SEX-SPECIFIC AGING

SEX DIFFERENCES IN SURVIVAL AND HEALTH IN A WILD PRIMATE POPULATION

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SUMMARY

Ageing influences the functioning of most living organisms in myriad ways and has profound consequences for their reproductive fitness and survival. Age-related changes in the functioning of an organism are thought to result from the weakened selection against deleterious mutations acting after the peak reproductive age, and selection optimizing resource allocation to reproduction versus self-maintenance. Consequently, life history theory predicts that the rate of senescence should be reduced and the age of onset of senescence delayed with increasing lifespan. A high risk of mortality from extrinsic causes should lead to earlier or faster senescence. Extensions of this theory lead to the prediction that the shorter-lived sex (e.g. males in most mammals) should show earlier senescence. Furthermore, the sex-specific selective pressures for lifespan and reproductive rate might be expected to lead to a preferential maintenance of traits that optimize immune function and self-maintenance in females, whereas male fitness may be better enhanced by a prolonged investment in competitive ability. Senescent declines might thus be expected to show trait- and sex-specific patterns. However, beyond comparative studies of age-related mortality rates, these predictions remain largely untested under natural conditions.

The aim of this thesis was to test these elemental predictions by studying sex-specific functional senescence (within-individual deterioration in physiological or physical functioning) in a sexually monomorphic, small-bodied primate species (gray mouse lemur, *Microcebus murinus*) that experiences a high risk of extrinsic mortality in its natural environment. To gain an understanding of the prevalence of senescence across traits, and how this is influenced by selective mortality of individuals in poor condition, I examined sex-specific age trajectories in four different components of health that together broadly indicate the overall functioning of the individual and its ability to cope with environmental demands: body mass, physical strength, gastrointestinal parasite burden and allostatic load (baseline glucocorticoid level). To estimate the significance of selective disappearance and for quantifying body mass senescence, I used long-term data (10-18 generations) from a captive breeding colony in Brunoy, France, and two wild populations in Kirindy, western Madagascar. I collected grip strength measurements and fecal samples over two dry and two rainy seasons from a long-term monitored, individually marked wild gray mouse

lemur population. To compare the patterns of senescence between wild and captive animals, I additionally measured grip strength of captive animals.

In the first study, selective disappearance of individuals with declining body mass was found in both, the captive and wild setting, whereas a senescent decline in the ability to regain lost body mass was found only in captivity. Some evidence was found that mortality in the wild was more condition-dependent in females than in males, and an intriguing reversal of the sex-bias in lifespan was detailed between the settings: females live longer than males in nature but males outlive females in captivity. This study also confirmed an overall female advantage in body mass, despite sex-specific, seasonal body mass fluctuation in the wild. In the second study, no sex differences were found in the age trajectories of hand grip strength, an indicator of physical functioning, in either the captive or wild setting. Contrary to other species that invariably report stronger males than females, females were equally strong or stronger than males in the gray mouse lemur. To test the prediction that immune function might decline at old age, with more pronounced declines expected in males, in the third study I estimated the age trajectories of gastrointestinal parasite burdens (parasite prevalence and morphospecies richness quantified via fecal egg counts) as a proxy of overall health in the wild population. Contrary to these predictions, parasite burden declined with age similarly in both sexes, possibly due to acquired immunity and selective mortality of individuals with impaired resistance to parasites. However, males initially experienced a higher parasite burden. The fourth study was motivated by the finding that in other species, a high allostatic load follows from senescent deterioration in the negative feedback regulation of glucocorticoids, and has detrimental consequences for health and fitness. After validating an assay to quantify glucocorticoid levels from gray mouse lemur feces, I tested the prediction that aged animals would experience a high allostatic load. The prediction was met only in aged females in the dry season, perhaps indicating a reduced ability of the aged animals to cope with the energetic demands that coincide with the ecological dry season.

In summary, I evidenced highly variable sex-, season-, and setting-specific patterns of senescence in the gray mouse lemur, with substantial differences in aging between the components of health measured. While senescent declines were observed in some parameters, an absence of senescence and even negative senescence were found in others. Body mass and parasite resistance were best preserved until old age, likely reflecting the importance of these indicators for survival and the associated strong, positive selective pressures for their maintenance. Contrary to the predictions of life history theory, wild males experienced generally a similar magnitude or less pronounced functional senescence relative to females. This implies that despite their early mortality, males do not experience earlier or faster

senescence in the included components of health, perhaps due to strong selective pressures for a "robust" male phenotype required for improved male fitness. However, age-related changes in further components of functioning, including reproductive success, should be examined in both sexes to confirm these patterns. In conclusion, the results of this thesis do not support the fundamental predictions of an earlier onset or higher rate of senescence in the wild population that experiences high extrinsic mortality risk, or of an earlier senescence in the shorter-lived sex. The rapid, selective mortality of individuals in a declining condition and the associated positive selection for better self-maintenance may counteract the selective pressures that act to reduce self-maintenance beyond the prime reproductive age. The fitness-enhancing traits and the significance of selective mortality may differ across species and between the sexes. Further investigation into sex-specific, multi-trait senescence across taxa can shed further light on mechanisms of lifespan determination and the patterns of senescence observed across the animal kingdom, including humans.

ZUSAMMENFASSUNG

Alterungsprozesse beeinflussen die Funktionsfähigkeit fast aller lebenden Organismen auf vielfältige Weise und folglich ihre reproduktive Fitness und Überlebenschancen. Es wird davon ausgegangen, dass altersabhängige Veränderungen der Funktionsfähigkeit durch die abgeschwächte Selektion gegen schädliche Mutationen nach dem Erreichen des Alters der höchsten Reproduktionsfähigkeit, sowie durch die Selektion einer optimalen Ressourcenallokation auf Reproduktion versus Selbsterhaltung (d.h. Überleben) verursacht werden. Infolgedessen sagt die *life history* Theorie voraus, dass sich, mit zunehmender Lebenserwartung, die Alterungsrate verlangsamt und der Beginn von Alterungsprozessen verzögert. Ein hohes Mortalitätsrisiko basierend auf extrinsischen Ursachen sollte dagegen zu einer früheren und schnelleren Seneszenz führen. Erweiterungen dieser Theorie führen zu der Vorhersage, dass die Seneszenz beim Geschlecht mit der kürzeren Lebenserwartung (z.B. das männliche bei den meisten Säugetieren) früher eintreten sollte. Zudem legen sie die Erwartung nahe, dass der geschlechtsspezifische Selektionsdruck auf Lebensdauer und Reproduktionsrate bei Weibchen zu einer bevorzugten Erhaltung von Merkmalen führen sollte, welche die Immunfunktion und die Selbsterhaltung optimieren, während die Fitness bei Männchen eher durch eine verstärkte Investition in die Wettbewerbsfähigkeit erhöht werden sollte. Der altersabhängige Verfall sollte daher merkmals- und geschlechtsspezifische Muster zeigen. Bis auf komparative Studien zu altersabhängigen Mortalitätsraten, sind diese Vorhersagen jedoch unter natürlichen Bedingungen weithin unerforscht.

Ziel dieser Arbeit war es diese grundlegenden Vorhersagen durch Untersuchungen zur geschlechtsspezifischen funktionalen Seneszenz (d.h. den individuellen Verfall in der physiologischen order physischen Funktionsfähigkeit) an einer sexuell monomorphen kleinen Primatenart (dem Grauen Mausmaki *Microcebus murinus*), die in ihrer natürlichen Umgebung einem hohen extrinsischen Mortalitätsrisiko ausgesetzt ist, zu überprüfen. Zum besseren Verständnis der Prävalenz von Seneszenz-Erscheinungen über Merkmale hinweg, und wie diese durch die selektive Mortalität von Individuen in schlechter Kondition beeinflusst wird, habe ich die geschlechtsspezifischen Alterstrajektorien von vier verschiedenen Gesundheitskomponenten untersucht, die gemeinsam einen weitreichenden Aufschluss über die allgemeine Funktionsfähigkeit eines Individuums geben, sowie über dessen Fähigkeit

Anforderungen seiner Umwelt zu bewältigen: Körpergewicht, physische Kraft, gastrointestinale Parasitenbelastung, sowie die allostatische Belastung (basales Glukokortikoidniveau). Um die Bedeutung der selektiven Mortalität abzuschätzen, sowie um die altersabhängige Abnahme des Körpergewichts zu quantifizieren, habe ich Langzeitdaten (10-18 Generationen) einer Gefangenschaftspopulation in Brunoy (Frankreich) und zweier freilebender Populationen in Kirindy, westliches Madagaskar, verwendet. Griffstärke-Messungen und Kotproben habe ich während zweier Trocken- und Regenzeiten von einer langzeitüberwachten, individuell markierten Freilandpopulation des Grauen Mausmakis gesammelt. Um die Alterungsmuster zwischen freilebenden und gefangenen Tieren vergleichen zu können, habe ich darüber hinaus Griffstärkemessungen an Tieren in Gefangenschaft durchgeführt.

In der ersten Studie konnte ein selektives Verschwinden von Individuen mit abnehmendem Körpergewicht sowohl in Gefangenschaft als auch im Freiland nachgewiesen werden, während eine altersabhängige Abnahme der Fähigkeit verlorenes Gewicht wieder zu gewinnen nur in Gefangenschaft beobachtet wurde. Es gab Hinweise darauf, dass die Mortalität der Weibchen im Freiland stärker konditionsabhängig war als die der Männchen, und es fand sich eine interessante Umkehrung des Geschlechtsunterschieds in der Lebensdauer in Abhängigkeit von der Umwelt: Im Freiland lebten die Weibchen länger als die Männchen, während in Gefangenschaft die Männchen die Weibchen überlebten. Diese Studie konnte zudem den Gesamt-Vorteil der Weibchen bezüglich des Körpergewichts, trotz geschlechtsspezifischer saisonaler Fluktuationen im Körpergewicht, bestätigen. In der zweiten Studie konnte ein Geschlechtsunterschied in den Alterstrajektorien der Handgriffstärke, einem Indikator der physischen Funktionsfähigkeit, weder in Gefangenschaft noch im Freiland gefunden werden. Im Gegensatz zu Studien an anderen Arten, die ausnahmslos einen Stärkevorteil für Männchen dokumentieren, waren beim Grauen Mausmaki die Weibchen genauso stark oder stärker als die Männchen. Um die Vorhersage zu testen, dass die Immunfunktion mit zunehmenden Alter abnimmt, wobei eine deutlichere Abnahme bei Männchen erwartet wurde, habe ich in der dritten Studie Alterstrajektorien der gastrointestinalen Parasitenbelastung (Parasiten-Prävalenz und Morphospeciesreichtum, quantifiziert mittels fäkaler Eizahlen) als allgemeinem Gesundheitsindikator erhoben. Entgegen dieser Vorhersagen, nahm die Parasitenbelastung mit dem Alter in ähnlicher Weise bei beiden Geschlechtern ab, möglicherweise aufgrund erworbener Immunität und selektiver Mortalität von Individuen mit verminderter Parasitenabwehr. Die Männchen zeigten jedoch zunächst eine erhöhte Parasitenbelastung. Die vierte Studie beruht auf dem Befund, dass bei anderen Arten eine altersbedingte Verschlechterung der negativen Rückkopplungsregulation des Glukokortikoidniveaus zu einer hohen allostatischen Belastung führen kann, was wiederum nachteilige Konsequenzen für Gesundheit und

Fitness mit sich bringen kann. Nach der Validierung eines Assays zur Quantifizierung des Glukokortikoidniveaus aus Kotproben des Grauen Mausmakis, habe ich die Vorhersage getestet, dass ältere Tiere eine erhöhte allostatischen Belastung erfahren. Diese Vorhersage traf jedoch nur auf ältere Weibchen in der Trockenzeit zu, was eventuell auf eine reduzierte Fähigkeit den energetischen Herausforderungen der Trockenzeit gerecht zu werden hindeuten könnte.

Abschließend zusammengefasst, konnte ich stark variable geschlechts-, saison-, und umweltspezifische Seneszenz-Muster beim Grauen Mausmaki nachweisen, wobei sich deutliche Unterschiede in der Seneszenz zwischen den gemessenen Gesundheitskomponenten zeigten. Während altersbedingte Verschlechterungen in einigen der Parameter beobachtet wurden, zeigten andere keinerlei Seneszenz, oder sogar negative Seneszenz. Das Körpergewicht und die Resistenz gegen Parasiten wurden bis ins hohe Alter aufrechterhalten, was vermutlich die Bedeutung dieser Indikatoren fürs Überleben und den damit assoziierten starken Selektionsdruck wiederspiegelt. Im Gegensatz zu den Vorhersagen der *life history* Theorie, zeigten die Männchen im Allgemeinen eine funktionale Seneszenz ähnlicher oder sogar geringerer Größenordnung relativ zu Weibchen. Dies impliziert, dass die Männchen, trotz der frühen Mortalität, keiner besonders früh einsetzenden oder raschen Seneszenz in den untersuchten Gesundheitskomponenten unterliegen, was auf einem starken Selektionsdruck auf einen „robusten" männlichen Phänotyp beruhen könnte. Um diese Muster zu bestätigen, sollten jedoch altersabhängige Veränderungen von weiteren funktionalen Komponenten, einschließlich des Reproduktionserfolgs, in beiden Geschlechtern untersucht werden. Abschließend bleibt festzuhalten, dass die Ergebnisse dieser Arbeit weder die fundamentale Vorhersage eines früheren Beginns oder einer höheren Rate der Seneszenz, noch die einer früheren Seneszenz des kurzlebigeren Geschlechts für eine freilebende Population mit hoher extrinsischer Mortalität unterstützen. Die rasche, selektive Mortalität von Individuen in einer abnehmenden Kondition, und die damit assoziierte positive Selektion auf eine bessere Selbsterhaltung könnte dem Selektionsdruck, der eine Selbsterhaltung nach dem produktivsten Alter reduziert, entgegenwirken. Die Fitnessfördernden Merkmale, und die Bedeutung der selektiven Mortalität könnten sich über Arten hinweg und zwischen den Geschlechtern unterscheiden. Um die Mechanismen zu beleuchten, die der Bestimmung der Lebensdauer und der Seneszenz über das Tierreich hinweg, einschließlich des Menschen, unterliegen, sind Untersuchungen zur geschlechtsspezifischen Seneszenz vieler Merkmale über Taxa hinweg nötig.

CHAPTER 1

GENERAL INTRODUCTION

All the world's a stage,
And all the men and women merely players:
They have their exits and their entrances;
And one man in his time plays many parts,
His acts being seven ages. At first the infant,
Mewling and puking in the nurse's arms.
And then the whining school-boy, with his satchel
And shining morning face, creeping like snail
Unwillingly to school. And then the lover,
Sighing like furnace, with a woeful ballad
Made to his mistress' eyebrow. Then a soldier,
Full of strange oaths and bearded like the pard,
Jealous in honour, sudden and quick in quarrel,
Seeking the bubble reputation
Even in the cannon's mouth. And then the justice,
In fair round belly with good capon lined,
With eyes severe and beard of formal cut,
Full of wise saws and modern instances;
And so he plays his part. The sixth age shifts
Into the lean and slipper'd pantaloon,
With spectacles on nose and pouch on side,
His youthful hose, well saved, a world too wide
For his shrunk shank; and his big manly voice,
Turning again toward childish treble, pipes
And whistles in his sound. Last scene of all,
That ends this strange eventful history,
Is second childishness and mere oblivion,
Sans teeth, sans eyes, sans taste, sans everything.

- William Shakespeare (As You Like It)

It is a fact of life that advancing age entails declines in the functioning of our bodies. In consequence of the wear and tear sustained by the body over the years, these age-related alterations tend to hamper physical and cognitive performance after the peak reproductive age has been passed. In addition to their effects on the individual, these changes have broad implications for modern human societies, in which average lifespan continues to increase and with it, the proportion of the society experiencing ailments of old age. The inescapability of old age (given a long lifespan) and its unwanted side effects have provoked a plethora of research aimed chiefly at diminishing old age decrepitude [Christensen et al. 2009]. Only quite recently has interest arisen in aging as a phenomenon, beyond its effects on humans. In fact, senescent decline was long considered a luxury of the protected anthropogenic environment reserved for humans and animals in their keep, whereas it was presumed that under natural conditions, any deterioration would quickly lead to death by environmental causes [Hayflick 2000]. A surge of research has followed the realization that actuarial (i.e. demographic senescence, the increasing risk of mortality with advancing age) as well as functional senescence (within-individual deterioration of physical or physiological functioning with advancing age) may be more prevalent also in natural populations than previously assumed [Nussey et al. 2013].

Despite these recent advances, the study of aging in the wild – including its ultimate causes, manifestations and consequences, variability among individuals and species, and the shaping of senescence by biological and environmental variables – is in its infancy. In this thesis, I will contribute to its maturation by exploring an intriguing, unresolved question from a life history perspective: how and why do the sexes differ in the way they age? To begin, I will summarize some key aspects of the study of senescence, including how and why senescent declines in functioning occur, whom they affect and with what consequences. In doing so, I identify several open questions regarding the variability found in senescent processes, especially between the sexes and across varying environments. I will also introduce the study species used in this thesis, the gray mouse lemur (*Microcebus murinus*), and illustrate its suitability for the study of functional senescence. Finally, I will outline the specific aims of this thesis and introduce the four distinct studies undertaken to achieve those aims.

WHY DO WE AGE?

It is thought that senescent deterioration ultimately occurs because natural selection is inefficient at pruning out maladaptive mutations that take effect only after the individual has already had an opportunity to pass on its genes [Medawar 1952; Williams 1957]. Although evidence has been found to support this idea, the exact genetic basis of the physiological changes associated with old age still remains unclear [Hughes et al. 2002; Kirkwood 2005; Moorad and Promislow 2009]. However, consistent support is found for a more general explanation offered for senescent declines in functioning, the *disposable soma* theory [Kirkwood 1977; Kirkwood 2002; Kirkwood 2005]. This theory is based on the

assumption of a limited pool of resources available for self-maintenance (i.e. somatic maintenance, prevention and repair of cellular damage) as well as all other functions, including growth and reproduction. Trade-offs that necessarily arise between self-maintenance and these other functions concomitantly shape the rate of aging [Kirkwood and Austad 2000; Lemaître et al. 2014; Selman et al. 2012; Westendorp and Kirkwood 1998].

The efficiency of self-maintenance is therefore at the core of senescent declines, and several molecular level mechanisms (e.g. the accumulation of reactive oxygen species [Selman et al. 2012] and the shortening of telomeres (repetitive nucleotide sequences at the ends of chromosomes) [Klapper et al. 2001; Monaghan 2014]) have been identified as potential proximate causes of age-related functional deterioration. In the absence of sufficient prevention and repair mechanisms (e.g. DNA repair, anti-oxidant action and telomerase activity), these molecular changes lead to cellular damage [Kirkwood 2011] that in turn can bring on changes in physiological processes, tissue composition and functional performance at old age. The resulting morbidity is reflected in aging-related diseases, disability and frailty and, in consequence, actuarial senescence in humans and the vast majority of non-human animal species [Gaillard 1994; Jones et al. 2014; Ricklefs 1998].

HOW VARIABLE IS SENESCENCE?

Interspecific patters of aging: the pace of life

Despite the conserved molecular mechanisms of senescence [Partridge and Gems 2002], immense variability exists in the rate of aging across species [Jones et al. 2014] and within species [Yang and Lee 2010]. The species-level variance has been attributed to variation in e.g. body size [Ricklefs 2010], mating systems and reproductive schedules [Ricklefs 2010], environment and means of escaping extrinsic hazard, such as flight [Healy et al. 2014], armor or weaponry ([Ricklefs 1998], but see [Moorad and Promislow 2010]). Trade-offs between reproduction and individual survival via self-maintenance are optimized differently across taxa with varying life histories [Jones et al. 2008].

A major determinant of the rate of aging is thought to be the level of extrinsic hazard [Ricklefs 1998; Williams et al. 2006]. In species that experience high extrinsic mortality risk (death by environmental causes, such as pathogens, predation or natural disasters), heavy investment into early reproduction would be expected, since the probability of survival to the next reproductive opportunity may be low [Jones et al. 2008] The resulting “fast pace of life”, or an “r-selected” life history [Austad 1997; Pianka 1970] more likely leads to reduced investment in somatic maintenance past reproductive maturity, leading to rapid senescent decline after sexual maturity is reached. In longer-lived animals with a slow pace of life (“K-selected” species), on the other hand, higher investment in somatic maintenance would be expected because lifetime reproductive success tends to accumulate over several reproductive events and may peak later in life. Senescence in fitness-promoting traits should consequently be delayed due to

a higher life expectancy and the potential for continued fitness gains [Jones et al. 2008] and, in some species, increasing reproductive success with age [Graves 2007].

Longitudinal data tends to be available predominantly for relatively large-bodied animals with slow life histories and consequently, such species are best represented in studies of within-individual senescence (ungulates [Nussey et al. 2011; Toïgo et al. 2006], sea birds [Pardo et al. 2013a]; seals [Hindle et al. 2009b]; baboons [Alberts et al. 2014; Altmann et al. 2010]; marmots [Tafani et al. 2013]). Still, a few studies have also been able to demonstrate senescence in natural populations of short-lived organisms, such as insects (antler flies [Bonduriansky and Brassil 2002]) and small fish (guppies [Reznick et al. 1990; Reznick et al. 2004]) and passerines (great tit [Bouwhuis et al. 2012]). Due to the emphasis on species from the slow end of the pace-of-life continuum with their relatively low risk of extrinsic mortality, it seems likely that the emerging patterns of senescence are biased towards overestimating the proportion of lifespan in which investment in survival dominates over reproductive investment. The detection of senescence may also be more likely in such species owing to the longer absolute time spent in old age due to their long lifespan.

Aging under natural versus protected conditions

The manifestations of morbidity that link the molecular changes to mortality have mainly been addressed in humans and laboratory rodents [Austad 1997]. Laboratory studies on the mechanisms of senescence benefit from the reduced heterogeneity among individuals and easier detection of senescent changes induced by the reduced mortality rates under protected conditions. However, to ultimately assess the relative contributions of biological and environmental factors in the shaping of life histories, including the onset and rate of senescence, it is necessary to also examine senescence in natural populations. Since the majority of aging studies are done in protected environments, it is essential also to evaluate how well the patterns observed in captivity compare to natural populations, to determine how much of the decline seen in captivity reflects intrinsically biological versus behavioral variation, and the interactions of individual quality and the environment. Besides its evolution-theoretic interest, knowledge about the basis of variance in senescence rates is required for human interventions of age-related disease and functional declines. Moreover, the mechanisms of senescence observed in current human and captive animal populations have evolved in interaction with environmental pressures similar to those experienced by wild populations of animals, for which sufficient functioning is vital for fitness and survival. Therefore, to understand the links between molecular self-maintenance, morbidity and mortality and the individual heterogeneity in these processes, it is vital to focus more attention on functional aging in nature.

In addition to intrinsic mortality by illness or physiological failures, morbid individuals may more likely succumb to mortality by extrinsic hazards, a process termed “selective disappearance” of lower quality individuals, [Bouwhuis et al. 2009; Hayward et al. 2013; Nussey et al. 2011; van de Pol and Verhulst 2006]. Aging individuals may therefore face an elevated mortality risk if their coping with extrinsic

pressures is impaired by senescent declines in immunological, physical or cognitive functioning. If individual lifespan is influenced by intrinsic quality, senescent decline would indeed be difficult to observe in natural populations, as it may be impossible to infer to what extent the observed variation (particularly in cross-sectional data) in a given trait reflects the higher quality of the subset of animals alive. This issue is likely at the core of the earlier conclusions about an absence of senescent decline in wild animals [Hayflick 2000; Williams 1957].

By using careful study designs, longitudinal data and appropriate methods of statistical inference, a number of studies have nevertheless been able to demonstrate senescence in the physiological and physical functioning of wild animals [Nussey et al. 2013]. However, the rates of senescence vary across traits [Hayward et al. 2013; Nussey et al. 2009; Walker and Herndon 2010] and senescent loss of function seems to be absent in some of the measured traits (e.g. [Gonzalez et al. 2012; Rueppell et al. 2007]). Due to the limited range of taxa studied thus far, and the paucity of studies measuring senescence in multiple traits, it is still unclear which aspects of the functioning of the organism are most affected and how environmental conditions and life history variation influence the detectability and rates of functional senescence. However, the fact that different rates of senescence exist across the traits measured might indicate that selective pressures to maintain functioning are stronger for some features than others, but little attention has been paid to this possibility [Bouwhuis et al. 2012; Burger and Promislow 2006]. The impact of the level of extrinsic mortality on senescence has been investigated using experimental manipulations and comparison of life history characteristics in different natural environments [Reznick et al. 1990; Reznick et al. 2004]. A comparison of captive and wild populations could be used to complement these approaches [Bronikowski et al. 2002; Lemaître et al. 2013; Magalhães et al. 2007; Promislow 1991; Ricklefs and Scheuerlein 2001], but this approach has thus far not been taken to assess how the environment shapes functional senescence.

Sex-specific patterns of morbidity and mortality

Differences in life history variation and aging rates also occur within species [Stearns 1992]. One of the key differences is the frequently observed sex-bias in longevity: in mammals, females tend to live longer than males [Bonduriansky et al. 2008], whereas in birds, the opposite is often (but not universally) true [Liker and Székely 2005]. Ultimately, sex differences in lifespan and senescence have been chiefly attributed to the differing strength of sexual selection on males and females [Clutton-Brock and Isvaran 2007; Liker and Székely 2005; Promislow 1992] and the consequent sex-specific selective pressures that lead to life history differences. Males, particularly in species with polygamous mating systems, attain higher lifetime fitness by investing heavily in reproduction in their peak reproductive age [Clutton-Brock and Isvaran 2007], whereas the fitness gains for females are more evenly distributed over successive reproductive events over their lifespan [Clutton-Brock and Isvaran 2007; Trivers 1972]. Following Bateman's principle of sex-specific reproductive variance [Bateman 1948], mammalian females (or, the sex that benefits from a prolonged reproductive lifespan) should live longer [Bonduriansky et al. 2008]

and experience delayed and/or less rapid senescence relative to males [Clutton-Brock and Isvaran 2007; Maklakov and Lummaa 2013; Williams 1957]. Based on the different reproductive strategies of the sexes, it might also be expected that different traits are preferentially preserved in the sexes over the lifespan: males would potentially benefit more from better competitive ability in peak reproductive age, including larger body, weapon or ornament size and stamina, whereas female success might be increased by investing in a longer healthy lifespan, predator avoidance and offspring care.

Proposed proximate causes for the sex differences include the effects of sex chromosomes and steroid hormones [Austad 2006]. The heterogametic sex hypothesis proposes that the heterogametic sex (e.g. male mammals, female birds) will suffer a disadvantage if slightly deleterious alleles are present on the shared chromosome, e.g. X in mammals. This follows from the compensatory capacity of the second copy of the chromosome in the homogametic sex that allows preferential expression of the better allele [Liker and Székely 2005], leading to prolonged survival of cells containing more beneficial alleles and, potentially, longer telomeres [Barrett and Richardson 2011]. Indeed, telomere length tends to decline faster in males than in females [Barrett and Richardson 2011]. The endocrinological explanation relies mainly on the stimulatory effects of estrogen and suppressive effects of testosterone on immune system functioning [Alexander and Stimson 1988; Baeza et al. 2011; Furman et al. 2014; Klein 2000], but see [Roberts et al. 2004]. Moreover, behavioral differences may explain part of the longevity bias: in humans, risky behaviors, such as substance abuse [Brady and Randall 1999] and sensation seeking [Rosenblitt et al. 2001] show male bias and a large proportion of the excess male mortality is explained by accidental or violent deaths [Owens 2002]. Similarly in other mammals, risky behaviors such as fighting [Trivers 1972] as well as dispersal [Greenwood 1980; Lawson Handley and Perrin 2007] are typically male biased and may contribute to the sex gap in longevity.

Empirical evidence for sex differences in functional senescence comes mainly from human studies [Oksuzyan et al. 2010; Oksuzyan et al. 2008], in which a seemingly contradictory pattern has been observed where “men die and women suffer” [Oksuzyan et al. 2010]. Despite their longer average life expectancy, women nevertheless succumb to frailty and disability [Kulminski et al. 2007], report worse health in surveys, seek medical attention and suffer from non-fatal conditions and disability more frequently than males [Oksuzyan et al. 2008]. Meanwhile, men retain better functioning throughout life and outperform women in measures of functioning positively associated with the projected life expectancy (e.g. handgrip strength, [Frederiksen et al. 2006]). While both, the average life expectancy and health expectancy (the proportion of lifespan without debilitating illness or disability) have increased over the past decades, the relative health expectancy continues to be lower for women than for men [Cambois et al. 2008; Jeune and Brønnum-Hansen 2008]. This phenomenon has been termed the male-female health-survival paradox (e.g. [Oksuzyan et al. 2009]). These paradoxical patterns have thus far not been fully resolved and it is unclear to what extent biological, social and behavioral factors cause the observed age-related changes in components of health in modern humans [Oksuzyan et al. 2010].

One possible solution to the paradox could be the definition of health used, since different aspects of immune function, somatic maintenance and physical functioning might be optimized differently by the sexes. For instance, while disability and frailty are common in elderly women, a strong body of evidence suggests a female advantage in immune system functioning in humans and other animals [Klein 2000; Nunn et al. 2009]. Males tend to have lower responses to general antigenic challenges, are more susceptible to infections by many parasites and non-lethal pathogens [Alexander and Stimson 1988; Klein 2000; Zuk and McKean 1996], and incur higher mortality from parasitic and infectious disease relative to females in humans [Owens 2002] and non-human animals [Klein 2004; Moore and Wilson 2002]. There is also some evidence that male mammals experience a faster rate of immunosenescence (age-related declines in immune function) than females (e.g. human: [Caruso et al. 2013; Goetzl et al. 2010; Wikby et al. 2008; Yan et al. 2010], macaque: [Zheng et al. 2014], rat: [De la Fuente et al. 2004]). The definition of health and the relative contributions of different components of health – acute illness and physiological and physical functioning – to mortality risk might therefore partly underlie the paradox. Surprisingly, this possibility has so far been largely overlooked [Alberts et al. 2014; Caruso et al. 2013]. An apparent health-mortality paradox might also be partially produced by sex differences in socioeconomic status, lifestyles and study participation as well as the accuracy of quantifying self-reported health [Oksuzyan et al. 2010; Oksuzyan et al. 2009].

Due to the potential confounding problems associated with human studies, our understanding of the health-survival paradox might be advanced by an assessment of sex-specific patterns in the senescence of different components of health, functioning and lifespan also in non-human species. In particular, it would be necessary to address the sex-specific selective pressures under which the observed patterns of senescence have evolved under natural conditions. Thus far few studies of wild animals have examined sex-specific senescence beyond mortality estimates (birds: [Pardo et al. 2013b; Saino et al. 2003], semi-captive chimpanzees: [Obanda et al. 2014], Soay sheep: [Hayward et al. 2009], bats: [Greiner et al. , 2014], marmots: [Tafani et al. , 2013]). However, with the exception of one comprehensive account on health and mortality in yellow baboons [Alberts et al. 2014], no study has explicitly addressed evidence for the paradox in animals. The existing studies on non-human mammals have found little evidence of the paradoxical patterns described in humans: rather, the shorter-lived males quite consistently experience earlier declines in health and fitness.

Indicators of functional senescence

Part of the variation in patterns of senescence between species and individuals may be a consequence of the variety of indicators of senescence used across studies. Hence it has been recommended that multiple traits be measured concomitantly to infer senescence [Nussey et al. 2009]. The variability in patterns of senescence across different parameters can provide insights into the significance of specific traits, their preservation via selective pressures and potential trade-offs involved. In addition to mortality rates [Ricklefs 1998; Ricklefs and Scheuerlein 2001], the traits related to reproductive performance are

often assessed because of their direct contribution to fitness outcomes [Hayward et al. 2013; Nussey et al. 2009]. Studies of non-reproductive measures of functional performance are also beginning to accumulate. These measures of functioning, e.g. maintenance of body composition and condition, stress resistance, immune function, sensory, physical and cognitive aptitude reflect the capacity of the individual to acquire and allocate resources, to defend resources and escape predation and other extrinsic challenges. Therefore, they indicate whole-organism performance that may experience senescence and directly indicate investment in somatic maintenance.

STUDY SPECIES: THE GRAY MOUSE LEMUR

The species chosen for this study, the gray mouse lemur (*Microcebus murinus,* Cheirogaleidae), is a small-bodied (ca. 60 g), strepsirrhine primate native to the forests of western and southern Madagascar. It has been established as a primate model of aging in captivity, and age-related declines have been found in several aspects of individual performance in captivity, many of which parallel human aging [Languille et al. 2012]. A particular advantage of the species is its short average lifespan for a primate (average: 2-3 years in the wild, 5-6 years in captivity) and onset of aging at 4-5 years (in captive males [Languille et al. 2012]), although their potential lifespan is substantially longer (maximum: 10-11 years in the wild, 13 years in captivity). This allows for large numbers of animals to be followed into old age within a relatively short time window. No studies exist of aging in the species under natural conditions and sex differences in aging have thus far not been addressed.

Despite its short lifespan relative to other primates, the species' potential longevity is high relative to its body size [Harvey and Clutton-Brock 1985; Ross 1998] (compared e.g. to mouse, maximum lifespan 2-3 years). The substantial increase in average lifespan in captivity compared to the wild suggests strong extrinsic mortality as a likely cause of death. This observation is supported by the high turn-over rates [Kraus et al. 2008] and evidence of heavy predation pressure [Goodman et al. 1993] found in nature. These selective pressures are likely highly influential in shaping life history evolution in the species and would be expected to lead to an early onset of senescent decline in functioning. The immense variation in adult lifespan especially in the wild suggests substantial heterogeneity in individual quality and provides an opportunity to trace broad patterns of individual differences in functioning despite the high mortality rates.

The species is particularly interesting for the study of sex-specific aging because it is sexually monomorphic, with a slight female size advantage, hence eradicating potential sex differences caused merely by a male-bias in body size that is typical for mammals. However, a clear female-bias in longevity is found in the wild [Kraus et al. 2008], whereas in captivity the sex bias is neutral or even reversed to favor males [Languille et al. 2012; Perret 1997]. The mating system is polygamous and the mating season is restricted to a few weeks per year, when males in the wild roam in search of receptive females, actively defend females [Eberle and Kappeler 2004b] and have an elevated risk of mortality [Kraus et al. 2008].

Male reproductive success tends to be influenced by body size and success in male-male competition [Eberle and Kappeler 2004b; Gomez et al. 2012]. The species is solitary living, hence reducing the potentially confounding effects of social status in a group.

STUDY AIMS AND APPROACHES

In the previous paragraphs I have outlined the state of the art in the study of functional senescence and identified several lacunas in the empirical data that must be addressed to explicitly test predictions of life history theory. In particular, I have expressed a need for further examination of the patterns of functional senescence in multiple traits in wild populations, where the fitness consequences of sufficient functioning are likely greater than in captivity; on exploring the importance of selective mortality in influencing the patterns of senescence observed; and assessing sex differences in senescence of different components of health to address the male-female health-survival paradox in non-human species. In light of these gaps, the aim of this thesis was to describe sex-specific age trajectories of several indicators of physical and physiological functioning in a wild primate population that is subject to high levels of extrinsic mortality.

The specific questions I set out to answer were:

1. *How prevalent and how variable is senescence?* In particular, can evidence of functional senescence be found in a short-lived primate in nature despite high mortality rates, and are the patterns consistent across traits? To what extent does selective disappearance of the lowest quality individuals affect the observed patterns of senescence? To what extent does the environment influence the prevalence (or detectability) of senescence? Is the ”coping” of aged animals influenced by environmental conditions?
2. *How do patterns of senescence differ for the sexes?* Do the sexes differ in their rates of senescence, and do these patterns differ across the components of health? If so, are the patterns consistent with the male-female health-mortality paradox?

To answer these questions, I examined the age trajectories of four different components of health: body mass, muscle strength, endoparasite burden and allostatic load (baseline glucocorticoid hormone levels). These indicators were selected because they reflect different aspects of general health and can significantly affect general coping, fitness and survival of wild individuals. I collected data on these indicators from wild animals during two dry and two rainy seasons (total of 11 months, 2010-2012) in Kirindy forest, Madagascar. To assess the influence of the environment and extrinsic mortality on patterns of senescence and to relate the findings from the wild population to those found in captivity, I also collected comparative data from captive gray mouse lemurs at a breeding colony in Brunoy, France. I

additionally utilized long term data collected over 10-18 years from two non-overlapping wild populations [Fredsted et al. 2005] in Kirindy and from the captive colony in Brunoy to rigorously examine within-individual change in body mass and to determine the ages of all individuals.

Body mass is a result of both physiological and behavioral processes and broadly reflects the resources available for physiological functioning, and the ability of the individual to acquire those resources. Therefore, it is a powerful indicator of whole-organism performance that is affected by environmental demands, illness and individual quality. In **Chapter 2**, I examined patterns of senescent loss of body mass using long term data from one captive and two wild gray mouse lemur populations. By estimating seasonal, sex-specific age trajectories of body mass and condition-dependence of mortality based on longitudinal data in both settings, I evaluated evidence for senescence and selective disappearance of individuals and whether these patterns differ for the sexes. Sufficient body mass is likely important for both sexes, but due to the males' earlier mortality, earlier and steeper senescent declines would be expected in males at least in the wild population.

In **Chapter 3**, I focused on a direct indicator of physical functioning, grip strength. Handgrip strength is a widely used measure of overall skeletal muscle strength and physical functioning in humans and laboratory mice, and has been shown to reliably predict longevity in these species [Cooper et al. 2010; Fahlström et al. 2012]. It may also reflect coping with environmental challenges via its dependence on nutrition and the catabolic effects chronic stress (high allostatic load) can have on muscle tissue. I measured grip strength of male and female gray mouse lemurs in a wild population and for comparison, collected cross-sectional data from the captive population. I used body mass-corrected strength to evaluate the effects of sex and environment on age trajectories of physical functioning. I wanted to particularly test whether males were stronger throughout life as predicted both by the health-survival paradox as well as the hypothesis that males should invest in competitive ability, e.g. strength and body condition, more than into immune function to improve their reproductive success.

Parasite burden, albeit an imperfect proxy for the functioning of the immune system, is commonly used as an indicator of health in studies of wild populations. **Chapter 4** assesses the effects of sex and age on the prevalence and species richness of gastrointestinal parasites in a wild gray mouse lemur population to evaluate indirect support for sex-specific rates of immunosenescence in the species. Higher parasite burdens would be expected for males according to Bateman's principle [Rolff 2002].

Finally, glucocorticoids are a class of hormones responsible for resource reallocation and responses to changing environmental conditions and life history stages. Their role in regulating body mass fluctuations, body composition and immune function, among other processes, make them an interesting indicator of an individual's ability to orchestrate the available resources. Furthermore, they are a good indicator of coping, as chronic elevation in glucocorticoids indicates prolonged physiological stress to the system, and comes with various harmful effects on fitness, health, somatic maintenance and survival [Ferrari et al. 2001; Juster et al. 2010]. Glucocorticoid metabolism changes at old age in some species

[Sapolsky 1985; Sapolsky et al. 1986], and the parallel, cumulative effects of aging and chronic stress [Frolkis 1993; Veldhuis et al. 2013] can have severe adverse effects on individual health and fitness. Because fecal glucocorticoid metabolites have not been previously measured in gray mouse lemurs, the method was first thoroughly validated. To exclude the possibility that aged animals might have altered glucocorticoid profiles simply due to their higher cumulative capture experience, I also assessed whether repeated capturing had long term consequences on the glucocorticoid profiles of wild individuals **(Chapter 5.1).** I then addressed the coping of aging individuals with environmental pressures by an examination of age-related changes in stress hormone levels (**Chapter 5.2**). For this study, fecal samples were collected from wild gray mouse lemurs in two dry and two rainy seasons to test the hypothesis that aged animals might be less able to cope with harsh environmental conditions and therefore experience higher allostatic load in the dry season.

In summary, in this thesis I aim to expand on the current knowledge of aging in the wild, with a specific focus on how mammalian males and females function within their environment, and how the patterns of senescence vary among traits. Besides providing valuable information on multiple-trait senescence in a wild vertebrate, these data can be used to test fundamental predictions of life history theory [Stearns 1992] and contribute to the understanding of how sex-biased mortality is linked to sex differences in morbidity, and what these patterns reveal about the investment of males and females into self-maintenance.

CHAPTER 2

SENESCENCE OR SELECTIVE DISAPPEARANCE? AGE TRAJECTORIES OF BODY MASS IN WILD AND CAPTIVE POPULATIONS OF A SMALL-BODIED PRIMATE

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ABSTRACT

Classic theories of ageing consider extrinsic mortality (EM) a major factor in shaping longevity and ageing, yet most studies of functional ageing focus on species with low EM. This bias may cause overestimation of the influence of senescent declines in performance over condition-dependent mortality on demographic processes across taxa. To simultaneously investigate the roles of functional senescence (FS) and intrinsic, extrinsic and condition-dependent mortality in a species with a high predation risk in nature, we compared age trajectories of body mass (BM) in wild and captive grey mouse lemurs (*Microcebus murinus*) using longitudinal data (853 individuals followed through adulthood). We found evidence of non-random mortality in both settings. In captivity, the oldest animals showed senescence in their ability to regain lost BM, whereas no evidence of FS was found in the wild. Overall, captive animals lived longer, but a reversed sex bias in lifespan was observed between wild and captive populations. We suggest that even moderately condition-dependent EM may lead to negligible FS in the wild. While high EM may act to reduce the average lifespan, this evolutionary process may be counteracted by the increased fitness of the long-lived, high-quality individuals.

KEYWORDS

Functional senescence, body mass, condition-dependent mortality, life-history evolution, lifespan, sex difference

INTRODUCTION

Actuarial senescence (AS, the increased risk of mortality with advancing age) is a well-defined demographic process in the vast majority of species [Gaillard 1994; Jones et al. 2014; Promislow 1991; Ricklefs 1998]. The increase in mortality likely results from functional senescence (FS, within-individual deterioration of physical or physiological functioning with advancing age), which, along with terminal disease or investment in reproduction at the expense of maintenance [Clutton-Brock 1984; Hoffman et al. 2010; Isaac and Johnson 2005; Weladji et al. 2010], can expose individuals to extrinsic hazards in a condition-dependent manner. Therefore, only high quality individuals may survive to an age where FS takes effect, making aging difficult to observe in cross-sectional studies of natural populations [Bouwhuis et al. 2009; Hayward et al. 2013; Nussey et al. 2011; Nussey et al. 2008; van de Pol and Verhulst 2006].

Classic theories on life-history evolution [Monaghan et al. 2008] posit that populations with high extrinsic mortality (EM) rates (random mortality from environmental causes) should have a reduced lifespan and age rapidly, and support for this pattern has been found with experimental and comparative work [Austad 1993; Bronikowski et al. 2002; Reznick et al. 2004; Ricklefs 1998; Stearns et al. 2000]. In spite of the supposed significance of extrinsic factors in shaping life-histories, aging research is still largely biased

towards captive animals living under standard, benign conditions (e.g. [Languille et al. 2012; Stearns et al. 2000; Takeda et al. 1981]). In the wild, studies of FS have largely focused on long-lived, large-bodied animals that face relatively low levels of environmental hazard (ungulates [Nussey et al. 2011; Toïgo et al. 2006], sea-birds [Pardo et al. 2013a]; seals [Hindle et al. 2009a]; primates [Altmann et al. 2010; Hoffman et al. 2010]). Because FS is more detectable when EM is low, this taxonomic bias may lead to the overestimation of the prevalence or intensity of FS compared to the influences of selective disappearance in the wild across species. Hence, the study of wild populations with high EM risk is essential for testing hypotheses on the evolution of lifespan and FS. To assess how declining individual performance versus the earlier mortality of low quality individuals shape demographic processes and hence selection pressures, it is necessary to simultaneously estimate functional declines and selective disappearance in a population. So far, few studies have taken this approach [Bouwhuis et al. 2009; Hayward et al. 2013; Nussey et al. 2011], and thus the relative importance of these processes within populations and across taxa is largely unresolved.

