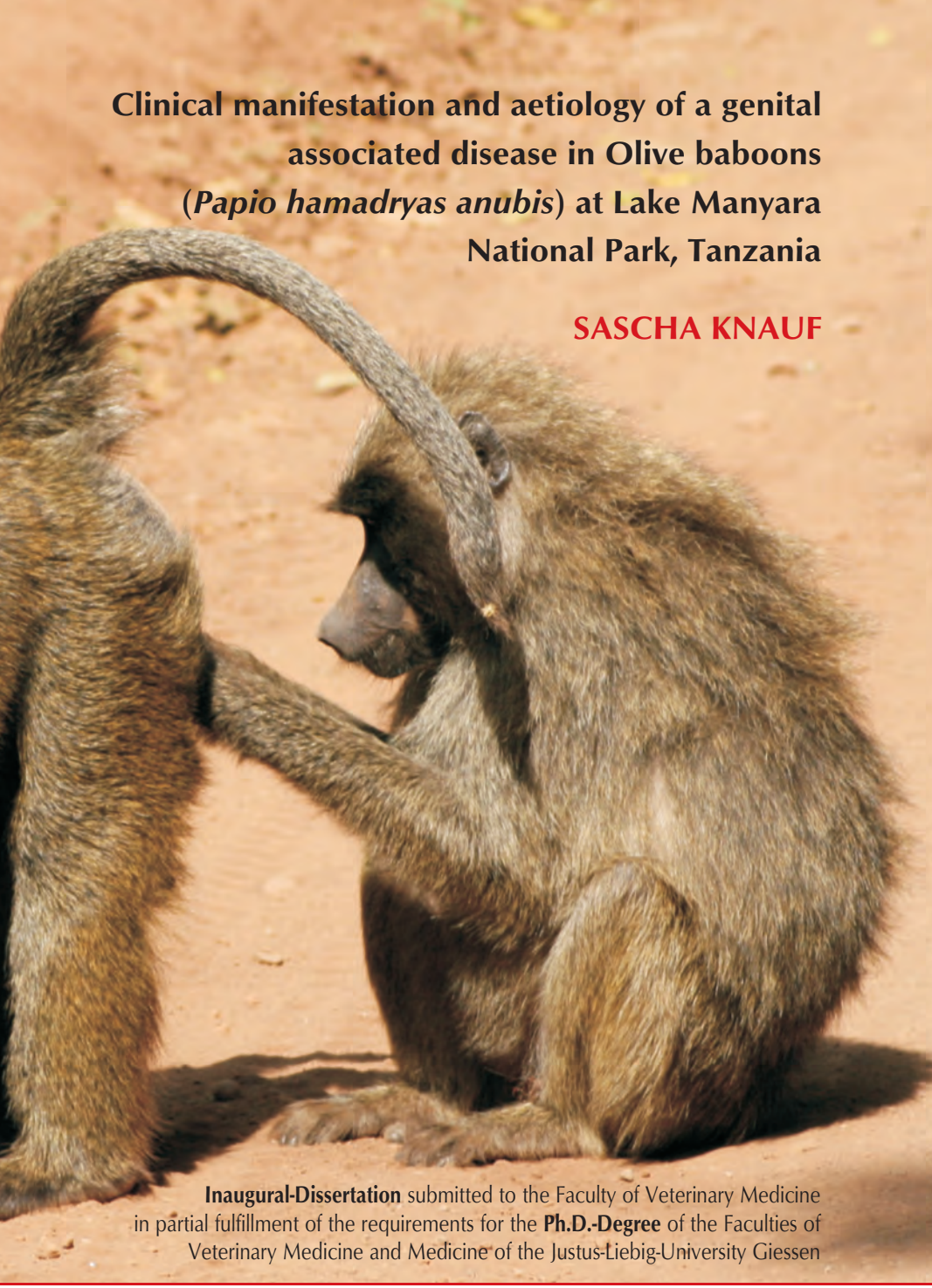
Clinical manifestation and aetiology of a genital associated disease in Olive baboons (*Papio hamadryas anubis*) at Lake Manyara National Park, Tanzania

SASCHA KNAUF



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by

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of
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Declarations

“I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus-Liebig-University of Giessen in carrying out the investigations described in the dissertation.”

Sascha Knauf

Dedication

To

my Family, Yvonne and our unborn child.

Contents

Abbreviations

AWF	African Wildlife Foundation
BLAST	Basic Local Aligment Search Tool
bp	Base pair
bw	Body weight
°C	Degrees Celsius
Cat	Catalogue
CNA	Clinically non-affected
COSTECH	Tanzania Commission for Science and Technology
dN	Non-synonymous substitution per site
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide tri-Phosphates
dS	Synonymous substitution per site
E	East
et al.	Et altera
GNP	Gombe Stream National Park
H&E	Haematoxylin-Eosin
HVH-2	Herpesvirus hominis 2
HVP-2	Herpesvirus papio 2
IN	Initial
IUCN	International Union for Conservation of Nature
JLU	Justus-Liebig-University of Giessen
kb	kilobases
kbp	kilobase-pairs
km	Kilometre
km^2	Square-kilometre
LCV	Lymphocryptovirus
LMNP	Lake Manyara National Park
λ	Lambda
M	Molar
min	Minute
ml	Millilitre
ML	Maximum Likelihood

mm	Millimetre
mM	Millimolar
MO	moderate
MP	Maximum Parsimony
Mt	Mount
µL	Microlitre
µm	Micrometer
µM	Micromolar
n	Quantity
neg	Negative
NHP	Nonhuman primate
nm	Nanometre
No(s)	Number(s)
P	Threshold probability value
PAS	Periodic-Acid-Shiff
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
polA	DNA polymerase I gene
pos.	Positive
R	River
RDP2	Recombination detection analysis from sequence alignments
RKI	Robert-Koch-Institute
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
S	South
SAS	Statistical Analysis Software
SA8V	Simian agent 8 virus
SD	Standard deviation
sec	Second
SEV	Severe
SIV	Simian Immunodeficiency Virus
SNP	Serengeti National Park
ssp.	Subspecies
STD	Sexually transmitted disease

SUA	Sokoine University of Agriculture, Morogoro, Tanzania
TANAPA	Tanzania National Parks
TAWIRI	Tanzania Wildlife Research Institute
U	Unit
UNESCO	United Nations Educational, Scientific and Cultural Organization
qRT-PCR	Quantitative Real-Time PCR
WHO	World Health Organization
WSS	Warthin-Starry-silver

List of papers (peer review)

1. Knauf, S.; Batamuzi, E.K.; Mlengeya, T.; Kilewo, M.; Lejora, I.A.V.; Nordhoff, M.; Ehlers, B.; Harper, K.N.; Fyumagwa, R.; Hoare, R.; Wehrend, A.; Kaup, F.J.; Leendertz, F.H.; Maetz-Rensing, K. (2010): Treponema infection associated with genital ulceration in wild baboons. J Vet Pathol, accepted.
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1. Knauf, S.; Batamuzi, E.K.; Mlengeya, T.; Leendertz, F.H.; Wehrend, A.; Kaup, F.J.; Maetz-Rensing, K. (2009): Investigating a sexual transmitted disease in Olive baboons (*Papio h. anubis*) at Lake Manyara National Park, Tanzania. 11. Tagung der Gesellschaft für Primatologie. Hannover. (**O**)
2. Harper, K.; Knauf, S.; Fyumagwa, R.; Hoare, R.; Leendertz, F.; Armelagos, G. (2009): Syphilis in the wild: an opportunity to learn more about the evolution of sexual transmission? 74th Cold Spring Harbor Symposium on Quantitative Biology, Evolution: The Molecular Landscape, Long Island, NY. (**P**)
3. Knauf, S.; Batamuzi, E.K.; Lendl, C.; Tacke, S.; Lawrenz, A.; Mlengeya, T.; Leendertz, F.H.; Maetz-Rensing, K.; Wehrend, A. (2009): Chemical immobilization of free ranging Olive baboon (*Papio h. anubis*) at Lake Manyara National Park, Tanzania. 11. Tagung der Gesellschaft für Primatologie. Hannover. (**P**)
4. Knauf, S.; Batamuzi, E.K.; Maetz-Rensing, K.; Leendertz, F.H.; Wehrend, A. (2009): Exfoliative vaginal cytology as a diagnostic tool for sexual cycle stages in nonhuman primates with sexually transmitted diseases. 42. Jahrestagung Physiologie und Pathologie der Fortpflanzung gleichzeitig 34. Veterinär-Humanmedizinische Gemeinschaftstagung, Reproduction in Domestic Animals, 44 (Supplement 1), 20. (**P**)
5. Knauf, S.; Batamuzi, E.K.; Mlengeya, T.; Leendertz, F.H.; Wehrend, A.; Kaup, F.J.; Maetz-Rensing, K. (2009): Histological findings of a sexually transmitted disease in wild Olive baboons (*Papio hamadryas anubis*) at Lake Manyara National Park, Tanzania. 3rd EWDA Student Workshop, Conference Center of Mérieux Fondation, Les Pensières, Veyrier-du-Lac, France. (**P**)
6. Knauf, S.; Mätz-Rensing, K.; Leendertz, F.H.; Batamuzi, E.K.; Wehrend, A. (2008): Exfoliative vaginal cytology in Olive Baboons (*Papio hamadryas anubis*) – A diagnostic tool for sexual-cycle stages. Proceedings of the EAWZV meeting at Leipzig 2008, 59 – 63. (**O**)
7. Knauf, S.; Blad-Stahl, J.; Batamuzi, E.K.; Leendertz, F.H.; Mätz-Rensing, K.; Kaup, F.J.; Wehrend, A. (2008): Differentialblutbilder bei genitalkranken

Anubispavianen (*Papio hamadryas anubis*). InLAB2008, Tierärztliche Praxis; 36 (K), A22. (**P**)

8. Knauf, S.; Mätz-Rensing, K.; Kaup, F.J.; Beardi, B.; Leendertz, F.H.; Batamuzi, E.K.; Wehrend, A. (2008): Histological findings of a venereal disease in free ranging Olive baboon (*Papio h. anubis*) at Lake Manyara National Park, Tanzania. 41. Jahrestagung Physiologie und Pathologie der Fortpflanzung gleichzeitig 33. Veterinär-Humanmedizinische Gemeinschaftstagung, Reproduction in Domestic Animals, 43 (Supplement 1), 19 – 20. (**P**)
9. Knauf, S.; Lawrenz, A.; Batamuzi, E.K.; Wambura, P.; Tacke, S.; Lendl, C.E.; Wehrend, A. (2007): Chemical immobilization in free ranging Olive baboon (*Papio h. anubis*) at Lake Manyara National Park. Proceedings of the 6th Scientific TAWIRI Conference 3rd-6th Dec. 2007 in Arusha, Tanzania, 505 – 510. (**O**)

1 Introduction

Sexually transmitted diseases (STDs) do have major influence on the demography and social structure of primates (Lockhart et al., 1996; Nunn et al., 2004; Smith and Dobson, 1992). Research in this particular field, especially in wildlife, has long been underrepresented in nonhuman primates (NHP). For example, in the wild syphilis caused by the spirochaete *Treponema pallidum* has only been suspected once (Wallis and Lee, 1999; Wallis, 2000). As a consequence it has not been considered as a potential reservoir of infection. In the context of a worldwide increasing incidence of STDs in humans (Da Ros and Schmitt, 2008), greater understanding of new diseases provide basic information for a broad array of research and provides opportunities to better understand the evolution, epidemiology and pathomechanisms at work. Results obtained might have consequences for future wildlife conservation strategies and disease eradication programs in humans and wildlife.

Lake Manyara National Park (LMNP) is part of the Serengeti – Ngorongoro Conservation Area – Lake Manyara – Tarangire National Park ecosystem. Due to the abundant wildlife and unique composition of different vegetation types the UNESCO Man and the Biosphere Program accredited LMNP as a biosphere reserve in 1981. It is among the smaller national parks in the United Republic of Tanzania although it doubled in size with the recent addition of Marang Forest. The size of the soda lake depends on the season but grossly covers two-thirds of the area (excluding Marang Forest), crowding an estimated number of 2,000 – 2,500 Olive baboons (*Papio hamadryas anubis*) to a high population density, the highest among Africa.

First sightings of a genital disease in baboons at LMNP were reported in 1994 (Mlengeya, 2004) and in 2003 the epidemic started to attract significant media attention (Hogan, 2003). Both genders show a characteristic exudative dermatitis of the ano-genital area, inevitably leading to a partial or complete mutilation of the external genitalia. The disease's overall prevalence was 10.5 % (0 – 29.1 %) based upon field observation of 13 baboon troops (n = 1,223 individuals) (Mlengeya, 2004). There was no correlation between sex and macroscopic signs of an infection, but the age distribution indicated that the disease affects only sexually mature animals (Mlengeya, 2004).

1.1 Taxonomy of the Olive baboon

The Olive baboon (*Papio hamadryas anubis*, Figure 1) is a member of the old world monkeys *Catarrhini*, suborder *Haplorrhini*, superfamily *Cercopithecoidea*, family *Cercopithecidae* and subfamily *Cercopithecinae*. It belongs to a group of monkeys with similar habitus: a characteristic dog-shaped nose and arms and legs of similar length, well adapted to the terrestrial locomotion. Baboons do have enlarged cheek pouches, which allow the animals to carry and temporarily store food. Currently, the genus *Papio* consists of only a single species with four different subspecies (Groves et al., 1993). However, there is lots of confusion and discussion surrounding the taxonomic status of the genus and many older publications upgraded current subspecies to species level. Thus, to avoid any kind of confusion surrounding the taxonomic status, this study makes use of the latest classification (Rowe, 1996).

Figure 1. Olive baboons (*P. h. anubis*) at LMNP. Animals were spotted near Marera River in the north of the park.

Species: Hamadryas baboon (*Papio hamadryas hamadryas*)
Subspecies: Yellow baboon (*P. h. cynocephalus*)
Guinea baboon (*P. h. papio*)
Olive baboon (*P. h. anubis*)
Chacma baboon (*P. h. ursinus*)

1.2 Reproductive biology

1.2.1 Behaviour

The social structure of Olive baboons is based on multimale-multifemale groups (Estes, 1991; Hoof, 1988; Melnick et al., 1987), often exceeding 200 group members. The average ratio of mature females to males is 2 – 3 : 1, whereas the number of subadults and juveniles frequently tops the average number of adults (Estes, 1991). Family bounds, friendships and alliances are an important part of the complex social system of the Olive baboon. Adult males leading the group run a rigid dominance based hierarchy. Females make up the stable core of the group, where as males emigrate during adolescence and often transfer rapidly between troops (Altmann, 1980; Packer, 1979a; Ransom and Ransom, 1971; Ransom, 1981). Offspring is ranked according to age in descending order; beginning with the youngest. In social relations between matrilines, the youngster takes the rank of the matriarch (Estes, 1991). By the age of two and a half years a female's rank is fixed for life. Males become dominant by the age of four, but have to mature for another three to five years before they can compete on equal terms with other males for reproductive opportunities (Estes, 1991). Females begin to reproduce by the age of five.

Males actively play godfather roles to their offspring and sometimes to infants other than their own: adult males are typically seen handling infants up to two years of age, when baboons become largely independent of their mothers in the juvenile stage. These and other social activities, which bring males in close contact with females, foster strong coalitions and social bonds between individuals. Thus, reproductive success of male Olive baboons is affected to a large extent by their nonsexual relationships with females (Estes, 1991; Hoof, 1988). Young males stay in close contact to their mothers until they are four years old: at which time they experience a growth spurt and become dominant over females. Baboons are sexually dimorphic with males exhibiting enlarged canines and a body mass significantly exceeding those of adult females (Estes, 1991). Low-ranking males do have the most to gain from emigration, since females tend to be receptive to dominant males that have recently joined the troop (Hausfater, 1975; Packer, 1979b).

1.2.2 Physiology of reproduction

Female baboons are non-seasonal, polyoestric (Ransom, 1981) with a tendency to a birth-rate that peaks in summer, at the beginning of the rain season. Gestation time is about 180 days (Melnick et al., 1987). The biphasic sexual cycle with a swelling- and a deturgescence phase is about 32.28 ± 4.26 (20 – 50) days long (Hendrickx and Kraemer, 1969) and divided into four separate stages: inflating, fully swollen, deflating and flat state (Ransom, 1981). Old world monkeys menstruate (Joslin, 2003), and menstruation as a consequence of endometrial desquamation is by definition set to be day 1 of the menstrual cycle (Meinecke et al., 2000). Shortly after menstruation, females enter the follicular dominated swelling phase were the ano-genital area becomes swollen. This is the time of the pro-oestrus, which is terminated by the ovulation in the fully swollen state, the time of oestrus. The following progesterone dominated phase is characterized by a deflating state of the sexual swelling leading to a flat state that determinates the menstrual cycle (Ransom, 1981).

Females in the late pro-oestrus often approach and present themselves to favored males, allowing even subadults to mount them (Estes, 1991). The level of a female's receptivity closely reflects her preference, which in turn affects the male's sexual performance. It is the sexual swelling that attracts males. Thus males rarely mate with subadult females, or females that are not in oestrus. Although females present themselves in pro-oestrus, both partners may take the initiative when the female comes into oestrus (Hall et al., 1965). Menopause in the baboon occurs at approximately 26 years of age (Luth et al., 2008).

During copulation females present themselves to the male in a quadrupedal position with tail deflected to the site. Males mount by, positioning their hind legs on the female's tarsi. Olive baboons copulate in a series of mounts each 30 minutes and males normally ejaculate during the first mount (Estes, 1991).

1.3 Diseases producing genital ulceration in nonhuman primates

The World Health Organization (WHO) classifies STDs as infections that are spread primarily through person-to-person sexual contact (WHO, 2007). STDs are furthermore split into infections that produce genital lesions (e.g. *Herpesvirus*

simplex, *T. pallidum*, *Haemophilus ducreyi*, *Klebsiella granulomatis*) and those, which can be sexually transmitted but do not generally produce genital lesions (e.g. Human Immunodeficiency Virus, Simian Immunodeficiency Virus (SIV), Human T-lymphotropic Virus Type, Cytomegalovirus).

1.3.1 Viral infections

In nonhuman primates STDs produced by viruses are basically caused by DNA-viruses of the family of *Herpesviridae* and *Papillomaviridae* (Ford et al., 1998). RNA-viruses of the family *Retroviridae* (e.g. SIV) are frequently transmitted by sexual contact, but do not cause genital lesions (Mansfield et al., 1998).

Virons of the family of *Herpesviridae* are 200 – 250 nm in diameter, and consist of a linear double-stranded DNA genome of 125 – 245 kbp packaged within an icosahedral capsid approximately 125 nm in diameter, embedded in a matrix which contains many virus-coded proteins, itself wrapped in a lipid membrane containing several viral glycoproteins (Davison, 2002). The gene encoding the glycoprotein B of primate alpha-herpesviruses (Black and Eberle, 1997) and the DNA polymerase gene (Prepens et al., 2007) are possible regions with alternating highly conserved and widely divergent DNA sequences that allow for differenciation. Herpesviruses remain latent in viable form within host cells after primary infection and there is an antigenic relationship among all members of the family (Pellett and Roizman, 2007). Three different subfamilies are recognized within the *Herpesviridae*: alpha- (e.g. Herpes simia), beta- (e.g. Cytomegalie) and gamma-herpesvirinae (e.g. Epstein-Barr). There is distinct species specificity among herpesviruses, even though cross-species transmission occurs frequently (Mansfield et al., 1998). Morbidity and mortality rates among different species of hosts differ significantly and in general mortality is higher in accidental hosts (Mansfield et al., 1998). Reactivation can occur from time to time from the latent stage to produce recurrent clinical lesions (Mayr and Kaaden, 2006). The particular mechanism for reactivation is currently unknown. The host is only infectious during the viraemic stage where virus replication and shedding takes place. In baboons, only *Herpesvirus papio 2* (HVP-2) has been documented to cause genital ulceration (Bigger and Martin, 2002). Some herpesviruses like the *Simian agent 8 virus* (SA8V) or the *Epstein-Barr virus* do have oncogenic potential

and sometimes produce cancer (Janeway et al., 2002; Martino et al., 1998). Syncytia and inclusion bodies of type Cowdry A can be found regularly in alpha-herpesvirus infections.

Cercopithecine herpesvirus 16 (HVP-2) is a member of the alpha-*herpesvirinae*. Historically the virus was set equal to the SA8V and sometimes is put on a level with the *Cercopithecine herpesvirus 2* (Mansfield et al., 1998). New studies define HVP-2 as “closely related vervet virus” and separate it from the SA8V (Rogers et al., 2005). Reasons for the confusion surrounding the taxonomic status are historically motivated as the SA8V was first isolated from a Vervet monkey (*Cercopitheceus aethiops pygerythrus*) in the late 50s (Malherbe and Harwin, 1958) and later on thought to be isolated from the baboon (Malherbe and Strickland-Cholmley, 1969a; Malherbe and Strickland-Cholmley, 1969b), too. According to the close phylogeny and antigenic characteristics, SA8V was discussed to be the pathogen that causes Herpes papionis (Mansfield et al., 1998). A retrospective study in the mid 90s showed evidence that SA8V differs from virus isolates which were gained from baboons in 1969 (Eberle et al., 1995). A comparison with “SA8V” isolates from a natural outbreak at the Southwest Foundation for Biomedical Research in 1985 (Levin et al., 1988) found that both outbreaks were caused by different viruses, sharing a high percentage of genetic and antigenic characteristics (Eberle et al., 1995). Baboon isolates were identical, but differed from those isolated from the Vervet monkey. In the following and with reference to the findings of Eberle et al. (1995) the papion “SA8V” is set equal to the HVP-2. Genetically the HVP-2 is closely related to the *Herpesvirus B*, although it has never been reported to infect humans (Rogers et al., 2005).

Two different transmission routes are known: via oral inoculation especially in neonates and juveniles (Kalter et al., 1978; Levin et al., 1988) and the common route via sexual contact (Levin et al., 1988; Payton et al., 2004). Infections are often asymptomatic in baboons (Mansfield et al., 1998) and its clinical manifestation was first recognized in the 1970s (Gary et al., 1973). Similar to lesions caused by *Herpesvirus simplex 2* in humans, natural infections with HVP-2 tend to cause genital ulceration in female and male baboons (Kaufman, 1986; Levin et al., 1988). There are reports about an increase in mortality rates in neonates (Eichberg et al., 1976; Rogers et al., 2005), but in most cases clinical

signs are restricted to the mucosa with subsequent viral persistence. Infected males sporadically show oral-genital, seldom oral and most often only genital lesions (Levin et al., 1988). In females genital lesions are also the most common clinical sign, but frequently included the ano-genital area around the base of the tail. In females, the oral manifestation was less frequently observed and oral-genital signs were seldom reported, when compared to male clinical manifestation (Levin et al., 1988). Corpus penis, prepuce and scrotum in males as well as vulva and perineum in female baboons showed small, and in females often confluent, laminar lesions. Multiple pustules, erythema and vesicles break down to from ulcers, which crust over and frequently get secondary bacterial infected (Levin et al., 1988; Mansfield et al., 1998; Martino et al., 1998). Untreated scar tissue might cause urethral obstruction (Singleton et al., 1995). Two days after experimental infection with HVP-2 into oral mucosa of healthy Olive baboons primary aphthae occurred at the site of inoculation, accompanied by secondary lesions at other sites of the oral mucosa (Rogers et al., 2005). Without exception and independent from the viral strain used, all with different virulence, oral lesions were observed within 21 days post infection. The aforementioned oncogenicity of HVP-2 was reported in a single case (Martino et al., 1998) where 4 females out of 60 baboons showed perineal neoplasia. Two lipomas, one spindle cell sarcoma with characteristics of an aggressive angiomyxoma and one benign angiomyxoma were diagnosed (Martino et al., 1998).

