The evolution of the Lepilemuridae-Cheirogaleidae clade

By

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Dedication

To my mothers'

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DECLARATION

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QUALIFICATION: Doctor of Philosophy

DECLARATION:

In accordance with Rule G5.6.3, I hereby declare that the above-mentioned thesis is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

Signature

Curswan Andrews

ABSTRACT

The Lepilemuridae and the Cheirogaleidae, according to recent molecular reconstructions, share a more recent common ancestor than previously thought. Further phylogenetic reconstructions have indicated that body size evolution in this clade was marked by repeated dwarfing events that coincided with changes in the environment. I aimed to investigate the morphological implications of changes in body size within the *Lepilemur*-cheirogaleid clade, testing four predictions.

Together with Dr. Couette, I collected data on the overall palate shape and predicted that shape is likely to be influenced by several factors including phylogeny, body size and diet. Geometric morphometric analyses revealed that, although a strong phylogenetic signal was detected, diet had the major effect on palate shape. In a similar vein, when examining the arterial circulation patterns in these taxa, I predicted that changes in body size would result in changes and possible reductions in arterial size, particularly the internal carotid artery (ICA) and stapedial artery (SA). Analyses with micro-computed tomography (CT) and 3D imaging indicated that changes in body size led to reduction of a functional stapedial artery in *Lepilemur*, making it an intermediate stage between the daubentoniid, lemurid and indriid species with large stapedial arteries, and the smaller bodied cheirogaleids with an alternative blood supply in the form of an enlarged ascending pharyngeal artery. *Lepilemur* is the smallest living folivorous primate, and likely to be at the threshold body size to be able to subsist on such a poor diet. To investigate shifts in dietary patterns that accompanied changes in body size, I chose to explore the reported behaviour of caecotrophy as a possible means for the sportive lemurs to derive additional nutrient from their food sources. I predicted that, if caecotrophy is a way to assist folivory at small body size, the energy contained in "caecotrophic" and latrine faecal samples should be different. Analyses showed significant

differences between the two types of faeces and, combined with an analysis of faecal bacterial diversity, support the occurrence of caecotrophy. Finally, I compared the digestive efficiency of two small, distantly related gummivorous primates that evolved their diets convergently. I studied the digestion of gum in *Microcebus griseorufus* and compared this with gum digestion in *Galago moholi*. I predicted that an evolutionary disposition to fermentation inherited from a folivorous ancestor would aid in the digestion of gum in mouse lemurs. Results indicated that retention time was prolonged by the presence of secondary compounds in *Microcebus* fed with *Commiphora* gum but relatively shorter (< 24 hrs) when fed *Alantsilodenron* gum, a preferred food. Despite the fact that *G. moholi* has an ansa coli, which is missing in *M. griseorufus* species, both are highly efficient at digesting gum.

These data provide some of the first indicators of how dietary changes from a larger-bodied folivorous ancestor to partially gummivorous, small-bodied descendants may have occurred in evolutionary time.

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CHAPTER 1: GENERAL INTRODUCTION

1.1. The evolution of Madagascar's primates

The lemurs of Madagascar (infraorders Lemuriformes and Chiromyiformes of Groves, 2001) form one of five groups of terrestrial mammals to have colonised the island continent and radiated *in situ* (Garbutt, 1999). The other groups include small- to medium-sized carnivores (Eupleridae), rodents (Nesomyinae), tenrecs (Tenrecidae) and dwarf hippopotamuses (Hippopotamidae). Bats, both Megachiroptera and Microchiroptera, make up the rest of the living mammal fauna, along with invasive species like sewer rats (*Rattus norvegicus*), house mice (*Mus musculus*), Asian musk shrews (*Suncus murinus*), deer (*Dama dama* and *Cervus timorensis*), bush pigs (*Potamochoerus larvatus*) and the small Indian civet (*Viverricula indica*) (Garbutt, 2007). The remaining mammals are domesticated animals: cows, pigs, sheep, goats, dogs and cats, in addition to humans.

The lemurs constitute one of the most diverse living radiations of primates. Living species have been classified into five different families (Cheirogaleidae, Daubentoniidae, Indriidae, Lemuridae and Lepilemuridae), while subfossil remains from at least three recently extinct families (Archaeolemuridae, Megaladapidae and Palaeopropithecidae) have been described (Godfrey and Jungers, 2002; Herrera and Dávalos, 2016). Recent phylogenetic reconstructions based on molecular data, some of which have included ancient DNA from subfossils, have indicated that Lepilemuridae and Cheirogaleidae share a more recent common ancestor than either group shares with any other lineage. This relationship implies several previously unexpected scenarios regarding the evolution of the clade: (i) there have been several marked changes in body size during the evolution of this group, from an ancestor close to 1 kg in body weight to descendants as small as 30 g (Masters *et al*., 2014);

(ii) that this series of dwarfing events has been accompanied by shifts both in diet (Andrews *et al*., 2016) and physiology (Génin and Masters, 2016), and possibly other biological features; (iii) that aspects of skull and gut anatomy may reflect or be related to the biological and behavioural changes that accompanied the diversification of this clade; and (iv) that characters like small body size and gummivory, previously viewed as ancestral states in primate and lemur evolution, may in fact be highly derived adaptations to late Cenozoic environments.

I investigated these scenarios in several ways. First, I studied the variation in palate shape among living strepsirrhine primates as it relates to diet and phylogeny (Chapter 2). Next, I compared patterns of basicranial circulation among strepsirrhine taxa to investigate the arterial changes that accompanied the evolution of the lepilemurid-cheirogaleid clade (Chapter 3). In Chapter 4 I explore the microbiome of *Lepilemur*, in an effort to understand how such a small animal is sustained by a folivorous diet, and, in Chapter 5, I investigate the digestive efficiency in mouse lemurs and galagos, two distantly related strepsirrhine groups that include significant quantities of gum in their diet. Chapter 6 summarises and integrates my findings.

1.2 Strepsirrhine phylogeny and evolution

Early in their radiation, primates separated into two major lineages that are now regarded as suborders: the Haplorhini (primates with simple nostrils) and the Strepsirrhini (primates with twisted nostrils) (Pocock, 1918; Hill, 1953). The Strepsirrhini have often been considered "primitive" because they share several traits that are considered to represent earlier stages in primate evolution (e.g. epitheliochorial placenta; lack of postorbital closure). However, these character states are not necessarily ancestral (Masters and Génin, 2016), and, as strepsirrhines

and haplorhines shared a common ancestor, the branches leading to the living crown groups are equally ancient.

Other than having twisted nostrils, the living Strepsirrhini can be characterised by the presence of a "tooth-comb" in the lower jaw in all taxa except *Daubentonia*. This is a modification of the lower anterior dentition whereby the incisors and canines have become elongated, thin, and rotated horizontally (Merrit, 2010). Additionally, the animals have a cartilaginous sublingua under the tongue, which serves to keep the tooth-comb free of detritus. The tooth-comb is used both in grooming and feeding, when it may be referred to as a "tooth-scraper" (Vaughn, 1986). The upper incisors are often reduced markedly in size and the medial teeth are separated by a relatively wide gap that contains the vomeronasal organ (Martin, 1990). In addition to the infraorders Chiromyiformes (Daubentoniidae) and Lemuriformes (Cheirogaleidae, Lepilemuridae, Indriidae and Lemuridae), the suborder Strepsirrhini includes the Afro-Asian Lorisiformes (Lorisidae and Galagidae) and the extinct infraorder Adapiformes that diversified within the forests of the northern hemisphere during the Eocene (Masters *et al*., 2013). Most adapiforms went extinct at the end of the Eocene, approximately 34 Ma, during the mass extinction known as the "Grande Coupure" (Fleagle, 2013), although a few lineages survived until the late Miocene in Asia.

Before the turn of the present century, there was little agreement regarding relationships among the diverse lineages of lemurs that make up the Malagasy crown group fauna (Yoder, 1997; DelPero *et al*., 2001). Data sets were limited in terms of both characters and species represented, and several nodes were difficult to resolve (e.g. Yoder, 1994, 1997; Stanger-Hall and Cunningham, 1998). Even the early reconstructions involving genetic sequence data, however, agreed on the position of *Daubentonia*, the aye-aye, which was placed as the basal divergence of the Malagasy clade (e.g. Yoder, 1994, 1997; Stanger-Hall and Cunningham, 1998). This placement was sustained in all subsequent molecular sequence

studies (DelPero *et al*., 2001; Pastorini *et al*., 2003; Roos *et al*., 2004; Yoder and Yang, 2004; DelPero *et al*., 2006; Horvath *et al*., 2008; Chaterjee *et al*., 2009; Perelman *et al*., 2011; Springer *et al*., 2012; Masters *et al*., 2013; Kistler *et al*., 2015; Herrera and Dávalos, 2016), but is not well supported by morphology (Groves, 1974, 2001) or by chromosomal structure (Picone and Sineo, 2012). Groves (2001) recommended that *Daubentonia* be classified in a separate infraorder from the other Lemuriformes, i.e. Chiromyiformes, which I follow here.

The position of the sportive lemurs (Lepilemuridae) within lemur phylogeny has been particularly inconsistent historically. Most authors prior to the 21st century assumed that the Lepilemuridae, Indriidae and Lemuridae formed a natural group (e.g. Groves, 2001). Considering the basicranial evidence, Szalay (1975, p. 109) postulated that the Cheirogaleidae were probably "derived from a lemuroid, a form not unlike *Lepilemur*", while Oxnard *et al.* (1990) proposed a relationship between *Lepilemur* and the cheirogaleids on the basis of similarities in postcranial anatomy. These similarities do not seem to have been generally recognised, however. For example, in their morphological study of lemur systematics, Groves and Eaglen (1988) concluded that *Lepilemur* was probably more closely related to the Indriidae than to any other lemuriform taxon, noting that their conclusion was consistent with existing literature that grouped *Lepilemur* with the extinct *Megaladapis*, that formed a sister group with Indriidae.

The morphological similarities between *Lepilemur* and *Megaladapis* are complex and intriguing. Most *Lepilemur* species weigh less than 1 kg, while *Megaladapis* is an extinct giant lemur that weighed around 50 kg, and is known only from subfossils. While other lemur genera show a single, horizontal facet for articulation between the lower jaw and the skull, the mandibles of *Megaladapis* and *Lepilemur* have both a horizontal and a vertical facet that are identical in structure. Additionally, both genera have lost both pairs of upper incisors, interpreted as an adaptation for leaf-cropping (Tattersall and Schwartz, 1975; Schwartz and

Tattersall, 1985). Microwear analyses of both genera confirm a folivorous diet. *Megaladapis* was unique in a projection of its maxillae that seems to have been associated with a mobile proboscis; nothing like this occurs in lepilemurs. For several years, *Lepilemur* and *Megaladapis* were classified together under the family Megaladapidae. However, studies of ancient DNA have challenged this idea. While some researchers reconstructed *Megaladapis* as the sister taxon to the family Lemuridae (Karanth *et al*., 2005; Kistler *et al*., 2015; divergence date 27 Ma), others placed it as the sister to all Malagasy lemuriforms excluding *Daubentonia* (Herrera and Dávalos, 2016; divergence date 42 Ma). Once again, morphological and genetic evidence provide different interpretations.

It was only when genetic data sets became more comprehensive in terms of taxa and DNA sequences sampled that a degree of consensus regarding the relationships of lemuriform lemurs (*sensu stricto*) began to emerge. This was also when molecular clock analysis for dating divergences became more sophisticated. All molecular reconstructions published within the last 12 years (DelPero *et al*., 2006; Horvath *et al*., 2008; Chatterjee *et al*., 2009; Springer *et al*., 2012; Kistler *et al*., 2015; Herrera and Dávalos, 2016) have indicated a close relationship between lepilemurs and the family Cheirogaleidae (mouse and dwarf lemurs) that indicates exclusive common ancestry. Few authors seem to have accorded much significance to this clade, so that precise estimates of its age have sometimes not been made or reported. However, on the basis of available data, the *Lepilemur*–cheirogaleid clade (hereafter the LC clade) appears to be between 28 and 37 million years old (Ma), with an average estimate of 31 Ma (Table 1.1, page 8). Reconstructions using mitochondrial DNA (Chatterjee *et al*., 2009; Springer *et al*., 2012; Masters *et al*., 2013) grouped *Lepilemur* and *Phaner* (fork-marked dwarf lemurs) as a clade that is sister to the remaining cheirogaleids. Nuclear DNA studies (Roos *et al.*, 2004; Perelman *et al.*, 2011; Herrera and Dávalos, 2016, see Figure 1.1 below).

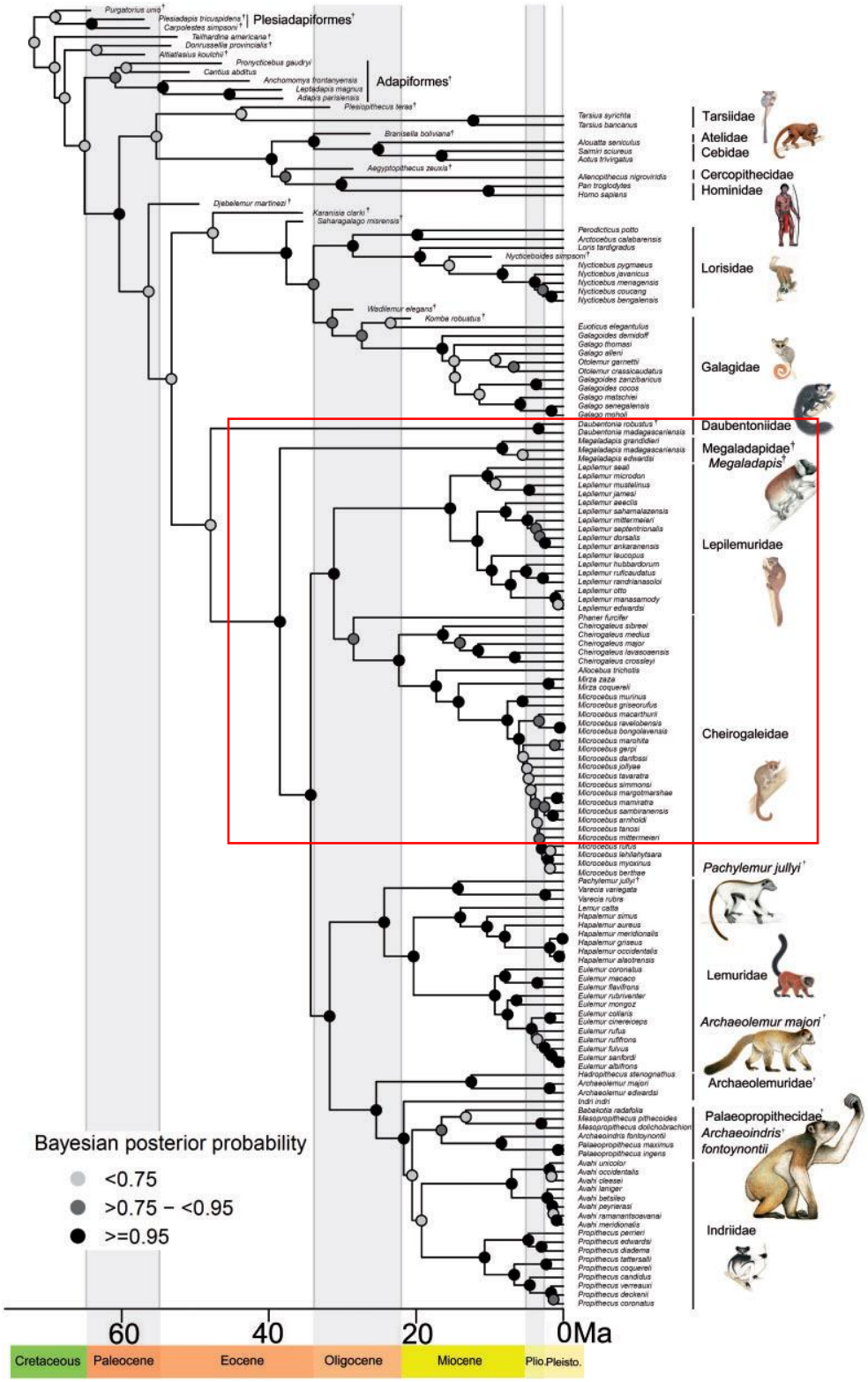

Figure 1.1 Phylogenetic reconstruction taken from Herrera and Dávalos, 2016, based on 421 morphological, 5767 protein-coding molecular characters. The relationship between Lepilemuridae –Cheirogaleidae clade is highlighted in the red square

However, they placed *Lepilemur* as the sister taxon to all the Cheirogaleidae including *Phaner*. In either reconstruction, the close affiliation of sportive lemurs and cheirogaleids is unassailable on the basis of currently available genetic evidence and Masters *et al.* (2013) advised that the sportive lemurs should be subsumed within the family Cheirogaleidae under their own subfamily (Lepilemurinae), as is the situation for the fork-marked dwarf lemurs (Phanerinae; Rumpler and Albignac, 1973). I follow this classification. The taxonomic hierarchy exists to summarize information regarding evolutionary descent, and monotypic families contain little systematic information, even if the $\geq$ 26 species that have been proposed for the genus *Lepilemur* are validated by future field research. If sportive lemurs shared a common ancestor with cheirogaleids more recently than either group shared with any other lemur taxon, their classification should reflect this fact.