The sexes often differ in their life-histories, EM hazard and aging processes [Altmann et al. 2010; Greiner et al. 2014; Jorgenson et al. 1997; Tafani et al. 2013], and female mammals typically enjoy longer lifespans than males [Bonduriansky et al. 2008]. Sex-specific life-history optimization and potential sexually antagonistic selection have been proposed as evolutionary mechanisms for the maintenance of life-history variation within a species [Maklakov and Lummaa 2013]. Therefore, a direct comparison of the sexes is essential for deciphering the evolutionary mechanisms behind senescence and lifespan determination.

In this study, we simultaneously assess the influences of intrinsic “background” mortality, extrinsic hazard and non-random mortality of the lower quality individuals in creating the observed patterns of FS and lifespan in a species that experiences high EM under natural conditions [Kraus et al. 2008]. We employ long-term body mass (BM) data from one captive and two wild populations (10-18 years covering 7-16 cohorts) of the gray mouse lemur (*Microcebus murinus*) to characterize variation in body condition. In mammals, including *M. murinus* [Castanet et al. 2004], body growth typically ceases around the age of sexual maturity [Karkach 2006] (6-8 months in *M. murinus* [Castanet et al. 2004; Némoz-Bertholet and Aujard 2003]). Thereafter, BM fluctuates in response to imbalances in energy acquisition and expenditure, cyclic seasonal changes [Dammhahn and Kappeler 2008; Murie and Boag 1984; Schmid and Kappeler 1998; Toïgo et al. 2006] or senescent muscle loss [Baumgartner 2000]. BM broadly reflects the resources available for allocation to physiological processes, making it a meaningful indicator of FS. We assess the influence of the environment on the rate of FS in the *M. murinus* by comparing patterns of BM decline under captive and wild conditions. Under the benign, captive conditions, any decline should be mainly owing to intrinsic rather than extrinsic causes. In wild animals these same intrinsic processes interact with the environmental hazards [Hayflick 2000; Kirkwood and Austad 2000; Promislow 1991] that likely remove low quality individuals rapidly from the population. This in combination with the modeling of condition-dependent survival permits an assessment of the relative importance of FS and selective disappearance in the species. While rates of AS have previously been found to be lowered in

captivity compared to natural populations [Bronikowski et al. 2002; Lemaître et al. 2013; Magalhães et al. 2007], this is, to our knowledge, the first study in which data on captive and wild populations spanning several generations have been juxtaposed for testing fundamental hypotheses of aging theory with respect to FS.

Given the high EM of *M. murinus* in the natural environment, we predicted average lifespan to be shorter in the wild than in captivity. If individuals are mainly removed from the population via random processes regardless of their condition, earlier or more dramatic FS might be expected in the wild [Bonduriansky et al. 2008]. If, however, extrinsic hazard selectively removes individuals in poor condition, evolutionary processes might instead lead to delayed FS or the selective survival of only the highest quality individuals (showing little senescent decline) to an old age. Assuming a weaker influence of condition on mortality in the predator and pathogen-protected captive colony, FS should in this case be more pronounced there. Consequently, aged animals in the wild should maintain a relatively high BM compared with captivity.

Condition-dependence of mortality is difficult to measure, particularly under natural conditions where the cause and exact timing of death are often unknown and fine-scale condition data difficult to obtain. Non-random mortality may act at different timescales: lifespan and body condition may be associated throughout life, or condition may decline shortly preceding death. While not perfect proof of condition-dependent EM (e.g. terminal declines in condition may indicate intrinsic causes, such as terminal illness), we expect selective disappearance to be indicated by prolonged survival of individuals in good body condition throughout life and by a terminal decline in BM. These intrinsic declines would probably render the individual more vulnerable to EM. As the same physiological processes presumably drive FS, senescent within-individual declines might nevertheless occur in both the wild and captivity. However, in presence of condition-dependent mortality, these declines should be relatively minor in the wild compared with captivity. In the wild, the coping of aged individuals may be especially compromised in the ecologically more demanding dry season when food and water availability decline considerably [Dammhahn and Kappeler 2008], which might lead to more severe BM senescence (i.e. age-related declines in body mass) during this period.

In the highly promiscuous mating system of *M. murinus* [Eberle and Kappeler 2004b; Eberle et al. 2007; Huchard et al. 2012], female reproductive skew is negligible [Eberle and Kappeler 2004a] and female lifetime fitness increases with lifespan. Longevity is slightly male-biased in our captive study population [Languille et al. 2012; Perret 1997] but strongly female-biased in the wild [Kraus et al. 2008]. Roaming by males during the mating season [Eberle and Kappeler 2004b] coincides with increased male mortality and, along with the age-associated increase in risk taking by males [Dammhahn 2012] likely drives the overall sex bias in longevity in the wild [Kraus et al. 2008]. Should this also induce male-biased selective disappearance, the wild males that survive to old age may be of exceptionally high quality, whereas the condition of long-lived captive males might be relatively low. Faster aging rates are predicted for the sex that experiences higher adult mortality [Bonduriansky et al. 2008], hence, we expected male BM to decline faster than that of females, especially in the wild.

METHODS

Study species

The gray mouse lemur is a small, sexually monomorphic primate that is emerging as a model species for aging [Languille et al. 2012]. For individuals that survive to adulthood (here, the second year of life), the average lifespan in captivity is approximately 5 years [Languille et al. 2012; Perret 1997] (maximum in our captive colony was 13.8 years) but only 2-3 years in the wild (lifespan of at least 10 years recorded in our study population). The annual turnover rate in nature is approximately 50% due to high levels of EM [Kraus et al. 2008] (mainly reptilian, avian and mammalian predation [Goodman et al. 1993]). BM shows cyclic annual fluctuation in response to changes in photoperiod [Perret and Aujard 2001] and resource availability [Dammhahn and Kappeler 2008]. Under natural conditions, individuals in sufficient condition utilize torpor to conserve energy during the dry season [Vuarin et al. 2013], whereas no spontaneous use of torpor is seen under usual captive conditions [Canale et al. 2011]. Data from captivity indicate the onset of FS at around age 5 years in males [Languille et al. 2012; Némoz-Bertholet and Aujard 2003; Perret 1997; Perret and Aujard 2001].

Body mass in the captive population

The captive colony in Brunoy, France, was set up nearly 40 years ago with *M. murinus* originating from Southwestern Madagascar. All individuals used for this study (258 individuals in 2000-2013) were born in captivity and were not involved in long-term experiments. We included only animals for which the entire lifespan was monitored, excluding transfers and animals that were still alive. Food and water was available *ad libitum* and the animals' BM was measured (precision ± 1 g) at least monthly throughout life, starting at weaning (age 4 months). Seasonality was induced with a change in photoperiod [Perret 1997] to promote physiological changes [Perret 1992]: the short day season (SDS; 6 months, light 10 out of 24 h) marks the onset of fattening, whereas the long day season (LDS; 6 months, light 14 out of 24 h) increases activity and initiates reproductive activity. Seasonal BM averages were calculated from 6-7 measurements (LDS/SDS) per individual per season for all analyses.

Body mass in the wild population

Gray mouse lemurs were studied in their natural habitat in Kirindy forest in central western Madagascar. The area experiences a distinct dry season in May - October (lean season, reproductive quiescence), and a rainy season in November - April (breeding and fattening), with associated changes in food availability [Dammhahn and Kappeler 2008]. Long term data were collected from two subpopulations inhabiting the study areas "N5" (2002-2012, n = 1144/159 (measurements/ individuals)) and "CS7" (1993-2011, n = 2324/436). The study sites are situated 3 km apart and the two subpopulations do not overlap [Fredsted et al. 2005].

Animals were captured with Sherman live traps (for details on capture and handling protocols see [Dammhahn and Kappeler 2008; Eberle and Kappeler 2004b]) 6-10 times per year with a minimum trapping effort of monthly captures at the end of the rainy season (March-May) and at the end of the dry season (September-November). Only data from the months March-November were used in the analyses to reduce the influence of female pregnancy. Unmarked animals were equipped with a subcutaneous transponder (Trovan EURO ID, Germany) for individual identification. The animals were almost exclusively first captured as juveniles (age less than 12 months), and the age estimates were confirmed by morphometrics. The few individuals for which age could not be estimated to the year with reasonable confidence (age at first capture presumably more than 2 years) were excluded from the analyses. Marked individuals were weighed monthly upon subsequent recaptures (precision ± 1 g). For the analyses, we used individuals born in 1993-2008 and captured for the last time at least 6 months before the last capture session included in the data set for each subpopulation.

Research in Madagascar was approved by the Ministère de l'Environment et des Eaux et Fôrets, MINEEF and complies with the applicable national laws of Madagascar. Research at the Brunoy breeding colony is authorized by the agreement DDPP, Essonne, France n° E91- 114-1.

STATISTICS

Modeling approach

Models were created separately for data from the wild and captivity and all analyses were performed in R version 3.0.1 [R Development Core Team 2014]. "Lifetime" models were created to describe age-trajectories of BM in adulthood (age range 1-11 years). The data sets included 1773 seasonal averages from 258 individuals (females: 1005/156, males: 768/102) in captivity ("C10" data set), and 3468 measurements of 595 individuals (females: 1665/292, males: 1803/303) from the two subpopulations in the wild ("W10"). The subpopulations were combined for the analyses, but the population identity ("CS7" or "N5") was included as a cofactor in all models to account for potential small-scale ecological effects, differences in capture effort and the different time windows for the data. We used only adult measurements (age 12 months or above) from animals with minimum lifespan of 1 year or above. All individuals included in the analyses are assumed to have reached adult structural size and sexual maturity. While the lifetime trajectories indicate the general patterns of change, we further examined the data for only the individuals that lived to be at least 5 years old to detail the changes that happen at old age and more accurately quantify rates of FS across seasons, settings and sexes. The final "old age" data sets included 316 observations from 70 wild individuals (W5, females: 244/50, males: 72/20) and 339 observations from 105 captive individuals (C5, females: 174/57, males: 165/48).

An *a priori* set of biologically meaningful candidate models (electronic supplementary material, Tables S1-S4) was created for each data set. Standard Akaike's information criterion values (AIC, for C10 and

W10) or AIC corrected for small sample size (AICc, calculated with the MuMIn package [Barton 2013], C5 and W5) and differences between models (AICΔ) were calculated for the candidate models. The associated AIC-weights (AICw) were used for model selection. When AICw for the best model was less than 0.9, all models with cumulative AICw of 0.95 or above ("confidence set") were used for multimodel inference [Burnham and Anderson 2002].

The year of measurement and subpopulation identity were included as fixed factors in all W10 and W5 models, and autocorrelation of measurements within an individual was included in all W10 candidate models. All continuous predictor terms were log-transformed, scaled (mean/SD) and centered [Grueber et al. 2011; Wood 2006] and BM was log-transformed. Further details on the statistical analyses are provided in electronic supplementary material, S1.

The seasonal patterns between the wild and captive populations do not directly correspond to each other, but for our purposes the factor of importance was the gain and loss of BM. Hence, seasonality based on the expression of maximum (SDS, rainy season) and minimum (LDS, dry season) annual BM provides an estimate of the animals' capacity to respond to the environmental cues and is used for across-setting comparison.

Lifetime body mass trajectories

To estimate flexible age trajectories of BM and control for the expected nonlinear seasonal BM fluctuation [Schmid and Kappeler 1998], we used Additive Mixed Modeling (GAMM, mgcv-package [Wood 2006]). For W10, a sex-specific smoother term for "day of year" was created to allow for sex-specific seasonal fluctuations [Schmid and Kappeler 1998]. For W10 and C10, a smoother term for age was used to describe the age-trajectories of BM. Alternative model terms allowed the age-trajectories to be sex or season-specific. Additional terms entered were "lifespan" (based on age at last capture or known date of death), season (wild: dry/rainy; captivity: SDS/LDS), a binary-coded variable "last season of life" to assess terminal change, and the interactions between sex and each of these three variables.

Body mass senescence

BM senescence was quantified in further detail using Linear Mixed Models (LMM, lme4-package [Bates et al. 2013]). An identical model set was built for the captive (C5, electronic supplementary material, Table S3) and wild (W5, electronic supplementary material, Table S4) populations, differing only in the random effects structure (both: individual nested within cohort, W5: year of measurement). Linear effects of age on BM were computed to obtain estimates of absolute change. Additional terms included in candidate models were lifespan, terminal change and interactions of age with sex and season as well as a three-way interaction of sex, season and age. For W5, the data were restricted to the months March-May and September-November.

RESULTS

Lifespan in captivity and the wild

Excluding juvenile mortality, captive males lived on average one season longer than females (males: 5.5 ± 1.7 years; females: 5.0 ± 1.5 years, t = -3.88, df = 330.4, p < 0.001, electronic supplementary material, Figure S1a). In contrast, average minimum lifespan of wild females was on average 7 months longer than that of males (males: 2.7 ± 1.4 years; females: 3.4 ± 2.0 years, t = 4.45, df = 421.66, p < 0.001, electronic supplementary material, Figure S1b). In the wild, the survival difference is probably a conservative estimate since males may be captured closer to their time of death than females due to their higher recapture probability [Kraus et al. 2008].

Lifetime trajectories of body mass in captivity (C10)

Model selection for C10 indicated a single best model (AICw > 0.99; electronic supplementary material, Table S1) that included the terms sex, season and their interaction, a season-specific smoother for age and the terms indicating condition-dependence: lifespan and last season. BM was consistently higher in SDS compared to LDS and females had on average higher BM in both seasons, with the sex difference being more pronounced in LDS. In LDS (Figure 1a), an asymptote in BM was reached in prime adulthood (approx. age 3 years) with no subsequent declines, but in SDS a decline in BM began at age 4-5 years (Figure 1b). Lifespan was positively associated with BM, and terminal decline was indicated by the negative influence of the last season of life on BM (Table 1; effect size based on back-transformed means: -5.5 g, i.e. 6% decline). No support was found for sex-specific age trajectories (i.e. sex-specific smoothers of age) or sex differences in terminal decline or condition dependence of lifespan.

Lifetime age-trajectories of body mass in the wild (W10)

In W10, the confidence set of models (cumulative AICw > 0.98) included 5 models (electronic supplementary material, Table S2), each of which contained the terms sex, sex-specific seasonal smoothers and season-specific smoothers of age. The seasonal patterns of BM differed for the sexes (electronic supplementary material, Figure S2) but overall, adult females were slightly heavier than males and both sexes weighed more in the rainy season than in the dry season (Table 1). BM continued to rise in adulthood until very old age in the rainy season (Figure 1d), but in the dry season, BM reached an asymptote or even declined slightly after peak values around 4-5 years of age (Figure 1c). No support was found for sex-specific age-trajectories of body mass.

Support for condition-dependent longevity was also indicated in the top models. Models incorporating an effect of last season on BM were 3 times more likely than those without. As expected, BM in the last season of life was on average lower than in other seasons (Table 1; back-transformed -1.4 g, i.e. 2% decline). Models incorporating the term lifespan also received some support (ΣAICw = 0.37), but,

contrary to our predictions, the negative association of lifespan with BM suggests that long-lived individuals actually had a slightly lower BM earlier in life than individuals that died at a younger age. Limited support (∑AICw = 0.19) was found for an interaction between sex and last season, with males having higher BM in the last season than females (Table 1).

Table 1: Parameter estimates for the terms included in the single best model for the captive population lifetime data (C10, ESM3: Table S1, AICw > 0.99, relative importance > 0.99 for all terms) and confidence set of models for the wild population lifetime data (W10, ESM3: Table S2, AICw > 0.98).

	Captive (C10)		Wild (W10)*		
Term	β	**SE**	β	**SE**	**Relative importance**
Sex (ref. female)	-0.134	0.017	-0.075	0.008	1
Season (ref. LDS)	0.149	0.006			
Lifespan	0.016	0.007	-0.008	0.005	0.37
Last season	-0.059	0.010	-0.018	0.007	0.75
Sex:Season	0.046	0.009			
Sex: Last season			0.012	0.011	0.19
s(Age):Season (LDS/dry)**	0.024	0.017	0.051	0.039	1
s(Age):Season (SDS/rainy)**	-0.032	0.027	0.296	0.039	1

Temporal within-individual autocorrelation and random structure (individual nested within cohort) applied to all models.
*Sex-specific seasonal smoother, subpopulation and year of measurement included as forced fixed variables.
**Estimated degrees of freedom for smoothers: SDS edf=3.42; LDS edf=2.15; rainy edf=2.281; dry edf=2.648

Body mass senescence in captivity (C5)

In C5, the confidence set (∑AICw >0.98, electronic supplementary material, Table S3) consisted of three models, each of which contained the terms sex, age, season and lifespan. The interaction terms age*season (∑AICw = 0.43) and lifespan*sex (∑AICw = 0.08) received limited support. Females were heavier in both seasons and BM was higher in both sexes in SDS (Table 2, electronic supplementary material, Figure S3). A steeper senescent decline was evident in SDS than in LDS (Figure 2a,b and Table 2). Strong support was found for condition-dependent longevity (∑AICw = 1.0), i.e. a positive relationship between BM and lifespan (Table 2), but unlike in C10, terminal decline in BM received no support in C5.

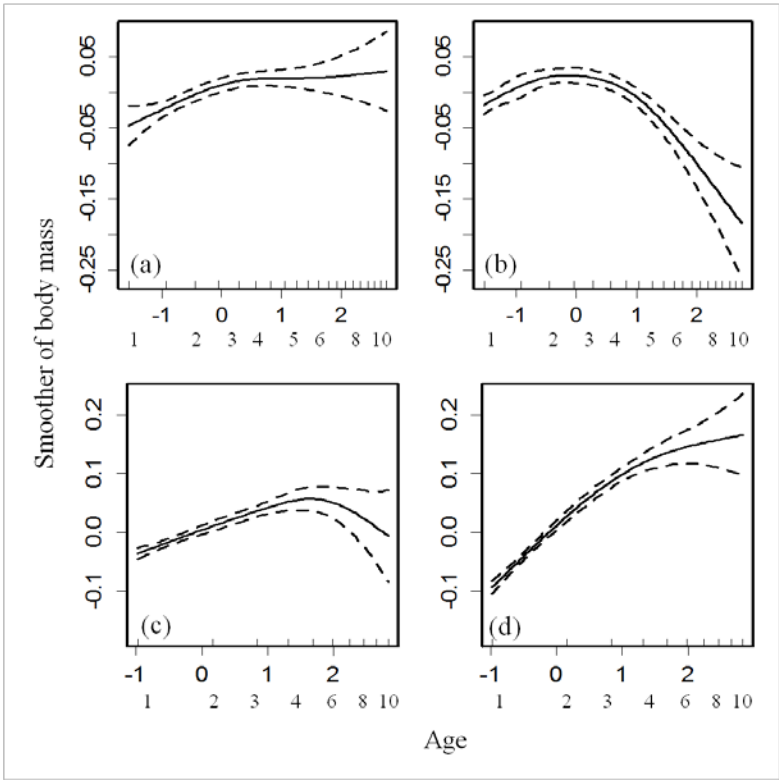

Figure 1: Age trajectories (smoothers of age based on scaled age (x-axis top row) with the corresponding chronological age in years (x-axis bottom row)) of body mass in LDS (a) and SDS (b) photoperiods in captivity (C10) and in the dry (c) and rainy season (d) in the wild (W10). The predictions are based on the best model in each setting. The smoother curves and their confidence bands reflect variation around mean body mass. Note that scales differ between captive (a-b) and wild (c-d) data.

Body mass senescence in the wild (W5)

In W5, a single model including the terms sex, season and their interaction received strong support (AICw = 0.91, electronic supplementary material, Table S4). BM differed between the sexes only in the rainy season, with females being heavier than males (Table 2, electronic supplementary material, Figure S3). Although a slight senescent decline seemed to be indicated by the lifetime age trajectory in the dry season (W10, Figure 2c), an age effect was not supported by the W5 model selection (Table S2), suggesting an overall plateau with no increase or decrease in BM at old age. The seasonal differences in age trajectories seen in W10 were not found in the old animals, which may be partially caused by the very low number of old males present in the rainy season. No evidence was found for an influence of terminal change or lifespan on body mass. The results were qualitatively similar when using GAMMs including the sex-specific seasonal trends as specified in W10.

Table 2: Parameter estimates for the terms included in the confidence set of models for the captive population aged animals (C5, Table S3, AICw > 0.98) and the single best model for the wild population aged animals (W5, Table S4, AICw > 0.90, relative importance > 0.90 for all terms).

	Captive (C5)*			Wild (W5)**	
Term	**β**	**SE**	**Relative importance**	**β**	**SE**
Season (ref. LDS/dry)	0.118	0.013	1	0.316	0.019
Sex (ref. female)	-0.124	0.025	1	0.011	0.038
Age	-0.053	0.009	1		
Lifespan	0.079	0.015	1		
Season:Age	-0.039	0.014	0.43		
Sex:Lifespan	0.064	0.030	0.08		
Season:Sex				-0.160	0.040

*Random effect of individual nested within cohort applied to all models.
**Random effects of individual nested within cohort and year of measurement applied to all models.
Subpopulation entered in all models as a forced fixed factor.

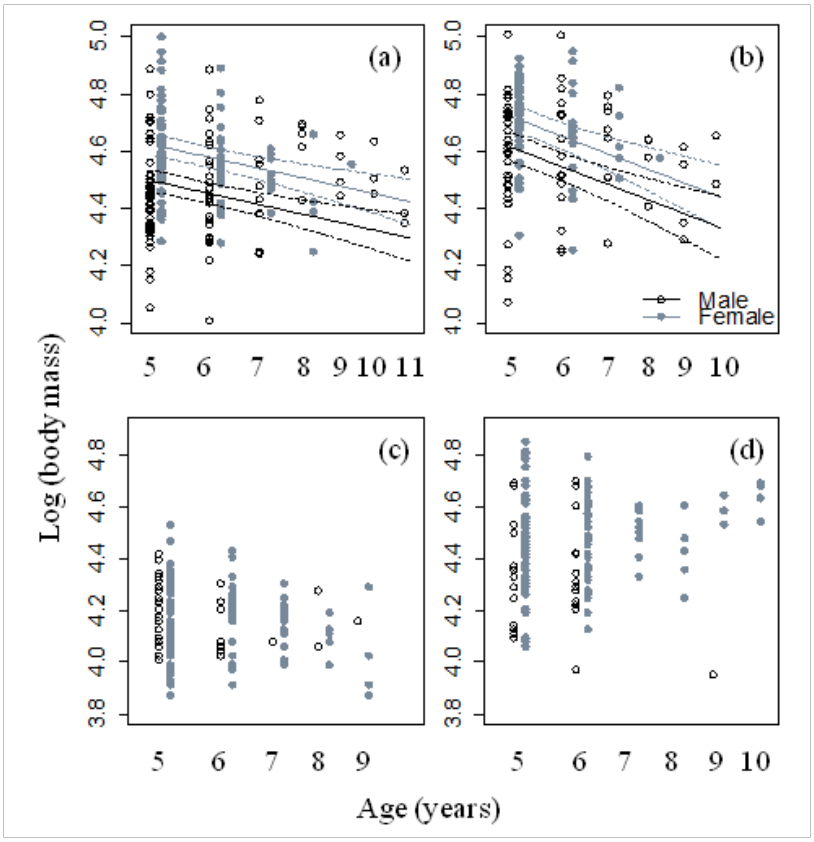

Figure 2: Body mass varied as a function of age in the aged animals in captivity (C5) LDS (a) and SDS (b), but not under natural conditions (W5) dry season (c) and rainy season (d). Solid line indicates the best fit based on the top model and dashed lines show the 95% confidence bands.

DISCUSSION

In this study, we tested fundamental hypotheses of aging theory by assessing the relative importance of condition-dependent disappearance and FS in shaping populations of a species that naturally experiences heavy predation pressure. By comparing longitudinal data from captive and natural populations we found evidence of selective disappearance operating in both settings, but the selective processes appear to be more rapid in the wild. FS was evident only in captivity. The disappearance of low BM individuals in captivity indicates intrinsic processes as a likely cause of the functional decline. In the presence of environmental hazard, these processes probably cause such rapid disappearance of individuals whose condition has declined, that FS is undetectable in nature even in this large dataset. This result is in stark contrast with studies of large-bodied herbivores with low EM risk, in which FS has been found after accounting for selective disappearance [Nussey et al. 2011]. We found no support for the prediction that aging occurs earlier in populations with high risk of EM. On the contrary, the oldest animals in the wild (unlike in captivity) remained in excellent condition relative to the population average.

Condition-dependent longevity and terminal declines in body mass

An energy imbalance can quickly render an individual susceptible to disease [Coop and Kyriazakis 1999] and predation [Murray 2002] or lower their success in resource competition. The end of life is therefore often characterized by a terminal decline in condition [Hoffman et al. 2010; Isaac and Johnson 2005; Karkach 2006; Tafani et al. 2013; Weladji et al. 2010]. Our results indicate that the mouse lemurs had a lowered BM in the season preceding death in both the captive (-6%) and wild (-2%) populations. As the decline in condition may be sudden but sampling was done only at a seasonal scale, the magnitude and importance of terminal decline especially in the wild is probably an underestimate. Selective mortality is presumably more rapid in the wild than in captivity, because weak individuals may more easily succumb to environmental hazards. Wild males had a slightly higher BM than females in their last season of life, which may indicate that female survival depends more on condition, whereas random external mortality may affect males more owing to their risky behaviours [Dammhahn 2012; Kraus et al. 2008].

BM throughout life was positively associated with lifespan in captivity, indicating that selective disappearance of low-quality individuals operated there also at a longer timescale. Unexpectedly, however, lifespan was negatively associated with BM in the wild, implying that heavier individuals may disappear from the population at an earlier age. The causes for this effect are unknown but it is possible that heavier or larger individuals incur higher mortality owing to higher predation pressure, parasite loads or, potentially, investment in reproduction. The lowest quality individuals in the wild might die as juveniles, thus being excluded from our sample, whereas their lifespan in captivity may be prolonged. However, the negative effect of BM on survival was quite weak, and heterogeneity in capture probabilities may contribute to the unexpected result, if individuals in a worse condition enter traps more frequently. Therefore, while the negative trend was highly unexpected, BM may have little influence on lifespan at a long timescale and rather operate mainly via rapid terminal declines in the wild.

This view is supported by the fact that the oldest surviving individuals in the wild were in an excellent condition. Random and condition-dependent mortality presumably both occur in the natural setting and their influences are difficult to disentangle. However, even moderate levels of condition-dependent mortality in the wild would lead to prolonged survival of high-quality individuals.

In the oldest animals, terminal changes were negligible in both the captive and wild setting and longevity was only condition-dependent in captivity, suggesting that the final declines in BM observed in the lifetime data are not directly associated with FS or terminal investment. The declines more likely follow from rapid processes such as illness, which may predispose the individual to a higher risk of EM, e.g. predation [Dowling 2012], and which the high-quality individuals may be able to escape and reach old age. The absence of condition-dependent longevity in the aged wild animals, while a negative connection was found in the lifetime data, may further indicate that any selective mortality of heavier or larger individuals occurs earlier in life.

The BM of an individual is largely dependent on body size, and the observed condition-dependent longevity might partially reflect body size variation. However, since our sample contained only mature individuals, which are considered to have reached adult size, we are confident that BM is a good approximation of the resources available for physiological maintenance in this species. In support of this, additional analyses (data not shown) of BM corrected for structural size (scaled mass index [Peig and Green 2009; Vuarin et al. 2013] using opportunistic measurements of adults in the wild) revealed no evidence for FS in body condition. Moreover, we found no support for individual variation (random slopes) in aging rates (electronic supplementary material).

Patterns of functional senescence differ in the presence and absence of natural hazard

BM fluctuations over the adult lifespan were consistently, strongly dependent on sex, season and age. Senescent declines in BM in captivity began at 4-5 years of age, coinciding with the onset of decline in physical functioning [Languille et al. 2012] found in other studies (balance performance [Némoz-Bertholet and Aujard 2003] and muscle strength (A. Hämäläinen, M. Dammhahn, F. Aujard, C. Kraus 2014, unpublished data), therefore probably reflecting muscle deterioration. It is plausible that wild animals which succumb to a similar degree of physiological senescence are more likely to succumb to EM. By contrast, we found no evidence of further changes in BM after the fifth year of life in the wild, even though the lifetime trajectories appeared to suggest a declining pattern (Figure 1c).

Seasonality modulated the age trajectories of BM across settings. In aged captive animals, the amplitude of seasonal BM fluctuation was diminished, which is attributable to a declined ability to regain BM. This pattern has been described earlier for captive aged males [Perret 1997; Perret and Aujard 2001], and was confirmed in this study for both sexes. In contrast, wild, aged animals' BM remained at a level similar to prime aged adults in the dry season (Figure 1, ESM4), and they regained BM in the rainy season even

more efficiently than younger animals, supporting the conclusion that the surviving old animals in the wild are of high quality. The observed plateau in BM in the dry season might reflect senescent muscle loss or longer periods of anorexia owing to intense torpor use by old individuals [Vuarin et al. 2013].

Despite the weak evidence for selective mortality at the long timescales in the wild, these differences between the captive and wild populations suggest that condition-dependent EM [Chen and Maklakov 2012; Williams et al. 2006] operates also in the natural population. In both settings, terminal processes eliminate individuals whose condition deteriorates, but this process may be intensified in the hazardous environment, as implied by the relatively high threshold condition apparently required for wild individuals to survive to old age. Unlike in species with low EM rates, this interaction probably eliminates individuals from the natural population before they show FS. The interplay of FS, selective disappearance of individuals in an inferior condition, and EM risk is likely a universal process [Carlson et al. 2007; Chen and Maklakov 2012; Williams and Day 2003; Williams et al. 2006], but its detection in natural conditions may be challenging. While captive research is vital for understanding mechanisms of senescence, information gained from captivity may be of limited relevance for wild animals in species whose life-histories are evolutionarily shaped by high EM. Despite high EM rates and AS, condition-dependent mortality may even lead to negligible FS under natural conditions [McNamara and Houston 1996; Williams and Day 2003].

Sex-specific patterns of seasonal body mass change

Our data confirm and further detail sex-specific patterns of seasonal BM fluctuation in the natural population [Schmid and Kappeler 1998] that likely reflect sex differences in seasonal energy requirements due to reproduction and torpor use [Schmid 1999]. Sex differences in aging rate are thought to stem from selection optimizing reproductive capacity for each sex [Bonduriansky et al. 2008; Maklakov and Lummaa 2013], leading males to show more rapid functional declines than females [Bowen et al. 2006; Hoffman et al. 2010; Tafani et al. 2013; Williams 1957]. However, our models indicated no support for sex differences in ageing in either captive or wild animals. It is possible that sex biases in mortality may lead to sex differences in FS, but these differences are masked by the strong seasonal effects and rapid terminal changes. Based on a visual inspection of the sex-specific age trajectories of BM (Figure 2; electronic supplementary material, Figure S4), the trends in senescent declines concur with the direction of each population's sex bias in mortality

Lifespan determination in captivity and nature

In support of previously formulated [Williams and Day 2003; Williams et al. 2006] and tested [Chen and Maklakov 2012] hypotheses, our results suggest that lifespan is determined at population level by an interaction of intrinsic mortality rate with environment influences. Condition-dependent EM can cause selection to favor improved somatic maintenance [Bronikowski and Promislow 2005; Chen and Maklakov 2012; Murray 2002; Williams and Day 2003] that increases predator-avoidance success and lifespan and

permits the manifestation of physiological aging only in the absence of extrinsic hazard As expected, the estimated lifespans of wild and captive M. murinus differ substantially: captive males live on average twice as long and females 50% longer than their wild counterparts. Such variation reflects highly plastic life histories that may have helped the species to adapt to the gradient of ambient temperatures found within its natural geographical distribution, and contribute to the proposed pace-of-life variation found across this range [Lahann et al. 2006]. Our results concur with previous studies [Lemaître et al. 2013; Magalhães et al. 2007] that have found longer lifespan and lower AS rates in captivity than in nature.

The particularly strong plasticity in *M. murinus* males results in a contrasting sex bias in lifespan across settings. This intriguing phenomenon may reflect stronger selection for robust males [Bonduriansky et al. 2008; Maklakov and Lummaa 2013], resulting from their higher EM, as suggested previously [Maklakov et al. 2009]. Alternatively, adjustment to captivity may evoke differential responses by males and females due to physiological differences associated with allocation to reproduction, somatic maintenance [Kirkwood 2002; Kirkwood and Austad 2000], hormone levels and susceptibility to disease [Alonso-Alvarez et al. 2007; Klein 2000], or torpor use [Turbill et al. 2011].

Theories of aging posit that molecular damage builds up at old age because selection is inefficient against deleterious effects that arise after individuals have passed on their genes [Monaghan et al. 2008]. However, if lifetime fitness is sufficiently enhanced by living longer, selection may favor somatic maintenance and counteract the accumulation of damage [Chen and Maklakov 2012; Maklakov and Lummaa 2013; Promislow 1991]. Gray mouse lemurs have a long potential lifespan relative to their body size and life-history characteristics [Harvey and Clutton-Brock 1985; Ross 1998], yet a distinctly shorter average lifespan in nature.

While high EM may be associated with evolutionary processes that shorten lifespan, these processes might be partially counteracted in *M. murinus* by their lifelong reproduction [Perret 1992; Perret 1997] and ability to efficiently fatten and use torpor in late adulthood. Overall, our results support the view that condition-dependent EM may shape FS and, eventually, lifespan. Further research on these simultaneous processes in other systems is required to identify the specific constraints on lifespan evolution, with emphasis on the influences that individual condition, environmental plasticity and the flexible nature of senescence may have on the observed patterns of senescence and lifespan.

CONCLUSIONS

Using long-term BM data from gray mouse lemurs, we demonstrate selective disappearance in both captive and wild populations. BM senescence is apparently absent in the wild, although clearly present in captivity. We identify condition-dependent mortality as a potentially significant demographic process that can influence senescent processes but has been largely overlooked in aging studies thus far. Its underestimation can lead to biased conclusions on the prevalence of FS and the evolution of lifespan. FS

is, indeed, sometimes undetectable in the wild since in species with high EM risk, the oldest individuals tend to be of high quality. Aging may also be masked by environmental variables, respond to subtle changes in conditions, or be altogether absent. An increased understanding of this variability will bring us closer to resolving open questions on life-history determinants across taxa.

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SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Details on the statistical analyses

Models were created separately for data from the wild and captivity and all analyses were performed in R version 3.0.1 (R development core team 2013). "Lifetime" models (ages 1-10 years) were used to describe patterns of body mass change over the lifespan under wild and captive conditions. Senescence was studied in further detail using only measurements at ages≥ 5 years in order to quantify the rates of senescence across settings and sexes since this age has been indicated as the beginning of senescent declines in captivity.

Because of the large number of factors considered and the consequently high number of plausible alternative models, we used an information-theoretic approach for model selection [Burnham and Anderson 2002]. An *a priori* set of candidate models was created including only biologically meaningful models (Tables S1-S4). Model selection was based on Akaike's Information Criterion values (AIC, for C10 and W10 data sets) or AIC corrected for small sample size (AICc, C5 and W5 data sets) and differences between the models (AICΔ) were calculated for the candidate models. The associated AIC-weights (AICw) and log-likelihood ratios were used for model selection. When AICw for the best model was < 0.9, all models with cumulative AICw of ≥ 0.95 ("confidence set") were used for multimodel inference [Burnham and Anderson 2002]. This confidence set was used to quantify the relative importance of each variable by calculating the sum of AICw of all of the models in which the term appeared. Variable estimates were computed based on the best model in the confidence set including a given variable since estimates were quantitatively very similar for a given term across the models included in the confidence set in all cases [Burnham and Anderson 2002].

All continuous predictor terms were log-transformed, scaled (mean/SD) and centered prior to analyses (as recommended in [Grueber et al. 2011; Wood 2006]) to more reliably estimate effects of polynomials included in interactions, main effects of terms included in interactions and smoother terms. Body mass was log-transformed to achieve normal distribution. The heterogeneity and distribution of the residuals of the model of highest complexity and each selected best model were confirmed by a visual examination of diagnostic graphs [Wood 2006]. Where outliers were suspected based on visual inspection of the diagnostic graphs, the models were run again after excluding these outliers (1-2 cases in W5 and C5 data sets). This exclusion had no influence on the conclusions and therefore these outliers were included in the final data analyses.

Lifetime body mass change with age

Because of the expected non-linear seasonal body mass fluctuation [Schmid and Kappeler 1998] and for describing flexible estimates of the age trajectories of body mass, we used Additive Mixed Modelling (GAMM, [Wood 2006]). First, we investigated the potential temporal autocorrelation between consecutive body mass measurements for an individual with the mgcv-package [Wood 2006]. This was done using a reduced model with body mass as the response variable, the year of measurement entered as a fixed effect and a sex-specific smoother of the date of measurement (wild; see below) or photoperiod (captive) as predictors and individual identity nested within cohort (year of birth) entered as a random factor. This model was run with and without an autocorrelation term of time (Julian date in the wild and age in seasons in captivity, to account for the difference in frequency and within-population synchrony of measurements) within the individual (function corAR1 [Pinheiro and Bates 2000]). The significance of the autocorrelation term in both settings was assessed by comparing the reduced and inclusive model using likelihood ratio tests (LRT). We found significant autocorrelation in the measurements in both settings (wild: LRT=489.89, $p<0.0001$, captive: LRT=27.14, $p<0.0001$). Therefore, the correlation structure was included in all candidate models for the examination of body mass over lifetime. Since multiple, non-nested random structures cannot be combined with correlation structures in currently available R packages, the random structure was defined as individual nested within cohort in both captivity and the wild. To account for potential subpopulation effects and annual fluctuations, the year of measurement and subpopulation identity were included as fixed factors in all W10 and W5 models.

For the W10 data set, a smoother term was created for “day of year” for each sex in order to allow for the known sex-specific non-linear seasonal trends in Kirindy [Schmid and Kappeler 1998] and alleviate possible variations in recapture rates of the sexes over the course of the year. The smoother terms were estimated using cubic regression splines to permit the computation of smoothers for the large sample [Wood 2006]. For both settings, a smoother term was also introduced for age (cubic regression spline, maximum number of knots set to 5) in order to describe the age-related changes in body mass.

Body mass senescence

The rate of decline at old age was quantified in further detail by modelling only data for individuals≥5 years (W5 and C5) using Gaussian Linear Mixed Models (LMM, R package lme4 [Bates et al. 2013]). This package was used to accommodate a crossed random effects structure in the wild (individual nested within cohort + year of measurement; captive: individual nested within cohort) to improve the explanatory power of models for this smaller data set. The conclusions were confirmed by model selection done with a) the inclusion of an autocorrelation structure using the nlme-package [Pinheiro et al. 2013], and b) GAMM of the W5 data, where sex-specific smoothers of the day of the year were fitted as described above for the lifetime models. The same confidence set of models was indicated in each modelling approach. Therefore, we show results based only on models with the crossed random structure. We also tested for inter-individual variation in rates of senescence with random slopes

[Grueber et al. 2011; van de Pol and Wright 2009] (using a model with age and season as fixed factors and, additionally, year and subpopulation in W5, including only individuals with more than two measurements available), but since models with random slope structures (with and without correlation of random slope and intercept) were not significantly better than ones without (LRT: all $p>0.07$ in captivity and the wild), we chose random intercept models for all analyses in the interest of parsimony.

An identical model set was built separately for the captive (Table S3) and wild (Table S4) populations, differing only in the random effects structure (see above). Only linear effects of log-transformed and scaled age on body mass were entered in the models to acquire estimates of absolute change. In the wild, data were restricted to the months March-May and September-November to exclude times of the year with low trapping probabilities and to reduce noise caused by ecological change during transition between seasons.

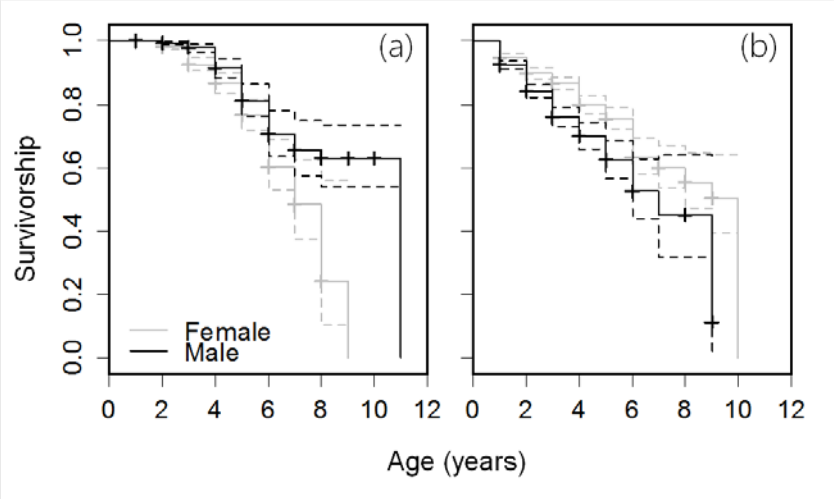

Figure S1: Sex-specific survivorship in the captive population (a) based on date of death and in the wild populations (b) based on age at last capture. Solid lines represent the proportion of the population surviving to a given age; dashed lines indicate 95% confidence intervals.

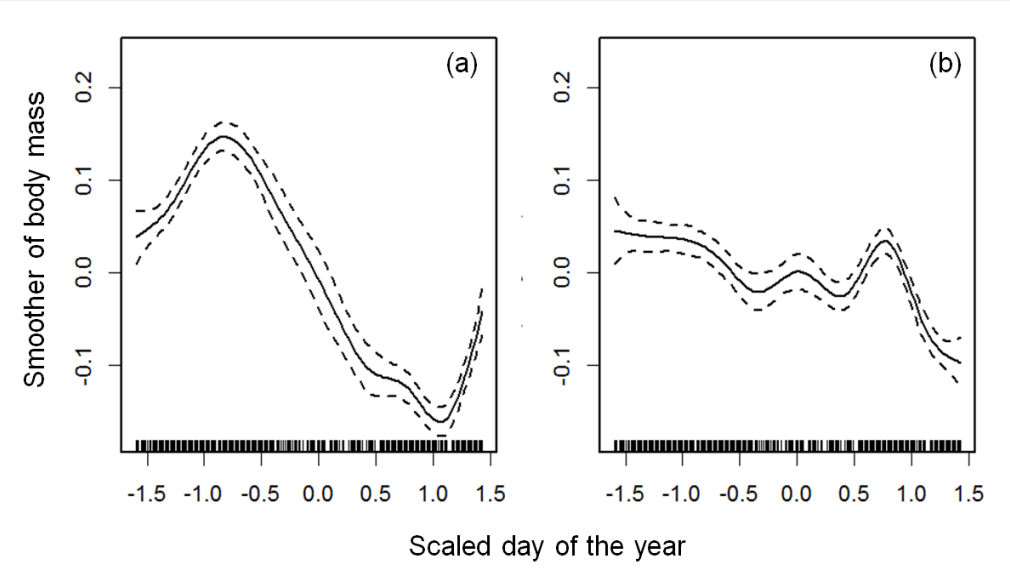

Figure S2: Seasonal body mass change for females (a) and males (b), from March to November in the natural population. The solid lines indicate the best estimate for a smoother of body mass over the course of the year, based on the model receiving highest support in model selection (first model, Table S2: s(day of year,by=sex) + sex + longevity + last season + s(age,by=season) + year + subpopulation), dashed lines show 95% confidence bands.