Herpesvirus hominis 2 (HVH-2) infections in NHPs lead to a diffuse inflammation of the vulva and cervical mucosa with multiple vesicles and oedema. Leucorrhoea and bleeding is a common clinical feature (Ford et al., 1998). The disease is self-limiting, although viral reactivation can occur at any time. Experimental intravaginal infection in White-fronted capuchins (*Cebus albifrons*) and *Callitrichidae* were possible and produced significant primary lesions associated with viraemia (Felsburg et al., 1973; Reeves et al., 1981). However, in baboons HVH-2 inoculation failed to produce clinical signs (Felsburg et al., 1973).

Most papillomaviruses do have pronounced species specificity and tissue tropism. They have circular double-stranded DNA genomes with sizes close to 8 kb. Three oncogenes, E5, E6 and E7 modulate the transformation process, two regulatory

proteins, E1 and E2 modulate transcription and replication, and two structural proteins, L1 and L2, compose the viral capsid (Munger and Howley, 2002). Infection can be more aggressive in non-adapted hosts (Mansfield et al., 1998). Major scientific concern arises from the ability of papillomaviruses to express trans-regulatory factors and early viral proteins that may influence cellular growth, leading to transformation of host cells (Mansfield et al., 1998). With its affinity to ceratinocytes the virus often causes malignant neoplasia of the cutis and mucosa. A massive hyperplasia of the Stratum spinosum and corneum is often accompanied by basophile intranuclear and acidophile intracytoplasmatic inclusion bodies in histology (Mansfield et al., 1998). Due to their potency to cause cervical carcinoma in women human papillomaviruses are recognized as major health concern (Gall, 2001) and there are first reports of suspected cases on viral associated benign and malign tumors in baboons (Sundberg et al., 1993). Condylomata acuminata is another common clinical manifestation in women (Gross et al., 1985; Gunter, 2003) but was never described for baboons. The finding of papillomavirus DNA in a squamous epithelial carcinoma of the penis with metastasis into regional lymphnodes in a Rhesus macaque (*Macaca mulatta*) (Kloster et al., 1988) as well as the description of venereal papillomas in an Eastern Black-and-white colobus (*Colobus guereza*) (O'Banion et al., 1987) presumes that NHPs are capable for papillomavirus infections leading to similar clinical manifestations and genital ulceration (Kloster et al., 1988). Polyposis and papillomatosis were mostly diagnosed as site-finding during post mortem examination and generally stayed undiagnosed in-vivo (Ruch, 1959).

1.3.2 Bacterial infections

Treponematosis has an ancient history in humans (Froment, 1994; Gray et al., 2006; Harper et al., 2008a; Harper et al., 2008b; Hudson, 1965) and treponemal infections in wild NHPs are not a new phenomenon. Great apes (Lovell et al., 2000) as well as monkeys have been identified to carry *Treponema* spec. and large numbers of infected baboons were identified in areas of West Africa (Fribourg-Blanc et al., 1963; Fribourg-Blanc and Mollaret, 1969). Clinical signs, when present at all, were usually described as small ulcers on the muzzle (Baylet et al., 1971a; Baylet et al., 1971b; Fribourg-Blanc and Mollaret, 1969; Fribourg-

Blanc and Siboulet, 1972). At Gombe Stream National Park (GNP) in Western Tanzania, *T. p.* ssp. *pertenue* was suspected, but not verified, to be the causative agent for a venereal disease in baboons (Wallis and Lee, 1999; Wallis, 2000). *Treponema pallidum* is a spiral shaped bacterium that belongs to the family of *Spirochaetaceae*, order *Spirochaetales*. The *pallidum*-genome is a circular chromosome of 1,000 kb, one of the smallest prokaryotic genomes, with a total of 1,041 predicted open reading frames, representing 92.9 % of total genomic DNA (Fraser et al., 1998). The organism cannot be grown on artificial media and it replicates slowly in tissue cultures and animal models with a replication time of approximately 30 hours (Fraser et al., 1998; Richens and Mabey, 2009).

Although the different subspecies of the spirochete ***T. pallidum*** are closely related in their phylogeny, their clinical manifestation differs widely. Most endemic and non-endemic forms do have their own clinical characteristics and therefore are named differently: bejel (ssp. *endemicum*), yaws (ssp. *pertenue*) and syphilis (ssp. *pallidum*). *T. carateum* is currently recognized as another species and causes pinta. Non-venereal strains (*T*. *p*. ssp. *endemicum* and *pertenue, T. carateum*) are mostly recognized as skin diseases of early childhood (Antal et al., 2002; Kapembwa, 2009), where as the ssp. *pallidum* causes syphilis and is the only known subspecies of the *pallidum*-complex that is adapted to a venereal transmission mode (Richens and Mabey, 2009). In humans, the clinical manifestation of syphilis and yaws is divided into three stages (Antal et al., 2002; Castro et al., 2007; Wenhai et al., 2004). In syphilis the primary stage, so called hard chancre, can be found at the side of inoculation. In the course of the disease the ulcer heals with or without treatment and if untreated the bacterium disseminates into other parts of the body (second stage), classically causing a palmar or solar rash (Richens and Mabey, 2009; Samuelson, 1999). The second stage is followed by a tertiary stage which could occur from weeks to years following the disappearance of the secondary stage: at which point several other organs (e.g liver, kidney, heart, central nervous system or bones) can be affected (Richens and Mabey, 2009; Samuelson, 1999). “Gumma”, a well-demarked ulcerative lymphocyte and plasma-cell aggregation, infiltrated by fibroblasts and epitheliod-cells, may appear on all parts of the body (Hay et al., 1990; Samuelson, 1999). Horizontal transmission of *T. p.* ssp. *pallidum* has been reported in humans (Beltrami and Berman, 2006).

Haemophilus ducreyi is endemic in Africa and is of major health concern, causing chancroid or soft sore in humans (D'Costa et al., 1985; Morse, 1989; Plummer et al., 1985; Richens and Mabey, 2009). The bacterium is a fastidious organism and requires complex media and growth conditions for culture (West et al., 1995). Its genome is composed of a single 1,700 kb chromosome with a total of 1,693 identified putative open reading frames (Spinola et al., 2002). There are no reports on naturally occurring infections in NHPs, but Pig-tailed macaques (*Macaca nemestrina*) were successfully inoculated with *H. ducreyi* into the foreskin. The animals developed lesions similar in appearance, histopathologic changes, and progression to those of human disease (Totten et al., 1994). Three to seven days after infection papules arise at the site of inoculation and rapidly form ulcers. Lesions are characteristically painful and in nearly half of the cases involve lymphadenopathy of regional lymphnodes (Jones et al., 1990; Morse, 1989; Richens and Mabey, 2009; Willis, 1990). Key factors for the extensive necrotising dermatitis produced by *H. ducreyi* infections are two bacterial toxins: a cell-associated haemolysin and a soluble toxin (Lagergard, 1992; Stevens et al., 1999).

The genus ***Chlamydia*** consists of amotile Gram-negative bacteria, which utilize host cell energy and therefore are obligate intracellular parasites (Selbitz, 2006). *Chlamydia trachomatis* has a 1,042 kb genome and a 7.5 kb plasmid (Stephens et al., 1998). Analysis of the chlamydial genome resulted in the identification of 894 likely protein-coding genes (Stephens et al., 1998). The infections caused salpingitis in Pig-tailed macaques (Cappuccio et al., 1994) where as epidermitis and urethritis has been observed in Vervet monkeys (*Chlorocebus aethiops*) after infection (Moller and Mardh, 1980). At least in humans a mucupurulent cervicitis has been described for *Chlamydia* infections, although compared to other clinical features (such as salpingitis) it is rarely seen (Oriel, 1983; Richens and Mabey, 2009).

1.3.3 Fungal and protozoal infections

Fungal and protozoal infections of the genitalia do not play a major role as STDs in NHPs. Genital ulceration has not been reported, yet. *Candida albicans* is known

as saprophytic yeast of the vagina in captive and wild baboons (al Doory, 1967; al Doory, 1968; al Doory, 1969) and may cause candidiosis in individual cases in immunocompromised hosts (Migaki et al., 1982).

Trichomonas vaginalis can be found in the vagina of NHPs, but in general does not cause macroscopic tissue alterations of the external genital (Kraemer et al., 1972), only three of eight artificially infected Pigtailed macaques showed erythema of the cervix and vagina (Patton et al., 2006). Vaginal discharge occurred in artificially inoculated new world monkeys (Street et al., 1983).

2 Materials and methods

2.1 Field site

Field investigation took place at LMNP (3° 35′ 0″ S, 35° 50′ 0″ E) in 2007. Lake Manyara is one of 15 national parks of the United Republic of Tanzania, located in Northern Tanzania, 126 km West of Arusha town (Figure 2). At the time when research was conducted its size was quoted to be around 330 km^2 [#] with two-third coverage by the lake (Figure 3). A variety of different vegetation types characterises the national park: a humid groundwater forest covers most of the northern area, where as savanna and more dry acacia woodlands dominate the southern parts. Baboons can be found in all parts of the park, but the groundwater forest and villages bordering LMNP in the North attract most of the animals. Two other primate species can be found at LMNP: the Blue monkey (*Cercopithecus mitis*) and the Vervet monkey (*Chlorocebus aethiops*). The genital ulcerative disease, however, does not clinically affect both species. The status of the primate community at LMNP is of "least concern" according to the IUCN Red List of Threatened Species (www.iucnredlist.org).

[#] Following the completion of fieldwork, LMNP doubled with the inclusion of Marang Forest in 2009, a huge forest reserve at the rift escarpment in the Southern part of the park.

Figure 2. Map of Tanzania. LMNP is located in the North, West of Kilimanjaro and Arusha. (Map source: http://www.tanzaniawildlifesafaris.com/img/tanzania-overview-map.gif)

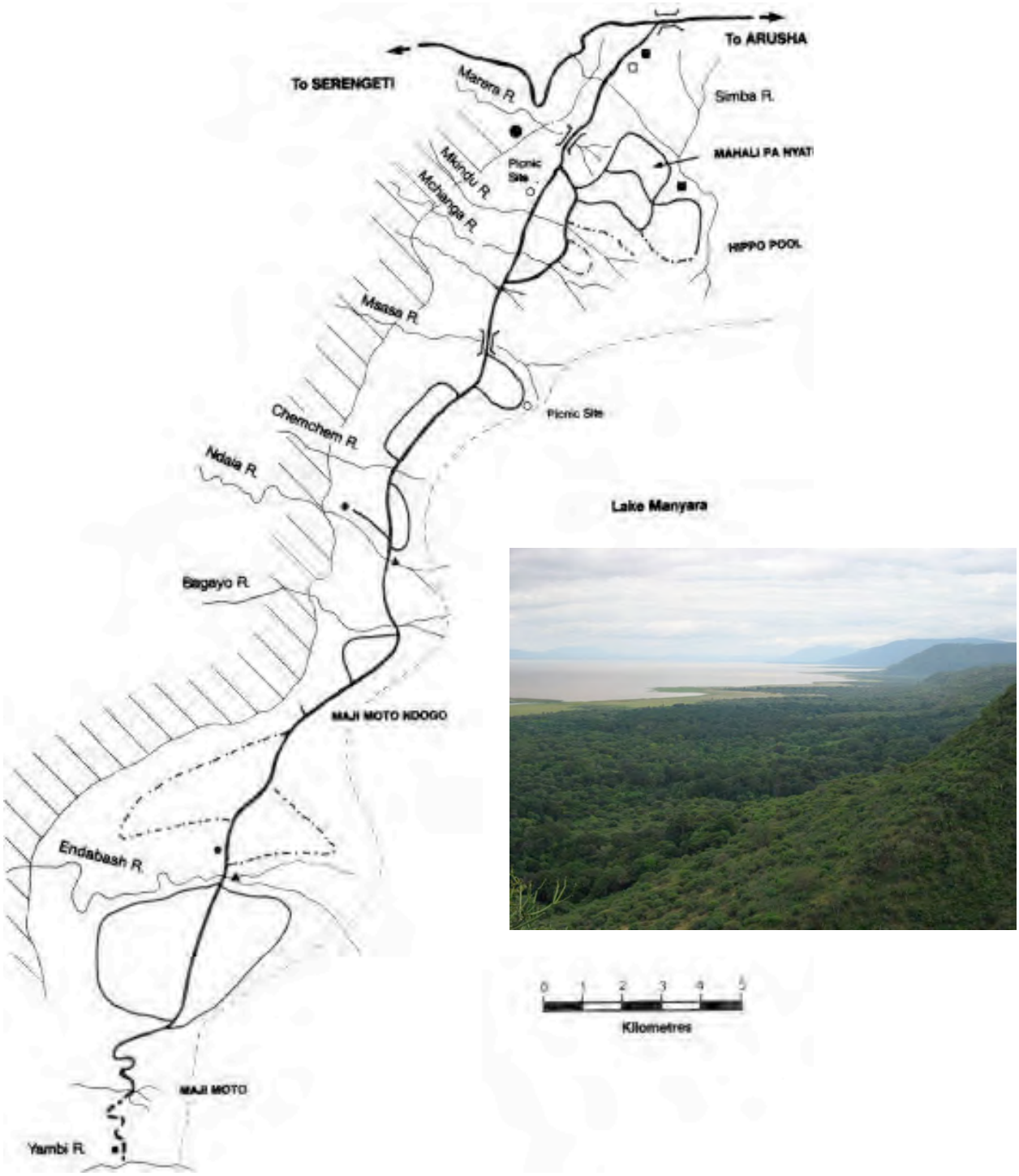

Figure 3. **Lake Manyara National Park** (reproduced from TANAPA/ AWF, Senelson, 1986). Photography showing the landscape along the East African Rift Valley from the North to the South.

2.2 Species

Sixty-three Olive baboons (Table 1), thirty-nine (Table 12: case Nos. 21 – 60 and 65) with clinical signs of infection and twenty-four (Table 12: case Nos. 1 – 20 and 61 – 64) without were sampled; two animals were recaptured (case Nos. 56 and 60, 58 and 59 are the same individual). Baboons with genital ulcerative lesions visible to the naked eye were categorised as “clinically affected”, those without any genital lesions were classified as “clinically non-affected”. Animals, which were affected by genital lesions, were furthermore divided into “initial”, “moderate” or “severe” stage, depending on the extent of macroscopic visible ulceration (Figure 4). The initial stage was characterized by minor efflorescences and beginning ulceration. The moderate stage was in-between initial and severe stage with genital ulceration clearly visible, compassing up to 50 % of the ano-genital area. The severe stage was documented in cases where ulceration and scarification covered the whole ano-genital area.

Since age-classification is challenging in wild baboons, especially when the animals are not observed for a longer period of time, some special parameters were used for gross estimation. Baboons were recorded as “neonate” (Table 12: case No. 64) from birth until fur-colour changed from dark- into olive-brown. When the baboon troop was moving, neonates were carried under the mother’s belly. “Subadults” were defined as children with olive-brown fur until they reached the weight of adult. The weight of female and male subadults (Table 12: case Nos. 4 – 9, 11 – 12, 18, 39 – 41, 53 and 62 – 63) was inbetween 1.5 and 10.0 kg (mean = 5.9, SD = 2.4, *n* = 15). Childrens were riding on their mother’s back like jockeys. “Adult females” (Table 12: case Nos. 1 – 3, 10, 21 – 25, 35 – 38, 42 – 49 and 61) had an average weight of 11.0 kg (SD = 1.7, *n* = 22), whereas “adult males” (Table 12: case Nos. 16, 26 – 27, 32 – 34, 50 – 51and 54 - 55) were recorded with an average weight of 16.5 kg (SD = 3.2, *n* = 11). Dominat males (Table 12: case Nos. 13 – 15, 17, 19 – 20, 28 – 31, 52, 56 – 58 and 65) had the longest canines and were found to be the heaviest baboons at LMNP (mean = 20.4 kg, SD = 2.3, *n* = 15).

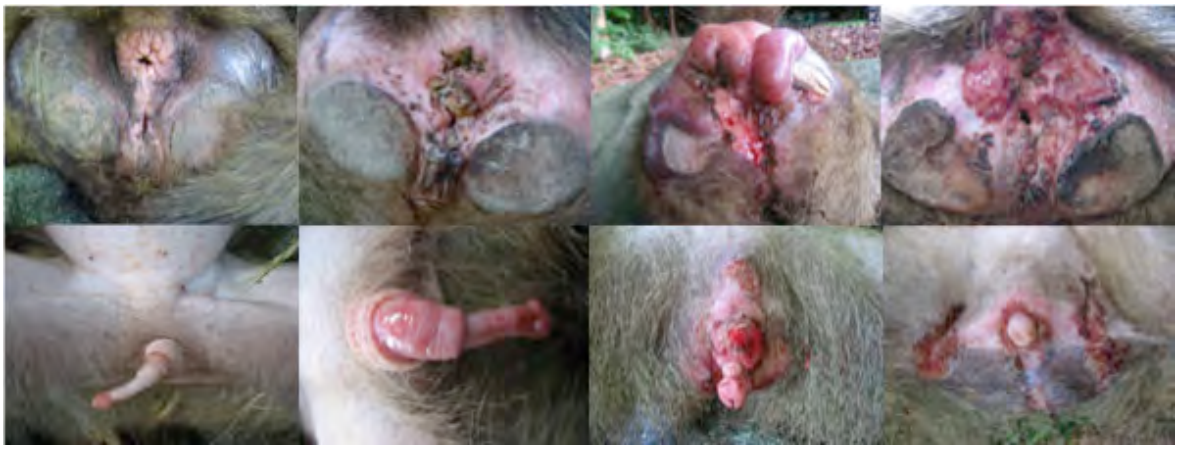

Figure 4. **Clinical infection stages.** From left to right: clinically non-affected, clinically affected: initial, moderate and severe stage. Females on top, males below.

Table 1. **Crosstab of sampled Olive baboons**. Ratio of gender, clinical infection stage and age.

***F*emale**				***M*ale**			
Group key	**Neonate**	**Subadult**	**Adult**	**Adult**	**Subadult**	**Neonate**	**Group key**
***F*1**	1	9	4	7	2	0	***M*1**
Group # 1: Clinically unaffected							
***F*2**	0	0	6	8	0	0	***M*2**
Goup # 2: Initial stage							
***F*5**	0	0	4	1	3	0	***M*5**
Group # 5: Clinically moderate affected							
***F*8**	0	0	8	8	1	0	***M*8**
Group # 8: Clinically severe affected							
***F*11**	0	0	0	1	0	0	***M*11**
Group # 11: Clinically genital and mouth alterations							
Total (n = 32)	1	9	22	25	6	0	**Total (n = 31)**
Total individuals n = 63[#]							

[#] Two animals were recaptured, resulting in 63 individuals and 65 cases (Table 12).

2.3 Field observations and sampling

Field trips made use of a 4 x 4 wheeled Land Cruiser (Toyota Inc., Torrance, CA, USA), which was driven and maintained by a local driver. An armed ranger from Tanzania National Parks (TANAPA) was present to secure and to support the research activities (Figure 5). Field investigations took place from 03-21 – 05-19-2007.

Figure 5. Research staff: veterinarian, driver and armed ranger.

Baboons were sought in all areas of LMNP (Figure 8, Appendix 7.5) starting at 8.00 am. Once a troop was spotted it was followed and the group size and overall prevalence of animals with genital ulcerations (visible to the naked eye) were estimated. Animals that were chosen for immobilisation were observed until they felt secure in a group setting. In general animals were not immobilized during midday heat (12.00 am – 2.00 pm), or later than 4.30 pm to guarantee enough time to recover until dawn. Field investigations were terminated with dawn around 6.00 pm. Individuals and samples were consequently marked with a unique identification key. The first number describes a running number of all immobilisations. All dart-hits were counted, even if the desired anesthesia was not achieved. Subsequently a running number was awarded only once. This first number was followed by a letter-number constellation that gives information about gender and clinical infection stage as it was documented in the field (Table 2). The

date of immobilization completed the identification scheme.

Example

Animal ID: 11^{1}-F1^{2}-26.03.07^{3}

[1]running number

[2]female, clinically non-affected

[3]date of immobilization

Table 2. **Code for gender and clinical infection stage**. Code used for gender (F/ M) and first categorization (1 – 13) of the clinical infection stage during field observation.

#	**F**emale	**M**ale	#
F1	Clinically non affected		**M1**
F2	Clinically affected - initial stage - genital		**M2**
F3	Clinically affected - initial stage - oral		**M3**
F4	Clinically affected - initial stage – oral/ genital		**M4**
F5	Clinically affected - moderate stage - genital		**M5**
F6	Clinically affected - moderate stage - oral		**M6**
F7	Clinically affected - moderate stage – oral/genital		**M7**
F8	Clinically affected - sever necrosis - genital		**M8**
F9	Clinically affected - sever necrosis – oral		**M9**
F10	Clinically affected - sever necrosis – oral/ genital		**M10**
F11	Clinically affected – oral and genital but lesions differ in severity (Please describe on the protocol)		**M11**
F12	…others		**M12**
F13	…others		**M13**

All baboons were immobilized according to state-of-the-art wildlife anaesthesia procedures (Figure 6). The immobilization protocol can be found in the appendix (Appendix 7.1). A mixture of ketamine (10 mg/kg body weight (bw)) and xylazine was used as appropriate drug (Knauf et al., 2007). Different dosages for xylazine, in a dose-range of 0.1 – 2.0 mg/kg bw, were tested to optimise the time from injection to the onset of anesthesia (induction time).

Figure 6. Baboon female at the moment when the anaesthetic was injected.

All anaesthetised patients were continuously monitored using a Nelcor OxiMax N-65 pulse-oximeter (Scil animal care company, Viernheim, Germany) starting at the time of discovery until fully recovered (Figure 7). Baboons were temporarily marked by shaving a part of their tail, after sampling has been completed.

Figure 7. Baboon monitored during anaesthesia.

Baboons were secured until they were awake and able to cope with the circumstances of their environment. Animals received a long-acting antibiotic (Duphamox LA 150 mg/ml, Pfizer GmbH, Berlin, Germany), an insect repellent (Butox, Intervet Deutschland GmbH, Unterschleissheim, Germany) and in some cases atipamezole in a dose of 1 ml/40 mg xylazine, 50% intramuscular and 50% subcutane (Antisedan, Janssen Animal Health, Neuss, Germany). A body condition score was used to document the animal's nutritional status (Table 3).

Table 3. **Body condition scoring**. Body condition scoring as it was used to categorize the Olive baboons at LMNP.

