UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

IRON STORAGE DISEASE PREVALENCE IN CAPTIVE RING-TAILED LEMURS

MARIA DO CARMO LOURO VASSALO SANTOS

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Doutor Rui José Branquinho de Bessa

Doutor Fernando Ribeiro Alves Afonso

Dr. João Inácio Louro Simões de Almeida

ORIENTADOR

Dr. João Inácio Louro Simões de Almeida

CO-ORIENTADOR

Doutor George Thomas Stilwell

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Iron storage disease prevalence in captive ring-tailed lemurs

Abstract

The present study aims to evaluate the prevalence of iron storage disease in captive ring-tailed lemurs' populations housed in different zoological parks in Portugal and relate it to the different diet regimens.

Eighteen animals were admitted to this study and then subdivided into three different groups, according to their zoological institution. Blood transferrin saturation level was measured for each animal. The diet given at each park was also analyzed and then related to the obtained transferrin saturation values.

It was verified that transferrin saturation value is high in 89% of the animals and the mean was higher than 55% (above the reference range) in all groups.

Despite the small sample size, it was evident that there is a high prevalence of iron storage disease in captive ring-tailed lemurs, which seems to be strongly related to the captive diet offered in zoological institutions.

Key words: Iron storage disease, hemosiderosis, ring-tailed lemurs, food, transferrin saturation

Prevalência de *iron storage disease* em lémures de cauda anelada mantidos em cativeiro

Resumo

O presente estudo tem como objectivo determinar a prevalência de *iron storage disease* em lémures de cauda anelada mantidos em condições de cativeiro e relacioná-la com a dieta fornecida aos lémures em diferentes parques zoológicos, em Portugal.

Para a realização do estudo, reuniu-se uma amostra de dezoito indivíduos divididos em três grupos, conforme o parque de onde provinham. Procedeu-se à colheita de sangue de cada animal com posterior análise da saturação de transferrina. Foi também analisada a dieta à qual os lémures são sujeitos em cada parque, relacionando-a mais tarde com os níveis de saturação de transferrina obtidos.

Foi constatado que o nível de saturação de transferrina dos indivíduos analisados é elevado em 89% dos animais, encontrando-se acima de 55% (valor máximo do intervalo de referência) em todos os grupos.

Apesar da reduzida amostra deste estudo, existe uma forte evidência da elevada prevalência de iron storage disease nos lémures de cauda anelada mantidos em cativeiro, o que parece estar fortemente relacionada com a dieta oferecida a esses animais nos respectivos parques zoológicos.

Palavras-chave: *Iron storage disease*, hemossiderose, lémures de cauda anelada, alimentação, saturação de transferrina

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Acronyms

AZA	Association of Zoos and Aquariums
BW	Body Weight
DFO	Deferoxamine
DM	Dry matter
EAZA	European Association of Zoos and Aquariums
EEP	European Endangered Species Programmes
ESB	European Studbooks
ISD	Iron Storage Disease
IUCN	International Union for Conservation of Nature
RCP	Regional Collection Plans
SF	Serum Ferritin
SI	Serum Iron
SSP	Species Survival Plan
TAG	Taxon Advisory Groups
TIBC	Total Iron Binding Capacity
TS	Transferrin Saturation

Internship report

The present study was developed during the 6th year of the Integrated Masters in Veterinary Medicine of the Faculty of Veterinary Medicine, University of Lisbon, under the supervision of Dr. João Almeida and Dr. George Stilwell.

The final masters' internship was realised at Badoca Park for a period of four months - about 700 hours. The shifts had the duration of eight hours per day. During this time, I had the opportunity to follow the park's veterinary Dr. João Almeida on his daily routine. This included the involvement in different fields of veterinary medicine, such as internal medicine, surgery, medical imaging and mainly anaesthesiology.

It was possible to participate in numerous anaesthesia procedures with wild mammals such as zebra, buffalo, wildebeest, oryx, wallaby, baboon, among others. Besides monitoring the patients during these procedures, I also had the chance to: prepare and administer subcutaneous, intramuscular and intravenous medication, including injectable anaesthetic darts; collect samples of blood and faeces; place intravenous catheters; measure blood pressure; realise a bronchoalveolar lavage, skin scraping, blood smears; wound cleansing and debridement; realisation and interpretation of blood and urine analysis and cytology; microscopically observe parasites.

Considering the field of internal medicine, I had the chance to participate in several medical cases regarding wild mammals (primates, ruminants, equines, marsupials, etc), reptiles (crocodiles and turtles) and numerous species of birds. It was possible to observe the animals and identify the clinical signs, when showed; elaborate a list of differential diagnosis and collaborate in the realisation of diagnostic methods and implementation of treatment.

Regarding medical imaging, I had the opportunity to help containing and positioning the animals and to realise and interpret radiographic exams and ultrasound exams.

Additionally I also joined the veterinary in several surgery procedures such as: orchiectomy, caesarean section, bone fractures resolution, nodule removal, tooth removal, among others.

Besides follow the responsible veterinary, I could also accompany the zookeepers on their daily routine, preparing the animals' food, feeding the animals and cleaning the animals' interior installations. I had the chance to get closed to the ring-tailed lemurs and feed them at least three days per week, during my internship.

Furthermore, I had the opportunity to take part in anaesthesiology formations for veterinary students and professionals.

The idea of choosing the subject "Iron Storage Disease in captive ring-tailed lemurs" came along with my special interest for this species. Additionally, the fact that this is a common but barely known syndrome that affects numerous species such as lemurs, black rhinos, tapirs, etc. made my supervisors and I consider it as an interesting opportunity to deepen this topic.

The overall internship gave me the chance to apply and enhance the veterinary knowledge learned during the last six years. It also provided practical and theoretical tools, allowing the development of notable scientific, technical and personal competences.

I. INTRODUCTION

Iron is an essential nutrient for all living organisms. Its multiple metabolic processes include oxygen transport, DNA synthesis and electron transport (Conrad, Umbreit, & Moore, 1999).
Iron balance is normally controlled by iron absorption, since its excretion is insignificant.
The absorption occurs through the upper intestine portion, under intestinal mucosa regulation (Gordeuk, Bacon, & Brittenham, 1987).
Despite being essential for most animals, iron can become toxic when excessive amounts of this nutrient are absorbed, which can lead to hemosiderin accumulation in the liver, spleen, lymph nodes, duodenum and other organs (Gonzales, Benirschke, Saltman, Roberts, & Robinson, 1984), causing iron storage disease.
Therefore, once absorbed, iron must be bound to proteins to prevent free radical formation (Conrad et al., 1999), since it can be associated with tissue damage and increased risk of neoplasia (Gonzales et al., 1984).
Spelman, Osborn and Anderson (1989) hypothesized that the increased bioavailability of iron in lemurs' captive diet, compared to their natural diet, may be the critical factor for the high incidence of hemosiderosis in this species.
However, despite the high levels of iron in the captive diets lead to an increased iron absorption, there are several dietary components that can influence iron's bioavailability.
Ascorbic acid is the most efficient enhancer of non-heme iron absorption (Teucher, Olivares & Cori, 2004), therefore diet components such as citrus fruits and other substances with vitamin C, will increase iron bioavailability and consequently, its absorption.
Contrary, phenolic compounds (polyphenols, tannins) are known to restrict iron absorption by constituting a complex with this nutrient in the gastro-intestinal lumen, turning it less bioavailable for cellular uptake (Brune, Rossander, & Hallberg, 1989).
For these reasons, several authors proposed a severe change in captive husbandry and diet practices.
Numerous studies have been made in order to evaluate the incidence of hemosiderosis in captive lemur populations, concluding that it is certainly a consistent postmortem finding in different captive lemur species (Wood, Fang, Hunt, Streich, & Clauss, 2003). However, there is still a lack of information about the ideal captive diet for this species. For this reason, it has been recently proposed that new studies comparing multiple facilities should be elaborated (Clauss & Paglia, 2012).
Considering this evidence, the present study compares the diet of three different zoological facilities and relates it to the transferrin saturation values obtained from all the ring-tailed lemurs housed at each institution.

Transferrin saturation was used to evaluate iron storage disease (ISD) status from each lemur collection. As well as in human medicine, this parameter indicates if excessive amounts of iron are being absorbed, prior to iron excessive deposition and tissue damage (Wood et al., 2003).

Therefore, this study intends to reveal the impact of the captive lemurs' diet on the transferrin saturation values obtained at the respective institution.

II. LITTERATURE REVIEW – Iron Storage Disease Prevalence in Captive Ring-tailed Lemus

1. Lemur

1.1. Origin

Madagascar is the only place where members of the Superfamily Lemuroidea can be found in the wild.

Situated off the southeast coast of Africa and separated from the continent by the 800 km-wide Mozambique Channel, the island of Madagascar, in the southwestern Indian Ocean, is the world's fourth largest island (Swindler, 2002), after Greenland, New Guinea and Borneo and the largest oceanic island (Schwitzer, Mittermeier, Davies, Johnson, Ratsimbazafy, Razafindramanana, Louis & Rajaobelina, 2013).

It has been separated from other landmasses for at least 88 million years, and from mainland Africa, its closest neighbour, for at least 130 million years (Schwitzer et al., 2013).

Madagascar is a 1650 km (1025 mi) long island split by a mountain chain running from north to south.

According to Pastorini, Thalmann and Martin (2003) the island can be divided into eight major zones of species distribution, each with distinctive climatic and vegetation characteristics and/or delimited by physical barriers. The authors consider that these climatic, vegetation and physical factors are important to understand the phylogeography of the Malagasy lemurs.

With 581,540 km^2, Madagascar's total land area is only about 7% of Brazil, the world's richest country in primates, and yet its primate diversity is comparable and its endemism much higher (Schwitzer et al., 2013).

Ganzhorn et al. (1999) suggest that a combination of long geographic isolation, poor soils, and low plant productivity, in an erratic and severe climate, could have played a major role in lemur evolution.

Until around 130 million years ago, Madagascar was attached to the African mainland as part of the super continent Gondwanaland (formed by Africa, South America, Australia, Antarctica, India, and Madagascar), but as Gondwanaland fractured, Madagascar moved away from Africa (Wild Madagascar, 2017). The flora and fauna of Madagascar underwent numerous adaptive radiations, due to the island's long isolation and low rates of colonization resulting in one of the world's most diverse biotas with remarkable levels of endemism (Myers, Mittermeier, Fonseca & Kent, 2000).

Endemism is extremely high, ranging from 55–100% at the species level, and at genus and family level it far surpasses any other hotspot, with more than 480 genera and 26 families

endemic to this island (Schwitzer et al., 2013).

Madagascar's endemic primates, the lemurs, are the most diversified element of a highly unusual fauna that displays an adaptive variety surpassing that of any comparable primate group, especially if the recently extinct "subfossil" forms are taken into account (Mertl-Millhollen, 2008).

As stated by Martin (2000), although lemurs have remained relatively primitive in many features, the adaptive array is remarkable. As an example, the author affirms that lemurs show a greater spectrum of dental formulae and molar morphology than all other living primates taken together, and there is also a considerable diversity in dietary habits, ranging from insectivory through frugivory to folivory and including the special dietary adaptations of the aye-aye.

The lemurs of Madagascar provide an excellent model for exploring evolutionary diversification (Pastorini et al., 2003).

The first lemur-like primates on the fossil record appeared roughly 60 million years ago in mainland Africa and crossed over to Madagascar shortly thereafter (Wild Madagascar, 2017).

As admitted by Schwitzer et al. (2013) the diversity of the lemur fauna of Madagascar is even more impressive when one looks at the giant lemur species that disappeared since the arrival of humans on the island some 2,000–2,500 years ago. These included at least 8 genera and 17 species, all of them larger than the surviving species.

Further, and of particular concern for conservation purposes, less than 10% of Madagascar remains sufficiently intact to serve as habitat for wild lemur populations, meaning that all of the country's primate diversity is confined to an area of approximately 50–60,000 km^2 (Mittermeier, Ganzhorn, Konstant, Glander, Tattersall, Groves & Rasoloarison, 2008).

Indeed, Madagascar is so important for primates that it is considered one of the four major biogeographic regions for primates, together with South and Central America, mainland Africa, and Asia, in spite of being only about 1.3–2.9% the size of each of the three continental regions (Schwitzer et al., 2013).

The Malagasy lemurs constitute one of six major natural groups of living primates. Lemurs species show remarkable diversity, both numerically and in terms of adaptation (Martin, 2000).

Classification within the Lemuriformes remains highly controversial and several different taxonomic schemes have been proposed. However, according to Pastorini et al. (2003), the living primates of Madagascar comprise five families: Lemuridae, Cheirogaleidae, Indriidae, Daubentoniidae and Lepilemuridae.

The authors assume that at present, a tentative consensus accepts four genera (*Eulemur*, *Hapalemur*, *Lemur*, and *Varecia*) in the family Lemuridae, which includes at least 10 species. The Cheirogaleidae family is currently classified into five genera (*Allocebus*, *Cheiro-galeus*,

Microcebus, *Mirza*, and *Phaner*), containing at least 13 species. The family Indriidae comprises at least seven species in three genera (*Avahi*, *Indri*, and *Propithecus*). The family Daubentoniidae contains only one extant lemur species (*Daubentonia madagascariensis*). *Lepilemur* is the only genus in the family Lepilemuridae and currently comprises a maximum of seven species (Pastorini et al., 2003).

All of lemur species enounced are endemic to Madagascar and just a few of them are kept in captivity in significant numbers. It means that the conservation of their unique habitat is an imperative strategy to protect the future of most species that will remain *in situ*.

As cited by Schwitzer et al. (2013), looking at the importance of Madagascar's primate fauna in another way, although the country is only one of 91 countries having wild primate populations, it alone is home to 15% of all primate taxa (103 of 682), 21% of all primate species (99 of 480), 19% of all primate genera (15/77), and 29% of all primate families (5/17) – a great responsibility for any one country and a concentration of unique primate species unmatched by any other nation.

As has been stated many times, the survival of Madagascar's unique biota, including its primates and ultimately the well-being of its people, depends on the continued presence of forests in the country (Harcourt & Thornback, 1990).

Madagascar, in the opinion of many, is the world's single highest priority biodiversity hotspot (Schwitzer et al., 2013).

1.2. The ring-tailed lemur

One of the most emblematic lemur species found in the island of Madagascar and the most common in captivity belongs to Lemuridae family, and is usually called ring-tailed lemur (Figure 1).

The scientific name of this small primate is *Lemur catta*. Lemur derives from the Latin word *lemurs*, which means ghosts or spectres, a reference to the animal's nocturnal habits and silent movements. The specific epithet *catta* refers to the animal's catlike form (Hanlon & Wilson, 2010).

Figure 1. The ring-tailed lemur (Original picture)

1.2.1. General characteristics

The conspicuous characteristic for which ring-tailed lemurs are known is their long tail, measuring about 60cm that has alternating bands of black and white rings (Mittermeier, Tatterson, Konstant, Meyers, & Mast, 1994).

Males and females are about the same size, in the wild measuring about 42.5cm from head to rump and weighing between 2207 and 2213g on average (Sussman, 2000).

As evidenced by Kappeler (1991), ring-tailed lemurs weigh slightly more in captivity than their wild counterparts with males weighing, on average, 2705g and females average 2678g.

Ring-tailed lemurs share unique dental characteristics with other members of the Superfamily Lemuroidea. They have specialized teeth in their lower jaw that form a dental comb. These long, narrow teeth project nearly straight forward from the jaw and this specialized dentition is thought to aid in grooming (Swindler, 2002).

Males and females are minimally dimorphic. Males can be easily identified by their hairless black scrotums and appear slightly larger in the head, upper arms, and shoulders. They have well-developed wrist and brachial glands. Both sexes utilize anogenital glands for scent marking (Cawthon, 2005).

The species is not considered territorial in a strict sense, but they will defend seasonal resources against other ring-tailed lemur troops, as cited by Sauther & Sussman (1993). They are diurnal and more terrestrial than other lemur species (Jolly, 1966).

Ring-tailed lemurs spend most of their time sleeping, sunbathing and resting, with males engaging in these activities slightly more than females (Rasamimanana, Andrianome, Rambeloarivony & Pasquet, 2006). The remainder of their time is spent feeding, moving, traveling, and grooming.