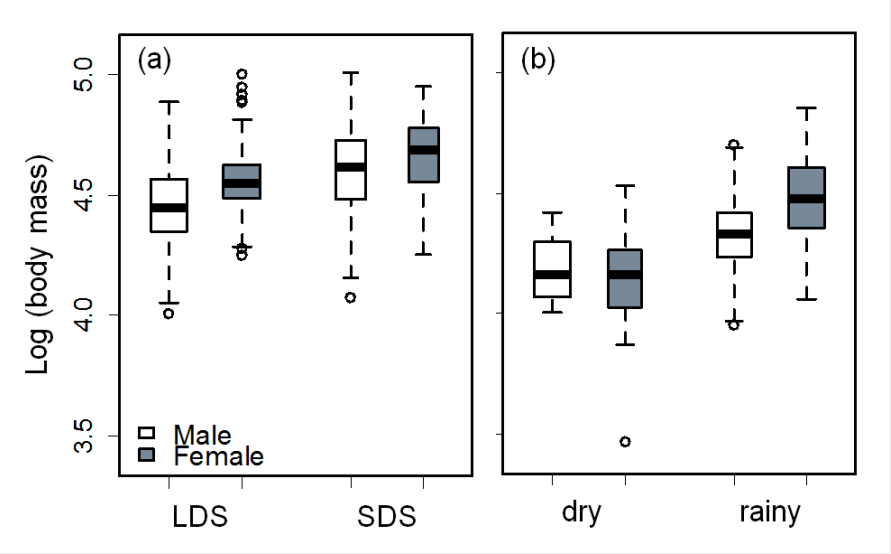

Figure S3: Body mass in captive (a) and wild (b) males and females (ages 5-10 years) in the different seasons. Shown are median, interquartile range, min–max range and outliers; box width corresponds to sample size.

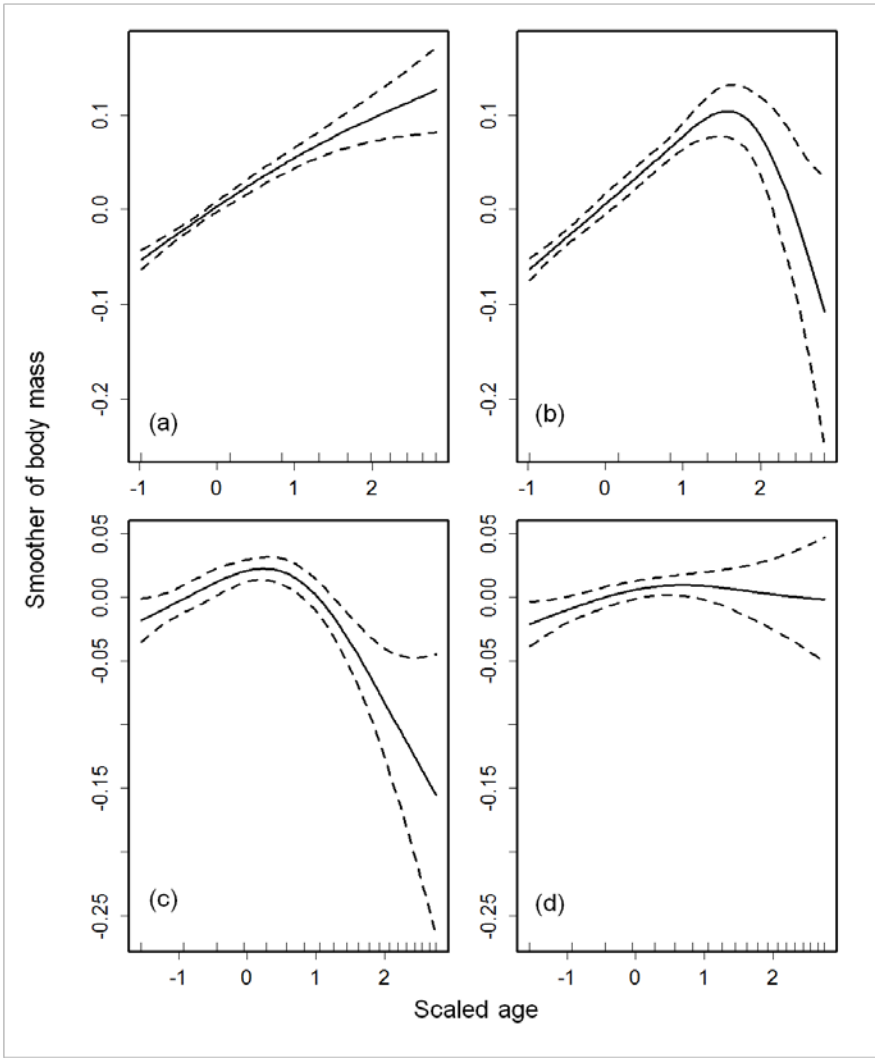

Figure S4: Age-specific smoothers of body mass over lifetime for wild females (a) and males (b) and captive females (c) and males (d). The solid lines indicate the best estimate for a smoother of body mass over the ages 1-11 years (scaled age shown) and dashed lines show 95% confidence bands. Estimates are based on models s(day of year, by=sex) + season + sex + longevity + last season + s(age, by=sex) + year + subpopulation) in the wild and season + sex + season:sex + longevity + last season + s(age,by=sex) in captivity. Note that sex-specific smoothers were unsupported by model selection, since season-specific smoothers received very strong support (Tables S1 and S2). Two separate smoothers of the same variable (e.g. sex- and season-specific smoother of age) cannot be included in the same model.

Table S1: Model selection for lifetime body mass data in captivity (C10). Set of candidate models for the captive population lifetime data (C10). The single best model (AICw > 0.99) in bold. N=1773 measurements from 258 individuals.

Model structure	df	AIC	AICΔ	LogLik	AICw
sex + longevity + last season + season:sex + s(age, by=season)	**14**	**-2469.55**	**0.00**	**1.000**	**1.000**
season + sex + longevity + last season + s(age, by=season)	13	-2444.06	25.50	0.000	0.000
season + sex + season:sex + s(age, by=season)	12	-2428.10	41.45	0.000	0.000
season + sex + s(age) + longevity + last season + season:sex + last season:sex	13	-2420.68	48.88	0.000	0.000
season + sex + s(age) + longevity + last season + season:sex	12	-2418.82	50.74	0.000	0.000
season + sex + s(age) + longevity + last season + season:sex + longevity:sex + last season:sex	14	-2418.77	50.78	0.000	0.000
sex + last season + last season:sex + s(age, by=season)	12	-2417.21	52.34	0.000	0.000
season + sex + s(age) + longevity + last season + season:sex + longevity:sex	13	-2417.05	52.50	0.000	0.000
season + sex + longevity + s(age, by=sex) + last season:sex	14	-2412.38	57.17	0.000	0.000
season + sex + longevity + last season + season:sex + s(age, by=sex)	14	-2412.23	57.33	0.000	0.000
season + sex + longevity + last season + s(age, by=sex) + longevity:sex + last season:sex	15	-2410.69	58.86	0.000	0.000
season + sex + s(age) + longevity + last season + last season:sex	12	-2410.49	59.06	0.000	0.000
season + sex + longevity + last season + season:sex + s(age, by=sex) + longevity:sex	15	-2410.23	59.33	0.000	0.000
sex + longevity + last season + s(age, by=season)	12	-2408.75	60.81	0.000	0.000
season + sex + last season + s(age, by=sex) + last season:sex	13	-2407.91	61.64	0.000	0.000
season + sex + s(age, by=season)	11	-2406.86	62.69	0.000	0.000
season + sex + s(age) + last season + last season:sex	11	-2406.25	63.30	0.000	0.000
sex + last season + s(age, by=season)	11	-2405.43	64.12	0.000	0.000
season + sex + s(age) + longevity + last season	11	-2401.36	68.19	0.000	0.000
season + sex + s(age) + longevity + last season + longevity:sex	12	-2399.57	69.99	0.000	0.000
season + sex + s(age) + longevity + season:sex	11	-2398.98	70.57	0.000	0.000
season + sex + longevity + last season + s(age, by=sex)	13	-2398.81	70.75	0.000	0.000
season + sex + longevity + last season + s(age, by=sex) + longevity:sex	14	-2396.82	72.73	0.000	0.000
season + sex + s(age) + season:sex	10	-2389.66	79.89	0.000	0.000
season + sex + s(age) + longevity	10	-2384.43	85.12	0.000	0.000
season + sex + last season + last season:sex	9	-2383.64	85.91	0.000	0.000
season + sex + s(age) + longevity + longevity:sex	11	-2382.63	86.93	0.000	0.000
sex + longevity + s(age, by=season)	11	-2382.40	87.15	0.000	0.000
season + sex + longevity + s(age, by=sex)	12	-2382.12	87.43	0.000	0.000
sex + longevity + longevity:sex + s(age, by=season)	12	-2380.62	88.94	0.000	0.000
season + sex + longevity + s(age, by=sex) + longevity:sex	13	-2380.13	89.43	0.000	0.000
season + sex + s(age)	9	-2375.13	94.42	0.000	0.000
sex + s(age, by=season)	10	-2373.13	96.43	0.000	0.000
season + sex + s(age, by=sex)	11	-2373.00	96.55	0.000	0.000
season + s(age, by=season)	10	-2372.30	97.26	0.000	0.000
last season + s(age, by=season)	10	-2368.18	101.38	0.000	0.000
season + sex + season:sex	8	-2366.66	102.90	0.000	0.000
season + s(age) + longevity + last season	10	-2359.92	109.64	0.000	0.000
season + sex + longevity + longevity:sex	9	-2358.32	111.23	0.000	0.000
season + sex	7	-2352.02	117.54	0.000	0.000
season + s(age) + longevity	9	-2342.76	126.79	0.000	0.000
longevity + s(age, by=season)	10	-2340.72	128.83	0.000	0.000
season + s(age)	8	-2340.39	129.17	0.000	0.000
season + s(age, by=sex)	10	-2339.72	129.84	0.000	0.000
s(age, by=season)	9	-2338.36	131.19	0.000	0.000
season + longevity + last season	8	-2338.26	131.29	0.000	0.000
season + longevity	7	-2319.07	150.48	0.000	0.000
Season	6	-2317.34	152.21	0.000	0.000
s(age)	7	-1776.47	693.09	0.000	0.000
s(age, by=sex)	9	-1760.83	708.73	0.000	0.000
last season	6	-1743.74	725.82	0.000	0.000
Sex	6	-1665.91	803.65	0.000	0.000
Longevity	6	-1632.49	837.07	0.000	0.000

Sex-specific seasonal smoother, subpopulation and year of measurement included as forced fixed variables. Temporal within-individual autocorrelation and random structure (individual nested within cohort) applied to all models.
s(age, by=season) = season-specific smoother of age; s(age, by=sex) = sex-specific smoother of age

Table S2: Model selection for lifetime body mass data in the wild (W10). Set of candidate models for the wild population lifetime data (W10). The confidence set of models indicated by model selection (cumulative AICw > 0.98) receiving most support for predicting the body mass in bold. N=3422 measurements from 593 individuals.

Model structure	df	AIC	AICd	L	AICw
s(Day of year, by=sex) + sex + longevity + last season + s(age, by=season)	**35**	**-5110.06**	**0.00**	**1.000**	**0.284**
s(Day of year, by=sex) + sex + last season + s(age, by=season)	**34**	**-5109.90**	**0.16**	**0.922**	**0.262**
s(Day of year, by=sex) + sex + s(age, by=season)	**33**	**-5109.20**	**0.86**	**0.650**	**0.185**
s(Day of year, by=sex) + sex + last season + last season:sex + s(age, by=season)	**35**	**-5109.06**	**1.01**	**0.604**	**0.172**
s(Day of year, by=sex) + sex + longevity + s(age, by=season)	**34**	**-5107.30**	**2.76**	**0.252**	**0.071**
s(Day of year, by=sex) + sex + longevity + longevity:sex + s(age, by=season)	35	-5105.32	4.74	0.093	0.027
s(Day of year, by=sex) + last season + s(age, by=season)	33	-5036.31	73.76	0.000	0.000
s(Day of year, by=sex) + longevity + last season + s(age, by=season)	34	-5034.31	75.76	0.000	0.000
s(Day of year, by=sex) + s(age, by=season)	32	-5033.61	76.46	0.000	0.000
s(Day of year, by=sex) + longevity + s(age, by=season)	33	-5033.02	77.05	0.000	0.000
s(Day of year, by=sex) + sex + longevity + last season + s(age, by=sex) + longevity:sex	36	-4997.70	112.37	0.000	0.000
s(Day of year, by=sex) + sex + last season + s(age, by=sex) + last season:sex	35	-4996.33	113.73	0.000	0.000
s(Day of year, by=sex) + sex + longevity + last season + s(age, by=sex)	35	-4996.19	113.87	0.000	0.000
s(Day of year, by=sex) + sex + s(age, by=sex)	33	-4995.86	114.21	0.000	0.000
s(Day of year, by=sex) + sex + longevity + last season + s(age, by=sex) + longevity:sex + last season:sex	37	-4995.75	114.31	0.000	0.000
s(Day of year, by=sex) + sex + longevity + s(age, by=sex) + last season:sex	36	-4994.98	115.09	0.000	0.000
s(Day of year, by=sex) + sex + longevity + s(age, by=sex) + longevity:sex	35	-4994.89	115.17	0.000	0.000
s(Day of year, by=sex) + sex + s(age) + last season + last season:sex	33	-4994.53	115.54	0.000	0.000
s(Day of year, by=sex) + sex + s(age)	31	-4994.51	115.55	0.000	0.000
s(Day of year, by=sex) + sex + s(age) + longevity + last season	33	-4994.42	115.64	0.000	0.000
s(Day of year, by=sex) + sex + longevity + s(age, by=sex)	34	-4994.08	115.99	0.000	0.000
s(Day of year, by=sex) + sex + s(age) + longevity + last season + longevity:sex	34	-4993.42	116.64	0.000	0.000
s(Day of year, by=sex) + sex + s(age) + longevity + last season + last season:sex	34	-4993.14	116.93	0.000	0.000
s(Day of year, by=sex) + sex + s(age) + longevity	32	-4992.69	117.38	0.000	0.000
s(Day of year, by=sex) + sex + s(age) + longevity + last season + longevity:sex + last season:sex	35	-4991.66	118.40	0.000	0.000
s(Day of year, by=sex) + sex + s(age) + longevity + longevity:sex	33	-4991.53	118.53	0.000	0.000
s(Day of year, by=sex) + s(age, by=sex)	32	-4930.38	179.69	0.000	0.000
s(Day of year, by=sex) + s(age) + longevity + last season	32	-4924.01	186.06	0.000	0.000
s(Day of year, by=sex) + s(age) + longevity	31	-4923.53	186.53	0.000	0.000
s(Day of year, by=sex) + s(age)	30	-4922.02	188.04	0.000	0.000
s(Day of year, by=sex) + sex + longevity + last season + longevity:sex + last season:sex	33	-4829.41	280.65	0.000	0.000
s(Day of year, by=sex) + sex + longevity + longevity:sex	31	-4824.31	285.75	0.000	0.000
s(Day of year, by=sex) + sex	29	-4778.52	331.55	0.000	0.000
s(Day of year, by=sex) + sex + last season + last season:sex	31	-4775.77	334.29	0.000	0.000
s(Day of year, by=sex) + longevity + last season + longevity:sex	31	-4772.45	337.61	0.000	0.000
s(Day of year, by=sex) + longevity + last season	30	-4769.50	340.56	0.000	0.000
s(Day of year, by=sex) + longevity	29	-4761.17	348.89	0.000	0.000
s(Day of year, by=sex)	28	-4696.35	413.71	0.000	0.000
s(Day of year, by=sex) + last season	29	-4694.46	415.60	0.000	0.000
s(age)	26	-3930.16	1179.90	0.000	0.000
sex	25	-3862.09	1247.97	0.000	0.000
longevity	25	-3800.88	1309.18	0.000	0.000
last season	25	-3788.04	1322.02	0.000	0.000
-	24	-3779.94	1330.12	0.000	0.000

Sex-specific seasonal smoother, subpopulation and year of measurement included as forced fixed variables. Temporal within-individual autocorrelation and random structure (individual nested within cohort) applied to all models.
s(age, by=season) = season-specific smoother of age; s(age, by=sex) = sex-specific smoother of age

Table S3: Model selection for body mass of aged animals in captivity (C5). Set of candidate models for the captive population aged animals (C5): confidence set of models (cumulative AICw>0.98) receiving most support for predicting the body mass in bold. N=339 measurements from 105 individuals.

Model structure	df	AICc	AICΔ	LogLik	AICw
season + sex + age + longevity	**8**	**-337.99**	**0.00**	**1.000**	**0.563**
season + sex + age + longevity + age:season	**9**	**-337.058**	**0.93**	**0.628**	**0.354**
season + sex + age + longevity + longevity:sex + age:season	**10**	**-334.054**	**3.94**	**0.140**	**0.079**
season + sex + age + longevity + longevity:sex + age:season + age:season:sex	12	-326.207	11.78	0.003	0.002
season + sex + age + longevity + season:sex + age:sex + age:season + age:season:sex	12	-324.444	13.55	0.001	0.001
season + sex + age + age:sex + age:season	9	-324.181	13.81	0.001	0.001
season + sex + age	7	-323.243	14.75	0.001	0.000
season + sex + age + age:sex	8	-323.14	14.85	0.001	0.000
season + age + longevity + age:season	8	-323.045	14.94	0.001	0.000
season + sex + age + age:season	8	-322.891	15.10	0.001	0.000
season + age + age:season	7	-316.865	21.12	0.000	0.000
season + age	6	-316.824	21.17	0.000	0.000
season + age + last season + age:season	8	-316.779	21.21	0.000	0.000
season + sex + age + season:sex + age:season	9	-315.364	22.63	0.000	0.000
season + sex + longevity	7	-312.688	25.30	0.000	0.000
season + sex + last season + last season:sex	8	-312.289	25.70	0.000	0.000
season + sex	6	-311.101	26.89	0.000	0.000
season + sex + age + season:sex + age:sex + age:season + age:season:sex	11	-310.406	27.58	0.000	0.000
season + last season	6	-309.367	28.62	0.000	0.000
season + sex + longevity + longevity:sex	8	-308.371	29.62	0.000	0.000
season + sex + age + last season + season:sex + age:sex + age:season + age:season:sex	12	-308.297	29.69	0.000	0.000
season + sex + age + last season + season:sex + age:sex + last season:sex + age:season + age:season:sex	13	-307.919	30.07	0.000	0.000
season + sex + season:sex	7	-303.797	34.19	0.000	0.000
Season	5	-302.028	35.96	0.000	0.000
season + longevity	6	-298.792	39.20	0.000	0.000
sex + last season	6	-288.892	49.10	0.000	0.000
last season	5	-280.309	57.68	0.000	0.000
sex + longevity	6	-269.882	68.11	0.000	0.000
sex + longevity + longevity:sex	7	-264.064	73.93	0.000	0.000
Sex	5	-262.05	75.94	0.000	0.000
sex + age + age:sex	7	-255.243	82.75	0.000	0.000
Longevity	5	-254.927	83.06	0.000	0.000
Age	5	-252.916	85.07	0.000	0.000

Random effects: individual identity nested within cohort.

Table S4: Model selection for body mass of aged animals in the wild (W5). Set of candidate models for the wild population aged animals (W5): single best model (AICw>0.90) in bold. N=316 measurements from 70 individuals.

Model structure	df	AICc	AICd	L	AICw
season + sex + season:sex	**7**	**-262.75**	**0.00**	**1.000**	**0.908**
season	7	-257.71	5.04	0.080	0.073
season + sex	7	-253.78	8.97	0.011	0.010
season + last season	7	-252.86	9.89	0.007	0.006
season + longevity	7	-249.21	13.54	0.001	0.001
season + age	9	-248.42	14.33	0.001	0.001
season + sex + age + season:sex + age:season	9	-246.84	15.91	0.000	0.000
season + sex + last season + last season:sex	8	-245.91	16.84	0.000	0.000
season + sex + age	9	-244.49	18.26	0.000	0.000
season + age + age:season	8	-243.56	19.19	0.000	0.000
season + sex + age + age:sex	9	-239.81	22.94	0.000	0.000
season + sex + age + age:season	10	-239.74	23.01	0.000	0.000
season + age + last season + age:season	8	-238.81	23.94	0.000	0.000
season + sex + longevity + longevity:sex	8	-238.45	24.30	0.000	0.000
season + sex + age + season:sex + age:sex + age:season + age:season:sex	8	-237.17	25.58	0.000	0.000
season + sex + age + longevity	10	-236.30	26.45	0.000	0.000
season + age + longevity + age:season	10	-235.40	27.35	0.000	0.000
season + sex + age + age:sex + age:season	10	-234.12	28.63	0.000	0.000
season + sex + age + last season + season:sex + age:sex + age:season + age:season:sex	9	-231.28	31.47	0.000	0.000
season + sex + age + longevity + season:sex + age:sex + age:season + age:season:sex	10	-228.97	33.78	0.000	0.000
season + sex + age + last season + season:sex + age:sex + last season:sex + age:season + age:season:sex	10	-227.82	34.93	0.000	0.000
season + sex + age + longevity + longevity:sex + age:season	10	-224.67	38.08	0.000	0.000
season + sex + age + longevity + longevity:sex + age:season + age:season:sex	11	-214.24	48.51	0.000	0.000
last season	12	-81.50	181.25	0.000	0.000
sex + last season	11	-76.84	185.91	0.000	0.000
sex	13	-75.01	187.74	0.000	0.000
longevity	14	-73.09	189.66	0.000	0.000
age	14	-70.24	192.51	0.000	0.000
sex + longevity	14	-69.53	193.22	0.000	0.000
sex + longevity + longevity:sex	15	-62.82	199.93	0.000	0.000
sex + age + age:sex	8	-61.10	201.65	0.000	0.000

Subpopulation identity entered as a forced fixed factor in all models. Random effects: individual identity nested within cohort + year of measurement

Age-specific body mass values from the raw data

Captivity

Table S5: Average age-specific body mass (mean ± SD) in captivity

	Males		Females	
Age (years)	LDs	SDs	LDs	SDs
1	NA	101.5 ± 15.3	NA	110.1 ± 13.6
2	82.3 ± 12.8	106.0 ± 17.8	94.7 ± 14.5	115.7 ± 16.3
3	85.5 ± 14.4	108.0 ± 19.2	98.3 ± 14.8	114.3 ± 15.2
4	89.0 ± 16.0	106.3 ± 18.7	98.8 ± 16.6	113.8 ± 13.5
5	85.4 ± 14.3	99.8 ± 19.1	98.6 ± 16.1	110.8 ± 14.6
6	87.5 ± 16.7	101.2 ± 22.9	94.6 ± 12.0	103.7 ± 17.7
7	89.3 ± 16.3	102.0 ± 16.4	91.3 ± 6.3	105.0 ± 13.4
8	101.6 ± 10.5	96.6 ± 10.2	84.8 ± 14.9	97.1 ± 0.0
9	94.5 ± 9.0	86.6 ± 13.5	95.0 ± 0	NA
10	93.0 ± 8.9	96.9 ± 11.5	NA	NA
11	83.4 ± 8.4	NA	NA	NA

Age at death: males= 4.7 ± 1.8; females= 4.2 ± 1.5

Mean lifetime body mass: males= 95.4 ± 18.8; females= 105.0 ± 17.0

Body mass in last season: males= 80.6 ± 15.7; females= 94.8 ± 15.4

Wild

Table S6: Average age-specific body mass (mean ± SD) in the natural population

	Males		Females	
Age (years)	Dry season	Rainy season	Dry season	Rainy season
1	62.2 ± 7.6	61.5 ± 7.7	62.4 ± 10.0	71.0 ± 11.8
2	64.9 ± 7.4	69.3 ± 11.8	64.6 ± 9.7	78.4 ± 11.4
3	65.4 ± 7.5	77.3 ± 14.1	63.9 ± 9.2	82.7 ± 11.9
4	67.1 ± 7.7	75.2 ± 13.2	64.9 ± 9.3	87.8 ± 16.5
5	67.4 ± 9.0	77.9 ± 14.2	64.8 ± 10.2	88.3 ± 16.0
6	63.9 ± 9.0	79.3 ± 16.3	66.3 ± 8.9	87.6 ± 14.2
7	59.0 ± 0.0	NA	66.0 ± 7.1	91.2 ± 7.04
8	65.0 ± 9.9	NA	61.4 ± 6.7	83.0 ± 10.3
9	59.0 ± 5.0	52.0 ± 0.0	56.8 ± 11.4	98.3 ±_ 5.5
10	NA	NA	NA	103.5 ± 6.9

Age at last capture: males= 2.7 ± 1.4; females= 3.4 ± 2.0

Mean lifetime body mass: males= 64.8 ± 9.6; females= 71.1 ± 14.5

Body mass in last season: males= 64.9 ± 9.6; females= 72.4 ± 16.2

CHAPTER 3

LOSING GRIP: SENESCENT DECLINE IN PHYSICAL STRENGTH IN A SMALL-BODIED PRIMATE IN CAPTIVITY AND IN THE WILD

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ABSTRACT

Muscle strength reflects physical functioning, declines at old age and predicts health and survival in humans and laboratory animals. Age-associated muscle deterioration causes loss of strength and likely directly influences fitness and survival of wild animals. However, the effects of age and life-history characteristics on muscle strength in wild animals are unknown. We investigated environment- and sex-specific patterns of physical functioning by measuring grip strength in wild and captive gray mouse lemurs. We expected more pronounced strength senescence in captivity due to rapid condition-dependent mortality in nature. Males were predicted to be stronger but experience more severe senescence than females, as found in other species. We found similar senescent declines in all groups except wild males, which showed little decline, presumably due to their early mortality. Captive animals were generally weaker and showed earlier declines than wild animals. Unexpectedly, females tended to be stronger than males, especially in the reproductive season in the wild. Universal intrinsic mechanisms (e.g. sarcopenia) likely cause the similar patterns of strength loss across settings. The female advantage in muscle strength merits further study; it may follow higher investments by males into reproduction, or be an adaptation associated with the female social dominance found in lemurs.

KEY-WORDS

Functional aging, grip strength, *Microcebus murinus*, natural population, sarcopenia, sex difference

INTRODUCTION

As an organism reaches an advanced age, senescent changes at the molecular level cause functional declines [Monaghan et al. 2008; Nussey et al. 2013] that likely contribute to the frequently observed increasing mortality risk at old age [Gaillard 1994; Ricklefs and Scheuerlein 2001]. Senescent declines in physical performance are well known from captive conditions [Demontis et al. 2013; Guarente and Kenyon 2000; Languille et al. 2012], but until quite recently senescence was thought to rarely manifest under natural conditions due to ecological pressures removing individuals from the population before age-related declines became evident [Hayflick 2000; Williams 1957]. Recent studies have nevertheless been able to show that ageing adversely affects the functioning of individuals that survive beyond the average lifespan in natural populations ([Nussey et al. 2008], but see [Rueppell et al. 2007]). However, survival tends to be condition-dependent in nature (snowshoe hares [Murray 2002], ungulates [Nussey et al. 2011]) hence selection may favor increased somatic maintenance [Maklakov and Lummaa 2013; Williams and Day 2003; Williams et al. 2006] that could increase predator-avoidance success, competitive ability and lifespan and thus reduce the age-related loss of function.

The physical functioning of an organism depends largely on the quality and quantity of its skeletal muscle. Aging-related declines in muscle mass, quality and strength have been studied in detail in

humans and, more recently, in animal model organisms [Demontis et al. 2013]. In humans, the age-related loss of skeletal muscle mass and function, termed sarcopenia [Cruz-Jentoft et al. 2010], commonly causes loss of body mass [Baumgartner 2000; Cohn et al. 1980] and muscle strength [Evans and Campbell 1993] in the elderly. Sarcopenia is reflected in functional measures, such as reduced balance performance [Laughton et al. 2003] and muscle strength [Cruz-Jentoft et al. 2010]. It is associated with frailty and disability [Janssen et al. 2002; Rantanen et al. 1999] and all-cause mortality in aged humans [Cooper et al. 2010; Gale et al. 2007; Ling et al. 2010; Metter et al. 2002; Rantanen 2003] as well as laboratory mice and rats [Carter et al. 2002; Fahlström et al. 2012; Ingram 2000]. The clinical consequences of sarcopenia make it an issue of increasing significance in the rapidly aging human societies and the topic of a large body of clinical research.

Muscle strength in adulthood and the rate of sarcopenia differs for the sexes in humans [Doherty 2001; Doherty 2003; Gallagher et al. 1997; Janssen et al. 2002]. Probably due to differences in body composition and the higher initial muscle mass of males, sarcopenia is more prevalent in women [Janssen et al. 2000] but causes higher rates of disability in men [Baumgartner et al. 1998]. This is consistent with the prediction of sex-specific rates of senescence postulated by life-history theory: in species in which males must allocate substantial energy into gaining access to females, males can be expected to show adaptations that improve competitive ability, but which may reduce longevity. The lifetime fitness of females, on the other hand, tends to be improved via better health, leading to a prolonged reproductive lifespan [Austad 2006; Bonduriansky et al. 2008; Maklakov and Lummaa 2013]. Consistent with these hypotheses, males typically experience earlier mortality [Bonduriansky et al. 2008] and tend to show more rapid senescent functional declines than females [Bowen et al. 2006; Galimberti et al. 2007; Hoffman et al. 2010; Tafani et al. 2013].

Although sarcopenia research has largely focused on humans, age-related declines in muscle mass and changes in muscle composition have also been found in mammal and invertebrate species in laboratory conditions [Altun et al. 2007; Demontis et al. 2013; Piccirillo et al. 2014] and in some wild populations [Hindle et al. 2009a; Hindle et al. 2009b; Hindle et al. 2010; Lailvaux et al. 2011], as well as in domestic animals [Freeman 2012]. The universality of this phenomenon suggests common underlying physiological mechanisms across taxa. Despite these advances, the significance of sarcopenia in nature is largely unknown. In wild animals, muscle atrophy would almost certainly lower the physical performance required for foraging, reproduction and predator avoidance and might directly impair survival or reproductive output of individuals in an inferior condition. Consequently, environmental hazard would cause the selective disappearance of individuals from a wild population in a condition-dependent manner, whereas, in the absence of natural risk factors, aged animals in captivity might exhibit declines in functioning and strength. The use of laboratory model species might therefore lead to an overestimation of the ecological relevance of functional declines, whereas measuring functional senescence in the wild might underestimate the magnitude of intrinsic declines, which are likely shaped by evolutionary processes such as condition-dependent mortality and sex-specific reproductive investment. Despite this, physical strength has rarely been assessed in wild animals (bite force in birds [Herrel et al. 2005] and small mammals: [Becerra et al. 2013; Becerra et al. 2011; Freeman and Lemen 2008; Santana et al. 2010], bite force and grip performance in reptiles (e.g. [Herrel et al. 2007; Herrel et al. 2013; McBrayer and Anderson 2007;

Silva et al. 2014; Wikelski and Trillmich 1994]). Even fewer studies have addressed sex differences in strength [Becerra et al. 2013; Becerra et al. 2011; Herrel et al. 2007; McBrayer and Anderson 2007; Silva et al. 2014] and none have explored senescent changes in strength. Strength may, however, be a better indicator of functional senescence than changes in muscle mass, since muscle strength directly influences the functioning of the individual, declines at a rate much higher than muscle mass [Goodpaster et al. 2006; Metter et al. 2002], and predicts mortality more reliably than muscle mass senescence in humans [Metter et al. 2002].

The purpose of this study was to clarify the roles of intrinsic and ecological factors in shaping functional senescence. To this end, we examined the age trajectories of grip strength in male and female gray mouse lemurs (*Microcebus murinus*, Cheirogaleidae, small-bodied (60 g) strepsirrhine primate, Figure 1) under captive and natural conditions. The gray mouse lemur is emerging as a primate model of aging in captivity as it is relatively short-lived and matures early, at 6-8 months of age [Castanet et al. 2004; Némoz-Bertholet and Aujard 2003], yet shows aging processes similar to humans [Languille et al. 2012]. Data from captivity reveal senescent declines in measures of functioning such as body mass and balance performance beginning around 4-5 years of age [Languille et al. 2012; Némoz-Bertholet and Aujard 2003], but no evidence of body mass senescence has been found in the wild [Hämäläinen et al. 2014a]. Grip strength and hand dexterity are particularly important for the species due to its ecology: it is an omnivorous, arboreal primate that experiences high predation pressure. Therefore, food acquisition and manipulation (e.g. capturing flying insects), substrate use and predator avoidance all involve the use of hand grip, making grip strength an ecologically relevant measurement of muscle strength in the gray mouse lemur.

The species is, overall, sexually monomorphic, hence size dimorphism should not cause significant differences in strength between the sexes, but sex-specific behaviors (female-biased torpor use [Schmid and Kappeler 1998] and male roaming in the mating season, [Eberle and Kappeler 2004b]) cause seasonal fluctuation in body mass sex dimorphism and, possibly, body composition. There is also a known male bias in mortality in the wild that escalates during the mating season [Kraus et al. 2008]. Due to these life-history differences, we were particularly interested in addressing the potential sex differences in physical functioning.

We established age trajectories of forelimb grip strength in a captive population that experiences no extrinsic mortality and should therefore reflect intrinsic functional deterioration in a manner comparable with studies on other captive model species. To test whether the oldest surviving animals in the wild also experience functional senescence, we examined grip strength in the natural environment. While senescent decline in grip strength in the wild animals might be absent or modest due to high rates of extrinsic mortality in the natural population that rapidly eliminates individuals with declining condition (as found for body mass senescence [Hämäläinen et al. 2014a]), captive animals were expected to show senescent declines in grip strength, since conditions in captivity are standardized, extrinsic mortality is absent and muscle-preserving exercise is limited. Because the seasonally varying resource availability, fluctuating energetic demands and the use of existing tissue (fat and muscle) for energy might have important consequences for physical strength, we measured strength in both sexes across ecological seasons in the wild. Based on sex differences found in other

species [Crabbe et al. 2003; Doherty 2001; Van Damme et al. 2008] we expected males to have higher muscle strength than females throughout life, but possibly lose strength at a more rapid rate towards old age.

METHODS

Study populations

For the captive part of the study, we used adult individuals from a breeding colony in Brunoy, France, where animals are maintained at a constant temperature and fed a balanced diet *ad libitum* (see [Némoz-Bertholet and Aujard 2003; Perret 1997] for details). All grip strength measurements were made within one week in February 2012. The animals were housed in 7 different rooms, and each room was in a slightly different stage of the annual photoperiodic schedule (enforced to trigger seasonal behaviors in captivity [Perret 1997; Perret and Aujard 2001]) in transition from long day to short day length conditions. This stage marks the onset of fattening, i.e. is roughly comparable to early rainy season in the wild.

The wild population (locally known as the "N5" population) has been monitored since year 2000 in a 25 ha study site of dry deciduous forest in Kirindy/CNFEREF, central Western Madagascar by researchers of the German Primate Center (DPZ). The area experiences pronounced seasonal variation with a distinct dry season (~May-November), and a rainy season (~November-April), with consequent variation in food availability [Dammhahn and Kappeler 2008], leading to substantial seasonal, sex-specific body mass fluctuation in the gray mouse lemurs [Hämäläinen et al. unpublished, Schmid and Kappeler 1998]. The brief mating season takes place at the end of the dry season (mid-October to November) and offspring are weaned by the end of the rainy season (March-April) [Eberle and Kappeler 2004a]. For long term data collection, trapping has been conducted at minimum 6 times per year (monthly in March-May and September-November) with Sherman live catch traps baited with small pieces of banana. All captured animals are equipped with an individual subcutaneous transponder (Trovan EURO ID, Germany) at first capture, typically as juveniles. The capture and handling protocols have been detailed elsewhere [Dammhahn and Kappeler 2008; Eberle and Kappeler 2004a; Hämäläinen et al. 2014b].

Quantifying grip strength

Grip strength was measured with a method adapted from rodent studies (e.g. [Ingram 1983; Justice et al. 2013; Smith et al. 1995]) where it has become a standard measure of muscle dexterity and has been shown to reliably predict overall motor function [Justice et al. 2013]. A force gauge (Chatillon LG Series Mechanical Force Gauge 50 N, 5 ± 0.025 kg) was mounted horizontally on a stand and attached to a metal grid. The gauge was set to record the maximum strength of a pull. The test subject was held by the rump and lifted so that their front paws were in reach of the metal grid, which usually induced the animal to grasp the bar closest to them (Figure 1). Once the animal was holding firmly with both hands, it was steadily but rapidly pulled horizontally away from the power gauge until it

released both hands from the bar. The procedure was repeated three times per individual in rapid succession, only recording grips where both hands of the animal were on the same bar of the grid. If the individual refused to grasp the grid, the testing was discontinued (< 5% of sessions). The maximum force was recorded for each grip attempt and the highest of these measurements was considered as the maximum grip strength of the animal at that time (e.g. [Gale et al. 2007; Herrel et al. 2013; Kallman et al. 1990]).

Figure 1. Grip strength testing method. A gray mouse lemur (*Microcebus murinus*) individual is held by the rump, allowed to grasp a metal bar and then pulled away from the bar until it releases its grip. The metal bar is attached to a power gauge that records the maximum pull force (in grams) exerted.

In captivity, 118 individuals (aged 2-11 years) were tested once each. In the wild, a total of 351 measurements from 142 individuals (aged 2-10 years) were collected over two late dry seasons (September-November, *N* = 151/75 (measurements/individuals)) and two late rainy seasons (March-May, *N* = 200/112) in the years 2010-2012. All measurements were made by the same person (AH). The body mass (with precision ± 1 g) of each individual was recorded within three days of the grip strength measurement with an electronic scale (captivity) or Pesola spring scale (wild).

Statistical analyses

We expected body mass to influence grip strength as has been shown for e.g. humans [Rantanen et al. 1998], mice [Justice et al. 2013] and marine iguanas [Wikelski and Trillmich 1994], hence we first explored this relationship via visual inspection of the data (Figure 2) and by linear regression models. We found a strong quadratic relationship between body mass and maximum grip strength in both, the captive (intercept: -283.267 ± 453.801, linear term β = 26.68 ± 8.55, *df* = 135, *t* = 3.12, *P* = 0.002, quadratic term: β = -0.11 ± 0.04, *t* = -2.76, *P* = 0.007) and the wild data (intercept= -38.25 ± 273.61, linear term: β = 28.01 ± 6.76, *df* = 348, *t* = 4.15, *P* < 0.001, quadratic term: β = -0.120 ± 0.039, *t* = -3.04, *P* = 0.003). We therefore used the "residual grip strength" calculated from a quadratic linear regression on body mass (separately for the captive and wild data set) as the response variable in all further analyses. This approach permitted us to account for the body mass effects while keeping the subsequent models simple enough to draw meaningful conclusions. To confirm the robustness of our

findings, the analyses were repeated using grip strength corrected in alternative ways as the response variable: these entailed the uncorrected maximum grip strength; a linear correction (as typically applied in most other studies, e.g. [Fahlström et al. 2012]); and, with a smaller sample size, grip strength corrected for body condition (body mass scaled to structural size using the scaled mass index [Peig and Green 2009; Vuarin et al. 2013]). In each case, the results were qualitatively similar (data not shown) to those based on the quadratic correction we applied. The residual models were used for inference on the influence of sex and age, but for demonstrative and comparative purposes, we also report "absolute grip strength" (maximum grip (g)) and "relative grip strength" (maximum grip (g)/body mass (g)).

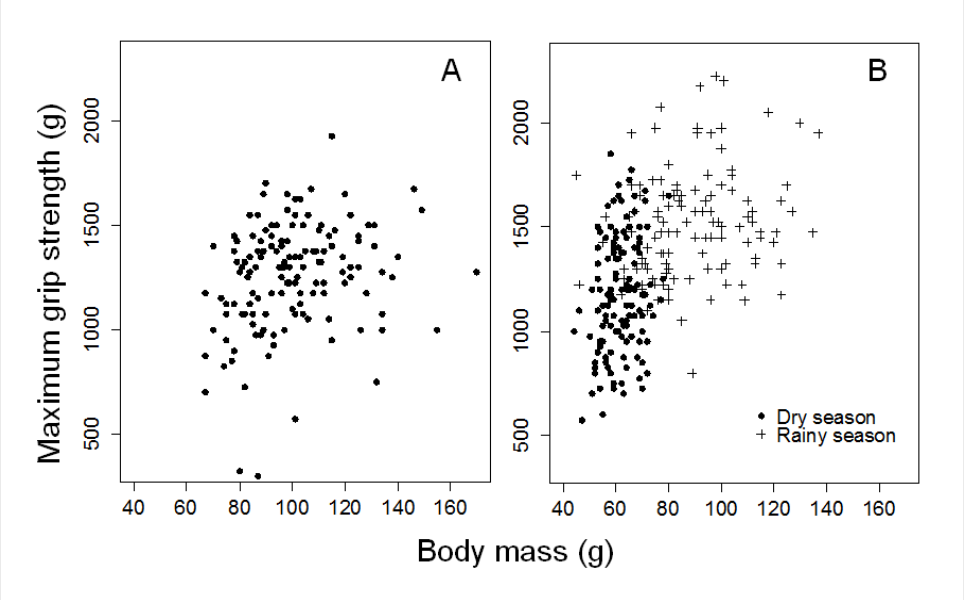

Figure 2: The relationship between body mass and maximum grip strength in captive (A) and wild (B) mouse lemurs.

To study the effects of sex and age on residual grip strength, we built linear mixed models (LMM) separately for the captive and wild data using the R-package *lme4* [Bates et al. 2013]. We fitted a complex model with restricted maximum likelihood and inferred effect sizes based on this model [Forstmeier and Schielzeth 2011]. In both data sets, the model included the terms sex, linear and quadratic age effects and their interaction terms with sex. For the wild data, the ecological season (dry or rainy) and its interactions with sex and age, the three-way interactions season*sex*age and season*sex*age^2, as well as the year of testing (2010-2011 or 2012) were additionally included in the fixed effects structure of the model. Because the group of animals within a given room in captivity and the photoperiodic stage they were exposed to at the time of testing could not be disentangled in our experimental setting, the group (room) was used as a random effect to account for the photoperiodic variation in captivity. In the wild data, individual identity was introduced as a random effect to account for repeated measurements of the same individuals. P-values based on

Satterthwaite's approximations were computed using the R package *lmerTest* [Kuznetsova et al. 2014]. The results were confirmed by backward selection [Zuur et al. 2009] in which the least influential term ($P > 0.05$) was removed in turn from the model and the reduced model compared with the inclusive model using Likelihood Ratio Tests (LRT). Both methods identified the same variables as significant.

Age and body mass were log-transformed, centered and scaled (mean/SD) prior to the analyses to improve the interpretability of polynomials and interactions [Grueber et al. 2011; Schielzeth 2010]. The normality and homogeneity of error assumptions were examined using residual plots for the most complex model for each data set. All analyses were performed in R version 3.0.3 [R Development Core Team 2014]. The significance level was set to $P \leq 0.05$

RESULTS

In terms of absolute strength, the captive and wild animals had similar levels of grip strength at a given age (Table 1). However, since the wild animals had, on average, a much lower body mass than the captive animals, the relative strength was higher in the wild than in the captive animals: on average, a mouse lemur female in prime adulthood (first age tertile corresponding to age 2-3 years) could withstand a force nearly 20 times its own body mass in the wild and 14 times its body mass in captivity. The loss of strength by the last age tertile (age ≥ 7 years) was higher in captivity (Table 1). Based on the age-specific averages, the decline in strength began slightly earlier in captivity than in the wild. In captivity, both absolute (1395 ± 217 g (mean ± SD)) and relative grip (15.1 ± 2.6) were at their highest at age 2 years. In the wild population, mean absolute strength (1398 ± 279 g) was highest at age 4, and mean relative strength (19.2 ± 5.2) at age 3 years (except a single individual measured at age 8 years with a relative strength of 19.8).

Table 1: Grip strength in wild and captive mouse lemurs in early adulthood and at old age. Shown are absolute and relative grip strength (mean ± SD) and the percentage decline in strength from the first to third age tertile, as well as predicted values from LMMs estimating residual strength for ages 2 and 7 years in the dry and rainy season.

		Absolute strength (g)			**Relative strength** [1]			**Predicted residual strength** [2]	
		1st tertile [3]	**3rd tertile** [4]	**% change**	**1st tertile** [3]	**3rd tertile** [4]	**% change**	**2 years**	**7 years**
Captive	Female	1430 ± 203	1100 ± 391	-23	14.3 ± 2.6	9.4 ± 3.5	-34	180.09	-161.53
	Male	1295 ± 181	1096 ± 310	-15	14.5 ± 2.8	11.2 ± 3.1	-23	127.98	-130.34
Wild	Female	1401 ± 308	1100 ± 242	-21	19.6 ± 5.2	13.9 ± 3.3	-29	182.45 / 190.34	-68.55 / -60.65
	Male [5]	1227 ± 348			18.0 ± 4.5			40.50 / 219.64	-210 / -31.35

[1] Maximum grip (g) / body mass (g)

[2] Predictions from LMMs (using untransformed age) for captive and wild data (dry season / rainy season) for ages 2 and 7 years, based on measurements made in year 2012 (*N* = 135).