Guided by the growing consensus among molecular phylogenies, Masters *et al*. (2014) explored skull allometries in the LC clade, and demonstrated that the adult skulls of mouse and dwarf lemurs closely reflect the size and shape of juvenile *Lepilemurs.* The small-bodied animals show typical paedomorphic traits: large heads, large eyes, and relatively short limbs. These observations are consistent with the interpretation that body size reduction in the Cheirogaleidae (including *Lepilemur*) evolved by means of progenesis (i.e. truncated development), an explanation that derives support from the markedly shorter gestation periods of small-bodied cheirogaleids compared with other lemurs, including lepilemurs.

Masters *et al*. (2014) proposed that a minimum of four dwarfing events occurred during the radiation of the LC clade: an initial reduction from a *Lepilemur*-sized ancestor to yield the dwarf taxa *Cheirogaleus major*, *Mirza* and *Phaner*, followed by "hyper-dwarfing" events to yield the smallest living taxa, *Cheirogaleus medius (s.l.), Allocebus* and *Microcebus*. Furthermore, it is highly likely that the living *Lepilemur* species are themselves the product

of phyletic dwarfing, as they are the smallest obligate folivores in the primate clade; Kay (1975) defined 500 g as the lowest viable body size for a folivorous primate. Reducing body size below this point, as apparently occurred in the mouse and dwarf lemurs, would necessitate changes in diet. Species of *Cheirogaleus* are highly frugivorous, as is evident from their very bunodont molars, while species of *Allocebus*, *Microcebus*, *Mirza* and *Phaner* combine varying degrees of gummivory with faunivory and frugivory. Only *Phaner* is regarded as an obligate gummivore.

My project consists of an investigation into the implications of the LC relationship for lemur evolution.

1.3 The primate fossil record and strepsirrhine evolutionary history

Understanding the evolutionary history of any group is always linked inextricably to the discovery and documenting of fossils. The primate fossil record is better than those of most groups because it concerns our own deep origins, but nevertheless it contains many serious gaps, which include some key periods of the evolution of primates. For instance, we know very little of the early evolution of primates (prior to the Eocene; Silcox *et al*., 2007). The oldest undoubted primate fossil is the 60 Ma *Altiatlasius* from the High Atlas of Morocco (Sigé *et al*., 1990), which consists of a ten small, loose teeth. The oldest known identifiably strepsirrhine and haplorrhine primates date back to the beginning of the Eocene (±55 Ma; Fleagle, 2013), when the adapiforms (strepsirrhine) and omomyids (haplorhine) radiated extensively across the northern continents of North America and Eurasia. Most of this diverse fossil fauna went extinct at the end of the Eocene, which is dated at 33.9 Ma. Some of the latest adapiforms have been recovered from Eocene – Oligocene fossil deposits in northern Africa (Simons and Miller, 1997; Marivaux *et al*., 2001; Benoit *et al*., 2013; Marivaux *et al*.,

Table 1.1. Comparison of divergence time estimates at key nodes in strepsirrhine phylogeny in millions of years (Ma). Numbers in brackets represent lower and upper 95% confidence intervals. CIs were not calculated by all authors for all nodes

Node	**Yoder and Yang (2004)**	**Horvath *et al*. (2008)**	**Chatterjee *et al*. (2009)**	**Perelman *et al*. (2011)**	**Springer *et al.* (2012)**	**Masters *et al.* (2013)**	**Kistler *et al*. (2015)**	**Herrera & Davalos (2016)**
Haplorhini/ Strepsirrhini	85 (77, 90)	-	67 (64,73)	87 (76, 99)	68 (63, 71)	69 (54, 85)	68 (60, 76)	64 (48, 70)
Crown Strepsirrhini	69 (61, 75)	75 (67, 84)	52 (48, 56)	69 (59, 77)	54 (53, 55)	58 (45, 71)	59 (52, 66)	61 (56, 67)
Lorisiformes	39 (38, 42)	39 (37, 42)	38 (37,3 9)	40 (35, 46)	35 (31, 37)	35 (28, 45)	38 (37, 41)	38 (32, 39)
Chiromyiformes - Lemuriformes	62 (58, 73)	66 (55, 75)	46 (41, 51)	59 (39, 77)	50 (49, 51)	48 (38, 61)	50 (42, 57)	55 (49, 61)
Lemuriformes	42 (35, 50)	39 (33, 46)	32 (29, 34)	39 (26, 50)	32 (27, 37)	33 (25,42)	31 (27,35)	42 (34,50)
Indriidae	39	36	21 (17, 25)	17 (10, 26)	18 (12, 26)	18 (12, 24)	17 (14, 20)	23 (17, 28)
Lemuridae	32 (26, 39)	23 (19, 29)	21 (18, 25)	26 (16, 37)	21 (15, 26)	17 (12, 23)	19 (16, 22)	26 (19, 33)
Lepilemur-cheirogaleid clade	37	30 (37, 25)	32 (29, 34)	33 (22, 44)	28	32 (23, 39)	28	31

2013). *Bugitlemur mathesoni* from early Oligocene deposits (30 Ma) in the Bugti Hills (Balochistan, Pakistan), was once viewed as a cheirogaleid, but is now generally regarded as an adapiform (Fleage, 2013). One family, the Sivaladapidae, survived in south-east Asia until the late Miocene, 5 Ma (Fleagle, 2013). We know very little of the transition between these early Euprimates to the modern forms that seem to appear suddenly in the Neogene.

The post-Eocene strepsirrhine fossil record is very scanty, while that of the haplorhines is better documented. The oldest lorisiform fossils (Lorisidae and Galagidae) are fragmentary remains from Egypt dated at 37 Ma (Seiffert *et al*., 2003), while a large number of very fragmentary fossils have been recovered from Miocene beds as widely dispersed as Namibia, Egypt and Ethiopia to East Africa (Tanzania, Uganda and Kenya) (Harrison, 2010). The Malagasy strepsirrhines are represented only by subfossils, 26,000 years old at most, and considered part of the modern fauna (Godfrey and Jungers, 2002); no discoveries of fossilised lemurs have been made on the island (Martin, 2003). Soligo and Martin (2007) have suggested that there are too many gaps in the primate fossil record (about 25 Ma are missing) to reconstruct the origins of primates adequately.

1.4 Primate origins and the evolution of body size and diet in Strepsirrhini

Diet co-evolves with body size and locomotion, and these additional characteristics can inform our interpretations of fossils. For instance, fossil primates found in the Fayum Depression of Egypt have features which suggest that Eocene prosimians followed a wide range of diets, including insectivory, frugivory, a mixture of both insectivory and frugivory, and folivory, all of which required specialist adaptations (Kirk and Simons, 2001). As a consequence of the many gaps in the fossil record, the origins of primates and their subsequent dispersal is one of the most contested subjects in primate evolution. Several

authors have contributed to the development of theories relating to the adaptive origins of primates (Smith, 1912; Jones, 1916; Szalay, 1968, 1972; Cartmill, 1974, 1992; Szalay and Dagosto,1988; Sussman, 1991), proposing different models to explain the evolution of the unique combination of primate characteristics and how they influenced the extant primate radiation. In almost all recent models, diet plays the central role – not that surprisingly, as dietary evolution is one of the corner-stones for explaining the emergence of mammalian lineages. The only recent model that does not refer to dietary adaption is that of Szalay and Dagosto (1988), who proposed that grasping extremities and nails on the digits evolved together with leaping adaptations to facilitate grasp-leaping locomotion. In all other models, the defining primate characteristics are viewed as feeding adaptations, usually for a single "ancestral diet", despite the diversity and versatility of modern primate dietary adaptations.

The goal of my project is to understand some of the dietary, physiological and ecological consequences of the dwarfing events that accompanied the radiation of the LC clade.

1.5 Research rationale and motivation

Recent phylogenetic reconstructions, as discussed above, have placed the Lepilemuridae with the Cheirogaleidae as sister taxa. This relationship has implications from an evolutionary perspective and this project aims to investigate some of the morphological changes related to a reduction in body size (Masters *et al.*, 2014). I will focus on aspects of cranial morphology and dietary ecology, and aim to shed light on the various adaptive features that evolved in these taxa. Furthermore, the information generated from this study could potentially be used to contribute to assessments of the conservation status of *Lepilemur*, as they are currently listed as data deficient (DD) on the IUCN's Red list based on an assessment done in 2008.

1.6. Project aims and objectives

I identified four objectives in this study. The first was to investigate variation in palate shape within the focal clade and compare them with the outgroups (Lemuridae or true lemurs) *Lemur* and *Varecia*. Palate shape is likely to be influenced by several factors including phylogeny, body size and diet, and I investigated the relative influence of these factors using geometric morphometrics. Objective two was to examine and describe the arterial circulation patterns using micro-computed tomography (CT) and 3D imaging. I predicted that changes in body size would result in changes and possible reductions in arterial size, and could help to explain the diversity in arterial patterns found among the Strepsirrhini. Objective three focused on the reported behaviour of caecotrophy in *Lepilemur* species (Charles-Dominique and Hladik, 1974). My working hypothesis was that energy contained in "caecotrophic" and latrine faecal samples should differ significantly and, combined with faecal bacterial diversity, may give an indication for the presence or absence of caecotrophy. Lastly, for objective four, I compared the digestive efficiency of two small gummivorous primates in an effort to understand the evolutionary changes associated with shifts in body size and diet. I predicted that an evolutionary predisposition to fermentation inherited from a folivorous ancestor (Andrews *et al*., 2016) would aid cheirogaleids in the digestion of gum.

CHAPTER 2: THE EVOLUTION OF PALATE SHAPE IN THE LEPILEMUR-CHEIROGALEIDAE CLADE (PRIMATES: STREPSIRRHINI)

2.2 Tracing patterns of variation in the palate within the *Lepilemur*-cheirogaleid clade

In primates, as in other mammals, variations in both cranial and dental morphology clearly carry a phylogenetic signal and convey systematic information (Fleagle *et al*., 2010, 2016; Masters *et al*., 2014; Masters and Couette, 2015; Clair and Boyer, 2016). Indeed, Lanèque (1992) recognized several taxa within the extinct Eocene strepsirrhine genus *Adapis* on the basis of variations in muzzle shape. Previous studies, however, have shown an overwhelming influence of diet on cranial, dental and mandibular variation in strepsirrhine primates (Ravosa 1989, 1992; Viguier, 2004; Scott, 2012; Ribeiro *et al*., 2013; Baab *et al.*, 2014; Marcé-Nogué *et al.*, 2017), as well as other mammalian taxa, including bats (Dumont, 1997, 2004, 2007; Dumont and O'Neal, 2004) and carnivores (Caumul and Polly, 2005).

In order to gain a clearer understanding of the dietary and morphological shifts that must have occurred during the radiation of the *Lepilemur*-cheirogaleid **(**LC) clade, I undertook a geometric morphometric study of palate shape among *Lepilemur* and cheirogaleid species, with specimens of *Lemur* and *Varecia* included as outgroups. A relationship between diet, tooth and snout morphology seems self-evident, but while the dentition has been extensively studied among lemuriform species (e.g. Maier, 1980; Swindler, 2002), the palate has received less focused attention, and has generally been studied as a partial aspect of cranial and craniofacial variation (Cheverud, 1982; Klingenberg *et al*., 2002; Frost *et al*., 2003; Lieberman *et al*., 2008; Baab *et al.*, 2014). Variations in palate shape and size are likely to influence and be influenced by a multitude of structural, ecological, behavioral and physiological factors (e.g. body weight, age and, to a lesser extent, sex, and vocal emissions),

as well as requirements for the acquisition and processing of food (Baab *et al*., 2014). Geometric morphometrics is an effective technique for characterising cranial structure and analysing patterns of morphological variation both between sexes of the same species (sexual dimorphism) and between species (e.g. O'Higgins and Dryden, 1993; Singleton, 2001; Hens, 2002, 2003, 2005; Plavcan, 2002). More importantly, currently available methods of statistical analysis allow the investigation of the relative significance of diverse influences in the evolution of different morphologies.

I investigated palate shape variation within a sample of strepsirrhine primate species with diverse diets and covering a wide range of body sizes, using geometric morphometrics. I advanced four alternative hypotheses:

1. Palate shape variation is largely driven by diet and foraging behaviour. If this is true, then morphological variation should be grouped by dietary categories and ecological adaptations for feeding.
2. Palate shape variation is primarily influenced by phylogeny; in this instance, palate variation should predominantly reflect evolutionary relatedness and recency of common ancestry.
3. Palate shape variation is largely an effect of body size, and allometries should explain much of the variation. However, allometric patterns are likely to be different from one clade to another, suggesting that size and phylogeny will not readily be separable.
4. Palate shape variation may reflect bioacoustic requirements, so that species that emit loud, long distance vocalisations may have similar shaped palates, while those taxa lacking loud calls may share common palate shapes.

2.2 Materials and Methods

2.2.1 Landmark data acquisition

This study is based on landmark coordinates of the palates of 359 specimens representing 8 genera and 16 species in the families Cheirogaleidae (including Lepilemurinae) and Lemuridae The skulls were housed in the primate collections of the Muséum national d'Histoire naturelle (Paris, France) and the Museum of Natural History (London, United Kingdom), and are listed in Table 2.1. Problems with my obtaining a visa to visit the UK prevented me from collecting data at the Natural History Museum, and Dr Sébastien Couette kindly took these coordinates on my behalf. Thirty-two landmark coordinates were collected in three-dimensions (3D) using a Microscribe G2X (Immersion corporation). The landmarks, defined in Table 2.2 and illustrated in Figure 2.1, highlighted the palatomaxillary, interpalatine and intermaxillary sutures, including the incisive fossa and, by extension, most of the dental row and palate width (across the cheek teeth). The upper incisors are absent in adult *Lepilemur* spp., complicating the acquisition of the LPI (lateral point of the incisors) landmarks for this genus. However, a bony notch was always present on the anterior portion of the palate in the position of the LPI, which we used to place the landmarks. In the case of damaged specimens, missing landmarks were estimated using the function 'estimate.missing' of the R package Geomorph (Adams and Otarola-Castillo, 2013). The function estimates the missing landmarks from undamaged specimens considered as a reference (Gunz *et al*., 2009).

2.2.2 Measurement error

I used Procrustes ANOVA (Goodall, 1991) to test the repeatability of data acquisition. Additionally, the percentage measurement error was calculated by analysing the variance between measurement sessions using the procedure proposed by Bailey and Byrnes (1990), and an estimation of error was performed landmark by landmark to identify the source of

Table 2.1. Sample composition noting the number of specimens representing each species, average body weight, dietary category/ies and use of territorial loud calls by the taxa

Taxon	***N***	***Body Weight (g)****	***Diet*** **	***Long-distance loud call***
Allocebus trichotis	1	92	Frugivory. Gummivory	No
Cheirogaleus adapicaudatus	5	282.5	Frugivory. Faunivory	No
Cheirogaleus major	34	400	Frugivory. Faunivory	No
Cheirogaleus medius	28	282.5	Frugivory. Faunivory	No
Lemur catta	7	2210	Frugivory. Folivory	No
Lepilemur dorsalis	7	550	Folivory	Yes
Lepilemur edwardsi	1	908	Folivory	Yes
Lepilemur leucopus	5	617	Folivory	Yes
Lepilemur microdon	20	952	Folivory	Yes
Lepilemur mustelinus	26	770	Folivory	Yes
Lepilemur ruficaudatus	56	771	Folivory	Yes
Microcebus murinus	93	61	Faunivory. Gummivory	No
Microcebus rufus	21	49	Faunivory. Gummivory	No
Mirza coquereli	8	326	Frugivory. Faunivory. Gummivory	No
Phaner furcifer	17	460	Faunivory. Gummivory	Yes
Varecia variegata	20	3520	Frugivory. Folivory	No

* From Fleagle (2013); ** Following Andrews *et al.* (2016)

measurement errors. I analysed the effect of intra-observer measurement error by measuring 166 specimens twice, and tested inter-observer variation by comparing coordinates taken by myself (CA) and Sébastien Couette (SC) on specimens of *Microcebus murinus* (n = 83) and *Lepilemur ruficaudatus* (n = 44), treating the coordinate taker as the independent factor. Both Student's t-test results were not significant (for *Microcebus murinus*: t = 0.89, df = 81, p = 0.35; for *Lepilemur ruficaudatus*: t = 0.91, df = 42, p = 0.39), indicating that measurements taken by CA and SC were not significantly different, and I combined the two datasets.

