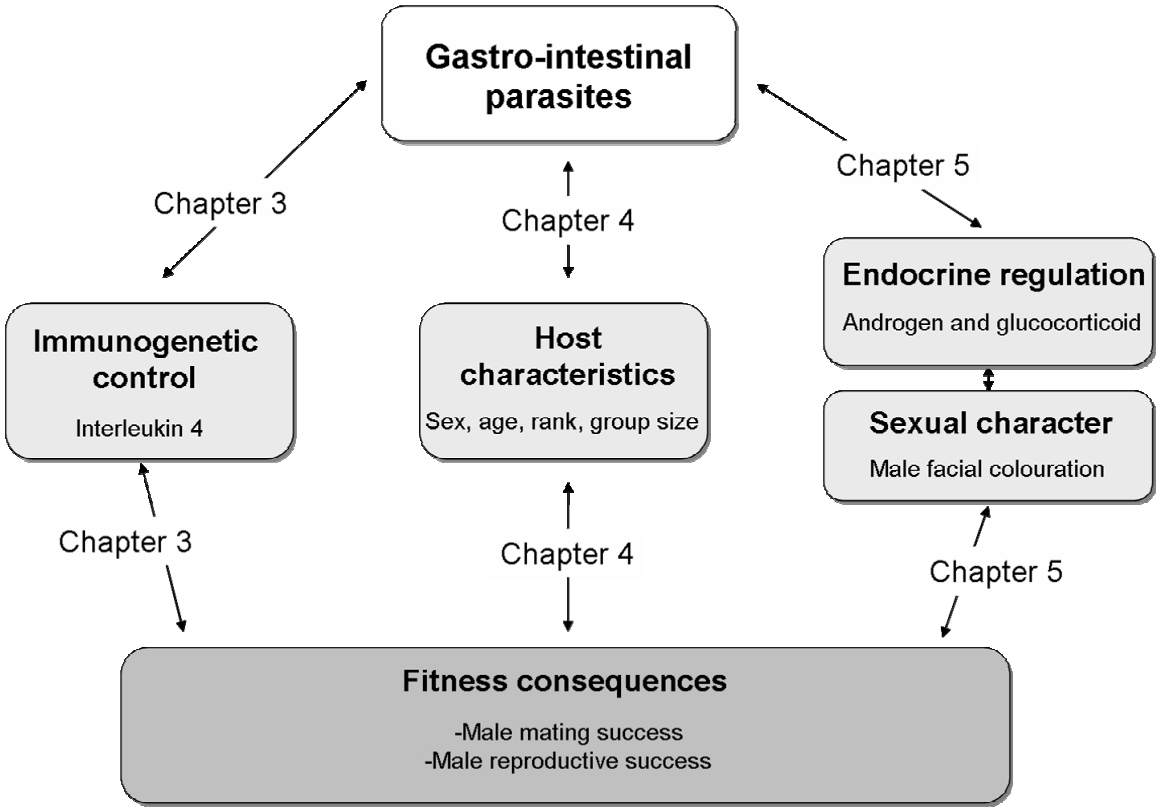


Figure 1. Organigram of the structure of the thesis

For an improved understanding of ultimate effects of parasite infection, it is important to first understand the proximate mechanisms regulating the variability and dynamics of susceptibility to parasite infections in the wild. Using a comprehensive dataset with multiple individually assignable parasite samples per individual, as well as information on age, sex, group size, social rank, and endocrine status (faecal androgen and glucocorticoid levels), we examined parasite infection patterns and host traits that may affect individual infection risk in **Chapter 4**. The longitudinal design with two study periods distributed over two consecutive years allows confirmation of potential general patterns. Naturally occurring changes in male steroid hormone levels facilitate the evaluation of the main prediction of the immunocompetence hypothesis, which postulates an immunosuppressive effect of androgens (Folstad and Karter 1992). As we could not measure immunocompetence non-invasively in the field, changes in parasite infection prevalence and intensity will be used as a proxy for the functioning of the immune system. Measuring female choice in a species with a promiscuous mating system is hampered due to the fact that any form of mate choice might be blurred by male reproductive tactics or moved to the

post-mating period and is thus difficult to evaluate (Kappeler and van Schaik 2004b). Still, several studies have already shown that mate choice exists in promiscuously mating species, too (e.g. Wolff and Macdonald 2004; Stumpf and Boesch 2005; Barelli *et al.* 2008). In order to assess the potential functional importance of parasite infection in red-fronted lemurs, we thus followed to approaches. First, we explored the association of observed mating success with individual parasite infection, which provides some indication if highly-parasitized males are discriminated during mating. Second, we analyse differences in parasite infections between males of different rank and reproductive success, as measured by genetic paternity analyses. By looking at the outcome instead of the sexual selection process itself, this will provide a test of the main predictions of the PMSS theory, namely that the less-parasitized males have increased reproductive success (Hamilton and Zuk 1982).

A core element of PMSS theory is the existence of a sexually selected trait with a signalling function that can honestly transmit information on male quality. We know, for example, from extensive studies on mandrills (*Mandrillus sphinx*, Setchell 2005) and rhesus macaques (*Macaca mulatta,* Waitt 2003) that the intensity of male colouration can play a major role in intra-sexual selection. In red-fronted lemurs, it has been suggested that females look longer at a bright red forehead of males in comparison to less colourful males and might thus prefer colourful males. However, the experimental setup of this study did not allow for the measure of the outcome of female choice (Cooper and Hosey 2003). In **Chapter 5**, we explore the signalling function and potential for condition-dependency of this male characteristic. As outlined above, the expression of a sexually selected signal may be regulated by hormones and the optimal expression may depend on the health status of the male (Folstad and Karter 1992). A potential quality signal in red-fronted lemurs should 1) vary in its expression between individuals, 2) reflect changes in quality on a short term basis, e.g. through hormone changes during the mating season and 3) be a predictor of reproductive success (Hamilton and Zuk 1982; Møller 1990a; Folstad and Karter 1992;). We test these hypotheses using digital quantifications of male facial colouration, steroid hormone data and reproductive success for the same individuals during the same period. Although sophisticated methods for measuring colouration in free-ranging animals by use of digital photography exist and are used in primatological studies (Setchell *et al.* 2006; Bergman and Beehner 2008; Higham *et al.* 2008), these studies have been restricted to skin colouration. The digital quantification of hair colour in wild primates is new, as nobody has, to our knowledge, measured hair colour beyond the measurement of luminance. Thus, in addition to the information needed on the potential of condition-dependency of male facial colouration, this part

of the study will evaluate and validate methods used to measure animal hair coloration in a free-ranging animals.

Each of the four core chapters of this thesis addresses one or several aspects of patterns, determinants and potential consequences of parasite infection susceptibility in red-fronted lemurs. By using an interdisciplinary approach, I hope to gain a better understanding of the proximate determinants of susceptibility to parasite infection and the strength of the driving force that parasites constitute in sexual selection processes in primates (**Chapter 6**).

2 Gastro-intestinal parasites of red-fronted lemurs in Kirindy Forest, western Madagascar

DAGMAR CLOUGH [1,2]

[1] Dept. of Behavioral Ecology and Sociobiology, German Primate Center, Göttingen, Germany
[2] Dept. of Anthropology and Sociobiology, University of Göttingen, Germany

Abstract

Although parasites are an important regulatory factor in animal populations, basic knowledge on the fauna of many vertebrate taxa is lacking. In particular, the parasite infections of primate species have gained little attention. Here I present data on the gastro-intestinal fauna of a population of wild red-fronted lemurs (*Eulemur fulvus rufus*; Primates: Lemuriformes) monitored over a total of 8 months during two consecutive field seasons in 2006 and 2007 in Kirindy Forest, western Madagascar. Using faecal samples for parasite analyses, I detected a minimum of ten parasite species including six nematodes (*Lemuricola vauceli*, *Trichuris* sp., two species of *Callistoura*, one trichostrongylid and one strongyloid-type egg), one anoplocephalid cestode and a dicrocoeliid trematode) as well as two protozoa species (*Entamoeba* sp. and *Balantidium coli*). In comparison to studies on other lemurs, the population in Kirindy Forest had the highest prevalence and parasite species richness ever recorded. Additionally, prevalence differed between the social groups studied. These findings lead to two conclusions: (1) short-term assessments of lemur health might underestimate the real parasite burden, and (2) it is important to extend the study to several social groups of a host population as groups may differ in parasite fauna due to minor microclimatic or habitat parameters.

Keywords

Helminth, protozoa, *Eulemur fulvus rufus*, primate, prevalence

2.1 Introduction

As primate species are important foci for conservation efforts, an understanding of the role that parasites play in wild populations will become vital for future conservation and management decisions (Altizer *et al.* 2003). The lemurs of Madagascar (Primates: Lemuriformes) are under particular threat due to habitat loss and the resulting increase in forest fragmentation which affects the whole island and its inhabitants (Ganzhorn *et al.* 1999; Sussman 1999). A series of short-term biomedical evaluations have provided information on lemur health, including parasite infection, for several species (Junge and Louis 2002; Dutton *et al.* 2003; Junge and Louis 2005a,b, 2007; Dutton *et al.* 2008), but the number of longitudinal studies assessing intestinal parasite infection over an extended period of time are limited (Muehlenbein *et al.* 2003; Raharivololona 2009; Wright *et al.* 2009; Schwitzer *et al.* submitted). Moreover, whereas the knowledge on parasite infections of other vertebrate taxa and anthropoid primates is being deepened, there is still a lack of baseline information about intestinal parasite infections for the majority of lemur species under natural conditions (Anderson 2000; Nunn *et al.* 2003; Junge and Sauther 2006; Nunn and Altizer 2006a; Bordes and Morand 2008).

The red-fronted lemur (*Eulemur fulvus rufus*) is a medium-sized, folivorous-frugivorous lemur species with a mean body mass of ca. 2 kg. Red-fronted lemurs are found in both moist lowland and montane forests in eastern Madagascar, as well as in dry deciduous forest in southern and western Madagascar (Mittermeier *et al.* 2006). According to the most recent IUCN Red List assessment, the red-fronted lemur is categorized as Near Threatened (NT), indicating that it is likely to qualify for a threatened category in the near future (IUCN Standards and Petitions Working Group 2008). The demography and behaviour of red-fronted lemurs has been studied extensively in Kirindy Forest (Wimmer 2002; Ostner *et al.* 2002; Kappeler and Erkert 2003; Ostner and Kappeler 2004; Kappeler and Port 2008; Port *et al.* 2009) and other places throughout its range (e.g. Sussman 1974, 1977; Overdorff 1998; Overdorff *et al.* 1999), yet information on parasite infection, a factor that is increasingly acknowledged as an important ecological and evolutionary force, is very limited for this species (Chabaud *et al.* 1965; Hugot *et al.* 1996; Hugot 1999; Junge and Louis 2005a).

Here I identify and quantify the gastro-intestinal parasites of four groups of wild red-fronted lemurs from faecal samples collected over a total of 8 months during two field studies in 2006 and 2007. Using a study design with multiple measurements per individual allowed for a good approximation of the real parasite load that is sustained by this lemur population.

2.2 Methods

During two study periods between March - July 2006 and 2007, I collected 735 faecal samples from a habituated population of red-fronted lemurs (*Eulemur fulvus rufus*) at Kirindy Forest, located some 60 km northeast of Morondava, western Madagascar (Figure 1).

The 60-ha study area is part of the field site operated by the German Primate Center (DPZ) and is situated within a forestry concession managed by the Centre National de Formation, d'Etudes et de Recherche en Environnement et Foresterie (CNFEREF) de Morondava. The forest is classified as dry deciduous forest and is subject to pronounced seasonality due to a dry season from March/April to October and a hot wet season from November to March/April. Mean annual temperature at Kirindy is 24.7°C (mean monthly maxima and minima of 30.7°C and 19.0°C) and mean annual precipitation is 767 mm/year with maximum and minimum values of 1511 mm and 390 mm (Sorg *et al.* 2004).

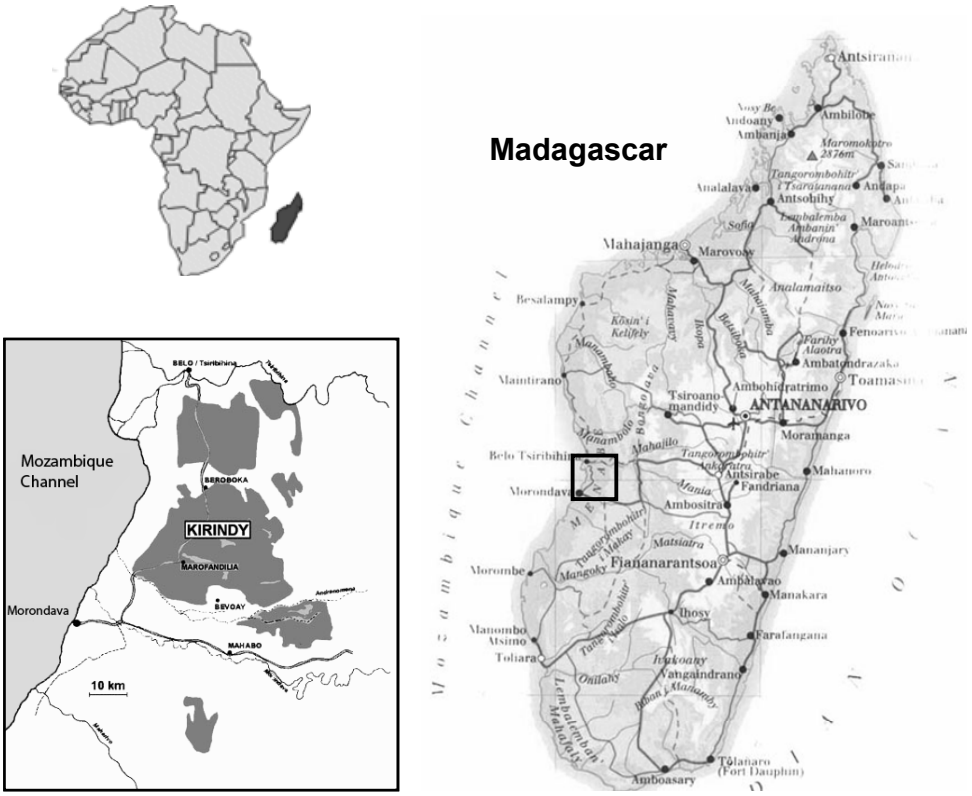


Figure 1. Location of the Kirindy study area, western Madagascar

Faecal samples for parasitological analyses were collected from four groups of red-fronted lemurs (group A, B, F, J) totalling 29 individuals over both years. Animals were habituated to human presence and marked with individual combinations of nylon collars and pendants or radio

collars which allowed for individual assignment of samples. Each individual was regularly and repeatedly sampled on a weekly basis throughout the study period. Home ranges of the four groups partially overlap (Scholz *et al.* 2004, Ostner *et al.* 2004).

Samples were collected immediately after defecation, placed in plastic tubes, pre-aliquoted with 10% neutral-buffered formalin (parasite analyses), labelled and wrapped with parafilm. Additionally, I used some faecal samples for coproculture, which helped species identification of helminth egg (Gillespie 2006). All samples were stored at ambient temperatures (25-35 C°) in the field and transported to the laboratories of the DPZ at the end of each field session. Samples were processed using a modified version of the formalin-ethyl-acetate sedimentation technique described in Ash and Orihel (1991). Approximately 5ml well-homogenized faecal material was strained into centrifuge tubes through a 400µm mesh size polyamide sieve. 10% formalin was added, bringing the total volume to 10ml. After adding 3ml ethyl-acetate I capped and shook the tube vigorously for 30s and centrifuged it for 10 min at 500x *g*. Before pouring off the supernatant consisting of ethyl-acetate, formalin and debris, the top layer of fat was removed from the centrifuge tube. The remaining sediment was subsequently analysed under a compound microscope or stored in Eppendorff tubes for later confirmation of identification. I used Lugol's solution to facilitate protozoan identification. Measurements of eggs, cysts and trophozoites were made to the nearest 0.1µm using an ocular micrometer fitted to a compound microscope. Processing of faecal samples and egg / cyst counts was done "blind" without knowledge of the identity of the sample but using only a numeric code instead.

I used two measures to control for the reliability of our egg/cyst detection method. First, the number of parasitic stages detected per one slide was directly compared to results derived from scanning three slides for the same sample. Correlation analyses proved that both methods resulted in comparable numbers (Pearson correlations per parasite species, $p<0.001$, r^2 range: 0.73 - 0.98, n=24), thus I applied the former, more economic method. Second, we conducted intra-observer and intra-sample reliability tests on repeatedly scanned samples, which confirmed that measures between and within samples were highly reliable and repeatable (Kendall's $W=0.9$; $p< 0.001$ n=8).

Species identification was based on morphological traits (colour, shape, size and content of eggs, cysts, trophozoites, larval and adult stages), identifying "morpho-species", which cannot replace preciseness of genetic analyses and might underestimate the true number of species encountered in a sample. Yet, for a parasitological-ecological study using non-invasive sampling of natural populations, these are often the only feasible measurements, and consequently morphospecies are widely used in primatology (Müller-Graf *et al.* 1996; Stoner 1996; Harf and

Sommer 2005; Muehlenbein 2005; Schad *et al.* 2005). Identification followed Chabaud and Choquet (1955), Chabaud and Petter (1958), Chabaud *et al.* (1961; 1964; 1965), Deblock and Diaouré (1962), Chabaud and Brygoo (1964), Brooks and Glen (1982) and Hugot *et al.* (1996). For the sake of simplicity, I use the term "species", yet implying morphospecies. For confirmation of parasite identification, I also collaborated with members of the Parasitic Worm Group, National History Museum, London. Due to the pre-dominance of eggs and larvae in most samples and the low numbers of parasite stages of some parasite taxa (e.g. trematode, cestode), I was not always able to identify parasite taxa to species level and the majority of our findings are presented at the genus or family level.

The degree of parasite infection will be presented as parasite prevalence (number of individuals infected as a percentage of the number of individuals examined, Margolis *et al.* 1982; Bush *et al.* 1997).

2.3 Results

Intestinal parasite infections

None of the faecal samples was diarrheic or showed traces of blood. Macroscopic parasites stages were detected in 22% of all samples (mean 3±2.4 SD) live parasite individuals excreted per infected faecal sample were detected. Additionally, I recovered the eggs and larvae of ten intestinal parasite species including six species of nematodes, one trematode, one cestode, and two protozoan species (Table 1).

Nematodes

Species identification of *Lemuricola vauceli* was based on eggs, larvae and adult worms (including gravid females) recovered from fresh faeces, which allowed identification to species level. Across both years, prevalence of infection with *Lemuricola vauceli* was 100%. *Callistoura* sp.1 was another pinworm parasite with 100% prevalence. Identification of *Callistoura* sp.1 eggs could not be pursued unambiguously to species level as I only detected eggs and larvae but no adult stages. However, the shape and size of eggs strongly suggested that they originated from adults of the species *Callistoura blanci.* Additionally, I recovered eggs of *Callistoura* sp.2, resembling *Callistoura brygooi* in both size and shape (Table 1), yet, minute warts on one of the poles, characteristic for this species (Chabaud and Petter 1958), were not visible on any of the eggs. *Callistoura* sp.2 was only detected in 4 of 29 individuals (14 %). *Trichuris* was identified on the basis of characteristic egg morphology (barrel-shaped, bipolar

plugs) and egg size. The strong host specificity of *Trichuris* parasites suggests that the species is *Trichuris lemuris* (Chabaud and Brygoo 1964). Prevalence of *Trichuris* was 31%. Trichostrongylid-type eggs were found in 24% of all individuals examined. Size of trichostrongylid eggs suggested identification as *Pararhabdonema* sp.. Thin-shelled strongyloid-type eggs were detected in only one individual. Egg morphology and filariform larvae closely resembled *Lemurostrongylus* sp., but low sample sizes prohibited more distinct identification.

Trematode

One trematode egg was recovered, which belongs to the family Dicrocoeliidae as characterized by its size and the presence of an operculum. Further identification was hampered by the low number of eggs in samples from only one individual. Larvae or adult stages were not detected.

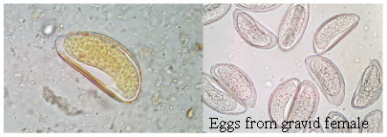
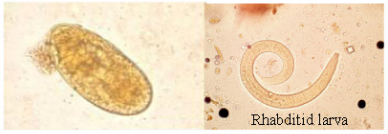
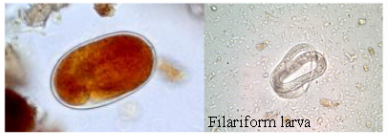
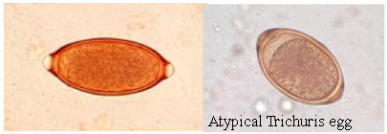
Cestode

Cestode eggs were found in three individuals. Size, colour and content pointed to classification within Anoplocephalidae, probably *Thysanotaenia lemuris* (Deblock and Diaouré 1962).

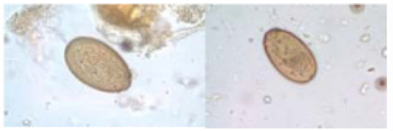
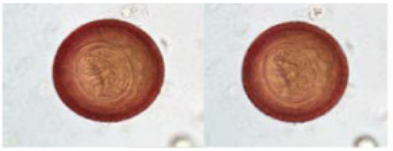
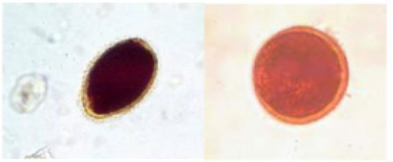
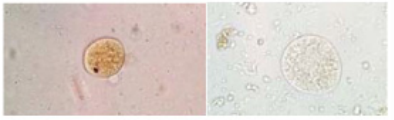
Protozoa

Two protozoan species were found in all individuals. Morphology of Entamoeba cysts most closely resembled *Entamoeba coli*, and preliminary molecular analyses confirmed the species identification. However, analyses also suggests the existence of undescribed *Entamoeba* spp. in the samples (Levecke B., unpublished results), which does not currently allow explicit identification and needs further exploration. The second protozoa species detected is *Balantidium coli*, which I identified via cysts and trophozoites detected in samples from all individuals (100%).

Table 1 (pages 16 and 17). Description of the parasites recovered from the faeces of red-fronted lemurs. Images on the left show representative eggs/cysts/trophozoites. Scale bar is given for the left column and represents 50µm. Right column of images provides additional information as indicated. References for identification are (1) Chabaud *et al.* 1965, (2) Chabaud and Petter 1958, (3) Chabaud *et al.* 1964, (4) Chabaud and Coquet 1955, (5) Déblock and Diaroué 1962.

Morphospecies	Phylum / Class	Order / Family	compatible species described in lemurs	Characteristics used for identification	Transmission cycle - Mode of infection	Image
Lemuricola vauceli [1]	Nematoda / Secernentea	Ascaridida / Oxyuridae	*Lemuricola vauceli*	Eggs (27-30 x 59-70 µm) and adult worms	Direct - Larvated eggs ingested	
Callistoura sp.1 [1, 2]	Nematoda / Secernentea	Ascaridida / Oxyuridae	*Callistoura blanci*	Operculated eggs (46-49 x 85-99 µm) and larvae	Direct - Larvated eggs ingested	
Callistoura sp. 2 [1, 2]	Nematoda / Secernentea	Ascaridida / Oxyuridae	*Callistoura* cf. *brygooi*	Operculated eggs (41-46 x 83-91 µm)	Direct - Larvated eggs ingested	
Trichostrongylidae sp.[4]	Nematoda / Secernentea	Rhabditida / Trichostrongylidae	*Pararhabdonema* sp.	Dense morulated and operculated egg, (37-38 x 70-79 µm), and rhabditid larvae	Direct - Skin penetration or ingestion of larvae	
Strongyloididae sp.[5]	Nematoda / Secernentea	Tylenchida / Strongyloididae	*Lemurostrongylus* sp.	Eggs (39-43x68-76 µm) and filariform larvae	Direct - Ingestion of third-stage larvae	
Trichuris sp. [3]	Nematoda / Adenophorea	Trichocephalida / Trichuridae	*Trichuris lemuris*	Eggs (36-41 x 71-86 µm)	Direct - Larvated eggs ingested	

50µm

Morphospecies	Phylum / Class	Order / Family	compatible species described in lemurs	Characteristics used for identification	Transmission cycle - Mode of infection	Image
Dicrocoeliidae sp.	Plathelminthes / Trematoda	Digenea / Dicrocoeliidae	-	Operculated eggs (24-25 x 42-44 µm)	Indirect - Oral ingestion of intermediate hosts	
Anoplocephalidae sp.[6]	Plathelminthes / Cestoda	Cyclophyllida/ Anoplocephalidae	*Thysanotaenia lemuris*	Eggs (Ø 68-73 µm)	Indirect - Oral ingestion of intermediate hosts	
Balantidium coli	Ciliophora / Litostomatea	Vestibuliferida / Balantiidae	*Balantidium coli*	Cysts (23-35 x 35-53 µm) and trophozoites (23-88 x 36-110 µm)	Direct - Cysts or trophozoite ingested	
Entamoeba sp.	Sarcomastigophora/ Amoebozoa	Entamoebida / Entamoebidae	*Entamoeba coli*, *Entamoeba* cf. *coli* (undescribed)	Cysts (Ø 17-25 µm)	Direct - Cysts ingested	

50µm

Table 2. Variation of parasite prevalence among groups and across both years studied. Columns for each group show the prevalence of infected adults per group (in %). Group sizes are given as number of adults per group in both years.