[3] First age tertile, age 2-3 years

[4] Last age tertile, age

[5] No wild males were alive in the third tertile.

In captivity, the negative effects of linear and quadratic terms of age were significant (Table 2): the animals' grip strength declined throughout adulthood, with the rate of decline accelerating after approximately 5 years of age (Figure 3 A, Table 2). There was no statistically significant sex difference in the rate of change for residual strength. In terms of absolute strength, however, females were initially stronger (females: 1430 g, males: 1295 g, i.e. a 9% difference) and lost 23% of strength from the first to the last age tertile, whereas males declined by only 15% in the same time frame (Table 1). Thus, contrary to our predictions and findings from other species, females were initially stronger than males, but declined more rapidly, with the absolute strength at old age converging for the sexes.

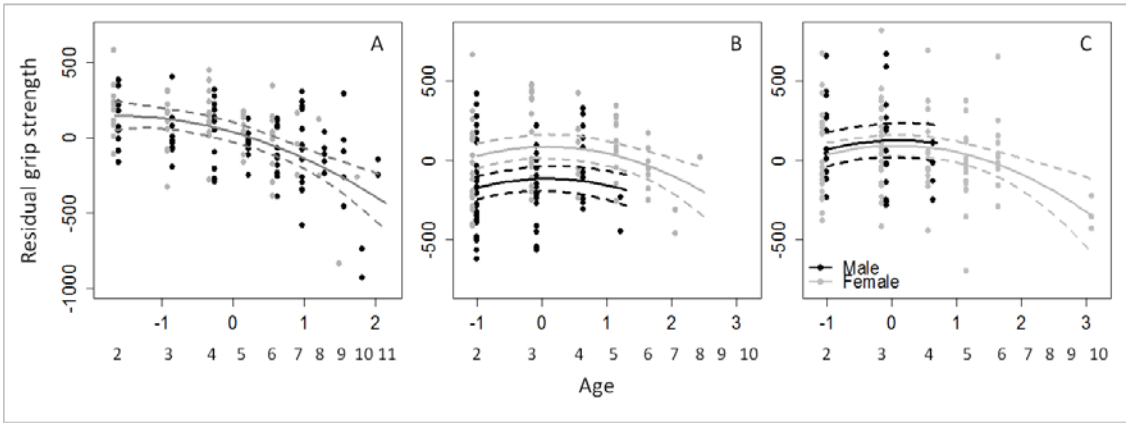

Figure 3: Residual grip strength as a function of age in captive animals (A) and wild males and females in the dry (B) and rainy (C) season. Solid lines show predictions from the best model for each setting and the dashed lines indicate the associated 95% confidence bands. X-axes represent scaled and centered age (top row) and the corresponding chronological age in years (bottom row).

In the wild, the terms quadratic age, the interaction term sex*season and the covariate year of measurement had significant effects (Table 2, Figures 3 B and 3 C). Overall, females had higher residual grip strength than males, but this effect was entirely due to the strong sex difference in the dry season (Figure 3 C), whereas the sex difference was negligible in the rainy season (Figure 3 B). Grip strength of both sexes showed little change until approximately 4-5 years of age, after which strength declined, particularly in the old surviving females. The patterns were similar for males until age 4-5 years, however, no data are available for males older than that due to high male mortality.

Table 2: Parameter estimates for variables influencing residual grip strength in captivity and in the wild based on the most complex model in each data set. The terms that were included in the final model after backward selection are shown in bold.

	Captivity [1]				**Wild** [2]			
Term	**β**	**SE**	**t**	**P**	**β**	**SE**	**t**	**P**
Intercept	**81.72**	**42.76**	**1.91**	**0.07**	**26.69**	**54.99**	**0.49**	**0.63**
Sex (ref. Female)	-58.85	52.26	-1.13	0.26	**-235.15**	**71.85**	**-3.27**	**0.001**
Age	**-164.49**	**33.91**	**-4.85**	**<0.001**	**14.55**	**36.72**	**0.40**	**0.69**
Age^2	**-60.80**	**30.64**	**-1.98**	**0.05**	**-73.00**	**33.47**	**-2.18**	**0.03**
Sex:Age	41.48	42.57	0.97	0.33	11.35	69.09	0.16	0.87
Sex: Age^2	11.81	37.88	0.31	0.76	102.31	86.26	1.19	0.24
Season (ref. dry)					**-48.51**	**56.26**	**-0.86**	**0.39**
Season:Sex					**236.77**	**99.66**	**2.38**	**0.02**
Season:Age					-46.70	46.35	-1.01	0.31
Season: Age^2					44.09	37.96	1.16	0.25
Season:Sex:age					-273.11	154.87	-1.76	0.08
Season:Sex: Age^2					-228.76	174.84	-1.31	0.19
Year					**160.62**	**36.62**	**4.39**	**<0.001**

[1] Group entered as random effect, SD= 45.27.
[2] Individual identity entered as random effect, SD= 100.30.

DISCUSSION

By quantifying sex-specific age trajectories of physical strength, we discovered evidence for senescent declines in physical performance in both captive and wild populations of gray mouse lemurs. Contrasting our expectation of negligible senescence in the wild population, the magnitude of the age-related decline in strength was generally similar in captive and natural environments. However, the decline in absolute strength began earlier in captivity, which may reflect differences in muscle use between the captive and wild animals, or result from the selective extrinsic mortality in the natural population. Surprisingly, females tended to have higher grip strength than males in both the captive and wild populations, and this result was statistically significant for the wild population in the dry season.

The influence of body mass on grip strength

Grip strength was strongly associated with body mass, the relationship being similar in captivity and in the rainy season in the wild. In the lean, dry season, however, the relationship between body mass and maximum grip strength was distinctly stronger (Figure 2). This suggests that the body condition of the individual is a strong correlate of strength. Sex-specific, seasonal body mass fluctuations are known from both wild and captive mouse lemurs and likely play an important role in their functioning and survival [Perret 1997; Perret and Aujard 2001; Schmid and Kappeler 1998]. The body mass fluctuation is likely caused mainly by the fat gain and loss due to the seasonal variation in the energy balance, but muscle is probably also lost during the lean, dry season in the wild [Giroud et al. 2010]. Increasing muscle mass increases metabolism and is therefore expensive to maintain [Piccirillo et al. 2014; Sparti et al. 1997; Suarez 1996], which may be a concern in the dry season when torpor-using individuals fast, and food – particularly protein – availability may be lowered for the active animals. Therefore, seasonality places constraints on the optimal body composition and may influence strength across seasons. Based on our data set, strength is better retained than body mass across seasons (mean seasonal change in body mass: 30% and in absolute strength: 22%), suggesting that the body mass variation is, indeed, mainly due to fat [Giroud et al. 2010], which has little influence on absolute strength. On average, wild mouse lemurs could resist forces nearly 20 times their own body mass, which probably reflects the ecological significance of gripping performance (climbing, use of hands for hunting) in the species.

Age-related decline in muscle strength suggests sarcopenia in captive and wild animals

Overall, our results indicate that the decline in body mass-corrected muscle strength began in early adulthood and the rate of decline accelerated at old age (from age 4-5 years on) in both the captive and wild population, indicating senescence. The initial absolute strength of both sexes was similar in captivity and the wild. Contrary to our prediction of negligible decline in the wild, the rate of decline in females was nearly identical across settings. While data on body composition would be required to address the processes leading to such similarities, our results are suggestive of strong internal control of muscle aging. Old males were absent in the wild data set due to male-biased selective disappearance [Hämäläinen et al. 2014a; Kraus et al. 2008], which rendered inference about age-related changes in males unreliable.

The magnitude of decline in grip strength was 15% (males) to 23% (females), whereas in humans, >28% declines were observed from age 30-39 to 70+ years [Kallman et al. 1990]). Based on the average values of strength across age categories, senescence may begin earlier in captive than in wild mouse lemurs. This might be a result of the intensive extrinsic mortality in the wild population that rapidly removes individuals whose condition declines, leaving only very high quality individuals to live to an old age. The differences across settings may also be influenced by potential disuse atrophy in captivity, body composition differences and differences in the structure of the data (single measure per individual in

captivity, repeated measures in the wild). Since our data was cross-sectional and survival is condition-dependent in the species, our results may underestimate the age effects. Still, cross-sectional and longitudinal data on measures of functioning have been shown to correlate strongly with each other in humans [Kallman et al. 1990] and in rats [Altun et al. 2007]. Our findings are dissimilar to a previous study recording bite force in captive gray mouse lemurs, in which it was found that bite force increases with age until the fifth year and then shows slight decline [Chazeau et al. 2013]. The reasons for the difference between the age trajectories of bite force and grip strength are unclear, but might reflect differing rates of senescence of the muscle groups or differing levels of jaw and hand muscle use in captivity.

Sarcopenia develops via the reduced regenerative capacity of the aging muscle [Demontis et al. 2013; Proctor et al. 1998; Renault et al. 2002], which leads to senescent loss of muscle mass and strength [Baumgartner 2000; Cohn et al. 1980; Kallman et al. 1990]. Together, the loss of lean mass and strength therefore strongly indicate sarcopenia. While studies on strength senescence are absent in wild populations, senescent declines have been found in lean body mass in various species (humans [Baumgartner 2000; Justice et al. 2013; Kyle et al. 2001], rhesus macaques [Colman et al. 2005; Roth et al. 2004], ungulates [Mysterud et al. 2001; Nussey et al. 2011] , seals [Hindle et al. 2009a], shrews [Hindle et al. 2009b; Hindle et al. 2010], mice and invertebrates [Demontis et al. 2013]). Pending confirmation by quantification of muscle function, this suggests that sarcopenia may be more prevalent across taxa than thus far appreciated.

In an earlier study [Hämäläinen et al. 2014a] we found that the gray mouse lemurs' ability to regain lost body mass is maintained until very old age in the wild, whereas a distinct senescent decline in seasonal fattening is seen in captivity. As the captive animals lose both body mass and muscle strength despite *ad libitum* food availability, these declines are likely due to physiological processes such as sarcopenia or muscle tissue remodeling, although an age-related reduction in feeding drive may contribute to the decline [Morley and Malmstrom 2013]. The fact that a senescent decline in grip strength was seen in the wild in both seasons despite the absence of body mass senescence also suggests that changes in the muscle tissue or the replacement of muscle with fat in the oldest animals could cause the observed patterns.

The discrepancy in the patterns of body mass and strength senescence suggests that strength declines more than body mass with advancing age, as found previously in humans [Goodpaster et al. 2006; Metter et al. 2002]. The surviving high quality individuals may be capable of maintaining a high body condition due to behavioral adjustments and experience. The decline in strength, however, likely results from physiological processes leading to muscle decomposition [Demontis et al. 2013], which individuals cannot compensate for. Strength may therefore be more susceptible than body mass to evolutionary influences, but the loss of strength may not be detrimental enough to be selected against at the age and rate at which it occurs in nature. As muscle function begins to decline around the prime reproductive age in most species studied, including gray mouse lemurs, it may reflect universal mechanisms leading to

declines in somatic maintenance, thereby lending support to the disposable soma hypothesis of senescence [Kirkwood and Austad 2000; Maklakov and Lummaa 2013; Monaghan et al. 2008]. It is also plausible that grip strength in the species is initially so high relative to body mass, that even after a 20% decrease in absolute strength, sufficient muscle strength remains for basic functions. Finally, torpor use by wild mouse lemurs during the dry season may be partially responsible for the loss of muscle seen in the aged females, which are also the demographic group that most frequently utilizes torpor [Schmid and Kappeler 1998; Vuarin et al. 2013]. The associated long periods of inactivity potentially lead to muscle atrophy (but see [Harlow et al. 2001]), after which muscle regeneration may be impaired in aged animals.

Weak males in the dry season

Males are on average stronger than females in all other mammal species tested so far [Becerra et al. 2011; Crabbe et al. 2003; Doherty 2001; Van Damme et al. 2008], as well as reptiles (including size-matched animals [Herrel et al. 2007; Lourdais et al. 2006; Silva et al. 2014]) and amphibians [Navas and James 2007; Peters and Aulner 2000]. Consistent with this, males typically have greater skeletal muscle mass than females (e.g. [Colman et al. 2005; Doherty 2001]) and sex differences exist in the muscle fiber type ratio [Doherty 2001; Van Damme et al. 2008]. Suggested proximate mechanisms for the sex difference include endocrine differences (growth hormone, testosterone, insulin-like growth factor 1) [Demontis et al. 2013; Herrel et al. 2007; Janssen et al. 2000] and ultimately, the male advantage might parallel evolutionary mechanisms leading to size dimorphism, such as male-male competition (Isaac 2005).

Contrary to previous research, we found that in the gray mouse lemur, females had significantly higher grip strength than males in the dry season in the wild. No significant sex difference was evident in the rainy season, possibly due to the survival of only the best quality males through the mating season, or the strain of pregnancy and lactation reducing female strength. The sexes may also differ in how they metabolize macronutrients: muscle function may be fuelled by carbohydrates and protein to a higher proportion by males than females, whereas females use more fat [Roepstorff et al. 2002; Tarnopolsky 2000] and therefore are perhaps better able to retain muscle tissue. Since females are known to fatten more efficiently than males in the rainy season [Schmid 1999], they also have larger quantities of fat to utilize during the lean season. Male body mass increases before the onset of the mating season, but this increase appears to be androgen-driven and possibly caused by the substantial increase in testes size [Schmid and Kappeler 1998] (possibly via catabolism of existing tissue) rather than fat or muscle gain, and therefore likely has little influence on physical strength. In captivity, females also tended to be stronger than males, although this result was statistically non-significant. However, the effect of photoperiodic season could not be assessed in the captive population due to the short time window of data collection and the potentially confounding group effect.

Unlike many of the species tested previously, the gray mouse lemur is thought to be sexually monomorphic, although sex-specific seasonal body mass fluctuation creates body mass differences

between the sexes, with females being slightly larger overall (Hämäläinen et al. unpublished, [Schmid and Kappeler 1998]). The structural size dimorphism is so small (e.g. 1.3 % for bizygomatic distance, Hämäläinen & Dammhahn, unpublished data), however, that it is unlikely to cause substantial sex differences in body mass-corrected strength (and our results were confirmed using a body size-scaled mass index). Socially, gray mouse lemur females are dominant over males [Génin 2003; Pagès-Feuillade 1988; Radespiel et al. 1998; Radespiel and Zimmermann 2001]. Female dominance is common within the lemur clade [Kappeler 1993] and has been suggested to result from an evolutionary advantage for females of having a higher resource acquisition potential in the ecologically demanding environment of Madagascar [Jolly 1984; Wright 1999; Young et al. 1990]. It is possible that the same selective pressures act to produce both dominance and higher physical strength levels. This is the first study of muscle strength in a female dominant species and raises caution against considering a male advantage in strength a universal truth and invites further research into the role of hormones, body size as well as social and mating systems in determining patterns of strength and sarcopenia.

According to life history theory, female lifetime reproductive success is enhanced by a longer reproductive lifespan, whereas males should invest more in reproduction in prime adulthood despite an increased associated mortality risk [Bonduriansky et al. 2008; Rolff 2002; Trivers 1972]. Gray mouse lemur males that are larger or have better fighting abilities tend to have higher reproductive success [Eberle and Kappeler 2004b; Gomez et al. 2012]. Hence a decline in strength might lower the reproductive success of old males and select against increased male longevity. This effect, together with a general increase of risk-taking (or boldness) with age in males but not females [Dammhahn 2012] might lead to the female-biased survival observed in the wild [Hämäläinen et al. 2014a; Kraus et al. 2008]. Interestingly, we found that in captivity, males showed less severe declines than females in absolute strength, whereas wild males suffered early mortality and tended to be weaker than wild females. These patterns concur with those found for body mass senescence [Hämäläinen et al. 2014a], supporting the hypothesis that males face more intense condition-dependent mortality and consequently their rate of senescence is lowered. In captivity, as the extrinsic risk is removed, males may therefore age slowly and outlive females.

Male grip strength was at its lowest (and significantly lower than that of females) during the reproductive season. This may reflect the increased energy demands due to mate search and defense at the expense of foraging, as indicated by a lowered body mass of males after the mating season [Hämäläinen et al. 2014a; Schmid and Kappeler 1998], and the associated catabolic effects of potentially elevated glucocorticoid levels [Hasselgren 1999]. Similarly, in seals, an indicator of muscle quality (myocyte density) was lower in males than in females in the breeding season, leading the authors to speculate that muscle stress due to territory defense may cause the female advantage [Hindle et al. 2009a]. Testosterone could mediate these seasonal differences in males as it is connected to higher muscle mass and/or strength [Huyghe et al. 2010; Schulte-Hostedde et al. 2003], but this is in contradiction with the pattern observed in our study: male physical strength was at its lowest when testosterone levels are highest (inferred from captive data [Perret 1992] and variation in relative tested size in the wild [Schmid

and Kappeler 1998]). Likewise, bite force was not correlated with testosterone levels in male gray mouse lemurs [Chazeau et al. 2013]. Therefore, a more likely explanation for the lower strength of males in the mating season is muscle loss due to energy requirements associated with the mating season and the coincident low food availability associated with the dry season.

CONCLUSIONS

We examined sex-specific age-trajectories of grip strength in captive and wild gray mouse lemurs to evaluate functional senescence in this small primate. We have demonstrated that functional declines occur under natural conditions in a manner similar to captive conditions, implying that sarcopenic loss of function is not an artifact of anthropogenic environment and raises confidence in the data obtained from captive studies of muscle function. However, our results indicate that potential differences in the onset of senescence and the selective pressures operating differently on the sexes should be taken into consideration when making captive-wild comparisons.

Sarcopenia is closely connected to grip strength in other species; however, it would be beneficial to confirm this connection in gray mouse lemurs and to specify whether the decline in strength results from a reduction of muscle mass or changes in muscle quality. Studies on body composition, biomarkers associated with sarcopenic processes found in other model species, muscle tissue composition, endocrinological mechanisms and the influences of diet and physical activity would be potential avenues of further research in the species. The study of sex differences in sarcopenia would be of particular value in the gray mouse lemur due to its lack of sexual dimorphism, and the slight female advantage in strength.

Further longitudinal studies are required to assess whether muscle condition correlates with longevity and fitness in wild animals, as in captive animals and humans. Such a connection would be highly influential for demographic processes in natural populations, and a detailed examination of this process in nature could lead to a better understanding of the prevalence and evolutionary basis of sarcopenia and, potentially, aid in finding additional ways to counter its effects.

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CHAPTER 4

SEX, SEASON, AGE AND BODY MASS – DETERMINANTS OF ENDOPARASITE BURDEN IN THE GRAY MOUSE LEMUR

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ABSTRACT

Introduction:

Sex differences in immune system profiles and prevalence or incidence of illness have been described in a number of species ranging from invertebrates to humans. Deterioration in immune function at old age (immunosenescence) is known to occur in humans and laboratory animals, but less is known about the sex-specific effects of immunosenescence. Despite its potentially critical importance for the population and disease dynamics and the fitness of wild animals, few studies have examined immunosenescence in natural populations, and studies of sex-specific effects of aging on health in wild animals are exceedingly scarce. Life history theory predicts that due to their shorter lifespan and higher investment into reproduction at the expense of immune defense, males might suffer from more rapid immunosenescence. In this study, we tested this hypothesis by examining sex-specific age trajectories of endoparasite burden (prevalence of common parasite morphotypes and morphotype richness measured via fecal egg counts), a commonly used indicator of health, in a wild gray mouse lemur (*Microcebus murinus*) population.

Results:

Contrary to the prediction of immunosenescence, both parasite prevalence and morphotype richness decreased at old age in the dry season, indicating acquired immunity by older animals or selective mortality of individuals with poor parasite resistance. A significant male bias in parasite burden was observed in the dry season, likely reflecting higher female investment in immune function as well as sex-specific behavioral repertoires. In the rainy season, sex and age had no effects on the parasite burden, with the exception of an increasing cestode prevalence in older males. This might suggest that heterogeneity in host susceptibility governs parasite distribution in the ecologically demanding dry season (mating season, low food availability), whereas stochastic processes affect parasite distributions more in the rainy season.

Conclusions:

These results provide rare information about the age-related patterns of health in a wild vertebrate population. The low parasite burden suffered by the aged individuals of both sexes suggests a potentially critical role of immune system functioning for the heterogeneity in longevity in wild animals.

KEYWORDS

Aging, helminth, immunosenescence, *Microcebus murinus*, parasite prevalence, parasite species richness

INTRODUCTION

The immune system provides some of the most important mechanisms for self-maintenance, and variation in its function can have substantial consequences for fitness. However, the development and maintenance of immune defenses and, especially, mounting an immune response are energetically expensive and take up resources otherwise available for processes such as growth and reproduction [Sheldon and Verhulst 1996]. Due to a limited pool of resources and various competing needs, the level of defense is not always sustained at the optimal level required to efficiently eradicate pathogens and parasites. The total resources available dictate how much energy can be allocated to immune defenses, and also determine the magnitude of trade-offs required to sustain health and to promote fitness. Successful resource allocation to the immune system may therefore depend on intrinsic factors such as genetic quality, physical condition, life history stage, sex and age of the individual, as well as extrinsic factors including pathogen encounter rates and energetic demands set by the environment [Beldomenico and Begon 2010; Holmes 1987; Wilson et al. 2002].

The functioning of the immune system changes as a function of age, from development of adaptive immunity beginning at birth to the deterioration of the system at old age [Albright and Albright 1994; Haberthur et al. 2010; Hayward 2013; Humphreys and Grencis 2002; Malaguarnera et al. 2001]. The latter phenomenon is known as immunosenescence, and is characterized by remodeling of the immune system, including a down-regulation of type Th2 immunity, which is involved in parasite resistance [Malaguarnera et al. 2001]. The age-associated accumulation of wear and tear or senescent declines in other physiological processes may amplify the negative effects suffered by immunosenescent individuals [Hayward et al. 2009; Palacios et al. 2010]. On the other hand, older animals have more likely developed adaptive immunity against recurring parasites [Tinsley et al. 2012; Wilson et al. 2002], but see [Humphreys and Grencis 2002]), hence individuals in good enough physical condition at old age might be even better able to resist some pathogens and thereby partially counteract the effects of immunosenescence. Most of the evidence for immunosenescence comes from studies on humans and laboratory animals, but a few studies have also demonstrated its occurrence in natural populations [Cichoń et al. 2003; Nussey et al. 2012; Palacios et al. 2007; Ujvari and Madsen 2006].

In addition to the within-individual change with age, there is inter-individual variation in the immune system profile and functioning (immunocompetence) and susceptibility to illness. This heterogeneity may have to do with differences in pathogen encounter rate [Benavides et al. 2012; Heitman et al. 2003] as well as the capacity to clear infections [Hayward 2013]. An individual's sex is one of the most important determinants of their immune function profile [Alexander and Stimson 1988]. In mammals, a male bias in parasite infection rates is common [Moore and Wilson 2002]. Ultimately, it is thought that sex differences in immune responses stem from sex-specific life history optimization: where males compete for females, they benefit more from optimizing reproductive success in their prime reproductive age, whereas female fitness is generally improved by a longer reproductive lifespan due to constraints on the number of offspring they can raise within one reproductive event. These constraints can lead females to invest more into health maintenance to enhance their longevity, whereas males sacrifice health and lifespan for improved competitive success [Clutton-

Brock and Isvaran 2007; Trivers 1972; Williams 1957]. Proximately, the sex differences are usually ascribed to pleiotropic effects of steroid hormones, especially the immunosuppressive effects of testosterone, although evidence is inconclusive [Adamo et al. 2001; Folstad and Karter 1992; Klein 2004; Poulin 1996; Roberts et al. 2004; Zuk and McKean 1996]. The sex bias in infection rates might also follow from differing behavioral repertoires, which may in turn be mediated by hormonal states [Hayward 2013].

Although sex differences in immune function are frequently described, little is known about sex-specific patterns of immunosenescence beyond human studies. Based on the existing research, the rate of decline in several immunological parameters tends to be slower in females relative to males in mammals (human: [Caruso et al. 2013; Wikby et al. 2008], macaque: [Zheng et al. 2014], rat: [De la Fuente et al. 2004]), and at least some invertebrates (cricket: [Adamo et al. 2001]), whereas immune responses seem to be stronger in males throughout life or similar for the sexes in birds (zebra finch: [Noreen et al. 2011], barn swallow: [Saino et al. 2003]). Studies examining sex differences in immunosenescence in natural populations are particularly scarce, but likewise suggest a female advantage in mammals, as the risk of illness or inflammation is higher in male primates (semi-captive chimpanzees: [Obanda et al. 2014], wild yellow baboons: [Alberts et al. 2014]), although no sex difference was found in senescence in parasite resistance in baboons [Alberts et al. 2014], and senescence in parasite resistance begins earlier in male Soay sheep [Hayward et al. 2009].

Endoparasites infect most natural populations, and parasite infection is a convenient proxy of general health in natural populations as it can be monitored in a minimally invasive manner via fecal egg counts. While many endoparasites evoke only moderate clinical symptoms, they may nevertheless incur substantial energetic costs due to immune defense investment required to clear or limit the infection [Moret and Schmid-Hempel 2000; Zuk 2002] as available resources are redirected into parasite expulsion, tissue maintenance and repair [Coop and Kyriazakis 1999; Holmes 1987]. Furthermore, endoparasites can impair nutrient absorption from food and simultaneously, the costs associated with parasite resistance are amplified by poor host condition due to a low nutritional status [Moret and Schmid-Hempel 2000] or other stressors [Marcogliese and Pietrock 2011]. Perhaps due to such demands on limited resources, trade-offs between reproductive performance and parasite resistance [Mills et al. 2010; Sheldon and Verhulst 1996; Zuk et al. 1996] as well as the rate of immunosenescence [Helle et al. 2004] have been discovered in several species. Hence, parasite infections have potentially far-reaching consequences for host survival and fitness.

Helminths are the most common and species-rich group of enteric macroparasites infecting mammals [Gillespie 2006]. Infective stages of helminths are primarily picked up by animals through food (via consuming intermediate hosts or directly from the environment on food plants or substrate [Kates 1965]) or transmitted via self- or allogrooming [Heitman et al. 2003; Hernandez and Sukhdeo 1995]. Seasonal variation in infection rate can be substantial [Altizer et al. 2006; Raharivololona and Ganzhorn 2010; Vicente et al. 2007] due to the life history characteristics of both, parasites (cycles of development, required environmental conditions such as sufficient water) [Kates 1965], and hosts (foraging behaviors, food items favored, body condition, patterns of ranging and social interactions). Macroparasite infections may increase the animal's chance of being infected further by other

pathogens and parasites [Graham 2008] and may expose the individual to a higher risk of predation [Hudson et al. 1992; Temple 1987]. There is ample evidence that infection by several parasite species strains the host system more than infection by one species only [Bordes and Morand 2009; Ezeamama et al. 2008; Petney and Andrews 1998].

Parasitological studies of strepsirrhine primate hosts are still scarce and their parasite communities poorly defined [Irwin and Raharison 2009]. Possibly due to the relatively low sampling effort, the parasite species richness infecting the taxon has been found to be lower than in other primate taxa [Nunn 2003; Walther et al. 1995]. The gray mouse lemur, *Microcebus murinus*, is a small strepsirrhine primate native to Madagascar. The species is nocturnal, solitary foraging, sexually monomorphic, omnivorous and mainly arboreal. The parasite communities of gray mouse lemurs have previously been described for the Mandena population in Southern Madagascar, and preliminary data are available also for the Kirindy population [Raharivololona and Ganzhorn 2009; Schwensow et al. 2010].

Seasonal differences in overall parasite loads and parasite communities might be expected for the species [Raharivololona and Ganzhorn 2010] due to strong climatic differences between ecological seasons and the associated food and water availability. The diet of mouse lemurs varies over the course of the year according to the availability of fruit and insects, with animal matter (mainly insects) being ingested most in the rainy season [Dammhahn and Kappeler 2008]. Seasonal behaviors also differ between the sexes: besides the characteristic life history differences associated with reproductive activities (males compete for females, females care for the young), female-biased nest sharing [Eberle and Kappeler 2006; Radespiel et al. 1998], allogrooming and resource use may have an influence on the observed parasite loads. Most adult females use regular torpor through most of the dry season between May and September [Schmid 1998; Schmid and Kappeler 1998], whereas most males remain active during this season. Males roam over large areas for receptive females in the mating season in October-November [Eberle and Kappeler 2002; Radespiel et al. 2001], which has been shown to coincide with increased male bias in mortality rates presumably due to the increase in risky behaviors [Kraus et al. 2008].

The purpose of this study was to examine the effects of advancing age on immunocompetence as indicated by endoparasite prevalence and parasite morphotype richness (at genus level) in a natural gray mouse lemur population. In particular, we examined whether these age effects differ for the sexes or are influenced by seasonal or body condition variation. Moreover, we characterize seasonal variation in the endoparasite community and seasonally varying host-specific traits as predictors of parasite loads.

Due to the immunosuppressive effects of testosterone and behavioral differences, we expected males to have higher overall parasite prevalence and morphotype richness [Folstad and Karter 1992; Klein 2004; Moore and Wilson 2002; Zuk and McKean 1996]. We expected more pronounced sex differences in the dry season, i.e. prior to and during the mating season, due to the differing behaviors and ecological pressures. In this season, the males' testosterone levels reach their annual peak [Perret 1985a; Perret 1985b], and roaming behavior may expose them to more infectious stages of parasites. Parasites might be generally more prevalent in the dry season when host body mass is

lowest, but the infective stages of certain species may be more prevalent in the rainy season, leading to higher infection rates of some morphotypes in rainy season. Since body condition can have a negative influence on immune defenses, parasite prevalence and morphotype richness was expected to correlate negatively with body mass in both sexes. Aging was expected to lead to increasing parasite prevalence and morphotype richness in reflection of immunosenescence. This increase might be faster in males due to their shorter life expectancy and the potentially associated reduction in investment in self-maintenance [Clutton-Brock and Isvaran 2007; Promislow 1992; Trivers 1972].

MATERIALS AND METHODS

Sample collection and analysis

Our study population (locally known as "N5") of gray mouse lemurs has been regularly monitored by live capturing in the dry deciduous forest in CNFEREF/Kirindy since 2002, establishing an individually marked, sexed and aged population. All individuals are individually, permanently marked with a subcutaneous transponder (Trovan EURO ID, Germany) at first capture, and weighed monthly at subsequent captures. Capture and handling protocols have been detailed in e.g. [Dammhahn and Kappeler 2008; Eberle and Kappeler 2004a]. Sampling for this study was done from animals captured in September - November 2010 and 2012 (transition phase from dry season to rainy season, hereafter "dry season") and in March – May 2011 and 2012 (transition from rainy to dry season, hereafter "rainy season").

Fecal samples were collected from captured animals during handling or from cleaned traps. Fresh feces (1-4 pellets) were weighed (range: 0.01 – 2.28g) and homogenized in 10% formaldehyde in eppendorf tubes. The samples were analyzed at the Institut Pasteur de Madagascar by trained laboratory technicians using the Ritchie's formol-ether concentration method [Ritchie 1948]. Parasite egg morphotypes found via microscopic examination of fecal smears were identified to the closest genus possible, in accordance with previously described identification criteria [Irwin and Raharison 2009; Raharivololona 2006; Raharivololona 2009]. When possible, photos were taken of representative egg morphotypes to aid identification. The presence of larva was also reported (N= 13 samples), but as no further species identifications could be made, these data were not further analyzed.

Ethical standards

All research reported in this study complied with animal care regulations and applicable national laws of Madagascar and was approved by the Ministère de l'Environment et des Eaux et Fôrets, MINEEF. The study was performed according to guidelines provided by the Association for the Study of Animal Behaviour (ASAB) and the Animal Behavior Society (ABS).

Statistics

Statistical analyses were conducted to examine predictors of parasite prevalence and morphotype richness. We chose not to attempt formal analyses of the intensity of infection, because of the difficulty of inferring intensity from fecal egg counts without the appropriate validations on temporal variation in the egg shedding rates of parasites (e.g. [Denwood et al. 2012; Shaw and Moss 1989; Tompkins and Hudson 1999]). Such validations would typically include counts of adult worms in the gut and investigations of parasite fecundity and life histories, which were not possible in the framework of this study.

To study the effects of age, sex, season and body mass on gastrointestinal parasite prevalence we used generalized linear mixed models (GLMM) with a binomial error structure and a logit link function using the R package *lme4* [Bates et al. 2014]. Owing to the seasonal variability in both extrinsic (weather conditions, available food items) and intrinsic (sex-specific, seasonal behaviors and body condition fluctuation) variables that might influence parasite infection rates, we first tested for seasonal differences in the prevalence of each of the common morphotypes by simplified models consisting only of the fixed factors season, sample mass and year of sampling, and individual identity as a random factor. Because of the complexity of the ensuing models, models were built separately for the dry and the rainy season to explore the effects of age, sex and body mass on parasite prevalence.

The prevalence of helminth parasites could meaningfully be statistically analyzed only for the three most common morphotypes: *Subulura*, *Trichuris* and *Hymenolepis* (Figure 1) and separate models were built using the presence of each of these as a response variable. Statistical analyses of the other morphotypes (recovered in <10 infected samples) were not possible due to the extremely low number of infections. To explore the possibility that the likelihood of infection with any of the rare morphotypes was influenced by age, sex or season, these morphotypes were grouped to form another response variable, the prevalence of “rare morphotypes”. The results from these cursory analyses are presented in the Appendix.

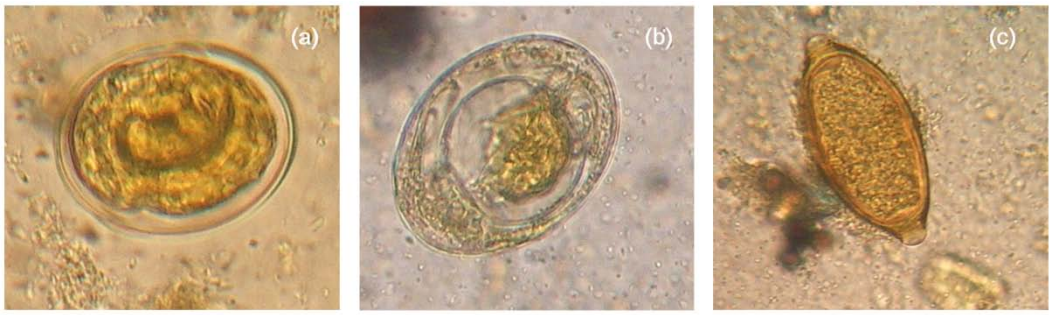

Figure 1: Microscopy photos of the three most common parasite egg morphotypes found in samples from *Microcebus murinus* in Kirindy forest. (a) *Subulura*, (b) *Hymenolepis*, (c) *Trichuris.*

The initial models included the fixed terms age, sex and their interaction, body mass and year of sampling, as well as sample mass. Individual identity was included as a random factor in all models to account for repeated sampling of the same individuals. Age, body mass and sample mass were log-transformed, and age was additionally scaled and centered prior to analyses to improve the interpretation of interaction terms [Schielzeth 2010]. When the interaction of age*sex and/or body mass had a non-significant effect ($P > 0.1$), the terms were removed from the model to avoid overparameterization [Engqvist 2005] and to improve sample size (not all samples could be matched to body mass measurements within a few days of the sample). For models of *Trichuris* prevalence, models had to be further reduced due to problems of convergence: the term year had to be dropped in all models and the term sex was dropped from the rainy season prevalence model.

Morphotype richness was calculated using all morphotypes present (based on different morphotypes), including nematode, trematode and cestode eggs as well as coccidian oocysts. Predictors of morphotype richness were studied with GLMMs with Poisson error distribution. Models were built separately for the dry and rainy season, with the same initial predictors and subsequent term reductions as detailed for parasite prevalence. The models were not overdispersed (goodness of fit tested in R package *aods3* [Lesnoff and Lancelot 2013]) or zero-inflated (tested with likelihood ratio tests using R package *glmmADMB* [Fournier et al. 2012; Skaug et al. 2014]).

RESULTS

Seasonal characteristics of the parasite community

Out of all samples (N= 470), in 262 samples (55.7%) at least one parasite morphotype was found. In total, 12 different helminth egg morphotypes were identified (Table 1). In addition to helminth parasites, coccidian protozoa were found in 2 (0.4%) samples. The majority (N= 8) of the helminth morphotypes were nematodes, and nematode morphotypes were also the most prevalent, with nematode eggs present in 179 (38.1%), cestode eggs in 139 (29.6%), and trematode eggs in 8 (1.7%) samples. The taxonomy, transmission routes and life history characteristics are unstudied in all parasites carried by gray mouse lemurs, but can be conjectured (Table 1) based on data available on related parasite species [Irwin and Raharison 2009].

The overall infection rates were essentially identical in the dry (55.7%) and in the rainy (55.8%) season, but seasonal differences were found in the prevalence of each of the common morphotypes. *Trichuris* and *Hymenolepis* were significantly more prevalent in the dry season (*Trichuris* dry: 16.2%, rainy: 6.5% of samples infected, $\beta = -2.345$, SE= 0.624, z= -3.755, P< 0.001; *Hymenolepis* dry: 32.4%, rainy: 26.3%, $\beta = -0.574$, SE= 0.280, z= -2.051, P=0.040), whereas *Subulura* was significantly more prevalent in the rainy season (dry: 26.1%, rainy: 33.6%, $\beta = 0.982$, SE= 0.274, z= 3.585, P< 0.001).

Table 1: Morphotypes of gastrointestinal helminth parasites found in *Microcebus murinus* in Kirindy forest and their prevalence (number and % of infected samples). Transmission routes are based on studies of related species.

Phylum	Family	Genus	Transmission route	Prevalence
Nematoda	Ascaridida	*Ascaris* sp.	Direct	3 (0.6 %)
	Subuluridae	*Subulura* sp.	Indirect [a]	141 (29.5 %)
	Capillaria	*Capillaria* sp.	Indirect [a]	3 (0.6 %)
	Oxyuridae	*Lemuricola* sp.	Direct	6 (1.2 %)
		Oxyuridae sp.	Direct	7 (1.5 %)
	Strongylida	*Oesophagostomum* sp.	Direct	9 (1.9 %)
		Strongylida sp.	Direct	1 (0.2 %)
	Trichuridae	*Trichuris* sp.	Direct	55 (11.5 %)
Cestoda	Hymenolepididae	*Hymenolepis* sp.	Indirect [a]	141 (29.5 %)
Trematoda	Fasciolidae	*Fasciolidae* sp.	Indirect [b]	1(0.2 %)
	Heterophyidae	*Metagonimus* sp.	Indirect [b]	6 (1.2 %)
	Opistorchiidae	*Opisthorchis* sp.	Indirect [b]	1 (0.2 %)

[a] insect intermediate host
[b] one or more intermediate hosts, first intermediate host typically snail

Predictors of morphotype prevalence

Morphotype-specific analyses indicated significant sex and age effects on the prevalence of *Subulura* in the dry season (Figure 2a), with higher prevalence in males and in young individuals, and a significant effect of the year of sampling in both seasons (Table 2). No age or sex effects were found in the rainy season (Figure 2d).

For *Trichuris*, no significant effect of any predictor was found in either season, and the fixed effects included in the best models explained only 0.26% and 0.14% of the variation in the rainy and the dry season, respectively. In the rainy season, however, the model had to be reduced to include only age and sample mass because of convergence issues; *Trichuris*-morphotype eggs were found only in samples collected in year 2012 from female hosts, with all other samples being negative (Table 2, Figure 2b,e).

The prevalence of cestode eggs of the *Hymenolepis* family was significantly higher in males and young animals in the dry season (Figure 2c), and a higher prevalence was, unexpectedly, associated with higher body mass. In the rainy season, the association with body mass was negative and a sex*age interaction indicated increasing prevalence of *Hymenolepis* eggs in older males, whereas no effect of age was apparent in females (Table 2, Figure 2f).

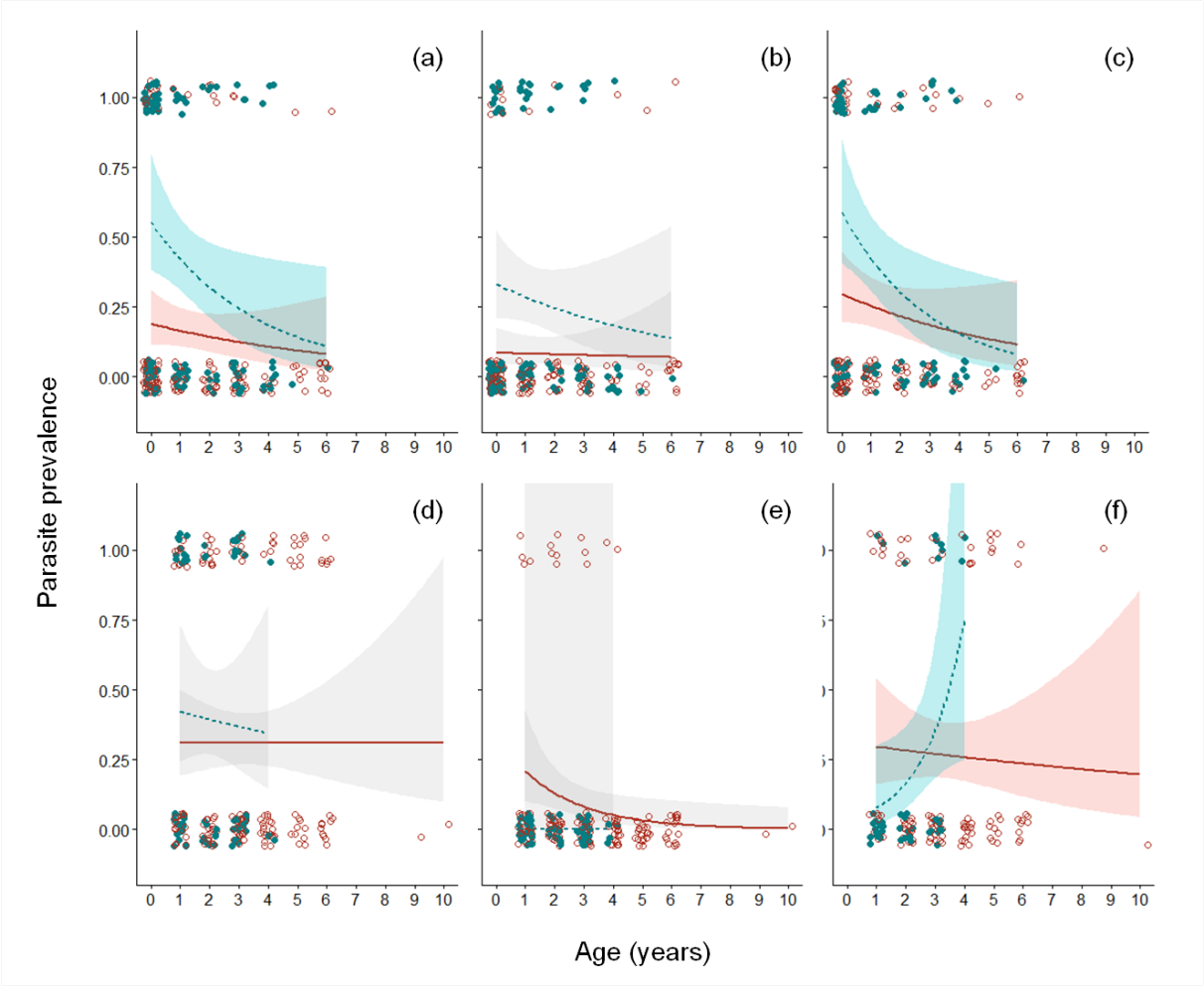

Figure 2: Prevalence (probability of infection) of the three most common parasite egg morphotypes in gray mouse lemurs in Kirindy forest: *Subulura* (a) dry season and (d) rainy season, *Trichuris* (b) dry and (e) rainy season and *Hymenolepis* (c) dry and (f) rainy season. Shown are all data points (with jitter introduced to the discrete variables for ease of interpretation) for males (solid symbol) and females (open symbol), and loess-smoothed prediction lines and 95% confidence bands for age effects for males (dashed line) and females (solid line). Significant age effects (a and c, trajectories not significantly different for the sexes), and a significant age*sex interaction (f) shown with colored confidence bands, non-significant relationships with gray confidence bands. See text and Table 2 for details.