Table 2.2. Landmarks used in this study (paired numbering to indicate symmetry of palate)

Landmark No.	**Abbreviation**	**Definition**
1 - 19	DM3	Point of distal M3, projected on to buccal alveolar margin
2 - 18	DDM3	Alveolar depth at M3 (internal to palate)
3 – 17	DDM2	Alveolar depth at M2 (internal to palate)
4 - 16	DDM1	Alveolar depth at M1 (internal to palate)
5 - 15	DDP3	Alveolar depth at P3 (internal to palate)
6 - 14	DDP2	Alveolar depth at P2 (internal to palate)
7 - 13	DDCP	Alveolar depth at canine (posteriorly)
8 - 12	DDCA	Alveolar depth at canine (anteriorly)
9 - 11	LPI	Lateral point of incisors (absent)
10	PR	Prosthion
20	STA	Staphylion
21 - 22	PPL/PPR	Most anterior point of posterior palate (left/right)
23	MPA	Maxopalatine
24	INC	Incisivion
25 - 27	APIF	Anterior-most point of incisive foramen
26 - 28	PPIF	Posterior-most point of incisive foramen
29 - 31	DDEP3	Alveolar depth at P3 (external)
30 - 32	DDEM3	Alveolar depth at M3 (external)

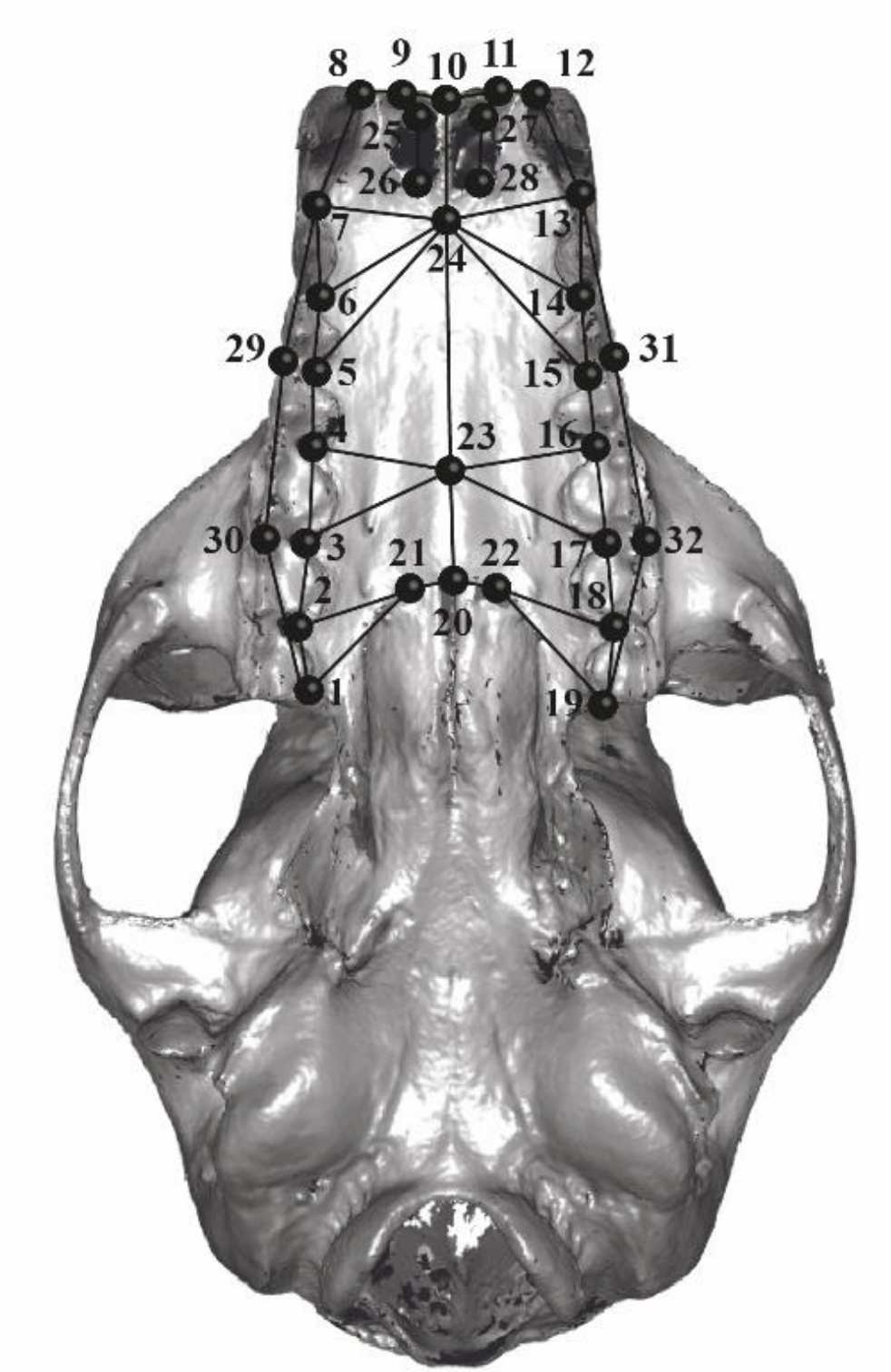

Figure 2.1. Illustration of the 32 landmarks defined to characterise the morphology of the palate in three dimensions. The species illustrated here is *Lepilemur ruficaudatus*. Written descriptions of the landmarks are presented in Table 2.2

2.2.3 Morphological variation

A Generalized Procrustes Analysis (GPA, Gower, 1975; Rohlf and Slice, 1990) was applied to the 3D landmark coordinates. A GPA is a translation that moves all specimens to the origin of the system, a scaling that separates size from shape and a rotation that optimizes the alignment of landmarks. The alignment of landmarks was computed using the Least Squares criterion (Bookstein, 1991; Rohlf and Marcus, 1993; Claude, 2008; Zelditch *et al*., 2012; Adams *et al*., 2013). The GPA method allows the separation of size (Centroid Size) and shape (Procrustes coordinates). Centroid size was used in preference to body weight as a proxy for body size as very few museum specimens have body weight data recorded. Centroid size is in fact a better proxy for size than body weight, particularly for a structure like the palate, which is prone to allometry; body weight estimates the size of the whole animal, and hence may over- or underestimate the size of the palate. A Principal Component Analysis (PCA) on the Procrustes coordinates allows the computation of a multivariate morphospace in which the position of each specimen characterises its shape. In this study, due to the unbalanced nature of the dataset (i.e. variable numbers of specimens for each taxon), I chose to use a Between Group PCA procedure (BGPCA; Mitteroecker and Bookstein, 2011), which computes the PCA on the group means and projects the specimens on to the principal components. This procedure reduces the risk of underestimating the intra- and intergroup variances.

2.2.4 Phylogeny

I assessed the influence of phylogeny on palate size and shape using the multivariate version of the K-mult method (Adams, 2014), which estimates the degree of phylogenetic signal in the dataset relative to a Brownian motion model of evolution. I used the phylogenetic tree proposed by Herrera and Dávalos (2016), which was based on 421 morphological characters,

two mitochondrial and four nuclear loci for a total of 5767 base pairs. In this phylogeny the LC clade was robustly supported, and its position and relationships were congruent with those advocated by other phylogenetic hypotheses (Chatterjee *et al.*, 2009; Perelman *et al.*, 2011; Springer *et al.*, 2012; Masters *et al.*, 2013; Federman *et al*., 2016). I included two species of the Lemuridae (*Lemur catta* and *Varecia variegata*) as the outgroup.

I investigated the distribution of morphological variation in the dataset relative to phylogeny with the help of a phylomorphospace (Sidlhauskas, 2008). In this analysis, the phylomorphospace consists of the projection of a phylogenetic tree on to the morphospace. If the morphological variation is structured by the phylogeny, each clade will occupy a distinct part of the morphospace. If not, there will be a great deal of overlap between clades in the phylomorphospace.

2.2.5 Allometries

I investigated the effect of size on shape (i.e. allometries) using several proxies for size. First, I used the centroid size directly computed from the landmark coordinates to describe the dimensions of the palate. Second, I measured the cranial length, from Prosthion (most anterior point of the palate in the sagittal plane) to Opisthocranion (most posterior point of the cranium in the sagittal plane), using digital calipers. Finally, I assembled data on body weight, calculated as the mean from males and females for each taxon from Fleagle (2013). I used a Phylogenetic Generalized Least Squares (PGLS) regression method to estimate the effect of the different size proxies on palate shape, taking phylogeny into account (Felsenstein, 1985; Rohlf, 2001; Rohlf, 2006; Revell, 2010; Adams, 2014).

I analysed five allometric patterns in total. As described above, I assessed the dependence of shape on (i) centroid size, (ii) cranial length, and (iii) body mass by comparing the observed slope to a predicted slope of 1 to test for allometry/isometry. Additionally, I

estimated the dependence of centroid size on (iv) cranial length and (v) body mass using a Reduced Major Axis regression model adapted to random variables (Jungers, 1985; Smith, 2009). All size variables were log-transformed for analyses.

2.2.6 Diet

I followed the dietary categories proposed by Andrews *et al*. (2016), who based their classification on the observations of Charles-Dominique (1977), Rowe (1996), Vinyard and Hanna (2005), Heymann (2011) and Fleagle (2013). Andrews *et al.* (2016) described four main dietary classes: (1) **faunivory** (including consumption of small vertebrates and invertebrates), (2) **folivory** (consumption of leaves, including flowers and occasionally unripe fruits), (3) **frugivory** (including granivory, consumption of fruits, seeds and buds) and, (4) **gummivory** (consumption of gum, nectar, honey and sap secretions). In the present study, food items consumed by some of the species showed an overlap between classes, and I created six dietary categories based on the percentage consumption of food from each category (Table 2.1).

2.2.7 Long-distance loud calls

Opera singers know that high notes are amplified by the palate (Lloyd, 2014). To investigate the hypothesis that the use of loud vocalisations may have an effect on palate shape in non-human primates, I coded all of the taxa included in the analysis in terms of the presence/absence of a long-distance territorial call. The calls were only present in *Lepilemur* and *Phaner*.

2.2.8 Statistical analyses

I conducted all statistics and treatments using R (R Core Team, 2015). I used the R package ‘openxlsx’ (Walker, 2015) to import the raw data, the packages ‘MASS’ (Venables and

Ripley, 2002) and ‘smatr’ (Warton *et al*., 2012) for linear models, ‘Geomorph’ (Adams and Otarola-Castillo, 2013) and ‘Morpho’ (Schlager, 2016a) for the geometric morphometrics, ‘phytools’ (Revell, 2012) and ‘ape’ (Paradis *et al.,* 2004) for phylogenetic analyses, and ‘Rvcg’ (Schlager, 2016b) and ‘RColorBrewer’ (Neuwirth, 2014) to visualize shape variations on 3D meshes.

2.3 Results

My observations revealed that palate shape contains multiple clues to clade identity among strepsirrhine primates. Indriids were clearly identified not only by their reduced number of cheek teeth (two premolars rather than three), but by a characteristic notch in the anterior margin of the palate. *Daubentonia* has a relatively narrow palate bearing only four cheek teeth and one incisor on each upper jaw. Cheirogaleid palates have a parabolic shape that is echoed to a degree in Galagidae, except for the fact that, in galagos, the palate is pinched in at the level of the P2s. Lemurids have elongated parabolic palates. In sportive lemurs, the snout is almost rectangular, with a square anterior margin and tooth rows that are almost parallel. There was little similarity between the palates of lepilemurs and the other cheirogaleids in my sample.

2.3.1 Measurement error

Many landmarks distributed along the dental row or defined by an intersection of structures had a measurement error of lower than 5%, whereas landmarks with lower precision (PPL/PPR, APIF and PPIF) presented high values of measurement error, reaching 30% in some cases (for APIF and PPIF). The overall error was lower than 10%, however, and we decided to validate the protocol and continue the analyses.

2.3.2 Palate shape variation

The results of the BGPCA are illustrated in Figure 2.2. The first Principle Component (PC1) explained 69.7% of the total shape variation, while PC2 explained 17.7% and PC3, 7.9 %. Only the two first PCs, totaling 84.7% of the variation, were used to visualise the morphospace (Figure 2.2), but all seven PCs were included in the multivariate analyses.

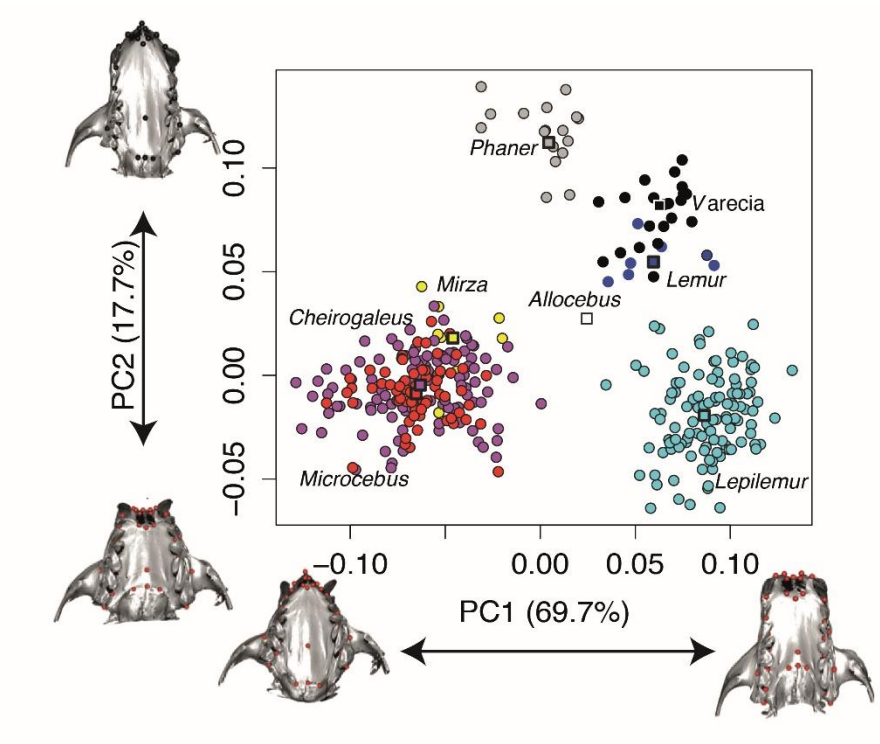

Figure 2.2. Illustration of the BGPCA morphospace using scores obtained from PC1 and PC2 to describe the shape of the palate. The morphological variation explained by the axes is illustrated by the extreme shapes. Squares represent species means

Positive scores of PC1 describe an almost rectangular palate shape elongated antero-posteriorly. The dental rows are parallel from the canines to the last molars. The distal margin of the M3s is very close to the postero-lateral part of the palatine bone (pyramidal processes, lesser palatine foramina). The anterior part, near the incisors, is rounded with a pinch at the infradental point (Figure 2.2). The negative values of PC1 describe palates with a relatively

wide, short and sharp snout. The dental rows are V-shaped and located anteriorly relative to the posterior part of the palatine bone. The genera *Lepilemur*, *Lemur* and *Varecia* present positive scores on this axis, while the genera *Mirza*, *Cheirogaleus* and *Microcebus* have negative scores (Figure 2.2). The two remaining genera (*Phaner* and *Allocebus*) have scores close to the origin.

Morphological variation along PC2 mainly concerns the relative length of the palate vault, antero-posteriorly extended along positive values of the axis. The incisors are located in a medial position, anterior to the canines, describing a pointy snout. The negative values of this axis characterize a relatively short and wide palate, with the incisors located posterior to the canines, and describing a short and slightly convex snout (Figure 2.2). On this axis, *Varecia*, *Phaner* and *Lemur* differ from the other genera, and are represented by positive scores.

In the morphospace the genus *Lepilemur* was clearly separated from the other genera, and there was considerable variation in palate shape within the sample. The palate of the single specimen of *Allocebus* was distinctive. *Varecia* and *Lemur* showed considerable overlap, with positive scores on PC1 and PC2, and could not be distinguished from one another on palate shape alone. Similarly, there was no clear distinction among the genera *Microcebus*, *Cheirogaleus* and *Mirza* in the morphospace.