Parasites	Group A n=7	Group B n=8	Group F n=6	Group J n=8	Overall n=29
Lemuricula vauceli	100	100	100	100	100
Callistoura sp. 1	100	100	100	100	100
Callistoura sp.2	14	25	17	0	14
Trichuris sp.	86	13	17	13	31
Strongyloididae sp.	0	0	33	0	7
Trichostrongylidae sp.	43	25	17	13	24
Dicrocoeliidae sp.	14	0	0	0	3
Anoplocephalidae sp.	14	0	33	0	10
Entamoeba sp.	100	100	100	100	100
Balantidium coli	100	100	100	100	100

Differences between social groups

Prevalence of *Lemuricola, Callistoura* sp.1*, Entamoeba and Balantidium* infections were generally high in all groups and did not show any variation between groups (Table 2). All other parasite taxa showed differences between groups. For example, no individual of group J showed any sign of infection with the oxyurid parasite *Callistoura* sp.2. Prevalence of *Trichuris* and trichostrongyloid infection was highest in group A. Trematode infections (Dicrocoeliidae sp.) were only detected in group A. Prevalence of strongyloid infections were only detected in group F.

2.4 Discussion

All red-fronted lemurs within the study population at Kirindy Forest were found to be co-infected with eight helminth and two protozoan taxa. 100% prevalence of infection for protozoan taxa Entamoeba spp. and Balantidium coli has not been recorded in such a magnitude before (Brack 1987; Faulkner *et al.* 2004; Junge and Louis 2005). Also, the number of unique parasite species detected in this study and 100% prevalence of infection of two nematode species exceeded numbers reported in other studies on wild Eulemur species (Nègre 2003; Faulkner *et al.* 2004; Junge and Louis 2005; Nègre *et al.* 2006; Schwitzer *et al.* submitted) and other lemur

species such as *Propithecus* spp. (Muehlenbein *et al.* 2003; Junge and Louis 2005; Loudon *et al.* 2007; Wright *et al.* 2009), *Varecia rubra* (Dutton *et al.* 2008), and *Lemur catta* (Dutton *et al.* 2003; Loudon *et al.* 2007). The only study to report similar parasite species richness was conducted on *Microcebus murinus* (Raharivololona 2009), where gastro-intestinal parasites of study animals had been assessed regularly over the course of several years and nine morphospecies had been identified although prevalence was not reported.

The ability to compare results of parasite infection in lemurs is certainly compromised by the application of divergent methodologies for faecal sample processing. (Gillespie 2006). Both flotation and sedimentation techniques are frequently applied yet might be targeting different parasite taxa. Ethyl-acetate sedimentation, the method used in this study, has been recommend as a reliable procedure to recover helminths and protozoa (Ash and Orihel 1991) and has proved to be a successful method for red-fronted lemur samples. Nevertheless, a comparison of our findings to results generated from other studies suggests that real parasite infection is potentially underestimated in studies assessing parasite infection over a relatively short period of time. The potential for underestimation is particularly problematic for pinworm (oxyurid) infections, as they are common in many primate species but eggs are not often seen in faecal analyses (Stuart and Strier 1995). Eggs could therefore easily be missed in studies using cross-sectional designs which might also be the case for other parasite taxa, including protozoan species. In addition, it has been shown that parasite diversity strongly correlates to sampling effort (Nunn *et al.* 2003)

Red-fronted lemur groups differed significantly in infection prevalence of four nematode (*Callistoura* sp.2, *Trichuris* sp., strongyloid and trichostrongylid parasite), one trematode (Anoplocephalidae sp.) and the cestode parasite (Dicrocoeliidae sp.). Part of the variability between groups might be explained by exposure to different habitat variables as the Kirindy River bisects the study area at its southern perimeter (see Scholz and Kappeler 2004 for more details). Although I did not specifically undertake analyses of home range distribution, group A was the group that spent most time close to the river (Clough, pers. observation). Close proximity to water might increase the transmission of trematode parasites via aquatic intermediate hosts or by higher interaction rates with other lemur groups visiting the water holes within their home range. A correlation of close proximity to rivers and parasite infections has already been reported by other studies on free-ranging howler monkeys (Stoner 1996). As animals living in cohesive groups spend more time in close proximity to other group members, transmission of parasites within groups within groups is likely which can result in a higher level of parasite similarity among group members (Altizer *et al.* 2003).

The majority of intestinal parasites detected in this study was characterized by a monoxenous life cycle (Table 1) in that they infect their host directly without the need of an intermediate host (Anderson 2000). This allows direct transmission of infectious parasite stages through the faecal-oral route via contaminated soil, fruits or water that have been in contact with faecal matter. The frequent change of vertical habitat use (i.e. time spent on the ground and in various forest strata, Sussman 1974) facilitate infection of red-fronted lemurs with free-living parasite stages and might explain their higher parasite infection prevalence in comparison to prevalence recorded for Verraux's sifakas (*Propithecus verreauxi),* a predominantly arboreal lemur species. Comparative parasitological studies on the sympatric sifaka population at Kirindy are currently under way but results from Muehlenbein *et al*., (2003) and Loundon *et al.* (2008) already indicate that the arboreal life-style of Verraux's sifakas may expose this species to a smaller range of parasites.

Pathogenicity of most parasite taxa detected in this study is not known due to the fact that they show a high specificity to their lemur hosts and differ from common parasites encountered by and known to veterinarians. However, extrapolating information from more common parasite species of the same taxonomic family or order provides some indication of the clinical importance of most of the species. Pinworm infections (such as *Lemuricola* and *Callistoura* infections in red-fronted lemurs) and trichuriasis (infection with *Trichuris*) can cause perianal itching, aggressiveness, dehydration, loss of weight and juvenile death. However, clinical symptoms depend on both the strength of infection and the condition of the host, and clinical manifestation can vary widely from asymptomatic to fatal infection (Fowler, 1993; Kaur and Singh, 2009). Strongyloid infections are viewed as dangerous infections in primates as fatalities have been reported for orang-utans chimpanzees, gibbons, patas monkeys and woolly monkeys (Fowler 2003). Trichostrongylid infections are mostly asymptomatic as are trematode infections (e.g. Dicrocoellidae sp.). Pathogenicity of cestode parasites (e.g. Anoplocephalidae sp.) strongly varies with species. Amoebic infections with *Entamoeba coli* are usually considered as apathogen and clinical symptoms are mainly known from infections with *Entamoeba histolytica*. Similar to *Balantidium coli* infections, these protozoan infections can occasionally cause severe diarrhoea or other gastro-intestinal disorders. However, it is most likely that both are well-adapted parasite species that opportunistically inhabit the gut (Fowler 1993). During the study period, the population was subject to intensive behavioural observations (Port *et al.* 2009) where every group was followed daily. Although prevalence of infection of some of the parasites was 100%, no clinical symptoms were apparent. Prevalence of more pathogenic parasites, such as strongyloid or cestode infections, was very low, which might provide an explanation for the fact that the animals did not show any obvious signs of disease.

Parasite infections are considered to be a critical component in conservation biology (May, 1988). Although red-fronted lemurs are not yet classified as endangered, the primary threat for these lemurs is habitat destruction (Mittermeier *et al.*, 2006). In Kirindy Forest, primates are also in indirect contact with humans through frequent tourist visits, researchers conducting field studies and villagers living in adjacent areas of the forest, which might increase the possibility of dynamic interplay of animals, humans and environment (Kaur and Singh 2009, Loudon 2008). Red-fronted lemurs showed a very high degree of parasite infection with high prevalence and the greatest parasite species richness to be detected in a study in Madagascar. Although I could not detect signs of clinical significance, and some parasite-host relationship might be of commensal nature, parasite infections might affect animals more severely when intrinsic or ecological stress increases. Additionally, the intensity of infection, not considered in this study, might act as an important factor which should be taken into account in future studies.

In summary, this study provides a detailed account of parasite infection in a free-ranging population of red-fronted lemurs. In comparison to other studies, the longitudinal design of the study may prove to be a better estimator of real parasite infection level than short-term cross-sectional designs: in this longitudinal study I detected the greatest parasites species richness than has been previously reported for red-fronted lemurs in particular and other lemur species in general. Parasite infection prevalence in Kirindy Forest was generally high, suggesting both commensal relationships between some of the parasite species and red-fronted lemurs might be reservoirs for them (Loudon *et al.* 2007; Kaur and Singh 2009). Whether the observed levels of parasite infection will have a biologically significant effect on the populations is not known. Increased ecological pressure and intrinsic stress can lead to increased parasite infections and therefore, subsequent studies are essential for monitoring the long term impact of parasite infection on lemur population viability. Differences between groups strongly suggests that sampling several groups per host population is required as micro differences in habitat or ranging behaviour can already lead to differences between groups and provide additional information.

2.5 Acknowledgements

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3 Susceptibility to helminth infections is associated with an *IL4* promoter polymorphism in wild red-fronted lemurs

DAGMAR CLOUGH[1, 2], PETER M. KAPPELER[1, 2], LUTZ WALTER [3]

[1]Dept. of Behavioral Ecology and Sociobiology, German Primate Center, Göttingen, Germany
[2]Dept. of Anthropology and Sociobiology, University of Göttingen, Germany
[3]Dept. of Primate Genetics, German Primate Center, Göttingen, Germany

Abstract

Susceptibility to parasite infections affects important fitness-related processes and traits, such as mate choice and survival, yet its genetic regulation remains poorly understood. Interleukin-4 (*IL4*) is known to play a central role in the humoral immune defence against parasite infections, inducing IgE switch and regulation of worm expulsion from the intestines. Recently, the evolutionary significance of single nucleotide polymorphisms (SNPs) in *IL4*-genes has been highlighted, yet empirical information on the effect of interleukin SNPs on helminth infections is lacking. Using a candidate-gene approach, we explored the association of *IL4*-gene promoter polymorphisms with nematode infection in a population of wild red-fronted lemurs (*Eulemur fulvus rufus*, Primates: Lemuridae), monitored over two study periods in consecutive years. Sequence analyses of lemur DNA detected a new lemur-specific C/T-promoter polymorphism significantly associated with parasite infection intensities. Carriers of the T/T genotype showed highest nematode infection intensities, suggesting a functional role of this SNP, yet long-term population analyses indicated higher reproductive success of T/T individuals than expected. This new molecular tool offers quick assessment of individual genetic constitution with regard to nematode infection intensity that is practically easy and very cost-efficient.

Keywords:

Interleukin-4, SNP, parasite, primate, *Eulemur fulvus rufus*

3.1 Introduction

Gastrointestinal parasite infections can impose high costs on both human and animal populations, increasing morbidity and mortality, particularly in hosts under ecological stress (Chan *et al.* 1994; Stephenson *et al.* 2000; Gillespie *et al.* 2005). Previous studies on the genetic control of parasite infection and resistance in natural population have focussed on genes of the major histocompatibility complex (MHC) as this highly polymorphic genetic system determines susceptibility and resistance to infectious diseases (Hill 2006). In general, individuals that are heterozygous at the MHC have a selection advantage and are more capable of combating diverse infections than MHC homozygotes (Carrington *et al.* 1999; Harf and Sommer 2005; Schad *et al.* 2005). A heterozygous advantage also applies to overall genetic diversity of individuals and supporting empirical evidence is accumulating from studies of various model systems (e.g. in seas lions, *Zalophus californianus*: Acevedo-Whitehouse *et al.* 2003; mountain white-crowned sparrows, *Zonotrichia leucophrys oriantha:* MacDougall-Shackleton *et al.* 2005; roe deer, *Capreolus capreolus*: Da Silva *et al.* 2009).

However, the magnitude of an immune reaction is not only regulated by a host's genetic diversity, but rather by the intensity of the elicited response. Recent discussion suggests that any genetic variation in resistance to parasites could also be associated with specific alleles of candidate-genes rather than heterozygosity per se (Hill 2006). Immunity to helminth infections is mainly mediated by CD4+ T-helper2-(T_H2) lymphocytes and promotion of T_H2 immune responses is essentially dependent on the cytokine interleukin-4 (IL-4) (Hotez *et al.* 2008). Presence of IL-4 not only induces and sustains T_H2 responses, but also initiates immunoglobulin (Ig) isotype switching to IgE, which plays an essential role in anti-parasite immunity (King and Mohrs 2009). Evidence is accumulating that single nucleotide polymorphisms (SNPs) in the promoter of the *IL4* gene can affect transcription, resulting in more or less IL-4 protein expression and, hence, in higher or lower IgE titers. Thus, SNPs can effectively influence the intensity of various infections (Rosenwasser and Borish 1997; Luoni *et al.* 2001; Kabesch *et al.* 2003; Basehore *et al.* 2004; Gyan *et al.* 2004; Verra *et al.* 2004; Paffen *et al.* 2008). In addition, IL-4 is known to play an important role in enteropathic expulsion of nematode worms and mucosal permeability varies after nematode infections (Lawrence *et al.* 1998; Zhao *et al.* 2003; Finkelman *et al.* 2004; Anthony *et al.* 2007). Despite its key role in the regulation of parasite infections and the evolutionary significance of its SNPs (Fumagalli *et al.* 2009), to our knowledge no study has yet investigated the importance of *IL4* promoter polymorphism in natural populations.

We studied red-fronted lemurs (*Eulemur fulvus rufus,* Primates: Lemuridae) inhabiting a dry deciduous forest in western Madagascar to examine the effect of *IL4* promoter polymorphism on parasite infections in a wild primate population. The population has been subject to long term demographic and behavioral studies (Ostner *et al.* 2002; Wimmer and Kappeler 2002; Kappeler and Erkert 2003; Ostner and Kappeler 2004; Kappeler and Port 2008) as well as to parasitological monitoring (Clough *et al.* unpublished data). The main objectives of our study were: (1) to identify promoter SNPs in the red-fronted lemur *IL4* gene, also focussing on polymorphic sites that had already been detected in human and non-human primates (Rosenwasser *et al.* 1995; Song *et al.* 1996; Takabayashi *et al.* 1999; Hackstein *et al.* 2001; Kabesch *et al.* 2003). (2) To associate the *IL4* SNP genotype in red-fronted lemurs with intensity of nematode infections but also on extracellular protozoa infections, as they can equally invoke a T_H2-type response triggered by cytokines (Zambrano-Villa *et al.* 2002). (3) To identify a possible functional role of the *IL4*-polymorphic allele in question on selective processes by exploring long-term fitness consequences between males with different allelic constitutions. Because this is the first time that the relation between immune-regulatory interleukin polymorphism and gastro-intestinal parasite infections in a wild primate species was studied, specific predictions could not be formulated *a priori*.

3.2 Methods

Study site and sample collection

Data were collected on a population of red-fronted lemurs at the study site of the German Primate Center (DPZ) in Kirindy Forest, western Madagascar. Detailed description of the study site can be found in Sorg *et al.* (2004). During two study periods between March and July in 2006 and 2007, 499 faecal samples were collected for parasitological analyses from 29 individually recognizable red-fronted lemurs of 4 social groups. Immediately after defecation individually assigned samples were taken and stored in labelled vials containing 10 % buffered formalin, and returned to the DPZ laboratories after each field season. Tissue samples for genetic analyses were collected routinely during an ongoing long-term study at the site.

Parasitological analyses

Faecal parasites samples were processed using a modified form of the formalin-ethyl-acetate sedimentation technique as described by Ash and Orihel (1991). Briefly, approximately 5ml homogenized faecal material was strained into centrifuge tubes through a polyamide sieve. 10%

formalin was added, bringing the total volume to 10ml. After adding 3ml ethyl-acetate we shook the tube vigorously for 30s, followed by centrifugation at 500x *g* for 10 min. Before pouring off the supernatant consisting of ethyl-acetate, formalin and debris, the top layer of fat was removed from the centrifuge tube. The remaining sediment was transferred to Eppendorf tubes and used for subsequent analyses.

Wet mounts were prepared using 20mg faecal sediment, analyzing individual samples for intestinal nematode and protozoa stages (eggs, larvae, cysts). Cysts, larvae and adult stages found in faecal samples were used for species identification (Clough *et al.*, unpublished data). Results on egg (nematodes) and cyst and trophozoite (protozoa) counts, respectively, were extrapolated to 1g faecal sediment (x50). As measuring units for parasite infection we used prevalence (the percentage of individuals infected within the population), PSR (parasite species richness, the number of parasite species detected within a hosts) and parasite infection intensity (the number of eggs / cysts per gram faeces).

Genetic analyses

IL4 promoter sequencing

DNA was isolated from faecal animal tissue samples using QIAamp® tissue kits (Qiagen). The fragment of the *IL4* gene promoter region was amplified using the following pair of primers: (1) forward 5'-CATACGAACCTGCTGGGAC-3' and reverse 5'-CAATCAGCAC GTCTCTTCCA-3'. Hot start PCR reaction was prepared in a total volume of 30µl with 10pmol of each primer, 166 µM dNTPs, and 2U Taq-DNA polymerase. Amplification was performed according to the following protocol: 5min at 92°C, 45 cycles of 92°C for 1min, 58°C for 1min and 1min at 72°C, and final elongation for 5min at 72°C. PCR products were purified with the Millipore DNA purification kit and sequencing was performed in both directions on an automated capillary sequencer (ABI 3130xl, Applied Biosystems) with same primers as mentioned above. Individual *IL4* sequences were aligned and examined for occurrence of SNPs using the biological sequence alignment editor BioEdit 7.0.9 (Hall 1999). The newly discovered SNP at position -485 bp upstream the transcription start has been submitted for publication in dbSSNP data base (ss -485C/T 42460308). The *IL4* promoter sequence is stored in the DDBJ/EMBL/Genbank database, accession number GQ221019.

Individual heterozygosity

Controlling for a potential heterozygosity effect we analysed additionally the effect of general genetic diversity on parasite infection using neutral markers. Following Schwensow *et*

al. (2007), we based our measurement on observed individual microsatellite heterozygosity ($H_{obs\text{-}ind}$), using the quotient of the number of heterozygous microsatellites loci per individual to the total number of typed loci. We used eleven microsatellites that had already been developed and established in earlier studies on the same population of red-fronted lemurs (Wimmer and Kappeler 2002; Kappeler and Port 2008) and calculated observed (H_{obs}) and expected and observed heterozygosity (H_{exp}) for every locus. Observed and expected heterozygosity, as well as deviations from Hardy-Weinberg-equilibrium were calculated using the allele frequency analyses in CERVUS 2.0.

Reproductive success

In order to explore a functional role of different *IL4* constitutions on reproductive success of males, we extended our dataset by including long-term paternity data from a total of 45 males collected between the years 1998 to 2007 of the same population. During this time period 59 offspring were born to the population of which genetic fathers could be identified. Paternity data as well as detailed methods on microsatellite-based paternity analyses have already been published elsewhere (Kappeler and Port 2008). Observed reproductive success of each male per social group and year was calculated with regard to individual genotype and compared to expected reproductive success, which was based on the distribution of males per group and year.

Statistical analyses

Variability in prevalence and PSR did not show any association with *IL4* genotype; hence analyses are only presented for parasite infection intensities. We used monthly means of faecal egg/cyst counts per individual accounting for natural occurring variations in parasitic excretions (Anderson and Schad 1985; Gillespie 2006). Individual variation was analysed using a general linear model with log-transformed response variables (nematode or protozoan infection intensities) and animal identity ("ID") and year as explanatory factors.

Exploring differences between individuals of different genotype, we used a linear mixed model approach (lmer in R, Bates *et al.* 2008) with square-root-transformed data on individual means, implementing the social group as random factor. Non-independence of repeated measurements per individual was accounted for by incorporating ID nested in group and crossed with years as random intercepts. P-values for mixed models were estimated using Markov chain Monte Carlo (MCMC) simulations (Baayen *et al.* 2008).

Relationships of individual heterozygosity and nematode and protozoan infection intensities were assessed using partial correlation analyses controlling for data sampling in different years. Observed and expected frequencies of successful paternities per offspring per male were compared between genotypes with χ^2 test statistics.

All statistical analyses were performed with software R (version 2.8.1, R Development Core Team 2008) and significant level was set at 0.05.

3.3 Results

IL4 promoter polymorphism in red-fronted lemurs

We sequenced a 529 bp fragment of the *IL4* promoter region of 29 individual red-fronted lemurs and identified a C/T polymorphism at position -485bp upstream of the transcription start site (Figure 1), which is not identical to known *IL4* promoter polymorphisms in human or non-human primates (Rosenwasser *et al.* 1995; Takabayashi *et al.* 1999; Hackstein *et al.* 2001; Kabesch *et al.* 2003). A polymorphic site at position -485 bp in primate *IL4* promoters has not been described so far. The -485C/T was the only SNP found within the promoter sequence of the population of red-fronted lemurs studied and all possible genotypes were present in the study population. Frequencies of the genotypes were C/C: 55.2%, C/T: 34.5%, T/T: 10.3% (n = 29).

Individual parasite infection and association with IL4 gene promoter polymorphism

Lemurs of both sexes were parasitized by at least four parasite species and we identified ten different parasite taxa in total, comprising six species of nematodes, one trematode, one cestode and two protozoan species (Clough *et al*. unpublished data). Most prominent parasite infections were caused by the nematode species *Lemuricola vauceli* and *Callistoura* sp. as well as the extracellular protozoa *Entamoeba* sp. and *Balantidium coli* with 100% prevalence each.

Intensity of individual nematode infections ranged from 0 to 3850 eggs per sample with means of 274 and 190 in the year 2006 and 2007, respectively. Numbers differed significantly between individuals ($F_{469,28} = 2.34$, $p<0.001$) and years ($F_{469,1} = 7.86$, $p<0.01$), but the interaction of both factors was not significant ($F_{450,19} = 0.66$. $p=0.86$). Similarly, the number of protozoan cysts per individual ranged from 0 to 41750 cysts per sample with means of 1813 and 1782 in the year 2006 and 2007, respectively, and the intensity of protozoan infections differed significantly between individuals ($F_{469,28} = 2.07$, $p<0.01$) and years ($F_{469,1} = 11.87$, $p<0.001$). Again, the interaction of both factors was not significant ($p=0.80$, $F_{450,19} = 1.14$).