Table 2: Predictors of the prevalence of different parasite morphotypes in fecal samples collected in dry and rainy season. Predictions are based on the final binomial models.

		Dry season						**Rainy season**					
		N (Samples/ individuals/ infected)	β	SE	z	P	R^2 (marginal/ conditional)	N (Samples/ Individuals/ infected)	β	SE	z	P	R^2 (marginal/ conditional)
Subulura	Intercept	253/ 113/66	-0.983	0.610	-1.610	0.107	0.224/ 0.224	217/ 93/121	-2.047	0.668	-3.063	0.002	0.176/ 0.281
	Sex		1.481	0.322	4.600	<0.001			0.388	0.423	0.919	0.358	
	Age		-0.500	0.614	-3.049	0.002			-0.232	0.299	-0.775	0.438	
	Year		-0.835	0.400	-2.087	0.037			1.841	0.441	4.179	<0.001	
	Sample mass		0.218	0.203	1.078	0.281			0.057	0.287	0.199	0.842	
Trichuris	Intercept	253/ 113/41	-5.592	2.200	-2.542	0.011	0.026/ 0.947	148/ 66/14 [a]	-20.976	12.078	-1.737	0.082	0.014/ 0.995
	Sex		2.094	1.420	1.475	0.14							
	Age		0.178	0.590	0.302	0.763			4.557	5.139	0.887	0.375	
	Year		-0.494	1.098	-0.450	0.653							
	Sample mass		0.863	0.538	1.603	0.109			-1.803	1.549	-1.164	0.245	

Table 2 continued

		Dry season						Rainy season					
		N (Samples/ individuals/ infected)	β	SE	z	P	R^2 (marginal/ conditional)	N (Samples/ Individuals/ infected)	β	SE	z	P	R^2 (marginal/ conditional)
Hymenolepis	Intercept	235/ 110/82	-1.618	0.676	-2.393	0.017	0.206/ 0.253	154/ 88/57	0.512	0.797	0.642	0.521	0.147/ 0.147
	Sex		0.736	0.331	2.224	0.026			-1.395	0.651	-2.145	0.032	
	Age		-0.844	0.197	-4.284	<0.001			0.029	0.391	0.074	0.941	
	Year		0.595	0.441	1.348	0.178			-0.638	0.471	-1.355	0.175	
	Sample mass		-0.067	0.198	-0.341	0.733			0.253	0.341	0.744	0.457	
	Body mass		3.883	1.220	3.184	0.001			-2.802	1.228	-2.282	0.023	
	Sex*Age								2.484	0.991	2.507	0.012	

[a] only infections in females in year 2012, hence non-convergence if year and sex appear as fixed terms

Predictors of parasite morphotype richness

Parasite morphotype richness ranged from 0 to 4 morphotypes in infected samples, and richness was similar for both seasons (average number of morphotypes present: dry: 0.8, rainy: 0.7, β= 0.017, SE=0.133, z= 0.130, P= 0.896). Based on the final model (Table 3, Figure 3), males carried on average twice as many parasite morphotypes than females in the dry season (average for males: 1.2, females: 0.6 genera), whereas no significant sex effect was found in the rainy season (average for both: 0.7 genera). No significant body mass effects were found on morphotype richness.

Table 3: Predictors of parasite morphotype richness measured by egg shedding in dry and rainy season. Presented predictions are based on the improved model after dropping non-significant terms age*sex and body mass. N= samples/ individuals

		β	SE	z	P	R^2 marginal/ conditional
Dry season	Intercept	-0.185	0.294	-0.631	0.528	0.197/0.274
N=253/113	Age	-0.264	0.086	-3.056	0.002	
	Sex (ref. female)	0.743	0.160	4.633	<0.001	
	Year	-0.189	0.193	-0.980	0.327	
	sample mass	0.160	0.095	1.696	0.090	
Rainy season	Intercept	-0.739	0.281	-2.626	0.009	0.089/0.129
N=217/93	Age	-0.133	0.088	-1.507	0.132	
	Sex (ref. female)	-0.079	0.191	-0.413	0.680	
	Year	0.619	0.194	3.191	0.001	
	sample mass	-0.011	0.138	0.076	0.939	

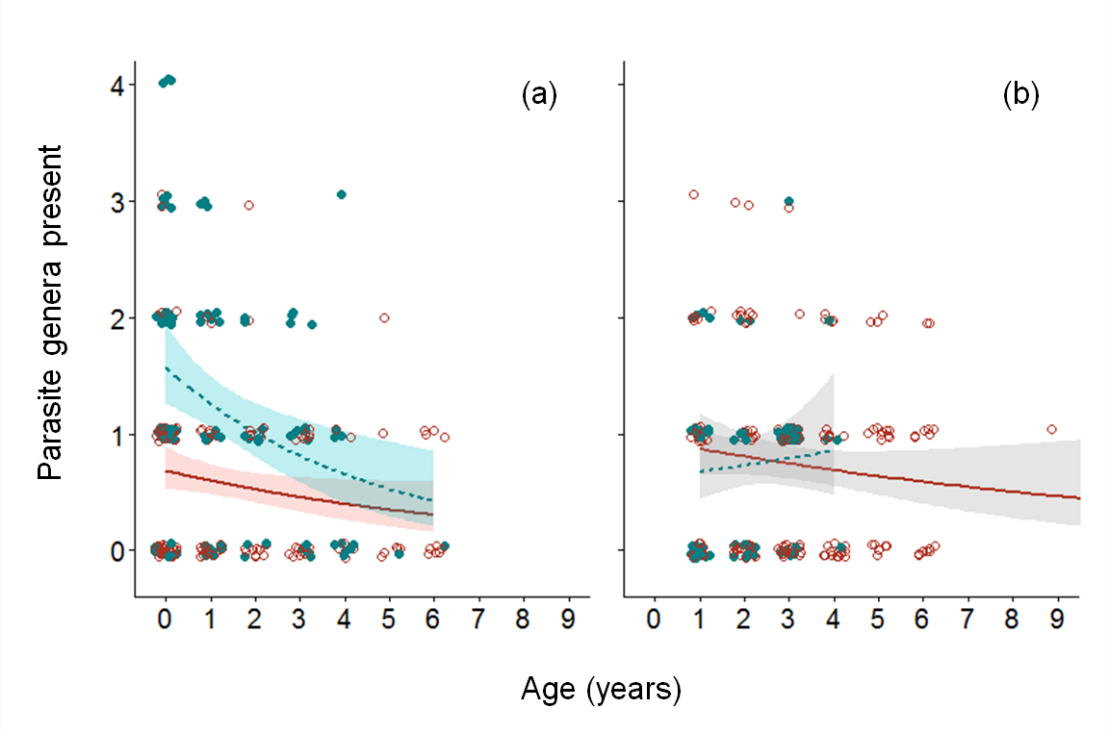

Figure 3: Parasite genus richness as a function of age for male (solid symbols, dashed line) and female (open symbols, solid line) gray mouse lemurs in (a) dry and (b) rainy season. Lines indicate loess smoothers of the age effects (significant decline (in color; P= 0.002) in dry season, non-significant (gray-scale) in rainy season) based on the final model in each season (Table 3), and shaded areas indicate 95% confidence intervals. Males had a significantly (P< 0.001) higher morphotype richness relative to females in the dry season, whereas no significant sex effect was found in the rainy season. The sex*age interaction was non-significant in both seasons. Both age and morphotype richness are based on discrete measures but jitter was introduced to improve interpretability.

DISCUSSION

In this study, we tested the hypothesis that aged wild animals and particularly males would suffer from a high parasite burden due to sex-specific immunosenescence. To this end, we investigated the seasonal effects of age and sex on the endoparasite burden of gray mouse lemurs. Overall, we found evidence of higher parasite prevalence and morphotype richness in males as expected based on immunosuppressive effects of testosterone or male-biased parasite encounter rates. The sex effect was stronger in the dry season, as predicted, for two of the common morphotypes. Contrary to our prediction of higher parasite prevalence and morphotype richness at old age, age effects were either absent or negative for nematodes, indicating a lower parasite burden at old age. A positive association between age and cestode prevalence was only found for males in the rainy season, offering limited support for the prediction of sex-specific immunosenescence.

Parasite community of gray mouse lemurs in Kirindy forest

Out of the 12 distinct helminth egg morphotypes found in this study, three morphotypes (the nematodes *Subulura* and *Trichuris* and a cestode *Hymenolepis*) accounted for the vast majority of the infections. The common morphotypes found in our study concur with those found previously using a smaller sample size in a different subpopulation in Kirindy forest [Schwensow et al. 2010], but parasite communities and prevalence of infections seem to differ between sites: in Mandena, southern Madagascar, 74-93% of all samples were infected with at least one parasite morphotype, with *Coccidia*, *Hymenolepis* and *Ascarididae* being the most common intestinal parasites [Raharivololona 2006; Raharivololona and Ganzhorn 2009]. The common morphotypes presumably represent both direct (*Trichuris*) and indirect (*Subulura*, *Hymenolepis*) routes of transmission [Irwin and Raharison 2009].

Of the rare morphotypes found in our study, only *Ascaris* sp. was also found in an earlier study in Kirindy [Schwensow et al. 2010] along with three unidentified nematode egg morphotypes. Most of the rare egg morphotypes of our study have been described previously for *M. murinus* [Irwin and Raharison 2009; Raharivololona 2006], but identifications have generally not been confirmed via study of adult worms. Hence, the actual taxonomic diversity is unknown and identifications can only be made based on egg morphology, which may at times be inaccurate [Irwin and Raharison 2009]. Consequently, while most of these recorded rare morphotypes likely represent genuine infections, some of them might be misclassifications due to difficulty of discriminating between closely related taxa based on egg morphology [Irwin and Raharison 2009]. Although we took every precaution against contamination by other intraspecific and heterospecific mammalian hosts by cleaning traps in between all captures, it is also possible that occasional contamination might have occurred by feces from other species (e.g. by insects or small reptiles visiting the traps, or undigested eggs of parasites of prey species being deposited in host feces [Irwin and Raharison 2009]).

Clinical effects of the specific parasites infecting mouse lemurs are unknown but their congeners generally induce at most mild pathogenic effects [Irwin and Raharison 2009; Raharivololona and Ganzhorn 2010], although particularly heavy infection or infection by multiple parasite species may be detrimental. Due to the non-invasive sampling, we have no reliable information of the intensity of infection (but see the Appendix), but morphotype richness may be indicative of increasing pathogenicity. Weakened individuals may nevertheless suffer from the additional energetic demands of fighting an infection. The (sex-specific) fitness and survival consequences of parasite infection rates in primates remain to be studied.

Predictors of parasite prevalence and morphotype richness

Older animals have lower parasite burdens

Declines in immune system function are commonly found at old age, but acquired immunity may counteract some of the detrimental effects [Shanley et al. 2009]. In this study, the observed age

effects (in one nematode and one cestode and overall morphotype richness) were negative in the dry season, indicating lower parasite burdens in older animals and suggesting acquired immunity rather than a senescent decline in parasite resistance. This seasonal pattern is contrary to our prediction that aged animals might suffer particularly high parasitism in the dry season due to impaired coping with the energetically demanding conditions. In the rainy season, a positive relationship between age and cestode prevalence was found for males, but not females, and no other parasite morphotypes were affected by age in rainy season. No age effects were evident in morphotype richness in the rainy season. In accordance with previous findings on patterns of aging in the same population [Hämäläinen et al. 2014a], therefore, it appears that the oldest animals are not in poorer condition than younger animals. This effect is possibly a result of non-random mortality with earlier disappearance of individuals with weaker immune defenses, or older animals may benefit from acquired immunity that leads to lower parasite prevalence [Wilson et al. 2002]. It is also noteworthy that, although the intensity of infection was not statistically analyzed due to the difficulty of interpretation (e.g. [Denwood et al. 2012; Shaw and Moss 1989; Tompkins and Hudson 1999]), a clear decline with age was also found in the total intensity of egg shedding (total egg counts as a function of age illustrated in the Appendix, Figure S1).

These results are in contrast with those from red-fronted lemurs in which age had little effect on the parasite burden [Clough et al. 2010], and with reports of higher parasite burdens in older animals in e.g. wild and captive rodents [Behnke et al. 1999; Humphreys and Grencis 2002], yellow baboons [Alberts et al. 2014], Soay sheep: [Hayward et al. 2009] and brown mouse lemurs [Zohdy 2012]. Similar to this study, indications of acquired immunity indicated by reduced parasite load at old age have been found in chacma baboons (declining species richness [Benavides et al. 2012]), gibbons [Gillespie et al. 2010], dogs and cats [Gates and Nolan 2009] as well as frogs [Tinsley et al. 2012], although prevalence is sometimes increased in exceptionally old individuals, possibly reflecting terminal immunosenescence.

We found an interaction of sex and age in *Hymenolepis* prevalence, with prevalence increasing with age in males. Similarly, an earlier onset of decline in parasite resistance was found in male in Soay sheep relative to females [Hayward et al. 2009]. However, due to the short male lifespan and high mortality during the mating season, the oldest males we sampled in the rainy season were only 4 year-olds, which is the age at which senescent declines in other measures of functioning start [Hämäläinen et al. 2014a; Languille et al. 2012; Némoz-Bertholet and Aujard 2003]. The elevated cestode prevalence of older males in the rainy season might result from acquisition of long-lived or slowly maturing parasites [Trouvé et al. 1998] during the mating season, when immune defenses may be lowered due to elevated testosterone levels. Therefore, the increase in parasite prevalence with age should be interpreted with caution as it may not indicate immunosenescence as much as, for instance, higher reproductive investment by older males in the preceding dry season, perhaps reflecting the increasing risk taking by males at advancing age [Dammhahn 2012]. One alternative explanation would be differential exposure due to dietary preferences of older males favoring potential cestode intermediate hosts, but any prey preferences remain to be demonstrated. Determining the pathogenicity of this parasite for mouse lemurs would be needed to evaluate its potential contribution to the higher male mortality in the species.

In summary, so far, no clear patterns of immunosenescence in wild populations have emerged. The variable results might reflect differing effects of various parasites and the immune responses they elicit, or behavioral or nutritional changes of hosts at old age that may influence encounter rates or resistance. Furthermore, it is possible that highly parasitized individuals succumb to mortality earlier than their more parasite resistant counterparts [Anderson 1979].

Males have higher parasite prevalence and carry more genera

Males had a higher prevalence of infection with the most common nematode and cestode morphotype in the dry season relative to females. Overall parasite morphotype richness was likewise higher in males in the dry season, whereas no sex difference was found in the rainy season. Our dry season results concur with the general male bias in parasite prevalence found across host taxa, the pattern being strongest for helminthiasis in mammals [Moore and Wilson 2002; Poulin 1996], see also [Morales-Montor et al. 2004]. It is also possible that due to potential sex differences in investment into different components of immune function [Lee 2006], females might suffer more from protozoan infections while males more often carry helminths [Clough et al. 2010]. Comparative studies have suggested male-biased sexual size dimorphism as an explanation for male-biased parasitism, since positive associations are found between sexual size dimorphism, sex-biased mortality and parasitism, as well as the intensity of sexual selection [Moore and Wilson 2002]. Consistently, females tend to experience higher parasite loads in species showing reversed sexual size dimorphism [Moore and Wilson 2002]. Since sexual size dimorphism in the gray mouse lemur is neutral or slightly female-biased, female-biased parasitism might also be expected; but, since potential for intense male-male competition exists (based on the polygynous mating system), males might be expected to suffer higher degrees of parasitism. Our results thus suggest a more important role for the mating system than body size.

Besides body size, explanations offered for the typical male-bias in parasitism are endocrinological (especially the immunosuppressive role of testosterone and cortisol), dietary and behavioral sex differences [Wilson et al. 2002; Zuk and McKean 1996] attributable to evolutionary mechanisms aimed at maximizing the reproductive output of each sex [Zuk and McKean 1996]. Both, the behavioral and hormonal explanations, fit the patterns found in our study. In the late dry season when our sampling took place, the males' testosterone levels are at their annual high [Perret 1985a; Perret 1985b] and males roam over larger areas in search of females [Eberle and Kappeler 2004b; Schmid 1999], which may increase their parasite encounter probabilities [Benavides et al. 2012; Bordes et al. 2009; Nunn 2003]. Unlike females, they also mainly remain active throughout the dry season [Schmid 1999], which may increase their ingestion of infective stages of parasites throughout the year. The youngest males may also suffer from higher parasite burdens due to their longer dispersal distances relative to females, and part of the high parasite burdens in the yearling males ("age 0") may be due to the increased travel distances preceding their first mating season [Schliehe-Diecks et al. 2012].

The mating season also coincides with the highest rate of male mortality, likely at least partially attributable to an increased risk of predation due to risky male behavior [Kraus et al. 2008]. Even sub-clinical parasite infections have been previously linked to reduced body mass gain, changes in body

composition, and reduced nutrient utilization efficiency [Holmes 1987], hence parasite infections may influence the males' general health status, increase energetic expenditure and consequently increase their predation risk or affect recovery after the mating season.

The absence of sex difference in our rainy season data (as opposed to male bias in the dry season), and the *Trichuris* infections exclusive to females in rainy season may reflect the effects of breeding on female parasite loads as well as the lower testosterone levels of males. Breeding has substantial energetic costs for females [Steketee 2003; Weigel et al. 1996], and together with the immunosuppressive effects of hormones produced during parturition and lactation [Barger 1993] might lead to elevated infection rates of females in the rainy season.

Body mass is associated with cestode prevalence

Since body condition broadly reflects the energetic status of an individual and, potentially, their nutritional status, it tends to be associated with the functioning of the immune response [Ezenwa 2004] and the ability to resist infection and compensate for damage caused by parasites [Wilson et al. 2002]. Furthermore, parasite infections may lead to a declining body condition [Beldomenico and Begon 2010], which renders it difficult to distinguish cause and consequence in observational studies. Consistent with either of these explanations, body mass correlates negatively with parasitism in e.g. red deer [Vicente et al. 2007] and pythons [Ujvari and Madsen 2006]. In this study, body mass was negatively associated with cestode prevalence in the rainy season, but surprisingly, this association was positive in the dry season. The contrasting effects of body mass on cestode infection rate are difficult to explain and might reflect complex interactions of sex, age and sex-specific seasonal fluctuations [Hämäläinen et al. 2014a] that could not be tested in detail due to limitations of sample size and sampling intervals. Furthermore, the longevity of parasitic helminths varies between a few weeks to several years, and maturation of the parasite within host can likewise take up to several months [Gems 2000] thus, the timing of infection cannot be reliably assigned. Body mass had no significant influence on the prevalence or richness of other morphotypes.

In the dry season, the body condition effect may have significance for male fitness. Males that are in a good body condition may be able to expend more energy into mating activities, while also allocating sufficient resources to cope with a parasite infection. This might indicate parasitism as a potential indicator of male "quality", sensu the Immunocompetence Handicap Hypothesis [Folstad and Karter 1992]. Males remain active during the dry season and therefore may encounter more infective parasite stages. However, a large proportion of male body mass in this season consists of seasonal testicular development [Schmid and Kappeler 1998], which may translate into reduced immunocompetence [Furman et al. 2014; Klein 2000].

Seasonality and other potential determinants of parasite burden

Our results indicate seasonal differences in the prevalence of each of the common parasite morphotypes: *Trichuris* and *Hymenolepis* were more common in the dry season and *Subulura* was more prevalent in the rainy season. The reasons for these differing patterns are impossible to deduce based on the available data, but may have to do with the (hitherto unknown) life cycles of the parasite species. Fluctuation in climatic conditions and in host and parasite communities may cause

temporal variation in parasite burdens (e.g. [Clough et al. 2010]), although evidence for annual fluctuations were limited in our data set spanning 2.5 years.

The fixed effects in our models of prevalence (or morphotype richness) explain up to 22% of the variation in the results, but in most cases much less. The random effects explain a larger proportion of the variance in some models (R^2_c, i.e. fixed and random effects together = 99.5% for *Trichuris* in rainy season), but little in others (none for *Hymenolepis* in the rainy season). Overall, only a relatively small proportion of the variance is explained by the models. The unexplained variation could be due to heterogeneity in behavior, territory characteristics, diet, social interactions (e.g. [Wilson et al. 2002]) and co-infections with other pathogens [Ezeamama et al. 2008; Petney and Andrews 1998]. Noise may be introduced by parasite egg shedding patterns, gradual change within seasons and other environmental fluctuations such as weather events and the activity of insects and other potential host species and the unknown delays to helminth egg shedding. Furthermore, it is possible that eggs decay in the sample and/or go undetected for other reasons [Dacombe et al. 2007], hence introducing additional inaccuracy to the predictions. These possible additional factors pose interesting directions for future research.

CONCLUSIONS

The parasite burden observed based on fecal samples reflects the combined consequences of which parasites the host has encountered as well as when and how successfully the parasite matured and reproduced within the host, and how effective the host's immune defenses were in clearing the infection. While it is very difficult to pull apart these factors in a minimally invasive study of a wild population, we could still draw broad inferences about population level predictors of the parasite burden. We found males to have generally higher parasite infection rates than females, and the oldest animals to suffer from the lowest parasite burden within this population, suggesting perhaps a role for acquired immunity and the prolonged survival of individuals that are able to resist parasite infection. Our results suggest that host susceptibility may govern parasite distribution in the ecologically demanding dry season, whereas stochastic processes have more influence on parasite distribution in the rainy season, as none of the host-specific traits were good predictors of the parasite burden. The seasonal differences in the predictors of parasite burdens are noteworthy for future studies of immunosenescence, as senescence may be overlooked if seasonal patterns are ignored.

While these results comprise one of the most thorough investigations into parasite loads of a lemur population in terms of the number of individuals sampled, it is only the first step towards understanding the parasite communities and variation among hosts in the parasite loads suffered. The taxonomy, life cycles and pathogenic effects of lemur parasites are virtually unstudied, as are the various host-specific determinants of parasite susceptibility and resistance.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

APPENDIX

Prevalence of rare parasite morphotypes

The parasite egg morphotypes with prevalence < 10 infected samples each (*Ascaris, Capillaria, Lemuricola,* other *Oxyuridae, Oesophagostomum Strongylida, Fasciolidae, Metagonimus* and *Opisthorchis* species) were grouped together into a "rare morphotype" response variable. No statistically significant seasonal differences were found in the prevalence of the rare parasite morphotypes (dry: 7.9%, rainy: 6.0%, β = -0.073, SE= 0.531, z= -0.138, P= 0.890). None of the predictors in the final model showed association with rare parasite prevalence, nor could the fixed effects in the models explain almost any of the variation in prevalence (R^2_m=0.003-0.006) (Table S1).

Table S1: Prevalence of all rare parasite morphotypes in the dry and rainy season. N= samples/ individuals/ infected.

		β	SE	z	P	R^2 marginal/ conditional
Dry season	Intercept	-7.034	2.326	-3.024	0.002	0.003/ 0.947
(N= 253/ 113/20)	Sex	-0.277	1.434	-0.193	0.847	
	Age	-0.273	0.627	-0.436	0.663	
	Year	-0.687	1.147	-0.599	0.549	
	Sample mass	0.087	0.577	0.151	0.88	
Rainy season	Intercept	-10.501	3.113	-3.373	<0.001	0.006/ 0.956
(N= 217/ 93/13)	Sex	0.337	1.953	0.173	0.863	
	Age	0.396	1.44	0.275	0.783	
	Year	-0.16	1.15	-0.139	0.889	
	Sample mass	-1.109	1.027	-1.08	0.28	

Intensity of parasite infection based on fecal egg counts

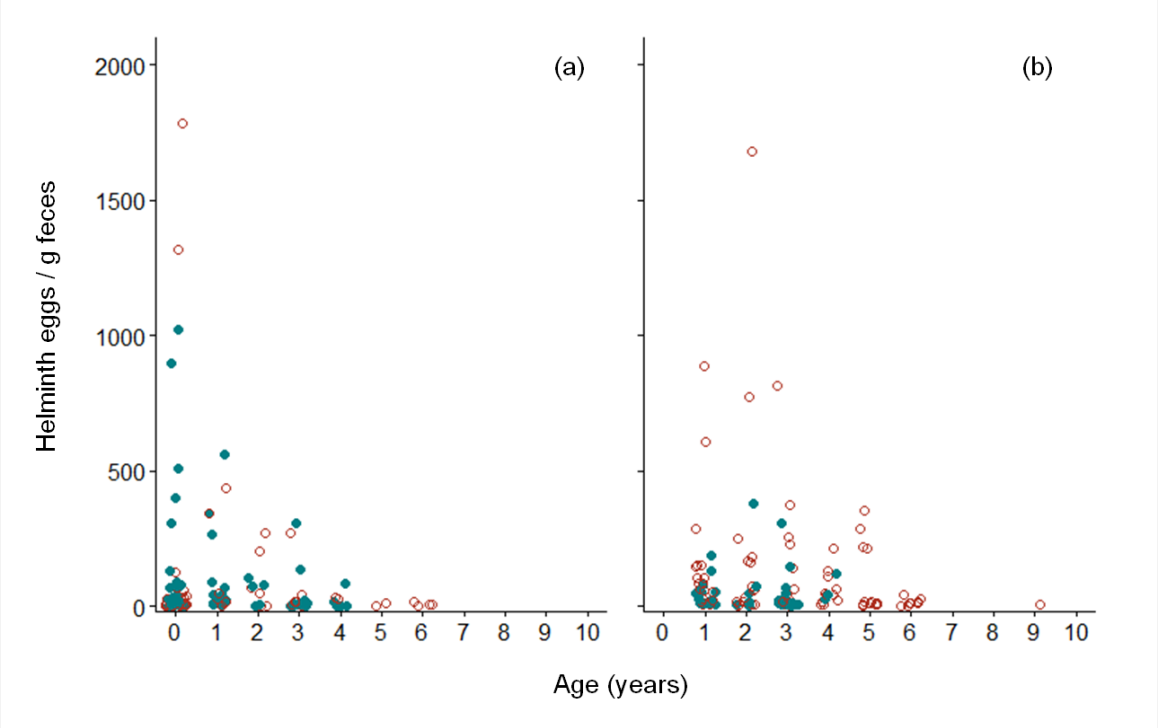

Figure S1: The total number of all helminth eggs recovered in the feces of male (solid symbols) and female (open symbols) gray mouse lemurs as a function of age in the (a) dry and (b) the rainy season. Data are shown for all samples infected with at least on type of parasite.

CHAPTER 5

5.1. EVALUATING CAPTURE STRESS IN WILD GRAY MOUSE LEMURS VIA REPEATED FECAL SAMPLING: METHOD VALIDATION AND THE INFLUENCE OF PRIOR EXPERIENCE AND HANDLING PROTOCOLS ON STRESS RESPONSES

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ABSTRACT

Reliable measurements of physiological stress are increasingly needed for eco-physiological research and for species conservation or management. Stress can be estimated by quantifying plasma glucocorticoid levels, but when this is not feasible, glucocorticoid metabolites are often measured from feces (FGCM). However, evidence is accumulating on the sensitivity of FGCM measurements to various nuisance factors. Careful species- and context-specific validations are therefore necessary to confirm the biological relevance and specificity of the method. The goals of this study were to: 1) establish and validate sampling methods and an enzymeimmunoassay to measure FGCM in the gray mouse lemur (*Microcebus murinus*); 2) explore causes of variability in the FGCM measurements, and; 3) assess the consequences of capturing and handling for free-living individuals by quantifying their stress responses via repeated fecal sampling within capture sessions. We further assessed the influence of different handling protocols and the animals' previous capture experience on the magnitude of the physiological response. Our validations identified the group-specific measurement of 11ß-hydroxyetiocholanolone as the most suitable assay for monitoring adrenocortical activity. The sample water content and the animal's age were found to significantly influence baseline FGCM-levels. Most captured animals exhibited a post-capture FGCM-elevation but its magnitude was not related to the handling protocol or capture experience. We found no evidence for long-term consequences of routine capturing on the animals' stress physiology. Hence the described methods can be employed to measure physiological stress in mouse lemurs in an effective and relatively non-invasive way.

KEYWORDS

physiological stress response, cortisol, fecal glucocorticoids, capture-and-handling, adrenocortical activity, prosimian, enzymeimmunoassay

1 INTRODUCTION

The assessment of physiological stress is increasingly used in many contexts of evolutionary biology, ecology and conservation to assess the health and coping of individuals or populations to environmental challenges [Ricklefs and Wikelski 2002; Romero 2004; Wikelski and Cooke 2006]. To this end, the capturing of animals is often necessary for physical examinations or sample collection. However, capturing and handling themselves are known to cause a stress response [Fletcher and Boonstra 2006; Möstl et al. 1999] that may introduce significant bias to studies of other phenomena if unaccounted for [Reeder and Kramer 2005]. The level of invasiveness of the handling procedures can influence the level of capture stress experienced by the animal [Bennett et al. 2012; Garcia et al. 2000] and within an individual, the magnitude of the physiological response to capture may change over subsequent captures via habituation or sensitization to the stressor [Boonstra 2013; Dickens and Michael Romero 2013; Fletcher and Boonstra 2006; Garcia et al. 2000; Lynn et al. 2010; Romero

2004; Walker et al. 2006]. Relatively little is known about the impact of capturing and handling procedures on animals despite the potential consequences for the research outcome and the welfare of the animals involved.

The physiological stress response – reflected by an increase in circulating glucocorticoid (GC) levels – facilitates appropriate reactions to and recovery from diverse challenges [Sapolsky et al. 2000]. Capturing may cause an acute elevation in stress hormone output, but it might also alter the long term stress physiology of the animal [Boonstra 2013; Clinchy et al. 2011] if a return to normal state is not achieved between capture events. While chronic stress may be adaptive in natural conditions in some circumstances [Boonstra 2013; Crespi et al. 2013], chronically elevated GC levels are typically associated with compromised health, reproduction and survival of individuals (reviewed in e.g. [Bonier et al. 2009a; Romero 2004]). Therefore, capturing may adversely affect especially those individuals that already have high GC levels prior to the capture [Collins 2001; Matthews et al. 2001] or have an impaired feedback system (e.g. due to old age) to facilitate the return back to baseline GC [Sapolsky et al. 1986].

The physiological stress response to capture can be measured via repeated blood sampling (e.g. [Lynn et al. 2010; Romero and Reed 2005], see also [Fletcher and Boonstra 2006]) or, alternatively, non-invasively using feces (FGCM) [Taylor 1971]. Fecal levels of GC metabolites reflect the baseline GC level at a delay of several hours and provide an integrated measurement of physiological stress levels over a period reflecting at minimum the animal's gut passage time [Harper and Austad 2000; Sheriff et al. 2010; Touma and Palme 2005]. FGCM may also better reflect the levels of biologically active, unbound GCs than total GC levels measured from blood [Breuner et al. 2013; Sheriff et al. 2011; Sheriff et al. 2010; Touma and Palme 2005].

Even though these advantages have led to the widespread use of FGCM in studies of natural populations, several caveats have recently been raised due to the sensitivity of FGCMs to potentially confounding factors [Goymann 2012]. The assessment of stress via FGCM measurements can be influenced by e.g. the ecological season [Huber et al. 2003; Romero 2002], the animal's diet [Goymann 2005], metabolic rate and gut bacterial community [Goymann 2012] and, not least importantly, the treatment of the samples and the analyses performed [Goymann 2005; Heistermann et al. 2006; Huber et al. 2003; Millspaugh and Washburn 2004; Möstl et al. 2005; Sheriff et al. 2011; Shutt et al. 2012]. Furthermore, the baseline GC level and the intensity of the endocrine stress response are known to vary among individuals based e.g. on their sex [Grueber et al. 2011; Wilson et al. 2002], age [Sapolsky et al. 1987], or prior experiences [Fletcher and Boonstra 2006; Garcia et al. 2000; Lynn et al. 2010; Walker et al. 2006]. As a result of these complex interactions, FGCM data tends to be "noisy" and, to draw meaningful conclusions, it is necessary to conduct careful species-specific validations of the methods and an evaluation of factors potentially confounding FGCM levels [Buchanan and Goldsmith 2004; Millspaugh and Washburn 2004; Möstl and Palme 2002; Romero 2004; Sheriff et al. 2011; Touma and Palme 2005].

In this study, we use FGCM measurements to quantify physiological stress in a small primate, the gray mouse lemur (*Microcebus murinus*). As GC excretion into feces has not been previously studied in the species, it was necessary to first select the most suitable assay for recording HPA axis activity

and to validate the method. Biological validations have been suggested as a method alternative to an ACTH-challenge for quantifying the hormonal stress response [Goymann 2005; Sheriff et al. 2011; Touma and Palme 2005]. Therefore we measured GC levels of wild and captive mouse lemurs before and after a known stressful event in three independent experiments. Based on these data, we selected an assay, assessed the lag-time to peak GC elevation and examined the influence of sample processing protocols on the FGCM measurements.

Following these validations, we examined stress responses to capture and handling of wild animals via repeated FGCM measurements. In a long-term monitored, routinely captured population of gray mouse lemurs (*Microcebus murinus*) (e.g. [Dammhahn 2012; Dammhahn and Kappeler 2009; Kraus et al. 2008]) some individuals voluntarily enter a trap up to 20 times per year and may be handled more than six times per year. The fact that mouse lemurs are easily re-trapped might suggest that the procedure is only minimally stressful to the individuals involved or that they habituate easily to trapping, in which case routine capturing may have few long-term consequences on their stress physiology. We hypothesized that the stress response to routine handling should be attenuated in animals with frequent capture experiences, in comparison to those individuals that enter traps less frequently and, if repeated capturing evokes chronic stress, this could translate to elevated baseline FGCM levels in the animals that are captured often. Additionally, the magnitude of the hormonal response should depend on the invasiveness of the handling regime the individual is subjected to [Bennett et al. 2012; Pitman et al. 1988]. To evaluate these effects, baseline FGCM and the change from baseline to response level FGCM were measured via repeated fecal sampling during capture sessions.

2 METHODS

2.1 The study population

The gray mouse lemur (*M. murinus*) is a small-bodied primate (average body mass ~60-80 g) that inhabits dry deciduous forests in Western Madagascar. The species is nocturnal, arboreal, sexually monomorphic and solitary living. The study population in the forest segment “N5” in Kirindy forest, Western Madagascar, has been intensively monitored since year 2001 for the purposes of long-term data collection (see e.g. [Dammhahn 2012; Dammhahn and Kappeler 2009; Kraus et al. 2008]). All individuals of the study population are individually marked with a subcutaneous transponder chip (Trovan). The animals are trapped using live-catch Sherman traps baited with banana. Routine capturing is conducted on three consecutive nights (“capture session”) monthly between March-May and September-November, in addition to which smaller scale captures may take place for the purposes of specific research projects.

2.2 Fecal sampling and field extraction

Upon each capture, fresh fecal samples were collected into polypropylene tubes from previously cleaned traps or when animals defecated during handling. The freshness of the feces was assessed based on the presence of a glossy surface on the pellets, since in the dry season the feces dry quickly after defecation. Because in most primates, a larger proportion of glucocorticoids are excreted via urine than via feces [Bahr et al. 2000; Wasser et al. 2000], any feces where urine contamination was suspected was not sampled. The time of day of collection and time lags to processing and extraction were recorded for each sample. Samples were extracted into ethanol in the field within 4 hours of sample collection adapting a protocol described by [Ziegler and Wittwer 2005] and modified by [Shutt et al. 2012]. Briefly, the freshest collected fecal pellets (total fecal mass of 0.15 – 0.8 g) were homogenized in a collection tube or on a petri dish with a metal rod, then a subsample of approximately 0.2 g (to the nearest 0.001 g) was weighed into an extraction tube and mixed with 2 ml of ~90% ethanol. For logistic reasons, the fecal suspensions were left to stand for 5-12 h, then vortexed for 2 min. Samples were finally centrifuged using a manually operated centrifuge (Hettich GmbH & Co. KG Tuttlingen, Germany) for 2 min [Shutt et al. 2012]. The supernatant was poured into a 2 ml polypropylene tube, sealed with parafilm and stored in a dark container at slightly below ambient temperatures until export to Germany, where samples were stored at -20° C until hormone analysis. The remaining fecal matter was sun-dried to a constant mass to obtain an estimate of the water content of the feces. The repeatability of the field processing and extraction procedure was assessed by splitting 16 homogenized samples collected in the wild into two subsamples and treating them the same way.

2.3 Method validations

2.3.1 Biological validation 1: Sampling series of wild animals in temporary captivity

We determined the validity of cortisol (CORT; [Palme and Möstl 1997]), corticosterone (CCST; [Heistermann et al. 2006]), and two group-specific enzymeimmunoassays (EIA) against cortisol metabolites with a 3α,11-oxo (11-oxoetiocholanolone: 3α,11-oxo-CM; [Möstl and Palme 2002]) and 3α,11β-dihydroxy structure (11ß-hydroxyetiocolanolone: 3α,11β-dihydroxy-CM; [Ganswindt et al. 2003]) to assess adrenocortical activity. All four assays have been successfully used to monitor GC output in various primate and non-primate species [Ganswindt et al. 2003; Heistermann et al. 2006; Martínez-Mota et al. 2008; Pirovino et al. 2011; Shutt et al. 2012; Wasser et al. 2000; Weingrill et al. 2011], including other lemurs (red-fronted lemur: [Ostner et al. 2008]; sifaka: [Fichtel et al. 2007]). For validation, we used the response to capture-and-handling stress (previously used e.g. by [Bosson et al. 2009; Dickens et al. 2009; Fletcher and Boonstra 2006]) in 4 individuals living in the camp area (thus being accustomed to human presence) to evaluate whether the increase in GC output is detected by the different fecal GC assays.

The animals were captured along paths in the field camp at sunset in October 2010 (2 males and 1 female) and in October 2012 (2 females; one female was used in both seasons). The captured individuals were handled briefly, first fecal samples were collected within 2 hours of capture to determine pre-capture control levels and the animal was allowed to re-enter the trap and left in a

safe area overnight. In the morning, the animals were handled for approximately 10 minutes for weighing, morphometric measurements and hair sampling. At dusk of the same day, the individuals were released into individual cages of approximately 1 m^3 that were kept inside a closed platform (for protection from predators) and furbished with branches, nest boxes and cover. Underneath each cage, plastic sheets were placed to facilitate collection of feces. The animals were held captive for 5 days. During this time they were fed with fruit and insects each morning and evening (approximately 12 h intervals) and all available fecal matter was removed from the cage at these occasions. At each collection, the freshest fecal pellets were homogenized and 0.2 g was extracted according to the protocol outlined in section 2.2.

2.3.2 Biological validation 2: Translocation of captive animals

Data from a translocation event was used to further assess the suitability of the four GC assays when a group of 17 animals (living in 8 subgroups of 1-3 individuals) was transported from Biopark d'Archamps (Archamps, France) to the German Primate Centre. Three fecal samples were collected from each subgroup of animals in the 5 days before the transport to record baseline GC levels and for 6 days thereafter to establish the GC response. Sleeping boxes were checked every morning and feces, if present, was collected in polystyrene tubes and stored at -20°C until further processing. Since animals within each subgroup shared nest boxes, we were unable to assign samples to specific individuals, thus, we assessed the stress-related changes in FGCM concentrations on the group level.

2.3.3 Biological validation 3: Repeated sampling of wild animals recaptured within a capture session

We collected repeated samples from wild individuals that were recaptured within capture sessions (see Section 2.1) to determine whether a capture-induced increase in the FGCM-levels could be seen in the days following a capture, and to assess the optimal delay to recording the maximum elevation in FGCM. Animals were sampled during handling on day 1 of the session, and subsequently samples were collected from animals recaptured one (“day 2”, 24 h delay, n=21), two (“day 3”, 48 h delay, n=23) or three (“day 4”, 72 h delay, data from additional capture nights, n=26) days after the first capture. For this analysis we only included cases where the animal was not captured on any of the intermediate days to exclude the possibility of cumulative stress due to repeated captures. Based on the magnitude of the change in FGCM from day 1 to the subsequent re-capture (see Section 3.1.5), and data from the other two biological validation series, a sampling interval of two days (~48 h, day 1 to day 3 of the session) was chosen for the subsequent study on capture-and-handling stress (Section 2.4).

2.3.4 Hormone analysis and HPLC

The fecal samples collected from captive mouse lemurs were processed and extracted following Heistermann et al. [1995]. In summary, we lyophilized and pulverized the feces and extracted an aliquot representing 0.04-0.06 g of fecal powder in 3 ml of 80 % methanol in water by vortexing the suspension for 10 min. Following centrifugation of the fecal suspension at 3000 rpm for 10 min, we recovered the supernatant and stored it at -20 °C until analysis. We analyzed fecal extracts for GC immunoreactivity with the four aforementioned EIA systems as described by [Heistermann et al. 2004; Heistermann et al. 2006]. Information on antibody characteristics, standards, and hormone

labels used as well as on other assay details, including data on assay sensitivities, is given in [Heistermann et al. 2006]. Intra- and inter-assay coefficients of variation (CVs) of high- and low-value quality controls were <10 % and <13 %, respectively, for all four assays.

Based on the outcome of the two biological validation tests (see Sections 3.1.1 and 3.1.2), the two group-specific assays were deemed most suitable for monitoring FGCM output. In order to characterize the patterns of metabolites measured by these two assays and evaluate any co-measurement of certain fecal androgens which could potentially be detected by antibodies raised against cortisol metabolites (see [Ganswindt et al. 2003; Heistermann et al. 2006]), we performed reverse-phase high pressure liquid chromatography analysis (HPLC). HPLC was carried out as described by [Möhle et al. 2002] and [Heistermann et al. 2006] using a fecal extract generated from a sample from one of the wild male study subjects. This sample was collected shortly prior to the onset of the mating season when the male had enlarged testes and presumably high levels of androgens [Aujard and Perret 1998]. We measured each HPLC fraction in the two group-specific FGCM assays to generate profiles of immunoreactivity.

Based on the combined validation and HPLC results, the 11ß-hydroxyetiocholanolone (3α,11β-dihydroxy-CM) EIA was used for the analysis of all fecal samples. The EIA was performed as described in detail by [Heistermann et al. 2004]. Prior to hormone measurement, we diluted extracts 1:50-1:300 (depending on concentration) in assay buffer and took duplicate aliquots to assay. Sensitivity of the assay was 1 pg/well. Serial dilutions of fecal extracts gave displacement curves parallel to those obtained with the 11ß-hydroxyetiocholanonole standard. Intra- and inter-assay coefficients of variation of high- and low-value quality controls were 6.5 % (high, n=16) and 7.4 % (low, n=16) and 10.4 % (high, n=24) and 11.9 % (low, n=24), respectively. All hormone concentrations are given as ng/g fecal wet weight.

2.3.5 Testing post-defecation change in FGCM levels

The delay from defecation to sample processing has been shown to potentially affect FGCM levels [Millspaugh and Washburn 2004; Möstl et al. 1999; Shutt et al. 2012] as a result of bacterial induced alterations on excreted metabolites. Dependent on field conditions and trapping designs it is not always possible to know the exact time of defecation or to immediately process samples. Since in our study, feces had to be stored unpreserved for up to 4 hours after defecation (see Section 2.2), we tested whether FGCM levels change as a function of the time elapsed between defecation and sample preservation in ethanol. For this, we recorded the time delays from collection to processing for all samples collected in the wild and tested statistically for time effects (from collection to preservation in ethanol; from collection to extraction; from preservation in ethanol to extraction). Additionally, we conducted a controlled degradation experiment in captivity at a mouse lemur breeding colony in Brunoy, France (MNHN; European Institutions Agreement No. 962773), where larger sample masses compared to the wild were obtainable. In total, we collected 11 fresh fecal samples (4 pooled samples from 2-3 individuals, individual samples from 7 animals) immediately after defecation and homogenized them well. Each sample was split into 2-7 0.2 g subsamples (depending on total sample mass obtained) which were placed in extraction tubes. 2 ml of 90 % ethanol was added to one of the subsamples within one hour after defecation (time 0 control). The

remaining samples were left at ambient temperature (ca. 22-24 °C) and ethanol was added to the samples at one to two hour intervals until 10 hours after defecation.