These animals live in social groups consisting of multiple males and females that are focused around a single dominant female (Jolly, 1966). The average group size is 13 individuals but it can range from 5 to 27 animals (Jolly, 1966; Sauther, Sussman, & Gould, 1999).
Females stay within their natal groups but males, begin to disperse when reaching three years, and repeat the migration every 3.5 years (Shire, 2012).
In the wild, it is rare for female ring-tailed lemurs to live past 16 years of age and the oldest known wild female was between 18 and 20 years old. Male life span is even less well-known, because of the social system, but have been recorded living to at least 15 years of age (Gould et al. 2003). In captivity, life span has reached 27 years (Jolly 2003).

1.2.2. Distribution

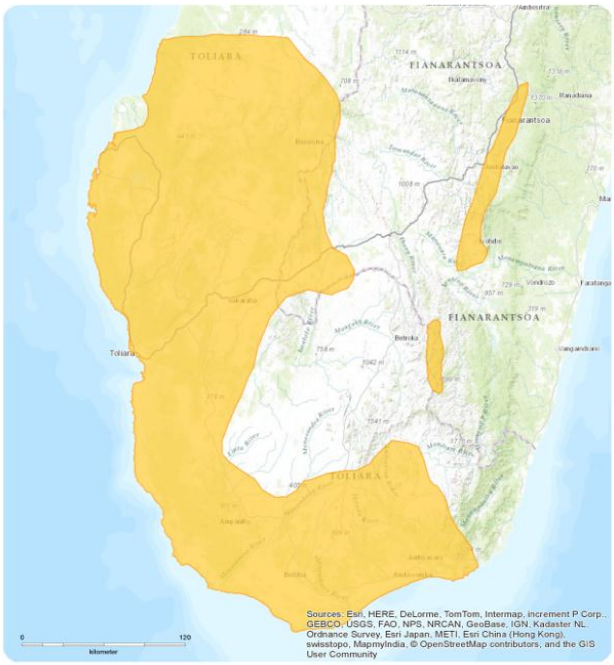
Lemur catta is found in the wild only in Madagascar (Hanlon & Wilson, 2010).
The diurnal ring-tailed lemur is found in dry brush, scrub and closed canopy forests of southern and southwestern portion of this island (Figure 2), and is probably the most terrestrial of all lemur taxa (Mittermeier, Konstant, Nicholl & Langrand, 1992).
Ring-tailed lemurs are patchily distributed throughout this portion of the island and they are found in a variety of habitats up to altitudes of 2600m (Cawthon, 2005).
Rainfall in the southern domain is sparse and irregular, ranging from 300-800 mm and the dry season is marked and very long (Mittermeier et al., 1994).
Because of the highly seasonal environment in which they live, wild ring-tailed lemurs must exploit a wide variety of food sources throughout the year (see below Diet).

Figure 2. *Lemur catta* distribution (IUCN, 2014)

1.2.3. Reproduction

Lemurs are sexually mature when they reach eighteen months of age. However, young males have a marked inferiority complex towards the older females and they do not usually mate till the age of 2.5 years (Basilewsky, 1965).

In captivity, where food is not limited, mothers produce their first offspring at an earlier age, have higher weight neonates, and shorter interbirth intervals, compared to mothers in the wild (Wright, 1999). This suggests that body condition and mother's nutritional state have a strong impact on female's reproduction.



Lemur's reproductive cycle is strongly correlated to a particular season of the year. In the wild births occur between August and November. The same happens in zoos in the southern hemisphere. In the northern hemisphere the situation is different, occurring between March and June.

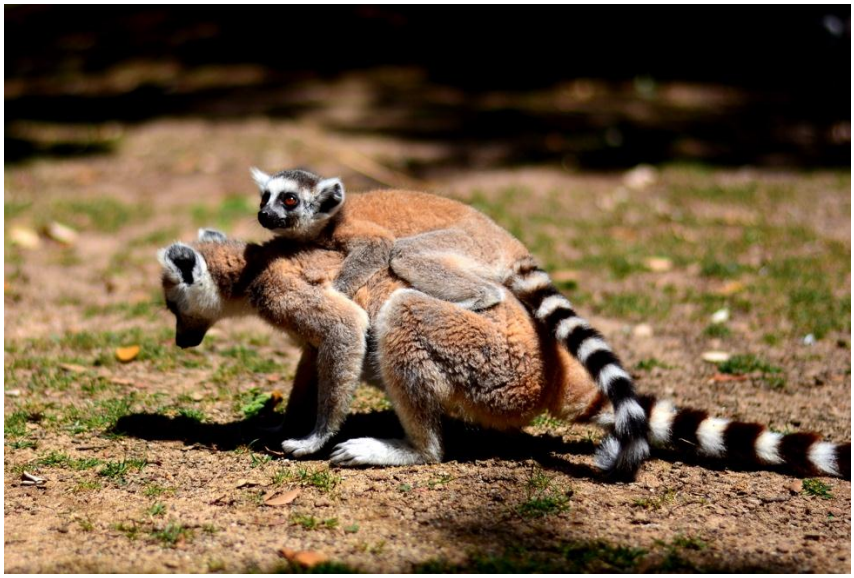
The gestation period range from 130 to 144 days (Hanlon & Wilson, 2010).

In the first days, young animals are seen attached to their mothers' bellies and at about fifteen days, they start to climb on their mother's back (Figure 3). When they reach one month, young lemurs begin to share some food with their mothers.

At six months they are quite independent, though they may continue to suckle till about five months and cling to their mother if danger threatens (Basilewsky, 1965).

Multiple births are rare in both wild and captive populations (Hanlon & Wilson, 2010).

Figure 3. Young lemur attached to mother's back (Original picture)



1.2.4. Diet

Lemur catta feeds primarily on fruit, but extensively on leaves as well (Hanlon & Wilson, 2010). Didiereaceae, an endemic plant family, and various species of *Euphorbia* are the dominant plant forms in their habitat (Mittermeier et al., 1994) and constitute part of their diet. One of the most important food sources for ring-tailed lemurs is the tamarind tree (*Tamarindus indica*) which is abundant in gallery and more open forests away from rivers, and produces fruits and leaves at alternating times of the year, providing a reliable food source throughout the year (Jolly et al., 2002; Mertl-Millhollen et al., 2003). Tamarinds leaves and seed pods can provide up to 50% of the total food consumed during particular seasons of the year and are considered a keystone resource for ring-tailed lemurs (Sauther, 1998; Jolly, 2003).

However, ring-tailed lemurs are best characterized as opportunistic omnivores so, besides ripe fruits and leaves they eat leaf stems, flowers, flower stems, plant exudates, spiders, spider webs, caterpillars, cicadas, insect cocoons, birds, chameleons, grasshoppers, and even dirt from termite mounds (Oda, 1996; Sauther et al., 1999; Jolly, 2003). Like most folivorous primates, ring-tailed lemurs supplement their diet by consuming soil, as it provides a considerable sodium intake (Ganzhorn, 1987).

Figure 4. Ring-tailed lemur eating vegetation (Source: http://etc.usf.edu)

1.2.5. Threats

The primary threats to forests of the southern domain are the collection of firewood and ornamental and medicinal plants, charcoal production and the uncontrolled use of the land for livestock, especially cattle and goats (Mittermeier et al., 1994).

As evidenced by (Wright, 1999) the widespread annual burning, still engrained in the Malagasy culture, has destroyed the seed banks and prevents restoration or recovery of tree cover in many areas. Subsequent over-grazing and the falling of trees for charcoal production further impact wild lemur populations (Andriaholinirina et al., 2014).

Additionally, much of their natural habitat has also been altered by human through deforestation to create settlements.

Actual predation pressure on ring-tailed lemurs is unknown. However, some potential predators include raptors, cat-like carnivores such as fossas and civets, various snakes, and brown lemurs, which have been recorded capturing and eating infant ring-tailed lemurs. Domestic cats introduced to Madagascar are also responsible for predation losses.

According to Kim Reuter (2016), two new independent studies estimate that there are only between 2,000 and 2,400 ring-tailed lemurs — perhaps the most charismatic of Madagascar's animals, and a flagship species of the country — left in the wild. This is a 95% decrease from the year 2000, when the last known population estimate was published. It also means that at present there are more ring-tailed lemurs in zoos around the world than remain in the wild.

In addition, the species is being extracted from the wild for the illegal pet trade, which provides private households with pets and businesses with lemurs for foreign tourists' amusement.

There is a suspected ring-tailed lemur population reduction of more than 50% over a three generation period (36 years, estimating the generation length to be 12 years) (Andriaholinirina et al., 2014).

Based on these premises, it is seriously necessary to implement conservation strategies that may prevent this species extinction.

1.3. Conservation

Fortunately, as evidenced by Kim Reuter (2016) the illegal trade of live lemurs out of Madagascar into the international market is strictly monitored. The author affirms that ring-tailed lemurs in zoos across the world have not been the victims of this trade; rather, they have been bred in captivity and are often incorporated in global breeding programs.

These breeding programs are part of a common conservation plan that integrates responsible entities, in order to safeguard healthy populations, hoping that one day they can be reintroduced in their wild habitat.

Habitat loss and hunting are the greatest causes of concern. Madagascar has undergone major habitat destruction in the last millennium, resulting in a complete loss of 80% of endemic habitat, and this deforestation has resulted in major erosion and drying of western and central habitats (Wright, 1999).

The ring-tailed lemur has a strong preference for gallery forests and for Euphorbia bush, but these habitats are already restricted in southern Madagascar and continue to decrease due to annual burning practices that help create new pasture for livestock (Andriaholinirina et al., 2014).

As affirmed by Jolly (2003), ring-tailed lemurs require some forest cover and are not successful at resettling in secondary growth areas once they have been cleared, therefore the total range occupied is large, but their distribution is patchy and dependent on forest cover.

Ring-tailed lemurs are currently listed as Endangered, since 2014, on the IUCN Red List (2017), and face a series of immediate threats from habitat loss and bushmeat hunting.

Satellite surveys of southern Madagascar indicate that *Lemur catta* habitat is disappearing at an alarming rate, as indicated by Mittermeier et al. (1994). The same author states that more surveys are needed to determine the distribution and sizes of remaining populations. Efforts should also be made to link captive breeding programs with conservation programs in the field.

Captive *Lemur catta* housed in zoos accredited by the Association of Zoos and Aquariums (AZA) are managed under the AZA Species Survival Plan (SSP) (Shire, 2012). The main goal of these plans is to use captive populations to ensure demographic stability and genetic diversity of a species that is threatened or endangered in the wild through breeding and management recommendations (AZA, 2017).

In addition, well-managed captive lemurs could contribute to global breeding programs (Reuter & Schaefer, 2016).

EAZA (European Association of Zoos and Aquariums) member institutions have established Taxon Advisory Groups (TAG) for all the different species of animals that are kept in zoos and aquariums. One of the main tasks of the TAGs is to develop Regional Collection Plans that describe which species are recommended to be kept, why, and how these species should be managed. The Regional Collection Plans also identify which species need to be managed in European Endangered Species Programmes (EEP) and European Studbooks (ESB) (EAZA, 2017).

Those mentioned breeding programmes, EEP and ESB, as well as the Regional Collection Plans (RCP), aim at conserving healthy populations of animals in captivity while safeguarding the genetic health of the animals (EAZA, 2017).

The purpose of these programs is to protect the future of the world's most vulnerable species, such as the *Lemur catta.* This animal, listed as Endangered in IUCN Red List Status 2017, is included in ESB program (IUCN, 2017).

The studbook keeper collects all the data on births, deaths, transfers, etc., from all the EAZA zoos and aquariums that keep the species in question. These data are entered in special computer software programs, which allow the studbook keeper to carry out analyses of the population (EAZA, 2017).

It is possible that EAZA zoos and aquariums may ask the studbook keepers for recommendations on breeding or transfers. By collecting and analysing all the relevant information on the species, the studbook keeper can judge if it is doing well in EAZA zoos and aquariums, or if maybe a more rigid management is needed to maintain a healthy population over the long term (EAZA, 2017).

These captive breeding programs can represent an important solution to restock the forested areas of Madagascar, one of the world's highest primate conservation priorities.

2. Captivity

The potential for zoos to contribute to conservation and education has increased, due to fragmentation and destruction of natural habitats (Rabb, 2004).

Conservation and education are considered two main goals for most zoos (Patrick, Matthews, Ayers & Tunnicliffe, 2007).

2.1. Adaptation

The import of ring-tailed lemurs into Europe started many decades ago, when these animals

were introduced in large numbers. As affirmed by Basilewsky (1965), even in the period between the two World Wars it was rare for a ship to land at Marseilles, coming from Madagascar, that did not have a pair of lemurs on board. At that time, there were lemurs in practically every zoo in the world.

Lemurs are a commonly held species in captivity with an estimated 3,318 ring-tailed lemurs housed in zoos and parks around the world, as registered in 2014 (Species 360, 2018), in addition to many more in smaller roadside collections, laboratories, and pet trade. The species is not only the most common lemur in captivity but indeed the most common of all captive primates (Andriaholinirina et al., 2014).

Ring-tailed lemurs are the most intensely studied of all lemurs. In addition, they are also the most easily recognizable lemurs.

The large number of captive individuals and the existence of ample literature covering species-specific behaviors in the wild make this an ideal population to study.

The ring-tailed lemur is one of the most suitable candidates for captive conditions, when talking about primates. The fact that they have relatively short generation times (compared to anthropoid primates), increases the chance that permanent behavioural changes, as a result of captivity, will become more widespread in the captive population in a shorter amount of time (Shire, 2012).

Like many captive primates, ring-tailed lemurs are typically held in an environment that does not mimic all the qualities of the wild habitat. Therefore, they do not have the full spectrum of behavioural stimuli as wild ring-tailed lemurs (Hosey, 2005; Tarou, Bloomsmith & Maple, 2005).

Despite all the efforts to simulate the wild habitat, the zoo staff cannot replicate a completely natural environment in captivity.

Hence, it is particularly important to study the requirements of individual species. The captive environment should offer the appropriate conditions to encourage the entire behavioural potential of an animal. This includes providing appropriate social conditions, which for primate species play a particularly important role. Primates living in groups have a set of cognitive capabilities and “emotional dispositions” (Netto & Van Hooff, 1986) that optimizes their inclusive fitness. Artificial social systems, such as those found in captivity, are less complex than those in the wild and may not provide adequate social interactions (Shire, 2012).

Therefore, social animals must be kept in an open-air enclosure living in a social group, with its subsequent troop activity, and provided with sufficient space to live a normal healthy life. To follow advice from field scientists on the lemurs' nutritional, social and territorial requirements is essential (Mallinson, 1967).

As cited by Carlstead (2000), one of the primary challenges of captive species management is assessing and coordinating husbandry protocols that facilitate the reproductive and

behavioural potential of all individuals in the captive population. The same author also mentioned that standardizing methods to describe and quantify behaviour of animals housed at different institutions is an essential tool for understanding intra-species behaviours.

Taking these facts into account, it is of singular importance that zoo vets and keepers have in mind the necessity of follow guidelines that demonstrate the correct practices to keep these animals in appropriate captive conditions.

When considering the effect of the zoo environment on a captive species, it is essential to first consider the natural history of the species and the behaviour of conspecifics in the wild (Hosey, Melfi & Pankhurst, 2013; Sherwen, Hemsworth, Butler, Franson & Magrath, 2015). Ring-tailed lemurs are characterized by their behavioural flexibility and adaptability (Sauther et al., 1999).

This species has proven to be easily kept in captivity and to breed readily in the right conditions.

Zoos and wildlife parks might be the future for this species, whose adaptability, social intelligence, opportunistic behaviour and ability to adjust to new environments, will certainly be the appropriate attributes for the success in captivity.

Figure 5. Attempt to mimic the wild habitat using a rope for young lemurs (Original picture)

2.2. Common problems in captivity

2.2.1. Obesity

According to Mallinson (1967), the requirements of lemurs in captivity were hardly

considered, so animals that need a lot of exercise, fresh air and sunshine were confined to small heated indoors accommodation, and kept singly or in pairs. As a result, some of them have become excessively fat and incapable of breeding, as affirmed by the author.