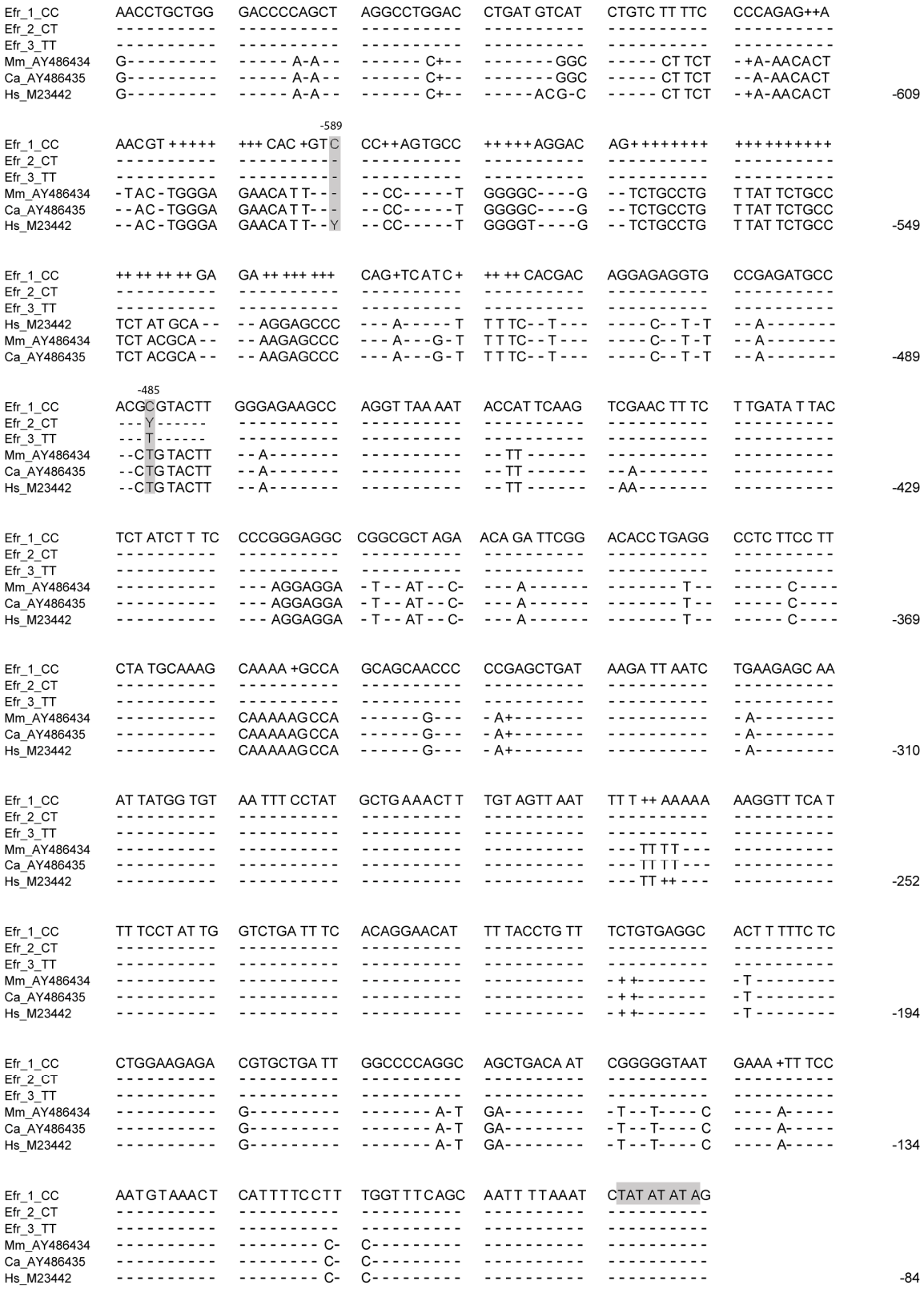

Figure 1. Alignment of *Eulemur fulvus rufus* IL4 promoter sequence (GenBank accession GQ221019) with published sequences of *Homo sapiens* (Hs, Arai *et al.* 1989), *Macaca mulatta* (Mm, Bostik *et al.* 2004) and *Cercocebus atys* (Ca, Bostik *et al.* 2004). Highlighted are the lemur -485CT SNP, the human -589CT SNP, and the TATA box. Gaps introduced to maximise homology are marked by "+". Nucleotides identical to the lemur sequence (Efr_1_CC) are shown by dashes. Nucleotide numbering is based on the human sequence.

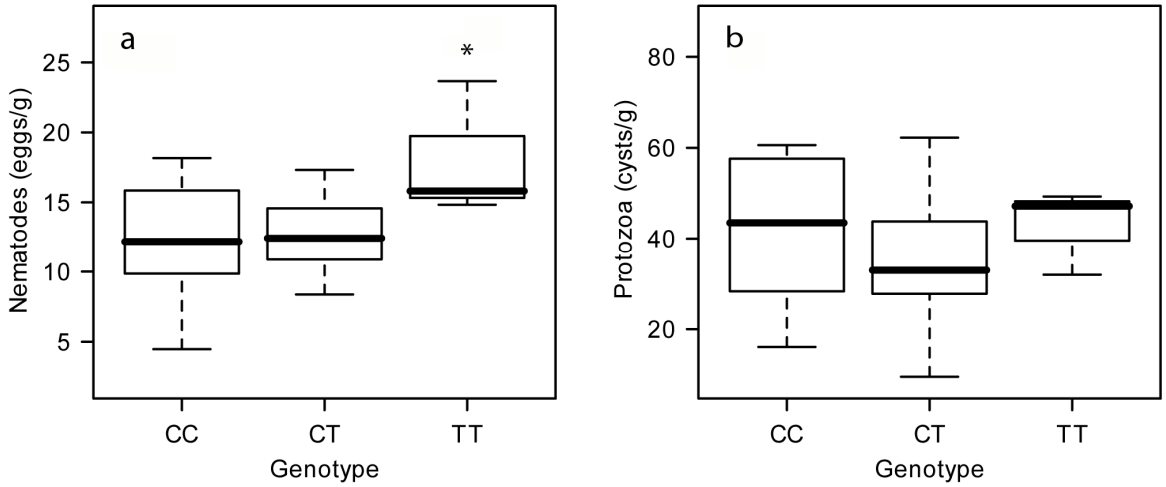

Figure 2. Box plots of nematode (a) and protozoa (b) infection intensities between individuals of different genotypes. Individuals with the T/T genotype showed significantly higher mean nematode intensities than others whereas protozoan infections did not differ significantly between genotypes. Response variables are depicted as sqrt-transformed data.

We found a significant association between the genotype and intensity of nematode infection (Figure 2a): individuals of the rare genotype T/T had a significantly higher nematode egg output than the more frequent genotypes C/T and C/C ($t_{24,2}$=2.20, $p<0.05$). The intensity of protozoan infection was not significantly different among individuals with different genotypes, however ($p_{CC\text{-}TT}$= 0.83, $p_{CT\text{-}TT}$=0.16; Figure 2b).

Table 1. Observed and expected heterozygosity of 11 microsatellite markers. None of the microsatellites deviated from Hardy-Weinberg-equilibrium (HW).

Locus	N	H_{obs}	H_{exp}	HW
Efr BW 02	10	0.821	0.798	ns
Efr 05	12	0.802	0.793	ns
Efr 08	16	0.922	0.895	ns
Efr BW F9	5	0.738	0.737	ns
Efr 24	9	0.847	0.778	ns
Efr 30	8	0.696	0.687	ns
Efr 37	9	0.756	0.723	ns
Efr 56	7	0.746	0.784	ns
Efr 81	7	0.733	0.737	ns
I3	14	0.839	0.825	ns
L2	18	0.898	0.888	ns
mean	10.45	0.790	0.786	ns

Individual heterozygosity

Eleven microsatellites that are distributed over the genome and exhibit 5 to 18 different alleles were analysed to study the genetic diversity at other, potentially neutral, loci. None of the 11 loci showed deviations from Hardy-Weinberg equilibrium (Table 1). Individual heterozygosity ($H_{obs\text{-}ind}$) ranged from 0.45 to 1 with mean $H_{obs\text{-}ind}$=0.81, and in contrast to effects of the IL4 genotype, this genetic diversity did not correlate with individual nematode or protozoa infection intensity (partial correlation, r^2= 0.024, p=0.115; Figure 3).

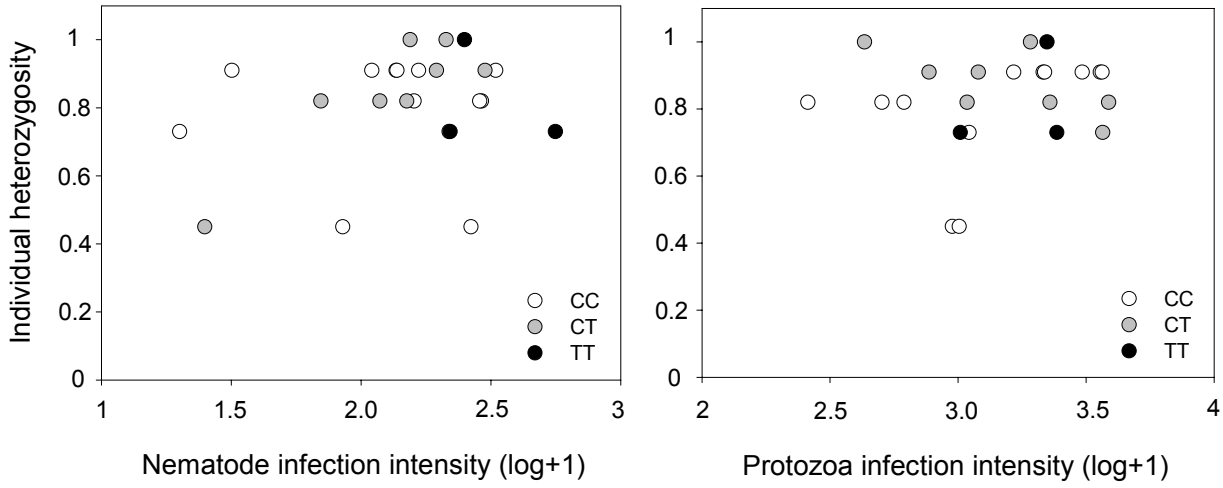

Figure 3. Nematode and protozoa infection intensities are not associated with individual heterozygosity. Data from 2007 only. Genotypes are displayed in different shades.

Fitness consequences of different IL4 constitutions

Over a time span of 10 years, frequency distributions of the three genotypes resembled earlier results with C/C: 53.5%, C/T: 37.2% and T/T: 9.3% (n = 45). Again, T/T was the rarest genotype observed in the population. Frequency distribution of genotypes did not deviate from a distribution as expected under Hardy-Weinberg equilibrium (Fisher's exact test, p=0.86, df=2). Observed reproductive success of males of different genotypes differed significantly from expected values. Individuals of the genotype T/T sired significantly more offspring than expected (χ^2=4.47, p<0.05, df=1), whereas observed paternity success in individuals of genotype CC and CT did not deviate from expectations (Figure 4).

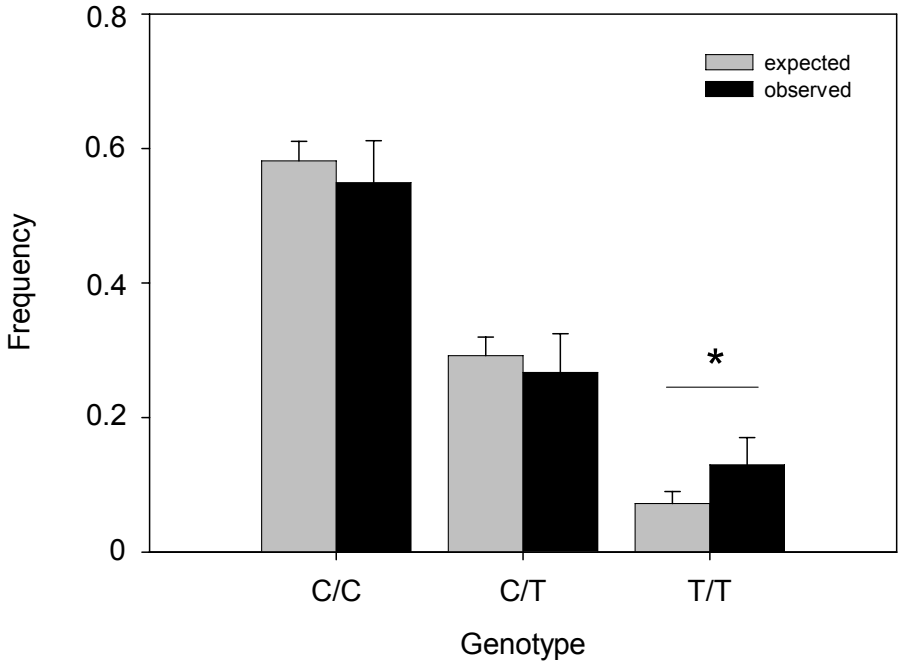

Figure 4. Observed frequencies of paternities differed significantly from expected patterns in animals of genotype TT.

3.4 Discussion

Understanding the genetic regulation of parasite resistance in natural population is of major importance for understanding host-parasite evolution and host sexual selection (Sorci *et al.* 1997). In past years, most effort has been devoted to study MHC diversity and compatibility as a key element of parasite resistance and a potential driving force in sexual selection (Potts *et al.* 1991; Wedekind *et al.* 1995; Penn *et al.* 2002; Harf and Sommer 2005; Schad *et al.* 2005; Milinski 2006; Schwensow *et al.* 2008). However, in some studies individual heterozygosity appeared to be a weak predictor of parasite infection, and the importance of specific alleles of candidate genes in regulation of parasite infection has been emphasized (Côté *et al.* 2005; Hill 2006). Cytokine genes are natural candidates due to their major regulatory role in helminth parasite susceptibility (Hotez *et al.* 2008) and their evolutionary significance is becoming apparent (Fumagalli *et al.* 2009). Nonetheless, empirical evidence for a relationship between cytokine gene polymorphisms and parasite susceptibility in natural population has been lacking.

We assessed the intensity of gastro-intestinal parasite infections through repeated and regular sampling of lemur faeces over more than 8 months in two consecutive years. This approach provided a good estimate of individual infection intensities measured as the number of parasitic stages per gram faeces, reducing the uncertainty related to the use of egg counts as measurement for parasite infection intensity (Gillespie 2006), and indicated that individual lemurs differed

significantly in both nematode and protozoan infection intensities. Sequence analyses of 52 genetically investigated animals detected a polymorphic site at position -485 bp in the promoter of the *IL4* gene. This SNP has not yet been reported in any other studies on human or non-human primates, suggesting that it is lemur specific. In contrast, all investigated lemur sequences were found to be invariable at position -589 bp, which defined the location of a SNP in the human *IL4* promoter that strongly affects IL-4 protein expression and, hence, IgE antibody levels. The -589C/T polymorphism is associated with various diseases such as malaria (Luoni *et al.* 2001; Gyan *et al.* 2004; Verra *et al.* 2004), atopic asthma (Rosenwasser and Borish 1997; Kabesch *et al.* 2003; Basehore *et al.* 2004), or myocardial infarction (Paffen *et al.* 2008). Our finding supports results from Rockman *et al.* (2003), which indicated that the polymorphic site at -589bp was strongly conserved in humans, thus being a derived characteristic that arose in the lineage leading to humans.

With regard to the -485CT polymorphism, we found different genotypes associated differently with nematode infection intensities: animals possessing the rare genotype (T/T) had the highest nematode egg output, which suggested that these individuals were compromised in their ability to fight infections by expelling worms from the intestines and thus lowering the number of eggs shed (Seivwright *et al.* 2007). In contrast, carriers of the genotype C/C and C/T seemed to perform better in the expulsion of nematode worms as their parasitic egg output was reduced. Protozoan infections did not differ significantly between genotypes, suggesting that the modulating effect of the observed *IL4* promoter polymorphism in our cohort appeared to be more prominent in nematode worm than protozoan infections. Such a differential response is known from experimental studies with infected mice (Yoshida *et al.* 1999), and results from a study conducted by Guo *et al.* (2008) suggested that IL-4 might even facilitate entamoebiasis by suppressing protective IFN-gamma production (T_H1 response).

Promoter SNP-modulated gene transcription, which leads to differential activity of a gene, is frequently based upon altered transcription factor binding properties at the site of the mutation (Li-Weber and Krammer 2003). Studies on the human -589CT polymorphism *in vivo* and *in vitro* provided evidence that a certain mutation in the allele bearing the binding site of the transcriptional factor NFAT results in altered transcription rates of *IL4* mRNA (Rosenwasser and Borish 1997; Wierenga and Messer 2000; Nakashima *et al.* 2002; Li-Weber and Krammer 2003; Rockman *et al.* 2003). Due to the lack of appropriate reagents and molecular tools in lemurs, experimental evidence cannot be provided, yet we hypothesize that the SNP detected in the lemur *IL4* gene promoter similarly affects *IL4* gene transcription, leading to lower *IL4* mRNA and protein levels in individual homozygous for the T allele. This would result in a decreased

T_H2 response, lower IgE levels and higher nematode infection intensity. Alternatively, the observed association could be due to linkage disequilibrium with the *IL13* and *IL5* genes, which are located just 12.5 kb and 132 kb upstream of *IL4* and are also key T_H2 cytokines (Anthony *et al.* 2007). The study provides a first empirical record of *IL4* regulation of parasite infections in natural populations, however, and laboratory-based studies confirming the functional role of the -485CT polymorphism are indicated.

Mean individual heterozygosity in the study population was higher than 0.8, suggesting that the population was not subject to inbreeding processes. In line with results from other studies (Côté *et al.* 2005; Ortego *et al.* 2007), findings from our study did not confirm a relationship between individual heterozygosity and parasite infection intensity, corroborating that intestinal parasite infection in lemurs was associated with one specific genotype of a candidate-gene rather than with heterozygosity per se. The power of these results is certainly constrained by the ability of estimating overall heterozygosity by use of a set of microsatellites (Slate *et al.* 2004; DeWoody and DeWoody 2005); however, using eleven microsatellite markers is comparable to most studies performed on vertebrates (Coltman *et al.* 1999; Côté *et al.* 2005; Ortego *et al.* 2007; Schwensow *et al.* 2007). Nevertheless, for further exploration of a heterozygosity-parasite correlation using alternative measures such as MHC diversity or genome-wide heterozygosity is desirable.

The presumably unfavourable association of the T/T genotype and highest nematode infection intensities in these individuals would lead to the conclusion of a positive frequency-dependent selection, giving an advantage to the more common genotypes that provide better resistance to parasites (Hamilton and Zuk 1982; Clayton 1991). However, long-term population analyses indicated a disproportional higher reproductive success of T/T individuals. Using 10 years of population genetic data, frequencies of T/T genotypes were still very low, which makes it difficult to disentangle the mechanisms behind this pattern; in particular, as our result is contradictory to other studies indicating that a certain immune genetic constitution can effectively impair hosts reproductive success (Milinski and Bakker 1990; Møller 1990c; Dobson and Hudson 1992). An explanation may be found in the counterbalancing function of IL-4: a regulatory polymorphism in the *IL4* promoter influences the activity of the cytokine and thus the balance of the T_H1/T_H2 ratio, resulting either in an increased T_H1 response, which might be advantageous when individuals are confronted with intracellular pathogens such as viruses or phagocytised bacteria, or an intensified T_H2 response, which is required when individuals are affected by extra-cellular parasites such as nematode parasites (Rockman *et al.* 2003). This suggests that the *IL4* promoter polymorphism is subject to balancing selection. Imbalanced

T_H1/T_H2 ratios are known to be responsible for increases in allergic disease in "parasite-free" industrialized areas in human (Yazdanbakhsh *et al.* 2002). In lemurs, it is likely that individuals with a potentially disadvantageous *IL4* genotype are positively affected by other aspects of the *IL4* biology, which could provide an explanation for the disproportionately higher fitness of these animals. Yet, it has to be emphasized that, although this is a plausible explanation, the *IL4*-gene may only be one out of many factors affecting variance in male reproductive success in a wild lemur population. Additionally, increased levels of nematode infection might not be a crucial point in selection processes of this particular population of red-fronted lemurs.

In conclusion, we detected a novel C/T polymorphism at position -485bp in the promoter area of the red-fronted lemur *IL4* gene that has not been reported before and is significantly associated with inter-individual variability in nematode infection encountered in this wild population of red-fronted lemurs. Carriers of the T/T allele were associated with higher nematode infection intensities than carriers of the genotype C/C or C/T, yet long-term population analyses also indicated above average reproductive success of the former. The methodological approach used here is open to a broad range of applications, requiring only species-specific validation. If the same relationship is found in other study systems, the analyses of promoter SNPs could provide an efficient scoring system for genetic variation in susceptibility to helminth infections.

3.5 Acknowledgements

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4 Host intrinsic determinants and potential consequences of parasite infection in free-ranging red-fronted lemurs (*Eulemur fulvus rufus*)

DAGMAR CLOUGH[1, 2], MICHAEL HEISTERMANN[3], PETER M. KAPPELER[1, 2]

[1]Dept. of Behavioral Ecology and Sociobiology, German Primate Center, Göttingen, Germany
[2]Dept. of Anthropology and Sociobiology, University of Göttingen, Germany
[3]Dept. of Reproductive Biology, German Primate Center, Göttingen, Germany

Abstract

Parasites and infectious diseases represent ecological forces shaping animal social evolution. Although empirical studies supporting this link abound in various vertebrate orders, both the study of the dynamics and impact of parasite infections and infectious diseases in strepsirrhine primates have received little empirical attention. We conducted a longitudinal parasitological study on four groups of wild red-fronted lemurs (*Eulemur fulvus rufus*) at Kirindy Forest, Madagascar, during two field seasons in consecutive years in order to investigate i) the degree of gastro-intestinal parasite infection on the population and individual level and ii) the factors potentially determining individual infection risk. Using a comprehensive dataset with multiple individually assignable parasite samples as well as information on age, sex, group size, social rank and endocrine status (faecal androgen and glucocorticoid levels) of all hosts, we examined parasite infection patterns and host traits that may affect individual infection risk. In addition, we examined whether parasite infection affects mating and reproductive success. Our results indicated high variability in parasite infection on individual and population level. Seasonality and group size were important determinants of variability in parasite infection. Variation in hormone levels was also associated with seasonal variation, parasite species richness and nematode infection intensity. These results indicate an immune-enhancing function of steroid hormones on nematode infections which has not been reported before from other vertebrates studied under natural conditions. Male mating and reproductive success were not correlated to any measure of parasite infection, which suggests a non-functional role of parasites in primate sexual selection.

Keywords

Helminth, protozoa, seasonality, hormones, reproductive success

4.1 Introduction

Parasites and pathogens can affect host populations dynamics in several ways, for example by increasing mortality, reducing competitive ability, or impairing individual fitness (Hudson *et al.* 1992; Møller 1997; Hudson *et al.* 1998). The abundance and magnitude of parasite infections is characterized by patterns of spatial and temporal aggregation (Anderson and May 1978; Shaw and Dobson 1995), determined by both variability in host encounter rates and susceptibility to parasites. Such patterns of infections have been investigated for example in gastro-intestinal parasites of baboons (*Papio cynocephalus*, Hausfater and Watson 1976; *Papio anubis*, Müller-Graf *et al.* 1996), mandrills (*Mandrillus sphinx,* Setchell *et al.* 2007), howler monkeys (*Alouatta palliata*, Stuart *et al.* 1998), chimpanzees (*Pan troglodytes*, McGrew *et al.* 1989; Huffman *et al.* 1997; Muehlenbein 2006) and several tamarin species (*Saguinus mystax, Saguinus fuscicollis, Callicebus cupreus*, Müller 2007). Host age, sex and variation in hormone concentrations as well as a species' social organisation have been identified as important determinants of parasite infection and seasonal variation in disease risk (see Nunn and Altizer 2006b, Huffman and Chapman 2009 for an overview). However, compared to other vertebrates (e.g. wood mouse, *Apodemus sylvaticus*: Behnke *et al.* 1999; Soay Sheep, *Ovis aries*: Coltman *et al.* 1999, 2001; yellow perch, *Perca flavescens*: Carney and Dick 2000), comprehensive studies that simultaneously explore the importance of several host intrinsic factors on parasite infection have been rarely conducted in primates (e.g. Freeland 1976; Müller-Graf *et al.* 1996; Stuart *et al.* 1998; Chapman *et al.* 2007). Furthermore, there is a particular lack of information on parasite infection patterns in strepsirrhine primates, which impedes our understanding on general patterns in primate parasite infection (Nunn and Altizer 2006a).

In the present study we examine parasite infection patterns and host traits that may affect individual infection risk in red-fronted lemurs (*Eulemur fulvus rufus*). Lemurs are interesting in this respect because they deviate in many morphological and socio-biological traits from patterns found in anthropoid primates (Kappeler 2000) allowing to develop specific predictions regarding expected patters of parasite infection in this taxon (as reviewed in Hudson and Dobson 1995; Altizer *et al.* 2003). First, group size, a major aspect of primate social organization, is expected to be positively correlated to parasite infection as greater host sociality facilitates transmission of contagious parasites that are spread directly from host to host (Freeland 1976; Côté and Poulin 1995; Nunn and Altizer 2006b). Parasite transmission in lemur groups is made particularly easy as they use toothcombs for auto- and allogrooming (Barton 1987) and grooming networks between mothers and juveniles (Kappeler 1993) but also between adult males and females (Port *et al.* 2009) facilitate contact with infectious stages caught in the fur of a grooming partner. We

therefore predict that variation in lemur group size should affect the prevalence, diversity and intensity of parasite infections.

Second, differences in parasite infection between the sexes are best explained by body size differences (Zuk and McKean 1996), differences in steroid hormone levels (Klein 2000, 2004) and mating systems that puts one sex at a disadvantage with regard to transmission of parasites (Moore and Wilson 2002). Unlike most other primates, lemurs show no dimorphism in body size (Kappeler 1990), and androgen levels do not differ significantly between sexes outside the mating season either (von Engelhardt *et al.* 2000; Drea 2007). Additionally, male and female red-fronted lemurs both mate multiply during the mating season so that we would not expect sex-biased parasitism in this species (Moore and Wilson 2002).