2.3.3 Phylogeny

The phylogenetic signal for palate shape in our sample was highly significant according to the K-statistic test (K-mult = 0.588, p = 0.001), as well as when computed using centroid size (K = 1.763, p = 0.001). The occupation of the phylomorphospace was similar to that of the BGPCA morphospace because of the strong phylogenetic effect on shape (Figure 2.3). The morphological variation described by the phylomorphospace and the BGPCA axes was

similar. The phylogenetic tree plotted into the morphospace was well structured between clades, with no overlapping of branches supporting the cheirogaleid subfamilies Cheirogaleinae, Lepilemurinae and Phanerinae, or the Lemuridae. Within the Lemuridae, represented here by two genera only and considered as an outgroup, the clade diversification is very low. In contrast, the Cheirogaleidae family presents an array of diverse palate shapes, with five different patterns. *Allocebus*, which looks superficially like a small version of *Phaner* (Masters *et al.*, 2014), is quite distinct from the other small cheirogaleid genera, and occupies a position intermediate between *Phaner* and *Lepilemur*. *Phaner* presents a diversification of palate shape that is orthogonal to the other members of the family. In the genus *Cheirogaleus*, the two species in my sample show very similar palate shapes. The genus *Microcebus* is the most diverse genus of the family, with different directions of shape diversification among species. In the subfamily Lepilemurinae, the six species included fold into the same part of the phylomorphospace, attesting to a similar pattern of shape diversification. *Lepilemur edwardsi* presents a potentially different palate shape from the other *Lepilemur* species, although the fact that this species is represented by a single specimen in my sample urges caution in interpreting this result.

2.3.4 Allometries

I investigated patterns of allometry in palate shape variation where palate shape was a multivariate matrix composed of the seven non-null PCs. The PGLS regression of palate shape and centroid size was significant ($F = 10.4$, $Z = 0.71$, $p = 0.013$), with a R^2 value of 0.53, indicating that an evolutionary allometry was present in the sample. I then analyzed the relationship between centroid size and the first two PCs independently. PC1 was significantly dependent on centroid size, with a slope of 0.185 and a R^2 value of 0.528 ($p < 0.001$), while PC2 was not. The PGLS relationship of palate shape and body weight was not significant ($F = 1.42$, $Z = 0.41$, $R^2 = 0.007$, $p = 0.172$), while that of palate shape and cranial length was (F

= 17.54, Z = 0.88, R^2 = 0.891, p < 0.01); PC1 was significantly dependent on cranial length (slope = 0.144, R^2 = 0.58, p < 0.001), but PC2 was not. The slope was different from 1, attesting to allometry rather than isometry.

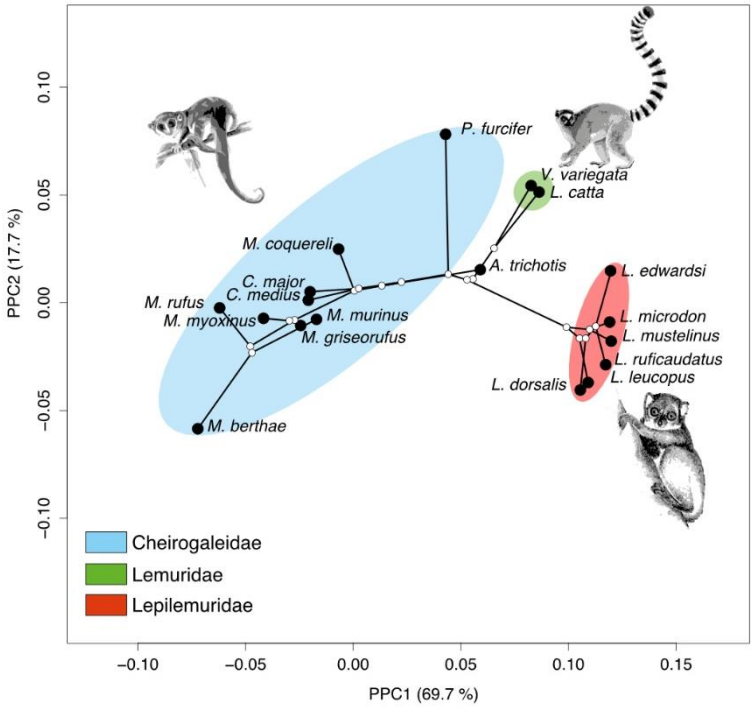

Figure 2.3. Phylomorphospace illustrating palate shape variation relative to phylogeny among genera of Cheirogaleidae and Lemuridae. Each segment is a branch of the phylogenetic tree, and black dots represent the mean specimen computed on palate shape variables for each species comprising the phylogenetic tree. White dots represent the nodes of the branches. Full names of the species are given in Table 2.1

With respect to the three proxies for body size, the relationship between centroid size and body weight (R^2 = 0.32, p = 0.012, slope = 0.01) and between body weight and cranial length (R^2 = 0.59, p = 0.001, slope = 0.7) were significant. In both cases, the slopes were different

from 0.67, the expected slope for isometry, attesting to an allometric pattern. The relationship between centroid size and cranial length was not significant (R^2 = 0.15, p = 0.1, slope = 0.007).

The allometric patterns described by regressing PC1 against centroid size were significantly different among genera (F = 1104, p < 0.001) (Figure 2.4). The coefficients of determination ranged from 0.42 to 0.46 in *Cheirogaleus*, *Microcebus* and *Phaner*, 0.63 to 0.64 in *Mirza* and *Lepilemur*, to 0.91 in *Lemur*. Three genera (*Lepilemur*, *Lemur* and *Varecia*) evinced positive slopes, while four (*Phaner*, *Cheirogaleus*, *Microcebus*, and *Mirza*) presented negative slopes (Figure 2.4).

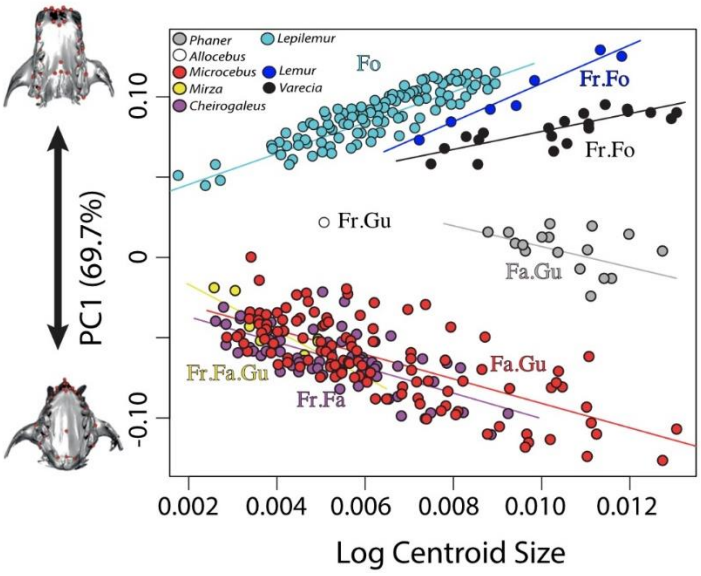

Figure 2.4. Allometric patterns described by the regression of the major axis of palate shape variation (PC1) against palate dimension (centroid size). Three genera (*Lepilemur*, *Lemur* and *Varecia* show positive slopes while the remaining genera show negative allometries. Dietary categories are included: Fo – Folivory; Fr.Fo – Frugivory. Folivory; Fr.Gu – Frugivory.Gummivory; Fa.Gu – Faunivory.Gummivory; Fr.Fa – Frugivory.Faunivory and Fr.Fa.Gu – Frugivory.Faunivory.Gummivory

2.3.5 Long-distance vocalisations

Only two of the genera included in my sample, *Phaner* and *Lepilemur* are known to use loud calls for distance communication. A possible influence of loud vocalization on palate forms cannot be excluded, because the two genera appear as outliers in all the analyses. I tested the loud calling behaviour using shape variation and long distance call as factors in a MANOVA. The two genera have different palate shapes and consequently the effect of call on shape variation was significant (p values < 0.001, Pillai Trace = 0.834, and F = 214.3). However, only the possible effect of vocalizations would be difficult to test for idiosyncratic reasons. The sound amplification hypothesis does not predict a clear, consistent trend, because of the complexity of the phenomenon of resonance. Thus, a larger sample and more specific predictions would be required for further investigation.

2.3.6 Patterns of covariation

I tested the covariation of diet with palate size and shape. I first ran the analysis without correcting for phylogenetic effect: log centroid size, diet and their interaction were significant with p values < 0.01, and R^2 values of 0.28 and 0.26 for shape/size and shape/diet, respectively. Multiple post hoc tests showed significant differences between folivory and all other dietary categories except frugivory/folivory. When I ran the test with a correction for phylogeny (phylogenetic regression), the effect of palate size on shape remained significant ($Z = 2.49$, $p = 0.016$), but with a low R^2 value (0.08). The effects of both dietary category and the interaction of size and diet on shape were significant ($Z = 1.89$, $R^2 = 0.04$, $p = 0.045$ for the effect of diet on shape, $Z = 1.41$, $R^2 = 0.06$, $p = 0.037$, for the effect of size and diet on shape). I can thus associate dietary categories with allometric patterns. The positive allometric pattern shared by the genera *Lepilemur, Varecia* and *Lemur* is explained, to some extent, by the consumptions of leaves. Similarly, the exclusion of leaves from the diet is

linked to a different allometric pattern, shared by the remaining genera in our sample. My result illustrates the strong effect of phylogeny on both shape and the dietary covariate.

2.4 Discussion

In this study, palate shape differed among dietary categories, most notably so in taxa categorised as folivorous. Size (allometry) also had a major influence on palate shape: the allometric pattern of palate variation in *Lepilemur* is very different from those of the other Cheirogaleidae and is more similar to those shown by Lemuridae spp., perhaps because *Lepilemur, Lemur,* and *Varecia* all include a significant proportion of leaves in their diet. Indeed, the allometric pattern is associated with the presence of large, square molars and premolars with strongly developed shearing crests characteristic of folivores (Swindler, 2002). This close similarity across families is at least partially an effect of my use of palate centroid size (which could be directly linked to diet) as a proxy for body size. If I had used body weight as the size proxy (as in many previous studies, e.g. Jungers 1985), the patterns may not have been so similar, because the body weights of *Lepilemur* spp. are much lower than those of Lemuridae spp.

The issue of body size is an essential part of the history of the LC clade. Masters *et al.* (2014) reconstructed the evolution of body mass among Strepsirrhini, and predicted that the LC clade ancestor was < 1000 g (reconstructed as 766 g) with a mixed diet of frugivory/folivory (Andrews *et al*., 2016). The authors proposed that body size evolution in this clade involved at least four dwarfing events; first, from a larger common ancestor, followed by three "hyper-dwarfing events" that led to the smallest species in the clade (Masters *et al.,* 2014). The dwarfing events would have been accompanied by dietary shifts, as lepilemurs appear to occupy the lowest viable size range for folivorous primates (Kay,

1975), with concomitant changes in palate shape. I hence concur that the ancestral diet for the *Lepilemur* - Cheirogaleidae clade was probably composed of fruits and leaves, based on the positive coordinates of the node on PC1. From this ancestral frugivorous/folivorous diet I hence infer a dietary shift to folivory for *Lepilemur* spp.; to faunivory/gummivory in *Phaner* and *Allocebus*; to frugivory/faunivory in *Cheirogaleus*; and to faunivory/gummivory in *Mirza* and *Microcebus.* This dietary diversity is reflected in the diverse palate shapes within the clade.

Dietary adaptations have been at the heart of theories on primate origins and early evolution, and cheirogaleids, with their penchant for faunivory-frugivory and gummivory, have long been held as model primate ancestors. Four scenarios of dietary evolution in strepsirrhines, sometimes extended to primates in general, have been proposed. (1) Cartmill's (1972, 1974, 1992) visual predation hypothesis centred on a small-bodied faunivorous/omnivorous ancestor that evolved its grasping extremities and forward-facing orbits by hunting insects at night, as mouse lemurs do. (2) Szalay (1968) favoured a larger-bodied frugivorous ancestor based on the size and dental structure of Palaeogene fossils. Sussman's (1991) angiosperm-primate diffuse coevolution model invoked an ancestor with similar dietary habits. (3) With a degree of prescience, Nash (1986) sought a link between exudativory and folivory well before the LC clade had much support. Building on Cartmill's visual predation model, she proposed a dietary transition series from faunivory to exudativory to folivory to frugivory. (4) Phylogenies based on molecular data, and increasingly sophisticated methods of analysing them, placed restrictions on potential evolutionary transformations. A fourth scenario, based on recent phylogenetic reconstructions, was proposed by Génin *et al.* (2010), Masters *et al.* (2014), Andrews *et al.* (2016) and Génin and Masters (2016). In this model, the reconstructed strepsirrhine ancestor was approximately 1 kg in body weight and followed a faunivorous/frugivorous diet. The ancestor to the non-

daubentoniid lemurs was a frugivore/folivore, and folivory constituted an essential precursor to the evolution of gummivory.

My analysis was similarly based on recent molecular phylogenetic reconstructions. One implication arising from these reconstructions is that the faunivory/frugivory practiced by mouse lemurs is a relatively recently derived diet. Furthermore, rather than being an ancestral or fall-back diet, gummivory has evolved convergently in independent cheirogaleid lineages. The palate morphology that supports obligate gummivory in the larger-bodied fork-marked dwarf lemurs (*Phaner*) is distinctly different from that seen in the small-bodied, facultative gummivores of the genus *Microcebus*.

Finally, although palate morphology retains a strong phylogenetic signal, diet appears to be even more important in defining palate shape. While a previous study (Masters *et al*., 2014) demonstrated close similarities in the ontogenetic allometries of overall skull shape between lepilemurs and other cheirogaleids, the present study points to a significant difference in palate shape. Size may account partially for the similarity in patterns of allometric growth of the palates of lepilemurs and lemurids, but diet appears to have had the overriding influence. The structural requirements for emitting loud calls cannot be excluded from influencing palate shape, and should be examined further.

CHAPTER 3: PATTERNS OF CRANIAL ARTERIAL CIRCULATION IN THE *LEPILEMUR*-CHEIROGALEID CLADE: IMPLICATIONS FOR THE PHYLETIC DWARFING SCENARIO

3.1 Introduction

In this chapter I examine carotid arterial patterns in strepsirrhine primates using micro-computed tomography, in order to trace the changes in basicranial anatomy that accompanied the evolution of the *Lepilemur*-cheirogaleid. My research into arterial circulation was not based on soft tissues, but on micro-CT scans of cleaned skulls; the enclosure of major arteries (internal carotid, stapedial and promontory) in a bony canal or grooves in the skull (Boyer *et al*., 2016) provides an opportunity for the reconstruction of arterial patterns using techniques of 3D imaging.

3.1.1 The morphology of the primate basicranium

The diverse nature of primate skull anatomy has received considerable attention, in part due to investigations of craniofacial variation and evolution (Lieberman *et al*., 2000). Much of the information that has been collected on cranial morphology has been linked to systematic or phylogenetic studies (e.g. Fleagle *et al.*, 2010; Masters and Couette, 2015), or have focused on dietary adaptations (Menegaz *et al*., 2010). The basicranium, the platform on which the brain develops and grows, plays an essential, functional role in the skull. Basicranial morphology in strepsirrhines is relatively diverse, and Szalay (1975) divided them into three major basicranial categories: (i) what he described as the "primitive strepsirhine pattern" shared by adapiforms, lemurids, indriids and daubentoniids, in which the stapedial is relatively large compared to the internal carotid; (ii) the cheirogaleid pattern, in which the stapedial is reduced and the main blood supply is carried by the ascending pharyngeal artery;

and (iii) the lorisiform pattern which resembles that seen in the cheirgogaleids except that the stapedial is apparently non-existent. The circulatory pattern shared by lorisiforms and cheirogaleids is unique among Primates (Cartmill, 1975; Coleman and Boyer, 2011). The sportive lemurs appear to form a fourth category, in which both the internal carotid and the stapedial arteries are reduced, but there is no ascending pharyngeal. The fork-marked dwarf lemurs, *Phaner* spp., which have sometimes been reconstructed as the sister-taxon to *Lepilemur*, probably have their major bloody supply via the ascending pharyngeal artery like other Cheirogaleidae, although this has not been documented in detail. The lepilemurs share their arterial pattern, with a stapedial either small or absent, with no other living lemur taxon, but seem to mirror the pattern of circulation seen in the extinct *Megaladapis*, with a completely absent stapedial artery and other subfossil giant lemurs including *Paleopropithecus* with a minute stapedial canal (MacPhee,1987). However, descriptions of the lepilemur arterial pattern have been rather inconsistent within the last decade, having first been described as a non-stapedial pattern (Coleman and Boyer, 2011), then as "other" (Benoit *et al*., 2013), and finally, as a stapedial pattern (Boyer *et al*., 2016).

Szalay (1975) provided a suite of basicranial characters that could be significant when distinguishing among strepsirrhine taxa. Using museum specimens, he was able to infer the relative diameters of the internal carotid, stapedial and promontory arteries. With the aid of schematic drawings, he recorded information regarding the place of entry of the carotid and ascending pharyngeal arteries into the bulla; the absence or presence of an anterior carotid foramen and enlarged ascending pharyngeal artery; the relative size, presence or absence, of the stapedial canal and artery, and the relative size of the promontory canal and artery. On the basis of their distribution among strepsirrhine taxa, he categorized these characters as ancestral or derived.