This is an important aspect to take into consideration, since one of the main health problems of captive ring-tailed lemurs is obesity. This generally occurs due to the selective consumption of preferred food items by alpha individuals in the group (Tyler, 2008).

Obesity is a major nutritional problem in captive lemurs and results from general overfeeding or overfeeding of highly palatable foods (Junge, Williams & Campbell, 2009). These authors suggest that providing excessive quantities of food, feeding inappropriate amounts of high-sugar and high-starch foods relative to primate biscuit, the overuse of food for environment enrichment, and the lack of exercise in captivity contribute to obesity.

It is quite common to see overweight or even obese lemurs in captivity (Goodchild, 2008). Indeed, studies comparing wild and captive lemurs have concluded that those kept in captivity are heavier than the ones found in the wild. The author states that this is a problem that faces many collections, probably due to a lack of knowledge and information.

A key characteristic of lemur physiology is a low basal-metabolic rate, also reflected in their behaviour. Folivorous species minimize energy expenditures to use a diet marginal in energy. Sportive lemurs spend up to 85% of their time eating or resting, with a resting metabolic rate that is among the lowest measured in mammals (Junge et al., 2009).

In addition, once obese lemurs become inactive and lethargic they do not burn off their excess energy. As a result, they will gain even more weight, intensifying the situation.

When it comes to breeding problems, and considering obese females, the cycle may not occur properly or the young are sometimes too large, which can carry serious difficulties for females trying to give birth.

Periodic weighing paired with well-chosen girth and skinfold measurements with animals of known linear dimensions would help zoos to monitor the effects of dietary and housing adjustments, established to enhance reproduction, condition and welfare (Carolina, Keynes, & Kingdom, 1995).

Besides causing breeding problems, obesity also leads to other health consequences. Coronary heart disease and diabetes are two of the main problems that can derive from obesity.

2.2.2. Diabetes

Information on the prevalence of diabetes mellitus in lemurs is limited. However, occasional case reports mentioning a high prevalence of diabetes in lemurs can be found.

It is unknown if there is a species or genetic predisposition in lemurs for developing this condition; however, obesity has been implicated as a risk factor in ring-tailed lemurs (Junge

et al., 2009).

Diamond (2003) suggested that “there is now a diabetes epidemic among captive populations of many primate populations” and that can be attributed to their “zoo lifestyle”.

The most common diabetes type among lemurs is Type II diabetes mellitus. This condition presupposes that there’s an inadequate use of insulin or the production of this hormone is compromised. Therefore, high levels of blood glucose are usually found.

Type II Diabetes mellitus is related to obesity and insulin resistance (Kuhar, Fuller & Dennis, 2012) which in turn may be related to a number of factors in captive animal husbandry, including stress, diet, lack of exercise, and lower fecundity (Wagner et al., 2006).

Nutritional management of diabetic lemurs consists of limiting the consumption of simple sugars and starches, increasing dietary levels of fibre, fat, and protein, and spreading feedings throughout the day to minimize fluctuation in blood glucose levels (Junge et al., 2009).

In the early stages of diabetes, nutritional management alone may provide sufficient control; however, as diabetes progresses, medical management with oral hypoglycemic agents or insulin may be necessary in addition to dietary modifications and weight management to control the condition (Junge et al., 2009).

2.2.3. Iron Storage Disease

When reflecting about common health problems in lemurs, it is extremely important and almost inevitable to consider iron storage disease, a syndrome usually referred to as hemosiderosis or hemochromatosis (Wood et al., 2003). This disease, has been studied since many decades ago, due to a high prevalence in captive lemurs.

Iron storage disease in lemurs has been reported since as early as the 1960s, and in the 1980s was demonstrated to be a consistent finding in postmortem investigations of captive lemurs (Wood et al., 2003). According to Junge et al. (2009), reports of hemosiderosis or excess iron accumulation in tissues of lemurs at necropsy initially appeared in the literature in the 1980s.

Excessive iron storage is a condition in which higher amounts of iron than normal are in circulation, iron is deposited within the body, or both. Sometimes, the finding is directly associated with clinical signs, disease, or mortality, but sometimes it is just an incidental finding at necropsy without evident involvement in the cause of death (Clauss & Paglia, 2012).

Spelman et al. (1989) found that captive lemurs were extremely susceptible to excess iron deposition (hemosiderosis) in the duodenum, liver and spleen, and they attributed this disease to a diet rich in iron and ascorbic acid, and poor in tannins.

Whatever the reasons for ISD susceptibility, reducing dietary iron levels to maintenance requirements of the species in question seems to be a logical preventive measure (Clauss &

Paglia, 2012).

3. Pathophysiology of Hemosiderosis or Iron Storage Disease

3.1. Iron metabolism

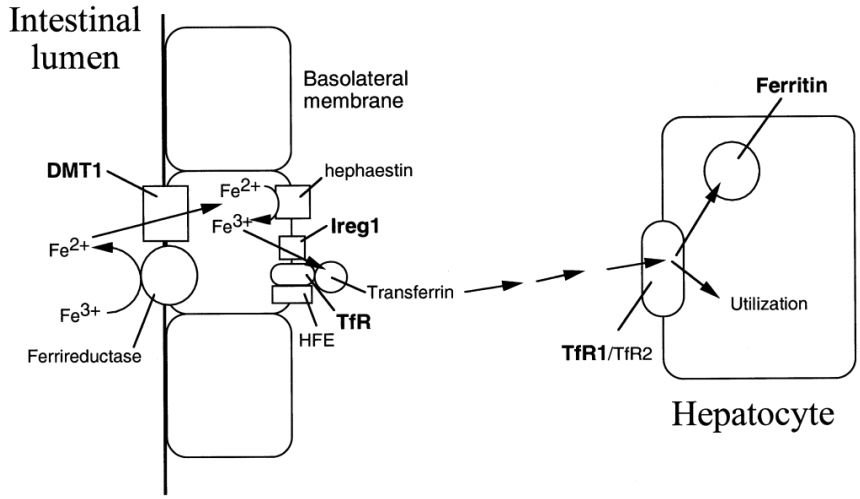
Iron is an essential mineral and is involved in many physiologic events, including oxygen transport, electron transport and DNA synthesis (Abbaspour, Hurrell & Kelishadi, 2014).
However, when ingested in excessive quantities, this element can become toxic. This toxicity involves many organs and can lead to a variety of serious diseases, such as liver disease, heart disease, diabetes mellitus, hormonal abnormalities dysfunctional immune system, etc. (Kang, 2001).
Iron is not easily excreted; therefore, absorption of dietary iron generally dictates body stores (Beard, Dawson & Piñero, 1996).
When iron absorbed by the body exceeds amounts needed for normal physiologic functions, the excess is stored in combination with the protein apoferritin, forming ferritin micelles. (Williams, Junge, & Stalis, 2008; Kumar, Abbas & Aster, 2017). Hemosiderin is a granular pigment that represents large aggregates of these ferritin micelles (Kumar et al., 2017). An excessive systemic load of iron that is characterized by abundant hemosiderin in a variety of tissues without impairment of the organ function is called hemosiderosis (Zachary, 2017).
Excessive accumulation of iron in tissues results in pathologic changes in those tissues, due to ferrous ion's (Fe^{2+}) ability to catalyse reactions that generate toxic free radicals. In addition, free iron readily damages tissues (Abbaspour et al., 2014).
Dietary iron contains both inorganic iron and the heme form.
This mineral is initially absorbed in the upper portion of duodenum. Iron is released from heme by heme oxygenase and enters the plasma as inorganic iron, to be bound to a transport protein, transferrin, which is responsible for iron's blood transportation (Figure 6) (Conrad, Umbreit & Moore, 1999).
Once absorbed, iron is recycled extensively so that iron losses are low (Conrad, Umbreit & Moore, 1994).
Most forms of dietary iron occur in the oxidized ferric (Fe^{3+}) state; however this form is poorly absorbed unless it is either reduced or chelated, because ferric iron is insoluble in aqueous solutions more alkaline than pH 3, whereas most ferrous iron remains soluble at neutral pH (Conrad et al., 1999).
There are two major chemical forms of iron in a mixed diet, and each is absorbed by a different mechanism (Morck & Cook, 1981). The same authors describe heme form as the

one found in haemoglobin and myoglobin and equivalent to 40% of the iron present in animal tissue. On the other hand, the iron in non-heme form, which is correspondent to the remaining 60%, must be released before it can be absorbed. Non-heme form can be found in vegetables and its availability is affected by other dietary components.

Iron is stored primarily as 2 non-heme compounds: ferritin and hemosiderin. These are found throughout the body but especially in the liver and spleen (Sheppard & Dierenfeld, 2002).

Factors affecting bioavailability and absorption are numerous and include the animal's iron status (i.e. iron deficient or replete), the animal's age and sex, the chemical form of iron in the diet, and levels of other dietary components including vitamins, minerals, fibre, and secondary plant compounds (polyphenols, tannins, phytates, etc.) (PTAG, 2003). Dietary constituents that solubilize iron enhance absorption, whereas compounds that either precipitate or polymerize iron decrease absorption (Conrad et al., 1999).

The conversion of dietary Fe3+ to the more "bioavailable" Fe2+ is enhanced by ascorbic acid (vitamin C), which provides enough reducing power to increase the absorption of non-heme iron by three to five fold (Monsen, 1982).



The extent of vitamin C's enhancement on iron absorption, however, depends on a number of factors including dietary levels of fibre, phosphates, and phytates (PTAG, 2003).

Figure 6. Iron absorption and transport scheme. Molecules shown in bold (DMT1. Ireg1, Tf R1 and ferritin) are regulated through iron-responsive elements (Templeton & Liu, 2003)

High dietary levels of manganese, copper, cobalt, cadmium, and zinc decrease absorption of iron, evidently by competing for binding sites (Sheppard & Dierenfeld, 2002). Therefore, high levels of dietary iron may interfere directly with absorption of copper, leading to secondary deficiencies of that nutrient.

In addition, plant polyphenols have been shown to decrease iron absorption by binding dietary iron, making it unavailable for uptake (PTAG, 2003).

Literature indicates that the excess intake of dietary iron and ascorbic acid (citrus fruits) combined with an insufficient amount of tannins ingested, can lead to an excessive accumulation of iron in the animal tissues, also named hemosiderosis.

It is important to distinguish between hemosiderosis and hemochromatosis. Iron accumulates first in the mucosal cells of the duodenum and then is preferentially stored in the liver, spleen, and bone marrow as hemosiderin (Glenn, Campbell, Rotstein & Williams, 2006).

When hemosiderin is detected in tissues with no evidence of toxicosis the condition is termed hemosiderosis. The term hemochromatosis is reserved for conditions in which there is functional or morphologic evidence of iron toxicosis (Williams et al., 2008) and in which pathologic changes have occurred (Junge et al., 2009). Hemochromatosis is an abnormally increased storage of iron within the body that can cause hepatic dysfunction (Zachary, 2017). This disease affects multiple tissues and can originate liver and heart disease, diabetes mellitus, neurodegenerative disorders, organ fibrosis and an increased risk of cancer, predominantly hepatic carcinomas.

3.2. Epidemiology

Excessive burden of iron, or iron storage disease, has been reported in a large variety of captive mammal species, including browsing rhinoceroses; tapirs; fruit bats; lemurs; marmosets and some other primates; sugar gliders; hyraxes; some rodents and lagomorphs; dolphins; and some carnivores, including procyonids and pinnipeds (Clauss & Paglia, 2012).

The disease occurs most commonly in species that, in the wild, feed primarily on fruits and insects, which are generally poor sources of dietary minerals (Sheppard & Dierenfeld, 2002).

The discovery of high susceptibility of lemurs to iron overload has generated a significant concern regarding this species. With natural diets low in iron, this species may have developed physiological mechanisms to compensate this scarcity, extracting dietary iron very efficiently (Spelman et al., 1989; Dierenfeld, Pini & Sheppard, 1992).

The disease has been particularly investigated in lemurs and marmosets (Clauss & Paglia, 2012). The same authors concluded, based in several surveys of lemur pathology, that there is a very high incidence of excessive iron storage in many lemur species with evident differences between free-ranging and captive specimens.

However, although *Lemur catta* is considered susceptible to ISD, differences among lemur species are evident and ring-tailed lemur tends to develop hemosiderosis to a lesser extent in captivity than other lemur species (Glenn et al., 2006).

3.3. Aetiology

Iron overload disorders represent a heterogenous group of conditions resulting from inherited

and acquired causes (Siah, Ombiga, Adams, Trinder & Olynyk, 2006).

A syndrome of excessive iron accumulation, leading to hemosiderin deposits (hemosiderosis) was first recognized in lemurs as early as the 1960's, but descriptive reports of the condition were not published until the 1980's (PTAG, 2003).

Reports of hemosiderosis in captive lemurs published in the 1980s described excessive iron deposits in tissues at necropsy in 67% to 100% of lemurs examined, whereas wild lemurs dying within a month of importation had no detectable hemosiderin deposits (Williams et al., 2008).

As referred, the aetiology of ISD can either be genetic, caused by heritable changes in iron uptake and storage, or acquired. Although the causes for the high incidence of this disease in lemurs were not completely clarified yet, there is strong evidence, based on several experimental studies, that supports the hypothesis of being related to nutritional causes.

Lemurs have physiological mechanisms to compensate the scarcity of dietary iron to which they are submitted in the wild. This can lead to an enhanced predisposition to ISD when the levels of dietary iron are above those found in standard captive diets (Spelman et al., 1989; Dierenfeld et al., 1992). One explanation is that the captive lemur ingests and stores more dietary iron than it is genetically capable of utilizing (Spelman et al., 1989).

The natural diet of affected species provides them with lower levels of available iron than the diet in captivity, meaning that those species did not have to develop mechanisms to protect them against iron overload. This is particularly important, when animal by-products are used as a protein source in commercial diets fed to frugivores and insectivores (Sheppard & Dierenfeld, 2002).

Manufactured complete feeds often inadvertently contain high amounts of iron, not because it is added deliberately, but because it is contained in various ingredients, especially in sources of other minerals, such as calcium carbonate or phosphorus sources, and because of small inevitable abrasions from the processing machinery (Clauss & Paglia, 2012). For example, commercial monkey biscuits fed to lemurs provide 15 times the human requirement of iron per kg (Gonzales et al., 1984).

As a link to diet has been proposed, some researchers have recommended altering the diets of captive lemurs to more closely mimic the presumed diet of wild lemurs (Williams et al., 2008).

Initial reports of iron overload in captive lemurs suggested that captive diets containing low levels of natural iron-binding ingredients (such as tannins) and high levels of iron and vitamin C were responsible for the high incidence of hemosiderosis observed.

Tannins are polyphenols that prevent the iron uptake by the duodenum mucosal cells. These secondary plant compounds act by chelating transition metals such as iron, which is bound to a hydroxyl group.

There are several plants that include these elements in their composition, as the tree

Tamarindus indica, predominant on the soils of Madagascar (Figure 7).

The tamarind dominates the gallery forest of western Madagascar and contains from 7 to 32% tannin in its leaves, pods, and bark (Figure 8) that are consumed by ring-tailed lemurs, constituting almost 50% of their natural diet (Spelman et al., 1989).

Such plants with high concentrations of tannins are not included in the diet of captive lemurs. In addition, the iron absorption and bioavailability is enhanced by ascorbic acid, also known as vitamin C, provided in many fruits and vegetables offered in captive diets.

The high levels of dietary ascorbic acid also increase the formation of free-radical reactions that produce the toxic effects of iron (Spelman et al., 1989).

Figure 7. *Tamarindus indica* tree (Richard & Francis, 2016)

Figure 8. Leaves, pods, seeds and barks from *Tamarindus indica* tree (Source: pfaf.org)

3.4. Symptomatology, post-mortem lesions and histopathology

The disease can be directly associated with clinical signs or mortality, but sometimes it is just an incidental finding at necropsy without evident involvement in the fatality (Clauss & Paglia, 2012).

Usually, the first clinical signs are caused by liver fibrosis, resulting in circulatory failure, ascites and hypoalbuminemia (Sheppard & Dierenfeld, 2002).

Besides liver disease, excess tissue iron deposition can lead to an increased risk of cancer, organ fibrosis, heart disease, decreased immunity, and diabetes mellitus (Glenn et al., 2006).