0	Very bad condition	Absolutely no subcutaneous fat, severe muscular weakness, skeletal system is prominent, cachexia
1	Bad condition	Same as score 0 but with almost no subcutaneous fat and moderate muscular weakness
2	Moderate	Underweight and with only a small percentage of subcutaneous fat but still in acceptable condition
3	Good	Physiological body mass, normal subcutaneous fat, muscular system well developed
4	Very good	In between score 3 and 5 but still physiologic
5	Adipose	Abnormal subcutaneous fat, body weight above normal, skeletal system not visible

Digital pictures of the perianal and genital area were taken while the animals were in dorsal recumbence for males and from caudal in females. Blood, genital smears and two skin samples were taken from clinically affected individuals. A sterile 6-mm biopsy punch (Heiland VET Vertriebsgesellschaft, Hamburg, Germany) was utilized to sample the ano-genital skin, excluding males with dermatitis affecting the corpus of the penis. In this particular case tissue samples were carefully taken with a sterile scalpel blade (Heiland VET Vertriebsgesellschaft, Hamburg, Germany) to prevent damage to the corpus cavernosum penis. Under general anaesthesia clinically affected individuals were sampled for one piece of tissue from the edge of the alteration and another one from the adjacent skin tissue that looked healthy. These samples, which included all three layers of the skin (epidermis, dermis and subdermis) were immediately preserved in 10 % buffered neutral Lilli's formalin (Appendix 7.4). Two more biopsies were taken from the edge of alteration, preserved in RNA-Later (Applied Biosystems Inc., Foster City, CA, USA) and frozen in liquid nitrogen. In clinically non-affected baboons only a single skin tissue sample of the ano-genital region was collected. Samples were cooled during the day utilizing a recoolX bag (Tex2recool GmbH, Osnabruck, Germany) including ordinary cooling elements taken from a freezer which

guaranted a constant low temperature under field conditions. In the evening samples were than moved into the appropriate custody (i.e. liquid nitrogen).

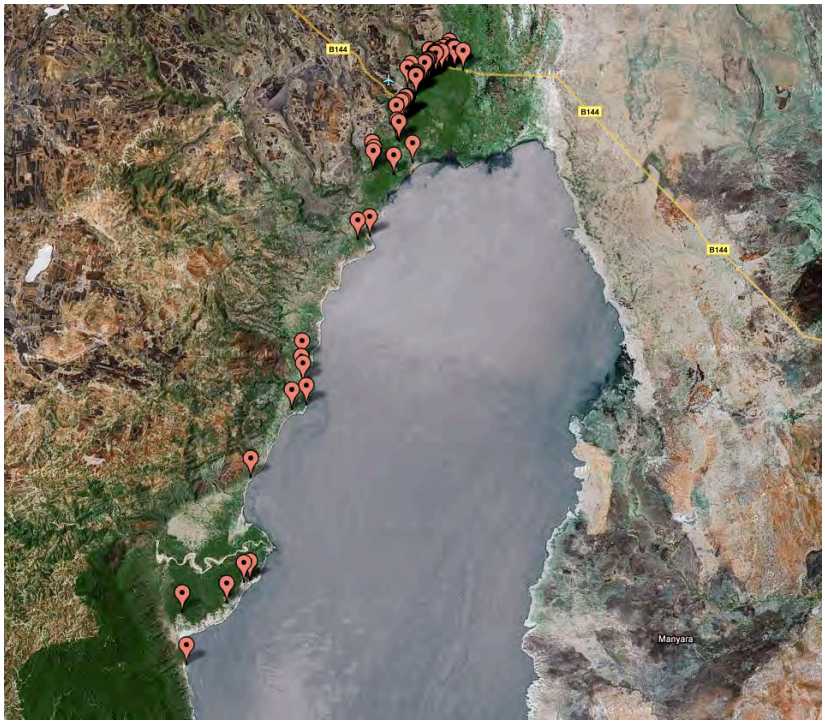

Figure 8. **Locations at LMNP were baboon immobilisations occured, bases upon GPS data taken at each field site (Appendix 7.5).** (Map source: Google Earth, Google Inc., Mountain View, CA, USA).

2.4 Vaginal exfoliative cytology

Vaginal exfoliative cytology was performed after proper cleaning of the female's external genital skin with a mucosa-protective disinfectant (Kodan, Schulke & Mayr, Norderstedt, Germany). The 140 mm speculum after Kilian was introduced to the vagina and straddled when fixed in a correct position. From each animal, two vaginal smears were collected from the dorsal vaginal vestibulum. This was done by introducing a sterile cotton swab into the vagina in between the straddled shanks of the speculum. In order to harvest enough epithelial cells the swab was moved towards the dorsal vestibulum under a slight back-and-forth rotation. Thereafter it was immediately rolled over a slide in two to three parallel lines. Exfoliated cells and mucous that adhered to the slide were dried at ambient temperature and stored in classic slide boxes. Silica gel (Silica gel orange, Carl Roth GmbH + Co KG, Karlsruhe, Germany) was used to reduce humidity. Two vaginal cytology specimens were stained with Papanicolaou (Papanicolaou staining system, Sigma-Aldrich, Steinheim, Germany, Appendix 7.2.5) and Diff-Quick staining solution (Haema Schnellfärbung, Niepötter-Labortechnik, Bürstadt, Germany, Appendix 7.2.1) according to the manufacturer's instruction. Staining was performed at the laboratory of the Clinic for Gynaecology and Andrology of Large and Small Animals. Finally, each slide was mounted with Euparal (Carl Roth GmbH + Co KG, Karlsruhe, Germany) in order to improve the integrity and prolong shelf-life. Slides were light-microscopically examined twice with a magnification of 200x (Leica DM R, Leica Microsystems, Wetzlar, Germany). Cells were scanned in a meandering course and evaluated using a standard technique (MacLennan and Wynn, 1971). The maturation index was calculated as described by Meisels (1967). The sum of 100 representative epithelial cells in one slide was counted: parabasal cells got a value of 0, intermediate cells 0.5 and superficial cells were considered as 1.0 per counted cell.

Digital pictures (2.3) were used to compare the cytological findings with the female's sexual swelling stage at the day when sampling was performed. Females with absolute no sexual swelling of the genital were recorded as "flat state", those with fully swollen genital as "fully swollen state". Females with minor swelling of the ano-genital skin were categorised as "inflating " or "deflating state".

2.5 Histology

Histology was performed at the German Primate Centre. Formalin preserved tissue samples were cut into two vertically asymmetrical pieces and embedded into paraffin (Hypercenter XP, Thermo Shandon GmbH, Frankfurt am Main, Germany) (Appendix 7.3). The paraffin blocks were subsequently cut into 4-µm thick layers using a microtome (Mikrotom HM 400R, Microm International GmbH, Walldorf, Germany) and submerged in a 40 °C water bath containing distilled water. Specimens were allowed to dry over night at 37 °C, after being transferred onto a slide (Superfrost plus, Gerhard Menzel Glasbearbeitungswerk GmbH & CoKG, Braunschweig, Germany).

Hematoxyline-Eosin (H&E, Merck KGaA and Thermo Shandon GmbH, Frankfurt, Germany, Appendix 7.2.4) and Periodic-Acid-Shiff (PAS, Merck KGaA, Darmstadt, Germany, Appendix 7.2.6) staining was performed in all specimens using a fully automated process (Varistain Gemini, Thermo Shandon GmbH, Frankfurt Germany). Warthin-Starry-silver (WSS) staining (Appendix 7.2.7) was also performed in 57 individuals (Table 12: case Nos. 1 – 57). Giemsa staining (Carl Roth GmbH & CoKG, Karlsruhe, Germany, Appendix 7.2.2) was applied in all cases in which bacterial structures were present. Staining followed the manufacturer's protocol. Each slide was examined in two separate rounds, under light-microscopy using a standardized meandering course, with starting magnification of 100x, followed by increasing magnification up to 1,000x.

2.6 Immunohistochemistry

Analyses were performed at the laboratories of the German Primate Centre. Paraffin-embedded tissue sections were heat pre-treated with citrate buffer (pH 6.0) followed by immunohistochemical analyses, using a rabbit-polyclonal antibody against *T. pallidum* (Cat. # GTX40465, Biozol Diagnostica Vertrieb, Eching, Germany) diluted 1:500. A tissue section from a patient infected with primary stage syphilis served as a positive control (provided by A. Weber, University of Zurich, Switzerland). Primary mouse-anti-human B-cell CD20 (M755, Dako, Hamburg, Germany) and rabbit-anti-human T-cell CD3 (A452, Dako, Hamburg, Germany) antibodies were used in dilutions of 1:300 and 1:50, respectively, in order to

differentiate B- and T-cells. Biotinylated secondary antibodies, streptavidin and diaminobenzidin (Cat. # 760-091, Detection Kit, Ventana/View DAB, Illkirch, France) were applied as chromagen according to the supplier´s instructions. Replacing the primary antibodies with sheep serum on a slide served as a negative control for the specificity of the antibody. B- and T-cell ratio and counts were estimated semi-quantitatively. Mayer`s hematoxylin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used to counterstain.

2.7 Fluorescence-in-situ-hybridization

A broad rRNA targeted oligonucleotide probe (EUB 338: 5'-GCT GCC TCC CGT AGG AGT-3', Amann et al., 1990) was used to illustrate prokaryote structures in-situ of five baboons with different infection status (Table 12: case Nos. 21, 37, 49, 59 and 60). Paraffin-coated slide specimens (2.5) were incubated in > 98 % Xylene (Carl Roth GmbH & Co KG, Germany) three times, five minutes each, under gentle movements; Xylene was renewed after each session. Drying was achieved under an extractor-hood. Specimens were then heated up to 50 °C and coated with 30 µl hybridization solution, containing 20 % formamid to achieve a better stringency. Incubation lasted for 4 hours at 50 °C in a chamber with high humidity. Before staining supernatant was removed by dipping the slides into destilled water. For nuclei counter-stain TOPRO staining solution (Invitrogen, Karlsruhe, Germany) was applied in a dilution of 1:750 with PBS buffer (pH 7.4) for 10 minutes. The supernatant was removed by bathing the specimens in destilled water. Finally items were covered with ProLong gold antifade reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Slides were examined with a fluorecence microscope (Leica DM IRE2, Leica, Wetzlar, Germany) at the laboratories of the Free University of Berlin.

2.8 DNA-extraction

Laboratory facilities of the Robert-Koch-Institute (RKI) were used to extract DNA from skin tissue biopsies conserved in RNA-Later. Skin samples were cut into small pieces and ground with Precellys-Keramik beads (peQlab Biotechnologie, Erlangen, Germany), followed by incubation with proteinase K for 4 to 6 hours at

56 °C. Remaining steps followed the manufacturer's protocol (NucleoSpin Tissue extraction kit, Macherey-Nagel, Düren, Germany). All washing steps were carried out twice.

2.9 Polymerase chain reaction

2.9.1 16S rRNA PCR

In six baboons (Table 12: case Nos. 17, 21, 26, 37, 49 and 59), covering all infection stages (one clinically unaffected animal and five clinically affected animals: two in the initial stage, one moderate case and two severe cases of genital ulceration), a 16S rRNA gene PCR (Muyzer et al., 1993) was used to detect a broad range of different prokaryotes. PCR was carried out following standard procedures. Primer sequences are listed in Table 4. Each 25-µL reaction mixture contained 3.00 mM $MgCl_2$, 200.00 µM dNTP, 0.10 µM of each primer, 0.50 U DFS-Taq DNA polymerase (Bioron, Ludwigshafen, Germany) with 2.50 µL of 10x PCR buffer (Bioron, Ludwigshafen, Germany) and template DNA in a concentration of 4.00 ng/µL. The thermocycler programme was as follows: one cycle of 10 minutes at 95.0 °C, 35 cycles of 30 seconds at 95.0 °C, 20 seconds at 56.0 °C and 40 seconds at 68.0 °C ending with one cycle of 7 minutes at 68.0 °C. The PCR products then underwent gel electrophoresis on a 1.5 % agarose-gel containing ethidium-bromide (Carl Roth, Karlsruhe, Germany) and were visualised under ultraviolet light. All positive products were then cloned (2.10) and sequenced (2.11). Resulting sequences were compared to the public GenBank database (NCBI, BLASTN 2.2.18+, Altschul et al., 1997). PCRs were performed at the RKI PCR facility.

2.9.2 PCRs for the detection of pathogens causing genital ulceration

Polymerase chain reactions were run in 57 Olive baboons (Table 12: case Nos. 1 – 57) to detect *T. pallidum* (the bacterium that causes yaws, syphilis and bejel) and to rule out the presence of *Klebsiella granulomatis* (the bacterium that causes donovanosis), *H. ducreyi* (the bacterium that causes soft chancre) and *Herpesvirus papio* 2 (the virus that causes baboon genital herpes), all known to

cause compatible clinical signs. In addition, 26 randomly chosen baboons (Table 12: case Nos. 1, 4, 7, 9 – 10, 12, 17, 21, 24, 26, 30 – 31, 35, 37 – 38, 40 – 42, 44 – 46, 48 – 49, 52, 54 and 59) were tested for the presence of any other herpesviruses with pan-herpes consensus PCR (Ehlers et al., 1999; Prepens et al., 2007). Primers for the detection of HVP2 were designed from an alignment considering 18 different sequences of alpha-herpesviruses. Primer sequences, annealing temperatures and method references are summarized in Table 4. PCR conditions for HVP2 followed the same protocol as that used to detect the other herpesviruses.

Amplifications were performed in 50 µ L reactions containing 0.5 mM primers (Invitrogen, Karlsruhe, Germany), 200 µM GeneAmp dNTPs (Applied Biosystems, Foster City, CA, USA), and 2.5 U AmpliTaq Gold polymerase with Gold Buffer and 3.0 mM $MgCl_2$ (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: one cycle of 94 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds, primer annealing at the appropriate temperature for 30 seconds, and 72 °C for 1 minute and 30 seconds; followed by a final extension for 7 minutes at 72 °C. Standard precautions to avoid DNA contamination were employed, including the use of negative controls, aerosol resistant pipette-tips, and a three-station PCR set-up protocol. Positive control for the detection of *H. ducreyi* was provided by S. Bruisten (Municipal Health Service Amsterdam, Netherlands). PCR products were prepared for sequencing using a gel extraction kit (Jetquick Spin Column Technique, Gel Extraction, Genomed GmbH, Löhne, Germany) following the manufacturer's instructions. PCRs were performed at the RKI PCR facility.

Table 4. **Primers (excl. *T. pallidum* primers).** Primers used for the detection of *K. granulomatis*[a], *H. ducreyi*[b], Pan-herpesvirus[c], HVP2[d] and 16S rRNA[e] targeting a broad range of different prokaryotes.

Gene	Primers (5' to 3') Forward Reverse	Anneal. temp. °C	Pro-duct size (bp)	Source
phoE[a]	CTATGACAGCAAGGATGGCGA CAGACCGAAGTCGAACTGATACTG	58	700	Carter et al., 1999
16S rRNA[b] Nested 1	CAAGTCGAACGGTAGCACGAAG TCATCTCTGAGTTCTTCTATG	58	960	Bruisten et al., 2001; Orle et al., 1996; Roesel et al., 1998
16S rRNA[b] Nested 2	TTCTGTGACTAACGTCAATCAATTTTG TCGGATTAAAGGGTGGGACCTT	58	309	Bruisten et al., 2001
dPol[c] Nested 1	GAYTTYG(N/I)AGYYT(N/I)TAYCC TCCTGGACAAGCAGCAR(N/I)YSGC(N/I)MT(N/I)AA GTCTTGCTCACCAG(N/I)TC(N/I)AC(N/I)CCYTT	46	737 475	Ehlers et al., 1999
dPol[c] Nested 2	TGTAACTCGGTGTAYGG(N/I)TTYAC(N/I)GG(N/I)GT CACAGAGTCCGTRTC(N/I)CCRTA(N/I)AT	46	228	Ehlers et al., 1999
HVP2[d] Semi-Nested 1	CTACCCCAGCATCATCCAGGC GCGGCCGATGGTCGTGAC	61	364	
HVP2[d] Semi-Nested 2	CGGGACTGGCTCGCCATG GCGGCCGATGGTCGTGAC	61	192	
16S rRNA[e]	AACGCGAAGAACCTTACCTG GACGGGCGGTGTGTAC	56	450	Muyzer et al., 1993

I = Inosine

Table 5. Primers and real-time primers[#], including probe[*] for the detection of *T. pallidum*.

Gene	Primers (5' to 3') Forward Reverse	Anneal. temp. °C	Product size (bp)	Source documen-ting changes
tpf1[a]	GAAAAAAATACCACAGCACCGC CAATGCCGTAGATGTGCCAGTG	57	171	Noordhoek et al. 1990
gpd[a]	AAGAACTTTCCCTCCTCCGTGC CGTTTGATACGCTTCAGCTCG	55	331	Cameron et. al. 1999
deoD[a]	GGTTACCAGAAAGGGCGTATTCC CGACCATTACACGACCATC	55	501	Centurion-Lara et al. 1998
cfpA[a]	GAGTCCCAATGTGTTTCATCC GAACGCACACTTGACTACCG	55	556	Harper et al. 2008a; Izard et al. 1999
tp92[a]	AGAGCCTGAAGCTCGGGTAT ACCGTGAACGACAACACAAA	55	1,030	Harper et al. 2008a; Cameron et al. 2000
tprI[b]	CGTCACCCTCTCCTGGTAGT ATCCCTCGCCTGTAAACTGA	60	1,830	Harper et al. 2008a; Centurion-Lara et al. 2006; Gray et al. 2006
tpf1 Nested	CGTGCCATTGCTGCTATCT TGCCGTAGATGTGCCAGTG	56	100	
gpd Nested	GTGGGTTGGAACAGACAACC CGTTTGCACATACACTAGATCC	55	161	
tp170 Nested	TAATGGCGTCCCCTTTGTTA GAAGCCACTACCGATGTGC	55	171	
cfpA Nested 1	GAGCGTCTGGACGTAATGG TAGGATGGCAATCTCCTTCG	55	189	
cfpA Nested *2*	CAATGTGTTTCATCCCGAAA CCTCCTTCGGCAGTTTAGTG	55	152	
tp92 Nested	GGTGGGCTTTGACTTTGAAC TTTTCTTTGTTTTTGGGATGC	55	980	
tprI Nested	GTGAGAGGAGGGGGAGTGA CACCATTGGAAAGGAAGGAG	55	599	
polA[c]	CGTCTGGTCGATGTGCAAATGAGTG TGCACATGTACACTGAGTTGACTCGG	65	394	
polA[d,#]	AGGATCCGGCATATGTCCAA GTGAGCGTCTCATCATTCCAAA	60		Leslie et. al. 2007; Leslie et al. 2008
polA[d,*]	ATGCACCAGCTTCGA	60		
c-myc[e,#]	GCCAGAGGAGGAACGAGCT GGGCCTTTTCATTGTTTTCCA	60		Griesche et al., 2008
c-myc[e,*]	6FAM-TGCCCTGCGTGACCAGATCC-BBQ	60		Griesche et al., 2008

[a] primers found in Harper et al. 2008; [b] primers found in Gray et al. 2006; [c] primers found in Liu et al. 2001; [d] primers found in Leslie et al. 2007; [e] primers found in Griesche et al. 2008.

2.9.3 Quantitative real-time PCR

A 67 bp sequence of the DNA polymerase I gene (*polA*) of *T. pallidum* (Leslie et al., 2007; Leslie et al., 2008) was targeted in DNA samples to run a quantitative real-time PCR (qRT-PCR) with a Stratagene Mx3000P (Applied Biosystems, Foster City, CA, USA). A PCR was run to gain the 67 bp product needed to create a dilution series. Isolation was achieved by agarose gel extraction (as described above), followed by cloning, using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany). For an increased rate of yield, emergent clones were cultured for another 24 hours in liquid lysogeny-broth medium (pH 7.0) plus 100 mg ampicillin per ml. Plasmid preparation followed the manufacturer's instructions in the NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany). Finally, a dilution series was prepared with λ-DNA (Fermentas, St. Leon-Rot, Germany) as stabilising medium. The real-time PCR utilised a standardised master mix composition and cycling conditions (Leslie et al., 2007; Leslie et al., 2008) and was carried out twice in each DNA sample. The ratio between means of copy numbers of the *T. pallidum polA* gene and the house keeping *c-myc* gene (Griesche et al., 2008) was used to check extraction success and to standardize results obtained from different individuals. Real-time PCRs were performed at the RKI PCR facility.

2.10 Cloning

For cloning, PCR products older than 2 hours were incubated with Platinum *Taq* polymerase (Invitrogen, Karlsruhe, Germany) using the manufacturer's guidance. All other steps complied with the TOPO TA cloning kit's protocol (Invitrogen, Karlsruhe, Germany). The 25-µL-PCR master mixture consisted of 4 mM $MgCl_2$, 200 µ M dNTP, 0.12 µ M of each primer (M13f, M13r, TOPO TA cloning kits, Invitrogen, Karlsruhe, Germany) and 1.25 U Platinum Taq DNA polymerase (Invitrogen, Karlsruhe, Germany) with 2.5 µL 10x PCR rxn buffer (Invitrogen, Karlsruhe, Germany). Cycling conditions were as follows: one cycle at 95.0 °C for five minutes; 35 cycles at 95.0 °C for 30 seconds, 55.0 °C for 30 seconds and 72.0 °C for one minute; and one cycle at 72.0 °C for seven minutes. At least 10 clones per culture were picked and processed for sequencing. Cloning was performed at the RKI research facility in Berlin.

2.11 Sequencing

Products were sent to the sequencing lab of the RKI. Technically, sequencing followed the dideoxy-method after Sanger (Sanger et al., 1977) using the PRISM Ready Reaction DiDeoxy Termination Cycle Sequencing Kit (ABI, Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Resulting sequences were processed using BLASTN 2.2.18+ (Altschul et al., 1997).

2.12 Statistics

Analyses were performed in collaboration with the Unit of Biomathematics and Data Processing at the Justus-Liebig-University utilising BMDP/ Dynamic Statistical Software (Release 7.0, Statistical Solutions Ltd., Cork, Ireland; Dixon, 1993) with arithmetic charts created on a personal computer using Microsoft Exel 2008 for MAC Software (Microsoft Deutschland GmbH, Unterschleißheim, Germany).

A correlation analysis was run to asses the association of xylazine dosage and induction time in all baboons and in addition separated, into age classes: neonates plus subadults, adult females, dominat adult as well as adult males.

Wilcoxon-Mann-Whitney test was performed in baboons with and without anatagonisation of xylazine with atipamezole to test for influence on the baboons' recovery time.

A two-way frequency table was used to describe and test for the association between different variables. The association between clinical infection and *T. pallidum* PCR results made use of two-tailed Fisher's exact test, where as the association between the clinical infection stage (non-affected, initial, moderate and severe) and *T. pallidum* PCR results utilized the exact Wilcoxon-Mann-Whitney U-test (Testimate V.6.0.65 Software, Statistics.com, Arlington, VA, USA). The statistical coherence between the clinical infection and histology, as well as clinical infection and immunohistochemistry results, were checked for significance utilising the two-tailed Fisher's exact test. The association between age and clinical infection stage was tested utilizing Spearman's rank correlation.

A two-way frequency table was considered to test for the association of *c-myc* gene copy numbers and genders, as well as the different clinical infection stages of the baboons. Kruskal-Wallis test and Spearman's rank correlation were than utilised to test for the association between the numbers of *T. pallidum* genome copies per house keeping *c-myc* gene copy (standardisation of extraction success) obtained from qRT-PCR of different genital ulceration stages.
The level of significance was set to $\alpha = 0.05$, thus *P* values were considered statistically significant at 0.05 or less.