Third, differences in social rank are usually associated with differential steroid hormone levels. High-ranking males usually exhibit higher testosterone levels compared to low-ranking males and also often experiencing more frequent aggressive interactions than subordinates (Dixson 1998; Bercovitch and Ziegler 2002; Setchell *et al.* 2008). In red-fronted lemurs, social rank is not reflected by differences in androgen or glucocorticoid levels between dominant and subordinate males (Ostner *et al.* 2002, 2008). Assuming an effect of steroid hormones on parasite infection patterns (Folstad and Karter, 1992; Dixson 1998; Bercovitch and Ziegler 2002), we would thus expect lemur males of different social ranks not to differ with regard to parasite infection.

Additionally, given the immune-modulatory function of steroid hormones (Weinstein and Bercovitch 1981; Grossman 1985; Alexander and Stimson 1988) there is evidence for an immunosuppressive effect of steroid hormones resulting in increased parasite infections in several taxa (Alexander and Stimson 1988; Zuk and McKean 1996; Klein 2004). However, ambiguous results from a variety of studies indicate that the pattern is not as clear and neutral associations (Hasselquist *et al.* 1999; Buttemer and Astheimer 2000; Tschirren *et al.* 2005) or even enhancing effects of the two steroid hormones on the immune system have been observed (Gross *et al.* 1980; Fleming 1985; Bilbo and Nelson 2001). In primates, information on the link between steroid hormones and parasite infection is limited to two studies, which showed a positive association of both testosterone and cortisol with total parasite species richness in wild chimpanzees (*Pan troglodytes*, Muehlenbein 2006) and, similarly, positive correlations between elevated cortisol levels and parasite infections in red colobus monkeys (*Procolobus badius*, Chapman *et al.* 2007). More data from other primate taxa studied in the wild are thus useful to extend our knowledge in this area and thereby improve our understanding of endocrine-parasite interactions in primates. In this respect, a study on red-fronted lemurs could be particularly

useful as male physiology in this species is characterized by predictable mating season increases in androgen and glucocorticoid output (Ostner *et al.* 2002; Ostner *et al.* 2008) and, furthermore, hormone levels can vary substantially between years (Clough *et al.,* 2009), which provides an ideal situation to examine the link between hormonal changes and parasite load in this species. If steroid hormones have a functional role in parasite infection in red-fronted lemurs, we would predict parasite infection to vary as a function of fluctuating hormone levels.

Furthermore, all males and females of a social group of red-fronted lemurs mate promiscuously during the mating season. Although dominant males achieve a higher reproductive success compared to subordinates (Kappeler and Port 2008), they do not succeed in complete monopolization of paternities and subordinate males gain a constant share of reproduction (29%, Kappeler and Port 2008). Assuming that females exercise mate choice and that parasite resistance is a relevant criterion of male quality (Hamilton and Zuk 1982; Able 1996; Loehle 1997), we would expect that females avoid highly-parasitized males and that reproductive shares are distributed between males according to their individual parasite resistance.

Finally, Madagascar harbours ecologically challenging primate habitats because of pronounced seasonality and unpredictability, which may negatively impact on lemur body condition, thus affecting parasite susceptibility (Chapman *et al.* 2007).

Using data collected during a field study of red-fronted lemurs, we report here prevalence, diversity and intensity of parasite infections as measured during consecutive time periods before, during and after two consecutive annual mating seasons. We explore determinants of individual parasite infection with regard to the predictions about the effects of group size, age, sex, social rank and steroid hormones on variation in parasite infection within and between years, and assess the effect of parasite infection on male mating and reproductive success.

4.2 Methods

Study site and host population

We studied a population of red-fronted lemurs (*Eulemur fulvus rufus*) in a 60 ha study area in Kirindy Forest, a dry deciduous forest 60 km northeast of Morondava, western Madagascar (44°40´E, 20°04'S; see Sorg *et al.* (2004) for further description of the study site). The study area is part of the field site of the German Primate Center (DPZ) and is managed within a forestry concession operated by the Centre National de Formation, d'Etudes et de Recherche en Environnement et Foresterie (CNFEREF). The area is subject to pronounced seasonality due to a

dry season from March to October and a wet season lasting from October to February, respectively.

During two consecutive field seasons between March and August in 2006 and 2007, we sampled all adult male and female red-fronted lemurs of four social groups. Field seasons were chosen to include the annual mating season, which takes place during 2-3 weeks in late May / early June and is characterized by marked elevations in androgen and glucocorticoid levels (Ostner *et al*. 2002; Ostner *et al.* 2008). All individuals were well habituated to human presence and marked with individual combinations of nylon collars and pendants for individual recognition. One animal per group was equipped with a radio-collar (Biotrack, Wareham, Dorset, UK) to facilitate ad hoc detection of a group. Individual information about sex and age was available due to long-term monitoring of the population. For individuals that had immigrated into the population, age was estimated at first capture of these individuals using tooth wear and sexual maturity. Individual social rank was determined by evaluating the outcome of agonistic interaction during focal observations (see Pereira and Kappeler 1997 for details), mating success was measured in terms of successful copulations using ad libitum protocols and reproductive success was determined by genetic paternity analyses (Port *et al.* 2009).

Faecal sample collection

For parasite and hormone analyses, faecal samples from all study animals were collected weekly over a duration of four sampling periods, including the mating season as well as 4 weeks before, and 4 and 8 weeks thereafter in both years (hereafter abbreviated as P1, P2, P3, and P4, see Table 1). Samples were collected immediately after defecation, placed in plastic tubes, pre-aliquoted with 10% neutral-buffered formalin (parasite analyses) or 90% ethanol (hormone analyses), labelled and wrapped with parafilm.

Parasite samples were collected for both sexes in both years. Concerning hormone analysis, males were sampled during both years (n_{males}=362), whereas female samples were only available for 2006 ($n_{females}$=161). Parasites and hormones samples were collected during the same time of day (11am-1pm) in order to account for a potential circadian effect on parasite egg shedding or hormone levels. Since long-term storage of red-fronted lemur faecal samples in alcohol at ambient temperature does not alter faecal androgen and glucocorticoid concentrations (Ostner *et al.*, 2008), samples were stored in alcohol in the shade (25-35C°) at the field site until they were returned to the laboratory at the end of each field season. Table 1 gives an overview on the number of focal animals per year, the number of samples collected, hours of behavioural

observation and the scheduling of the sampling periods before, during, and after the mating season in 2006 and 2007.

Table 1. Study group composition, sample collection, behavioural hours and sampling periods during field seasons 2006 and 2007

	2006	**2007**
Adult males	14	13
Adult females	11	11
Parasite samples	386	532
Hormone samples	383	271
Behavioural observation hours	322	475
P1: Pre-mating (- 4 weeks)	10.04.-08.05.06	26.03.-22.05.07
P2: Mating season	09.05.-01.06.06	23.05.-10.06.07
P3: Post-mating I (+ 4 weeks)	02.06.-26.06.06	11.06.-15.07.07
P4: Post-mating II (+8 weeks)	26.06.-23.07.06	16.07.-26.08.07

Parasite analyses

Faecal samples were processed using a modified version of the formalin-ethyl-acetate sedimentation technique described in Ash and Orihel (1991). Briefly, approximately 5ml homogenized faecal material was strained into centrifuge tubes and 10% formalin was added until the total volume was at 10ml. After adding 3ml ethyl-acetate, we shook the tube vigorously for 30s and centrifuged it for 10 min on 2200rmp. Before pouring off the supernatant consisting of ethyl-acetate, formalin and debris, the top layer of fat was removed from the centrifuge tube. The remaining sediment was used for subsequent analyses. Details of the methods as well as on the identification of parasite species can be found elsewhere (Clough submitted). Wet mounts of individual samples were prepared with 20 mg of sediment and one drop of Lugol's solution on a microscope slide and all eggs and larvae were counted. One slide was systematically scanned for each sample and results of egg and larvae (helminths) as well as cyst and trophozoite (protozoa) counts were extrapolated to 1g faecal sediment (x50).

Measurements of parasite infection

We use parasite species prevalence, richness and infection intensity as measurements of parasite infection. Prevalence is the number of individuals infected, given as a percentage of the number of individuals examined per unit of interest (e.g. per group or per year). Parasite species richness (PSR) is the number of different parasite species documented in each host and is also given per unit. Parasite infection intensity is the number of faecal eggs (helminth parasites) or cysts and trophozoites (protozoan parasites) per gram faecal sediment (definitions follow

Margolis *et al.* 1982; Bush *et al.* 1997). Due to parasite-specific variation in egg shedding, there has been some discussion about the reasonable use of faecal egg counts (FEC) as a measure of infection intensity (Anderson and Schad 1985; Gillespie 2006). We accounted for natural occurring variations in parasitic excretions by using monthly medians of faecal egg/cyst counts per individual and pooling the data for all nematode infections (FEC_nem) and all protozoan infections (FEC_pro) to generate the response variables for statistical analyses (see below).

Hormone analyses

Prior to hormone measurement, samples were first homogenized in their original ethanolic solvent by squashing them with a metal stick and subsequently extracted twice as described by Barelli *et al.* (2007). Following extraction, the remaining faecal pellets were dried in a vacuum oven at 50°C and the dry weight of individual samples was determined. The supernatant was used for measurements of immunoreactive testosterone and 5ß-reduced cortisol metabolites (3α,11-oxo-CM) using microtitreplate enzymeimmunoassays (EIA), which have previously been validated for monitoring androgen and glucocorticoid status in lemurs (Kraus *et al.* 1999; von Engelhardt *et al.* 2000; Fichtel *et al.* 2007), including red-fronted lemurs (Ostner *et al.* 2002; Ostner *et al.* 2008). The assay procedures have been described in detail by Kraus *et al*. (1999) and Ostner *et al.* (2008), respectively. Sensitivity of both assays at 90% binding was 0.5 pg (androgen) and 3 pg (glucocorticoid) per well. Intra-and inter-assay coefficients of variation (CV) of high and low value quality controls (QCs) were 6.2 % (n=16) and 7.6 % (n=24; high), and 10.2% (n=16) and 11.2 % (n=24; low) for androgens across both years. Intra-and inter-assay CV values for glucocorticoid measurements were 7.5 % (n=16) and 9.5 % (n=30; high) and 9.8 % (n=16) and 14.7 % (n=30; low), respectively. All hormone values are expressed as mass per gram dry faecal weight (ng/g) and we used median values per individual per period for statistical analyses.

Statistical analyses

Variability and determinants of prevalence were analysed using generalised linear mixed models (GLMM) with binomial error (link=logit). In this model, non-independence of repeated measurements of individuals was accounted for by incorporating animal identity (ID) nested within social group (Paterson and Lello 2003). Because year of collection could potentially also affect parasite infection, we included year in each model before we fitted other terms. Further terms that were included as fixed effect factors were group size measured as the number of adults and subadults per group (factor with four levels, ranging from 4-7 individuals) sex

(factor), rank (factor: dominant or subordinate), age class (factor with three levels: 1 (3-5 yrs), 2 (6-9 yrs), 3 (10-14 yrs)), and period of sampling (factor with four levels: P1 (four weeks before mating), P2 (during mating), P3 (four weeks after mating) and P4 (eight weeks after mating), see Table 1 for scheduling of periods). Due to low numbers of positive samples or consistently high prevalence (100%) of some of the parasite taxa, statistical comparison of prevalence could only be conducted for *Lemuricola* and *Trichuris* infections. To determine the extent of sex bias in prevalence, the prevalence of each parasite taxon in females was subtracted from the respective prevalence in males (Moore and Wilson 2002). A one sample t-test was used to test whether the mean value of sex bias over all parasite taxa differed from zero, which would imply no difference between sexes.

Individual differences in parasite species richness (PSR) was analyzed using a general linear model (GLM) which incorporated individual identity ("ID") and group as explanatory factors and year as a fixed effect covariate. Next, we applied a GLMM with Gaussian errors (link=identity; analogous to a linear mixed effect model LMM) to analyze determinants of PSR. The distribution of the response variable PSR complied with normality and homogeneity of variance was given (as checked in plots of the error (residuals) against predicted values (fits)). Again implementing repeated measurements of PSR per individual, random and fixed effects initially fitted were the same as for prevalence analyses.

Analyses of variability and determinants of parasite infection intensities were conducted separately for nematode and protozoan parasites. Both response variables showed high degrees of overdispersion (Φallnem= 114.1, Φallpro= 988.69, see Hudson and Dobson 1995, Bolker *et al.* 2009) which could not be improved by applying a GLMM with quasi-error structure as advised for use of parasitological data (Paterson and Lello 2003). We therefore used transformed response variables with square-root (FEC_nem) and log (FEC_pro+1). After transformation, distributions of response variables were no longer different from the normal distribution (see Appendix 1). Homogeneity of variances was checked using residual plots. Random and fixed effect factor structure complied with model structure for prevalence and PSR analyses.

Variability in steroid hormones between sexes, ranks and within years was analysed using a GLMM (link=identity) with log+1-transformed response variable, sex, rank and period as fixed effects, year as fixed co-variate and ID nested within group as a random factor. Differences between sexes were only analyzed for androgen and glucocorticoid levels in 2006 as hormone data for females were not available for 2007.

The effect of steroid hormones on parasite infection was analyzed using linear mixed effects models on individual means of males in both years. Originally fitted factors were: Response-

PSR, FEC_nem or FEC_pro; Fixed effect – androgen or glucocorticoid, period, year; Random – ID and group.

An association of parasite infection on mating and reproductive success was analysed using the same response and random effect model structure as defined above for PSR, nematode and protozoa infection intensity, but implementing proportion of mating observed or reproductive success and year as fixed factors. Also, we explored whether subordinate males that sired an infant differed in their parasite loads from subordinates that were not successful in siring infants using subordinate success (factor with two levels) as a further fixed factor in the GLMMs. This was particularly interesting with regard to the fact that about 30% of subordinates reap a regular share of paternities (Kappeler and Port 2008).

Model simplification was conducted by step-wise removal of non-significant parameters. Nested models with different and fixed effects were compared using likelihood-ratio tests with ML estimation (Zuur *et al.* 2009), which was also used to confirm lack of contribution of eliminated variables. All statistical analyses were undertaken in R 2.8.1 (R Development Core Team 2008), GLMMs and LMMs were conducted using the package lme4 (Bates *et al.* 2008).

4.3 Results

Variability in parasite infections within and across years

We recovered ten intestinal parasite species from red-fronted lemur faecal samples, representing six species of nematodes, one trematode, one cestode and two protozoan species). The nematodes included *Lemuricola vauceli*, two species of *Callistoura* sp., *Trichuris* sp., one trichostrongyloid-type and one strongyloid parasites species. An anoplocephalid cestode and a dicrocoellid trematode could not be identified further; we also identified two protozoan parasites, likely *Entamoeba coli* and *Balantidium coli.* Further details on parasite identification can be found elsewhere (Clough submitted).

Across both years, the number of co-infecting parasites per individual (PSR) ranged from 4 to 7 with a mean of 2.66 (± 1.13 SD) parasites species. Intensity of individual nematode infections ranged from 0 to 7500 eggs per sample with a mean of 252 (± 494 SD) nematode eggs/g faeces. Intensity of individual protozoa infections ranged from 0 to 41750 cysts or trophozoites per sample with a mean of 1593 (± 3686 SD) protozoa stages/g faeces. Host individuals differed significantly from each other in individual PSR ($p<0.001$, $F_{158,28}=2.73$), nematode infection intensity ($p<0.05$, $F_{158,28}=1.80$) and protozoan infection intensity ($p<0.01$, $F_{158,28}=2.18$).

Table 2. Variation in parasite prevalence, PSR, and nematode and protozoan infection intensities between years. Columns for each year show the prevalence of infected adults year (in %). Parasite species richness, nematode and protozoa faecal egg counts are given as mean ± S.E. of untransformed values.

		2006	2007
Prevalence per parasite (%)	*Lemuricola vauceli*	100	88
	Callistoura sp.1	100	100
	Callistoura sp.2	12	4
	Trichuris sp.	24	29
	"Strongyloid"	8	0
	"Trichostrongyloid"	12	17
	Dicrocoellid sp.	4	0
	Anoplocephalid sp.	4	8
	Entamoeba sp.	100	100
	Balantidium coli	100	100
Parasite species richness	PSR	2.8 (±0.07)	2.4 (±0.08)
Parasites infection intensity (eggs/g)	FEC_Nem	214 (±21.3)	130 (±17.6)
	FEC_Pro	1350 ±287.5)	776 (±114.2)

Whereas prevalence for the nematode parasite *Callistoura* sp.1 and the protozoan parasites *Entamoeba* and *Balantidium* was 100% in both years, prevalence for the other parasites was generally lower and differed between years (Table 2). Specifically, infection prevalence of three nematode and one trematode species decreased in 2007, whereas *Trichuris* sp., the trichostrongyloid-type parasites and cestodes infection prevalence increased in 2007 (Table 2). Differences between years were, however, not statistically significant (Table 3 a,b). Similarly, within years, prevalence of *Lemuricola vauceli, Callistoura* sp.1*, Trichuris* sp., *Entamoeba* sp. and *Balantidium coli* showed no significant variation between the different seasonal periods studied (Table 2, Table 3 a,b; term: period). In all remaining parasite species, numbers were too small to test for a within-year seasonal effect. Parasite species richness (PSR) decreased significantly from 2006 to 2007 (Table 2 and 3), but showed no significant variation within each study year (Table 3c). Nematode infection intensities were substantially lower in 2006 compared to 2007 (Table 2 and 3d). In addition, the interaction of variability within and between years was significant and showed variable patterns (Table 3d, year x period interaction, Figure 1a and description below). Intensity of protozoan infections was also markedly lower in 2006 (Table 2

and 3e) and showed variable patterns within the two years, too (Table 3e, Figure 1b, description below).

Determinants of parasite variability: group size, age, sex and social rank

Group size ranged from 4 to 7 adult individuals per group and did not affect prevalence of *Lemuricola* or *Trichuris* infection (Table 3 a,b), but had a significant effect on PSR (Table 3c): bigger groups harboured more parasite species. A higher number of adults and subadults per group also tended to result in a higher nematode infection intensity (Table 3d), but group size did not co-vary systematically with protozoan infection intensities (Table 3e). Age did not have a significant effect on any of the measures of parasite infection in red-fronted lemurs (Table 3a-e). Only *Trichuris* infection prevalence showed a tendency of increased prevalence in older individuals (Table 3b).

Table 3. General linear mixed effects model of (a*) Lemuricola vauceli* and (b) *Trichuris* sp. infection prevalence, (c) parasite species richness, and (d) nematodes and (e) protozoa infection intensities. Significant terms are highlighted in bold. P-values were estimated by comparison with reduced models not containing the term in question (likelihood-ratio test).

Parasite infection	term	df	χ^2-value	p-value	Effect
(a) Lemuricola prevalence	**Sex**	**1**	**4.62**	**<0.05**	**males > females**
	Year	1	0.69	0.40	no effect
	Period	3	0.85	0.84	no effect
	Group size	3	1.47	0.69	no effect
	Age	2	1.46	0.48	no effect
	Rank	1	<0.001	0.997	no effect
(b) Trichuris prevalence	**Age**	**2**	**5.58**	**0.06**	**tendency: older > younger**
	Year	1	2.30	0.13	no effect
	Period	3	1.76	0.62	no effect
	Group size	3	0.20	0.98	no effect
	Sex	1	0.26	0.61	no effect
	Rank	1	0.97	0.32	no effect
(c) Species richness	**Year**	**1**	**13.72**	**< 0.001**	**2006 > 2007**
	Group size	**3**	**11.36**	**< 0.01**	**increasing with groupsize**
	Period	3	6.32	0.10	no effect
	Age	2	1.28	0.26	no effect
	Sex	1	0.36	0.55	no effect
	Rank	1	1.73	0.19	no effect

Table 3. Continued

Parasite infection	term	df	χ^2-value	p-value	Effect
(d) FEC_nem	**Year**	**1**	**34.41**	**< 0.001**	**2006 > 2007**
	Period	**3**	**21.52**	**< 0.001**	**variable**
	Group size	**3**	**7.40**	**0.06**	**increasing with groupsize**
	Year x period	**7**	**37.92**	**< 0.001**	**variable**
	Age	2	1.69	0.43	no effect
	Sex	1	0.17	0.68	no effect
	Rank	1	0.09	0.77	no effect
(e) FEC_pro	**Year**	**1**	**10.56**	**< 0.05**	**2006 > 2007**
	Period	**3**	**3.93**	**< 0.05**	**variable**
	Sex	**1**	**6.62**	**< 0.05**	**females > males**
	Year x period	**7**	**31.99**	**< 0.001**	**variable**
	Year x sex	**3**	**18.36**	**< 0.001**	**variable**
	Rank	**1**	**4.20**	**< 0.05**	**dominant > subordinate**
	Group size	3	1.79	0.62	no effect
	Age	2	1.13	0.29	no effect

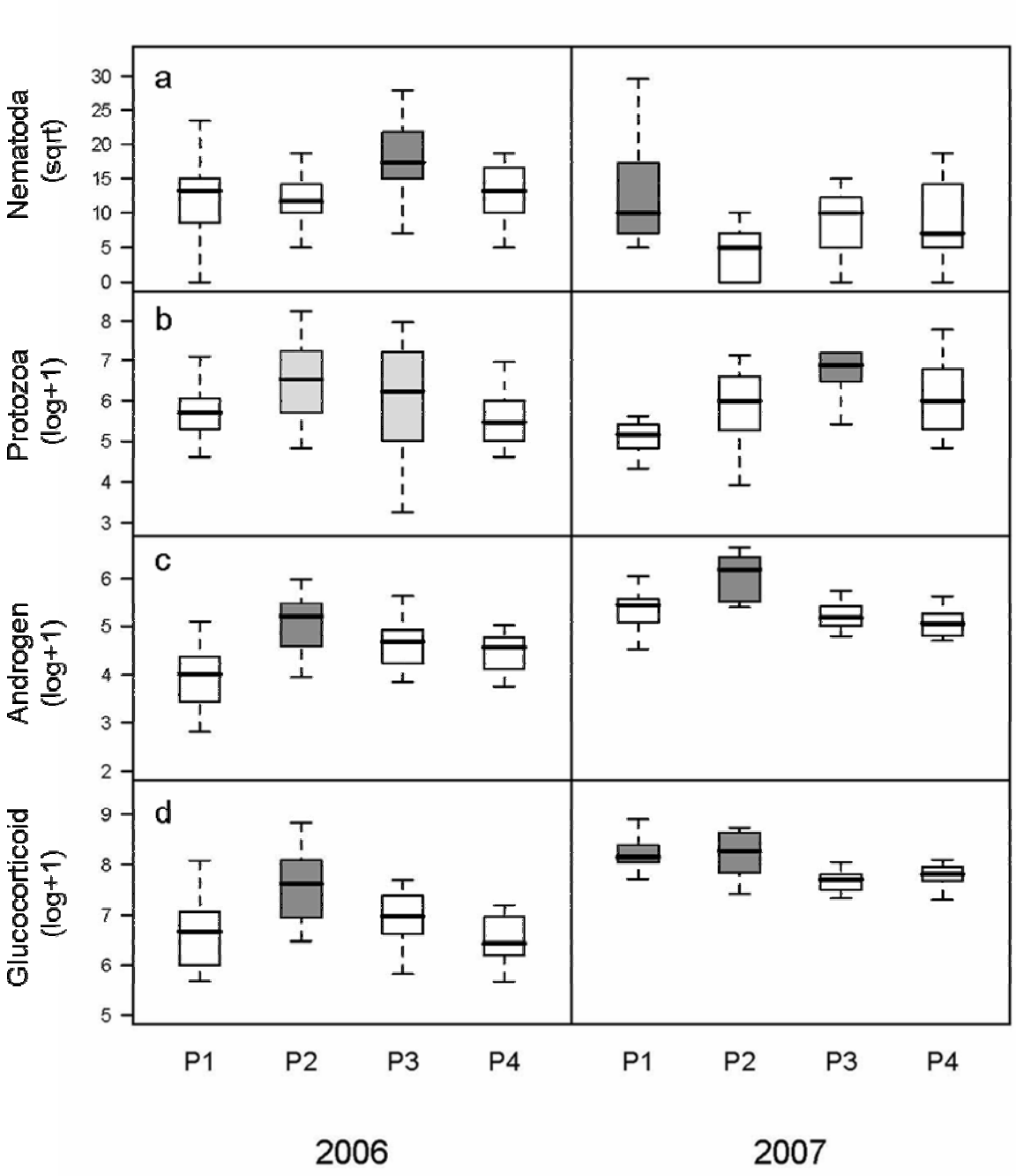

Figure 1. Temporal variation in a) nematode infection intensity (FEC_nem), b) protozoa infection intensity (FEC_pro), c) androgen and d) glucocorticoid levels between four-week periods before (P1), during (P2), four-weeks after (P3) and eight weeks after (P4) the mating season in male red-fronted lemurs. Data are depicted in transformed values as used for statistical analyses. Periods that differed significantly from others within a year are highlighted in dark-grey ($p<0.05$). Light-grey colouration depicts periods with highest levels, yet no significant difference.