As reviewed in Chapter 1, recent phylogenetic reconstructions based on molecular data strongly support the grouping of the Lepilemuridae and Cheirogaleidae as a clade (LC clade) with a single common ancestor (Chatterjee *et al*., 2009; Perelman *et al*., 2011; Masters *et al*., 2013; Herrera & Dávalos, 2016). This reconstruction indicates that there were at least four dwarfing events during the evolution of the LC clade (Masters *et al*., 2014). Body size changes have significant implications regarding circulatory systems and their capacity to supply oxygen and nutrients to organs, particularly the brain. This fact led me to question whether the transitions in basicranial circulatory patterns that accompanied the diversification of this clade were at least partially influenced by allometric factors.

3.1.2 The allometry of basicranial circulation canals

One common explanation for allometric patterns concerns the surface area/volume ratio (McNab, 2002). Given a linear increase in the size of a character, its volume will increase as a cubic function of that value, while the surface area increases as the square of the value: hence, volumes increase more rapidly than surface areas do. Hence, I decided to investigate a consequence of this principle: i.e. that dwarfed taxa should have proportionally smaller circulation canals than their larger ancestors. All things being equal, the volume of the canal necessary to supply the brain with blood should be proportionally lower in small animals, even if the size of the brain decreases allometrically. This may explain the absence of stapedial arteries in the smallest strepsrirrhines. I chose to examine the basicranial circulation of *Lepilemur* and *Phaner*, in particular, because they should be intermediate between large lemurs (which all have stapedial arteries) and Cheirogaleidae (which all lack stapedial arteries). Because of the lack of consensus regarding the presence of the stapedial artery in *Lepilemur* (Coleman and Boyer, 2011; Benoit *et al*., 2013; Boyer *et al*., 2016), I focused on this genus, as well as the fork-marked dwarf lemur, *Phaner*, because little has been published regarding its basicranial circulation.

In this chapter, I describe the patterns of basicranial circulation among a sample of strepsirrhine species. The advent of technological advances for studying morphology, like micro-CT scanning and 3D imaging, have allowed researchers to get a more detailed view of primate basicranial morphology (Coleman and Boyer, 2011; Benoit *et al.*, 2013; Boyer *et al*., 2016). I therefore employed micro-CT scanning techniques combined with 3D imaging to ascertain strepsirrhine circulatory patterns, and to explore and to clarify the evolution of basicranial circulation associated with dwarfing in cheirogaleids.

3.2 Materials and Methods

3.2.1. Sample

The sample of scans I analysed consisted of 15 specimens representing 7 genera and 10 species (Table 3.1). Most scans were obtained from a collection maintained by Dr Sébastien Couette at the Université de Bourgogne in Dijon, France (including specimens of *Cheirogaleus*, *Galago*, *Lemur*, *Lepilemur*, *Microcebus* and *Phaner*). The original specimens were housed in the Muséum national d'Histoire naturelle in Paris. An additional specimen of indri (*Indri indri*) was provided by Prof. José Braga, from the Muséum d'Histoire Naturelle de la ville de Toulouse, that was scanned at CIRIMAT at the Université Paul Sabatier, France. All scans were of high resolution (Image Voxel Size (μm) = 29.352), to enable location of the arteries, which presented as bony canals within the petrosal bones of the specimens.

3.2.2 Observations

The scans were transferred to Avizo 8.1 (Visualization Sciences Group, 2009). Once the cropped regions were separated - including only the petrosal region of the skull - using

features within Avizo 8.1, the specimens were segmented starting with the identification of the internal carotid, stapedial (where present), promontory, and ascending pharyngeal artery (where present) (Figure 3.1).

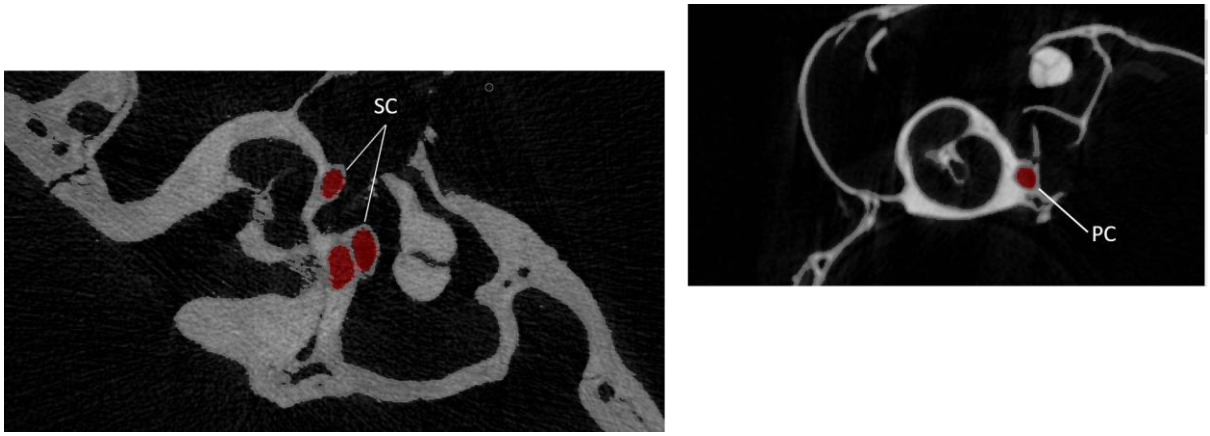

Figure 3.1 Illustration of the main arteries housed in bony canals in the cranium of *Lemur catta*. Highlighted here are the promontorial canal (PC), stapedial artery (SC)

3.2.3. Data analysis

Because my sample size was relatively small, I decided to analyse my data in the context of the larger data set regarding primate arterial patterns reported by Boyer *et al*. (2016). There was an inconsistency in their data set – notably an inversion of values for Endocranial Volume (ECV) between *Cheirogaleus major* and *Cheirogaleus medius* – which I took into account. I also included data regarding ECV, measured using polypropylene balls, taken from Masters *et al.* (2014). I used a Pearson correlation coefficient to test the degree of significance of the linear correlation I observed between ICA and ECV volumes. On the basis of this expanded data set, I regressed the volume of the Internal Carotid Artery (ICA), determined as the mean of 3 measurements at different points along the canal, against ECV, and calculated a 95% confidence interval relative to the regression line. All taxa that fell

below or above the 95% confidence interval would be categorised as having a relatively small or relatively large ICA. Lastly, I plotted the changes in arterial patterns on a simplified phylogeny adapted from Herrera and Dàvalos (2016).

Table 3.1. Taxa and samples included in this study, including source collection

Taxon	***N***	**Collection**
Cheirogaleus medius	1	MNHN, Paris
Cheirogaleus major	2	MNHN, Paris
Galago senegalensis	1	MNHN, Paris
Indri indri	1	MNHN, Toulouse
Lemur catta	1	MNHN, Paris
Lepilemur leucopus	1	MNHN, Paris
Lepilemur ruficaudatus	2	MNHN, Paris
Microcebus murinus	2	MNHN, Paris
Microcebus rufus	1	MNHN, Paris
Phaner furcifer	3	MNHN, Paris

3.3 Results

3.3.1 Description of arterial pathways

All three *Lepilemur* specimens I investigated had a small stapedial artery (SA) (Figure 3.2), indicating that they are anatomically intermediate between true lemurs (with a large stapedial artery) and cheirogaleids (with no stapedial artery). *Phaner* specimens lacked an SA, as did the other cheirogaleids. All of these taxa receive their major supply of blood to the brain through an alternate route, i.e. through the enlarged ascending pharyngeal artery (Figure 3.2).

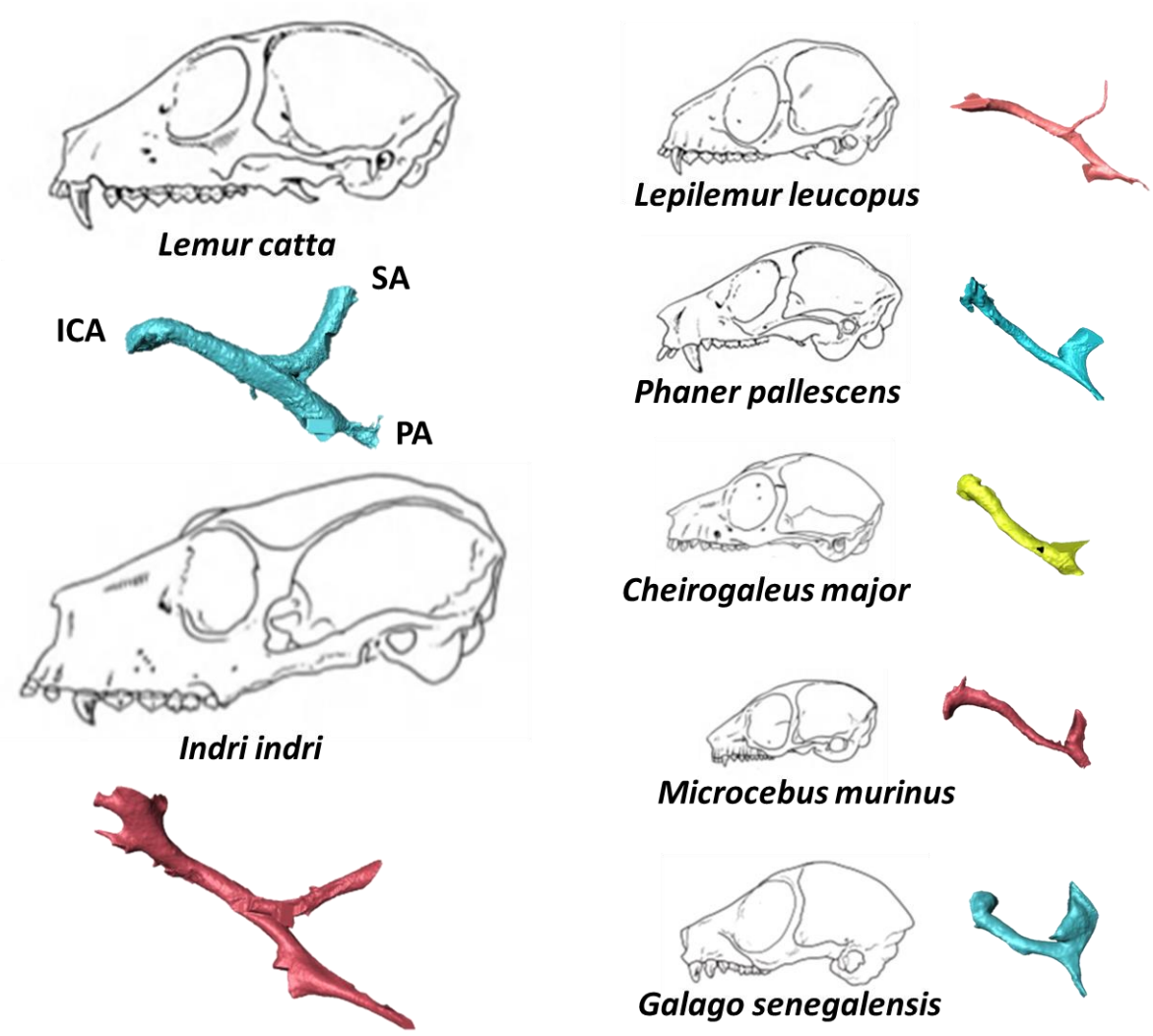

Figure 3.2. Arterial patterns of representative genera among the study specimens in ventro-lateral view (ICA: internal carotid artery; SA: stapedial artery; PA: promontory artery

In the case of the Lemuridae (*Lemur*) and Indriidae (*Indri*), the major blood supply is via a very large stapedial artery (Figure 3.2). *Lepilemur*, however, does not share its arterial pattern with any of the other taxa in this study, having a reduced stapedial artery and an enlarged promontory artery (Figure 3.2)

As was to be expected, the size of the cranial arteries and the size of the brain were highly positively correlated: the ICA volume showed a linear correlation with the ECV values of $R^2 = 0.9664$, $P < 0.001$, $N = 66$). The correlation allowed me to calculate size-

independent residuals. The method yielded negative residuals for all the strepsirrhines in the sample, all lower than the lower 95% confidence interval. In the case of non-cheirogaleids, small ICAs can be explained by the presence of the SA. In contrast, the proportionally small sizes of ICAs in cheirogaleids is explained by a reduction of body size that made the SA unnecessary. I propose a scenario of loss of the SA as a consequence of phyletic dwarfing, a scenario that may also apply to the lorisoids who all lack a SA (Fig. 3.3), particularly if the ascending pharyngeal was independently acquired in the two clades.

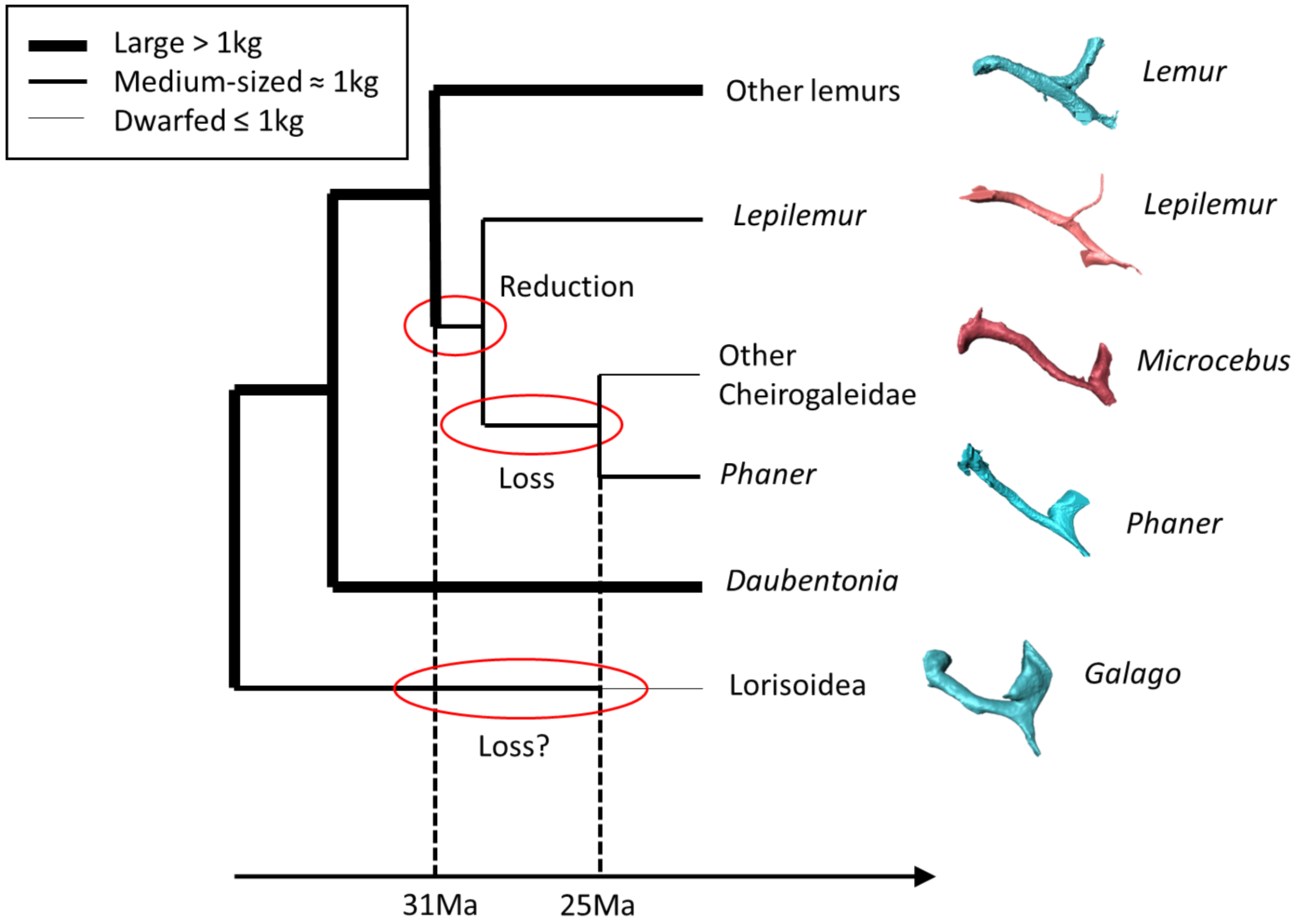

Figure 3.3 Simplified phylogenetic reconstruction of Strepsirrhini showing the evolution of stapedial arterial patterns based on Herrera and Dàvalos (2016)

3.4. Discussion

This study confirms that the loss of stapedial artery in the Cheirogaleidae occurred in steps, as it is much reduced in *Lepilemur*. However, the complete loss of the stapedial artery probably occurred during the dwarfing event that occurred after the dwarf lemurs had diverged from the sportive lemurs. According to divergence dates estimated by Springer *et al*. (2012), Masters *et al*. (2013) and Herrera and Dávalos (2016), the lineage leading to the extant dwarf and mouse lemurs diverged from sportive lemurs between 28 and 21 Ma (Figure 3.3).