The histopathology of this condition has been well described and follows a characteristic pattern: clusters of hemosiderin appear first in the phagocytic cells of the duodenum (the normal site of iron absorption) and accumulates in liver, kidney, spleen, and bone marrow. In severe cases, this pigment also accumulates in the parenchymal cells and interstitial areas of various organs, particularly the liver (Spelman et al., 1989). In this situation, when iron overload becomes more pronounced, hepatic cell necrosis and periportal fibrosis can occur.

At necropsy, considerable liver damage and disease are often observed in lemurs, with subsequent staining revealing the presence of iron in the damaged liver and other organs (Lowenstine & Munson, 1999; Dorrestein, Sa, Ratiarison & Mete, 2000; Smith, 2000).

Additionally, there are several reports that relate a high incidence of neoplasia in animals with ISD, enouncing hepatocelullar carcinoma as the most common necropsy finding.

It is now clearly recognized that chronic iron storage disease can entail serious health

consequences for many captive wild mammals, such as lemurs. Therefore, the implementation of prophylactic programs against ISD becomes particularly indispensable.

Figure 9. Deposits of hemosiderin found in the liver of a black-and-white ruffed lemur (Adapted from Burnum, 2016)

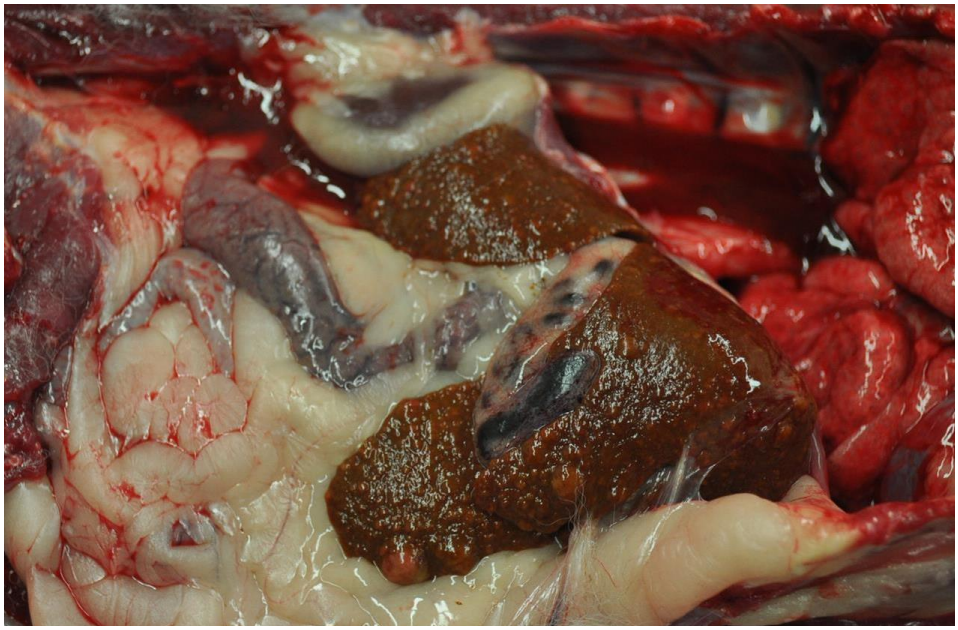
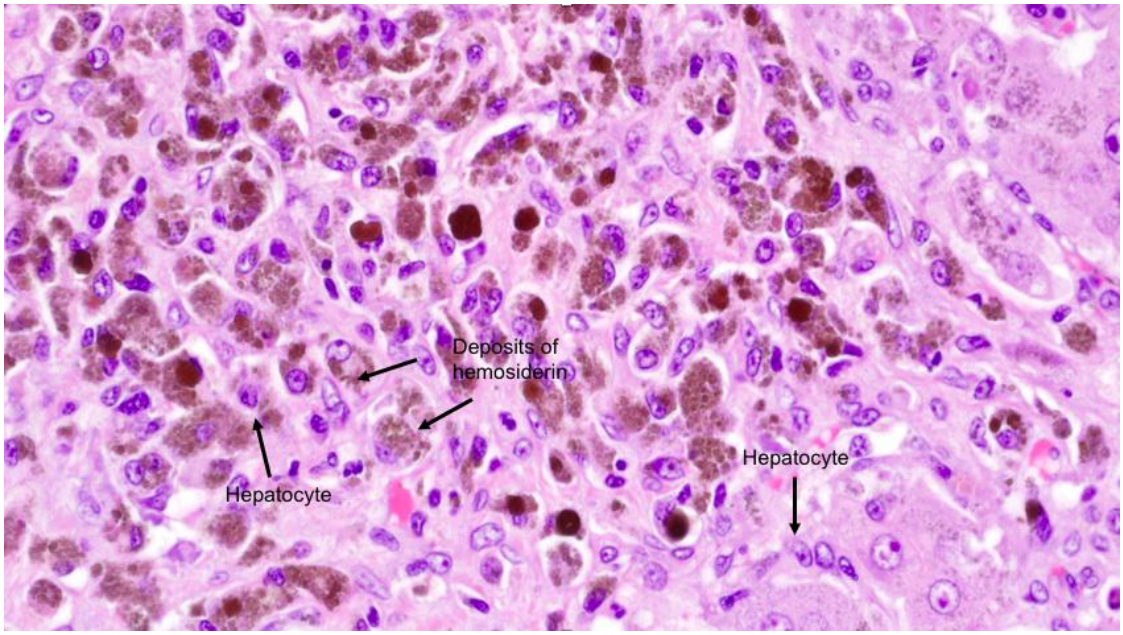


Figure 10. Histopathologic findings: Deposits of hemosiderin in the hepatocytes of a black-and-white ruffed lemur (Adapted from Burnum, 2016)



3.5. Diagnosis

Evaluating the iron status of lemurs is difficult, and hemosiderosis is most consistently diagnosed at post-mortem examination (Williams et al., 2008).

The definitive antemortem diagnosis of iron storage disease can only be made by hepatic biopsy (PTAG, 2003), a non-practical procedure to be done in zoos that would not be well tolerated by the animals. The lack of a non-invasive method to evaluate iron status in captive lemurs limits investigators' ability to effectively screen animals for the presence of ISD, and to detect the condition early when treatment protocols are most effective (Williams et al., 2006). Nevertheless, a complete investigation of lemur iron status should be a recurrent preventive strategy implemented in most lemur husbandry programs. This comprises diagnostic tests, including total serum iron (SI), total iron binding capacity (TIBC), ferritin and percent transferrin saturation (%TS).

Measurements of SI concentration, TIBC, ferritin concentration, and transferrin saturation are routinely used to evaluate iron status in humans and various domestic animals (Smith, 1997; Bassett, Halliday, Bryant, Dent, & Powell, 1988; Kang, 2001).

Although serum iron tests can be used to diagnose iron metabolism disorders, they are not specific for iron storage disease. Additionally, it is important to take into consideration that the time of the day, the recent ingestion of a meal, the regular consumption of iron-containing supplements, chronic inflammation, infection, liver disease and malignancy can all influence the measurement of iron analytes in serum (Williams et al., 2006).

Complicating this issue, serum ferritin concentration is usually measured using an immunologic assay that is species specific. When ferritin assays are unavailable, transferrin saturation is a useful and readily available backup option for monitoring iron status (Clauss & Paglia, 2012).

Transferrin saturation is an assessment of tissue iron supply, calculated using serum iron expressed as a percentage of total iron binding capacity (Williams et al., 2008). It represents the percentage of all binding sites in the serum that contain iron; as it becomes saturated, iron is deposited in the liver (Dutton, Junge, & Louis, 2003).

Transferrin saturation decreases in iron deficiency and in chronic disease states associated with infection, inflammation, and malignant disease. Values increase in response to increased iron absorption from the gastrointestinal tract and increased demand for hematopoiesis (Williams et al., 2008).

The %TS value is regarded as a highly reliable diagnostic parameter in human hemochromatosis (O'Hara, Cavanagh, Cassidy & Cullina, 2003). The %TS gives a quantified indication of whether excessive amounts of iron are being absorbed from the diet. This can reveal the problem prior to excessive iron deposition and the damage to internal organs that would, over time, result from that excessive absorption (Wood et al., 2003).

Non-dietary factors should be considered as they can also affect %TS values. Recent blood loss (e.g., from parturition, injury or phlebotomy), certain malignancies and inflammation falsely decrease %TS, while hepatitis, pregnancy, contraceptive agents, hyperthyroidism and recent ingestion of iron-containing supplements cause %TS to increase (Williams et al.,

2006)

Williams et al. (2008) concluded that transferrin saturation was positively correlated with hepatic iron concentrations in ruffed and ring-tailed lemurs, but not in black lemurs, according to their study.

While the number of studies regarding this subject is increasing, researchers are still facing a lack of information concerning iron metabolism in lemurs.

Normal reference ranges for serum iron, TIBC and serum ferritin do not currently exist and the utility of these tests as predictors of total body iron stores in lemurs remains to be determined (PTAG, 2003).

Although Gonzales et al. (1984) previously used human standards to evaluate iron parameters in lemurs, it has never been determined experimentally whether human reference ranges for serum ferritin or %TS can be extrapolated to lemur species. In humans, persistent elevations of ferritin 4400ng/mL and TS 45–70% are considered risk factors for developing iron overload and warrant additional testing (Edwards, Griffen, Goldgar, Drummond, Skolnick & Kushner 1988; Finch, Bellotti, Stray, Lipschitz, Cook, Pippard & Huebers, 1986; Kang, 2001; Witte, Crosby, Edwards, Fairbanks & Mitros, 1996).

According to (Wood et al., 2003), the %TS in animals is normally above 15% and may rise to 50%, in dietary iron sufficiency.

Dutton et al. (2003) reported mean values of 71 μg/dl for SI, 241 μg/dl for TIBC and 41 ng/ml for ferritin, measured in a population of ring-tailed lemurs in the Tsimanampetsotsa Strict Nature Reserve in Madagascar. Using these values, it is possible to calculate an average of approximately 30% for TS in this free-ranging population.

Lemurs with serum iron and transferrin saturation above 27 μmol/L (150 μg/dl) and 50% respectively, but serum ferritin below 100 μg/L (ng/ml), are considered to be at risk to have accumulated toxic levels of iron (Crawford, Andrews, Chavey, Dunker, Garner & Sargent, 2005). In these cases, the authors recommend that every effort should be made to reduce dietary iron uptake and iron indices, and physiologic parameters should be evaluated frequently. However, considering the same values for SI and TS, but serum ferritin consistently above 100 μg/L (ng/ml), the authors consider that liver biopsies should be performed to assess the extent of iron deposition and tissue damage, since the animals in this case may have already accumulated excess iron.

Therefore, despite the lack of reliable information regarding reference ranges for %TS in captive ring-tailed lemurs, it is commonly accepted, based on existing literature, that the upper limit for these animals is 55%. As previously mentioned, it is important to remember that the %TS is a useful iron status indicator, but not a definitive diagnostic method of ISD.

For this reason lowering the %TS in a lemur is not the same as treating it for ISD. If a lemur has already developed considerable iron stores, lowering the %TS will stop it from increasing more, but will only provide a very slow decrease of body's overall iron storage (Wood et al.,

2003).

Taking this into account, it is important to consider the viable options of treatment for these animals, in order to decrease the iron storage.

Table 1. Transferrin Saturation (%TS) baseline values in human medicine and their interpretation (Wood et al., 2003).

% TS	Diagnosis	Indicative of
<15%	Dietary iron insufficiency	Iron responsive anemia
15-25%	Boundary low	Boundary deficiency
25-44%	Normal iron intake	Normal healthy animal diet
45-55%	Boundary high	Boundary excess
>55%	Iron overload	Hemosiderosis/Hemochromatosis

3.6. Treatment

The treatment of affected individuals includes phlebotomy, the application of iron chelators, and a reduction of (available) dietary iron levels (Clauss & Paglia, 2012).

Phlebotomy has been used in birds, rhinoceroses, dolphins and lemurs. It has been established as an effective therapeutic measure for human genetic hemochromatosis (Witte, 1997). The periodic blood draws likely lowered the amount of iron available for storage in the *Lemur catta* (Glenn et al., 2006).

Phlebotomy and chelation therapy have been performed with some success in individual lemurs (Clauss & Paglia, 2012).

Chelation may be used alone or in conjunction with phlebotomy (Miller & Fowler, 2014).

The gold standard for chelation therapy is deferoxamine (DFO), but the fact that this drug must be given parenterally, has spurred the search for oral agents capable of mobilizing iron safely. Two such drugs have emerged, deferiprone and deferasirox (Beutler, 2007).

A combination of chelation (DFO 10mg/kg, IM, every other day for 4 weeks) and phlebotomy (10ml/kg, weekly) resulted in decreased SI, serum ferritin (SF), TS, bilirubin and bile acids in a lemur with severe hemochromatosis; however, the animal died of liver failure and hepatocellular carcinoma (Miller & Fowler, 2014).

As demonstrated, the current ISD therapy for captive wild animals is not a practical measure to implement in zoological facilities.

For this reason, it is necessary to adopt preventive strategies that aim to reduce the incidence of hemosiderosis in susceptible captive species.

3.7. Prevention

It is possible that the tendency to develop iron storage disease varies among institutions depending on diet and husbandry protocols, and possibly the genetic stock of the collection (Williams et al., 2006). However there is a general agreement that the cause of hemosiderosis in the lemur is dietary (Spelman et al., 1989). Thereby, reducing the dietary iron levels to maintenance requirements of the species in question seems to be a logical, preventive measure (Clauss & Paglia, 2012).

This can be considered the first step when talking about ISD prevention in captive lemurs.

Dietary manipulation, decreasing iron (<100ppm DM basis), limiting vitamin C, and/or adding ingredients that bind iron, is the most important means of preventing iron absorption and overload in zoological species (Miller & Fowler, 2014).

It is clear that the captive diet for lemurs has the potential to increase iron uptake and thus enhance any physiological differences in iron metabolism possessed by these primates (Spelman et al., 1989).

There is one report of a massive dietary intervention that reduced the amount of iron and vitamin C, and presumably also decreased iron availability by adding sources of tannin to the diet, thereby reducing the serum transferrin saturation when comparing measurements before and after the intervention (Clauss & Paglia, 2012). In their study, Wood et al. (2003) also noticed a considerable difference in three different lemur species by comparison of %TS values before and after the dietary change.

When feeding animals potentially susceptible to ISD, commercial feeds high in iron should be avoided, since conventionally produced pelleted feeds generally contain higher levels of iron than are found in natural herbivore forages (Clauss et al., 2002). However, a reduction in the use of pelleted feeds carries the risk of reducing a well-balanced diet item from the overall ration, and should be implemented with particular caution (Wood et al., 2003).

Lemur diets should be balanced and nutritionally complete in concordance with the current standards for primates published by the National Research Council (PTAG, 2003).

Junge et al. (2009) considered as a guideline, the total amount fed should be approximately 2.0% to 2.5% by dry weight of the animal's ideal body weight, with manufactured biscuit accounting for 80% to 85% of the dry matter, and the fruit and vegetable portion making up the remaining 15% to 20%. The authors recommend feeding approximately 25g per day of primate biscuit per kilogram of ideal body weight plus 35g per day of fruit and vegetables mix

per kilogram of ideal body weight.

Despite the metabolism and dietary requirements are undefined for most species, the National Research Council estimates the adequate dietary iron concentration (dry matter basis) for nonhuman primates to be 100 mg/kg (PTAG, 2003).

Iron concentrations as low as 65 ppm DM basis for lemurs have been recommended, but it is difficult to formulate balanced diets this low (Miller & Fowler, 2014).

The fact that it is the iron-limited diets that are more difficult to produce and therefore expensive, rather than iron-fortified diets, may contribute to a reluctance of changing feeding regimes (Clauss & Paglia, 2012).

A safe upper limit for dietary iron has not been established for nonhuman primates. However, special attention should be paid to the use of mineral supplements, in particular calcium sources that may contain high iron levels, and mineral supplements without iron should be used (Clauss & Paglia, 2012). Supplements are not needed if the animals are fed a well-balanced diet in proper proportions and amounts (PTAG, 2003).