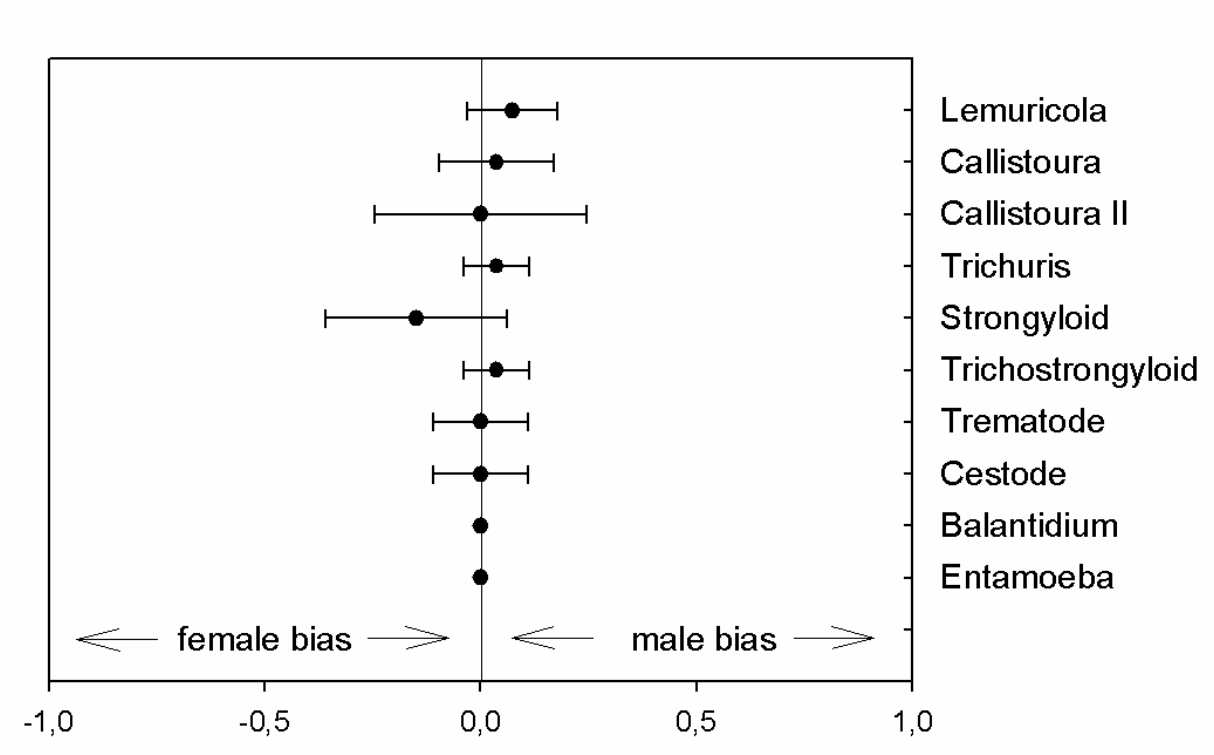

Figure 2. Effect size and 95% confidence interval in parasite prevalence of red-fronted lemurs

Prevalence of *Lemuricola* infections was higher in males than in females, yet the difference was statistically significant only for 2007, because in 2006 the entire population was infected with *Lemuricola* (Table 2 and 3a). In contrast, strongyloid infections were detected more often in females in both years (Figure 2), but these differences could not be tested statistically as overall prevalence of strongyloid infections in the population was low. Considering all parasite taxa, effect sizes in prevalence did not show a sex-bias in parasite infections (t_9=-0.128, p=0.90, Figure 2). Sex did not have a significant effect on parasite species richness (Table 3c) or nematode infection intensities (Table 3d). The effect of sex on protozoan infection intensity was mainly dominated by a significant interaction of sex and year (Table 3e, year x sex interaction): In 2006, females had protozoan counts that were about twice as high as those of males (mean ± SE of untransformed values: males: 965±380.9; females=1831±431.9), whereas in 2007, female counts were slightly lower than those of male counts (males: 813±167.6; females= 729±148.2). Still, overall protozoan infection intensity in females was higher than in males (Table 3e).

Prevalence of *Lemuricola* and *Trichuris* infections, PSR and nematode infection intensities were not associated with male social rank (Table 3 a-d). Intensities of male protozoan infection were significantly higher in dominant than in subordinates males (Table 3e), but variance in

subordinate males was high and results should be therefore interpreted with caution (SD dominant: 108.36; subordinate= 300.63).

Hormone effects on parasite infection

Influence of sex and rank on faecal hormone concentrations

Sex had a significant effect on hormone levels with males exhibiting higher faecal androgen and glucocorticoids levels compared to females (androgen: $t_{1,99}$= -3.55, p<0.01, glucocorticoid: $t_{1,99}$= -2.33, p<0.05). In contrast, and in support of previous data from the same population (Ostner *et al.* 2002; Ostner *et al.* 2008) males of different ranks did not differ in androgen or glucocorticoids levels (androgen: $t_{1,105}$= -0.22, p=0.83, glucocorticoid: $t_{1,105}$= 0.08, p=0.90).

Temporal variation in male hormones and parasite infection

Androgen and glucocorticoid levels of males were significantly elevated during the mating season in 2006 compared to the other periods (androgen: $t_{3,50}$ = 5.98, p<0.001; glucocorticoid: $t_{3,55}$ = 4.01, p<0.001; Figure 1c). The same pattern was found in 2007 (androgen: $t_{3,50}$ = 4.22, p<0.001), with the exception that glucocorticoid levels were already elevated in the period preceding mating, so that both periods, P1 and P2, were characterized by significantly higher glucocorticoid concentrations compared to P3 and P4 ($t_{P1,2\text{-}P3,4}$ = 5.36, p<0.001, Figure 1d).

In 2006, changes in parasite infection intensities seemed to partially lag behind the mating season elevations in androgen and glucocorticoid levels. As depicted in Figure 1a, nematode infection intensities in 2006 increased 4 weeks after the mating season (period P3: $t_{P3\text{-}P1,2,4}$ = 2.26, p<0.05). Intensity of protozoa infection was higher during both the mating period and the period thereafter (periods P2, P3, Figure 1b), however, values in P2 and P3 did not differ significantly from values of other periods ($t_{P2,3\text{-}P1,4}$=1.6, p=0.10). Both nematode and protozoan infection intensity decreased during P4, 8 weeks after the mating period (Figure 1b). In 2007, the pattern was more variable. Similar to 2006, nematode and protozoan infection intensities in 2007 were higher in P3 (and thus lagged behind the mating season rise in androgens and glucocorticoids) and levelled off in P4 (Figure 1a,b, protozoa: $t_{P3\text{-}P1,2,4}$ = 4.59, p<0.001). However, in 2007 nematode infection intensity was already high in the period preceding the mating period ($t_{P1\text{-}P2,3,4}$ = 2.67, p<0.01), a pattern not observed in 2006 (Figure 1a,b), but in correspondence with elevated glucocorticoid levels seen during this period (Figure 1d). Time-variant controlled correlation of male androgen and glucocorticoids levels with nematode and protozoa infection intensities were not significant (r^2 range=0 – 0.15; p_{range} = 0.14 -0.97).

Between years, mean individual PSR and parasite infection intensity showed a direct association with increased hormone levels in 2007 (Figure 3). A comparison of models with fixed factors androgen and year, and year only, respectively, confirmed that including androgen in the model improved the fit of the model for PSR (χ^2=6.96, p<0.01, df=1) and nematode infection intensity (χ^2=4.81, p<0.05, df=1) tremendously, suggesting a strong negative effect of androgen levels on PSR ($t_{1,27}$ =-2.83) and a weaker effect on nematode infection intensities ($t_{1,27}$ =-2.32). Also, incorporating glucocorticoid to a model containing only year improved the model fitted to the PSR data significantly (χ^2=6.59, p<0.05, df=1), and had a strong effect on the model regarding nematode infection intensity (χ^2=3.04, p=0.08, df=1). This suggests a negative effect of glucocorticoids on PSR ($t_{1,27}$=-2.76), and an indicated negative effect on nematode infection intensity ($t_{1,27}$=-1.85). Between-year variability in protozoa infection intensity was neither correlated to androgen nor glucocorticoid levels, and including these factors did not add significantly to the fit of the model (androgen: χ^2=0.86, p=0.35, df=1; cortisol: χ^2=3.55, p=0.06, df=1; Figure 3).

The impact of parasite infections on mating and reproductive success

We observed 62 copulations in 2006 and 97 copulations in 2007. The proportion of copulations per males within the respective social group was not significantly associated with any measure of male parasite infection. The number of offspring born to the population in 2006 and 2007 ranged from 0-3 infants per group per year and dominant males sired a significantly bigger proportion of infants than did subordinate males (t=-2.25, p<0.05). Parasite infection of subordinate males did not differ as a function of their reproductive success (p-values: $p_{Lemuricola}$=0.54; $p_{Trichuris}$=0.78; p_{PSR} =0.33, p_{nem} =0.90 p_{pro} =0.27). Similarly, reproductive success of both dominant and subordinate males was neither associated with PSR (t=-0.53, p=0.50) nor to the intensity of nematode (t=-0.95, p=0.41) or protozoa infections (t=-0.69, p=0.41).

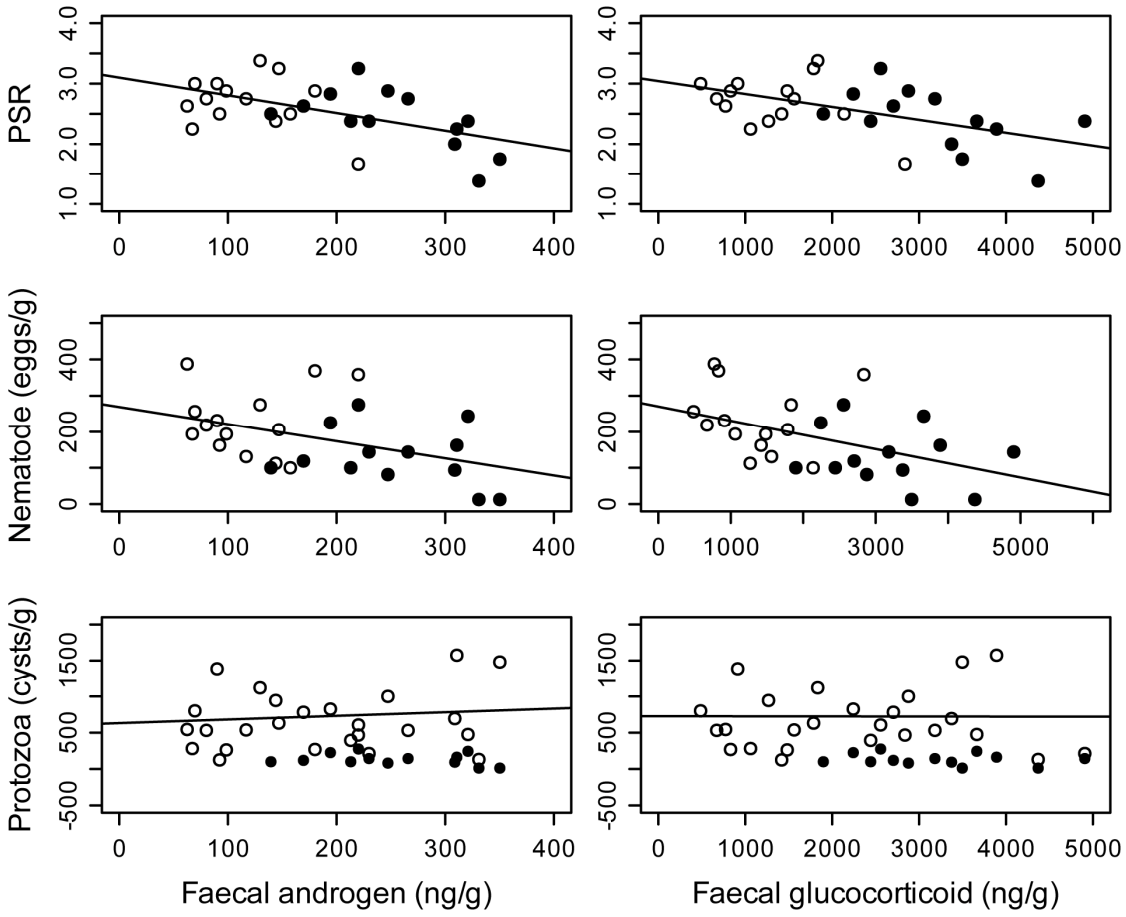

Figure 3. Associations of parasite species richness (PSR), nematode and protozoa infection intensity with faecal androgen and glucocorticoid levels in 2006 (white) and 2007 (black). Lines indicate trends.

4.4 Discussion

The number of parasitological studies in primatology has increased during the past years, yet detailed studies considering determinants of parasite infections are still limited (see Nunn and Altizer 2006a; Huffman and Chapman 2009 for recent overviews). In particular in strepsirrhine species such as lemurs, information on patterns of parasite infections and their intrinsic determinants are scarce, with most previous work for this taxon focusing on biomedical assessments of lemur health (Dutton *et al.* 2003; Junge and Louis 2005; Dutton *et al.* 2008) or effects of habitat disturbance on parasite infections (Schad *et al.* 2005; Wright *et al.* 2009; Schwitzer *et al.* submitted). A design with multiple samples per individual and repeated over two consecutive years provided a good approximation of the real parasite burden in a wild population of red-fronted lemur. This enabled us to analyse the effect of various determinants of parasite

infections simultaneously and provided new information on the significance of various potential determinants of parasite infection in this species.

The effect of group size

Group size had a strong positive effect on nematode infection intensity and PSR. A positive correlation of intestinal parasite species with group size has also been reported for mangabeys (Freeland 1979) and baboons (McGrew *et al.* 1989), whereas other studies on three tamarin species (Müller 2007) and a meta-analysis on anthropoid primates (Vitone *et al.* 2004) provided only limited support. Recent theoretical models suggest that clustering of hosts into smaller groups with little dispersal among groups might actually reduce disease risk within groups (Wilson *et al.* 2003), a pattern which might be particularly relevant for group-living lemurs (Nunn and Altizer 2003). Our findings support the idea that more animals in a group translate to higher parasite diversity and intensity (Anderson and May 1979; Côté and Poulin 1995; Arneberg 2002), a link that might be facilitated in red-fronted lemurs by i) the infection with several species being highly contagious parasites (Clough submitted) and ii) the use of the toothcomb for grooming purposes, which both makes parasite transmission easy.

The effect of age

Older individuals are expected to harbour a greater diversity of parasites because they should already have encountered more parasite species throughout their lifetime than younger individuals (Bell and Burt 1991). In addition, immune-competence tends to vary over a life-time and generally declines with age, suggesting higher parasite susceptibility in older individuals (Morand and Harvey 2000). In our study on red-fronted lemurs, however, age was not an important determinant of any measurement of parasite infection. Only prevalence of *Trichuris* infection was higher in individuals of the oldest age-class. Similarly, Stuart *et al.* (1998) detected age-related patterns in parasite prevalence in wild howlers only in one parasite species, whereas the overall pattern between individuals of different age classes was inconspicuous. In red-fronted lemurs, the non-significant association of overall parasite infection and age could be mainly due to two reasons. First, parasite infections of juvenile animals could not be included in this study, which allowed only comparison of adult age classes. Differences between these adult age classes and younger individuals might be more pronounced, as known for example from several species of baboons where adults had higher parasite prevalence and intensities than subadults or immatures (Miller 1960; Hausfater and Watson 1976; Dunbar 1980). Second, adult red-fronted lemurs in Kirindy are subject to predation pressure by large predators such as the fossa

(*Cryptoprocta ferox*) or aerial raptors (e.g. Madagascar harrier hawk, *Polyboroides radiatus*). If increased parasite susceptibility in older individuals was associated with a higher risk of predation, it would be difficult to detect under natural conditions.

The effect of sex and social rank

There was little evidence for a sex-biased pattern in parasite infection. Prevalence of one nematode parasite was significantly higher in males, but all other measurements (PSR and infection intensities) did not differ between males and females. This overall pattern supported our prediction that, due to the polgynandrous mating system and lack of sexual dimorphism in body size (Zuk and McKean 1996; Moore and Wilson 2002), red-fronted lemurs should not exhibit sex differences in parasite infection. Lack of differences in parasite infection between the sexes has also been reported from mantled howler monkeys *Alouatta palliata* (Stoner 1996) and olive baboons *Papio anubis* (Müller-Graf *et al.* 1996). Such sex differences were commonly discussed with regard to mating systems (Moore and Wilson 2002) and differences in steroid hormone levels (Zuk and McKean 1996; Klein 2004). In red-fronted lemurs, androgen and glucocorticoid levels in males were significantly higher than in females throughout the year and therefore endocrine status is unlikely to explain the lack of sex-biased parasitism. However, we cannot exclude the possibility that other hormones, such as progesterone or estrogens, have immune-regulatory effects, too (Klein 2004), blurring an existing modulatory effect of male steroid hormones on parasite infection.

Prevalence, PSR and nematode and infection intensities did not differ between males of different rank, but there was an indication that dominant males encountered higher protozoan levels than subordinates. Increased parasite load in dominant males has been reported in several other primate host species (Hausfater and Watson 1976; Freeland 1981; Halvorsen 1986) and have mainly been attributed to rank-related differences in androgen levels and their associated behaviour patterns such as ranging behaviour or aggression (Zuk and McKean 1996; Bercovitch and Ziegler 2002). Because in red-fronted lemurs social rank is not reflected in androgen and glucocorticoid levels (this study and Ostner *et al.* 2002; Ostner *et al.* 2008), the lack of a clear rank-related difference in parasite parameters may therefore not be surprising and is in line with our prediction. The differences seen in protozoa infection intensity might be due to behavioural or immunological differences between males of different rank, but more data are needed for a more detailed analysis.

Temporal variation in parasite infection and its association with hormone levels

All measurements of parasite infection in our study were subject to temporal variation within and between years, and part of the variability could be explained by natural variation in steroid hormone levels. Generally, an immune-suppressive function of steroid hormones is assumed as part of the immunocompetence hormone hypothesis (ICHH, Folstad and Karter 1992; reviewed in Hillgarth and Wingfield 1997; Braude *et al.* 1999). In our study, seasonal increases in male androgen and glucocorticoid levels during the mating season were followed by a time-lagged increase in nematode infection, which would be in line with the idea of an immune-suppressive effect of either or both of the two hormones, resulting in higher parasite susceptibility within years. The time-lagged response of parasite infections to endocrine changes can be explained by prepatence time, i.e. the parasite-specific time needed from infection of a host to excretion via faeces. Information on prepatence time is not available for lemur-specific parasites, yet estimations based on parasite species belonging to the same genera as the most abundant nematodes detected in this study (*Lemuricola* and *Callistoura*) suggest time periods of 4-6 weeks for oxyurid species (Mehlhorn and Piekarski 2002), which concurs with the time lag span detected in our study. However, we could not statistically confirm an association of endocrine changes with changes in parasite infection within years, and the distinct time-lagged pattern from 2006 was only visible as a trend in the second year. Factors that are directly linked to the seasonality of the mating season, such as changes in habitat use or grooming frequencies might be more directly linked to an increase in parasite infections in both sexes than to androgens or glucocorticoids. Seasonality in parasite abundance, which is extremely difficult to measure in natural populations, might also play an important role here. Variation in infection intensities due to temporally varying environments have been observed in other studies of parasite communities in wild hosts and patterns may differ between parasite species (Dobson 1990; Pence 1990; Müller-Graf *et al.* 1996).

Long-term changes in males' steroid hormone levels indicated a negative association with parasite species richness and nematode infection intensities in red-fronted lemurs, suggesting an immune-enhancing (rather than immune-suppressive) function of androgens and glucocorticoids, thus contradicting predictions of the ICHH (Folstad and Karter 1992). Strongest evidence for the ICHH *in vivo* were found from studies that manipulated individual hormone levels directly (see Roberts *et al.* 2004 for review), whereas a meta-analysis of experimental studies found no significant effect of testosterone on several immune parameters including endoparasite counts in mammals (Roberts *et al.* 2004). An immune-enhancing function of testosterone has been reported from manipulated house sparrows (Evans *et al.* 2000) and hamsters (Bilbo and Nelson

2001), but, to our knowledge, there is no study reporting such an association under entire natural conditions. Data on parasite infection and hormone levels in the months between the two study periods are not available, but would be essential to get a better understanding of the cause-and-effect relationship between the two parameters. Additional factors might help explain the interplay of seasonal changes in parasite prevalence and/or host susceptibility with variation in steroid hormone levels (Rubenstein and Hauber 2009). For example, nutrient supply or body condition is a conceivable factor, in particular as we have some indication that body mass of some individuals was higher in 2007 than in other years (Kappeler, unpublished data). When nutrient supplies are adequate, infections cause little or no effect on host condition or fitness (Coop and Holmes 1996; Milton 1996; Chapman *et al.* 2007). Moreover, improved nutrition can have a positive effect on steroid hormone levels (Volek *et al.* 1997), providing a potential explanation for the increase reported in our study in 2007.

Effect of parasite infection on mating and reproductive success

One theory of parasite-mediated sexual selection assumes that females avoid mating with parasitized males in order to protect themselves from infection (contagion indicator hypothesis, Able 1996; Loehle 1997), particularly with directly transmitted parasites. However, our data do not support this hypothesis because mating success was not associated with the intensity or species richness of males' parasite infection. Similarly, male reproductive success was not associated with any measure of parasite infection, and subordinate males that sired offspring did not differ in their parasite load from others. A main assumption of parasite-mediated sexual selection theory is that females choose less-parasitized males (Hamilton and Zuk 1982; Folstad and Karter 1992). Although we only identified the outcome but not the source of any form of bias in reproductive success (i.e., sperm competition or female choice), we found some evidence that reproductive success in red-fronted lemurs is not a function of individual parasite infection. Fitness advantages of less-parasitized males are known from other vertebrate taxa (Borgia and Collis 1989; Milinski and Bakker 1990; Møller 1990; Ehman and Scott 2002), but no primatological study has yet reported evidence for parasite-mediated sexual selection in primates.

Conclusions

In this study we explored the effects of group size, age, sex, social rank and endocrine status on parasite infection in a wild population of red-fronted lemurs. All measures of parasite infection were subject to strong seasonal variation within and between years and were positively

related to group size, while sex and rank explained only little variation. Accounting for lemur characteristic traits such as morphology, social organisation, sexual size monomorphism, and a promiscuous mating system helped to understand detected patterns. Variations in androgen and glucocorticoid levels between years were strongly negative associated with some measures of parasite infection, suggesting an immune-enhancing function of the two hormones. We also propose that further factors, such as nutrient supply or body condition, may explain the immune-enhancing pattern. Parasite infections did not appear to have important fitness consequences. Although we simultaneously incorporated several determinants of parasite infection into our models, there remains considerable variation in levels of parasite infection not accounted for by our model. We suggest that aspects, up to now neglected in such studies, such as parasite population dynamics, should be explicitly included in future models.

4.5 Acknowledgements

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5 Individual facial colouration in red-fronted lemur males: a condition-dependent ornament?