The predicted linear correlation between Internal Carotid Artery volume and brain volume indicates that the loss of stapedial artery occurred because of the redundancy of blood supply to the brain in the smallest species, as an effect of a smaller volume relative to surfaces for exchanges (oxygen and nutrients). The body mass-based allometries of arteries and cranial volume are consistent with this interpretation: artery diameter in mammals shows a negative allometry (allometric exponent = 0.375, according to Dawson, 2014); and cranial volume in cheirogaleids also shows a negative allometry (allometric exponent = 0.59 according to Masters *et al.*, 2013). If the loss of one artery was the consequence of phyletic dwarfing it is also likely to have resulted from mechanical constrains on development, as small mammals have relatively much larger arteries than large mammals. In other words, cheirogaleids may have lost one artery to fit in a relatively much smaller neck.

This has interesting implications on the other groups of primates that lack stapedial arteries, such as the lorisoids and the anthropoids. In the case of the lorisoids, it implies that their ancestor may also have been a dwarfed form. This is also indicated by a number of other anatomical, behavioural and physiological traits shared between the small-bodied cheirogaleids and galagids (Charles-Dominique and Martin, 1970), and by the convergent

evolution of gummivory in the two clades (see Chapter 5). As shown on Figure 3.3, these proposed dwarfing events may have occurred at the same time as in the *Lepilemur*-cheirogaleid clade or later, coeval with the more recent event of hyper-dwarfing in cheirogaleids (i.e. the emergence of *Microcebus* and secondary dwarfing in *Cheirogaleus*: Masters *et al.*, 2014). Late dwarfing also occurred during the Miocene in South American callitrichines (Marivaux *et al.*, 2016). There is no evidence that haplorrhine primates ever had stapedial arteries, but it is also possible that they could have lost them in a much older dwarfing event.

However, the dwarfing hypothesis does not explain the case of the very small *Tarsius*, in which a stapedial artery is present (Boyer *et al.*, 2016). Another case seems to contradict the generality of my conclusion: the stapedial artery seems to have been lost in at least one not particularly small adapiform from the middle Eocene, *Hesperolemur* (Gunnell, 1995).

CHAPTER 4: FOLIVORY IN SPORTIVE LEMURS: INSIGHTS INTO DIGESTION AND THE GUT MICROBIOME OF *LEPILEMUR LEUCOPUS*

4.1 Dietary diversity in the *Lepilemur*-cheirogaleid clade

Compared with other mammal orders, primates have relatively diverse feeding ecologies, perhaps as a result of their mostly arboreal lifestyles. Most primates consume a variety of plant parts (herbivory), which may include leaves (folivory), fruits (frugivory), seeds (granivory), and nectar and exudates (exudativory, including gummivory) (Richard, 1985); but some species feed on animal prey, ranging from insects (mouse lemurs) to small vertebrates (tarsiers) (Jablonski and Crompton, 1994; Génin, 2008; Génin *et al*., 2010). Many dietary adaptations can be interpreted in terms of diffuse co-adaptation between primates and their food items. For instance, coevolution between primates and angiosperms leading to frugivory is believed to have been generalised in the Eocene as primates became major seed dispersers (Sussman, 1991; Andrews *et al*., 2016). This explains why fruits are much easier to digest and much less toxic when ripe than leaves. Interestingly, the digestion of leaves also involves coevolution as it requires symbiotic bacteria believed to have derived from parasites. Such coevolution has occurred convergently in many lineages of ruminants and hindgut fermenters among mammals, and even in some birds (McNab, 2002).

In this chapter, I examine the validity of an evolutionary scenario proposed to explain a potential adaptation among sportive lemurs that is unique among primates: the phenomenon of adaptive caecotrophy described by Hladik and Charles-Dominique (1971, 1974; Hladik *et al*., 1971). Caecotrophy is generally described as the adaptive or functional consumption of faeces, as compared to coprophagy that is often observed in captivity and labelled a pathological behaviour (Flurer and Zucker, 1988). Génin and Masters (2016) interpreted this

dietary adaptation as a consequence of a physiological rule known as Kay's threshold: relatively large body size is required for digesting leaves (Kay, 1974; Kay and Davies, 1994; McNab, 2002). If indeed sportive lemurs evolved from a larger folivorous ancestor, then decreased body size would have led to caecotrophy (adaptive coprophagia or caecophagy in McNab, 2002) in convergence with other small folivores such as lagomorphs, some rodents and marsupials (Herron, 2002; Liu *et al.*, 2007; Karasov and Douglas, 2013; Crowley *et al.*, 2017).

The controversy surrounding *Lepilemur* caecotrophy started with a short chapter in an unpublished PhD thesis by Russell (1977), in which the author stated that Hladik and Charles-Dominique had mistaken anogenital grooming for caecotrophy. Russell (1977) offered limited evidence in support of his claim:

(1) he included some anecdotal observations of ano-genital grooming;

(2) he offered his interpretation of the presence of unidentified bacteria in the *Lepilemur* caecum as an effect of putrefaction;

(3) he described the presence of "undigested plant material" that he observed in "many thousands of faeces", but did not quantify or photograph (Russell, 1977: 76).

The main thesis defended by Russell (1977) was that Hladik and Charles-Dominique wrongly claimed that sportive lemurs are particularly energy efficient. Nash (1998) reported her work on the energy budget of *Lepilemur petteri* (regarded as another population of *L. leucopus* at the time), in which she confirmed the high energetic efficiency view. Unfortunately, she also failed to observe caecotrophy, and concluded that studies in captivity would be required to obtain a definitive answer, however complicated by the fact that *Lepilemur* survives poorly in captivity (Nash, 1998).

Typically, caecotrophic faeces are very different from secondary faeces, reportedly excreted after reingestion and consequent digestion, a difference not observed by Charles-Dominique and Hladik (1971). However, the recent observations that sportive lemurs defecate in latrines used for socio-territorial communication, perhaps in combination with urine, may provide another method for testing the caecotrophy hypothesis (Irwin *et al*., 2004; Dröscher and Kappeler, 2014). Indeed, animals only visit latrines in the night-time (Dröscher and Kappeler, 2014), suggesting that latrine faeces may be secondary faeces that can be compared with diurnal or fresh faeces. Using a similar comparison of distinct soft (caecotrophic) and hard (secondary) faeces in rabbits, Zeng *et al.* (2015) identified two groups of bacteria probably involved in caecal fermentation associated with caecotrophy: Ruminococcaceae (Firmicutes, Clostridia) and *Akkermansia* spp. (Verrucomicrobia). Both belong to taxa commonly found in primate guts, including lemurs and humans (Clayton *et al*., 2018).

According to Charles-Dominique and Hladik, ingesting their diurnal faeces allows lepilemurs to increase their protein assimilation, as in other caecotrophs (Hladik and Charles-Dominique, 1971; Hladik *et al*., 1971; Hladik and Charles-Dominique, 1974; Chivers and Hladik, 1980; Liu *et al*., 2007; Génin and Masters, 2016). Therefore, a potentially good method of investigating caecoptrophy would be based on a comparison of the nitrogen content of diurnal or hypothetically caecotrophic faeces, and nocturnal faeces found in latrines. If caecotrophy is effective, I would expect less nitrogen in nocturnal faeces than in diurnal faeces.

Pioneering studies have documented the gut microbiome of a number of mammals, including their coevolution with their respective hosts (Drasar and Barrow, 1985; Ley *et al*., 2008a; Ley *et al*., 2008b). This has extended to human and non-human primates (Frey *et al*., 2006; Bo *et al*., 2010; Szekely *et al*., 2010; Mallot and Amato, 2018). Much of the work has

investigated the important roles played by the microbiome in various physiological activities, including factors that could contribute to shifts in the composition of the microbial community. This makes an understanding of the diversity found within the gut, and the interaction between host and microbiome, so much more important. The aforementioned studies include some great apes like the *Gorilla* species (Frey *et al*., 2006; Ochman *et al*., 2010; Bittar *et al*., 2014) and chimpanzees (Uenishi *et al*., 2007; Szekely *et al*., 2010), baboons (Nakamura *et al*., 2009; Ren *et al*., 2015), and smaller guenons (McCord *et al*., 2014), including captive and wild populations. However, very few studies exist for members of the Strepsirrhini, and these are limited to ring-tailed lemurs (Fogel, 2015; Bennet *et al*., 2016), ruffed lemurs (McKenney *et al*., 2015), sifakas (Fogel, 2015) and the pygmy slow loris (Bo *et al*., 2013). Interestingly, these studies showed that primates share a number of their flora, and that the gummivorous *Nycticebus* probably used a variety of bacteria including *Acinetobacter*; *Alkalibacterium* (Proteobacetria); *Corynebacterim* (Actinobacteria); *Clostridium*, *Eubacterium* and *Bacillus*, to digest gum. The method of DNA barcoding used by these studies has its limitations, however, as many common taxa might have been spread by domesticated animals, whereas endemic micro-flora may not be identified. This would explain the surprising result of almost identical gut floras in *Lemur catta* and *Propithecus verreauxi* (Fogel, 2015; Bennet *et al*., 2016).

As ethical policies have changed in recent years and veterinarians have become increasingly involved in primatological studies, primatology journals have begun to publish more studies on primate parasites (Pederson *et al*., 2005; Gillespie, 2006; Chapman *et al*., 2006; Teichroeb *et al*., 2009; Srivathsan *et al*., 2016). Indeed, parasites may be used to test a variety of fundamental hypotheses, including phylogenetic hypotheses. Paulian (1961) was probably the first to observe that lemurs have original, endemic ecto-parasites, different from those found on endemic carnivores and rodents, possibly as the result of their older presence

on the island of Madagascar. Unlike ecto-parasites, intestinal parasites are passed on by secondary hosts or by the direct ingestion of faeces. In arboreal folivores, such as koalas (*Phascolarctos cinereus*), and probably at least one lemur, the indri (*Indri indri*), infants acquire their capacity to digest and detoxify leaves progressively, by grooming or allo-caecotrophy (Rabemananjara, pers. com.).

For this study I made the two following predictions:

1) Faecal analyses: If *Lepilemur leucopus* uses caecotrophy, latrine faeces should have lower caloric content and at least a lower protein content than diurnal faeces. If *L. leucopus* does not use caecotrophy, the two types of faeces should show the same composition in terms of carbohydrates, proteins and secondary compounds.
2) Micro-floral analysis: If *Lepilemur leucopus* practises caecotrophy, it may have an original, endemic micro-flora particularly abundant in fresh faeces; or it may have typical bacteria found in other caecotrophs like rabbits (such as Ruminococcaceae and *Akkermansia* spp.). If *L. leucopus* does not use caecotrophy, its micro-flora should be similar to that of *Lemur catta* and *Propithecus verreauxi* present in the same site.

4.2 Materials and Methods

4.2.1 Study site, animal capture and handling

The field study was conducted at the site of the earlier studies of *Lepilemur* caecotrophy, i.e. the Berenty Private Reserve of southeastern Madagascar (Hladik and Charles-Dominique, 1971, 1974; Hladik *et al*., 1971; Russell, 1977). To ensure capture and consequent collection of samples (see below regarding permits and animal ethics approval), I followed individuals for one hour before dawn in the spiny forest, to determine their sleeping sites. Animals were visited in their sleeping sites the following morning when captures were performed between

08:30 am and 12:30 pm. Each individual was immobilized by remote injection using a blowpipe (Pneu-Dart blo-jector kit) in association with a hypodermic syringe tranquilizer (PneuDart, P-type projectiles, 3/8" length needles). Based on body weights estimated for the lepilemurs, darts were loaded with an anaesthetic combination of Ketamine (Ketamidor® 7 mg/kg) and Medetomidine (Dormitor® 0.04 mg/kg). All anaesthetics were administered by a qualified veterinarian. Once darted, animals were observed closely and caught delicately in a net upon falling. Under anaesthetic, lemurs were sexed, weighed (Pesola® spring scale) and their age estimated prior to receiving a brief health examination.

a)  **b)** 

Figure 4.1 (a) *Lepilemur leucopus* in sleeping site (Picture: DR Roberts) and (**b)** darting of individual in the spiny forest (Picture: CA Andrews)

The examination consisted of a general health assessment and measurement of body temperature, investigation for the presence or absence of external parasites, pathology symptoms and possible injuries related to immobilization procedures. Thereafter, the animals

were kept individually in secure sleeping bags and transported to the field station, where they were kept until release later the same day at dusk. I collected faecal material obtained during handling and, upon release, from the handling bags. Half of the samples were stored in sterile vials containing 95% ethanol, and the other half were dried and preserved using silica gel. All were stored first at 0°C during the field period (less than one month) and then at -20°C until further analyses. Additionally, dried faeces were collected from identified latrines in the forest for comparative calorimetric analyses. Furthermore, the rest of the fresh samples were stored in vials containing 10% formalin for future gastro-intestinal parasite identification.

4.2.2. Laboratory analyses

Calorimetric analysis

The dried faeces – both fresh and latrine samples – were ground in the laboratory and pressed into pellets. These pellets were weighed and subjected to bomb calorimetric combustion – a measure of the calorific value of samples - to determine their energy content (Parr Instrument Company, 2013). To calculate the residual non-digestible energetic content, I used the following formula:

Non-digested fibres (converted in kJ/g) = Caloric content measured by calorimetry – 4 x (% Crude Proteins + % Carbohydrates) (Rothman *et al.*, 2011).

Nutrient content analyses

A subsample of the dried faeces was analysed at the University of Hamburg, Germany, for nutrient content and subjected to four biochemical assays. Protein availability was determined through the Kjedahl assay, simple sugars were measured using HPLC (high performance liquid chromatography; Rothman *et al*., 2011), while condensed tannins and phenolic concentrations were measured using a photometer. Using the concentrations of

phenolics to correct for this compaction we estimated the increase in digestive efficiency enabled by caecotrophy.

DNA Extraction

Samples were sent to Inqaba Biotechnical Industries, Pretoria (Tshwane), a commercial next generation sequencing service provider, for DNA sequencing. Genomic DNA samples were PCR amplified using a universal primer pair (341F and 785R – targeting V3 and V4 of the 16S rRNA gene). Resulting amplicons were gel purified, end paired and illumina specific adapter sequences were ligated to each amplicon.

Following fluorometric quantification, the samples were individually indexed, and another Ampure bead based purification step was performed. Amplicons were then sequenced on illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit. For each sample, 20 Mb of data (2 x 300 bp long paired end reads) were generated. A BLAST-based data analysis was performed using a data analysis pipeline developed in-house by Inqaba. The top hit for every BLAST result (i.e genus and species name) was counted and a record was kept of how many times each species appeared as a hit.

4.2.3 Statistical analyses

All statistical tests were performed in SYSTAT. I used *t*-tests to compare the chemical compositions of the two kinds of *Lepilemur* faeces: the latrine faeces collected in the forest (secondary faeces) and the potentially caecotrophic, fresh faeces that were collected from captured individuals.

4.2.4 Ethical considerations

This research complied with standard protocols for animal handling and capture approved by the Research Ethics Committee: Animal of the Nelson Mandela University (A17-SCI-ZOO-012), the management of Berenty Private Reserve and the legal requirements of Madagascar. All immobilization and handling procedures were performed with the assistance of an experienced wildlife veterinarian and professional darter.

4. 3 Results

4.3.1 Captures and general condition of animals

I captured 11 white-footed sportive lemurs (6 males and 5 females), including 3 sub-adults, one of which was a female resting with her mother, in 10 different sleeping sites (*Alluaudia ascendens* and *Salvadora angustifolia* tree holes and forks). Animals were always inactive but alert. Animals weighed on average 588 ± 31g (420-710 g), and had relatively high body temperatures (37.9 ± 0.3°C). They had no visible ecto-parasites, and no visible intestinal parasites were found in the faeces. One parasite (*Ciliobacteria protist*) was detected by the microbiological analysis in two individuals (Table 4.1).

4.3.2 Chemical composition of faeces

Although they do not differ in size or shape, diurnal faeces are much softer than latrine faeces; they are dark avocado green whereas latrine faeces are paler and brown. However, the two kinds of faeces become more similar in texture and appearance when dried. The comparison of the chemical composition of the two types of faeces confirmed the main

prediction of the caecotrophy hypothesis, with significantly lower protein content in the latrine faeces than in the diurnal faeces (Table 4.2).