2.13 Phylogenetic analysis

PCR products were characterised in six different polymorphic regions (*deoD* [*tp0170*/ *pfs*] (Centurion-Lara et al., 1998), *gpd* [*tp0257*/ *glpQ*] (Cameron et al., 1999), *tp92* (Cameron et al., 2000; Harper et al., 2008b), *tprI* (Centurion-Lara et al., 2006; Gray et al., 2006; Harper et al., 2008b), *cfpA* (Harper et al., 2008b; Izard et al., 1999), and *tpf1* [*tp1038*] (Noordhoek et al., 1990)) previously determined to aid in differentiation between the *T. pallidum* subspecies. Polymorphisms, which fell within regions in which no signature of recombination was detected, were concatenated and aligned in ClustalX version 1.83 (Thompson et al., 1997). Recombination was ruled out using genomic searches for possible donor regions at highly polymorphic sites, utilising the program RDP-2: Recombination Detection Analysis from Sequence Alignments (Martin et al., 2005) and by analysis of dN/dS ratios to detect recombination events (Harper et al., 2008b). All excluded polymorphisms were located in hypervariable regions of *tp92* and included nucleotide sites 1,530 – 1,606, 1,801 – 1,827, 2,111 – 2,135 and 2,359 – 2,373. Kimura's two-parameter was chosen as the appropriate model of nucleotide substitution utilising by Modeltest Software (Posada and Crandall, 1998).
An alignment was used to create phylogenies constructed in Phylogenetic Analysis Using Parsimony (PAUP; Swofford, 2003) using Maximum Parsimony (MP) and Maximum Likelihood (ML) methods, with 1,000 replicates used to obtain bootstrap support and starting trees obtained through random step-wise addition. *T. paraluiscuniculi,* the agent that causes rabbit syphilis*,* was used as an outgroup. Tree bisection and reconnection was used for the branch-swapping algorithm. The MP tree was chosen for display, with bootstrap support displayed at all nodes that

received greater than 50 %, using both methods. References and sources for the strains that were used for analysis, excluding the LMNP simian strains, can be found in Harper, 2008b. Phylogenetic analysis were performed in collaboration with the Robert Wood Johnson Health and Society Scholar Program at the Columbia University NY, New York, USA.

3 Results

3.1 Field observations and sampling

The group size of observed baboon troops varied from 8 to 250 individuals (observations *n* = 78, mean = 104, SD = 57). The estimated prevalence of genital ulceration within each observed group varied widely. Exact data were not possible to obtain, as many baboon troops were only found in dense vegetation and the genital health status was only visible in animals close to the observer. In addition, observation time was limited, as baboons found researchers showing interest in the group suspicious, especially at the end of the field research activities, when the animals were more alerted to darting activities than in the beginning. Therefore short observation periods followed by a smooth and quick immobilization of individuals were a necessity. Single injected baboons went down 4 – 5 minutes after injection with a mean ketamine dosage of 11.4 mg/kg bw (*n* = 38, SD = 5.8 mg/kg bw). The induction time for all baboons, regardless of their age and gender, was not significantly influenced by the dosage of xylazine (dose range of 0.1 – 2.0 mg/kg bw, *n* = 38, mean = 0.3 mg/kg bw, SD = 0.3 mg/kg bw) used for immobilisation (*P* = 0.33). This was also the case when data were grouped into adult females (*n* = 14, *P* = 0.96) and males (*n* = 15, *P* = 0.28). However, correlation-analysis indicated a slight significant association of xylazine dosage and induction time in subadults (*n* = 6, *P* = 0.037).

In addition, the antagonisation of xylazine with atipamezole in single injected baboons had no significant effect on the recovery time (*n* = 23, *P* = 0.20) when compared to those without antagonisation (*n* = 14). The early antagonisation of xylazine, however, caused cataleptic stages in one animal (69-F5-09.05.07).

3.2 Gross pathological findings

Healthy female Olive baboons show sexual swelling of the ano-genital area correlated with hormonal changes in the course of their sexual cycles (Knauf et al., 2009). In oestrus, when the vaginal exfoliative cytology was predominated by superficial cells (3.3), the sexual turgescence reaches its maximum; the labia vulvae are closed and the vulva itself is separated from the anus by a 1 – 2 cm

long perineum. In healthy males, the penis is non-pigmented; the glans penis is well-demarcated and similar in shape to that of humans.

In clinically affected individuals of both genders, the skin of the ano-genital region displays a moderate to severe necrotizing dermatitis with severe mutilation of the outer genital structures. In the course of infection, a cloacae may appear in severely affected females (Table 12: case No. 46; Figure 10, Picture 1), while males tended to lose the glans or even the complete corpus of the penis (Table 12: case No. 52; Figure 10, Picture 1). Progressive scarification of the tissue leads to a vagina and anus that are permanently ajar in females, sexual swelling is no longer possible, while in males it promotes phimosis. The age of the baboon was not significantly correlated with the extent of genital ulceration (n = 57, Spearman's r_S = -0.105, P = 0.44).

3.3 Vaginal exfoliative cytology

According to the different cell types (i.e. superficial, intermediate, parabasal and basal cells) it was possible to differentiate the diverse phases of the female's sexual cycle. During menstruation mainly erythrocytes were part of the cell population. Intermediate, some few parabasal as well as superficial cells were commonly found. Mucus, cellular debris and leukocytes were frequently found. In females with maximal tumescence the cytological picture was characterized through a predominant population of superficial cells (Table 12: case No. 37; Figure 9, Picture 1 – 2). The maturation value rose up to 100 (mean = 97.7, SD = 2.3, n = 5, Table 12: case Nos. 10, 22, 37, 43 and 48). In females with moderate perineal tumescence the maturation index showed a mean of 67.6 (SD = 27.1, n = 2, Table 12: case Nos. 2 and 25). Basal and parabasal cells were found to be rare in this phase; intermediate cells were the most common cell type. Non-turgescent females had a mean maturation index of 7.5 (SD = 15.3, n = 16, Table 12: case Nos. 1, 3, 5 – 6, 8 – 9, 21, 23 – 24, 38, 44, 46 – 47, 49, 61 and 63) and the cytological picture was mainly characterized by intermediate and parabasal cells (Table 12: case Nos. 21 and 44; Figure 9, Pictures 3 – 6). In 23 (77 %) of 30 sampled female Olive baboons the vaginal exfoliate cytology corresponded well to the perineal swelling of the female's genitalia. In one case the light-microscopic feature was doubtful as it differed from the macroscopic sexual swelling phase

(Table 12: case No. 42). In six cases a diagnosis was not possible due to a lack of cells or staining of bad quality (Table 12: case Nos. 4, 7, 11 –12, 36 and 45).

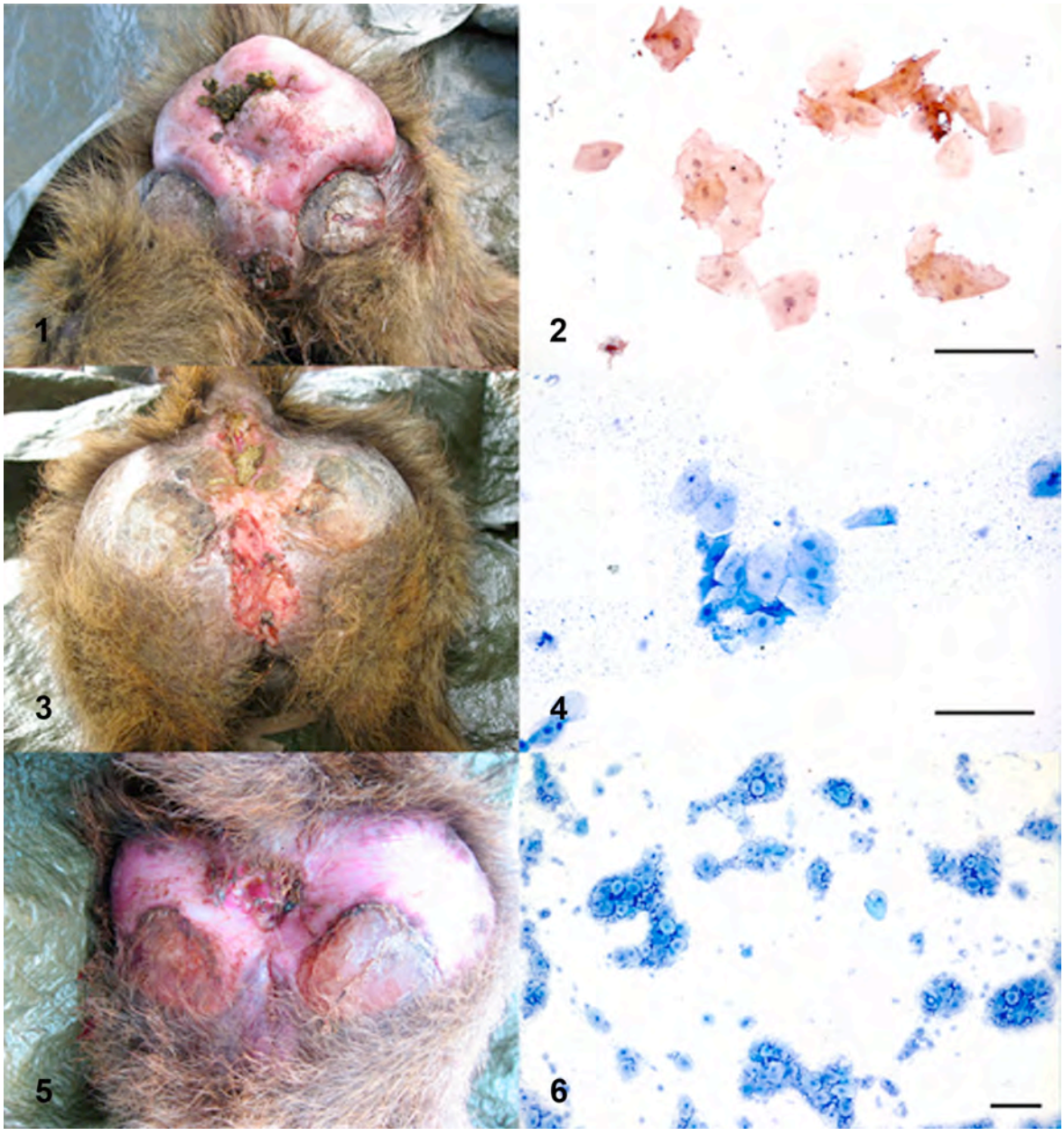

Figure 9. Vaginal exfoliative cytology.

Picture 1. Female Olive baboon (18-F5-28.03.07), case No. 37 (Table 12), ano-genital region. Prominent sexual tumescence, indicating oestrus. Macroscopic view.

Picture 2. Corresponding cytological picture (18-F5-28.03.07), case No. 37 (Table 12). Superficial cells and multiple sperms. The maturation index was calculated 100.00. Papanicolaou-Shorr staining. Bar, 100 µm.

Picture 3. Female Olive baboon (21-F8-04.04.07), case No. 44 (Table 12), ano-genital region. Severe genital ulcerated female with no signs of sexual swelling. Macroscopic view.

Picture 4. Corresponding cytological picture (21-F8-04.04.07), case No. 44 (Table 12). Cytological picture characterized by intermediate cells. The maturation index was calculated 29.75. Diff-Quick staining. Bar, 100 µm.

Picture 5. Female Olive baboon (8-F2-25.03.07), case No. 21 (Table 12), ano-genital region. Moderate perianal ulceration. No sexual swelling is present. Macroscopic view.

Picture 6. Corresponding cytological picture (8-F2-25.03.07), case No. 21 (Table 12). Cytological picture mainly characterized by parabasal and basal cells. The maturation index was calculated 11.50. Diff-Quick staining. Bar, 100 µm.

3.4 Histology

The microscopic findings displayed a uniform manifestation (Table 12: case Nos. 1 – 7, 10 – 13, 15, 17 – 19 and 21 – 57). Skin lesions were characterized by irregular epidermal proliferation of different extent. The epidermis developed hyperkeratosis and hypertrophy of the epidermal rete pegs, which branch and project deeply into the corium. The exaggerated elongation of rete-ridges was accompanied by acanthosis, acantholysis and exocytosis. In some cases, small intraepidermal microabscesses were found. In the underlying dermal layer, a moderate to severe mixed-cell infiltration, composed mainly of lymphocytes and histiocytes and single giant cells was present. The cellular reaction was most marked around the dermal blood vessels resulting in superficial and deep perivascular dermatitis. In severe cases, the overlaying epidermis was ulcerated (Table 12: case No. 44; Figure 10, Picture 6) and a chronic granulomatous reaction ensued, starting from the ulcer margins. The centre of the lesion was filled with cell detritus, neutrophil granulocytes and macrophages. The surface was covered with a dried serosanguineous discharge. In several cases a moderate to severe tissue eosinophilia was demonstrable (Table 12: case No. 50; Figure 10, Picture 5). No inclusion bodies of type Cowdry A were found. Fungal infections could be excluded by the results of PAS and WSS staining. The histochemical silver staining method (WSS) failed to demonstrate intralesional bacteria. A relatively high percentage of clinically healthy animals showed clear signs of a beginning dermatitis, characterised by mild epidermal hypertrophy and elongation of the rete ridges (Table 12: case Nos. 1 – 7, 10 – 13, 15 and 17 – 19).

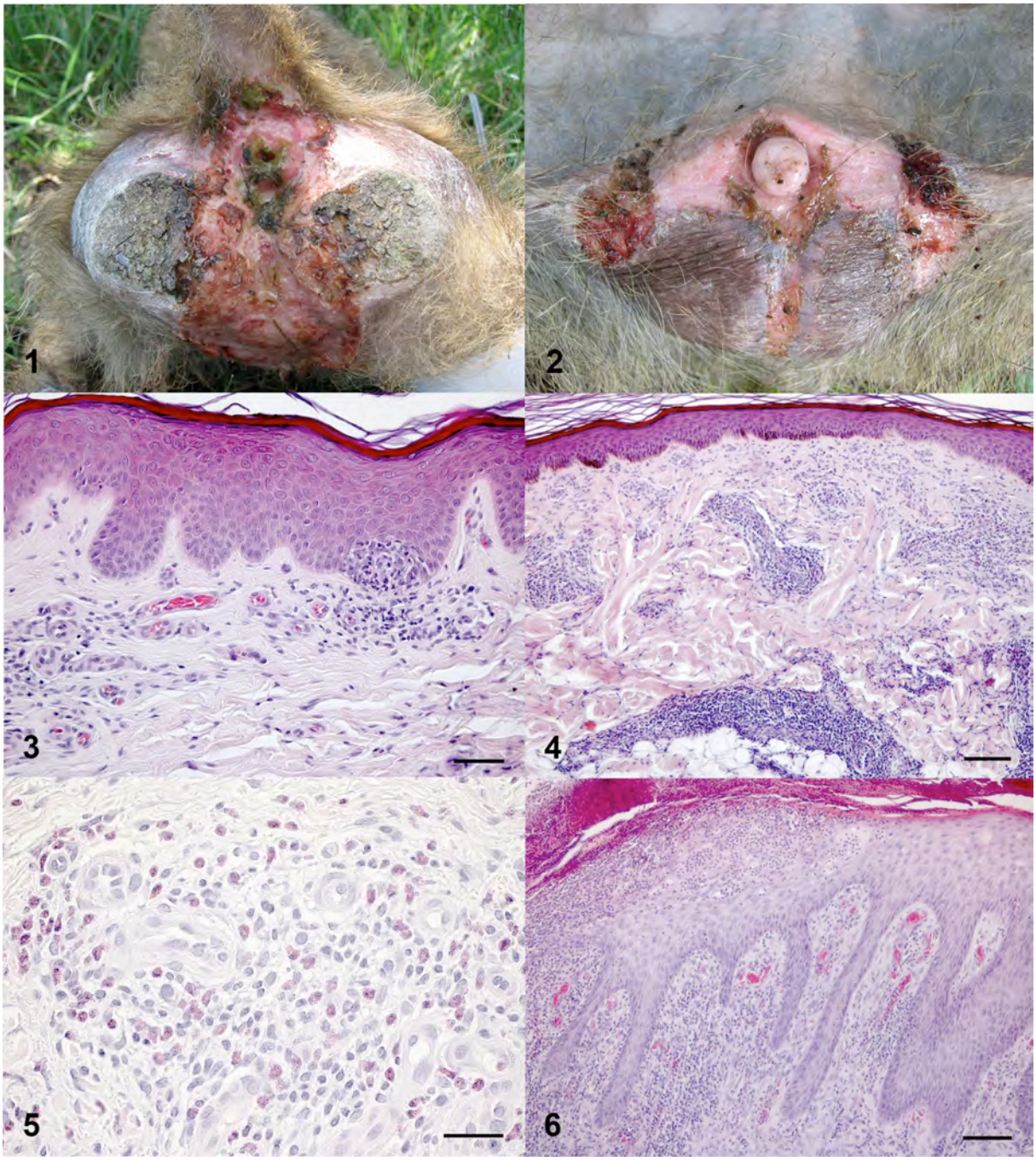

Figure 10. Gross pathological and histopathological findings.

Picture 1. Female Olive baboon (49-F8-19.05.07), case No. 46 (Table 12), ano-genital region. Severely affected female with massive destruction of the outer genital. Macroscopic view.

Picture 2. Male Olive baboon (33-M8-12.04.07), case No. 52 (Table 12), genital area. Severely affected male with massive destruction of the genital. The glans penis is already missing. Macroscopic view.

Picture 3. Skin tissue biopsy of the genital (53-M2-21.04.07), case No. 31 (Table 12). Moderate irregular epidermal proliferation and focal exocytosis with mixed-cell infiltration. H&E stain. Bar, 50 µm.

Picture 4. Skin tissue biopsy of the genital (41-F1-16.04.07), case No. 6 (Table 12). Severe perivascular mixec-cell infiltration. H&E stain. Bar, 100 µm.

Picture 5. Skin tissue biopsy of the genital, (3-M8-22.03.07), case No. 50 (Table 12). Tissue eosinophilia, as found in most of the clinically affected baboons. H&E stain. Bar, 30 µm.

Picture 6. Skin tissue biopsy of the genital, (21-F8-04.04.07), case No. 44 (Table 12). Excessive irregular epidermal proliferation and ulceration. H&E stain. Bar, 100 µm.

3.5 Immunohistochemical findings

Immunohistochemistry demonstrated small numbers of spirochete-like bacteria in 16 of 65 cases (Table 12: case Nos. 58 – 63 are not displayed and were negative, in case Nos. 64 – 65 no tissue sample was taken). Two animals (Table 12: case Nos. 2 and 10) had no macroscopic genital lesions, but proved to be positive for *T. pallidum* by PCR. The remaining 14 animals (Table 12: case Nos. 24 – 25, 30, 37, 39 – 41, 44, 46, 51 – 52, 54 – 55 and 57) had signs of genital ulceration. Treponemes were significantly more often depicted in clinically affected than in clinically non-affected baboons (n = 57, two-tailed Fisher's exact test, P = 0.032, Table 6).

Table 6. Association of immunohistochemical findings and clinical signs of infection.

Immunohistochemistry	**Clinical signs of infection**		**Total**
	Negative	**Positive**	
Negative	18	23	41
Positive	2	14	16
Total	20	37	57

Spirochetes were mainly distributed in the centre of the lesion, showing a clear tropism for the epidermis-dermis region (epitheliotropic pattern) and displaying vasculotropic pattern. They were arranged in small clusters. A single organism was about 6.0 – 15.0 µm in length and 0.15 µm in diameter. A characteristic spiral-like shape with 8 – 15 corkscrew spirals was clearly visible (Table 12: case No. 39; Figure 11).

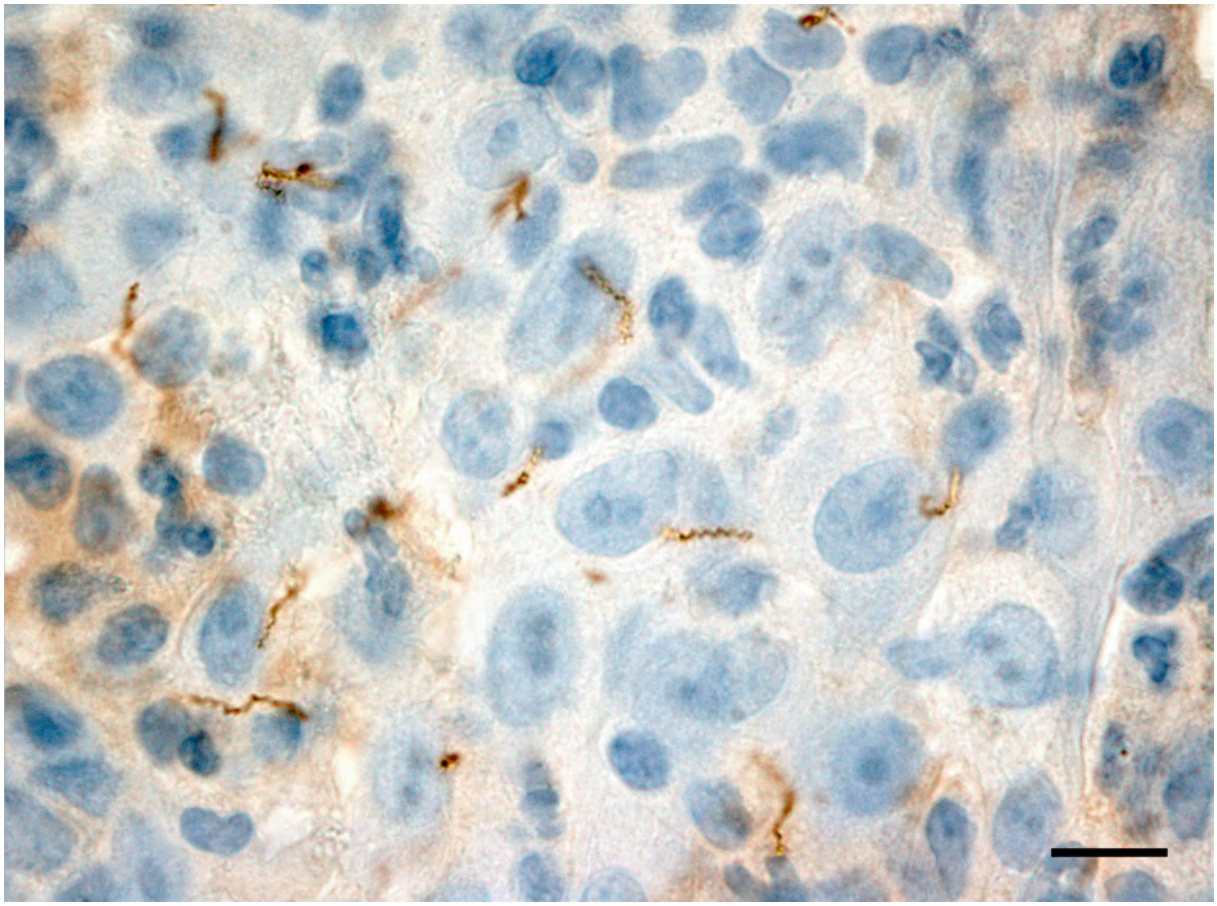

Figure 11. Immunohistochemical detection of *Treponema pallidum*. Skin tissue biopsy of the genital (20-M5-03.04.07), case No. 39 (Table 12). The characteristic spiral-like shape of the *sphirochete* is visible. Rabbit polyclonal antibody against *T. pallidum*, with epithelial cells slightly counterstained with Mayer's hematoxylin. Bar, 10 µm.