As previously mentioned the high dietary levels of vitamin C enhance the iron absorption and can contribute to the development of hemosiderosis. For this reason, Spelman et al. (1989) suggest that the use of citrus fruits in lemur diets should be critically assessed, as well as other fruits and vegetables rich in vitamin C.

Feeding a well-balanced plant-based diet containing species-appropriate fibre levels will likely minimize excessive iron absorption in diurnal lemurs (Junge et al., 2009), since dietary levels of fibre, phosphates and phytates can influence the extent of vitamin C's enhancement on iron absorption.

The addition of locally available fresh leaves and browse is beneficial for diurnal species as a means of increasing fibre in the diet, stimulating natural feeding behaviours, and providing environment enrichment (Junge et al., 2009).

As referred before, it is known that wild lemur diets have higher amounts of tannins, when compared to captive lemur diets. Therefore, iron-binding compounds should be given with the main feed or within an hour after feeding, but not before (Wood et al., 2003).

Indeed, polyphenols such as tannic acid, in the form of tea (leaves or infusion) or tamarind (pods or juice), has been used in birds, lemurs, and fruit bats. This was shown to be effective in lemurs by following %TS (Miller & Fowler, 2014). However, tannins do not affect only iron availability and their addition to the captive diet may lower the digestibility and nutrient availability of other diet components as well, resulting in deficiencies in other micronutrients, not having the desired impact on iron absorption (Zutrition, 2017).

While some investigators have recommended adding tea, beans, or other tannins sources to captive lemur diets in an effort to reduce dietary iron absorption, there is currently insufficient evidence to support these recommendations (Junge et al., 2009).

Hence, the deliberate inclusion of tannins or other polyphenol sources, although successful

in individual experiments and not evidently harmful in others, cannot be recommended at present without further research (Clauss & Paglia, 2012).

Despite the results of many surveys, however, prophylactic programs against ISD have not been implemented in most lemur husbandry programs (Wood et al., 2003).

Adjusting the dietary iron levels so that requirements are not exceeded would represent the most practical approach if limiting iron in diets was not so difficult or against historical feeding traditions (Clauss & Paglia, 2012). Nevertheless, changing captive diets is a crucial step to take in most lemur husbandry programs but not the only key factor for a successful ISD prevention.

Screening protocols based on blood measurements (in tandem with medical training programs that reduce the need for anaesthetic intervention), should be instigated for susceptible species (Crawford et al., 2005). Measurement of serum ferritin concentration in conjunction with transferrin saturation may constitute a non-invasive approach for evaluating iron status in lemurs (Williams et al., 2008).

Diagnosis of this disease has remained consistently at the point of necropsy, and routine screenings that would detect the disease early enough to instigate therapeutic measures are not part of a standard management protocol (Wood et al., 2003). In addition, the investigation of ISD at necropsy of susceptible animals should be implemented as a routine procedure, to estimate the incidence of this disease in the facilities and to increase documented cases of negative findings.

Post that, although results indicate that further investigations should be conducted, there are some practical measures to adopt that seem to be a logical first step.

Current feeding regimes should either be adjusted within reasonable boundaries right away, such as reducing iron levels to maintenance recommendations of model species, with accompanying health monitoring measures, or (further) adjusted on the basis of necropsy findings or screening of live animals (Clauss & Paglia, 2012).

Studies of lemurs housed at multiple institutions are needed to gain a better understanding of the prevalence, severity and clinical impact of iron overload in captive lemurs (Williams et al., 2008).

III. STUDY – Iron Storage Disease Prevalence in Captive Ring-tailed Lemurs

The present study was carried out in three different zoological facilities and included all the ring-tailed lemurs housed at these institutions.
In order to facilitate the analysis of the diet influence, the individuals were divided into three different groups, according to the zoological facility in which they live.

1. Goals

This master's dissertation aims to evaluate the prevalence of ISD in ring-tailed lemurs housed at several institutions from different regions of Portugal and relate it to their captive diet.
The survey may contribute to better understand possible causes for the high prevalence of this disease in susceptible species, such as lemurs. In addition, the present study intends to expose potential strategies that can contribute to ISD prevention.

2. Material and methods

2.1. Sample

The present study was conducted to assess the prevalence of hemosiderosis in captive ring-tailed lemurs in Portugal. The study was carried out in three different zoological facilities and included all the ring-tailed lemurs housed at these institutions. In order to facilitate statistical analysis, the individuals were categorised into three different groups, according to the zoological facility in which they live.
The animals were divided into three groups, correspondent to the three different zoological facilities. Each group had a different number of animals included:

- Park 1: 10 animals
- Park 2: 2 animals
- Park 3: 6 animals

2.2. Institutions description

2.2.1. Park 1

Park 1 is situated in the southwestern region of Portugal in the Alentejo. It includes 600 wild animals of 75 different species in an area of 90ha.

The park is home to ten ring-tailed lemurs, cohabiting with four red-bellied lemurs (*Eulemur rubriventer*). All animals are kept in an open enclosure during the day, spending the night in an indoor installation, separated by species.
The park is a member of the European Association for the Study and Conservations of Lemurs. It is also a consortium of the European Zoological Gardens and Universities, aiming to the implementation of research projects to promote the conservation of different lemur species.

2.2.2. Park 2

This park is situated in the north region of Portugal and is the home to 100 different species of animals, including mammals, reptiles, birds, amphibians and fishes.
Characterized by its pedagogical component, the zoo aims to collaborate in different educational programs, promoting the realization of several activities that intend to sensitize people for the protection of biodiversity.
Similarly, this zoological park contributes to different research programs, through the implementation of partnerships with other zoos and universities.
The institution houses two ring-tailed lemurs living in an indoor enclosure.

2.2.3. Park 3

Located in the north of Alentejo region, in the southwest of Portugal, the park houses 350 animals from 60 different species, in an area of 20ha.
Nature conservation and protection, as well as education for sustainability are their main purposes.
The park is home for eight ring-tailed lemurs, living in an open enclosure during the day. At night, all the lemurs are closed in an indoor installation.

2.3. Sample collection

The blood was collected by external saphenous venipuncture to a serum separator tube and then centrifuged for serum obtaining. The animals were not submitted to anaesthesia and all the samples were collected under minimal stress conditions. Zookeepers from the institution were responsible for animals' capture, using an appropriate net (Figure 11). Immediately after that, the animals were identified using a microchip scanner, and the blood was collected while the zookeeper contained the animal (Figure 12).
The animals' information was registered in an identification form (Appendix 4).
The laboratory analysis was duplicated to increase accuracy in animals from which it was possible to collect more than 1ml of blood.

The blood samples were centrifuged and freezed at minus 20ºC. Samples were sent to the laboratory DNATech for transferrin saturation analysis.
Diets given to the animals in the different institutions were analysed and compared and then related to the prevalence of iron storage disease in the respective zoological facility.

Figure 11. Zookeeper capturing a ring-tailed lemur (Original picture)

Figure 12. Blood collection by external saphenous venipuncture (Original picture)

3. Statistical Analysis

All data collected during the study were loaded onto the program Microsoft Office Excell (Microsoft Office® 2011 for Mac). The statistical analysis was performed using the extension R *Commander*, from software R®, version 3.0.1.

The one-way statistical test ANOVA was used to determine if there were any significant differences between the analysed groups (Park 1, Park 2 and Park 3).

Statistical differences were set at $p<0.05$ for a 95% confidence interval.

The statistical analysis was conducted by calculating sample features of each group (mean, standard deviation, maximum, minimum and median) for transferrin saturation level.

4. Results

4.1. Sample description

All the sample participants (n=18) were submitted to the same protocol, which estimated the levels of transferrin saturation verified at the moment of blood collection.

The sample included 18 animals, 13 males and 6 females, all from the same species - *Lemur catta,* living in captive conditions. Animals' average age was 5.8 years (minimum 1 year and maximum 14 years).

4.2. Blood values

The results obtained from all the individuals are registered in Table 2, separated by groups. It was possible to identify the animal by its microchip identification number.

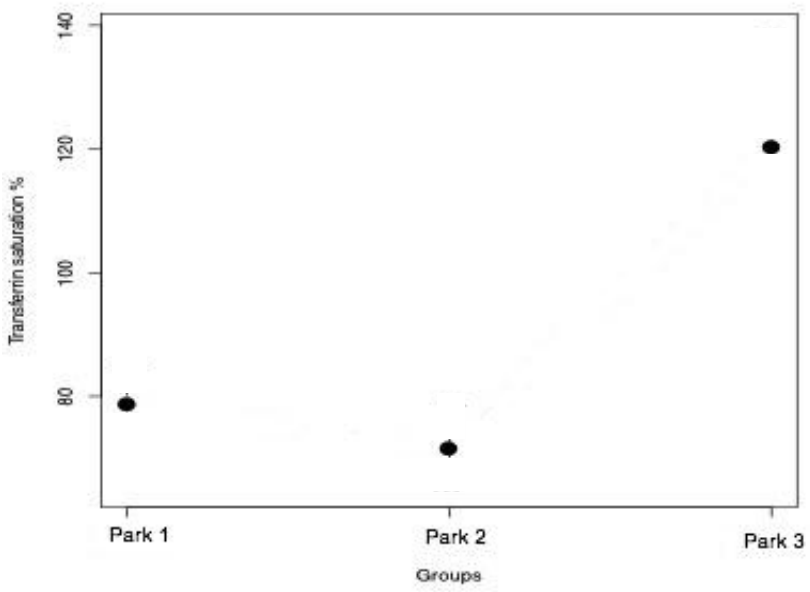
The average transferrin saturation values varied between parks. However, these differences were not significant when comparing Park 1 with Park 2 ($p = 0.8977$) or between Park 2 and Park 3 ($p = 0.1161$).

Only the difference between the groups Park 1 and Park 3 values were statistically different ($p = 0.0404$).

Table 2. Individual transferrin saturation values (Reference values for this species: 20 – 55%). Blood samples were duplicated to increase accuracy when possible.

Institution	Animal identification	Sample 1	Sample 2
Park 1	852	100.3%	
	764	83.2%	100%
	891	91%	88.3%
	784	89.3%	92.7%
	451	48.9%	49.6%
	455	68.9%	70.5%
	588	103.2%	
	742	52.9%	
	751	85.1%	83.4%
	757	56%	56.3%
	Average	78.8%	
Park 2	754	65%	
	759	70%	87%
	Average	71.75%	
Park 3	7630	99.7%	
	6250	71.8%	
	7621	176.8%	
	1108	145.2%	
	6387	70.8%	
	0443	157.6%	
	Average	120.32%	

Graphic 1. Average transferrin saturation values for the different groups



4.2.1. Results presentation per group

All groups analysed demonstrated high average levels of transferrin saturation, considering that the reference range is between 20% and 55% (Table 2 and Graphic 1).

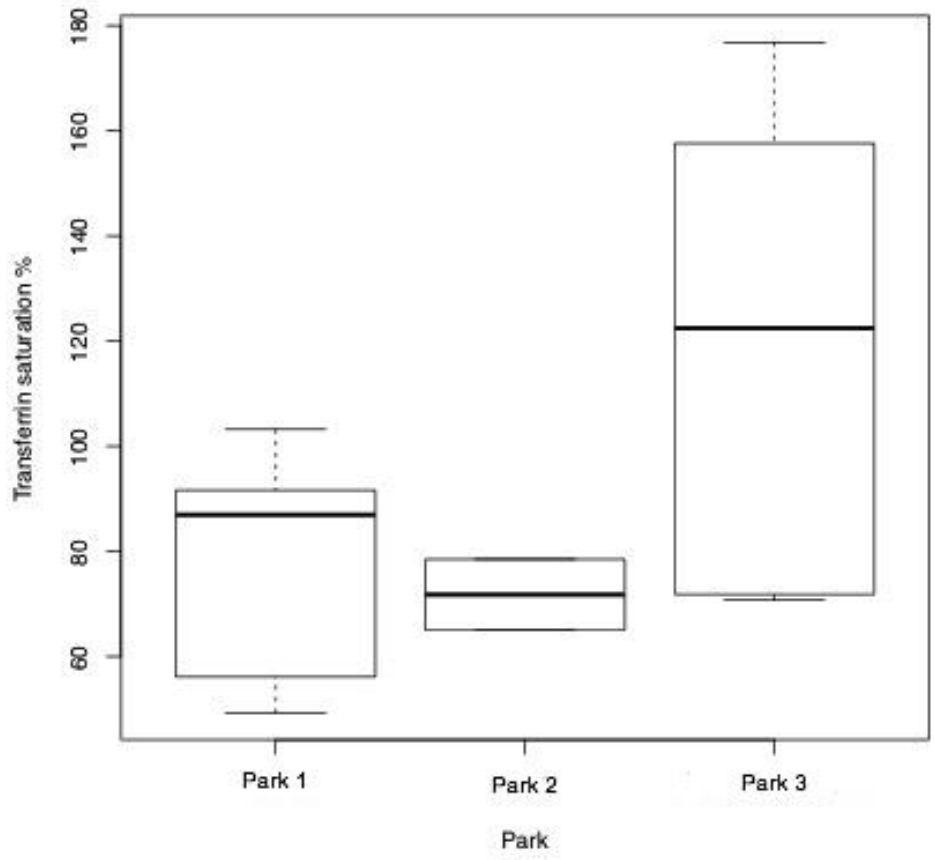
The group Park 3 revealed the highest average level of transferrin saturation, with a mean of 120.32%. On the other hand, the group Park 2 was considered the one with the lowest average level of transferrin saturation, with an average of 71.75%.

The group Park 1 showed intermediate values, with an average level of transferrin saturation of 78.80%.

Table 3. Sample features for transferrin saturation

	Transferrin saturation (%)				
	Mean	**Standard deviation**	**Minimum**	**Maximum**	**Median**
Park 1	78.80	20.16	49.25	103.2	86.95
Park 2	71.75	9.5	65.00	78.50	71.75
Park 3	120.32	45.67	70.80	176.80	122.45

Graphic 2. Results presentation per group



4.3. Diets description

Most of the food given to the lemurs at the parks is offered by supermarkets, as it is reaching the expiry date. This is valid mainly for fruits and vegetables and also for some cereals and supplements, with exception for dry food.

To have a detailed description of the animals' diet, all the food given was minutely described for 15 consecutive days (Table 5, 7 and 11). During this time, the zookeeper registered the type and amount of fruit, vegetables or cereals given, as well as any eventual supplement offered to the animals.

Considering this information, it was possible to elaborate a specific graphic for each park, which allowed to observe the relative percentage of each class of food given to the animals.

4.3.1. Park 1

At Park 1, fruits are the main components of lemurs' diet, representing 91.7% of the food given to the animals. Although constituting a significant minor proportion, vegetables also

represent an important element of their diet (7.47%). The cereals given to the lemurs are counted as just 0.83% of their feed.

It was possible to identify all the types of fruits, vegetables and cereals given to the animals at the park (Table 4).