Table 4.1. *Lepilemur* gastrointestinal flora compared with other primates and one known caecotrophic species (the domestic rabbit)

	% phylogenetic lineage								
Taxa	***Lepilemur leucopus***	***Propithecus verreauxi****	***Lemur catta****	***Nycticebus pygmaeus*** **	***Homo sapiens****	***Gorilla beringei*** **	***Pan troglodytes *****	***Papio* spp.****	***Oryctolagus cuniculus*****
Actinobacteria	0.10	<5	<1	5.2	0.2	5.3	3.3	2.4	0.9
Bacteroidetes	6.44	25 – 30	10 – 15	17.2	47.7	1.1	40.0	10.3	36.4
Firmicutes	1.39	35 - 40	20 - 25	43.1	50.8	71.0	49.2	81.7	56.0
Fusobacteria					0.08			5.2	
Lentisphaerae						3.2			
Planctomycetes						1.1			
Proteobacteria	0.06	<5	<10	34.5	0.6		6.7	0.4	6.1
Spirochetes	1.62	<1	<1			1.1	0.8		
Euryarchaeota		<1	<5						
Tenericutes									0.6
Verrucomicrobia	0.02				0.6	17.2			
Unclassified bacteria	90.9	20 - 25	50 - 55	0	0.02	0	0	0	0
Ciliophora (Protist)	0.05								

*Ranges estimated from figure provided by Fogel (2015); **Taken from Bo *et al.* (2010); ***From Crowley *et al.* (2017).

Table 4.2. Comparison of chemical composition in diurnal faeces (hypothetical caecotrophic faeces) and latrine faeces (hypothetical secondary faeces)

Faeces type	**Protein (% dry matter)**	**Carbohydrates (% dry matter)**	**Phenolics (CT%ATE/g)**	**Non-digested fibres (kJ/g)**
Fresh	4.00 ± 0.25	1.20 ± 0.18	0.37 ± 0.04	3.8 ± 0.3
Latrine	2.88 ± 0.08	1.23 ± 0.07	0.68 ± 0.06	7.4 ± 0.4
t	5.73	0.17	4.37	6.75
df	5	14	13	11
P	0.005	0.864	0.001	< 0.001

I found no trace of tannins in the faeces despite the fact that the animals' diet was rather rich in tannins (for instance, *Alluaudia* flowers consumed during the period of this study are particularly rich in tannins, Gould *et al.*, 2009). In contrast, I found non-tannin phenolics in higher concentrations in latrine faeces than in diurnal faeces, suggesting an effect of compaction, which was confirmed by an almost identical increase in non-digested fibres in the latrine faeces.

4.3.3 Microflora analysis

The fresh faeces of sportive lemurs had a unique bacterial flora. While they contained a number common bacteria also found in other primates, the majority of bacterial species (> 90% in all 15 faeces collected from 11 individuals) was not even identified to family level by DNA barcoding methods, indicating that they are probably endemic, and possibly involved in caecotrophy. As shown in Table 4.1, Bacteroidetes and Firmicutes usually make up most of

primate gut bacteria, but gummivorous slow lorises also use Proteobacteria, probably for digesting gum. In contrast, *Lepilemur* guts are characterised by extremely low proportions of Firmicutes compared to all other primates investigated.

4.4 Discussion

This study is largely consistent with the observations of Hladik and Charles-Dominique (*loc. cit*.) confirming that *Lepilemur leucopus*, and probably all sportive lemurs, use caecotrophy to increase the absorption of proteins, resulting in a 54% increase in digestive efficiency. Moreover, I suggest that the bacteria responsible for caecal fermentation are endemic to lemur guts due to high number of unknown bacteria detected in the faecal samples (> 90%), and should be subject to further identification.

Although the bacteria responsible were not identified, my analysis shows that the bacterial flora of *Lepilemur leucopus* is absolutely unique among all the investigated primates. Moreover, unknown bacteria were also found in the faeces of other lemurs like *Propithecus verreauxi* (24%) and *Lemur catta* (52%) that occur in the same region of southern Madagascar, but they occurred in much lower proportions (Fogel, 2015; Bennett *et al*., 2016). All other primates share very similar bacterial flora, with differences in proportions associated with specialisations.

In fact, these possibly endemic bacteria are probably those observed directly by Hladik *et al.* (1971); but reinterpreted as effect of putrefaction. However, these two interpretations were not exclusive knowing that the process of putrefaction is initiated by intestinal bacteria (Hyde *et al.*, 2013).

This confirms aforementioned lemurs have an endemic gut flora and perhaps have acquired a more diverse bacterial flora as the result of exchanges with domesticated animals. Similarly, in the case of the sportive lemurs, the detection of original bacterial flora is another strong argument supporting the hypothesis of derived caecotrophy in *Lepilemur*. Indris (*Indri indri*) are likely to acquire their intestinal bacterial flora by ingesting some of their mother's faeces as juveniles (Rabemananjara and Guzzo, pers. obs.). This suggests that allo-caecotrophy may have served as precursor for caecotrophy in *Lepilemur* ancestors, making caecotrophy a possible example of paedomorphic behaviour in the *Lepilemur*-Cheirogaleidae clade.

One observation that I made upon visual examination of the faeces was that the latrine faeces appeared, indeed, more fibrous, a character that I first attributed to desiccation. In fact, the "undigested plant material" mentioned by Russell (1977) as evidence that sportive lemurs do not practise caecotrophy turned out to be undigested fibres, that were present in double the concentration in the latrine faeces relative to diurnal faeces. This supports the hypothesis that latrine faeces can be regarded as secondary, hard faeces, contrasting with the much softer, greener faeces I collected during the daytime from captured animals, and that I regard as caecotrophic faeces. This has interesting implications on the use of latrines (Irwin *et al*., 2004; Dröscher and Kappeler, 2014), as the odours perceived by the animals are likely to be a combination of secretions from anal glands and products of bacterial fermentation.

Other studies have confirmed the Hladik – Charles-Dominique hypothesis (Hladik and Charles-Dominique, 1971, 1974; Hladik *et al*., 1971; Chivers and Hladik, 1980). Notably, Nash (1998) and Dröscher (2014) also observed the remarkable energetic efficiency of *Lepilemur leucopus*, which allows animals to occur at exceptionally high population densities and to use remarkably small home ranges despite a very poor diet.

The use of DNA barcoding in this analysis, and prior studies of primate microbiota, has been very limited as material like primers identify what has already been detected, leaving new identifiable species as unknown in the literature. One way of compensating for this would be a combination of traditional methods like histology in combination with sequencing technology to enhance our understanding of the important role microbes play in digestion and overall animal health.

CHAPTER 5: FROM FOLIVORY TO GUMMIVORY: COMPARING THE DIGESTIVE EFFICIENCY OF *GALAGO MOHOLI* AND *MICROCEBUS GRISEORUFUS*

5.1 Introduction

The consumption of gums (soluble exudates) by primates, or gummivory has been considered a fall-back feeding strategy employed in the face of persistent adverse environmental conditions and dietary scarcity (Lambert, 2007; Marshall *et al*., 2009; Rosenberger, 2013). Fall-back foods have been defined as either low in quality, but available when more desirable food is not (Bearder and Martin, 1980; Lambert, 2007; Porter *et al*., 2009; Rosenberger, 2013), or high in quality, but rare (Lambert, 2007). More recent research, however, indicates that gums are not necessarily lower in energy content than fruit, although the gums of different tree species may vary widely in composition (Génin *et al*., 2010), and may confer health benefits; e.g. pygmy slow lorises in captivity show ill health when their diets lack exudates (Starr and Nekaris, 2013).

The evolutionary scenario proposed by Masters *et al.* (2014) and Génin and Masters (2016) reverses the fall-back diet narrative by suggesting that many partial gummivores like the smallest cheirogaleids and the galagos may have had more gummivorous ancestors. Moreover, exudativory is a dietary syndrome not limited to gum consumption but also including nectar, honey, and the secretions produced by sap-eaters (Flatidae, Homoptera) (Andrews *et al*., 2016). Cases of convergence also include the South America callithrichines and Australian possums of the Petaudidae family, suggesting that the exudativory syndrome evolved in regions subject to El Niño-induced droughts (Génin *et al*., 2010). Here I examine the hypothesis of Andrews *et al.* (2016) and Génin and Masters (2016) that the dietary

evolution of cheirogaleids was a by-product of phyletic dwarfing resulting in a shift from folivory to gummivory.

What makes the fall-back diet particularly difficult to apply to small nocturnal strepsirrhines is that gummivory seems to have evolved early in the history of these groups. Using the method of Bayesian ancestral character state reconstruction, Andrews *et al.* (2016) suggested that gummivory probably evolved in convergence in at least four lineages of primates on four different landmasses: the cheirogaleids (Madagascar), the slow lorises (Southeast Asia), the galagos (Africa) and the callithrichines (South America). Interestingly, the two most spectacularly convergent groups of hyper-specialised gum scrapers, the fork-marked dwarf lemur (*Phaner* spp.) and the needle-clawed galago (*Euoticus* spp.) (Forbanka, 2018), probably diverged from the other members of their respective families in the early Oligocene, at the time of the first dwarfing event (Figure 5.1). This time corresponds to the Grande Coupure, a major mass-extinction event caused by a drastic cooling and drying period that led to the extinction of most of the northern adapiforms (Fleagle, 2013). Andrews *et al.* (2016) demonstrated that the early evolution of gummivory coincided with the spread of major gum-producing trees, especially the Mimosoidea and the Combretaceae.

A good indication that gummivory evolved convergently in cheirogaleids and galagids is the very simplified gut of *Microcebus* which lost its ansa coli, probably as another example of paedomorphic anatomical simplification. In *Microcebus*, fermentation occurs in the caecum (Hill and Rewell, 1948) whereas lesser galagos use caeco-ansal fermentation for digesting the complex β-linked polysaccharides found in gum and the exoskeletons of insects (Caton *et al.,* 2000). This observation of what appear to be very different mechanisms of gum digestion suggests that gummivory evolved from different ancestral states in these two lineages: i.e. folivorous in the *Lepilemur*-cheirogaleid clade, and more faunivorous in the lorisoid ancestor (Andrews *et al.*, 2016). The latter authors concluded that the difficult

digestion of chitin (in galagos) and leaves (in ancestral cheirogaleids) pre-adapted these ancestors to the digestion of gum.

One problem posed by phylogenetic reconstructions of diet is that the method is based on the assumption that ancestral animals fed on modern food items. This assumption is largely false as large edible fleshy fruits were rare prior to the Eocene epoch when the generalisation of seed dispersal by frugivores occurred (Sussman, 1991). Indeed, many plant parts like fruits, in particular, are the result of a long coevolution with animals. Before the Cretaceous-Palaeocene boundary, primates probably fed mainly on flowers, and nectarivory was likely to have been the first exudate consumed by animals. Because of the spread of resinous gymnosperms at that time, there are good reasons to believe that some late Cretaceous or early Palaeocene animals fed on resins high in secondary compounds, as precursors of gummivory (Andrews *et al.*, 2016). Indeed, gummivores may also have co-evolved with gum-producing trees, as they also tend to feed on the insects infesting the trees producing gums. Because gum foragers may gouge out some xylophagous larvae (or allow other animals to do so), Andrews *et al*. (2016) proposed that gummivory may benefit the trees in way similar to many "cleaner species" observed among fish and birds. A prediction derived from this hypothesis is that the mimosoid soluble gum preferred by the mouse lemurs (Génin, 2008; Génin *et al.*, 2010) should be more digestible than Burseraceae resinous gum that contains terpenes (Génin *et al.*, 2010).

I made three predictions:

(1) Reddish-grey mouse lemurs (*Microcebus griseorufus*) should digest gum efficiently, at least the soluble mimosoid gums known to be a seasonal keystone resource (>75% of the diet in the dry season) (Génin, 2008).

(2) If soluble gums evolved from resins, the digestion of soluble mimosoid gums should be more efficient than the digestion of Burseraceae resinous gums.

(3) Due to the absence of an ansa coli, digestive efficiency should be lower in *Microcebus griseorufus* than in *Galago moholi*, due to a shorter retention time of food in the gut.

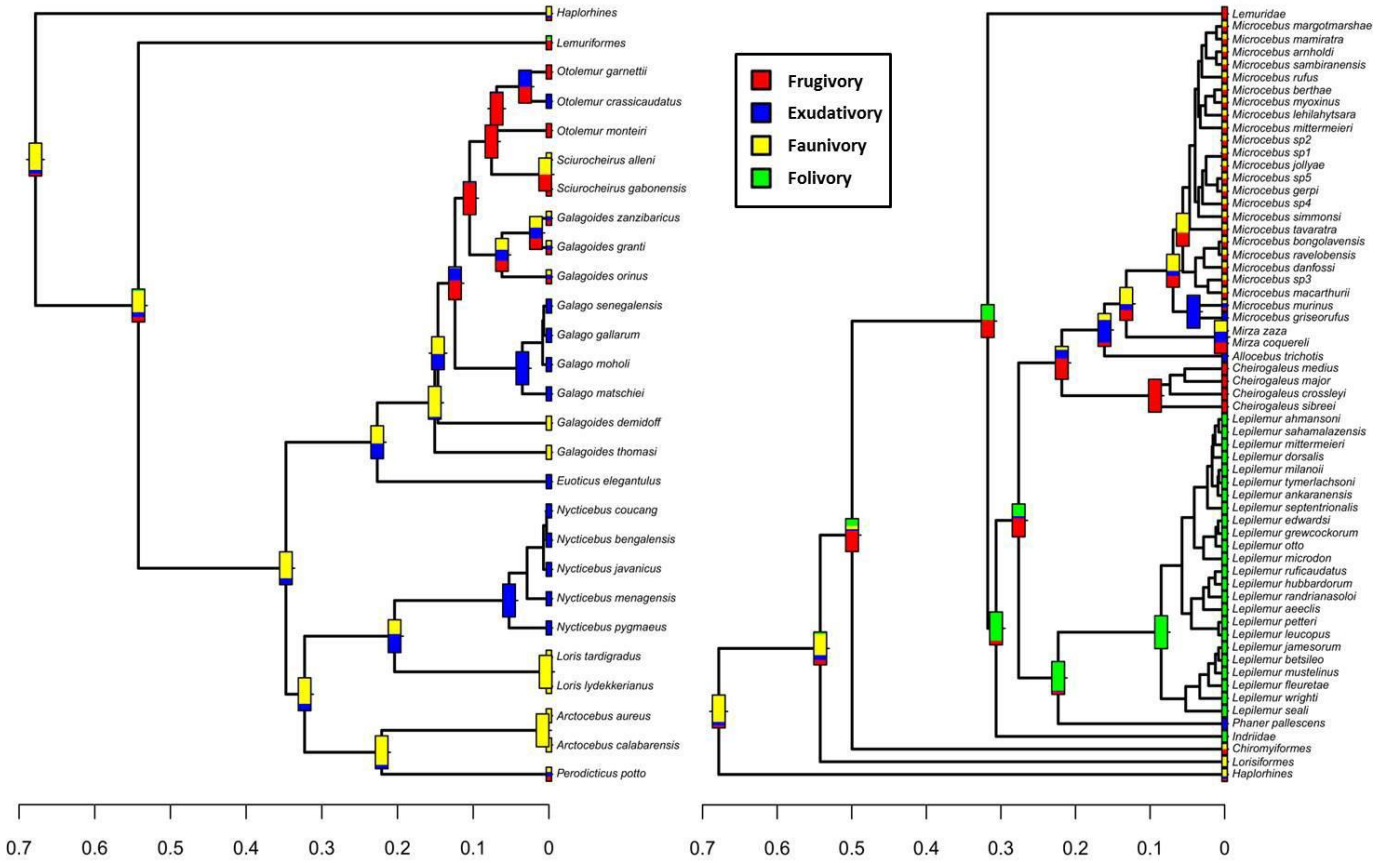

Figure 5.1 Phylogenetic reconstruction of ancestral dietary patterns of (left) Afro-Asian Lorisoidea and (right) Malagasy *Lepilemur*-Cheirogaleidae (taken from Andrews *et al*., 2016)

5.2 Materials and Methods

5.2.1 Gum digestion trials in Galago moholi *and* Microcebus griseorufus

Six reddish-grey mouse lemurs (*Microcebus griseorufus*) were captured in the same site of spiny forest as the sportive lemurs (see Génin, 2008, 2010 for capture methods). Animals were transferred to individual square cages (1 m^3) each containing a nest-box and a bowl of water. Six southern lesser galagos (*Galago moholi*) from the Ithumela Primate Sanctuary, north of Pretoria (Tshwane), were transferred to similar cages and subjected to the same experimental protocol. The galagos were all animals born in the wild and rescued as adults by the Ithumela Primate Sanctuary. The gums I tested were those most commonly consumed by the animals. For the mouse lemurs, I collected gums from *Alantsilodendron alluaudianum* (Fabaceae, Mimosoidea) and *Commiphora orbicularis* (Burseraceae) in the site of capture (Génin, 2008); and for the galagos, I collected gums from *Vachellia* (=*Acacia*) *karroo* (Fabaceae, Mimosoidea) from the surroundings of the Ithumela Primate Sanctuary (also used by Caton *et al.*, 2000).

To compare the total amounts of gum and banana consumed by the animals, animals were always fed *ad libitum*, as revealed by leftovers. However, an exception was the *Alantsilodendron* gum that the animals depleted in a few instances. This made the comparison of total consumption difficult.