An immunological differentiation between B- and T-cells was done to characterise the inflammatory-cell reaction. In all cases, more T- than B-cells were present among the lymphocytic inflammatory-cell population. In this study 10 – 20 % of the inflammatory-cells were CD20 positive B cells and 70 to 80 % CD3 positive T-cells. The T-cells were diffusely distributed in the altered dermis, were frequently found in close association with the epidermal-dermal junctions or were seen to infiltrate the inflamed epidermis. B-cells were found to a lesser extent. They were located in the deeper dermal parts and prefer to accumulate around blood vessels or to form follicular aggregates. Follicular aggregates with clear CD20 positive

centre and CD3 positive mantel zone were frequently found in deeper dermal parts.

3.6 Fluorescence-in-situ-hybridization

The fluorescence-in-situ-hybridization did not reveal any useful information about bacteria that might play a role in the pathogenesis of the genital associated disease. Signals of rod-shaped to coccoid bacteria were clustered in the tissue of a single baboon (Table 12: case No. 60). Unpleasant background staining was accompanied by illuminating tissue fibers in two other animals (Table 12: case Nos. 21 and 37). Since EUB 338 binds to a broad range of different prokaryotes it is not surprising that bacteria, especially in the upper part of ulcerated skin wounds, were present. However, spirochete-like bacteria were not visible.

3.7 Polymerase chain reactions

The 16S rRNA PCR generated a wide range of different bacterial sequences, but no cluster or specific pathogen associated with genital ulceration could be identified (Table 7).

Table 7. List of bacterial sequences found in six Olive baboons utilising 16S rRNA PCR.

Bacterium	Quantity of sequence findings in 6 baboons[#]	Animals per 6 baboons
Arcanobacterium sp.	2	2
Citrobacter sp.	1	1
Clostridium sp.	2	2
Corynebacterium sp.	6	4
Enterobacter sp.	1	1
Flexibacteriaceae	1	1
Haemophilus sp.[a]	3	2
Klebsiella sp.[b]	4	1
Mamoricola sp.	1	1
Nocardioides sp.	1	1
Peptoniphilus sp.	3	1
Prevotella sp.	2	1
Pseudonocardia sp.	1	1
Spirobacillus sp.	1	1
Uncultured bacterium	5	4
Butyrivibrio sp.	1	1

[#] Table 12: case Nos. 17, 21, 26, 37, 49 and 59; [a] Tests for *H. ducreyi* were negative; [b] Tests for *K. granulomatis* were negative.

Thirty-eight baboons were tested positive by PCR and/ or qRT-PCR for *T. pallidum* (Table 12). Baboons with positive *T. pallidum* PCR result showed significantly more often clinical signs of the infection (n = 57, two-tailed Fisher's exact test, P < 0.0001) than those that were negative in PCR (Table 8).

Table 8. Association of PCR results and clinical signs of infection.

PCR	Clinical signs of infection		Total
	Negative	Positive	
Negative	14	5	19
Positive	6	32	38
Total	20	37	57

A comparison of the different clinical infection stages with *T. pallidum* PCR results revealed significant differences between the described genital ulceration stages (*n* = 57, exact Wilcoxon-Mann-Whitney U-test, *P* < 0.0001, Table 9).

Table 9. Crossstab of PCR results and different clinical infection stages used to perform an exact Wilcoxon-Mann-Whitney U-Test.

Scores	Clinical infection stage				Valid No.	Mean rank
	CNA[#]	Initial	Moderate	Severe		
Groups	10.5	27.5	38.0	49.5		
Negative	14	4	0	1	19	16.13
***n* = 19**	73.68 %	21.05 %	0 %	5.26 %		
Positive	6	10	7	15	38	35.43
***n* = 38**	15.79 %	26.32 %	18.42 %	39.47 %		

[#] Clinically non-affected

Olive baboons with histological signs of an infection were significantly more often positive in *T*. *pallidum* PCR (*n* = 57, two-tailed Fisher's exact test, *P* = 0.004) than those without characteristic histological alterations (Table 10).

Table 10. Association of histological alterations and clinical signs of infection.

Histological alterations indicating an infection	Clinical signs of infection		Total
	Negative	Positive	
Negative	5	0	5
Positive	15	37	52
Total	20	37	57

The house keeping *c-myc* gene was not influenced by gender (two-way frequency analysis, *P* = 0.20), but showed a significant association of its quantity to the different clinical infection stages (two-way frequency analysis, *P* < 0.0001, Figure 12). The interaction effect between gender and stage had a tail probability of 0.43.

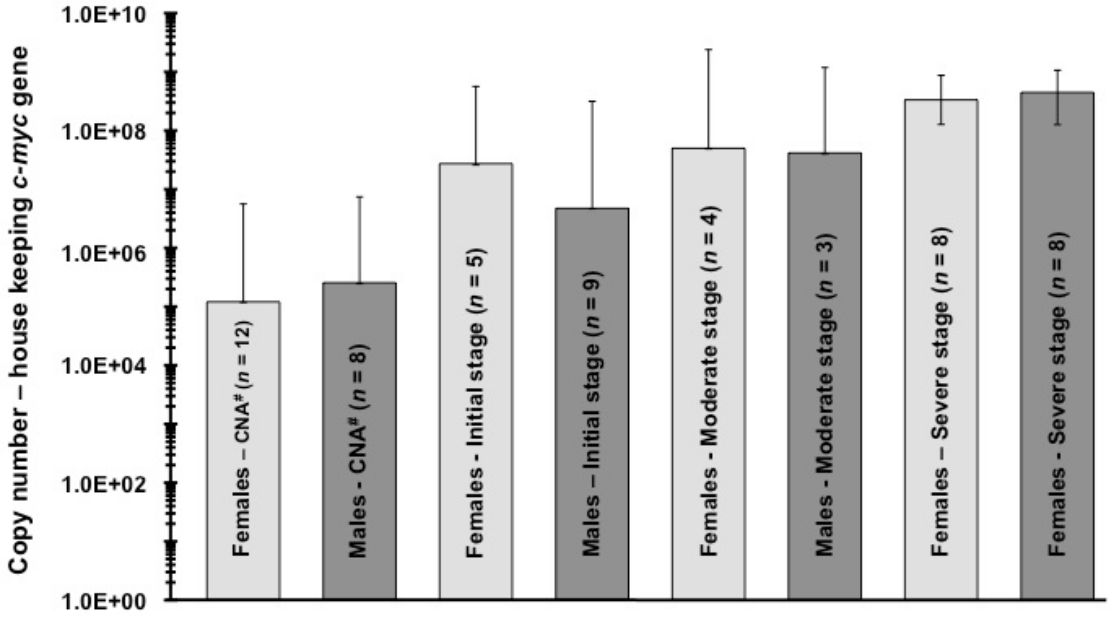

Figure 12. Test for usefulness of the *c-myc* house keeping gene. The copy number is influenced by the clinical infection stage, but not by gender.

[#] Clinically non-affected.

The number of *T. pallidum* genome copies per house keeping gene does significantly correlate with the severity of infection (n = 57, Kruskal-Wallis test, P = 0.043, Table 11).

Table 11. Crossstab of rank sums for different clinical infection stages (group).

Group	Frequency	Rank sum
Clincally non-affected	20	436,0
Initial stage	14	416,5
Moderate stage	7	281,5
Severe stage	16	519,0

Baboons which show a moderate clinical infection stage (mean rank sum 40.21) had the highest number of *T. pallidum* gene copies, followed by severe affected animals (mean rank sum 32.43) and animals in the clinical initial stage (mean rank sum 29.75). Clinically non-affected baboons had the lowest number of *T. pallidum* genome copies (mean rank sum 21.80). The ratio of *T. pallidum* gene copies per house keeping c-myc gene copy increases significantly with the clinical infection stage (n = 57, Spearman's r_S = 0.312, P = 0.018).

Particularily worth mentioning is also that 30 % of the Olive baboons without genital ulceration (clinically non-affected) were PCR positive for *T. pallidum* (Table 12: case Nos. 2, 10 – 12, 15 and 18).

Table 12. Summary of clinical manifestation, histological signs of infection, immunohistochemistry and PCR results in 57 Olive baboons from LMNP.

Case	Baboon ID #	Gender[$]	Clinical stage[§]	Clinical signs	Histo*	Immuno-histo-chemistry	PCR[a]	*polA*[b#] gene, mean quantity	*c-myc*[c#] gene, mean quantity	Ratio polA/c-myc gene quantity
1	9-F1-25.03.07	F	CNA	-	+	-	-	0.00E+00	7.87E+08	0.00E+00
2	11-F1-26.03.07	F	CNA	-	+	+	+	1.85E+01	8.25E+04	2.23E-04
3	14-F1-27.03.07	F	CNA	-	+	-	-	0.00E+00	2.35E+05	0.00E+00
4	22-F1-05.04.07	F	CNA	-	+	-	-	0.00E+00	3.27E+08	0.00E+00
5	37-F1-15.04.07	F	CNA	-	+	-	-	0.00E+00	7.97E+05	0.00E+00
6	41-F1-16.04.07	F	CNA	-	+	-	-	0.00E+00	2.81E+08	0.00E+00
7	43-F1-16.04.07	F	CNA	-	+	-	-	0.00E+00	3.97E+06	0.00E+00
8	58-F1-24.04.07	F	CNA	-	-	-	-	0.00E+00	5.46E+04	0.00E+00
9	59-F1-25.04.07	F	CNA	-	-	-	-	0.00E+00	2.98E+08	0.00E+00
10	61-F1-26.04.07	F	CNA	-	+	+	+	1.12E+01	2.24E+08	5.00E-08
11	62-F1-26.04.07	F	CNA	-	+	-	+	9.87E+00	2.94E+05	3.36E-05
12	76-F1-18.05.07	F	CNA	-	+	-	+	1.25E+01	1.57E+05	7.96E-05
13	6-M1-24.03.07	M	CNA	-	+	-	-	0.00E+00	2.38E+08	0.00E+00
14	19-M1-03.04.07	M	CNA	-	-	-	-	0.00E+00	8.71E+05	0.00E+00
15	27-M1-09.04.07	M	CNA	-	+	-	+	1.01E+01	1.98E+05	5.10E-05
16	31-M1-11.04.07	M	CNA	-	-	-	-	0.00E+00	7.67E+04	0.00E+00
17	35-M1-13.04.07	M	CNA	-	+	-	-	0.00E+00	1.80E+05	0.00E+00
18	44-M1-17.04.07	M	CNA	-	+	-	+	1.17E+01	4.42E+05	2.65E-05
19	65-M1-04.05.07	M	CNA	-	+	-	-	0.00E+00	3.76E+04	0.00E+00
20	79-M1-19.05.07	M	CNA	-	-	-	-	0.00E+00	1.92E+03	0.00E+00
21	8-F2-25.03.07	F	IN	+	+	-	+	0.00E+00	2.06E+08	0.00E+00
22	32-F2-11.04.07	F	IN	+	+	-	+	1.08E+03	2.35E+08	4.60E-06
23	34-F2-13.04.07	F	IN	+	+	-	+	7.44E+02	1.03E+06	7.22E-04
24	50-F2-19.04.07	F	IN	+	+	+	+	1.32E+02	2.84E+08	4.65E-07
25	57-F2-24.04.07	F	IN	+	+	+	-	0.00E+00	1.07E+06	0.00E+00
26	10-M2-26.03.07	M	IN	+	+	-	+	3.36E+02	3.90E+04	8.62E-03
27	28-M2-09.04.07	M	IN	+	+	-	-	0.00E+00	7.13E+05	0.00E+00

28	29-M2-09.04.07	M	IN	+	+	-	-	0.00E+00	1.33E+05	0.00E+00
29	30-M2-11.04.07	M	IN	+	+	-	-	0.00E+00	1.41E+05	0.00E+00
30	47-M2-18.04.07	M	IN	+	+	+	+	6.46E+02	3.90E+08	1.66E-06
31	53-M2-21.04.07	M	IN	+	+	-	+	1.22E+01	1.46E+08	8.36E-08
32	55-M2-23.04.07	M	IN	+	+	-	+	4.62E+01	3.77E+08	1.23E-07
33	60-M2-25.04.07	M	IN	+	+	-	+	2.06E+01	7.54E+08	2.73E-08
34	68-M2-08.05.07	M	IN	+	+	-	+	1.42E+02	1.39E+05	1.02E-03
35	4-F5-23.03.07	F	MO	+	+	-	+	4.70E+03	1.68E+05	2.80E-02
36	7-F5-25.03.07	F	MO	+	+	-	+	2.98E+03	7.85E+08	3.80E-06
37	18-F5-28.03.07	F	MO	+	+	+	+	0.00E+00	1.77E+08	0.00E+00
38	69-F5-09.05.07	F	MO	+	+	-	+	1.04E+02	2.65E+08	3.92E-07
39	20-M5-03.04.07	M	MO	+	+	+	+	1.98E+03	9.15E+05	2.16E-03
40	40-M5-16.04.07	M	MO	+	+	+	+	6.96E+03	3.70E+08	1.88E-05
41	70-M5-10.05.07	M	MO	+	+	+	+	9.88E+01	2.11E+08	4.68E-07
42	2-F8-22.03.07	F	SEV	+	+	-	+	1.98E+01	2.34E+08	8.46E-08
43	15-F8-27.03.07	F	SEV	+	+	-	+	1.46E+03	5.54E+08	2.64E-06
44	21-F8-04.04.07	F	SEV	+	+	+	+	4.62E+02	3.79E+08	1.22E-06
45	26-F8-06.04.07	F	SEV	+	+	-	+	1.26E+03	4.08E+08	3.09E-06
46	49-F8-19.04.07	F	SEV	+	+	+	+	3.89E+03	2.96E+08	1.31E-05
47	52-F8-21.04.07	F	SEV	+	+	-	+	7.18E+02	2.55E+08	2.82E-06
48	56-F8-23.04.07	F	SEV	+	+	-	+	2.31E+01	6.59E+08	3.51E-08
49	78-F8-19.05.07	F	SEV	+	+	-	-	0.00E+00	1.50E+08	0.00E+00
50	3-M8-22.03.07	M	SEV	+	+	-	+	4.63E+01	3.66E+08	1.27E-07
51	16-M8-28.03.07	M	SEV	+	+	+	+	3.30E+02	3.25E+08	1.02E-06
52	33-M8-12.04.07	M	SEV	+	+	+	+	7.63E+01	4.80E+08	1.59E-07
53	39-M8-15.04.07	M	SEV	+	+	-	+	2.31E+03	4.76E+08	4.85E-06
54	54-M8-21.04.07	M	SEV	+	+	+	+	0.00E+00	2.56E+08	0.00E+00
55	63-M8-27.04.07	M	SEV	+	+	+	+	9.39E+01	5.41E+08	1.73E-07
56	67-M8-07.05.07	M	SEV	+	+	-	+	4.94E+01	6.66E+08	7.42E-08
57	74-M8-16.05.07	M	SEV	+	+	+	+	5.27E+02	6.23E+08	8.46E-07

[$] F = Female, M = male;

[§] CNA = clinically non-affected, IN = initial, MO = moderate, SEV = severe;

* Histological signs of an infection;

[a] PCR indicates positives from PCR (Liu, et al. 2001) and/ or real-time PCR (Leslie, et al. 2007); case No. 58 (not listed here; 64-M8-04.05.07) is the same individual as case No. 59 (not listed here; 71-M8-10.05.07) and was recaptured after antibiotic treatment, skin biopsy was taken after antibiotic treatment, PCR results are not taken into account; case No. 56 is the same individual as case No. 60 (not listed here; 72-M11-11.05.07) and was recaptured after antibiotic treatment, second skin biopsy was taken after antibiotic treatment, PCR results are not taken into account; DNA extraction failed in three animals, case Nos. 61-63 (not listed here; 25-F1-05.04.07, 75-M1-17.05.07 and 77-F1-18.05.07); in case Nos. 64-65 (not listed here; 51-F1-19.04.2010 and 66-M8-05.05.07) no tissue samples were taken;

[b] DNA polymerase I gene (polA), quantitative real-time PCR (Leslie, et al. 2007); house keeping gene, human *c-myc* gene, quantitative real-time PCR (Griesche, et al. 2008);

[#] each real-time PCR was carried out twice.

In contrast to the *T. pallidum* findings, molecular biological tests for *K. granulomatis* and *H. ducreyi* were negative. The PCRs targeting herpesvirus were positive in 23 of 26 tested individuals (Table 13: 19 clinically affected [17 of those PCR pos.] and 7 clinically non-affected [2 of those PCR pos.]) and revealed a variety of sequences, ranging from only one animal infected with *Cercopithecine herpesvirus 16* (HVP-2) (Table 12: case No. 45) to 20 infected with *Papio hamadryas* lymphocryptovirus *1 – 3* (LVC 1 – 3, Table 12: case Nos. 1, 7, 9, 10, 12, 21, 24, 26, 30, 35, 38, 40 – 42, 44, 46, 48, 52, 54 and 59) and two with Baboon cytomegalovirus (Table 12: case Nos. 31 and 49). Only three animals (Table 12: case Nos. 4, 17 and 37) were PCR negative for herpesvirus infection.

Table 13. Herpesviruses found in 26 Olive baboons at LMNP. Three animals were negative (not displayed).

Herpesvirus	GenBank accession number	Animal ID	Case No. (Table 12)	Genital infection stage
PhamLCV1	AY174069	56-F8-23.04.07	48	Severe
		33-M8-12.04.07	52	Severe
PhamLCV2	AF534229	9-F1-25.03.07	1	CNA[#]
		43-F1-16.04.07	7	CNA
		59-F1-25.04.07	9	CNA
		76-F1-18.05.07	12	CNA
		8-F2-25.03.07	21	Moderate
		4-F5-23.03.07	35	Moderate
		69-F5-09.05.07	38	Moderate
		40-M5-16.04.07	40	Moderate
		2-F8-22.03.07	42	Severe
		49-F8-19.04.07	46	Severe
		54-M8-21.04.07	54	Severe
		71-M8-10.05.07	57	Severe
PhamLCV3	EU11846	61-F1-26.04.07	10	CNA
		50-F2-19.04.07	24	Moderate
		10-M2-26.03.07	26	Moderate
		47-M2-18.04.07	30	Moderate
		70-M5-10.05.07	41	Moderate
		21-F8-04.04.07	44	Severe
Baboon CMV	AF387664	53-M2-21.04.07	31	Mild
		78-F8-19.05.07	49	Severe
HVP-2	NC007653	26-F8-06.04.07	45	Severe

[#] CNA = Clinically non-affected

3.8 Phylogenetic analysis

Using previously published information regarding polymorphic sites useful in distinguishing the subspecies, LMNP simian strains are genetically most closely related to the non-venereal human subspecies *T. pallidum* ssp. *pertenue* (which causes yaws), and *endemicum* (which causes bejel/ endemic syphilis) strains (Table 14). Trees constructed without *tprI* and *tp92*, which contribute an inordinate number of polymorphisms (64 %) to the alignment, are qualitatively similar to the one displayed in Figure 13. Sequences found in three individuals from LMNP were identical, resulting in only one brunch on the tree and genomic information on three different genes was deposited in GenBank under accession numbers FJ896486, FJ896487 and EU683034.

Table 14. Polymorphisms included in phylogenetic analysis.

Strain/ Gene	*deoD*		*gpd*		*tp92*													*tprI*			*cfpA*			*tpF-1*	
Bosnia[a]	T	T	A	G	C	C	T	T	G	T	A	C	C	C	G	T	C	G	T	G	A	C	A	G	G
Iraq[a]	T	T	A	G	C	C	T	T	G	T	A	C	C	C	G	T	C	G	T	G	A	C	A	G	G
Brazza-ville[b]	T	T	A	G	C	C	C	T	G	T	A	C	-	C	G	T	C	A	T	G	A	C	A	G	G
CDC1[b]	T	T	A	G	C	C	T	T	G	T	A	C	-	C	A	T	C	A	T	A	A	C	A	G	G
CDC2[b]	T	T	A	G	C	C	T	T	G	T	A	C	-	C	G	T	C	A	T	G	A	C	A	G	G
CDC2575[b]	T	T	A	G	C	C	T	T	G	T	A	C	-	C	A	T	C	A	T	A	A	C	A	G	G
Gauthier[b]	T	T	A	G	C	C	C	T	G	T	A	C	-	C	G	T	C	A	T	G	A	C	A	G	G
Ghana[b]	T	T	A	G	C	C	T	T	G	T	A	C	-	C	A	T	C	A	T	A	A	C	A	G	G
Pariaman[b]	T	T	A	G	C	A	T	T	G	T	A	C	-	C	G	T	C	A	T	G	A	C	A	G	G
Samoa D[b]	T	T	A	G	C	C	T	T	G	T	A	C	-	C	G	T	C	A	T	G	A	C	A	G	G
Samoa F[b]	T	T	A	G	C	C	T	T	G	T	A	C	-	C	G	T	C	A	T	G	A	C	A	G	G
Chicago B[c]	T	C	A	A	C	C	T	T	G	T	A	C	A	C	G	T	C	G	G	A	A	C	G	G	A
Dallas[c]	T	C	A	A	C	C	T	T	G	T	A	C	A	C	G	T	C	G	G	A	G	C	G	G	A
Grady[c]	T	C	A	A	C	C	T	G	G	T	A	C	C	C	G	T	C	G	G	A	A	C	G	G	A
Haiti B[c]	T	C	A	A	T	C	T	T	G	T	A	C	C	C	G	T	C	G	G	A	A	C	G	G	A
Madras[c]	T	C	A	A	C	C	T	T	G	T	A	C	A	C	G	T	C	G	G	A	G	C	G	G	A
Mexico A[c]	T	C	A	A	C	C	T	G	G	T	A	C	A	C	G	T	C	G	G	A	A	C	G	G	A
Nichols[c]	T	C	A	A	C	C	T	T	G	T	A	C	-	C	G	T	C	G	G	A	G	C	G	G	A
Philadel-phia 1[c]	T	C	A	A	C	C	T	G	G	T	A	C	C	C	G	T	C	G	G	A	A	C	G	G	A
South Africa[c]	T	C	A	A	C	C	T	G	G	T	A	C	-	C	G	T	C	G	G	A	A	C	G	G	A
TPC A[d]	C	T	G	G	C	C	T	T	A	T	A	C	-	-	G	C	T	-	-	-	A	T	A	A	G
TPC H[d]	C	T	G	G	C	C	T	T	A	C	A	G	-	-	G	C	T	-	-	-	A	T	A	A	G
TPC M[d]	C	T	G	G	C	C	T	T	A	T	A	G	-	-	G	C	T	-	-	-	A	T	A	A	G
Baboon: SNP	**C**	**T**	**A**	**G**	**C**	**C**	**T**	**T**	**G**	**T**	**A**	**C**	**-**	**C**	**G**	**T**	**C**	**A**	**T**	**G**	**A**	**C**	**A**	**A**	**G**
Baboon: LMNP	**T**	**T**	**A**	**G**	**C**	**C**	**T**	**T**	**G**	**T**	**G**	**C**	**-**	**C**	**G**	**T**	**C**	**A**	**T**	**A**	**A**	**C**	**A**	**G**	**G**
Baboon: Guinea	T	T	A	G	C	C	T	T	G	T	A	C	-	C	G	T	C	A	T	G	A	C	A	A	G
Synonymous or Nonsynonymous Substitution	**S**	**S**	**S**	**S**	**N**	**N**	**N**	**N**	**S**	**N**	**N**	**N**	**N/D**[e]	**D**	**N**	**N**	**S**	**N**	**N**	**N**	**N**	**S**	**S**	**S**	**N**
Nucleotide Residue	744	759	459	579	1,592	1,964	1,966	1,967	2,010	2,101	2,209	2,326	2,388	2,382-2,399	2,405	2,408	2,421	137	143	151	92	121	303	117	122

[a] *T. p*. ssp. *endemicum* (bejel/ endemic syphilis); [b] *T. p*. ssp. *pertenue* (yaws); [c] *T. p.* ssp. *pallidum* (syphilis); [d] *T. paraluiscuniculi* (rabbit syphilis); [e] Deletion from nucleotide residues 2,384 – 2,398.