Table 4. List of fruits, vegetables and cereals given to the lemurs at Park 1

Fruits	Vegetables	Cereals
Apple	Mixed salad (lettuce and carrot)	Seed bread
Banana	Leek	Raisin bread
Pear	Beaded lettuce	
Annona	Endives	
Melon	Green salad	
Mango	Carrot	
Rockmelon	Spinach	
Persimmons		
Watermelon		
Grapes		
Pineapple		
Strawberry		
Plum		

Graphic 3. Constituents of lemurs' diet at Park 1

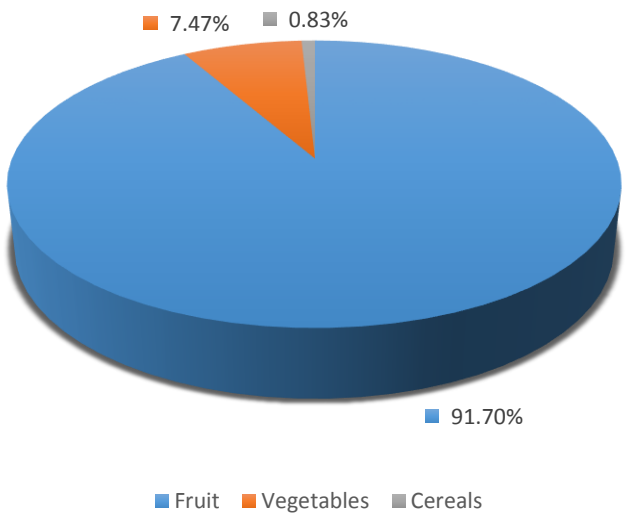


Table 5. Diet analysis for ten animals in Park 1

		Type of food	Amount (pieces)	Unit weight (g)	Total weight (g)
Day 1	A.M.	Red apple	5	130	650
		Banana	5	115	575
		Pear	5	230	1150
	P.M.	Seed bread	1	100	100
		Banana	4	115	460
		Mixed salad (lettuce and carrot)	2	175	350
		Annona	3	220	660
		Pink lady apple	2	185	370
		Melon	0.50	2000	1000
Day 2	A.M.	Red apple	5	130	650
		Banana	5	115	575
		Mango	2	500	1000
	P.M.	Leek	3	200	600
		Beaded Lettuce	0.50	400	200
		Mixed salad (lettuce and carrot)	1	175	175
		Annona	2	220	440
		Banana da Madeira	6	100	600
		Rockmelon	0.50	1250	625
Day 3	A.M.	Red Apple	3	130	390
		Banana	6	115	690
		Melon	0.25	2000	500
	P.M.	Raisin bread	1	100	100
		Persimmons	4	300	1200
		Endives	3	150	450
		Mango	1	500	500
		Melon	0.25	2000	500
		Leek	4	200	800
		Banana	4	115	460
Day 4	A.M.	Red apple	5	130	650
		Banana	2	115	230
		Watermelon	0.25	2500	625
	P.M.	Red apple	6	130	780
		Melon	0.25	2000	500
		Brunches of grapes	2	400	800
		Leek	4	200	800
Day 5	A.M.	Melon	1	2000	2000
		Red apple	5	130	650
		Pineapple	0.50	1300	650
	P.M.	Rockmelon	0.50	1250	625

		Strawberry	11	20	220
		Pear	6	230	1380
		Melon	0.25	2000	500
		Red apple	4	130	520
		Mango	3	500	1500
		Green salad	2	175	350
Day 6	A.M.	Melon	0.25	2000	500
		Red apple	6	130	780
	P.M.	Persimmons	8	300	2400
		Strawberry	30	20	600
		Brunches of grapes	2.50	400	1000
		Banana	4	115	460
		Green salad	1	175	175
Day 7	A.M.	Red apple	9	130	1170
		Strawberry	24	20	480
		Brunches of grapes	2.50	400	1000
	P.M.	Strawberry	10	20	200
		Pear	5	230	1150
		Melon	0.50	2000	1000
		Red apple	4	130	520
		Mango	3	500	1500
		Banana	2	115	230
		Green salad	2	175	350
Day 8	A.M.	Annona	1	220	220
		Red apple	4	130	520
		Pineapple	0.13	1300	162.5
		Banana	4	115	460
		Mango	1	500	500
	P.M.	Mango	4	500	2000
		Persimmons	5	300	1500
		Annona	2	220	440
		Pineapple	0.50	1300	650
		Seed bread	1	100	100
		Leek	5	200	1000
Day 9	A.M.	Red apple	5	130	650
		Plum	13	60	780
		Melon	0.75	2000	1500
	P.M.	Pear	3	230	690
		Seed bread	1	100	100
		Rockmelon	0.50	1250	625
		Mango	3	500	1500
		Persimmons	8	300	2400
Day 10	A.M.	Red apple	4	130	520
		Plum	12	60	720

		Rockmelon	0.50	1250	625
	P.M.	Carrot	5	150	750
		Banana	5	115	575
		Rockmelon	0.50	1250	625
		Seed bread	1	100	100
		Plum	10	60	600
Day 11	A.M.	Melon	0.50	2000	1000
		Pineapple	0.50	1300	650
		Red apple	6	130	780
		Plum	8	60	480
	P.M.	Pear	6	230	1380
		Strawberry	12	20	240
		Red apple	5	130	650
		Pineapple	0.50	1300	650
		Plum	10	60	600
Day 12	A.M.	Mango	1	500	500
		Red apple	7	130	910
		Plum	10	60	600
	P.M.	Persimmons	6	300	1800
		Red apple	4	130	520
		Melon	0.50	2000	1000
		Seed bread	0.50	100	50
Day 13	A.M.	Banana	4	115	460
		Melon	0.50	2000	1000
		Red apple	4	130	520
		Plum	2	60	120
	P.M.	Persimmons	5	300	1500
		Mango	3	500	1500
		Brunches of grapes	2	400	800
		Red apple	4	130	520
		Spinach	1	200	200
Day 14	A.M.	Pear	5	230	1150
		Brunches of grapes	1.50	400	600
		Melon	0.25	2000	500
	P.M.	Mango	2	500	1000
		Banana	5	115	575
		Plum	8	60	480
		Red apple	4	130	520
		Persimmons	2	300	600
		Seed bread	1	100	100
Day 15	A.M.	Banana	4	115	460
		Red apple	4	130	520
		Rockmelon	0.75	1250	937.5
		Pear	3	230	690

	P.M.	Leek and carrot soup	1	350	350
		Mixed salad (lettuce and carrot)	1	175	175
		Seed bread	1	100	100
		Banana	4	115	460

4.3.2. Park 2

Fruit is also the main constituent of lemurs' diet at Park 2, representing 64.12% of the food given. At this park, vegetables are also an important food source, constituting almost 31.65% of their diet.

The park also provides dry food to the animals, although in a much smaller proportion than the other enounced food classes (0.84%).

Cereals represent the smallest proportion of the food given, calculated as just 0.25%.

Occasionally the lemurs receive food supplements that account as 3.13% of their diet.

As shown in Table 6, it was possible to identify all the types of fruits, vegetables, dry food, cereals and supplements given to the animals at the park.

Table 6. List of fruits, vegetables and cereals given to the two lemurs at Park 2

Fruits	Vegetables	Dry food	Cereals	Supplements
Tomato	Spinach	Dog dry food	Cerelac (baby food)	Pine nuts
Strawberry	Carrot			Seeds mix
Apple	Leek			Cat wet food
Pear	Pepper			
Banana	Endives			
Rockmelon	Cucumber			
Grapes	Lettuce			
Mango				
Fruit purees				
Papaya				
Passionfruit				

Graphic 4. Constituents of lemurs' diet at Park 2

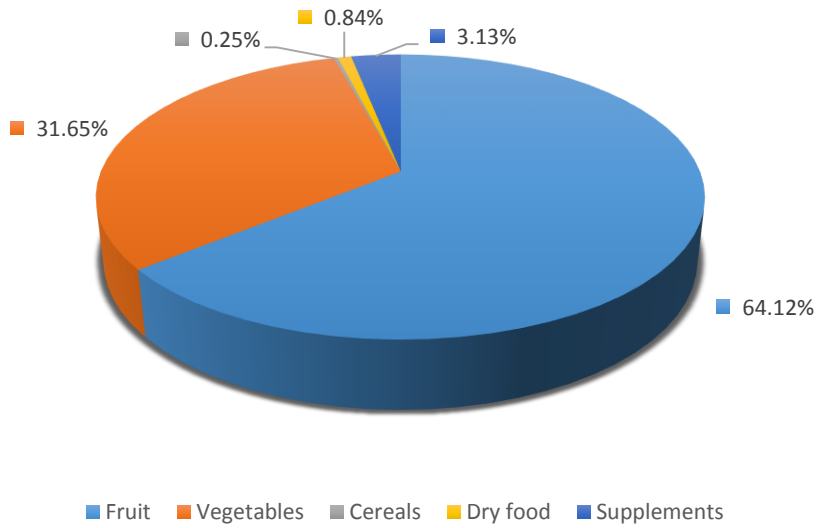


Table 7. Diet analysis for two animals in Park 2

	Type of food	Amount	Unit weight (g)	Total weight (g)
Day 1	Tomato	1	100	100
	Spinach	15	60	900
	Strawberry	2	20	40
	Apple	1	130	130
	Pear	1	230	230
	Banana	1.5	115	172.5
	Rockmelon	0.25	1250	312.5
	Carrot	2	150	300
Day 2	Brunch of grapes	1	400	400
	Pear	0.5	230	115
	Mango	0.5	500	250
	Banana	2	115	230
	Leek	2	200	400
	Carrot	0.5	150	75
	Tomato	1	100	100
	Pepper	0.5	210	105
	Apple	1	130	130
	Seeds mix	1	100	100
	Fruit purees	2	100	200
Day 3	Endive	1	150	150
	Pear	1	230	230
	Banana	2	115	230
	Cucumber	0.5	350	175

	Papaya	0.5	515	257.5
	Pepper	0.25	210	52.5
	Apple	1	130	130
	Seeds mix	1	100	100
	Cerelac pap	1	30	30
Day 4	Pepper	0.5	210	105
	Banana	1.5	115	172.5
	Apple	1	130	130
	Pear	2	230	460
	Carrot	2	150	300
	Brunch of grapes	1	400	400
	Tomato	1	100	100
	Lettuce	1	430	430
	Pine nuts	1	70	70
Day 5	Banana	2	115	230
	Pear	1	230	230
	Apple	1	130	130
	Brunch of grapes	1	400	400
	Rockmelon	0.5	1250	625
	Mango	1	500	500
	Carrot	1	150	150
	Dog dry food	1	100	100
Day 6	Rockmelon	0.25	1250	312.5
	Cucumber	0.5	350	175
	Strawberry	3	20	60
	Apple	1	130	130
	Lettuce	1	430	430
	Papaya	0.25	515	128.75
	Mango	0.33	500	165
	Passionfruit	1	160	160
	Wet cat food	1	100	100
Day 7	Tomato	1	100	100
	Spinach	15	60	900
	Strawberry	2	20	40
	Apple	1	130	130
	Pear	1	230	230
	Banana	1.5	115	172.5
	Rockmelon	0.25	1250	312.5
	Carrot	2	150	300
Day 8	Brunch of grapes	1	400	400
	Pear	0.5	230	115
	Mango	0.5	500	250
	Banana	2	115	230
	Leek	2	200	400

	Carrot	0.5	150	75
	Tomato	1	100	100
	Pepper	0.5	210	105
	Apple	1	130	130
	Seeds mix	1	100	100
	Fruit purees	2	100	200
Day 9	Endive	1	150	150
	Pear	1	230	230
	Banana	2	115	230
	Cucumber	0.5	350	175
	Papaya	0.5	515	257.5
	Pepper	0.25	210	52.5
	Apple	1	130	130
	Seeds mix	1	100	100
	Cerelac pap	1	30	30
Day 10	Pepper	0.5	210	105
	Banana	1.5	115	172.5
	Apple	1	130	130
	Pear	2	230	460
	Carrot	2	150	300
	Brunch of grapes	1	400	400
	Tomato	1	100	100
	Lettuce	1	430	430
	Pine nuts	1	70	70
Day 11	Banana	2	115	230
	Pear	1	230	230
	Apple	1	130	130
	Brunch of grapes	1	400	400
	Rockmelon	0.5	1250	625
	Mango	1	500	500
	Carrot	1	150	150
	Dog dry food	1	100	100
Day 12	Rockmelon	0.25	1250	312.5
	Cucumber	0.5	350	175
	Strawberry	3	20	60
	Apple	1	130	130
	Lettuce	1	430	430
	Papaya	0.25	515	128.75
	Mango	0.33	500	165
	Passionfruit	1	160	160
	Wet cat food	1	100	100
Day 13	Tomato	1	100	100
	Spinach	15	60	900
	Strawberry	2	20	40

	Apple	1	130	130
	Pear	1	230	230
	Banana	1.5	115	172.5
	Rockmelon	0.25	1250	312.5
	Carrot	2	150	300
Day 14	Brunch of grapes	1	400	400
	Pear	0.5	230	115
	Mango	0.5	500	250
	Banana	2	115	230
	Leek	2	200	400
	Carrot	0.5	150	75
	Tomato	1	100	100
	Pepper	0.5	210	105
	Apple	1	130	130
	Seeds mix	1	100	100
	Fruit purees	2	100	200
Day 15	Endive	1	150	150
	Pear	1	230	230
	Banana	2	115	230
	Cucumber	0.5	350	175
	Papaya	0.5	515	257.5
	Pepper	0.25	210	52.5
	Apple	1	130	130
	Seeds mix	1	100	100
	Cerelac pap	1	30	30

4.3.3. Park 3

At park 3, fruits and vegetables are also the main constituents of lemurs' diet, representing 46.77% and 31.76% of their nutrition, respectively. However, at this park there is a significant higher proportion of dry food (Table 10) given to the animals (11.34%), comparing to the other parks. Additionally, cereals (3.84%) and variable supplements (6.28%) are also provided to the animals in relatively high quantity.

Table 8. List of fruits, vegetables and cereals given to the lemurs at Park 3

Fruits	Vegetables	Cereals	Supplements
Apple	Lettuce	Bread	Boiled egg
Banana	Cucumber	Seeds mix	Dog dry food
Papaya	Potato	Rice	Natural yogurt
Kiwi	Broccoli	Spaghetti	
Melon	Cabbage		
Mango	Cauliflower		
Rockmelon	Purple cabbage		
Persimmon	Sweet potato		
Grapes	Spinach		
Strawberry	Carrot		
Tomato	Kale		

Table 9. Foods avoided at Park 3

- Strawberry
- Orange
- Plum
- Cabbage
- Citrus fruits
- Mushrooms

Table 10. Analytical composition of lemurs' dry food - Marmoset / New World monkeys, 4 mm pellets (Complete feed for Marmosets – NHP)

Analytical constituents	%	Nutritional Additives	Per kg
Crude Protein	26.10	Vitamin A (E 672)	18000 (IU)
Crude oils and fats	7.00	Vitamin D3 (E671)	3000 (IU)
Crude ash	6.80	Vitamin E	120 (mg)
Calcium	1.00	Vitamin C (stabilized; Ascorbyl monophosphate calcium sodium salt)	2900 (mg)
Phosphorus	0.70	Iron, Ferrous sulphate monohydrate (E1)	100 (mg)
Sodium	0.20	Zinc, Zinc sulphate monohydrate (E6)	50 (mg)
Lysine	1.70	Manganese, Manganous-(II)-sulphate monohydrate (E5)	30 (mg)
Met + Cyst	1.10	Copper, Copper-(II)-sulphate pentahydrate (E4)	5 (mg)
		Selenium, Sodium selenite (E8)	0.1 (mg)
Energy	**MJ/kg**	Iodine, Calcium iodate anhydrous (E2)	2.0 (mg)
ME	15.6	Cobalt, Cobaltous carbonate monohydrate (E3)	2.0 (mg)
Composition: Grains and grain products, oil seed products, sugar, milk and dairy products, vegetable oils, fruits (dried), minerals, flavours.			