After the addition of the ethanol, each subsample was briefly stirred in order to immerse the entire fecal sample in the solvent. All tubes were stored at ambient temperature until the following morning (within 18 h of sample collection), and samples were then extracted as described in 2.2., with two exceptions: the precipitate was allowed to settle instead of centrifuging the samples, and the pellets were oven-dried instead of sun-drying.

2.4 Physiological response to capturing and different handling regimes

The stress response to capture and handling in the wild study population was evaluated during five monthly 3-day capture sessions in the non-reproductive season (March-July 2012) (see Section 2.1). In each session, traps were set before dusk and closed at dawn. In March-May, traps were additionally monitored in the evening when a subset of the captured animals was subjected to brief handling on site of capture for a physical strength experiment. All captured animals were transported to the field camp. In the morning following the first night of the capture session ("day 1"), each individual was subjected to handling and fecal samples were collected for establishing the baseline FGCM level. At dusk, the animals were released at the capture location.

To assess the magnitude of the physiological stress caused by capture and handling, repeated samples were collected from the individuals that were captured on days 1 and 3 of the capture session (n=65) and the change in FGCM from day 1 to day 3 was used to quantify the stress response. On day 1 each individual was subjected to one of three handling protocols: minimum handling, repeated handling or anesthesia (Table 1). On day 3, they were only let out of the trap into a fabric bag to facilitate fresh feces collection. Many individuals (n=38) were also captured on day 2, intermediate to the two sampling events, but were not handled.

Anesthesia was avoided whenever possible to minimize any adverse effects of the chemical treatment and therefore, only unmarked individuals were anesthetized in order to insert a transponder and gain tissue samples. Hence, we cannot fully disentangle the effects of anesthesia, first time capture and age (presumably, the youngest individuals of the cohort are anesthetized) as the size and complexity of the data set did not permit a detailed analysis of the possible interactions of these factors.

The animals' ages (juvenile, <1 year or adult, >1 year old) and individual capture histories were drawn from the routine capture data. Lifetime capture experience was calculated as the total number of capture events from birth to June 2012 (median=6, range: 1-95), divided by the age in years (range: 0-10) to adjust prior experience for differences in exposure to traps.

Table 1: Description of the handling regimes used and associated sample sizes.

Regime	Procedure	~Handling time	Sample size
Minimum handling	Body mass measurement only. The animal is released from the trap into a fabric bag in the morning and weighed using a Pesola scale, then allowed to re-enter the trap.	2 minutes	47
Repeated handling	The animal was handled briefly for a physical strength experiment on the night before the routine processing and handled again in the morning according to the minimum handling regime.	5 minutes + 2 minutes	13
Anesthesia	Unmarked individuals were briefly anesthetized with 0.2 ml subdermally administered Ketamin-solution (Ketanest 100, Parke-Davis), then tissue sampled by cutting small pieces of the ears, equipped with a subcutaneous transponder microchip (Trovan) and hair sampled using pet clippers. Morphometrics and body mass were measured.	25 minutes	12

2.5 Statistical analysis

2.5.1 Validation of a fecal glucocorticoid assay

A linear mixed effects model [Möstl and Palme 2002] was used to estimate post-defecation change in FGCM in untreated feces in the controlled experiment in captivity. The log-transformed sample FGCM was used as the response variable and FGCM at time 0 (control), number of hours after collection and their interaction were included as covariates, weighted by the number of samples in the series. Sampling series identity was added as a random effect.

For samples collected in the wild, the influence of lag times from sample collection to the different stages of processing (preservation in ethanol and extraction) on the sample FGCM level were calculated using linear regression, with fecal water content included as a control variable. Protocol repeatability was assessed by calculating Spearman's correlation coefficients for 16 duplicate samples. The optimal lag time to recording the maximum hormonal response to capture and handling (change from day 1 to day 2, 3 and 4, with no intermediate capture events) was assessed using one-tailed paired t-tests.

2.5.2 Baseline FGCM and the stress response to capturing and handling

Linear mixed models [Pinheiro and Bates 2000] were used to examine the factors causing variation in baseline FGCM and in the stress response to capturing and handling. All mixed models were built using the R-program package lme4, employing the function lmer with a Gaussian error distribution and an identity link [Bates et al. 2012], and individual identity was used as a random effect. Variables were log-transformed when necessary to meet assumptions of normality, homoscedasticity and linearity of relationships, which were assessed using residual plots. Model stability was confirmed by calculating variance inflation factors and by excluding data points one by one and comparing the resulting estimates and fitted values with the inclusive model. The P-values were obtained via

Markov Chain Monte Carlo (MCMC)-estimation [Baayen 2011] or, for factorial variables with 3 or more levels, via likelihood ratio tests (LRT). The relationship between the individual's baseline FGCM level and the magnitude of the response was studied with simple linear regression, controlling for the possible influence of second day captures.

Two potential nuisance factors: fecal water content (water%) and the time of day of sampling were included as control variables in the models. Fresh fecal mass was used instead of dry mass in samples collected from the wild to adjust for a higher measurement error in weighing very small dry sample masses. The water% (difference in mass between fresh and dried sample) showed high variability and strongly, negatively influenced the measured FGCM level (β=-3.430, SE=0.613, df=130, X^2=30.510, P<0.001). Sample water content was therefore controlled for in all models. A part of the baseline samples were collected in the evening before the handling and all other samples in the morning during handling. As it is known from many species that the diurnal rhythms can cause variation on GC-levels over the course of the day [Bosson et al. 2009; Rimbach et al. 2013], we also included the time of day of sample collection in the baseline model.

To study how the baseline FGCM was influenced by variables pertaining to the individual (age, sex, lifetime capturing experience) and the sampling event (month, time of day, water%) we used all baseline samples collected during the field season (March-July 2012, including also cases where the individual was not recaptured) in order to increase the sample size, resulting in n=277 observations from 145 individuals.

To estimate the influences of lifetime capture experience and handling regime on the magnitude of the stress response to capture and handling, we created interaction terms of each of these variables with the sampling stage (baseline vs. response sensu [Liang and Zeger 2000] and [Liu et al. 2009]) and modeled their influence on the sample FGCM level.

Since the influences of age, experience and regime cannot be separated in the anesthesia regime (only juvenile animals were anesthetized) we restricted the above response model to cases from the minimum and repeated handling regimes. Therefore, we constructed a separate model to assess the difference between the anesthesia and minimum regime in juveniles only (n=53 observations from 27 individuals), including regime and intermediate capture as interaction terms with stage, and water% as control. Capture experience was 0 for all anesthetized individuals and was therefore excluded from the model.

From the baseline analyses (see Section 3.2) it was evident that water% explained a large part of the variation in the FGCM levels and was therefore included as a control variable in the stress response model. Furthermore, age and the potential cumulative effect of an intermediate capture between samplings (second day capture) were controlled for by including their interactions with stage. Due to missing values, the total number of cases included in the model was 55 observations from 28 individuals. To account for the low sample size and model complexity, we attempted to improve the model fit by removing the least significant interaction terms (above P=0.1) one at a time based on LRT [Engqvist 2005].

Although baseline values were not significantly influenced by the time of day of sampling (see Section 3.2), it was further examined in stress response models in order to control for possible time differences between the repeated sampling events in stress response models (evening samples n=6). For this purpose the models were re-run after excluding the 6 cases of evening sampling. The exclusion did not affect the model outcomes and therefore all cases were included in the analyses and the time of day of sampling removed from the final models.

Means are given with standard errors and significance levels were set to P=0.05 for all tests. All analyses were done in R version 2.15.1. [R Development Core Team 2012].

3 RESULTS

3.1 Validation of a fecal glucocorticoid assay

3.1.1 Biological validation 1

In absolute terms, the highest levels of fecal GCs were measured by the group-specific 11-oxoetiocholanolone assay whilst the lowest concentrations were detected by the CCST assay (Table 2; Figures 1 and 2). Nevertheless, in all 5 cases of captured wild animals, all four assays measured a similarly strong response to the capture and handling as reflected by an on average 8-9-fold increase in FGCM levels (Table 2; Figure 1). FGCM levels started to rise within 24 hours of the capture, with median lag times to peak FGCM response ranging from 38 to 50 hours across the four assays (Figure 1; Table 2). There was considerable individual variation in the timing of the peak FGCM response in all assays; however, the two group-specific assays were more consistent in this respect than the CORT and CCST assays (Table 2). FGCM levels usually returned to pre-stress levels by day 4 (Figure 1).

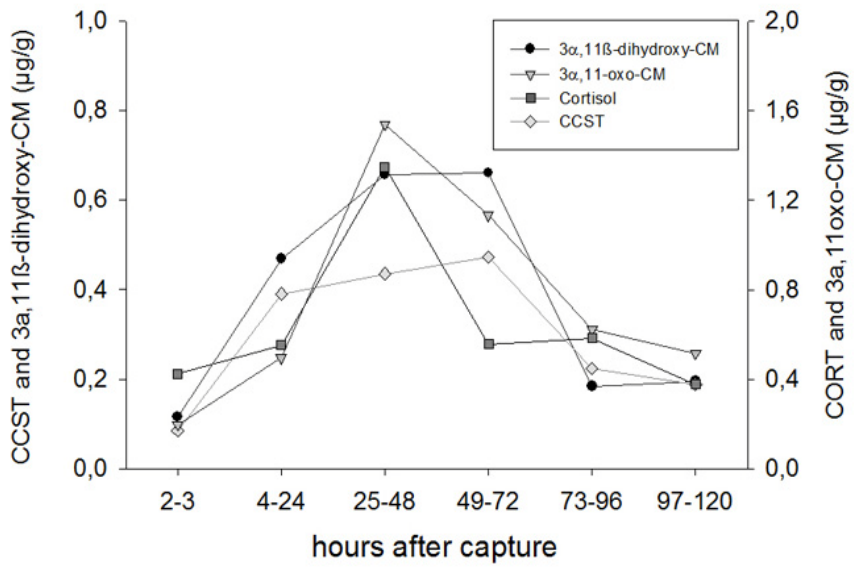

Figure 1: Change in immune-reactive FGCM levels as measured by four glucocorticoid EIAs in response to capture and handling. Data points represent median values calculated for 24 h intervals across the 5 cases examined.

Table 2: Fecal glucocorticoid concentrations (as detected by four different GC assays) in response to capture stress in wild mouse lemurs

Individual	3a,11ß-dihydroxy-CM[#]				3a,11oxo-CM[#]				Cortisol				CCST			
	Pre[a]	Peak[b]	Delta[c]	Lag[d]	Pre[a]	Peak[b]	Delta[c]	Lag[d]	Pre[a]	Peak[b]	Delta[c]	Lag[d]	Pre[a]	Peak[b]	Delta[c]	Lag[d]
06DF B6BB*	0.21	1.68	8.00	62.50	0.50	4.50	9.00	62.50	0.42	1.44	3.40	38.30	0.12	0.87	7.40	62.50
06E9 B40D	0.10	1.11	11.10	50.30	0.20	2.06	10.30	38.30	0.51	6.13	12.00	38.30	0.09	1.37	16.10	38.30
06E9 CDDE	0.12	3.12	27.00	50.30	0.09	7.47	79.30	38.30	0.17	2.06	12.10	26.30	0.06	0.52	8.50	15.80
06DF B6BB*	1.23	8.90	7.20	45.50	2.51	9.36	3.70	45.50	0.46	1.94	4.20	45.50	0.36	1.41	3.90	45.50
CAMP F1	0.02	0.13	7.60	34.50	0.05	0.44	8.80	34.50	0.11	0.99	9.00	69.50	0.06	0.86	14.30	69.50
Median	**0.12**	**1.68**	**8.00**	**50.30**	**0.20**	**4.50**	**9.00**	**38.30**	**0.42**	**1.94**	**9.00**	**38.30**	**0.09**	**0.87**	**8.50**	**45.50**

[a] baseline levels (i.e. levels within 2 h after capture) in µg/g wet feces (see Methods)

[b] peak levels in response to capture in µg/g wet feces

[c] x-fold increase of peak levels above baseline concentrations

[e] lag time in hours between time of capture and peak response

* animal was captured twice (in 2010 and 2012)

[#] group-specific assay

3.1.2 Biological validation 2

The FGCM response to the translocation event was generally similar to that seen in the captured animals. Following translocation, a clear increase in FGCM levels was detected by the different assays with the exception of the cortisol measurement which demonstrated a decrease (Figure 2). The FGCM increase measured by the two group-specific EIAs was similar in magnitude (about 4 fold) and more pronounced than that of the CCST assay (about 2 fold). In terms of timing, both the start of the FGCM rise (on average at day 3 post-transport; Figure 2) as well as the occurrence of peak levels (day 4 post-transport, Figure 2) was delayed by about two days compared to the situation observed in the wild animals. This delay was most likely due to the fact that all animals were completely inactive and did not feed on the first day after arrival and remained very inactive with only small amounts of food consumed in the days thereafter. It is conceivable that metabolic rate and gut passage time were thus slowed down, resulting in a delayed hormone metabolism and excretion pattern. Additionally, the fact that animals were only sampled once per day likely contributed to the pronounced delay times in the GC responses observed.

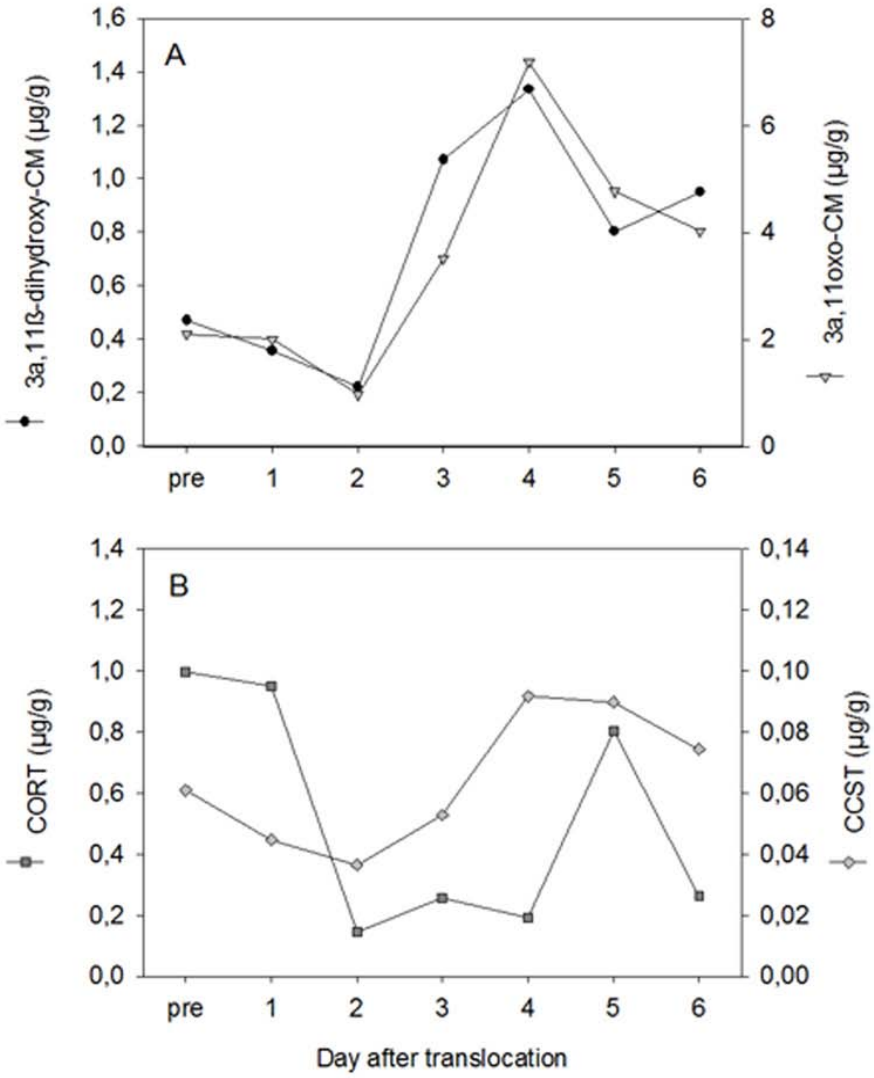

Figure 2: Change in immunoreactive FGCM levels as measured by (A) the two group-specific assays and (B) the cortisol and CCST assays in response to translocation. Data points represent median values calculated for 24 h intervals across the 8 groups of animals examined. Pre = pre-transport baseline FGCM levels.

3.1.3 HPLC

Since the two group-specific FGCM assays appeared to be of similar value for monitoring adrenocortical activity, we performed an HPLC analysis to characterize the immunoreactive metabolites present in the feces and measured by the two assays. HPLC immunoreactivity profiles indicated the presence of several distinct peaks between fractions 9 and 31 in both assays (Figure 3), at positions where cortisol metabolites elute in our HPLC system [Heistermann et al. 2006]. The

presence of abundant immunoreactivity found at fractions 25 (11ß-hydroxyetiocholanolone EIA) and 30 (11oxoetiocholanolone EIA) at the elution positions of 11ß-hydroxyetiocholanolone and 11-oxoetiocholanolone standards, respectively, indicate that these two cortisol metabolites are abundant in the feces of grey mouse lemurs. In the 11oxo-etiocholanolone assay, however, the major peak of immunoreactivity was detected around fraction 50 at a position where in other primate species metabolites of testosterone elute [Möhle et al. 2002], indicating a substantial co-measurement of steroids not deriving from cortisol in the 11-oxoetiocholanolone EIA. Based on the combined results from validations 1 and 2 and the HPLC data, we selected the 11ß-hydroxyetiocholanolone assay for all further analyses.

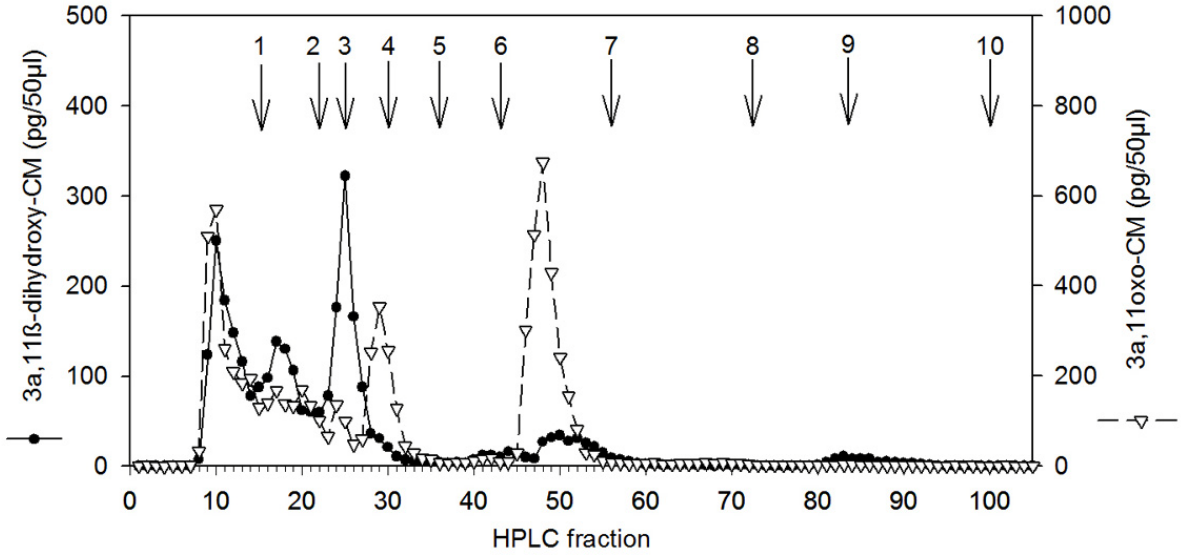

Figure 3: HPLC profiles of immunoreactivity detected with the (A) 3α,11β-dihydroxy-CM and (B) 3α,11-oxo-CM EIA in a fecal extract of a wild male gray mouse lemur. Arrows and numbers indicate the associated elution positions of reference standards: 1) cortisol (fraction 14/15), 2) corticosterone (22/23), 3) 11ß-hydroxyetiocolanolone (24/25), 4) 11-oxoetiocholanolone (29/30), 5) 5β-androstane-3,11,17-trione (36), 6) testosterone (43), 7) androstenedione, dehydroepiandrosterone (55/56), 8) epiandrosterone, 5β-dihydrotestosterone (72), 9) 5β-androstane-3α-ol-17-one (82/83) and 10) androsterone (100).

3.1.4 Fecal sampling method

The protocol repeatability assessment revealed a highly significant correlation between the FGCM levels measured in duplicate subsamples (for ng/g fresh feces r_S=0.92, P<0.001, n=16). In samples collected from the wild (n=289), FGCM results were not significantly influenced by differences in the time lags from collection to preservation in ethanol (range: 0.5-4 h, β=-0.138, SE= 0.126, t=-1.090,

P=0.276), from collection to extraction (6-14 h, β=0.194, SE=0.125, t=1.558, P=0.120) or from preservation in ethanol to extraction (5-12 h, β=-0.147, SE=0.127, t=-1.158, P=0.248).

3.1.5 Biological validation 3

Paired samples from repeated captures within a capture session (change from day 1 to day 2, 3 or 4) revealed a significant elevation from baseline FGCM at each time interval, with a 2-day lag (samples from day 1 and day 3) showing the strongest and most consistent effect (Table 3, Figure 4), confirming the results on GC-response time lags derived from biological validation 1 (camp animals, Sections 1.2.3.3 and 1.3.1.1). Consequently, the day 1 to day 3 change in FGCM was used to quantify the stress response to capture and handling in the subsequent experiment.

Table 3: Differences between baseline and response FGCM level, measured by the 11ß-hydroxyetiocholanolone assay, at a delay of 1-3 days. Paired samples from recaptures within a capture session.

	Approximate time difference	**One-tailed paired t-test**	**df**	**P**
Day 1 vs. day 2	24 h	t= 3.449	20	0.001
Day 1 vs. day 3	48 h	t= 4.159	27	0.0001
Day 1 vs. day 4	72 h	t= 1.832	16	0.043

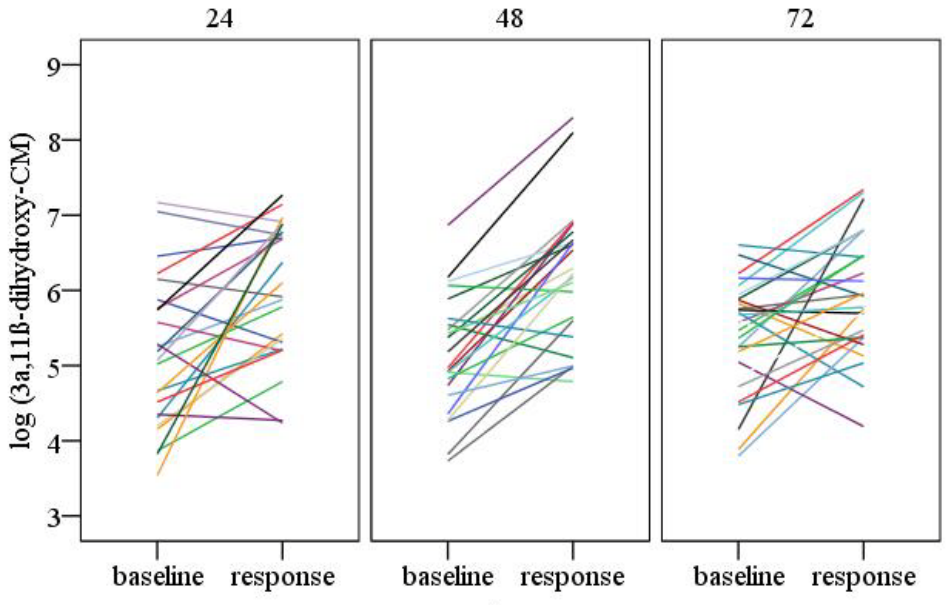

Figure 4: Change in FGCM levels, detected by 11ß-hydroxyetiocholanolone assay, from baseline (day 1) to a consecutive sample collected (A) 24, (B) 48 or (C) 72 hours after the baseline sample. For statistics see Table 3.

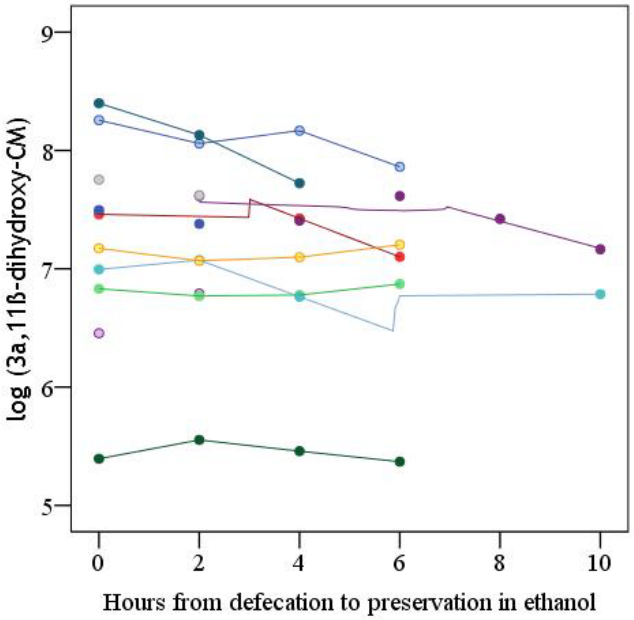

Figure 5: Post-defecation changes over time in FGCM levels in untreated feces, based on the experiment in captivity, detected by 11ß-hydroxyetiocholanolone assay. Lines (95% loess smoothers) represent sampling series of fecal samples divided into several subsamples that were preserved in ethanol at different delays after defecation.

3.1.6 Post-defecation changes in FGCM

The profiles of change in FGCM over time (Figure 5) illustrate that the delay from sample collection to preservation in ethanol did not induce statistically significant changes in the sample FGCM (baseline FGCM(t0): β=0.950, SE=0.027, t=34.93, P<0.001, hours to ethanol added: β=0.118, SE=0.046, t= 2.57, P=0.292, interaction: β=-0.020, SE=0.006, t=-3.13, P=0.121).

3.2 Correlates of baseline FGCM

The percentage of water negatively influenced the FGCM level in a sample (Table 4). The overall baseline FGCM decreased slightly over the course of the season, but the overall influence of the month of capture was not statistically significant (month of capture: X^2_4=6.962, P=0.138). Adults had on average higher baseline FGCM values than juveniles (Table 4), but the time of day of sampling, the animal's sex and previous capture experience had no significant influence on the FGCM level (Table 4).

Table 4: Variables influencing the baseline FGCM levels (day 1 level). P-values are based on MCMC-tests, significant results in bold. n=277 observations from 145 individuals

Fixed effects		**Estimate**	**SE**	**t**	**P**
Intercept		**8.040**	**0.485**	**16.573**	**<0.001**
Water%		**-3.661**	**0.620**	**-5.906**	**<0.001**
Time of day (ref. Morning)		0.295	0.151	1.948	0.087
Month (ref. March)	April	-0.355	0.199	-1.783	0.068
	May	-0.486	**0.203**	**-2.392**	**0.008**
	June	**-0.519**	**0.232**	**-2.239**	**0.008**
	July	-0.365	0.231	-1.583	0.067
Age category (ref. Adult)		**-0.404**	**0.136**	**-2.959**	**0.002**
Sex (ref. Female)		0.081	0.126	0.645	0.613
Capture experience		0.023	0.074	0.305	0.727

3.3 Physiological response to capturing and handling

In the capture and handling experiment, an increase in the FGCM level from day 1 to day 3 was evident in 78% (61/78) of the sample pairs (3.30±2.81 fold increase from baseline, t_{paired}=6.12, df=77, P<0.001). The magnitude of the response was positively influenced by the baseline FGCM level (β=0.449, SE=0.144, $F_{2,45}$=9.406, P=0.002). The individuals that showed a decline from day 1 to day 3 had higher than average baseline FGCM concentrations and mainly represented the minimum handling regime (15/17 cases).

In the stress response model, the interaction terms *stage x regime* (LRT: X^2_1=0.007, P=0.934) and *stage x experience* (X^2_1=0.028, P=0.867) were the least significant terms and were removed from the model in this order. In the final model, the magnitude of the stress response tended to be slightly higher for the animals that were captured also on the intermediate day (P=0.068, Table 5). The response strength was not significantly influenced by the animal's age), handling regime or capture experience (Table 5).

In the model for anesthesia vs. minimum handling (juveniles only), the *regime x stage* interaction was non-significant (LRT: X^2_1=0.356, P=0.551). After removing the interaction term, the anesthesia main effect was significant (reference value: no anesthesia, β=-0.662, SE=0.269, t=-2.463, P=0.009), indicating that anesthetized animals had overall lower FGCM values than the minimum handling juveniles.

Table 5. Variables influencing the FGCM response to capture and handling, final model. Interaction terms are indicated with "x". P-values are based on MCMC-tests. N=55 observations from 28 individuals.

Fixed effects	**Estimate**	**SE**	**t**	**P**
Intercept	5.777	0.909	6.357	<0.001
Stage	0.464	0.410	1.133	0.335
Water%	-0.830	1.144	-0.726	0.476
Age category (ref. Adult)	0.264	0.295	0.895	0.413
Intermediate capture (ref. none)	-0.101	0.343	-0.294	0.782
Capture experience	-0.125	0.187	-0.671	0.514
Regime (ref. Minimum)	0.334	0.282	1.187	0.253
Intermediate capture (ref. none) x Stage	0.909	0.419	2.171	0.068
Age category (ref. Adult) x stage	-0.677	0.352	-1.922	0.118

4 DISCUSSION

In this study, we successfully established a group-specific 11ß-hydroxyetiocholanolone assay for monitoring the physiological stress response in the grey mouse lemur via fecal samples and applied this method to explore the influence of capture and handling on the hormonal stress response in wild animals. These data showed no evidence of habituation or sensitization of individuals as a response to frequent capture and handling. In addition, the results indicate that more invasive handling did not cause a stronger stress response in the animals. Our study provides important new information for field researchers interested in using fecal glucocorticoid analysis techniques to monitor adrenocortical activity in mouse lemurs and suggests that routine capturing by trapping and brief handling at monthly intervals does not cause chronic stress to animals.

4.1 Validation of a fecal FGCM assay

A major aim of this study was to validate a reliable assay for measuring fecal glucocorticoids as a measure of physiological stress in gray mouse lemurs which is of primary importance before any application [Buchanan and Goldsmith 2004; Heistermann et al. 2006; Touma and Palme 2005]. Our tests of physiological validity suggest that at least three of the four glucocorticoid EIAs tested are generally suitable for the detection of changes in adrenocortical activity in the species through fecal GC metabolite analysis. However, the two group-specific assays appear to be more sensitive in detecting stress-related changes in glucocorticoid output as shown by a more consistent response across contexts compared to the CCST and, in particular, the CORT assay. Thus, the group-specific assays appear to be better suited for assessing adrenocortical activity in gray mouse lemurs.

Moreover, HPLC immunoreactivity peaks co-eluting with 11ß-hydroxyetiocholanolone and 11-oxoetiocholanolone standards indicated the presence of 3α,11ß-dihydroxylated GC metabolites and 11,17-dioxoandrostanes, both of which have also been reported as abundant fecal glucocorticoid

metabolites in other primate and non-primate species (e.g. [Ganswindt et al. 2003; Heistermann et al. 2006; Ostner et al. 2008; Palme and Möstl 1997]). Since group-specific glucocorticoid assays measure a broad spectrum of steroids, there is, however, a higher risk of co-measurement of androgen metabolites [Ganswindt et al. 2003; Schatz and Palme 2001] compared to more specific assays. Our HPLC data indicate that, similar to findings in the chimpanzee [Heistermann et al. 2006], this is likely the case concerning the 11-oxoetiocholanolone assay which detected peak amounts of immunoreactivity at a position where in our HPLC system metabolites of testosterone (but not of cortisol) elute [Ganswindt et al. 2003; Heistermann et al. 2006; Möhle et al. 2002]. The results also indicate a small potential for co-measurement of androgen metabolites by the 11ß-hydroxyetiocholanolone assay. For this study, however, this is likely of negligible importance given that the vast majority of samples of our capture stress experiments were collected when animals are reproductively quiescent and sex hormone levels are probably low [Aujard and Perret 1998], thus FGCM levels in the experiments were unlikely affected by the animals' reproductive state. In contrast, the sample used for HPLC came from a male in prime reproductive condition (shortly before onset of the short mating season, with maximum testes size) when androgen levels are clearly elevated [Aujard and Perret 1998]. For measuring FGCM in our samples, we therefore chose the 11ß-hydroxyetiocholanolone assay which has proven to be a versatile assay to monitor adrenocortical activity in many other species of primates [Fichtel et al. 2007; Heistermann et al. 2006; Martínez-Mota et al. 2008; Ostner et al. 2008; Pirovino et al. 2011; Rimbach et al. 2013; Shutt et al. 2012; Weingrill et al. 2011] and non-primates [Ganswindt et al. 2003; Möstl et al. 2002].

Our data from the capture-validation study as well as from the repeated capture experiment of wild animals indicate a 2-day delay in FGCM excretion from stressor to peak response, with levels starting to rise within 24 hours of the stressful event. Although this time lag in fecal glucocorticoid excretion is within the range of those reported for many other primate species [Heistermann et al. 2006; Pirovino et al. 2011; Shutt et al. 2012; Weingrill et al. 2011; Whitten et al. 1998], it is distinctly longer than expected given the small body size of the species and a gut passage time (which usually determines the lag time between steroid secretion in blood and appearance of the metabolites in feces [Palme 2005]) of less than 24 h [Raharivololona 2009]. The reason for this long delay in peak FGCM excretion is unknown, but may, at least in part, be explained by cumulative stress of capture with up to 20 h restraint in the trap [Fletcher and Boonstra 2006; Garcia et al. 2000] and subsequent handling and housing in a novel environment (validation animals). As suggested by our translocation data, the extent of activity and food consumption appears also to have a marked effect on the time course of FGCM excretion, probably by altering general metabolic rate and processes, which might affect hormone excretion patterns [Goymann 2012; Morrow et al. 2002]. There was no indication of reduced activity and feeding in our wild study animals, however. Finally, a pronounced and delayed enterohepatic recirculation of glucocorticoids prior to excretion of the metabolites into the feces [Lindner 1972; Möstl and Palme 2002; Symonds et al. 1994] may also contribute to the long lag time, although this remains completely speculative.

Collectively, these data suggest that peak FGCM output responses in the grey mouse lemur can generally be predicted to occur 1-2 days after exposure to a stressor. There was, however, considerable variation among individuals in the time to peak FGCM elevation. This variation was

particularly evident for the CORT and CCST assay, whilst time lags for the two group-specific assays were more consistent, a finding also seen for lowland gorillas [Shutt et al. 2012]. The reason for this is not clear but may be related to differing influences of factors affecting metabolic processes and the production of the various types of cortisol metabolites measured by the different assays. Further studies on stress responsiveness and GC-metabolism in the species, including an ACTH-challenge test and the assessment of post-peak decline in the FGCM in a larger number of animals will be required to identify the causes of the long delays and the heterogeneity of responses.

Most wild-caught individuals were able to return from the peak elevation to near the baseline within 2-3 days. Typically, habituation to captivity is assessed in time periods of months rather than days, particularly when behavioral aspects are studied and very few studies exist on the short term (physiological) habituation to captivity. One such study by [Dickens et al. 2009] showed that wild-caught chukar partridge (*Alectoris chukar*) can return from stress response GC levels to near the baseline levels in approximately 9 days after capture and subsequent captivity. Short term studies such as ours provide evidence towards a more rapid physiological habituation than would be expected based on behavioral measures of habituation, which may reflect protective mechanisms against chronically elevated cortisol levels. There is some evidence that stabilizing conditions, such as an improvement in the predictability of food acquisition may decrease a stress response and could possibly reduce the time required to return to baseline when wild-caught animals are held in temporary captivity and fed at regular intervals [Romero 2004]. On the other hand, the diet in captivity differed from the natural diet which could potentially also influence the change in measured FGCM levels [Behie et al. 2010; Dantzer et al. 2011; Goymann 2005; Goymann 2012; Millspaugh and Washburn 2004] from baseline to subsequent measurements in our study. Overall, our study joins many others in stressing the importance of validation across contexts: we have shown that the delay from the stressor to recording the peak stress response via feces differed between experiments using captive and wild animals. This source of uncertainty should be considered when conducting GC research in conditions that differ from the validation setting.

4.2 Post-defecation FGCM change in unpreserved feces

Our experiment illustrates that FGCM concentrations in grey mouse lemurs are generally unaffected when preservation of the sample in alcohol takes place within 10 h of defecation. To date, few studies have investigated hormone change in feces between defecation and fixation. Most of these studies demonstrated either an increase (cattle, horses and pigs [Möstl et al. 1999]; Borneon orangutans [Muehlenbein et al. 2012], or decrease (brown hyena [Hulsman et al. 2011]; lowland gorilla [Shutt et al. 2012]) in FGCM concentrations within a few hours when samples were stored unpreserved at ambient temperature. Our findings are therefore noteworthy and imply that immediate fecal preservation is not absolutely necessary to obtain reliable FGCM results for mouse lemurs (see also [Rehnus et al. 2009] for mountain hares). This is of particular importance for studies in the wild where immediate immersion of the feces after defecation in ethanol may be difficult to accomplish. In our setting, most individuals are captured in the first hours of the activity period and the period between defecation to preservation of the sample in ethanol is largely standardized. However, as defecation time is often not accurately known, only fresh-looking feces were collected

to minimize the time interval between defecation and fecal preservation and the risk of sample degradation. Confirming the findings from captive experiment, no effect of the sampling-to-preservation period was found on FGCM results in the samples collected from wild animals.

4.3 Correlates of baseline FGCM

Adult wild mouse lemurs had significantly higher baseline FGCM levels than juveniles, which agrees with the trend found in humans, some non-human primates and rodents [Gunnar and Quevedo 2007; Romeo 2010; Sanchez et al. 2001; Sapolsky and Altmann 1991; Sapolsky and Meaney 1986] and has been explained with the maturation and senescence of the adrenocortical system. The individual's sex or time of day of the sampling (morning vs. evening) had no influence on the FGCM levels. Uncontrollable variables such as diet, metabolic rates or bacterial communities may cause variation between individuals in the measured GC titres [Goymann 2012] and could be partially responsible for the observed, substantial inter-individual variation. The water content of the feces significantly negatively influenced the baseline level. The influence of water content probably merely signifies the solid mass of the feces into which GC could be excreted or, alternatively, it may reflect the hydration status of the animal, which itself can cause a stress response [Kiss et al. 1994; Ulrich-Lai and Engeland 2002].

4.4 Influence of handling stress on the hormonal stress response

As expected, the capture-and-handling protocol in general acts as a stressor, causing on average a three-fold increase in FGCM levels from baseline within 2 days of the stressor. This is significantly lower than the responses recorded in the validation series conducted in the wild, where the peak GC elevation to stressors (capture, handling and subsequent temporary captivity) resulted, on average, in an 8-fold increase from baseline GC and the delay to peak FGCM response ranged between approximately 34-63 h after capture. The "trap happy" behavioral response (i.e. increased capturing probability) of most individuals in the population [Kraus et al. 2008] that leads to repeated voluntary trap entry may further indicate that the protocol causes them comparatively low levels of stress.

Individuals with higher baseline FGCM levels typically showed a higher response to the stressor than individuals with low baseline levels. This implies that animals with higher baseline FGCM levels maintain the capacity to react adequately to stressful stimuli and do not show a desensitization of the HPA axis (associated with an attenuated stress response) as reported for animals that are severely or chronically stressed [Rich and Romero 2005]. High FGCM baseline levels, measured prior to an acute stressor, are typically interpreted as a sign of chronic stress (but see [Cyr and Romero 2009; Dickens and Michael Romero 2013]), which in turn is known for its multiple detrimental influences on health, fitness and survival [Crespi et al. 2013; Romero 2004]. The samples collected on the day of first capture were considered to represent the baseline level, but, since the animals could not be monitored prior to the captures, we cannot rule out the occurrence of natural stressors prior to the capture and handling that may have elevated the pre-handling FGCM levels. Therefore the minority of cases that showed a decline in FGCM from baseline to day 3 likely reflect recovery from another stressor rather than a decline in GC level as a response to capture and handling.

Contrary to our predictions, prior capture experience had no influence on the baseline FGCM level or on the magnitude of the stress response, which indicates a lack of habituation (or sensitization) to the repeated stressor. Few studies have previously explored the potential for habituation to repeated handling but the available studies (mainly from captive conditions, e.g. [Dobrakovová et al. 1993; Jones and Waddington 1992]) suggest that repeated handling can lead to habituation when done regularly at relatively short intervals (several times per week). Longer intervals (several weeks) between stressors typically do not lead to habituation (e.g. [Desportes et al. 2007; Dickens and Michael Romero 2013; Tort et al. 2001]) since long delays between similar stressors may render the stressor too unpredictable for the animal to habituate to [Dickens and Michael Romero 2013; Fowler 1999; Koolhaas et al. 2011]. In our study population, the capturing is usually conducted at monthly intervals with seasonal breaks (reproduction and the high dry season) and handling is done on the first day only, after which recaptured animals are only transported and fed, which may further decrease the predictability of the process for the animal. It has been suggested that habituation may also take the form of a more rapid recovery from the stressor even when the magnitude of the response remains similar [Sheriff et al. 2010] but unfortunately this possibility could not be addressed in our experimental design. Further support for the lack of habituation is suggested by the exploratory analyses of repeated measurements of the stress response (data not shown) for a small number of animals (n=9) that could be repeatedly sampled in more than one capture session. While the sample size was insufficient for meaningful statistical analyses, these data suggested no systematic changes in the magnitude of the response within an individual from one capture session to another. The trapping frequency of an individual may be influenced by the exact location of its home range (e.g. center or edge of the study area) and it can be associated with certain personality traits, which may also influence the individual's stress levels [Koolhaas et al. 1999; Montiglio et al. 2012]. However, explorative analysis of our (limited) data showed no patterns suggesting that individuals with lower baseline levels or lower stress responses on the first capture of the season would enter traps more frequently later on in the season.

Also in contrast to our predictions, the handling regime had no significant influence on the physiological response evoked at a two-day delay from the stressor, although lowest values were generally measured for animals in the anesthesia regime (probably due to these animals being the youngest of the cohort, or due to changes in metabolism induced by anesthesia) and highest for those individuals that were handled repeatedly. Handling is, however, only a short part of the protocol and likely contributes only partially to the variation in the response strength: individuals may experience differing levels of stress due to confinement in the trap and transport. This, along with some degree of cumulative stress induced by intermediate captures and the "noisy" data may mask any fine scale differences between the handling regimes.

5 CONCLUSIONS

The successful validation of a fecal glucocorticoid metabolite assay (11ß-hydroxyetiocholanolone) in grey mouse lemurs permitted us to use the routine recapturing of animals to measure stress induced

by capture and handling without the need to draw blood or to restrain animals beyond the capture events. Routine capturing does not seem to induce chronic stress or lead to habituation in animals captured at monthly intervals. Bearing in mind the restrictions posed by the data, we also found no indications of the invasiveness of the handling influencing the magnitude of the stress response. Overall, the data suggests that regular capturing does not lead to long-term changes in the stress physiology that could interfere with the study of other phenomena in the species using a capture-and-handling design, or risk the well-being of the study animals. The methods we describe will be useful for the monitoring of health and overall physiological status of individuals or populations of wild mouse lemurs efficiently and in a relatively non-invasive manner.