DAGMAR CLOUGH[1,2,3*], MICHAEL HEISTERMANN[3], PETER M. KAPPELER[1,2]

[1]Dept. of Behavioral Ecology and Sociobiology, German Primate Center, Göttingen, Germany
[2]Dept. of Anthropology and Sociobiology, University of Göttingen, Germany
[3]Dept. of Reproductive Biology, German Primate Center, Göttingen, Germany

Abstract

Studies of individual variation in conspicuous skin colouration in primates have suggested that colour indicates male quality. Although primate fur colour can also be flamboyant, the potential condition-dependence and thus signalling function of fur remain poorly studied. We studied sources of variation in sexually dichromatic facial hair colouration in red-fronted lemurs (*Eulemur fulvus rufus*). We collected data on 13 adult males in Kirindy Forest, Madagascar, during two study periods in 2006 and 2007, to determine whether variation in facial colouration is correlated with male age, rank, androgen status, and reproductive success. We quantified facial colouration using standardized digital photographs of each male, assessed androgen status using faecal hormone measurements, and obtained data on reproductive success through genetic paternity analyses. Male facial colouration showed high individual variation, and baseline facial colouration was related to individual androgen status but not to any other parameter tested. Colour did not reflect rapid androgen changes during the mating season. However, pronounced long-term changes in androgen levels between years were accompanied by changes in facial hair colouration. Our data suggest that facial hair colouration in red-fronted lemur males is under proximate control of androgens and may provide some information about male quality, but it does not appear to function as a prominent predictor of male reproductive success.

Keywords

Colouration, facial hair, androgen, condition-dependent trait, *Eulemur fulvus rufus*

5.1 Introduction

In many primate species males and females can be distinguished by striking colour differences. Such sexual dichromatism ranges from whole body colouration (e.g. silver backs in male gorillas) to locally restricted areas such as whiskers, blazes or ocular markings (Dixson 1998; Gerald 2003). In contrast to between-species colour differences, which may function in species recognition or concealment (Andersson 1994; Bradley and Mundy 2008), colour ornaments that differ between the sexes carry the potential to convey quality information to mating partners or to competitors of the same sex (Andersson 1994). Colouration is under complex regulatory control by multiple distinct factors which interact at different level to determine pigmented phenotype (Hearing 1999; Slominski *et al.* 2004a). This makes coloured ornaments potentially more malleable than other visual ornaments, facilitating to convey various kinds of "quality" information which may be of particular interest in intra- and inter-sexual competition. Theoretical work and research on other vertebrates have demonstrated that coloured ornaments carry costs (e.g. increased predation risk, agonistic interactions or nutritional constraints), so that fully developed ornaments can act as honest signals of individual quality (see, for example, Zahavi 1975; Rohwer and Ewald 1981; Hamilton and Zuk 1982; Folstad and Karter 1992; Veiga and Puerta 1996; Maynard-Smith and Harper 2003).

Historically, most research on signalling functions of colour ornaments has been conducted on birds and fish (Milinski and Bakker 1990; Hill 1991; Wedekind 1992; Buchholz 1995), but recent work has begun to focus on colour ornaments as signals in primates. Studies of primate skin colouration have shown that the development of bright colouration can vary with age (Setchell and Dixson 2001b; Setchell *et al.* 2008), dominance rank (Gerald 2001; Setchell and Wickings 2005) and reproductive state (Bergman and Beehner 2008; Higham *et al.* 2008) of both males and females. Androgens are indirectly responsible for reddening of facial and anogenital skin in male rhesus macaques via aromatization to estrogens, which in turn increase vascularization and epidermal blood flow (Vandenbergh 1965; Rhodes *et al.* 1997; Waitt *et al.* 2003). Similarly, in mandrills the degree of the red colouration of the facial skin has been shown to be androgen related and appears to be used as a "badge of status" (Setchell and Wickings 2005). Females of both species showed a preference for the brightest coloured males as mating partners (Waitt *et al.* 2003; Setchell 2005; Gerald *et al.* 2007), and males seem to judge competing males with regard to their colour signal (Setchell and Dixson 2001a; Setchell *et al.* 2008). These observations strongly suggest that certain primate skin colourations act as ornaments in inter - and intrasexual communication and selection.

Flamboyant colouration in primates is, however, not restricted to skin colour, as sexual dichromatism can also be found in pelage and facial hair colouration, where one sex usually shows more conspicuous colouration than the other (see Gerald 2003 for a review). Studies of the adaptive significance and physiological correlates of primate hair colouration are rare (Bradley and Mundy 2008), presumably due to the general view that hair colouration is a permanent cue that does not vary in form or intensity, rather than a signal that conveys quality traits (Hauser 1996; Gerald 2003). However, hair colour is under the control of multiple agents including androgens (Slominski *et al.* 2004a), which suggest hair colour to fluctuate between and within individuals as a function of changes in hormone levels.

Hair colour in primates is based on the melanin-based pigments, eumelanin and phaeomelanin (Castanet and Ortonne 1996; Slominski *et al.* 2004a), which are produced in the hair follicle bulb during melanogenesis (Castanet and Ortonne 1996). During the anagen (growth) phase of hair, androgens can affect hair colour without complete molt by altering the production of specific regulatory factors in the hair bulb (Randall 2000; Randall 2008). These factors may in turn alter the activity of other follicular cells, including melanocytes, resulting in a shifted ratio of eumelanin to phaeomelanin, affecting hair colour development (Slominski *et al.* 2004a,b). Studies of black lemurs (*Eulemur macaco macaco*) appear to confirm this physiological underpinning, as manipulation of androgen levels affect hair colouration in both sexes (Asa *et al.* 2007; A. Yoder, pers.comm. cited in Barthold *et al.* 2008). These findings are backed by results of studies on lion mane colouration (West and Packer 2002), as well as further evidence from patas monkeys (*Erythrocebus patas*, Loy 1974; Palmer *et al.* 1981), showing that hair colour can vary individually and temporally with regard to endocrine function. Yet, due to a lack of studies exploring causal relationships, it remains unknown whether primate hair colouration functions as a signal of quality by mirroring changes in the condition of the bearer.

In this study, we investigated individual variation in facial hair colouration in red-fronted lemurs (*Eulemur fulvus rufus*), and explored whether colour changes occur within one individual, a pre-condition for a possible condition-dependency. The lemur genus Eulemur is well-known for its sexual dichromatism (Mittermeier *et al.* 2006; Bradley and Mundy 2008), in which males usually display more striking patterns. In red-fronted lemurs, males exhibit conspicuous facial colouration characterized by a rufous crown, white patches around the eyes, a black nasal stripe that extends up from the face dividing the crown, and a uniformly grey body pelage. In contrast, female body pelage is rufous coloured and female facial colouration is dominated by dark and white patches of hair (see Figure 1 for images of both sexes). Male facial colouration varies between individuals with crown colour ranging from pale, cream-coloured to

rufous, bright red colour (Figure 1), and results of an experimental study suggested female *Eulemur fulvus* sp. prefer the brightest males (Cooper and Hosey 2003). However, we do not know if facial colouration varies with changes in male condition.

Figure 1. Above: Sexual dichromatism in facial coloration between male (left) and female (right) red-fronted lemurs. Below: Individual variation in facial coloration of red-fronted lemur males. This study focused on the coloured forehead, which ranges in colour from pale to rufous.

Old-world anthropoid primates and New World howler monkeys (*Alouatta* spp.) are known to possess uniform trichromatic vision, where both sexes have three divergent photopigment genes (Jacobs 1993; Surridge *et al.* 2003). Recent studies of primate colour vision have provided evidence that along with most New World primates and some diurnal lemur species, red-fronted lemurs, possess a second form of trichromatic vision, known as polymorphic trichromacy (Surridge *et al.* 2003; Bradley *et al.* 2008). In contrast to uniform trichromacy, polymorphic

trichromacy is based on two photopigment genes, one of which is a polymorphic X-linked gene. Thus heterozygous females can be either dichromatic or trichromatic whereas homozygous females and males can only be dichromatic (Jacobs 1999; Tan and Li 1999). Red and orange reflectance spectra, as seen in male facial colouration, would be cryptic to all males and homozygous females, but conspicuous to heterozygous, trichromatic females (Sumner and Mollon 2003).

There is no information on either the signalling function of hair colouration or the physiological correlates of this colourful trait under natural conditions. Androgen levels in this species vary according to reproductive season (Ostner *et al.* 2002; Ostner *et al.* 2008), providing a "natural experiment", enabling us to test whether facial crown red hair colour of male red-fronted lemurs is androgen dependent; if facial colouration is able to rapidly mirror changes in males' androgen status, seasonal changes in androgen levels should result in hair colour changes in the face.

Although methods for measuring skin colouration in free-ranging animals are available and used widely in primate skin colouration studies (Gerald *et al.* 2001; Setchell *et al.* 2006; Bergman and Beehner 2008; Higham *et al.* 2008), the quantification of hair colour in wild primates, which is characterized by changing reflection properties (Sumner and Mollon 2003), is still in its infancy, and nobody has, to our knowledge, measured hair colour using digital photography beyond the measurement of luminance. The present study therefore has the following aims:

(1) to adjust and validate methods used to measure animal colouration to assess hair colour in a free-ranging primate;
(2) to explore the signalling function of hair colouration by analyzing individual variation in facial colouration within and between male red-fronted lemurs in relation to intrinsic variables (age, social rank) and their consequences (reproductive success);
(3) to investigate whether facial hair colour is related to male androgens level under natural conditions both short-term (over a period of four months surrounding the mating season) and long-term (between two consecutive study periods).

5.2 Methods

Study site and animals

We studied red-fronted lemurs at the German Primate Centre (DPZ) field site in Kirindy Forest, a dry deciduous forest in Western Madagascar. The site is managed within a forestry concession operated by the Centre National de Formation, d'Etudes et de Recherche en Environnement et Foresterie (CNFEREF), Morondava. We collected data from all adult males living in four social groups of red-fronted lemurs during two field seasons between March and July in 2006 ($n_{2006}=11$ males) and 2007 ($n_{2007}=13$). Of these males, eight were present during both years and could be used for direct comparisons between years; the remaining eight males, either left (n=5) or joined (n=3) their group in the second year. All individuals were well habituated to human presence and were marked with individual combinations of nylon collars and pendants or radio collars for individual recognition. As part of an ongoing long-term study, one animal per group was equipped with a radio-tag (Biotrack, Wareham, Dorset, UK), which facilitated *ad hoc* detection of the group.

Red-fronted lemurs live in multi-male-multi-female groups and have a polygynandrous mating system. Reproductive skew within a group is usually high, as one male per group, the dominant or "central male", usually achieves the highest reproductive success (Ostner and Kappeler 1999; Kappeler and Port 2008). During the short mating season (3-4 weeks per year) each female is receptive for only 1-2 days and gestation length lasts about 122 days (Izard *et al.* 1995). Our study periods included the time before, during and after the mating season, which took place from mid May to early June in both years.

Collecting digital photos

We took photographs of focal males with a Canon EOS 20D digital SLR camera (8.2 megapixels, fitted with a Canon EF 55-200mm 1:4.5-5.6 II USM lens), which allowed full manual control over metering and exposure. As the area of interest was the forehead of males, we used full-frontal shots for later analyses from males that were sitting at eye level of the observer. We attempted to capture male colouration every week. To guard against major variation in light environment related to time of day and weather conditions (Endler 1993), we restricted data acquisition to a time window from 11am to 2pm on days with blue sky. Accounting for exposure and light differences in the forest required calibration (Gerald *et al.* 2001). After taking the photographs, we used the "sequential method" (Bergman and Beehner

2008), which consisted of taking a shot of the standard colour chart right after the animals left the spot. We used a qp coloursoft 201 colour chart (QPcard AB, Gøteborg, Sweden). Colours on this chart are mixed separately from pigments and are extremely fade resistant, and the chart was also protected from discolouration by being stored in a black folder when not in use.

As the preferred forest habitat of red-fronted lemurs is spatially heterogeneous in light intensity, we used a flash (internal EOS 20D flash and external flash Canon speedlite 430EX) to adapt to varying distances and light intensities. All images were taken within a mean distance of d=118 (±22.5) cm and a later analysis of the relationship between distance to object and colour score (see below for computation of colour score) confirmed that these two factors were not related (p=0.92, Linear Mixed Model, LMM: dependent variable – distance; fixed covariate - colour score; random factor - individual ID). For technical reasons the flash had to be substituted in the second year by an external flash. Testing the integrity of colour measurements under altered shooting conditions, we compared repeated measures between years of the colour score of 5 different standard colour patches on colour charts used in both years. Results of repeated measurement analyses of variance confirmed that colour scores did not differ between years and the substitution of the flash did not have a statistically significant effect on colour measurements ($F_{1,174}$=0.00, p=0.9997, Figure 4a). All images were stored as 12bit RAW files and further processed using Adobe Photoshop CS3.

Colour calibration and measurement

RAW files of paired sets of images of males and matching colour standards were opened in Adobe Photoshop CS3 RAW converter and we set CIE temperature to a fixed value of 5400K (adapting to the ambient light condition at shooting). As both images were shot using similar manual settings (i.e. similar exposure time and aperture) and as we purposely underexposed all images by one or two stops in the field in order to avoid clipping of channels (Stevens *et al.* 2007; Bergman and Beehner 2008), optimal exposure of images was not possible to achieve in the field and exposure had to be corrected manually using the RAW converter. Starting with the colour chart image we corrected the image's exposure by balancing the colour histogram (e.g. exposure +0.15) and repeated this procedure with the male colour image (again exposure +0.15). This method provided us with optimally exposed colour charts; a prerequisite for successful subsequent calibration. Proceeding to the Photoshop surface, we combined matched pairs of male facial portraits and colour chart images in one single image and calibrated the combined image following a methodology modified from Gerald *et al*. (2001): We used the Photoshop "adjust level" command to reset the combined image to pure white (maximum Red (R), Green

(G) and Blue (B) values) as obtained from the colour chart. Next we took mean R, G, and B values of the black square on the colour chart and reset the image accordingly.

To explore the reliability of our method, we tested 40 corrected images for linearity and RGB equalization, following methods described by Stevens *et al*. (2007). We used linear regression to analyze the relationship between RGB values measured from the seven step grey scale on the colour chart, and the known nominal reflection values for these seven squares and explored the relationship between measured greyscale value and nominal reflection value for the seven squares in comparison to predicted values.

For analyses of male facial hair colouration, we cropped a standard area of the male's forehead (60% of the total height) of each corrected image and used the rectangular "marquee" tool to select two measuring areas ("Patch") per image, to the left and right of the median axis of the forehead. Colour of these two patches ("left" and "right") was then measured within the RGB colour space and average RGB scores for each patch were obtained using the Photoshop "histogram" command. We applied a standardized Principal Component Analyses (PCA; *princomp* in *R*) to obtain one single component that explained most of the variability contained in these three colour channels. The first principal component vector (PC1) explained 98.4 % of the variance and provided the quantification of males' facial colouration, which we refer to as "colour score".

To compare potential condition-dependence of the facial colouration with the colouration of other body parts, we also measured colouration of a grey control patch located on the ruff of males using the same method.

Acquisition of intrinsic variables and reproductive success

Information on individual social rank was obtained from focal animal behavioural observations of all four groups of red-fronted lemurs (630 observation hours), and evaluated independently for both years. The age of most individuals was known from long-term monitoring of the population. For individuals that had immigrated into the population, age was estimated at first capture of these individuals using tooth wear and sexual maturity. Information on reproductive success was obtained via genetic paternity analyses of infants conceived during the mating season. Tissue samples of 16 weaned offspring were collected routinely during an ongoing long-term study and we assigned paternities using eleven nuclear markers (microsatellites) developed and established in earlier paternity studies on the same population (Wimmer and Kappeler 2002; Kappeler and Port 2008) and the genetic software CERVUS 2.0 (see Kappeler and Port 2008 for a detailed description of methods).

Hormone analyses

Male androgen status was determined from analyses of faecal samples, which were collected once a week, resulting in 32 samples per study animal (n_{total}= 512 samples of 16 males in four social groups) before, during, and after the mating season. Samples were collected directly after defecation 11am-1pm - the time of day when animals were most likely to stay close to the ground and individual assignment of faeces was straightforward. As it was previously shown that storage of red-fronted lemur faecal samples in alcohol did not affect faecal androgen concentrations (Ostner *et al.* 2008), samples were placed in tubes containing 90% ethanol, labelled, wrapped with parafilm, and stored in the shade at ambient temperatures at the field site (25-35 °C). At the end of each field season samples were transported to the endocrine laboratory.

Prior to hormone measurement, samples were homogenized in their original ethanolic solvent by squashing them with a metal stick (including a 3 ml methanolic rinse of the original sample tube) and subsequently extracted twice as described by Ziegler *et al*. (2000), with the modification that samples were vortexed in each of the two extractions for 10min on a multi-tube vortexer instead of shaking them overnight on a horizontal shaker. Following extraction the remaining faecal pellets were dried in a vacuum oven at 50°C and the dry weight of individual samples was determined. The supernatant was used for measurements of immunoreactive testosterone using a microtitreplate enzyme-immuno-assay (EIA), which has previously been validated for monitoring androgen status in lemurs (Kraus *et al.* 1999; von Engelhardt *et al.* 2000), including the red-fronted lemur (Ostner *et al.* 2002). The assay procedure has been described in detail by Kraus *et al*. (1999). Sensitivity of the assay at 90% binding was 0.5 pg per well. Intra-and inter-assay coefficients of variation of high and low value quality controls (QCs) were 6.2 % (n=16) and 7.6 % (n=24) (high) and 10.2% (n=16) and 11.2 % (n=24) (low), respectively. QC values from measurements of samples from both years did not differ significantly between years (QC_{high}: p=0.191, $t_{1,22}$=1.348; QC_{low}: p=0.64, t $t_{1,22}$=-0.475). All hormone values are expressed as mass per gram of dry weight (ng/g).

Statistical analyses

We collected a total of 112 images of 15 individual males in 2006 and 2007. Images were about equally distributed over time with 16 images taken during the pre-mating period (four weeks prior to mating), 28 images during the mating period, 26 images four weeks after, and 30 images eight weeks after the mating period. However, due to unbalanced sample sizes between individuals, we used different analyses and datasets to address our study aims as follows:

(1) Validation of the colour assessment method was conducted using a dataset of images that was collected during main data collection in the field. For each of six individuals we took three photographs at the same day and location (of these only one image per individual was used in the main analyses, see below). Assuming correct calibration of the photographs, colour scores of individual images taken during the same day should contribute less to total variance in colour scores (first component of the PCA) than the identity of the individual. This was tested by applying a Random Effects Model for the Analysis of Variance Components (Brown and Mosteller 1991) using "Colour Score" as response and incorporating male identity ("Male ID") and "Photo Number" as two random factors.

(2) Individual variation in facial hair colouration was first analyzed by applying a two-way nested Analysis of Variance followed by Variance Component Analysis with Colour Score as response and both Male ID and Patch (two factor levels: left and right) as explanatory variables. Error structure reflected the nested design of repeated measures per individual. Due to unbalanced numbers of individual images in 2006 we restricted this analysis to a dataset comprising all images collected in 2007 (n_{2007}=63) for which all model assumptions were met and the error structure was normally distributed.

To determine whether male facial colour variation was related to group membership, age, rank or reproductive success, we applied a LMM to the full dataset of images collected in 2006 (n_{2006}=37) and 2007 (n_{2007}=63). Full model structure was: Response – Colour Score; Fixed effect factors –Age, Rank, Reproductive Success, and Year. Incorporating Male ID as random effect factor accounted for repeated measurements per individual male.

(3) To investigate temporal patterns in colour variation in relation to hormone levels we first applied two LMMs to the total dataset exploring variability in male facial colouration and androgen levels with regard to short-term (within a 4-month study period, including the mating season) and long-term (between years) temporal changes using the following model structure: Response – Colour Score or Androgen Level; Fixed effects – "Season", Year; Random effect – Male ID. A direct relationship between androgen levels and male facial colouration was further explored by Spearman-Rank correlations on individual means of males that were part of the study population in both years. We used the variability in colouration of grey patches (n=60; LMM: Response – Colour $Score_{grey}$; Fixed effect – Year; Random effect – Individual ID) to control for androgen-dependent colour change (Cotton *et al.* 2004).

All statistical analyses were undertaken in *R 2.8.1* (R Development Core Team 2008). We used the "nlme"- package (Pinheiro *et al.* 2008) to estimate LMM parameters with restricted maximum likelihood (REML) as model convergence could not be achieved with maximum

likelihood (ML). Model simplification was conducted by step-wise removal of non-significant parameters.

5.3 Results

Validation of the colour assessment method

Results of 120 regressions (3 colour channels x 40 images) showed very high degrees of linearity (r^2 range: 0.98-1, $mean_{Red}$= 0.997 ± 0.005 SD, $mean_{Green}$= 0.996 ± 0.005, $mean_{Blue}$= 0.996 ± 0.005, n=120). In addition, the degree of RGB equalization was very high. The absolute value of the difference between the RGB-values in all seven grey squares was 0-16 (0.00%-6.27% of a maximal possible difference of 255) and the mean ± SD difference from equality was 2.95 ± 2.59 (1.16%, n=840). Grey values from calibrated images showed a close fit to required values (see Appendix 2). Statistical validation of the hair colouration assessment method (Variance Component Analysis) revealed that only 4 % of the total variation in colour score measurements was attributable to the error variability within a series of photos of one individual, whereas 96 % was attributable to differences between individuals.

Individual variation in facial hair colouration

Standardized colour scores of facial hair colouration ranged from -153.24 to 107.85 (mean = 0.00, SD =57.78). A high colour score indicated a rufous red colour. Colour scores differed significantly between individuals ($F_{12, 126}$= 14.13, p<0.001; Figure 2), but the location of the colour measurement on the forehead ("side") did not have a significant effect ($F_{1,126}$=0.01, p=0.94). Male ID explained 97.6 % of the overall variability whereas patch location contributed only 2.4 % to the explanation of existing variability in the response variable. We collapsed levels of this factor in subsequent analyses.

Social groups (groups A, B, F, J; Figure 2) did not differ systematically with regard to male facial colouration (t_{all_groups} < 1.64, p_{all_groups} > 0.20), and there were rufous and pale individuals in each social group. Individual differences in colouration between males were not related to age ($t_{1,63}$ =0.41, p= 0.68; age range: 3-12 yrs), dominance rank ($t_{1,63}$=1.05, p= 0.32; ranks: dominant or subordinate) or reproductive success ($t_{1,63}$=1.20, p=0.23; 0-3 infants per yr/male). Thus in this analysis, model simplification indicated best fit for a simple model with year as a covariate (see below for differences between years) and Male ID as a random effect.

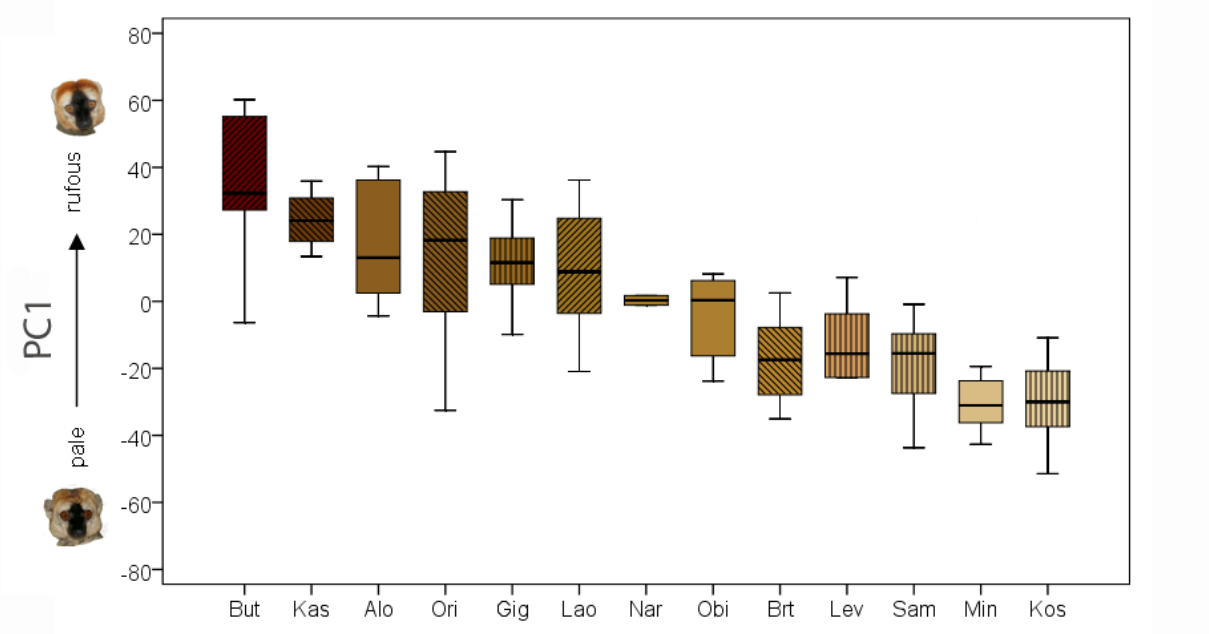

Figure 2. Individual red-fronted lemur males differed significantly from each other in facial hair coloration. Social groups all included both pale and rufous males (group A: , B: ,F: , J:). Data for 2007.