Graphic 5. Constituents of lemurs' diet at Park 3

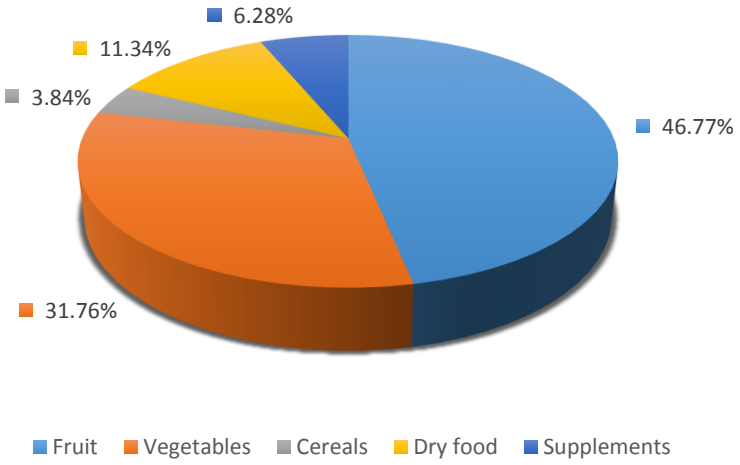


Table 11. Diet analysis for six animals in Park 3

		Type of food	Amount	Unit weight (g)	Total weight (g)
Day 1	A.M.	Cucumber			480
		New World pellet			320
	P.M.	Apple			580
		Melon			700
		Cucumber			640
		Boiled egg	4	50	200
Day 2	A.M.	Lettuce			480
		New World pellet			320
	P.M.	Grapes			425
		Persimmons			855
		Spinach			200
		Carrots			440
		Seeds mix			160
Day 3	A.M.	Lettuce			480
		New World pellet			320
	P.M.	Banana			400
		Papaya			680
		Grapes			200
		Sweet potato			640
		Bread	2	40	80
Day 4	A.M.	Lettuce			480
		New World pellet			320
	P.M.	Mango			530
		Papaya			750

		Tomato			640
		Dog dry food			200
Day 5	A.M.	Lettuce			480
		New World pellet			320
	P.M.	Grapes			400
		Apple			880
		Broccoli			640
		Natural yogurt	4	120	480
Day 6	A.M.	Potato			280
		Broccoli			200
		New World pellet			320
	P.M.	Banana			860
		Kiwi			420
		Lettuce			640
		Rice			800
Day 7	A.M.	Cabbage			480
		New World pellet			320
	P.M.	Persimmons			350
		Rockmelon			500
		Apple			430
		Lettuce			640
		Seeds mix			500
Day 8	A.M.	Cauliflower			480
		New World pellet			320
	P.M.	Apple			530
		Rockmelon			750
		Tomato			640
		Boiled egg	4	50	200
Day 9	A.M.	Broccoli			480
		New World pellet			320
	P.M.	Apple			700
		Banana			580
		Sweet potato			640
		Seeds mix			160
Day 10	A.M.	Tomato			480
		New World pellet			320
	P.M.	Grapes			1280
		Lettuce			640
		Bread	2	40	80
Day 11	A.M.	Purple cabbage			480
		New World pellet			320
	P.M.	Apple			800
		Tomato			480
		Carrots			640

		Dog dry food			200
Day 12	A.M.	Cabbage			480
		New World pellet			320
	P.M.	Kiwi			640
		Apple			640
		Cabbage			640
		Natural yogurt	4	120	480
Day 13	A.M.	Sweet potato			480
		New World pellet			320
	P.M.	Papaya			700
		Strawberries			580
		Carrots			400
		Broccoli			240
		Spaghetti			800
Day 14	A.M.	Sweet potato			480
		New World pellet			320
	P.M.	Apple			1280
		Lettuce			640
		Seeds mix			500
Day 15	A.M.	Cucumber			480
		New World pellet			320
	P.M.	Grapes			780
		Tomato			500
		Lettuce			640
		Boiled egg	4	50	200

4.4. Amount of iron and vitamin C

The content of iron and vitamin C were also discriminated for each type of food (Appendix 5, 6 and 7), which allowed the estimation of the total amount given to the animals, regarding these components. It was calculated based on the “Nutritive value of foods” (Gebhardt & Thomas, 2002) and analytical composition of the foods given, when available. The assessment of tannin content in the food offered to the animals was not achievable, since it is not defined for most fruits and vegetables.

Table 12. Amount of iron and vitamin C given to the animals

Amount per day	Park 1	Park 2	Park 3
Iron (mg/kg BW)	3.44	9.7	9.4
Vitamin C (mg/kg food)	249	213	506

*Considering the ideal body weight for *L. catta* (2.6kg)

5. Discussion

The intention of the present study was to analyze the levels of transferrin saturation of all the ring-tailed lemurs housed at three different zoological parks and relate the obtained values to the diet which the animals are submitted to.

Hemosiderosis has been reported in captive lemurs since the decade of 1960s, as noticed by pathologists at San Diego Zoo, when deposits of hemosiderin were found in the liver, spleen, lymph nodes, duodenum and other organs (Gonzales et al., 1984). Subsequently, researchers began to explore possible causes for the incidence of hemosiderosis in susceptible animals.

It is commonly accepted that the main cause for the high prevalence of ISD in lemurs is related to their captive diet, composed of several substances that may contribute to enhance the iron absorption, such as the high dietary levels of iron and ascorbic acid (citrus fruits) (Spelman et al., 1989).

Additionally, diets of captive lemurs have fewer substances that reduce iron availability, such as tannins, compared to the foods that the animals find in the wild (Clauss & Paglia, 2012).

For these reasons, our study included a diet analysis, focusing on the amount of fruit, vegetables, cereals, dry food and supplements given to the animals in each park, and its iron and vitamin C content.

Although the diagnosis of this condition requires a liver biopsy, it is possible to evaluate the animals' iron status using blood tests such as ferritin and transferrin saturation, similarly to human medicine (Williams et al., 2008). Transferrin saturation is an indicative of tissue iron reserves and it has been demonstrated to be positively correlated with hepatic iron deposition in ring-tailed lemurs (Williams et al., 2008).

Transferrin saturation represents the ratio between serum iron and total iron binding capacity (TS=SI/TIBC) (Ghosh & Collier, 2012) and the TIBC corresponds to the maximum amount of iron that can be bound to transferrin. Under physiologic circumstances, nearly all iron is bound to plasma proteins. However, under excessive iron conditions, transferrin is fully saturated and the plasma may contain iron which is not bound to plasma proteins ("free iron") (Zanen, Adriaansen, Bommel, Posthuma & Jong, 1996) leading to increased values of transferrin saturation, occasionally over 100%.

The present study revealed a high prevalence of ISD in almost all the animals housed at all the participant zoological facilities, considering that the reference range for transferrin saturation varies between 20 and 55%.

Although some animals revealed normal values of transferrin saturation, the average calculated for all the parks evaluated demonstrated a high prevalence of ISD in captive ring-tailed lemurs housed at these parks in Portugal.

As demonstrated, the average of iron ingested per day at Park 1, Park 2 and Park 3 was 3.44 mg/kg BW, 9.7 mg/kg BW and 9.4 mg/kg BW, respectively.

According to the “Dietary References Intakes” from the Institute of Medicine of the National Academies, U.S., the recommended daily allowance for a man weighing approximately 65 kg is 8 mg of iron per day. Considering the ideal body weight for ring-tailed lemurs to be 2.6 kg, it is reasonable to affirm by extrapolation that the lemurs housed at the enounced parks are consuming high levels of dietary iron, particularly at Park 2 and Park 3.

Despite this evidence, there is a significant difference between the transferrin saturation mean values obtained at Park 3 and the mean values from the other parks. One possible explanation for the higher values obtained at Park 3 is related to the increased percentage of dry food given to the animals housed at this park, leading to increased amounts of iron and vitamin C offered to the lemurs.

According to the National Research Council, the appropriate concentration of dietary iron should be 100 mg/kg DM (PTAG, 2003), which corresponds to the iron concentration in the commercial diet given at Park 3 (Table 10). However, it has been recently proposed that the iron concentrations for lemurs should be as low as 65 ppm (65 mg/kg), although it is difficult to formulate diets with this concentration (Miller & Fowler, 2014).

Alternatively, it has been recommended to feed approximately 25 g of monkey pellets per kilogram of ideal body weight per day (Junge et al., 2009). Considering the ideal body weight of a captive ring-tailed lemur to be approximately 2.6 kg (Kappeler, 1991), and the total amount of monkey biscuits given to the eight lemurs at Park 3 to be 320 g per day, it can be considered as an adequate amount per animal (15.4 g per kilogram of ideal body weight). However, even feeding the animals with appropriate amounts of dry food, it was still verified high levels of transferrin saturation after blood analysis.

It is considered by the National Research Council that the recommended vitamin C concentration for ring-tailed lemurs is 111 mg of vitamin C per kg of food (Moury & Campbell, 2001). This means that an excessive amount of vitamin C is being provided in the food from all the studied ring-tailed lemurs, as evidenced in table 12. It is important to notice that this concentration is significantly higher at Park 3, when comparing to the other parks. The reason for this difference is the fact that the lemurs from the enounced park consume a larger proportion of dry food in their diet (11.34%), which has a concentration of 2900 mg of vitamin C per kg of food, leading to a substantial increment in the total amount of vitamin C present in the food given to the lemurs.

Consequently, the higher values of %TS detected Park 3 may be related to both iron and vitamin C concentration levels, incremented by the larger proportion of dry food offered to the animals.

Therefore, it is essential to reformulate the diet given to the animals, in order to achieve smaller levels of dietary iron and vitamin C.

Diets formulation should be based on species adaptation, considering their nutrition in the wild and their common captive health problems. The fact that almost all zoological institutions in Portugal feed their animals (especially primates) using food offered by supermarkets, makes food selection more difficult, which leads to an inadequate diet.

For this reason, it is essential that the mentioned institutions follow strategies to implement a species-specific diet for all the animals.

A list of avoided foods should be applied for each species, based on their metabolism and nutrition adaptation in captive conditions. This could eventually be realized by a nutrition specialist, preferably a veterinarian.

Considering ISD, it is crucial that lemurs' diet formulation avoids iron rich foods (Appendix 1) and vitamin C rich foods (Appendix 2) and contains high levels of tannin rich food sources (Appendix 3). This should be considered the first step to reduce the incidence of hemosiderosis in this species, thus reducing their mortality.

As previously mentioned, ISD in lemurs is associated with hemosiderin accumulation in liver, kidney, spleen and bone marrow, often leading to a stage of generalized hemosiderosis in interstitial tissue and parenchymal cells, producing extensive tissue damage (Spelman et al., 1989), which can lead to an increased mortality risk (Mainous, Wells, Carek, Gill, & Geesey, 2004).

Therefore, it is essential to make zoological parks aware of the necessity of creating balanced diets, adapted to the species in question, since it can effectively decrease animals' mortality.

6. Study limitations and future considerations

The most preeminent limitation of this study was the sample size. Despite the initial intention of gathering animals from five different zoological parks, there were two facilities that discarded the participation on the present study due to animal husbandry possible interference. Furthermore, the sample size was different for each institution.

Increasing the sample size could be beneficial to have more accurate results.

Diet analysis was another limitation almost as relevant as the previously mentioned. As the fruit and vegetables are offered by supermarkets it is difficult to accurately define the diet, as it varies according to availability. This variation makes it extremely challenging to analyze the relative percentage of fruit, vegetables, cereals, dry food and supplements given to the animals.

Additionally, the significant food variation made it extremely difficult to accurately analyze relevant diet components, such as iron, ascorbic acid and tannins, considered essential factors for the incidence of ISD.

A deeper investigation of diet components, quantifying the exact amount of iron, ascorbic acid and tannins would be a useful approach to determine its influence on transferrin saturation levels. However, the initial goal of this study was to provide a practical analysis of the diet given and to suggest the respective revision based on comparative results.

The lack of information regarding this subject was considered an additional limitation.

Unfortunately, the number of studies including this species is limited, thus reference values for hematological components are not completely defined yet.

Despite the initial goals of this study did not include a reassessment of the results obtained, it would be interesting to analyze the levels of transferrin saturation after the implementation of the suggested diet changes.

Conclusion

It was extremely challenging to take specific conclusions from this study, due to the sample size. However, it is strongly evident that ISD is currently present in almost all lemurs' collections in Portugal.

Although the causes are not completely defined, it is reasonable to conclude that food is probably the main risk factor for the high incidence of hemosiderosis in this species.

As referred before, it is known that ISD can carry serious health problems for susceptible species, such as lemurs and it is also considered an important mortality cause for these animals. For this reason, further research should be conducted in order to reformulate the diets given to the animals, as an approach to reduce the incidence of ISD.

During this study, it was verified among zoos and wildlife parks that there was no consciousness about the impact that ISD can have in lemurs' health, neither the diet's influence on this disease.

It could be interesting to develop an awareness campaign to sensitize wildlife veterinarians and zookeepers about this subject, in order to create strategies that decrease the prevalence of ISD in captive populations.

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Appendix 1. List of iron-rich food sources (Source: http://healthline.com and http://dietitians.ca)

Vegetables	Beans
	Lentils
	Peas
	Soybeans
	Potatoes
	Mushrooms
	Palm hearts
	Asparagus
	Turnips
	Beets
	Spinach
	Kale
	Swiss chard
	Collard greens
	Beet greens
Fruits	Prune
	Olives
	Mulberries
	Dried apricots
Whole grain	Quinoa
	Oats
Nuts and seeds	Pumpkin seeds
	Sesame seeds
	Cashews
	Pine nuts

Appendix 2. List of vitamin C food sources (Sources: http://healthaliciousness.com and http://health.com)

Vegetables	Bell peppers
	Chiili peppers
	Broccoli
	Peas
	Kale
	Cauliflower
	Brussels sprouts
Fruits	Papaya
	Strawberries
	Pineapple
	Kiwi
	Mango
	Orange
	Tomato

Appendix 3. List of tannin-rich food sources (Source: Rao & Prabhavathi, 1982; Serrano, Puupponen-Pimiä, Dauer, Aura & Saura-Calixto, 2009)

Vegetables	Chickpea	
	Green gram	
	Red gram	
	Cowpea	
	Kidney bean	
	Lentils	
Fruits	Apple	Mango
	Apricot	Medlar
	Avocado	Nectarine
	Banana	Peach
	Blackberries	Pear
	Blueberries	Persimmon
	Cherries	Pineapple
	Cloudberries	Plum
	Cranberries	Pomegranate
	Currants	Prune
	Dates	Quince
	Grapes	Raspberries
	Lime	Strawberries
Cereals	Barley	
	Red sorghum	
Condiments	Coriander	
	Tamarind	
	Turmeric	
	Chili powder	
	Tea	

Appendix 4. Animal identification form

Institution:

Animal ID:		
Birth date:	Sex:	
Date collected:	Time:	
Food given:	Yes/Time:	No:
Comments:		

Appendix 5. Iron and vitamin C content in the food offered at Park 1

		Type of food	Iron content (mg/100g)	Total iron (mg)	Vit C content (mg/100g)	Total Vit C (mg)
Day 1	A.M.	Red apple	0.1	0.65	6	39
		Banana	0.4	2.3	8.7	50.025
		Pear	0.1	1.15	4	46
	P.M.	Seed bread	1.67	1.67	0.2	0.2
		Banana	0.4	1.84	8.7	40.02
		Mixed salad (lettuce and carrot)	0.775	2.7125	9.4	32.9
		Annona	0.6	3.96	16	105.6
		Pink lady apple	0.1	0.37	6	22.2
		Melon	0.2	2	42	420
Day 2	A.M.	Red apple	0.1	0.65	6	39
		Banana	0.4	2.3	8.7	50.025
		Mango	0.2	2	28	280
	P.M.	Leek	1	6	12	72
		Beaded Lettuce	0.9	1.8	9.2	18.4
		Mixed salad (lettuce and carrot)	0.775	1.35625	9.4	16.45
		Annona	0.6	2.64	16	70.4
		Banana da Madeira	0.4	2.4	8.7	52.2
		Rockmelon	0.2	1.25	42	262.5
Day 3	A.M.	Red Apple	0.1	0.39	6	23.4
		Banana	0.4	2.76	8.7	60.03
		Melon	0.2	1	42	210
	P.M.	Raisin bread	1.67	1.67	0.2	0.2
		Persimmons	2.5	30	66	792
		Endives	0.8	3.6	6.5	29.25
		Mango	0.2	1	28	140
		Melon	0.2	1	42	210
		Leek	1	8	12	96
		Banana	0.4	1.84	8.7	40.02
Day 4	A.M.	Red apple	0.1	0.65	6	39
		Banana	0.4	0.92	8.7	20.01
		Watermelon	0.2	1.25	10	62.5
	P.M.	Red apple	0.1	0.78	6	46.8
		Melon	0.2	1	42	210
		Brunches of grapes	0.3	2.4	11	88
		Leek	1	8	12	96
Day 5	A.M.	Melon	0.2	4	42	840
		Red apple	0.1	0.65	6	39
		Pineapple	0.3	1.95	15	97.5