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5.2. THE STRESS OF GROWING OLD: SEX- AND SEASON-SPECIFIC EFFECTS OF AGE ON ALLOSTATIC LOAD IN WILD GRAY MOUSE LEMURS

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ABSTRACT

Chronic stress (i.e. long term elevation of glucocorticoid levels) and aging have similar, negative effects on the functioning of an organism. Deterioration in glucocorticoid metabolism at old age may therefore lead to impaired coping of aged individuals under energetically demanding conditions, as found in humans. The coping of aged animals is unstudied under natural conditions, although the ability to appropriately respond to stressors is likely integral for the fitness and survival of wild animals. To assess the effect of age on stress profiles in response to naturally fluctuating energetic demands, we measured glucocorticoid metabolites in fecal samples (fGCM) of wild gray mouse lemurs (*Microcebus murinus*) in different ecological seasons. If the coping of aged individuals is impaired under energetically demanding conditions, they were expected to demonstrate elevated allostatic load (higher fGCM) in the dry season, when food and water availability are low and mating takes place. Consistently, we found elevated fGCM levels aged females in the dry season. Males, the shorter-lived sex, showed no age effects, but male body mass correlated positively with fGCM in the dry season. The sex- and season-specific predictors of fGCM may reflect the differential investment of males and females into reproduction and longevity and indicate adaptive responses to predictable changes in energetic demands, or non-adaptive deterioration in glucocorticoid functioning. A review of prior research demonstrates highly contradictory patterns in senescent changes in glucocorticoid metabolism, emphasizing the necessity of considering sex differences and context-specificity in evaluating age-related changes in glucocorticoid metabolism.

KEYWORDS

Coping, glucocorticoid metabolites, senescence, sex difference, trade-off

INTRODUCTION

Glucocorticoid hormones (GC) are pivotal mediators of an array of physiological processes that allow the organism to adaptively respond to varying energetic demands set by the environment, and to requirements associated with various life-history stages [Crespi et al. 2013]. GCs facilitate physiological and behavioral mechanisms that directly promote survival [Sapolsky et al. 2000], by redirecting resources (mainly by regulating blood glucose levels). GCs can adaptively fluctuate in preparation or in response to certain predictable energetic demands: for instance, basal GC fluctuation mediates daily activity cycles and prepares the system for changing ecological seasons and associated behaviors, such as breeding activities [Boonstra et al. 2014; Reeder and Kramer 2005; Romero 2002; Sapolsky et al. 2000]. GCs also play an important role in responding to unpredictable, acute stressors (real or anticipated perturbations to homeostasis) such as predator attacks, which usually provoke rapid, short-term elevations in GCs above the baseline level [Sapolsky et al. 2000].

Normal, predictable variation in energetic demands should not lead to harmful effects in healthy individuals, as long as the variation stays within a range of conditions that the organism can cope

with (termed “regulatory range“ by [Koolhaas et al. 2011]). However, an individual’s ability to react adaptively within this range might be influenced by illness, energy deficiency or maladaptive alterations in GC metabolism, leading to a decreased range of manageable conditions. If the energy demands placed on the system exceed the organism’s capacity to react adaptively, the continued glucocorticoid activity leads to a negative energy balance and an increasing allostatic load [McEwen and Wingfield 2003] that can have negative consequences for functioning, including immune defenses, cognition and reproductive performance [Ferrari et al. 2001; Juster et al. 2010]. Therefore, although long-term elevation in GCs may in some circumstances adaptively promote survival at the expense of less essential functions [Boonstra 2013], it is often considered non-adaptive.

The ability to cope with challenges might also be lowered at old age due to senescent changes in GC regulation. Aging organisms typically experience deteriorating physical and physiological functioning due to for example accumulation of oxidative damage, shortened telomeres and altered endocrinological profiles [Monaghan et al. 2008]. Senescent changes have also been found in the activity of the Hypothalamic-Pituitary-Adrenal (HPA) axis that regulates the levels of GCs in circulation [Sapolsky et al. 1987; Veldhuis et al. 2013; Vitale et al. 2013; Wilkinson et al. 1997; Wilkinson et al. 2001]. In the laboratory rat, senescent deterioration in HPA activity occurs in the negative feedback mechanisms that down-regulate GCs after the stressor has ended, and may lead to chronic GC-elevation [Sapolsky et al. 1986]. Many of the effects of chronic stress on physiological functioning parallel those associated with normal aging [Frolkis 1993; Veldhuis et al. 2013] hence, stress can accelerate age-related processes of deterioration.

The regulatory range in GC levels (*sensu* [Koolhaas et al. 2011]) of old individuals might narrow due to this deterioration. Hence, their coping may be impaired when unusually high demands are placed on the system. Disproportionately severe consequences are suffered by aged humans compared to younger individuals when faced with the same stressor [Frolkis 1993; Graham et al. 2006; Kiecolt-Glaser and Glaser 2001]. In natural populations of animals, environmental demands may compromise the coping of individuals in a deteriorating condition. However, due to the paucity of studies examining the effects of aging on adrenocortical activity in wild animals, little is known about the prevalence and significance of aging on HPA-activity in natural populations. The influence of varying environmental conditions on the coping of aged wild individuals is virtually unknown.

In this study, we evaluate support for the coping hypothesis in a natural population of gray mouse lemurs (*Microcebus murinus*), a small-bodied primate in which senescent declines have previously been found in measures of physical functioning (e.g. deterioration in body mass [Hämäläinen et al. 2014a; Perret 1997; Perret and Aujard 2001] and physical strength (Hämäläinen et al. unpublished, [Chazeau et al. 2013]). These senescent changes show season-specific patterns, which may be suggestive of compromised coping with the fluctuating energetic demands at old age. The native habitat of *M. murinus* in Western Madagascar is strongly seasonal and the species shows a range of adaptations to cope with the environmental demands, such as seasonal breeding, efficient fattening during times of high food availability and torpor use to conserve energy during the lean season [Eberle and Kappeler 2002; Schmid and Kappeler 1998]. The dry season is presumably highly energetically demanding due to the characteristically low food and water availability [Dammhahn

and Kappeler 2008], and the coinciding, short mating season [Eberle and Kappeler 2002; Eberle and Kappeler 2004b]. In the rainy season, food and water availability are high and breeding is finished by late rainy season [Eberle and Kappeler 2004a]. Adaptive responses to this seasonal variation likely have high significance for the fitness and survival of individuals. It seems plausible that differences between individuals in coping are most pronounced in the time of higher environmental demands to the physiology, and the dry season might therefore be a critical time for those individuals whose functioning is compromised. Seasonality was therefore used as an indicator of environmental demands in our study.

To evaluate the seasonal changes in allostatic load we quantified fecal GC metabolite (fGCM) levels in two dry and two rainy seasons. fGCM provides an integrated measure of GC excretion over a longer time period than baseline GCs measured in blood (at least 24h in *M. murinus*, [Hämäläinen et al. 2014b], opposed to a few minutes in blood [Romero and Reed 2005]) and is thought to reflect the levels of biologically active, unbound GC [Breuner et al. 2013]. Therefore, it broadly reflects the individual's current allostatic load and the functioning of the HPA-axis [McEwen and Wingfield 2003; Sheriff et al. 2011]. We sampled animals ranging from age at maturity to the maximum age in the population. Animals in better body condition might be able to cope with the stressors better than leaner individuals; hence we also assessed the influence of body mass on individual GC profiles. The sexes typically differ in their HPA-activity due to the differing effects of gonadal steroid hormones on HPA-activity [Handa et al. 1994]. Additionally, behavioral and life history differences may influence GC levels [Bokony et al. 2009]. Therefore, both sexes were sampled to assess sex-specific effects of aging and seasonal fluctuations on GC profiles.

Levels of fGCM may be generally higher in the dry season due to the energetic demands likely imposed on all animals. However, following our predictions of an interaction between stress and aging, we expected to find fGCM to be elevated in the aged animals in dry season beyond the presumably adaptive fGCM levels of younger individuals, as an indication of a declined ability to adaptively respond to increased energetic stress. Based on results from our validation study [Hämäläinen et al. 2014b], we expected no overall sex-differences in fGCM levels, but aging might influence the sexes differentially, perhaps affecting females more, as found previously (e.g. [Veldhuis et al. 2013]). We assumed prolonged nutritional stress or illness to be indicated by low body mass, and therefore predicted body mass to correlate negatively with fGCM concentrations.

MATERIALS AND METHODS

The study population

The gray mouse lemur (Cheirogaleidae, small-bodied (60 g) strepsirrhine primate) has a relatively short lifespan (average of 2-3 years in the wild [Kraus et al. 2008], maximum age in our study population 11 years, personal observation) and matures early (6-8 months of age [Castanet et al.

2004; Némoz-Bertholet and Aujard 2003]). High extrinsic mortality risk (mainly by predation [Goodman et al. 1993]) leads to rapid selective disappearance of individuals in a declining condition [Hämäläinen et al. 2014a], and there is a known male bias in mortality in the wild that escalates during the mating season likely due to the males' roaming for estrous females [Kraus et al. 2008].

The study population (locally known as the "N5" population) has been monitored since 2000 in a 25 ha study site of dry deciduous forest in Kirindy/CNFEREF, Central Western Madagascar by researchers of the German Primate Center. The area experiences pronounced seasonal variation with a distinct dry season (~May-November), and rainy season (~November-April), with consequent variation in food availability [Dammhahn and Kappeler 2008]. The energetic demands along with sex-specific behaviours (female-biased torpor use [Schmid and Kappeler 1998] and male roaming in the mating season, [Eberle and Kappeler 2004b]) lead to substantial seasonal, sex-specific body mass fluctuation in *M. murinus* [Schmid and Kappeler 1998]. The brief mating season takes place between mid-October and November and offspring are typically weaned by March-April [Eberle and Kappeler 2004a; Hämäläinen et al. 2014a].

For long term data collection, trapping and body mass measurements have been conducted at minimum 6 times per year (monthly in March-May and September-November) with Sherman live catch traps baited with small pieces of banana. Recapture rates are high during these months and no evidence has been found for long-term influences of the capture-and-handling protocols on the animals' stress physiology [Hämäläinen et al. 2014b]. All captured animals are equipped with an individual subcutaneous transponder (Trovan EURO ID, Germany) at first capture, typically as juveniles. Hence, the age of all individuals can be estimated to the year with a high level of accuracy. The capture and handling protocols have been detailed in e.g. [Dammhahn and Kappeler 2008; Eberle and Kappeler 2004a].

Sample collection and hormone analysis

A total of 369 fecal samples from 171 individuals were collected over four field seasons: in September-November (late dry season, mating season) 2010 and 2012 and in March-May (late rainy season, non-reproductive) 2011 and 2012. All individuals included in the sample were assumed to be sexually mature (age >6 months), since juveniles of the season were excluded from the rainy season sample. Animals were sampled either within a few hours after trap entry at night, or on the following morning after a night spent in a trap. Capturing induces an acute stress response that is measurable 24-72 h after capture [Hämäläinen et al. 2014b]; hence samples were always collected within the "baseline" period, (i.e. within 12 h of the first monthly capture) and should be unaffected by the capture event. The time of day of fecal sampling does not influence fGCM levels in the species ([Hämäläinen et al. 2014b], confirmed for the data used in this study). Fresh feces was collected from cleaned traps, avoiding urine contamination (details in [Hämäläinen et al. 2014b]) and stored in ethanol within a few hours of collection.

The methods of collection, extraction and fGCM analysis have been previously validated [Hämäläinen et al. 2014b]. Briefly, approximately 0.2 g of feces (range: 0.07 – 0.36 g) was subsampled and

homogenized in 2 ml of 80-90% ethanol. The fecal suspension was then vortexed, centrifuged, and the supernatant stored for future hormone analyses. The pellet was dried to a constant mass to obtain an estimate of feces water content. Duplicate aliquots of the fecal extracts were measured as detailed in [Hämäläinen et al. 2014b; Heistermann et al. 2004] using a validated enzymeimmunoassay for 11ß-hydroxyetiocholanolone [Ganswindt et al. 2003], a major metabolite of cortisol in gray mouse lemur feces [Hämäläinen et al. 2014b]. All hormone concentrations are given as ng/g fecal wet weight.

Statistical analyses

Explorative analyses of the data was conducted by visual inspection of the data and smoothers of age and body mass (GAM, R-package *mgcv*, [Wood 2006]) to examine the shapes of the relationships between fGCM and age or body mass. The relationships were found to be approximately linear and the exploration further indicated that 3-way interactions of sex and season with age or body mass might be present (data not shown). Given our interest in the seasonal patterns of GC across the range of ages and the two sexes, and the potentially non-linear within-season trends, we proceeded to analyse the data separately for the seasons to reduce the complexity of the models.

The influences of sex, age, body mass and within-season variation on fGCM levels were analyzed separately for each season using Linear Mixed Models (LMM, R-package lme4, [Bates et al. 2014]). The fixed effects structure of each full model included the terms sex, age, body mass and month of sampling, and all associated two-way interaction terms (sex*age, sex*body mass, age*body mass, month*age, month*sex and month*body mass). The nuisance variables year of sampling (2010-2011 or 2012) and feces water content (water%) were added as fixed factors to all models to account for yearly variation and the known influence of fecal water content on fGCM levels in a sample [Hämäläinen et al. 2014b]. Individual identity was included as a random effect to account for repeated sampling of the same individuals.

Due to the modest sample size and substantial “noise” typical for hormone data, we removed non-significant interaction terms (Likelihood Ratio Tests, LRT, P >0.1) one at a time from the full model to reduce over-parameterization and to identify significant effects [Pinheiro and Bates 2000]. Predictions based on the full models indicated similar patterns as the reduced models, and are presented in the electronic supplementary material.

Age and body mass were log-transformed, centered and scaled (mean/SD) prior to the analyses to improve the interpretability of interactions [Schielzeth 2010]. The normality and homogeneity of error assumptions were examined using residual plots for the most complex model for each data set. Satterthwaite estimation was used to compute P-values (*lmerTest*-package, [Kuznetsova et al. 2014]). To gain an estimation of significance for factors with more than 2 levels (month, month*body mass), LRT was used. Marginal and conditional R^2-scores [Nakagawa and Schielzeth 2013] were computed using the *r.squaredGLMM*-function of the *MuMIn*-package [Barton 2014]. All analyses were performed in R version 3.0.3 [R Development Core Team 2014] and statistical significance was set at $P \leq 0.05$.

RESULTS

In the rainy season model, the only interaction term remaining after model simplification was month*body mass (Table 1, Fig 1a). In the dry season model, the interaction terms sex*age and sex*body mass were retained. The main effects of the terms sex, age, month, body mass and the control variables water% and year of sampling were retained in the models regardless of their significance (Table 1).

Results from the season-specific models indicate that fGCM-levels were higher at old age in the dry season as predicted based on the coping hypothesis. However, this relationship was much stronger in females, as indicated by the interaction term sex*age (Table 1, Fig 1b). A positive association was found between body mass and fGCM levels in males in dry season, whereas the trend for females was negative, as indicated by the interaction term sex*age (Table 1, Fig 2b). In the rainy season, the term month*body mass indicated that body mass was negatively associated with fGCM in March and May, but in April, this relationship was leveled or even reversed (Table 1, Fig 2a). The nuisance variables water% and year of sampling had statistically significant effects in each season.

Table 1. Predictors of fGCM in the dry and the rainy season based on the best model after exclusion of non-significant interaction terms.

	Dry [a]				**Rainy** [b]			
	β	**SE**	**t**	**P**	**β**	**SE**	**T**	**P**
Intercept	6.522	0.468	13.945	<0.001	7.694	0.672	11.450	<0.001
Water%	-1.451	0.444	-3.269	0.001	-4.028	0.724	-5.565	<0.001
Year	0.309	0.144	2.153	0.033	0.867	0.249	3.482	<0.001
Month2 [c]	-1.134	0.140	-0.962	0.338 [d]	-0.616	0.250	-2.462	0.015
Month3 [c]	0.080	0.245	0.327	0.744 [d]	-0.066	0.344	-0.192	0.847
Sex (ref. female)	0.416	0.332	1.253	0.212	0.201	0.242	0.832	0.409
Age	0.760	0.187	4.069	<0.001	0.128	0.176	0.724	0.472
Body mass	-0.435	0.341	-1.274	0.205	-0.521	0.291	-1.789	0.075
Sex:Age	-0.667	0.221	-3.024	0.003				
Sex:Body mass	0.812	0.384	2.117	0.036				
Month2:Body mass					0.762	0.282	2.698	0.008 [e]
Month3:Body mass					-0.087	0.395	-0.220	0.826 [e]

[a] N= 170/126, final model $R^2_{marginal}$= 0.243, $R^2_{conditional}$= 0.560
[b] N= 204/76, final model $R^2_{marginal}$= 0.300, $R^2_{conditional}$ = 0.507
[c] Month within season: Reference value 1=March & September, 2=April & October, 3=May & November
[d] Month LRT: X_2^2= 1.098, P= 0.578
[e] Month*body mass LRT: X_2^2= 12.187, P=0.002

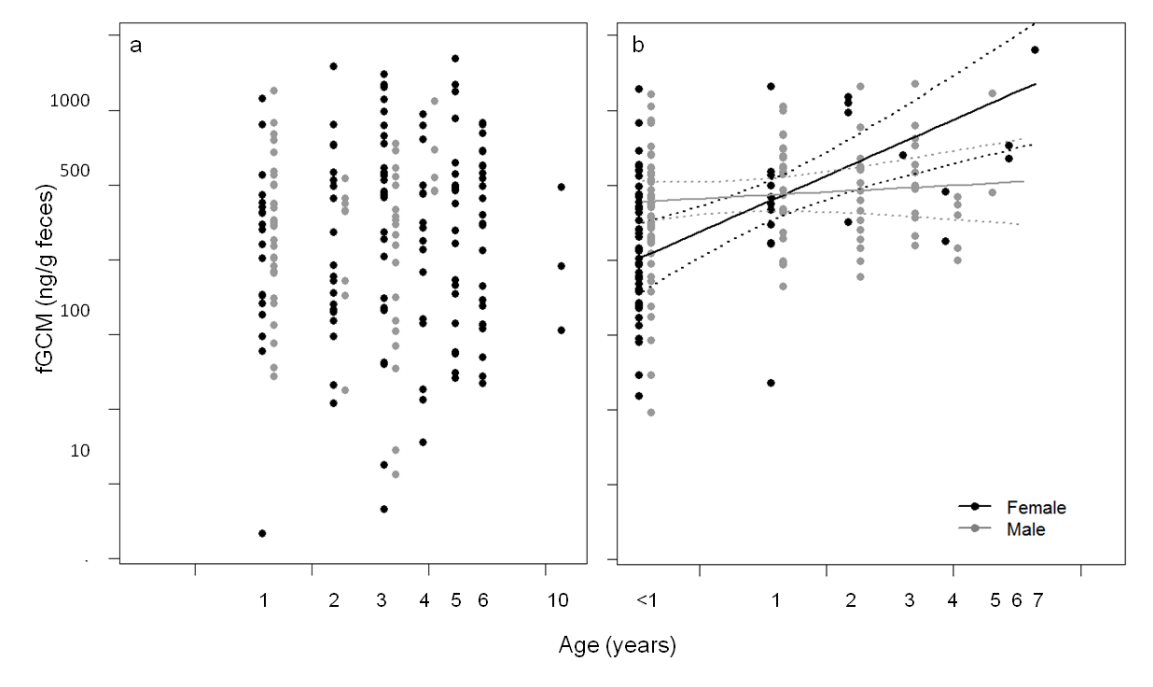

Figure 1: Age did not influence on fGCM-levels of wild *Microcebus murinus* in the (a) rainy season, but was positively associated with fGCM in (b) dry season in the females (sex*age interaction, *P* =0.003). Solid lines indicate prediction lines from the final model for year 2012 (predictions for 2010/2011 have slightly lower values but lines are parallel), and dotted lines indicate associated 95% confidence intervals. Data points are shown for both years. The predictions are based on scaled and centered age but corresponding chronological age in years is presented on x-axis. Note log scale used on y-axis.

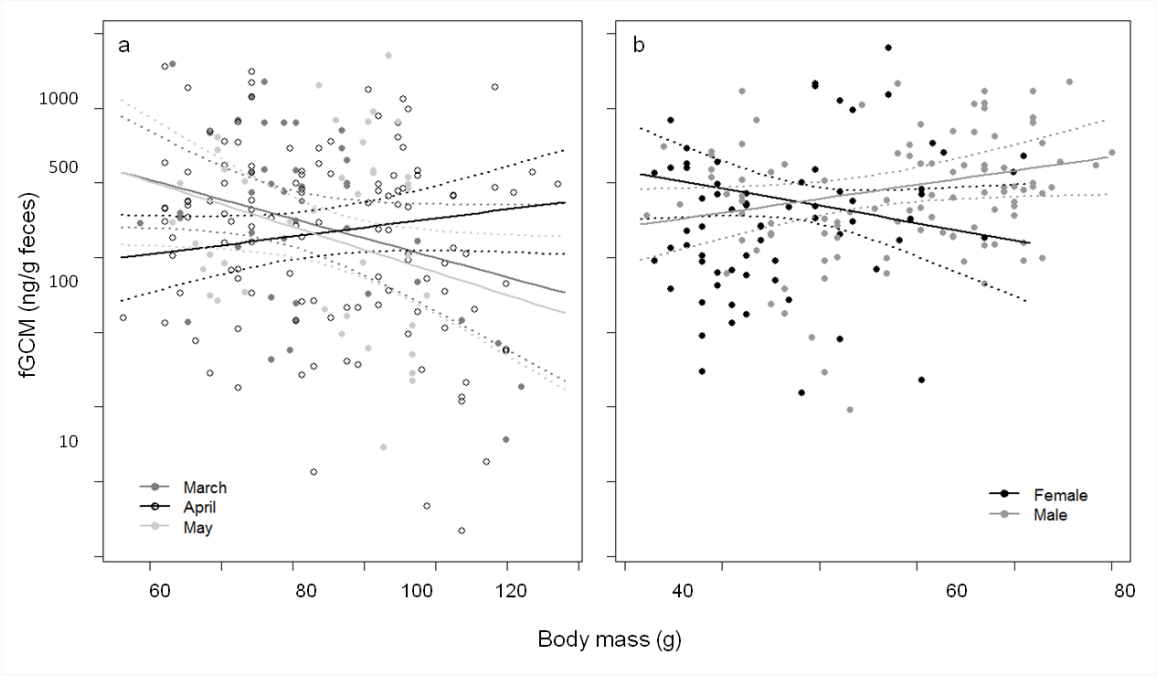

Figure 2: Body mass as a predictor of fGCM-levels. A significant interaction of month*body mass (P = 0.002; prediction lines shown only for females, the patterns are similar for both sexes) was found in (a) the rainy season, and in (b) the dry season an interaction of sex*body mass was significant (P = 0.036). All data points are shown, prediction lines are only for year 2012 (predictions for 2010/2011 have slightly lower values but lines are parallel). Solid lines indicate predictions from the best models for each season, and dotted lines show the associated 95% confidence intervals. The predictions are based on scaled and centered body mass but corresponding untransformed body mass (grams) is presented on x-axis. Note log scale used on y-axis.

DISCUSSION

In this study, we tested the coping hypothesis of aging, evaluating whether there are physiological indications that the performance of aged individuals in a natural population of gray mouse lemurs is impaired during energetically demanding environmental conditions. In support of this hypothesis, we found a sex-specific, seasonal age effect, with old females showing significantly higher stress hormone (GC) levels than young females in the dry season, when intrinsic and extrinsic factors both may increase the allostatic load. No age effect was found in males in the dry season or in either sex in the non-reproductive, rainy season, when food availability is high.

Elevated fGCM in aged females during the dry season

Old individuals may be able to operate equally well as young ones when conditions are good and stressors minimal, but may be affected more when the energetic demands reach marginal levels

along their regulatory range due to intensive or frequent stressors. Our finding of elevated fGCM in aged females during the dry season is consistent with this hypothesis, suggesting that high energetic demands associated with the dry season function as a more intensive stressor for aged females than animals at younger ages. The adrenocortical regulation of GCs in circulation can change at old age via a reduced efficiency of the negative feedback mechanisms of the HPA-axis, or an increased duration of the stress response [Sapolsky et al. 1983; Sapolsky et al. 1984]. These changes are thought to operate via a deterioration of the hippocampus [Jacobson and Sapolsky 1991] or glucocorticoid resistance at the brain level [Mizoguchi et al. 2009]. The increases in baseline GC levels observed in the aged females in our study might therefore reflect the deterioration of the negative feedback mechanisms [Mizoguchi et al. 2009; Sapolsky et al. 1983; Sapolsky et al. 1986] that lead to chronic GC elevation when high energetic demands are placed on the system. Evidence of impaired coping during chronic stress at old age has also been found in humans [Kudielka et al. 2009] and rats [Shoji and Mizoguchi 2010].

While the pattern of high GCs in the dry season fits the coping hypothesis, alternative explanations for the observed pattern are conceivable. The highest fGCM concentrations were found in females that had passed the average survival age in the wild (~2 years) and which regularly utilize torpor proportionally more than other demographic groups [Schmid and Kappeler 1998]. It has been suggested that arising from torpor might coincide with high GC levels to induce re-feeding and boost metabolism after the long periods of anorexia, or in preparation for the impending mating season [Boonstra et al. 2014; Romero 2002]. In mouse lemurs, energetic status (particularly in females arising from torpor) is likely at its lowest around the time of the mating season, hence elevated GCs in older adult females might also reflect an adaptive response to reasonably predictable seasonal fluctuation. In this case, the high GCs in the old females might reflect the increased longevity of the highest quality individuals that are able to up-regulate their GC metabolism to meet these demands. Elevated GC level may be a consequence of the low food availability and the long period of anorexia by hibernating individuals, but might be beneficial for rapidly improving body condition because GCs increase gluconeogenesis and stimulate foraging behavior [Sapolsky et al. 2000]. Due to constraints on the timing of breeding, gray mouse lemur females have a limited time between acquiring the necessary body condition after terminating torpor use and becoming pregnant. However, since we found no effect of month (or interactions of month with age or sex) on fGCM in the dry season, it seems more likely that fGCM was elevated in the aged females not only upon arousal from torpor but throughout the dry season sampling, hence lending support for the coping hypothesis.

Although elevated GCs may be essential for survival through the harsh season, hypercortisolism can interfere with reproductive behaviors [Sapolsky et al. 2000; Wingfield and Sapolsky 2003]. Baseline GC elevation has been linked to reproductive failure or suppression of reproduction in times of high stress [Goutte et al. 2010a; Sheriff et al. 2009]. Female receptive behavior is also negatively affected by high GC [Sapolsky et al. 2000], hence mating in gray mouse lemurs might be delayed until GC has been lowered from post-torpor levels in response to re-feeding. Unfortunately, we do not have information on the breeding decisions or reproductive success of our study subjects to evaluate the extent to which these constraints affect wild gray mouse lemur females.

Sex and context influence the effects of aging on HPA-axis function

While age-related changes in coping have been previously explicitly addressed in few studies of non-human animals, and never in field studies, there are indications that age-related changes frequently occur in the HPA-axis function of vertebrates. However, the patterns of aging in GC metabolism differ substantially across species, and also across contexts and sexes within species. Some of these findings are summarized in Table 2 to demonstrate the variability in outcomes from previous research. While almost every conceivable result is found on the effects of sex and age on GC metabolism across (and sometimes within) species, the one measure that seem to reflect moderately consistent directionality at aging is baseline GC, which is typically either unchanged or elevated at old age. The trend in negative feedback efficiency, on the other hand, is for unchanged or decreasing responsiveness to efficient down-regulation of GCs across studies. These findings combined seem to support the hypothesis that impaired negative feedback efficiency of the HPA-axis may act to chronically elevate baseline GC level [Jacobson and Sapolsky 1991]. For the rest of the measures detailed in Table 2 (influence of aging on the GC response to a stressor, sex differences in baseline and response GC as well as sex-specific effects of aging), the main conclusion is a remarkable absence of general patterns.

Table 2: Evidence for the effects of sex and aging on GC-levels at baseline and during acute stress response in mammals and birds. + denotes a positive association, - a negative one, 0 no difference or non-significant effect and NA lack of data. M= males, F= females. Matrix: B=blood, F=feces, S=saliva, U=urine.

	Sex differences		**Effect of aging**			**Sex-specific aging**	**Matrix**	**Setting**	**References**
Species	**Baseline**	**response**	**Baseline**	**response**	**Feedback #**				
Human (Homo sapiens)	F+	F+	+	+	NA	NA	B	Clinical	[Heuser et al. 1994]
	NA	NA	NA	+	NA	M+(free GC), F+ (total GC)	B, S	Clinical, review	[Kudielka and Kirschbaum 2005]
	0	0	+	-	NA	F- (response)	S	Psychological stress	[Nicolson et al. 1997]
	0	NA	0	+	NA	F+	B & S	Clinical, psychological stress, review	[Otte et al. 2005]
	0 / M+	0 / F+ / M+	NA	0 / - / +	-	0 / F+ / M+	NA	Review	[Veldhuis et al. 2013]
Rhesus monkey (Macaca mulatta)	NA (F only)	NA (F only)	+	0	-	NA (F only)	B	Captive	[Gust et al. 2000]
	NA (F only)	NA (F only)	0	0 / +	-	NA (F only)	B	Captive	2002; Goncharova and Lapin 2004]
Hamadryas baboon (Papio hamadryas)	NA (M only)	NA (M only)	0	NA	-	NA (M only)	B	Captive	[Goncharova and Lapin 2004]
Yellow baboon (Papio cynocephalus)	0	0	+	NA	-	0	B	Wild	[Sapolsky and Altmann 1991]
	NA	NA	+	NA	NA	0	F	Wild	[Alberts et al. 2014]
Dog (Canis familiaris)	NA	NA	+	+	0	NA	B	Captive	[Reul et al. 1991]
	NA	NA	+	+	-	NA	B	Captive	[Rothuizen et al. 1993]

Table 2. continued

Species	Sex differences: Baseline	Sex differences: response	Effect of aging: Baseline	Effect of aging: response	Effect of aging: Feedback #	Sex-specific aging	Matrix	Setting	References
Mouse (*Mus musculus*)	F+	NA	0 / +	NA	NA	M+ (baseline + begins earlier)	F	Captive	[Touma et al. 2004]
Rat (*Rattus norvegicus*)	NA	NA	+	NA	-	0	B	Captive	[Sapolsky 1992; Sapolsky et al. 1986]
	F+	NA	NA	NA	NA	NA	B	Captive	[Critchlow et al. 1963]
	NA	F+	NA	NA	NA	NA	B	Captive	[Kitay 1961]
	NA (Male only)	NA (Male only)	0?	0 / +	NA	NA (Male only)	B	Captive	[Herman et al. 2001]
	NA	NA	0	0 / -	NA	F- (response - at old age)	B	Captive	[Brett et al. 1983]
	NA (Male only)	NA (Male only)	0	NA	+ / -	NA (males only)	B	Captive	[Mizoguchi et al. 2009]
	NA (males only)	NA (males only)	0	0	+	NA (males only)	B	Captive	[Kasckow et al. 2005]
Red-backed vole (*Myodes rutilus*)	NA (males only)	NA	0	NA	-	NA (males only)	B?	Wild	Fletcher et al. unpublished in [Boonstra et al. 2014]

Table 2. continued

	Sex differences		Effect of aging			Sex-specific aging	Matrix	Setting	References
Species	**Baseline**	**response**	**Baseline**	**response**	**Feedback #**				
California mouse (*Peromyscus californicus*)	0	0	0	0	0 / + (M)	M- (response + at old age)	B	Captive	[Harris and Saltzman 2013]
Tree shrew (*Tupaia belangeri*)	NA (males only)	NA (males only)	0 / + (+ until 200 days, 0 after)	NA	NA	NA (males only)	U	Captive	[Van Kampen and Fuchs 1998]
Florida scrub-jay (*Aphelocoma coerulescens*)	0	0	0	- / + (general -, + in oldest age class)	NA	0	B	Wild	[Wilcoxen et al. 2011]
Common tern (*Sterna hirundo*)	0	0	0	-	NA	NA	B	Wild	[Heidinger et al. 2006; Heidinger et al. 2008]
Snow petrel (*Pagodroma nivea)*	M+	M+	0?*	0 / + (+ in oldest animals)	NA	NA	B	Wild	[Goutte et al. 2010b]

* Limited support from model selection for an age effect (based on graphics, negative trend at least in females), effects not reported
# - = resistance to negative feedback at old age

This variability might in part reflect the fact that few of the studies are directly comparable due to differences in the methodology used, parameters measured, demographic groups studied, the research setting (captivity, wild) and potential differences introduced by phylogeny or social/mating structure of the species studied. A major issue with quantifying GCs in blood has been that the potential significance of the dynamics of corticosteroid-binding globulins (CBG) [Breuner and Orchinik 2002; Breuner et al. 2013] was overlooked in older studies, rendering those results difficult to interpret. In blood, part of the GCs are bound to CBG and only the unbound fraction is thought to be biologically active, whereas fecal GC metabolites reflect the levels of active GCs in circulation [Breuner et al. 2013; Sheriff et al. 2010]. The measurement of total GCs estimates the cumulative concentration of free and bound GCs. This is potentially significant, because the fluctuation of CBGs may significantly influence the levels of active GCs in blood [Breuner and Orchinik 2002]. Excreted GC, as used in our study, might therefore be a useful addition to the methodology, when allostatic load to the system is of interest. However, we advocate thorough validation of these methods prior to using them for inference about individual state, since much is already known about the potential confounding factors influencing these measurements [Goymann 2012; Hämäläinen et al. 2014b; Sheriff et al. 2011]. Overall, this variability in study designs makes it at present impossible to predict the directionality of effects and should alert the scientific community to the species- and context-specificity of HPA-axis aging.

Females (at least in the most studied organisms) tend to show more pronounced changes at advancing age compared to males (Table 2), probably because the sexes differ in their GC metabolism and the way that aging influences the HPA pathways [Kudielka and Kirschbaum 2005]. This in combination with life history differences may lead to the sex-specific patterns of aging observed in this study. The absence of age-effects in males could also be due to higher male mortality [Kraus et al. 2008], which eliminates males from the population before they show senescent declines. Male mortality is elevated in the mating season [Hämäläinen et al. 2014a; Kraus et al. 2008], the time when they also experience higher GC levels. Due to the detrimental effects of chronic GC elevation on health [Glaser and Kiecolt-Glaser 2005], chronic stress may contribute to the selective disappearance of individuals [Pride 2005]. Due to the shorter life expectancy of males compared to females, the life history characteristics of males are likely shaped by the associated adaptive pressures, leading perhaps to higher reproductive investment by males in early life at the expense of survival.

GCs have been implicated as possible mediators of trade-offs between survival and reproductive effort due to their role in resource reallocation [Ricklefs and Wikelski 2002; Wingfield and Sapolsky 2003]. Because an individual's future reproductive potential typically decreases with advancing age, it has been proposed that – in contrast to the coping hypothesis – aged individuals might exhibit lower fGCM compared to young animals during the mating season (perhaps via changes in adrenal capacity, [Heidinger et al. 2008]) to facilitate higher investment in reproduction (*sensu* CORT-trade-off hypothesis [Boonstra et al. 2001; Patterson et al. 2014; Wingfield and Sapolsky 2003]. The few studies that have thus far addressed this hypothesis have found limited support for it [Harris 2012; Harris and Saltzman 2013; Heidinger et al. 2006; Heidinger et al. 2008]. Our study also fails to find evidence in favor of this idea, suggesting that gray mouse lemurs may not be able to compensate for

their declining reproductive value by down-regulating their overall GC production at old age. Due to the paucity of studies, it is at this stage impossible to evaluate the causes of the discrepancies between the studies, but potential lines of future research might be the examination of differences between captive and wild studies, the influences of breeding status and annual fluctuations as well as different mating systems on the patterns of aging observed. Further study of these topics on various taxa may improve our understanding of the circumstances under which GC production is down- or up-regulated at old age.

Age-related physiological deterioration may expose aged individuals to extrinsic causes of mortality at a higher rate than their younger conspecifics, contributing to the increasing risk of mortality with advancing age observed in most species. However, individuals that live to old age in nature might be of high quality or have an HPA phenotype that promotes self-maintenance (perhaps at the expense of reproduction early in life). Therefore, the oldest surviving individuals may experience less pronounced senescent changes than would be expected without selective mortality of individuals with HPA activity that promotes early fitness at the expense of survival. Due to the potential significance of deteriorating physiological functioning at old age on survival probability, it is likely that the detectability of senescence is difficult in natural populations compared to captive conditions [Hämäläinen et al. 2014a] and might contribute to the relatively low number of studies on wild animals reporting significant age-related changes in HPA axis functioning (Table 2). Stressful conditions experienced early in life can also influence the phenotype later in life, and could influence long-term health and longevity via oxidative stress and telomere activity [Monaghan 2014], hence, the old individuals in our sample may be influenced by life events much prior to the start of our study. Longitudinal following of the same individuals at a longer time scale might help disentangle some of these effects.

Sex- and season-specific predictors of fGCM levels

As expected, the predictors of fGCM levels differed between the dry and rainy season in the gray mouse lemur. Seasonal fluctuations are thought to be predictable for animals in their natural habitat due to the strong adaptive value of being able to anticipate changing energetic needs and rapidly adjust to them [Landys et al. 2006; Romero 2002]. The causes for the seasonal differences are likely related to food availability and breeding activities; nutritional or hydration stress and the energetic needs associated with the mating season likely cumulatively increase the allostatic load experienced by the animals. Seasonally fluctuating food availability might directly induce an elevation in GC in preparation for, or in response to the increasing energetic demands due to nutritional stress. Evidence for this connection has been found in e.g. baboons [Gesquiere et al. 2008; Gesquiere et al. 2011] and sea birds [Kitaysky et al. 1999]. Quantification of nutritional stress or manipulation of breeding efforts would be needed to clarify the cause of the season effects.

Since the energetically most demanding reproductive behaviors by males (roaming and competing for females) occur during the mating season, the higher fGCM levels found in gray mouse lemur males in the dry season might reflect adjustments to predictable, seasonal energetic demands. Our results indicate that heavier males have somewhat higher fGCM than lighter males (and females of a

similar body mass), whereas a negative trend was observed for females. This unexpected result might indicate that heavier males invest more resources into breeding and the increase in fGCM reflects the higher energetic strain they incur. A similar result might follow from a direct investment into testis volume: testis size increases prior to the mating season [Schmid and Kappeler 1998] and contributes substantially to male body mass. Males with larger testes may have higher testosterone levels or have an advantage in sperm competition. Therefore, it is plausible that fGCM positively correlates with reproductive success in males. Males that invest most in time and energy into roaming tend to forego foraging (personal observations), hence they may have to utilize existing tissue for energy, which would require elevating GCs. A similar strategy has been proposed for semelparous marsupials [Boonstra 2005]. The CORT-adaptation hypothesis [Bonier et al. 2009a; Bonier et al. 2009b] proposes that GC levels should be elevated during breeding to promote fitness when energetically expensive behaviors improve reproductive success, hence our results for males may offer support for this hypothesis. For females, the period of gestation and lactation (December-February) likely poses higher energetic costs associated with breeding. Unfortunately, this period could not be addressed in our study due to logistic and ethical limitations on capturing breeding females. The results for males should also be interpreted with caution, since the effects of the mating activities and low food availability in the dry season cannot be disentangled in our experimental design. Breeding season values of GCs tend to be elevated in many vertebrate taxa, although the evidence for this pattern is weakest in mammals [Romero 2002].

In the rainy season, no effects of age or sex on fGCM were found. This implies that all demographic groups experience similar GC exposure during the time of high food availability. We found an unexpected month*body mass interaction in the rainy season, with the generally negative trend between body mass and fGCM being leveled or reversed in April. This result seemed to be driven by a few females aged 3-4 years for which fGCM was higher than expected in April. Their elevated fGCM might reflect fattening at the end of the rainy season in preparation for torpor use, a strategy used by individuals that are in good enough physical condition to conserve energy during the dry season [Vuarin et al. 2013]. GCs are thought to mediate pre-hibernation hyperphagia since moderately elevated GCs in circulation stimulates foraging behavior and the storage of energy [Reeder and Kramer 2005; Romero 2002; Willis and Wilcox 2014] and April would be the time for this effect to occur in gray mouse lemurs.

Some alternative explanations besides food availability and mating activities also exist for the seasonal differences observed in gray mouse lemur fGCM levels. An overall effect of “metabolic stress” due to harsh environmental conditions, as found in geladas [Beehner and McCann 2008] might drive the seasonal effects. As the nuisance variable sample water content shows a highly significant, negative association with GC, it might indicate hydration stress (in addition to dilution effects of fGCM in feces). This line of reasoning is supported by the fact that the influence of water on fGCM was substantially stronger in the dry season (Table 1), when water availability is extremely low. Seasonal differences might also exist in predation pressure between the dry and rainy season, although this has not been quantified. Predator exposure is a potential cause of stress, and is known to alter stress physiology [Fischer et al. 2014]. Seasonal differences may exist in the relative predator

and prey abundance, and due to food scarcity, mouse lemurs may have to engage in more risky behaviors when foraging and face higher predation risk.

The seasonal patterns we observed are surprisingly different from those found in an earlier study on captive *M. murinus* [Perret and Predine 1984], in which GC showed the highest annual peak at the onset of fattening (comparable to ~April-May in the wild), with a smaller increase in females during estrous. It was lowest during the "active" season that coincides with lower body mass and breeding, in complete contradiction to the findings of our study. These differences might have to do with the differing energetic demands experienced by wild and captive animals, or the matrix tested (blood vs. feces), since only total GC (and not free GC or CBG) were quantified by [Perret and Predine 1984], whereas the fGCM measured in our study are thought to reflect the free proportion of GC in circulation [Sheriff et al. 2011].

CONCLUSIONS

The seasonal age-effect found in this study for gray mouse lemur females may reflect impairment of the physiological stress response to high energetic demands at old age. Alternatively, the elevated dry season GC in aged females might be an adaptive response to emergence from extended periods of torpor and facilitate fast recovery by promoting foraging behavior and/or generation of energy from existing tissue. While our results cannot resolve the cause for the phenomenon, they highlight the sex-specific, differential performance of aged individuals across seasons.

Since natural selection acts most strongly early in life, coping with stressors at old age may not be within the scope of selection. However, the detection of this effect requires the establishment of age trajectories of HPA-axis function under environmentally harsh and benign circumstances. Due to the role of glucocorticoids in mediating survival-reproduction trade-offs, an improved understanding of sex- and context specific changes in the regulation of these hormones over the individual lifespan has great potential in advancing our understanding of the mechanisms underlying life-history variation between and within species. We invite further studies into the coping of aged animals across contexts to evaluate the significance of senescent changes in GC metabolism under natural conditions, ideally combining measures of performance with quantification of HPA-axis functioning.

Despite the increasing number of studies examining the effects of aging on GC metabolism, no clear patterns have thus far emerged on the directionality of age effects across the sexes, seasons or taxa (but see [Hau et al. 2010]). The contradicting age effects found across studies highlight the importance of context and the complexity of the HPA-axis function in determining the allostatic load suffered by aged individuals. Combined with the highly variable methodology and the young, evolving theory, this complexity manifests as the current disarray of results. Integrating the existing hypotheses into a single theoretic "stress of aging" framework would be essential for reconciling the causes underlying this variation.

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SUPPLEMENTARY MATERIAL

Table S1: Predictors of log (fGCM) in the dry and the rainy season based on the full models.