Temporal variation in facial colouration with regard to androgen levels

During both 4-month study periods including the mating season, male facial colour scores showed no consistent temporal variation (short-term variation: $t_{3,100} = -0.27$, $p=0.80$). However, colour scores increased significantly between the years 2006 and 2007 with all males displaying consistently higher mean colour scores in 2007 (long-term variation: $t_{1,100} = 12.23$, $p<0.01$, Figure 3c). Colour scores of grey control patches also varied between years ($t_{1,100} = 9.34$, $p<0.01$, Figure 3b), but the effect size explaining the difference between grey patches between years was smaller than that explaining the difference between red patches between 2006 and 2007 (66.49±6.79 S.E vs. 98.89±7.25 S.E.).

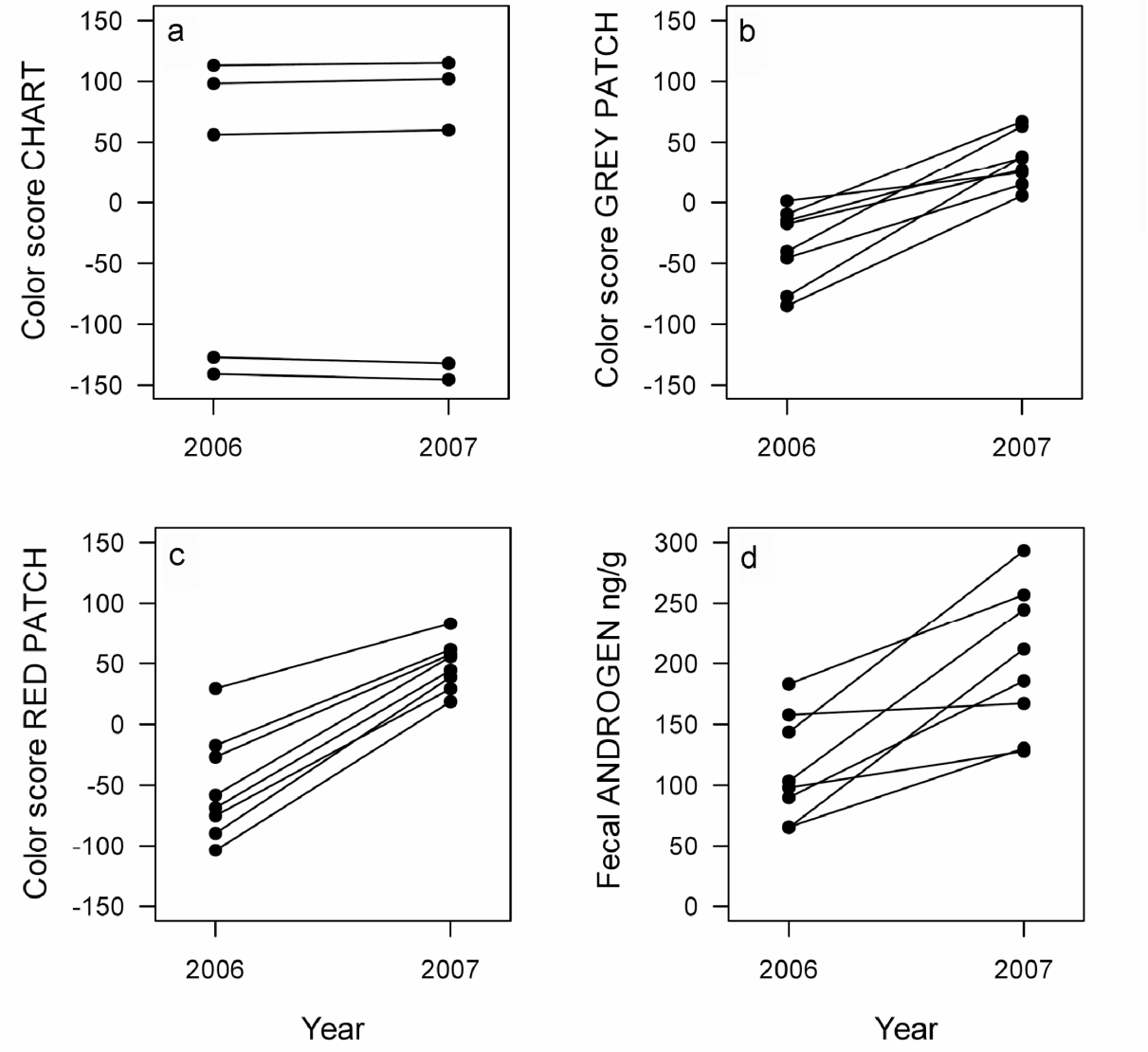

Figure 3. Individual variation in hair colouration and androgen level between years. Lines link repeated measures of the same individual in different years. **a.** Colour scores of colour chart standard patches did not differ between years. **b.** Colour scores of grey control patches were significantly higher in 2007. **c.** Colour scores of red patches increased significantly between years, and effect size explaining the difference between red patches between years was greater than that explaining the difference between grey patches between years. **d.** Androgen levels were significantly increased in 2007 compared with 2006.

Faecal androgen levels increased significantly during the mating season in both years ($t_{mating} = 8.54$, $p<0.001$). Androgen levels were also significantly higher in 2007 than in 2006 across all individuals ($t_{1,98} = 6.12$, $p<0.001$; Figure 3d) and levels in 2007 were also higher compared to those measured in an earlier study in 1999 (Ostner *et al.* 2002). Correlation analyses revealed a positive relationship between individual androgen level and mean individual colour

score, and this relationship was significant in both years ($p_{2006}<0.05$, $r^2=0.58$, $p_{2007}<0.05$, $r^2=0.59$; Figure 4).

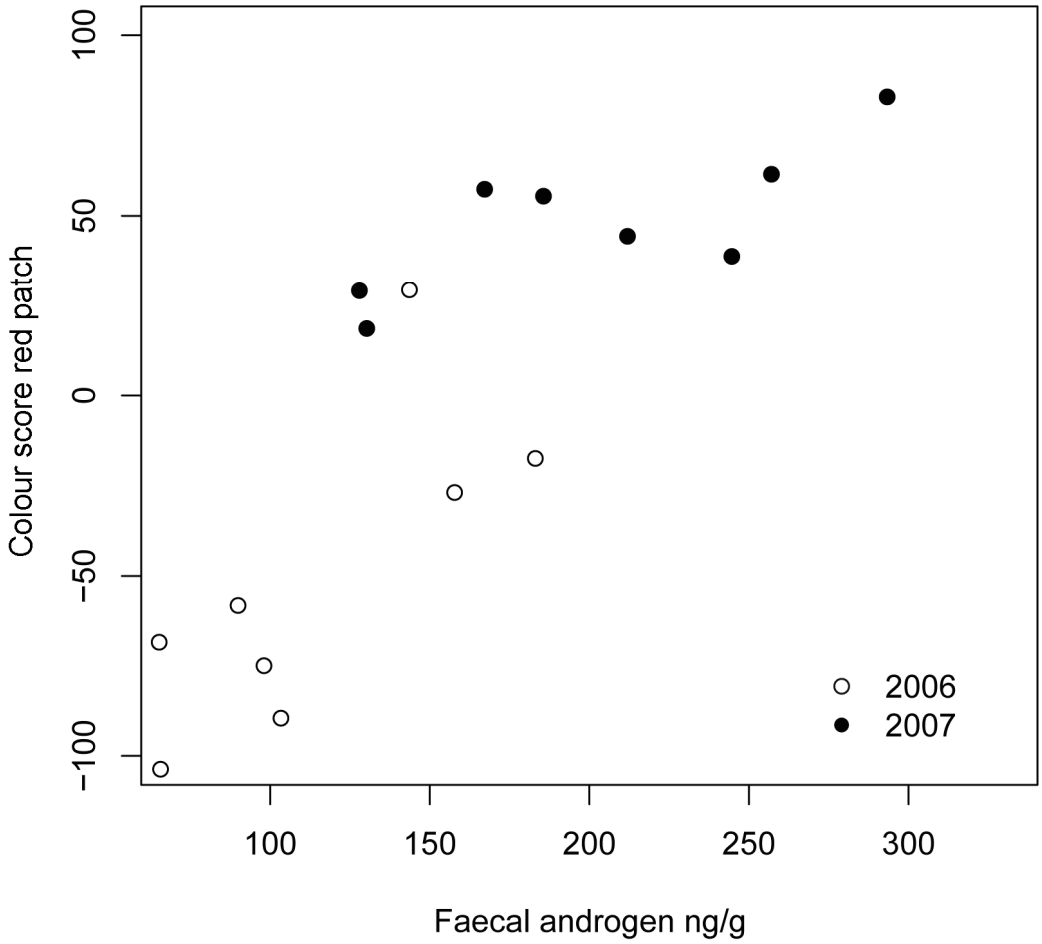

Figure 4. Mean individual colour scores of red-fronted lemur males were positively correlated with mean faecal androgen levels in both years. Both colour scores and androgen levels were higher in the year 2007.

5.4 Discussion

Measuring primate hair colouration

An increased interest in the functional meaning of primate colour paired with the use of digital photography as a method for the quantification of animal colour (as opposed to spectrophotometry, see Zuk and Decruyenaere 1994 for a review) gave rise to a spate of studies on the proximate and ultimate function of colouration in free-ranging subjects (Setchell 2005; Setchell *et al.* 2006; Bergman and Beehner 2008; Higham *et al.* 2008). However the majority of studies have focused on measuring skin colouration, while studies of the functional significance and physiological correlates of primate hair colouration are underrepresented (but see Sumner

and Mollon 2003; Bradley and Mundy 2008). We measured primate facial hair colouration digitally in free-ranging red-fronted lemurs and explored its functional significance. We found that: i) correcting for even minor deviations from optimal exposure; ii) including information from multi-dimensional colour spaces; and iii) using repeated measurements per individual, gave reliable results for quantifying facial hair colouration in free-ranging lemurs. Statistical validation confirmed that this method provided highly reliable measurements of hair colour and we therefore recommend our method for application in further studies of hair colouration.

Signalling character of facial colouration

Digital colour assessments confirmed our visual impression that individual forehead colouration in red-fronted lemurs varies significantly among males. Yet, in contrast to other studies investigating the signalling function of male skin colouration (Gerald 2001; Setchell and Wickings 2005; Bergman and Beehner 2008), we found no support for status-dependent male colouration with regard to age or rank. We do not know if females would have chosen the brightest male as suggested by Cooper and Hosey (2003) and preferences for a particular stimulus may not translate directly into mating decisions in a system where potential mates are associated year-round (Setchell and Kappeler 2003). Our results indicate that individual male reproductive success is not a function of male facial colouration. In addition, rank and consequently reproductive success (Wimmer and Kappeler 2002; Kappeler and Port 2008), are not mirrored by differences in androgen levels (Ostner *et al.* 2002). Thus, the outcome of reproductive competition appears to be influenced by complex traits beyond simple male ornaments. Colour differences between individuals may be based on genetic differences, operating as an identity rather than a quality signal (Dale 2000; Dale *et al.* 2001). In support of this hypothesis juvenile males strongly resemble their father in facial colour intensity (DC, pers. observation).

Physiological regulation of facial colouration

Baseline male androgen levels were positively correlated to individual colour scores in both years, suggesting that, like skin colouration in other species (Wickings and Dixson 1992; Setchell and Dixson 2001b), forehead colour in red-fronted lemur males is influenced by testosterone. However, we found no evidence that sudden increases in androgen levels resulted in short-term variation in male facial hair colouration. Given existing reports of hair colour change in manipulative studies (Asa *et al*. 2007; A. Yoder, pers.comm. cited in Barthold *et al*. 2008), this result was surprising, as we expected rapid changes in hair colouration to co-occur

with the pronounced changes in androgen levels during the mating season. Our finding suggests that hair physiology in lemurs does not allow such rapid changes in pigmentation, possibly due to cyclic melanogenesis (Slominski and Paus, 1993). As current knowledge of hair physiology is derived from studies on mice and humans, further research on primate melanogenesis is indicated. Due to our study design, we cannot exclude that facial colouration changes occurred in a time-delayed manner, as we were only able to collect colour data up to eight weeks after the significant androgen increase during the mating season and colour scores did not vary significantly during this time periods. Nevertheless, results of this study suggest that hair colouration does not provide reliable short-term cues about male condition.

In contrast, comparison between years revealed an increase in colouration in all males from 2006 to 2007. This colour intensification was not obvious to the observer during field work, but a period of absence from the field between the two study periods prevented direct comparison. Colour changes of the red forehead were accompanied by increases in androgen levels, and grey body colour also intensified between years. However, the effect size for grey patches was smaller than for red patches, suggesting a greater condition-dependence of red patches (Cotton *et al.* 2004).

Combining the above information on androgen and colouration, our results indicate that the regulating effect of androgens is less clear-cut in hair than described for skin colouration. A certain threshold level of androgens may be required to induce noticeable changes in hair colouration (see also Asa *et al*., 2007). Similar findings were reported by Roulin and colleagues (2008) for barn owls, in which glucocorticoids altered phaeomelanin production in feathers only during prolonged periods of stress, but where baseline hormone levels were not correlated with colour.

Although the present study suggested a positive association between androgen level and colour score, there was still unexplained variability left between years. As hair colouration is under the control of multiple factors, we briefly discuss some other possible influences. In an experimental study on house sparrows, Veiga and Puerta (1996) proposed that nutritional constraints determine the expression of not only carotinoid, but also melanin-based colouration, based on diet quality and fat reserves. Consequently, differences in diet composition or body constitution of red-fronted lemurs may also influence the intensity of colouration; however, data on these variables were not available for this study. Along the same lines, male health (i.e. immune status, parasite infection intensities) and other physiological challenges may act on the expression of facial hair colouration. In subsequent analyses we are thus planning to analyze intestinal parasite infection patterns between years.

Adaptive function of facial colouration

Our data suggest that male facial colouration conveys some information on male androgen status, which might contain subtle cues about male quality (e.g. with regard to immunocompetence, Folstad and Karter 1992). However, as males with the brightest colours did not necessarily have the highest reproductive success, this preliminary study using a small sample of males (but from several social groups) could not shed light on the possible adaptive function of this potential colour ornament. A promising approach for future studies might include information about the visual system because only some trichromatic red-fronted lemur females could perceive the red colour signal (Bradley *et al.* 2008), whereas other (dichromatic) females may not possess the physiological abilities to do so (Tan and Li 1999). Unfortunately, we were not able to gather information on the genetic constitution of visual abilities of the females in our study population, but might include this in future studies.

Summary and conclusions

Quantitative assessment of male facial hair colouration in red-fronted lemurs showed that males differ with regard to their individual colouration, but this colour trait did not have the potential to provide reliable short-term cues to changes in male androgen levels. Intrinsic factors did not explain the conspicuous individual variability in male colouration, and mean individual androgen levels explained only part of the existing variability. However, our findings suggest that prolonged increases in androgen levels were reflected in changes in male forehead colouration. Although we cannot exclude other factors possibly affecting long-term colour changes, facial hair colouration appears to be partially under proximate control of androgens and may provide a long-term quality signal. Information on further male quality traits as well as individual visual abilities of females should be considered in future experimental studies of female choice.

5.5 Acknowledgements

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6 Susceptibility to parasite infection in a wild primate species. General discussion

Primates are among the best-studied vertebrates and over the past decades a multitude of information has been compiled on their ecology, behaviour, and evolutionary biology (e.g. Crook and Gartlan 1966; Wrangham 1980; Terborgh and Janson 1986; Dixson 1998; Fleagle 1999; Kappeler and van Schaik 2004). Whereas the effects of parasites on individual survival, social evolution and sexual selection have been demonstrated in various other vertebrate groups (e.g. birds: Hillgarth 1990; Møller 1990a,b; Zuk *et al.* 1990, fish: Kennedy *et al.* 1987; Milinski and Bakker 1990 and amphibians: Ressel and Schall 1989; Hausfater *et al.* 1990), the regulatory effect of infectious diseases is one of the last frontiers in primate socio-ecology (Heymann 1999; Kappeler and van Schaik 2002; Setchell and Kappeler 2003; Nunn and Altizer 2006a). Furthermore, empirical data on proximate determinants and ultimate effects of variability in parasite infection in primates is very limited and partially not available (Nunn and Altizer 2006a).

The aim of this thesis was to investigate patterns, determinants and consequences of variation in individual disease risk in red-fronted lemurs by combining detailed information on individual parasite infection, genetic components of parasite susceptibility, and variation in steroid hormone levels. Red-fronted lemurs mate promiscuously and although dominant males obtain the greatest share of reproductive success, approximately one third of all offspring produced are sired by subordinate males. Recently, it has been shown that these paternities do not appear to be reproductive concessions given to subordinates by dominant individuals as an incentive to remain in the group (Kappeler and Port 2008), leaving room for alternative explanations for the distribution of reproduction among males, including the reproductive interests of females. In order to get a picture of the importance of parasite infection in red-fronted lemurs, I empirically tested some predictions of the parasite-mediated sexual selection theory and established a basis for further discussion on the relevance of parasite infections in primates.

Patterns of parasite infection in red-fronted lemurs

Red-fronted lemurs harboured a broad range of gastro-intestinal parasites including a minimum of eight unique helminth parasites and two protozoan species (**Chapter 2**). This level of parasite species richness was comparable with results from previous studies on free-ranging primates (Nunn *et al.* 2003). However, parasite species richness and, in particular, maximal prevalence of two protozoan and two nematode parasites exceeded results reported from other lemur studies (Dutton *et al.* 2003; Junge and Louis 2002; 2005a;b; Nègre 2003; Raharivololona 2009; Schwitzer *et al.* submitted). Although comparisons between studies can be problematic due to different methodologies and sampling efforts, results suggested a high degree of parasite infection in this free-ranging population (**Chapter 2**). I could not observe any clinical symptoms in the population; however, high levels of parasitism are known to be of particular importance during times of increased social or environmental stress (Gillespie *et al.* 2005). Variability of parasite infection differed significantly between individuals (**Chapter 3 and 4**), groups (**Chapter 2 and 4**), within and between years (**Chapter 4**), and part of the variation in nematode infections could be explained by differential genetic constitution and changes in steroid hormone levels (**Chapter 3 and 4**, results discussed below). Variability in protozoan infection intensities was not accounted for by differences in genetic or hormone constitution and there is evidence that red-fronted lemurs act as a reservoir for these protozoan species (**Chapter 2**). The majority of parasites recovered from red-fronted lemur samples are subject to direct transmission cycles, and thus, the risk of infection can represent a potential cost during mating or other social contacts.

Genetic regulation of susceptibility

The nematode infection intensity of red-fronted lemurs was significantly associated with a newly detected Interleukin-4 (*IL4*) gene promoter polymorphism (**Chapter 3**). *IL4* plays a central role in the humoral defence against and expulsion of helminth infections (Hotez *et al.* 2008) by inducing and sustaining T_H2 responses, but also by initiating immunoglobulin isotype switching to IgE, which plays an essential role in anti-parasite immunity (King and Mohrs 2009). Single nucleotide polymorphisms in the promoter area have been shown to affect IL-4 protein expression and, hence, IgE titres. Results represented in **Chapter 3** strongly suggest a functional role of the detected polymorphism, as individuals with the negatively-associated, and simultaneously rarest genotype in the population, appeared compromised in their ability to fight infections by expelling worms from the intestines, resulting in a lower number of eggs being shed (Seivwright *et al.* 2007). The few studies that have explored genetic principles of parasite

resistance in wild primates have focussed on the Major Histocompatibility Complex (Knapp 2005; Schad *et al.* 2005; Schwensow *et al.* 2008; Wedekind *et al.* 1995) as this highly polymorphic genetic system determines susceptibility and resistance to infectious diseases (Hill 2006). However, the magnitude of an immune reaction is not only regulated by a host's immune genetic diversity, but also by the intensity of the elicited response. Our findings confirmed that parasite resistance was also associated with a specific allele of a candidate-gene (i.e. *IL4* polymorphism) - making *IL4* a promising tool for future studies looking for suitable genetic markers of parasite resistance.

However, although *IL4* seemed to be an appropriate marker for parasite resistance, results from long-term population analyses strongly indicated that an increased susceptibility to nematode parasites did not necessarily result in adverse fitness effects (**Chapter 3**). Male carriers of the supposedly disadvantageous genotype had a disproportionately higher reproductive success than expected under random mating. This result may be explained by multiple functions of *IL4* (as discussed in **Chapter 3**) and we assume that other aspects of *IL4* biology, i.e. mounting a T_H1-type immune response, which is elicited when individuals are confronted with intracellular pathogens such as viruses or bacteria, play an important, if not the crucial role in this host-parasite system. In addition, we were only able to investigate one genetic system that is important for parasite resistance. Alternative genetic marker systems such as MHC diversity or genome-wide heterozygosity might provide further insights into the regulation of susceptibility to parasite infection.

The immune-regulatory effect of hormones

A regulatory effect of steroid hormones on the immune system and, as a consequence, on parasite infections, is usually expected to be reflected in sex-biases in parasite infection (Klein 2000). In red-fronted lemurs, males and females differed in androgen and glucocorticoid levels, yet overall prevalence of parasite infection was not biased towards one sex. However, as progesterone and oestrogen are also known to regulate the functioning of the immune system, this may be considered a poor test of the immune-modulatory function of steroid hormones. However, an increase in male androgen levels and glucocorticoid levels (**Chapter 4 and 5)** between years was significantly associated with decreased parasite species richness and nematode infection levels, suggesting that increased androgen levels might indicate better parasite resistance or better overall condition (**Chapter 5**). The immune-enhancing effect of steroid hormones indicated by our results contradicts the main premise of the immunocompetence hypothesis, which centres on the immunosuppressive effect of steroid

hormones (Folstad and Karter 1992). In addition, baseline androgen levels were significantly associated with facial colouration intensity and long-term changes in androgen levels were accompanied by changes in facial hair colouration: all males turned redder with increased androgen levels (**Chapter 4**). This interplay of parasite infection, male steroid hormone levels and male colouration does not support the assumptions of the immunocompetence model as only the incorporation of a component that results in physiological trade-off guarantees the honesty of the signal to be used for quality assessment and can prevail as an evolutionary stable strategy (Zahavi 1975). Instead, we suggest that in red-fronted lemurs additional factors, namely nutrient supply or body condition may explain the immune-enhancing pattern and future studies should incorporate such information (e.g. Westneat and Birkhead 1998). Empirical evidence for the effect of steroid hormones on parasite infections in other primate species can be reduced to two studies (Chapman *et al.* 2007; Muehlenbein 2006) and further examples from other species would be necessary to improve our understanding of general relationships. However, mixed results from non-primate studies (see Roberts *et al.* 2004 for review) indicate that the concept of an immune-suppressive effect of androgens modulating a trade-off between immune function, parasite infection and fitness-related traits will have to be reconsidered before further field studies are set up (Braude *et al.* 1999; Getty 2002; Ryder 2003; Siva-Jothy 1995; Westneat and Birkhead 1998).

Signalling character of primate colouration

Charles Darwin developed his theory of sexual selection based on the observation that males often bear elaborate sexual traits, which seem difficult to explain directly within the context of natural selection (Darwin 1871; 1876). According to the theories of PMSS (see Chapter 1), females can rely on such traits as indicators of male health and then proceed to judge them accordingly (Able 1996; Hamilton and Zuk 1982; Loehle 1997). Due to their highly-developed visual system, primates are most suitable candidates to attribute a functional role of colour signals (Bradley and Mundy 2008). An experimental study conducted by Cooper and Hosey (2003) suggested that females across various *Eulemur fulvus* ssp. prefer brightly coloured males, however this study was restricted to confronting females with male images of different brightness. Our analyses of male facial colouration (**Chapter 5**) showed a high degree of individual variation in the colour trait, however, individual variation was not accounted for by male age or social rank and did not appear to function as a reliable predictor of reproductive success because a male's share of paternities was not associated with the intensity of the forehead's red colouration. Besides the possibility that male facial colouration has not the

potential to hold an ultimate function in this system, there might also be a proximate explanation for this result. Whereas the predictions of our study (**Chapter 5**) were based on the assumption that female red-fronted lemurs at Kirindy possess polymorphic trichromatic colour vision as confirmed from preliminary studies (Bradley *et al.* 2008), we now know that all females that were present in the population during the time of this study were functionally dichromatic (Bradley, unpublished data), implying that both males and females were red-green colour blind and thus unable to assess differences in red colour intensity.