My initial project also included testing both species with the same kind of gum. For this, I chose the gum that I could collect in large amounts, the gum of *Vachellia karroo* collected in South Africa and tested on the mouse lemurs. Unfortunately, the animals did not feed on this gum and the experiment had to be terminated.

a)

b)

Figure 5.2 (a) Gum of *Commiphora* spp. in Berenty; **(b)** lay-out of cages (including nest box, branches and linoleum lining) for feeding trials (Photos: DR Roberts)

The galagos of the Ithumela Sanctuary were fed before nightfall, and most mouse lemurs were captured early in the evening, when they feed only on small pieces of banana that form the bait in the Sherman traps. Hence, the feeding experiments started on the second evening after transfer into the trial cages, as animals were all assumed to have empty guts at that time. This was confirmed by the absence of faeces in the cages for the next 2-3 days. Because Caton *et al*. (2000) found that *Vachellia karoo* gums were retained by *Galago*

moholi guts for > 24 h, I started by feeding the animals gums (as much as could be gathered) until the first faeces were collected (after 2-3 nights). I provided them with water for one night, before feeding them banana *ad libitum* – for instance if there were leftovers, the animals had more than what they could consume - for one more night. After 5-6 nights I released the animals at dusk, and any late faeces excreted while the animals were in the release traps were recovered. To facilitate the collection of faeces, I placed a plastic tray at the bottom of each cage. Following an environmental enrichment technique (Huber and Lewis, 2011), the food provided was spread in the cages and placed in small cavities on branches. The food items as well as the subject animals were weighed before the feeding trials, and all faeces collected were weighed and placed in a drying oven at 43°C for 30 hours to ensure complete desiccation of the samples. After desiccation, the faeces were weighed again and stored in airtight bags in the refrigerator for further analyses.

5.2.2 Chemical analyses

All samples (food, including gum and banana, and faeces), were ground in the laboratory at the University of Hamburg, Germany, and pressed into pellets. These pellets were weighed and subjected to bomb calorimetric combustion – "measuring calorific values of solid and liquid combustible samples" (Parr Instrument Company). A subsample was analysed for nutrient content and subjected to four biochemical assays. Protein availability was determined through the Kjedahl assay, simple sugars were measured using HPLC (high performance liquid chromatography; Rothman *et al*., 2011), while condensed tannins and phenolic concentrations were measured using a photometer.

The digestive efficiency (DE) of banana and gum by *Microcebus griseorufus* and *Galago moholi* was calculated as follows:

Digestibility [%] = Gross Energy Feed – Gross Energy Faeces/ Gross Energy Food *100

5.2.3 Data analyses

All statistical tests were performed in SYSTAT. I used Repeated Analysis of Variance to compare the digestive efficiency of banana (trial 1) and gum (trial 2) in the two species. $P < 0.05$ was considered the level of statistical significance.

5.2.4 Ethical considerations

This research complied with standard protocols for animal handling and capture approved by the Research Ethics Committee: Animal (A17–SCI–ZOO-012) of the Nelson Mandela University, the management of Berenty Private Reserve and the legal requirements of Madagascar. All immobilisation and handling procedures were performed with the assistance of an experienced wildlife veterinarian.

5.3 Results

5.3.1 Gum feeding

The collection of large amounts of *Vachellia* (= *Acacia*) *karroo* gum allowed me to test 6 *Galago moholi* individuals. In contrast, the collection of gum at Berenty yielded smaller samples, allowing me to test only 2 individuals with *Alantsilodendron alluaudianum* gum (the most frequently consumed gum, but consumed in smaller amounts at a time); and 4 individuals with *Commiphora orbicularis* gum (more rarely consumed, but sometimes consumed in large amounts after a tree is injured) (Génin, 2008). Animals of both species consumed the gum but in small amounts compared with the banana (Table 1).

Interestingly, animals consumed much more *Alantsilodendron* gum (7.4 ± 0.4g per trial) than *Commiphora* gum (5.6 ± 0.8g per trial), despite greater availability of the latter.

Table 5.1. Chemical contents of some gums consumed by *Microcebus griseorufus* and *Galago moholi*

	Crude Protein (%)	**Carbohydrates (%)**	**Tannins (CT%ATE/g)**	**Phenolics (CT%ATE/g)**	**Energy Content (kJ/g)**	**Reference**
Vachellia karroo	1.1	59.6	0.24	0.09	14.0	This study
Commiphora orbicularis	2.9	52.4	0.05	0.18	16.5	This study
Alantsilodendron alluaudianum	21.0	29.5	0	0	8.45	Génin *et al*. 2010
Banana (SA)	3.9	60.0	0.25	0.18	14.5	This study
Banana (MD)	5.1	73.0	0.00	0.26	14.6	This study

* SA – South Africa, MD – Madagascar.

5.3.2 Digestive efficiencies

I observed no overall difference in the animals' digestive efficiency of gum and banana ($F1/10 = 2.76$; $P = 0.128$), and no overall difference in digestive efficiency between *Microcebus griseorufus* and *Galago moholi* ($F1/10 = 1.93$; $P = 0.195$) (Table 2). However, there was a significant interaction between the two factors ($F1/10 = 5.42$; $P = 0.042$): *Galago moholi* showed a relatively higher digestive efficiency of the gum than the banana when compared with *Microcebus griseorufus*. This last result was a consequence of low digestive efficiency of *Alantsilodendron* gum by mouse lemurs compared with *Commiphora* gum, a result that contradicted my prediction of better digestion of soluble gum. Despite a small sample size (two animals tested), this result was confirmed by direct observations of short retention times of *Alantsilodendron* gum (< 24h), whereas all the other trials revealed longer retention times (in excess of 36h) in both species.

Table 5.2. Digestive efficiency (DE) measured in *Galago moholi* and *Microcebus griseorufus*

	Gum type	**Retention time gum (h)**	**Total Gum consumed (g)**	**Retention time banana (h)**	**Total banana consumed (g)**	**Gum %DE**	**Banana %DE**
Galago moholi	*Vachellia karroo**	>36 h	19.9±2.1	< 24 h	43.1±1.5	93.5±2.7	90.6±3.1
Microcebus griseorufus	*Commiphora orbicularis***	>36 h	5.6±0.8	< 24 h	33.9±1.8	88.7±2.7	95.1±0.6
	*Alantsilodendron alluaudianum****	< 24 h	7.0±0.4			55.8±8.4	

*Average±SEM; *N*=6 ; **Median±SEM, *N*=4 ; *** Median±SEM, *N*=2;

5.4 Discussion

My study of the mouse lemurs of the xerophytic forest, *Microcebus griseorufus*, revealed a remarkable ability to digest gums, despite very short, simplified guts. Caecal fermentation was revealed by long retention times similar to those observed in the African southern lesser galago (*Galago moholi*) for one of the two gums tested, the resinous *Commiphora* gum. Curiously, the most frequently consumed and probably preferred gum, the gum of the small mimosoid *Alantsilodenron alluaudianum* (75% of the diet in the late dry season) appeared to be less digestible and was eliminated in less than 24 h. The reason that the animals prefer *Alantsilodendron* gum may be for its short retention time and lower toxicity (evident in reduction or absence of tannins), but is more likely to be related to its high protein content (Table 5.1) or its generally more generous exudations (Génin *et al.*, 2010). McNab (2002) observed that animals rarely maximise retention times but rather adapt them to their daily rhythms, which also explains why *Commiphora* gum is generally consumed at the end of the night (Génin *et al*., 2010). Porter *et al.* (2009) investigated the selection of exudates by *Callimico goeldii*, and proposed that exudates that were more difficult to digest were eaten later in the day and digested overnight. Heymann and Smith (1999) drew similar conclusions regarding gum-feeding in two *Saguinus* species (*S. mystax* and *S. fuscicollis*), in that gum-feeding generally occurred later in the day.

The long caecal retention of *Commiphora* gum may be explained by the presence of terpenes that give them their characteristic resinous smell. Secondary compounds are known to delay digestion and prolong gut retention in ruminant mammals (Acamovic and Brooker, 2005). Animals are probably capable of effective detoxification: indeed, Génin (pers. comm.) observed 5 cases of dying *Commiphora orbicularis* producing very large amounts of gum, always consumed by animals throughout the night. However, animals avoid the white gum of

the most toxic species of resinous gum producer *Commiphora simplicifolia* (Génin *et al.*, 2010)

The relative importance of gum in the diets of the Cheirogaleidae, which all consume gum in various proportions, indicates that they became partial gummivores secondarily, probably from a more gummivorous ancestor (Andrews *et al.*, 2016). This indicates that mouse lemurs were pre-adapted to gummivory although they may use gum as fall-back foods. They clearly prefer fruits to gum, and switch to fruits when they are available. They also defend patches of fruit. The consumption of insects by all gummivores, including specialists, also suggests the necessity of complementing diets with proteins.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1 The palaeoenvironmental context to the emergence of the *Lepilemur* – cheirogaleid clade

In Table 1.1, I summarised divergence dates for major nodes in primate phylogeny estimated using a diverse range of sequences and techniques. The average age that has been calculated for the split between the Haplorhini and the Strepsirrhini from these studies is 72.6 Ma, which is much older than the first undoubted primate fossil (i.e. *Altiatlasius*, 60 Ma; Sigé *et al*., 1990). This is not too surprising as fossil dates are always minimal ages: a fossil cannot reasonably be assumed to be the oldest member of its lineage. The average estimate for the emergence of the crown Strepsirrhini (i.e. Chiromyiformes, Lemuriformes and Lorisiformes) is 62 Ma, in the early Palaeocene. The average divergence estimated for the split between Chiromyiformes (the aye-aye, *Daubentonia*) and the Malagasy Lemuriformes is 54.5 Ma – at the beginning of the Eocene. This is the same period when the Adapiformes began to be preserved as fossils across the northern continents.

The lemuriform radiation appears to have begun around 36 Ma, towards the end of the Eoecene. The *Lepilemur*-Cheirogaleid clade has an estimated average age of 31 Ma, shortly after this divergence. The Eocene was a period of unusually warm and wet climate, when broad-leaved forests were spread widely across North America and Eurasia. The epoch came to an abrupt end at 33.9 Ma, when climates became drier and much colder (Fleagle, 2013). This climate change has been linked to the first formation of the Antarctic ice sheet (Zachos *et al*., 2001). It is also possible that climates became a lot more unpredictable during this period, as the ice sheet did not become stabilised under the late Miocene. Masters *et al*. (2013), citing de Wit (2003), further suggested that the Eocene-Oligocene transition might

have witnessed the uplift of the Malagasy highlands, related to the mantle plume-induced uplift that occurred during this period in East Africa.

If these reconstructions are correct, they suggest that the fauna of Madagascar experienced both dramatic climatic changes and topographic changes around the same time period. When climate change is linked to complex topography, environmental and habitat shifts are particularly rapid and intense (Cracraft, 1985; Masters *et al.*, 1995). Such environmental factors would be conducive to the initial dwarfing event that caused a larger-bodied leaf-eating *Lepilemur* ancestor to reduce its body size to cope with unpredictable food resources, rainfall and temperatures. Later dwarfing events appear to have taken place throughout the Miocene: both *Cheirogaleus* and *Phaner* emerged around 24 Ma, at the beginning of the epoch; *Allocebus* and *Mirza* appear to have diverged between 18 and 16 Ma; and the smallest-bodied lemurs, the mouse lemurs (*Microcebus*), only radiated around 8 Ma – once again, as the Antarctic ice sheet caused dramatically drier, cooler climates, and East Africa began another phase of uplift (Corti, 2009).

My study examined the anatomical and dietary consequences of the emergence of the *Lepilemur*-Cheirogaleidae clade and explored four possible consequences of proposed repeated phyletic dwarfing events. My investigation into the shape of the palate revealed that this character did not follow other aspects of skull morphology in reflecting close similarities in ontogenetic size and skull shape between lepilemurs and other cheirogaleids. It hence does not reproduce the pattern of parallel dwarfism reported by Masters *et al.* (2014), but rather reveals very different adaptive forces. This supports the idea of a brutal shift in selection regimes, from one driving changes in life history and body size (primarily a response to environmental unpredictability), to another forcing dietary changes – perhaps while subjected to acoustic constraints. This in turn suggests that dietary changes are often by-products of other changes allowed by previous adaptations. If my interpretation is correct, such pre-

adaptations included ancestral allo-caecotrophy (as a forerunner to *Lepilemur* caecotrophy), folivory (as a prelude to cheirogaleid gummivory) or insectivory (as a precursor to lorisoid gummivory).

My investigation into the arterial circulation patterns of the LC clade supported the prediction that changes in body size led to reduction of a functional stapedial artery in *Lepilemur*, making it an intermediate stage between the daubentoniid, lemurid and indriid species with large stapedial arteries, and the smaller bodied cheirogaleids with an alternative blood supply in the form of an enlarged ascending pharyngeal artery. This shift possibly occurred under the influence of dramatic changes in the environment, whereby broad-leaved forests disappeared in the face of a drier, colder climate.

My study on the white-footed sportive lemur (*Lepilemur leucopus*) presented indirect evidence in support of Hladik's hypothesis of caecotrophy. I found that the rapid passage (< 12h) of food through the very short guts of *Lepilemur* allows the animals to produce diurnal caecotrophic soft faeces during the morning following a night of feeding. These faeces contrast with nocturnally-produced, hard faeces that are deposited in latrines that I interpreted as secondary faeces because of their lower protein content. Moreover, the latrine faeces had twice the amount of phenolics found in the diurnal, fresh faeces, strongly suggesting compaction. Furthermore, I found that the composition of the faecal bacterial flora, although the largest portion was unknown and possibly endemic to lemur guts, aids in the digestion and maximises the extraction of protein and other nutrients during periods of rest, further suggesting caecotrophy. Interestingly, caecotrophy by *Lepilemur* can also be interpreted as paedomorphic behaviour that derived from infantile allo-caecotrophy, used by folivores to acquire the bacteria necessary for digesting and detoxifying leaves. Overall, this study supports the dwarfing hypothesis: reduction of body size around Kay's folivory limit of 500 g led to the evolution of caecotrophy in *Lepilemur.*

The evolution of exudativory from a folivorous ancestor was tested in *Microcebus griseorufus* using the digestive efficiency of gum as a proxy. This confirmed that a further reduction of body size probably led to this dietary shift, which may have arisen by pre-adaption of the gastrointestinal tract to fermentation during ancestral folivory (Génin and Masters, 2016). Interestingly, the convergent evolution of exudativory in African and Asian strepsirrhines appears to have benefited from a similar pre-adaptation, not to an ancestral folivorous diet, but rather to an insectivorous diet which poses similar digestive challenges (Andrews *et al.*, 2016).

Throughout this study, the data suggested that phyletic dwarfism in the *Lepilemur*-cheirogaleid clade was accompanied by various changes related to morphology, physiology and behaviour. This includes changes in palate shape in relation to shifts in diet with a strong phylogenetic effect. The arterial circulation patterns possibly followed shifts in body size with reduction and eventual loss of the stapedial artery in the LC clade. Furthermore, the shifts in diet necessitated by dwarfing support the hypothesis that caecotrophy in *Lepilemur*, the smallest folivorous primate, was accompanied by the evolution of endemic bacteria that play an essential role in the digestion of plant material by means of fermentation in a large caecum. This gastrointestinal adaption to folivory suggests that hyper-dwarfs, like *Microcebus*, benefited from this pre-adaption during the evolution of gummivory. This indicates that exudativory evolved early in the history of cheirogaleids and was retained in *Microcebus*, perhaps because of its highly adaptive value of fall-back diet used during recurrent but unpredictable dry periods.

6.2 Future Studies

This study centred on the premise of dwarfing and morphological and physiological changes associated with a reduction in body size, and though the data collected here provides support for the predictions set out in Chapter 1, further research would add to the reconstruction I put forward here. One potentially fruitful avenue of research would involve following up the study of caecotrophy in order to collect more direct evidence of its occurrence, for example, by filming this elusive behaviour, which is probably mistaken for ano-genital grooming. One of the problems related to obtaining such evidence is that it would be most easily obtained using captive animals, but *Lepilemur* are known to survive poorly under conditions of captivity because of their folivorous diet (Nash 1998). A possible way around this problem is through the use of tinted glass nest boxes prepared as specific observation posts. During the course of her behavioural study, Dröscher (2014) placed nest boxes in the forest patches where *Lepilemur* occur in the Berenty Reserve, and these are still being used by the animals. Further future studies should also include testing the hypothesis of folivory as a precursor to gummivory in this group by investigating the bacterial flora of the small-bodied Cheirogaleidae, and comparing it with the gut flora of other lemurs in the form of a survey of lemur intestinal bacteria that would aid in identifying endemic forms.

The dataset used to investigate changes in arterial circulation could be supplemented with soft-tissue dissections of the taxa as some of the arteries supplying blood to the brain, like the vertebral artery, are not evident in the micro-CT scans I analysed. Including other strepsirrhines, like *Daubentonia*, as well as haplorrhine taxa, would allow for a more extensive comparison of arterial patterns.

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