	Dry [a]				Rainy [b]			
	β	SE	t	P	β	SE	t	P
Intercept	6.419	0.362	17.740	<0.001	7.339	0.713	10.289	<0.001
Water%	-1.495	0.453	-3.298	0.001	-3.903	0.740	-5.278	<0.001
Year	0.400	0.153	2.607	0.010	0.909	0.257	3.532	<0.001
Month2 [c]	-0.060	0.234	-0.258	0.797	-0.207	0.293	-0.707	0.480
Month3 [c]	0.402	0.435	0.924	0.357	0.043	0.370	0.117	0.907
Sex (ref. female)	0.226	0.226	0.998	0.320	-0.209	0.549	-0.380	0.704
Age	0.883	0.194	4.549	<0.001	0.043	0.259	0.165	0.869
Body mass	-0.378	-0.378	-1.611	-1.611	-0.507	0.235	-2.154	0.032
Age*Month2	-0.297	0.189	-1.569	0.119	-0.015	0.286	-0.053	0.958
Age*Month3	-0.225	0.315	-0.713	0.477	-0.066	0.321	-0.206	0.837
Sex*Month2	-0.113	0.295	-0.384	0.702	0.710	0.563	1.262	0.208
Sex*Month3	-0.621	0.764	-0.813	0.418	-0.107	0.731	-0.146	0.884
Age*Body mass	-0.145	0.101	-1.437	0.153	0.113	0.120	0.943	0.347
Sex:Age	-0.617	0.203	-3.039	0.003	0.102	0.312	0.325	0.746
Sex:Body mass	0.526	0.237	2.218	0.028	0.364	0.242	1.503	0.135
Month2:Body mass	0.179	0.149	0.915	0.362	0.631	0.222	2.848	0.005
Month3:Body mass	0.149	0.571	0.260	0.795	-0.252	0.404	-0.623	0.534

[a] N= 170/126, full model $R^2_{(marginal)}$=0.258, $R^2_{(conditional)}$=0.508

[b] N= 204/76, full model $R^2_{(marginal)}$=0.313, $R^2_{(conditional)}$=0.525

[c] Month within season: Reference value 1=March & September, 2=April & October, 3=May & November

CHAPTER 6

GENERAL DISCUSSION

Before thirty, men seek disease; after thirty, diseases seek men.

- Chinese Proverb

It`s not how old you are, it`s how you are old.

- Jules Renard

Old age isn't so bad when you consider the alternative.

- Maurice Chevalier

The aim of this thesis was to address largely unresolved, fundamental questions of life history theory: how are patterns of senescence influenced by the selective mortality of individuals in a poor condition, and how does functional senescence differ between the sexes? I aimed to shed light on these questions by examining sex-specific patterns of senescence and selective disappearance in a natural population of the gray mouse lemur (*Microcebus murinus*). In the previous chapters I have presented original research on aging in four different components of health in the gray mouse lemur. In this final chapter, I will first summarize the main findings of the four studies that make up the body of this thesis and then discuss the broader implications of these findings for life history theory. In particular, I will discuss how selective disappearance shapes senescence, the variable patterns of change found across the health components, and the influence of the environment on the observed patterns. I will then evaluate to what extent patterns of senescence differ between the sexes and whether the observed patterns offer support for the male-female health-survival paradox. Since this hypothesis has been developed almost exclusively in modern humans, I will also specifically assess the implications my results could have for resolving the paradox in humans. Finally, I will propose lines of future research to achieve a better understanding of the causes and consequences of sex-specific senescence and survival.

FUNCTIONAL SENESCENCE IN THE GRAY MOUSE LEMUR

One of the key aims of this thesis was to examine the ways in which selective disappearance of low quality individuals affects patterns of senescence. In **Chapter 2**, I used longitudinal body mass data and survival estimates for captive and wild mouse lemurs to assess these simultaneous processes. I discovered strong indications of senescent decline in body mass in captivity, operating via impaired ability of the aged animals to regain lost body mass, whereas no evidence of body mass senescence could be found in the wild population. Although no obvious sex differences were apparent in the rates of senescence in either setting, females were on average slightly but significantly heavier than males throughout. Moreover, I was able to show that selective mortality of individuals with declining body mass was operating in both settings. Selective disappearance seemed to occur more rapidly in the wild, providing a possible explanation for the negligible senescent declines in body mass in the natural population. Some evidence was found suggesting that body condition might be a more important determinant of mortality in females than in males.

In contrast with the apparent lack of senescent decline in body mass in the longitudinal data, cross-sectional measurements of body mass-corrected grip strength revealed senescent declines in the physical strength of wild females as well as both sexes in captivity (**Chapter 3**). No sex differences were apparent in the age trajectories of strength, although only tentative conclusions could be drawn for males due to their earlier mortality. Contrary to the prediction of higher investment in physical functioning and better strength maintenance in males, the sexes exhibited either similar magnitude of strength or a female advantage in this monomorphic species.

Illness is likely a leading cause of direct mortality in the wild [Anderson 1979], and compromised health may further increase the risk of extrinsic mortality. In **Chapter 4**, I tested the hypothesis that due to immunosenescence and the associated impairment in the clearance of parasite infections, aging gray mouse lemurs would experience higher endoparasite loads. Contrary to this prediction, the oldest animals were shedding almost no helminth eggs, possibly suggesting parasite resistance via acquired immunity. High macroparasite burdens suffered by young individuals may lead to selective mortality, leaving only the parasite resistant individuals with the best immune function to live to old age. Similar to findings from other taxa, male gray mouse lemurs tended to have higher parasite prevalence and morphotype richness than females. However, this pattern emerged only in the dry season. Sex differences in the age trajectories were found only for tapeworm prevalence in the rainy season, with a higher proportion of older adult males harboring cestodes, whereas no age effect was apparent in females.

Because no assay had been previously validated for quantifying glucocorticoid metabolites in gray mouse lemur feces, the suitability of the sampling and assaying method was ascertained (**Chapter 5.1**) before using fecal glucocorticoids as a measure of allostatic load in the species. To exclude the possibility that repeated captures might have long-term consequences on the baseline glucocorticoid levels of the animals, I also tested whether capture history influenced the baseline levels and found no evidence of such a connection. Aging-related impairments have been found in the negative feedback regulation of glucocorticoid excretion in other species, leading to chronically elevated glucocorticoid levels [Sapolsky and Altmann 1991; Sapolsky et al. 1986]. Therefore, I tested the hypothesis that higher allostatic load (indicated by high baseline glucocorticoid levels) might be found in aged animals in reflection of such impairment in glucocorticoid regulation (**Chapter 5.2**). I expected aged individuals to experience particularly high allostatic load in the dry season if senescence is manifested as a lowered ability to cope with harsh environmental conditions. As predicted, old age was associated with elevated baseline glucocorticoid levels in females (but not in males) in the dry season, which coincides with low food availability, recovery from prolonged torpor and the mating season, all of which can be physiologically stressful and demand resource reallocation. Glucocorticoids are essential for facilitating this reallocation, however, the highly elevated levels found in aged females compared to younger females and males may reflect reduced feedback efficiency of glucocorticoids. The ensuing prolonged exposure to these hormones is potentially detrimental to health or reproduction in aged females. While age effects were apparent only in females, body mass was positively associated with male glucocorticoid levels in the dry season, perhaps indicating higher reproductive investment by males in better body condition.

The main findings of these studies are summarized below in Table 1.

Table 1: Summary of the key findings from Chapters 2 to 5: sex-specific patterns of senescence in four components of health. + indicates evidence of senescence, - evidence of negative senescence (i.e. improvement with age), n.s. non-significant change in the parameter with age

Chapter	Parameter	Setting/season	Initial sex difference	Male senescence	Female senescence	Sex-specific age trajectories	Other findings / clarifications
2	Body mass	Captive long day	F +	n.s.	n.s.	n.s.	Longvity male-biased in captivity, female-biased in the wild
		Captive short day	F +	+	+	n.s.	
		Wild dry	M +	n.s.	n.s.	n.s.	
		Wild rainy	F +	-	-	n.s.	
3	Grip strength	Captive	n.s	+	+	n.s.	
		Wild dry	F +	+	+	n.s.	
		Wild rainy	n.s.	n.s. / +	+	n.s.	Age trajectories parallel but oldest males in the data set 5 years old
4	Parasite burden	Wild dry	M +	-	-	n.s.	Body mass positively associated with cestode prevalence
		Wild rainy	n.s.	n.s. / +	n.s.	n.s. / +	Age-related increase in male cestode infection, oldest males in data set 6 years old; body mass positively associated with cestode prevalence
5	Allostatic load	Wild dry	n.s.	n.s.	+	+	Male body mass positively associated with fGCM level
		Wild rainy	n.s.	n.s.	n.s.	n.s.	

EVIDENCE OF SENESCENCE AND SELECTIVE DISAPPEARANCE

It is evident from the results from these studies that the age trajectories of the four components of health are not uniform, and the patterns are invariably affected by sex, season and/or setting. In this section, I will discuss the potential causes and evolutionary implications of these broad patterns of senescence and mortality.

Signs of senescence

The mammalian organism is complex, and no aspect of its physiology acts in isolation. Hence, measuring several different aspects of functioning can lead to a better understanding of whole-organism senescence [Burger and Promislow 2006; Nussey et al. 2008]. While evidence of senescence was found in several of the parameters measured in wild gray mouse lemurs, the age trajectories differed for the measured traits. It seems intuitive that the traits that affect fitness the most should be best maintained to old age (although these may differ for the sexes and species, see section *Sex differences in aging*). Body mass and parasite resistance were maintained or even improved up until old age. These traits are likely associated with avoidance of predation and disease, as well as the ability to thermoregulate and avoid starvation; hence deterioration in them may lead to rapid mortality. Alterations in muscle strength or glucocorticoid metabolism, which were more affected by aging, might have only moderately harmful effects on individual performance and therefore incur weaker selective pressures. Muscle maintenance is costly [Piccirillo et al. 2014; Sparti et al. 1997; Suarez 1996] and fat may be a more limiting resource than muscle for aged females due to their intense torpor use [Schmid 1999; Vuarin et al. 2013]. Although grip strength reflects overall physical functioning and frailty well in humans and mice [Justice et al. 2013; Metter et al. 2002], no empirical data exists on how well it reflects overall functioning or stamina in other species. It is also plausible that experience and cognitive skills can help individuals to compensate for declines in strength, for instance via better knowledge of their habitat or prey capturing technique [Goerlitz and Siemers 2007; Lührs et al. 2009]. Likewise, I cannot completely exclude the possibility that elevated glucocorticoid levels in the dry season could be adaptive for the aged females by facilitating rapid recovery from torpor (see **Chapter 5.2**). Measuring reproductive performance was not possible in the framework of this thesis, but maintenance of reproductive traits might also be subject to positive selection. In other species, the evidence for this scenario is mixed. Reproductive senescence occurs frequently (e.g. bighorn sheep females [Bérubé 1999]; antler fly males [Bonduriansky and Brassil 2002]; great tit females [Bouwhuis et al. 2009]; common lizard females [Massot et al. 2011]), but it is apparently absent in some species (snow petrel [Berman et al. 2009]; common brushtail possum [Isaac and Johnson 2005]) and some traits [Hayward et al. 2013].

Similar to our findings, other studies that have simultaneously explored senescence in several traits have also observed trait-specific patterns of senescence [Hayward et al. 2013; Massot et al. 2011; Rueppell et al. 2007; Wright et al. 2008], or reported a decoupling of the rate of demographic senescence from the rates of senescence in reproductive [Bouwhuis et al. 2012; Nussey et al. 2008], and other functional traits [Burger and Promislow 2006; Rueppell et al. 2007]. Depending on the relative fitness benefits and costs of prolonged survival and reproductive output, traits may be

differentially preserved across species, in reflection of the level of somatic maintenance [Kirkwood and Austad 2000]. In addition to trade-offs between reproductive investment and somatic maintenance, I propose that preferential somatic maintenance could lead to preferential investment within the "somatic traits" into the most critical biochemical pathways and cell types. However, the paucity of studies that have measured senescence in multiple traits still hinders conclusions on the broader patterns of trait-maintenance.

Aging in different environments

Aging in the wild is now a widely recognized phenomenon [Nussey et al. 2013], but little information exists on how similar the aging processes are between natural and anthropogenic environments. The available evidence from the three most commonly used model species for aging research indicates a remarkable influence of laboratory conditions on the species' longevity compared with animals derived from natural populations. House mice derived from wild populations live up to 24 % longer compared with standard laboratory stock, and exhibit better functioning in several biochemical pathways associated with longevity [Miller et al. 2002]. Similarly, in *Drosophila melanogaster*, the longevity of wild-derived individuals matches that of a strain selected for long lifespan, exceeding that of control lines by 60 % [Linnen et al. 2001]. Contrary to these patterns, the nematode *Caenorhabditis elegans* lives 10 times longer on agar than a more natural substrate [Van Voorhies et al. 2005]. In both the wild and captivity, the accumulation of molecular damage would be expected to lead to physiological and physical deterioration at old age, but the level of deterioration may be influenced by for example resource availability and lifelong trade-offs among growth, reproduction and somatic maintenance. These characteristics typically differ between captive and wild populations and may cause the substantial difference found in these model species and also in body mass senescence of gray mouse lemurs across settings (**Chapter 2)**. However, little difference was found in the age trajectories of grip strength between wild and captive mouse lemurs (**Chapter 3**). This suggests that the mechanisms of muscle deterioration are largely constrained by intrinsic mechanisms, as might be expected given the remarkably preserved mechanisms of sarcopenia evidenced across taxa [Demontis et al. 2013]. Together, **Chapters 2** and **3** demonstrate that depending on the trait measured, different conclusions may be reached on the comparability of aging processes across settings.

If the preferential maintenance of fitness-maximizing traits causes these differences, one might expect similar age trajectories between wild and captive animals in traits that confer little survival advantage, whereas a relatively more pronounced decline may be detected in captivity in traits that are most vital for survival in the wild. For example, immune system function might show decline of a higher magnitude under protected conditions compared with natural populations. The types of species most extensively used as laboratory model organisms ("r-selected" species with a fast pace of life) are typically subject to high extrinsic mortality in their natural environments [Austad 1997]. Hence, they may experience especially pronounced senescent deterioration in survival-enhancing traits under protected conditions than would be possible in the natural environment with its associated extrinsic hazards. Although the studies presented in this thesis demonstrate the value of captive-wild comparisons in assessing the relative importance of different evolutionary mechanisms,

such studies can be hampered by differences in the type of long term measurements available, differences in life histories due to restricted breeding of captive animals, and limited availability of data for wild animals. Potential differences in patterns of senescence might also be introduced by the physiological effects of, for example, torpor use [Turbill et al. 2012; Vuarin et al. 2013] or calorie or nutrient intake [Colman et al. 2014] that consistently differ between the settings and may influence senescent processes (discussed in **Chapters 3** and **5.2**).

A reduced ability of aged animals to cope with environmental challenges can be an important manifestation of aging in the wild [Cotto and Ronce 2014; Coulson et al. 2001; Kirkwood and Austad 2000] and increase mortality risk. Support for this view is offered by the dry season plateau in body mass (**Chapter 2**) and high allostatic load in females (**Chapter 5**), and the observed terminal declines in body mass (**Chapter 2**). Interestingly, it appears that the ecological effects of seasonality are so pervasive that they carry over to photoperiodic cycles in captivity despite the otherwise standard conditions, leading to photoperiod-specific age trajectories of body mass (**Chapter 2**). A similar phenomenon has been described previously for immunological profiles of song sparrows [Greenman et al. 2005]. It would be worthwhile to consider this phenomenon in studies of physical and physiological performance in captivity, as the interpretations might depend on the photoperiodic season in which measuring is done.

Intrinsic, extrinsic and selective mortality

Senescence was previously thought to not occur or be impossible to observe in the wild because aged animals would succumb to extrinsic mortality once their condition began to decline [Hayflick 2000]. Direct evidence of such selective disappearance was found by examining condition-dependent mortality in **Chapter 2**, and selective mortality may also contribute to the absence of senescence in body mass and parasite resistance in both sexes. In contrast to the wild population, substantial functional senescence was observed in captivity (**Chapters 2** and **3**). Together, these patterns suggest a critical role for extrinsic hazards and non-random mortality in regulating the magnitude of functional senescence in natural populations. Similar to our findings, studies that estimated functional senescence as well as selective disappearance simultaneously in free-living females of a passerine bird [Bouwhuis et al. 2009] and three ungulate species [Hayward et al. 2013; Nussey et al. 2011] found evidence for both processes. Female guppies derived from natural populations experiencing high predation risk showed better escape responses and reduced aging compared to a low-predation population, which the authors suggested could result from selective mortality [Reznick et al. 2004].

As shown for the first time in **Chapter 2** of this thesis, these coexistent forces may also differ for the sexes. In addition to a reversed sex bias in lifespan between the wild and captive population, body mass had an apparently stronger influence on female mortality, whereas male mortality might be more affected by random processes or reflect condition-dependent mortality too rapid to show up in our measurements. As both, functional senescence and lifespan, were more similar across the settings in females compared with males, it appears that female lifespan is determined to a larger extent by intrinsic mechanisms. In contrast, the substantial disparity found in patterns of senescence

and lifespan in males across settings indicates primarily extrinsic mortality, as males disappeared from the natural population at an earlier age and in better physical condition than females. Interestingly, such a sex bias in the sources of intrinsic versus extrinsic mortality has also been found in humans of the general population [Owens 2002] and also when comparing nuns and monks living under more "protected" conditions [Luy 2009]. The similar patterns suggest at least partially intrinsically determined, endocrinological or behavioral causes for these differences. The higher incidence of death by extrinsic causes may reflect the typically male-biased sensation-seeking behaviors in humans [Rosenblitt et al. 2001], and dispersal and risky mating behaviors in many male mammals [Greenwood 1980; Lawson Handley and Perrin 2007; Trivers 1972]. The demands set for male functioning for them to escape extrinsic mortality may exceed those for females, and with the removal of natural hazards, costly male behaviors and demands for repair from disease and injury are reduced (see also section *Of mouse lemurs and men: towards resolving the (human) health-survival paradox*). Resources thus released for somatic maintenance can potentially lead to a disproportionate increase in male lifespan. This idea might also explain the finding that the males of some vertebrate and invertebrate species outlive females under captive conditions [Austad 2006; Maklakov et al. 2009]; **Chapter 2]**, but rigorous comparison of captive and field data from more species will be required to test this hypothesis, and to assess the conditions required for this phenomenon to arise. At this time I also cannot exclude the possibility that artificial selection for some male or female traits (e.g. high female fecundity) in captivity has inadvertently led to a larger increase in male lifespan relative to females in gray mouse lemurs.

Conclusions on variable senescence and selective mortality

In summary, evidence of both, senescent decline in functioning and selective disappearance of individuals in a declining condition, were discovered in the gray mouse lemur. The patterns of senescence differed across components of health, supporting the hypothesis that in this species with a high extrinsic mortality risk, traits that are linked to improved survival in nature are likely best maintained to old age. In species such as the gray mouse lemur, that face very high extrinsic mortality and seasonal reproduction, survival to the next reproductive event may be limiting for fitness, leading to a better maintenance of traits that enhance survival. While this thought is contrary to the central prediction that species with high extrinsic mortality should invest more in current reproduction than in survival [Ricklefs 1998; Williams et al. 2006], it is consistent with theoretical models suggesting that selective mortality of low quality individuals may even reduce the rate or delay the onset of senescence [McNamara and Houston 1996; Williams and Day 2003].

Senescence may be universally shaped by selective disappearance, but life history variation (e.g. pace of life) [Jones et al. 2008] and the common sources of mortality likely dictate which traits are subject to the strongest selective pressures. The strength of the selective pressures may also depend on the age of the individual. For example in male antler flies, selection was stronger for survival in young flies and for reproductive performance at old age [Bonduriansky and Brassil 2002]. Currently, the data on patterns of senescence in multiple traits within species and across taxa are insufficient to draw conclusions on how mortality rates and mating systems influence preferential trait maintenance [Bouwhuis et al. 2012; Hamel et al. 2010].

Sex differences in patterns of mortality suggest that intrinsic mechanisms may regulate female mortality to a large extent, whereas male mortality more often follows from extrinsic causes. Seasonal differences in the age trajectories offered limited support for the hypothesis that aging in the wild might be manifested as impaired coping with energetically demanding conditions. This was particularly apparent in females, which can apparently live to an older age if they remain in good condition and are able to cope with energetic demands set by the environment. It should be noted that inference on preferential trait maintenance is necessarily largely based on females since they were the only sex showing senescent declines in some but not other traits, whereas wild males experienced negligible senescence in essentially all measured traits.

SEX DIFFERENCES IN FUNCTIONAL AGING

While male-biased functional decline is expected when males are the shorter-lived sex [Graves 2007; Williams 1957], it is unclear whether different features of functioning should decline at similar rates. Sex-specific patterns of senescence were found in some components of health measured in this thesis, but these differences were not uniform across the traits studied. In this section, I will discuss the possible causes and consequences of the sex differences observed in the gray mouse lemur and relate them to those found in other species, with special reference to humans because most of previous studies on the topic were done in humans. The two hypotheses I aimed to test were 1) whether sex biases in the aging of different components of health reflect the same evolutionary mechanisms that presumably produce the sex bias in longevity and 2) whether the patterns of sex-specific senescence present in the gray mouse lemur parallel the health-mortality paradox described in humans.

Hypothesis 1 stems from the prediction that each sex should optimize the traits most beneficial for their fitness [Vinogradov 1998]. Consequently, I expected to see better physical functioning (grip strength) throughout life in males, whereas females should maintain better general health, especially immunocompetence (lower parasite burden) until old age. Hypothesis 2 is based on the observations that in humans, men seem to remain in good health and physical condition until old age (if they avoid early mortality via e.g. acute illnesses and accidents) [Frederiksen et al. 2006; Kulminski et al. 2007] although they tend to have lower overall immunocompetence [Nunn et al. 2009]. Paradoxically, women live longer but in worse health due to debilitating but non-lethal conditions [Kulminski et al. 2007; Oksuzyan et al. 2008]. If the basis of this paradox is intrinsic and extends to other species beyond humans, male mouse lemurs might be expected to show better functioning and/or reduced senescent decline in health relative to females despite their shorter lifespan. I will next discuss evidence for each hypothesis in turn.

Strong males and healthy females?

Overall, wild gray mouse lemur females seemed to invest more in immune function over physical strength, as predicted by the first hypothesis. However, the patterns were inconsistent across the focal health indicators and the ecological seasons. Declining functioning in aged females was

suggested by the patterns of grip strength and glucocorticoid levels, whereas patterns of body mass and parasite loads contrast this interpretation by rather improving or remaining unchanged at old age. Contrary to the prediction of earlier or more rapid aging in males than in females [Bonduriansky et al. 2008; Kirkwood and Rose 1991; Williams 1957], the dry season patterns of senescence in wild male gray mouse lemurs mainly paralleled females (but males' glucocorticoid levels remained unchanged with age). It should be noted that the estimation of senescence in wild males in all measurements besides body mass is tentative due to the absence of very old males from the short term sample owing to the males' short average lifespan (in 2010-2012, maximum age of sampled males was 4-6 years depending on the data set). However, the observed patterns suggest that the males' shorter lifespan has not led to an earlier onset of senescence compared with females. Since evidence of earlier senescence was also not found in captivity, where males live long enough for senescent declines to be more easily detectable, it seems likely that early functional senescence in male gray mouse lemurs has been selected against.

Where sex-specific patterns were found, differences tended to be larger in the dry season compared with the rainy season, perhaps because of more divergent behavioral repertoires and endocrinological states of the sexes during the dry season data collection period. Male grip strength was significantly lower (**Chapter 3**) and their parasite loads higher (**Chapter 4**) than in females in the dry season. This might indicate reduced investment by males in both immune defenses and muscle tissue maintenance in favor of other functions more directly related to reproductive performance, such as sperm production or roaming intensity. Although grip strength is expected to indicate overall physical functioning, it may therefore not be directly related to male gray mouse lemurs' reproductive success, which is likely influenced largely by the intensity of roaming and defending females. Rather, higher body mass and the associated expendable energy reserves could better improve male performance. Larger males indeed enjoy better reproductive success [Eberle and Kappeler 2004a], and, as no senescence was found in male body mass, selective pressures likely promote its maintenance in the wild. In the rainy season, excess energy may be used by males for recovery from any parasites acquired and body condition lost during the mating season, whereas female maintenance costs are likely elevated due to their prolonged investment in breeding, hence leveling the sex bias in health.

A strategy of terminal investment [Clutton-Brock 1984] might be more beneficial for male fitness than sparing energy for post-mating recovery, especially given the males' high mortality risk during the brief mating season [Kraus et al. 2008]. The accumulation of high parasite loads by mouse lemur males in the dry season (**Chapter 4**) is likely associated with such a strategy, and possibly leads to excess male mortality. Evidence for terminal investment into reproduction has been found in males of e.g. the blue-footed booby [Velando et al. 2006] and a damselfly [González-Tokman et al. 2013]. In certain marsupial mice, such a strategy leads to the death of all males shortly after the mating season, whereas females can survive up to twice as long [Cockburn 1989]. Selective pressures likely promote energy-intensive behaviors particularly in older males, whose residual reproductive value may be lowered due to their shorter projected remaining lifespan [Clutton-Brock 1984]. In support of this, male boldness, or risk-taking behavior, has been found to increase with age in gray mouse lemurs [Dammhahn 2012]. Behavioral correlates of the health parameters measured here, as well as

measuring further indicators of energy metabolism would be useful for evaluating variation in male reproductive investment.

The selection for robustness can be linked by evolutionary mechanisms with terminal investment. Males that survive high, (potentially terminal) reproductive investment and also maintain sufficient body condition and avoid injury, illness and predation may be of exceptionally high quality, and lead to parallel selective pressures for reproductive investment and improved survival. This may follow if individuals of high "genotypic quality" are able to gain enough resources to reduce the necessity to trade off survival for reproductive performance [Kodric-Brown and Brown 1984]. A positive association between reproductive success and survival has sometimes been observed in other species [Alatalo et al. 1991; Kimber and Chippindale 2013; Kodric-Brown and Brown 1984], and is thought to indicate the high genetic quality of the male [Kodric-Brown and Brown 1984]. This mechanism may also act in the gray mouse lemur, as male competitive ability and larger size improve their reproductive performance [Eberle and Kappeler 2004b; Gomez et al. 2012], but detailed information on age-specific reproductive performance of wild individuals would be needed to confirm this conclusion. Heavy investment in energy-intensive behaviors by males in good body condition might be reflected as their higher glucocorticoid levels during the dry season (**Chapter 5.2**). The (presumably) most robust males in the wild population live twice as long as the average adult male (typically 5-6 years, compared to average 2.7 years, Hämäläinen et al. unpublished). Despite the potential longevity advantage of the healthiest males, male lifespan might be limited by the number of mating seasons they can survive, particularly if they increase their investment with age. As onset of senescence occurs around age 5 years in both sexes and across several traits in both captivity and the wild, this constraint on male longevity likely has had a significant influence on mechanisms of senescence in the species. This conclusion is also supported by the fact that up until age 4-5 years, both sexes followed similar age trajectories of health, and only beyond that did wild females experience declines in some traits.

Roaming and fighting for females likely involve an increased risk of predation and pathogen encounters in the reproductive season [Kraus et al. 2008], leading wild males to typically die in prime adulthood. Due to the high energetic demands and risky behaviors required for reproductive success, males may experience strong selection for "robustness" [Bonduriansky et al. 2008; Maklakov et al. 2009; Maklakov and Lummaa 2013]. The higher fitness of males in good condition, or an improving reproductive success with age could lead to selection against male functional senescence [Bonduriansky et al. 2008; Graves 2007]. An examination of sex-specific reproductive senescence and mortality in captive black field crickets has likewise demonstrated that female reproductive senescence is faster and lifespan shorter, whereas males show little senescence [Maklakov et al. 2009]. In a more natural environment, female crickets outlive males and are the only sex to show senescence [Zajitschek et al. 2009]. These findings and **Chapter 2** of this thesis are to my knowledge the only two studies examining sex-specific patterns of senescence and lifespan in both captive and natural environments. The remarkably similar results in these two widely differing taxa are a strong incentive to expand examination of this phenomenon to other species to discover the prerequisites for sex-specific life history flexibility of such magnitude.

The rarity of studies examining senescence in the same trait in both sexes limits inference on general sex-specific patterns of senescence. The focus on reproductive senescence may be the cause for this lack of studies, as reproductive traits typically differ for the sexes, and male reproductive success is often difficult to measure reliably. The use of functional traits that are not directly related to reproduction is therefore a useful additional approach for clarifying sex-specific patterns of functional senescence. The few studies in addition to this thesis that have measured senescent declines in both sexes have found contrasting patterns. In some, males exhibit an earlier onset or faster rate of senescence (body mass in marmots [Tafani et al. 2013]; several health indicators in yellow baboons [Alberts et al. 2014]). In others, more pronounced declines are seen in females (breeding success in the guillemot [Reed et al. 2008] and in the cricket [Maklakov et al. 2009]; incidence of injury in yellow baboons [Alberts et al. 2014]). Interspecific variation in sex-specific mortality risk and energetic demands may partially explain these patterns. The sex differences may follow taxonomic or mating system variation (although these are difficult to pull apart in comparative studies, e.g. [Clutton-Brock and Isvaran 2007]). However, the focal traits likely also play a large role in the direction and detectability of sex differences in senescence, as demonstrated in this thesis for gray mouse lemurs, and by Alberts et al. [2014] for baboons.

Of mouse lemurs and men: towards resolving the (human) health-survival paradox

Our measurements of health broadly included immunological, endocrine and physical functioning, yet we found scant direct support for the health-survival paradox in wild mouse lemurs. The body mass and grip strength of the longer-lived females exceeded those of males throughout and continued to surpass the population average at old age, indicating their good overall functioning. However, since little evidence was found of early-onset senescence in males, we cannot fully ignore the possibility that mouse lemurs might show patterns of senescence similar to humans, albeit with much more exaggerated sex differences in longevity and health due to species-specific life histories. The exceptional longevity of captive males indicates potential for excellent male functioning, although the rate and onset of senescence appear to be similar for the sexes in captivity.

As discussed in section *Intrinsic, extrinsic and selective mortality*, the relaxation of extrinsic mortality and the limited opportunities for captive males to express the risky behaviors that seem to account for much of natural male mortality [Kraus et al. 2008] have led to a large increase in male lifespan relative to females in captivity. It might be argued that similar processes occur in human societies: a reduction in the sex gap in mortality is seen in humans when male-biased causes of mortality – risky male behaviors and infectious disease – are reduced, leaving a larger proportion of men to survive to an old age. For instance, the longevity gap was less pronounced (but not completely eradicated) in cultures such as the late 19th century Mormons, where for cultural reasons, male behaviors were characterized by lower risk-taking, and female lifespan was reduced by high fecundity [Alberts et al. 2014]. Similarly, in German monasteries of nuns and monks, where many life style differences and sex-specific behaviors are equalized, the sex gap in life expectancy was significantly smaller than in the general population [Luy 2003; Luy 2009]. The other side of the coin is seen in contemporary Russian society, where a large sex gap in survival is found, presumably because commonly male-

biased risky behaviors, such as excessive alcohol consumption, are particularly prevalent in the society [Alberts et al. 2014]. Differences between countries in social gender roles (e.g. position in the labor force, access to health care, and socioeconomic status) and lifestyle choices [Crimmins et al. 2010] likely lead to the observed population level differences found among EU countries in the sex gap in relative health expectancy [Jagger et al. 2008; Van Oyen et al. 2013], although the overall (male-biased) mortality from infectious disease and parasite infections [He et al. 2005; Owens 2002] has been reduced with improving health care [Kruger and Nesse 2006].

In primordial societies, men most successful in accumulating resources were able to engage in polygynous relationships and thereby father more offspring [Gems 2014]. Human males are capable of reproducing to a later age than females, and it has been suggested that due to female menopause, male senescence could be attenuated due to higher fitness gains by males at older ages relative to females [Gems 2014; Tuljapurkar et al. 2007]. Women, on the other hand, might continue to gain indirect, post-reproductive fitness benefits by caring for grandoffspring [Lahdenperä et al. 2004; Shanley and Kirkwood 2001]. When these benefits can be gained despite some level of frailty, selection for physical functioning at old age might be weakened in women, offering a possible explanation for the observed patterns. In gray mouse lemurs, menopause has not been reported under captive conditions. Rather, both sexes can reproduce until very old age [Perret 1992; Perret 1997] and anecdotal information also suggests that aged females are able to reproduce under natural conditions (Melanie Dammhahn, personal communication). As some evidence has been found of nest sharing and even allomaternal care in the species [Eberle and Kappeler 2006], it is theoretically possible that the presence of a grandmother could improve the survival of the young, either via direct care or e.g. thermoregulatory benefits. However, as the extrinsic hazards are high, it seems likely that frail individuals would face increased mortality, hence leaving only the best quality females to potentially transfer benefits to their grandoffspring. These differences could be a possible partial explanation for the observed species differences in sex-specific health and mortality. The species- and sex-specific selective pressures would be expected to lead to robust and short-lived males in both species (with high intensity, potentially terminal investment reducing lifespan further in mouse lemurs), and to long-lived females that differ in their frailty due to the extrinsic hazards of their environments. While post-reproductive lifespan in common among mammals [Cohen 2004] and some fish [Reznick et al. 2005], evidence of substantial grandmaternal care that could lead to adaptive benefits of prolonged post-reproductive life in non-human animals is essentially missing [Fedigan and Pavelka 2001; MacDonald Pavelka et al. 2002; Packer et al. 1998; Reznick et al. 2005]. Instead, the post-reproductive life typically coincides with the period of care required for the last born offspring to reach independence [Cohen 2004; Packer et al. 1998]. Further information on the potential direct and indirect benefits of grandmothers and the significance of their condition and reproductive status [Packer et al. 1998] for grandoffspring fitness will be required to further assess the relevance of this phenomenon for non-human species.

Similar to our findings, no paradoxical patterns between functioning and survival have been demonstrated in yellow baboons. Instead, baboon males experience similar or more pronounced declines relative to females in essentially all indicators of functioning and have shorter longevity than females [Alberts et al. 2014]. In studies of other polygynous mammals, male-biased declines also

dominate [Greiner et al. 2014; Hayward et al. 2009; Obanda et al. 2014], and female-biased longevity is the norm [Promislow 2003]. Interestingly, in pair-living mammals and birds, sex differences in (reproductive) senescence are sometimes eliminated or female senescence rate may exceed that of males [Clutton-Brock and Isvaran 2007; Liker and Székely 2005; Promislow 2003], possibly because the costs of reproduction, including parental care, are relatively similar for the sexes in pair-living species [Clutton-Brock and Isvaran 2007]. Data on patterns of sex-specific functional senescence are still too taxonomically restricted to permit phylogenetically controlled inference, but it seems plausible that the mating system, and thus the strength of sexual selection, influences variation in sex-specific functional senescence, as found for actuarial senescence [Clutton-Brock and Isvaran 2007].

Conclusions on sex-specific senescence

No equivocal evidence was found in gray mouse lemurs for either of the two alternative hypotheses: 1) preferential investment in strength maintenance in males and immune investment in females, or 2) healthy males that die young and morbid, long-lived females, as described in humans. However, partial evidence was gained in favor of each in the wild population. In support of hypothesis 1, females tended to exhibit better health than males in three of the four focal parameters, but negligible sex differences were found in the maintenance of both strength and parasite resistance to old age. In favor of hypothesis 2, the shorter-lived males showed little senescent loss of functioning, but only moderate senescence was found in females. In the captive population, body mass and grip strength both declined similarly in both sexes, contradicting both of the tested hypotheses. I propose that these differing patterns may be reconciled by three evolutionary mechanisms that operate simultaneously: strong selective pressures leading to a robust male phenotype; the preferential maintenance of traits associated with improved survival; and the selective disappearance of individuals that do not meet these requirements. Such mechanisms might also explain some of the interspecific and sex-specific variability in patterns of senescence across taxa and merit further study.

The weak evidence for senescent decline in males in the wild population and their significantly improved longevity in captivity likely indicates selective pressures for their robustness and simultaneously reflects their high natural mortality rates. Overall, it appears that risky behaviors by both gray mouse lemur males and human men account for at least part of the excess male mortality, but the significance of the behavioral variation differs across environments and human societies. The positive association between reproductive success with age in human men and their ensuing robustness, combined with the female menopause and potentially reduced selection against frailty in human females, might contribute to the observed health-mortality paradox (which may be amplified by the relatively recent transition to pair-living and the prolonged lifespan in modern humans). However, it is not very likely to manifest in other species. The social organization and mating system variation likely contributes to the differences in patterns of aging and lifespan observed between these two primate species and beyond.

Little evidence was found in these studies of sex differences in trait maintenance at the ages that could be reliably compared, but this conclusion is tentative due the mainly cross-sectional or

intermediate-term data. Furthermore, other traits, such as reproductive success or demographic senescence, might be subject to larger sex differences.

OUTLOOK

While evidence for the life history trade-offs predicted by the disposable soma theory [Kirkwood and Rose 1991] has begun to accumulate for natural populations, enormous scope remains for testing theories of senescence. For instance, likely due to limitations of available longitudinal data, little attention has thus far been given to heterogeneity in life-history strategies and how it might explain variance in patterns of senescence within populations. Long-term studies of age-specific reproductive output, including the quality of offspring produced and, potentially, senescence of the progeny, could indicate whether the fitness benefits of old age reproduction can maintain high potential lifespan (see also [Promislow 1991]), or whether the often high variability in lifespan within a species is purely down to chance. Furthermore, theoretical modeling of the benefit of continued reproduction might help clarify the conditions under which such lifespan extension might evolve.

Studies of sex-specific senescence will be needed in more species to test the possibility that traits are preferentially maintained in males versus females, perhaps depending on the mating system of the species. In particular, the “robust male” hypothesis should be further explored using comparative and experimental approaches, since it may explain a part of the human paradox as well as patterns observed in other animals. Given that males are the sex more often used in laboratory studies due to their “less complex” life histories unhampered by pregnancy – whereas female reproductive success is easier to study in the wild, creating the opposite bias for wild mammals – it would be of particular importance to expand these sex-specific investigations to include the other sex, and to examine the way captivity influences each sex. Ideally, the sex-specific costs of breeding in the wild should then also be quantified to assess the magnitude of the reproduction-survival trade-offs potentially incurred due to environmental variation.

To rigorously evaluate how functioning influences mortality, it would be necessary also to document the causes of mortality. This may be prohibitively difficult in small-bodied, wild vertebrates, and may be better accomplished in large scale studies of invertebrates under semi-natural conditions or in intensely monitored, semi-free ranging vertebrates, although such settings involve a risk of too simplistic a view of the extrinsic hazards and often a reduced predation pressure. By allowing natural genetic variability but reducing extrinsic hazards, wild-derived organisms studied under laboratory conditions could prove useful for examining the influence of interactions between the environment and the phenotype on patterns of senescence. The proximate mechanisms associated with functional senescence: telomere dynamics, nutritional metabolism, mitochondrial activity and oxidative damage could be examined across settings to further assess the influence of environment and life history restrictions on mechanistic senescence.

For gray mouse lemurs, further insights into sex-specific senescence could be gained via the study of e.g. body composition, sex hormone levels and further indicators of health. Longitudinal studies of

multiple indicators of functioning are needed to resolve the interconnectedness of the different parameters because, due to non-random mortality, stochastic annual fluctuations and cohort effects, within-individual associations may not translate into comparable cross-sectional age trajectories. To determine which traits best indicate overall functioning, further comparison and connections between the different components of health also merit further study. For example, the overall patterns of glucocorticoid exposure and parasite burden did not coincide in gray mouse lemurs as would be expected if high allostatic load acutely reduced parasite resistance via impaired immune function [Klein 2004]. However, there may be delays in the actions of glucocorticoids on parasite infections, and the effects may differ between parasite species. Therefore, individual-based analyses are needed to examine these associations. Furthermore, endoparasite load inferred from fecal egg counts is a rough albeit widely employed indicator of general health. While endoparasites may cause morbidity, it is also likely that bacterial or viral illnesses and co-infections that could not be measured within this thesis are more direct mediators of morbidity and mortality than macroparasites alone. Future studies would benefit from further, more direct investigation of immune function, including markers representing the functioning of the different arms of the immune system. Testosterone levels, which may have sex-specific immunosuppressive effects, could unfortunately not be quantified in our study because we could not exclude the possibility of cross-reactivity of testosterone with glucocorticoids in the available testosterone assays for fecal samples. The measurement of sex steroids, glucocorticoids and thyroid hormone may be possible from for example hair samples, and may provide additional means of evaluating age- and sex-specific reproductive investment.

While the gray mouse lemur is highly flexible in its life history traits and able to adapt to a range of environmental conditions [Lahann et al. 2006], it seems plausible that a difficulty coping with particularly challenging conditions might lead to reduced fitness in populations under high environmental or anthropogenic pressures. This might also quantitatively influence the patterns observed in cross-sectional data, which are sensitive to annual fluctuation. Harsh years may cause especially pronounced disappearance of individuals with an impaired ability to cope with the environmental demands, including aged animals. Age effects on coping by more sensitive species would be required to assess the broader significance of this phenomenon and its implications for population viability in changing environments and due to anthropogenic pressures.

Finally, as the study of senescence in the wild is still a young field, the accumulating research on taxonomically, geographically, and demographically diverse patterns of functional aging will eventually provide sufficient material for phylogenetically controlled comparative analyses. This will permit broader testing of the theoretical framework, including the influence of mating systems, preferential trait maintenance and phenotypic plasticity on patterns of senescence.

CONCLUSIONS

In this thesis I have demonstrated that senescence can be observed in both, longitudinal and cross-sectional studies of functioning, in a wild vertebrate which experiences high rates of extrinsic mortality. The environment and sex interacted to create patterns of senescence: both sexes showed similar levels of decline in the captive colony, but in the wild, seasonal sex differences were found either in the absolute values or the age-trajectories in all of the focal parameters. Of the components of health measured, survival in the wild was apparently most enhanced by investment in body mass maintenance and parasite resistance. The short-lived males tended to have similar or worse initial performance relative to females, but showed little evidence of senescence. Overall, the results of this thesis do not support the fundamental prediction of life history theory of an earlier onset or higher rate of senescence in the shorter-lived sex. The rapid, selective mortality of individuals in a declining condition and the associated positive selection for self-maintenance may counteract the selective pressures that act to reduce somatic maintenance beyond the prime reproductive age.

Via a comparative approach I could provide rare data on the comparability of senescence patterns between captivity and the wild for a model species of aging, and take a step towards assessing the relative importance of non-random mortality in shaping senescence. Selective mortality is presumably a universal (but rarely quantified) phenomenon in wild populations, and there is also evidence of it in captivity and in humans. The theoretical predictions on how selective mortality should influence senescence have rarely been tested (see [Austad 1993; Reznick et al. 2004]), and not previously examined explicitly for both sexes in a species. In this thesis I discovered sex-specific patterns in the interplay of senescence and selective mortality for the first time in a mammal, and under truly natural conditions. Strong sexual selection for good physical condition, combined with the potential benefits of terminal investment in reproduction, may lead to attenuated male senescence and the potential for life expansion in the absence of extrinsic mortality. It is likely that only males in good condition can withstand health challenges and also be able to reproduce, further leading to robustness selection. If similar pressures are molding senescence in men, the human male-female health-survival paradox might be explained by “robust men” that remain in good physical condition, yet succumb to mortality by extrinsic and behavioral causes. Good health should similarly improve female lifetime fitness, albeit to a different extent. These patterns combined (and any potential sexual conflict associated with the differing optima of the sexes [Bonduriansky et al. 2008; Promislow 2003]) act to determine the species-specific lifespan.

While functional senescence is being increasingly reported in wild populations, it is noteworthy that the probability of observing senescence may be reduced by both, the actual negligible senescence in the trait measured, or confounding variables such as non-random mortality or seasonal differences in coping, that mask existing senescent declines. It is possible that a publication bias for positive findings of aging, along with taxonomic bias towards species in which senescent declines may be more prevalent or easier to observe, may lead to an underestimation of the variability of senescence across traits. The patterns of decline in different components of individual performance could eventually be used to explain life history variation between the sexes and across taxa.

In this thesis, I have provided the first account of multiple-trait, sex-specific functional senescence in a highly predated vertebrate in the wild. I have discussed the most likely explanations for the observed patterns by examining evidence for the shaping of senescence by extrinsic mortality, selective disappearance, sex-specific selective pressures, and preferential trait maintenance. In doing so, I have synthesized, and further developed these central theories of life history evolution. Results of this thesis broaden the current view of functional senescence and are useful in formulating novel hypotheses to guide future research into the evolution of (variable) senescence.

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I hereby declare that I have written this thesis independently and with no other aids or sources than quoted.

Göttingen,

Anni Hämäläinen