Lack of an association could also be due to the choice of the "wrong" variable (Møller 1990a). For example, we have looked at variation in red colouration, while other attributes such as the size of the forehead patch or the black nasal strip could be more important (see Figure 1 in Chapter 3). Furthermore, because most primates live in stable social groups, and know each other from regular interactions and associations, it is likely that selective pressures for arbitrary phenotypic traits such as male colourations are low and might be more important in species with ephemeral mating groups or extra-pair mating (Kappeler and van Schaik 2002; Setchell and Kappeler 2003; Snowdon 2004).

Compared to primate hair colour, the signalling function of brightly coloured skin in primates has been studied in great detail (see Dixson 1998 for an overview). Little is known about the proximate effects that cause variability in trait expression or maintain the signal (Setchell and Kappeler 2003), however there is empirical evidence, for instance, from rhesus macaques (Waitt *et al.* 2003) and mandrills (Setchell 2005) that male colouration have a function in intersexual competition. Setchell and colleagues (2007) also investigated parasite infections of the same population, but there was only little interindividual variation in parasite infection, which could be due to residual effects of antiparasite treatment of the semi-captive population (Setchell *et al.* 2007, Setchell, pers. comm.). For future studies, I suggest that in species where the expression of skin colour ornaments is already known to vary between and within individuals as a function of male status or condition (e.g. in red uakaris: Ayres 1986; vervets: Gerald 2001; hamadryas baboons: Kummer 1968; mandrills: Setchell and Dixson 2001), assessments of colour signals in free-ranging populations should include measurements of parasite infection such as those presented in this study, in order to contribute to a better understanding of the signalling function of primate colourations.

Effect of parasite on reproduction

According to the main prediction of the PMSS theory, females would avoid mating with parasitized males, and males with high parasite levels should be impaired in their reproductive success. We tested several aspects of parasite infection with regard to their effect on mating and/or reproductive success (**Chapter 4**). The mating and reproductive success of males was not correlated to any measure of parasite infection, which provides some evidence against a direct benefit model of PMSS (e.g. parasite-avoidance model, see Chapter 1). In addition, subordinate males that were able to reproduce successfully did not differ in parasite infection compared to other males. Female reproductive success was not impaired by any means as all adult females that were present during the mating season were observed to give birth.

Disease risk is one ecological force among many that shape primate mating patterns and other factors, such as resource competition, predation or sexual conflict, might be of more importance (Nunn and Altizer 2006a). In addition, reproductive success might not be the best method to assess the importance of PMSS, as the survival of juvenile offspring, and thus, the successful transfer of genes to the next generation, is ultimately the best measure of fitness (Cockburn 1995). Nevertheless, this has rarely been applied and offspring survival with regard to parasite infection is difficult to measure in the wild.

Parasite-mediated sexual selection in primates

Although (i) high individual variation in parasite infection, (ii) genetic basis of parasite susceptibility and (iii) the condition-dependent variation of male colouration complied with some predictions of PMSS theory (see Chapter 1 and Folstad and Karter 1992; Hamilton and Zuk 1982; Møller 1990a), complementing the picture with results on (iv) the immune-enhancing effect of steroid hormones and (v) the neutral association of mating and reproductive success with parasite infection measures, did not support the hypothesis that reproductive success is a function of parasite infection, or that females use male colouration to assess their disease resistance (Hamilton and Zuk 1982). The approach leading to the latter finding has limited explanatory power as this thesis did not aim to study mechanisms of female choice but focussed on the outcome of any form of sexual selection. Still, results confirm that male colouration does not appear to function as a prominent predictor of male reproductive success, which provides a basis for further studies on mate choice mechanisms. Results from the *IL4* study, highlighting enhanced reproductive success of strongly-infected males, further suggest that the resistance to intracellular parasites and pathogens might be more of an issue than gastro-intestinal parasites,

and should be included in future studies trying to evaluate the overall parasite and pathogen burden in primates and other vertebrate hosts.

Results of this thesis did not support overall predictions of the PMSS hypothesis, yet results provide indications as to why findings did not comply with expectations. One main aspect is the parasite community encountered by red-fronted lemurs. The high prevalence and intensities of several parasite species and the overall good condition of the population, regardless of these high levels of parasitism, suggests that parasites are well-habituated to their hosts and vice versa, which might be due to a close co-evolution of oxyurid parasites and lemur hosts (Hugot 1999). Generally in primates, endoparasite infections are considered not deleterious to their host, which might explain why the mechanism of PMSS may simply not have as much of an impact as on birds, fish or insects (Fiennes 1967). Additionally, although the design of the study allowed for repeated sampling of the same individuals over more than 8 months, our findings can naturally only provide a partial insight into the total diversity of parasite and pathogen infections encountered by these lemur hosts. Collecting and analysing long-term data over several years, while at the same time including information on individual mortality, would be important in order to get a more complete picture, but is beyond the scope of a doctoral thesis.

Next, the need for directly selected communication cues of individual quality may not be of utmost importance in long-lasting primate groups. Information about potential mates might be accumulated over longer periods of time and might be based on other aspects such as protection, paternal care or competitive abilities (Setchell and Kappeler 2003; Snowdon 2004). It has been reported from other red-fronted lemur groups that similar to the well-known intersexual friendships in baboons (Smuts 1985), red-fronted lemur females may also favour mating with male companions, which they know from regular interactions and associations (Kappeler 1993; Pereira and McGlynn 1997)

Finally, results of this study show that the interplay of parasite infection, hormones and the immune system is not very clear cut, which suggests additional variables (e.g. parasite population dynamics, host condition, environmental seasonality) to be taken into account when studying such a complex aspect as PMSS (Clayton 1991; Møller 1990a).

Conclusions

The comprehensive approach of this thesis demonstrated that gastro-intestinal parasite infections in red-fronted lemurs are very heterogeneously distributed within and between individuals and between seasons. We found that susceptibility to nematode infection is partially regulated by a genetic polymorphism, and longitudinal hormone and parasite data collected

across two years showed that both parasite species richness and nematode infection intensities were negatively correlated to changes in steroid hormone levels. Contrary to predictions from theoretical models and empirical results of studies on other vertebrate taxa (Able 1996; Clayton 1991; Folstad and Karter 1992; Hamilton and Zuk 1982; Møller 1990a), long-term population analyses and information on male reproductive success suggested that male infections do not play an important role in the outcome of sexual selection. Although all individuals of the study population were strongly infected with various parasite species and the intensity of infection varied between and within individuals, parasite infections were not reflected in mating and reproductive success and parasite infection was not honestly indicated by male colouration. Evidence suggests that PMSS in primates may not have such a large impact as in birds, fish or invertebrates, as (1) parasite infections are often considered not deleterious (Fiennes 1967), (2) living in stable social groups with regular social interactions might prevents the need for a direct sexually selected communication signal (Kappeler and van Schaik 2002; Snowdon 2004), and (3) other factors such as parasite populations dynamics or host condition and nutrition status could blur the effect of parasites on a host population (Clayton 1991).

I used a comprehensive approach which aimed to incorporate simultaneously the most important intrinsic factors affecting susceptibility to parasite infection, and provided new insights in the hormonal and genetic regulation of parasite infection in red-fronted lemurs. Extrapolation of these results to other primate taxa might be impeded by the fact that the data presented reflect only a small part of the big variation that is found in natural parasite-host systems and also that primates are extremely diverse with regard to their environment, their social system, or the parasite community they may host (Stuart and Strier 1995). Nevertheless, results of the study provide a good basis for further investigations that aim to unravel the impact of parasite infection on primate populations. I propose that comparative studies should be conducted in other group-living, potentially polyandrous, primate species to determine the generality of these findings. Beside the investigation of patterns and determinants of parasites infections, as well as the evaluation of PMSS processes in red-fronted lemurs, this thesis introduced new methodological approaches in immune genetic assessment of parasite resistance (the use of *IL4* marker) and the quantification of hair colour in free-ranging subjects. These advances provide a good basis for further studies in other primate species.

Finally, detailed analyses of parasite infection both at the population and individual level indicated that although I included a comprehensive set of determinants of parasite infection and used a repeated design, variability that was not explained by statistical models was still high. More attention needs to be paid to factors that cause variability and heterogeneity in parasite

infections, including environmental seasonality and parasite population dynamics, for a better understanding of primate-parasite interaction under natural conditions.

Summary

Parasites and infectious diseases represent an ecological force shaping animal social evolution. Particular attention has been paid to parasite infection as a driver of mate choice in models of sexual selection as one sex may advertise parasite resistance via elaborate sexual selected phenotypic signals. Although empirical studies supporting the importance of parasite infection in natural populations abound in various vertebrate orders, relatively little is known about both dynamics and impact of parasite infections and infectious diseases in natural populations of primates. More specifically, there is a particular lack of baseline parasitological information in strepsirrhine primate species. Using an interdisciplinary approach that combined individual parasite, genetic, hormone and sociobiological information, I studied patterns and proximate determinants of gastro-intestinal parasite susceptibility in a wild population of red-fronted lemurs (*Eulemur fulvus rufus*). Male red-fronted lemurs display a more conspicuous facial colouration than females do and there is considerable inter-individual variation in this trait among males. In a second step, I examined if strong parasite infections have negative consequences on male reproductive and whether male colourations is a condition-dependent ornament that can be provide information on male quality. During two 4-month field studies in 2006 and 2007, I collected several types of data on 29 adult individually marked male and female lemurs in Kirindy Forest, western Madagascar. Data on parasite infection (prevalence, species richness, infection intensity) and hormone (androgen and glucocorticoid) levels were obtained by analysing faecal samples that were collected non-invasively once per week. Data on mating and reproductive success were obtained through focal animal observations and genetic paternity analyses.

The entire population was infected by a minimum of 10 unique parasite species including 8 helminth and 2 protozoa species. Prevalence of the majority of parasite infection and parasite species richness was higher than ever recorded from other lemur species. The four social groups studied differed partially in their parasite fauna, which was probably due to minor differences in habitat parameters. Comparisons to other lemur studies indicated that short-term assessments of lemur health might underestimate the real parasite burden, and the application of long-term studies such as conducted during this project is advised. Part of individual variability in parasite infection could be explained by a newly detected genetic polymorphism in the *IL4* gene promoter that regulates immune response against nematode infections and facilitates worm expulsion. Carriers of a particular genotype showed highest nematode infection intensities,

suggesting a functional role of the *IL4* polymorphism, yet individual reproductive success appeared not impaired and long-term population analyses indicated higher reproductive success of these individuals than expected. Host intrinsic factors such as group size, age, sex and rank explained only a part of variation in parasite infection susceptibility on population level and lemur characteristic traits (morphology, social organization, mating system) helped to understand detected patterns. Male mating and reproductive success were not correlated to any measure of parasite infection, which suggests a non-functional role of gastro-intestinal parasite in red-fronted lemur sexual selective processes. Additionally, male facial colouration, although under proximate control of androgens, did not appear to function as prominent predictor of reproductive success.

The population was subject to strong seasonal variation in parasite infection levels between years, which was significantly associated to changes in androgen and glucocorticoid levels. Contrary to predictions of the immunocompetence hypothesis, which assumes immune-suppressive action of androgens (Folstad and Karter 1992), results indicate an immune-enhancing effect of androgen and glucocorticoid levels, leading to a decrease in parasite species richness and nematode infection intensity. Although the comprehensive approach applied in this thesis provided valuable new insights, I propose that additional factors such as body condition or nutrient supply but also information on parasite population dynamics need to be incorporated into future models in order to further improve our understanding of the multi-faceted interactions of primate hosts and parasites under natural conditions.

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Zusammenfassung

Parasiten stellen wichtige ökologische und evolutionäre Faktoren dar, die einen starken modulierenden Effekt auf natürlich vorkommenden Tierpopulationen ausüben können. Vor allem im Zusammenhang mit Mechanismen der sexuellen Selektion, wird der Einfluss von Parasiten auf die Wirtspopulation als treibende Kraft angesehen, da angenommen wird, dass potentielle Paarungspartner mit Hilfe von ausgefallenen sexuell selektierten phänotypischen Merkmalen ihre Resistenz gegenüber Parasiteninfektionen signalisieren. Obwohl viele empirische Studien in den verschiedensten Vertebratentaxa auf die Wichtigkeit von Parasiteninfektionen in natürlichen Population hinweisen, so ist doch bisher noch sehr wenig über die Mechanismen und den Einfluss von Parasitenbefall bei Primaten bekannt. Vor allem bei den Strepsirrhini (Feuchtnasenaffen) gibt es große Lücken bezüglich basis-ökologischer Daten zum Parasitenbefall.

Mit Hilfe eines interdisziplinären Ansatzes, der parasitologische, genetische, soziobiologische und Hormondaten vereint, habe ich zunächst Muster und Determinanten von gastrointestinalem Parasitenbefall bei frei lebenden Rotstirnmakis (*Eulemur fulvus rufus*) untersucht. In einem nächsten Schritt habe ich mögliche Konsequenzen von individuellem Parasitenbefall auf den Paarungs- und Reproduktionserfolg männlicher Rotstirnmakis erforscht. Da Männchen dieser Art durch inter-individuell variierende Gesichtsfärbungen auffallen, war ein zusätzliches Ziel dieser Arbeit, die Konditionsabhängigkeit des Ornaments und damit eine mögliche Signalwirkung bezüglich der Männchenqualität zu evaluieren. Während zwei jeweils viermonatiger Feldaufenthalte in den Jahren 2006 und 2007 habe ich verschiedenartige Daten von 29 individuell markierten männlichen und weiblichen Rotstirnmakis im Kirindy Wald in West-Madagaskar aufgenommen. Parasiten- (Prävalenz, Artenreichtum, Infektionsintensität) und Hormondaten (Androgen und Glukokortikoide) wurden mit Hilfe wöchentlich gesammelter Kotprobenanalysen, Information zum Paarungs- und Reproduktionserfolg durch Fokustierverhaltensprotokolle und genetische Vaterschaftsanalysen ermittelt. Die gesamte Population war mit mindestens 10 unterschiedlichen Parasitenarten befallen, wovon 8 Arten den Helminthen, 2 weitere Arten den Protozoen zugeordnet werden. Prävalenz der meisten Parasiteninfektionen, sowie die Anzahl der verschiedenen Parasitenarten pro Individuum, überstiegen alle Werte, die bislang bei anderen Untersuchungen zum Parasitenbefall bei Lemuren berichtet wurden. Die vier untersuchten Rotstirnmaki-Gruppen unterschieden sich zudem teilweise in ihrer Parasitenfauna, was auf geringe Habitatunterschieden zurückzuführen

ist. Im Vergleich zu anderen Studien, die Parasitenbefall bei Lemuren mit Hilfe eines horizontalen Designs untersucht haben, geben die Ergebnisse meiner Studie Hinweise darauf, dass solche Kurzzeitaufnahmen das wahre Ausmaß des Parasitenbefalls unterschätzen, und dass eine longitudinale Herangehensweise, wie in dieser Studie beispielhaft vorgestellt - im besten Fall über mehrere Jahre durchgeführt- für zukünftige Studien von Vorteil wäre. Ein Teil der individuellen Variabilität im Parasitenbefall konnte durch genetische Polymorphismen im Promoter des *IL4* Gens erklärt werden. *IL4* reguliert die Immunantwort gegen Nematodenbefall und begünstigt den Ausstoß von Würmern aus dem Magen-Darm Trakt. Träger eines bestimmten *IL4* Genotyps hatten den stärksten Nematodenbefall, was darauf hindeutet, dass der genetische Polymorphismus eine funktionelle Rolle in der Parasitenabwehr spielt. Allerdings zeigten genetische Langzeitpopulationsanalysen, dass, entgegen den Erwartungen, diese Tiere in ihrem Reproduktionserfolg nicht negativ beeinflusst waren, sondern sogar einen höheren Reproduktionserfolg hatten als erwartet, was auf andere, positive Effekte von *IL4* rückschließen lässt. Weitere wirts-intrinsische Faktoren wie Gruppengröße, Alter, Geschlecht und sozialer Rang erklärten nur einen Teil der Variation im Parasitenbefall. Lemuren-spezifische Charakteristika (Morphologie, soziale Organisation und Paarungsystem) halfen, diese Muster zu verstehen. Paarungs- und Reproduktionserfolg der Männchen war nicht mit Parasiteninfektion korreliert. Dieses Ergebnis deutet darauf hin, dass gastro-intestinale Parasiten bei Rotstirnmakis keine funktionelle Rolle in Prozessen der sexuellen Selektion innehaben. Die männliche Gesichtsfärbung, die zwar, wie hier gezeigt, unter der proximaten Kontrolle von Androgenen steht, schien auch nicht als Prädiktor für den männlichen Reproduktionserfolg zu fungieren.

Der Parasitenbefall der Rotstirnmakipopulation unterlag starken saisonalen Schwankungen zwischen den Untersuchungsjahren und diese Variabilität konnte teilweise auf Änderungen im Androgen - und Glukokortikoidhaushalt der Männchen zurückgeführt werden. Im Gegensatz zu den Vorhersagen des Immunokompetenzmodells (Folstad und Karter 1992), das von einer immunsuppressiven Wirkung von Androgenen ausgeht, weisen die Ergebnisse dieser Arbeit darauf hin, dass Androgene und Glukokortikoide vermutlich eine immunfördernde Funktion haben, die zu erniedrigtem Parasitenbefall führt. Zum besseren Verständnis der Interaktionen zwischen Primatenwirten und Parasiten schlage ich vor, dass in zukünftigen Modellen zwar ein ähnlich umfangreicher Ansatz gewählt wird, wie er hier vorgestellt wurde; allerdings würde die Integration von zusätzlichen Faktoren wie z.B. Körperverfassung oder Ernährungszustand des Wirtstieres, aber auch Information zur Populationsdynamik der verschiedenen Parasitenarten in zukünftigen Modellen das Verständnis der komplexen Parasit-Wirt Beziehungen in natürlichen Populationen komplimentieren.

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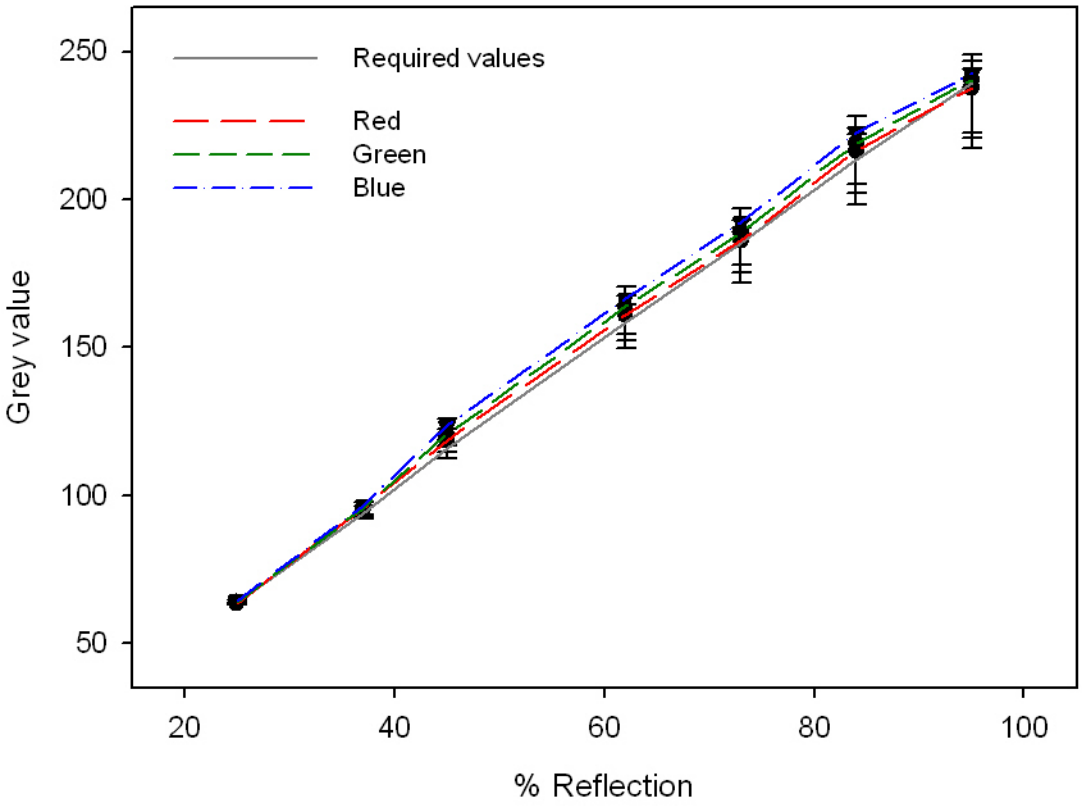
Appendix

	Response	Mean	Variance	Minimum	Maximum	P(norm.)	Skewness
a)	FEC_nem	175.53	38030.63	0	1525	<0.001	2.81
	FEC_pro	1079.95	4931242.2	0	20900	<0.001	6.08
b)	Sqrt (FEC nem)	11.34	47.15	-	-	0.269	0.52
	Log (FEC_pro +1)	2.67	0.35	-	-	0.248	-0.83

Appendix 1. Dispersion of the two response variables FEC_nem (nematode eggs/g) and FEC_pro (protozoa cysts/g) a) before and b) after square-root (nematodes) and logarithmic (protozoa) transformation, respectively. P(norm.): Probability that the distribution was significantly different from the normal distribution. After transformation, distributions of response variables were no longer different from the normal distribution.



Appendix 2. Greyscale values of a set of calibrated colour images show a close fit to the required values in all three colour channels (red, green, blue).

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Curriculum vitae

Personal information

Name	Dagmar Clough (née Lorch)
Date of birth:	05.03.1977
Place of birth:	Esslingen am Neckar, Germany
Citizenship:	German
Marital status:	married to Yann Clough

Educational history

2005-2009	PhD studies at the Dept. of Behavioral Ecology and Sociobiology, German Primate Centre and Dept. Sociobiology and Anthropology; University of Göttingen under the supervision of Profs. Peter Kappeler, Eckhard Heymann and Matthias Schaefer
2004	Degree in Biology (Diplom) at the Friedrich Schiller University, Jena
2003 - 2004	Diploma project at the Friedrich Schiller University, Jena, in cooperation with the Australian National University, Canberra, under supervision of Prof. Stefan Halle and Dr. Diana Fisher: "Sex-specific variation in ectoparasite infestation and diversity on the Brown Antechinus, *Antechinus stuartii*
2000 - 2003	Graduate biology studies at the Friedrich-Schiller University of Jena, Germany
1999 - 2000	Graduate biology studies at University of Aberdeen, UK
1997 - 1999	Undergraduate biology studies at the Eberhard-Karls University, Tübingen, Germany
1996	Completion of secondary education at Schloßgymnasium Kirchheim/Teck

Publications

Clough D. Gastro-intestinal parasites of red-fronted lemurs at Kirindy Forest, western Madagascar. Submitted to *Journal of Parasitology*

Clough D, Kappeler PM, Walter L. Susceptibility to helminth infections is associated with an *IL4* promoter polymorphism in wild red-fronted lemurs. Submitted to *Molecular Ecology*

Clough D. Heistermann M, Kappeler, PM. Host intrinsic determinants and potential consequences of parasite infection in free-ranging red-fronted lemurs (*Eulemur fulvus rufu*s). Prepared for submission to *American Journal of Physical Anthropology*

Schwitzer N, **Clough D**, Zahner H, Kaumanns W, Kappeler P, and Schwitzer C. Parasite prevalence in blue-eyed black lemurs (*Eulemur flavifrons*) in differently degraded forest fragments. Submitted to *Endangered Species Research*

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Port M, **Clough D**, Kappeler PM (2008): Market effects offset the reciprocation of grooming in free-ranging redfronted lemurs, *Eulemur fulvus rufus.* Animal Behaviour 77, 29-36

Lorch D, Fisher DO, Spratt M (2007): Variation in ectoparasite infestation on the brown antechinus, *Antechinus stuartii*, with regard to host, habitat and environmental parameters. Australian Journal of Zoology, 55, 169-176

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Desweiteren erkläre ich, dass ich mich nicht anderweitig einer Doktorarbeit ohne Erfolg unterzogen habe und dass diese Arbeit in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen hat.

Die Publikationen, wie sie in den Kapiteln 2-5 repliziert sind, wurden von mir selbst verfasst. Peter Kappeler leitete alle Arbeiten als Dissertationsbetreuer an. Lutz Walter entwickelte die Idee der Untersuchung zu *IL4*-Polymorphismen und Michael Heistermann betreute die endokrinologischen Arbeiten. Alle Koautoren wirkten bei der Finalisierung der Manuskripte mit.

Göttingen, den 20.Juli 2009

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Dagmar Clough