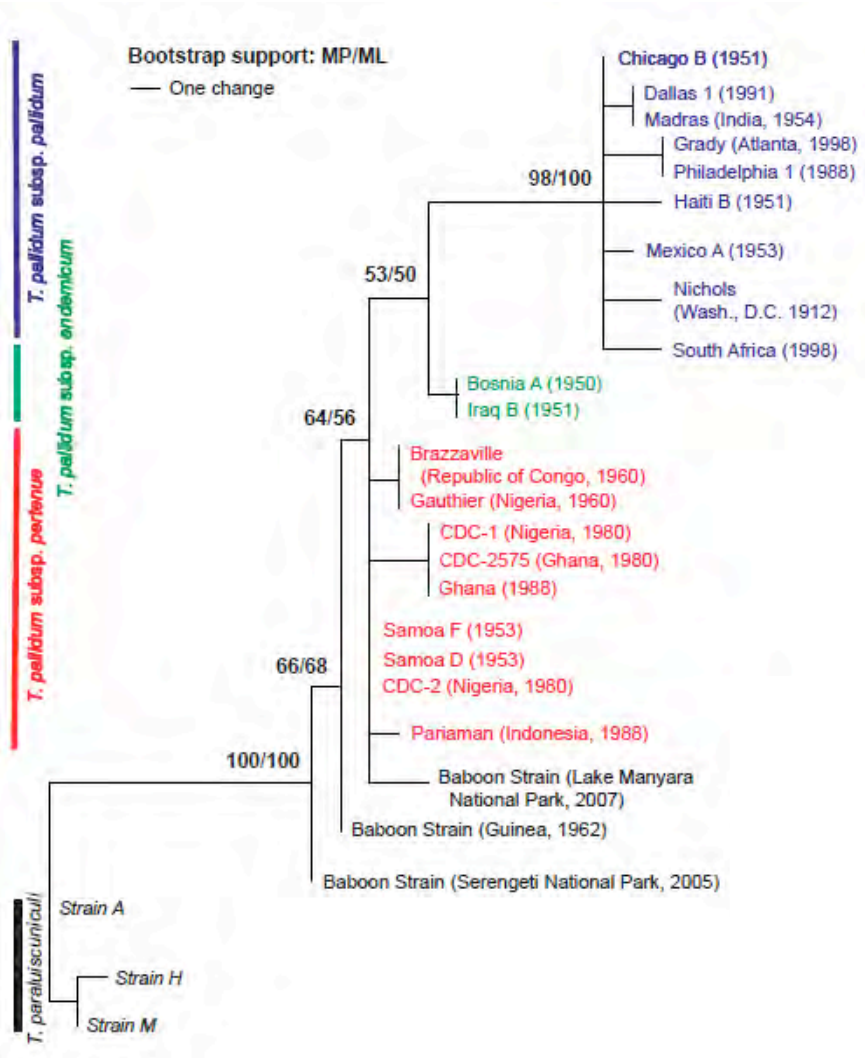

Figure 13. **Phylogenetic tree**. Simian strains of *T. pallidum* found in Tanzania are distinct from syphilis-causing strains found in humans. The phylogeny demonstrates that the *T. pallidum* strains present in Tanzanian baboons (denoted by arrows) are distinct from human ssp. *pallidum* strains and occupy ancestral positions on the tree. The phylogeny is based on 25 polymorphisms in six concatenated regions of the *Treponema* genome. Nodes with greater than 50 % bootstrap support using Maximum Parsimony and Maximum Likelihood methods are labelled as such.

4 Discussion

The genital associated disease in LMNP's baboons was first reported in 1994 (Mlengeya, 2004) and shows similarities to an infection in Olive baboons at GNP, 700 km away from Manyara (Figure 2), first reported in 1989 (Wallis and Lee, 1999; Wallis, 2000). Since 2004 there are some additional cases of genital ulceration in baboons at Serengeti National Park and Ngorongoro Conservation Area. Unfortunately, there are no long-term data available from GNP (Collins, pers. comm.) or any other site. Thus, the time at which the infection first appeared cannot be rigorously assessed and the missing information on transmission mode and potential reservoirs does not allow any conclusions on the diseases temporal and special distribution. At present the link to other areas in Africa, especially outside Tanzania, where genital ulceration in baboons is reported, Mt. Kenya in Kenya (Fischer, pers. comm., 2006) and in Nigeria (Wallis, pers. comm., 2008), cannot be made. However, the timing of the first reports of the disease at each site in Tanzania, along with the continued absence of the disease in two nearby Kenyan sites, Masai Mara and Amboseli National Parks (Harper, unpublished data), suggests that this disease may be relatively new in these populations and in the process of spreading.

T. pallidum is an obligate pathogen which requires inoculation with only 50 organisms in order to initiate an infection (Magnuson et al., 1956). The demonstration of *T. pallidum* in clinical specimens was and still is a challenging task; this bacterium cannot be grown on artificial media, cannot be maintained in tissue culture for more than a brief period of time and replicates slowly in animal models (Richens and Mabey, 2009). Furthermore it is highly susceptible to drying (Richens and Mabey, 2009) and thus requires high quality sampling. The presence of *Treponema* in the tissue of baboons affected by a genital disease was demonstrated using both immunohistochemistry and advanced molecular biological tests, specific to the bacterium. The combination of methods that were used to test and differentiate treponemes in this study is conventional to those used in human medicine (French et al. 2009; Little, 2005). Serology has not been performed, yet, since the different *Treponema* sp. are morphologically identical, and all are known to induce reactivity in the standard serological tests used for

syphilis diagnosis in humans (Larsen et al., 1995; Nandwani and Evans, 1995; Mulligan et al., 2008; Young, 2000).
Though *T. pallidum* has a long-term history of genital ulceration in human beings (Froment, 1994; Hudson, 1965), treponemal infections in wild nonhuman primates have never been reported to cause similar outward signs, apart from the aforementioned suspected cases at GNP (Wallis and Lee, 1999; Wallis, 2000).
Sampled animals received a single shot of long-acting antibiotics to prevent secondary infections at the side of retained biopsy wounds. At the time of sample collection the aetiology of the disease was unclear, thus antibiotics were not considered to treat animals against the genital associated disease, but to prevent skin wounds from additional bacterial infections.

H&E and Giemsa staining were used to evaluate tissue alterations typical for the treponemal infection. The main characteristic findings were irregular epidermal proliferation with acanthosis and acantholysis, epidermal ulceration and perivascular dermatitis with mixed-cell infiltration and tissue eosinophilia. All of these observations can equaly be found in humans infected with *T. pallidum* ssp. *pallidum* (Engelkens et al., 1993; Martin-Ezquerra et al., 2009). The predominant T-cell population which characterised the lymphocytic inflammatory-cell population in all infected baboons might be an incidence that the simian strain isolated from baboons at LMNP differs from what is currently known from *T. pallidum* ssp. *pertenue*. This of course gives a clear contrast to what is found genetically (3.8). In human yaws hardly any T- or B-cells are detectable (Engelkens et al., 1993). Syphilitic lesions caused by *T. pallidum* ssp. *pallidum* on the other hand are clearly filled with T- and B-cell populations (Engelkens et al., 1993).
Syphilis and yaws infection can be partitioned into three stages: primary, secondary and tertiary (Antal et al., 2002; Castro et al., 2007; Engelkens et al., 1993; Martin-Ezquerra et al., 2009; Richens and Mabey, 2009; Wenhai et al., 2004). Currently, it is not possible to categorise the different grades of infection observed in the Olive baboons at LMNP into the defined stages established in humans, even though it seems likely that within the sample size of histological specimens from 62 individuals (Table 12: two animals were recaptures and one neonate was not sampeled for skin biopsy), of both sexes, all age classes and varying severity in terms of the macroscopic signs of the infection, all potential

stages of the infection in baboons were represented. However, at LMNP the clinical manifestation appears strictly limited to the ano-genital region. No alterations outside this area (such as rash in different parts of the body, soft raised condyloma lata, or deformation of the bones) were seen. It is possible that the simian strains described here lack the ability to disseminate or cause clinical manifestations elsewhere in the body; alternatively, it is also conceivable that baboons respond to *T. pallidum* infection differently than do humans. It is also open to question whether the baboon's lifespan allows the disease to progress to a level comparable to the tertiary stage in humans or African apes (Lovell et al., 2000). Serology and PCR of blood results are currently under investigation and will provide additional information on the epikrisis of the disease at LMNP. Furthermore it might lighten the open question concerning the pathogens ability to disseminate in its host organism.

The reliable and sensitive *T. pallidum*-specific PCR assay demonstrated that 86 % (32 out of 37) of the baboons with genital lesions and 30 % (6 out of 20) of the clinically non-affected individuals (without genital ulceration) were positive for the bacterium. It is possible that the relatively low positive rate achieved using rabbit polyclonal antibodies against *T. pallidum* (spirochetes were demonstrated immunohistochemically in 39 % (15 out of 38) of PCR pos. animals and 5 % (1 out of 19) of PCR negative animals) could be caused by antigenic differences between the baboon strains and those used to develop commercially available tests. Because simian strains have been little studied thus far, the sensitivity and specificity of the tests available have not been validated for them. Validation could be of interest for future studies in NHPs, but was not essential for the diagnostic of genital ulceration in Olive baboons at LMNP. The combination of different detection methods in context with modern molecular biological tests provided enough alternatives.

In the past, silver staining was most commonly used for the detection of *Treponema* sp. in histological specimens from human samples. Since detection rates are extremely variable but in general low, ranging anywhere from 0 % (Behrhof et al., 2008; Wenhai et al., 2004), 33 % (Jeerapaet and Ackerman, 1973), 41 % (Sutton et al., 2001) to 50 % (Martin-Ezquerra et al., 2009) it is not surprising that in our case WSS staining failed to depict the spirochetes. Based upon the very low sensitivity and specificity of WSS staining, some authors have made use

of Dieterle staining with some better results (Behrhof et al., 2008). However, at least in Germany, Dieterle staining has been taken off the market and therefore is no longer available.

In both human yaws and syphilis, spirochete numbers can change significantly over the course of infection (Engelkens et al., 1993; Martin-Ezquerra et al., 2009; Wenhai et al., 2004). This is also akin for baboons at LMNP where the quantity of treponemes in the skin tissue biopsy differed significantly between clinical infection stages. The number of treponemes in moderate affected baboons exceeds the one found in severely affected animals, which might indicate a competition of secondary bacterial infections with the primary cause of genital ulceration in the course of the disease.

The copy number of the house keeping gene was not influenced by the gender of the animal, but showed a significant association with the clinical infection stage, which in fact questiones the usability of the gene for standardisation of extraction success and comparability in the detection of *T. pallidum*. The increase in copy numbers of the housekeeper with severity of clinical infection stages presumably constitutes with an increase in inflammatory cells. Although the properties of the house keeping *c-myc* gene are obviously insufficient, the differences of *T. pallidum* genome copies between the different clinical infection stages are statistically not anhilated. The ratio was calculation by diviation of *T. pallidum* over *c-myc* gene copies. This however attenuates the real increase of the *T. pallidum* gene copies with rising severity of the infection, but does not rule it out completely. In fact this indicates that the statistical interpretation is still authentic and practicable, countervailing the bad properties of the *c-myc* house keeping gene.

RNA-Later as a nucleic acid fixative especially designed for RNA protection, can also be used to conserve DNA samples (protocol is available from the manufacturer). Under field conditions it is often necessary to minimize sample size, while enhancing sample usability. RNA-Later allows the protection of RNA and DNA in a single sample which was proved by adequate yields of DNA from the tissue extracts that were used for PCR screening.

The combination of laboratory results with field observation data allows a maximum of diagnostic interpretation. Animals without clinical and histological signs of an infection and, which in addition are negative for treponemes in immunohistochemistry and PCR, can be diagnosed as *T*. *pallidum* non-infected (*n*

= 5, Table 12: case Nos. 8 – 9, 14, 16 and 20). In contrast animals with genital ulceration, histological findings that indicate an infection and positive immunohistochemistry as well as PCR results can be classified as *T*. *pallidum* positive (*n* = 13, Table 12: case Nos. 24, 30, 37, 39 – 41, 44, 46, 51 – 52, 54 – 55 and 57). Baboons, which are positive in all tests for *T. pallidum*, but failed to depict treponemes in immunohistochemistry, can also be classified as *T*. *pallidum* positive (*n* = 19, Table 12: case Nos. 21 – 23, 26, 31 – 36, 38, 42 – 43, 45, 47 – 48, 50, 53 and 56). As previously discussed, the sensitivity of immunohistochemistry seems not to be high and false negative animals are likely. In the case of animals without clinical signs of an infection, but characteristic histological findings as well as a positive PCR result for *T*. *pallidum* (*n* = 6, Table 12: case Nos. 2, 10 – 12, 15 and 18), it is either plausible that the infection is in its prodromal stage or in some cases the clinical manifestation underlies individual differences. Olive baboons with or without clinical signs of an infection but positive histology and negative immunohistochemistry and PCR results for *T*. *pallidum* (*n* = 13, Table 12: case Nos. 1, 3 – 7, 13, 17, 19, 27 – 29, 49 and 53) need to be interpreted carefully. It is likely that samples of those animals decreased in quality during transport and storage in the field. The preservation of samples in formalin is much less defective under field conditions than for cryo-conservation. Another plausible scenario would be that the biopsy taken for histology included clusters of treponemes in the skin, but the biopsy for PCR failed to incorporate treponemes. In rare cases a local trauma with secondary infection might mimic a *T*. *pallidum* associated genital ulceration. In the single case where a female showed clinical and histological signs of a manifest infection and treponemes that were visualised by immunohistochemistry (*n* = 1, Table 12: case No. 25), the biopsy for histology again might have included clusters of the pathogen and the skin sample for extraction not. The animal should be considered as *T*. *pallidum* positive.

PCR results proved to be the most sensitive method for the diagnosis of treponemal infections in NHPs. Although false positive results are unlikely, false negative test results can occure with respect to the quality of the sample.

Three different subspecies of *T. pallidum* are recognized, based on the clinical characteristics of the diseases they cause in humans: yaws (ssp. *pertenue*), bejel (ssp. *endemicum*, also known as endemic syphilis) and syphilis (ssp. *pallidum*). *T. carateum* causes pinta and is currently recognized as another species. Although

the clinical manifestations of these diseases vary widely, the different subspecies of the spirochete *T*. *pallidum* are so closely related that only in recent years has genetic differentiation between them become possible (Gray et al., 2006; Harper et al., 2008a; Harper et al., 2008b). The non-sexually transmitted subspecies (*pertenue* and *endemicum*) are recognized as diseases of early childhood (Antal et al., 2002; Kapembwa, 2009), while ssp. *pallidum* causes syphilis in humans and is the only known subspecies of the pallidum-complex with adaption to a venereal transmission mode (Richens and Mabey, 2009). In this particular context it is interesting that the LMNP simian strains are genetically most closely related to non-venereal human *T. pallidum* strains (ssp. *pertenue*), although the clinical manifestation in baboons differs in its association to the genital. Mlengeya (2004) found only sexually active baboons to be clinically affected which was not the case in this study. The age distribution of clinically affected baboons observed in the field, compared to the different stages of genital ulceration, was not statistically significant. Thus the transmission mode of *T. pallidum* found in LMNP baboons is not clear, yet.

In terms of genetical variation and phylogeny the genomic results, but not the description of gross-pathology, are consistent with analysis of a *T. pallidum* strain obtained decades ago from a baboon in Guinea (Harper et al., 2008b). Two of the three simian strains examined are phylogenetically distinct from the human clade. The basal position of baboon strains in the phylogeny (Figure 13), confirmed by both ML and MP tree-building methods used, could indicate that treponemal strains in nonhuman primates may predate human strains. Since sequences were similar in all three baboon-isolates, there is only one brunch for LMNP simian strains allocated in the phylogenetic tree. It is pretty normal, at least in humans, to have so little variation within a geographical area like a national park. The bootstrap support that was used to build a Majority Rule Consensus tree (Figure 13), showing all nodes that received greater than 50 % replicates, is quite low but conventional (Knoop and Müller, 2009). Since sequence similarities are based on only a few single nucleotide polymorphisms with extraordinarily high rate of evolutionary changes in a genus that has been characterised by very little variation (Mulligan et al., 2008) there is lots of ambiguity among the evolution of “subspecies” that may represent a biological continuum, rather than discrete agents (Mulligan et al., 2008). Nevertheless two possible hypotheses are worth to

discuss: a) spp. *pertenue*-like strains have evolved to produce syphilis-like manifestations more than once, resulting in baboon strains that cause genital ulceration but fall outside of the monophyletic ssp. *pallidum* clade; or b) the Tanzanian simian strains have recently begun to produce syphilis-like manifestation in response to host or environmental changes, indicating the potential for genital ulceration is present in both ssp. *pallidum* and *pertenue*. The genome-wide analysis using microarray and whole genome restriction mapping in *T. pallidum* ssp. *pallidum* (strain Nichols) and *T. paraluiscuniculi* (strain Cuniculi A) already indicates an overall sequence similarity in the range of 98.6 – 99.3 % (Strouhal et al., 2007).

If a novel *T. pallidum* baboon strain emerged recently in Tanzania and spread rapidly across the country, one would expect isolates to be nearly identical genetically, especially given the low level of polymorphism typically found among strains of this bacterium and the small number of genes examined in this study. In contrast to this strains causing the disease in LMNP and SNP were genetically distinct, differing at four of 25 polymorphisms used in the phylogeny (Table 14). While it is possible that recent, rapid evolution of the pathogen could explain the polymorphism, the fact that two synonymous substitutions in the LMNP strain are shared by human strains of ssp. *pertenue* makes this possibility seem unlikely, as it would require convergent evolution at sites that are presumably neutral. Other explanations that reconcile the high level of polymorphism with the recent appearance of the disease include a genetically heterogeneous bacterial population which seeded outbreaks at the sites studied, recent changes in host or environmental characteristics that fostered genital manifestations of the disease, or some combination of these.

The results demonstrate that herpesviruses do not play a causal role in the infection present in baboons at LMNP. Though HVP-2 had previously been considered the causative agent for the genital disease at LMNP (Fyumagwa et al., 2003; Mlengeya, 2004), in our study it was only found in one individual. Thus, there was no correlation between HVP-2 prevalence and clinical manifestations, making it unlikely that herpesviruses play a role in the pathology of this disease. The high number of Lymphocryptovirus 1 – 3 positive baboons is not surprising, as the prevalence of latent LCV infections in wild nonhuman primates is known to be

high (Ehlers et al., 2003). LCVs in nonhuman primates can cause symptoms similar to Epstein-Barr virus infections in humans; producing malignant lymphoma (Deinhardt et al., 1978; Rangan et al., 1986) or hairy leukoplakia (Baskin et al., 1995), especially in Makaks coinfected with SIV. The viruses have never been documented in association with genital ulceration. Furthermore, LCVs were found to be equally prevalent in infected vs uninfected animals at LMNP. Future phylogenetic analysis of herpesviruses found in LMNP's baboons would be an interesting story, but do not provide important information to the aetiology of the current genital associated disease.

The bunch of different bacterial species that were found from 16S rRNA PCR is not surprising since skin wounds are most often secondary contaminated, regardless of its original source. The missing cluster of pathogens known to cause genital ulceration within the sequences that were gained from this methode and at the same time the presents of *T. pallidum* that correlates with the clinical infection stage, underlines the theory that Treponemes are the causative agent of the genital ulcers in Olive baboons at LMNP.

H. ducreyi is endemic in Africa and as previously mentioned (1.3.2) of major health concern for humans. Although natural infections are not reported, yet, the artificial infections proved to produce lesions similar to human infections providing justification to include the pathogen into a standardised molecular biological screening for the aetiology of genital ulceration in NHPs.

Not yet described in NHPs, but of regional importance in human genital infections and endemic for areas in Africa (O'Farrell, 2002; Richens, 2006), *K. granulomatis* should be included in the screening for STDs in monkeys and great apes, too. *K. granulomatis* is responsible for donovanosis, a disease that ultimately leads to genital ulceration in humans. The bacterium is an encapsulated Gram-negative coccobacillus and an intracellular parasite that can be grown in tissue cultures (Carter et al., 1997). Three to forty days after exposure a small papule grows and ruptures to form slowly extending granulomatous lesions that are characteristically pain free (O'Farrell, 2002). Ulcers are often elevated above the level of the surrounding skin and bleed easily on contact (O'Farrell, 2002). Extra genital manifestations are described as well (O'Farrell, 2002; Richens and Mabey, 2009). Donovan-bodies, intracellular coccoid to rod-shaped granula, are pathognomonic for the infection and their light-microscopic illustration is the golden diagnostic

standard (Richens, 2006; Richens and Mabey, 2009). All baboon samples were negative for Donovan bodies in histology and did not reveal any PCR products for *K. granulomatis*.

Non-infectious diseases, as well as common bacterial infections, need to be considered in differential diagnosis of individual cases of genital ulceration in nonhuman primates. The skin of the ano-genital area is exceedingly exposed to the environment. In times of sexual swelling female baboons do have an increased risk of trauma associated skin wounds. In males the penis sometimes gets injured during fighting. In both genders the genital is in direct contact to the ground when the animal sits in an upright position. All these factors may contribute to secondary bacterial infection of previously traumatised skin. In a single case, foreign bodies in the vagina, grass and stones, that were incidental findings at necropsy, have been described for a captive Rhesus macaques (Ford et al., 1998).

In general, ketamine and xylazine combinations are useful for field immobilization of Olive baboons. The combination has synergistic effects, balances the drugs individual side effects and brings about a safe anaesthetic state, with extremely wide safety margins. Animals recover spontaneously and an antidote is not necessary. Ketamine is sufficient as a sedative, although the addition of xylazine is needed for appropriate muscle relaxation. Very low α_2-adrenoceptor-agonist dosages are almost completely metabolised after 30 minutes. The small drug volume, when xylazine dry substance is diluted in ketamine 10 % solution, and the adequate central analgesic effect of ketamine, contribute to the usefulness of the drug combination. Both components are non-terratogenic which is a big advantage for field immobilization of female baboons, as in most cases it is unclear whether the animal is pregnant. In general a dosage of 0.2 mg/kg bw xylazine in combination with 10 mg/kg bw ketamine provides good results for a sampling procedure of 30 – 45 minutes with minor surgery. Since xylazine had no effect on the induction time in Olive baboons it does not provide any further potential to minimize the time from injection until the animal was fully anaesthesised. The slight significant association of xylazine dosage and induction time in subadults is caused by a single individual which received a very high dosage of xylazine (> 2.0 mg/kg bw).