	P.M.	Rockmelon	0.2	1.25	42	262.5
		Strawberry	0.5	1.1	57	125.4
		Pear	0.1	1.38	4	55.2
		Melon	0.2	1	42	210
		Red apple	0.1	0.52	6	31.2
		Mango	0.2	3	28	420
		Green salad	0.9	3.15	9.2	32.2
Day 6	A.M.	Melon	0.2	1	42	210
		Red apple	0.1	0.78	6	46.8
	P.M.	Persimmons	2.5	60	66	1584
		Strawberry	0.5	3	57	342
		Brunches of grapes	0.3	3	11	110
		Banana	0.4	1.84	8.7	40.02
		Green salad	0.9	1.575	9.2	16.1
Day 7	A.M.	Red apple	0.1	1.17	6	70.2
		Strawberry	0.5	2.4	57	273.6
		Brunches of grapes	0.3	3	11	110
	P.M.	Strawberry	0.5	1	57	114
		Pear	0.1	1.15	4	46
		Melon	0.2	2	42	420
		Red apple	0.1	0.52	6	31.2
		Mango	0.2	3	28	420
		Banana	0.4	0.92	8.7	20.01
		Green salad	0.9	3.15	9.2	32.2
Day 8	A.M.	Annona	0.6	1.32	16	35.2
		Red apple	0.1	0.52	6	31.2
		Pineapple	0.3	0.4875	15	24.375
		Banana	0.4	1.84	8.7	40.02
		Mango	0.2	1	28	140
	P.M.	Mango	0.2	4	28	560
		Persimmons	2.5	37.5	66	990
		Annona	0.6	2.64	16	70.4
		Pineapple	0.3	1.95	15	97.5
		Seed bread	1.67	1.67	0.2	0.2
		Leek	1	10	12	120
Day 9	A.M.	Red apple	0.1	0.65	6	39
		Plum	0.2	1.56	10	78
		Melon	0.2	3	42	630
	P.M.	Pear	0.1	0.69	4	27.6
		Seed bread	1.67	1.67	0.2	0.2
		Rockmelon	0.2	1.25	42	262.5

		Mango	0.2	3	28	420
		Persimmons	2.5	60	66	1584
Day 10	A.M.	Red apple	0.1	0.52	6	31.2
		Plum	0.2	1.44	10	72
		Rockmelon	0.2	1.25	42	262.5
	P.M.	Carrot	0.4	3	10	75
		Banana	0.4	2.3	8.7	50.025
		Rockmelon	0.2	1.25	42	262.5
		Seed bread	1.67	1.67	0.2	0.2
		Plum	0.2	1.2	10	60
Day 11	A.M.	Melon	0.2	2	42	420
		Pineapple	0.3	1.95	15	97.5
		Red apple	0.1	0.78	6	46.8
		Plum	0.2	0.96	10	48
	P.M.	Pear	0.1	1.38	4	55.2
		Strawberry	0.5	1.2	57	136.8
		Red apple	0.1	0.65	6	39
		Pineapple	0.3	1.95	15	97.5
		Plum	0.2	1.2	10	60
Day 12	A.M.	Mango	0.2	1	28	140
		Red apple	0.1	0.91	6	54.6
		Plum	0.2	1.2	10	60
	P.M.	Persimmons	2.5	45	66	1188
		Red apple	0.1	0.52	6	31.2
		Melon	0.2	2	42	420
		Seed bread	1.67	0.835	0.2	0.1
Day 13	A.M.	Banana	0.4	1.84	8.7	40.02
		Melon	0.2	2	42	420
		Red apple	0.1	0.52	6	31.2
		Plum	0.2	0.24	10	12
	P.M.	Persimmons	2.5	37.5	66	990
		Mango	0.2	3	28	420
		Brunches of grapes	0.3	2.4	11	88
		Red apple	0.1	0.52	6	31.2
		Spinach	3.6	7.2	28	56
Day 14	A.M.	Pear	0.1	1.15	4	46
		Brunches of grapes	0.3	1.8	11	66
		Melon	0.2	1	42	210
	P.M.	Mango	0.2	2	28	280
		Banana	0.4	2.3	8.7	50.025
		Plum	0.2	0.96	10	48

		Red apple	0.1	0.52	6	31.2
		Persimmons	2.5	15	66	396
		Seed bread	1.67	1.67	0.2	0.2
Day 15	A.M.	Banana	0.4	1.84	8.7	40.02
		Red apple	0.1	0.52	6	31.2
		Rockmelon	0.2	1.875	42	393.75
		Pear	0.1	0.69	4	27.6
	P.M.	Leek and carrot soup	0.85	2.975	11.5	40.25
		Mixed salad (lettuce and carrot)	0.775	1.35625	9.4	16.45
		Seed bread	1.67	1.67	0.2	0.2
		Banana	0.4	1.84	8.7	40.02

Appendix 6. Iron and vitamin C content in the food offered at Park 2

	Type of food	Iron content (mg/100g)	Total iron (mg)	Vit C content (mg/100g)	Total Vit C (mg)
Day 1	Tomato	0.4	0.4	19	19
	Spinach	3.6	32.4	28	252
	Strawberry	0.5	0.2	57	22.8
	Apple	0.1	0.13	6	7.8
	Pear	0.1	0.23	4	9.2
	Banana	0.4	0.69	8.7	15.0075
	Rockmelon	0.2	0.625	42	131.25
	Carrot	0.4	1.2	10	30
Day 2	Brunch of grapes	0.3	1.2	11	44
	Pear	0.1	0.115	4	4.6
	Mango	0.2	0.5	28	70
	Banana	0.4	0.92	8.7	20.01
	Leek	1	4	12	48
	Carrot	0.4	0.3	10	7.5
	Tomato	0.4	0.4	19	19
	Pepper	0.43	0.4515	150	157.5
	Apple	0.1	0.13	6	7.8
	Seeds mix	8.8	8.8	1.7	1.7
	Fruit purees	3	6	35	70
Day 3	Endive	0.8	1.2	6.5	9.75
	Pear	0.1	0.23	4	9.2
	Banana	0.4	0.92	8.7	20.01
	Cucumber	0.2	0.35	4	7
	Papaya	0.25	0.64375	62	159.65
	Pepper	0.43	0.22575	130	68.25
	Apple	0.1	0.13	6	7.8
	Seeds mix	8.8	8.8	1.7	1.7
	Cerelac pap	8.5	2.55	65	19.5
Day 4	Pepper	0.43	0.4515	150	157.5
	Banana	0.4	0.69	8.7	15.0075
	Apple	0.1	0.13	6	7.8
	Pear	0.1	0.46	4	18.4
	Carrot	0.4	1.2	10	30
	Brunch of grapes	0.3	1.2	11	44
	Tomato	0.4	0.4	19	19
	Lettuce	0.9	3.87	9.2	39.56
	Pine nuts	5.5	3.85	1.6	1.12
Day 5	Banana	0.4	0.92	8.7	20.01

	Pear	0.1	0.23	4	9.2
	Apple	0.1	0.13	6	7.8
	Brunch of grapes	0.3	1.2	11	44
	Rockmelon	0.2	1.25	42	262.5
	Mango	0.2	1	28	140
	Carrot	0.4	0.6	10	15
	Dog dry food	8	8	20	20
Day 6	Rockmelon	0.2	0.625	42	131.25
	Cucumber	0.4	0.7	4	7
	Strawberry	0.5	0.3	57	34.2
	Apple	0.1	0.13	6	7.8
	Lettuce	0.9	3.87	9.2	39.56
	Papaya	0.25	0.321875	62	79.825
	Mango	0.2	0.33	28	46.2
	Passionfruit	1.6	2.56	30	48
	Wet cat food	0.4	0.4	9	9
Day 7	Tomato	0.4	0.4	19	19
	Spinach	3.6	32.4	28	252
	Strawberry	0.5	0.2	57	22.8
	Apple	0.1	0.13	6	7.8
	Pear	0.1	0.23	4	9.2
	Banana	0.4	0.69	8.7	15.0075
	Rockmelon	0.2	0.625	42	131.25
	Carrot	0.4	1.2	10	30
Day 8	Brunch of grapes	0.3	1.2	11	44
	Pear	0.1	0.115	4	4.6
	Mango	0.2	0.5	28	70
	Banana	0.4	0.92	8.7	20.01
	Leek	1	4	12	48
	Carrot	0.4	0.3	10	7.5
	Tomato	0.4	0.4	19	19
	Pepper	0.43	0.4515	150	157.5
	Apple	0.1	0.13	6	7.8
	Seeds mix	8.8	8.8	1.7	1.7
	Fruit purees	3	6	35	70
Day 9	Endive	0.8	1.2	6.5	9.75
	Pear	0.1	0.23	4	9.2
	Banana	0.4	0.92	8.7	20.01
	Cucumber	0.4	0.7	4	7
	Papaya	0.25	0.64375	62	159.65
	Pepper	0.43	0.22575	150	78.75

	Apple	0.1	0.13	6	7.8
	Seeds mix	8.8	8.8	1.7	1.7
	Cerelac pap	8.5	2.55	65	19.5
Day 10	Pepper	0.43	0.4515	150	157.5
	Banana	0.4	0.69	8.7	15.0075
	Apple	0.1	0.13	6	7.8
	Pear	0.1	0.46	4	18.4
	Carrot	0.4	1.2	10	30
	Brunch of grapes	0.3	1.2	11	44
	Tomato	0.4	0.4	19	19
	Lettuce	0.9	3.87	9.2	39.56
	Pine nuts	5.5	3.85	1.6	1.12
Day 11	Banana	0.4	0.92	8.7	20.01
	Pear	0.1	0.23	4	9.2
	Apple	0.1	0.13	6	7.8
	Brunch of grapes	0.3	1.2	11	44
	Rockmelon	0.2	1.25	42	262.5
	Mango	0.2	1	28	140
	Carrot	0.4	0.6	10	15
	Dog dry food	8	8	20	20
Day 12	Rockmelon	0.2	0.625	42	131.25
	Cucumber	0.4	0.7	4	7
	Strawberry	0.5	0.3	57	34.2
	Apple	0.1	0.13	6	7.8
	Lettuce	0.9	3.87	9.2	39.56
	Papaya	0.25	0.321875	62	79.825
	Mango	0.2	0.33	28	46.2
	Passionfruit	1.6	2.56	30	48
	Wet cat food	0.4	0.4	9	9
Day 13	Tomato	0.4	0.4	19	19
	Spinach	3.6	32.4	28	252
	Strawberry	0.5	0.2	57	22.8
	Apple	0.1	0.13	6	7.8
	Pear	0.1	0.23	4	9.2
	Banana	0.4	0.69	8.7	15.0075
	Rockmelon	0.2	0.625	42	131.25
	Carrot	0.4	1.2	10	30
Day 14	Brunch of grapes	0.3	1.2	11	44
	Pear	0.1	0.115	4	4.6
	Mango	0.2	0.5	28	70
	Banana	0.4	0.92	8.7	20.01

	Leek	1	4	12	48
	Carrot	0.4	0.3	10	7.5
	Tomato	0.4	0.4	19	19
	Pepper	0.43	0.4515	150	157.5
	Apple	0.1	0.13	6	7.8
	Seeds mix	8.8	8.8	1.7	1.7
	Fruit purees	3	6	35	70
Day 15	Endive	0.8	1.2	6.5	9.75
	Pear	0.1	0.23	4	9.2
	Banana	0.4	0.92	8.7	20.01
	Cucumber	0.4	0.7	4	7
	Papaya	0.25	0.64375	62	159.65
	Pepper	0.43	0.22575	150	78.75
	Apple	0.1	**0.13**	6	7.8
	Seeds mix	8.8	8.8	1.7	1.7
	Cerelac pap	8.5	2.55	65	19.5

Appendix 7. Iron and vitamin C content in the food offered at Park 3

		Type of food	Iron content (mg/100g)	Total iron (mg)	Vit C content (mg/100g)	Total Vit C (mg)
Day 1	A.M.	Cucumber	0.4	1.92	4	19.2
		New World pellet	10	32	290	928
	P.M.	Apple	0.1	0.58	6	34.8
		Melon	0.2	1.4	42	294
		Cucumber	0.4	2.56	4	25.6
		Boiled egg	1.2	2.4	0.1	0.2
Day 2	A.M.	Lettuce	0.9	4.32	9.2	44.16
		New World pellet	10	32	290	928
	P.M.	Grapes	0.3	1.275	11	46.75
		Persimmons	2.5	21.375	66	564.3
		Spinach	3.6	7.2	28	56
		Carrots	0.4	1.76	10	44
		Seeds mix	8.8	14.08	1.7	2.72
Day 3	A.M.	Lettuce	0.9	4.32	9.2	44.16
		New World pellet	10	32	290	928
	P.M.	Banana	0.4	1.6	8.7	34.8
		Papaya	0.25	1.7	62	421.6
		Grapes	0.3	0.6	11	22
		Sweet potato	0.6	3.84	2.4	15.36
		Bread	1.7	1.36	0.1	0.08
Day 4	A.M.	Lettuce	0.9	4.32	9.2	44.16
		New World pellet	10	32	290	928
	P.M.	Mango	0.2	1.06	28	148.4
		Papaya	0.25	1.875	62	465
		Tomato	0.4	2.56	19	121.6
		Dog dry food	8	16	20	40
Day 5	A.M.	Lettuce	0.9	4.32	9.2	44.16
		New World pellet	10	32	290	928
	P.M.	Grapes	0.3	1.2	11	44
		Apple	0.1	0.88	6	52.8
		Broccoli	0.7	4.48	89.2	570.88
		Natural yogurt	0.1	0.48	0.9	4.32
Day 6	A.M.	Potato	0.8	2.24	19.7	55.16
		Broccoli	0.7	1.4	89.2	178.4
		New World pellet	10	32	290	928
	P.M.	Banana	0.4	3.44	8.7	74.82
		Kiwi	0.3	1.26	98	411.6
		Lettuce	0.9	5.76	9.2	58.88

		Rice	0.2	1.6	2	16
Day 7	A.M.	Cabbage	1	4.8	36.6	175.68
		New World pellet	10	32	290	928
	P.M.	Persimmons	2.5	8.75	66	231
		Rockmelon	0.2	1	42	210
		Apple	0.1	0.43	6	25.8
		Lettuce	0.9	5.76	9.2	58.88
		Seeds mix	8.8	44	1.7	8.5
Day 8	A.M.	Cauliflower	0.4	1.92	48.2	231.36
		New World pellet	10	32	290	928
	P.M.	Apple	0.1	0.53	6	31.8
		Rockmelon	0.2	1.5	42	315
		Tomato	0.4	2.56	19	121.6
		Boiled egg	1.2	2.4	0.1	0.2
Day 9	A.M.	Broccoli	0.7	3.36	89.2	428.16
		New World pellet	10	32	290	928
	P.M.	Apple	0.1	0.7	6	42
		Banana	0.4	2.32	8.7	50.46
		Sweet potato	0.6	3.84	2.4	15.36
		Seeds mix	8.8	14.08	1.7	2.72
Day 10	A.M.	Tomato	0.4	1.92	19	91.2
		New World pellet	10	32	290	928
	P.M.	Grapes	0.3	3.84	11	140.8
		Lettuce	0.9	5.76	9.2	58.88
		Bread	1.7	1.36	0.1	0.08
Day 11	A.M.	Purple cabbage	0.8	3.84	57	273.6
		New World pellet	10	32	290	928
	P.M.	Apple	0.1	0.8	6	48
		Tomato	0.4	1.92	19	91.2
		Carrots	0.4	2.56	10	64
		Dog dry food	8	16	20	40
Day 12	A.M.	Cabbage	1	4.8	36.6	175.68
		New World pellet	10	32	290	928
	P.M.	Kiwi	0.3	1.92	98	627.2
		Apple	0.1	0.64	6	38.4
		Cabbage	1	6.4	36.6	234.24
		Natural yogurt	0.1	0.48	0.9	4.32
Day 13	A.M.	Sweet potato	0.6	2.88	2.4	11.52
		New World pellet	10	32	290	928
	P.M.	Papaya	0.25	1.75	62	434
		Strawberries	0.5	2.9	57	330.6

		Carrots	0.4	1.6	10	40
		Broccoli	0.7	1.68	89.2	214.08
		Spaghetti	1.3	10.4	2	16
Day 14	A.M.	Sweet potato	0.6	2.88	2.4	11.52
		New World pellet	10	32	290	928
	P.M.	Apple	0.1	1.28	6	76.8
		Lettuce	0.9	5.76	9.2	58.88
		Seeds mix	8.8	44	1.7	8.5
Day 15	A.M.	Cucumber	0.4	1.92	4	19.2
		New World pellet	10	32	290	928
	P.M.	Grapes	0.3	2.34	11	85.8
		Tomato	0.4	2	19	95
		Lettuce	0.9	5.76	9.2	58.88
		Boiled egg	1.2	2.4	0.1	0.2