Apart from minor harassments against researches, especially when the immobilized baboon was picked up in a way that was visible to the group, there was no problem concerning fithing or overt aggression against immobilized individuals or those animals, which recovered from anaesthesia. The complex social system of Olive baboons is largely influenced by troop composition and relationship between individual males. In some cases it was possible to observe baboons, especially males, guarding and animating females during their recovery phase to follow the troop (Figure 14). Animation was achieved by calling and precursory walking.

Figure 14. Baboon male (left) guarding and animating his female (right) to follow the nearby troop. The troop (approx. 80 members) is hiding beyond the dense vegatation to the left.

Vaginal exfoliative cytology is a useful tool in NHPs with massive destruction of the perineal and rump skin (e.g. caused by an STD). It is a simple and easy way to gain useful information on the females' current reproductive state. Although in wildlife the procedure requires immobilised individuals and therefore is essentially invasive, the technique produces a rapid bioessay on the females' sex-hormonal status. As previously demonstrated in laboratory primates (MacLennan and Wynn, 1971), different sexual cycle stages correlated with characteristic cytological findings, although further studies need to test for the association of sexual swelling, cytological findings and additional hormonal analysis from sera and

faecal samples of Lake Manyara's baboons. The maturation value allows a classification of the sexual cycle stage, especially when the classification has to be done by technicians with little expertise.

The quality of samples was a limiting factor. Under tropical conditions, moisture can lead to fungal contamination of stored samples, which has a direct influence on sample quality and the value of laboratory interpretation. Silica gel in combination with an airtight box can be used to keep samples dry. Slides should be protected from sun light at any time. In general, both Papanicolaou as well as Diff-Quick staining provided appropriate results for the analysis of vaginal exfoliative cytological specimens. In older vaginal smears the classic Papanicolaou staining seemed to have a slight disadvantage, as cell populations were more bleached when compared with those stained with Diff-Quick staining solution. The deterioration of staining quality can be efficiently reduced when fresh air-dried samples are stained. Vaginal epithelial cells seem to lose their staining quality over time. Diff-Quick staining technique is relatively easy to perform and requires less time than Papanicolaou staining. Samples of good quality provide reliable results and whenever exact hormon values are not required, may represent an acceptable non-invasive compromise for cost and time-consuming hormone analysis in the laboratory, especially under tropical conditions.

In conclusion, a detailed description of histological findings in wild baboons consistent with syphilis-like clinical signs is presented. Clinical manifestation is associated with the consistent finding of a strain of *T. pallidum* most closely related to the human strains of *T*. *p*. ssp. *pertenue*. Epidemiologically, it is interesting that the prevalence of the disease (based on histological findings) is much higher than expected from macroscopic observations in the field. The range in different baboon troops at LMNP has been estimated at 7.1 – 29.1 % (Mlengeya, 2004). The histological findings (Langhans-Giant-cells, granulomatous tissue, irregular epidermal proliferation and scarification) indicate that the disease is chronic in many animals. Further studies are needed to describe the pathogenesis of this disease in baboons, covering the time between initial exposure and the appearance of first clinical signs, until recovery or death. According to what is known from human inoculation experiments with the Fribourg-Blanc simian strain, there could be a potential for zoonotic transmission (Centurion-Lara et al., 2006),

underscoring the necessity for additional research to gain insight into the disease's transmission dynamics, as well as its temporal and spatial distribution. Several large-scale human treponematosis eradication programs have been run in the late 50s and early 60s without any sustainable effect (Arya and Bennett, 1976; Guthe et al., 1972) and NHPs have only recently been considered as a potential reservoir for human infection (Centurion-Lara et al., 2006).

5 Summary (extended)

The aim of the study was to investigate a genitally associated disease and to describe its clinical manifestation and aetiology in baboons at Lake Manyara National Park in the United Republic of Tanzania.

Lake Manyara National Park is located in the northern part of the country, 160 km northwest of the Mt. Kilimanjaro. It is among the smallest protected areas, but belongs to the extended ecosystem of the Serengeti, Ngorongoro Conservation Area, Lake Manyara and Tarangire National Park. The area is famous for its baboon population, the highest in Africa with an estimated number of 2,000 – 2,500 individuals inhabiting an area of roughly 110 km^2. First sightings of baboons with severe genital ulceration were reported in 1994. This PhD study made use of 63 immobilised and sampled Olive baboons (*P. h. anubis*), 39 of which displayed outward signs of genital ulceration and 24 of those looking healthy. Animals with genital lesions were grouped into “initial”, “moderate” or “severe” affected. Immobilisation and sampling followed a standardised protocol. A combination of ketamine (10 mg/kg bw) and xylazine in a dose-range of 0.1 – 0.2 mg/kg bw was used as anaesthetic. Baboons were continuously monitored using a Nelcor OxiMax N-65 pulse-oximeter, starting at the time of discovery until fully recovered. The field site was left not before the animal regained consciousness. Photo documentation of genital lesions and the sampling procedures were conducted under general anaesthesia. Baboons were sampled for blood, oral and genital swaps and skin tissue biopsies. In addition, a vaginal exfoliative cytology was performed in female baboons. Sample storage and preservation followed the principle of prevention of loss through cooling-chain breakdown and protection of sample quality under extreme tropical conditions.

In the laboratory tissue samples were prepared for histological and molecular biological analysis. Formalin preserved tissue was embedded into paraffin and cut into 4-µm thick layers. All slides specimens were stained with Hematoxyline-Eosin, Periodic-Acid-Shiff and Warthin-Starry-silver, according to the manufacturer’s protocol. In some cases an additional Giemsa staining was performed. The light-microscopic examination was done twice, with stepwise increasing magnification from 100x to 1,000x.

Immunohistochemistry for the detection of *Treponema pallidum* made use of rabbit-polyclonal antibodies diluted 1:500. Furthermore primary mouse-anti-human B-cell CD20 and rabbit-anti-human T-cell CD3 antibodies were used in dilutions of 1:300 and 1:50, respectively, to differentiate B- and T-cells semi-quantitative. Biotinylated secondary antibodies, streptavidin and diaminobenzidin were applied as chromagen according to the supplier´s instructions.

Fluorescence-in-situ-hybridisation was used in baboons with and without genital ulceration and of different stages of clinical infections. The technique utilised the probe EUB 338 to illustrate a broad range of prokaryote structures in-situ in paraffinised tissue slides.

DNA-extraction from RNA-Later conserved tissue samples was achieved by usage of a commercially available extraction kit. A broad screening for bacteria with 16S rRNA PCR was followed by PCR assays for specific pathogens. Products were cloned and sequenced. Sequences were compared with those of existing prokaryotes to the public GenBank database. In addition, all DNA-extracts were tested for the presence of *T. pallidum* (the bacterium that causes yaws, syphilis and bejel), *Klebsiella granulomatis* (the bacterium that causes donovanosis), *Haemophilus ducreyi* (the bacterium that causes soft chancre) and *Herpesvirus papio* 2 (baboon genital herpes), all known to cause genital ulceration in primates. Furthermore 26 baboons were tested for the presence of any other herpesviruses with pan-herpes consensus PCR. Products were gel-extracted and sequenced.

A real-time PCR was used for the quantitative detection of *T. pallidum*. Primers were set to include a 67 bp sequence of a highly conserved part of the DNA polymerase I gene.

Phylogenetic analysis made use of six different polymorphic regions previously determined to aid in differentiation between the *T. pallidum* subspecies. An alignment was used to create phylogenies constructed in Phylogenetic Analysis Using Parsimony (PAUP) software. Polymorphisms, which fell within regions in which no signature of recombination was detected, were concatenated and aligned in ClustalX version 1.83. Recombination was ruled out using genomic searches for possible donor regions at highly polymorphic sites, utilising Recombination Detection Analysis from Sequence Alignments Software, and by analysis of dN/dS ratios to detect recombination events. All excluded polymorphisms were located in hypervariable regions of the *tp92* gene.

Phylogenetic trees were constructed in PAUP using *Treponema paraluiscuniculi,* the agent that causes rabbit syphilis*,* as an outgroup. Maximum Parsimony and Maximum Likelihood methods were applied, with 1,000 replicates used to obtain bootstrap support and starting trees were obtained through random, step-wise addition.

The group size of observed baboon troops varied from 8 to 250 individuals. Observation of genital ulcerated animals was sometimes difficult due to dense vegetation or limited access to the residence. Baboons went down 4 – 5 minutes after injection of the anaesthetic. The induction time (time from injection until full anaesthesised) was not significantly influenced by the dose of xylazine and the antagonisation of xylazine in a dosage range of 0.1 – 2.0 mg/kg bw, in single injected animals, had no significant effect on the recovery time (time from injection until fully recovered).

The macro- and microscopic findings of infected baboons displayed a uniform character. The chronic-active ulcerative disease was accompanied by a massive destruction of the genitalia. Progressive scarification of the tissue lead to a vagina and anus that are permanently ajar in females, while males displayed a substantial to complete loss of the penis through autoamputation and mutilation of the external reproductive organ. The age of the baboons was not significantly correlated with the extend of genital ulceration.

According to the characteristic picture of the vaginal exfoliative cytology it was possible to assign genital ulcerated female baboons, which did not show any or reduced physiological sexual swelling of the ano-genital area, to the different sexual cycle stages.

The histology showed a chronic-active inflammatory-cell composition, most often condensed around the dermal blood vessels, resulting in superficial and deep perivascular dermatitis. Skin lesions were characterised by irregular epidermal proliferation of different extent with acanthosis, acantholysis and exocytosis. In severe cases the overlaying epidermis was ulcerated and a chronic granulomatous reaction ensued. The morphologic illustration of the spirochete in tissue samples was possible in 16 cases by immunohistochemistry. Significantly more often in clinically affected than clinically non-affected baboons. The pathogene was mainly distributed in the centre of the lesion, showing a clear tropism for the epidermis-dermis region (epitheliotropic pattern) and displaying a

vasculotropic pattern. A single organism was characteristic spiral-like shaped and about 6.0 – 15.0 µ m in lengh. Warthin-Starry-silver and fluorescence-in-situ-hybridisation were not able to depict treponemes. In all cases, more T- than B-cells were present among the lymphocytic inflammatory-cell population.

16S rRNA PCR found a broad, but not meaningful, spectrum of bacteria. Thrirty-eight baboons tested positive for *T. pallidum* by PCR and/ or quantitative real-time PCR. Baboons with positive PCR result showed significantly more often clinical signs than those that were negative in PCR. In addition Olive baboons with histological signs of an infection were significantly more often positive in *T. pallidum* PCR than those without histological alterations. Baboons of moderate clinical infection stage had the highest number of *T. pallidum* gene copies, followed by those of severe and clinically initial stage. Clinically non-affected animals had the lowest number of *T. pallidum* copies. Particularily worth mentioning is also that 30 % of the animals without genital ulceration were PCR positive for *T. pallidum*.

Molecular biological tests for *K. granulomatis* and *H. ducreyi* were negative in all samples. Only one baboon out of 57 (63 individuals: in three baboons DNA extraction failed, two were not sampled for skin tissue and in one animal only RNA was extracted) was positive for *Herpesvirus papio* 2. In addition a variety of different herpesviruses were found in 23 of 26 tested individuals. Sequence analysis of the herpesvirus genomes revealed a high prevalence of lymphocrypto- and Baboon cytomegaloviruses.

This study is the first detailed description of macroscopic, histological and molecular biological findings of a genital associated disease in baboons, caused by *T. pallidum*. In comparison to previously published information regarding polymorphic sites useful in distinguishing the subspecies and epidemiologically it is interesting that the Lake Manyara National Park simian strain is genetically most closely related to non-venereal human *T. pallidum* strains (ssp. *pertenue*), raising the question if the potential for predominat genital associated ulceration is present in treponemes that fall outside the monophyletic ssp. *pallidum* clade, too. Results gained from this study indicate that the prevalence of the disease in reality is much higher than previously estimated by field observations. Though treponemal infections in wild nonhuman primates are not a new phenomenon, they never have been considered to cause genital associated diseases. Further research is needed

to understand the disease's transmission dynamics, as well as its temporal and spatial distribution, especially in the context of proven zoonotic transmission of non-venereal Fribourg-Blanc simian strains of West Africa.

5.1 **Zusammenfassung** (extended German summary)

Ziel der vorliegenden Arbeit ist die Erforschung einer Geschlechts-assoziierten Erkrankung bei Pavianen im Lake Manyara Nationalpark der Vereinigten Republik Tansania.

Der Lake Manyara Nationalpark liegt im Norden des Landes, 160 km nordwestlich des Mt. Kilimanjaro. Er ist eines der kleineren Schutzgebiete, gehört aber zum großen Gesamtökosystem des Serengeti, Ngorongoro Conservation Area, Lake Manyara und Tarangire Nationalparks. Bekannt ist das Gebiet für seine Pavianpopulation, die mit geschätzten 2.000 – 2.500 Tieren als die Höchste Afrikas gilt. Im Jahre 1994 wurde erstmals von einer Erkrankung berichtet, die bis heute bei den Anubispavianen des Lake Manyara Nationalparks zu einer schweren ulzerativen Genitalveränderung führt. Im Rahmen der PhD Arbeit wurden 63 Anubispaviane (*P. h. anubis*), 39 mit genitaler Läsion und 24 gesund erscheinende Tiere, narkotisiert und beprobt. Tiere, die an einer klinisch manifesten Genitalinfektion litten, wurden in die Stadien „initial“, „moderat“ und „hochgradig“ eingeteilt. Die Immobilisation und Probennahme erfolgte nach einem zuvor festgelegten Schema (Anhang 7.2). Mit einem Gemisch aus Ketamin (10 mg/kg Körpergewicht) und Xylazin in unterschiedlichen Dosierungen (0,1 – 2,0 mg/kg Körpergewicht) erfolgte die Narkoseeinleitung mittels Distanzinjektion. Vom Zeitpunkt des Auffindens bis zum Aufwachen des Pavians ermöglichte das Pulsoximeter Nelcor OxiMax N-65 die Überwachung der Narkose. Erst nach vollständiger Wiederherstellung des Bewusstseins wurde der Patient sich selbst überlassen. An den narkotisierten Tieren erfolgte eine Fotodokumentation der Veränderungen, sowie die Probenentnahme. Den Tieren wurde Blut, Abstriche von Mund und Genital sowie Hautgewebe entnommen. Zusätzlich wurde von weiblichen Tieren eine exfoliative Vaginalzytologie angefertigt. Die Probenkonservierung folgte den Prinzipien des Vermeidens von Kühlkettenverlusten und der Sicherung bestmöglicher Probenqualität unter extremen tropischen Bedingungen.

Im Labor erfolgte die Aufarbeitung der Gewebeproben zur histologischen und molekularbiologischen Untersuchung. Formalin fixiertes Gewebe wurde paraffinisiert, zur histologischen Begutachtung in 4-µm dicke Schichtpräparate geschnitten und auf Objektträger aufgezogen. Alle Schnitte wurden entsprechend

den Angaben des Herstellers mit Hematoxylin-Eosin, Periodic-Acid-Shiff und Warthin-Starry-silver gefärbt. In einzelnen Fällen erfolgte zusätzlich eine Giemsafärbung. Die lichtmikroskopische Auswertung gelang in zwei separaten Durchgängen, mit schrittweiser Vergrößerung von 100 bis 1.000-fach.

Der immunohistologische Nachweis von *Treponema pallidum* wurde mit Kaninchen-polyklonalen Antikörpern in einer Verdünnung von 1:500 durchgeführt. Zur semiquantitativen Unterscheidung der B- und T-Zellpopulation kamen primäre Maus-anti-Mensch B-Zell CD20 und Kaninchen-anti-Mensch T-Zell CD3 Antikörper in einer Verdünnung von 1:300 und 1:50 zum Einsatz. Biotinylierte Sekundärantikörper, Streptavidin and Diaminobenzidin, ermöglichten den Färbenachweis und wurden entsprechend den Angaben des Herstellers eingesetzt.

Die Fluoreszenz-in-situ-Hybridisierung erfolgte bei Pavianen mit und ohne genitaler Ulzeration und unterschiedlichem Infektionsgrad. Die verwendete Sonde EUB 338 diente dem Nachweis prokaryotischer Strukturen im histologischen Schnitt.

Die Extraktion von DNA zum Nachweis des Erregergenoms mittels PCR erfolgte aus RNA-Later fixierten Gewebeproben unter zur Hilfenahme eines kommerziellen Extraktionskits. Einem breit angelegten Nachweis von Bakterien mittels 16S rRNA PCR folgte der spezifische Nachweis einzelner Pathogene. Produkte der 16S rRNA PCR wurden geklont und nach erfolgter Sequenzierung in einer öffentlichen GenBank mit existierenden prokaryonten Sequenzen verglichen. In allen DNA-Extrakten wurde nach dem Genom von *T. pallidum* (dem Erreger der Frambösie, Syphilis und Bejel), *Klebsiella granulomatis* (Erreger der Donovanose), *Haemophilus ducreyi* (Erreger des weichen Schanker) und *Herpesvirus papio* 2 (Genitalherpes des Pavians) gesucht. Eine pan-Herpes Konsensus PCR bei 26 Pavianen diente dem Nachweis weiterer Herpesviren. PCR-Produkte wurden aus dem Agarosegel extrahiert und sequenziert.

Der quantitative Nachweis von *T. pallidum* erfolgte mittels real-time PCR. Die Primer erfassten dabei einen 67 Basenpaar großen konservierten Genomabschnitt auf dem DNA Polymerase I Gen. Phylogenetische Analysen wurden an sechs verschiedenen polymorphen Regionen im Genom von *T. pallidum* vorgenommen, anhand derer sich die einzelnen Unterarten unterscheiden lassen. Polymorphismen in Regionen ohne Hinweis auf Rekombination wurden verknüpft

und mit dem Program ClustalX Version 1.83 untereinander verglichen. Rekombinationen wurden unter zur Hilfennahme der Software Recombination detection analysis from sequence alignments (RDP2) zum Vergleich genomischer Sequenzen aus möglichen Donorregionen in hochpolymorphen Regionen, ausgeschlossen. Zusätzlich erfolgte die Analyse des Verhältnisses von nicht-synonymen und synonymen Substitutionen pro Region (dN/dS) zur Erkennung von Rekombinationsereignissen. Alle ausgeschlossen Polymorphismen lagen in hypervariablen Regionen des *tp92* Gens. Stammbäume wurden mit der Phylogenetic Analysis Using Parsimony Software erstellt. Der Erreger der Kaninchensyphilis *T. paraluiscuniculi* diente dabei als Außengruppe. Zum Einsatz kamen die phylogentischen Methoden der Maximum Parsimony und Maximum Likelihood mit einem 1.000-fach replizierten Aligment zur Bootstrap-Analyse und zufallsbedingter schrittweiser Addition.

Paviangruppen hatten Größen zwischen 8 und 250 Individuen. Das Beobachten genitalerkrankter Tiere gestaltete sich aufgrund der Vegetation und dem oft versperrten Zugang zum Aufenthaltsort der Tiere schwierig.

Etwa 4 – 5 Minuten nach der Injektion von Ketamin und Xylazin lagen die Tiere in Narkose. Die Phase der Einleitung (Zeitpunkt der Injektion bis das Tier in Narkose lag) war unabhängig von der verwendeten Xylazindosierung (Dosisbereich 0.1 – 2.0 mg/kg bw). Das Antagonisieren des α_2-Adrenozeptoragonisten Xylazin führte nicht zu einem verfrühten Wiedereinsetzen des Bewusstseins.

Makroskopisch und histologisch zeigte sich bei infizierten Tieren ein einheitliches Bild. Chronisch-aktive Genitalulzerationen gingen einher mit einer teilweise massiven Entstellung des Genitales. Weibliche Tiere zeigten narbig kontrahierte und dadurch offenstehende Vaginen, männliche Tiere wiesen einen Substanzverlust des Penis, bis hin zur vollständigen Autoamputation und Verstümmelung des Reproduktionsorgans auf. Es ergab sich kein Zusammenhang zwischen Alter und Schweregrad der Erkrankung.

Weibliche Paviane, deren Genitalbereich stark ulzeriert war, und keine physiologische Sexualschwellung mehr erkennen lies, konnten anhand des zytologischen Bildes der exfoliativen Vaginalzytologie den einzelnen Phasen des Geschlechtszyklus zugeordnet werden.

Histopathologisch ergab sich bei beiden Geschlechtern ein chronisch aktives Entzündungbild mit vorwiegend perivaskulärer Anhäufung gemischtzelliger

Entzündungszellen. Daraus ergibt sich eine oberflächliche und tiefe perivaskuläre Dermatitis. Hautveränderungen waren durch eine irreguläre epidermale Proliferation unterschiedlicher Ausprägung mit Akanthose, Akantholyse und Exozytose gekennzeichnet. In schweren Fällen war die Epidermis ulzeriert und das verbleibende Gewebe zeigte eine chronisch-granulomatöse Entzündungszellreaktion. Die morphologische Darstellung des Spirochäten in Gewebeproben der Haut gelang in 16 Fällen mittels Immunohistologie. Treponemen wurden signifikant häufiger bei klinisch erkrankten, als klinisch gesund erscheinenden Tiere gefunden. Der Erreger war hauptsächlich im Zentrum der Läsion nachweisbar, mit ausgeprägtem Tropismus für den Bereich der Epidermis und Dermis und der Nähe zu Blutgefäßen. Eine einzelne Treponeme ist 6,0 – 15,0 µ m lang und zeigt die charakteristische Spiraldrehung in der Längsachse. Mittels Warthin-Starry-silver-Färbung und Fluoreszenz-in-situ-Hybridisierung konnten keine Spirochäten im Gewebe dargestellt werden. In allen Fällen waren mehr T- als B-Zellen innerhalb der Lymphozytenpopulation des Entzündungszellinfiltrats erkennbar.

Die 16S rRNA PCR generierte ein breites, aber nicht aussagekräftiges Spektrum an Bakterien. Achtunddreißig Paviane wurden mittels *T. pallidum* PCR und/ oder quantitativer Real-time PCR positive getested. Paviane mit positivem PCR Ergebnis zeigen signifikant häufiger Genitalulzerationen als Tiere mit negativem Ergebnis. Zugleich zeigt sich, dass Anubispaviane mit histologischen Anzeichen für eine Infektion signifikant häufiger positive PCR Resultate haben, als solche ohne Anzeichen von Gewebeveränderungen. Paviane mit moderater klinischer Ausprägung hatten die höchsten *T. pallidum* Kopienzahlen, gefolgt von denen mit hochgradiger und initialer Ausprägung. Klinisch nicht infizierte Tiere hatten die niedrigsten *T. pallidum* Kopienzahlen. Besonders erwähnenswert ist vor allem, dass 30 % der Tiere, die im Feld als klinisch gesund klassifiziert wurden, mit *T. pallidum* infiziert waren.

Molekularbiologische Nachweise *von K. granulomatis* und *H. ducreyii* verliefen bei allen Proben negativ. Ein einziger Pavian von ingesamt 57 Tieren (63 Individuen: bei drei Tieren war die DNA Extraktion nicht erfolgreich, von zwei Tieren gibt es keine Hauptprobe und bei einem Tier wurde nur RNA extrahiert) wurde HVP-2 PCR positiv getestet. Eine Vielzahl getesteter Paviane war jedoch mit anderen Herpesviren infiziert (23 von 26 Tieren). Sequenzanalysen der

Herpesviruskonsensus-PCR ergaben eine hohe Prävalenz für Lymphocrypto- und Pavian Cytomegaloviren.

Erstmals konnte eine detaillierte Beschreibung makroskopischer, histologischer und molekularbiologischer Befunde einer Geschlechtserkrankung bei Pavianen, verursacht durch *Treponema pallidum*, bei Pavianen erfolgen. In Anlehnung an zuvor veröffentlichten Informationen über polymorphe Regionen im Genom von Treponemen, die zur Unterscheidung von Unterarten geeignet sind und epidemiologisch von großem Interesse, ist die nahe genetische Verwandtschaft des Treponemenisolates mit den nicht venerischen Treponemaarten (*T. pallidum* ssp. *pertenue*). Die einheitliche klinische Manifestation des Erregers lässt damit den Schluß zu, dass das Potential für eine genitale Manifestation auch in nicht venerischen Treponemen vorhanden sein kann. Die einzelnen Untersuchungen zeigen, dass die ursprünglich über Feldbeobachtungen ermittelte Prävalenz deutlich höher liegt. Erkrankungen bei nicht wildlebenden menschlichen Primaten verursacht durch Treponemen sind kein neues Phänomen, wurden zuvor aber niemals mit einer Erkrankung der Geschlechtsorgane in Verbindung gebracht. Weitere Forschung ist nötig, um epidemiologische Fragestellungen zu klären, zumal Infektionsversuche mit nicht venerischen Pavianisolaten aus Westafrika ein zoonotisches Potential bereits belegt haben.

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7 Appendix

7.1 Immobilisation protocol

Immobilisation Form and Sample List

Baboon Investigation Study – LMNP

animal ID#: _____;Pic #1: _____; Pic #2: _____;

investigator/ gunner: _____; date: _____.2007;

species: Olive baboon (*Papio hamadryas anubis*); location: _____;

group:_____; location of capture (pref. GPS Coordinates): _____;

terrain: _____; troop size: _____ ; ambient temp. (°C): ___;

Immobilisation drugs used

drug: Ketamin; dosage used: _____ mg/ kg KM; Vol.: _____ml; applic. modus : IM;

drug: Xylazin; dosage used: _____ mg/ kg KM; Vol.: _____ml; applic. modus : IM;

drug: Atropin; dosage used: _____ mg/ kg KM; Vol.: _____ml; applic. modus : IM;

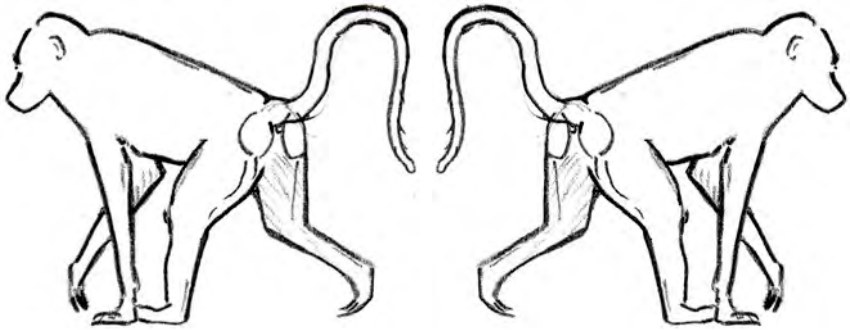
Behaviour at time of injection: (run, walk, stand, etc.) _____;

application modus: (blowpipe, immobilisation-gun) _____;

first time of injection: _____;

time of following injection: _____; reason: _____

drug: Ketamin; dosage used: _____ mg/ kg KM; Vol.: _____ml; administration route: IM;



side of injection (please mark)

time of initial effect (ataxia and first signs of sedation): _____;

time when animal goes down:_____;

time when down (head down): _____;

time when arrive at immob. baboon: _____;

How many darts failed, drugs lost:

_____

_____

Signalement

body condition score (0-5): _____; body weight:_____ kg

male ☐ female ☐ female lactating Y ☐ N ☐

age: _____; permanent incisors: Y ☐ N ☐

other emergency treatment: _____

temporarily identification : (i.e. haircut shoulder plus silver spray)_____

_____

Vital parameters

time:_____; temp. (°C): _____ ; hfq: _____ bpm; respiration:_____ per min;

time:_____; temp. (°C): _____ ; hfq: _____ bpm; respiration:_____ per min;

other vitals:_____

_____

Clinical examination

skin and hair: _____ NAD

mucosa: _____ NAD

lymphnodes: _____ NAD

cardio-vascular-system: _____ NAD

respiratory system: _____ NAD

digestive tract: _____ NAD

urinary system: _____ NAD

reproductive and genital system: _____ NAD

_____

Reversal time of immobilisation

reversal drug: recovery time: _____

drug: _____; dosage used: _____ mg/ kg KM; Vol.: _____ml; applic. modus : IM;

additional drugs:

drug: **Duphamox**; dosage used: _____ mg/ kg KM; Vol.: _____ml; applic. modus : IM;

drug: _____; dosage used: _____ mg/ kg KM; Vol.: _____ml; applic. modus : IM;

general comment to anaesthesia: _____

Sampling

K^+-EDTA blood sample (min. 2 x 9 ml): quantity _____ ID# _____ ☐

plasma (frozen) ☐ ID# _____

buffy coat (frozen) ☐ ID# _____

plasma (dried) ☐ ID# _____

buffy coat (dried) ☐ ID# _____

blood smear ☐ slide ID# _____

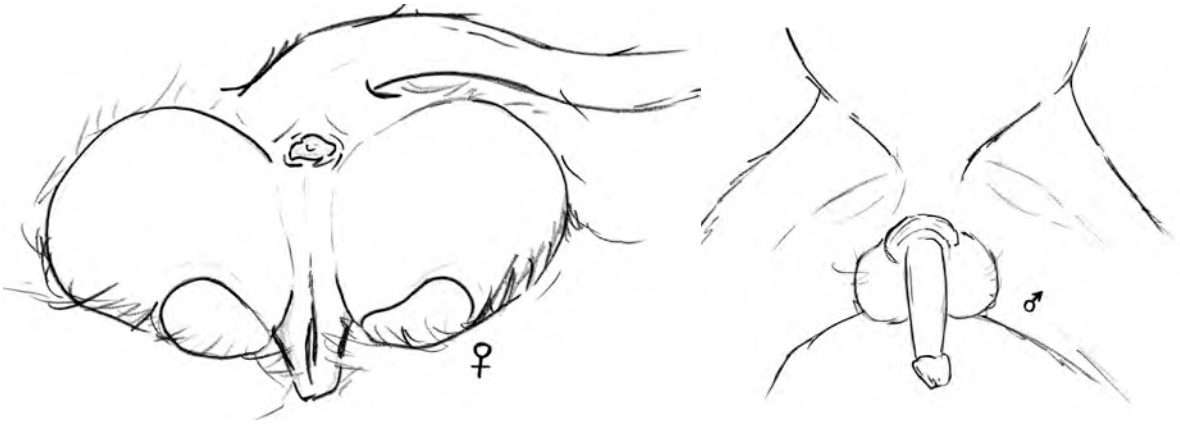
Swabs

genital (min. 2) frozen ☐ ID# _____; RNA-later ☐ ID# _____

face (min. 2) frozen ☐ ID# _____; RNA-later ☐ ID# _____

Urine (sterile sampled) ☐ ID# _____; **faeces** ☐ ID# _____;

Skin biopsy (min. 3 per animal): ☐



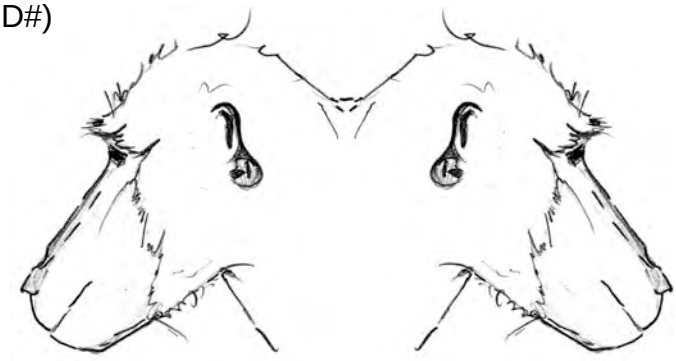
(Please mark location with ID#)

ID# _____; (frozen) ☐

ID# _____; (RNA-later) ☐

ID# _____; (formalin) ☐

ID# _____; (formalin) ☐*



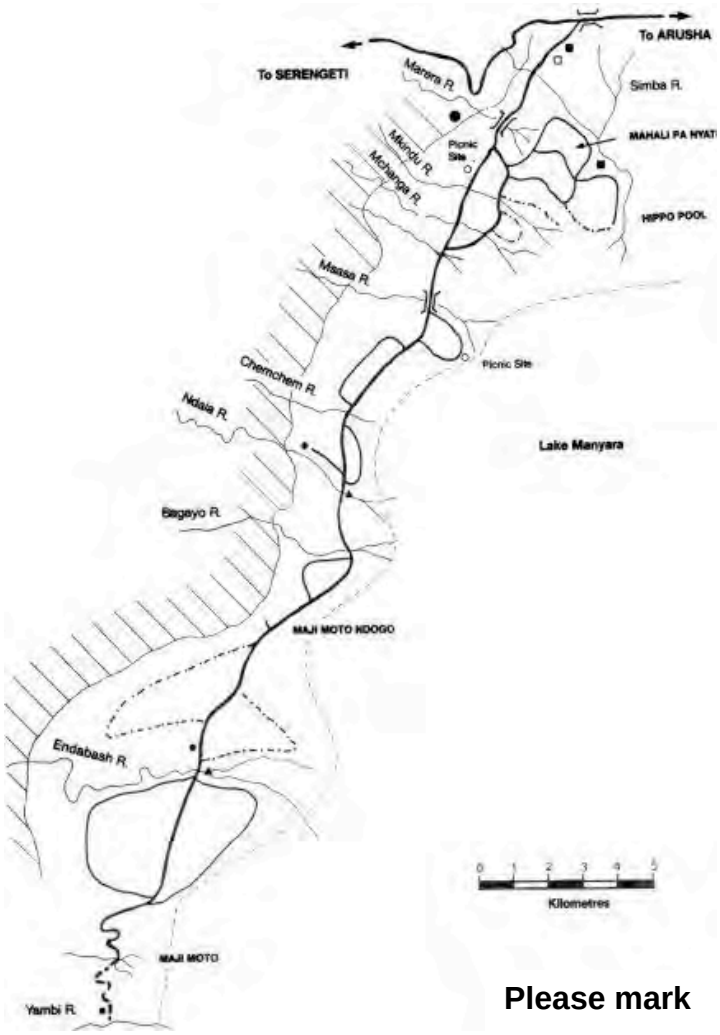
left right

* *tissue from healthy skin*

Exfoliate vaginal cytology

slide ID# _____;

other comments



Team

Veterinarian: med. vet. S. Knauf

Driver:

Accompanying persons:

Please mark location of capture

7.2 Staining protocols

7.2.1 Diff-Quick staining solution

Fixing solution (I)	5 x 1 sec
Fixing solution (II)	10 x 1 sec
Fixing solution (III)	10 x 1 sec

Washing step, under running water (tap water (pH 7,2))

Air-drying

Item coverage with Eukitt (O. Kindler GmbH, Freiburg, Germany)

7.2.2 Giemsa staining

Deparaffinization und rehydration

Xylene	5 min
Xylene	2 min
Xylene	2 min
100 % ethanol	2 min
96 % ethanol	2 min
80 % ethanol	1 min
70 % ethanol	1 min
Aqua destillata	1 min

10 % Giemsa staining solution (Appendix 7.2.3)

in 0.1 mol phosphate buffer (pH 6.8)	25 min
Aqua destillata	a few sec

Differentiation in 0.5 % acetic acid (1 ml concentrated acetic acid + 200 ml distilled water) until slides are shiny red

96 % ethanol	a few sec
Isopopropanol	2 x 5 min
Xylene	1 min
Xylene	3 min
Xylene	3 min

Item coverage with Eukitt (O. Kindler GmbH, Freiburg, Germany)

7.2.3 Giemsa working solution

Working solution (phosphate buffer (pH 7.2))

Giemsa concentrate	10 drops
Destilled water	10 ml

7.2.4 Hematoxyline-Eosin staining

Deparaffinization und rehydration	
Xylene	5 min
Xylene	2 min
Xylene	2 min
100 % ethanol	2 min
96 % ethanol	2 min
80 % ethanol	1 min
70 % ethanol	1 min
Destilled water	1 min
Nucleus staining with Hemalaun after Mayer (Merck KGaA, Darmstadt, Germany)	3 min
Running water	10 min
Counter stain with aqueous eosin (Thermo Shandon GmbH, Frankfurt am Main, Germany)	5 min
Washing step, under running water	5 sec
Dehydration	
70 % ethanol	1 min
80 % ethanol	1 min
96 % ethanol	1 min
100 % ethanol	1 min
100 % ethanol	2 min
Xylene	1 min
Xylene	3 min
Xylene	3 min

Item coverage with Eukitt (O. Kindler GmbH, Freiburg, Germany)

7.2.5 Papanicolaou-Shorr staining

Ascending ethanol series	
Ethanol absolute	15 min
80 % ethanol	15 min
70 % ethanol	15 min
50 % ethanol	15 min
Destilled water	15 min
Hematoxyline staining	6 min
Washing step with tap water, until free of cords	
Ammoniac-alcohol	1 min
Shorr-staining solution	2 min
Descending ethanol series	
50 % ethanol	15 min
70 % ethanol	15 min
80 % ethanol	15 min
Ethanol absolute	15 min
Item coverage with Eukitt (O. Kindler GmbH, Freiburg, Germany)	

7.2.6 Periodic-Acid-Schiff staining

Deparaffinisation and rehydration	
Xylene	5 min
Xylene	2 min
Xylene	2 min
100 % ethanol	2 min
96 % ethanol	2 min
80 % ethanol	1 min
70 % ethanol	1 min
Destilled water	1 min
0.5 % periodic acid	10 min
Destilled water, running water	10 min
Schiff´s reagent	15 min
(Merck KGaA, Darmstadt, Germany)	

Destilled water	a few sec
Washing step, under running water (tap water (pH 7.2))	10 min
Nucleus staining with Hemalaun after Mayer	30 sec
(Merck KGaA, Darmstadt, Germany)	
For blueing, washing step under running water	10 min
Dehydration in ascending ethanol series	
70 % ethanol	1 min
80 % ethanol	1 min
96 % ethanol	1 min
100 % ethanol	1 min
100 % ethanol	2 min
Xylene	1 min
Xylene	3 min
Xylene	3 min

Item coverage with Eukitt (O. Kindler GmbH, Freiburg, Germany)

7.2.7 Warthin-Starry silver staining

Deparaffinisation und rehydration	
Xylene	5 min
Xylene	2 min
Xylene	2 min
100 % ethanol	2 min
96 % ethanol	2 min
80 % ethanol	1 min
70 % ethanol	1 min
Destilled water	1 min
Impregnation of specimens with 1 % silver nitrate solution, preheated to + 45 °C	
(1 g $AgNO_3$ in 100 ml double distilled water)	60 min
Coating of slides with developing solution (7.2.7.1)	1 - 2 min
Interruption as soon as color intensity is sufficient (= light brown)	
Washing step with + 60 °C warm tap water	
Dehydration in ascending ethanol series	
70 % ethanol	1 min

80 % ethanol	1 min
96 % ethanol	1 min
100 % ethanol	1 min
100 % ethanol	2 min
Xylene	1 min
Xylene	3 min
Xylene	3 min

Item coverage with Eukitt (O. Kindler GmbH, Freiburg, Germany)

7.2.7.1 Developing solution

2 % silver nitrate solution (2 g $AgNO_3$ in 100 ml double destilled water)
5 % galantine (5 g dry substance galantine in 100 ml double destilled water.)
0.15 % hydrochinon solution (0.15 g hydrochinon in 100 ml double destilled water)
Each ingredient is preheated to 45 °C and mixed together shortly before usage. Double destilled water must be acidified to pH 4.0 (e.g. with 0.4 % citric acid).

7.3 Hypercenter XP protocol

- Water (demineralized) for 2 h at room temperature
- Ascending ethanol series (50 % – 70 % – 80 % – 96 % – 96 % – 100 % – 100 %), each step lasting for at least 45 min at 35 °C in vacuum
- 2 x chloroform for 1.5 h or 1 h at room temperature in vacuum
- 2 x paraffin for 1.5 h at 60 °C in vacuum

7.4 Fixation solution

<u>4 % neutral buffered formalin</u>

35 % formaldehyde solution	114 ml
Destilled water	386 ml
Stock solution A (phosphate buffer)	100 ml
Stock solution B (phosphate buffer)	400 ml

10 % neutral buffered formalin

35 % formaldehyde solution	285 ml
Destilled water	215 ml
Stock solution A (phosphate buffer, Appendix 7.4.1)	100 ml
Stock solution B (phosphate buffer, Appendix 7.4.1)	400 ml

7.4.1 Phosphate buffer

Stock solution A (0.2 M)

Natriumdihydrogenphosphate monohydrate	27.6 g
(Cat. # 6346, Merck KGaA, Darmstadt, Germany)	
double destilled water	ad 1000 ml

Stock solution B (0.2 M)

Di-natriumhydrogenphosphate dehydrate	
(Cat. # 6580, Merck KGaA, Darmstadt, Germany)	35.6 g
double destilled water	ad 1000 ml

Working solution (0.1 M, pH 7.4 – 7.6)

Stock solution A	10 ml
Stock solution B	40 ml
double destilled water	50 ml

7.5 GPS Data

Data were collected at each sampling site, utilizing a Garmin eTrex H (Gramin Deutschland GmbH, Gräfelfing, Germany).

Baboon ID #	Case # (Table 12)	South	East
9-F1-25.03.07	1	3°22'24.0''	35°50'11.4''
11-F1-26.03.07	2	3°23'33.4''	35°49'24.6''
14-F1-27.03.07	3	3°29'41.4''	35°46'58.2''
22-F1-05.04.07	4	3°22'20.7''	35°50'51.8''
37-F1-15.04.07	5	3°23'33.7''	35°49'23.1''
41-F1-16.04.07	6	3°23'39.9''	35°49'15.1''
43-F1-16.04.07	7	3°22'52.2''	35°49'45.3''
58-F1-24.04.07	8	3°22'56.6''	35°49'43.3''
59-F1-25.04.07	9	3°36'37.1''	35°44'12.7''
61-F1-26.04.07	10	3°24'03.6''	35°49'19.5''
62-F1-26.04.07	11	3°24'01.1''	35°49'17.0''
76-F1-18.05.07	12	3°22'37.8''	35°49'57.8''
6-M1-24.03.07	13	3°29'19.2''	35°46'59.1''
19-M1-03.04.07	14	3°26'25.6''	35°48'19.4''
27-M1-09.04.07	15	3°22'15.8''	35°50'35.7''
31-M1-11.04.07	16	3°23'28.0''	35°49'30.7''
35-M1-13.04.07	17	3°32'08.9''	35°45'45.8''
44-M1-17.04.07	18	3°24'32.8	35°48'41.0
65-M1-04.05.07	19	3°22'40.0''	35°49'35.9''
79-M1-19.05.07	20	3°22'20.4''	35°50'03.5''
8-F2-25.03.07	21	3°23'27.8''	35°49'30.9''
32-F2-11.04.07	22	3°23'27.9''	35°49'30.9''
34-F2-13.04.07	23	3°22'16.6''	35°50'38.4''
50-F2-19.04.07	24	3°22'33.1''	35°50'02.3''

57-F2-24.04.07	25	3°22'34.5''	35°50'00.2''
10-M2-26.03.07	26	3°22'24.2''	35°50'11.4''
28-M2-09.04.07	27	3°23'28.1''	35°49'28.5''
29-M2-09.04.07	28	3°35'08.4''	35°45'10.2''
30-M2-11.04.07	29	3°30'23.8''	35°47'06.0''
47-M2-18.04.07	30	3°22'20.5''	35°50'3.5''
53-M2-21.04.07	31	3°22'56.0''	35°49'45.0''
55-M2-23.04.07	32	3°22'17.8''	35°50'39.0''
60-M2-25.04.07	33	3°34'39.0''	35°45'35.8''
68-M2-08.05.07	34	3°22'45.4''	35°49'32.0''
4-F5-23.03.07	35	3°24'51.4''	35°49'11.7''
7-F5-25.03.07	36	3°22'20.1''	35°50'15.3''
18-F5-28.03.07	37	3°30'30.4''	35°46'44.5''
69-F5-09.05.07	38	3°24'45.6''	35°48'41.7''
20-M5-03.04.07	39	3°22'24.1''	35°50'11.3''
40-M5-16.04.07	40	3°23'39.9''	35°49'15.0''
70-M5-10.05.07	41	3°22'39.1''	35°49'55.7''
2-F8-22.03.07	42	3°22'23.7''	35°50'11.2''
15-F8-27.03.07	43	3°22'24.5''	35°50'12.3''
21-F8-04.04.07	44	3°22'23.4''	35°50'10.7''
26-F8-06.04.07	45	3°23'27.8''	35°49'30.8''
49-F8-19.04.07	46	3°22'22.1''	35°50'20.6''
52-F8-21.04.07	47	3°22'15.8''	35°50'23.9''
56-F8-23.04.07	48	3°22'24.1''	35°50'11.4''
78-F8-19.05.07	49	3°24'34.5''	35°49'39.4''
3-M8-22.03.07	50	3°22'7.5''	35°50'31.4''
16-M8-28.03.07	51	3°35'22.5''	35°44'6.5''
33-M8-12.04.07	52	3°22'14.1''	35°50'28.7''
39-M8-15.04.07	53	3°22'24.4''	35°50'11.3''

54-M8-21.04.07	54	3°22'56.1''	35°49'43.5''
63-M8-27.04.07	55	3°22'16.2''	35°50'36.9''
67-M8-07.05.07	56	3°26'20.3''	35°48'36.4''
74-M8-16.05.07	57	3°34'36.0''	35°45'43.7''
64-M8-04.05.07	58	3°22'24.6''	35°50'11.7''
71-M8-10.05.07	59	3°22'55.1''	35°49'43.9''
72-M11-11.05.07	60	3°24'40.2''	35°48'43.2''
25-F1-05.04.07	61	3°29'51.2''	35°47'00.6''
75-M1-17.05.07	62	3°22'20.5''	35°50'03.4''
77-F1-18.05.07	63	3°22'35.1''	35°49'59.4''
51-F1-19.04.07	64	3°22'33.1''	35°50'02.3''
66-M8-07.05.07	65	3°22'24.0''	35°50'11.1''

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